Direct Identification of Tyrosine 474 as a Regulatory Phosphorylation Site for the Akt Protein Kinase*§

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Understanding the regulation of Akt has been of major interest for elucidating the control of normal cellular physiology as well as malignant transformation. The paradigm for activation of Akt involves phosphatidylinositol 3-kinase-dependent membrane localization followed by activating phosphorylation of Thr-308 and Ser-473. Many of the activating signals for Akt involve the stimulation of receptor and non-receptor tyrosine kinases, and the most potent activator known is the tyrosine phosphatase inhibitor pervanadate, highlighting a possible role for tyrosine phosphorylation in the regulation of the enzyme. In this study we show that activation of Akt by pervanadate or serum is associated with tyrosine phosphorylation of Akt. In addition, in SKOV3 ovarian carcinoma cells that exhibit high basal levels of Akt activity, Akt was tyrosine-phosphorylated in the basal state, and this phosphorylation was further enhanced by both pervanadate and insulin-like growth factor-1. We have used NH2-terminal sequencing and phosphate release analysis to directly identify Tyr-474 as the site of tyrosine phosphorylation. Substitution of Tyr-474 with phenylalanine abolished tyrosine phosphorylation of Akt and resulted in up to 55% inhibition of Akt activation, indicating phosphorylation at Tyr-474 is required for full activation of the kinase. Our data identifies a novel regulatory mechanism for this pleiotropic enzyme that may be applicable to the AGC family of protein kinases given the conserved nature of the COOH-terminal hydrophilic motif containing Tyr-474.

The protein kinase Akt plays key regulatory roles in a range of physiological processes including glucose metabolism (1, 2), cell survival (3–5), proliferation (6, 7), migration (8, 9), and angiogenesis (10–12). Furthermore, the kinase is inappropriately regulated in a number of tumors (13–16). Understanding the regulation of Akt has thus been of major interest for elucidating the control of normal cellular physiology as well as malignant transformation. The paradigm for activation of Akt involves phosphatidylinositol 3-kinase (PI3K)1-dependent membrane localization followed by activating phosphorylation of Thr-308 in the activation loop of the kinase and Ser-473 at the COOH terminus (4, 5, 17). Binding of the PH domain of Akt to PI3K-generated phosphatidylinositol 3,4,5-trisphosphate releases the autoinhibitory function of this domain allowing phosphorylation of Thr-308 by PDK1, while the mechanism of Ser-473 phosphorylation remains to be determined definitively. Many of the activating signals for Akt involve the stimulation of receptor and non-receptor tyrosine kinases, and the most potent activator known is the tyrosine phosphatase inhibitor pervanadate, highlighting a possible role for tyrosine phosphorylation in the regulation of the enzyme. Recent reports show that the tyrosine kinases Syk and Btk are required for B cell receptor induced activation of Akt (18), and Chen et al. (19) have demonstrated that activation of Akt induced by EGF or v-Src transformation requires tyrosine phosphorylation. In addition, dephosphorylation of active Akt with the tyrosine phosphatase PTPβ markedly reduced kinase activity (19). Chen et al. (19) assigned the tyrosine phosphorylation to two sites near the activation loop of the kinase based on mutational analysis (Tyr-315 and Tyr-326). That mutation of these sites has major effects on activity, and phosphorylation is not surprising given the essential nature of this region of the enzyme. To enable direct biochemical identification of regulatory sites of tyrosine phosphorylation we have utilized pervanadate, the most potent activator of Akt to maximize the stoichiometry of phosphorylation. In this report we show that activation of Akt by pervanadate or serum was associated with tyrosine phosphorylation of Akt. In addition, in SKOV3 ovarian carcinoma cells that exhibit high basal levels of Akt activity, Akt was tyrosine-phosphorylated in the basal state, and this phosphorylation was further enhanced by both pervanadate and insulin-like growth factor-1 (IGF-1). We have used NH2-terminal sequencing and phosphate release analysis to identify Tyr-474 as the site of tyrosine phosphorylation. Substitution of Tyr-474 with phenylalanine abolished tyrosine phosphorylation of Akt and resulted in up to 55% inhibition of Akt activation, indicating that phosphorylation at Tyr-474 is required for full activation of the kinase. Phosphorylation site analysis reveals that mutation of this site or the neighboring regulatory site Ser-473 to Phe and Ala, respectively, inhibits phosphorylation of Thr-308 concomitant with decreased activation of the kinase. * In vitro studies confirm these mutations render the kinase insensitive to activation by PDK1. As corresponding carboxyl-terminal residues are found in many other members of the AGC kinase family, these data identify a novel regulatory mechanism for this pleiotropic enzyme that may be applicable to related AGC protein kinases.

