BIOCHEMISTRY
including biophysical chemistry & molecular biology

ARTICLE

pubs.acs.org/biochemistry

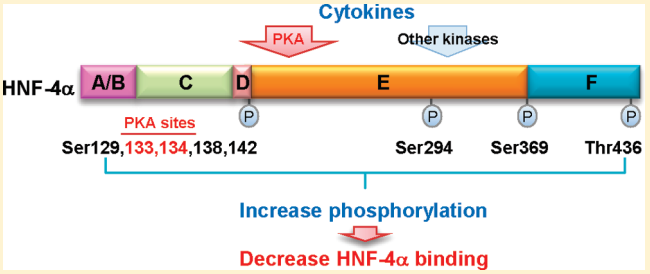
Quantitative Analysis of Cytokine-Induced Hepatocyte Nuclear Factor-4α Phosphorylation by Mass Spectrometry

Zhongyan Wang,[†,§] Erdjan Salih,[‡,§] and Peter A. Burke*[,†]

[†]Department of Surgery, Boston University School of Medicine, Boston, Massachusetts 02118, United States

[‡]Department of Periodontology and Oral Biology, Boston University Henry M. Goldman School of Dental Medicine, Boston, Massachusetts 02118, United States

ABSTRACT: Hepatocyte nuclear factor-4α (HNF-4α), a liver-enriched transcription factor, is essential for liver development and function. HNF-4α regulates a large number of liver-specific genes, many of which are modulated by injury. While HNF-4α function is regulated by phosphorylation, only a limited number of phosphorylation sites in HNF-4α have been identified, and the roles of HNF-4α phosphorylation after injury are unexplored. To address these issues, we have carried out an extensive quantitative mass spectrometry (MS)-based analysis of HNF-4α serine and threonine phosphorylation in response to cytokine stimulation. Studies were performed in HNF-4α-enriched HepG2 cells treated with cytokines for 3 h or left untreated, followed by chemical derivatization of the phosphoserine and phosphothreonine residues using stable isotopic variants of dithiothreitol (DTT) and MS analysis. This has allowed the identification and relative quantification of 12 serine/threonine phosphorylation sites in HNF-4α. Eight of these phosphorylation sites and their sensitivity to cytokine stimulation have not been previously reported. We found that cytokine treatment leads to an increase of HNF-4α phosphorylation in several phosphopeptides. The phosphorylation of HNF-4α mediated by protein kinase A (PKA) significantly reduces HNF-4α binding activity, which mimics the repressive effect of cytokines on HNF-4α binding, and the inhibition of PKA activity by PKA inhibitor can partially recover the reduced HNF-4α binding activity induced by cytokines. These results suggest that the mechanism that alters HNF-4α binding after cytokine stimulation involves modulation of specific HNF-4α phosphorylation dependent, in part, on a PKA signaling pathway.



Hepatocyte nuclear factor-4α (HNF-4α) is a liver-enriched transcription factor, giving it the potential to regulate hundreds or even thousands of targets. The importance of HNF-4α to liver differentiation and function has been demonstrated by cell-type-specific gene ablation in mutant mice. When HNF-4α was deleted in fetal development, hepatocyte precursors lost their epithelial phenotype.[1] When the gene was deleted in the adult liver, defects in metabolism were most prominent.[2] HNF-4α is a member of the ligand-dependent nuclear receptor family of transcription factors, making it an interesting target for drug development. The native ligand for HNF-4α remains controversial, and multiple molecules have been proposed, ranging from palmitoyl-coenzyme A to linoleic acid.[3,4] The agonists and antagonists have been investigated as a means to manipulate HNF-4α function as a potential therapeutic approach.[5] HNF-4α has been shown to contribute to human disease. A dominant mutations in the *HNF4A* gene cause both maturity onset diabetes of the young[6] and neonatal macrosomia and hyperinsulinemic hypoglycemia.[7] Previous work by our group has demonstrated that HNF-4α plays a role in the liver's response to systemic injury and the development of the acute phase response.[8−10]

To fully understand how HNF-4α accomplishes these important physiologic functions in the liver, knowledge of the mechanisms regulating HNF-4α function itself is required. Protein phosphorylation is a common post-translational modification among transcription factors and has been shown to play a function role in nuclear localization, DNA binding, and transactivation.[11,12] Previous studies have demonstrated that HNF-4α protein can be phosphorylated by multiprotein kinases at multiple sites. Protein kinase A (PKA) phosphorylates HNF-4α at serine (Ser, S) 133 and 134 in the DNA binding domain (DBD) and impairs its DNA binding activity.[13] AMP-activated protein kinase phosphorylates HNF-4α at S304 in the ligand binding domain (LBD) and impairs its dimerization and DNA binding activity.[14] p38 kinase phosphorylates S158 in the LBD of HNF-4α in response to inflammatory redox, causing an increase in DNA binding and transactivation.[15] It has been reported by our group that the activation of Janus kinase-2 pathway is involved in HNF-4α phosphorylation that may account for the rapid decrease in HNF-4α DNA binding and transactivation after injury.[9]

Despite the acceptance that phosphorylation is a critical modification involved in many cellular events, and the recent advances in mass spectrometry (MS) technology to identify phosphoproteins, the role of phosphorylation and phosphoproteins in physiological processes cannot be considered a static process. Protein phosphorylation is dynamic and specific depending on both the site and state of phosphorylation. However,

Received: April 11, 2011
Revised: May 10, 2011
Published: May 20, 2011

qualitative and quantitative analyses and understanding of protein phosphorylation remain a challenge for a number of reasons.[16] First, phosphopeptides are usually detected with low efficiency or not at all by MS probably due to their acidic character. Second, the phosphorylation may not be stoichiometric at a given site in a protein; i.e., the phosphopeptide of interest may be of low abundance compared to the unmodified peptide. Third, phosphorylated residues may interfere with the enzymatic digestion of the protein resulting in peptide fragments that are either too large or too small for effective analysis by MS. Fourth, phosphopeptides do not efficiently fragment during MS/MS CID (collision-induced dissociation) for sequence identification. Therefore, only a few phosphorylation sites in HNF-4α have been mapped, and quantitative analyses of HNF-4α phosphorylation as well as the significance of its phosphorylation in response to an inflammatory stimulus have not been well characterized. In the current study, we have utilized the overexpressed FLAG-tagged HNF-4α model to generate sufficient quantities of HNF-4α to allow its reliable detection, followed by chemical derivatization of phosphoserine and phosphothreonine residues with either dithiothreitol (DTT) or deuterated [D_6]DTT form for quantification.[16−19] This enables the cytokine sensitive phosphorylation sites in HNF-4α to be identified, and the extent of phosphorylation to be quantitatively analyzed by liquid chromatography−electrospray ionization−tandem mass spectrometry (LC-ESI-MS/MS) in a cytokine-stimulated HepG2 cell culture model. Our results show that HNF-4α can be phosphorylated at various serine and threonine sites in a peptide-dependent manner in response to cytokine treatment. The impact of *in vitro* phosphorylation of HNF-4α by several protein kinases on HNF-4α binding activity was also defined. Utilizing novel means facilitate us, for the first time, to map qualitatively and quantitatively HNF-4α's phosphorylation profile in a cytokine-induced inflammatory response model of injury. It also extends current knowledge about the quantitative changes in HNF-4α phosphorylation during inflammatory response and provides important insight into the mechanism regulating HNF-4α function and the hepatic response to systemic injury.

