Insulin Regulation of Gene Expression through the Forkhead Transcription Factor Foxo1 (Fkhr) Requires Kinases Distinct from Akt†

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ABSTRACT: Insulin inhibits expression of certain liver genes through the phosphoinositol (PI) 3-kinase/Akt pathway. However, whether Akt activity is both necessary and sufficient to mediate these effects remains controversial. The forkhead proteins (Foxo1, Foxo3, and Foxo4, previously known as Fkhr or Afx) are transcriptional enhancers, the activity of which is inhibited by insulin through phosphorylation-dependent translocation and nuclear exclusion. Others and we have previously shown that the forkhead protein Foxo1 is phosphorylated at three different sites: S^{253}, T^{24}, and S^{316}. We have also shown that T^{24} fails to be phosphorylated in hepatocytes lacking insulin receptors, and we have suggested that this residue is targeted by a kinase distinct from Akt. In this study, we have further analyzed the ability of Akt to phosphorylate different Foxo1 sites in control and insulin receptor-deficient hepatocytes. Expression of a dominant negative Akt (Akt-AA) in control hepatocytes led to complete inhibition of endogenous Akt, but failed to inhibit Foxo1 T^{24} phosphorylation and, consequently, insulin suppression of IGFBP-1 promoter activity. Conversely, expression of a constitutively active Akt (Akt-Myr) in insulin receptor-deficient hepatocytes led to an overall increase in the level of Foxo1 phosphorylation, but failed to induce T^{24} and S^{316} phosphorylation. These data indicate that the Foxo1 T^{24} and S^{316} kinases are distinct from Akt, and suggest that the pathways required for insulin regulation of hepatic gene expression diverge downstream of PI 3-kinase.

Insulin plays an important role in the regulation of gene expression (*1*). The mechanisms of insulin action on gene regulation are presumably diverse, since insulin has opposing effects on different genes. For example, insulin increases transcription of genes encoding enzymes involved in glucose transport, oxidation (GLUT1,[1] GLUT4, glucokinase, and hexokinase II), and fatty acid synthesis (fatty acid synthase and acetyl-CoA carboxylase), while inhibiting transcription of genes required for glucose synthesis and glycogenolysis (reviewed in ref *1*).

Recently published evidence from numerous laboratories has indicated that inhibition of gene expression by insulin is mediated through the PI 3-kinase pathway (*2*−*7*). Among the PIP_3-activated kinases, Akt has been extensively investigated (*8*−*10*). Its substrates include enzymes regulating glycolysis (*11*), glycogen synthesis (*9*, *12*), cAMP production (*13*), protein synthesis (*14*), and gene expression (*3*, *4*, *15*). Others (*5*, *7*, *16*−*20*) and we (*6*, *21*) have shown that forkhead transcription factors (*22*) regulate gene expression in an insulin (or IGF1) suppressible manner and are phosphorylated by PIP_3-dependent kinases. Insulin-dependent phosphorylation of Foxo1 proteins results in their nuclear exclusion and inhibition of transcription (reviewed in ref *23*).

It is unclear whether insulin regulation of Foxo1 phosphorylation is mediated exclusively through Akt, or whether Foxo proteins represent the merging point of different signaling pathways. In a broader context, it is important to determine whether insulin signaling downstream of PI 3-kinase requires activation of multiple serine/threonine kinases. Thus, understanding the specificity of Foxo1 phosphorylation in a physiologic cellular context has important ramifications for our understanding of insulin action.

There are three potential Akt phosphorylation sites conforming to the canonical R-X-R-X-X-(S/T)-Φ sequence (*24*) on Foxo1 and related proteins. There is general agreement in the literature on the phosphorylation of S^{253} (or homologous residues in other species or forkhead isoforms), which is located within a nuclear localization sequence in the forkhead domain (*16*). However, phosphorylation of two additional sites, T^{24} and S^{316}, remains controversial (*23*). While several groups reported that both sites are phosphorylated in an Akt-dependent fashion (*16*−*20*, *25*), Kops et al. failed to detect T^{28} phosphorylation of human Foxo4 expressed in NIH 3T3 cells (*7*), and S^{316} phosphorylation of Foxo1 and Foxo3 was questioned by both in vitro (*17*) and in vivo findings (*16*).

In a previous study, we demonstrated that all three Akt consensus phosphorylation sites of murine Foxo1 are phosphorylated in response to insulin in a PI 3-kinase-dependent manner in SV40-transformed hepatocytes derived from

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[1] Abbreviations: GLUT1, glucose transporter 1; GLU4, glucose transporter 4; PIP, phosphatidylinositol phosphate. For simplicity, we shall refer to the amino acid numbering of murine Foxo1. T^{24} corresponds to T^{28} in Foxo4 and T^{32} in Foxo3. S^{253} corresponds to S^{193} in Foxo4 and S^{253} in Foxo3. S^{316} corresponds to S^{258} in Foxo4 and S^{315} in Foxo3.

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normal mice (*21*). In contrast, residue T^{24} failed to be phosphorylated in hepatocytes derived from insulin receptor (IR) knockout mice, while phosphorylation of residue S^{316} could not be conclusively addressed (*6*). Since these cells express a sizable number of IGF1 receptors, which possess virtually overlapping signaling capabilities and are able to activate Akt (*26*), these observations raised the question of whether T^{24} and S^{316} are indeed Akt substrates.

To further address this point, we used two complementary approaches: overexpression of dominant negative Akt (Akt-AA) or constitutively active Akt (Akt-Myr). In both instances, we compared T^{24} phosphorylation in control and IR-deficient hepatocytes (*6*). Phosphate incorporation onto T^{24} was assessed with phosphopeptide-specific antibodies and by direct ^{32}P labeling of cells expressing wild-type (WT) or S253D/S316A mutant Foxo1. This mutant was chosen because we had previously shown that T^{24} phosphorylation requires prior phosphorylation of S^{253}. Replacement of aspartate for S^{253} generates a "constitutively active" mutant Foxo1 in which T^{24} can be phosphorylated independently of S^{253} (*6*). The results presented herein are consistent with the idea that kinases other than Akt are required for insulin regulation of Foxo1 phosphorylation. Moreover, Brunet et al. have recently identified the serum- and glucocorticoid-inducible kinase SGK as a T^{28} and S^{315} kinase in the related protein Foxo3 (*27*), thus providing further support for our findings.

