Characterization of Insulin Inhibition of Transactivation by a C-terminal Fragment of the Forkhead Transcription Factor Foxo1 in Rat Hepatoma Cells

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The forkhead transcription factor Foxo1 controls the expression of genes involved in fundamental cellular processes. In keeping with its important physiological roles, Foxo1 activity is negatively regulated in response to growth factors and cytokines that activate a phosphatidylinositol 3-kinase (PI 3-kinase) protein kinase B (PKB/Akt) pathway. PKB/Akt-mediated phosphorylation of Foxo1 has been shown to result in the inhibition of target gene transcription and to trigger the export of Foxo1 from the nucleus, which is generally believed to explain the subsequent decrease of transcription. In the present study, using a chimeric protein in which a C-terminal fragment of Foxo1 (amino acids 208–652) containing the transactivation domain is fused to the yeast Gal4 DNA binding domain, we present evidence showing that insulin can directly regulate transactivation by Foxo1 in H4IIIE rat hepatoma cells. Insulin inhibition of Foxo1-(208–652)-stimulated transactivation is mediated by PI 3-kinase but in contrast to full-length Foxo1, does not require either of the two PKB/Akt phosphorylation sites (Ser253 and Ser316) present in the protein fragment. Using mutational and deletion studies, we identify two potential phosphorylation sites, Ser219 and Ser499, as well as a 15-amino acid region located between residues 350 and 364 that are critical for insulin inhibition of transactivation by Foxo1-(208–652). We conclude that the transcriptional activity of Foxo1 is regulated at different levels by insulin: transactivation, as well as DNA binding and nuclear exclusion. These different regulatory mechanisms allow the precise control of transcription of Foxo1 target genes by insulin.

The forkhead transcription factor Foxo1 is a member of the FOXO subfamily of hepatocyte nuclear factor-3/forkhead ("winged helix") transcription factors (1–4). Foxo1 was initially identified in an alveolar rhabdomyosarcoma following a chromosomal translocation that fused its C-terminal transactivation domain (amino acids 211–655) to the DNA binding domain of Pax3 (5). Other members of the FOXO subfamily were subsequently identified and include FOXO3a (6) and FOXO4 (7, 8), as well as the Caenorhabditis elegans ortholog of FOXO1, Daf-16 (9, 10).

The FOXO1 transcription factors regulate the expression of a wide variety of genes involved in the control of key cellular processes, such as cell survival, apoptosis (11–16), cell cycle progression (17–22), DNA repair (21), protection against oxidative stress (22, 23), and differentiation (24, 25). Forkhead proteins also influence insulin sensitivity (26) and participate in the transcriptional control of genes regulated by insulin, including phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase, and insulin-like growth factor-binding protein-1 (IGFBP-1) (26–31). The FOXO proteins recognize the insulin response element (IRE) located in the promoter of these genes and activate transcription (27, 29, 32, 33).

Members of the FOXO subfamily share several structural features. In addition to the highly conserved central DNA binding forkhead domain (Fox Box), they contain a C-terminal transactivation domain and three consensus phosphorylation sites for the serine/threonine protein kinase B/Akt (34). For example, in Foxo1 used in the present study, these sites are located at Thr24, Ser253, and Ser316 (35). In vivo, the activity of these transcription factors is tightly regulated by phosphorylation of their PKB/Akt sites in response to insulin, insulin-like growth factor-I (IGF-I), and other growth factors that activate a PI 3-kinase/phosphoinositide-dependent kinase-1 (PDK-1) pathway leading to PKB/Akt activation. PKB/Akt-mediated phosphorylation of the forkhead proteins was shown to result in inhibition of target gene transcription. Phosphorylation of the PKB/Akt sites also caused the redistribution of the FOXO proteins from the nucleus to the cytoplasm (12, 13, 36, 37). The resulting decrease in nuclear FOXO proteins has been proposed as a possible mechanism for the inhibition of FOXO-stimulated transcription (13).

