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AMP-activated Protein Kinase Regulates HNF4α Transcriptional Activity by Inhibiting Dimer Formation and Decreasing Protein Stability*

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AMP-activated protein kinase (AMPK) is the central component of a cellular signaling system that regulates multiple metabolic enzymes and pathways in response to reduced intracellular energy levels. The transcription factor hepatic nuclear factor 4α (HNF4α) is an orphan nuclear receptor that regulates the expression of genes involved in energy metabolism in the liver, intestine, and endocrine pancreas. Inheritance of a single null allele of HNF4α causes diabetes in humans. Here we demonstrate that AMPK directly phosphorylates HNF4α and represses its transcriptional activity. AMPK-mediated phosphorylation of HNF4α on serine 304 had a 2-fold effect, reducing the ability of the transcription factor to form homodimers and bind DNA and increasing its degradation rate *in vivo*. These results demonstrate that HNF4α is a downstream target of AMPK and raise the possibility that one of the effects of AMPK activation is reduced expression of HNF4α target genes.

The transcription factor HNF4α[1] (NR2A1), a member of the nuclear receptor superfamily, plays a key role in regulating the expression of metabolic genes in multiple tissues including the liver, intestine, kidney, and endocrine pancreas (for review see Ref. 1). In humans, a nonsense mutation in a single allele of the *HNF4α* gene causes an inherited form of diabetes known as maturity onset diabetes of the young (MODY) (for a review see Ref. 2). This syndrome is characterized by insufficient insulin secretion (3, 4), indicating a significant defect in pancreatic function. Surprisingly, there is not a major deficit in liver function, despite the important role of HNF4α in hepatic gene expression (5). These observations suggest that HNF4α regulates pancreatic genes involved in glucose sensing and/or insulin production. This hypothesis is supported by experiments demonstrating that HNF4α regulates expression of genes encoding enzymes of glucose metabolism and insulin secretion (6–8). Because MODY1 patients have a single null allele that does not produce a dominant-negative acting protein (9), the physiological abnormalities in these patients must be caused by a relatively mild (~50%) decrease in the amount of HNF4α protein. Taken together, these observations raise the possibility that any modification of HNF4α that causes a reduction in its transcriptional activity could have significant effects on pancreatic function.

HNF4α has also been shown recently to play an important role in the regulation of hepatic glucose output, a key component of the maintenance of plasma glucose levels. Yoon *et al.* (10) recently demonstrated that the transcriptional regulation of gene for the key gluconeogenic enzyme phosphoenolpyruvate carboxykinase by cAMP was mediated by the transcriptional co-activator PGC-1 acting through HNF4α. These findings suggest that HNF4α may play a role in the transcriptional response of the liver to metabolic hormones and that factors regulating the activity of HNF4α could have an effect on hepatic glucose output.

AMPK is the mammalian homolog of the yeast SNF1 protein kinase (11). In yeast, the SNF1 complex is a nuclear protein that regulates expression of genes involved in glucose metabolism and is activated by phosphorylation when glucose is removed from the growth medium (12). In mammalian cells, AMPK acts as a fuel sensor that monitors AMP and ATP levels. It is activated by high AMP:ATP ratios during states of low energy charge. Once activated, the enzyme reduces the activity of ATP-consuming anabolic pathways and increases the activity of catabolic ATP-producing pathways, acting to reestablish normal cellular energy balance (for review see Ref. 13). Depending on the tissue, a variety of physiological circumstances can cause AMPK activation including hypoglycemia (14), ischemia (15), heat shock (16), and exercise (17–19). AMPK activation has been associated with several key aspects of metabolism including exercise-induced glucose uptake and fatty acid oxidation in muscle (17, 20, 21), reduced lipid (22, 23) and glucose synthesis in liver (12, 24), and altered glucose-stimulated insulin secretion in the endocrine pancreas (14, 25).

AMPK is a heterotrimer consisting of a catalytic α subunit and two non-catalytic β and γ subunits (26, 27) that exist in multiple isoforms (28–30). Kinase activity is regulated allosterically by AMP binding and also by direct phosphorylation mediated by an uncharacterized AMP-sensitive protein kinase. A potential role of AMPK in the nucleus is suggested by several observations. The first is the similarity of the mammalian kinase to the yeast SNF1 transcriptional regulator, as described above. Second, AMPK heterotrimers containing the α2 subunit isoform are preferentially found in the nucleus (31). In addition, AMPK has been shown to phosphorylate the tran-

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[1] The abbreviations used are: HNF4α, hepatic nuclear factor 4α; AMPK, AMP-activated protein kinase; GST, glutathione *S*-transferase; CMV, cytomegalovirus; CHO, Chinese hamster ovary; MODY, maturity onset diabetes of the young; AICAR, 5′-aminoimidazol-4-carboxoxamide 1-β-D-ribofuranoside; BHK, baby hamster kidney; GFP, green fluorescent protein; EGFP, enhanced GFP.

scriptional coactivator p300 and to regulate its ability to mediate nuclear receptor transcriptional activity (32).

