The G115S mutation associated with maturity-onset diabetes of the young impairs hepatocyte nuclear factor 4 α activities and introduces a PKA phosphorylation site in its DNA-binding domain

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HNF4 α (hepatocyte nuclear factor 4 α) belongs to a complex transcription factor network that is crucial for the function of hepatocytes and pancreatic β -cells. In these cells, it activates the expression of a very large number of genes, including genes involved in the transport and metabolism of glucose and lipids. Mutations in the HNF4 α gene correlate with MODY1 (maturity-onset diabetes of the young 1), a form of type II diabetes characterized by an impaired glucose-induced insulin secretion. The MODY1 G115S (Gly¹¹⁵ $\rightarrow$ Ser) HNF4 α mutation is located in the DNA-binding domain of this nuclear receptor. We show here that the G115S mutation failed to affect HNF4 α -mediated transcription on apolipoprotein promoters in HepG2 cells. Conversely, in pancreatic β -cell lines, this mutation resulted in strong impairments of HNF4 α transcriptional activity on the promoters of LPK (liver pyruvate kinase) and HNF1 α , with this transcription factor

playing a key role in endocrine pancreas. We show as well that the G115S mutation creates a PKA (protein kinase A) phosphorylation site, and that PKA-mediated phosphorylation results in a decreased transcriptional activity of the mutant. Moreover, the G115E (Gly¹¹⁵ $\rightarrow$ Glu) mutation mimicking phosphorylation reduced HNF4 α DNA-binding and transcriptional activities. Our results may account for the 100 % penetrance of diabetes in human carriers of this mutation. In addition, they suggest that introduction of a phosphorylation site in the DNA-binding domain may represent a new mechanism by which a MODY1 mutation leads to loss of HNF4 α function.

Key words: hepatocyte nuclear factor 4 α (HNF4 α), mass spectrometry (MS), maturity-onset diabetes of the young 1 (MODY1), pancreatic β -cell, phosphorylation, protein kinase A (PKA).

INTRODUCTION

HNF4 α (hepatocyte nuclear factor 4 α) is a nuclear receptor (NR2A1; nuclear receptor 2A1) that is expressed in liver, kidney, intestine and endocrine pancreas [1]. HNF4 α is involved in a complex transcription factor network that is crucial for the function of hepatocytes and pancreatic β -cells [2–4]. In these cells, it activates the expression of a very large number of genes directly, including those involved in the transport and metabolism of lipids and glucose [1,5]. The key role of HNF4 α in these metabolisms has been highlighted by invalidation of its gene in mice [6,7]. In pancreatic β -cells, HNF4 α activates the insulin gene promoter directly [8] and is required for glucose-induced insulin secretion [9]. Further underscoring the importance of HNF4 α in pancreatic β -cells, mutations in the HNF4 α gene have been identified in patients with MODY1 (maturity-onset diabetes of the young 1), characterized by an impaired glucose-induced insulin secretion [10]. Several groups, including ours, have shown that MODY1 mutations affect HNF4 α functions differently, as reviewed by Ryffel [11]. In addition, we showed previously that D126Y (Asp¹²⁶ $\rightarrow$ Tyr) and D126H (Asp¹²⁶ $\rightarrow$ His) mutations associated with type II diabetes impair HNF4 α transcriptional activity markedly in pancreatic β -cells [12]. These mutations are localized in the DBD (DNA-binding domain), consisting of two zinc fingers and a C-terminal extension where the T and A boxes are found. An additional MODY1-associated mutation, G115S (Gly¹¹⁵ $\rightarrow$ Ser), is also localized in the highly conserved DBD of HNF4 α [13]. All carriers of this mutation had low insulin secretion and precociously developed diabetes [13].

HNF4 α is a phosphoprotein [14], and activities of nuclear receptors are regulated by phosphorylation [15]. Tyrosine phosphorylation is required for HNF4 α nuclear localization [16], and serine and threonine kinases also play key roles in HNF4 α function. The cAMP-dependent PKA (protein kinase A) phosphorylates HNF4 α at serine residues 133 and 134 located in the A box, thus resulting in a loss of HNF4 α DNA-binding and transactivation activities [17]. AMPK (AMP-activated protein kinase) phosphorylates HNF4 α at Ser³⁰⁴, leading to decreased HNF4 α dimerization, DNA binding and stability [18]. These effects probably account for the decrease in HNF4 α target gene transcription observed upon AMPK activation [19].