The present study was initiated to understand the mechanism of insulin inhibition of transactivation of the IGFBP-1 promoter by Foxo1 and, in particular, to identify the sites in the forkhead protein that are mediating the insulin effect. We have used a fusion protein in which the yeast Gal4 DNA binding domain (1–147) was coupled to a C-terminal fragment of Foxo1 (Foxo1-(208–652)) that includes the transactivation domain and corresponds to the FOXO1 fragment (amino acids 211–655) originally identified in an alveolar rhabdomyosarcoma (5). The Foxo1-(208–652) fusion protein was shown to stimulate
transcription in an insulin-inhibitable manner (38). Here, we demonstrate that in rat hepatoma cells, insulin inhibition of Foxo1-(208–652)-stimulated promoter activity occurs at the transcription level rather than by affecting subcellular localization. We show that insulin inhibition is mediated through a PI 3-kinase pathway, but that the two consensus PKB/Akt phosphorylation sites present in the protein are not required. Using mutational and deletion studies, we identify two additional potential phosphorylation sites, Ser319 and Ser499, as well as a 15-amino acid region located between residues 350 and 364 that are critical for insulin inhibition of transactivation by Foxo1-(208–652). Thus, the inhibition of Foxo1 by insulin is complex and involves multiple sites. The direct regulation of the transactivation activity of Foxo1-(208–652) by insulin provides an additional mechanism by which insulin promotes Foxo1 inhibition, thereby regulating the expression of genes controlled by this transcription factor.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes were purchased from Invitrogen. Taq polymerase was obtained from PerkinElmer Life Sciences-Applied Biosystems (Foster City, CA). Human recombinant insulin (Humulin U-100) was obtained from Eli Lilly & Co. (Indianapolis, IN). LY294002, PD08059, and SB203580 were purchased from Calbiochem (San Diego, CA).

**Cell Lines**—H4IIE rat hepatoma cells (39) were grown as monolayer cultures in low glucose Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) and antibiotics (50 units/ml of penicillin and 50 μg/ml of streptomycin).

**Plasmid Constructs**—The rat IGFBP-1 promoter-luciferase reporter plasmid (p925GL3) and the expression vector pCMVS/c-Myc-Foxo1, which contains the entire 652 amino acid coding region of Foxo1 were previously described (35, 38). pG5E1b, a plasmid encoding a luciferase gene driven by an E1b promoter and five copies of the Gal4 binding domain, was previously described (35, 38). pG5E1b, a plasmid encoding a luciferase gene driven by an E1b promoter and five copies of the Gal4 binding domain, was previously described (35, 38). pG5E1b, a plasmid encoding a luciferase gene driven by an E1b promoter and five copies of the Gal4 binding domain, was previously described (35, 38).

**Cell lysates for luciferase and β-galactosidase assays** were prepared according to the manufacturer’s instructions (Tropix, PE Applied Biosystems, Bedford, MA) using a Lumat LB 9507 luminometer (EG&G Berthold, Germany). Luciferase and β-galactosidase activities were measured in the same tube using a Low Light chemiluminescent reporter gene assay kit, allowing 1 h at room temperature for luciferin fluorescence to decay before measuring β-galactosidase activity. Assays were performed in duplicate.

**Immunoprecipitation and Western Blot Analysis**—H4IIE cells were transfected with GFP-tagged Foxo1 (208–652) and Foxo1 (208–652) Ser256/Ala261, Ser319Ala, Ser499Ala using the LipofectAMIN™ Plus reagent (Invitrogen) according to the manufacturer’s instructions. After 48 h, cells were switched to serum-free medium overnight. Forty-eight hours after transfection, the medium was replaced with serum-free Dulbecco’s modified Eagle’s medium containing 1% bovine serum albumin with or without recombinant human insulin (0.25 μg/ml), and the incubation was continued for 20 h before harvest. In some experiments, transfected cells were preincubated for 30 min with LY294002 (50 μM), a selective inhibitor of PI 3-kinase (45, 46), before insulin was added. Transfection efficiency was monitored by cotransfecting pRSV-β-galactosidase (40 ng) kindly provided by Dr. P. Yen (NIDDK, National Institutes of Health). In each experiment, the total amount of DNA was adjusted by adding empty expression vectors.

**Luciferase and β-galactosidase Assays**—Cell lysates for luciferase and β-galactosidase assays were prepared according to the manufacturer’s instructions (Tropix, PE Applied Biosystems, Bedford, MA) using a Lumat LB 9507 luminometer (EG&G Berthold, Germany). Luciferase and β-galactosidase activities were measured in the same tube using a Low Light chemiluminescent reporter gene assay kit, allowing 1 h at room temperature for luciferin fluorescence to decay before measuring β-galactosidase activity. Assays were performed in duplicate.

