Phosphatidylinositol 3-Kinase Signaling Inhibits DAF-16 DNA Binding and Function via 14-3-3-dependent and 14-3-3-independent Pathways*

In Caenorhabditis elegans, an insulin-like signaling pathway to phosphatidylinositol 3-kinase (PI 3-kinase) and AKT negatively regulates the activity of DAF-16, a Forkhead transcription factor. We show that in mammalian cells, C. elegans DAF-16 is a direct target of AKT and that AKT phosphorylation generates 14-3-3 binding sites and regulates the nuclear/cytoplasmic distribution of DAF-16 as previously shown for its mammalian homologues FKHR and FKHRL1. In vitro, interaction of AKT-phosphorylated DAF-16 with 14-3-3 prevents DAF-16 binding to its target site in the insulin-like growth factor binding protein-1 gene, the insulin response element. In HepG2 cells, insulin signaling to PI 3-kinase/AKT inhibits the ability of a GAL4 DNA binding domain/DAF-16 fusion protein to activate transcription via the insulin-like growth factor binding protein-1-insulin response element, but not the GAL4 DNA binding site, which suggests that insulin inhibits the interaction of DAF-16 with its cognate DNA site. Elimination of the DAF-16/1433 association by mutation of the AKT/14-3-3 sites in DAF-16, prevents 14-3-3 inhibition of DAF-16 DNA binding and insulin inhibition of DAF-16 function. Similarly, inhibition of the DAF-16/14-3-3 association by exposure of cells to the PI 3-kinase inhibitor LY294002, enhances DAF-16 DNA binding and transcription activity. Surprisingly constitutively nuclear DAF-16 mutants that lack AKT/14-3-3 binding sites also show enhanced DNA binding and transcription activity in response to LY294002, pointing to a 14-3-3-independent mode of regulation. Thus, our results demonstrate at least two mechanisms, one 14-3-3-dependent and the other 14-3-3-independent, whereby PI 3-kinase signaling regulates DAF-16 DNA binding and transcription function.

In Caenorhabditis elegans, genetic evidence indicates that an insulin-like signaling pathway, which includes an insulin/IGF-1-like receptor (DAF-2), phosphatidylinositol 3-kinase (PI 3-kinase; AGE-1), and protein kinase B (also known as AKT) controls life cycle, metabolism, and longevity (1–5). This pathway negatively regulates the activity of DAF-16, a member of the Forkhead (FKH) family of transcription factors (3, 6–8).

In mammalian cells, insulin/IGF-1 signaling via PI 3-kinase and AKT mediates diverse effects on cell metabolism, growth, and survival (9–11). Biochemical studies to date suggest that PI 3-kinase is important to the metabolic actions of insulin including its effects on gene transcription. A common DNA sequence, referred to as the insulin response element (IRE), binds members of the Forkhead transcription factor family and mediates the negative effect of insulin on transcription of the insulin-like growth factor binding protein-1 (IGFBP-1) and phosphoenolpyruvate carboxykinase (PEPCK) genes (12). In hepatoma cells, insulin-inhibition of IRE-directed gene transcription is mediated via a PI 3-kinase-dependent signaling pathway (13). Accordingly, work in several laboratories aimed at identifying the downstream targets of insulin signaling to the nucleus has focused on the role of mammalian homologues of DAF-16, FKHR, FKHRL1, and AFX in mediating the negative effect of insulin/IGF-1 signaling on gene transcription. In the absence of insulin/IGF-1, FKHR and FKHRL1, and AFX in mediating the negative effect of insulin/IGF-1 signaling on gene transcription. In the absence of insulin/IGF-1, FKHRL1 (14), AFX (15), and FKHR (16–18) activate gene transcription via the IGFBP-IRE. Insulin/IGF-1 signaling (19–21) or overexpression of AKT (17, 19) stimulates phosphorylation of these factors and inhibits their activating effect (16, 17).

The prevailing view of the mechanism underlying insulin/IGF-1 inhibition of FKHR1 and other DAF-16 homologs is that phosphorylation of FKHR1 by AKT at two sites, Thr-32 and Ser-253 promotes retention of these proteins in the cytoplasm (14). AKT preferentially phosphorylates substrates that carry the RXXXXS motif, which is contained within certain consensus 14-3-3 binding motifs RSXSXXP, or RXXXSXXP where S represents phosphoserine (22). Hence, AKT phosphorylation of its target proteins may create 14-3-3 binding sites. For example, the AKT site at T32 in FKHR1 is a 14-3-3 consensus binding sequence; AKT phosphorylation of FKHR1 at sites Thr-32 and Ser-253 promotes interaction of FKHR1 with 14-3-3 and cytoplasmic retention of FKHR1 (14). The 14-3-3

* This work was supported by National Institutes of Health NCI Grant CA75818-1, by National Institutes of Health Grant DK57200A01, by institutional support from Massachusetts General Hospital, and by National Institutes of Health Training Grant T32 DK07028–24 (to N. N.) and Grants AG05790 (to N. N.) and AG14161 and GM50012 (to S. O. and G. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: IGF, insulin-like growth factor; FKHR, forkhead; PKB, protein kinase B; PI 3-kinase, phosphatidylinositol 3-kinase; IGFBP-1, insulin-like growth factor binding protein-1; IRE, insulin response element; PEPCK, phosphoenolpyruvate carboxykinase; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; WT, wild-type.
family of proteins has also been shown to play a role in nuclear export and/or cytoplasmic retention of the yeast protein Cdc25 (23–25). In addition to promoting changes in cellular localization, binding of 14-3-3 to certain of its target proteins directly affects their activity. For example, 14-3-3 can stimulate the catalytic activity of the serine/threonine kinase c-Raf-1 (26, 27), the DNA binding activity of p53 (28), and other targets (29–31).