Despite the 100 % penetrance of diabetes in human carriers of the G115S mutation, the functional consequences of this mutation have not yet been investigated. In the present paper, we show that the G115S mutation affects DNA binding and transcriptional activities of HNF4 α . We also provide some evidence that this mutation introduces a PKA phosphorylation site that might contribute to the impairment.

MATERIALS AND METHODS

Plasmid constructs

Plasmid pcDNA3.1 HNF4 α 2 described in [20] was used as a template to create pcDNA3.1 HNF4 α 2-G115S, -G115E, -SS133-134AG and -SS133-134AG G115S constructs by site-directed mutagenesis using the QuikChange^{} kit from Stratagene according to the manufacturer's recommendations. Plasmids pET-28a(+)

Abbreviations used: AF, activation function; DBD, DNA-binding domain; ESI, electrospray ionization; HNF, hepatocyte nuclear factor; LPK, liver pyruvate kinase; Luc, luciferase; MODY, maturity-onset diabetes of the young; PKA, protein kinase A; PKA C α , catalytic subunit C α of PKA; PKI, PKA inhibitor; PP1, protein phosphatase 1; RXR, retinoid X receptor; TTR, transthyretin.

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HNF4 α R154X and pET-28a(+) HNF4 α R154X G115S, used to express recombinant His-tag/thrombin/T7-tag HNF4 α derivatives, were obtained by inserting a PCR fragment encompassing human HNF4 α R154X and R154X G115S cDNA into the *EcoRI/HindIII* sites of pET-28a(+) (Novagen). Plasmids pSV-PKA C α (PKA C α is the catalytic subunit C α of PKA) and pCMV-PKI (PKI is PKA inhibitor) were gifts from Dr P. Sassone-Corsi and Dr M. D. Uhler respectively. The human HNF1 α promoter (−341/+183) and rat (L4L3)₃×-96 LPK (LPK is liver pyruvate kinase) promoter were gifts from Dr G. Bell and Dr M. Raymondjean respectively. The human apolipoprotein AI and CIII promoters were described previously [12,20]. The (TTR)₄× TATA Luc (where TTR is transthyretin and Luc is luciferase) reporter plasmid containing four copies in the same orientation of the HNF4 α -response element of the TTR gene upstream of the TATA box was cloned by the strategy described previously in [21] to obtain the (AIIJ)₄×-TATA Luc vector.

Cell culture and transient transfection assays

HeLa and HepG2 cells were maintained as described in [20] and plated in 24-well plates at a density of 2×10^5 cells and 5×10^5 cells respectively. HIT-T15 and β TC3 cells were maintained and plated in 24-well plates as described previously in [22] and [12] respectively. Rin m5F cells were maintained and plated as in [23]. Cells were transfected as described in [12], except that in Rin m5F cells, the amounts of plasmids were 50 ng for the HNF4 α -expressing vectors, 800 ng for the reporter vector and 10 ng for the *Renilla* luciferase expression vector. In experiments performed in the presence of PKA C α and PKI expression vectors, amounts of plasmids are indicated in the legend of Figure 5.

Western blot analysis

Western blot assays were performed as described in [20] and revealed with the α 455 HNF4 α antiserum [24] or the horseradish-peroxidase-conjugated anti-(T7 tag) antibody (Novagen).

Expression of His₆-human HNF4 α R154X and R154X G115S

Bacteria, *Escherichia coli* strain BL21(DE3)pLysS (Promega), were transformed with the pET-28a(+) HNF4 α R154X and R154X G115S plasmids and were grown for 8 h in 3 ml of LB (Luria-Bertani) broth in the presence of 25 μ g/ml kanamycin. To improve production of the His₆-human HNF4 α R154X recombinant protein, the medium was supplemented with glucose (0.5 % final concentration). Aliquots of 6 ml of these respective media were inoculated with 2 ml of precultures, and bacteria were grown with agitation in 250 ml flasks at 37 °C to a D_{595} of 0.6. Bacteria were grown further in 600 ml of media at 37 °C to a D_{595} of 0.6. Expression of recombinant proteins was induced with 0.4 mM IPTG (isopropyl β -D-thiogalactoside) for 3 h at 37 °C. Bacteria were harvested by centrifugation at 700 g for 20 min and stored for further use at −80 °C.

