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Multiple post-translational modifications in hepatocyte nuclear factor 4α

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ABSTRACT

To investigate the role of post-translational modifications (PTMs) in the hepatocyte nuclear factor 4α (HNF4α)-mediated transcription, we took a comprehensive survey of PTMs in HNF4α protein by mass-spectrometry and identified totally 8 PTM sites including newly identified ubiquitilation and acetylation sites. To assess the impact of identified PTMs in HNF4α-function, we introduced point mutations at the identified PTM sites and, tested transcriptional activity of the HNF4α. Among the point-mutations, an acetylation site at lysine 458 was found significant in the HNF4α-mediated transcriptional control. An acetylation negative mutant at lysine 458 showed an increased transcriptional activity by about 2-fold, while an acetylation mimic mutant had a lowered transcriptional activation. Furthermore, this acetylation appeared to be fluctuated in response to extracellular nutrient conditions. Thus, by applying an comprehensive analysis of PTMs, multiple PTMs were newly identified in HNF4α and unexpected role of an HNF4α acetylation could be uncovered.

1. Introduction

Protein post-translational modifications (PTMs) are one of the most efficient biological signals for expanding the genetic code and play key roles in regulating cellular physiology such as protein transportation, DNA repair and gene transcription [1,2]. The significance of protein PTMs in key cellular events is increasingly being shown by proteomic analysis with improvements in mass spectrometric approaches [3]. However, there are little reports that illustrate all of PTMs in a given protein, since identification of PTMs remains as a challenging issue attributed to complicated combination of multiple PTMs and their low abundance *in vivo* [4]. To date, PTM analyses using mass spectrometry have been carried out mostly by affinity-based enrichments of modified proteins and peptides, such as the phospho-specific IMAC column or immunoprecipitation using PTM specific antibodies [3]. However, recent advances in nonrestrictive sequence alignment make it possible to identify PTMs without prior specification, enabling comprehensive analysis of PTMs [4–6].

Hepatocyte nuclear factor 4α (HNF4α, NR2A1) is an orphan nuclear receptor that is expressed mainly in liver, and less in kidney, small intestine, colon and pancreas [7–9]. HNF4α is required for development of the liver and for controlling the expression of many genes involved in key metabolic pathways such as gluconeogenesis and fatty acid transportation [9]. In humans, heterozygous mutation of HNF4α gene causes maturity onset diabetes of young 1 (MODY-1), supporting the importance of HNF4α in the energy metabolism [10,11]. HNF4α binds as a homodimer to its recognition site direct repeat 1 and 2 (DR1 and DR2), and on binding to DNA, HNF4α recruits transcriptional co-activators and positively regulates the expression of target genes such as apolipoprotein CIII (ApoCIII) [9,12,13]. Though physiological significance of HNF4α in animal models is well characterized [14–19], regulation of HNF4α cellular functions still remains to be examined. Accumulating recent findings have uncovered that functions of epigenetic regulators are under controls of PTMs in response to cellular signaling and nutrient [20–22], while little is known about PTMs for regulating HNF4α function.

Here we performed unrestricted comprehensive analysis of PTMs in HNF4α and we identified 8 modification sites including previously unknown acetylation and ubiquitilation sites. To verify the impact of PTMs, we generated point mutants for the PTM sites to test transcriptional activity of HNF4α. Unexpectedly, we found that newly identified acetylation at K458 is inhibitory for transcription function of HNF4α.

2. Material and methods

2.1. Plasmid and antibodies

The expression plasmid for full-length HNF4α cDNA was cloned from a cDNA library of HepG2 cells and inserted into a pcDNA3 vector (Invitrogen, Carlsbad, CA). The expression vectors of HNF4α

Abbreviations: PTM, post-translational modification; HNF4α, hepatocyte nuclear factor 4α; MODY-1, maturity onset diabetes of young 1; DR, direct repeat; ApoCIII, apolipoprotein CIII; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CID, collision-induced dissociation; ETD, electron transfer dissociation; FDR, false discovery rate; qPCR, quantitative reverse transcription-PCR.

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point mutants were generated from a pcDNA3–FLAG–HNF4α vector. 8xHNF4 binding site-tk Luc. Vector, which contain eight copies of HNF4-binding sites encompassing the nucleotides −156 to −138 region of mouse transthyretin promoter, was kindly provided by Dr. Akiyoshi Fukamizu (University of Tsukuba) [23]. Anti-HNF4α antibody (H1415) was purchased from PPMX (Tokyo, Japan). Anti-Acetyl lysine antibody were purchased from Millipore (Bedford, MA). Anti-HNF4α and Anti-actin antibodies were purchased from Santa Cruz Biotechnology (Santa-Cruz, CA). Anti-FLAG antibody was purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Cell culture and transfection

HepG2 cells were obtained from the ATCC (HB-8065) and cultured in DMEM (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum and antibiotics. The cells were grown at 37 °C in 5% CO_2. For transfection, we used Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Glucose treatment was performed as described [24].

2.3. Purification of the endogenous HNF4α protein and immunoprecipitation

For purification of HNF4α protein, we cross-linked the antibodies with Protein G dynabeads (Invitrogen). First, 2 μg of antibodies were incubated with 30 μl of Protein G dynabeads in BC100 buffer (20 mM HEPES [pH 7.6], 100 mM KCl, 0.2 mM EDTA, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride) at 4 °C. Beads were washed 3 times by BC100 and twice by 0.2 M triethanolamine buffer (pH 8.2). Then, 20 mM dimethyl pimelimidate (DMP), freshly dissolved in a 0.2 M triethanolamine buffer was added to the beads for cross-linking. Cross-linking reaction was performed for 1 h at room temperature. The reaction was stopped by replacing the buffer with 50 mM Tris–HCl (pH 7.5) for 15 min at room temperature. Beads were washed 3 times by BC100 and once glycine–HCl (pH 2) and then 3 times by BC100.