MATERIALS AND METHODS

Cell Culture and Antibodies. Culture of SV40-transformed mouse hepatocytes has been described in previous publications (*6*, *21*, *26*, *28*). Cells were maintained at 33 °C in alpha-modified Eagle's medium (AMEM) supplemented with 4% fetal calf serum (FCS), 2 mM glutamine, and 10 nM dexamethasone. Anti-HA monoclonal antibody 12CA5, anti-FLAG monoclonal antibody M2, anti-c-Myc monoclonal antibody 9E10, and anti-Akt polyclonal antibody were obtained from Boehringer Mannheim, Sigma, Santa Cruz, and New England Biolab, respectively. Rabbit polyclonal antibodies against the human Foxo1 S^{256} phosphopeptide, antiphospho GSK-3α/β ($Ser^{21/9}$), and anti-Akt pS^{473} were from Cell Signaling Techonologies. Monoclonal anti-GSK3β antibody was from Transduction Laboratories. Antiphospho Foxo4 T^{28} and S^{319} antibodies were a gracious gift of M. Comb (Cell Signaling Technologies). Antibodies were used for Western blotting at 1:1000 dilutions. For simplicity, we refer to the three anti-Foxo1 antibodies as anti-pT^{24}, -pS^{253}, and -pS^{316} (i.e., we use the amino acid residue number of mouse Foxo1).

Immunofluorescence. Hepatocytes were seeded onto four-well slide culture chambers (Lab Tek) and incubated in serum-free medium for 12 h prior to insulin (100 nM) or IGF1 (200 ng/mL) stimulation for the indicated time. Cells were fixed using a solution of 2% paraformaldehyde in 150 mM NaCl and 2 mM $MgCl_2$ and permeabilized in 0.5% Triton X-100. Endogenous Akt was visualized with anti-Akt polyclonal antibody and FITC-conjugated AffiniPure donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.) (*6*).

Detection of Insulin- and IGF1-Dependent Foxo1 Phosphorylation with Antiphosphopeptide Antibodies. Confluent monolayers of SV40-tranformed hepatocytes were incubated overnight in serum-free AMEM. Thereafter, insulin (100 nM) or IGF1 (200 ng/mL) was added for 15 min. At the end of incubation, cells were solubilized and processed for immunoprecipitation with anti-c-Myc antibody as described in previous publications (*6*, *21*). Alternatively, cell extracts were immunoprecipitated sequentially with anti-HA and anti-FLAG monoclonal antibodies to isolate HA-tagged Akt-WT and FLAG-tagged Akt-AA, respectively (*13*, *29*). Immune complexes were resolved via 8% SDS−PAGE and transferred to nitrocellulose filters. The membranes were sequentially reprobed with antiphospho Foxo1 T^{24}, S^{253}, S^{316}, and anti-Foxo1 (*6*, *21*) antisera, or with anti-Akt pS^{473} and anti-Akt antisera to normalize the amount of phosphate incorporated into each band for the amount of protein applied to the gel (*6*, *21*). Detection of the immune complexes was achieved with horseradish peroxidase-conjugated anti-rabbit IgG (ECL, Amersham, Buckinghamshire, U.K.). All experiments were repeated three to five times.

Coexpression of Foxo1 and Akt in Hepatocytes. Expression vectors encoding wild-type or mutant Foxo1 (pCMV5/c-myc WT Foxo1, pCMV5/c-myc T24A/S316A, pCMV5/c-myc T24A/S253D, or pCMV5/c-myc S253D/S316A) were described in previous publications (*6*, *21*). Adenoviral vectors encoding HA-tagged Akt-WT (AxCAAkt-WT), a FLAG-tagged mutant Akt in which Thr^{308} and Ser^{473} are replaced with alanine (AxCAAkt-AA), and a constitutively active Akt (AxCAAkt-Myr) have been described previously (*13*, *29*). Transient transfections with Foxo1 expression vectors were carried out with Lipofectamine (Gibco-BRL) according to the manufacturer's protocols using 10 μg of plasmid DNA. Twelve hours after transfection, cells were infected with recombinant adenoviruses (*13*, *29*). Experiments were carried out 48−72 h after infection with adenoviruses. Each experiment was repeated three to six times.

Luciferase and β-Galactosidase Assays. Transfections were carried out on cells at a 70−80% confluence using 1.5 μg of plasmid DNA and 2 μg of IGFBP-1/luciferase reporter gene (p925GL3). Plasmid pRSV-β-galactosidase (20 ng) was used as an internal control of transfection efficiency (*30*). After transfection, cells were treated with insulin or IGF1 and assayed for luciferase and β-galactosidase activity after addition of D-Luciferine (Sigma) using a Lumat LB 9507 luminometer (EG&G, Berthold, Germany). β-Galactosidase activity in cell lysates was assayed according to the manufacturer's instructions (Tropix, Bedfold, MA) as described previously (*6*, *31*). In cotransfection experiments, recombinant adenovirus encoding Akt was added to cells 12 h after transfection with expression vectors encoding Foxo1s. Forty-eight hours later, cells were incubated with serum-free medium containing 0.1% BSA in the presence or absence of insulin (100 nM) for 16 h. Luciferase activity was determined in triplicate samples and normalized for β-galactosidase activity (relative light units) in the same sample.

Statistical Analysis. Following densitometric scanning of the autoradiograms, descriptive statistics and analysis of variance were calculated using the Statsview software (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Subcellular Localization of Endogenous Akt in Insulin- or IGF1-Treated Hepatocytes. Previously, we showed that

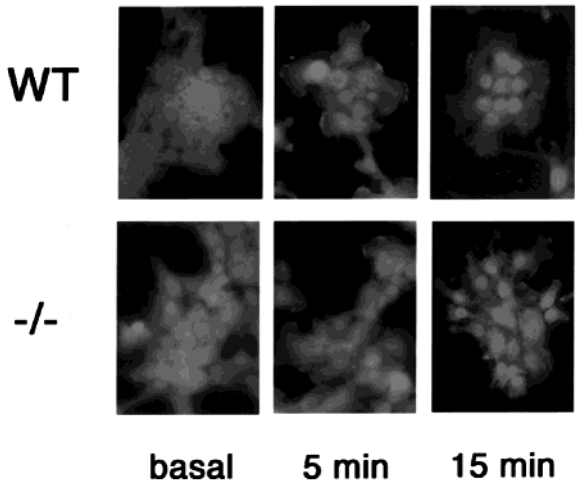

FIGURE 1: Subcellular localization of endogenous Akt in SV40-transformed hepatocytes. Hepatocytes derived from control (WT) or IR-deficient mice were seeded onto slide culture chambers and incubated in serum-free medium for 12 h prior to addition of insulin (100 nM) or IGF1 (200 ng/mL) for the indicated times. Endogenous Akt was visualized with anti-Akt polyclonal antibody and FITC-conjugated anti-rabbit IgG. In the top panels, WT hepatocytes were treated with insulin; in the bottom panels, IR-deficient hepatocytes were treated with IGF1.

insulin stimulates Akt activity in control hepatocytes to an extent similar to that of IGF1 in IR-deficient hepatocytes (*26*). Nevertheless, Foxo1 phosphorylation on T^{24} differs in the two cell types (*6*). It is not clear whether Akt phosphorylates Foxo1 in the cytoplasm or in the nucleus (*32*, *33*). However, to rule out the possibility that the failure to phosphorylate T^{24} is due to impaired Akt translocation, we examined the subcellular localization of endogenous Akt in response to insulin or IGF1 in both cell lines. In the basal state, Akt immunoreactivity did not localize to a specific cellular compartment (Figure 1, left panels). After stimulation of control hepatocytes with insulin (100 nM) or of IR-deficient hepatocytes with IGF1 (200 ng/mL), some Akt immunoreactivity could be found localized near the plasma membrane in both cell types (Figure 1, middle panels). After 15 min, immunoreactivity revealed an intense pattern of nuclear staining (Figure 1, right panels), consistent with the notion that Akt undergoes nuclear translocation with similar kinetics in both cell types (*32*, *33*).