It has been reported previously (33) that AMPK activation reduces HNF4α target gene transcription. These findings, together with the fact that both proteins participate in the regulation of similar metabolic pathways, raise the possibility that HNF4α is directly regulated by AMPK. The experiments described here examine the possibility that AMPK phosphorylates HNF4α and regulates its transcriptional activity.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The HNF4α expression construct pH4c-WT contains a C-terminal FLAG-tagged full-length human HNF4α cDNA sequence cloned into the CMV promoter-based vector pCDNA3.1. The S304A and S304D mutants were introduced into pH4c-WT using the Quik-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions, and confirmed by DNA sequencing, to produce the plasmids pH4c-304A and pH4c-304D. The pH4c series plasmids were used in experiments presented in Figs. 1, 2*B*, 3*A*, 3*D*, 4*A*, and 5. For experiments designed to examine HNF4α protein stability (Fig. 4, *B* and *C*), full-length N-terminal FLAG-tagged wild-type, 304A, and 304D cDNA sequences were cloned into the tetracycline-regulated vector, pTRE-shuttle2 (Clontech, Palo Alto, CA), to form pH4i-WT, pH4i-304A, and pH4i-304D. GFP-HNF4α protein was expressed from a pCDNA3.1 vector containing an N-terminal fusion of the EGFP coding sequences (Clontech, Palo Alto, CA) fused to the full-length HNF4α cDNA. GST-HNF4α protein was produced from a pET23a vector (Novagen, Madison, WI) containing an N-terminal fusion of glutathione *S*-transferase sequences and the full-length HNF4α cDNA. The pET.HNF4/A–D plasmids contain PCR-amplified subfragments of HNF4α (Fig. 1*C*) as His and T7 tag N-terminal fusions in the pET28a vector. The same subfragments were also cloned into the pCMV-Tag2b plasmid (Stratagene, La Jolla, CA) for expression in eukaryotic cells. The HNF4α-responsive reporter plasmid pL854 contains promoter sequences from the human *apoCIII* gene (−854 to +22) linked to luciferase in the pGL3-basic vector (Promega, Madison, WI). The β-galactosidase plasmid contains a CMV-driven β-galactosidase gene.

Cell Culture, Transfection, and Reporter Assay—Transient transfections for *in vivo* labeling were conducted in a baby hamster kidney cell line, BHK-21, which was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Chinese hamster ovary (CHO) cells were used in transfection for transcription assays and nuclear extract preparation. CHO cells were maintained in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 10% fetal bovine serum. All transfections were carried out with LipofectAMINE 2000 (Invitrogen). Transfections for reporter assays were conducted in 24-well plates in triplicate for each condition. Cells were harvested at 20 h post-transfection using lysis buffer (Promega, Madison, WI), and the lysates were analyzed with Tropix Dual Light Luciferase and β-galactosidase assay kit (Tropix, Inc., Bedford, MA) using an EG&G Berthold Microlumat 96P luminometer.

In Vitro and in Vivo Phosphorylation—*In vitro* phosphorylation assays were conducted with *in vitro* translated proteins produced using the TnT Quick-Coupled transcription/translation system (Promega, Madison, WI). TnT reactions were carried out for 60 min at 30 °C and then immunoprecipitated by anti-FLAG (Sigma) or anti-T7 (Novagen Inc., Madison, WI) antibodies, and protein A/G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). After washing five times with HNTG buffer (20 mM HEPES, pH 7.5, 50 mM NaCl, 5% glycerol, 1% Triton, 1.5 mM $MgCl_2$, 1 mM EDTA), the purified beads were subjected to an *in vitro* phosphorylation reaction using 100 milliunits of purified rat liver AMPK (Upstate Inc., Lake Placid, NY) per reaction. Reactions were carried out in a buffer containing 5 mM HEPES, pH 7.5, 0.1 mM dithiothreitol, 0.25% Nonidet P-40, 7.5 mM $MgCl_2$, 50 μM ATP, 10 μCi of [γ-^{32}P]ATP, and a mixture of protein kinase C/protein kinase A inhibitors, with or without of 300 μM AMP, and incubated at 30 °C for 30 min. The reactions were stopped by washing the beads three times with HNTG buffer and adding 30 μl of 2× SDS loading buffer (Invitrogen). The protein products were separated on SDS-PAGE, transferred to nitrocellulose membranes, which were then subjected to autoradiography and Western blot analysis using an anti-FLAG antibody conjugated with horseradish peroxidase (Sigma), and detected with the SuperSignal West Pico chemiluminescent system (Pierce).

In vivo phosphorylation labeling was conducted in BHK cells. Wild-type pCMV.HNF4/C and pCMV.HNF/C.S304A mutant, along with the control vector, were transfected in 100-mm plates. Each plate was split into two 60-mm plates 16 h post-transfection. *In vivo* labeling was conducted with 1 mCi (37 MBq) of [^{32}P]orthophosphate per plate for 1 h at 40 h post-transfection, with or without of 500 μM 5′-aminoimidazol-4-carboxoxamide 1-β-D-ribofuranoside (AICAR). Cells were harvested in 600 μl of HNTG buffer and mixed for 30 min at 4 °C. The cell lysates were immunoprecipitated with anti-FLAG monoclonal antibody (Sigma) and 50 μl of protein A/G-agarose beads for 2 h at 4 °C. The beads were washed five times with same buffer. Bound proteins were eluted in 30 μl of 2× SDS loading buffer at 95 °C for 5 min, separated on 4–12% gradient SDS-PAGE gel, transferred to nitrocellulose membrane, and detected by autoradiography or Western blot using anti-FLAG antibody conjugated with horseradish peroxidase as described above.

Gel Mobility Shift and Pull-down Assays—CHO cells transfected with full-length, wild-type, or mutant HNF4α were harvested at 20 h post-transfection, with or without 12 h treatment of AICAR. Nuclear extracts were prepared essentially as described (34). The recombinant HNF4α proteins were produced *in vitro* using TnT Quik-Coupled transcription/translation system as described by the manufacturer (Promega, Madison, WI). For dimerization experiments, where different HNF4α proteins were produced from two plasmids, templates producing wild-type or mutant HNF4α proteins were used in the co-translation reactions at ratios determined in pilot translations to produce equal amounts of protein. For GST pull-down assays, 25 μl of translation reactions were incubated with 15 μl of glutathione-Sepharose 4B (Amersham Biosciences) in BC-150 buffer (20 mM Tris-HCl, pH 7.5, 150 mM KCl, 0.01% Nonidet P-40, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor mixture (Roche Diagnostics)) for 30 min at room temperature on a rotary platform. The beads were washed five times with BC-150 buffer, boiled in 25 μl of SDS sample buffer, and resolved on 4–20% gradient gels (Invitrogen). Gels were stained with Coomassie Blue, incubated in Amplify (Amersham Biosciences) for 30 min, dried, and fluorographed.

