Functional properties of the R154X HNF-4α protein generated by a mutation associated with maturity-onset diabetes of the young, type 1

B. Laine[a,*], J. Eeckhoute[a], L. Suaud[a], I. Briche[a], H. Furuta[b], G.I. Bell[b], P. Formstecher[a]

[a] *Unité 459 INSERM, Laboratoire de Biologie Cellulaire, Université H. Warembourg, 1 Place de Verdun, F 59045 Lille, France*
[b] *Howard Hughes Medical Institute, University of Chicago, 5841 South Maryland Avenue, MC1028, Chicago, IL 60637, USA*

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Abstract Mutations in the hepatocyte nuclear factor 4α (HNF-4α) gene are associated with one form of maturity-onset diabetes of the young (MODY1). The R154X mutation generates a protein lacking the E-domain which is required for normal HNF-4α functions. Since pancreatic β-cell dysfunction is a feature of MODY1 patients, we compared the functional properties of the R154X mutant in insulin-secreting pancreatic β-cells and non-β-cells. The R154X mutation did not affect nuclear localisation in β-cells and non-β-cells. However, it did lead to a greater impairment of HNF-4α function in β-cells compared to non-β-cells, including a complete loss of transactivation activity and a dominant-negative behaviour.

Key words: Hepatocyte nuclear factor 4α; Hormone nuclear receptor; Maturity-onset diabetes of the young; Diabetes mellitus; Pancreatic β-cell

1. Introduction

Mutations in the hepatocyte nuclear factor-4α (HNF-4α) are the cause of the type 1 form of maturity-onset diabetes of the young (MODY) (OMIM 125850) which is a monogenic form of diabetes characterised by an early age at onset (usually before 25 years), autosomal dominant inheritance and abnormal pancreatic β-cell function [1]. HNF-4α regulates normal pancreatic β-cell function and controls expression of genes involved in glucose transport and metabolism [2]. It also regulates the expression of transcription factors such as HNF-1α [3–5].

Like other members of the hormone nuclear receptor superfamily, HNF-4α has a modular structure consisting of functional domains named A–F (Fig. 1A). HNF-4α contains two transactivation function modules AF-1 and AF-2 located at the amino-terminal end of domain A and the carboxy-terminal end of domain E, respectively [6,7]. Domain E is involved in dimerisation [8] and interactions with other transcription factors [9,10] and coactivators [11–14].

Studies of functional properties of several diabetes-associated mutations of HNF-4α have shown that mutations can have variable effects on the ability of HNF-4α to transactivate a reporter gene [2,15–18]. The R154X mutation generates a protein that has an intact DNA binding domain but lacks the E-domain [19]. Lausen et al. have recently described some of the properties of R154X in non-pancreatic β-cells [16]. The results presented here extend their studies and describe functional properties of the mutant protein that may explain how this mutation leads to abnormal HNF-4α function in pancreatic β-cells and could contribute to the development of the MODY1 form of diabetes.

2. Materials and methods

2.1. DNA Constructs

The wild-type and R154X human HNF-4α2 were cloned in pcDNA3.1/HisB (Invitrogen). The p enhanced green fluorescent protein (EGFP)-HNF-4α2 and pEGFP-R154X were cloned in pEGFP-C1 (Clontech). The HNF-4α 1–358 construct was obtained by a method similar to that used to prepare the HNF-4ΔF [20] with the oligonucleotide 5′-CGTTCTAGATTAGTCAATCTTGGCCATGC-3′ as the antisense primer. The HNF-1α, apolipoprotein CIII (apoCIII), -186 liver pyruvate kinase (LPK) and $(L4L3)_{3x}$-96 LPK promoter–reporter constructs have been described previously [18,21]. The $(PEPCK)_{4x}$TATA box construct which includes four copies of the HNF-4 response element in the rat PEPCK promoter (nucleotides −456 to −436) cloned upstream of the TATA box of the adenovirus major late promoter and luciferase reporter gene was prepared as described in [20]. The sequences of all constructs were verified by DNA sequencing.

