Developmentally Regulated N-terminal Variants of the Nuclear Receptor Hepatocyte Nuclear Factor 4α Mediate Multiple Interactions through Coactivator and Corepressor-Histone Deacetylase Complexes

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To understand the mechanisms governing the regulation of nuclear receptor (NR) function, we compared the parameters of activation and repression of two isoforms of the orphan receptor hepatocyte nuclear factor (HNF) 4α. HNF4α7 and HNF4α1 differ only in their N-terminal domains, and their expression in the liver is regulated developmentally. We show that the N-terminal activation function (AF)-1 of HNF4α1 possesses significant activity that can be enhanced through interaction with glucocorticoid receptor-interacting protein 1 (GRIP-1) and cAMP response element-binding protein-binding protein (CBP). In striking contrast, HNF4α7 possesses no measurable AF-1, implying major functional differences between the isoforms. Indeed, although HNF4α1 and HNF4α7 are able to interact via AF-2 with GRIP-1, p300, and silencing mediator for retinoid and thyroid receptors (SMRT), only HNF4α1 interacts in a synergistic fashion with GRIP-1 and p300. Although both isoforms interact physically and functionally with SMRT, the repression of HNF4α7 is less robust than that of HNF4α1, which may be caused by an increased ability of the latter to recruit histone deacetylase (HDAC) activity to target promoters. Moreover, association of SMRT with HDACs enhanced recruitment of HNF4α1 but not of HNF4α7. These observations suggest that NR isoform-specific association with SMRT could affect activity of the SMRT complex, implying that selection of HDAC partners is a novel point of regulation for NR activity. Possible physiological consequences of the multiple interactions with these coregulators are discussed.

Members of the nuclear receptor (NR) family superfamily regulate a broad array of physiological processes (1). Their effects are elicited by either activation or repression of a set of genes that harbor DNA motifs recognized by NRs. Although mechanisms of activation are well understood today, repression is beginning to be recognized as a key player in homeostasis (2–4).

Gene activation is generally associated with relaxed chromatin structure, which is facilitated by acetylation and methylation of histone N-terminal tails through the recruitment of acetyltransferases or methyltransferases (5, 6). Recruitment of these coregulators to target promoters occurs mainly through direct physical interaction with one of the two activation function modules of NRs, the ligand-dependent AF-2 (7). The role of the other AF, AF-1, is less well studied. Nevertheless, increasing evidence suggests that AF-1 is involved in recruitment of coactivators such as Src-1, GRIP-1, and CBP (8–11). The mechanisms of cooperative action of the two AFs within a single NR are by and large poorly understood, but it has been shown for estrogen receptor α that the transcription intermediary factor TIF2 mediates such cooperation (12).

Gene repression is associated with recruitment of histone deacetylases (HDACs) that remove acetyl groups from histones, provoking the recondensation of chromatin (5, 6). Repression is achieved by recruiting corepressors to regulatory regions through direct physical association with NRs or other transcription factors (13–17). The corepressors SMRT (silencing mediator for retinoid and thyroid receptors) and NCoR (nuclear receptor corepressor) contain two receptor interacting domains (RID1 and RID2) that mediate interactions with NRs (18, 19). In most cases, interaction with the RIDs occurs in the absence of the corresponding ligand (20). Moreover, different NRs exhibit a preference for association with NCoR or SMRT (21) and for one RID within the corepressors (18, 22, 23). SMRT and NCoR (other additional components of the corepressor complexes, including HDACs, through their silencing domains (4, 16, 24). The search for partners of SMRT and NCoR has revealed an increasing number of HDACs.

HDACs have been classified on the basis of their relatedness to yeast homologs. The mammalian class I HDACs (HDAC1, 2, and 3) are related to RPD3, and HDACs of class II (HDAC4, 5, 6, and 7) share similarities with HDA1 (25–28). There seems to be a selectivity for association with HDACs because it has recently been established that SMRT functions as an activating cofactor for HDAC3 but not for HDAC4, 5, or 7 (29, 30). Instead, HDAC4 needs to be bridged to a SMRT-HDAC3 complex to
become active (29). Regulated binding of HDACs might play a role in determining SMRT corepressor activity on promoters, thereby constituting a critical level of regulation for NR activity which integrates the diversity of HDACs in the current model for repression of transcription. No data concerning the occurrence and consequences of recruitment of HDAC family members to different promoters have been documented.

HNF4 is a member of the NR superfamily and is highly expressed in the liver (31). A key role for HNF4α in development and hepatic differentiation and homeostasis has been demonstrated unambiguously (32–34). A number of isoforms of HNF4α resulting from alternative splicing and alternative promoter usage have been identified (35), but their roles in development and differentiation have not been examined. The activity of HNF4α1, the first isoform identified, has been shown to be repressed by SMRT (36) and enhanced by association with CBP/p300 and GRIP-1 (37–39). HNF4α2, a C-terminal splice variant, exhibits enhanced interaction with coactivators (38). We showed recently that HNF4α7, resulting from alternative promoter usage, is expressed during the early stages of liver development, prior to HNF4α1, and that it exhibits transcriptional activity different from that of HNF4α1 (40). Beyond these findings, nothing is known about functional differences in the HNF4α isoforms.