The synthetic double-stranded oligonucleotides used in DNA binding reaction were labeled with [α-^{32}P]dCTP using the Rediprime-II random labeling system (Amersham Biosciences). The double-stranded DNA binding probe contains the proximal HNF4-binding site from the human *apoCIII* gene (35). DNA binding reactions were carried out in 20 mM HEPES, pH 7.9, 60 mM KCl, 3% Ficoll, 0.5 mM $MgCl_2$, 0.06% Nonidet P-40, 1 mM dithiothreitol, 1 μg of double-stranded poly(dI-dC), with 20,000 cpm of labeled probe (~0.25 ng), and either with 2–5 μg of nuclear extract or 1 μl of TnT reaction. Reactions were incubated for 20 min at room temperature and then analyzed on precast 6% DNA retardation gels (Invitrogen) in 0.5× TBE at 125 V for 45 min at room temperature.

Protein Stability Experiments—For the experiments shown in Fig. 4, *B* and *C*, CHO cells were transfected in 12-well plates as described above but using LipofectAMINE Plus (Invitrogen) with 1 ng of the tetracycline-regulated HNF4α expressing plasmids (pH4i-WT, pH4i-304A, and pH4i-304D) together with 20 ng of pTET-OFF and 40 ng of pEGFP-N1 (Clontech, Palo Alto, CA) per well. 18 h after transfection doxycycline was added (1 μg/ml) to the culture media to stop transcription of recombinant HNF4α and cells were harvested 2, 4, 6, and 8 h later by lysis in HNTG buffer. Gel electrophoresis and Western blot analysis to detect FLAG-HNF4α and GFP protein were carried out as described above.

RESULTS

Activation of AMPK in cultured hepatocytes with the AMP analog AICAR caused a decrease in the activity of HNF4α target gene expression and a reduction in the amount of HNF4α (33). To explore the possibility that these effects are due to direct regulation of HNF4α by AMPK, we tested whether AMPK could phosphorylate HNF4α. An *in vitro* phosphorylation assay containing full-length HNF4α and partially purified AMPK was carried out. The results (Fig. 1*A*) demonstrate that HNF4α was efficiently phosphorylated by AMPK, showing approximately the same degree of phosphorylation as a similar amount of the well characterized AMPK substrate hormone-sensitive lipase.

Mapping AMPK Phosphorylation Site on HNF4α—When the HNF4α amino acid sequence was screened for the consensus target sequence for AMPK phosphorylation, three potential sites were found (Fig. 1*B*) with the best match to a site surrounding serine 304. To determine whether any of these sites were targets for AMPK phosphorylation, they were each mu-

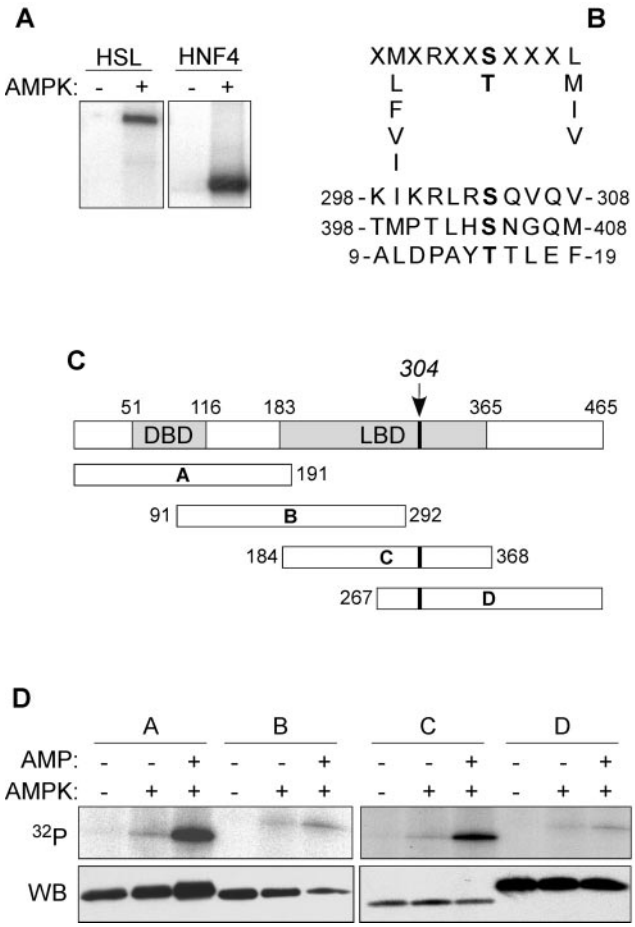

FIG. 1. **HNF4α is phosphorylated by AMPK *in vitro*.** *A*, autoradiogram of full-length human HNF4α phosphorylated by AMPK in an *in vitro* kinase reaction. HNF4α protein produced in an *in vitro* transcription/translation system was incubated with purified rat liver AMPK and [γ-^{32}P]ATP. For comparison, the same quantity of hormone-sensitive lipase (*HSL*), a known substrate of the kinase, was phosphorylated in a parallel reaction. *B*, amino acid sequence of the consensus phosphorylation site for AMPK (53), showing the three best matches in the human HNF4α sequence (*bottom*). Potential phosphorylation residues are shown in *boldface*. The amino acid numbers are based on GenBank™ entry XM029795. *C*, schematic map of the HNF4α protein showing the location of the DNA binding domain (*DBD*) and ligand binding domain (*LBD*) and the location of the serine 304 residue. The four sub-fragments of the receptor that were used in phosphorylation studies are shown. *D*, *in vitro* phosphorylation of each of the four HNF4α subfragments shown in *C*, in the presence or absence of AMP as indicated. *Lower panels* are Western blots (*WB*) showing total protein amounts in the reactions.