**Immunoprecipitation and Western Blot Analysis**—H4IIE cells were transfected with GFP-tagged Foxo1 (208–652) and Foxo1 (208–652) Ser256/Ala261, Ser319Ala, Ser499Ala using the LipofectAMIN™ Plus reagent (Invitrogen) according to the manufacturer’s instructions. After 48 h, cells were switched to serum-free medium overnight before incubation with or without recombinant human insulin (0.25 μg/ml) for the indicated times. Lysis was performed using 0.5 ml of lysis buffer containing 150 mM NaCl, 0.8 mM MgCl2, 5 mM EGTA, 50 mM Heps, pH 7.5, 10% glycerol, 1% Triton X100, 10 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Lysates clarified by centrifugation at 13,000 rpm for 10 min at 4 °C, were either directly separated by SDS-PAGE and transferred onto nitrocellulose membrane, and/or first immunoprecipitated with anti-GFP antibody (Covance). Western blots were detected using anti-FKHR antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Nuclei from HEK-293T human embryonic kidney cells transfected with GFP-tagged Foxo1 (208–652) or Foxo1 (208–652) Ser256/Ala261, Ser319Ala, Ser499Ala and FLAG-tagged p300 were prepared using the nuclei EZ Prep kit (Chemicon International, Temecula, CA). The expression of GFP antibody, proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-FLAG antibody to detect p300 (Covance).

**Immunofluorescence**—H4IIE cells were transiently transfected with p300 was a generous gift from Dr. M. Kawabata (Cancer Institute of Japanese Foundation for Cancer Research, Tokyo, Japan). The sequence of all constructs was confirmed by automated DNA sequencing using a rhodamine fluorescent terminator sequencing kit (PerkinElmer Life Sciences).

**Site-directed Mutagenesis**—Mutations were introduced using a PCR-based method (QuikChange™ site-directed mutagenesis kit; Stratagene, La Jolla, CA following the manufacturer’s instructions. The two consensus PKB/Akt phosphorylation sites (Ser256 and Ser261) in pM2-Foxo1-(208–652) and pM2-Foxo1-(208–652379–460) were mutated to alanine. Ser319Thr, Ser329Thr and Thr330Ser, Ser379Thr, Thr384Ser, Ser395Thr, and Ser499Thr were substituted with alanine in pM2-Foxo1-(208–652379–460) and pM2-Foxo1-(208–652)/H11032/Ala317/Ala364/Ala379 (400). Ser319 and Ser499, alone or together, were also mutated to alanine in pM2-Foxo1-(208–652) and pCEF-GFP-Foxo1-(208–652). Mutated oligonucleotides used for DNA amplification are available on request. The introduction of mutations was confirmed by DNA sequencing.
the LipofectAMINE Plus reagent (Invitrogen) using c-Myc-tagged full-length Foxo1, GFP-tagged Foxo1-(208–652), Gal4DBD/Foxo1-(208–652) or the corresponding empty vector. After 48 h, cells were serum-starved overnight, before incubation with recombinant human insulin (1 μg/ml) 1 h at 37 °C. Then, cells were washed twice with phosphate-buffered saline, fixed with 2% paraformaldehyde and mounted with mounting medium from Molecular Probes. Cells transfected with c-Myc-tagged full-length Foxo1, were serum-starved overnight, treated with or without insulin, and fixed as described above, before permeabilization with Triton X-100 and detection with anti-c-Myc antibody (clone 9E10) (Covance) followed by incubation with goat anti-mouse FITC-labeled secondary antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA). Cells were visualized using a fluorescence microscope.