To contribute to understanding the mechanisms governing the regulation of NR function, we have compared the parameters of activation and repression of the two isoforms of HNF4α which differ only in their N-terminal domains. We show here that the N-terminal AF-1 of HNF4α1 possesses significant activity that can be enhanced through interaction with GRIP-1 and CBP. In striking contrast, HNF4α7 possesses no measurable AF-1, implying major functional differences between isoforms. Indeed, we were able to show that although HNF4α1 and HNF4α7 are both able to interact via the AF-2 with GRIP-1, p300, and SMRT, only HNF4α1 is able to interact in a synergistic fashion with both GRIP-1 and p300. Although both factors show physical and functional interaction with SMRT and HDACs, the repression of HNF4α7 is less robust than that of HNF4α1. Our determination that they recruit HDAC activity in a different fashion suggests a biochemical basis for their different roles in transactivation of target genes. Our observations strongly suggest that both qualitative and quantitative differences ranging from the nature of the recruited coregulator to the selectivity of its association with direct effectors of histone modification such as HDACs can act simultaneously to modulate transcriptional activity of NRs.

EXPERIMENTAL PROCEDURES

Plasmids—The empty pCB6 vector (41) and the CMV.HNF4α1.VSV (42) and CMV.HNF4α7.VSV expression vectors (40) containing the full-length rat cDNAs for the corresponding isoforms are described elsewhere. The apoA-I reporter pZL.HIV.LTR.AI-4 (38) and the apoC-III.Luc reporter (41) are described elsewhere. The pSG424 plasmid contains the EcoRI sites of the pGEX-3x-6His vector (Amersham Biosciences). The cDNA with the 6His tag in 3′ derived from the pGEX-3x-6His vector was then excised by EcoRI and Xhol and ligated into the pRSET5d vector (52). The constructs were verified by sequencing. The GST-SMRT fusion proteins constructs, GST-SMRT.RID1 (amino acids 1055–1495), from human SMRT have been described previously (19).

Transient Transfections—HNF4α-deficient NIH3T3 and 293T cells (40, 53) were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum at 37 °C under a 5% CO2 atmosphere. For transient transfection assays, 2 × 106 cells were seeded in six-well plates and transfected by the calcium phosphate coprecipitation procedure described next. To examine the effect of promoter reporter and various amounts of expression vectors as indicated in the figure legends. Equivalent molar amounts of empty vector were added to equalize the DNA amount. Glycerol shock was carried out 5 h later, and cells were harvested 24 h after the shock. β-Galactosidase activity was measured by the standard colorimetric method, and luciferase activity was determined with a Berthold LuminoMetric 9501 as described (54). For the one-hybrid assay, the indicated amounts of the pSG424 or the GAL4.HNF4α plasmids along with 1 µg of Tpl.GAL4.Luc, various amounts of CMV.SMRT, and 100 ng of CMV.β-gal were used. For TSA treatment, 100 ng of trichostatin A (Sigma) or vehicle (dimethyl sulfoxide) was added 6 h before harvesting. For immunoprecipitations, 6 × 105 cells were seeded 1 day before transfection and were transfected by the same procedure with 15 µg of expression vectors as indicated in the figure legends.

Immunoprecipitations and Western Blot Analysis—Transfected cells were rinsed with ice-cold phosphate-buffered saline, resuspended in 1 ml of lysis buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40, 5% glycerol), and incubated for 30 min at 4 °C. Lysates were cleared by centrifugation and incubated for 1 h with either the e445 antisera (31) directed against the C-terminal region of HNF4α1/α7, or with an anti-SMRT antibody (sc-1612, Santa Cruz Biotechnology) and then overnight with protein A or protein G PLUS-agarose (Santa Cruz Biotechnology). Beads were pelleted by centrifugation, washed with the same buffer, and resuspended in 20 µl of some buffer. For the double pulldown experiments, the lysis buffer was 200 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40, 5% glycerol. Beads were washed four times with lysis buffer and twice with 0.01% Nonidet P-40 NET-N (20 mM Tris-Cl, pH 8.0, 1 mM EDTA, 100 mM NaCl). Whole cell extracts and immunocomplexes were subjected to Tris-glycine SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (Millipore). The primary antibodies were a monoclonal anti-VSV antibody coupled to peroxidase (Roche Molecular Biochemicals), the e445 antisera, the anti-SMRT antibody, and an anti-GAL GALB DBD antibody (sc-577, Santa Cruz Biotechnology). When required, goat anti-rabbit or donkey anti-goat horseradish peroxidase antibodies were used. Peroxidase activity was detected by ECL (Amersham Biosciences).

GST Pulldown Assays and Protease Digestion—All protein–protein interaction analyses were performed as described previously (38) using agarose-immobilized GST fusion proteins. [35S]Methionine-labeled proteins were produced with the TNT system (Promega) using pSET.HNF4α1.6His, pSET.HNF4α7.6His, pETV859.HDAC3.FLAG (48), or pL67.HDAC4.FLAG (49) plasmid. Where indicated, 2 µl of the TNT product was incubated for 10 min on ice with 200 ng of double-stranded oligonucleotides in 100 µM HEPES, 10 mM MgCl2, 2.5 mM EGTA, 5 mM dithiothreitol, and 2% Ficol. The apoc-II oligonucleotide used is 5′-TGAGGCGCTGGCGAAGGCTCAGTCG-3′. The two GAL-A/B digestion assays and the HNF4α and HNF4α7 constructs were prepared by inserting the sequence encoding the A/B domains of rat HNF4α1 or HNF4α7 into-frame into the EcoRI sites of the pSG424 vector. For the GAL4.HNF4α.A/B constructs, rat cDNAs encoding truncated HNF4α1 and HNF4α7 proteins were cloned into the EcoRI sites of the pSG424 plasmid. The constructions were verified by sequencing. The CMV.β-gal plasmid (California Biotechnology Inc.) contains the lacZ gene under control of the CMV promoter.