Specificity of Antiphosphopeptide Antibodies to Foxo1. The specificity of the three antiphosphopeptide antibodies employed to detect Foxo1 phosphorylation is shown in Figure 2. All three antibodies cross-reacted with cMyc-tagged Foxo1 immunoprecipitated from transiently transfected hepatocytes (Figure 2A, lanes 1 and 2). In contrast, each of the phosphopeptide-specific antibodies failed to recognize the Foxo1 mutant in which the respective site had been mutated to alanine (lanes 3−6). Furthermore, the three antibodies failed to react with recombinant Foxo1 produced by in vitro translation of rabbit reticulocyte lysate (Figure 2B, lane 2), demonstrating that the antibodies only react with the phosphorylated amino acid residues.

Foxo1 Phosphorylation in Hepatocytes Expressing Dominant Negative Akt-AA. We first studied the effect of a dominant negative Akt on Foxo1 phosphorylation in normal hepatocytes. To this end, cells expressing WT Foxo1 were transduced with pAxCAAkt-WT (Figure 3, lanes 1 and 2)

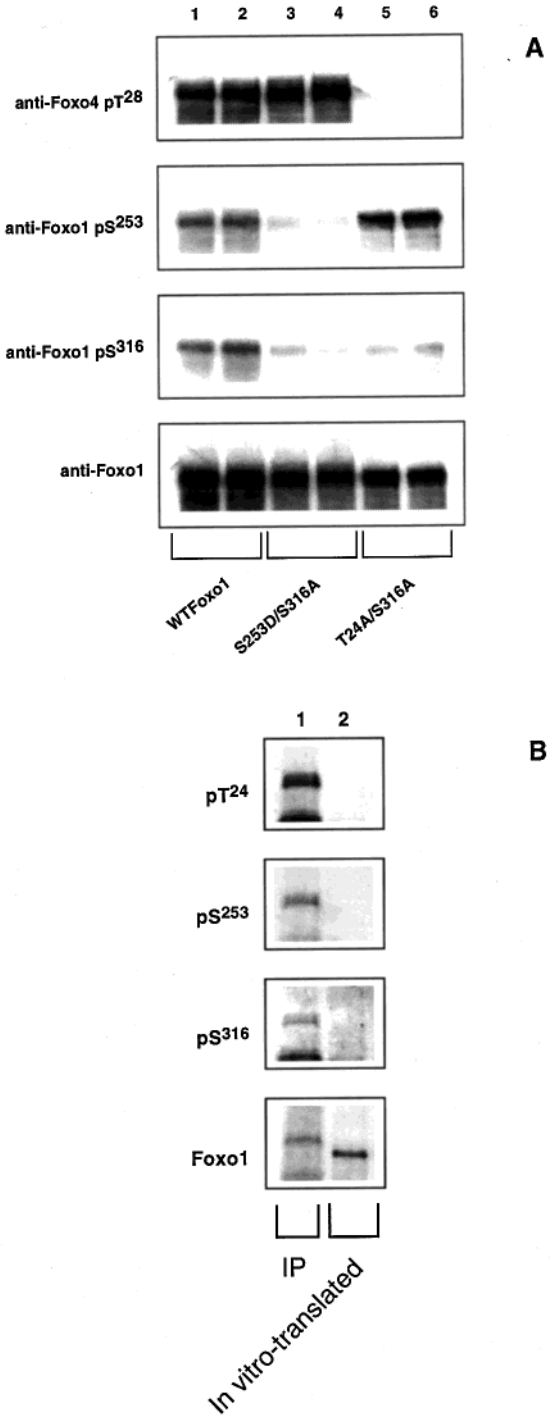

FIGURE 2: Specificity of antiphosphopeptide antibodies to Foxo1. (A) Hepatocytes from normal mice were transiently transfected with expression vectors encoding WT (lanes 1 and 2) and S253D/S316A (lanes 3 and 4) or T24A/S316A mutant Foxo1 (lanes 5 and 6). Thereafter, Foxo1 was immunoprecipitated with an anti-cMyc antibody and analyzed by immunoblotting with phospho-specific antibodies raised against the corresponding peptides of human Foxo4 (T^{28}) or human Foxo1 (S^{256} and S^{318}). For simplicity, the antibody nomenclature is adapted to the phosphorylation sites of mouse Foxo1 (T^{24}, S^{253}, and S^{316}). Each immunoprecipitation was performed in duplicate. (B) Foxo1 was isolated by immunoprecipitation from transfected hepatocyes as in panel A (lane 1) or was obtained by in vitro translation using rabbit reticulocyte lysate (lane 2). The immunoblot with antiphospho Foxo1 antibodies was carried out as described for panel A.

or cotransduced with pAxCAAkt-WT and Akt-AA (Figure 3, lanes 3 and 4) (*29*). In cells overexpressing Akt-WT,

