Identification of a Proline-rich Akt Substrate as a 14-3-3 Binding Partner*

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Akt (also called protein kinase B) is one of the major downstream targets of the phosphatidylinositol 3-kinase pathway. This protein kinase has been implicated in insulin signaling, stimulation of cellular growth, and inhibition of apoptosis as well as transformation of cells. Although a number of cellular proteins have been identified as putative targets of the enzyme, additional substrates may play a role in the varied responses elicited by this enzyme. We have used a combination of 14-3-3 binding and recognition by an antibody to the phosphorylation consensus of the enzyme to identify and isolate one of the major substrates of Akt, which is also a 14-3-3 binding protein. This 40-kDa protein, designated PRAS40, is a proline-rich Akt substrate. Demonstration that it is a substrate of Akt was accomplished by showing that 1) PRAS40 was phosphorylated in vitro by purified Akt on the same site that was phosphorylated in insulin-treated cells; 2) activation of an inducible Akt was alone sufficient to stimulate the phosphorylation of PRAS40; and 3) cells lacking Akt1 and Akt2 exhibit a diminished ability to phosphorylate this protein. Thus, PRAS40 is a novel substrate of Akt, the phosphorylation of which leads to the binding of this protein to 14-3-3.

The phosphatidylinositol (PI)3-kinase pathway is activated by many extracellular growth factors including insulin (1, 2). A number of approaches have implicated this pathway in the subsequent responses elicited by this hormone. One of the major downstream targets of the lipid products generated by this enzyme (PI3,4,5-P3 and PI3,4-P2) is the Ser/Thr kinase called Akt or protein kinase B. Which of the particular responses elicited by the PI3-kinase are actually caused by Akt has been the subject of numerous studies, and these works have implicated Akt in many of the responses including stimulation of glucose uptake and cell growth as well as inhibition of apoptosis (3–5). The Akt enzyme phosphorylates proteins on Ser or Thr residues in the motif RXRXX(S/T) (6). A number of substrates of Akt that contain this motif have been identified. These substrates include glycogen synthase kinase (GSK)-3, several mammalian homologs of the Caenorhabditis elegans DAF-16 transcription factor, the anti-apoptotic protein BAD, phosphodiesterase 3B, a Rab GTPase-activating protein, ATP-citrate lyase, and most recently, tuberous sclerosis complex-2 (1, 2, 7–10).

The phosphorylation of a substrate by Akt will in several cases (i.e. BAD, the DAF-16 homologs, and tuberous sclerosis complex-2) result in the subsequent binding of the substrate to another protein called 14-3-3 (11–13). 14-3-3 is one of a family of seven related proteins that in some cases (i.e. BAD, the DAF-16 homologs) can induce a change in the subcellular localization of a protein, whereas in other cases this protein can activate or inhibit the intrinsic enzymatic activity of a protein (i.e. Raf) (14, 15). The binding of 14-3-3 to a protein also has been proposed to regulate either the proteolysis and/or the phosphorylation state of the bound protein. Most important for the purposes of the present discussion has been the finding that the 14-3-3 proteins bind to phosphoserine- and phosphothreonine-containing motifs in a sequence-specific manner (15–17). The particular motif recognized by these proteins includes the consensus phosphorylation site of Akt, a phosphorylated Ser/Thr with an Arg residue present at position −3 or −4.

In the present work we have used a combination of the 14-3-3 protein and anti-pAkt substrate antibodies (18) to screen and isolate substrates of Akt. The major 14-3-3 binding protein observed in cells after insulin treatment was a 40-kDa molecule. This protein (designated PRAS40) was purified, sequenced, and identified as a proline-rich molecule without any major homology to other proteins in the data base and also lacking any recognizable domains. We have demonstrated that this protein is a substrate of Akt by showing that it can be phosphorylated in vitro with purified Akt, that the activation of an inducible Akt (called mer-Akt) (19) was alone sufficient to induce PRAS40 phosphorylation, and that the phosphorylation of this protein was decreased in cells lacking Akt1 and Akt2 (3, 4).