The pSET.HNF4α1.6His and pSET.HNF4α7.6His vectors contain the full-length cDNA of rat HNF4α1 and HNF4α7, respectively. These plasmids were constructed as follows. The cDNAs for each of the isoforms were amplified by PCR and ligated into the HindIII site of the pGEX-3x-6His vector (Amersham Biosciences). The DNA with the 6His tag in 3′ derived from the pGEX-3x-6His vector was then excised by EcoRI and Xhol and ligated into the pRSET5d vector (52). The constructs were verified by sequencing. The GST-SMRT fusion proteins constructs, GST-SMRT.RID1 (amino acids 1055–1291) and GST-SMRT.RID2 (1291–1495), from human SMRT have been described previously (19).

RESULTS

HNF4α1 and HNF4α7 are isoforms of HNF4α resulting from alternative promoter usage leading to the transcription of alternative coding first exons (Fig. 1A). The two promoters are sequentially transcribed during development (40). Distal to the amino acids encoded by the first exon, the two proteins are
The A/B domain of HNF4a7 Lacks the AF-1 Function

The A/B domain of NRs is located at the N-terminal region and harbors AF-1 (Fig. 1B), one of the two activation function modules. The AF-1 of HNF4a1 has been shown to interact with members of the basal machinery and with CBP (8), providing a surface of contact with transcriptional coregulators. The A/B domain of HNF4a1 consists of 49 amino acids, and its AF-1 has been mapped to amino acids 1–24 (56). The AF-1 exhibits an overall negative charge and shows autonomous transactivation accounting for 40% of HNF4a1 activity as tested by the one-hybrid assay (56). As shown in Fig. 1B, the corresponding region in HNF4a7 contains completely unrelated noncharged amino acids. Thus, we first questioned whether the A/B domain of HNF4a7 possesses an AF-1 function.

The regions corresponding to the A/B domains of HNF4a1 and HNF4a7 were fused to the DBD of GAL4, and one-hybrid assays were performed to test transactivation potentials. 293T cells were transfected with 300 ng of one or the other of the GAL4.HNF4.A/B constructs and a reporter for GAL4. As has been shown previously, the A/B domain of HNF4a1 showed a robust transactivation activity in this assay, provoking a 120-fold activation compared with the GAL4 construction (Fig. 1C). This activity is caused entirely by the presence of the AF-1 domain (8). In striking contrast, the HNF4a7/A/B construct showed no activity, and increasing the GAL4.HNF4a7/A/B plasmid amount up to 5 μg still did not result in transactivation of the reporter gene (data not shown). Nonetheless, it is possible that the A/B domain of HNF4a7 is constitutively repressed by endogenous HDAC activity. To exclude this possibility we measured the transcriptional activity of the GAL4.HNF4a7.A/B construct in the presence of the HDAC inhibitor TSA and still failed to detect transactivation (data not shown). The lack of activity was not the result of the absence of the protein because the two fusion proteins were detected by Western blot analysis using an anti-GAL4 DBD antibody (Fig. 1D). These experiments demonstrate unambiguously that HNF4a7 does not contain an AF-1 function.

It could not be ruled out, however, that the A/B region of HNF4a7 tethers coactivators to target promoters. To test this hypothesis we performed the same one-hybrid experiments in the presence of CBP or GRIP-1, which belong to different families of coactivators.

Coactivators Increase HNF4a7 Activity Only through Its AF-2

CBP and GRIP-1 Coactivate the AF-1 of HNF4a1—NIH3T3 cells were cotransfected with 500 ng of pSG424, GAL4.HNF4a1.A/B, or GAL4.HNF4a7.A/B plasmid, the Tp1.GAL4.Luc reporter plasmid, and 5 μg of an expression vector for GRIP-1 or CBP. CBP elicited a modest but reproducible increase in the transcriptional activity of the GAL4.HNF4a1.A/B construct (Fig. 2A, left panel). In contrast, even in the presence of CBP, the A/B domain of HNF4a7 failed to display substantial activity (Fig. 2A). In the case of the p160 family member GRIP-1, an increase of 5.9-fold of activity was elicited for the construct containing HNF4a1.A/B, but no stimulation of activity of the GAL4.HNF4a7.A/B construct was observed (Fig. 2A, right panel).

GRIP-1 Coactivates HNF4a7 through Its AF-2—The AF-2 module of NRs is located in the E domain and, in the case of HNF4a1, has been shown to interact functionally with GRIP-1 (38). To verify that the AF-2 of HNF4a7 is functional and can
Luciferase activity was normalized to amounts of the pSG5.GRIP-1 plasmid and the Tp1.GAL4.Luc reporter. 100 ng of the GAL4 plasmids was cotransfected with increasing amounts of GRIP-1 does not coactivate the A/B domain of HNF4.

