Construction and Characterization of a Conditionally Active Version of the Serine/Threonine Kinase Akt*

Aimee D. Kohn‡, Andreas Barthel‡, Kristina S. Kovacina‡, Annegret Boge‡, Brenda Wallach‡, Scott A. Summers§, Morris J. Birnbaum§, Pamela H. Scott¶, John C. Lawrence, Jr.§, and Richard A. Roth‡‡

From the ‡Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, California 94305, and the ¶Howard Hughes Medical Institute, University of Pennsylvania School of Medicine, Clinical Research Building, Philadelphia, Pennsylvania 19104-6148, and the †Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Akt is a serine/threonine kinase that requires a functional phosphatidylinositol 3-kinase to be stimulated by insulin and other growth factors. When directed to membranes by the addition of a src myristoylation sequence, Akt becomes constitutively active. In the present study, a conditionally active version of Akt was constructed by fusing the Akt containing the myristoylation sequence to the hormone binding domain of a mutant murine estrogen receptor that selectively binds 4-hydroxytamoxifen. The chimeric protein was expressed in NIH3T3 cells and was shown to be stimulated by hormone treatment 17-fold after only a 20-min treatment. This hormone treatment also stimulated an approximate 3-fold increase in the phosphorylation of the chimeric protein and a shift in its migration on SDS gels. Activation of this conditionally active Akt resulted in the rapid stimulation of the 70-kDa S6 kinase. This conditionally active Akt was also found to rapidly stimulate in these cells the phosphorylation of properties of PHAS-I, a key protein in the regulation of protein synthesis. The conditionally active Akt, when expressed in 3T3-L1 adipocytes, was also stimulated, although its rate and extent of activation was less then in the NIH3T3 cells. Its stimulation was shown to be capable of inducing glucose uptake into adipocytes by stimulating translocation of the insulin-responsive glucose transporter GLUT4 to the plasma membrane.

Akt is a serine/threonine kinase that contains a pleckstrin homology (PH)1 domain at its amino terminus (1, 2). The PH domain is a protein module found in many signal transduction proteins that can mediate either protein-lipid or protein-protein interactions (1, 2). The kinase activity of Akt is stimulated by a number of different growth factors, including insulin and platelet-derived growth factor (3–5). Several studies have shown that Akt stimulation requires prior activation of phosphatidylinositol 3-kinase (PI 3-kinase) (3–5), possibly due to a direct interaction of the Akt PH domain with these lipids products (6) or, alternatively, due to the phosphorylation of Akt by a distinct Ser/Thr kinase, which is activated by PI 3-phosphates (7).

Further interest in Akt has been stimulated by the finding that this enzyme can induce a variety of biological responses. In particular, Akt has been proposed to positively regulate the 70-kDa S6 kinase (4) and negatively regulate the GSK-3 kinase (8). Moreover, Akt has been shown to be capable of stimulating the differentiation of 3T3-L1 cells into adipocytes (9, 10) and to inhibit apoptosis of neuronal cells, as well as fibroblasts (11, 12). Finally, Akt has been shown to stimulate lipogenesis (9) and to induce glucose uptake into adipocytes by stimulating GLUT4 translocation to the plasma membrane (9, 13).

To determine whether a particular biological response can be mediated via Akt, the above studies made use of a constitutively active form of Akt in which the enzyme is targeted to membranes via the addition of either the src myristoylation signal or a myristoylated gag sequence (4, 8–13). Studies have the problem that the Akt kinase activity is unregulated, and thus the kinase is active as soon as it is expressed in cells. In contrast, the responses one is attempting to mimic, such as stimulation of glucose uptake, are stimulated by insulin within minutes. In addition, such studies require one to utilize different populations of cells to compare a particular response; for example, cells expressing the constitutively active Akt must be compared with control cells.

In this report, we describe and characterize a conditionally active form of the Akt molecule. This conditionally active form of Akt was created by fusing the hormone binding domain (HBD) of a mutant murine estrogen receptor (14) to a variant of Akt that lacks its PH domain but contains a src myristoylation signal at its amino terminus (myrAkt Δ4–129) (15). The Akt construct without the HBD has unregulated constitutive kinase activity (15). This mutant HBD of the estrogen receptor, which has also been used to make a conditionally active myc (14), does not bind 17β-estradiol but is responsive to 4-hydroxytamoxifen (14). In the present work, we show that the kinase activity of the Akt-estrogen receptor fusion protein (myrAkt Δ4–129-ER) is dependent on 4-hydroxytamoxifen (HT). This conditionally active form of Akt was used to demonstrate that acute activation of Akt was sufficient to stimulate the phosphorylation of PHAS-I (phosphorylated heat- and acid-stable protein) and to induce its dissociation from eIF4E, a key step in the regulation of protein synthesis.

