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Convergence of Peroxisome Proliferator-activated Receptor γ and Foxo1 Signaling Pathways*

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The forkhead factor Foxo1 (or FKHR) was identified in a yeast two-hybrid screen as a peroxisome proliferator-activated receptor (PPAR) γ-interacting protein. Foxo1 antagonized PPARγ activity and vice versa indicating that these transcription factors functionally interact in a reciprocal antagonistic manner. One mechanism by which Foxo1 antagonizes PPARγ activity is through disruption of DNA binding as Foxo1 inhibited the DNA binding activity of a PPARγ/retinoid X receptor α heterodimeric complex. The *Caenorhabditis elegans* nuclear hormone receptor, DAF-12, interacted with the *C. elegans* forkhead factor, DAF-16, paralleling the interaction between PPARγ and Foxo1. *daf-12* and *daf-16* have been implicated in *C. elegans* insulin-like signaling pathways, and PPARγ and Foxo1 likewise have been linked to mammalian insulin signaling pathways. These results suggest a convergence of PPARγ and Foxo1 signaling that may play a role in insulin action and the insulinomimetic properties of PPARγ ligands. A more general convergence of nuclear hormone receptor and forkhead factor pathways may be important for multiple biological processes and this convergence may be evolutionarily conserved.

Peroxisome proliferator-activated receptor (PPAR)[1] γ is a member of the nuclear hormone receptor (NHR) superfamily that includes ligand-activated receptors for steroid hormones, vitamins, fatty acids, and other lipophilic signaling molecules (reviewed in Ref. 1). Compared with the expression pattern of other PPAR family members (PPARα and PPARδ), PPARγ exhibits a relatively restricted expression pattern in adipose tissue (2–4), macrophages (5–7), and, in obese states, in the liver (8). PPAR family members function only in the context of PPAR/retinoid X receptor (RXR) heterodimeric complexes (4). PPARγ is a critical regulator of adipogenesis (9) and is required for this cellular differentiation process (10–12). Antidiabetic thiazolidinediones are potent PPARγ ligands (13) that likely exert their insulinomimetic properties by acting through PPARγ, although how this leads to increased insulin sensitivity is not completely understood.

Foxo1 (also known as FKHR) is a winged helix transcription factor that, along with other Foxo family members including Foxo3 (FKHRL1) and Foxo4 (AFX), is implicated in several biological processes including but not limited to cell cycle regulation, apoptosis, and glucose homeostasis (reviewed in Refs. 14 and 15). Foxo1 is expressed in insulin-responsive tissues such as liver, skeletal muscle, and adipose tissue (16). Insulin and other growth factors promote phosphorylation of Foxo factors on one or more phospho-acceptor sites resulting in exclusion and/or export from the nucleus. This appears to serve as the primary molecular switch that regulates Foxo factor activity. Growth factors induce activation of Akt/protein kinase B, which then phosphorylates Foxo factors (17–21), but other kinases may also be involved (22, 23). Recent evidence suggests that the phosphorylation status of Foxo1 might also regulate transcriptional activation properties in addition to regulating cytolocalization (24). Insulin-induced inactivation of Foxo1 appears to be one of the mechanisms through which insulin suppresses gluconeogenic gene expression as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, two enzymes involved in gluconeogenesis, are encoded by Foxo1 target genes (25, 26). While regulation of phosphoenolpyruvate carboxykinase via this mechanism is controversial, recent evidence from *Foxo1*± mice supports such a mechanism for regulation of glucose-6-phosphatase (27).

Extensive work in *Caenorhabditis elegans* has culminated in the identification of at least three conserved signaling pathways that influence metabolism, aging, stress tolerance, and/or cellular differentiation processes. Elucidation of complex interactions between *C. elegans* insulin, transforming growth factor-β, and NHR pathways has proven useful to understanding homologous vertebrate pathways (reviewed in Refs. 28 and 29). In fact, clues as to the identification of Foxo factors as Akt substrates came from genetic analysis of the homologous pathway present in *C. elegans*. The nematode forkhead factor, DAF-16, most closely resembles members of the mammalian Foxo family (30, 31), and DAF-16 is a likely ortholog of at least one Foxo factor (32–34). Interestingly, detailed genetic analyses of *daf-2* (insulin receptor), *daf-16* (Foxo factor), and *daf-12* (NHR) suggest interactions among these signaling components, but the molecular basis for such genetic interactions is largely unknown.