Bars represent the fold induction relative to transcriptional activity of the GAL4 plasmid alone. Numbers above the bars represent the fold induction relative to transcriptional activity of GAL4.HNF4a.A/B constructs in the absence of coactivators. Results are the mean ± S.E. of three independent experiments.

be coactivated, we used a longer fusion protein, GAL4.HNF4a7.A/F, which includes the A/B through the E domains (Fig. 2B). NIH3T3 cells were cotransfected with GAL4, GAL4.HNF4a7.A/B, or GAL4.HNF4a7.A/F with various amounts of GRIP-1 expression vector and the Tp1.GAL4.Luc plasmid. Again, the A/B domain fused to GAL4 failed to induce any transcriptional activity (Fig. 2C). However, the GAL4.HNF4a7.A/F construct did elicit a transcriptional activity that was augmented in a dose-dependent manner upon addition of increasing amounts of GRIP-1. Thus, GRIP-1 is able to coactivate HNF4a7, but only through the E domain, and presumably AF-2.

For the estrogen receptor α and glucocorticoid receptor, for which the agonists are well characterized, the AF-1 and AF-2 modules have been shown to synergize through the action of coregulators (9, 12). We wondered whether the AF-1 and AF-2 of HNF4α are able to synergize via the p160 and p300 coactivators. Moreover, if the AF-1 is necessary for the simultaneous action of these two families of coregulators, then no synergy should be observed for HNF4α7 in the presence of GRIP-1 and p300.

**HNF4a1, but Not HNF4a7, Responds Synergistically to the p160 and p300 Coactivators**

293T cells were cotransfected with the CMV.HNF4a1.VSV or CMV.HNF4a7.VSV plasmid encoding full-length proteins, expression vectors for p300 and/or GRIP-1, and the apoA-I reporter plasmid. Both HNF4a1 and HNF4a7 transactivated the apoA-I reporter (Fig. 3). p300 coactivated both isoforms by 9-fold and 8-fold for HNF4a1 and HNF4a7, respectively (Fig. 3). GRIP-1 showed similar behavior, coactivating both isoforms, HNF4a1 by 2-fold and HNF4a7 by 4-fold (Fig. 3). When both coactivators were cotransfected with the reporter, HNF4a1 activity was increased 15-fold, which represents a synergistic rather than an additive effect compared with the effects provoked by either coactivator alone. In the case of HNF4a7, activity was increased 12-fold, which corresponds to
HNF4

apoC-III reporter contains the region /H11002 activity was normalized to internal control /H9251 extracts were immunoprecipitated with the /H9251

pressor activity for the A/B domain of HNF4 transfection assays (36). In that work, a potential role in core-SMRT of an HNF4 because deletion of this domain restored responsiveness to /p300 and GRIP-1 act independently of the A/B domain. Thus, through the combined action of GRIP-1 and p300, the two AF modules of HNF4 appear to synergize. However, this is not the case for HNF4 /H9251 in vitro migrating band as a result of the /H9251 protein was calculated with a PhosphorImager using 10% of the input of the reaction as reference. We systematically observe a second, fast SDS-PAGE autoradiographies representative of several independent experiments. The percentage of labeled HNF4 /H9251 the two AF modules of HNF4 alone. Thus, through the combined action of GRIP-1 and p300, there therefore compared the ability of SMRT to repress activity of HNF4α1 and HNF4α7. Expression vectors containing full-length HNF4α1 or HNF4α7 were cotransfected along with increasing amounts of CMV.SMRT in 293T cells. We chose to use the apoA-I and the apoC-III reporters that are more avidly activated by HNF4α1 than by HNF4α7 (Fig. 4A and Ref. 40). The relevance of the HNF4 sites for the activation of these promoters is underscored by recent findings that HNF4α controls the constitutive expression of both the apoA-I and apoC-III genes in vivo (33, 57), neither of which is expressed in fetal liver (58) when HNF4α7 activity is predominant (40).

Cotransfection with SMRT had no effect on the activity of either of the reporters in the presence of the pCB6 empty vector only, but it induced a dose-dependent decrease of the activity elicited by HNF4α1 and by HNF4α7 on both of the reporters (Fig. 4A). The effect of SMRT on the activity of both HNF4α isoforms was seen even when only 25 ng of CMV.SMRT was used in the transfections. The maximal amount of SMRT repressed the HNF4α1-mediated activation of the apoC-III and apoA-I reporters by 87 and 55%, respectively (Fig. 4A, top

the additive value for the induction elicited by each coactivator alone. Thus, through the combined action of GRIP-1 and p300, the two AF modules of HNF4α1 appear to synergize. However, this is not the case for HNF4α7, which possesses only one activation function module. Hence, synergy seems to be dependent on the presence of the AF-1 in HNF4α1. Moreover, the synergistic effect was lost when a construct bearing a mutation in the NR boxes of GRIP-1 was used (Fig. 3, GRIP-1 mut), thus indicating that the synergy is also dependent on the NR boxes that interact with the AF-2 (10). From these experiments we can conclude that in the context of the full-length HNF4α1, p300 and GRIP-1 act independently of the A/B domain.