The on-line version of this article (available at http://www.jbc.org) contains Supplementary Fig. 4.

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‡ The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; PH, pleckstrin homology; IGF-1, insulin-like growth factor-1; GST, glutathione S-transferase; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; HPLC, high performance liquid chromatography.

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EXPERIMENTAL PROCEDURES

Materials—The peptide substrate RPRAAF, and carboxyl-terminal peptide from Akt1 (CRHRHPFFQFSYSSASTA) was used to raise polyclonal antibodies, which were synthesized by Auspep Pty Ltd. IGF-1 was purchased from ICN, and wortmannin and Affi-Gel 10 resin were from Sigma.

Plasmids and Transfections—pECE HA-Akt1 (20) was subcloned as a KpnI/EcoRV fragment into a pCDSAS vector containing GST, in-frame with GST-Akt1 Phe-474 (GST-PHAkt1, GST-ΔPHAkt1, GST-ΔPHAkt1 Ala-473, and GST-ΔPHAkt1 Ala-473/Phe-474 mutants were created with the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. The kinase inactive construct HA-Akt K179A had the essential catalytic domain lysine residue mutated to alanine. All constructs were confirmed by sequencing. The Myc-tagged PKD1 was generated as described previously (19). Transfections were performed on COS1 cells grown to 50–80% confluence using FuGENE (Roche Molecular Biochemicals) at a ratio of 3 μl of FuGENE/1 μg of DNA according to the manufacturer’s protocol.

Cell Culture and Stimulation—COS1 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum. The transformed ovarian epithelial cell line SKOV3 was cultured in RPMI with 10% fetal calf serum. Prior to stimulation all cells were cultured in DMEM for at least 24 h. Serum-starved cells were then incubated at 37°C in a 5% CO2 atmosphere with various stimuli under optimized conditions. Following stimulation, cells were rinsed twice in ice-cold phosphate-buffered saline and harvested in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM EDTA, 50 mM β-glycerophosphate, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride), Dounce homogenized, and cleared by centrifugation at 4°C for 10 min at 13,000 × g. The supernatant (cell extract) was retained. The protein concentration of cell extracts was determined using the Bio-Rad DC Protein Assay with bovine serum albumin as a protein standard.

Antibodies—Sheep and rabbit polyclonal antibodies directed to the COOH-terminal residues of Akt1 (CRHRHPFFQFSYSSASTA) were affinity-purified on an Affi-Gel 10 resin (Sigma) coupled to the COOH-terminal peptide. The bound antibody was eluted with 7M urea, 1 M Tris-HCl, pH 1.9 buffer (formic acid:glacial acetic acid:H2O; 50:156:1794 v/v), and phosphoamino acids were separated by electrophoresis as described previously (23). Phospho-specific antibodies recognizing Akt phosphorylated on Ser-473 or Thr-308 were obtained from New England Laboratories Inc.) was used for immunoblotting, and 1:2000 dilution, or Akt1 antibodies at 1:3000 (v/v) dilution, phospho-specific antibodies recognizing Akt phosphorylated on Ser-473 or Thr-370 at 1:2000 (v/v) dilution, or Akt1 antibodies at 5 μg/ml as indicated. Bound antibodies were visualized by enhanced chemiluminescence. The location of the antibody heavy chain is indicated (Ab). The results shown are representative of three to five independent experiments.