**EXPERIMENTAL PROCEDURES**

**Constructs**—To make an estradiol-dependent human Akt protein kinase, the hormone binding domain of a mutant murine estrogen...
receptor (ER) (a gift from Dr. Martin McMahon) that no longer binds 17β-estradiol but is activated by the synthetic steroid 4-hydroxysteroido
tifen (14) was first subcloned into the pWZLneo retroviral vector (16),
which carries a neomycin resistance gene, using the EcoRI and SacI
restriction sites at the 5'- and 3'-ends, respectively. In addition, the
polymerase chain reaction was used to modify myrAktΔ4–129 and
A2myrAktΔ4–129 (15) by replacing the stop codon after the hemag-
globulin (HA) epitope tag with an in-frame EcoRI site. The fragment
obtained by the polymerase chain reaction was confirmed by sequen-
cing. The Akt constructs were then fused to the estrogen receptor in
pWZLneo using the 5' BanHI and 3' EcoRI restriction sites, resulting
in the formation of myrAktΔ4–129-ER and A2myrAktΔ4–129-ER.
A p70 S6 kinase clone (a gift from Dr. Gerry Crabtree) was modified
to encode a myc epitope-tag at its 5' end and subcloned into the pWZL-
neo retroviral vector using standard molecular biological techniques.

**Retroviral Infection—**NIH3T3 fibroblasts and 3T3-L1 preadipocytes
were infected with either myrAktΔ4–129-ER or A2myrAktΔ4–129-ER
as described previously (15).

NIH3T3 cells infected with either myrAktΔ4–129-ER or A2myrAktΔ4–129-ER were infected with the pWZLneo retroviral vector expressing
the myc epitope-tagged p70 S6 kinase construct. The cells
were infected using the same protocol noted above, except that the cells
were not subjected to additional drug selection.

Akt, p70 S6, PI 3-Kinase Assays, PHAS-I Shift, and eIF4E Dissocia-
tion—The Akt immunoprecipitations and immunoblotting were per-
formed as described previously (9), except that a single 100-mm plate
was used for each treatment, and each plate was lysed in 400 µl of lysis
buffer. In most experiments, a peptide (sequence GRPRSSSSFAEQG)
assay was utilized to measure Akt kinase activity as described (8),
except that a 40% SDS-polyacrylamide gel was utilized to separate the
incorporated label from free ATP. This assay was linear for up to 90
min, and all the values determined were within the linear range for Akt
levels. To detect phosphorylation of Ser473, immunoblotting was per-
formed with the phospho-Ser473 Akt antibody from New England
Biolabs.

The expressed p70 S6 kinase was immunoprecipitated using a mono-
clonal antibody directed against the myc epitope tag (Babco) that was
preadsorbed to protein G-Sepharose. The nonspecific background was
measured by incubating lysates with normal mouse immunoglobulin
that was also preadsorbed to protein G-Sepharose. The kinase activity
was measured as described previously (15). 3X Laemmli sample buffer
(45 µl) was also added to the remaining beads. The bound protein was
eluted by incubating for 4 min at 100 °C, and these samples were
electrophoresed on 10% SDS-polyacrylamide gels. The gels were trans-
ferred and immobiloblotted using the anti-myc antibody to detect p70 S6
kinase.

The PI 3-kinase assay using pure phosphatidylinositol (Sigma) as a
substrate was performed as described previously (17), except that cells
were serum-starved for 16 h before being treated and lysed. The
PHAS-I shift and eIF4E dissociation were measured by PHAS-I immu-
noblotting whole cell extracts and m'Tet-SPH-Sepharose bound ma-
terial, respectively, as described previously (8).

**3T3-L1 Preadipocytes and Adipocytes—**3T3-L1 preadipocytes infected
with either myrAktΔ4–129-ER or A2myrAktΔ4–129-ER were cultured,
selected, and differentiated as described previously (9). These
cell lines were used to measure glucose uptake and to isolate crude
membrane fractions for detection of GLUT1 and GLUT4 expression as
described previously (9). Translocation of GLUT4 to the plasma mem-
brane was performed and quantitated as described previously (19, 20).

In Vivo Labeling of myrAktΔ4–129—Medium was replaced with
Krebs-Ringer bicarbonate buffer containing 10 mM glucose and
[U-14C]-glucose (500 µCi/triplicate). After 3 h at 37°C/5%CO2, NIH3T3
cells expressing myrAktΔ4–129-ER were treated without or with 1 µM
HT for additional 40 min, placed on ice, washed with ice-cold HBS and
lysate with 500 µl of lysis buffer (50 mM Heps, pH 7.4, 1% Triton X-100,
1 mM phenylmethylsulfonil fluoride, 1 mM NaVO4, 300 mM NaF, 10 mM
NaF, 10 mM β-glycerol phosphate, 10 µg/ml aprotinin). Lysates were
immunoprecipitated with monoclonal anti-HA (12CA5) bound to Protein-A agarose beads. Beads were washed, and bound proteins were eluted
and analyzed by SDS-polyacrylamide gel electropho-
resis and transferred to nitrocellulose. To confirm which band was
Akt, the membrane was immunoblotted with anti-HA antibodies. The
autoradiographs were scanned, imported into Adobe Photoshop, and
quantitated after subtracting a background region of the gel.