RESULTS

Insulin Inhibition of Foxo1-(208–652)-stimulated Transactivation Does Not Result from Nuclear Exclusion—The nuclear export of the FOXO proteins was proposed as a mechanism to explain the decrease of transcription of their selected target genes in response to growth factors (13). We previously reported that insulin potently inhibits transactivation by a C-terminal fragment of Foxo1, residues 208–652 (38). Here, we examined whether insulin regulates the subcellular localization of Foxo1-(208–652) in H4IIE cells in the same way that it affects the distribution of full-length Foxo1 (residues 1–652). As shown in Fig. 1, in contrast to full-length Foxo1, which was mainly localized to the nucleus in the absence of insulin, GFP-Foxo1-(208–652) was predominantly cytoplasmic in all cells. When insulin was added, we observed, as reported by others (36, 37), a dramatic redistribution of full-length Foxo1 to the cytoplasm with almost no nuclear staining remaining, whereas the subcellular localization of GFP-Foxo1-(208–652) showed little if any change. Similar results were obtained with GFP-Gal4DBD/Foxo1-(208–652). Thus, unlike full-length Foxo1, changes in the subcellular localization of Foxo1-(208–652) in response to insulin cannot account for the inhibition of transcription observed (38). Our results suggest that inhibition of the C-terminal Foxo1 fragment by insulin occurs at the transactivation level.

Insulin Inhibition of Foxo1-(208–652)-stimulated Transactivation Does Not Require the Two Consensus PKB/Akt Phosphorylation Sites—Several studies reported that insulin inhibition of transcription stimulated by full-length Foxo1 requires the phosphorylation of three consensus sites (Thr24, Ser253, and Ser316). Foxo1-(208–652) is not required for inhibition of promoter activity by insulin. A, H4IIE cells were transfected with GFP-tagged wild-type Foxo1-(208–652) or a mutant of Foxo1 in which the two consensus PKB/Akt sites were mutated to alanine (Foxo1-(208–652/Ser253Ala/Ser316Ala)). After 48 h, cells were switched to serum-free medium overnight before incubation with recombinant human insulin (0.25 μg/ml) for the indicated times. GFP-tagged Foxo1 proteins were immunoprecipitated (IP) with anti-GFP antibody followed by immunoblotting (WB) with anti-phospho-FKHR Ser253 antibody. The expression levels of Foxo1 proteins were examined by immunoblotting total cellular lysates with anti-FKHR antibody. B, H4IIE cells were transfected with the pGL2E1b luciferase reporter plasmid (2 μg) and an expression vector for wild-type Foxo1-(208–652), Foxo1-(208–652/Ser253Ala/Ser316Ala), or the empty expression vector containing only the Gal4DBD (3 μg), as indicated. Cells were either untreated or treated with recombinant human insulin (0.25 μg/ml). Luciferase activity was determined as described under “Experimental Procedures.” Relative luciferase activity in the absence of insulin is taken as 100%. The mean ± S.E. of three independent experiments is shown.
Ser^{216} by PKB/Akt (11, 13, 48). In contrast to what has been shown for full-length Foxo1, we suggested in a previous publication (38) that PKB/Akt was not involved in insulin inhibition of Foxo1-(208–652)-stimulated transactivation. To explore further the mechanism by which insulin inhibits Foxo1 activity, we examined whether a Foxo1-(208–652) double mutant (Foxo1-(208–652/Ser253Ala/Ser316Ala)) that cannot be phosphorylated at its two PKB/Akt phosphorylation sites (Ser^{253} and Ser^{316}) (Fig. 2A) is inhibited by insulin. A yeast Gal4 promoter system (40, 41) was used in which the transactivation domains of wild-type and double mutant Foxo1-(208–652) were fused to the Gal4 DNA binding domain and cotransfected with a reporter plasmid (pG5E1b) containing a luciferase gene coupled to five copies of the Gal4 binding elements (Fig. 2B). Basal pG5E1b promoter activity was negligible in H4IIE cells so that luciferase activity reflected only transactivation by Foxo1 C-terminal fragments. As shown in Fig. 2B, insulin inhibited transactivation of the Gal4 promoter by Foxo1-(208–652) by ~70%. Moreover, substitution of Ser^{253} and Ser^{316} with alanine in Foxo1-(208–652) had no effect on insulin inhibition. These results indicate that Foxo1-(208–652) contains the information necessary for insulin inhibition and that neither of the two consensus PKB/Akt phosphorylation sites in this fragment is required for insulin inhibition of Foxo1-(208–652)-mediated transactivation.