The Thr-32 and Ser-253 sites are conserved within DAF-16 (Thr-54 and Ser-243/245) (14), FKHR (Thr-24, Ser-253) (32), and AFX (Thr-28, Ser-258) (15). Accordingly, regulation of nuclear export by growth factor signaling to PI 3-kinase and AKT has been demonstrated for FKHR (13, 29, 30), and AFX (14). Binding activity of p53 (28), and other targets (29–31).

The Thr-54 site in DAF-16 would function as a 14-3-3 binding site and, if so, whether PI 3-kinase signaling would regulate the interaction of C. elegans DAF-16 with elements of the mammalian nuclear import/export machinery as is the case for the mammalian homologs of DAF-16.

We therefore examined the effect of AKT phosphorylation and 14-3-3 association on several aspects of DAF-16 function, including its ability to localize to the nucleus, bind DNA and activate transcription. We find evidence for PI 3-kinase-dependent inhibition of DAF-16 DNA binding activity via 14-3-3-dependent and 14-3-3-independent mechanisms. Thus, our observations suggest a more complex mode of DAF-16 regulation than previously anticipated.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Reagents**—The DAF-16a1 HindIII/XhoI insert from pGEM-FLAG-DAF-16a1 was ligated into the HindIII/XhoI site of pcDNAs (+) (Invitrogen) to generate pcDNAs-Flag DAF-16a1. The DAF-16a1 BstEII insert from pGEM-FLAG-DAF-16a1 was ligated into the BamHI site of pGEX-TT-1 (Amersham Pharmacia Biotech) to generate pGEX-DAF-16a1. Phosphorylation site mutants were prepared using the QuickChange site-directed mutagenesis kit (Stratagene). The DAF-16a1 BstEII insert from pGEM-FLAG-DAF-16a1 was ligated into the BamHI site of the GAL4 DNA binding domain plasmid to generate GAL4-DAF-16a1 derivatives. The rat IGF-BP-1 promoter (14) was expressed in 293 cells and subsequently affinity-purified on GST beads (Amersham Pharmacia Biotech). Kinase assays were performed using pGEX-14-3-3, pGEX-GST-14-3-3, and the pGEX-GST-14-3-3 dimerization mutant has been described previously (26, 27). The pEGB-GST-AKT was a gift from J. R. Woodgett (Toronto, Canada). Specific DAF-16 antibodies were produced in rabbits using the Forkhead DNA binding domain of the mammalian homologs of DAF-16. Accordingly we also find that DAF-16 binds specifically to the 3′UTR of the gene (15, 16).

**Kinase Assay**—For experiments to phosphorylate DAF-16 in vitro, GST-DAF-16 proteins were purified from bacteria and GST-AKT was expressed in 293 cells and subsequently affinity-purified on GSH beads (Amersham Pharmacia Biotech). Kinase assays were performed using 2 μg of GST-AKT as the kinase and 2 μg of GST-DAF-16 or DAF-16 mutant as the substrate in a kinase buffer containing 40 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5 mM MgCl2, 2 mM dithiothreitol, and 100 μM ATP (cold assay) supplemented with [γ-32P]ATP (10–20 μCi/reaction) (hot assay) at 30 °C for 30 min.

**Protein Interaction Assays**—Myc epitope-tagged 14-3-3 expressed in 293 cells was absorbed to anti-Myc epitope antibodies (clone 9E10) pre-coupled to protein-A beads and incubated with 2 μg of AKT- phosphorylated wild-type and mutant GST-DAF-16 for 90 min at 4 °C. Following extensive washes, the associated proteins were separated on SDS-PAGE and phosphorylated DAF-16 was detected by autoradiography. Both wild-type and mutant GST-DAF-16 variants were detected by anti-GST immuno blotting.

**Electrophoretic Mobility Shift Assay**—Samples containing 2 μg of GST-DAF-16 or 5–10 μg of nuclear extracts, treated as indicated in the figure legends were incubated with 50,000 cpm of [32P]-labeled IGFBP-IRE probe (caaaaaaatttcattgaa) or G-C/A-C mutant probe (caaaa-gaagacctttgaa) for 15 min at 4 °C in a buffer containing 40 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol, 50 mM KCl, 10% glycerol, 0.1% bovine serum albumin, and 1 μg of poly(dG/dC) in each sample. For competition assays, 10× cold IRE or mutant IRE was added prior to the addition of [32P]-labeled IRE probe. For supershift assays, the reaction was pre-incubated with 1 μg of either specific DAF-16 antibody (for detection of GST fusion proteins) or M2 antibody (for detection of the FLAG tag for detection of DAF-16 expressed in mammalian cells) for 15 min at 4 °C prior to the addition of [32P]-labeled IRE probe. To demonstrate inhibition of DNA binding by 14-3-3, DAF-16 (2 μg) was phosphorylated with GST-AKT (2 μg) for 30 min at 30 °C, followed by addition of 14-3-3 (2 μg). The reaction was further incubated at 4 °C for 15 min, at which time labeled [32P]-IRE probe was added. Samples were resolved on 4% Tris-glycine PAGE at 100 V for 3 h. Nuclear and cytoplasmic extracts were prepared using the NE-PER kit (Pierce) according to the manufacturer’s instructions.