**SMRT Corepresses HNF4α1 More Robustly than HNF4α7**

Previous work from one of our laboratories showed that the corepressor SMRT represses HNF4α1 activity in transient transfection assays (36). In that work, a potential role in corepressor activity for the A/B domain of HNF4α1 was suggested because deletion of this domain restored responsiveness to SMRT of an HNF4α1 protein truncated in the F domain. We therefore compared the ability of SMRT to repress activity of HNF4α1 and HNF4α7. Expression vectors containing full-length HNF4α1 or HNF4α7 were cotransfected along with increasing amounts of CMV.SMRT in 293T cells. We chose to use the apoA-I and the apoC-III reporters that are more avidly activated by HNF4α1 than by HNF4α7 (Fig. 4A and Ref. 40). The relevance of the HNF4 sites for the activation of these promoters is underscored by recent findings that HNF4α controls the constitutive expression of both the apoA-I and apoC-III genes in vivo (33, 57), neither of which is expressed in fetal liver (58) when HNF4α7 activity is predominant (40).

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**Fig. 4.** SMRT corepresses HNF4α1 more robustly than HNF4α7 and is recruited by both isoforms. A, 293T cells were cotransfected with 200 ng of empty (pCB6), HNF4α1, or HNF4α7 expression vector, increasing amounts of CMV.SMRT, and 1 μg of the indicated reporter. The apoC-III reporter contains the region −821 to +24 from the human apoC-III gene driving the expression of the firefly luciferase gene. Luciferase activity was normalized to internal control β-gal activity. The mean ± S.E. of two independent experiments performed in triplicate is shown. Below each graph, repression by increasing amounts of SMRT for each isoform is expressed as percent activity, 100 being basal activity of HNF4α1 or HNF4α7 on each reporter. B, Western blot analysis of transfected HNF4α isoforms using the α445 antiserum. 15 μl of the same protein extracts used for determining luciferase activity was separated by SDS-PAGE and analyzed by Western blot. The percentage of labeled HNF4α isoforms was seen even when only 25 ng of CMV.SMRT was used in the transfections. The maximal amount of SMRT repressed the HNF4α1-mediated activation of the apoC-III and apoA-I reporters by 87 and 55%, respectively (Fig. 4A, top

**C.** CMV.HNF4α1.VSV, CMV.HNF4α7.VSV, and/or CMV.SMRT as indicated. Whole cell extracts were immunoprecipitated with the α445 antiserum. Immunoprecipitate complexes (IP) were separated by SDS-PAGE and analyzed by Western blot with an anti-VSV or an anti-SMRT antibody. D, HNF4α1 and HNF4α7 interact with the RID2 of SMRT in vitro. A GST pulldown assay was performed using in vitro translated 35S-HNF4α1 or 35S-HNF4α7 and bacterially purified GST.RID1 and GST.RID2 proteins. Shown are SDS-PAGE autoradiographies representative of several independent experiments. The percentage of labeled HNF4α bound to the GST fusion protein was calculated with a PhosphorImager using 10% of the input of the reaction as reference. We systematically observe a second, fast migrating band as a result of the in vitro translation of HNF4α1 which probably corresponds to partial translation products that were not taken into account for quantification.
pression of the two isoforms. The repression of HNF4α by SMRT was much more gradual than that of HNF4α1. Western blot analysis of extracts from transfected cells revealed that these differences are not the result of different amounts of protein because both HNF4α1 and HNF4α7 are present at equivalent amounts in transfected 293T cells (Fig. 4B).

These results show that NR isoforms can be repressed differently by SMRT. We then examined whether both isoforms interact in vivo and in vitro directly with SMRT and whether differences in the affinity with which they interact with SMRT could account for the results observed in the cotransfection experiments.

To explore whether physical interaction between the HNF4α isoforms and SMRT takes place in vivo, communoprecipitation experiments were performed. 293T cells were cotransfected with the same amount of either the CMV.HNF4α1.VSV or the CMV.HNF4α7.VSV expression vector with or without CMV.SMRT. Whole cell extracts were prepared and immunoprecipitated with the α445 antiserum directed against the C-terminal region of HNF4α1 (which is shared with HNF4α7). Immunoprecipitates were analyzed by Western blot using an anti-VSV and an anti-SMRT antibody. As shown in Fig. 4C, SMRT coprecipitated with both HNF4α1 and HNF4α7 (Fig. 4C, lanes 6 and 10).

SMRT contains two silencing domains responsible for transcriptional repression and two independent RIDs that display different affinities for members of the NR family (18, 23). RID1 is contained within the region spanning amino acids 1055–1291, and RID2 is delimited by amino acids 1291–1495 (23). The ability of HNF4α1 and HNF4α7 to bind to RID1 or RID2 was tested by GST pulldown assays. GST.RID1 and GST.RID2 proteins were bacterially produced, purified, and incubated with in vitro translated 35S-HNF4α1 or 35S-HNF4α7. As shown in Fig. 4D, both HNF4α1 and HNF4α7 bind to RID2 but not to RID1 of SMRT. Indeed, previous studies have shown that HNF4α1 interacts with RID2 of SMRT in vitro (36). Moreover, the association of HNF4α1 and HNF4α7 protein bound to GST.RID2 is equivalent to 14% of the input for each of the isoforms, indicating that in vitro, HNF4α1 and HNF4α7 bind with the same affinity to RID2 of SMRT.