**FIG. 1. Schematic representation of the Akt constructs de-
scribed in this study.** A, unregulated Akt alleles: Akt constructs
described previously (9) that bear a myristoylation signal sequence
(myr) at the amino terminus lack the amino-terminal pleckstrin homol-
domains of Akt (14–29) and possess a carboxyl-terminal HA epitope tag.
The kinase activity of myrAktΔ4–129 is constitutive and unregulated.
B, conditional Akt alleles: conditionally active Akt alleles that are fused
at their carboxyl terminus to the hormone binding domain of an ER.
The conditional Akt alleles were generated as described under “Exper-
imental Procedures.”

**RESULTS**

**Production of a Conditionally Active Akt Kinase—**Several strategies exist to make a conditionally active Akt kinase, the activity of which can be regulated. Tetracycline-regulatable systems have been described in which tetracycline can be added to suppress or induce expression of a given construct (21). In addition, one could use drug-induced dimerization systems exploiting synthetic bivalent membrane permeable compounds that bind to drug binding domains fused to intracellular signaling molecules (22). Finally, conditionally active forms of the transcription factor Myc and the protein kinases RafI and Abl have been made by fusing them with the HBD of steroid receptors, particularly the estrogen receptor (23–25).

Although we were unable to create a conditionally active form of Akt using the tetracycline-regulatable or drug-induced dimerization systems (data not shown), we were able to generate such an enzyme by constructing a HBD fusion protein.

We have previously described a constitutively active form of Akt (myrAktΔ4–129) created by attaching the src myristoyla-
tion signal to the amino terminus of a variant of Akt that lacked its PH domain and carried a HA epitope tag at its carboxyl terminus (15). We also described a control construct (A2myrAktΔ4–129) that was not constitutively active in which the myristoylated glycine at the second amino acid position was converted to alanine to eliminate the membrane-targeting function of the src myristoylation signal sequence (15). To generate a conditionally active form of Akt, the myristoylated Akt construct and the mutant control lacking the myristoylated glycine were fused in-frame to the HBD of a mutant form of the murine estrogen receptor (myrAktΔ4–129-ER and A2myrAktΔ4–129-ER) (Fig. 1) that no longer binds 17β-estradiol but is still responsive to a synthetic steroid HT (26). These fusion proteins were subcloned into a retroviral vector that carried a neomycin resistance gene (16).

NIH3T3 cells were infected with retroviruses encoding the different forms of Akt, drug-selected, serum-starved for 16 h, and then treated for increasing amounts of time with HT. The Akt fusion protein was immunoprecipitated from cell lysates using a monoclonal antibody directed against the HA tag, and

**FIG. 1. Schematic representation of the Akt constructs de-
scribed in this study.** A, unregulated Akt alleles: Akt constructs
described previously (9) that bear a myristoylation signal sequence
(myr) at the amino terminus lack the amino-terminal pleckstrin homol-
domains of Akt (14–29) and possess a carboxyl-terminal HA epitope tag.
The kinase activity of myrAktΔ4–129 is constitutive and unregulated.
B, conditional Akt alleles: conditionally active Akt alleles that are fused
at their carboxyl terminus to the hormone binding domain of an ER.
The conditional Akt alleles were generated as described under “Exper-
imental Procedures.”

**RESULTS**

**Production of a Conditionally Active Akt Kinase—**Several strategies exist to make a conditionally active Akt kinase, the activity of which can be regulated. Tetracycline-regulatable systems have been described in which tetracycline can be added to suppress or induce expression of a given construct (21). In addition, one could use drug-induced dimerization systems exploiting synthetic bivalent membrane permeable compounds that bind to drug binding domains fused to intracellular signaling molecules (22). Finally, conditionally active forms of the transcription factor Myc and the protein kinases RafI and Abl have been made by fusing them with the HBD of steroid receptors, particularly the estrogen receptor (23–25).

Although we were unable to create a conditionally active form of Akt using the tetracycline-regulatable or drug-induced dimerization systems (data not shown), we were able to generate such an enzyme by constructing a HBD fusion protein.

We have previously described a constitutively active form of Akt (myrAktΔ4–129) created by attaching the src myristoyla-
tion signal to the amino terminus of a variant of Akt that lacked its PH domain and carried a HA epitope tag at its carboxyl terminus (15). We also described a control construct (A2myrAktΔ4–129) that was not constitutively active in which the myristoylated glycine at the second amino acid position was converted to alanine to eliminate the membrane-targeting function of the src myristoylation signal sequence (15). To generate a conditionally active form of Akt, the myristoylated Akt construct and the mutant control lacking the myristoylated glycine were fused in-frame to the HBD of a mutant form of the murine estrogen receptor (myrAktΔ4–129-ER and A2myrAktΔ4–129-ER) (Fig. 1) that no longer binds 17β-estradiol but is still responsive to a synthetic steroid HT (26). These fusion proteins were subcloned into a retroviral vector that carried a neomycin resistance gene (16).

NIH3T3 cells were infected with retroviruses encoding the different forms of Akt, drug-selected, serum-starved for 16 h, and then treated for increasing amounts of time with HT. The Akt fusion protein was immunoprecipitated from cell lysates using a monoclonal antibody directed against the HA tag, and
were electrophoresed and blotted with a phospho-specific Akt antibody. A control immunoprecipitate using normal mouse Ig is also shown (lane C).

