The activity of the activation function 2 of the human hepatocyte nuclear factor 4 (HNF-4 α) is differently modulated by F domains from various origins

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Hepatocyte nuclear factor 4 (HNF-4) is a member of the nuclear hormone-receptor superfamily, which plays an important role in the regulation of several genes involved in numerous metabolic pathways. HNF-4 contains a DNA-binding domain located in domain C and two activation-function domains, designated AF-1 and AF-2, located in domains A/B and E, respectively. The seven isoforms of human HNF-4, termed $\alpha 1$ – $\alpha 6$ and γ , differ mainly by their A/B and F domains. The high sequence variability of the F domain led us to investigate whether this domain modulates the transcriptional activity of HNF-4. Using constructs having the same core receptor and different F domains, we observed that the F domains of HNF-4 modulate the transactivating activity of the full-length HNF-4. A more precise analysis using HNF-4 α AF-2 fused to GAL4 protein and various F domains demonstrated that F domains of isoforms $\alpha 3$ and γ

exhibited inhibitory effects on the activation function AF-2 but that their inhibition behaviours were weaker than that of HNF-4 $\alpha 2$ F domain, which has been reported previously. The presence of domain F results in a decreased interaction with the co-activator glucocorticoid receptor-interacting protein 1. For a given F domain, the modulating effects on the full-length HNF-4 as well as on the AF-2 depended on the target promoters. Our results suggest that the presence of domain F results in conformation changes in HNF-4 AF-2 or in its spatial environment, which probably modify the interaction of the AF-2 activation domain with co-factors and transcription factors bound to *cis*-elements of the target promoters.

Key words: activation domain, AF-2, C-terminal repression domain, isoform, nuclear receptor.

INTRODUCTION

The transcription factor hepatocyte nuclear factor 4 (HNF-4) is a member of the nuclear hormone-receptor superfamily and plays an important role in the regulation of several genes involved in numerous metabolic pathways [1,2]. HNF-4 gene-disruption experiments in mouse embryos [3,4] as well as HNF-4 over-expression in developing *Xenopus* larvae [5] have documented its crucial role in embryogenesis. The perfect correlation between HNF-4 expression and differentiation of hepatic and enterocytic-like cells indicates the role of HNF-4 in cell differentiation [6,7]. Like other nuclear hormone receptors, HNF-4 has a modular structure consisting of six distinct domains with defined functions called A to F from the N-terminal to the C-terminal ends. Seven isoforms of human HNF-4 have been described up to now [8–11]. Isoforms $\alpha 1$, $\alpha 2$ and $\alpha 3$ (using the nomenclature given by Furuta et al. [8]) differ only in the F domain: by comparison with isoform $\alpha 1$, isoform $\alpha 2$ exhibits an insertion of 10 amino acids (aas) in the F domain [9] and isoform $\alpha 3$ has a F domain of completely different sequence [10]. Isoforms $\alpha 4$, $\alpha 5$ and $\alpha 6$ have F domains corresponding to those of isoforms $\alpha 1$, $\alpha 2$ and $\alpha 3$, respectively, but differ from these three isoforms in an insertion of 30 aas in the domain A/B [8,11]. These α isoforms are products of the same gene and arise from alternative splicing of the HNF-4 α mRNA, whereas HNF-4 γ is the product of another gene and exhibits numerous sequence differences in the A/B and F domains [11].

HNF-4 behaves as a homodimer that binds to a direct-repeat-1 response element [12] and with weaker affinity to a direct-

repeat-2 response element [13]. Phosphorylation affects DNA-binding and transactivation potential of HNF-4 $\alpha 1$ [14,15].

Nuclear hormone receptors have two transactivation-function regions; the first one, denoted activation function 1 (AF-1), is ligand independent and located in the domain A/B, whereas the second one, denoted AF-2, is ligand dependent and located in domain E. The transactivation function AF-2 comprises a highly conserved module $\phi\phi XE\phi\phi$ (where ϕ corresponds to a hydrophobic residue and X to a non-conserved aa), denoted AF-2 activation domain (AF-2 AD), which displays an amphipathic α -helix structure [16]. The AF-2 AD module is likely to interact with proteins that are distinct from basal transcription factors and are thought to act as transcriptional co-activators or co-repressors (for a review see [17]). The AF-2 AD of HNF-4 α is essential for the transcriptional activity of AF-2, which requires the presence of the sequence 128–366 (spanning domains D and E) for full activity [18]. The AF-2 of HNF-4 is involved in direct protein–protein interactions with other transcription factors, such as chicken ovalbumin upstream-promoter transcription factor I (COUP TF-I) and HNF-1 [19,20], and with the co-activator cAMP-response-element-interacting-protein binding protein (CBP) [21]. The functional role of the F domain of nuclear receptors remains unclear; nevertheless, a role for this domain has been documented in several reports. First, the F domain of oestrogen receptor exhibits an important role in modulating the magnitude of gene transactivation by oestrogens and anti-oestrogens, and in determining the effectiveness of anti-oestrogens in suppressing oestrogen-stimulated transactivation of genes [22]. Second, two retinoic-acid-receptor (RAR) mutants,