2.2. Cell culture and transient transfection assays

COS-1 and HEK 293T cells (2×10^5 cells and 5×10^5 cells per 6 well dish, respectively) were grown as in [18]. HIT-T15 cells (ATCC CRL-1777) (5×10^5 cells per 6 well dish and passages 69–80) were grown in RPMI 1640 medium containing 4 mM glucose and supplemented with 10% FCS, 10 mM HEPES pH 7.4, 100 U/ml penicillin and 100μg/ml streptomycin. Transfections and luciferase assays were carried out as described previously [18].

2.3. Subcellular localisation of HNF-4α

Transfection of cells and confocal laser scanning microscopy were carried out as indicated in [22] using an oil Pl Apo 100× (NA = 1.4) objective.

2.4. Western blot assays

Western blot assays were carried out as in [17] using either the α-455 antiserum raised against the carboxy-terminal sequence of HNF-4α [23] or the anti-Xpress antibody (Invitrogen) which recognises the Xpress-epitope tag of proteins expressed from the pcDNA3.1/HisB.

2.5. Electrophoretic mobility shift assays (EMSA)

EMSA were performed as in [20]. Proteins were in vitro synthesised as indicated in [18].

*Corresponding author. Fax: (33)-3-20 62 68 84.
E-mail: blaine@lille.inserm.fr

Abbreviations: HNF-, hepatocyte nuclear factor; MODY, maturity-onset diabetes of the young; EGFP, enhanced green fluorescent protein; apoCIII, apolipoprotein CIII; LPK, liver pyruvate kinase; EMSA, electrophoretic mobility shift assays; NLS, nuclear localisation signal

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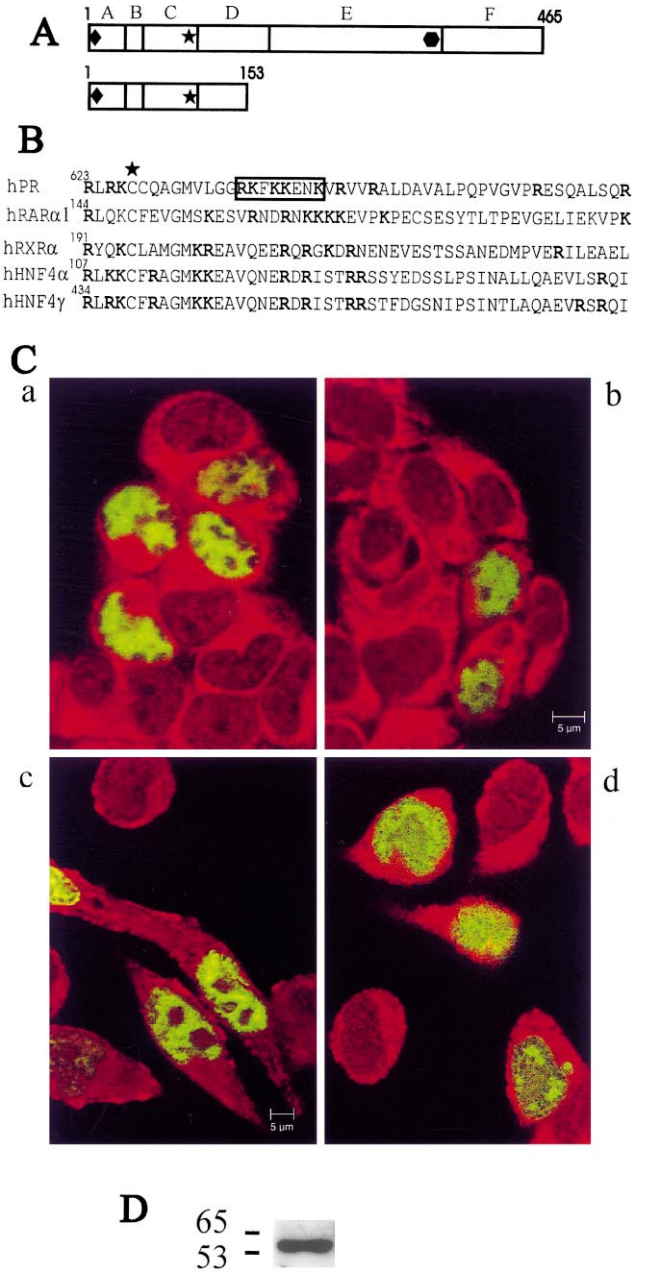