Purification of His₆-human HNF4 α R154X and R154X G115S

All steps of purification were performed at 4 °C. The bacterial pellets were resuspended in 7 ml of buffer A [20 mM Tris/HCl, pH 8.0, 200 mM NaCl, 0.1 % Triton X-100, 0.5 mM PMSF and 70 μ l of protease inhibitor cocktail (Sigma)] containing 5 mM imidazole. Cells were lysed by three sonication cycles on ice. After digestion with DNase I (40 units) in the presence of Nonidet P40 (0.02 %) for 1 h, lysates were centrifuged at 17 600 g for 30 min. The soluble cellular material was incubated with gentle agitation for 1 h with 500 μ l of Talon metal-affinity resin

(Clontech), which had been extensively washed with buffer A. The resin was then washed three times for 10 min with 3 ml of buffer A containing 20 mM imidazole. A fourth wash was performed in buffer A containing 40 mM imidazole. Proteins were eluted using 300 μ l of buffer A containing 0.5 M imidazole for 15 min at room temperature (25 °C). This elution step was repeated twice and glycerol was added to eluates (20 % final concentration). Recombinant proteins were quantified using the Bio-Rad Bradford protein assay kit and stored at −80 °C.

In vitro phosphorylation of recombinant HNF4 α

Purified His₆-tagged recombinant proteins (50 μ g) were incubated for 30 min at 30 °C with 40 units of the catalytic subunit of PKA (Promega) and 4 mM ATP, in 100 μ l of 25 mM Tris (pH 7.5), 5 mM β -glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na₃VO₄ and 10 mM MgCl₂.

Endoproteinase Arg-C digestion

The phosphorylated His₆-tagged recombinant proteins were digested for 16 h with the endoproteinase Arg-C (Roche) using a substrate/enzyme ratio of 50:1 (w/w), according to the supplier's recommendations, except that the incubation buffer contained 4 M urea. The reaction was stopped by addition of 1 μ l of methanoic (formic) acid.

HPLC-ESI (electrospray ionization)-MS coupling

Protein digests (2.4 nmol in 200 μ l) were analysed by reverse-phase HPLC coupled with ESI-MS using protocols, equipment and the software to plot, acquire and treat the ESI-MS data described in [25]. A linear gradient of 0–80 % acetonitrile in 0.05 % trifluoroacetic acid (40 min) was used to elute peptides. The column effluent was split 1:5 to give a flow rate of 40 μ l/min into the electrospray nebulizer. Fractions of the remaining effluent were recovered for subsequent direct nano-ESI-MS analysis after concentration under vacuum in a SpeedVac apparatus.

Nano-ESI-MS

Analyses were performed on a PE Sciex (Foster City, CA, U.S.A.) API 3000 tandem quadrupole mass spectrometer equipped with a Protana (Odense, Denmark) nano-electrospray source. Samples (2–3 μ l) were deposited in the gold/palladium-coated glass nano-electrospray capillaries. Product ion spectra of the appropriate positively charged precursor ions were recorded to identify peptides with nitrogen as the collision gas and collision energies of 40–45 eV. Mass spectra were acquired at unit resolution with step size of 0.1 a.m.u. (atomic mass unit) and a total scan time of 3 s. Thirty spectra were summed in MCA (Multi Channel Analyser) mode. The potential of the spray needle capillary was held at +800 V and orifice voltage was at +70 V. The computer programs Analyst 1.3.2 and Bioanalyst 1.3 were used to plot, acquire and treat the ESI-MS data.

EMSAs (electrophoretic mobility-shift assays)

EMSAs were performed as in [26] using HNF4 α proteins over-expressed in HeLa cells. Nuclear extracts were prepared as in [27]. A radiolabelled double-stranded oligonucleotide encompassing the HNF4 α -response element of the transthyretin gene [24] was used as probe. When indicated, nuclear extracts were treated with 7.5 units of recombinant PP1 (protein phosphatase 1) at 30 °C for 20 min according to the manufacturer's instructions (New England BioLabs).

Table 1 Loss of HNF4 α transcriptional activity by the G115S mutation

The effects of the G115S mutation were analysed on the human HNF1 α and rat (L4L3)₃×96LPK promoters in HeLa cells, and in three pancreatic β -cell lines, β TC3, Rin m5F and HIT T15 cells. Cells were transfected as indicated in the Materials and methods section. Fold transactivation refers to basal activities of the promoters (cells transfected with the empty expression vector). Results are means $\pm$ S.E.M. for three independent experiments performed six times. *** P < 0.001; ** P < 0.01 compared with wild-type HNF4 α . ND, not determined.