HepG2 cells nuclear extracts were immunoprecipitated with Protein G dynabeads coupled with each indicated antibody for 2 h. The immunoprecipitates were washed by BC100 and eluted with 0.1 M glycine–HCl buffer (pH 2). The eluates were boiled with Leammli sample buffer and then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and SYPRO-Ruby (Invitrogen) staining according to the manufacturer's instruction or Western blotting with the indicated antibodies.

2.4. Mass spectrometric analysis

HNF4α was excised from the gel and analyzed by LC-MS/MS [25].The gel piece of HNF4α was cut into small pieces to increase surface and put into a 0.6 mL tube. Initially, an aliquot of 100 μL of 100% ACN was added to the tube to cover the gel piece completely and incubated for at least 15 min. The gel piece was dried completely in a Centrifugal concentrator VC-96R (Taitec, Saitama, Japan). Reducing cysteines was carried out with a 10 mM dithiothreitol solution in 0.1 M ammonium bicarbonate, pH 8.6, for 60 min at 56 °C. The same volume of a 55 mM solution of iodoacetamide in 0.1 M ammonium bicarbonate buffer, pH 8.6, was added and incubated in darkness for 45 min at room temperature to alkylate cysteine residues. The reduction/alkylation solutions were replaced by 100 mM ammonium bicarbonate buffer for 10 min. Gel piece was washed and dried in ACN followed by Centrifugal concentrator.

The dried gel piece was re-swollen with 25 ng/μL trypsin gold (Promega) solution buffered in 50 mM ammonium bicarbonate. The gel piece was incubated for 16 h (overnight) at 37 °C. The supernatant was transferred to new 0.6 mL tubes, and the gel piece was extracted again with 50 μL of 0.5% formic acid/50% ACN for 20 min in a sonication bath. This step was performed two times. Samples in extraction buffer were pooled in a 0.6 mL tube and evaporated in a Centrifugal concentrator, and then 40 μL HPLC grade water (Wako) was added for nano-LC–ESI-MS/MS analysis.

A total of 10 μL of extracted peptides was analyzed by ESI-MS/MS using an LTQ velos Orbitrap ETD instrument (Thermofischer Scientific, Pittsburgh, PA). The HPLC used was a DiNa system (KYA tech corporation, Tokyo, Japan) equipped with C-18 ESI capillary column (100 μm × 150 mm, NIKKYO technos, Tokyo, Japan). The gradient consisted of (A) 0.1% formic acid in 2% ACN, (B) 0.1% formic acid in 80% ACN: 0–100% B from 0 to 40 min, 100% B from 41 to 45 min and 0% B from 46 to 55 min. The flow rate was 300 nL/min from 0 to 55 min. MS spectra were recorded from a range of m/z 350–1500 at resolution 60000, followed by data-dependent collision-induced dissociation (CID) MS/MS spectra and electron transfer dissociation (ETD) MS/MS spectra generated from five highest intensity precursor ions. The voltage between ion spray tip and transfer tube was set to 1800 V. More than 2 charged peptides were chosen for MS/MS experiments.

2.5. Computational analysis for PTMs

For protein identification, spectra were processed using Proteome Discoverer Version 1.2 (Thermofisher Scientific) against SEQUEST, and subjected to a cutoff of 5% false discovery rate (FDR). Database used was the NCBI human protein database with a 10 ppm mass cutoff for MS and a 0.5 Da cutoff for MS/MS spectra. Carbamidomethyl cysteine was set as a fixed modification.

For PTM identification, searches were performed by using the Modiro v1.1 (Protagen) software against FASTA format of HNF4α isoform b amino acid sequence. Searching parameters were set as follows: two maximum missing cleavage sites, a peptide mass tolerance of 15 ppm for peptide tolerance, 1.5 Da for fragment mass tolerance, modification 1 of carbamidomethyl(C). Positive protein identification was first of all listed by spectra view and subsequently each identified peptide was considered significant based on the 0.2 Da delta value, ion-charge status of peptide, b- and y-ion fragmentation quality and significant scores.

2.6. Luciferase assay

At 40% confluence, HepG2 cells were transfected with the indicated plasmids in 12-well trays. The total amount of DNA was adjusted by supplementing it with empty vector. Luciferase activities were determined using the dual Luciferase Reporter Assay system (Promega). Renilla luciferase was used as a reference to normalize transfection efficiencies in all experiments. All values are means +/− standard deviations from at least three independent experiments.

2.7. RNA isolation, cDNA synthesis, and quantitative reverse transcription-PCR (qPCR)

Toral RNA was extracted with Trizol (Invitrogen), and cDNA was synthesized using Prime script (Takara, Tokyo, Japan). Reverse transcription of 2 μg total RNA was carried out with 0.2 μg of oligo(dT) 15 primer for 50 min at 50 °C, and the PCR was performed using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA).

qPCR was performed using Sybr Premix EX Taq (Takara) with the Thermal Cycler Dice Real Time System II (Takara) according to the manufacture's instruction. qPCR primer sets predesigned for for ApoCIII and Rplp0 (36B4) gene were purchased from Takara. All values are means +/− standard deviations from at least three independent experiments.