The chimera was immunoprecipitated, and its enzymatic activity was measured *in vitro* using a GSK-3 peptide as substrate. A control of the myrAkt–4–129-ER treated with control vehicle (ethanol) is also shown (∆). HT, HT-induced shift in Akt. The immunoprecipitated myrAkt–4–129-ER from NIH3T3 cells treated for the indicated periods of time with HT were separated on a 10% SDS-polyacylamide gel, and the Akt protein was detected by Western blot using a polyclonal anti-HA antibody. A control immunoprecipitate using normal mouse Ig is also shown (lane C). D, HT-induced phosphorylation of myrAkt–4–129-ER. Metabolically labeled NIH3T3 cells expressing myrAkt–4–129-ER were treated or not treated with 1 μM HT for 40 min and lysed, and the lysates were immunoprecipitated with the anti-HA antibodies. E, HT-induced phosphorylation of Ser473 in myrAkt–4–129-ER. NIH3T3 cells expressing myrAkt–4–129-ER were treated or not treated with 1 μM HT and lysed, and the lysates were immunoprecipitated with the anti-HA antibodies (HA). A control immunoprecipitate using normal mouse Ig is also shown (lane C). The precipitates were electrophoresed and blotted with a phospho-specific Akt antibody.

The kinase activity was measured *in vitro* using a synthetic peptide as substrate (∆). Exposure of cells to HT for only 20 min caused the kinase activity of myrAkt–4–129-ER to increase 17-fold. The kinase activity continued to increase with longer exposures to HT to more than a 50-fold activation (Fig. 2A). In contrast, the kinase activity of myrAkt–4–129-ER treated with ethanol alone and the activity of A2myrAkt–4–129-ER treated with either ethanol or HT was barely elevated compared with control immunoprecipitates using normal mouse immunoglobulin.

Studies on the Mechanism of Activation of the Conditionally Active Akt—As shown above, although the levels of the myrAkt–4–129-ER protein did not change after HT treatment of cells, the protein did shift to a higher molecular weight on SDS gels (Fig. 2B), consistent with an increase in the phosphorylation state of the protein.

To determine whether PI 3-kinase had any role in the activation of the conditionally active form of Akt, NIH3T3 cells that had been infected with myrAkt–4–129-ER were treated for 20 min with increasing concentrations of the PI 3-kinase inhibitor wortmannin (27). These cells were then treated for 10 min with HT to induce the Akt kinase activity. The basal PI 3-kinase activity was measured in anti-p85 antibody precipitates prepared from nonstimulated cells to verify the inhibitory effect of wortmannin. MyrAkt–4–129-ER was immunoprecipitated from cell lysates prepared from cells that had been treated with HT, and its kinase activity was measured *in vitro* to determine whether its activation was dependent on PI 3-kinase. At 1 nM wortmannin, a slight increase in Akt kinase activity was observed, similar to a slight increase in Akt activity previously observed with 1 nM wortmannin after insulin treatment (5). However, at higher wortmannin concentrations, the activation of myrAkt–4–129-ER was inhibited, and this paralleled the inhibition of PI 3-kinase activity (Fig. 3). A structurally unrelated inhibitor of PI 3-kinase, LY294002 (28), was also found to block the activation of myrAkt–4–129-ER (data not shown).

To determine whether the activating site Ser473 was phosphorylated, the immunoprecipitated myrAkt–4–129-ER was blotted with a phospho-specific antibody directed against this site. HT treatment greatly stimulated the ability of the phospho-specific antibody to recognize this chimera (Fig. 2E), demonstrating that HT treatment stimulates an increase in the phosphorylation of Ser473.

The Conditionally Active Akt Rapidly Stimulates p70 S6 Kinase—Prior studies have shown that constitutively active forms of Akt stimulate the enzymatic activity of p70 S6 kinase. To determine whether the conditionally active Akt also stimulated p70 S6 kinase activity, NIH3T3 cells infected with myrAkt–4–129-ER were treated for 40 min with either HT or ethanol, and lysates were separated on a 10% SDS-polyacrylamide gel, and the Akt protein was detected by Western blot using a polyclonal anti-HA antibody. A control immunoprecipitate using normal mouse Ig is also shown (lane C). The precipitates were electrophoresed and blotted with a phospho-specific Akt antibody.

The protein did shift to a higher molecular weight on SDS gels (∆). HT, HT-induced shift in Akt. The immunoprecipitated myrAkt–4–129-ER from NIH3T3 cells treated for the indicated periods of time with HT were separated on a 10% SDS-polyacrylamide gel, and the Akt protein was detected by Western blot using a polyclonal anti-HA antibody. A control immunoprecipitate using normal mouse Ig is also shown (lane C). D, HT-induced phosphorylation of myrAkt–4–129-ER. Metabolically labeled NIH3T3 cells expressing myrAkt–4–129-ER were treated or not treated with 1 μM HT for 40 min and lysed, and the lysates were immunoprecipitated with the anti-HA antibodies. E, HT-induced phosphorylation of Ser473 in myrAkt–4–129-ER. NIH3T3 cells expressing myrAkt–4–129-ER were treated or not treated with 1 μM HT and lysed, and the lysates were immunoprecipitated with the anti-HA antibodies (HA). A control immunoprecipitate using normal mouse Ig is also shown (lane C). The precipitates were electrophoresed and blotted with a phospho-specific Akt antibody.

