Hepatocyte Nuclear Factor-4 Is a Novel Downstream Target of Insulin via FKHR as a Signal-regulated Transcriptional Inhibitor*

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Experimental Procedures

Previous studies have shown that FKHR, a member of the forkhead family of transcription factors, acts as a DNA binding-independent cofactor of nuclear receptors, including estrogen, retinoid, and thyroid hormone receptors, in addition to the original function as a DNA binding transcription factor that redistributes from the nucleus to the cytoplasm by insulin-induced phosphorylation. Here, we demonstrated the physical interaction of FKHR with hepatocyte nuclear factor (HNF)-4, a member of steroid/thyroid nuclear receptor superfamily, and the repression of HNF-4 transactivation by FKHR. FKHR interacted with the DNA binding domain of HNF-4 and inhibited HNF-4 binding to the cognate DNA. Furthermore, the binding affinity of HNF-4 with phosphorylated FKHR significantly decreased in comparison to that with unphosphorylated FKHR. Therefore, a phosphorylation of FKHR by insulin followed by its dissociation from HNF-4 and the redistribution of FKHR from the nucleus to the cytoplasm would expect to induce the transactivation of HNF-4 by facilitating to the access of HNF-4 to its DNA element. Indeed, most intriguingly, insulin stimulation reversed the repression of HNF-4 transcriptional activity by phosphorylation-sensitive (wild-type) FKHR, but not by phosphorylation-deficient FKHR. These results suggest that insulin regulates the transactivation activity of HNF-4 via FKHR as a signal-regulated transcriptional inhibitor.

HNF-4, a member of the steroid/thyroid nuclear receptor superfamily, is a transcriptional factor which expresses in the liver, intestine, kidney, and pancreatic β-cells (1, 2). It contains several functional domains: a ligand-independent activation domain (AF1), a zinc finger DNA binding domain, and a ligand-dependent activation domain (AF2) (3). HNF-4 binds to a specific DNA element as a homodimer and regulates the expression of many genes, involved in glucose, fatty acid, and cholesterol metabolisms (4–6). The blood glucose levels are controlled by the balance of two opposing hormones, glucagon and insulin. Whereas glucagon decreases the activity of HNF-4, the effect of insulin on that of HNF-4 has not fully been understood yet (7–9).

FKHR, a forkhead family member, is a transcriptional factor and regulates the expression of multiple genes, such as key enzymes of gluconeogenesis (10, 11). Insulin has a dynamic effect on the localization of FKHR, when phosphorylated by Akt at the three residues of FKHR: Thr-24, Ser-253, and Ser-316. Once phosphorylated, the cytoplasmic retention is induced, leading to inhibit the transactivation activity. On the other hand, in the absence of insulin, FKHR is dephosphorylated and localized to the nucleus, where FKHR binds to the specific DNA element, resulting in transactivation of the target genes (12–15).

Recently, it has been reported that FKHR activates or represses the transactivation by nuclear receptor family members as a DNA binding-independent cofactor (16, 17). In the present study, we analyzed the interaction of FKHR with HNF-4, the effect of FKHR on the transactivation mediated by HNF-4, and its molecular mechanism. FKHR associates with HNF-4 in vitro and in vivo and represses the transactivation by HNF-4 through the decrease in its DNA binding affinity. Interestingly, the inhibitory effect is canceled by insulin, resulting from the dissociation of HNF-4 from phosphorylated FKHR that subsequently translocates to the cytoplasm. This suggests the possibility that HNF-4 is a novel downstream target of insulin via FKHR as a signal-regulated transcriptional inhibitor.

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‡ The abbreviations used are: HNF, hepatocyte nuclear factor; HA, hemagglutinin; GST, glutathione S-transferase; DTT, dithiothreitol; CA, constitutive active; DN, dominant negative; EMSA, electrophoretic mobility shift assay; DMEM, Dulbecco’s modified Eagle’s medium; PI3K, phosphatidylinositol 3-kinase; AF2, activation function 2.

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Cell Culture and Transfections and Reporter Gene Assays—HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transfections were performed by FuGENE-6 (Roche Molecular Biochemicals). Twenty ng of pCMV-β-gal plasmid were included in each transfection experiment to control for the efficiency of transfection. To ensure equal DNA amounts, empty plasmids were added in each transfection. The luciferase activity was measured with an ARVO™SX (Wallac Berthold). The values were normalized to β-galactosidase activity as an internal control.