Our results demonstrate that SMRT interacts with both of the HNF4α isoforms in vivo and in vitro. However, these results do not explain the differences observed regarding the repression of transactivation by HNF4α1 or HNF4α7. Therefore, to determine whether the AF-1 domain of HNF4α1 plays a role in SMRT repression we examined whether SMRT is able to interact functionally with this domain and repress its activity.

**SMRT Represses the Activity of AF-2 but Not AF-1 of HNF4α**

One-hybrid experiments using the GAL4.HNF4α1.A/B construct were performed using increasing amounts of CMV.SMRT. Addition of the SMRT expression vector did not decrease the activity elicited by the A/B domain of HNF4α1 (Fig. 5A). Even when a 20-fold excess of CMV.SMRT compared with the GAL4.HNF4α1.A/B construct was used (400 ng), no repression was observed. These results show that SMRT does not repress the activity of the AF-1 domain. On the contrary, when the GAL4.HNF4α1.ΔF and GAL4.HNF4α7.ΔF constructs that include the AF-2 of both isoforms were cotransfected with increasing amounts of CMV.SMRT, repression of transcriptional activation of both HNF4α1 and HNF4α7 was observed (Fig. 5B). Thus, SMRT action is mediated solely through repression of the AF-2 activity within full-length HNF4α1 and HNF4α7.

Because the difference in repression of HNF4α1 and HNF4α7 by SMRT cannot be explained by differences in the AF-1 or by recruitment of SMRT by the two isoforms, and because SMRT has been shown to tether different HDACs to constitute the corepressor complex (4, 16, 26, 30, 59), we considered two hypotheses to explain our results. One, HNF4α7 could preferentially recruit inactive SMRT complexes to target promoters. Alternatively, the interaction of the NR isoform with SMRT could affect the transcriptional repression elicited by the recruited SMRT complex. In both cases, the ability of the two isoforms to recruit HDAC activity to target promoters should be different.

**Endogenous HDAC Activity Is Recruited to Target Promoters Differently by HNF4α1 and HNF4α7**

As a first approximation, we sought to determine whether HNF4α1 and HNF4α7 differ in their ability to recruit HDAC activity by analyzing their transcriptional activities on HNF4 reporters in the absence or presence of an inhibitor of HDAC activity, TSA. 293T cells were transiently transfected with expression vectors encoding full-length HNF4α1 or HNF4α7 together with the apoC-III or the apoA-I reporters and treated with TSA. The basal activity of both reporters was increased slightly upon TSA treatment (2.9- and 3-fold for the apoC-III and apoA-I reporters, respectively) as seen from the results...
obtained with the empty pCB6 vector (Fig. 6A). The addition of TSA had a dramatic effect on transactivation of the apoC-III reporter by HNF4α. The activity was induced 44-fold compared with HNF4α1 activity in the absence of TSA (Fig. 6A, left panel). This result indicates that HNF4α1 is highly repressed by association with endogenous HDAC activity. On the contrary, the addition of TSA increased only slightly, by 7.7-fold, the HNF4α1 activity on the apoC-III promoter. Thus, HNF4α1 recruits less HDAC activity than HNF4α1 to the apoC-III promoter. For the apoA-I reporter, the effects elicited by TSA were smaller than with the apoC-III reporter (Fig. 6B, right panel), and its addition had the same impact on the transcriptional action (Fig. 6B). In the absence of any HDAC (Fig. 6C), the SMRT/H18528—HDAC complexes were used. Because HDAC3 migrates at the same position as HNF4α7 (compare input lanes for HDAC3 and HNF4α7), we used nonlabeled HDAC3 (lanes 3–5) for further incubations with 35S-HNF4α. An 35S-HDAC3 control (lane 6) was performed in parallel. Both HNF4α1 and HNF4α7 bound to the SMRT-HDAC3 complex (Fig. 6D, lanes 4 and 5). Surprisingly, however, the presence of HDAC3 greatly increased the amount of HNF4α1 bound to the complex (from 0.1 to 1.5% of the input, compare lanes 1 and 4 in Fig. 6D) and provoked no change for HNF4α7 (compare lanes 2 and 5). Thus, it appears that the presence of HDAC3 either renders more stable the HNF4α1-SMRT-HDAC3 complex or induces an increase in affinity for HNF4α1.

In lanes 7–10 of Fig. 6D, SMRT-35S-HDAC4 complexes were used for incubation with 35S-HNF4α1 and 35S-HNF4α7. Both HNF4α isoforms were able to bind to these complexes (Fig. 6D, lanes 9 and 10), and no differences were observed concerning the binding of the two isoforms. Again, the presence of the deacetylase appears to stabilize or increase the affinity for HNF4α1 only (compare lanes 1, 2, and 9, 10). Thus, both HNF4α isoforms can associate to the two types of SMRT/HDAC class I and class II complexes in vitro. Moreover, association of SMRT with HDACs enhanced recruitment of HNF4α1 but not of HNF4α7. Thus, the presence of an HDAC affected the binding of one but not the other NR isoform.