Fig. 1. R154X mutant is localised in the nucleus. A: Schematic representation of HNF-4α2 and R154X mutant. Numbers indicate amino acid positions at the amino- and carboxy-terminus of these proteins; functional domains A–F are shown at the top; diamonds and hexagons depict activation function modules AF-1 and AF-2, respectively. The star depicts the last cysteine residue of the DNA binding domain (DBD) localised in domain C. B: The NLSs in the C/D-domain junction of nuclear receptors. The last cysteine residue in the DBD is indicated by a star. Basic residues are in bold. The box corresponds to a basic motif that is conserved in the steroid receptor subfamily [24]. Numbers on the left indicate the positions in the amino acid sequences. C: Subcellular distribution of EGFP-HNF-4α2 and EGFP-R154X in HIT-T15 (a and b, respectively) and in HeLa cells (c and d, respectively). The fluorescence images were observed by confocal laser scanning microscopy. Bars indicate 5 μm. D: HNF-4α expression in HIT-T15 cells. Western blot of HIT-T15 whole cell extracts (5 μg) revealed with the α-455 antiserum. Numbers indicate the molecular mass of protein markers in kDa.

3. Results

3.1. R154X mutant is localised in the nucleus

Nuclear translocation of nuclear receptors involves motifs termed nuclear localisation signals (NLSs). The NLS1 which is localised at the C/D-domain junction, displays a constitutive nuclear targeting activity. However, complete nuclear translocation requires the cooperation with another NLS located in the ligand binding domain E (for review see [24]). The C/D-domain junction of HNF-4α contains a sequence with 10 basic residues that is strictly conserved between HNF-4α and HNF-4γ and is similar to clusters of basic residues that constitute the NLS1 of other nuclear receptors (Fig. 1B). The R154X has the C/D-domain element but lacks the E-domain element (Fig. 1A). Our first goal was to investigate the subcellular localisation of R154X.