The kinase activity was measured *in vitro* using a synthetic peptide as substrate (∆). Exposure of cells to HT for only 20 min caused the kinase activity of myrAkt–4–129-ER to increase 17-fold. The kinase activity continued to increase with longer exposures to HT to more than a 50-fold activation (Fig. 2A). In contrast, the kinase activity of myrAkt–4–129-ER treated with ethanol alone and the activity of A2myrAkt–4–129-ER treated with either ethanol or HT was barely elevated compared with control immunoprecipitates using normal mouse immunoglobulin.

The immunooblotted protein was also analyzed by immunooblotted using antibody directed against the HA epitope. Treatment of cells with HT did not change the expression level of myrAkt–4–129-ER. However, HT treatment was associated with the appearance of a slower migrating band (Fig. 2B), consistent with an increase in the phosphorylation state of the protein.

Studies on the Mechanism of Activation of the Conditionally Active Akt—As shown above, although the levels of the myrAkt–4–129-ER protein did not change after HT treatment of cells, the protein did shift to a higher molecular weight on SDS gels (Fig. 2B), consistent with an increase in phosphorylation. To directly test this, metabolically labeled cells expressing myrAkt–4–129-ER were treated for 40 min with either HT or ethanol and lysed, and the lysates were immunoprecipitated with anti-HA antibodies. An approximate 3-fold increase (range, 1.8–3.4; n = 3) in labeling of the myrAkt–4–129-ER was observed with HT treatment (Fig. 2D).

To determine whether the activating site Ser473 was phosphorylated, the immunoprecipitated myrAkt–4–129-ER was blotted with a phospho-specific antibody directed against this site. HT treatment greatly stimulated the ability of the phospho-specific antibody to recognize this chimera (Fig. 2E), demonstrating that HT treatment stimulates an increase in the phosphorylation of Ser473.

To determine whether PI 3-kinase had any role in the activation of the conditionally active form of Akt, NIH3T3 cells that had been infected with myrAkt–4–129-ER were treated for 20 min with increasing concentrations of the PI 3-kinase inhibitor wortmannin (27). These cells were then treated for 10 min with HT to induce the Akt kinase activity. The basal PI 3-kinase activity was measured in anti-p85 antibody precipitates prepared from nonstimulated cells to verify the inhibitory effect of wortmannin. MyrAkt–4–129-ER was immunoprecipitated from cell lysates prepared from cells that had been treated with HT, and its kinase activity was measured *in vitro* to determine whether its activation was dependent on PI 3-kinase. At 1 nM wortmannin, a slight increase in Akt kinase activity was observed, similar to a slight increase in Akt activity previously observed with 1 nM wortmannin after insulin treatment (5). However, at higher wortmannin concentrations, the activation of myrAkt–4–129-ER was inhibited, and this paralleled the inhibition of PI 3-kinase activity (Fig. 3). A structurally unrelated inhibitor of PI 3-kinase, LY294002 (28), was also found to block the activation of myrAkt–4–129-ER (data not shown).

The Conditionally Active Akt Rapidly Stimulates p70 S6 Kinase—Prior studies have shown that constitutively active forms of Akt stimulate the enzymatic activity of p70 S6 kinase.
activation, NIH3T3 cells expressing myrAktΔ4–129-ER or A2myrAktΔ4–129-ER with HT were treated with either control vehicle or increasing concentrations of wortmannin for 20 min, after which the cells were treated for 10 min with 1 μM HT. After the cells were lysed, the cell lysates were adsorbed with either monoclonal anti-HA antibody 12CA5 or normal mouse Ig and assayed for kinase activity using myelin basic protein as a substrate, as described under “Experimental Procedures.” To measure PI 3-kinase activity, PI 3-kinase was immunoprecipitated from NIH3T3 cells infected with myrAktΔ4–129-ER that had also been treated with either control vehicle or increasing concentrations of wortmannin for 20 min. PI 3-kinase was isolated using an antibody directed against the 85-kDa regulatory subunit, and its activity was measured as described under “Experimental Procedures.” The values obtained were normalized to that obtained in the absence of wortmannin (100%) and are means ± S.E. (n = 3).

NIH3T3 cells already expressing either myrAktΔ4–129-ER or A2myrAktΔ4–129-ER were subsequently infected with a myc-epitope-tagged p70 S6 kinase that had also been subcloned into a retroviral vector. The cells were serum-starved for 16 h and then treated for 10 min with HT or with ethanol, the control vehicle. The p70 S6 kinase was immunoprecipitated using a monoclonal antibody directed against the myc epitope tag, and its kinase activity was measured in vitro using the 40S ribosomal subunit as a substrate. Induction of Akt kinase activity by treating cells that co-expressed myrAktΔ4–129-ER with HT was associated with a 3- and 7-fold increase in p70 S6 kinase activity after a 10- and 60-min stimulation, respectively (Fig. 4). In contrast, treating cells that co-expressed A2myrAktΔ4–129-ER with HT did not cause an increase in p70 S6 kinase activity compared with control-treated cells (Fig. 4). The stimulation of p70 S6 kinase after 60 min with HT in cells coexpressing myrAktΔ4–129-ER chimera was comparable to the stimulation achieved using 2 μM platelet-derived growth factor as substrate (Fig. 4). The increased activity of p70 S6 kinase observed in the context of the induced myrAktΔ4–129-ER was also associated with the appearance of hyperphosphorylated forms of p70 S6 kinase (data not shown), consistent with prior studies of this enzyme (29, 30).