Phosphoamino Acid Analysis—Phosphopeptides corresponding to approximately 100 pmol were hydrolyzed for an hour at 95°C with 50 μl of 5.7 N HCl. HCl was removed by drying, resuspending into 100 μl of pH 1.9 buffer (formic acid:glacial acetic acid:H2O; 50:156:1794 v/v), and drying again. The dried hydrolyzed peptides were resuspended into 2–5 μl of pH 1.9 buffer together with phosphoamino acid standards and spotted on a TLC plate. Phosphoamino acids were separated by electrophoresis at 1000 V for 90 min in (1:2) pH 1.9 buffer pH 3.5 buffer (pyridine/glacial acetic acid:H2O, 110:189 v/v) and visualized with ninhydrin (0.1% w/v in ethanol) or using a PhosphoImager and ImageQuant software (Molecular Dynamics).

Phosphate Release and NH2-terminal Sequencing of Purified Phosphopeptides—Selected phosphopeptides corresponding to at least 400 pmol were covalently coupled to a Sequelon arylamine membrane and analyzed on a Hewlett-Packard G1000A protein sequencer as described previously (23). Fractions were collected at each cycle of Edman degradation and 32P radioactivity counted. The chemical sequence of the phosphopeptides was identified on the same protein sequencer by solid phase sequencing as described previously (23).

RESULTS

Akt is maximally activated by treatment of COS1 cells and Swiss 3T3 fibroblasts with the potent tyrosine phosphatase inhibitor, pervanadate (20). We hypothesized that tyrosine phosphorylation of endogenous Akt may play a role in its activation and assays for this possibility by Western blot analysis of Akt immunoprecipitates from pervanadate-treated COS1 cells using anti-phosphotyrosine antibodies (Fig. 1a). Pervanadate treatment induced marked tyrosine phosphorylation of Akt in COS1 cells. Furthermore, tyrosine phosphorylation of Akt in pervanadate-treated COS1 cells was abolished by the PI3K inhibitor wortmannin, indicating either that the responsible tyrosine kinase is PI3K-dependent or that, like Thr-308 phosphorylation, the tyrosine phosphorylation requires a phoshoinositide-induced conformational change in the kinase (5). Akt appears to play a major role in the biology of ovarian...
epithelial cells and 40% of ovarian tumors display elevated levels of Akt activity (16). We have demonstrated that the ovarian carcinoma cell line SKOV3 exhibits elevated basal Akt activity that can be further stimulated by pervanadate and growth factors (data not shown). Consistent with these data, Akt in these cells exhibits a low but reproducible basal level of tyrosine phosphorylation that is further stimulated by pervanadate and IGF-1 treatment (Fig. 1b). Similar patterns of Thr-308 and Ser-473 phosphorylation were observed, consistent with a regulatory role for tyrosine phosphorylation of Akt in SKOV3 cells. The Ser-473 phospho-specific antibody gave a more intense signal that is likely to reflect both more robust phosphorylation of this site and the higher sensitivity of this antibody. In both cases, Akt was detected, indicating they are not a prerequisite for tyrosine phosphorylation of the kinase.

To directly identify the site(s) of this tyrosine phosphorylation of Akt, we chose COS1 cells, because the response was most robust, and these cells can be transiently transfected to achieve expression levels of the kinase sufficient for biochemical characterization. Transiently transfected cells were metabolically labeled with $^{32}$P and the immunoprecipitated Akt subjected to tryptic phosphopeptide mapping. Phosphopeptides were separated by HPLC and further analyzed by thin layer chromatography as described previously (24) (Fig. 2). A range of phosphopeptides (A–L) was observed, with peptides E, F, G/H, I/J, and K all generated in response to pervanadate treatment (compare Fig. 2, a and b). This was particularly interesting as the only regulated phosphorylation sites positively identified so far reported are Thr-308 in the activation loop and Ser-473 in the COOH-terminal regulatory region of the kinase (25). Phosphopeptides E, F, G/H, and K were stimulated by serum treatment (20). To confirm these phosphopeptides were not artifacts of the over expression system, phosphopeptide maps were generated from endogenous kinase (Fig. 2d). As with serum stimulation, phosphopeptides E, F, G/H, and K were all enhanced by pervanadate treatment, indicating the existence of at least two novel regulated phosphorylation sites in Akt in addition to Thr-308 and Ser-473.