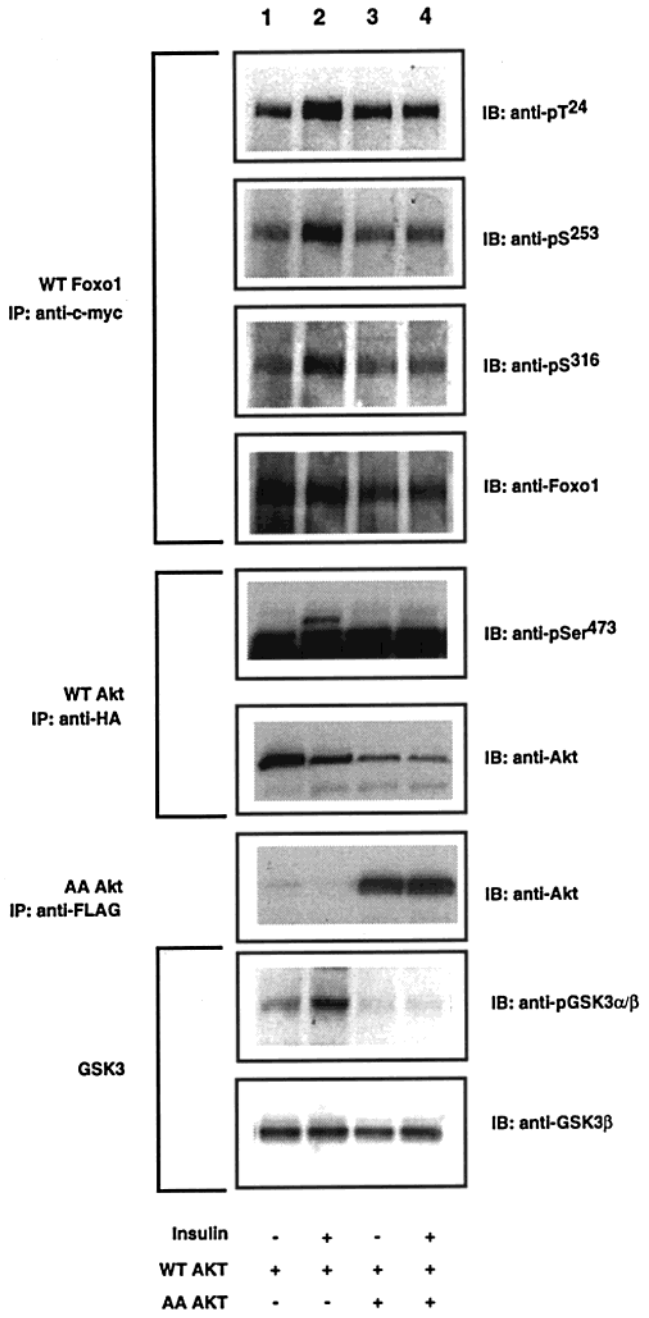

FIGURE 3: Akt-AA inhibits Foxo1 phosphorylation in SV40-transformed hepatocytes. Control hepatocytes were transiently transfected with WT c-Myc-tagged Foxo1. Twelve hours after transfection, cells were infected with adenoviral vectors encoding HA-tagged Akt-WT in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of FLAG-tagged Akt-AA. Foxo1 phosphorylation was assessed in the absence (lanes 1 and 3) and presence of insulin (lanes 2 and 4). Transfected Foxo1 was immunoprecipitated with anti c-myc antibody 9E10, and phosphate incorporation into each site was assessed using antiphosphopeptide antibodies against T^{24} (top panel), S^{253} (second panel from the top), and S^{316} (third panel from the top). The total amount of Foxo1 was measured by stripping the blots and reprobing them with an anti-Foxo1 antiserum (fourth panel from the top) (*21*). Akt phosphorylation was measured in anti-HA immunoprecipitates (*29*) by immunoblotting with an anti-pS^{473} antibody (fifth panel from the top). The total amount of Akt was measured by stripping the blots and reprobing them with anti-Akt antiserum (fourth panel from the bottom). Expression of Akt-AA was assessed using anti-FLAG immunoprecipitation of cell extracts followed by immunoblotting with anti-Akt antiserum (third panel from the bottom). Akt kinase toward endogenous substrates was measured by immunoblotting total cellular lysates with anti-$pS^{21/9}$ GSK-3 α/β antibody (second panel from the bottom). The total amount of GSK3β was determined by immunoblotting whole cell lysates with a monoclonal anti-GSK3β antibody (bottom panel). The results from one of four independent experiments are shown.

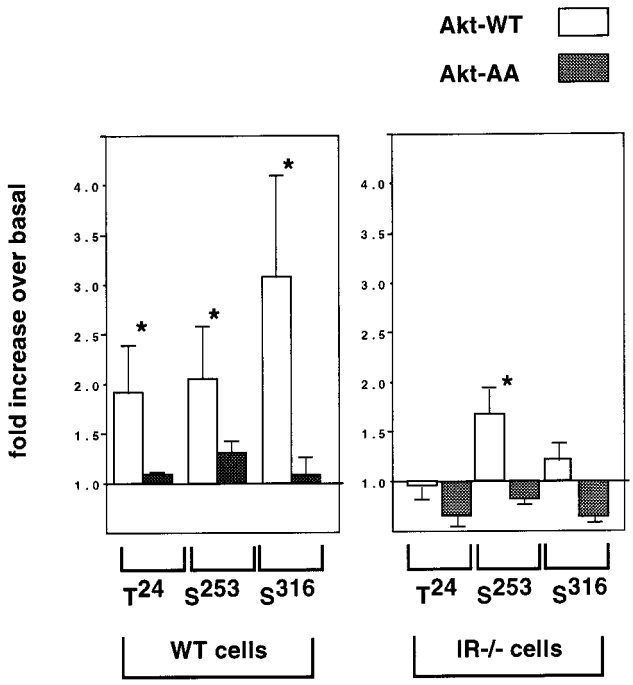

FIGURE 4: Quantitative analysis of insulin-induced Foxo1 phosphorylation. Data from transfection experiments in cells transduced with Akt-Wt and Akt-AA were analyzed by scanning densitometry of the autoradiograms. The order of magnitude increase in immunoreactivity with the different antiphosphopeptide antibodies following insulin stimulation was compared in cells expressing Akt-WT (white bars) or Akt-WT and Akt-AA (hatched bars). The data represent the means ± standard deviations from four independent experiments. An asterisk indicates a statistically significant difference as determined by ANOVA ($P < 0.05$) between the effect of Akt-WT and Akt-AA.

addition of insulin to the culture medium resulted in a readily detectable increase in the level of Foxo1 phosphorylation on all three sites, as assessed by phospho-specific antibodies (Figure 3, first three panels from the top). Quantitation of insulin's effect on the phosphorylation of each site in different experiments is shown in the left panel of Figure 4 (white bars). Coexpression of Akt-AA and Akt-WT led to an ~70% decrease in the amount of phosphate incorporated into S^{253} (Figure 3, second panel from the top, lanes 3 and 4; Figure 4, left panel, hatched bars). Consequently, phosphorylation of the additional two sites in response to insulin was also inhibited (Figure 3, first and third panels from the top, lanes 3 and 4). Akt phosphorylation on S^{473} was abolished (Figure 3, fifth panel from the top, lanes 3 and 4), as was GSK-3β phosphorylation (second panel from the bottom, lanes 3 and 4), consistent with the notion that Akt activity was effectively inhibited by Akt-AA. No alterations of cellular morphology or increased cell death was observed. The inhibition of T^{24} phosphorylation in cells expressing Akt-AA should be ascribed to the inhibition of S^{253} phosphorylation, which we have shown to be a prerequisite for T^{24} phosphorylation (*6*).