**Transfections**—For transcriptional analysis, HepG2 cells were transfected using the CaPO4 method in 30-mm six-well plates with IGFBP-LUC (15 μg) reporter plasmid and pcDNA3-DAF-16 variants (2 μg) or pcDNA3 control vector (2 μg) per 1.5 ml of precipitate. The RSV-β-galactosidase vector (2 μg) was used to control for transfection efficiency. In the experiments described in Figs. 3 and 5, 2 μg of GAL4 DNA binding domain control vector or GAL4-DAF-16 fusion protein vector variants cotransfected with either the IGFBP-luciferase reporter gene or a luciferase reporter gene driven by five GAL4 DNA binding sites cloned upstream of the TK109 promoter. Cells were shocked for 1 min with 10% Me2SO and the incubation continued in the absence of serum. Insulin was added during the last 16 h of the incubation.

For the DAF-16/14-3-3 association experiments, 293 cells were transfected using LipofectAMINE (Life Technologies, Inc.) in 10-cm plates with 2 μg each of GST-14-3-3 or GST-AKT and 4 μg of pcDNA3-DAF-16 variants. For the DAF-16 localization and DNA binding experiments, 293 cells were transfected with 5 μg of the pcDNA3-DAF-16 variants or pcDNA3 alone.

**RESULTS**

**AKT Phosphorylates DAF-16 and Promotes Its Association with 14-3-3**—Consistent with the genetic data that positions DAF-16 downstream of the PI 3-kinase-regulated serine/threonine kinase AKT in C. elegans, there are four consensus AKT phosphorylation sites in DAF-16 (Fig. 1A). As has been established for the mammalian DAF-16 orthologs FKHR (16, 19, 32), FKHR1 (14), and AFX (15, 34), AKT can phosphorylate DAF-16 on at least three of its four potential AKT sites, and these sites serve as the only AKT-phosphorylation sites in vitro (Fig. 1B, top). Phosphospecific antibodies generated against 14-3-3-binding consensus sequences can specifically recognize DAF-16 phosphorylated by AKT but not unphosphorylated DAF-16 (Fig. 1B, compare lane 2 to lane 1). This antibody recognizes phosphorylation of DAF-16 at threonine 54 (Fig. 1B, middle, lane 2 versus lane 3). Phosphorylation of recombinant prokaryotic GST-DAF-16 by AKT induces its binding to recombinant mammalian 14-3-3 in vitro (Fig. 1C). This association is inhibited by a competitor phosphopeptide corresponding to a 14-3-3 binding site on c-Raf1 but not by the unphosphorylated form of the peptide (compare lane 2 with lanes 3 and 4). The association with 14-3-3 is also inhibited by mutation of the AKT-phosphorylation sites on DAF-16 (compare lane 2 with lanes 5, 7, and 8). In particular, the AKT-phosphorylation site at threonine 54, a site matching closest to the 14-3-3 binding consensus, represents a site whose phosphorylation is indispensable for 14-3-3 binding in vitro (compare lane 2 with lane 5).

**14-3-3 Association with Wild-type DAF-16 Inhibits Its DNA Binding Activity**—Homologues of DAF-16 bind and activate transcription through the IRE in the IGFBP gene (14, 16). Accordingly we also find that DAF-16 binds specifically to the 3′UTR of the gene (15, 16). The association with 14-3-3 is also inhibited by mutation of the AKT-phosphorylation sites on DAF-16 (compare lane 2 with lanes 5, 7, and 8). In particular, the AKT-phosphorylation site at threonine 54, a site matching closest to the 14-3-3 binding consensus, represents a site whose phosphorylation is indispensable for 14-3-3 binding in vitro (compare lane 2 with lane 5).
**Fig. 1.** AKT phosphorylates DAF-16 on four distinct sites and mediates 14-3-3 binding. A, linear map of DAF-16 protein showing the amino acid sequence of the putative AKT consensus (RXXRXXS) phosphorylation sites at Thr-54, Ser-240, Thr-242, and Ser-314. The indicated mutants were constructed for expression in both mammalian and bacterial cells: 1A (T54A); 2A (S240A and T242A); 3A (T54A, S240A, and T242A); 4A (all four sites mutated to alanine). B, phosphorylation of GST-DAF-16 in vitro by AKT. Recombinant prokaryotic GST-fused DAF-16 (lanes 1 and 2) and the indicated mutants (lanes 3–6), 2 μg each, were incubated in a kinase buffer containing 2 μg of active recombinant mammalian GST-AKT (lanes 2–6) or vehicle (lane 1) for 40 min at 30 °C. Following SDS-PAGE, the samples were blotted with phosphopeptide antibodies against degenerated 14-3-3 binding consensus (S(p)XXRXXS) (a gift from M. Comb). Autoradiogram showing GST-DAF-16 phosphorylation (top) and immunoblot showing reactivity with the phosphospecific antibodies (middle) and an anti-GST immunoblot showing equal protein loading (bottom) are presented. C, in vitro binding of AKT phosphorylated GST-DAF-16 to 14-3-3. Unphosphorylated GST-DAF-16 (lane 1), AKT-phosphorylated GST-DAF-16 (lanes 2–4 and 9), or the indicated GST-DAF-16 mutants (lanes 5–8) were incubated with immobilized Myc-tagged 14-3-3 (prepared using anti-Myc antibodies and protein A beads) from 293 cells (lanes 1–8) or control beads (lane 9) in the presence of competitor 14-3-3 binding phosphopeptide (PP, 1 μm, lane 3) or unphosphorylated control peptide (P, 1 μm, lane 4) for 2 h at 4 °C. Following washes to remove nonspecific binding, bound proteins retained on the immobilized Myc-tagged 14-3-3 beads were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and tested for phospho-GST-DAF-16 by autoradiography (top). Anti-GST immunoblot (middle) was used for the detection of both DAF-16 WT and AKT site mutant DAF-16 derivatives bound to the Myc-14-3-3 column. The DAF-16 mutants 3A and 4A are only partially phosphorylated or not at all and can not be detected by autoradiography. A Coomassie stain of the blot (bottom) is shown to demonstrate equal 14-3-3 input.