MATERIALS AND METHODS

Cell Culture and Treatments. HepG2 cells (ATCC # HB-8065), human hepatoma cells, were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), and 10% heat-inactivated fetal bovine serum (Mediatech, Herndon, VA) at 37 °C in a humidified atmosphere with 5% CO_2.

The inflammatory response in HepG2 cells was stimulated with a cytokine mixture consisting of 1 ng/mL of recombinant human interleukin (IL)-1β, 10 ng/mL of IL-6, and 10 ng/mL of tumor necrosis factor (TNF)-α (PeproTech, Rocky Hill, NJ) in serum-free medium for 3 h.

Plasmid Construction, Lentivirus Packaging, and Cell Infection. The expression plasmid of pCMV-FLAG-hHNF-4α was kindly provided by Dr. Paul C. Kuo, Duke University Medical Center.[15] For lentiviral expression, the FLAG-hHNF-4α construct was cloned into pCDH-CMV-MCS lentivector (System Bioscience, Mountain View, CA) between *XbaI* and *Bam*HI. The resulted plasmid was purified and verified by DNA sequencing. Lentivector expression system was purchased from System Biosciences. Packaging and production of lentivirus were performed according to the manufacturer's protocol. Briefly, the lentivector containing the FLAG-hHNF-4α expression construct was co-transfected with the packaging plasmids into 293NT cells using Lipofectamine Plus (Invitrogen, Carlsbad, CA). 48 h after transfection, the virus-containing supernatants were harvested in a 15 mL sterile, capped conical tube and centrifuged at 3000 rpm at room temperature for 15 min to pellet cell debris. The viral supernatants were further clarified by filtration through a 0.45 μm low protein-binding filter (Millipore, Bedford, MA) and then concentrated by poly(ethylene glycol) (PEG) precipitation according to the manufacturer's protocol (System Bioscience). HepG2 cells were infected with FLAG-hHNF-4α lentivirus in the presence of 5 μg/mL Polybrene (Sigma, St. Louis, MO) for 12 h and then replaced with fresh complete medium (with serum and antibiotics, without Polybrene). Two days after infection, the cells were treated with or without cytokines for 3 h.

Immunoprecipitation of FLAG-hHNF-4α Fusion Protein. Cell lysates from HepG2 cells, infected by FLAG-hHNF-4α lentivirus and followed by treatment with or without cytokines, were prepared by incubating cells in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) for 20 min at 4 °C and centrifugation at 8200*g* for 10 min at 4 °C. The protein concentration of the lysates was determined using the Bradford protein assay (Bio-Rad, Hercules, CA). For immunoprecipitation of FLAG-tagged HNF-4α and its associated proteins, cell lysates, containing 2 mg of protein, were incubated with 60 μL of EZview red anti-FlAG M2 affinity gel (Sigma) overnight at 4 °C with constant rotation. Beads were then washed three times using the TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and one time with PBS. After final washing, the beads were stored in 100 μL of PBS and followed by processing for MS analysis.

Qualitative and Quantitative Identification of Phosphorylation Peptides by MS Analysis. To define the phosphorylated peptides at a qualitative level, the FLAG-affinity beads, containing the enriched FLAG-HNF-4α protein, were digested with trypsin, according to a previously described procedure,[18,20] and then analyzed directly using LTQ (linear ion trap quadropole)−LC−tandem mass spectrometer (Thermo Electron Co., San Jose, CA). The phosphorylated peptides were identified by the signature mass difference of 80 Da (HPO_3) between theoretical and found peptide mass.

Quantitative identification of phosphopeptides was performed by utilizing a bifunctional thiol reagent, DTT and its deuterated [D_6]DTT form, to derivatize phosphoserine/phosphothreonine residues, followed by MS analysis. After trypsin digestion, the cytokine-untreated control and cytokine-treated samples were processed as follows. Controls were derivatized by light DTT and the cytokine-treated group by heavy (deuterated)-[D_6]DTT under base-catalyzed conditions which convert the phosphoserine/phosphothreonine residues to a DTT-derivative as described previously.[17−19,21] In brief, the tryptic peptides were incubated in 0.3 M NaOH with 10 mM DTT (or [D_6]DTT) at 50 °C for 1 h. At the end of this chemical reaction, an equal amount of protein from control and cytokine-treated samples were mixed, the pH of the solution was adjusted to pH ∼2 by addition of dilute HCl, and the peptides were freed of salts and excess DTT using a microspin C-18 reversed phase column. The eluted peptides from the C-18 columns were freeze-dried using Speed-Vac (Savant, Farmingdale, NY), suspended in 20 μL of Buffer A (0.25% formic acid/H_2O), and then subjected to LC-ESI-MS/MS analysis as recently described.[19] The raw MS/MS data were searched for DTT (136.2 Da) and [D_6]DTT (+6 Da, 142.2 Da)

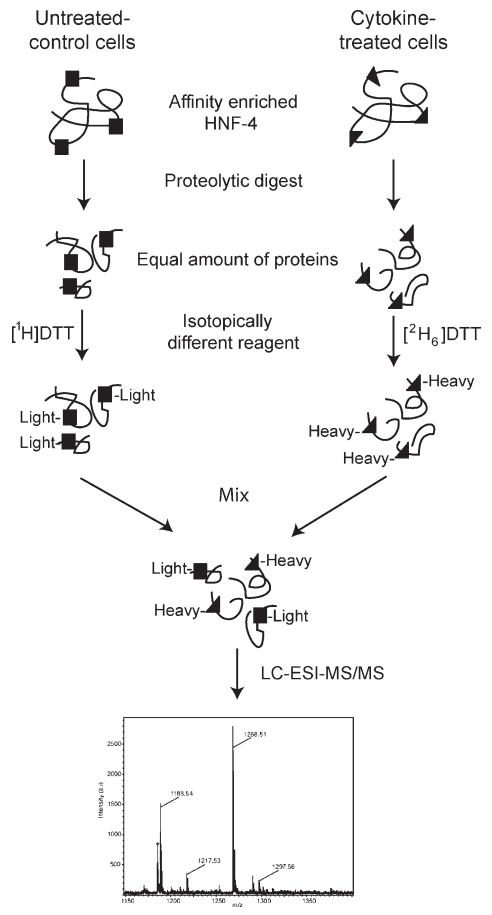


Figure 1. Quantitation of phosphopeptides using MS. The schematic diagram shows the strategy for relative quantitative phosphopeptide analysis in cytokine-treated and untreated cells. The essential principle is to treat two samples to be compared with two different stable isotopic forms of the same reagent, DTT. The quantitation and identification are achieved by MS analysis of the mixture of peptides derived from such samples.

modifications which allowed identification of not only phosphorylation sites but also the paired phosphopeptides, one derived from control and the other from cytokine-treated cells. The relative quantification of the phosphorylated peptides/sites between control and cytokine-treated groups was achieved automatically using Bioworks 3.3.1 software and the PepQuan option which gives the ratio of peak areas of the related doublets of the phosphopeptide ions separated by mass equivalent to the difference in the total isotopic mass of the reagent, i.e., 6 Da for a singly charged peptide. Figure 1 illustrates the general steps and procedures in going from phosphoprotein to DTT-derivatized peptides for relative quantitative LC-MS/MS analysis of phosphoproteins/peptides.