The Conditionally Active Akt Stimulates Glucose Uptake and GLUT4 Translocation in 3T3-L1 Adipocytes—The unregulated constitutively active Akt has previously been shown to stimulate glucose uptake in 3T3-L1 adipocytes and induce the translocation of GLUT4 to the plasma membrane (9, 13). To test whether the conditionally active Akt could also stimulate this response, we infected 3T3-L1 preadipocytes with either myrAktΔ4–129-ER or A2myrAktΔ4–129-ER. The kinase activity of the Akt fusion proteins was measured in immunoprecipitates prepared from differentiated adipocytes that had been treated with either HT or ethanol, the control vehicle, for different periods of time by utilizing a peptide as exogenous substrate (8). This assay showed no activation of Akt in the adipocytes after a 1-h stimulation, but after 6 h, there was a 20-fold stimulation (Fig. 2C), and the activity remained elevated after 16 h. In contrast, the A2myrAktΔ4–129-ER construct was not significantly stimulated at any of the times tested, and the myrAktΔ4–129-ER was not activated in the absence of HT (Fig. 2C). The slower rate of activation of myrAktΔ4–129-ER with HT in the adipocytes could be due to the lower level of expression of these constructs in the adipocytes in comparison to the NIH3T3 cells or for some other unknown reason.

To measure the enzymatic activity of the expressed myrAktΔ4–129-ER in comparison to the endogenous Akt, 3T3-L1 adipocytes expressing myrAktΔ4–129-ER were stimulated with HT for 16 h or 10 min with 100 nM insulin and lysed, and the expressed Akt and endogenous Akt were immunoprecipitated with the anti-HA antibody and an antibody to the PH domain of Akt, respectively. The immunoprecipitates were tested for kinase activity in vitro using the peptide as substrate (8). The immunoprecipitated endogenous Akt had approximately 5 times as much activity as the expressed myrAktΔ4–129-ER.

We next evaluated the ability of this conditionally active Akt to regulate glucose transport. The uptake of 2-[3H]deoxyglucose was measured in differentiated adipocytes expressing either myrAktΔ4–129-ER or A2myrAktΔ4–129-ER that had been treated for various periods of time with HT. Exposure of cells expressing myrAktΔ4–129-ER to HT for 1 h or less did not result in any significant change in glucose uptake (Fig. 5A), consistent with the low activation of Akt enzymatic activity at these times in adipocytes (Fig. 2C). In contrast, the 16-h stimulation was associated with a 2.5-3-fold increase in 2-[3H]deoxyglucose uptake compared with both control-treated cells and cells expressing A2myrAktΔ4–129-ER that were control-treated or treated with HT (Fig. 5A). Incubations of
cells expressing A2myrAkt Δ4–129-ER with HT for shorter periods of time (2–6 h) gave a partial stimulation of glucose uptake. This increase in glucose uptake was not associated with a change in the level of expression of GLUT4, as determined by immunoblotting crude total membrane fractions (Fig. 5A, inset).

To assess whether activation of this conditionally active Akt could stimulate GLUT4 translocation to the plasma membrane, the sheet assay was utilized (19, 20). Cells expressing either myrAkt Δ4–129-ER or A2myrAkt Δ4–129-ER were treated or not for various periods of time with HT and sonicated, and the amount of plasma membrane GLUT4 was measured. Exposure of cells expressing myrAkt Δ4–129-ER to HT for 2 h or less did not result in any significant change in surface GLUT4 (Fig. 5B, consistent with the low activation of Akt enzymatic activity at these times in adipocytes (Fig. 2C). After 16 h, HT stimulated a 3-fold increase in the amount of surface GLUT4 in the cells expressing myrAkt Δ4–129-ER but not those expressing A2myrAkt Δ4–129-ER (Fig. 5B). After a 6-h stimulation, a smaller increase in surface GLUT4 was also observed in the cells expressing myrAkt Δ4–129-ER. Insulin stimulated GLUT4 translocation approximately 7–8-fold in both cell types (Fig. 5B, inset). Thus, the conditionally active Akt could regulate 2-[3H]deoxyglucose uptake and GLUT4 translocation in 3T3-L1 adipocytes.

