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 multiple 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)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 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
were maintained and induced to differentiate as described previously (35). Construction of the 3T3-L1 adipocyte library was described previously (21). Foxo1AAA/His212 (His212 mutated to Arg) was expressed 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 297–467, 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 (26). 293T cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and transfected using standard calcium phosphate reagents. Quantities of plasmids transfected per 6-cm plate were: 100 ng 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 DNAAs 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. 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/potato/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 297–467, respectively. Construction of the 3T3-L1 adipocyte library was described previously (35).

**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. 1A). 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. 1A). 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 PPARβ) in the absence of any PPAR ligands (Fig. 1B). 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. 2A). 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. 2A). Foxo1 protein was also detectable in mouse adipose tissue (EWAT, Fig. 2A). PPARγ was highly induced during adipogenesis as expected (Fig. 2A; 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. 2B). 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-
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Fig. 1. Identification of Foxo1 as a PPARγ-interacting protein. A, yeast two-hybrid quantiative 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 plasmids 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 (Foxo1 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+Fboxo1) 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 His212 residue has been replaced by arginine (Foxo1AAA/His212). The His212→Arg mutation has been shown to markedly impair the DNA binding activity of Foxo1 (40) and we confirmed these results (data not shown). Therefore, Foxo1AAA/His212 represents a constitu-
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**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/PPARγ 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/PPARα 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. 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 NHRs 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.

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*a* P. Dowell and M. D. Lane, unpublished results.
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

![Identification of Foxo1 as a PPARγ-interacting Protein](http://www.jbc.org/)

**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 RXR, 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.

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include PPARγ coactivator (PGC-1α) leading to the possibility of competition between PPARγ and Foxo1 for PGC-1α functions. We have not yet examined this competition. 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 Foxo2 (50), Foxo1 (49), and Foxo2 (51), also appear to regulate adipogenesis or adipocyte function at some level. In mice, adipose-selective overexpression of Foxo2 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 Foxo2 in a preadipocyte cell line prevents adipogenesis in vitro, while Foxo2−/– 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/His12 (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 competition between 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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Fig. 6. DAF-16 interacts with DAF-12. In vitro protein interaction assays were conducted as described in the legend to Fig. 1B. 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.
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