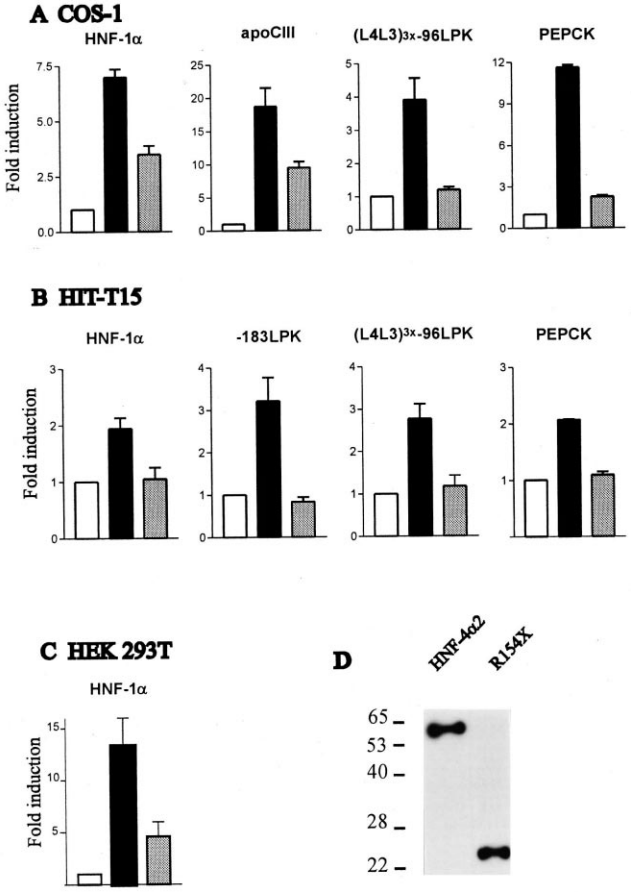

Fig. 2. Transactivation activities of R154X mutant. A, B and C: activities in COS-1, HIT-T15 and HEK 293T cells, respectively. apoC-III, apoCIII promoter; HNF-1α, HNF-1α promoter; -183 LPK, LPK promoter; $(L4L3)_{3x}$-96 LPK, three tandem repeats of the adjacent glucose and HNF-4α response elements upstream of the LPK promoter; $(PEPCK)_{4x}$-TATA, four copies of the HNF-4α response element from the PEPCK gene upstream of the TATA box. These promoters (1 μg) were cotransfected with 50 ng of HNF-4α expression vectors. White, black and grey columns represent the activities obtained in the absence or in the presence of wild-type and R154X HNF-4α, respectively. Fold induction refers to the activity with no HNF-4α derivative. Data are means ± S.D. for at least three experiments. D: Western blotting. Nuclear proteins were resolved by SDS–PAGE and transferred on a PDVF membrane. The HNF-4α proteins were detected using the anti-Xpress antibody. Numbers indicate the molecular mass of protein markers in kDa.

EGFP-HNF-4α2 and EGFP-R154X that have the EGFP fused in frame to HNF-4α2 and R154X, respectively, were expressed in HIT-T15 pancreatic β-cells. Both proteins were exclusively localised in the nucleus of cells expressing these proteins and no green fluorescence was detected in the cytoplasm (Fig. 1C, a and b). As previously reported, EGFP alone is uniformly distributed in the cell and has to be fused to a protein that translocates to the nucleus to accumulate in this cell compartment [22,25]. Since HIT-T15 cells express endogenous HNF-4α, as determined by Western blot (Fig. 1D), the nuclear accumulation of EGFP-R154X may result from its passive transport as a heterodimer formed with the wild-type HNF-4α [26]. Therefore, the nuclear localisation studies were carried out using HeLa cells which do not express HNF-4α and again, the expression of both fluorescent proteins was restricted to the nucleus (Fig. 1C, c and d).

3.2. Transactivation activity of R154X

In the COS-1 kidney cell line, R154X failed to transactivate $(L4L3)_{3x}$-96 LPK and $(PEPCK)_{4x}$ TATA promoters which contain tandem repeats of the HNF-4α response elements of the LPK and PEPCK genes (Fig. 2A). However, it was able to moderately activate the HNF-1α and apoCIII promoters (50% when compared to HNF4-α2). A moderate transactivation activity of R154X on the HNF-1α promoter was also observed in the other kidney cell line HEK 293T (Fig. 2C). The differences in transactivation activities between HNF-4α and R154X were not due to differences in their expression as determined by Western blotting (Fig. 2D). By contrast, R154X could not transactivate any of the tested promoters in HIT-T15 cells (Fig. 2B). Similar transactivation activities were observed using HNF-4α2 and R154X lacking the Xpress-epitope tag (data not shown), ascertaining that this tag does not modulate the transcriptional activity of HNF-4α.