Cell lines	Promoters	Fold transactivation		P
		HNF4 α WT	HNF4 α G115S	
HeLa	HNF1	2.98 $\pm$ 0.38	0.90 $\pm$ 0.17	***
	(L4L3) ₃ ×96LPK	37.78 $\pm$ 7.55	23.40 $\pm$ 8.39	**
β TC3	HNF1	3.90 $\pm$ 0.52	2.73 $\pm$ 0.30	
	(L4L3) ₃ ×96LPK	28.71 $\pm$ 1.63	7.16 $\pm$ 0.80	***
Rin m5F	HNF1	3.85 $\pm$ 0.73	1.20 $\pm$ 0.25	***
	(L4L3) ₃ ×96LPK	6.06 $\pm$ 0.74	1.27 $\pm$ 0.24	***
HIT T15	HNF1	7.33 $\pm$ 0.23	0.51 $\pm$ 0.03	***
	(L4L3) ₃ ×96LPK	ND	ND	

Data analysis

Statistical analyses were performed by Student's t test for unpaired data using Prism software. Statistical significance has been considered at *** P < 0.001, ** P < 0.01 and * P < 0.05.

RESULTS

Effects of the G115S mutation on HNF4 α transcriptional activities

The effects of the MODY1-associated HNF4 α G115S mutation on HNF4 α transcriptional activities were investigated using the HNF1 α promoter and the (L4L3)₃×96LPK promoter containing three copies of the HNF4 α -response element of the LPK gene upstream of the LPK minimal promoter. The transcription factor HNF1 α is crucial for the function of pancreatic β -cells [5,28–30]. Expression of LPK in pancreatic islets is low and is subject to controversy [31–34]; however, because it closely parallels that of HNF4 α [32], this nuclear receptor appears to be a key regulator of the LPK promoter. The G115S mutation resulted in significant losses of HNF4 α transcriptional activities in most assays, including pancreatic β -cell lines where this activity was abolished (Table 1). Western blotting analyses indicated that these impairments were not due to lower expression of the G115S mutant, as shown in Figure 1.

It has been reported that some MODY1 patients have decreased serum levels of various proteins involved in lipid homeostasis, including apolipoproteins (as reviewed in [10] and [35]). These defects may result from transcriptional impairments in hepatocytes, since conditional inactivation of HNF4 α in rat liver results in reduced mRNA levels of apolipoproteins [6]. This led us to investigate the effects of the G115S mutation on the HNF4 α -mediated activation of promoters of apolipoproteins AI and CIII in the human hepatoma HepG2 cell line. However, the G115S mutation failed to impair the HNF4 α transcriptional activity on the apolipoprotein CIII promoter, and a very mild loss was observed on the apolipoprotein AI promoter (Table 2). The slight decrease in activity was not due to loss of protein expression (Figure 1C).

The serine residue in HNF4 α G115S is a substrate for PKA

With a serine residue at position 115, the HNF4 α mutant contains a RXS (Arg-Xaa-Ser) consensus site for phosphorylation by PKA [36]. This led us to analyse whether PKA phosphorylates the serine residue at position 115 in the mutant HNF4 α . HNF4 α

Figure 1 Expression of wild-type (WT) and G115S HNF4 α in HeLa, β TC3 and HepG2 cells (panels A, B and C respectively)

Western blots were revealed with the α 455 HNF4 α antiserum [24].

Table 2 Effects of the G115S mutation on HNF4 α transcriptional activities on apolipoprotein promoters in HepG2 cells

Cells were transfected as indicated in the Materials and methods section. Fold transactivation refers to basal activities of the promoters (cells transfected with the empty expression vector). Results are means $\pm$ S.E.M. for three independent experiments performed six times. * P < 0.05 compared with wild-type HNF4 α .

Promoters	Fold transactivation		P
	HNF4 α WT	HNF4 α G115S	
Apolipoprotein AI	2.81 $\pm$ 0.28	1.65 $\pm$ 0.21	*
Apolipoprotein CIII	5.20 $\pm$ 0.39	4.83 $\pm$ 0.45	