We examined whether AKT phosphorylation and/or subsequent association of DAF-16 with 14-3-3 could alter the ability of DAF-16 to bind its target IRE site. Phosphorylation of DAF-16 by AKT did not by itself affect DAF-16-DNA binding (Fig. 2B, compare lanes 1 and 3); however, the addition of 14-3-3 to AKT-phosphorylated DAF-16 resulted in an almost complete inhibition of DAF-16 DNA binding activity (Fig. 2B, compare lanes 3 and 4). The addition of 14-3-3 had no effect on DAF-16 DNA binding when AKT was omitted (Fig. 2B, compare lanes 2 and 4), or when ATP was omitted (Fig. 2C, compare lanes 7 and 3) from the kinase reaction. Moreover, the competitor 14-3-3 binding phosphopeptide selectively blocked the ability of 14-3-3 to inhibit DAF-16 DNA binding while the unphosphorylated version had no effect (Fig. 2B, compare lanes 5 and 6) demonstrating the requirement of the 14-3-3-phosphopeptide binding domain for the inhibition. Thus, the ability of 14-3-3 to inhibit DAF-16 DNA binding required the association of 14-3-3 with phospho-DAF-16. The DNA binding activity of DAF-16 mutants impaired in their ability to bind 14-3-3, DAF-16 5A (T54A), and DAF-16 4A (T54A, S240A, T242A, S314A) was unaffected by AKT/T14-3-3 (Fig. 2D, compare lanes 1 and 2 with lanes 4 and 5 and lanes 7 and 8). Conversely, the DNA binding activity of the DAF-16 2A (240/242A) mutant that retains the ability to bind 14-3-3 was inhibited (Fig. 2D, lanes 10 and 11). Although the DAF-16 (S314A) mutant retains the ability to bind 14-3-3 following AKT phosphorylation (data not shown), 14-3-3 does not inhibit its ability to bind DNA (Fig. 2D, lanes 12 and 13). The inability of the dimerization-deficient 14-3-3 mutant to inhibit DAF-16 DNA binding (Fig. 2C, compare lane 3 with lane 6), together with the ability of wild-type 14-3-3 to inhibit mutant DAF-16 2A (240/242A) or (S314A) DNA binding, suggests that dimeric 14-3-3 interacts with DAF-16 at sites Thr-54 and Ser-314. This interaction may, in turn, mask the forkhead DNA binding domain of DAF-16.