The aim of the present study was to isolate proteins expressed in adipocytes that interact with PPARγ and to examine the functional consequences of such interactions. Here we report the identification of Foxo1 as a PPARγ-interacting protein. Functional assays indicate that Foxo1 antagonizes PPARγ signaling and that PPARγ antagonizes Foxo1 signaling suggesting a reciprocal antagonistic interaction between these two

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[1] The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; NHR, nuclear hormone receptor; RXR, retinoid X receptor; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; HA, hemagglutinin; GST, glutathione *S*-transferase; ER, estrogen receptor; AR, androgen receptor; CBP, cAMP-responsive element-binding protein-binding protein.

transcription factors. In protein interaction assays, the *C. elegans* NHR, DAF-12, interacted with the *C. elegans* forkhead factor, DAF-16, in a manner analogous to the interaction between PPARγ and Foxo1 suggesting that NHR-forkhead factor interactions and cross-talk may be conserved throughout evolution. Considering the prominent roles played by PPARγ and Foxo1 in vertebrate insulin signaling pathways, and that of DAF-12 and DAF-16 in the homologous nematode insulin-like pathway, these findings may provide a foundation for a better mechanistic understanding of insulin action.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening and 3T3-L1 Adipocyte Library Construction—The yeast two-hybrid screen was initiated by growing a 100 ml culture of the yeast reporter strain L40 transformed with the PPARγ bait plasmid (pBTM116/PPARγ ligand binding domain, encoding a LexA DNA binding domain fused to the PPARγ ligand binding domain residues 182–505) at 30 °C to an $A_{600} > 0.8$. An aliquot of the frozen yeast strain BY4734 transformed with a 3T3-L1 adipocyte library was thawed and mated with the bait-transformed reporter strain in a 50-ml volume of 2× YPDA (yeast extract/peptone/dextrose/alanine) + 50 μg/ml kanamycin for 20 h at 30 °C with gentle shaking (50 rpm). The resulting diploid cells were then plated on 50 100-mm culture plates containing synthetic medium lacking tryptophan (to select for the reporter strain), leucine (to select for the library plasmid), and histidine (to select for HIS3 reporter activity). Library plasmids were recovered from colonies that grew on the screening plates, shuttled into *Escherichia coli* and sequenced. The regions of Foxo1 and RXRα isolated from the screen encompassed amino acids 436–652 and 257–467, respectively. Construction of the 3T3-L1 adipocyte library was described previously (35).

Cell Culture and Transient Transfections—3T3-L1 preadipocytes were maintained and induced to differentiate as described previously (36). 293T cells were maintained in Dulbecco's modified Eagle's medium + 10% fetal bovine serum and transfected using standard calcium phosphate reagents. Quantities of plasmids transfected per 6-cm plate were: 100 ng of β-galactosidase-encoding plasmid, 100 ng of luciferase reporter plasmid, and 100–200 ng of transcription factor-encoded expression plasmid (*i.e.* Foxo1, PPARγ, RXRα cDNAs as indicated in the figure legends to Figs. 3 and 4). 293T cells were transfected and 24 h later the medium was changed to Dulbecco's modified Eagle's medium without serum. Insulin-like growth factor-I (IGF-I des-(1–3), a naturally occurring IGF-I derivative with decreased affinity for IGF-I-binding proteins as described in Ref. 37) and rosiglitazone, where indicated, were added at the time the cells were switched to serum-free medium. 24 h later (48 h post-transfection) the cells were harvested and assayed for luciferase and β-galactosidase activity using chemiluminescent detection kits (Tropix). Luciferase reporter assays were conducted in triplicate and luciferase activity normalized for transfection efficiency using β-galactosidase activity.

Plasmid Constructs—The PPARγ yeast two-hybrid bait plasmid was constructed by subcloning a PPARγ cDNA fragment encoding amino acids 182–505 into pBTM116 in-frame with the encoded LexA DNA binding domain. Foxo1 wild type and Foxo1AAA cDNAs were as described previously (21). Foxo1AAA/His^{212} (His^{212} mutated to Arg) was constructed by site-directed mutagenesis of the Foxo1AAA cDNA using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer's instructions. All Foxo1 cDNAs contained HA-epitope tags and were subcloned into the eukaryotic expression vector pSG5 (Stratagene) as BamHI fragments. The Foxo1-responsive luciferase reporter was constructed by ligating three copies of the Foxo1 binding site from the IGFBP-1 gene promoter into the luciferase reporter vector pGL3promoter (Promega). The PPARγ-responsive luciferase reporter was constructed by ligating two regions of the 422/aP2 gene promoter into pGL3Basic (Promega). The first 422/aP2 region encompasses an enhancer from −5400 to −4900, which contains the PPARγ binding site (ARE7; Ref. 4). The second 422/aP2 region encompasses the 5′-proximal promoter elements (−248 to +24) including basal promoter elements. Additional details on plasmid constructions and other plasmids described can be obtained upon request.

DNA Binding Assays—The PPARγ/RXRα binding site from the 422/aP2 gene enhancer element (ARE7) was radioactively labeled. PPARγ, RXRα, and Foxo1 proteins were produced *in vitro* using a rabbit reticulocyte lysate system (TNT coupled lysate system, Promega). The labeled DNA probe was incubated with 0.2 μl of PPARγ and RXRα programmed lysate in the absence and presence of 5 μM rosiglitazone and allowed to bind for 10 min at room temperature with buffer conditions as described previously (36). 1.0 μl of luciferase-programmed lysate (negative control) or Foxo1-programmed lysate was then added, and after 10 min at room temperature the binding reactions were electrophoresed on a non-denaturing polyacrylamide gel.

