Probing the effect of MODY mutations near the co-activator-binding pocket of HNF4α

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Synopsis

HNF4α (hepatocyte nuclear factor 4α) is a culprit gene product for a monogenic and dominantly inherited form of diabetes, referred to as MODY (maturity onset diabetes of the young). As a member of the NR (nuclear receptor) superfamily, HNF4α recruits transcriptional co-activators such as SRC-1α (steroid receptor co-activator-1α) and PGC-1α (peroxisome-proliferator-activated receptor γ co-activator-1α) through the LXXLL-binding motifs for its transactivation, and our recent crystal structures of the complex provided the molecular details and the mechanistic insights into these co-activator recruitments. Several mutations have been identified from the MODY patients and, among these, point mutations can be very instructive site-specific measures of protein function and structure. Thus, in the present study, we probed the functional effects of the two MODY point mutations (D206Y and M364R) found directly near the LXXLL motif-binding site by conducting a series of experiments on their structural integrity and specific functional roles such as overall transcription, ligand selectivity, target gene recognition and co-activator recruitment. While the D206Y mutation has a subtle effect, the M364R mutation significantly impaired the overall transactivation by HNF4α. These functional disruptions are mainly due to their reduced ability to recruit co-activators and lowered protein stability (only with M364R mutation), while their DNA-binding activities and ligand selectivities are preserved. These results confirmed our structural predictions and proved that MODY mutations are loss-of-function mutations leading to impaired β-cell function. These findings should help target selective residues for correcting mutational defects or modulating the overall activity of HNF4α as a means of therapeutic intervention.

Key words: diabetes, gene expression, hepatocyte nuclear factor 4α (HNF4α), LXXLL motif, maturity onset diabetes of the young (MODY), mutation, nuclear receptor (NR)

INTRODUCTION

HNF4α (hepatocyte nuclear factor 4α) plays an essential role in the development and function of vital organ cells such as hepatocytes and pancreatic β-cells by regulating expression of multiple genes involved in organ development, nutrient transport and diverse metabolic pathways. Conditional targeted gene disruption of HNF4α results in marked metabolic disregulation and increased mortality [1–3], and the mutations on Hnf4α are directly linked to the onset of MODY (maturity onset diabetes of the young), a monogenic and dominantly inherited form of diabetes that is mainly characterized by the impairment of glucose-stimulated insulin secretion from the β-cells [4]. HNF4α is a novel member (NR2A1) of the NR (nuclear receptor) superfamily [5], which comprises all-cysteine zinc-finger DBD (DNA-binding domain), lipophilic LBD (ligand-binding domain) and additional domains with activation function. HNF4α displays a unique mode of ligand-induced transactivation [6] and still remains as an orphan NR even though the recent crystal structures of HNF4α LBD alone [7] and in complex with the co-activator fragments [8,9] revealed fatty acids as potential structural ligands for HNF4α, and the recent affinity-aided identification of LA (linoleic acid; C18:2ω6) as an exchangeable, yet non-transactivational endogenous ligand for mammalian HNF4α [10].

In addition to ligand binding, the NR-LBD has an additional role of recruiting many other proteins with transactivation function such as co-activators/co-repressors and mediators which in turn modify the chromatin structure and bring

Abbreviations used: DBD, DNA-binding domain; DMEM, Dulbecco’s modified Eagle’s medium; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; GC, gas chromatography; GST, glutathione transferase; HNF4α, hepatocyte nuclear factor 4α; IC, isothermal titration calorimetry; LBD, ligand-binding domain; MODY, maturity onset diabetes of the young; NR, nuclear receptor; PGC-1α, peroxisome-proliferator-activated receptor γ co-activator-1α; SRC, steroid receptor co-activator; TEV, tobacco etch virus; wt, wild-type.