To characterize and identify these phosphopeptides, each was further purified by preparative 2-dimensional thin layer electrophoresis and chromatography and then subjected to phosphoamino acid analysis, phosphate release analysis, and NH$_2$-terminal sequencing as described previously (24). Phosphoamino acid analysis revealed peptides E and I/J contained phosphothreonine, peptides A–D and F contained phosphoserine, and peptides K and L contained phosphohistidine (Fig. 3a). Phosphate release analysis and NH$_2$-terminal sequencing enabled positive identification of all peptides except G/H and I/J (Fig. 3b) and are summarized in Table I.

Phosphopeptide E, present in both epitope-tagged and endogenous kinase, was identified as being phosphorylated on Tyr-474, adjacent to the known regulatory phosphorylation site Ser-473 (phosphopeptide F). This is an exciting finding, directly identifying a regulatory tyrosine phosphorylation site in Akt for the first time. The I/J peptides were absent from endogenous maps (Fig. 2d) and maps of GST-tagged Akt (data not shown) and run as a single spot when subjected to performic acid oxidation, indicating they are differentially oxidized forms of the same peptide. This phosphopeptide contained phospho-tyrosine, but did not yield phosphate release or NH$_2$-terminal sequence data. Taken together this data indicate I/J may correspond to phosphorylation of the blocked NH$_2$-terminal he-maggutatin (HA)-tagged peptide, a possibility to be aware of when analyzing anti-phosphorysiney blotting of HA-tagged proteins. Phosphopeptides F and K correspond to the known regulatory sites Ser-473 and Thr-308, respectively. The intensities of each of the regulated spots were determined by analysis of the original image files using Scion Image for Windows acquisition and analysis software (Scion Corporation, Frederick, MD). The peptides included in the analysis are circled in the relevant figures. Tyr-474 is phosphorylated to a similar extent as Thr-308 and Ser-473 in response to pervanadate (Fig. 2b; 1224:1082:1410 units, respectively). Similar levels of relative
phosphorylation were also observed upon serum stimulation (Fig. 2c; 398:223:414 units, respectively), although incorporation in to all sites was low. Tyr-474 is also significantly phosphorylated in endogenous Akt (Fig. 2d; 557 units versus 1614 for Thr-308 (35%) and 2321 for Ser-473 (24%)) although to lower levels than observed for overexpressed Akt.

The peptides A/B and L contain Ser-124 and Thr-450, respectively, known sites that are constitutively phosphorylated (25),
consistent with the lack of pervanadate-induced phosphorylation observed for these peptides. Peptides A and B and C and D run as single spots when subjected to performic acid oxidation (data not shown). Peptides C and D are doubly phosphorylated on Ser-124 and Ser-129 and are not evident in the endogenous kinase. The relative phosphorylation of Thr-450 was significantly lower in Akt from serum treated Cos1 cells compared with resting cells. The basis for this difference is unclear but may reflect selective effects of serum on the phosphorylation of Ser-124. None-the-less, as mutation of Thr-450 has little effect on kinase activation (26), it is unlikely that lack of phosphorylation of Thr-450 accounts for the poor activation of Akt by serum.