Insulin Inhibition of Foxo1-(208–652)-stimulated Transactivation Is Mediated by PI 3-Kinase—To determine which signaling pathway is involved in the insulin inhibition of Foxo1-(208–652), specific inhibitors of PI 3-kinase (LY294002), MAP kinase kinase (PD98059), and p38 MAP kinase (SB203580) were used (Fig. 3A). Inhibition of PI 3-kinase abolished the ability of insulin to decrease Foxo1-(208–652)-stimulated transactivation. By contrast, neither MAP kinase kinase nor p38 MAP kinase had any effect on insulin inhibition of Foxo1-(208–652).

To confirm the involvement of PI 3-kinase in insulin inhibition of transactivation stimulated by the Foxo1 C-terminal fragment, Foxo1-(208–652) was cotransfected together with the wild-type regulatory subunit of PI 3-kinase, p85α, or a dominant negative mutant of p85α (Δp85), that contains a deletion that prevents it from binding to and activating the p110 catalytic subunit (42, 43). When Δp85 was overexpressed, in contrast to wild-type p85, insulin inhibition of transactivation of the Gal4 promoter by Foxo1-(208–652) was greatly decreased (Fig. 3D). Taken together, these results indicate that insulin inhibition of Foxo1-(208–652)-stimulated transactivation is mediated by PI 3-kinase but does not require the PKB/Akt phosphorylation sites.

Ser^{378} and Ser^{499} Play a Critical Role in Insulin Inhibition of Transactivation by Foxo1-(208–652)—To identify the site(s) responsible for insulin inhibition of transactivation by Foxo1-(208–652), progressive N-terminal deletions of Foxo1 fused to the Gal4 DNA binding domain were generated (Fig. 4). The different C-terminal Foxo1 fragments tested, residues 256–652, 317–652, and 501–652, significantly stimulated Gal4 promoter activity in the absence of insulin (data not shown). As shown in Fig. 4, insulin inhibited transactivation of the Gal4 promoter by Foxo1-(256–652) and -(317–652) to the same extent as Foxo1-(208–652) (~70%). In contrast, with the shorter C-terminal Foxo1 fragment, 501–652, the insulin inhibitory effect was lost, and instead a slight stimulation of transactivation (~1.5-fold) was observed. These results suggest that the site(s) critical for insulin inhibition of transcription activation by Foxo1-(208–652) is (are) located between amino acids 317 and 500.

The region 317–500 of Foxo1 contains numerous potential phosphorylation sites: 43 Ser/Thr and 7 Tyr. A majority of them, 25 Ser/Thr and 6 Tyr, are located between amino acids 379 and 460 (Fig. 5A). This observation prompted us to ask whether insulin inhibition of transactivation by Foxo1-(208–652) is affected when amino acids 379–460 were deleted. As shown in Fig. 5B, transactivation of the Gal4 promoter by Foxo1-(208–652/Ser^{379Ala/Ser^{460Ala}}) was inhibited by insulin to the same extent as wild-type Foxo1-(208–652). To exclude the possibility that the two PKB/Akt phosphorylation sites present in the fragment might compensate for the deletion, we also analyzed the effect of this deletion in the double mutant (Foxo1-(208–652/Ser^{216Ala/Ser^{316Ala}}Δ379–460)) (Fig. 5B). In either case, deletion of residues 379–460 had no effect on insulin inhibition.

We next carried out mutational studies to investigate the role of the potential phosphorylation sites present in the regions flanking amino acids 379–460: residues 317–378 and 461–500 (Fig. 5A). Five groups of Ser/Thr-Ala mutations (designated A–E) were tested in Foxo1-(208–652/Ser^{216Ala/Ser^{316Ala}}Δ379–460). As shown in Fig. 5C, insulin inhibition still was observed when groups B, C, and D, which contain a total of 12 Ser/Thr, were mutated. Insulin inhibition, however,
was abolished when Ser319Ala/Thr320Ala (Group A) or Ser499Ala (Group E) were mutated. Similar results were obtained when these point mutations were introduced into Foxo1-(208–652), which possess functional PKB/Akt sites (data not shown). Of the 2 residues mutated in Group A, Ser319 is most likely to be involved since it has a high probability of being phosphorylated (49).