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the main transcriptional machinery to the initiation site [11,12]. Well-known co-activators for HNF4α include NCoA (nuclear receptor co-activator)/SRC-1 (steroid receptor co-activator-1)/p160 family members and PGC-1α (peroxisome-proliferator-activated receptor γ co-activator-1α), whose interactions are mediated by a small pentapeptide motif (LXXLL), termed the NR-box [13]. The co-activator-binding pocket is made of several core hydrophobic residues and the surrounding residues that facilitate the interactions with the signature LXXLL motifs [8,9].

While HNF4α MODY mutations are sporadically found throughout the sequence, the D206Y and M364 mutations are found directly near the co-activator-binding pocket, and these substitutions are expected to influence specific aspects of protein structure and/or function. These mutations cause inflicted individuals to have abnormal glucose tolerance along with other typical diabetic symptoms as a result of β-cell dysfunction including very low insulin secretion [14,15]. To better understand the exact molecular basis of these MODY mutations, we have cloned and overproduced both the full-length and the truncated versions of the wt (wild-type) and the MODY mutants of HNF4α and conducted a set of functional/biochemical/biophysical studies. The results presented here clearly show that each residue plays a distinctive role in protein stability and molecular interactions resulting in a different degree of impairment in overall transactivation, supporting the notion that MODY mutations are loss-of-function ones.

**EXPERIMENTAL**

**Construction of overexpression vectors for wt and mutants, and protein purification**

The luciferase reporter plasmids, pCMV Sport6 co-activator harbouring the full-length cDNA of human PGC-1α (MED25 mediator of RNA polymerase II transcription, subunit 25 homologue) or SRC-1α, pcDNA3 HNF4α FL containing the full-length of human HNF4α, and firefly reporter vector pGL3 (BA1), were constructed as described previously [9]. For in vitro studies with the recombinant proteins, both the HNF4α-LBD (142–368) and the PGC-1α fragment (74–219) containing all three LXXLL motifs were cloned into pET41a (Novagen) with a GST (glutathione–agarose beads (Invitrogen) for TEV digestion (1:50 molar ratio of TEV to substrate) from glutathione–agarose beads (Invitrogen) after overnight incubation at 4°C in the buffer containing 50 mM Tris/HCl (pH 8.0), 0.5 mM EDTA and 2 mM DTT, and further purified by ion-exchange chromatography. The purified proteins were estimated by SDS/PAGE to be at least 98 % pure as judged by staining with Coomassie Brilliant Blue on a 8–25 % gradient gel. Fractions were pooled; concentrations were measured by UV absorption and stored at −80°C as a 10 % (v/v) glycerol stock.

**Site-directed mutagenesis analysis**

The QuikChange® multi site-directed mutagenesis kit (Stratagene) was used to generate the constructs with each point mutation of HNF4α-LBD according to the manufacturer’s instructions. The plasmid templates used in the mutations were pET41a GST–HNF4α-LBD and pcDNA3 HNF4α-FL, PGC-1α NR-box mutants were generated in a similar manner by substituting each LXXLL motif with the LXXAA sequence. The plasmid templates used in the mutagenesis protocol were pET41a GST–PGC-1α (74–219) and pCMV Sport6 PGC-1α-FL. All the generated constructs with the mutated sequences were verified by DNA sequencing.

**Cell cultures**

The HeLa cell line was cultured in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10 % fetal bovine serum (Invitrogen), 50 units/ml penicillin, 50 μg/ml streptomycin and 0.1 mM non-essential amino acids (Invitrogen).