Abbreviations used: aa, amino acid; AF, activation function; AF-2 AD, activation function-2 activation domain; EMSA, electrophoretic mobility shift assay; HNF, hepatocyte nuclear factor; apo, apolipoprotein; TK, thymidine kinase; GST, glutathione S-transferase; GRIP1, glucocorticoid receptor-interacting protein 1; COUP TF, chicken ovalbumin upstream-promoter transcription factor; CBP, cAMP-response-element-interacting-protein binding protein; RAR, retinoic acid receptor; RXR, retinoid X receptor; TAE, Tris-acetate/EDTA.

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differing only by the presence or absence of the F domain, exhibit a significant difference in transactivation potential [23]. According to Hadzopoulou-Cladaras et al. [18], the F domains of rat HNF-4 α 1 and HNF-4 α 2 exhibit an inhibitory effect on HNF-4 α transcriptional activity. We have shown recently that a mutation resulting in the Val-393 $\rightarrow$ Ile substitution, which is located in the F domain of human HNF-4 α and co-segregates with the maturity-onset diabetes of the young (MODY1) form of diabetes, impairs the transactivation activity of the protein [24]. The F domain of HNF-4 is the most variable domain among isoforms within a species, such as human, or among different species. The proximity of the F domain to the AF-2 AD module prompted us to investigate how its high sequence variability modulates the transcriptional activities of full-length HNF-4 and of its activation function AF-2.

EXPERIMENTAL

Plasmid constructions

HNF-4 isoforms and chimaeras

Human HNF-4 α 2 cDNA [9] and rat HNF-4 α 1 cDNA [1] were cloned in the *Eco*RI site of pSG5 (Stratagene). In all constructs, positions are given taking the adenine of the initiator methionine as position 1. In the course of screening a human liver cDNA library [9], a 5'-truncated cDNA of HNF-4 α 3 was isolated and used to clone the full-length human HNF-4 α 3 in pSG5 with the following strategy: the fragment *Msc*I (position 1082)–*Bam*HI (polylinker of the pSG5) in the pSG5 HNF-4 α 2 construct was replaced by the corresponding fragment from HNF-4 α 3. This fragment, spanning sequence 1047–1354 of isoform α 3, was prepared by PCR using the primer couple 1/2 (Table 1). The chimaera Ch-hu F γ , consisting of the domain F of human HNF-4 γ fused in-frame to domains A–E of human HNF-4 α 2, and the chimaera Ch-rat F α 1, consisting of the domain F from rat HNF-4 α 1 fused in-frame to domains A–E of human HNF-4 α 2, were generated using a similar strategy. Fragments 2044–2350 of human HNF-4 γ [11] and 1082–1400 from rat HNF-4 α 1 [1] were synthesized with primer couples 3/4 and 1/5, respectively. The

human HNF-4 α 2 cDNA was also cloned in the *Eco*RI site of the pcDNA3 vector (Invitrogen). To generate the pDNA HNF-4 Δ F (aas 1–369), the fragment –25/+1126, prepared by PCR using the HNF-4 α 2 cloned in pUC19 as a matrix and oligonucleotides 16 (reverse of pUC19) and 13, was inserted in the *Eco*RI/*Xba*I sites of the pcDNA3 vector. The pcDNA HNF-4 Fu (with the F domain replaced by a short peptide with the unrelated sequence Ser-Arg-Gly-Pro-Tyr-Ser-Ile-Val-Ser-Pro-Lys-Cys-OH) was prepared in a similar way using the antisense primer 17, where the stop codon was omitted in order to use a stop codon TAG present in the pcDNA3 vector, then introducing the peptide Ser-Arg-Gly-Pro-Tyr-Ser-Ile-Val-Ser-Pro-Lys-Cys-OH in-frame with the sequence 1–369 of HNF-4 α .

Reporter constructs

The human apolipoprotein CIII (apoCIII) promoter (–1390/+21), cloned previously in the pBLCAT5 plasmid [24], was prepared by PCR using the primer 6 and the forward primer of pBLCAT5, primer 7 (Table 1). The *Kpn*I–*Hind*III fragment was inserted in the *Kpn*I and *Hind*III restriction sites of the pGL3 luciferase reporter vector (Promega). The mouse HNF-1 promoter (–281/+14) was prepared by PCR using the primer couple 8/9 with the mouse HNF-1 promoter (*Pst*I–*Nhe*I, –1300/+157) as DNA template and cloned in the *Kpn*I and *Hind*III restriction sites of the pGL3 vector. Double-stranded phosphorylated oligonucleotides, encompassing the HNF-4 response elements from the human apoAII gene (site J, denoted AIIJ, nucleotides –735 to –716) and the human apoCIII gene (site B, denoted CIIIB, nucleotides –96 to –61), and ending with *Bam*HI and *Bgl*II sites, were cloned as multi-copies in the same orientation into the pIC 20H vector. To generate the plasmids (AIIJ) $\times$ 4-TATA and (CIIIB) $\times$ 2-TATA, the *Sac*I–*Xba*I fragment containing either four copies of the AIIJ site or two copies of the CIIIB site, respectively, was subcloned in the *Sac*I and *Nhe*I restriction sites of the TATA box luciferase vector [25]. Alternatively, a *Hind*III–*Hind*III fragment of the pIC 20H vector containing three copies of the AIIJ site was inserted in the