All spectra from MS/MS were searched against the human database and the human HNF-4α amino acid sequence using the SEQUEST algorithm with "No Enzyme" specificity, which would include identification of full, half, and no tryptic peptides.[22] Search parameters for qualitative identification of phosphopeptides with no chemical derivatization included a modification of 80 Da on serine and threonine residues representing the presence or absence of phosphate on a given peptide. For qualitative chemical derivatization condition with DTT, the search algorithms included the DTT (+136.2 Da) modification only on serine and threonine residues. The 136.2 Da modification is a unique mass addition to a phosphopeptide as a result of the reaction of DTT (+154.2 Da) with dehydroalanine for phosphoserine and dehydroaminobutyric acid for phosphothreonine. The loss of phosphate group as phosphoric acid ($-H_3PO_4$, −98 Da) includes an 18 Da contribution from the hydroxy group of serine/threonine residue during base-catalyzed β-elimination. Hence, the net mass addition by DTT (154.20 Da) to any peptide in the human database would be 136.2 Da (154.2 Da − 18 Da).[17,19,23] For relative quantification, the search identified peptides with mass addition 136.2 Da for light labeled (control) and +6 Da (i.e., 142.2 Da) for the heavy labeled (cytokine-treated). In addition to the search parameters and criteria used,[19] the identified phosphopeptide sequences were evaluated manually by examining each of the identified phosphopeptide MS/MS data for the quality and the confidence through the *b* and *y* ion fragment series.

Electrophoretic Mobility-Shift Assays (EMSAs). The preparation of nuclear extracts from HepG2 cells and EMSA were performed as described previously.[10] Briefly, EMSAs were performed in binding buffer containing 5−10 μg of nuclear extracts, 2 μg of poly(dI-dC), 100 μg/mL BSA, 1 mM DTT, 25 mM Tris (pH 7.5), 50 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 10% (v/v) glycerol, 0.05% NP40, and ^{32}P-labeled oligonucleotide probe. The oligonucleotide corresponding to the HNF-4α binding site in the transthyretin (*TTR*) gene promoter region −154 to −136 (5′-CTAGGCAAGGTTCATATTT-3′) was used based on *TTR* gene as a well-known HNF-4α target gene[24] and its classification as an acute phase protein with significant changes after injury.[25]

In Vitro Kinase Assays. Reactions were carried out in the binding buffer, described above under the procedure of EMSA, supplemented with 200 μM ATP and 25−50 ng of kinases in the presence of 5−10 μg of nuclear extracts for 30 min at 30 °C. Specific inhibition of PKA activity was performed by pretreatment of the cells with 20 mM of H89 (dihydrochloride) for 30 min and treatment with cytokines for 3 h. The kinases and PKA inhibitor were purchased from the following: PKA catalytic subunit, Casein Kinase (CK) I, and CKII were from New England Biolabs (Ipswich, MA); Protein kinase C (PKC) was from Promega (Madison, WI). PKA inhibitor, H89, was from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunoblot Analysis. Nuclear extracts from HepG2 were isolated for immunoblot analysis using antibodies against HNF-4α (Santa Cruz Biotechnology) or β-actin (Sigma) as described previously.[10]

RESULTS

Mass Spectrometry Characterization of Human HNF-4α. To enrich HNF-4α level, overexpression of HNF-4α in HepG2 cells was performed by infection with FLAG-hHNF-4α lentivirus. Figure 2 shows that HNF-4α protein levels were enhanced approximately 4−6-fold compared to endogenous HNF-4α in HepG2 cells (Figure 2B, column 1 vs 2 or 3). We noticed that protein level of HNF-4α after cytokine stimulation is slightly higher than nonstimulation condition, but no statistical significance between them (Figure 2B, column 2 vs 3) was detected ($p > 0.05$).

To analyze HNF-4α protein by MS, the cytokine-treated or -untreated, FlAG-tag immunoprecipitated HNF-4α-enriched cell lysates were digested with trypsin and then were subjected to LTQ−LC−tandem mass spectrometric analysis either directly

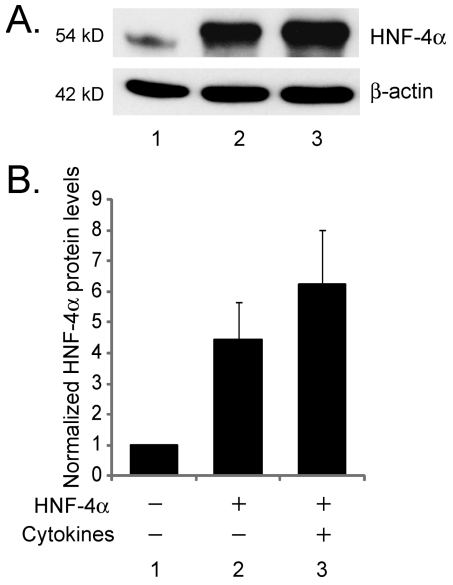


Figure 2. Overexpression of HNF-4α in HepG2 cells. The cells were infected with FLAG-hHNF-4α lentivirus and then treated with cytokines or left untreated as described under Materials and Methods. (A) The protein expression levels of HNF-4α assayed by Western blot in native HepG2 cells (lane 1), and HNF-4α overexpressed HepG2 cells untreated (lane 2), or treated with cytokines for 3 h (lane 3) are shown. β-actin expression is included as an internal loading control. (B) The bar graph shows the densitometric analyses from three separate experiments performed as described in (A). Values represent normalized HNF-4α protein levels (mean ± SD), native HepG2 cells (lane 1) are set as 1. No significant difference is found in HNF-4α concentration in the HNF-4α overexpressed HepG2 cells treated with or without cytokines (lane 2 vs 3, $p > 0.05$).

or following chemical derivatization by DTT and [D_6]DTT. The data generated by MS/MS analysis, searches against human database and HNF-4α protein sequence led to an identification of 87% of the peptide sequences of human HNF-4α (Figure 3). This relatively high coverage ensured that we were able to visualize the majority of potential phosphorylation sites in HNF-4α.

Qualitative Identification of Phosphopeptides and Phosphorylation Sites in HNF-4α by LC-MS/MS Analysis. The HNF-4α-enriched tryptic peptides were directly analyzed by MS. From the masses of the peptide fragments, sequence data were determined by comparison with the known sequence. The phosphorylated peptides were identified by the presence of species with a theoretical peptide mass of +80 Da (+1 phosphate group or multiples of). In HepG2 cells, under both untreated-control and cytokine-treated conditions, the phosphopeptide ^{132}RS*SYEDS*SLPSINAL^{146} (*indicates the potential phosphorylated residue, i.e., at S133 and S138) was detected. An additional peptide, ^{410}TPETPQPS*PPGGSGS*EPY^{427} (S417 and S424) was only identified after cytokine treatment. However, no phosphorylation at any tyrosine residues was identified by using +80 Da mass modification search.

While phosphopetides can be detected by searching the peptide mass shifts of 80 Da corresponding to putative phosphopeptides, specific MS/MS approaches are required to identify the phosphorylation site(s) in such peptides. An inherent problem limiting analysis of tryptic phosphopeptides is their poor detection in the positive-ion mode of MS as well as poor fragmentation during MS/MS by CID to determine peptide sequences.