**Transient transfection and luciferase assay**

The full-length cDNA of human HNF4α wt or mutant was subcloned into the pcDNA3(+)Neo vector (Invitrogen), and the reporter vector pGL3-(BA)1, containing one copy of the HNF4α response element (−64 to −52) within the promoter of human Hnf1α (−298 to the first AGT) was constructed and used for luciferase assays in the absence or presence of transfected co-activators (PGC-1α, MED25 or SRC-1α). HeLa cells were transfected using Opti-MEM and Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer’s recommendations. Briefly, a total of 30 ng of pcDNA3 HNF4α (wild-type or MODY mutants) and 100 ng of pcCMV Sport6 PGC-1α (or 50 ng of pcCMV Sport6 MED25 or 250 ng of pSG5.HA SRC-1α), 50 ng of pGL3 (BA1), and 10 ng of pRL-TK (control Renilla luciferase vector) were used for transfection of 1 × 10⁵ cells seeded on 24-well plate 1 day before transfection. At 48 h after transfection, cells were washed with 1×PBS and lysed with luciferase lysis buffer supplied with the Luciferase assay kit (Promega). Luciferase activity was measured using Dual Luciferase assay system (Promega) and Lmax Luminometer (Molecular Devices). All values were normalized by the relative ratio of firefly luciferase activity and Renilla luciferase activity. At least three inde-
dependent transfections were performed in quadruplicate. Results were expressed as means ± S.E.M. for at least four independent groups. Statistical significance was determined by one-way ANOVA followed by the Student–Newman–Keuls method using Sigma Stat 3.1 software (Systat Software). A probability value \( P < 0.05 \) was considered statistically significant.

**Preparation of the nuclear extracts and subsequent comparative EMSA (electrophoretic mobility-shift assay) analysis for wt and mutants**

A total of 3 \( \mu \)g of wt or mutant HNF4α plasmid was transfected into HeLa cells. The HeLa cells were harvested 24 h after transfection, and the nuclear extracts were prepared using NucBuster protein extraction kit (Novagen) according to the manufacturer’s protocol. The loading amounts of HNF4α wt and the mutants were adjusted to an equal amount after quantification by an ELISA assay and visualization by a Western blot. For EMSA, synthetic 15 bp oligonucleotides containing the HNF4α response element within the Hnf1α promoter were labelled with an IR dye (IRDye 700 phosphoramide) (LI-COR Biosciences). EMSA were performed on a 6 % native polyacrylamide gel using a Tris/glycine/EDTA buffer and analysed with the Odyssey Infrared Imaging System (LI-COR Biosciences).

**Protein stability assays (pulse–chase experiment)**

Stabilities of the wt and the mutants in cultured cells were measured by a means of pulse–chase experiments. At 24 h after transfection, HeLa cells were serum-starved for 1 h before being incubated in DMEM minus methionine/cysteine residue for 30 min and then labelled with 100 \( \mu \)Ci/ml Trans 35S labelling mix (MP Biomedicals) for 30 min at 37°C. Labelling medium was removed thereafter and the 35S-labelled cells were incubated in DMEM containing 10 % FBS for the indicated periods of time (0–18 h) and lysed. The nuclear extracts were prepared using NucBuster protein extraction kit (Novagen). Proteins were immunoprecipitated with anti-HNF4α antibody (Santa Cruz) and Protein A–Sepharose beads (GE Healthcare), resolved by SDS/PAGE, and incorporated radioactivity was analysed by autoradiography using BioMax film (Eastman Kodak) or storage phosphor screens (type GP, GE Healthcare) for quantification with a Typhoon phosphorimager equipped with the Image-Quanta software (GE Healthcare).

**GST pull-down assays for HNF4α/PGC-1α interactions**

wt and the mutant HNF4α-LBD (142–368) GST fusion proteins were produced in *E. coli* while the full-length 35S-labelled PGC-1α proteins were produced with a TNT® reticulocyte lysate *in vitro* transcription and translation kit (Promega). Approx. 1 \( \mu \)g of fusion protein was mixed with 30 \( \mu \)l of the *in vitro* translate in a binding buffer containing 50 mM Tris/HCl buffer (pH 8.0), 100 mM NaCl, 0.5 mM EDTA and 0.1 % Nonidet P40. Binding was performed for 4 h at 4°C and the mixtures were loaded on to the beads containing glutathione. Immobilized GST protein was detected by SDS/PAGE and Western blotting using Coomassie Brilliant Blue staining and probing with a GST-specific antibody (GE Healthcare). The beads were then washed multiple times with the binding buffer and resuspended in SDS/PAGE sample buffer. After electrophoresis, the 35S-labelled proteins were detected by autoradiography.