Table 1 PCR primers used for the preparation of constructs

Lower-case letters correspond to nucleotides added to introduce cloning sites. Positions are shown relative to the adenine of the initiator methionine in HNF-4 α and γ cDNAs as position 1, or to position 1 as the transcription start sites in promoters.

Primer no.	Sequence	Position
1	5'-TCGAGCAGATCCAGTTCATCA-3'	1047/1067
2	5'-gacggatcctctAGACAGGTTAAGCAACTTGC-3'	1354/1335
3	5'-acgtggccAAAATTGACAATCTACTTCAG-3'	2044/2064
4	5'-cgtggatcctctagaGTGCCGTTCTGAAGTAAACAC-3'	2350/2330
5	5'-gtcggatCCCCAGCGGCTTGCTAGAT-3'	1400/1382
6	5'-cacggtaCCACTTTCTCAGGCCTC-3'	–1390/–1374
7	5'-GAAAATCTCGCCAAGCTCC-3'	720/702
8	5'-agcggtagcAAGCATCCTGGAGAGTG-3'	–281/–265
9	5'-tagtaagcttCAGCGCCAGTGAATCAG-3'	+14/–4
10	5'-gatccGCCAAGATTGACAACCTGTTGCAGGAGATGCTGCTGGGAGGGTCCI-3'	1082/1126
11	5'-ctagaGGACCTCCAGCAGCATCTCTGCAACAGGTTGCAATCTTGCCg-3'	1126/1082
12	5'-ctaggatccATCAGCACTCGAAGGTCAAG-3'	401/420
13	5'-cgttctagattaGGACCTCCAGCAGCAT-3'	1126/1109
14	5'-tcgggatccGCCAAGATTGACAACCTG-3'	1082/1099
15	5'-agttctaGAGGCACAGACAGGTTAAGC-3'	1361/1342
16	5'-GTAAACGACGGCCAGT-3'	379/395
17	5'-cgttctagaGGACCTCCAGCAGCAT-3'	1126/1109

*Hind*III restriction site of pBLCAT4 reporter vector containing the minimal thymidine kinase (TK) promoter (−105/+51), to generate the (AIIJ) × 3-TK vector.

GAL4–HNF-4 chimaeras

GAL4–human HNF-4 α AF-2 AD (A355–S369) was generated by inserting a double-stranded phosphorylated oligonucleotide (oligonucleotides 10 and 11) encompassing the sequence 1082–1126 of human HNF-4 α cDNA into the *Bam*HI and *Xba*I sites of the pGAP vector [26], expressing the DNA-binding domain (aa 1–147) of the yeast GAL4 protein. The fragments encompassing the HNF-4 α AF-2 AD flanked with the F domains of either HNF-4 α 2 (residues 355–465), HNF-4 α 3 (residues 355–408) or HNF-4 γ (residues 681–774) were prepared by PCR with the primer couples 14/5, 14/15 and 14/4, respectively. These fragments were cloned into the *Bam*HI and *Xba*I sites of the pGAP vector to generate the plasmids GAL4 AF-2 AD F α 2, GAL4 AF-2 AD F α 3 and GAL4 AF-2 AD F γ , respectively. Fragments encompassing the human HNF-4 α AF2 alone (residues 128–369) or flanked with F domains of either HNF-4 α 2 (residues 128–465), HNF-4 α 3 (residues 128–408) or HNF-4 γ (residues 128–369 from HNF-4 α 2 linked to residues 696–774 from HNF-4 γ) were prepared by PCR with the primer couples 12/13, 12/5, 12/15 and 12/4, respectively. PCR to prepare this latter fragment was performed using the chimaera Ch-hu F γ as a DNA template. These fragments were cloned into the *Bam*HI and *Xba*I sites of the pGAP vector to generate the plasmids GAL4 AF-2, GAL4 AF-2 F α 2, GAL4 AF-2 F α 3 and GAL4 AF-2 F γ , respectively. The two luciferase reporter constructs (GAL) × 5 TATA LUC [27] and UAS_{tk}LUC [28] contained five and two copies of the GAL4-binding element upstream of the TATA box and the TK promoter, respectively. All PCR fragments used in these constructs were generated with the *Pfu* DNA polymerase (Stratagene) and all constructs were verified by DNA sequencing analysis.