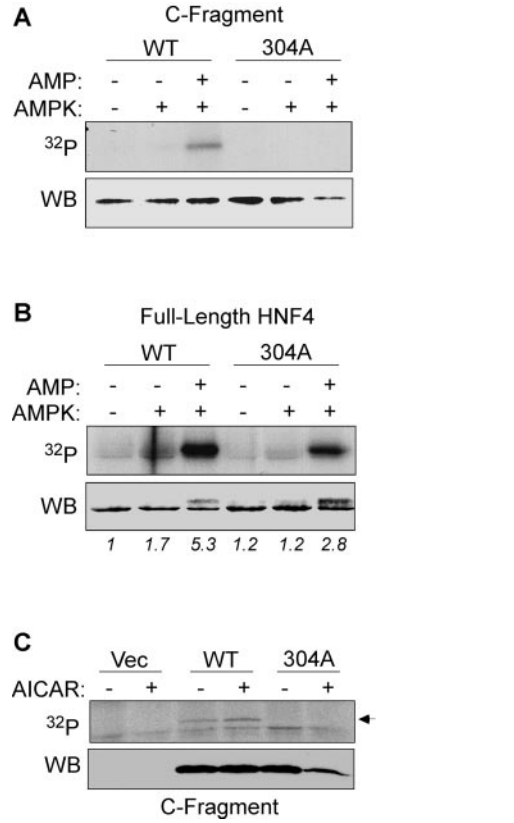

FIG. 2. **AMPK phosphorylates HNF4α at serine 304.** *A*, autoradiogram of the HNF4α C fragment phosphorylated *in vitro* by AMPK. Wild-type and Ser-304 mutant C fragments of HNF4α produced in an *in vitro* transcription/translation system were incubated with purified rat liver AMPK and [γ-^{32}P]ATP, in the presence or absence of AMP as indicated. *Lower panels* are Western blots (*WB*) showing total protein amounts in the reactions. *B*, phosphorylation of full-length wild-type and Ser-304 mutant HNF4α by AMPK *in vitro* as in *A*. *Numbers below* each lane indicate the degree of phosphorylation relative to the wild-type protein in the absence of kinase and AMP. The degree of phosphorylation was calculated from quantified autoradiograph and Western blot bands as the ratio of phosphorylated to total protein. *C*, *in vivo* phosphorylation of the HNF4α C fragment by AMPK. BHK cells were transfected with either vector (*Vec*) alone or constructions producing FLAG-tagged wild-type or S304A mutant HNF4α C protein. Transfected cells were treated with the AMPK activator AICAR as indicated and [^{32}P]orthophosphate. HNF4α C fragment protein was immunoprecipitated with anti-FLAG antibodies and subjected to electrophoresis, transfer to nitrocellulose, and autoradiography (*upper panel*) or Western analysis (*lower panel*).

tated to alanine and examined in an *in vitro* phosphorylation assay. None of the mutants abolished phosphorylation by AMPK (data not shown), indicating that there are multiple AMPK phosphorylation sites on HNF4. To examine phosphorylation of individual segments of the protein independently, four overlapping fragments of HNF4α were generated from subclones of the HNF4α cDNA (Fig. 1*C*). When *in vitro* AMPK assays were conducted, all four subfragments were phosphorylated to some degree (Fig. 1*D*) and showed the expected enhancement of phosphorylation in the presence of AMP (an allosteric activator of the kinase). However, when the results were normalized to the total amount of protein in the assay, the HNF4/C fragment (containing the Ser-304 site) showed the highest degree of AMP-dependent phosphorylation (data not shown). A significant amount of phosphorylation was also observed with the HNF4/A fragment. The HNF4/A fragment contains one of the three potential consensus sites for AMPK (Fig. 1*B*). However, mutation of threonine 15 to alanine in this sequence did not reduce the phosphorylation of the HNF4/A fragment, suggesting the presence of one or more cryptic AMPK sites in this part of the protein.

To determine whether the C fragment is phosphorylated at the Ser-304 residue, the S304A mutant was transferred to the C fragment and examined in an *in vitro* phosphorylation assay. As presented in Fig. 2*A*, mutation of serine 304 completely abolished phosphorylation of the C fragment. Although the Ser-304 site is also present in the D fragment (Fig 1*C*), it does not appear to be a good substrate for the kinase in this context as this fragment is phosphorylated to a much lower degree than the C fragment (Fig. 1*D*). When the S304A mutation was examined quantitatively in the context of the full-length protein, it reduced phosphorylation by ~50% (Fig. 2*B*). Together, these results suggest that S304A is a phosphorylation site for AMPK *in vitro* and that additional sites are present in both the N and C termini of protein.

In vivo, HNF4α is a phosphoprotein and is a substrate for multiple kinases (36, 37). To explore the phosphorylation of HNF4α by AMPK *in vivo*, wild-type and S304A versions of the