Western Blotting and in Vitro Protein-Protein Interaction Assays—Whole cell and nuclear extracts for immunoblot analyses were prepared as described previously (36). GST pull-down assays were as described (38). The GST-Foxo1 fusion protein encoded amino acids 436–652 of Foxo1 (the Foxo1 region identical to that identified during the two-hybrid screen). The GST-DAF-12 fusion protein encoded the entire A1 isoform, and the labeled DAF-16 in pull-down experiments (Fig. 6) was the B isoform. The Foxo1 and HA-tag antibodies were purchased from Upstate Biotechnology (catalog numbers 07-176 and 07-221, respectively).

RESULTS

Identification of Foxo1 as a PPARγ-interacting Protein—A yeast two-hybrid screen was conducted to identify PPARγ-interacting proteins. One clone isolated from a 3T3-L1 adipocyte library encoded a C-terminal fragment of the forkhead transcription factor Foxo1. A second clone encoded a C-terminal fragment of RXRα, the heterodimeric partner of PPARγ. As indicated by a quantitative β-galactosidase reporter assay in the yeast two-hybrid system, both Foxo1 and RXRα interacted with PPARγ (Fig. 1*A*). Rosiglitazone, a synthetic thiazolidinedione and PPARγ ligand, increased the reporter activity when Foxo1 was examined suggesting that rosiglitazone stimulates the interaction between PPARγ and Foxo1 (Fig. 1*A*). *In vitro* protein interaction assays were conducted to verify the identification of Foxo1 as a PPARγ-interacting protein. A GST-Foxo1 fusion protein interacted much more efficiently with PPARγ than with other PPAR family members (PPARα and PPARδ) in the absence of any PPAR ligands (Fig. 1*B*). The ability of rosiglitazone to stimulate the interaction between Foxo1 and PPARγ was not as pronounced in this assay as compared with that in the two-hybrid assay.

Foxo1 Is Expressed in the 3T3-L1 Preadipocyte Cell Line and Responds to Insulin—Because the Foxo1 clone was isolated from an adipocyte library, we examined the protein expression level of this transcription factor during adipogenesis, a cellular process that is highly dependent on PPARγ function. 3T3-L1 preadipocytes were induced to differentiate and whole cell extracts were prepared every 48 h after induction. Foxo1 protein was detectable in uninduced preadipocytes (*D0*, Fig. 2*A*). The expression level increased progressively in differentiating cells with a peak around day 4 followed by a slight decrease in fully differentiated 3T3-L1 adipocytes (day 8 (*D8*) in Fig. 2*A*). Foxo1 protein was also detectable in mouse adipose tissue (*EWAT*, Fig. 2*A*). PPARγ was highly induced during adipogenesis as expected (Fig. 2*A*; Refs. 2–4). Thus, Foxo1 and PPARγ are coexpressed during adipogenesis.

Several investigators have shown that Foxo family members are excluded from the nucleus in response to insulin or other growth factors (see Introduction). At least one mechanism involves insulin/growth factor-stimulated activation of Akt which in turn phosphorylates Foxo proteins. The amount of Foxo1 protein present in nuclear extracts from insulin-treated 3T3-L1 adipocytes was much less than that compared with untreated adipocytes (Fig. 2*B*). These results suggest that in 3T3-L1 adipocytes, insulin inactivates Foxo1 by preventing accumulation of Foxo1 in the nucleus. This response is consistent with that observed in other cell lines and tissues upon exposure to insulin or other growth factors.

PPARγ Antagonizes Foxo1—Transcription factor-driven reporter assays were used to examine the effects of PPARγ on Foxo1 activity and vice versa. 293T cells were chosen to conduct these assays because 3T3-L1 preadipocytes and adipocytes transfect at a relatively low efficiency and often yield inconsis-