Cell culture and transient-transfection assays

COS-1, HepG2 and HeLa cells (2×10^5 cells per 35-mm dish) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and containing 4.5 g/l glucose. Cells were transfected by the polyethylenimine method (Euromedex, Strasbourg, France) using 500 ng of reporter plasmid and 250 ng of pSG5 vector expressing HNF-4, or 100 ng (in COS-1 cells) or 50 ng (in HeLa cells) of pGAD vector expressing GAL4–HNF-4 chimaeras according to the manufacturer's recommendations. To normalize for differences in transfection efficiencies a cytomegalovirus-driven β -galactosidase expression plasmid (250 ng) was transfected simultaneously. Luciferase assays were performed as indicated in [25].

Electrophoretic mobility shift assays (EMSAs)

Nuclear extracts were prepared from transfected COS-1 cells (3×10^6 cells per 100-mm dish, with 10 μ g of appropriate expression plasmid). Nuclei and nuclear extracts were prepared as indicated in [29]. Protein concentrations were determined by the Bradford assay (Bio-Rad) according to the manufacturer's recommendations. DNA–protein complexes formed with the indicated labelled oligonucleotides were studied by EMSA performed in 1 × TAE buffer (TAE; 40 mM Tris-acetate/1 mM EDTA) [29]. The specificity of the retarded bands was ascertained by competition with the corresponding unlabelled oligonucleotides.

In vitro protein–protein interaction assays

Growth and expression, in the bacterial strain JM109, of glutathione S-transferase (GST)–glucocorticoid receptor-interacting protein 1 (GRIP1) was performed using the pGEX 2-TK GRIP1 vector coding for the sequence 730–1121 [30]. Pull-down assays were performed as described [30] using the immobilized GST–GRIP1 and ³⁵S-labelled HNF-4 α 2 or its deletion mutants HNF-4 Δ F and HNF-4 Fu, which were synthesized *in vitro* with the TNT-Coupled Reticulocyte Lysate System (Promega).

Secondary structure predictions

The secondary structures of F domains of HNF-4 isoforms were predicted using the Antheprot computer software developed by Deléage et al. [31]. This software utilizes the algorithms of Fasman, Garnier et al., Levin et al., Deléage et al. and Gibrat et al., of which the references are indicated in [31]. Only structures predicted by at least three of these algorithms were considered in the joint prediction of secondary structures presented below.

RESULTS

Domain F modulates the transactivating activity of HNF-4 α

To test how the high variability of primary structure of F domain can affect the transactivating potential of HNF-4, we have prepared four constructs containing the A–E domains of human HNF-4 α 2 and differing in the F domains (Figure 1A). Two constructs correspond to human HNF-4 isoforms α 2 and α 3. When compared with HNF-4 α 2, the F domain of HNF-4 α 3 strongly differs in size (40 versus 98 aas) and in sequence, with only three common residues [10]. The two other constructs were chimaeras consisting of domains A–E of HNF-4 α 2 linked to the domain F from either rat HNF-4 α 1 (denoted Ch-rat F α 1) or human HNF-4 γ (denoted Ch-hu F γ). These F domains showed significant sequence differences when compared with that of human HNF-4 α 2: in rat HNF-4 α 1 five conservative substitutions and four non-conservative substitutions occurred in addition to a deletion of 10 aas [32]. The F domain of human HNF-4 γ exhibited 15 conservative substitutions, 31 non-conservative substitutions and 17 deletions [11]. By using these constructs, it was possible to determine the influence of various F domains on the transactivating activity of the core receptor.

Transactivation efficiencies of these HNF-4 isoforms and chimaeras are shown in Figure 1(B). On the apoCIII promoter, the various isoforms and chimaeras exhibited similar transactivation potentials except the chimaera Ch-hu F γ , which appeared to be slightly more efficient. When compared with isoform α 2, stronger transactivation potentials of the chimaera Ch-hu F γ were also observed on (CIIIB) × 2-TATA and (AIIJ) × 3-TK promoters. Compared with HNF-4 α 2, HNF-4 α 3 exhibited marked differences, which varied from one promoter to the other: transactivating activities of HNF-4 α 3 were markedly weaker (about 50%) on HNF-1, (CIIIB) × 2-TATA and (AIIJ) × 4-TATA promoters but were similar on the apoCIII promoter and twice as strong on the (AIIJ) × 3-TK promoter. The fact that these changes for a given isoform or chimaera depended on the promoters suggests that differences in transactivating activities from one isoform or chimaera to the other were not due to differences in their expression. To verify this hypothesis, we could not use Western-blotting assays due to the lack of proper antisera: the HNF-4 α 455 antisera had been raised against the very C-terminal peptide of rat HNF-4 α 1 [1] and could not recognize F domains of isoforms α 3 and γ . So, expression of HNF-4 isoforms and chimaeras was checked, as already performed [33], by EMSA assays, taking advantage of the fact that

Figure 1 Modulation of transactivating activities of full-length HNF-4 by its F domain