**Insulin Inhibition of DAF-16 Activity Is Mediated at the Level of DNA Binding**—We have shown that AKT phosphorylation of DAF-16 WT allows association of 14-3-3 and that this association inhibits binding of DAF-16 to DNA. In HepG2 cells, insulin inhibits transcription activation by DAF-16 and this effect requires the AKT/T14-3-3 sites (16, 21). When activity was assessed by guest on July 26, 2018http://www.jbc.org/Downloaded from
and of ATP, followed by a 30-min incubation with GST-14-3-3 (lanes 2–5) and resolved on a 4% nondenaturating gel as described under "Experimental Procedures." An autoradiogram of the gel is presented. The positions of DAF-16/DNA complexes and complexes supershifted with antibody are indicated. B, AKT phosphorylation of DAF-16 and 14-3-3 association prevents DAF-16 binding to IRE DNA. GST-DAF-16 (2 μg) was incubated in a kinase buffer containing 2 μg of active GST-AKT (lanes 3–6) or vehicle (lane 1 and 2) for 40 min at 30 °C, followed by a 30-min incubation with prokaryotic recombinant GST-14-3-3 (lanes 2 and 4–6), or vehicle (lanes 1 and 3). The presence of competitor phosphopeptide (pp, 1 mM, lane 5) or unphosphorylated peptide (p, 1 mM, lane 6) is indicated. GST-DAF-16 was assayed for DNA binding as in panel A. C, Inhibition of DAF-16 binding to the IRE requires active AKT and an intact 14-3-3 dimer. GST-DAF-16 (2 μg) was incubated in a kinase buffer containing 2 μg of active GST-AKT in the presence (lanes 1–6) or absence (lanes 7–10) of ATP, followed by a 30-min incubation with GST-14-3-3 (lanes 2–5 and 7–9), vehicle (lane 1), or a dimerization-deficient GST-14-3-3 (dm, lanes 6 and 10) in the presence of competitor phosphopeptide (pp, 1 mM, lanes 4 and 8) or unphosphorylated peptide (p, 1 mM, lanes 5 and 9). The samples were assayed for DNA binding as in panel A. D, inhibition of DAF-16 binding to the IRE by AKT/14-3-3 requires intact AKT sites Thr-54 and Ser-314 on DAF-16. GST-DAF-16 (lanes 1–3), GST-DAF-16(T54A) (lanes 4–6), GST-DAF-16(4A) (lanes 7–9), GST-DAF-16(314A) (lanes 10–12), and GST-DAF-16(2A) (lanes 13–15), 2 μg each, were incubated in a kinase buffer containing 2 μg of active GST-AKT (lanes 2, 5, 8, 11, and 14) or vehicle (all others) for 40 min at 30 °C followed by 30-min incubation with prokaryotic recombinant GST-14-3-3 (lanes 2, 5, 8, 11, and 14) or vehicle (all others). The samples were assayed for binding to mutant (lanes 3, 6, 9, 12, and 15) or wild type (all others) 32P-IRE probes as in panel A.

4A, which fails to bind 14-3-3, was not affected by insulin (Fig. 3, panel B, compare bars C and D). By contrast, although the GAL4-DAF-16 and GAL4-DAF-16 4A fusion proteins activated transcription similarly when assessed on the GAL4 DNA binding site (bars E–H), neither wild-type GAL4-DAF-16, nor GAL4-DAF-16–4A were inhibited by insulin (panel B, bars E and F and bars G and H).

The observation that GAL4-DAF-16 responds to insulin when its activity is assessed using an IRE site, but not a GAL 4 site, indicates that the response of this fusion protein is analogous to that of the native DAF-16 protein. If insulin’s action to inhibit DAF-16 activity resulted from a direct effect on the intrinsic transcription activity of GAL4-DAF-16 or from nuclear export of GAL4-DAF-16, we would expect to see the negative effect of insulin on both the GAL4 and the IRE DNA binding sites. Inasmuch as we observe the inhibitory effect of insulin on the IRE alone, we conclude that insulin’s effect is mediated at the level of DAF-16 DNA binding. Furthermore, the observation that GAL4-DAF-16 is resistant to insulin signaling when the protein is tethered to the GAL4 DNA target site suggests that 14-3-3 inhibition of DAF-16 DNA binding may be a first step in the negative regulation of DAF-16 activity allowing subsequent changes in DAF-16 subcellular localization to occur.

PI 3-Kinase Signaling Regulates DAF-16/14-3-3 Interaction and Consequent Subcellular Distribution—Our in vitro DNA binding results imply that the association of DAF-16 with 14-3-3 plays a crucial role in the negative regulation of DAF-16 DNA binding. As HepG2 cells do not express sufficient DAF-16 to enable detection by DNA binding assay, we were unable to study direct effects of insulin on DAF-16 DNA binding in these cells. However, coexpression studies of GST-tagged 14-3-3 and Flag-tagged DAF-16 proteins in 293 cells demonstrate that 14-3-3 and DAF-16 can associate both in serum-deprived cells and in cells growing exponentially in serum (Fig. 4A, compare lanes 2 and 4). Treatment of serum-starved cells with the PI
3-kinase-specific inhibitor LY294002 caused a marked decrease in 14-3-3/DAF-16 association (Fig. 4A, compare lane 2 with 3 and lane 11 with 12). These findings suggested that PI 3-kinase signaling to AKT and phosphorylation of DAF-16 could regulate its association with mammalian 14-3-3 as it does for FKHR1 (14).

Accordingly, we found that the 14-3-3/DAF-16 association depended on the presence of the AKT-phosphorylation site at residue Thr-54 on DAF-16 (Fig. 4A, lanes 13–15) in vivo, as is the case in vitro. However, the alanine mutations at residues 240/242, which did not prevent association of DAF-16 2A with 14-3-3 in vitro, greatly reduced 14-3-3 association in vivo (lanes 16–18). Mutation of site Thr-54 and sites Ser-240/Thr-242 in DAF-16 completely prevented DAF-16/14-3-3 association in 293 cells (Fig. 5). The identity of DAF-16 overexpressed in HEK 293 cells was demonstrated by supershift experiments using antibodies (Fig. 5). The expression of wild-type DAF-16 (Fig. 5, lower panel), DAF-16 2A and DAF-16 4A (Fig. 5, upper panel) was increased in DAF-16 protein in the LY294002 treated nuclear extracts due to nuclear translocation, because extracts containing equal amounts of DAF-16 were employed (Fig. 5, lower panel; compare lanes 4 and 6). Thus, the increase in DAF-16 DNA binding activity shown reflects an increase in its specific DNA binding activity indicating that LY294002 can prevent negative regulation of DAF-16 DNA binding activity by a PI 3-kinase-mediated mechanism.