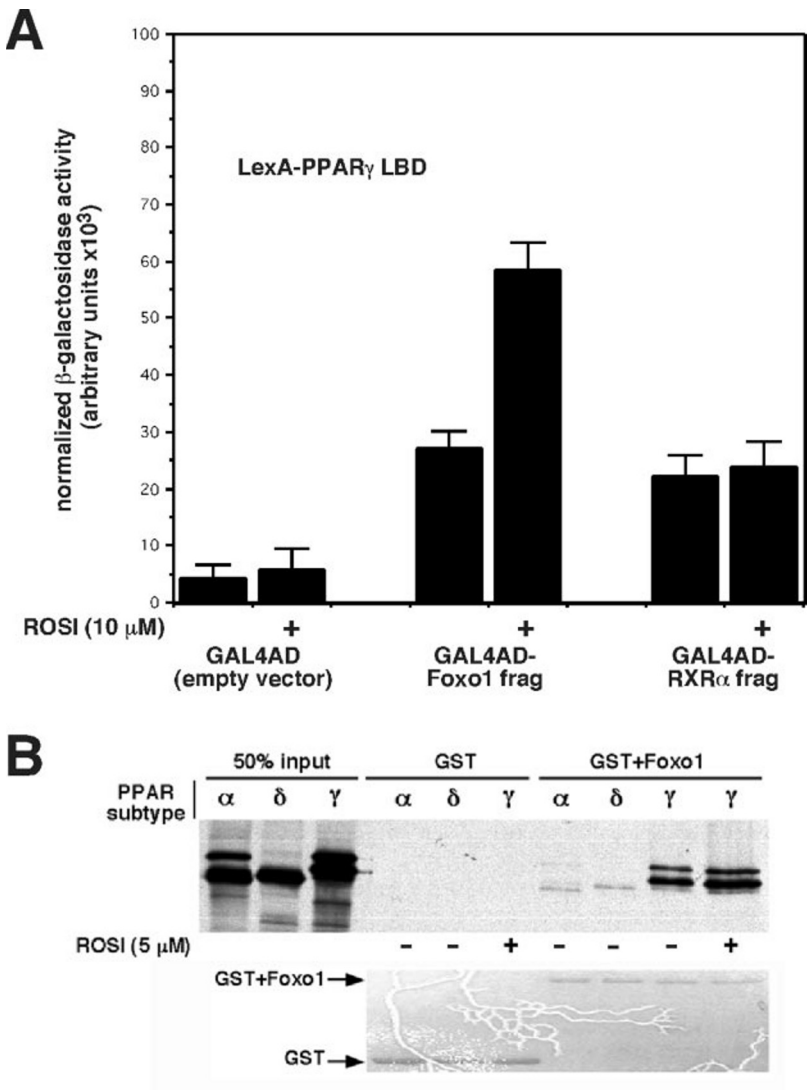

FIG. 1. **Identification of Foxo1 as a PPARγ-interacting protein.** *A*, yeast two-hybrid quantitative β-galactosidase assay. As indicated, various combinations of bait and prey plasmids were transformed into a yeast reporter strain containing an integrated LexA-driven β-galactosidase reporter gene. The bait plasmid pBTM116-PPARγ encodes a LexA DNA binding domain-PPARγ ligand binding domain (*LBD*) fusion. The prey plasmids pAD-GAL4–2.1-Foxo1 and pAD-GAL4–2.1-RXRα encode GAL4 activation domain (*AD*) fusion proteins with Foxo1 amino acids 436–652 (F*oxo1 frag*) and RXRα amino acids 257–467 (*RXRα frag*), respectively. Transformed yeast were grown in the absence or presence of rosiglitazone for 18 h before determining β-galactosidase activity, which was normalized to yeast cell number. *B*, *in vitro* protein interaction assays. GST or GST fused to Foxo1 amino acids 436–652 (*GST+Foxo1*) was bound to glutathione-Sepharose beads and incubated with radioactively labeled PPARs in the absence and presence of rosiglitazone as indicated. The beads were washed and bound proteins eluted. Half the amount of PPARs added to each reaction is shown in the *first three lanes*. The *bottom panel* shows a Coomassie stain of the gel demonstrating equal GST and GST + Foxo1 loading per binding reaction.

tent results. The Foxo1 binding sequence from the IGFBP-1 gene (25) was placed upstream of a minimal promoter and luciferase reporter gene. When cotransfected with a Foxo1 expression vector, this luciferase reporter construct was activated 16-fold (Fig. 3). Cotransfection with increasing amounts of expression vectors encoding PPARγ and RXRα resulted in a dose-dependent inhibition of Foxo1-driven reporter activation (Fig. 3, ~50% inhibition at maximal amount of PPARγ and RXRα cotransfected). As expected, treatment with IGF-I inhibited Foxo1-driven luciferase reporter activity (Fig. 3, ~50% inhibition). Further inhibition was observed when PPARγ and RXRα were cotransfected in combination with IGF-I treatment (Fig. 3, ~85% inhibition). Surprisingly, rosiglitazone alone inhibited Foxo1 activity slightly (Fig. 3, ~20% inhibition) perhaps due to low but significant amounts of endogenous PPARγ. Rosiglitazone further augmented the inhibitory effect of PPARγ and RXRα on Foxo1 activity (Fig. 3, ~80% inhibition). Combined treatment with IGF-I and rosiglitazone attenuated Foxo1 activity (~65% inhibition) and, in combination with PPARγ and RXRα, reduced Foxo1 activity to near basal levels (Fig. 3, >90% inhibition). Thus, a functional PPARγ complex (*e.g.* a PPARγ/RXRα heterodimer, see Introduction) repressed Foxo1 activity.

Foxo1 Antagonizes PPARγ—The effect of Foxo1 on PPARγ activity was examined in a manner similar to that described above. The PPARγ/RXRα binding site within the 422/aP2 gene (encoding a lipid-binding protein that is highly induced during adipogenesis; Refs. 4 and 39) was placed upstream of a minimal promoter and luciferase reporter gene. As expected, either PPARγ or RXRα alone did not strongly stimulate reporter activity (Fig. 4). However, when cotransfected with both PPARγ and RXRα, the luciferase reporter gene was induced ~10- and 25-fold in the absence and presence of rosiglitazone, respectively (Fig. 4). Cotransfecting increasing amounts of Foxo1 resulted in a dose-dependent inhibition of reporter activity both in the presence and absence of rosiglitazone (Fig. 4, ~50% inhibition at maximal amount of Foxo1 cotransfected). This latter effect of Foxo1 was markedly reduced in the presence of IGF-I (Fig. 4, *right group* of *bars*), which is expected to prevent nuclear accumulation of Foxo1 (17–21). In contrast, IGF-I treatment did not alter the inhibitory effect of a constitutively active Foxo1 (Foxo1AAA). The Foxo1AAA mutant in which the three principal Akt phosphorylation sites have been replaced by alanine is resistant to growth factor-induced phosphorylation and cannot be inactivated by nuclear exclusion (17–21). Consistent with this assumption, Foxo1AAA was even more potent than wild type Foxo1 in inhibiting PPARγ/RXRα activity (Fig. 4, ~70% *versus* ~50% inhibition, respectively).