(A) Schematic representation of HNF-4 isoforms and chimaeras. The four constructs in the pSG5 vector contain the A-E domains of HNF-4α2 and F domains from various origins, which are depicted by different patterns and shading. Human HNF-4α2 and α3 correspond to natural isoforms, whereas Ch-rat Fα1 and Ch-hu Fγ correspond to chimaeras containing F domains from rat isoform α1 and human isoform γ, respectively. (B) Reporter assays. The effects of F domains were analysed on five different promoters: human apoCIII promoter (apoCIII), mouse HNF-1 promoter (HNF-1), apoCIII and apoAII HNF-4-responsive elements upstream of the TATA box [(CIIIB) × 2-TATA and (AIIJ) × 4-TATA, respectively] and apoAII HNF-4-responsive element upstream of the TK promoter [(AIIJ) × 3-TK]. These promoters were co-transfected in COS-1 cells with either non-programmed pSG5 or pSG5 programmed with the various HNF-4 isoforms or chimaeras indicated in (A). For calculation of transactivations, the reporter activities were divided by the reporter activity obtained with the promoter co-transfected with the non-programmed pSG5 within each set of tested promoters. Transactivations are shown with S.D. from at least three experiments performed in triplicate. (C) Expression levels of HNF-4 isoforms and chimaeras. COS-1 cells were transfected with 10 μg of the appropriate expression plasmids per 100-mm plate. The amounts of HNF-4 isoforms and chimaeric proteins were analysed by EMSA: nuclear extracts (3 μg) were incubated with 0.2 ng of ³²P-labelled CIIIB and the DNA-protein complexes were resolved by PAGE in TAE buffer at 4 °C.

DNA-binding activity of HNF-4 is not affected by the F domain. Indeed, DNA-binding activities of full-length HNF-4 and of HNF-4 mutants lacking part or all of the F domain have been reported to be identical [18]. Results presented in Figure 1(C) did not show significant differences in intensities of the retarded bands, indicating that differences in transactivation cannot be explained by differences in protein amounts. Therefore, our results indicated clearly that the F domain of HNF-4 modulates differently the transactivating activity of this nuclear receptor and that this modulation depends on the origin of the F domains and of target promoters.

Domain F modulates the activity of the activation function AF-2 of HNF-4α

Given the proximity of the F domain to the activation function AF-2, we further wanted to test whether the influence of the F domain on the transactivation activity of the HNF-4 core

receptor could result from a direct modulation of the activity of AF-2. In fact, HNF-4 exhibits a close aa sequence similarity to retinoid X receptor RXRα, another nuclear receptor [34]. The activation function AF-2 of RXRα includes an AF-2 AD module displaying an autonomous transcriptional activity [16]. The structural similarities between HNF-4 and RXRα prompted us to test the effects of HNF-4 F domains on both the proximal AF-2 AD module and full-length AF-2. Therefore the AF-2 AD (residues A355–S369) or the full-length AF-2 (residues I128–S369) of HNF-4α2, linked or not to various HNF-4 F domains, were fused to the yeast GAL4 DNA-binding domain. The study was focused on the F domains of HNF-4 isoforms α2, α3 and γ (Figure 2A), which displayed the largest differences in transactivation when tested in the full-length HNF-4 context (Figure 1). These GAL4–HNF-4 chimaeras bind to GAL4-response elements placed upstream of the TATA box or TK promoter, eliminating potential complications arising from altered dimerization and DNA-binding activities of HNF-4-derived

Figure 2 Modulation of the AF-2 activity by the F domains

(A) Schematic representation of the constructs used: chimaeras expressing the yeast GAL4 DNA-binding domain (1–147) fused to the HNF-4 α AF-2 AD (355–369) or the HNF-4 α AF-2 (128–369) and F domains of various origins, depicted by different patterns. Reporter assays. COS-1 and HeLa cells, (B) and (C) respectively, were transiently transfected with the (GAL4) $\times$ 5-TATA box or (GAL4) $\times$ 2-TK promoter luciferase reporter plasmids (GAL4-TATA and GAL4-TK, respectively) and the vectors expressing the chimaeras. For calculation of transactivations, the reporter activities were divided by the reporter activity obtained with the co-transfected pGAD vector expressing only the GAL4 DNA-binding domain within each set of tested promoters. SD, standard deviations from three experiments performed in triplicates. (D) Expression levels of GAL4 chimaeras. COS-1 cells were transfected as indicated in the Experimental section with the chimaeras presented in panel (A). Nuclear extracts (6 μ g) were incubated with 0.2 ng of 32 P-labelled upstream poly(A) $^{+}$ signal UAST 17MX double-stranded oligonucleotide carrying the GAL-4 DNA-binding sequence [28] and DNA–protein complexes were resolved by PAGE as in Figure 1(C).