**Fatty acid analyses**

wt and the mutant recombinant HNF4α-LBD (142–368) proteins prepared from *E. coli* (20 mg) were combined with a 10-fold excess (v/v) of 15 % NaOH in 50 % methanol and heated for 30 min at 100°C. Esterification was accomplished by combining the cooled solutions with 20 vol. of 46 % methanol in 6 M HCl and heating for 10 min at 80°C. The fatty acid methyl esters were extracted into 50 % methyl t-butyl ether in hexane and analysed by GC (gas chromatography) (Sherlock MIS; 170–270°C at 5°C/min) at the Bacterial Identification and Fatty Acid Analysis Laboratory, University of Florida.

**Protein preparation and ITC (isothermal titration calorimetry)**

The ITC experiments were performed using a high-precision VP-ITC titration calorimeter (MicroCal/GE Healthcare). Equivalent molar amounts of both purified recombinant proteins, HNF4α-LBD (142–368) and PGC-1α fragment (74–219), were mixed in the reaction buffer (20 mM Tris (pH 8.0), 100 mM NaCl and 5 % glycerol) and then degassed for 5 min prior to sample loading. No exogenous ligands were added for the experiments. Protein–protein interactions were quantitatively measured by this method. Briefly, a defined amount of protein was titrated against 1:20 molar concentration of the binding partner, PGC-1α, in the thermostatically controlled cell by means of a syringe via 30–40 individual injections. Both samples were prepared in the same buffer and pre-heated to 5°C below the actual experimental temperature. The experiments were performed at 20°C, and the stoichiometry, thermodynamic parameters and binding affinity were calculated by fitting the data using the software Origin 7.0 (MicroCal/GE Healthcare). The binding isotherm was fitted by a non-linear least-square regression using the one-set-of-sites model since this model fits the best. The binding Gibbs free energy (\( \Delta G \)) was calculated from enthalpy changes (\( \Delta H \)) and association constant (\( K_a \)) using the equation: \( \Delta G = -RT\ln K_a \), where \( R \) is the gas constant and \( T \) is the absolute temperature in Kelvin. Meanwhile, the stoichiometry (n) of the interaction was determined from the one-set-of-sites model.

**RESULTS AND DISCUSSION**

**MODY mutations near the co-activator-binding pocket of HNF4α**

Our previous crystal structures of HNF4α-LBD [7–9] revealed that two MODY point mutations are found near the LXXLL
Figure 1 Close-up view of the LXXLL motif-mediated co-activator-binding pocket of HNF4α

Side chains of the selected MODY mutations for the current studies (D206Y and M364R) are highlighted as a ball-and-stick model (grey sticks and labels). The bound SRC-1α LXXLL motif peptide (blue) and PGC-1α LXXLL motif fragment (red) shown as a ribbon diagram have nearly identical binding modes towards HNF4α. Hydrogen bonds are indicated as broken lines. The hydrophobic residues lining the LXXLL motif-binding groove and the ‘charge-clamp’ residues (Lys194 and Glu363) making hydrogen bonds with the backbone atoms of the bound peptides are also highlighted as yellow and green sticks respectively. These structures have been deposited in PDB (protein data bank; accession codes 1P2L and 3FS1).

motif-binding pocket (Figure 1). They are the D206Y and M364R mutations. These mutations were identified from the MODY patients who had increased birth weight and low blood-sugar level at birth, and reduced β-cell function, but near normal insulin sensitivity throughout life [14,15]; however, functional and biochemical studies of these mutants have not been conducted.

The Asp^{206} residue is located on the first turn of the helix H4 at the fringe of the LXXLL motif-binding pocket, and forms a set of hydrogen bonds with the side-chain hydroxy group and the backbone amino group of Ser^{294} (Figure 1). This residue should help anchoring the helix H4 for optimal recognition of the LXXLL motif. Substitution to a bulky tyrosine residue at this position will likely disrupt the organization of the local structure by causing steric hindrance and breakup of the hydrogen bonds.