Similar results were obtained in a parallel set of experiments, in which we analyzed the effects of Akt-AA on Foxo1 phosphorylation in IR-deficient hepatocytes. Addition of IGF-1 to cells transduced with pAxCAAkt-WT resulted in a 2-fold increase in the level of S^{253} phosphorylation, but not in increased levels of T^{24} and S^{316} phosphorylation (Figure 5, top three panels, lanes 1 and 2). This small increase

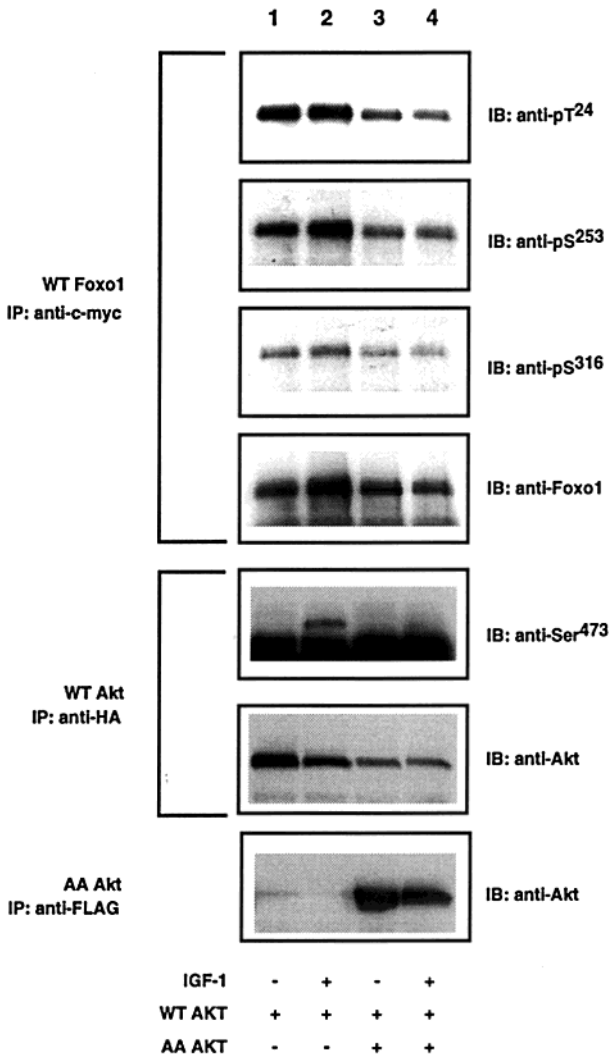

FIGURE 5: Effect of Akt-AA on Foxo1 phosphorylation in IR-deficient SV40-transformed hepatocytes. IR-deficient hepatocytes were transfected with c-Myc-tagged Foxo1 as indicated in Materials and Methods. Twelve hours after transfection, cells were transduced with pAxCAAkt-WT (encoding HA-tagged Akt) in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of pAxCAAkt-AA (encoding FLAG-tagged Akt-AA). Foxo1 phosphorylation was assessed in the absence (lanes 1 and 3) and presence of IGF-1 (lanes 2 and 4). Foxo1 was immunoprecipitated with anti c-Myc antibody 9E10, and phosphate incorporation into each site was assessed using antiphosphopeptide antibodies against T^{24} (top panel), S^{253} (second panel from the top), and S^{316} (third panel from the top). The total amount of Foxo1 was measured by stripping the blots and reprobing them with an anti-Foxo1 antiserum (fourth panel from the top) (*21*). Akt phosphorylation was measured in anti-HA immunoprecipitates (*29*) by immunoblotting with an anti-pS^{473} antibody (fifth panel from the top). The total amount of Akt was measured by stripping the blots and reprobing them with anti-Akt antiserum (second panel from the bottom). Expression of Akt-AA was assessed using anti-FLAG immunoprecipitation of cell extracts followed by immunoblotting with anti-Akt antiserum (bottom panel). The results from one of four independent experiments are shown. The results from all experiments are summarized in Figure 4.

was consistently observed in four independent experiments (Figure 4, right panel), and agrees with our previous observations (*6*). These findings confirm, using a different approach, that these cells lack IGF1-stimulated T^{24} kinase activity. They also provide evidence for a lack of S^{316} kinase activity, as we previously tentatively showed (*6*). Cotransduction of pAxCAAkt-AA with pAxCAAkt-WT resulted in inhibition of S^{253} phosphorylation (Figure 5, second panel from the top, lanes 3 and 4; Figure 4, right panel, empty bars) and Akt phosphorylation, as shown by the lack of pS^{473} immunoreactivity (Figure 5, fifth panel from the top, lanes 3 and 4). Predictably, T^{24} and S^{316} were not phosphorylated (Figure 5, top panel and third panel from the top, lanes 3 and 4), since we have shown them to be hierarchically dependent on S^{253} phosphorylation.

T^{24} Phosphorylation Is Not Inhibited by Akt-AA in Control Hepatocytes. To investigate whether T^{24} is a direct substrate of Akt, we studied the ability of Akt-AA to inhibit phosphorylation of an S253D/S316A mutant Foxo1, in which T^{24} is the sole remaining site of insulin-dependent phosphorylation in murine SV40-transformed hepatocytes (*6*). Thus, the prediction would be that insulin stimulation of T^{24} phosphorylation would be unaffected in this mutant after Akt inhibition. In cells transduced with Akt-WT, the level of T^{24} phosphorylation in response to insulin was increased ~60% (Figure 6A, lanes 1 and 2). Following transduction with Akt-AA, the level of T^{24} phosphorylation was still increased ~60% in response to insulin (Figure 6A, lanes 3 and 4). Data from six different experiments are summarized in Figure 6B. These findings indicate that the insulin-stimulated T^{24} kinase is not inhibited under conditions in which Akt autophosphorylation and its ability to phosphorylate GSK3β are totally abolished. These findings confirm previous reports that Akt inhibition does not completely abolish Foxo1 kinase activity in other systems (*7*, *16*), as well as our previous observations that the patterns of Foxo1 phosphorylation are qualitatively different in normal hepatocytes and IR-deficient hepatocytes (*6*).

A Constitutively Active Akt Fails To Restore Foxo1 T^{24} Phosphorylation in IR-Deficient Hepatocytes. To conclusively rule out the possibility that Akt is the T^{24} kinase, we investigated whether this residue can be phosphorylated by a constitutively active Akt-Myr in hepatocytes expressing cMyc-tagged Foxo1. Expression of Akt-Myr resulted in a 3−4-fold increase in the level of S^{253} phosphorylation in both WT (Figure 7, top panel, lanes 1 and 2) and IR-deficient hepatocytes (lanes 3 and 4), after correction for the amount of total Foxo1 expressed in each cell line (Figure 8). (Note that in WT cells expressing Akt-Myr, Foxo1 levels are ~30% higher than in control cells, thus leading to an apparently greater effect of Akt-Myr on Foxo1 phosphorylation.) In contrast, the level of T^{24} phosphorylation was increased 40% in WT cells, but was unaffected in IR-deficient cells (Figure 7, second panel from the top). The level of S^{316} phosphorylation was increased ~5-fold in control hepatocytes (Figure 7, third panel from the top, lanes 1 and 2), but not in IR-deficient hepatocytes (lanes 3 and 4). The results of four independent experiments are summarized in Figure 8, and are consistent with the possibility that Akt is not the T^{24} kinase.