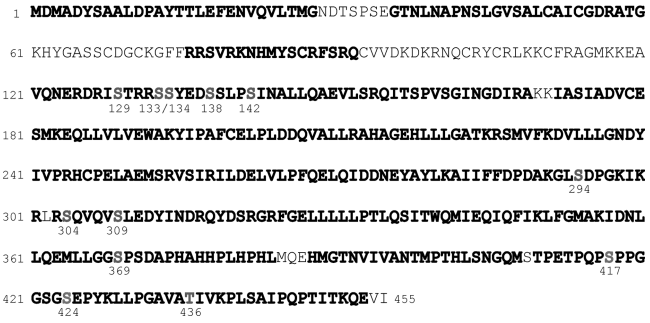


Figure 3. Proteolytic coverage maps of human HNF-4α. The amino acid sequence of human HNF-4α is shown. The peptides identified by MS analysis were shown in bold black color. Phosphorylated residues are exhibited in gray color. The identified peptides cover 87% of HNF-4α protein (396/455 amino acids).

This latter case is due to high lability of the phosphoserine/phosphothreonine moiety, whereby the phosphate group is the first to dissociate from the peptide during CID, and the peptide backbone remaining becomes resistant to fragmentation. Some of the limitations and complexity of analyzing the native phosphopeptides by MS/MS approaches without chemical derivatization have been highlighted recently.[26,27] The outcome is that many phosphopeptides remain undetected when analyzed in this manner. One of the more effective methods is to first convert the phosphopeptide to a DTT-derivative, followed by LC-MS/MS analysis.[17−19,23] Application of the derivatization procedures enabled us to identify six unique tryptic phosphopeptides with a total of 12 phosphorylation sites, including 11 serine sites (S129, S133, S134, S138, S142, S294, S304, S309, S369, S417 and S424) and 1 threonine sites (T436) (Table 1 and Figure 3). Among them, eight sites are novel, previously uncharacterized, while the sites of S133, S134,[13] S304,[15,28] and S417[29] have been reported.

Quantitative Identification of Phosphopeptides and Phosphorylation Sites in HNF-4α by LC-MS/MS Analysis. Although defining the precise sites of phosphorylation yields important information that can be related to the biological function for a given phosphoprotein, the quantitative evaluation of the extent of phosphorylation at a given site is critical for the interpretation of biological significance. Evaluation of HNF-4α phosphorylation events in response to cytokine stimulation would be useful to dissect their functional role in regulating the hepatic inflammatory and injury responses.[9,10,30] Cytokines IL-1 and IL-6, in conjunction with TNF-α, have been found to participate in the modulation of a broad group of liver acute phase proteins during periods of inflammation;[25,31] a classic acute phase response in our cell culture model has been demonstrated utilizing the mixture of cytokines (IL-6, IL-1β, and TNF-α).[9,10] In order to determine phosphorylation events in response to cytokine stimulation, we looked at changes in the relative amount of HNF-4α phosphorylation between untreated control cells and the cells treated with cytokines. We chose a 3 h time point for cytokine treatment based on our previous findings[10] that a classic acute phase response could be induced by cytokines, and the binding activity of HNF-4α is significantly altered after 3 h of cytokine exposure. To quantify phosphorylation events, the cells were transfected with a FLAG-tagged expression construct encoding HNF-4α and then treated with cytokines. Immunoprecipitates derived from equal amount of protein lysates from untreated-control

Table 1. Phosphorylation Sites in HNF-4α Determined by Mass Spectrometry and Kinase Prediction Algorithms

phosphopeptide[a]	site	predicted kinase[b]	NetPhos (score)
R.DRIS*TRRSSYEDSSLPSIN.A	S129	PKC	0.87
R.DRISTRRS*SYEDSSLPSIN.A	S133	PKA/cdc2	0.53/0.50
R.DRISTRRSS*YEDSSLPSIN.A	S134	RSK/DNAPK/PKA/CKII	0.63/0.52/0.55
R.DRISTRRSSYEDS*SLPSIN.A	S138	cdc2/CKI	0.52
R.DRISTRRSSYEDSSLPS*IN.A	S142	unknown	
F.FDPDAKGLS*DPGKIK.R	S294	CKII	
R.S*QVQVSLEDYINDRQYDSRG.R	S304	RSK/PKA	0.53/0.61
R.SQVQVS*LEDYINDRQYDSRG.R	S309	CKI/cdc2	0.52/0.53
G.MAKIDNLLQEMLLGGS*P.S	S369	cdc2/GSK3	0.53/0.52
TPETPQPS*PPGGSGSEPY	S417	cdc2/GSK3/cdk5	0.53/0.52/0.61
TPETPQPSPPGGSGS*EPY	S424	CKII	
K.LLPGAVAT*IVKP.L	T436	unknown	

[a] The amino acids followed by a superscript asterisk (*) denote phosphorylated residues. [b] The protein kinases were defined by our personal experience of kinase consensus sequences, and the Web-based phosphorylation prediction program, NetPhos (Technical University of Denmark, www.cbs.dtu.dk/services/NetPhos). PKC, protein kinase C; PKA, protein kinase A; Cdc2, cell division cycle protein kinase 2; RSK, ribosomal S6 kinase; DNAPK, DNA-dependent protein kinase; CKI (II), casein kinase I (II); GSK3, glycogen synthase kinase-3; Cdk5, cell division protein kinase 5.

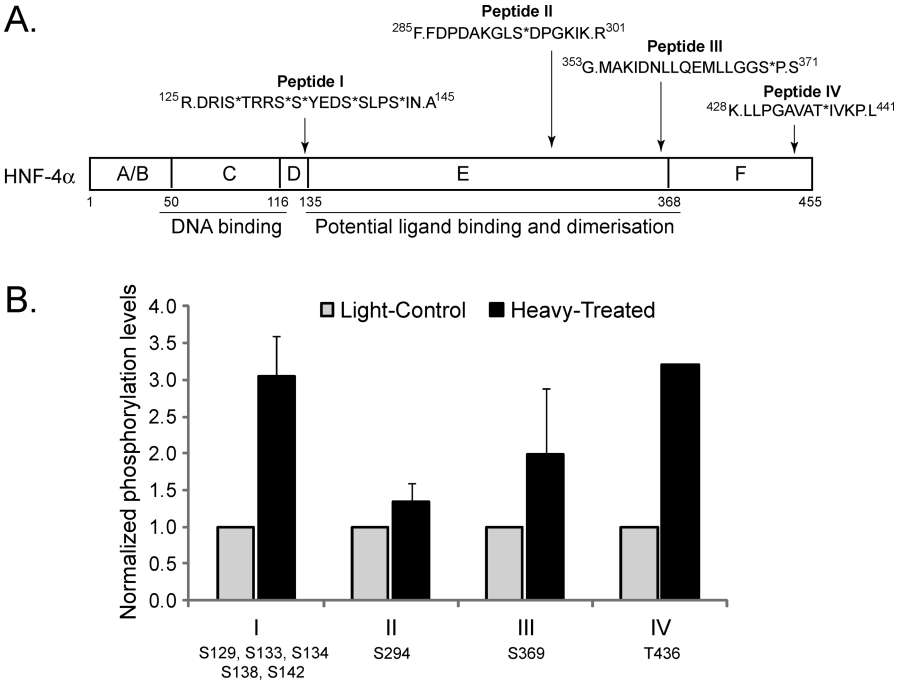


Figure 4. Quantitative MS analysis of HNF-4α phosphorylation in response to cytokine stimulation. The changes in four HNF-4α phosphopeptides (I to IV) were detected by using HNF-4α enrichment, DTT/[D_6]DTT labeling and MS/MS analysis as described under Materials and Methods. (A) Schematic representation of the human HNF-4α illustrating the functional domains. The sequence and location of identified phosphopeptides are shown. (B) The levels of phosphorylation in HNF-4α are presented as the relative ratio of light (control, DTT-labeled, gray column) and heavy (cytokine-treated, [D_6]DTT labeled, black column). The data are mean ± CI (confidence interval at 95% confident level) from three different experiments, and controls are set as 1.