molecules in which some sequences have been deleted. On the GAL4 TATA box, no transactivation activity was detected with the GAL4–AF-2 AD module with or without F domains (Figure 2B). Using a longer fragment (W340–S369), corresponding to that used in the study of the thyroid hormone receptor AF-2 AD [35], neither gave any transactivating activity (results not shown), a result consistent with that of Hadzopoulou-Cladaras et al. [18]. These results indicate that, whatever its length, HNF-4 AF-2 AD module does not exhibit autonomous transactivation activity, a feature shared with that of the oestrogen receptor [36], whereas the AF-2 AD of the thyroid hormone receptor and of RAR α and RXR α are able to transactivate autonomously [16,35]. On the GAL4 TATA box, no significant differences in transactivating activities were observed between GAL4 AF-2 F α 3 and GAL4 AF-2 F γ , whereas GAL4 AF-2 F α 2 exhibited a weaker activity (Figure 2B). On the GAL4 TK promoter, the three constructs with F domains from isoforms α 2, α 3 and γ exhibited significant activity differences in COS-1 cells (Figure 2B) and in HeLa cells (Figure 2C). Therefore, our results indicate that the HNF-4 AF-2 activity is modulated differently by F domains

from various origins. In addition, similarly to the effects of F domains on the transactivating activities of the HNF-4 core receptor (domains A–E), the effects of F domains on the AF-2 depended on the target promoters. Indeed, in COS-1 cells, compared with GAL4 AF-2 F α 3, GAL4 AF-2 F γ exhibited a similar activity on the GAL4 TATA box but a stronger activity on the GAL4 TK promoter. EMSA performed with the GAL4-binding sequence as DNA probe (Figure 2D) showed that the weaker transactivation activities of GAL4 AF-2 F α 2 on the two promoters were not due to a lower expression of the protein.

It must be emphasized that in COS-1 cells the construct without the F domain (GAL4 AF-2) activated much more than constructs with the various F domains (Figure 2B), showing that the three F domains studied exhibited inhibitory effects on AF-2 activity. Note that slighter inhibitory effects of HNF-4 α 2 and α 3 F domains were observed in HeLa cells (Figure 2C). The strongest inhibitory effect was observed with HNF-4 α 2 F domain. Different results were reported concerning the inhibitory effect of the HNF-4 α 2 F domain in the context of full-length HNF-4:

Figure 3 Transactivating activities of full-length HNF-4α2 and its deletion mutants

(A) Schematic representation of constructs: HNF-4α2 and its mutants where the F domain was either deleted (HNF-4 ΔF) or replaced by a 12-aa-long peptide (HNF-4 Fu). (B and C) Activities in COS-1 and HepG2 cells, respectively. The transactivating activities of constructs depicted in (A) were studied on mouse HNF-1 (HNF-1) and human apoCIII (apoCIII) promoters and on promoters constituted of HNF-4-responsive elements of apoAII and apoCIII genes upstream of the TATA box [(AIIJ) × 4-TATA and (CIIIB) × 2-TATA, respectively]. For calculation of transactivations, the reporter activities were divided by the reporter activity obtained with the promoter co-transfected with the non-programmed pcDNA3 vector within each set of tested promoters. SD, standard deviations from three experiments performed in triplicates. (D) Expression levels of HNF-4 deletion mutants. COS-1 cells were transfected with 10 μg of the appropriate expression plasmids per 100-mm plate. The amounts of HNF-4α2 and its mutants HNF-4 ΔF and HNF-4 Fu were analysed by EMSA: nuclear extracts (3 μg) were incubated with 0.2 ng of ³²P-labelled CIIIB and the DNA-protein complexes were resolved by PAGE in TAE buffer at 4 °C. (E) Sequence similarities between repressor domains. Rat HNF-4α1 and human progesterone receptor (PR) repressor domains (boxed sequences) are from [33]. Unbroken line and dashed line represent mutations that completely and partially disrupted the repressor domain activity, respectively [33]. Asterisks denote identical aa residues or conservative mutations.

upon removal of the F domain, Hadzopoulou-Cladaras and co-workers [18] observed an 8-fold increase in HNF-4 activity, whereas Kristis and co-workers [10] did not observe any change in activity. In addition, Hadzopoulou-Cladaras and co-workers have suggested that HNF-4α1 and α2 F domains may mask critical residues of the AF-2 for interaction with a cofactor [18]. This led us to compare the activities of human HNF-4α2 with those of two mutants where the F domain has been either deleted (construct denoted HNF-4 ΔF in Figure 3A) or replaced by a short peptide with the unrelated sequence Ser-Arg-Gly-Pro-Tyr-Ser-Ile-Val-Ser-Pro-Lys-Cys-OH (construct denoted HNF-4 Fu in Figure 3A). Replacement of HNF-4α2 F domain, which is 98 aa long, by a 12-aa-long peptide should give insight into this hypothesis. In order to compare our results with those of