3.3. Effects of R154X HNF-4α on the activities of the wild-type HNF-4α2

MODY1 patients express both wild-type and mutant forms of HNF-4α. It is therefore of interest to determine whether the reduced expression of HNF-4α target genes is due to haploinsufficiency or to an impaired activity of the wild-type

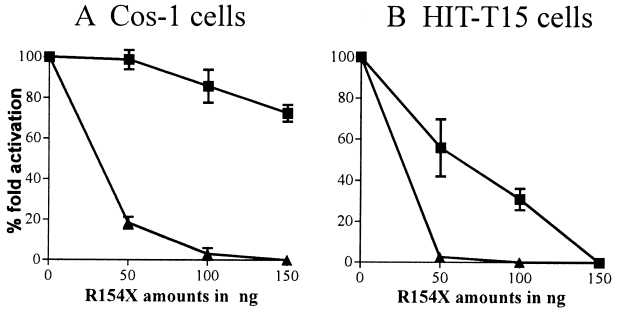

Fig. 3. Effects of R154X on the transactivation activity of HNF-4α2. COS-1 cells (A) and HIT-T15 cells (B) were transfected with constant amounts of $(L4L3)_{3x}$-96LPK reporter (1 μg) and HNF-4α2 expression vector (50 ng) together with indicated amounts of vectors expressing either R154X mutant (squares) or HNF-4(1–358) (triangles). In every case vector DNA (pcDNA3.1His) was added as necessary to achieve a constant amount of transfected DNA. Results are expressed as percentage of the activity of the promoter when activated by HNF-4α2 alone. Data are means ± S.D. of three experiments performed in quadruplicate.

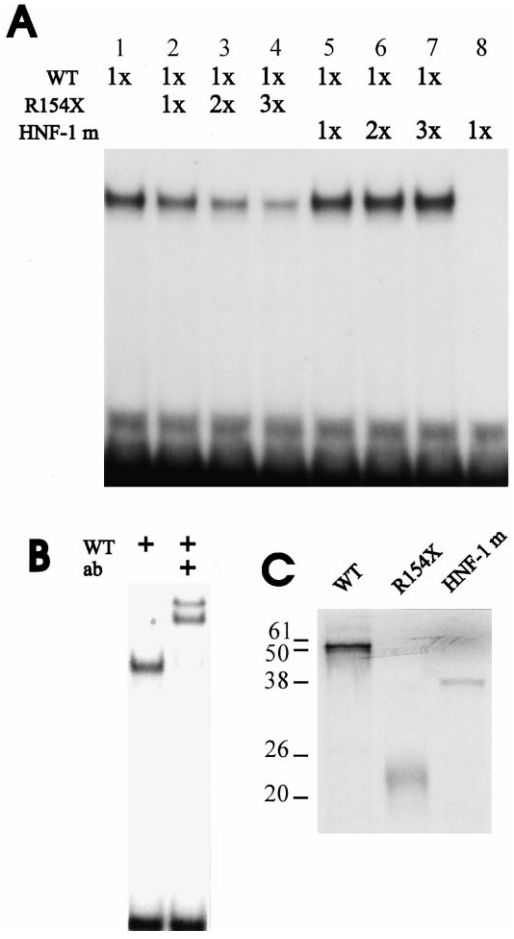

Fig. 4. Effects of R154X on the DNA-binding activity of HNF-4α2. ^{32}P-labelled LPK L3 was incubated with in vitro synthesised HNF-4α2 (WT), R154X and HNF-1α P291fsinsC (HNF-1 m) using the fold molar amounts of R154X and HNF-1α P291fsinsC over HNF-4α2 indicated in A. B: Supershift of the HNF-4α2-LPK L3 complex yielded by incubation with 0.1 μl of α-455 antiserum containing the antibodies raised against the carboxy-terminal sequence of the protein (ab). Control of protein synthesis performed in the presence of [^{35}S]methionine is presented in panel C where numbers on the left indicate the molecular mass of the markers in kDa. 1μl of protein synthesis was loaded except 2 μl of HNF-1α P291fsinsC due to its low content in methionine residues (4 versus 19 in Xpress-tag-HNF-4α2).