and treated-cells were trypsin digested, labeled with either "light" DDT (control) or "heavy" DTT (treated), mixed, and analyzed by LC−MS/MS. These data are summarized in Figure 4, which shows four identified phosphopeptides exhibiting changes in the relative ratio of phosphorylation (control vs treatment) after cytokine stimulation. The phosphopeptide I (^{125}R.DRIS*-TRRS*S*YEDS*SLPS*IN.A^{145}) has five phosphorylated sites (S129, S133, S134, S138, and S142); a comparison of the relative peak ratio (control vs treated) revealed an increment change in the ratio to 1:3 at 3 h, indicating that after a 3 h treatment with cytokines, phosphorylation on these five serine residues within this peptide as a whole was increased 3-fold compared to the untreated controls. The phosphopeptide III (^{353}G.MAKIDNLL-QEMLLGGS*PS^{371}, S369) and phosphopeptide IV (^{428}K.LLP-GAVAT*IVKP.L^{441}, T436) showed similar patterns as phospho-peptide I, having a higher phosphorylation ratio (1:2−3) after 3 h of cytokine treatment compared to untreated controls. The peptide II, ^{285}F.FDPDAKGLS*DPGKIK.R^{301}, containing one phosphorylated

dx.doi.org/10.1021/bi200540w |*Biochemistry* 2011, 50, 5292–5300

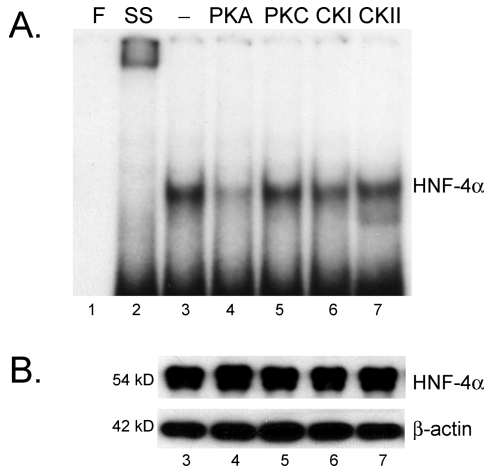


Figure 5. Effect of *in vitro* phosphorylation by specific kinases on HNF-4α−DNA binding activity. (A) Nuclear proteins extracted from HepG2 cells were incubated without (—) or with several kinases (PKA, PKC, CKI, and CKII) and subjected to EMSA using [α-^{32}P]dATP-labeled oligonucleotide probe based on the HNF-4α specific binding site in the promoter of *TTR* gene. HNF-4α antibody was used for supershift of the antibody−protein−DNA complex (SS, second lane). F, free probe (first lane). (B) Protein expression of HNF-4α was examined by Western blot using the same nuclear extracts as those used for EMSA.

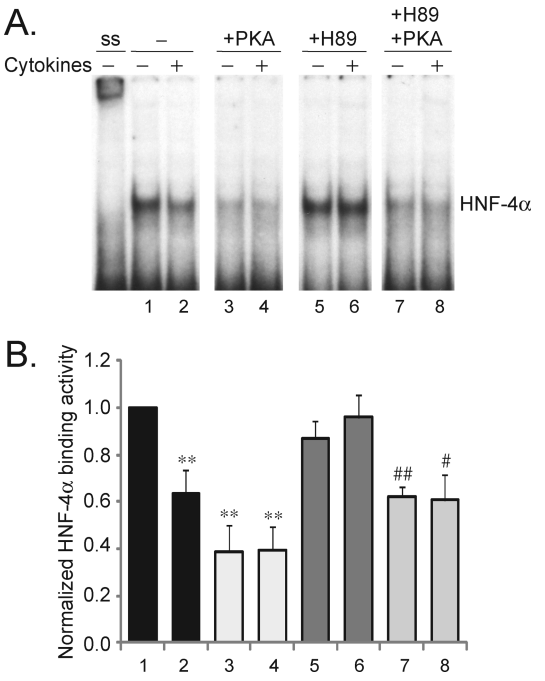


Figure 6. HNF-4α DNA binding activity is modulated by PKA. (A) HepG2 cells were pretreated with H89 (PKA inhibitor) for 30 min and then treated with cytokines for 3 h (+) or left untreated (−). Nuclear proteins were prepared and then incubated with PKA *in vitro*. EMSAs were performed using [α-^{32}P]dATP-labeled oligonucleotide probe as described in Figure 5. HNF-4α antibody was used for the supershift (SS). (B) The bar graph shows the densitometric analyses of normalized HNF-4α binding activity from three separate experiments as described in (A). Values represent mean ± SD, and the untreated-HepG2 cells (column 1) are set as 1. $^{**}p < 0.01$ indicates significant difference compared to the untreated-HepG2 cells (column 1); $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ denote significant difference compared to the absence of PKA inhibitor, H89 (columns 3 and 4).

site at S294, revealed only a slight increase in phosphorylation in response to cytokine treatment. The phosphopeptide ^{410}TPET-PQPS*PPGGSGS*EPY^{427}, identified by +80 Da mass modification search, was also detected by DTT derivatization, but no ratio (control vs treatment) was found, which may be due to a low abundance of this phosphopeptide in controls causing it to be under our detection thresholds.

Taken these data together, the quantitative analysis of HNF-4α phosphorylation demonstrates that HNF-4α is a phosphoprotein under either untreated or cytokine-treated condition. Cytokine stimulation alters the amount of existing phosphoproteins and changes the levels of phosphorylation of a given phosphopeptide as well.

PKA-Dependent Phosphorylation Regulates HNF-4α Binding Activity in Cytokine-Induced Inflammatory Response. The liver-enriched transcription factor HNF-4α belongs to the nuclear receptor superfamily,[32] whose members exhibit a modular structure with six distinct functional domains (A-F). HNF-4α contains a DNA-binding domain located in domain C, a putative ligand-binding and a dimerization domain in domain E, and two activation-function domains, designated AF-1 and AF-2, located in domains A/B and E, respectively.[24,33] As shown in Figure 4, the identified phosphorylation sites are scattered in the D, E, and F functional domains of HNF-4α. These sites provide a potential link with the modulation of HNF-4α function under various physiological and pathological conditions. Therefore, identifying the corresponding protein kinases for a given phosphorylation site will shed further light on the cellular mechanisms involved in HNF-4α phosphorylation and the potential signaling pathways involved. The potential protein kinases for the identified phosphopeptides of HNF-4α were defined by our personal experience of kinase consensus sequences as well as utilizing the Web-based phosphorylation prediction program, NetPhos (Technical University of Denmark).[34,35] As shown in Table 1, HNF-4α is a potential substrate for multiple kinases, including the basophilic serine/threonine kinase group (protein kinase A and protein kinase C), the acidophilic group (glycogen synthase kinase-3), and the proline-dependent group (cell division cycle protein kinase 2).