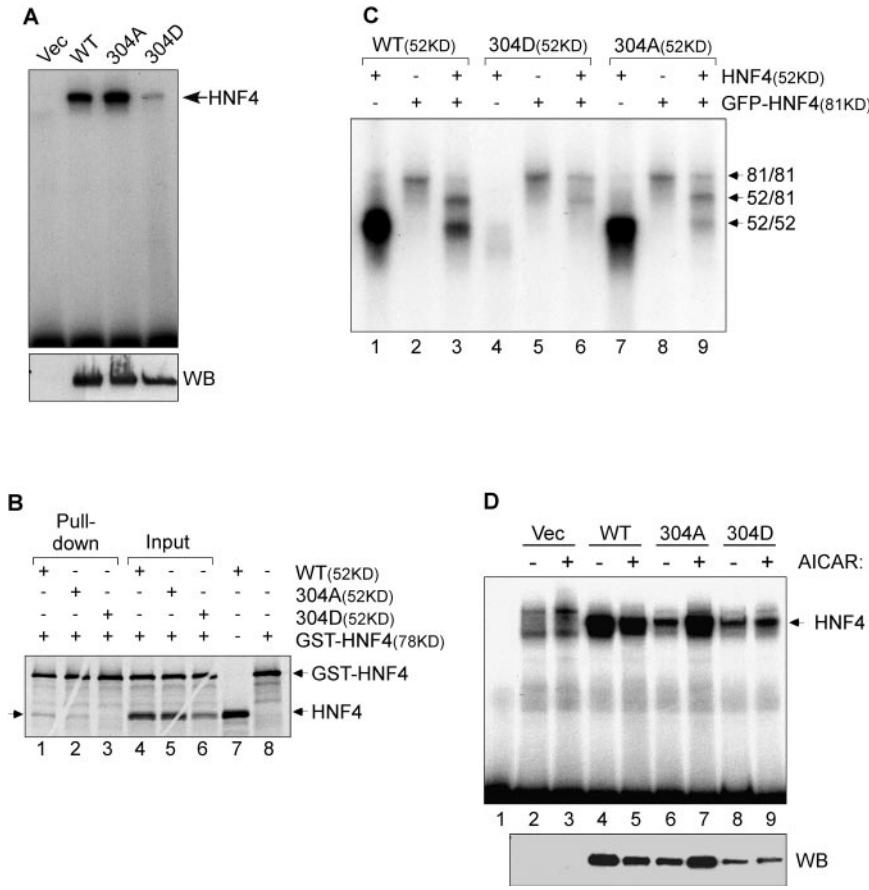

FIG. 3. **Phosphorylation at Ser-304 blocks dimerization and DNA binding.** *A*, the phosphomimetic HNF4α S304D mutation is defective in its ability to bind DNA. *In vitro* transcription/translation reactions were programmed with plasmids producing full-length wild-type, S304A, or S304D HNF4α proteins (pH4c series plasmids) or with empty vector (*Vec*). The DNA binding activities of the three HNF4α proteins were analyzed in a gel mobility shift assay using the proximal HNF4α-binding site of the apoCIII promoter as labeled probe. The *lower panel* is a Western blot showing the input amount of HNF4α protein used in the DNA binding reactions. *B*, the S304D mutant HNF4α protein is defective in its ability to form HNF4α homodimers. A GST pull-down assay was performed with *in vitro* produced GST-HNF4α fusion protein (wild-type, full-length). The plasmid producing the 78-kDa GST fusion protein was co-translated with plasmids producing 52 kDa wild-type, S304A, or S304D as indicated in the presence of [^{35}S]methionine. *Lanes 1–3* were subjected to GST pull-down procedure using glutathione beads, whereas *lanes 4–6* were loaded directly on the gel without pull-down. *Lanes 7* and *8* are translations of the two input plasmids alone. *C*, gel mobility shift assay showing that the phosphomimetic HNF4α S304D mutation is defective in forming functional dimers. *In vitro* translated GFP-HNF4α fusion protein (81 kDa) and 52-kDa wild-type, S304A, and S304D mutants were co-translated as indicated and analyzed in a gel mobility shift assay. The DNA-protein complexes with each of the possible dimer combinations is indicated on the *right.* Equivalent amounts of each protein were used the DNA binding reactions as determined by Western blot analysis (data not shown). *D*, gel shift analysis of wild-type and mutant HNF4α proteins produced *in vivo*. CHO cells were transfected with the indicated plasmids, treated with the AMPK activator AICAR as indicated, and nuclear extracts were prepared and subjected to gel mobility shift analysis using the apoCIII promoter proximal HNF4α-binding site as a probe. Equivalent amounts of nuclear extract were loaded on each lane. *Lower panel* is a Western blot showing exogenous HNF4α amounts at the time of harvest (20 h post-transfection) using an anti-FLAG antibody.

HNF4/C fragment were cloned into a eukaryotic expression vector and transfected into baby hamster kidney (BHK) cells. *In vivo* phosphorylation labeling was carried out with these transfected cells in the presence or absence of AICAR. AICAR is converted in cells to the AMP analog ZMP (5′-aminoimidazole-4-carboxamide-1-*E*-D-ribofuranotide), which activates the kinase (38). The wild-type HNF4/C fragment was phosphorylated during the labeling reaction, and the treatment of the cells with AICAR caused an ~2-fold increase of phosphorylation (Fig. 2*C*). This phosphorylation was completely absent when the S304A version of the fragment was used, indicating that Ser-304 is phosphorylated *in vivo* by AMPK. *In vivo* phosphorylation experiments using the full-length HNF4α protein showed the same degree of phosphorylation for the wild-type and Ser-304 mutant protein (data not shown). This may be due to a combination of high basal phosphorylation of HNF4α by multiple kinases *in vivo* (36, 37) and the likelihood that there are additional AMPK phosphorylation sites on HNF4 (Fig. 1*D*).