To further address the role of Akt in S^{316} phosphorylation, WT and −/− cells were transiently transfected with the double mutant T24A/S253D, which can only be phosphorylated on S^{316}, and transduced with Akt-WT or Akt-AA. Addition of Akt-WT increased the level of S^{316} phosphorylation in WT cells ~30% (Figure 9, lanes 1 and 2). The increase was abolished by the addition of Akt-AA (lanes 3 and 4) (please note that the amount of protein is slightly higher in the insulin-stimulated lane). In contrast, S^{316}

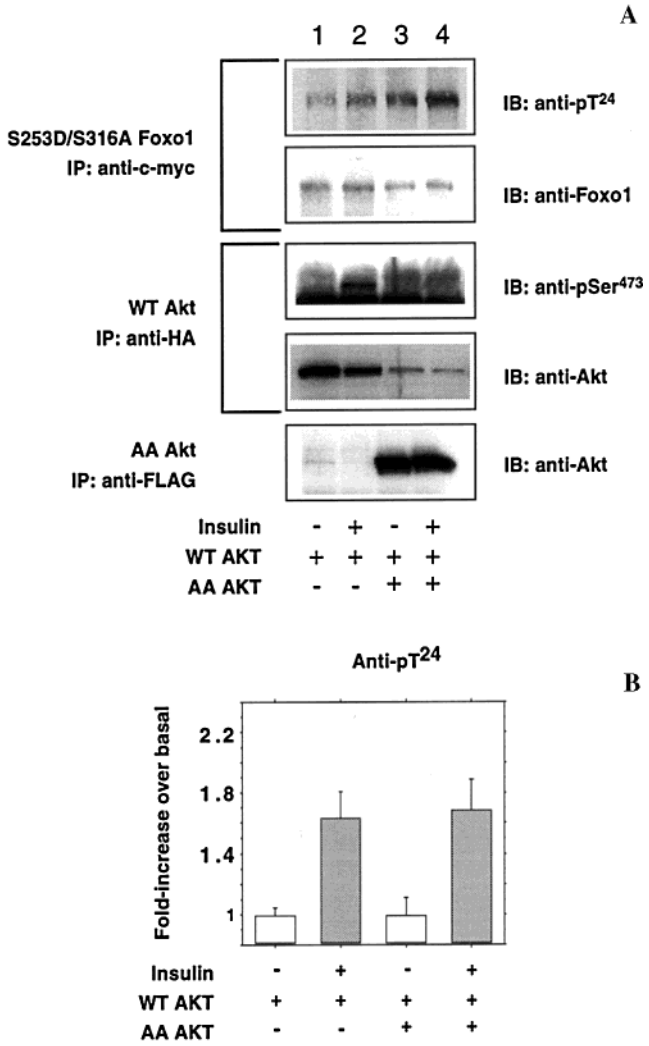

FIGURE 6: Akt-AA fails to inhibit phosphorylation of an S253D/S316A mutant Foxo1 in normal hepatocytes. (A) WT hepatocytes were transiently transfected with S253D/S316A mutant c-Myc-tagged Foxo1. Twelve hours after transfection, cells were transduced with pAxCAAkt-WT (HA-tagged) and pAxCAAkt-AA (FLAG-tagged). Insulin-induced phosphorylation was studied following overnight incubation in the absence of serum. In the top panel, S253D/S316A Foxo1 was immunoprecipitated with anti-cMyc antibody 9E10 and blotted with anti-pT24 antiserum (top panel). In the second panel from the top, membranes were reprobed with an anti-Foxo1 antiserum to normalize the protein content of each lane. In the third panel from the top, Akt phosphorylation was assessed using a pS^{473} antiserum. Expression of WT and expression of Akt-AA were detected by immunoprecipitation with anti-HA and anti-FLAG antibodies, respectively (bottom two panels). (B) Analysis of insulin-induced T^{24} phosphorylation in cells transduced with Akt-WT (lanes 1 and 2) or Akt-AA (lanes 3 and 4) using immunoblotting with antiphospho T^{24} antibody. Data from six independent determinations were analyzed using NIH Image 1.6, following scanning densitometry of the autoradiograms.

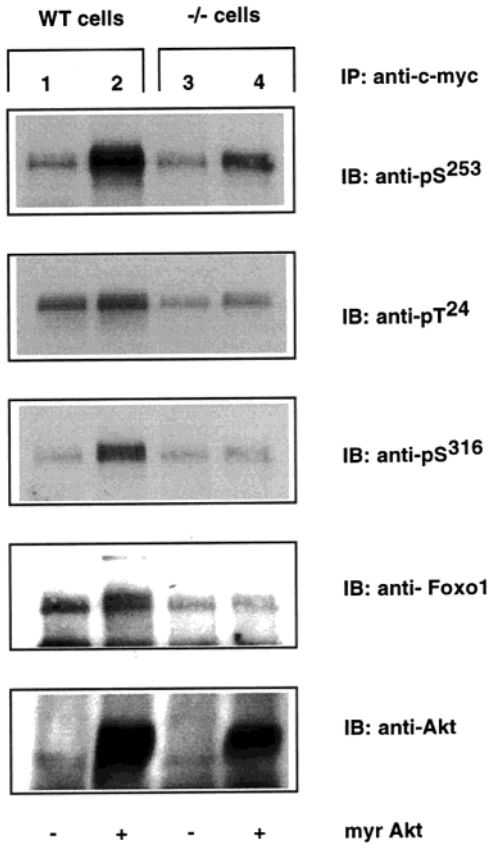

FIGURE 7: Foxo1 phosphorylation by constitutively active Akt-Myr. Control (lanes 1 and 2) and IR-deficient hepatocytes (lanes 3 and 4) were transduced with pAxCAAkt-Myr (lanes 2 and 4) after transient transfection with Foxo1 (lanes 1−4). Phosphate incorporation into Foxo1 was assessed using antiphosphopeptide antisera. Cell extracts were immunoprecipitated with anti c-myc antibody, and immunoblotted with antiphosphopeptide antibody S^{253} (top panel), T^{24} (second panel from the top), and S^{316} (third panel from the top). Membranes were then reprobed sequentially with anti-mouse Foxo1 antiserum (*21*) (second panel from the bottom) or with anti-Akt polyclonal antibody (bottom panel) to normalize the protein content of each lane.

phosphorylation was nearly undetectable in −/− cells (lanes 5−8). Interestingly, Akt-Myr was detected by immunoblotting with anti-Akt antibody following immunoprecipitation of Myc-tagged Foxo1 (Figure 7, bottom panel). These data indicate that Akt is co-immunoprecipitated with Foxo1, suggesting that Akt becomes physically associated with Foxo1, as previously shown in PC-12 cells (*34*).