Hadzopoulou-Cladaras et al. [18], these constructs were prepared in the pcDNA3 vector, a parent vector to that used by those authors. On the HNF-4-responsive elements linked to the TATA box [(CIIIB) × 2-TATA box and (AIIJ) × 4-TATA box], HNF-4α2 and HNF-4 ΔF exhibited similar activities in COS 1 and HepG2 cells (Figures 3B and 3C, respectively). HNF-4 ΔF activities were slightly stronger than those of HNF-4α2 on apoCIII and HNF-1 promoters in COS 1 cells (Figure 3B) and lower differences of activities between these two constructs were observed in HepG2 cells (Figure 3C). So, our results showing moderate inhibitory effects of HNF-4α2 F domain in the context of HNF-4 core receptor are closer to those of Kristis et al. [10] than to those of Hadzopoulou-Cladaras et al. [18]. These discrepancies with the results of the latter authors are not due to

Figure 4 Interaction *in vitro* of HNF-4 α 2 and its deletion mutants with the co-activator GRIP1

³⁵S-labelled HNF-4 α 2 and its deletion mutants were incubated with bacterially expressed GST-GRIP1 bound to glutathione-Sepharose (GST-Sepharose) beads. After extensive washing of beads, input and bound proteins were analysed by SDS/PAGE. The input lanes represent 5% of proteins used in pull-down experiments. Proteins were visualized by autoradiography and quantified on a PhosphorImager.

cell models since, on the apoCIII promoter, the transactivating activities of HNF-4 α 2 and HNF-4 Δ F were identical in C33 cells (results not shown). Unexpectedly, when compared with HNF-4 Δ F, HNF-4 Fu exhibited transactivating activities markedly weaker on all the tested promoters except on the (CIIB) $\times$ 2-TATA box in HepG2 cells (Figure 3C). When compared with HNF-4 α 2, HNF-4 Fu exhibited weaker transactivating activities except on the HNF-1 promoter in COS-1 cells and (CIIB) $\times$ 2-TATA box in HepG2 cells. These differences in transactivating activities were not due to differences in protein expression, as shown in Figure 3(D).

The involvement of the AF-2 AD of several hormone-nuclear receptors in protein-protein interactions with co-activators led us to check whether the HNF-4 α 2 F domain and the peptide Fu impair the interaction of HNF-4 with the co-activator GRIP1. With this aim, pull-down experiments were performed with the immobilized GST-GRIP1 and labelled HNF-4 α 2 or its two deletion mutants, depicted in Figure 3(A). A strong interaction was observed between GRIP1 and HNF-4 Δ F (Figure 4). When compared with HNF-4 Δ F, HNF-4 α 2 gave a faint band and HNF-4 Fu gave a band of intermediate intensity (Figure 4). Quantification of the bands and normalization to the inputs (1/20 of proteins used in pull-down assays) indicated that the amounts of HNF-4 Δ F, HNF-4 Fu and HNF-4 α 2 bound to GRIP1 were 100, 60 and 12%, respectively. These results showed that interaction of HNF-4 with GRIP1 is impaired markedly by the HNF-4 α 2 F domain and by the peptide Fu but that HNF-4 Fu interacts more efficiently than HNF-4 α 2 with GRIP1.

DISCUSSION

Our data show that F domains of the various HNF-4 isoforms modulate the transactivating activity of the HNF-4 core receptor (domains A-E) and exhibit inhibitory effects on the activation function AF-2. An inhibitory effect of rat HNF-4 α 1 and α 2 F domains has been reported previously [18,33]. Our results extend these observations by demonstrating that F domains of isoforms α 3 and γ also repressed the activity of the activation function AF-2 to different extents. In addition, we observed that, for a given F domain, the modulating effects on the HNF-4 core

receptor as well as on the activation function AF-2 depended on the target promoters.