We also examined the effect of PI 3-kinase inhibition on regulation of DAF-16 AKT site mutants that do not bind 14-3-3 in vivo, DAF-16 2A and DAF-16 4A (Fig. 5C, upper panel). Both LY294002 and wortmannin increased the DNA binding activity of wild-type DAF-16 (Fig. 5C, upper panel, compare lane 4 to lanes 5 and 6). In exponentially growing cells mutants of DAF-16 impaired in 14-3-3 binding, DAF-16 2A, 3A, and 4A were confined strictly to the nucleus (Fig. 5C, lower panel; compare lanes 9 and 10, lanes 11 and 12, and lanes 13 and 14). Nevertheless, similar to DAF-16 WT (Fig. 5C, lanes 4 and 5), the DNA binding activity of DAF-16 2A and DAF-16 4A was enhanced by PI 3-kinase inhibition (Fig. 5C, upper panel, compare lanes 7 and 8 and lanes 9 and 10).

In the insulin-responsive HepG2 cell line, serum inhibits the effect of endogenous factors on IGFBP gene transcription by 90% (Fig. 5D, bar B) relative to the activity seen in serum-deprived cells (bar A). The PI 3-kinase inhibitor enhances IG-
FBP-1 gene transcription 2-fold above that seen in serum-starved cells (compare bars A and C). DAF-16 activates the IGFBP promoter (compare bar A to bar D) and serum inhibits the activity of exogenous DAF-16 by 50% (compare bars B and D, while LY294002 increases DAF-16 activity 2.5-fold over control levels (compare bars D and F).

The transcriptional activity of both wild-type and mutant derivatives of DAF-16 was similarly regulated by PI 3-kinase inhibition in HepG2 cells (Fig. 5E). Whether wild-type or mutant DAF-16 derivatives are expressed in the pcDNA expression system (panel E) or as GAL4 fusion proteins compare (panel E), their activity is stimulated above basal in response to LY294002 (Fig. 5E, bars B, D, and F). However, when activity is assessed using the GAL4 DNA binding site to direct gene expression, LY294002 does not activate the GAL4-DAF-16 derivatives (Fig. 5E, bars B, D, and F). Thus, we conclude that the stimulatory effect of LY29004 is also mediated at the level of DNA binding in vivo.

The enhancing effect of LY294002 on GAL4-DAF-16 WT is greater than its enhancing effect on GAL4-DAF-16 3A and 4A.
PI 3-kinase inhibition enhances DAF-16 DNA binding and transcriptional activity via an AKT/14-3-3 site-independent pathway. A, identification of DAF-16 DNA binding activity in 293 cell nuclear extract. Nuclear extract was isolated from 293 cells expressing Flag epitope-tagged DAF-16 (M2-DAF-16) (lanes 3–5) or pcDNA alone (lanes 1 and 2) and assayed for binding to the 32P-labeled IGFBP-IRE as in Fig. 2A. Preimmune serum (PI, lanes 1 and 4) or anti-Flag antibody (M2, lanes 2 and 5) was used to supershift DAF-16/DNA complexes. The location of the DAF-16/DNA complex and M2/DAF-16/DNA complex (supershift) is indicated. B, inhibition of endogenous PI 3-kinase activity enhances binding of DAF-16 to IRE DNA. Upper panel, nuclear extracts of 293 cells expressing Flag-epitope-tagged DAF-16 (M2-DAF-16) (lanes 2 and 3) or vehicle (lane 1) grown in serum (lanes 1 and 2) or serum-deprived in the presence of LY294002 (10 μM, lane 3) were prepared as in Fig. 4B and assayed for binding to the IGFBP-IRE as described in Fig. 2A and “Experimental Procedures.” Lower panel, expression of DAF-16 in the nuclear (N) and cytoplasmic (C) fractions of the extracts shown was determined by anti-Flag immunoblotting. C, inhibition of endogenous PI 3-kinase with LY294002 enhances binding of DAF-16 AKT site mutants to IRE DNA. Upper panel, nuclear extract was isolated from 293 cells transfected with pcDNA alone (lanes 1–3), Flag-epitope-tagged DAF-16 (lanes 4–6), DAF-16 2A (lanes 7 and 8), or DAF-16 4A (lanes 9 and 10). Cells were grown in serum (lanes 1, 4, 7, and 9) or serum-deprived in the presence of LY294002 (10 μM, lanes 2, 5, 8, and 10) or wortmannin (10 nM, lanes 3 and 6). Binding to IGFBP-IRE was assayed as in Fig. 2A. Lower panel, expression of DAF-16 in the nuclear (N) and cytoplasmic (C) fractions was determined by anti-Flag immunoblotting. D, serum growth factors regulate DAF-16 transcription activation. Insulin-responsive HepG2 hepatoma cells were cotransfected with a luciferase reporter gene under the control of the native IGFBP promoter (15 μg) and pcDNA3-DAF-16 (2 μg/ml) (bars D–F) or a control pcDNA3 vector (2 μg/ml) (bars A–C) together with RSV-β-galactosidase to correct for transfection efficiency. 4 h after transfection, cells were changed to serum-containing media (bars B and E) or serum deprivation media (starved) (bars A, C, D, and F) in the absence (bars A and D) or presence (bars C and F) of LY294002 (10 μM). Cells were harvested and assayed for luciferase (Promega kit) and β-galactosidase (Tropix kit) expression according to the manufacturers instructions. The mean ratios ± S.E. of luciferase/β-galactosidase triplicates are presented. E, inhibition of endogenous PI 3-kinase activity enhances transcription activity of DAF-16 WT and AKT site mutants DAF-16 3A and DAF-16 4A on the IGFBP-IRE. HepG2 cells were transiently cotransfected with an expression vector encoding the wild-type GAL4-DAF-16 (bars A and B) or mutant GAL4-DAF-16 derivatives 3A (bars C and D) or 4A (bars E and F) (2 μg), the IGFBP-luciferase reporter gene (15 μg), and the RSV-β-galactosidase reporter gene (2 μg). Control cells growing exponentially in serum were stimulated with vehicle (bars A, C, and E) or serum-starved cells were stimulated with LY294002 (bars B, D, and F). The effect of LY294002 is shown as the percentage of control value. F, inhibition of endogenous PI 3-kinase activity does not affect transcriptional activity of DAF-16 WT or mutants on the GAL4 site. Cells were grown (bars A, C, and E) or serum-deprived in the presence of LY294002 (10 μM) (bars B, D, and F). Luciferase activity was normalized for β-galactosidase gene expression and is presented as the percentage of the serum value for each plasmid.