Functional Effects of HNF4α Phosphorylation—To explore potential functional effects of phosphorylation of HNF4α at serine 304, an aspartic acid residue was introduced at this site by *in vitro* mutagenesis to generate a charge mimic of the phosphoserine residue. The effect of phosphorylation at Ser-304 on the ability of HNF4α to form functional homodimers and bind to DNA was examined in a gel mobility shift assay with wild-type and the phosphomimetic S304D versions of HNF4α proteins synthesized by *in vitro* translation. Both the wild-type and S304A version of HNF4α bound to an HNF4α-response element derived from the human apoCIII promoter (Fig. 3*A, 2nd* and *3rd lanes*). However, the S304D mutant HNF4α showed a dramatically reduced ability to bind to DNA despite similar amounts of protein present in the reaction (Fig. 3*A, 4th lane*). Gel mobility shift experiments using HNF4α protein that was phosphorylated by AMPK *in vitro* showed only a mild reduction in DNA binding activity (data not shown). The effect of phosphorylation on the ability of HNF4α to bind DNA would be less pronounced than the S304D mutation if less than 100% of the HNF4α protein is phosphorylated in the *in vitro* kinase reaction. Although the efficiency of these reactions is unknown, it is likely that only a fraction of the HNF4α molecules become

phosphorylated at Ser-304 leaving the non-phosphorylated protein free to form dimers and bind DNA. In addition, it is also possible that dimers composed of one phosphorylated and one non-phosphorylated HNF4α molecule are competent to bind DNA.

Because HNF4α binds DNA as a homodimer, the defective DNA binding of the S304D mutant could be due either to reduced dimer formation or to a diminished affinity of mutant dimers for DNA. To examine directly the effect of the mutant on dimerization, in the absence of DNA, an *in vitro* association assay using ^{35}S-labeled HNF4α monomers of different sizes was carried out. A fusion protein of GST and wild-type HNF4α (protein size 78 kDa) was co-translated with either wild-type or mutant full-length HNF4α protein (52 kDa). The GST-HNF4α fusion protein was purified out of the reaction with a glutathione resin together with dimerized 52-kDa wild-type or mutant HNF4α. The results (Fig. 3*B*) demonstrated that wild-type and S304A HNF4α readily formed dimers with the wild-type GST-HNF4α protein (*arrow, lanes 1* and *2*). In contrast, the S304D protein showed a reduced ability to interact with the wild-type GST-HNF4α (compare *lane 3* to *lanes 1* and *2*). Densitometry analysis of the Western blot demonstrated that the lower input amount of S304D protein (*lane 6*) did not account for the reduction in the amount of S304D bound to wild-type GST-HNF4α protein (data not shown).

To examine further the ability of dimers composed of one unphosphorylated monomer and one phosphorylated monomer to form and bind to DNA, wild-type and S304D mutant HNF4α proteins of different sizes were mixed together and the resulting dimers analyzed by gel mobility shift. The results (Fig. 3*C*) demonstrate that dimers composed of one wild-type GFP-HNF4α molecule (81 kDa) and one S304D molecule (52 kDa) can form and are competent to bind DNA (*lane 6*) but with less efficiency than dimers of wild-type GFP-HNF4α and either wild-type or S304A HNF4α proteins (Fig. 3*C*, compare *lane 6* to *lanes 3* and *9*). As was observed in Fig. 3*A*, homodimers of S304D HNF4α failed to form (Fig. 3*C*, compare *lane 4* to *lanes 1* and *7*). Taken together, these results demonstrate that, *in vitro*, a negative charge on serine 304 inhibits dimer formation and that the effect is more pronounced when both monomers contain the additional negative charge.

To examine the effects of Ser-304 phosphorylation on dimer formation and DNA binding *in vivo*, wild-type, S304A, and S304D expression constructs were transfected into CHO cells (which have very low amounts of endogenous HNF4). After transfection, AMPK was activated by AICAR treatment, and nuclear extracts were prepared for gel mobility shift assays. As observed *in vitro* the S304D phosphomimetic mutant showed reduced DNA binding activity (Fig. 3*D*, compare *lane 8* to *lane 4*). In addition there is a mild reduction in DNA binding of the wild-type protein upon AICAR treatment (Fig. 3*D*, compare *lanes 4* and *5*) that did not occur in the phosphorylation-defective S304A protein (Fig. 3*D*, compare *lanes 6* and *7*). However, much of this reduction in DNA binding activity correlates with changes in the amount of HNF4α present in the various transfections (Fig. 3*D, lower panel*). For example, the amount of S304D protein is significantly lower than the wild-type protein (Fig. 3*D*, compare *lane 8* to *lane 4, lower panel*). Likewise, the amount of wild-type HNF4α is reduced after AMPK activation (Fig. 3*D*, compare *lanes 4* and *5, lower panel*). The reduction of HNF4α amounts after AICAR treatment did not occur with the phosphorylation-defective S304A mutation (Fig. 3*D*, compare *lanes 6* and *7, lower panel*) suggesting that the effect is due to phosphorylation at serine 304. These changes in HNF4α protein amounts suggest that addition of a negative charge to serine 304 reduces the stability of the HNF4α protein in these cells.