HNF-4α by a dominant negative activity of the mutant, thus decreasing the available amount of efficient HNF-4α. This is particularly important in pancreatic β-cells where normal HNF-4α expression is lower than in liver or kidney [27]. This led us to investigate whether R154X could alter the transactivation activity of the wild-type protein. In COS-1 cells, the activity of HNF-4α2 on the $(L4L3)_{3x}$-96 LPK promoter was not affected by the presence of an equal amount of R154X but larger amounts caused a slight reduction in activity (Fig. 3A). A much stronger repression of the wild-type HNF-4α2 transactivation activity was observed upon addition of HNF-4α(1–358), an HNF-4α mutant lacking the AF-2 module and having a strong dominant negative activity [6] (Fig. 3A). Interestingly, a stronger dominant-negative effect of R154X was observed in HIT-T15 cells (Fig. 3B).

These results led us to analyse, by EMSA, the effect of R154X on the binding activity of HNF-4α2 on the HNF-4α response element of the LPK gene (LPK L3) [21]. The band-

shift yielded by wild-type HNF-4α2 (Fig. 4A, lane 1) was specific since it was supershifted by the α-455 antibody raised against HNF-4α (Fig. 4B). When identical molar amounts of HNF-4α2 and R154X were incubated with the LPK L3 probe, we observed a 20% decrease in the DNA binding activity of HNF-4α2 (Fig. 4A, lane 2). The DNA binding inhibition was clearly concentration-dependent (Fig. 4A, lanes 3 and 4). Both wild-type and R154X proteins were efficiently expressed (Fig. 4C). This decrease was specifically due to R154X since it was not observed upon addition of the unrelated protein human HNF-1α P291fsinsC (Fig. 4A, lanes 5–7). This truncated HNF-1α lacks the domain located between residues 280 and 440 which is required for HNF-1α–HNF-4α interaction [10]. HNF-1α P291fsinsC which yielded a band at its expected molecular size (37 kDa) in SDS–PAGE (Fig. 4C) could not bind the LPK L3 probe (Fig. 4A, lane 8).

4. Discussion

Our results showing that the R154X HNF-4α mutant was efficiently translocated to the nucleus indicate that the E-domain of HNF-4α is not required for efficient nuclear targeting.

R154X exhibited strong impairment in transactivation activity, the extent of which varied with target promoters. In COS-1 cells, the transactivation activity of R154X was completely lost on two promoters and remained moderate (50% of that induced by wild-type HNF-4α2) on two other promoters tested. It has been previously shown that the transactivation activity of HNF-4α is extinguished by deletion of the carboxy-terminal end of its E-domain but is recovered by deletion of the full-length E-domain in the context of a chimera GAL4-HNF-4α on a promoter having a GAL4 binding site [6]. Our results with R154X extend this finding by indicating that HNF-4α lacking the full-length E-domain can have a moderate transactivation activity on the natural apoCIII and HNF-1α promoters.

The ability of the R154X to transactivate the HNF-1α promoter was cell-type specific: this promoter was activated in the kidney cell lines COS-1 and HEK 293T but not in the β-cell line HIT-T15. Synergies between HNF-4α and other transcription factors have been documented [28–30] and the cell-type differences observed on the HNF-1α promoter may reflect differences in the expression of cell-specific transcription factors.

The complete loss of transactivation of the HNF-1α promoter by R154X in HIT-T15 cells is probably of physiological importance since HNF-1α expression is required for normal pancreatic β-cell function and mutations in the HNF-1α gene are associated with another form of MODY, MODY3 [31]. In HIT-T15 cells, none of the four promoters tested was transactivated by R154X, indicating a more severe impairment of transactivation activity of this mutated protein in β-cells than in non-β-cells. In addition, the R154X mutant may also exert a dominant-negative effect on the activity of wild-type HNF-4α in β-cells. Thus, both loss-of-function and dominant-negative behaviour of R154X could affect β-cell function thereby leading to the β-cell dysfunction observed in MODY1 patients. The other nonsense HNF-4α mutant Q268X does not exhibit a dominant negative activity in non-β-cells [2,15] and determination of such a behaviour in β-cells will be of interest. The functional properties of R154X likely account for the clinical severity of diabetes observed in patients harbouring this mutation [19].

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