On the other hand, the Met^{364} residue lining the LXXLL motif-binding groove plays a key role in recognition of the first leucine residue through van der Waals and hydrophobic interactions (Figure 1). In addition, the Met^{364} residue is placed right next to the strictly conserved Glu^{363} residue that provides one end of the ‘charge clamp’ [16]. Replacement with a highly charged arginine residue at this position will likely disrupt the hydrophobic environment of the pocket and the LXXLL motif interactions. In the present study, we set out to test the predicted functional disruptions by these mutations and to gain molecular insight into additional roles played by these key residues.

Mutational effects on their overall transactivation

HNF4α is a gene-specific transcription factor and the measurement of its overall transactivation is the ultimate readout for its functional activity. Thus, as a first step in gauging the effect of these MODY mutations in vivo, we measured the overall transcriptional activities of the wt and the mutants by performing luciferase reporter assays on the Hnf1α (another MODY gene) promoter, and the results are shown in Figure 2(A). HNF4α alone as well as specific co-activator-mediated transactivations such as PGC-1α, MED25 (a newly identified transcriptional mediator component for HNF4α; G.B. Rha and Y.-I. Chi, unpublished work) and SRC-1α-mediated transactivations were measured. Like all other loss-of-function MODY mutations [17–19], these mutations caused a reduction in the overall transactivation in all cases of repeated assays. While the M364R mutation caused a drastic reduction (over 70%), the D206Y mutant produced a smaller effect (~20% reduction). This effect by D206Y substitution was even smaller than the ones caused by the MODY mutations on the fringe of HNF4α-DBD [17] and supports the notion that even subtle disruption of HNF4α molecular function can cause significant impacts in afflicted MODY patients [17].

Similar comparative levels and patterns of transcriptional reduction were observed for HNF4α alone and all three co-activators-mediated transactivations that we tested (Figure 2A).

NR co-activators typically contain more than one LXXLL motif, and each NR has a different preference for different LXXLL motifs [20,21]. The HNF4α–PGC-1α interaction is no exception and our recent studies revealed that HNF4α displays a preference for the PGC-1α LXXLL motifs (NR-boxes) in the order of NR-2>NR-3>NR-1, yet requires all three for its full activity [9]. In order to investigate, as a next step, whether or not these mutations alter HNF4α’s ability to specifically recognize individual LXXLL motifs (thus reducing its overall transactivation), we measured the transcriptional activities with the wt PGC-1α and each of the NR-box mutants containing one or two (or three) non-functional LXXLL motifs (Supplementary Figure S1 at http://www.bioscirep.org/bsr/031/bsr0310411add.htm). We chose PGC-1α for our studies because it represents a novel transcription co-activator and its partnership with HNF4α plays a central role in gluconeogenesis and onset of diabetes [22,23]. For NR-box mutants, we mutated one, two or all three of the individual LXXLL motifs to LXXAA (Supplementary Figure S1B), and measured their overall transcriptional activities. As shown in Figure 2(B), the MODY mutants retained the same pattern of LXXLL motif usage as the wt and did not alter the selectivity towards the multiple LXXLL motifs of PGC-1α, and the apparent reduction in overall transcription is believed to be the result of other molecular defects. The M364R mutant could not be used in these comparative studies, owing to its low level of overall transcription, even in the presence PGC-1α.