Furthermore, neither S319A nor S499A alone was sufficient to abolish insulin inhibition, suggesting that the 379–460 region could compensate for the loss of one of these two sites (Fig. 5D). However, when S319A and S499A were simultaneously mutated in Foxo1-(208–652), insulin no longer inhibited trans-
activity stimulated by this fragment. Together, these results indicate that Ser\(^{319}\) and Ser\(^{499}\) are important for insulin inhibition of Foxo1-(208–652)-stimulated transactivation. The presence of the 379–460 region can compensate for mutation of one of these two sites, but insulin inhibition is lost when both sites are mutated even though residues 379–460 are present.

Mutation of Ser\(^{319}\) and Ser\(^{499}\) could regulate transactivation by altering the interaction of Foxo1 with coactivators/corepressors. Nasrin (66) previously reported that the coactivator p300/CBP (CREB-binding protein) binds to full-length Daf-16 and FOXO1. As shown in Fig. 6, p300 interacted with Foxo1-(208–652). Alanine substitution of Ser\(^{319}\) and Ser\(^{499}\) in the C-terminal transactivation domain of Foxo1, however, did not affect the binding to p300, indicating that these serines are not involved in the interaction with the coactivator.

**Residues 350–364 of Foxo1-(208–652) Are Required for Insulin Inhibition of Transactivation**—As a parallel approach, we also analyzed the ability of insulin to inhibit transactivation stimulated by progressive N-terminal deletions of Foxo1. The different fragments tested originated at different points in the 317–500 region but terminated at residue 652. As seen in Fig. 7A, insulin potently inhibited transactivation of the Gal4 promoter by the Foxo1 317–652, 332–652, and 350–652 fragments. When further truncations were tested (Foxo1-(365–652), -(412–652), -(476–652), and -(501–652)), insulin inhibition was lost and instead a stimulation of transactivation was observed. These results suggest that the region 350–364 of Foxo1-(208–652) is necessary for insulin inhibition. Moreover, our results also suggest that insulin stimulation of Foxo1 activity is masked in Foxo1-(208–652) and can only be observed in the shortest Foxo1 fragments.

To confirm the importance of the 350–364 region in insulin inhibition, we examined the effect of insulin on Foxo1-(208–652)-stimulated transactivation when amino acids 350–364 were deleted (Foxo1-(208–652) \(\triangle 350–364\)) (Fig. 7B). Deletion of these fifteen residues in Foxo1-(208–652) was sufficient to abolish insulin inhibition. Taken together, these results indicate that, in addition to Ser\(^{319}\) and Ser\(^{499}\), the region 350–364 in Foxo1-(208–652) is also critical for insulin inhibition. Moreover, mutation of the potential phosphorylation sites (4 Ser and 1 Thr) present in the 350–364 region did not affect Foxo1-(208–652)-stimulated transactivation, indicating that phosphorylation of these sites is not required for insulin inhibition (Fig. 5, A and C).

**DISCUSSION**

The FOXO forkhead proteins induce the transcription of genes involved in fundamental cellular processes (reviewed in Ref. 51). In keeping with their important physiological roles, the activity of these transcription factors is highly regulated. Although their regulation by insulin and IGF-1 has been most intensively studied (13, 48, 52), there is now a growing body of evidence indicating that other growth factors (epidermal growth factor (53); nerve growth factor (54); and platelet-derived growth factor (55)) and cytokines (interleukin-2, (56); interleukin-3 (57), erythropoietin (58, 59); thrombopoietin (60); and transforming growth factor-\(\beta\) (61)) also inhibit FOXO activity by similar mechanisms. All of these signaling molecules activate PKB/Akt, which phosphorylates Foxo1 at three consensus sites. The phosphorylation of Foxo1 at these sites results in its exclusion from the nucleus (12, 13, 36, 37), which, according to the generally accepted model, responsible for the decrease of its transcriptional activity (13).

In this report, we demonstrate that a C-terminal fragment of Foxo1, residues 208–652, is a potent activator of transcription when fused to the Gal4 DNA binding domain. This is likely to be physiologically relevant, as a fusion protein of Pax3 with the human counterpart of this fragment identified in alveolar rhabdomyosarcomas (5) is also known to enhance transcription (62). Consistent with what has been described for full-length Foxo1 (36, 38), we found that insulin strongly inhibits transactivation by Foxo1-(208–652) (\(-70\%\)) in H4IIE rat hepatoma cells. Our results, however, contrast with the absence of insulin regulation reported for a comparable C-terminal fragment of FOXO4, another member of the FOXO subfamily (52). The discrepancy may reflect differences between the transactivation domains of the two related transcription factors or differences in the insulin responsiveness of the cell lines used (mouse fibroblasts overexpressing insulin receptors (52) or H4IIE rat hepatoma cells in the present study).