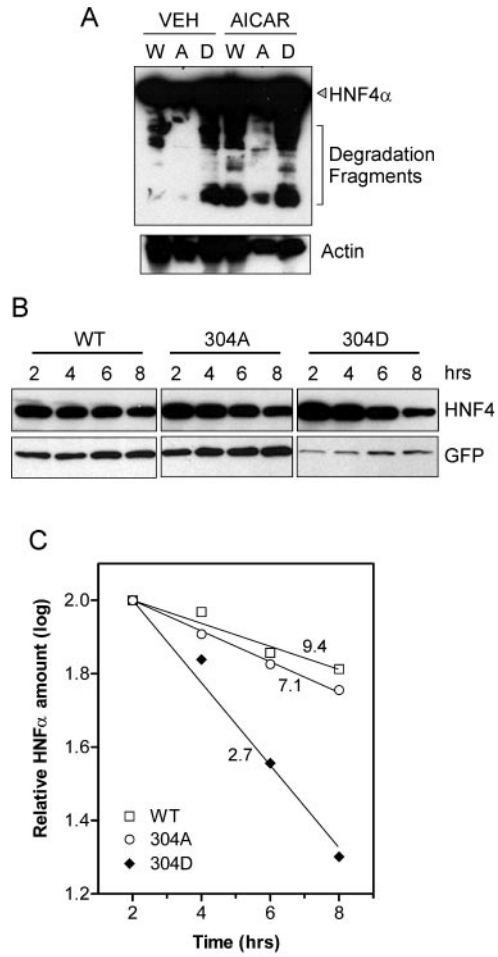

FIG. 4. **Phosphorylation of HNF4α on Ser-304 reduces protein stability.** *A*, Western blot showing the effect of AMPK activation on the appearance of proteolytic degradation fragments of HNF4α from CHO cells transiently transfected with the indicated pH4c series construction (*W*, wild type; *A*, S304A; *D*, S304D). These construct(ion)s produce C-terminal FLAG-tagged HNF4α. AICAR (500 μM) was present as indicated during the last 24 h of the transfection, with a second addition 2 h before the cells were harvested. *Upper panel*, anti-FLAG antibody, showing full-length HNF4α and smaller molecular weight fragments. *Lower panel* is the same membrane reprobed with anti-β-actin antibodies as loading control. *B*, Western blot showing the effect of S304 mutations on the rate of HNF4α protein degradation. CHO cells transiently transfected with pH4i plasmids (pH4i-WT, pH4i-304A, and pH4i-304D) producing N-terminal FLAG-tagged HNF4α protein under the control of a tetracycline-regulated promoter. Cells were treated with doxycycline 18 h post-transfection to shut off expression of HNF4α protein and harvested at the indicated times thereafter. Western analysis was performed using an anti-FLAG antibody to detect HNF4α and reprobed with anti-GFP antibody to detect the amount of EGFP protein produced from a co-transfected pEGFP-N1 reference plasmid. *C*, results from a densitometric analysis of the blots in *B*. The HNF4α:GFP ratio was calculated for each lane, and the data for each construction was normalized to the 2-h time point that was set to 100. The data are plotted as the log values of the relative HNF4α protein amount. The calculated half-life values from regression lines are shown in hours.

Potential effects of AMPK activation on HNF4α protein stability were further explored by examining the appearance of proteolytic products of wild-type and mutant HNF4α proteins in transiently transfected CHO cells treated with AICAR. Western blot analysis shows that in the absence of AICAR detectable amounts of degradation fragments appear in only lysates from cells transfected with the phosphomimetic S304D HNF4α (Fig. 4*A*). The activation of AMPK by AICAR addition

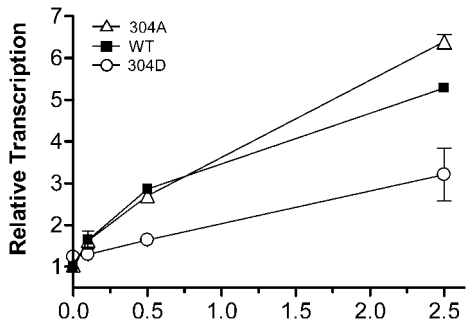

FIG. 5. **Phosphomimetic S304D mutation reduces transcriptional activity of HNF4α.** CHO cells were transfected with an HNF4α-responsive luciferase reporter and increasing amounts (in micrograms) of either wild-type, S304A, or S304D HNF4α producing plasmids as indicated. Relative transcription was calculated as the ratio of luciferase to β-galactosidase activity (from a cotransfected reference plasmid) normalized to the activity of the vector control. Values are the means of three replicates, and *error bars* represent standard deviation.

induced the appearance of degradation fragments from the wild-type but not the phosphorylation-defective S304A protein (Fig. 4*A*). These findings indicate that phosphorylation of HNF4α on serine 304 increased the proteolytic degradation of the protein. In addition, these results strongly support the conclusion that AMPK phosphorylates HNF4α on serine 304 *in vivo*. To measure the rate of degradation of wild-type and mutant HNF4α proteins, a tetracycline-regulated expression system was used to transiently express wild-type and 304 mutant HNF4α in transfected CHO cells. 18 h post-transfection, HNF4α expression was specifically extinguished by the addition of doxycycline, and the amount of HNF4α protein remaining in the cells was determined at regular intervals over the following 8 h. The results (Fig. 4, *B* and *C*) demonstrate that the phosphomimetic S304D mutant protein was degraded more rapidly then either the wild-type or S304A proteins. Taken together, the *in vitro* and *in vivo* results suggest that phosphorylation of HNF4α at Ser-304 has two effects on HNF4: to reduce its ability to form functional dimers, and to reduce the stability of HNF4α *in vivo*.

The HNF4α dimerization and DNA binding results presented above predict that the AMPK-mediated phosphorylation of HNF4α on serine 304 would have a negative effect on its transcriptional activity. To determine whether this is the case, plasmids expressing wild-type and Ser-304 mutant proteins were transfected into CHO cells together with an HNF4α-responsive reporter plasmid. As predicted from the DNA binding studies, the S304D mutant showed significantly less transcriptional activity than the wild-type HNF4α protein (Fig. 5). The S304A mutant was slightly more active than the wild-type protein suggesting the possibility that there is a basal level of phosphorylation that reduces the activity of the wild-type protein. These results indicate AMPK-negative regulates HNF4α activity by phosphorylation of serine 304 and suggest that AMPK activity could influence the expression of HNF4α target genes.

DISCUSSION

Here we report that the transcription factor HNF4α is a novel substrate of AMP-activated protein kinase. Given the central role of AMPK in mediating the response to changes in cellular energy balance and the fact that HNF4α regulates genes involved in energy metabolism, these findings suggest a mechanism for how the expression of a specific set of metabolic genes might be linked to cellular energy status. Although we have not directly demonstrated that AMPK regulates HNF4α-mediated gene expression *in vivo*, a previous study reported that activation of AMPK activity in primary liver cells correlated with reduced expression of HNF4α target genes (33). Our findings offer a potential mechanistic explanation for the relationship between HNF4α target gene down-regulation and AMPK activation. The kinase phosphorylates HNF4α on serine 304 (Figs. 1 and 2), which causes a reduction in the ability of HNF4α to form dimers and bind DNA (Fig. 3). *In vivo*, addition of a negative charge to this residue, by substitution with an aspartic acid residue, reduced the ability of HNF4α to activate transcription from a cotransfected HNF4α-dependent reporter (Fig. 5).