Effects of a Dominant Negative Akt on IGFBP-1 Promoter Activity. To correlate Akt phosphorylation of Foxo1 with the latter's ability to affect gene expression in an insulin-dependent manner, we performed reporter gene assays using an insulin response element derived from the IGFBP-1 promoter (*30*). Previously, we showed that the lack of T^{24} phosphorylation in IR-deficient hepatocytes abolished IGF1 inhibition of IGFBP-1 promoter activity (*6*). In this study, we compared the ability of dominant negative Akt-AA to suppress insulin inhibition of IGFBP-1 promoter activity mediated by WT and mutant Foxo1. In cells transfected with reporter vector alone, insulin did not inhibit, and indeed slightly increased, luciferase activity (Figure 10). This is probably due to the low levels of luciferase activity detected in the absence of Foxo1 (∼20-fold lower than in cells transfected with Foxo1). In the absence of Akt-AA, expression of WT Foxo1 was associated with a 50% inhibition of IGFBP-1 promoter activity following insulin stimulation, as previously reported (*31*). Coexpression of Akt-AA was associated with an ∼20% inhibition by insulin of IGFBP-1 promoter activity mediated by WT Foxo1. These findings are consistent with the level of inhibition of S^{253} phosphorylation by the same mutant (Figure 3, second panel from the top). As previously shown, expression of S253D/S316A mutant Foxo1 reduced the effect of insulin in inhibiting IGFBP-1 to ∼80% (Figure 9) (*6*). This effect was not altered

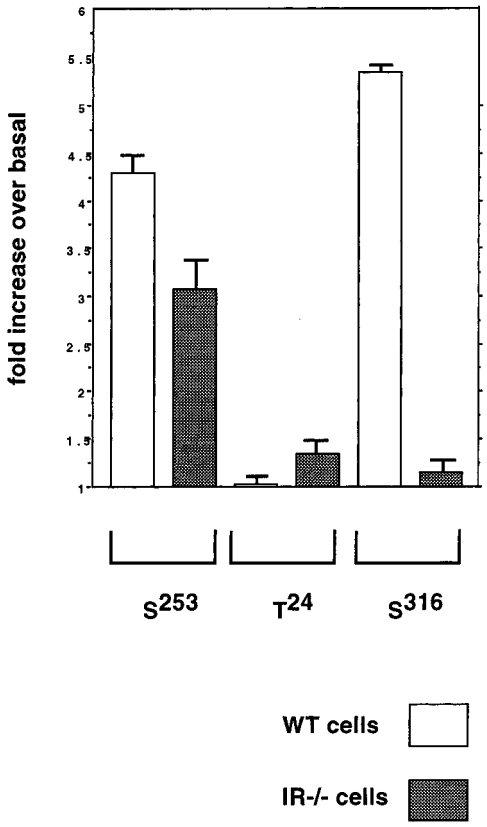

FIGURE 8: Quantitative analysis of Foxo1 phosphorylation induced by Akt-Myr. Data from four independent experiments with Myr-Akt were analyzed by scanning densitometry of autoradiograms as described in the legend of Figure 4. The order of magnitude increase in immunoreactivity with the different antiphosphopeptide antibodies was compared in control (white bars) and IR-deficient cells (hatched bars) expressing Akt-Myr.

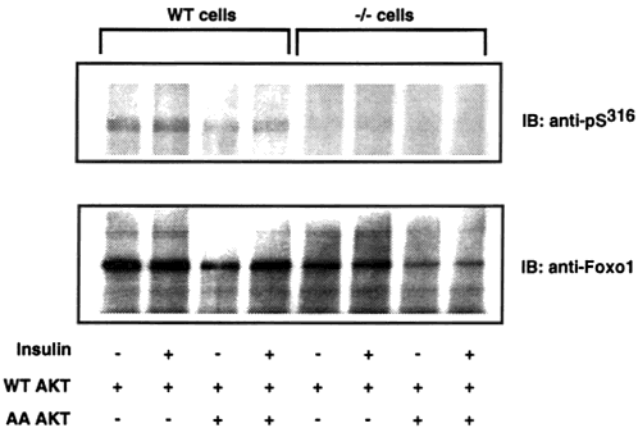

FIGURE 9: S^{316} phosphorylation in cells expressing the double mutant T24A/S253D. WT and −/− cells were transiently transfected with T24A/S253D mutant Foxo1, and then transduced with Akt-WT or Akt-AA adenovirus. Phosphorylation of S^{316} was assessed under basal and insulin-stimulated conditions by immunoblotting with a phospho-specific antiserum (top panel). Thereafter, the blots were stripped and reprobed with anti-Foxo1 antiserum to normalize protein content (bottom panel). Results from a representative experiment are shown.

by coexpression of Akt-AA, indicating that the inability of Akt to phosphorylate T^{24} partially preserves insulin inhibition of IGFBP-1 promoter activity. In contrast, the effect of insulin on IGFBP-1 promoter activity was completely abolished by expression of an S253A mutant Foxo1 (Figure 10), which does not to undergo phosphorylation in response to insulin (*6*, *21*). The conclusion of these experiments is that a component of insulin's effect on IGFBP-1 gene expression mediated through Foxo1 is Akt-independent. It consists of a non-Akt S^{253} kinase, as suggested by Kops (*7*) and Brunet (*17*), and of a non-Akt T^{24} kinase, as suggested by us (*6*) and Brunet et al. (*27*).

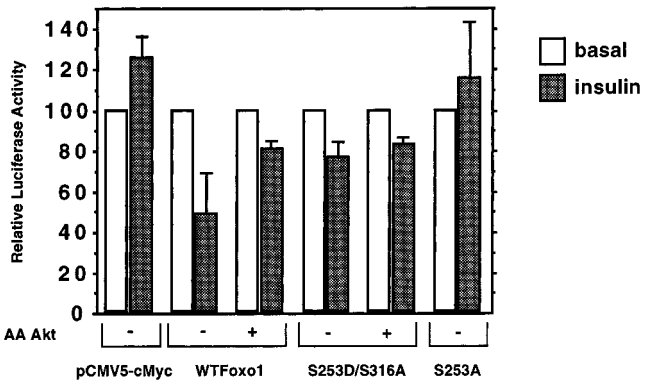

FIGURE 10: Effect of Akt-AA on IGFBP-1/luciferase promoter activity induced by WT and mutant Foxo1. WT hepatocytes were cotransfected with WT or S253D/S316A or S253A mutant Foxo1 and IGFBP-1/luciferase reporter gene (p925GL3). Plasmid pRSV-β-galactosidase was used to control transfection efficiency (*30*). Akt-AA was added 12 h after transfection, and luciferase and β-galactosidase activities were measured as described in Materials and Methods. Luciferase activity in the absence of insulin (white bars) was normalized to 100%, and the effect of insulin was calculated as the percent of basal (hatched bars) (*31*). The data represent means ± standard deviations from five independent experiments.