We found no evidence of alternative tyrosine-phosphorylated peptides in the HPLC profile of tryptic digestions of endogenous or overexpressed Akt from pervanadate stimulated COS1 cells (data not shown). While the yields of phosphopeptides during the tryptic digestion and purification were high (see figure legends 2 and 3), it is impossible to exclude the selective loss of individual peptides. To further examine the possibility of alternate tyrosine phosphorylation sites, we constructed a mutant GST-tagged Akt with Tyr-474 substituted with Phe. This substitution abolished tyrosine phosphorylation of Akt consistent with Tyr-474 being the major site of tyrosine phosphorylation (Fig. 4a). Furthermore, this substitution resulted in 37 and 55% inhibition of pervanadate- and IGF-1-stimulated activity, respectively (Fig. 4b), consistent with the partial inhibition of Akt activity observed following incubation with PTPβ (19). These effects were not due to differences in expression...
levels as indicated by Western blotting (Fig. 4c). Similar results were obtained using endothelial nitric oxide synthase as protein substrate for Akt (see Supplementary data).

To investigate the mechanism of regulation via phosphorylation of Tyr-474, we examined the effects of its mutation to phenylalanine on phosphorylation of Ser-473 and Thr-308 (Fig. 4c). Western blotting with phospho-Ser-473 antibody reveals that the Phe-474 mutant is phosphorylated at Ser-473 at slightly lower levels than wild type GST-Akt, indicating the mutation has not caused major changes in the local conformation. Thr-308 phosphorylation in the Phe-474 mutant was significantly lower than in wild type. The hydrophobic motif containing Ser-473 and Tyr-474 is conserved in the AGC family of protein kinases and has been implicated in binding PDK1 (27). Furthermore, the equivalent tyrosine residue in the kinase PRK2 is required for PDK1 interaction as is an adjacent NH2-terminal acidic residue. To test whether the reduction in Thr-308 phosphorylation was due to effects of the mutation on PDK1 binding, each Akt construct was co-transfected with PDK1. The Phe-474 mutation had little effect on PDK1 binding following pervanadate stimulation (Fig. 4d, upper panel). In addition, immunoprecipitates of endogenous PDK1 from pervanadate-treated cells contained similar amounts of mutant and wild type GST-Akt (Fig. 4d, lower panel). This observation is consistent with recent data showing that the region of PDK1 that interacts with the hydrophobic motif of AGC kinases is not required for activation of Akt (28). These results indicate that phosphorylation of Tyr-474 plays a more subtle role in regulating Akt activity rather than causing a major perturbation of PDK1 binding.

We failed to detect any peptide doubly phosphorylated on Ser-473 and Tyr-474, as would be expected if the two sites cooperated in activating the kinase. While this may be due to selective loss of this peptide, given the complex pattern of phosphorylation of the kinase, it is also possible that a complex interplay exists between the regulatory phosphorylation sites. Indeed, as with the Phe-474 mutant, mutation of Ser-473 to Ala resulted in a marked reduction in Akt activity (25). This reduction correlates with decreased phosphorylation of both Thr-308 and Tyr-474 compared with Ser-124 and Thr-450. To compare the relative intensities of Tyr-474 and Thr-308 phosphorylation in the wild type (Fig. 2b) and Ala-473 mutant (Fig. 5a) kinase, the intensity of each was expressed as a ratio of the intensity of Ser-124 phosphorylation. The peptides included in the analysis are circled in the relevant figures. Tyr-474 was phosphorylated by guest on July 24, 2018http://www.jbc.org/Downloaded from
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...phosphorylation observed in the ovarian tumor-derived SKOV3 cell line.

It is clear that phosphorylation of Tyr-474 plays an important role in regulating Akt activity, although the detailed mechanism of this regulation remains to be elucidated. Our in vivo data indicate that the mutation of either Ser-473 or Tyr-474 markedly reduces Thr-308 phosphorylation and kinase activation. This was confirmed in vitro where substitution of either residue markedly reduced PDK1-induced activation of Akt PH domain mutants. The finding that the double mutant abolished activation implies Ser-473 and Tyr-474 may cooperate in facilitating activation of the kinase by PDK1. We have shown this mechanism to be dependent on PI3K activity. Given the effects of PP2 on Akt activity, it is likely that this PI3K dependence is due to the requirement for PH domain-mediated localization of Akt close to membrane localized Src family kinases as recently reported for DAPK-1 (31).