To evaluate whether specific kinases could modulate HNF-4α function during an inflammatory response, we have selected several commercial available kinases to test their effects on HNF-4α binding. These kinases, including PKA, PKC, CKI, or CKII, were used in an *in vitro* kinase assay. Specific kinases were incubated with nuclear extracts from untreated HepG2 cells, and EMSAs were performed with a probe containing the HNF-4α binding site derived from the promoter region of *TTR* gene. As shown in the Figure 5A, among the kinases tested, only PKA was found to produce a significant decrease in HNF-4α binding activity. None of the kinases used had any significant effect on HNF-4α protein concentrations assayed by Western blot (Figure 5B), suggesting that the decrease in intensity of HNF-4α−DNA complex induced by PKA was related to a loss of DNA-binding activity and not of protein concentration.

To further explore the role of PKA in HNF-4α function during the inflammatory response, HepG2 cells were pretreated with the specific PKA inhibitor, H89, and then treated with cytokines. Figure 6 shows that cytokines could significantly reduce HNF-4α binding activity compared to control ($p < 0.01$) (Figure 6B, column 1 vs 2). PKA caused a dramatic decrease in HNF-4α binding in both untreated and cytokine-treated cells ($p < 0.01$)

dx.doi.org/10.1021/bi200540w |*Biochemistry* 2011, 50, 5292–5300

(Figure 6B, column 1 vs 3 and 4), and the PKA-induced decrease in binding activity was effectively blocked by the *in vivo* addition of the PKA inhibitor, H89 ($p > 0.05$) (Figure 6B, column 1 vs 5 and 6). When the nuclear extracts prepared from PKA inhibitor-treated cells were incubated with PKA *in vitro*, HNF-4α binding activity was suppressed compared to untreated control (Figure 6B, column 1 vs 7 and 8) but significantly higher than PKA alone in the absence of PKA inhibitor ($p < 0.05$) (Figure 6B, column 3 vs 7, and column 4 vs 8).

These results suggest that during a cytokine-induced inflammatory response, HNF-4α function is regulated, in part, by alterations in its binding to DNA, and this alteration is secondary to modulation of HNF-4α phosphorylation. The change in amount of phosphopeptide is partially due to the activation of a signaling transduction pathway involving the phosphorylation of HNF-4α by PKA.

DISCUSSION

Post-translational modification of transcription factors by phosphorylation provides a rapid cellular response to environmental changes. Previous studies have suggested that serine/threonine and tyrosine phosphorylation were required for DNA-binding activity and consequently for transactivation efficiency of HNF-4α.[36,37] The major focus of this work is the application of mass spectrometric techniques to identify the phosphorylation sites in the HNF-4α protein, to identify and quantify the relative changes in phosphoprotein abundance after exposure to inflammatory condition, and, most importantly, to explore how the changes in phosphorylation after cytokine stimulation affects HNF-4α function.

It has been accepted that phosphorylation is a crucial modification involved in many cellular events; however, the determination of phosphopeptide abundance, the localization of phosphorylation sites, and the way the phosphoproteins and phosphorylation events are modulated by or in response to significant changes in homeostasis remains a challenge. In the present study, specific phosphopeptide identification and quantitative phosphorylation state/site determination was achieved using stable isotopic variants of bifunctional thiol agent, DTT.[19,21,38] This approach enhances our ability to not only identify novel or new phosphorylation sites in HNF-4α protein but also provide a means to look for the changes in the concentration of phosphorylated protein over a biologically relevant time frame. Isotopic DTT derivatization allows a simple and direct qualitative and quantitative MS analysis for several reasons. First, since DTT does not cleave off, the DTT−phosphoserine/phosphothreonine derivatives are resistant to CID fragmentation during MS/MS analysis, permitting precise and more sensitive localization of the residue on which the phosphate group resided. Second, DTT−phosphoserine/phosphothreonine derivatives produce unambiguous MS/MS spectra with excellent fragmentation and are not complicated by the loss of the phosphate. Third, the +136.2 Da modification of the phosphopeptides by DTT derivatization is a unique differential mass addition, greatly enhancing the identification of original phosphopeptide amino acid sequences and pinpointing unequivocally the precise site(s) of phosphorylation. Finally, perhaps most importantly, DTT and its isotopic variant in conjunction with MS analysis permit relative quantitative analysis of HNF-4α phosphorylation. Despite the advantage of thiol reagent, certain precaution should be taken to avoid the identification of false positive phosphorylation due to low levels (1−2%) of conversion of O-glycosylation sites to dehydroalanine. On the basis of the fact that HNF-4 expressed in insect cells and rat liver nuclei is not substantially glycosylated,[36] the procedure of deglycosylation was not necessary in our experiments.

Table 1 and Figure 3 display the location of the phosphorylation sites in HNF-4α identified in this study. A total of 12 phosphorylation sites (including 11 serine and 1 threonine sites) were identified, indicating that HNF-4α is able to be phosphorylated at multisites in a human hepatic cell line. The multisite phosphorylations may provide a sophisticated means of regulating HNF-4α function. For example, phosphorylation likely affects HNF-4α's DNA-binding activity. Our previous work[9,10] has demonstrated, utilizing both a cytokine-induced cell culture injury model and various animal injury models, that injury leads to a rapid and reversible decrease in the binding activity of HNF-4α. HNF-4α−DNA binding activity is restored when the nuclear proteins extracted from HepG2 cells treated with cytokines or liver tissue 3 h after injury are incubated *in vitro* with CLP (calf intestinal alkaline phosphatase), suggesting that the decrease in HNF-4α binding after cytokine stimulation or injury is due to a post-translational modification by phosphorylation. In the current study, we further show, for the first time, that there are quantitative changes in phosphorylation of HNF-4α after cytokine treatment. Figure 4 illustrates that the phosphopeptide ^{125}R.DRIS*TRRS*S*YEDS*SLPS*IN.A^{145} is phosphorylated at S129, S133, S134, S138, and S142 sites under control condition and that overall phosphorylation of this phosphopeptide increases 3-fold after 3 h of cytokine treatment. Three other peptides were identified, ^{285}F.FDPDAKGLS*DPGKIK.R^{301}, ^{353}GMAKIDNLLQEMLLGGS*PS^{371}, and ^{428}K.LLPGAVAT*IVKP.L^{441}, that also exhibit similar changes in phosphorylation pattern after cytokine stimulation, but phosphorylation levels varied among these peptides. These results collaborate our previous findings[9,10] and provide further insight into possible mechanisms of HNF-4α regulation after injury.

To investigate the biological significance of individually identified phosphorylation site, the potential sites of specific protein kinases were searched and verified. One of the identified peptides ^{125}R.DRIS*TRRS*S*YEDS*SLPS*IN.A^{145} is most interesting, because it contains the S133 and S134 phosphorylation sites, which are located in a region near the DNA-binding domain of HNF-4α (Figure 4A) downstream of two zinc finger motifs and within the A-box region. This region has been shown to be required for high-affinity DNA binding of HNF-4α.[39] A consensus PKA-dependent phosphorylation site (^{131}RRSS^{134}) also lies in this region.[40] *In vitro* kinase assays clearly show that the binding affinity of HNF-4α can be strongly repressed by PKA-induced phosphorylation in the cytokine-treated or untreated nuclear extracts, and inhibition of PKA activity in cells with PKA inhibitor has a recovery effect (Figure 6). Our findings are also in line with the work by Viollet et al.,[13] showing that the binding affinity of HNF-4α is suppressed by PKA-induced phosphorylation, but not in HNF-4α mutants produced by mutation of HNF-4α at S133 and S134 sites. While six potential PKA sites are present in HNF-4α, identified by NetPhos (Technical University of Denmark, www.cbs.dtu.dk/services/NetPhos), three (S133, S134, and S304) of them were detected in our current study; however, only the phosphopeptide containing the PKA sites at S133 and S134 showed a change in phosphorylation level after cytokine treatment (Figure 4). Given the *in vitro* effect of PKA treatment on the inhibition of HNF-4α binding (Figures 5 and 6), it is suggested that the phosphorylation sites at S133 and/or S134 are likely candidates/targets for PKA phosphorylation of HNF-

4α. Thus, we propose a plausible mechanism for the decrease in HNF-4α binding seen in an inflammatory or injury response. PKA, activated by cytokines, phosphorylates HNF-4α at S133/S134 residues, which leads to a significant decrease in HNF-4α binding and transactivation activity, affecting HNF-4α regulated genes in the liver after injury.