DISCUSSION

Consensus and Controversy with Regard to Regulation of Forkhead Proteins by Akt. Although the presence of HNF3 binding sites in the IGFBP-1 and PEPCK promoters suggested that forkhead transcription factors play a role in insulin regulation of these genes (*35*, *36*), mechanistic evidence linking forkhead proteins to insulin action was obtained from genetic complementation studies in *Caenorhabditis elegans*. In this simple metazoan, mutations of the Foxo1 homologue Daf-16 gene rescue with complete penetrance null mutations of the insulin/IGF1 receptor homologue Daf-2 and prevent dauer formation (*37*−*39*). Likewise, null Daf-16 alleles can completely rescue the dauer phenotype due to inactivating Akt mutations, suggesting that Akt signaling in *C. elegans* is entirely mediated through Daf-16 (*40*). On the other hand, activating Akt mutations do not fully suppress the Daf-2 dauer phenotype, suggesting that Daf-16 mediates both Akt-dependent and -independent pathways (*40*). Thus, the genetic evidence in *C. elegans* suggests that both Akt and additional kinases are required for physiologic regulation of Foxo proteins.

Of the three potential Akt sites in mammalian Foxo proteins, S^{253} has been consistently identified by various groups to be a direct Akt substrate (*23*). Evidence for Akt regulation of the T^{24} and S^{316} sites is discordant, as is the functional consequence of the phosphorylation of these two sites (*6*, *7*, *16*, *31*). For example, the T^{28} site of Foxo4 has been reported to be phosphorylated and to be required for nuclear export in CHO cells (*19*), but not in NIH 3T3 cells (*7*). Likewise, the S^{316} site is phosphorylated in hepatocytes (*5*, *6*, *18*, *21*), but not in CV1 cells (*16*). Moreover, regardless

of its phosphorylation state, this site appears not to be required for nuclear export in hepatocytes (*6*) and CV1 cells (*16*), whereas it affects nuclear export in 293 cells (*17*).

While undoubtedly some of these discrepancies can be explained by the different experimental systems (use of heterologous cell lines, levels of Akt expression, and different Foxo1 isoforms), it is possible that some underlie important physiologic differences in the regulation of Foxo1 proteins in response to insulin (*41*).

In this respect, it is important to emphasize that we analyzed the effect of inhibition of endogenous Akt activity on the phosphorylation of Foxo1 in a physiologic cell context. In fact, we have previously shown that permanent cultures of neonatal hepatocytes transformed with a temperature-sensitive mutant SV40 (tsA) (*42*) retain many of the metabolic features of differentiated hepatocytes and represent a useful model for investigating the specificity of insulin signaling (*6*, *26*, *28*, *43*, *44*). Our conclusions are wholly consistent with data for *C. elegans* suggesting that Daf-16 is the merging point of Akt-dependent and -independent signaling pathways (*40*).

Other signaling pathways have also been implicated in the regulation of Foxo1 function. We have shown that a region between amino acids 256 and 318 in Foxo1 plays a role in preventing stochastic phosphorylation of the three Akt sites (*6*), while Kops et al. (*7*) have suggested that Foxo4 is targeted by kinases in the Ras/Ral pathway.

Candidate T^{24} and S^{316} Kinases. In this study, we have provided further evidence that the Foxo1 T^{24} and S^{316} kinases are distinct form Akt. Similar data were obtained by Kops et al. in a different system. In that study, the authors used a dominant negative Akt (PKB caax), in which the CAAX membrane-targeting signal of Ki-Ras is inserted next to S^{473} and prevents Akt phosphorylation. This mutant Akt inhibited insulin-dependent Foxo4 phosphorylation only to a limited extent (*7*). More recently, Brunet et al. (*27*) showed that expression of a K179M mutant Akt in 293 cells failed to inhibit S^{315} phosphorylation and had at best a modest effect on T^{32} phosphorylation of Foxo3. However, since phosphorylation of endogenous Akt was not assessed, those data can only be interpreted as being indirectly supportive of the present data. The same study identifies the serum- and glucocorticoid-inducible kinase SGK1 as a T^{24} and S^{315} Foxo3 kinase in HEK293 cells. This is consistent with our previous observation that the T^{24} kinase is wortmannin-inhibitable (*6*). Moreover, the related kinase SGK3 (CISK) has also been shown to phosphorylate Foxo3, although the sites have not been determined (*45*). Interestingly, the manuscript by Brunet et al. shows that the atypical PKCζ is not a Foxo1 kinase. These findings provide an explanation for our previous observation that insulin inhibition of the PEPCK promoter in hepatoma cells, a potential Foxo1 effect (*46*), is not completely suppressed by inhibition of Akt or by a dominant negative PKCζ (*47*).

Relevance of the Findings to the Specificity of Insulin Action. An important question in insulin action is how specificity of insulin signaling is achieved at the molecular level. For example, while IR and IGF1R have highly conserved signaling pathways, their in vivo roles are substantially different, as indicated by the phenotype of the relevant knockout mice (*48*−*50*). Such differences have been generally ascribed to their different tissue distribution and ligand binding properties (*51*). However, studies from our laboratory have recently suggested that the two receptors may possess intrinsic differences in their signaling capabilities (*26*, *28*, *52*). In SV40-transformed hepatocytes, activation of IRs leads to inhibition of glucose production, whereas activation of IGF1Rs does not (*28*). Since Foxo1 can potentially mediate the inhibitory effect of insulin on the G-6-Pase promoter (J. Nakae, T. Kitamura, and D. Accili, manuscript submitted for publication) and (*53*, *54*), the different Foxo1 phosphorylation patterns elicited by insulin and IGF1 may have a direct physiological reverberation.

In conclusion, we provide further evidence that the T^{24} and S^{316} Foxo1 kinases in hepatocytes are distinct from Akt and suggest that the T^{24} kinase is required for insulin signaling to regulate gene expression. Further analysis of this kinase may provide insight into the specificity of insulin signaling in liver and into the pathogenesis of the insulin-resistant state characteristic of non-insulin-dependent (type 2) diabetes mellitus.

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