**Experimental Procedures**

Materials and Cell Lines—LY294002 and rapamycin were from Calbiochem. The 14-3-3-GST expression constructs were a gift of Dr. Andrey Shaw, and the fusion protein was purified as described (17). Cloning enzymes and competent DH5α cells were from Invitrogen. Plasmid purification kits were from Qiagen (Valencia, CA). Pfu Turbo and the QuikChange XL mutagenesis kit were from Stratagene (La Jolla, CA). Enhanced chemiluminescence detection reagents were from Pierce. Porcine insulin was from Roche Molecular Biochemicals, goat anti-mouse and anti-rabbit phosphatase-conjugated antibodies were from Promega, anti-mouse and anti-rabbit antibodies coupled to

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The abbreviations used are: PI, phosphatidylinositol; PRAS40, proline-rich Akt substrate of 40 kDa; GST, glutathione S-transferase; wt, wild type; PDGF, platelet-derived growth factor; PKB, protein kinase B; EST, expressed sequence tag; MEF, mouse embryonic fibroblasts; HA, hemagglutinin; GSK, glycogen synthase kinase; myr-Akt, myristoylated Akt; mer, myristoylated estrogen receptor.
Proline-rich Akt Substrate of 40 kDa

**PLATE 1**

Identification of a 40-kDa phosphoprotein as an insulin-responsive 14-3-3-binding protein. Panel (a), binding to 14-3-3 columns. H4IIE cells were treated without or with 100 nM insulin for 10 min and then lysed with lysis buffer (50 mM HEPES, 150 mM NaCl, 1 % Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 5 mM β-glycerol phosphate, 10 mM NaF, 100 mM okadaic acid, 10 μg/mL aprotinin, and 10 μg/mL leupeptin). Lysates were centrifuged at 10,000 × g for 15 min at 4 °C, and the supernatants were incubated with 14-3-3-GST bound to glutathione-agarose beads at 4 °C. The beads were washed three times with HEPES-buffered saline, pH 7.6, and the bound proteins eluted by the addition of 1 ml of 0.5 % Empigen BB. Eluted proteins were concentrated and then analyzed or purified on either SDS-PAGE gels or via two-dimensional gel electrophoresis. To find the position of the PRAS40 band, gels were transferred to nitrocellulose and blotted with anti-pAkt substrate antibodies. The PRAS40 bands were digested with trypsin, and the tryptic peptides were sequenced by microcapillary liquid chromatography mass spectrometry/mass spectrometry on an ion trap mass spectrometer at both Thermo Finnigan and the Harvard Microchemistry Facility.

**RESULTS AND DISCUSSION**

Identification and Isolation of a 40-kDa Protein That Binds 14-3-3, the Phosphorylation of Which Is Induced by Insulin—A rat hepatoma, H4IIE, was treated with insulin and lysed, and the lysates were either probed directly or after purification on 14-3-3 affinity columns. Total lysates as described above were also analyzed by SDS-PAGE, and the transferred blots were probed with either the wt 14-3-3 or the mutant 14-3-3 (R56,60A), and the bound 14-3-3 was detected as described under "Experimental Procedures." c, the role of PI 3-kinase in the insulin-stimulated phosphorylation of the 40-kDa protein. H4IIE cells were treated with insulin in either the absence or presence of 1 μM wortmannin or 1 μM rapamycin. The cells were lysed, the lysates were absorbed with 14-3-3, and the material that bound and was eluted from a 14-3-3 affinity column was analyzed by SDS-PAGE and immunoblotting with an anti-pAkt substrate antibody. d, two-dimensional gel analysis of the 40-kDa protein. H4IIE cells were treated without or with insulin and lysed, and the 14-3-3-bound proteins were analyzed by two-dimensional gel electrophoresis and transferred to nitrocellulose, and the blots were probed with the anti-pAkt substrate antibody.

insulin-treated H4IIE cells or PRAS40 precipitates from PC-3 cells were separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking in 5 % nonfat dry milk in Tris-buffered saline buffer, membranes were incubated with purified 14-3-3-GST (10 μg/ml). Bound 14-3-3-GST was detected using either an anti-GST (Covance) or an anti-14-3-3 antibody (Santa Cruz) and the appropriate secondary antibody.