The Conditionally Active Akt Rapidly Stimulates PHAS-I Phosphorylation in NIH3T3 Cells—In addition to regulating glucose uptake, insulin stimulates protein synthesis in a variety of cell types (31). The constitutively active Akt, myrAkt, was found to activate protein synthesis to a level comparable to insulin stimulation.1 One mechanism whereby insulin has been found to regulate protein synthesis is via the phosphorylation of the eIF4E-binding protein, PHAS-I (31). The nonphosphorylated form of PHAS-I inhibits protein synthesis by tightly binding to the mRNA cap-binding protein, eIF4E. When PHAS-I is phosphorylated on the appropriate sites, it releases eIF4E, which is then free to participate in translation initiation (31). The phosphorylation of PHAS-I has been shown to be controlled by a rapamycin-sensitive pathway involving the mammalian target of rapamycin, mTOR (31). There is also evidence that PI 3-kinase is an upstream element in this pathway, but the role of Akt in the control of PHAS-I phosphorylation is not known.

Experiments were therefore performed to investigate the effect of the conditionally active Akt on the phosphorylation of PHAS-I and its association with eIF4E. NIH3T3 cells expressing myrAkt Δ4–129-ER (MER-3T3) or control cells were treated with 1 μM HT and lysed, and the lysates were either analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting for PHAS-I or first incubated with m7GTP-Sepharose to determine the amount of PHAS-I complexed with eIF4E. Phosphorylation of PHAS-I has been shown to markedly decrease its electrophoretic mobility in SDS gels (18). By gel shift analysis, HT treatment of MER-Akt-expressing cells was found to rapidly stimulate PHAS-I phosphorylation to an extent comparable to insulin (Fig. 6A). In contrast, HT was without effect in the control cells (Fig. 6A), whereas insulin stimulated the shift of PHAS-I in both cell types. In addition, tamoxifen stimulated a dissociation of PHAS-I from eIF4E in the MER-Akt cells but not in the control cells, as detected by a decrease in the amount of the PHAS-I/eIF4E complex bound to the m7GTP-Sepharose (Fig. 6).

DISCUSSION

The results presented here demonstrate that fusion of the hormone binding domain of the estrogen receptor to an activated form of Akt renders the kinase activity of this protein dependent on the addition of exogenous HT. Using HT, the kinase activity can be rapidly turned on in several cell types, including NIH3T3 cells (Fig. 2A), a mouse hepatoma cell line, and primary myoblasts.2 The induction of Akt kinase activity correlates with the phosphorylation of this protein, including at a key regulatory site, Ser473 (Fig. 2, B, D, and E), consistent
with the hypothesis that phosphorylation regulates the enzymatic activity of this chimera as it does the wild type enzyme (32). The rapid activation of the chimera with HT treatment is consistent with the previously described model, in which hormone binding stimulates the release of the 90-kDa heat shock protein from the HBD, thereby allowing access of other proteins (e.g. a kinase) to the chimeric molecule (33). An alternative model would be that the HBD could induce oligomerization of the chimera, and this oligomerization could be sufficient to result in transphosphorylation and activation of Akt. This did not appear to be the case because A2myrAkt Δ4–129-ER, which only lacks the glycine that is myristoylated, was not activated. Thus, the Akt chimera must be present in a membrane compartment to be activated. Presumably, this is due to the presence of a kinase in the membrane that is responsible for phosphorylating Akt. This kinase could be the recently described Akt kinase kinase, which is stimulated by PI 3-phosphates. Alternatively, it may be due to the lower levels of expression of the Akt chimera in these cells or for some other reason.

The generation of a conditionally active Akt allowed us to investigate downstream targets of this enzyme. Prior studies have shown that expression of different constitutively active forms of Akt induce various subsequent biological responses, including stimulation of the p70 S6-kinase and glucose uptake (4, 8–13). In the present study, we could show activation of the p70 S6-kinase activity in NIH3T3 cells expressing myrAkt Δ4–129-ER after only a 10-min stimulation with HT (Fig. 4), a time very close to that required for induction of Akt enzymatic activity in these cells (Fig. 2A). These results indicate that the activation of the 70-kDa S6 kinase by Akt is an immediate consequence of stimulation of Akt kinase activity, rather than an event secondary to long-term expression of a constitutive active kinase.

In 3T3-L1 adipocytes, the activation of myrAkt Δ4–129-ER was considerably slower than in NIH3T3 cells (Fig. 2C), and the maximal amount of activity was less. A detectable increase in glucose uptake was observed after 4- and 6-h incubations with HT. After 16 h, glucose uptake was stimulated approximately ¼ as well as a maximal dose of insulin. The extent of stimulation of glucose uptake after HT treatment corresponded with the extent of GLUT4 translocation and the relative amounts of Akt stimulated by HT in comparison to insulin. That is, the amount of Akt kinase activity and glucose uptake observed with a 16-h HT stimulation of cells expressing myrAkt Δ4–129-ER was approximately ¼ the maximal amount of glucose uptake and endogenous Akt kinase activity observed in insulin-treated cells. It is possible that the longer period of time required to maximally stimulate Akt activity and glucose uptake in these cells allowed other processes to occur in addition to Akt activation, raising the question of whether Akt activation alone is sufficient for stimulation of glucose uptake. For example, it was possible that Akt activation stimulates the release of a secreted molecule that feeds back to activate glucose uptake. However, the supernatants of these cells were incapable of stimulating glucose uptake in 3T3-L1 adipocytes, indicating that this increase in glucose uptake was at least not due to the release of a secreted factor. Recent additional evidence of a role for Akt in mediating the insulin-induced increase in glucose uptake has come from studies showing that expression of an inactive Akt can hinder the ability of insulin to stimulate GLUT4 translocation in adipocytes (35).