To examine whether the antagonistic effect of Foxo1 is dependent on Foxo1 binding to DNA, we examined a Foxo1AAA mutant in which the conserved His^{212} residue has been replaced by arginine (Foxo1AAA/His^{212}). The $His^{212} \rightarrow$ Arg mutation has been shown to markedly impair the DNA binding activity of Foxo1 (40) and we confirmed these results (data not shown). Therefore, Foxo1AAAA/His^{212} represents a constitu-

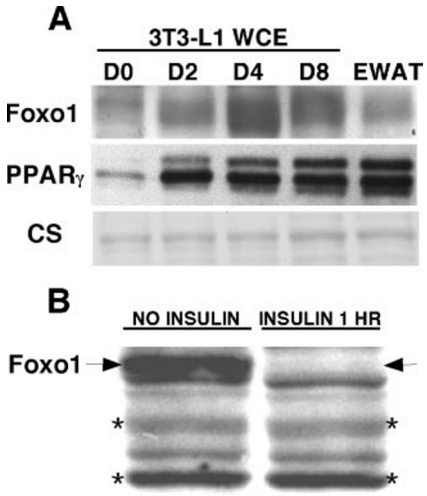

FIG. 2. **Foxo1 expression during adipogenesis and effect of insulin on nuclear localization.** *A*, whole cell extracts (*WCE*) from 3T3-L1 preadipocytes were prepared before differentiation (day 0 = *D0*) and 2, 4, and 8 days (*D2*, *D4*, and *D8*, respectively) after induction of adipogenesis. The 3T3-L1 extracts, and that from mouse epididymal white adipose tissue (*EWAT*), were subjected to immunoblot analysis with Foxo1 and PPARγ antibodies as indicated. *CS panel*, Coomassie stain of a section of the blot demonstrating equal protein loading. *B*, 3T3-L1 adipocytes at day 8 of differentiation were serum-starved for 3 h then incubated in the absence or presence of 167 nM insulin in serum-free medium for 1 h. Nuclear extracts were prepared and subjected to immunoblot analysis with Foxo1 antibody. Foxo1 immunoreactive bands, or lack thereof, are indicated with *arrows*. Nonspecific bands are indicated with *asterisks* and shown to demonstrate equal protein loading.

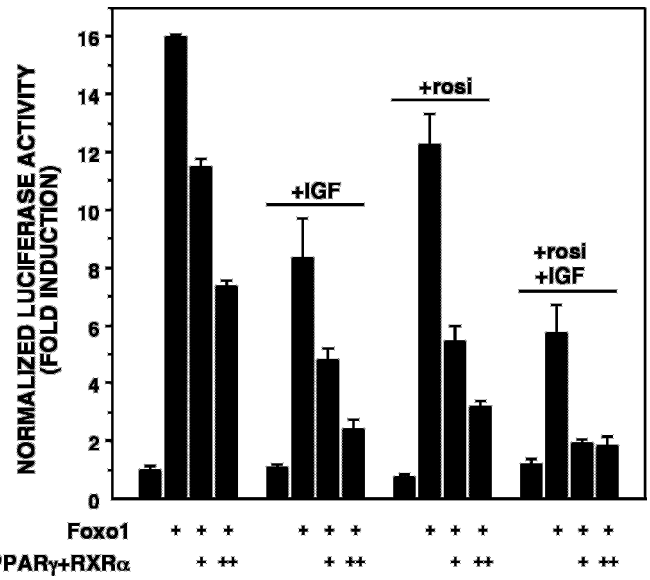

FIG. 3. **PPARγ antagonizes Foxo1.** 293T cells were cotransfected with a Foxo1-responsive luciferase reporter (100 ng), β-galactosidase plasmid (100 ng), and various combinations of expression plasmids: empty expression plasmids to establish basal activity, 200 ng of Foxo1 expression plasmid, 100 or 200 ng of PPARγ and RXRα expression plasmids (as indicated by "+" and "++," respectively). Transfected cells were exposed to IGF-I (*IGF*) (20 ng/ml) or rosiglitazone (*rosi*) (5 μM) as indicated, harvested, and assayed for β-galactosidase and luciferase activity. Luciferase activity was normalized to β-galactosidase activity to control for transfection efficiency and plotted as fold induction relative to basal activity in the absence of IGF-I and rosiglitazone.

tively nuclear, but DNA binding-defective, transcription factor. Cotransfection of Foxo1AAA/His^{212} inhibited PPARγ/RXRα-driven reporter activity to roughly the same extent as that of Foxo1AAA (Fig. 4). Thus, Foxo1 antagonizes PPARγ activity without a requirement for DNA binding. All three forms of Foxo1 examined were expressed at similar levels suggesting that the more potent antagonism exhibited by Foxo1AAA and Foxo1AAA/His^{212} was not due simply to higher expression levels (Fig. 4, *inset*).