**FIG. 1.** Identification of a 40-kDa phosphoprotein as an insulin-responsive 14-3-3-binding protein.
Proline-rich Akt Substrate of 40 kDa

FIG. 2. Schematic of the deduced sequence of the 40-kDa protein (PRAS40). Scaled-up preparations of the 40-kDa 4-3-3 bound rat protein were electrophoresed on SDS-PAGE and digested with trypsin, and the resulting peptides were analyzed by mass spectrometry. Five peptides were identified that matched the deduced sequence of a mouse protein in the data base (gi:12834425). This protein was highly homologous to that of a human protein (gi:14150199). The single predicted Akt phosphorylation site (Thr-246) and the proline-rich regions are indicated in a schematic of the PRAS40.

(a) autoradiography

(b) blot: α-pAkt substrate

Fig. 3. In vitro phosphorylation of PRAS40 by Akt. Four μg of purified PRAS40-GST fusion protein or GST alone were incubated with a constitutively active Akt immunoprecipitated from transfected cells. After a 30-min incubation at 30 °C in the presence of 10 mM MgCl₂ and 5 μM ATP (containing 2 μCi of [γ-32P]ATP), the reaction mixtures were analyzed by SDS-PAGE followed by transfer to nitrocellulose and autoradiography. a, the reaction was also performed in the presence of either 0 or 2.5 mM ATP, and the products were analyzed by immunoblotting with the anti-pAkt substrate antibody (b).

ability to bind phosphorylated peptides (17). To look for other proteins that could bind to 14-3-3 in the total lysates but that may not have been recovered in the 14-3-3 affinity column, total lysates from insulin-treated cells were probed with the 14-3-3 in a far Western. Again, the major insulin-stimulated 14-3-3 binding protein observed in the total lysates was a 40-kDa protein (Fig. 1b).

To determine which signal cascade was responsible for the insulin-stimulated increase in 14-3-3 binding to the 40-kDa protein, H4IIE cells were treated with various inhibitors before insulin stimulation. Wortmannin, an inhibitor of the PI 3-kinase/Akt pathway, was found to largely block the insulin-stimulated increase in phosphorylated 40-kDa protein in the 14-3-3 eluates. In contrast, rapamycin, an inhibitor of the downstream kinase mammalian target of rapamycin, had only a slight effect on the amount of phosphorylated 40-kDa protein (Fig. 1c). In contrast, an inhibitor of the mitogen-activated protein kinase pathway (PD 098059) had no effect on the phosphorylation of the 40-kDa protein (data not shown).

To further characterize this 40-kDa protein, two-dimensional gel electrophoresis was used. The isoelectric point (pI) of the protein was estimated by this procedure to be ~4.5 (Fig. 1d). This point was considerably different from any previously identified substrate of Akt in this size range (i.e. glycogen synthase kinase-3β has a pI of 8.9). A similar 14-3-3-binding protein was also observed in 3T3-L1 adipocytes as well as in various transformed cells like the prostate cancer cell line PC-3 (data not shown).

The above results suggested that the 40-kDa protein may be a novel substrate of Akt and a major 14-3-3 binding protein. We therefore isolated sufficient amounts of this protein for tryptic digestion and identification by mass spectrometry. Five peptides from the isolated protein were found to match a predicted mouse protein gi:12834425 of unknown function and a predicted M₉ of 27,500 and pI of 4.6. A human homolog (gi:14150199) of this protein was also present in the EST data base. The deduced sequences of these proteins contained a consensus Akt phosphorylation site (Thr-246) but no other recognizable motif (Fig. 2). It was, however, highly proline-rich, with 15% of its amino acids being proline (versus 5% for a typical protein), and these proline-rich regions are potential SH3 and/or WW domain binding partners (21). The protein has therefore been named PRAS40, for proline-rich Akt substrate of 40 kDa.