was phosphorylated *in vitro* and cleaved to identify the peptide containing the putative phosphorylated serine residue at position 115. Identification of this serine residue is easier in a short peptide containing a single amino acid residue with a hydroxy group. Such a peptide (12 residues) can be obtained by cleavage of the protein at arginine residues at positions 113 and 125. Thus cleavage of HNF4 α G115S with endoproteinase Arg-C would yield a peptide containing a single phosphoserine residue. Peptides were identified by MS, an accurate method to analyse potential phosphorylation sites [37]. The mass spectrometric response of a phosphopeptide may be attenuated relative to its unphosphorylated counterpart. Because this phenomenon is enhanced in the presence of other peptides, we reduced the number of peptides obtained after proteolysis considerably using the naturally occurring mutant R154X, which is a truncated form of HNF4 α ending at position 153. This mutant contains the entire DBD, which is expected to adopt a conformation identical with that found within the full-length protein. By chromatography on a Talon metal-affinity resin, we obtained fractions highly enriched in bacterially produced His-tag HNF4 α R154X and His-tag HNF4 α R154X G115S (Figures 2A and 2B). These proteins were phosphorylated *in vitro* by PKA α . Proteins were digested with endoproteinase Arg-C, and peptides were analysed by reverse-phase HPLC–ESI-MS coupling. The ESI-MS analysis of peptides yielded from the His-tag HNF4 α R154X revealed a doubly charged peak at m/z 681 (Figure 2C), a value characterizing the expected peptide overlapping the sequence

Figure 2 PAGE, Western blot and MS analysis of His-tag HNF4α R154X and His-tag HNF4α R154X G115S

PAGE (A) and Western blot (B) analyses of bacterially produced His-tag HNF4α R154X and His-tag HNF4α R154X G115S obtained after chromatography on a Talon metal-affinity resin. In (A), the gel was stained with Coomassie Blue. In (B), blots were revealed with the horseradish-peroxidase-conjugated anti-T7 tag antibody. MM, molecular-mass markers (values in kDa). ESI-MS analyses in the range 600–800 *m/z* for digests with endoproteinase Arg-C of phosphorylated His-tag HNF4α R154X and His-tag HNF4α R154X G115S are shown in panels (C) and (D) respectively. Names of peptides are indicated above the *m/z* values of doubly charged peaks. Peptide sequences and theoretical molecular masses are presented in Table 3. All samples were prepared in duplicate, and multiple injections were examined with identical results.

114–125 (peptide C1 in Table 3). The ESI-MS analysis of the peptides yielded from His-tag HNF4α R154X G115S showed doubly charged peaks at *m/z* 696 and 736 (Figure 2D). These *m/z* values characterize peptides overlapping the sequence 114–125 with an unphosphorylated and a phosphorylated serine residue respectively (peptides D1 and D3 in Table 3). These peptides are specific to the His-tag HNF4α R154X G115S, since they were found neither in the His-tag HNF4α R154X digest (Figure 2C) nor in experiments performed in the absence of HNF4α derivatives (blank assay; results not shown). We also detected doubly charged peaks at *m/z* 689 in panel C (peptide C2 in Table 3) and at *m/z* 704

Table 3 Masses of doubly charged peaks and sequences of corresponding peptides obtained from His-tag HNF4α R154X and His-tag HNF4α R154X G115S

The peptide names include a letter indicating the panel of Figure 2 where their ESI-MS analyses were shown. M, theoretical molecular masses of peptides.

Proteins	Peptide names	M	<i>m/z</i> of (<i>M</i> + 2H) ²⁺
His-tag HNF4α R154X	Peptide C1	A ¹¹⁴ GMKKEAVQNER ¹²⁵	1360 681
	Peptide C2	A ¹¹⁴ GMKKEAVQNER ¹²⁵	1376 689
His-tag HNF4α R154X G115S	Peptide D1	A ¹¹⁴ SMKKEAVQNER ¹²⁵	1390 696
	Peptide D2	A ¹¹⁴ SMKKEAVQNER ¹²⁵	1406 704
	Peptide D3	A ¹¹⁴ SMKKEAVQNER ¹²⁵	1470 736
	Peptide D4	A ¹¹⁴ SMKKEAVQNER ¹²⁵	1486 744

and 744 in panel D (peptides D2 and D4 in Table 3). These masses revealed an oxidized form of methionine in these peptides. The presence of a methionine, a marker residue, argues further for the accurate identification of the peptides of interest.