Foxo1 Inhibits PPARγ DNA Binding Activity—The effect of Foxo1 on PPARγ DNA binding activity was examined as a potential mechanism for the observed antagonism of PPARγ activity by Foxo1. PPARγ/RXRα heterodimers readily form a detectable protein-DNA complex when incubated with a known PPARγ response element and the presence of ligand stimulates DNA binding activity (Fig. 5, compare *lanes 1* and *3*) when receptor concentrations are low as described for PPARα (see Ref. 41 for details). Adding Foxo1 to the binding reaction significantly reduced the formation of the PPARγ/RXRα/DNA complex both in the presence and absence of rosiglitazone (Fig. 5, compare *lanes 1* and *2* and *lanes 3* and *4*). These results suggest that Foxo1 antagonizes the transcriptional activation properties of PPARγ, at least in part, by inhibiting PPARγ DNA binding activity.

DAF-12 Interacts with DAF-16—Studies using *C. elegans* as a model organism suggest genetic interactions between *daf-2* (an insulin-like receptor), *daf-16* (a forkhead factor), and *daf-12* (a NHR, see Introduction). These findings, combined with our present results, led us to investigate whether DAF-12 and DAF-16 interact in a manner similar to that observed for PPARγ and Foxo1. DAF-12 was produced as a GST fusion protein and examined for interaction with several labeled proteins. DAF-12 did not interact with luciferase but a strong interaction was observed with DAF-16 (Fig. 6, *lanes 7* and *12*). Interestingly, DAF-12 also interacted with mammalian Foxo factors and with PPARγ, although these interactions were much weaker compared with that with DAF-16 (Fig. 7, *lanes 8–11*). None of the labeled proteins interacted with GST alone demonstrating the specificity of the interactions (Fig. 7, *lanes 1–6*). Thus, DAF-12 interacts with DAF-16 paralleling the interaction between PPARγ and Foxo1. Considering the weak but detectable interaction between DAF-12 and mammalian Foxo factors, it seems plausible that interactions between NHRs and forkhead factors may be conserved through evolution.

DISCUSSION

There are precedents for convergence of NHR and forkhead factor signaling pathways. In one study, Foxo1 antagonized estrogen (ER), progesterone, and glucocorticoid receptor activities while enhancing retinoic acid and thyroid hormone receptor activities (42). In a second study, Foxo1 enhanced ER activity, while ER antagonized Foxo1 activity (43). In a third study, the androgen receptor (AR) antagonized the ability of Foxo1 to bind DNA and to activate transcription (44). We report here that Foxo1 antagonized the ability of PPARγ to bind DNA and to activate transcription and that PPARγ antagonized Foxo1 activity. In addition, we have observed a strong ligand-dependent interaction between another NHR, liver X receptor α, and Foxo1.[2] While some of the findings of the aforementioned studies are conflicting, perhaps due to functional analyses in different cell lines, the evidence supporting convergence (either protagonistic or antagonistic) of NHR and forkhead factor signaling pathways is prevalent, and in all cases protein-protein interactions between NHRs and forkhead factors were found. Our observation that DAF-12 and DAF-16 interact *in vitro* suggests that convergence of these pathways may also occur in nematodes. Furthermore, the finding that DAF-12 can interact with both *C. elegans* and mammalian forkhead factors indicates that such protein-protein interactions may be conserved through evolution.

[2] P. Dowell and M. D. Lane, unpublished results.