Transcription factors interact with many different proteins including other transcription factors, coregulators, and components of the basal transcription complex to regulate gene transcription. These interactions potentially are controlled or modified by the state of protein phosphorylation. It has been reported that the AF-2 domain (spanning the amino acids 128−366 region) and the F domain of HNF-4α play an important role in the interaction with coactivators and corepressors.[41,42] We have previously shown that cytokine treatment does not significantly alter the protein levels of HNF-4α and its coactivator PGC-1α (peroxisome-proliferator-activated receptor-γ coactivator-1α), but it does reduce the recruitment of PGC-1α to HNF-4α and thereby decrease HNF-4α transcriptional activity.[43] The identified phosphopeptide ^{353}GMAKIDNLLQEMLLGGS*^{369}PS^{371} is located in the vicinity of the AF-2 domain and has a higher rate of phosphorylation in cells treated with cytokines compared to control (Figure 4). PGC-1α has been reported to bind to the AF-2 domain of HNF-4α.[44] The identification of a phosphorylation site at S369 in close proximity to the AF-2 domain, and the detection of a cytokine induced changes in the abundance of this phosphopeptide may represent another potential mechanism for transcriptional control by HNF-4α via an alteration in the interaction of PGC-1α and HNF-4α during the inflammatory response. However, further work will be needed to provide direct evidence of the role of HNF-4α phosphorylation in coregulator recruitments.

In summary, we have mapped the phosphorylation sites in the liver-specific transcription factor HNF-4α. Twelve unique phosphorylation sites were identified by LC-MS/MS analysis. More importantly, we found that the phosphorylation level of HNF-4α is regulated by cytokines, and a PKA signaling pathway may be involved in the modification of HNF-4α function during the inflammatory response. Our study also suggests that the present of a particular HNF-4α phosphopeptide is not enough to significantly change the binding activity, which requires a significant change in quantity of a particular phosphopeptide initiated by stimulation, such as cytokine treatment, to affect HNF-4α activities. A system that relies on modulation of phosphoproteins would be one that could be easily ramped up and down and could explain the observation of the rapid and reversible changes in HNF-4α binding activity that are seen in animal models of injury. In order to better understand the dynamic changes of phosphorylation in phosphorylation-mediated signaling pathway, kinetic studies of cytokine induced inflammatory responses in both cell culture and animal injury models are essential. Our future studies will be directed at exploring the dynamics of phosphorylation after injury and its effects on the activities of HNF-4α and their impact on regulating the liver's response to injury.

AUTHOR INFORMATION

Corresponding Author

*Tel: 617-414-8056. Fax: 617-414-7398. E-mail: peter.burke@bmc.org.

Author Contributions

§These authors contributed equally to this work.

Funding Sources

This work was supported by NIH grant (R01DK064945, to P.A.B.) and by NIDCR grant (DE018448, to E.S.).

ACKNOWLEDGMENT

We greatly appreciate the gift of pCMV-FLAG-hHNF-4α plasmid from Dr. Paul C. Kuo, Duke University Medical Center, Durham, NC.

ABBREVIATIONS

HNF-4α, hepatocyte nuclear factor-4α; MS, mass spectrometry; DTT, dithiothreitol; CID, collision-induced dissociation; LC-ESI-MS/MS, liquid chromatography−electrospray ionization−tandem mass spectrometry; protein kinase A, PKA; DBD, DNA binding domain; LBD, ligand binding domain; PGC-1α, peroxisome-proliferator-activated receptor-γ coactivator-1α.

REFERENCES

(1) Parviz, F., Matullo, C., Garrison, W. D., Savatski, L., Adamson, J. W., Ning, G., Kaestner, K. H., Rossi, J. M., Zaret, K. S., and Duncan, S. A. (2003) Hepatocyte nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis. *Nature Genet. 34*, 292–296.

(2) Hayhurst, G. P., Lee, Y. H., Lambert, G., Ward, J. M., and Gonzalez, F. J. (2001) Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol. Cell. Biol. 21*, 1393–1403.

(3) Hertz, R., Magenheim, J., Berman, I., and Bar-Tana, J. (1998) Fatty acyl-CoA thioesters are ligands of hepatic nuclear factor-4alpha. *Nature 392*, 512–516.

(4) Yuan, X., Ta, T. C., Lin, M., Evans, J. R., Dong, Y., Bolotin, E., Sherman, M. A., Forman, B. M., and Sladek, F. M. (2009) Identification of an endogenous ligand bound to a native orphan nuclear receptor. *PLoS One 4*, e5609.

(5) Kaestner, K. H. (2010) Making the liver what it is: the many targets of the transcriptional regulator HNF4alpha. *Hepatology 51*, 376–377.

(6) Yamagata, K., Furuta, H., Oda, N., Kaisaki, P. J., Menzel, S., Cox, N. J., Fajans, S. S., Signorini, S., Stoffel, M., and Bell, G. I. (1996) Mutations in the hepatocyte nuclear factor-4alpha gene in maturity-onset diabetes of the young (MODY1). *Nature 384*, 458–460.

(7) Pearson, E. R., Boj, S. F., Steele, A. M., Barrett, T., Stals, K., Shield, J. P., Ellard, S., Ferrer, J., and Hattersley, A. T. (2007) Macrosomia and hyperinsulinaemic hypoglycaemia in patients with heterozygous mutations in the HNF4A gene. *PLoS Med. 4*, e118.

(8) Burke, P. A., Drotar, M., Luo, M., Yaffe, M., and Forse, R. A. (1994) Rapid modulation of liver-specific transcription factors after injury. *Surgery 116*, 285–292; discussion 292−293.

(9) Li, X., Salisbury-Rowswell, J., Murdock, A. D., Forse, R. A., and Burke, P. A. (2002) Hepatocyte nuclear factor 4 response to injury involves a rapid decrease in DNA binding and transactivation via a JAK2 signal transduction pathway. *Biochem. J. 368*, 203–211.

(10) Wang, Z., and Burke, P. A. (2007) Effects of hepatocyte nuclear factor-4alpha on the regulation of the hepatic acute phase response. *J. Mol. Biol. 371*, 323–335.

(11) Hunter, T., and Karin, M. (1992) The regulation of transcription by phosphorylation. *Cell 70*, 375–387.

(12) Karin, M., and Hunter, T. (1995) Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Curr. Biol. 5*, 747–757.

(13) Viollet, B., Kahn, A., and Raymondjean, M. (1997) Protein kinase A-dependent phosphorylation modulates DNA-binding activity of hepatocyte nuclear factor 4. *Mol. Cell. Biol. 17*, 4208–4219.