Analysis of fragmentation patterns was performed with tandem MS (MS/MS) for peptides C1, D1 and D3. Their ESI-MS/MS spectra presented in Figures 3(A)–3(C) show the presence of abundant *y*-type fragment ions and revealed the sequence KKEAVQNE. This sequence is found specifically between amino acids at positions 117–124 in both His-tag HNF4α R154X and His-tag HNF4α R154X G115S (Table 3). From the mass and partial sequence features, we can infer that these peptides correspond to the expected ones. Interestingly, fragmentation of the peptide D3 containing the phosphorylated serine residue yielded a fragment of *m/z* at 687 (Figure 3C), indicating an apparent loss of 49 (doubly charged peak) under moderate fragmentation conditions. Such a loss corresponds to release of a phosphoric acid (mass 98) and provides a signature for phosphopeptides [37,38]. A subsequent fragmentation of the fragment of *m/z* 687 confirmed the sequence KKEAVQNE (Figure 3D). These results taken together and the fact that the sequence 114–125 contains a single serine residue allowed us to assign a phosphorylatable serine at position 115. Our findings thus confirm that the serine residue introduced by the G115S mutation in HNF4α is a substrate for PKA.

Introduction of a negative charge at position 115 by either the G115E mutation or PKA-mediated phosphorylation affects HNF4α functional activity

Since serine at position 115 in HNF4α G115S can be phosphorylated *in vitro* by PKA, we investigated in a cellular context the effects of the introduction, by the G115E mutation, of a constitutive negative charge mimicking phosphorylation and of PKA expression on HNF4α transcriptional activity. To avoid any artifactual effect of PKA on the promoter activity, notably on the (L4L3)₃–96 LPK promoter, which contains a cAMP-responsive site at positions –57/–47 [39], experiments were performed on a minimal promoter consisting of four copies of the HNF4α-response element of the TTR gene upstream of the TATA box [(TTR)₄TATA]. The TTR site was preferred to other HNF4α-binding sites because it does not bind RXRα (retinoid X

Figure 3 ESI-MS/MS ion-scanning of peptides overlapping the sequence 114–125 of His-tag HNF4 α R154X and His-tag HNF4 α R154X G115S

(A), (B) and (C) ESI-MS/MS of peptides C1, D1 and D3 respectively. Noteworthy, the fragment of m/z value 687 obtained in (C) was derived from an apparent loss of 49 that is a signature of a phosphorylated serine or threonine residue. (D) ESI-MS/MS of the doubly charged peptide of m/z value 687 obtained in (C) and mentioned above. The partial sequence of these peptides was determined as KKEAVQNE that is specifically found in the HNF4 α sequence 114–125. Ion-scanning was performed in duplicate. The MS/MS spectra were the sum of 30 spectra in MCA mode.

receptor α) homodimers, a known PKA target [24,40], thus eliminating possible artifacts related to phosphorylation of endogenous RXR α . On this promoter, HNF4 α transcriptional activity was markedly impaired by the G115S and G115E mutations in both HeLa and Rin m5F cells (Figures 4A and 4B). These decreases were not due to lower protein expression levels (Figure 4C).

Since Gly¹¹⁵ is located in the HNF4 α DBD, we investigated the consequences of G115S and G115E mutations on HNF4 α DNA-binding activity. HNF4 α G115S and G115E bound the TTR probe poorly compared with wild-type HNF4 α (Figure 4D, first three lanes). However, the loss of DNA binding is less marked than that of transactivation activity. In transfection assays, we cannot exclude that in addition to a partial, but significant, loss of DNA binding, a loss of recruitment of a co-activator may contribute to the strong impairment observed. Alternatively, the mutation may enhance recruitment of a co-repressor. Indeed, although

Figure 4 The G115S and G115E mutations impair HNF4 α -mediated activation of the (TTR)_{4x} TATA promoter

Transcriptional activation in HeLa cells (A) and Rin m5F cells (B). Cells were transiently transfected as indicated in the Materials and methods section. Fold transactivation refers to basal activity of the promoter (cells transfected with the empty expression vector). Results are means $\pm$ S.E.M. for three independent experiments performed in triplicate. *** P < 0.001 compared with wild-type (WT) HNF4 α . (C) Western blotting revealed with the α 455 HNF4 α antiserum of wild-type, G115S and G115E HNF4 α expressed in HeLa cells. (D) DNA-binding activities analysed by EMSA performed with the HNF4 α -response element of the TTR promoter and HNF4 α proteins overexpressed in HeLa cells. The arrow denotes the DNA–HNF4 α complexes. In the last three lanes, nuclear extracts were treated with PP1 before incubation with the probe.