Surprisingly, our results show that the subcellular localization of Foxo1-(208–652) is not significantly affected by insulin, whereas under the same conditions insulin promotes the redistribution of full-length Foxo1 to the cytoplasm. Therefore, changes in the subcellular localization of Foxo1-(208–652) are unlikely to account for the inhibition of its transcriptional activity by insulin, and suggest that insulin is directly regulating transcription.

Transcriptional regulation by insulin may reflect an altered binding of the transcription factor to the promoter region or the regulation of transactivation. These possibilities are not mutually exclusive, as described for the regulation of the pancreatic islet homeobox transcription factor PDX-1 by glucose (63). Phosphorylation of members of the FOXO subfamily, Daf-16 (64) and FOXO1 (65), in response to insulin have been shown to inhibit their binding to the IRE in the promoter of target genes. In the present study, using a yeast Gal4 system (40, 41) in which the level of promoter activity is strictly determined by the transactivation domain of Foxo1, we provide the first direct demonstration that the insulin effects can also be mediated by inhibition of transactivation.

Mutation and deletion studies were performed to identify the sites involved in insulin inhibition of Foxo1-(208–652)-stimulated transactivation. As we demonstrate in this report, and in contrast to what has been described for full-length Foxo1, the two PKB/Akt consensus phosphorylation sites (Ser\(^{253}\) and Ser\(^{214}\) contained in Foxo1-(208–652) are not necessary for inhibition of its transactivation activity by insulin. Indeed, a Foxo1-(208–652) mutant in which the two PKB/Akt sites were mutated, and more extensive N-terminal deletions of Foxo1 (for example, Foxo1-(317–652)) that do not contain those sites, are
still strongly inhibited by insulin.

We have identified three sites in Foxo1-(208–652) that are critical for insulin inhibition: Ser\textsuperscript{319} and Ser\textsuperscript{499}, as well as a 15-amino acid region located within residues 350 to 364 (Fig. 8). Mutation of Ser\textsuperscript{319} and Ser\textsuperscript{499} together was sufficient to abolish insulin inhibition. These two serines act in concert and are presumably phosphorylated. The kinase(s) that phosphorylate them remain to be identified. Phosphorylation of Ser\textsuperscript{319} by casein kinase I (equivalent to Ser\textsuperscript{322} of FOXO1) can be excluded, however, since it requires prior phosphorylation of Ser\textsuperscript{253} (36). Our results also indicate that the 379–460 region of Foxo1 can compensate for the mutation of either Ser\textsuperscript{319} or Ser\textsuperscript{499}, but not for the mutation of both sites (Fig. 8). We do not know yet whether the same or different sites in Foxo1-(379–460) compensate for the mutation of the two serines. Both Foxo1-(208–652) and Foxo1-(208–652/Ser\textsuperscript{319}Ala/Ser\textsuperscript{499}Ala) bind to the coactivator p300, indicating that mutation of these serines does not abolish insulin inhibition of transactivation by disrupting interactions with the coactivator.

Residues 350–364 are also critical for insulin inhibition of Foxo1-(208–652) activity. Mutation of the Ser/Thr residues contained in this region does not prevent inhibition of transactivation by insulin, suggesting that phosphorylation of these particular sites is not required for insulin action. Whether insulin acts in Foxo1-(350–364) by another type of post-translational modification or by promoting the interaction with a protein (that may or may not be phosphorylated) remains to be determined. In addition, it will be interesting to examine whether Ser\textsuperscript{319}/Ser\textsuperscript{499} and residues 350–364 are sequential components of the same inhibitory pathway or belong to separate inhibitory pathways that mediate insulin action. For example, the 350–364 domain may be required for phosphorylation of Ser\textsuperscript{319} or Ser\textsuperscript{499}. This is reminiscent of the ability of the Foxo1-(256–317) domain to inhibit phosphorylation of Thr\textsuperscript{244} and Ser\textsuperscript{316} following phosphorylation of Ser\textsuperscript{253} (36).