Mutational effects on their protein stability or target gene recognition

The structural integrity of a transcription factor is essential for its vital functions such as DNA target recognition and additional...
Probing the effect of MODY mutations

Figure 2 Measurement of transactivation potential exerted by the MODY mutants compared with the wt
(A) Overall transcriptional activity by the MODY mutants compared with the wild-type HNF4α alone or in combination with known co-activators/mediator. Standard luciferase-based transcripational reporter assays were conducted using HeLa cells transfected with the wt or mutated pcDNA3 HNF4α and the luciferase reporter construct containing one copy of the HNF4α response element (−64 to −52) within the promoter of human Hnf1α (pGL3-HNF1α-298). CTL in the first lane refers to an empty vector and all data have been normalized against the Renilla luciferase activity. The next three lanes are transfected with the HNF4α-expressing vector only while the remaining lanes are the results of double transfection of HNF4α and the respective PGC-1α (MED25- or SRC-1α-) expressing vectors. Values are means ± S.E.M. (n = 4), and the mutational effects are more pronounced for the M364R mutant in all cases. (B) PGC-1α LXXLL motif selectivity by HNF4α wt and the MODY mutant. Overall transactivation potential exerted by the PGC-1α wt and the NR-box mutants toward HNF4α were measured. The mutated NR-boxes of PGC-1α are indicated in the names of each mutant and schematically shown in Supplementary Figure S1(B). CTL in the first lane refers to an empty vector and the second lane refers to transfection with HNF4α alone, while the remaining lanes refer to double transfection with HNF4α and PGC-1α. Only the D206Y mutant (dark grey bars) was used in this comparative study with the wt (light grey bars) because M364R showed a very low level of transcriptional activity. The D206Y mutant had no impact on the selectivity towards PGC-1α multiple LXXLL motifs.

protein interactions. DBD is the most conserved structural and functional element of NRs and consists of two non-equivalent zinc-finger motifs, each containing four highly conserved cysteine residues co-ordinating the binding of a zinc ion. This results in the formation of a tertiary structure containing three main helices among which the N-terminal helix (helix I) directly and specifically interacts with the major groove of each DNA half-site sequence element, whereas the C-terminal helix (helix III) overlays the N-terminal helix in a perpendicular fashion and contributes to stabilization of the overall protein structure by forming a hydrophobic core [17]. Although the D206Y and M364R mutations are situated within the LBD and NRs are modular proteins in which individual domains are believed to act independently, there can be considerable allosteric regulations between the modular domains and DNA binding can be affected by the domains other than the DBD as revealed by a full-length NR structure in complex with DNA [24]. Thus, we also examined DNA binding activities of the mutants by using the nuclear extracts containing HNF4α-FL (instead of the truncated recombinant HNF4α proteins) to examine the allosteric effect of these mutations on DNA binding.

First, protein stabilities of the wt and the mutants were assessed by in vivo pulse–chase experiment on HeLa cells and the results are shown in Figure 3(A). Even though the differences were
Figure 3  Additional characterization and functional studies of the MODY mutants

(A) Lifetime of the HNF4α mutants compared with the WT. Left panel: raw data: HeLa cells were labelled with [35S]-methionine/cysteine for 30 min (0 h) and pulse-chased for various lengths of time (3–18 h) in the presence of excess non-radioactive methionine/cysteine residue. Samples were immunoprecipitated under normal stringency conditions using polyclonal anti-HNF4α antibody and subjected to SDS/PAGE followed by autoradiography. Right panel: fraction of initial protein: percentage of the remaining protein (relative value) is calculated by the relative amount of the protein at a given post-chase time to the initial amount at 0 h as determined by the intensity of each band using the Image-Quanta software.

(B) Retained DNA-binding activities of the mutants revealed by EMSA. The first two lanes (from left) were used as control (DNA probe only and nuclear extract from the transfection with the empty vectors). The wt is shown in lane 3 and the mutants are shown in the subsequent set of lanes (lanes 4 and 5). The lower bands correspond to free DNA while the upper bands represent the shifted HNF4α-DNA complex. The Western blot (top panel) compares the amount of wt and mutated forms of HNF4α in the loaded nuclear extracts. (C) Similar compositions of fatty acids bound to HNF4α-LBD.
marginal, the M364R mutant (light grey line with triangles on each data point) showed more rapid degradation in mammalian cell lines compared with the wt, while the D206Y mutant retained comparable stability.