The association of HNF4α with HDACs was constitutive as revealed by the HDAC assays on immunoprecipitated HNF4α (Fig. 6B). It is possible that once HNF4α translocates to its target, association with a SMRT/HDAC complex is determined by the promoter context. In that case, the affinity of the complex could be affected by specific DNA binding mediated by the NR partner. Indeed, binding of a NR to its target site on DNA could allosterically modulate its interaction with other proteins by provoking the acquisition of a different “DNA-bound” conformation. We tested this hypothesis indirectly by analyzing the sensitivity of both isoforms to protease digestion in the absence and presence of DNA.

**HNF4α Isoforms and Coregulator Interaction**

Because the amino acid composition of the two isoforms is different at the A/B domain, it could be anticipated that differences occur in the folding of the two proteins. We analyzed the protease sensitivity exhibited by both isoforms alone or after incubation with the apoC-III oligonucleotide (Fig. 7). Digests were carried out with trypsin on in vitro translated 35S-HNF4α1 and 35S-HNF4α7. No major differences concerning protection of the longer fragments of HNF4α1 compared with HNF4α7 were observed in the absence of DNA (compare relative band intensities in lanes digested with 1 ng/µl in Fig. 7, no DNA). Thus, no significant differences in resistance to trypsin digestion were observed between HNF4α1 and HNF4α7 in the absence of DNA. However, a slight protection of long fragments was observed upon trypsin digestion in the presence of the apoC-III oligonucleotide for HNF4α1 but not for the HNF4α7 isoform (compare lanes digested with 1 and 5 ng/µl with and without DNA in Fig. 7). These results suggest that HNF4α1 may fold differently upon DNA binding compared with its native “unbound” conformation. Such a change could confer a
FIG. 6. HNF4α1 and HNF4α7 recruit different HDAC activities to target reporters and associate with SMRT-HDAC complexes in vitro. A, 293T cells were cotransfected with 200 ng of empty (pCB6), HNF4α1, or HNF4α7 expression vector and 1 μg of the indicated reporter. Cells were incubated with or without 100 ng of TSA for 8 h before harvesting. Luciferase activity was measured and normalized to the internal control β-gal activity. Numbers above the bars indicate the fold difference of transactivation obtained in the presence of TSA. Shown are representatives of two independent experiments performed in triplicate.

B, endogenous HDAC activity coprecipitates with HNF4α. 293T cells were transfected with HNF4α1 or HNF4α7 expression vectors. Whole cell extracts were precipitated with the α445 antiserum, and immunocomplexes or crude lysate (equivalent to 1/250 of the volume used for each immunoprecipitation reaction) were assayed for HDAC activity in the absence or presence of the nonspecific inhibitor sodium butyrate.

C, diagram for the experiment performed in D. SMRT-HDAC complexes were prepared by incubating immunoprecipitated (IP) SMRT first with in vitro translated HDACs and then with in vitro translated HNF4α. As controls,
immunoprecipitated SMRT was incubated with in vitro translated HNF4α1 and HNF4α7 in the absence of HDACs. D, HNF4α associates with class I and class II HDAC-SMRT complexes in vitro. 293T cells were transfected with the CMV-SMRT plasmid. Whole cell extract was precipitated with an anti-SMRT antibody, and protein G-agarose beads were washed extensively. First, the SMRT-containing beads were incubated with in vitro translated HDAC3 (lanes 3–5), 35S-HDAC3 (lane 6), or 35S-HDAC4 (lanes 8–10). After this first incubation, beads were washed extensively to remove nonbound proteins and were subsequently incubated with either 35S-HNF4α1 (lanes 4 and 9), 35S-HNF4α7 (lanes 5 and 10), or empty vector-TNT product (lanes 1–2, 6–8). The SMRT interaction with 35S-HNF4α1 and 35S-HNF4α7 in the absence of HDACs is shown in lanes 1 and 2. Complexes were resolved by SDS-PAGE and exposed for autoradiography and PhosphorImager quantification. The percentage of bound protein was calculated using the values corresponding to the 10% input as reference. We systematically observe a second, fast migrating band as a result of the in vitro translation of HNF4α1 (which probably corresponds to partial translation products) which was not taken into account for quantification. The presence of SMRT was revealed in the same filter using an anti-SMRT antibody (bottom).
main (30). Nevertheless, the deacetylase activating domain of SMRT activates HDAC3 but not HDAC4 upon recruitment to the complex (30). Another mechanism of regulation of the corepressor complex activity has been documented in which binding of the NR to SMRT through domains other than RID prevents the assembly of the other partners of the complex (65). Here, even though recruitment of SMRT takes place, the activity of the NR is not repressed, a phenomenon known as "anti-repression" (65).

Thus, SMRT-mediated repression must be considered a multistep process, and each step is potentially open to regulation. First, SMRT is recruited to the DNA-bound NR, and then the complex is assembled by association of mSin3A (16) or TBL1 (4) and selective recruitment of HDACs. The HDACs are in turn specifically activated by interaction with SMRT, and repression can then occur.