In the rat HNF-4 α 1 and α 2, the inhibitory function has been attributed to a short repression sequence located in the C-terminus (aa 430-441) [33]. This sequence is lacking in the F domains of HNF-4 α 3 and γ , suggesting that another mechanism is involved in the repression activity of F domains of these isoforms. Similarly, the involvement of an additional mechanism in the repression activities of F domains of isoforms α 1 and α 2 can be inferred from the observation that the N-terminal half of these F domains (aa 369-410) also exhibited a repression effect on the HNF-4 core receptor [18]. The AF-2 AD module located at the extreme C-terminus of AF-2 is known to play a main role in the transactivating activities of nuclear receptors and to be a site of interactions with cofactors [17]. In addition, HNF-4 AF-2 has been shown to be involved in direct protein-protein interactions with other transcription factors such as COUP TFs and HNF-1 [19,20], or with the co-activator CBP [21]. Masking of the surface close to the AF-2 by the F domains of HNF-4 α 1 and α 2 has been proposed as a model to explain their inhibitory effects [18]. Indeed, given their proximity to the AF-2 AD module, it is tempting to speculate that a large F domain will impinge more strongly than a small one on the surface of interaction with effectors that mediate the transcriptional activity. But in fact there is no direct relation between the lengths and inhibitory effects of F domains on transactivation activities of both HNF-4 core receptor and its activation function AF-2. Constructs with HNF-4 α 3 F domain and Fu peptide (40 and 12 aa long, respectively) exhibited weak transactivation activities. The strong inhibitory effect of peptide Fu is not due to its short length, which is identical to that of the truncated F domain spanning only the first 12 aas of HNF-4 α 1 F domain in the construct HNF-4 1-380 used by Iyemere et al. [33], since these authors showed that this latter construct and HNF-4 1-368 (lacking the complete F domain) exhibited identical transactivation activities [33]. On the other hand, peptide Fu impaired the interaction of the HNF-4 core receptor with the co-activator GRIP1. The peptide Fu contains three identical aas and exhibits two conservative substitutions when compared with the repressor motif of the C-terminal end of the rat HNF-4 α 1 F domain [33] (Figure 3E). These residues are placed at positions where mutations disrupt completely the repressor-domain activity [33]. In addition, among these five aa residues, mutation of residues at positions 920 and 923 in the progesterone receptor abolished the repressive behaviour of this motif [37], thus underlining the crucial role of these residues in this repressive function. This sequence similarity probably accounts for the very weak transcriptional activities of HNF-4 Fu. HNF-4 Fu, which interacted more strongly with the co-activator GRIP1 when compared with HNF-4 α 2, exhibited weaker transactivating activities. This unexpected result may be due to a stronger interaction with a co-repressor, as suggested for the repressor domain of the progesterone receptor [37].

Interestingly, the effects of F domains on transcriptional activities depended on the nature of target promoters. Similar observations have been reported by Hadzopoulou-Cladaras et al. in the context of the HNF-4 core receptor: the activity of their mutant HNF-4 1-410 was close to that of HNF-4 α 1 and α 2 on the apoCIII promoter but was also close to that of the mutant lacking the complete F domain on apoB promoter [18]. These authors also reported that the repressing activity of the HNF-4 α 2 F domain was stronger on apoCIII than on apoB promoters (8-fold and 3-fold, respectively). It is worth pointing out that the repressive effects of the F domain were more pronounced on the complex promoters (HNF-1 and apoCIII) than on promoters

Figure 5 Prediction of secondary structures of F domains from HNF-4 isoforms

F domains from rat HNF-4 α 1 (rat α 1), human HNF-4 α 2 (hu α 2), human HNF-4 α 3 (hu α 3) and human HNF-4 γ (hu γ) are shown. For convenience of alignment of primary structures, the insertion of 10 aas in HNF-4 α 2, which does not give rise to predicted secondary structure, is presented above the sequence common to isoforms α 1 and α 2. The joint predicted structures obtained from five methods using the Antheptot program [31] are presented. The highly conserved motif $\phi\phi X E \phi\phi$ encompassed in the AF-2 AD module is boxed. Positions of aas of the F domains are given taking as first residue the glycine corresponding to the first codon of the ninth exon, which codes for the N-terminal half of the F domains [44]. Helices, chevrons and bent-line symbols represent α -helices, β -sheet strands and β -turns, respectively.

constituted of only HNF-4-responsive elements linked to the TATA box. Similarly, compared with HNF-4 α 2, HNF-4 α 3 exhibited significant differences in transactivating activities on the HNF-4-responsive element from the apoAII gene linked to the TK promoter or the TATA box. This suggests that F domains modulate the contribution of transcription factors bound to the *cis* elements of the target promoters in the complex interplay of interactions between HNF-4 and other effectors. Therefore it appears that F domains may play a role in modulating protein-protein interactions between HNF-4, co-activators or co-repressors and transcription factors bound to the promoter through changes in the conformation of the activation function AF-2. Synergies between HNF-4 and other transcription factors have already been documented [38–40] and support this assumption. The effects of F domains may occur through structural constraints modifying the environment in the vicinity of critical residues of the AF-2. Such structural constraints probably result from differences in secondary structures of the F domains as predicted by five algorithms (see the Experimental section). In isoform α 3 F domain, the N-terminal half has a high potential to form three consecutive β turns and the C-terminal half is probably in a coil structure (Figure 5). The F domains of human and rat HNF-4 α 1 and α 2 have great potential to form one short α -helix and two β -sheet strands, whereas the F domain of HNF-4 γ would be significantly more structured with a high α -helix content in its C-terminal third and two β -sheet strands in the median part (Figure 5).

Our results, together with other studies on different nuclear receptors [22,23,41], underline that the F domain is not as inert for nuclear-receptor function as thought previously. Furthermore, very recently, it has been shown that different isoforms of a single nuclear-hormone-receptor class differ markedly in their

interactions with co-repressors, which can account for their different activities [42]. The expression patterns of HNF-4 isoforms vary from one tissue to another [10,11,43]. In addition, we have shown that expression of several HNF-4 isoforms also varies during cell differentiation [7]. Therefore, the different transactivation activities of HNF-4 isoforms, which differ mainly in their F domains, most probably have a physiological impact on cell life.

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