As a next step, we measured their abilities to bind one of HNF4α’s target genes and another MODY gene Hnf1α promoter sequence in vitro by EMSA experiments using nuclear extracts from HNF4α-FL-infected HeLa cells that do not contain endogenous HNF4α. HNF1α works very closely with HNF4α to control the development of vital organs such as liver and pancreas [25]. As shown in Figure 3(B), they all have the similar DNA-binding activities to the wt, indicating that these mutations have very little effect on DNA interaction/target gene recognition, and the potential allosteric regulations between the HNF4α-LBD (ligand-binding domain) and the HNF4α-DBD are not affected by these mutations. Likewise, it is also believed that the nuclear localization of this protein is not affected by these two MODY mutations because the primary NLS (nuclear localization signal) sequences of HNF4α are located far outside of the LBD and only found within the DBD [26]. It is believed that nuclear import is mediated by isolated recognitions between the nuclear-targeting signals and their cognate receptors [27].

**Mutational effects on their ligand selectivity and the recruitment of co-activators and mediators**

HNF4α exhibits constitutive transactivation activity in that no exogenous ligand is needed for HNF4α to activate transcription [28]. Although its *bona fide* physiological role remains unknown, long-chain fatty acids (a mixture of fatty acids in the case of bacterially expressed HNF4α [7,29] and LA (C18:2ω-6 fatty acid) in the case of HNF4α expressed in mammalian tissues [10]) are proven to be apparent functional/structural ligand for HNF4α. The exact composition of bound fatty acids from bacterially expressed and purified recombinant HNF4α proteins has been previously analysed by running GC [7].

To gain further insight into the mutational effect on ligand selectivity, we analysed, by the same method, the identities and the relative quantities of the bound lipids for HNF4α wt and the mutants. Fatty acids were extracted from human HNF4α-LBD wt or the mutants, converted into the corresponding methyl esters, and analysed by GC. Their chromatograms and the relative amounts of each fatty acid (in a separate table) are provided. 12:0, 14:0 and 16:0 are saturated lauric, myristic and palmitic acids respectively; 16:1ω7c is monounsaturated palmitoleic acid, cyclo refers to a cyclopropyl group, 20H and 30H have hydroxyl groups at the 2 or 3 positions respectively and iso has an extra methyl group on the penultimate carbon. Both fatty acids are listed when mixtures could not be resolved. They all show the similar patterns of ligand selectivity. (D) Decreased binding of PGC-1α LXXLL motifs to the mutants measured by GST pull-down in *in vitro* binding studies. The recombinant proteins of GST–HNF4α-LBD wt or mutant and the *in vitro*-translated and 35S-labelled PGC-1α (full-length) bearing all the three LXXLL motifs (Supplementary Figure S1). The results are shown in Figure 3(D) and they are in good agreement with the transcription assays. The mutants showed noticeably reduced bindings to PGC-1α, and the impairment was more pronounced for the M364R mutant that is directly within the binding groove and just one amino acid downstream of the ‘charge-clamp’ residue Glu561 [8]. Local re-arrangements and weakening of the ‘charge-clamp’ could further contribute to the pronounced effect by the M364R substitution. These findings indicated that the major mutational effects by the D206Y and M364R MODY mutations are the reductions in their ability to recruit co-activators, leading to reduced transcriptional activities. These interactions were further examined by more quantitative ITC experiments. The binding energetic of each protein was measured with the recombinant proteins of HNF4α-LBD wt or mutant and PGC-1α bearing all three LXXLL motifs. Purification of each recombinant protein to homogeneity and accurate measurement of its concentration enabled quantitative determination of binding affinity and associated thermodynamic parameters on binding. The calorimetric titrations and the fitting results of the binding experiments are shown in Figure 3(E). In accordance with
the results of the transcription assays and the pull-down experiments, the quantitative analysis of in vitro bindings also showed a similar pattern of binding affinities. Binding of the wt HNF4α-LBD to PGC-1α ($K_a = 2.72 \times 10^5 \text{M}^{-1}$) was almost 2-fold and 4-fold stronger than that of the D206Y mutant ($K_a = 1.28 \times 10^5 \text{M}^{-1}$) and the M364R mutant ($K_a = 0.674 \times 10^5 \text{M}^{-1}$) respectively. These mutants did not completely ablate the binding, consistent with the notion that HNF4α is essential for development and subtle disruption of HNF4α molecular function can cause significant impacts in afflicted MODY patients [17]. Any severe impairment can be lethal to the embryos and we would never see those individuals.