In the present study, we also utilized the conditionally active Akt to determine whether this enzyme can modulate the phosphorylation of PHAS-I, a key regulator of protein synthesis, as well as its association with eIF4E (31). A constitutively active Akt, myrAkt, can increase protein synthesis in 3T3-L1 adipocytes to the same extent as insulin and stimulate an increase in leptin production in these cells (36). One mechanism by which insulin and other growth factors regulate protein synthesis is a stimulation in phosphorylation of PHAS-I and its subsequent release from eIF4E (31). Prior studies have demonstrated that insulin stimulates a phosphorylation of PHAS-I and its subsequent release from eIF4E, thereby increasing the amount of eIF4E available to participate in translation of capped mRNAs (31). In the present study, we showed that activation of the conditionally active Akt caused a shift of the PHAS-I protein within 10 min of stimulation (Fig. 6). This phosphorylation was associated with a release of PHAS-I from eIF4E. Thus, acute activation of Akt was sufficient to mimic the ability of insulin to stimulate PHAS-I phosphorylation and release of eIF4E, indicating that Akt is upstream of the kinase(s) responsible for phosphorylation of PHAS-I.

In summary, the present study provides further evidence that Akt can directly regulate activation of the 70-kDa S6 kinase and glucose uptake. In addition, we provide the first...
evidence that activation of Akt can regulate the phosphorylation of PHAS-I, as well as its association with eIF4E, providing a potential mechanism whereby insulin may regulate protein synthesis.

Acknowledgments—We thank Dr. Martin McMahon for the HB domain construct, Dr. Garry Nolan for the Phoenix retroviral packaging cell line and the retroviral vectors, and Drs. Gerry Crabtree and John Blenis for the p70 S6 kinase clones.

REFERENCES
1. Haslam, R. J., Kolde, H. B., and Hemmings, B. A. (1993) Nature 363, 309–310
2. Shaw, G. (1996) Bioessays 18, 35–46
3. Franke, T. F., Yang, S. I., Chan, T. O., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) Nature 376, 599–602
4. Kohn, A. D., Kovacina, K. S., and Roth, R. A. (1995) EMBO J. 14, 4288–4295
5. Franke, T. F., Kaplan, D. R., Cantley, L. C., and Toker, A. (1997) Science 275, 665–668
6. Alessi, D., James, S., Downes, C., Holmes, A., Gaffney, P., Reese, C., and Cohen, P. (1997) Curr. Biol. 7, 261–269
7. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovic, M., and Hemmings, B. A. (1995) Nature 378, 785–789
8. Kohn, A. D., Summers, S. A., Birnbaum, M. J., and Roth, R. A. (1996) J. Biol. Chem. 271, 31372–31378
9. Magun, R., Burgering, B. M., Coffer, P. J., Pardasani, D., Lin, Y., Chabot, J., and Sorisky, A. (1996) Endocrinology 137, 3590–3593
10. Dudek, H., Datta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R., Cooper, G. M., Segal, R. A., Kaplan, D. R., and Greenberg, M. E. (1997) Science 275, 31515–31524
11. Kozma, S. C., and Thomas, G. (1994) Semin. Cancer Biol. 5, 255–260
12. Lawrence, J. C., and Abraham, R. T. (1997) Trends Biochem. Sci. 22, 345–349
13. Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J. C., Frech, M., Cron, P., Cohen, P., Lucocq, J. M., and Hemmings, B. A. (1996) EMBO J. 15, 6514–6515
14. Scherrer, L., Picard, D., Massa, K., Harmon, J., Simons, S. J., and Yamamoto, K. (1993) Biochemistry 32, 5381–5386
15. Cong, L. N., Chen, H., Li, Y., Zhou, L., McGibbon, M. A., Taylor, S. I., and Quon, M. J. (1997) Mol. Endocrinol. 11, 1881–1890
16. Bartel, A., Kohn, A. D., Luo, Y., and Roth, R. A. (1997) Endocrinology 138, 3877–3888
17. Liu, L., Chen, H., Li, Y., Zhou, L., McGibbon, M. A., Taylor, S. I., and Quon, M. J. (1997) Mol. Endocrinol. 11, 1881–1890
Construction and Characterization of a Conditionally Active Version of the Serine/Threonine Kinase Akt

Aimee D. Kohn, Andreas Barthel, Kristina S. Kovacina, Annegret Boge, Brenda Wallach, Scott A. Summers, Morris J. Birnbaum, Pamela H. Scott, John C. Lawrence, Jr. and Richard A. Roth

J. Biol. Chem. 1998, 273:11937-11943.
doi: 10.1074/jbc.273.19.11937

Access the most updated version of this article at http://www.jbc.org/content/273/19/11937

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 15 of which can be accessed free at http://www.jbc.org/content/273/19/11937.full.html#ref-list-1