Very recently, crystallographic studies have indicated that the COOH-terminal hydrophobic motif undergoes a Tyr-474 phosphorylation-dependent interaction with the NH2-terminal lobe of the kinase that results in activation of the enzyme (32). Peptide analogues of this region and the equivalent region from the related kinase PRK2 (27) activate the kinase by mimicking this interaction, binding with and simultaneously stabilizing the activated conformation of the kinase (32). Importantly, substitution of the homologous residue to Tyr-474 in the PRK2 peptide reduced its ability to activate Akt by 90%. This observation indicates a key role for the conserved tyrosine in regulating the affinity of the hydrophobic domain with the NH2-terminal lobe, consistent with our findings that mutation of Tyr-474 inhibits activation of the kinase.

The findings presented in this study are based on direct chemical identification of the phosphorylation site. However, they are in direct conflict with those of Chen et al. (19) who used mutational analysis to assign the tyrosine phosphorylation to two sites near the activation loop of the kinase (Tyr-315 and Tyr-326). It is not surprising that mutating these sites has major effects on activity and phosphorylation given the essential nature of this region of the enzyme. While it is possible that the different stimuli employed result in phosphorylation of alternative sites, we found no evidence of alternative tyrosine-phosphorylated peptides in the HPLC profile of tryptic digests of wild type or Phe-474 mutant Akt from serum- or pervanadate-stimulated COS1 cells. Despite efficient recovery of phosphorylated peptides throughout the isolation procedure, it is possible alternative tyrosine-phosphorylated peptides have been selectively lost. However, substitution of Tyr-474 with Phe abolished tyrosine phosphorylation of Akt consistent with Tyr-474 being the major site of tyrosine phosphorylation.

These in vivo experiments are invaluable as, unlike phosphospecific antibodies, they allow assessment of the relative phosphorylation of the known and novel regulatory sites. It is important to note this assessment is predicated on the assumption that individual phosphopeptides are recovered with similar efficiencies during the digestion and mapping process. As observed above, this is a reasonable assumption given the high efficiency of overall recovery of 32P, incorporated into peptides; however, some selective loss of individual peptides is possible. Tyr-474 is phosphorylated to a similar level as Thr-308 and Ser-473 in response to pervanadate or serum and is significantly phosphorylated in endogenous Akt. This is an exciting and significant observation that is reinforced by the functional role for phosphorylation of Tyr-474 indicated by structure function analysis using the Phe-474 mutant. Furthermore, corresponding tyrosine residues are found in many other members of the AGC kinase family including p70S6K, S6K2, and protein kinase Cα, γ, and ε (24, 33), suggesting this may be a common regulatory mechanism for this family of mitogen-stimulated protein kinases, providing a general mechanism linking tyrosine phosphorylation and growth factor signaling.

While the mechanism by which tyrosine phosphorylation regulates Akt activity remains elusive, it appears to involve a complex interplay between the regulatory phosphorylation sites. The mutation of either COOH-terminal site affects the phosphorylation of the other two regulatory sites without marked effects on PDK1 binding. Phosphorylation of Tyr-474 may, in fact, contribute to the long-standing difficulty in establishing the mechanism of regulation of Ser-473 phosphorylation (34). The complexity of these regulatory events reflects the plethora of cellular stimuli that feed into the Akt signaling pathway (3–5) and raises the possibility of agonist-specific pathways differentially activating the kinase. Furthermore, the activation process may be more complicated still, with a family of proteins homologous to the product of the T cell lymphoma 1 locus (TCL1) being capable of binding and activating Akt (35). The role of phosphorylation in this process remains to be ascertained. Definitive examination of the role of Tyr-474 in the regulation of Akt and the interplay between its phosphorylation and that of Ser-473 and Thr-308 awaits the generation of phospho-specific antibodies to both the Tyr-474 and Ser-473/Tyr-474-phosphorylated peptides and identification of the kinases responsible for phosphorylating these two COOH-terminal residues. These reagents will be invaluable in understanding the process of Akt activation in both normal and transformed cells.

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