In all cases, ITC measured the global thermodynamic parameters of a system that is consistent with a combined contribution by the hydrophobic interactions (positive TΔS or negative TΔA; entropically favourable) and the formation of hydrogen bonds through the ‘charge-clamp’ (negative ΔH; enthalpically favourable). Finally, the best fit of the binding stoichiometry (N) was one within experimental error in all measurements, confirming a 2:2 stoichiometry of the complex since the functional unit of HNF4α is a homodimer [30].

Conclusions

HNF4α represents a unique member of the NR family as it displays unique DNA, ligand and co-Regulator-binding properties [7–10,17] as well as an auto-regulatory mechanism [28]. Many NRs have been successfully targeted for therapeutic intervention and the target sites include a ligand-binding pocket, a DNA-binding surface and a co-Regulator-binding interface [31]. Since HNF4α is known as a key regulator of vital organ cells such as hepatocytes and pancreatic β-cells, agonists or antagonists to modulate various metabolic pathways such as glucose homeostasis and drug metabolism can be designed and optimized by directly targeting these key interaction sites [32]. Recently, small-molecule regulators that interact with the HNF4α-LBD have been identified and shown to be effective, albeit toxic [33]. For further development, naturally occurring disease-causing point mutations and the encoded single amino acid substitutions at these sites can be very instructive as to the functional roles played by each residue and the strategy for improved design of selective modulators [34,35].

The two HNF4α variants carrying the respective single amino acid substitutions of MODY have been selected for these extended studies. The selective mutations are structurally located near the co-activator-binding pocket, and thus believed to influence co-activator recruitment. Our findings confirm these predictions and prove that the principal molecular bases of their reduced transcriptional activities are their impaired ability to recruit the co-activators. Although the neighboring residues such as Asp206 also play a role, the actual make-up of the hydrophobic groove seems to play a more crucial role in LXXLL motif recognition and co-activator recruitment. These obtained results are one step towards better understanding of the structure and function of HNF4α and a better strategy for targeting this protein, especially its interaction with co-activators, for therapeutic intervention [36,37].

AUTHOR CONTRIBUTION

Geun Bae Rha performed the experiments and contributed to the discussion. Guangteng Wu performed the experiments and contributed to then discussion. Young-In Chi performed the experiments, contributed to the discussion and wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Probing the effect of MODY mutations near the co-activator-binding pocket of HNF4α

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Figure S1 Schematic diagram and sequence alignment of PGC-1α functional regions

(A) Functional domain structure of PGC-1α and its three NR boxes containing the LXXLL motif or its derivatives. Relative positions of the residues comprising the LXXLL motif and the flanking regions by the conventional numbering system are shown at the bottom in parenthesis. The characteristic serine and leucine residues of the class III NR-box are also indicated by circles. Regulatory phosphorylation sites are also indicated. Abbreviations used: AD, activation domain; NRs, nuclear receptor boxes; LXXLL motifs; ID, inhibitory domain; RS, serine/arginine-rich region; RRM, RNA recognition motif.

(B) Schematic representation of PGC-1α NR-box mutants. One, two or all three LXXLL motifs were mutated to LXXAA sequences to make them non-functional. Mutated NR-boxes are indicated by cross marks in the Figure and accordingly implied in the names of each mutant.

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