Verification That the Identified cDNA Encodes for the 40-kDa Substrate of Akt and That Thr-246 Is the Major Phosphorylation Site—PRAS40 was first expressed as a GST fusion protein in E. coli. This fusion protein was found to be phosphorylated by Akt in vitro, either by inclusion of radioactive ATP in the kinase reaction and autoradiography (Fig. 3a) or by reaction with the anti-pAkt substrate antibody used to screen the total cell lysates (Fig. 3b). These results indicate that PRAS40 is readily phosphorylated in vitro and that this phosphorylation leads to the recognition of PRAS40 by the anti-pAkt substrate antibody.

The PRAS40-GST protein was also used to generate a polyclonal antibody to the protein. This antibody was capable of immunoprecipitating from lysates of insulin-treated H4IIE cells a protein that ran in an identical position on SDS-PAGE as the 14-3-3 bound material and the phosphorylation of which was stimulated by insulin (Fig. 4a). In addition, an identical anti-pAkt substrate-reactive band was immunoprecipitated with an antibody generated to the carboxyl-terminal 19 amino acids of the deduced sequence of PRAS40 (Fig. 4a). The antibody to PRAS40 also immunoprecipitated a similar band, the phosphorylation of which was stimulated by insulin in 3T3-L1 adipocytes (Fig. 4b).

An HA-epitope-tagged version of PRAS40 was also ex-
pressed transiently in human embryonic kidney cells (293 cells). Lysates of cells expressing the PRAS40 cDNA showed a phosphorylated 40-kDa band by blotting with the anti-pAkt substrate antibody, the levels of which were greatly increased in comparison with cells transfected with plasmid alone (Fig. 5). The phosphorylation of this band was further increased when the cells were also transfected with a constitutively active version of Akt (called myr-Akt) (22) (Fig. 5). Wortmannin, but not rapamycin, almost completely inhibited the phosphorylation of the expressed PRAS40 (Fig. 5), as was previously observed for the endogenous protein. Wortmannin did not block the phosphorylation of the expressed PRAS40 when the constitutively active Akt was also co-expressed. These results are consistent with PRAS40 being a direct substrate of Akt.

To determine whether the predicted Akt consensus phosphorylation site (Thr-246) was in fact the site recognized by the anti-pAkt substrate antibody, the levels of which were greatly increased in comparison with cells transfected with plasmid alone (Fig. 5). The phosphorylation of this band was further increased when the cells were also transfected with a constitutively active version of Akt (called myr-Akt) (22) (Fig. 5). Wortmannin, but not rapamycin, almost completely inhibited the phosphorylation of the expressed PRAS40 (Fig. 5), as was previously observed for the endogenous protein. Wortmannin did not block the phosphorylation of the expressed PRAS40 when the constitutively active Akt was also co-expressed. These results are consistent with PRAS40 being a direct substrate of Akt.

Detection of PRAS40 mRNA in Various Tissues—Northern blot analyses of a number of tissues confirmed the presence of PRAS40 in all of the tissues tested. Western blot analyses of various rat tissues tested, the highest levels of both mRNAs were observed in brain, heart, liver and muscle. However, a PRAS40 band was readily detected in almost all the tissues tested, the highest levels of both mRNAs were observed in brain, heart, liver and muscle. However, a PRAS40 band was readily detected in brain, heart, liver, muscle, placenta, spleen and stomach, and was not observed in testis.