most cofactors bind to the activation functions AF-1 and AF-2, an increasing number of transcriptional partners bind the DBD of nuclear receptors [41–44]. Note that both mutants display very similar activities. This led us to check whether removal of the phosphate group on serine would restore DNA binding of the G115S mutant. As previously observed on the HNF4 α -binding site of the LPK promoter, treatment of nuclear extracts with the serine/threonine protein phosphatase PP1 increased DNA binding of all HNF4 α proteins (Figure 4D, last three lanes), which is explained by dephosphorylation of serine residues at positions 133 and 134 [17]. Interestingly, the increase in DNA binding of the G115S mutant was stronger than that of the G115E mutant, suggesting that phosphorylation of S115 contributes to the loss of DNA binding. Note, however, that DNA binding of the G115S mutant was not completely recovered by the PP1 treatment (compare binding of wild-type and G115S HNF4 α). Therefore the losses of transcriptional activity and DNA binding do not reflect a complete phosphorylation of S115, but rather would result from a combination of the introduction of a negative charge and an additional mechanism that we are unable to determine.

To investigate the impact of PKA expression on Ser¹¹⁵, we took into account that serine residues at positions 133 and 134 in

HNF4 α are PKA phosphorylation sites and that mutations of these residues in alanine and glycine respectively abolished the PKA-mediated decrease in activation of transcription by HNF4 α [17]. So, to analyse more specifically the effects of the G115S mutation, we engineered constructs with the same mutations: HNF4 α SS133-134AG and HNF4 α SS133-134AG G115S. We verified that mutations at positions 133 and 134 did not alter HNF4 α expression (Figure 5B) and transcriptional activity on the (TTR) $_{4\times}$ TATA promoter, as they failed to alter activation of the (L3) $_{4\times}$ -54/+11 LPK promoter [17]. Note that, again, in the SS133-134AG protein, the G115S mutation impaired HNF4 α -mediated activation of transcription dramatically (Figure 5A). The basal activity of the (TTR) $_{4\times}$ TATA promoter was not affected by overexpression of PKA C α , as shown in Figure 5(C) (compare bars 1, 4 and 7). In the absence of the PKA C α expression vector, the (TTR) $_{4\times}$ TATA promoter was activated 3.83-fold by HNF4 α SS133-134AG compared with 1.77-fold by HNF4 α SS133-134AG G115S (Figure 5C). The weaker activation by HNF4 α SS133-134AG when compared with that observed in Figure 5(A) may be due to depletion of factors of the basal transcription machinery by the promoter present in the co-transfected empty expression vector for PKA C α , a phenomenon often observed in assays using multiple expression vectors. When PKA C α was overexpressed, the difference in promoter activation by the two HNF4 α proteins was enlarged markedly and, noteworthily, activation by HNF4 α SS133-134AG G115S was abolished (Figure 5C, bars 6 and 9). Because these HNF4 α proteins only differ in the G115S mutation, the opposite changes induced by the PKA expression are probably attributed to phosphorylation of Ser¹¹⁵ by PKA. The specificity of these PKA-mediated effects was controlled using PKI, a specific PKA inhibitor. To better visualize changes due to the G115S mutation, the activity of HNF4 α SS133-134AG G115S was expressed relative to that of HNF4 α SS133-134AG, which was set to 100%. In the absence of the PKA C α expression vector, the activity of HNF4 α SS133-134AG G115S was enhanced markedly upon PKI expression (Figure 5D, compare bars 2 and 4), thus indicating that the low activity of HNF4 α SS133-134AG G115S could be due, at least in part, to phosphorylation of the Ser¹¹⁵ residue by endogenous PKA. When PKI and PKA C α expression vectors were co-transfected, the significant loss of HNF4 α SS133-134AG G115S activity due to PKA C α overexpression was abrogated (compare bars 6 and 8 in Figure 5D). We verified and confirmed by cell titre analysis that overexpression of PKA C α and PKI did not affect cell viability (results not shown). Our findings suggest strongly that PKA-mediated phosphorylation of the serine residue at position 115 contributes to the impairment of HNF4 α transcriptional activity by the G115S mutation.