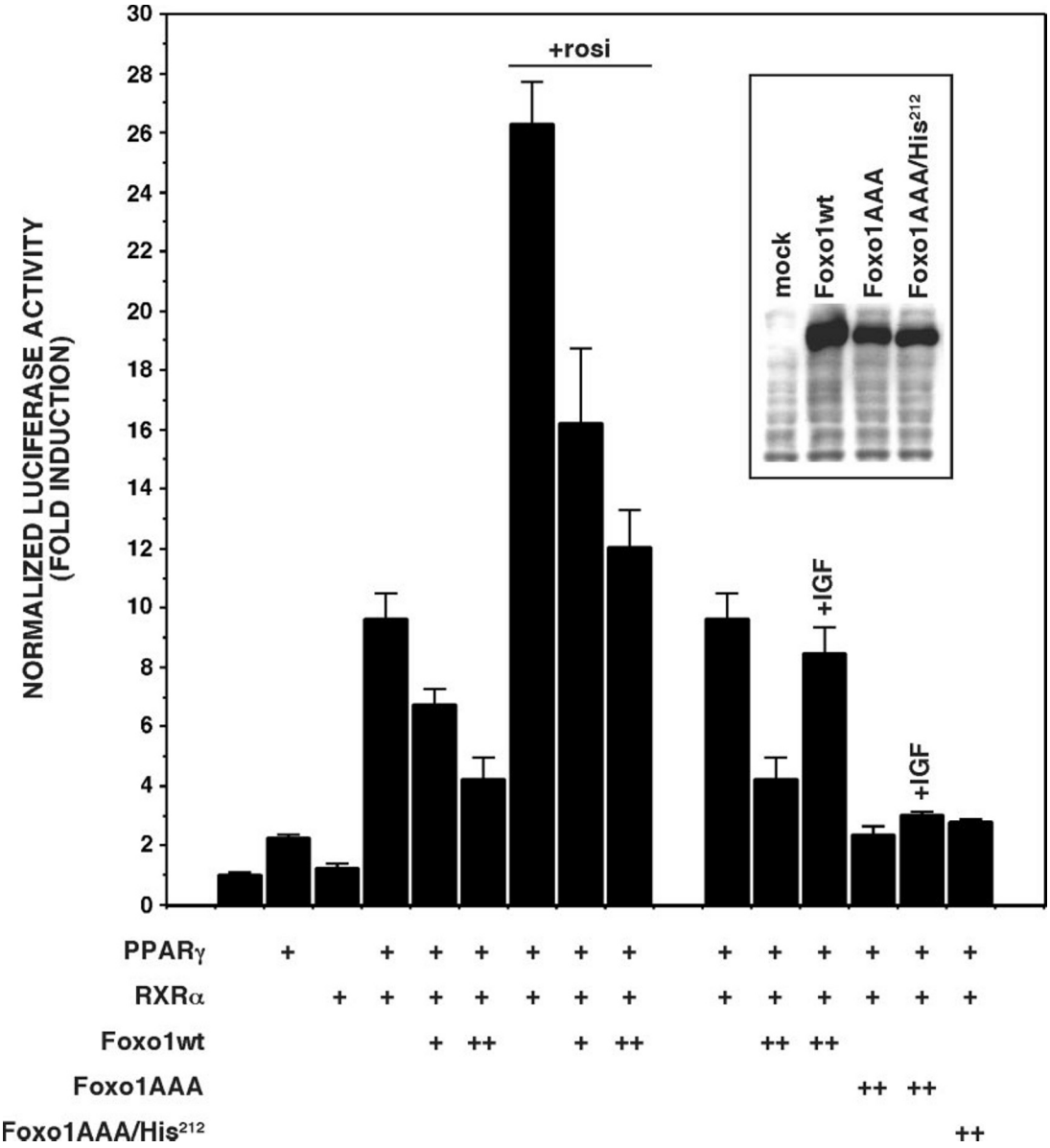

FIG. 4. **Foxo1 antagonizes PPARγ.** 293T cells were cotransfected as described in the legend to Fig. 3 with the indicated plasmids but with a PPARγ-responsive luciferase reporter in place of the Foxo1-responsive reporter. The DNA amounts transfected were as described in the legend to Fig. 3 (*i.e.* 100 ng of reporter, 100 ng of β-galactosidase plasmid, 200 ng of PPARγ and/or RXRα, and 100 or 200 ng of Foxo1 as indicated by "+" and "++," respectively). Transfected cells were exposed to IGF-I (*IGF*) (20 ng/ml) and rosiglitazone (*rosi*) (5 μM) as indicated, harvested, and assayed for β-galactosidase and luciferase activity. Addition of IGF-I alone did not significantly alter PPARγ/RXRα activation of the luciferase reporter (data not shown). *Inset*, whole cell extracts from 293T cells transfected with equal amounts of the indicated Foxo1 expression plasmids were subjected to immunoblot analysis with HA antibodies to demonstrate equal expression levels. All expressed Foxo1 cDNAs contain HA-epitope tags.

Evidence is presented to suggest that Foxo1 inhibits PPARγ DNA binding activity, thus offering one mechanism whereby Foxo1 antagonizes PPARγ activity. A similar mechanism may be responsible for AR-dependent antagonism of Foxo1, because Foxo1 DNA binding activity was perturbed by AR (44). Competition for limiting amounts of coactivator proteins represents an additional and plausible mechanism, because both NHRs (45, 46) and forkhead factors (34) utilize p300/cAMP-responsive element-binding protein-binding protein (CBP) and steroid receptor coactivator (SRC-1). CBP, SRC-1, and AIB1 were unable to relieve antagonism of Foxo1 by AR (44). Likewise during our experiments CBP, p300, and SRC-1 were unable to relieve the reciprocal antagonism between PPARγ and Foxo1,[2] which does not support competition for some coactivators as a mechanism. Recent evidence indicates that the list of coactivator proteins shared by NHRs and forkhead factors should be extended to

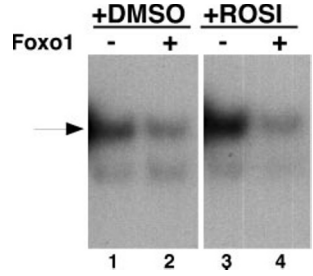

FIG. 5. **Foxo1 inhibits the DNA binding capacity of PPARγ.** PPARγ and RXRα were incubated with a labeled probe in the absence (+*DMSO*) and presence of rosiglitazone (+*ROSI*) and the resulting complexes allowed to assemble. Foxo1 (or luciferase programmed lysate as a negative control) was then added where indicated and examined for effects on the ability of PPARγ/RXRα heterodimers to bind DNA. The PPARγ-RXRα-DNA complex is indicated with an *arrow*.