We showed that the HNF4α1 and HNF4α7 isoforms are repressed in a different fashion by the corepressor SMRT. Although both isoforms interact with the same region of SMRT in vitro and with apparently similar affinity, the activity of HNF4α7 is significantly less repressed in cotransfection experiments. It can be considered that HNF4α7 exhibits a mild form of antirepression compared with HNF4α1. Using the criterion of inhibition of endogenous HDAC activity by TSA, we found that both isoforms are able to recruit HDAC activity to two target promoters, but the degree of relief of inhibition was both isoform- and target promoter-dependent. Such differences of recruitment of HDAC activity could be explained by association with SMRT complexes displaying different composition/activities. In other words, HNF4α7 could preferentially recruit inactive SMRT complexes such as SMRT-HDAC4 (29) instead of SMRT-HDAC3. In support of this hypothesis, our data indicate that within a NR-SMRT-HDAC complex, the presence of class I or class II HDAC may increase binding of a particular isoform of a NR. Moreover, we showed that both isoforms are able to associate with SMRT-HDAC3 and SMRT-HDAC4 complexes in vitro. Because the activity displayed by these deacetylases differs (29), association with specific SMRT-HDAC complexes within the cell could represent a critical step in the regulation of NR activity.

Differences in protein folding resulting from DNA binding of the two isoforms could also selectively affect the recruitment of SMRT complexes. In this sense, activity of the corepressor complex may be dependent not only on the NR isoform, but on the DNA binding site as well. Indeed, HNF4α1, unlike HNF4α7, exhibited enhanced protease resistance upon specific binding to DNA (Fig. 7).

The AF-1 of NR provides a surface of contact for coactivators, but its role in repression remains to be established. Removal of the A/B domain does not affect SMRT binding to HNF4α1 in vitro, but a truncated protein lacking both the A/B and F domains showed increased binding of SMRT (36). It can be speculated that the A/B domain is involved in regulating formation of the F domain, which could then affect functional interactions with SMRT (36). Indeed, changes in the conformation of the C-terminal domain of other NRs can regulate association of the receptor with SMRT (18, 22, 66). We have shown that the AF-1 of HNF4α1 is not affected by SMRT, whose main target for repression is the AF-2 of both isoforms. For the NRs whose ligands are well characterized, repression by SMRT-NCoR occurs only for the unliganded receptor, providing a mechanism to reinforce ligand inducibility. For the orphan receptors such as HNF4α or COUP-TF, the unresponsiveness of the AF-1 to repression could provide a basis to explain their constitutive activity (67). Moreover, in the case of HNF4α isoforms, only HNF4α1 possesses a functional AF-1 that could act to antagonize SMRT repression.

Transcription factors whose activity is modulated by coactivators and corepressors can be controlled by the physiological levels of expression of coregulators. Messenger levels of SMRT, NCoR, and coactivators can vary from one cell type to another and during differentiation (68, 69). Indeed, in the liver, SMRT transcripts are entirely down-regulated during a transitory period at birth,2 at the very stage when expression of HNF4α1 is dramatically induced (40) and the liver must assume the functions necessary to render metabolism of the newborn independent of maternal circulation. Thus, a number of genes involved mainly in lipid and glucose metabolism, which are also HNF4α targets (35), must be activated within a short time. We proposed previously that HNF4α1 could be involved in activation of expression of hepatic neonatal functions because it is expressed mainly in adult liver and because it transactivates robustly reporter genes for adult hepatic functions (40). The down-regulation of SMRT could facilitate the activation of these genes through the action of HNF4α1 in the absence of a corepressor that inhibits its activity. Further, in adult liver, when rapid metabolic changes are required in response to nutritional state, SMRT is present in concert with the HNF4α isoform that is more sensitive to SMRT repression.

We have compared the functional and physical interactions with coregulators of HNF4α isoforms whose expression is developmentally regulated. The embryonic isoform, HNF4α7, possesses only one activation function, AF-2, that can interact with both coactivators and corepressors. The adult isoform, HNF4α1, possesses two activation functions, AF-1 being able to interact only with coactivators and AF-2 with both classes of coregulators (Table I). What could be the utility to the organism of expressing NR isoforms with only one or with two activation domains? One potentially important difference was documented: synergy between two different families of coactivators occurs only for HNF4α1. If coregulators can be associated simultaneously with two domains, cases not only of synergy but also of antagonism between coactivators and corepressors should be possible.

The fetal liver is in a relatively constant environment, but the adult liver is constantly aggregrated, by nutritional needs and metabolics, such that under some circumstances HNF4 target genes may need to respond simultaneously to induction and to repression. In this context, the HNF4α1 bipartite factor that is subject to synergy or to antagonism in its transcriptional capacities may be optimal. In any case, the existence of HNF4α isoforms that show differences in the recruitment of coregulators and their effectors in a site/promoter-specific context for activation and repression adds a new level of complexity and of flexibility to hepatocyte-specific gene regulation by a NR.

Acknowledgments—We thank G. Hautbergue for providing the pGEX and pSET, M. L. Privalsky for all of the SMRT constructs, S. Emiliani for xenopus expression vector, M. R. Stallcup for the GRIP-1 plasmids, and Marion Mathieu for kindly providing the histone H4 acetylated peptide. We thank C. Mulet for DNA plasmid preparation and A. Israel for generously sharing facilities. We are particularly indebted to M. R. Stallcup, C. Deschatrette, D. Faust, G. Hayhurst, and R. Weil for helpful discussions and to M. D. Ruse for sharing results prior to publication.

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