DISCUSSION

The G115S mutation in HNF4 α resulted in a significant loss of HNF4 α transcriptional activities on promoters of HNF1 α and LPK in most cell lines tested, notably in pancreatic β -cell lines. In pancreatic β -cells, ATP and other factors generated from pyruvate essentially serve to promote insulin exocytosis [28]. HNF1 α is required for the metabolism–insulin secretion coupling in pancreatic β -cells [29,30]. Taking into account that MODY1 is characterized mainly by a defect in insulin secretion, our results are in line with the 100% penetrance of MODY1 diabetes in carriers of the G115S mutation. In sharp contrast with the strong impairment observed in pancreatic β -cells, the G115S mutation did not significantly decrease the HNF4 α -mediated activation of apolipoprotein promoters in HepG2 cells. This result is in agreement with

Figure 5 PKA overexpression contributes to the impairment of HNF4 α transcriptional activity due to the G115S mutation

(A) and (B) Controls to show that mutations at positions 133 and 134 do not affect HNF4 α transcriptional activity on the (TTR) $_{4\times}$ TATA promoter and HNF4 α expression. Experimental conditions were as in Figures 4(A) and 4(C) respectively. WT, wild-type. (C) Effect of PKA C α expression on transcriptional activities of HNF4 α SS133-134AG and HNF4 α SS133-134AG G115S. HeLa cells were co-transfected with 500 ng of (TTR) $_{4\times}$ TATA Luc, 50 ng of vector expressing HNF4 α derivatives and the indicated amounts of vector expressing PKA C α . The total amount of transfected DNA was equalized with the corresponding empty expression vectors. Fold induction refers to the activity of the promoter in the absence of HNF4 α derivatives. (D) Control to show the specific action of PKA on repression of HNF4 α transcriptional activity using PKI expression. HeLa cells were transfected as in (C) plus 50 ng of vector expressing PKI or its empty vector. Activity of HNF4 α SS133-134AG G115S is expressed relative to that of HNF4 α SS133-134AG, which was set at 100%. Results are means $\pm$ S.E.M. for three independent experiments performed six times. *** P < 0.001.

the normal triacylglycerol level of patients carrying this mutation [13] and more generally with the fact that so far no severe defects have been detected in the liver of MODY1 patients [10,35]. The

stronger effect of these mutations in endocrine pancreas than in liver may be due to the much lower expression of HNF4 α in pancreatic islets compared with that in liver [32]. Because of the limiting amount of HNF4 α in pancreatic β -cells, loss of function of the product of one HNF4 α allele would switch off the regulatory loop required for the normal function of pancreatic β -cells, as proposed in the haploinsufficiency model of Ferrer [4].

We show that the G115S mutation creates a PKA phosphorylation site and its PKA-mediated phosphorylation contributes to the impairment of HNF4 α transcriptional activity. Furthermore, introduction of a constitutive negative charge that mimics phosphorylation at position 115 results in significant losses of DNA binding and transactivation activities of HNF4 α . Gly¹¹⁵ is located in the C-terminal extension of the DBD. The three-dimensional structure of the HNF4 α DBD remains to be determined; nevertheless, HNF4 α and RXR α share closely related structures [45]. NMR studies of the RXR α DBD indicate that the glycine residue at the equivalent position (Gly¹⁹⁹) is located in a sharp loop (four residues in length) between helices α 2 and α 3 [46]. These amphipathic helices form, with helix α 1, a hydrophobic core that contributes to the stabilization of the DBD conformation. Two basic residues located in the vicinity of Gly¹⁹⁹ (Lys²⁰¹ and Arg²⁰²) form electrostatic bonds with the DNA backbone. In HNF4 α , the basic character of these residues is conserved, since two lysine residues are encountered at the equivalent positions. Importantly, the glycine residue is strictly conserved in almost all nuclear receptors and probably plays a crucial role. In the G115S HNF4 α mutant, Ser¹¹⁵ is likely to be accessible for phosphorylation, since, based on a conserved three-dimensional structure of the DBD, it would be exposed in a loop. Through introduction of a negative charge, phosphorylation of Ser¹¹⁵ may hinder the electrostatic interaction between HNF4 α and DNA. It is worth pointing out that PKA C α is expressed in pancreatic β -cells, where it plays a crucial role [47,48]. Several mechanisms have been described to explain how mutations in HNF4 α impair the function of this nuclear receptor. They include: (i) deletion of the activation function AF-2 by nonsense mutations such as R154X and Q268X [11], (ii) partial loss of DNA binding by mutations in the DBD such as D126Y and D126H [12], and (iii) impaired recruitment of co-activators and other transcriptional activators by the E276Q mutation [20,49]. Even if we could not determine the added effect of phosphorylation of Ser¹¹⁵ beyond that of natural sites, our results suggest that introduction of a phosphorylation site in the DBD may represent an additional mechanism by which a MODY1 mutation may contribute to the loss of HNF4 α function. Because HNF4 α activates a large number of genes, including that of insulin, a reduced activity of HNF4 α may affect β -cell function.

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