Verification That PRAS40 Is a 14-3-3-binding Protein—To determine whether the expressed PRAS40 could bind 14-3-3, transiently expressed protein was immunoprecipitated from
293 cells via the use of antibodies to the added epitope tag. The precipitates were analyzed by immunoblotting for 14-3-3. Insulin was found to stimulate the amount of 14-3-3 present in the precipitates (Fig. 7a). To determine whether the endogenous PRAS40 was also capable of directly binding 14-3-3, we used the prostate cancer cell line called PC-3 that lacks the inositol 1,4,5-triphosphate lipid phosphatase PTEN and thus has constitutively active Akt (23). Precipitates of PRAS40 from these cells could directly bind 14-3-3 in a far Western (Fig. 7b). To determine whether this was also regulated by phosphorylated PRAS40, we treated these cells overnight with a reversible PI 3-kinase (LY294002) (24) and then either lysed the cells or washed out the inhibitor and incubated the cells for an additional 2 h. As seen in the anti-pAkt blot of these lysates, the overnight incubation with LY294002 resulted in a more than 95% decrease in phosphorylated Akt that was almost completely reversed after a subsequent 2-h incubation without inhibitor (Fig. 7b). The 14-3-3 did not bind to PRAS40 from cells treated with LY294002 but did bind PRAS40 after removal of the inhibitor and the 2-h incubation (Fig. 7b). This correlated with the phosphorylation of the PRAS40 (Fig. 7b). These results indicate that 14-3-3 can bind directly to PRAS40 and that this binding is regulated by PRAS40 phosphorylation.

Demonstration That PRAS40 Is an in Vivo Target of Akt—Because insulin as well as LY294002 washout will activate various Ser/Thr kinases downstream of inositol 1,4,5-triphosphate in addition to Akt (1, 2), we sought a method to test whether activation of Akt alone was sufficient to stimulate phosphorylation of PRAS40. To this end, we expressed PRAS40 in mammalian cells that contain a conditionally active Akt. This expressed variant Akt can be activated by treatment of the cells with 4-hydroxytamoxifen, a treatment that should not activate any other downstream targets of PI 3-kinase (19). Treatment of these cells with 4-hydroxytamoxifen stimulated the phosphorylation of wt PRAS40 but not the mutant PRAS40 in which Thr-246 was mutated (Fig. 8). These results demonstrate that activation of Akt alone is sufficient to induce phosphorylation of PRAS40.

To determine whether endogenous Akt is responsible for the phosphorylation of PRAS40 in nontransfected cells, we used MEFs from mice lacking either Akt1 or Akt2, or both Akt1 and Akt2 (3, 4). Phosphorylation of endogenous PRAS40 was greatly stimulated by PDGF in the MEFs from the wt mice and those lacking Akt2 (Fig. 9a). MEFs lacking Akt1 showed some decrease in the PDGF-stimulated phosphorylation of PRAS40 (approximately a 30% decrease), whereas those lacking both Akt1 and Akt2 showed the largest decrease (70%) in PDGF-stimulated phosphorylation (Fig. 9a). These values were consistent with the decreases observed in the PDGF-stimulated phosphorylation of GSK-3 (Fig. 9b), another substrate of Akt (25), as well as the amount of PDGF-stimulated phosphorylated Akt present in the four cell lines (Fig. 9b). These results implicate endogenous Akt in the PDGF-stimulated phosphorylation of PRAS40.

CONCLUSIONS

The present studies have identified a new substrate of Akt. Demonstration that PRAS40 is a substrate of Akt was accomplished by showing that it could be phosphorylated in vitro by Akt at the same site where it is phosphorylated in vivo after activation by insulin. Moreover, we showed that activation of an inducible Akt was alone sufficient to stimulate its phosphorylation. Finally, we showed that cells lacking Akt1 and Akt2 exhibit a diminished ability to phosphorylate this protein. This protein appears to be the major 14-3-3 binding protein which is responsive to insulin. It is also quite similar in size to a recently reported 14-3-3 binding protein (whose sequence is unknown) that is responsive to growth factors and nutrients in PC-12 cells (26). Although the function of this protein is not known, the presence of several proline-rich regions may allow it to interact with various SH3 or WW domain-containing proteins, thereby modifying the function of these molecules.

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