Fig. 5. PI 3-kinase inhibition enhances DAF-16 DNA binding and transcriptional activity via an AKT/14-3-3 site-independent pathway. A, identification of DAF-16 DNA binding activity in 293 cell nuclear extract. Nuclear extract was isolated from 293 cells expressing Flag epitope-tagged DAF-16 (M2-DAF-16) (lanes 3–5) or pcDNA alone (lanes 1 and 2) and assayed for binding to the 32P-labeled IGFBP-IRE as in Fig. 2A. Preimmune serum (PI, lanes 1 and 4) or anti-Flag antibody (M2, lanes 2 and 5) was used to supershift DAF-16/DNA complexes. The location of the DAF-16/DNA complex and M2/DAF-16/DNA complex (supershift) is indicated. B, inhibition of endogenous PI 3-kinase activity enhances binding of DAF-16 to IRE DNA. Upper panel, nuclear extracts of 293 cells expressing Flag-epitope-tagged DAF-16 (M2-DAF-16) (lanes 2 and 3) or vehicle (lane 1) grown in serum (lanes 1 and 2) or serum-deprived in the presence of LY294002 (10 μM, lane 3) were prepared as in Fig. 4B and assayed for binding to the IGFBP-IRE as described in Fig. 2A and “Experimental Procedures.” Lower panel, expression of DAF-16 in the nuclear (N) and cytoplasmic (C) fractions of the extracts shown was determined by anti-Flag immunoblotting. C, inhibition of endogenous PI 3-kinase with LY294002 enhances binding of DAF-16 AKT site mutants to IRE DNA. Upper panel, nuclear extract was isolated from 293 cells transfected with pcDNA alone (lanes 1–3), Flag-epitope-tagged DAF-16 (lanes 4–6), DAF-16 2A (lanes 7 and 8), or DAF-16 4A (lanes 9 and 10). Cells were grown in serum (lanes 1, 4, 7, and 9) or serum-deprived in the presence of LY294002 (10 μM, lanes 2, 5, 8, and 10) or wortmannin (10 nM, lanes 3 and 6). Binding to IGFBP-IRE was assayed as in Fig. 2A. Lower panel, expression of DAF-16 in the nuclear (N) and cytoplasmic (C) fractions was determined by anti-Flag immunoblotting. D, serum growth factors regulate DAF-16 transcription activity. Insulin-responsive HepG2 hepatoma cells were cotransfected with a luciferase reporter gene under the control of the native IGFBP promoter (15 μg) and pcDNA3-DAF-16 (2 μg/ml) (bars D–F) or a control pcDNA3 vector (2 μg/ml) (bars A–C) together with RSV-β-galactosidase to correct for transfection efficiency. 4 h after transfection, cells were changed to serum-containing media (bars B and E) or serum deprivation media (starved) (bars A, C, D, and F) in the absence (bars A and D) or presence (bars C and F) of LY294002 (10 μM). Cells were harvested and assayed for luciferase (Promega kit) and β-galactosidase (Tropix kit) expression according to the manufacturers instructions. The mean ratios ± S.E. of luciferase/β-galactosidase triplicates are presented. E, inhibition of endogenous PI 3-kinase activity enhances transcription activity of DAF-16 WT and AKT site mutants DAF-16 3A and DAF-16 4A on the IGFBP-IRE. HepG2 cells were transiently cotransfected with an expression vector encoding the wild-type GAL4-DAF-16 (bars A and B) or mutant GAL4-DAF-16 derivatives 3A (bars C and D) or 4A (bars E and F) (2 μg), the IGFBP-luciferase reporter gene (15 μg), and the RSV-β-galactosidase reporter gene (2 μg). Control cells growing exponentially in serum were stimulated with vehicle (bars A, C, and E) or serum-starved cells were stimulated with LY294002 (bars B, D, and F). The effect of LY294002 is shown as the percentage of control value. F, inhibition of endogenous PI 3-kinase activity does not affect transcriptional activity of DAF-16 WT or mutants on the GAL4 site. Cells were grown (bars A, C, and E) or serum-deprived in the presence of LY294002 (10 μM) (bars B, D, and F). Luciferase activity was normalized for β-galactosidase gene expression and is presented as the percentage of the serum value for each plasmid.