(14) Hong, Y. H., Varanasi, U. S., Yang, W., and Leff, T. (2003) AMP-activated protein kinase regulates HNF4alpha transcriptional activity by

inhibiting dimer formation and decreasing protein stability. *J. Biol. Chem. 278*, 27495–27501.

(15) Guo, H., Gao, C., Mi, Z., Wai, P. Y., and Kuo, P. C. (2006) Phosphorylation of Ser158 regulates inflammatory redox-dependent hepatocyte nuclear factor-4alpha transcriptional activity. *Biochem. J. 394*, 379–387.

(16) Salih, E. (2005) Phosphoproteomics by mass spectrometry and classical protein chemistry approaches. *Mass Spectrom. Rev. 24*, 828–846.

(17) Salih, E. (2003) Synthesis of a radioactive thiol reagent, 1-S-[3H]carboxymethyl-dithiothreitol: identification of the phosphorylation sites by N-terminal peptide sequencing and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal. Biochem. 319*, 143–158.

(18) Salih, E., and Fluckiger, R. (2004) Complete topographical distribution of both the in vivo and in vitro phosphorylation sites of bone sialoprotein and their biological implications. *J. Biol. Chem. 279*, 19808–19815.

(19) Salih, E., Siqueira, W. L., Helmerhorst, E. J., and Oppenheim, F. G. (2010) Large-scale phosphoproteome of human whole saliva using disulfide-thiol interchange covalent chromatography and mass spectrometry. *Anal. Biochem. 407*, 19–33.

(20) Salih, E., Ashkar, S., Gerstenfeld, L. C., and Glimcher, M. J. (1997) Identification of the phosphorylated sites of metabolically 32P-labeled osteopontin from cultured chicken osteoblasts. *J. Biol. Chem. 272*, 13966–13973.

(21) Salih, E. (2004) Emergence of Phosphoproteomics through Combination of Mass Spectrometry and Classical Protein Chemistry Approaches in *The Chemistry and Biology of Mineralized Tissues* (Landis, W. L., and Sodek, J., Eds.) pp 208−211, University of Toronto Press, Toronto, Canada.

(22) Eng, J. K., M., A., and Yates, J. R., III (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass Spectrom. 5*, 976–989.

(23) Salih, E. (2003) In vivo and in vitro phosphorylation regions of bone sialoprotein. *Connect. Tissue Res. 44* (Suppl 1), 223–229.

(24) Sladek, F. M., Zhong, W. M., Lai, E., and Darnell, J. E., Jr. (1990) Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. *Genes Dev. 4*, 2353–2365.

(25) Baumann, H., and Gauldie, J. (1994) The acute phase response. *Immunol. Today 15*, 74–80.

(26) Villen, J., Beausoleil, S. A., and Gygi, S. P. (2008) Evaluation of the utility of neutral-loss-dependent MS3 strategies in large-scale phosphorylation analysis. *Proteomics 8*, 4444–4452.

(27) Boersema, P. J., Mohammed, S., and Heck, A. J. (2009) Phosphopeptide fragmentation and analysis by mass spectrometry. *J. Mass Spectrom. 44*, 861–878.

(28) Gourdon, L., Lou, D. Q., Raymondjean, M., Vasseur-Cognet, M., and Kahn, A. (1999) Negative cyclic AMP response elements in the promoter of the L-type pyruvate kinase gene. *FEBS Lett. 459*, 9–14.

(29) Daigo, K., Kawamura, T., Ohta, Y., Ohashi, R., Katayose, S., Tanaka, T., Aburatani, H., Naito, M., Kodama, T., Ihara, S., and Hamakubo, T. (2010) Proteomic analysis of native hepatocyte nuclear factor-4alpha (HNF4alpha) isoforms, phosphorylation status, and interactive cofactors. *J. Biol. Chem. 286*, 674–686.

(30) Sladek, F. M. (1993) Orphan receptor HNF-4 and liver-specific gene expression. *Receptor 3*, 223–232.

(31) Ganapathi, M. K., Rzewnicki, D., Samols, D., Jiang, S. L., and Kushner, I. (1991) Effect of combinations of cytokines and hormones on synthesis of serum amyloid A and C-reactive protein in Hep 3B cells. *J. Immunol. 147*, 1261–1265.

(32) Gronemeyer, H., Gustafsson, J. A., and Laudet, V. (2004) Principles for modulation of the nuclear receptor superfamily. *Nat. Rev. Drug Discov. 3*, 950–964.

(33) Jiang, S., Tanaka, T., Iwanari, H., Hotta, H., Yamashita, H., Kumakura, J., Watanabe, Y., Uchiyama, Y., Aburatani, H., Hamakubo, T., Kodama, T., and Naito, M. (2003) Expression and localization of P1 promoter-driven hepatocyte nuclear factor-4alpha (HNF4alpha) isoforms in human and rats. *Nucl. Recept. 1*, 5.

(34) Blom, N., Gammeltoft, S., and Brunak, S. (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J. Mol. Biol. 294*, 1351–1362.

(35) Blom, N., Sicheritz-Ponten, T., Gupta, R., Gammeltoft, S., and Brunak, S. (2004) Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics 4*, 1633–1649.

(36) Jiang, G., Nepomuceno, L., Yang, Q., and Sladek, F. M. (1997) Serine/threonine phosphorylation of orphan receptor hepatocyte nuclear factor 4. *Arch. Biochem. Biophys. 340*, 1–9.

(37) Ktistaki, E., Ktistakis, N. T., Papadogeorgaki, E., and Talianidis, I. (1995) Recruitment of hepatocyte nuclear factor 4 into specific intranuclear compartments depends on tyrosine phosphorylation that affects its DNA-binding and transactivation potential. *Proc. Natl. Acad. Sci. U.S.A. 92*, 9876–9880.

(38) Vora, S. R., Guo, Y., Stephens, D. N., Salih, E., Vu, E. D., Kirsch, K. H., Sonenshein, G. E., and Trackman, P. C. (2010) Characterization of recombinant lysyl oxidase propeptide. *Biochemistry 49*, 2962–2972.

(39) Jiang, G., and Sladek, F. M. (1997) The DNA binding domain of hepatocyte nuclear factor 4 mediates cooperative, specific binding to DNA and heterodimerization with the retinoid X receptor alpha. *J. Biol. Chem. 272*, 1218–1225.

(40) Kennelly, P. J., and Krebs, E. G. (1991) Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem. 266*, 15555–15558.

(41) Hadzopoulou-Cladaras, M., Kistanova, E., Evagelopoulou, C., Zeng, S., Cladaras, C., and Ladias, J. A. (1997) Functional domains of the nuclear receptor hepatocyte nuclear factor 4. *J. Biol. Chem. 272*, 539–550.

(42) Ruse, M. D., Jr., Privalsky, M. L., and Sladek, F. M. (2002) Competitive cofactor recruitment by orphan receptor hepatocyte nuclear factor 4alpha1: modulation by the F domain. *Mol. Cell. Biol. 22*, 1626–1638.

(43) Wang, Z., and Burke, P. A. (2008) Modulation of hepatocyte nuclear factor-4alpha function by the peroxisome-proliferator-activated receptor-gamma co-activator-1alpha in the acute-phase response. *Biochem. J. 415*, 289–296.

(44) Yoon, J. C., Puigserver, P., Chen, G., Donovan, J., Wu, Z., Rhee, J., Adelmant, G., Stafford, J., Kahn, C. R., Granner, D. K., Newgard, C. B., and Spiegelman, B. M. (2001) Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature 413*, 131–138.