In addition to the effects on HNF4α dimerization and DNA binding, phosphorylation of HNF4α on Ser-304, or conversion of Ser-304 to an aspartic acid, also caused an increase in the degradation rate and a reduction in the amount of HNF4α protein (Fig. 3*D* and Fig. 4). Our results are consistent with previous findings showing that activation of AMPK in primary hepatocytes resulted in a reduction in the amount of endogenous HNF4α (33). Taken together, our findings suggest a model in which one of the branches of the AMPK signaling pathway results in the phosphorylation of HNF4α on serine 304, leading to both a reduction in the amount and activity of HNF4α and a subsequent reduction in the expression of HNF4α target genes.

Although the work reported here is focused on the phosphorylation of the serine 304 residue in HNF4α, it is clear that this is not the only site on HNF4 that is phosphorylated by AMPK. The HNF4/A fragment from the N-terminal half of the protein was strongly phosphorylated *in vitro* on a site that we were not able to map. This site (or sites) may be responsible for the remaining phosphorylation of the full-length protein that occurs when the Ser-304 residue is mutated to alanine (Fig. 2*B*). We are currently mapping the AMPK site in the N terminus of HNF4α so that we can determine their functional significance. In addition to the regulation of HNF4α activity that we describe here, it has been reported previously that HNF4α DNA binding activity is repressed by protein kinase A-mediated phosphorylation of residues in the DNA binding domain (37). It has also been reported that phosphorylation of HNF4α on tyrosine residues affected its transcriptional activity and subnuclear localization (39). These findings, together with those reported here, suggest that that HNF4α is a highly regulated transcription factor and that multiple signaling pathways influence its activity.

An interesting question concerning the phosphorylation of HNF4α by AMPK *in vivo* is the cellular location in which it occurs. HNF4α is strongly associated with the nucleus, whereas most of the known AMPK substrates are cytoplasmic enzymes. Although it is possible that HNF4α is also phosphorylated by in the cytoplasm, the most appealing compartment for the interaction would be the nucleus. Several observations support this possibility. First, specific isoforms of the kinase have been reported to be present in the nucleus (31, 40). Second, other transcriptional components have been reported to be potential substrates of the kinase; the co-activator p300 (32) and the carbohydrate-response transcription factor ChREBP (41). Finally, the yeast homolog of mammalian AMPK, SNF-1, is present in the nucleus (for review see Ref. 13) and is associated with chromatin, possibly in a gene-specific pattern (42). Together these observations suggest the intriguing possibility that at least some version of mammalian AMPK may be a component of the transcriptional complex on specific genes that are regulated by cellular energy balance.

What would be the physiological outcome of reduced HNF4α target gene expression? The answer to this question depends on the tissue being considered. In the endocrine pancreas, reduction of HNF4α activity would be predicted to have significant effect on the ability of the islet to secrete the appropriate

amount of insulin. This possibility is illustrated by the phenotype of MODY1 patients who have only one normal allele of the *HNF4α* gene and presumably a reduced amount of active HNF4α protein (3). These patients have a defective insulin secretion in response that appears to be a primary effect of reduced HNF4α levels in pancreatic β-cells (4, 43). Consistent with the apparent role of HNF4α in pancreatic physiology are the observations that HNF4α regulates a variety of β-cell genes involved in glucose sensing and insulin secretion (6–8).

Like HNF4α, AMPK also clearly plays a role in the physiology of the endocrine pancreas. It is likely that the β-cell senses glucose concentration as fluctuations in the amount of intracellular ATP generated by glucose oxidation. The insulin secretory machinery gauges the amount of insulin to secrete by sensing these ATP level fluctuations (for review see Ref. 44). It is possible that the changes in cellular energy balance in β-cells that occur in normal physiology have an effect on HNF4α phosphorylation and activity and that fluctuation in HNF4α activity are part of the normal physiological regulation of the islet. Consistent with this possibility is the observation that low glucose levels induce AMPK activity in cell lines derived from pancreatic β-cells and the suggestion that changes in kinase activity are involved in insulin secretion (25). Alternatively, it is possible that AMPK-mediated changes in HNF4α activity are relevant in pathological conditions where AMPK is aberrantly activated and HNF4α activity is suppressed below normal levels. This situation could potentially result in a physiological circumstance analogous to the MODY1 phenotype.

Another tissue where AMPK-mediated changes in HNF4α target gene expression could have a significant metabolic effect is the liver. It has been understood for many years that HNF4α plays an important role in the liver, regulating genes involved in lipid (45), amino acid (46, 47), and glucose metabolism (48, 49) and the coagulation cascade (50). It has been reported recently that HNF4α plays a key role in the induction of hepatic gluconeogenesis by mediating the transcriptional effects of the co-activator PGC1 (10). On the other hand, the reduction of HNF4α levels due to haploinsufficiency in MODY1 patients did not cause major liver abnormalities (5, 51), although changes in amounts of some liver-derived serum proteins were observed (52).

It is clear that an AMPK-mediated phosphorylation of HNF4α could potentially have important effects on liver and pancreatic islet physiology. We are currently examining HNF4α phosphorylation in hepatic and pancreatic cell lines to determine whether AMPK-dependent phosphorylation of Ser-304 occurs under more physiological conditions and to ascertain the potential metabolic role of the phosphorylation events described here.

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AMP-activated Protein Kinase Regulates HNF4α Transcriptional Activity by Inhibiting Dimer Formation and Decreasing Protein Stability

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