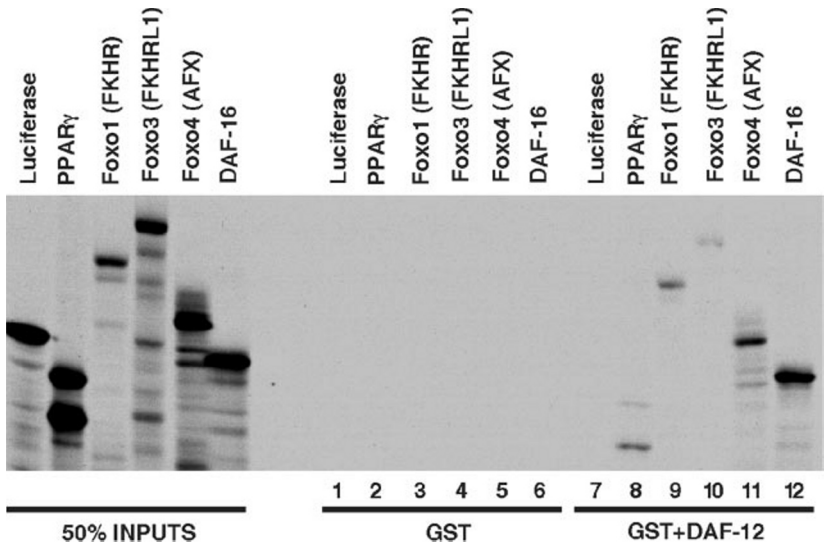

FIG. 6. **DAF-16 interacts with DAF-12.** *In vitro* protein interaction assays were conducted as described in the legend to Fig. 1*B*. Radioactively labeled proteins examined for interaction with GST and GST+DAF-12 include luciferase (negative control), PPARγ, Foxo1, Foxo3, Foxo4, and DAF-16 as indicated.

include PPARγ coactivator (PGC-1α (47)) leading to the possibility of competition between PPARγ and Foxo1 for PGC-1α which we have not yet examined. This could represent a mechanism in some tissues like brown adipose where PPARγ (4), Foxo1 (16), and PGC-1α (48) are coexpressed. However, this seems an unlikely mechanism in other tissues like white adipose where PPARγ (4) and Foxo1 (16, 49) are expressed but PGC-1α (48) is relatively absent. It will be important to design future experiments aimed at defining the precise molecular mechanisms whereby NHR and forkhead factor signaling pathways converge.

Adipogenesis is a cellular differentiation process that is highly regulated by PPARγ (see Introduction). Several forkhead factors, including Foxc2 (50), Foxo1 (49), and Foxa2 (51), also appear to regulate adipogenesis or adipocyte function at some level. In mice, adipose-selective overexpression of Foxc2 prevents diet-induced obesity and insulin resistance (50). Expression of a constitutively active Foxo1 (termed Foxo1AAA in our experiments) in an established preadipocyte cell line prevents adipogenesis *in vitro* and *Foxo1*± mice are less susceptible to diet-induced insulin resistance (49). Expression of Foxa2 in a preadipocyte cell line prevents adipogenesis *in vitro*, while *Foxa2*± mice exhibit enhanced susceptibility to diet-induced obesity (51). Forkhead factor activation of downstream target genes has been proposed as a likely mechanism for the effects of these factors on adipocyte biology and energy homeostasis. As reported by Nakae *et al*. (49), we have also observed that Foxo1AAA inhibits adipogenesis. Surprisingly, a constitutively active, DNA binding-defective forkhead factor, Foxo1AAA/His^{212} (as described in the legend to Fig. 4), inhibited adipogenesis as effectively as DNA binding competent Foxo1AAA.[2] We are currently testing the hypothesis that at least some of the antiadipogenic effects of Foxo1 result from antagonistic convergence of Foxo1 and PPARγ signaling and that these effects are not solely dependent upon activation of Foxo1 target genes. Furthermore, it is of interest to determine whether other forkhead factors, like Foxc2 and Foxa2, influence or are influenced by PPARγ or other NHRs.

When interpreting the results presented here, it is interesting to consider several points about insulin action, PPARγ and forkhead factors. Both insulin and PPARγ are proadipogenic (9, 52), while constitutively active Foxo1 is antiadipogenic (49). Both PPARγ activation (through thiazolidinedione ligands; Ref. 53) and Foxo1 inactivation (through hepatic expression of a dominant negative Foxo1; Ref. 54) improve fasting hyperglycemia in diabetic rodents. Both insulin (19) and PPARγ (described here) negatively impinge on Foxo1 signaling. Thus, it appears that in many instances, insulin and PPARγ function cooperatively in opposition to Foxo1 and vice versa. Convergence of PPARγ and Foxo1 signaling may represent an important mechanism regulating adipogenesis, glucose homeostasis, and insulin sensitivity.

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