on the IGFBP-IRE (Fig. 5E, compare bar B to bars D and F), which suggests that DAF-16 WT is subject to both 14-3-3-dependent and independent regulation by LY294002 in vivo. The ability of LY294002 to enhance the activity of DAF-16 AKT/14-3-3 site mutants that are confined strictly to the nucleus (Fig. 5C, lower panel, lanes 9–14, DAF-16 2A, 3A, and 4A) indicates that a PI 3-kinase-responsive, 14-3-3/AKT site-independent mechanism can control DAF-16 DNA binding and transcription activity.

**DISCUSSION**

Our results reveal the existence of at least two mechanisms that cooperate to inhibit DAF-16 DNA binding in response to factors that activate PI 3-kinase-dependent signaling path-
ways. First, we show that in addition to its proposed role in promoting nuclear export/cytoplasmic retention of forkhead proteins, 14-3-3 can directly inhibit binding of AKT-phosphorylated DAF-16 to DNA (Table I and Fig. 6, pathway I).

Second we describe a novel PI 3-kinase-dependent pathway that inhibits the DNA binding activity of DAF-16 4A, an AKT/14-3-3 site mutant that cannot bind 14-3-3 and is not subject to PI 3-kinase-dependent nuclear export (Table I and Fig. 6, pathway II). The ability of endogenous PI 3-kinase signaling to prevent DAF-16 DNA binding independent of 14-3-3 may involve a phosphorylation-dependent interaction of DAF-16 with an interacting protein. This cofactor could have an analogous function to 14-3-3 and inhibit DAF-16 DNA binding activity in response to PI 3-kinase signaling. On the other hand, a cofactor that acts to stabilize DAF-16 DNA binding activity might dissociate from DAF-16 in response to PI 3-kinase signaling. In a third scenario, a non-AKT kinase (or phosphatase) downstream of endogenous PI 3-kinase could directly phosphorylate DAF-16 4A and inhibit their ability to bind DNA.

In HepG2 cells, we find that insulin inhibition of DAF-16 function occurs via an AKT/14-3-3 site-dependent pathway (Fig. 6, pathway I), consistent with the observed ability of dimeric 14-3-3 to bind AKT phosphorylated DAF-16. Our observation that insulin fails to inhibit the activity of GAL4-DAF-16 bound to the GAL4 DNA site, as opposed to the IRE DNA site, implies that GAL4-DAF-16 is not subject to insulin-mediated inhibition of DNA binding or nuclear export when it is tethered to GAL4 DNA. Thus, we propose that, in HepG2 and 293 cells, growth factors that regulate PI 3-kinase activity may act primarily to inhibit DAF-16 DNA binding via an interaction with PI 3-kinase-dependent signaling pathways. Under conditions in which PI 3-kinase is inactive, DAF-16 is found in the nucleus and is bound to DNA. Pathway I, following growth factor stimulation and activation of PI 3-kinase, AKT phosphorylates DAF-16 on Thr-54, Ser-240/242, and Ser-314, 14-3-3 binds the Thr-54 and Ser-314 sites and prevents the interaction of DAF-16 with DNA. DAF-16 is then translocated to the cytoplasm. Pathway II, endogenous PI 3-kinase signaling to DAF-16 WT and DAF-16 4A, which lacks all four AKT sites, does not bind 14-3-3, is not exported from the nucleus but, like DAF-16 WT, is subject to DNA binding regulation by the PI3 kinase inhibitor LY294002. LY294002 enhances DNA binding and transcription activity of both DAF-16 WT and 4A and therefore mediates its effect at least in part via an AKT site/14-3-3-independent pathway. Again regulation by LY294002 of GAL4 DAF-16 WT and 4A on an IRE but not a GAL4 DNA site, indicates that this effect is mediated primarily at the level of DNA binding.
14-3-3-dependent and -independent Regulation of DAF-16

The proposed model of multistep regulation of DAF-16 at the level of DNA binding as well as regulation of subcellular localization by 14-3-3 underscores the complexity of the PI 3-kinase signaling pathways to forkhead proteins. Analogous results have been described for PHO4, where four distinct phosphorylation sites cooperate to regulate nuclear import, nuclear export, and transcription activation of the target gene for PHO5 (38). Understanding the complex regulation of DAF-16 and its mammalian homologues will provide valuable insights into the mechanism that underlie the diverse effects of insulin on the metabolism, growth, and survival of its target tissues.

Acknowledgments—We thank Joseph Avruch, Jack Rogers, and Phil Daniel for critical reading of the manuscript. We thank Simin Nui for construction of GALA-DAF-16 plasmids.

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Phosphatidylinositol 3-Kinase Signaling Inhibits DAF-16 DNA Binding and Function via 14-3-3-dependent and 14-3-3-independent Pathways
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J. Biol. Chem. 2001, 276:13402-13410.
doi: 10.1074/jbc.M010042200 originally published online December 20, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M010042200

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