Insulin inhibition of Foxo1-(208–652)-stimulated transactivation is dependent on PI 3-kinase, but the kinase(s) downstream of PI 3-kinase that are involved in the insulin response remain unknown. Several lines of evidence (the results presented here and Ref. 38) suggest that PKB/Akt is unlikely to be involved. The possibility that PKB/Akt might act indirectly, by phosphorylating a coactivator that interacts with Foxo1 (67), is excluded by the fact that coexpression of constitutively active PKB/Akt with Foxo1-(208–652) does not mimic the effect of insulin (38). Candidate kinases other than PKB/Akt downstream from PI 3-kinase known to be involved in insulin signaling include the serum- and glucocorticoid-induced protein kinase (SGK) (68–70), the mammalian target of rapamycin (mTOR), and the atypical protein kinase C variants (PKC-β and -δ, Ref. 71). Direct phosphorylation of the C-terminal fragment of Foxo1 by SGK is unlikely to be involved in insulin inhibition of transactivation since SGK recognizes the same consensus phosphorylation sites as PKB/Akt (69, 70, 72) and we have shown that these sites are not essential for insulin inhibition. mTOR has been reported to mediate insulin inhibition of FOXO-stimulated transcription in some cell lines. The mTOR inhibitor, rapamycin, decreased insulin inhibition of IGFBP-1 gene expression in rat hepatocytes (73), had no effect on insulin inhibition of IGFBP-1 promoter activity in HepG2 cells (74), and partially decreased insulin inhibition of transcription by FoxO1 in H4IIE cells (75). Finally, the atypical PKCs have been shown to mediate insulin stimulation of glucose transport in adipocytes and in muscle (76–78), but do not induce phosphorylation of Ser\textsuperscript{253} in FOXO3a (72). Whether these PKC variants or an insulin receptor-specific kinase that phosphorylates Foxo1 at Thr\textsuperscript{244} in hepatocytes (36, 79) might phosphorylate other sites in Foxo1 is presently unknown.

An unexpected result from the mutation and deletion approaches used in the present study, was the demonstration that insulin stimulates transactivation by Foxo1-(365–652) and smaller C-terminal Foxo1 fragments (Fig. 9).\textsuperscript{3} Stimulation was observed with the shortest Foxo1 fragment, 501–652, in-

\footnote{3 Characterization of the enhancement of Foxo1-(501–652)-stimulated transcription by insulin will be reported separately (V. Perrot and M. M. Rechler, unpublished data).}
indicating the presence of a stimulatory site (St) in this region. The deletion of an inhibitory site (Inh) located within amino acids 350–364 in Foxo1-(208–652) abolished insulin inhibition, but was not sufficient to stimulate transcription. Furthermore, in the absence of this inhibitory site, the enhancement of transactivation in response to insulin was masked unless amino acids 208–350 were also deleted, suggesting the presence of an additional regulatory site, designated suppressor of stimulation (Sup), in this region.

In summary, these data provide compelling evidence to demonstrate that insulin regulation of transactivation by Foxo1-(208–652) is complex and involves multiple sites as well as different mechanisms. Insulin inhibition of Foxo1-(208–652)-stimulated transactivation is mediated by PI 3-kinase, and requires Ser319/Ser499 acting in concert and amino acids 350–364. Moreover, our results also indicate that in contrast to full-length Foxo1, the PKB/Akt phosphorylation sites are not necessary for insulin inhibition of Foxo1-(208–652) activity. Further N-terminal truncations unmask a stimulatory site located within the C terminus of Foxo1, amino acids 501–652, leading to an increase of transcription by Foxo1 in response to insulin. In this study, we demonstrate that insulin can directly regulate transactivation by Foxo1-(208–652). Therefore, the transcriptional activity of Foxo1 is regulated at different levels by insulin: transactivation, as well as DNA binding (64, 65) and nuclear exclusion (13). This model is consistent with the reported multistep regulation of other transcription factors. In yeast, for example, the availability of phosphate regulates the transcription factor Pho4 at the levels of nuclear import, nuclear export and transcription (80, 81). Thus, the direct regulation by insulin of Foxo1-stimulated transactivation provides an additional mechanism to allow the precise control of transcription of FOXO target genes.
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