Plasmids—The hHNF-4 a2 cDNA was subcloned into pcDNA3 tagged with the HA epitope (pcDNA3HA). A series of HNF-4 deletion fragments were generated by PCR and subcloned into pGEX 4T-1 (Amersham Biosciences) or pcDNA3HA. The pGAL4-HNF-4 vector was made by subcloning hHNF-4 a2 cDNA tagged with the Gal4 DNA binding domain (amino acids 1–147) of pGEB9 (Clontech) at the N terminus into pcDNA3. pGAL4 control vector, pHNF4-tk-Luc, pG5b-Luc, pcDNA3-FLAG-FKHR-wild-type, and 3A mutant were described previously (18–20). A new versatile PCR strategy was used to generate pcDNA3-FLAG-FKHR-3D mutant and pcDNA3-FLAG-FKHR-525D mutant (21).
FIG. 1. In vivo and in vitro binding of HNF-4 to FKHR. A, in vitro binding assay, using 293T whole cell extracts that expressed HA-tagged full-length HNF-4 or an empty control plasmid, with GST protein alone or GST FKHR fusion protein. Bound proteins were analyzed by SDS-PAGE gel electrophoresis and subjected to immunoblotting with anti-HA antibody (12CA5, Roche Molecular Biochemicals). B, coimmunoprecipitation of endogenous HNF-4 using anti-FKHR antibody. Nuclear extracts from HepG2 cells were immunoprecipitated with anti-FKHR antibody or preimmune as a control. C, a schematic diagram of HNF-4 and its deletion mutants used in this study. Hatched area, activation function 1; DBD, DNA binding domain. D, in vitro interaction of GST-FKHR with HNF-4. Whole cell extracts from 293T cells transfected with HA tagged HNF-4 deletion mutants were incubated with GST protein alone or GST FKHR fusion protein. Bound proteins were analyzed as described in A. E, in vitro binding assay using in vitro translated 35S-labeled FKHR and GST or GST-HNF-4 deletion mutants. Bound proteins were analyzed by SDS-PAGE using the imaging analyzer.

FIG. 2. Mechanism of the repression of HNF-4 mediated transactivation by FKHR. A, repression of HNF-4 activity by FKHR. HepG2 cells were cotransfected with 100 ng of HNF4-tk-Luc reporter plasmid and 3 ng of HNF-4 expression plasmid together with 10 or 20 ng of the expression vectors for either FKHR wt or 3A mutant. The results are presented as arbitrary units (1 × 10^5). All values represent the mean of triple samples. B, effects of FKHR on the transactivation of GAL4-HNF-4. HepG2 cells were transfected with 100 ng of pG5b-Luc reporter plasmid and 3.3 ng of GAL4 or GAL4-HNF-4 expression plasmid together with 10 or 20 ng of FKHR expression plasmid. The results are presented as arbitrary units (1 × 10^5). All values represent the mean of triple samples. C, inhibition of HNF-4 binding to its cognate DNA by FKHR. In vitro translated HNF-4 was incubated with the 32P-labeled probe in the presence or absence of bacterially expressed GST-FKHR.
GST Pull-down Assay—GST-FKHR and GST-HNF-4 full-length and deletion mutant fusion proteins were prepared as described previously (19). [35S]Methionine-labeled FKHR was prepared by in vitro translation with the TNT-coupled reticulocyte lysate system (Promega) with T7 RNA polymerase. 293T whole cell extracts that transiently expressed full-length HNF-4 or its deletion mutants were prepared with 1x lysis buffer (0.1% Tween 20, 200 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 20 mM HEPES, pH 7.9, 20 mM DTT). For GST pull-down assay, [35S]methionine-labeled FKHR or 200 µg of 293T whole cell extracts were incubated with about 20 µg of GST fusion protein bound to glutathione-Sepharose beads at 4 °C for 4 h in 1 ml of the HEPES binding buffer (20 mM HEPES, pH 7.9, 200–300 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% Tween 20, 5% glycerol, protease inhibitors). After washing the beads with HEPES binding buffer, the pull-down complexes were fractionated by SDS-PAGE. The gels were performed immunoblotting with rat anti-HA monoclonal antibody or analyzed using the imaging analyzer.

Immunoprecipitation—Approximately 200 µg of HepG2 nuclear extracts were immunoprecipitated with anti-FKHR antibody in IP buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 1 mM DTT, 5% glycerol, protease inhibitors). Immunoblotting of immunoprecipitates was done using anti-FKHR antibody or anti-HNF-4 antibody as described previously (19).

In Vitro Kinase Assay—293T cells were seeded and transfected with 2 µg of the constitutive active (CA) or dominant negative (DN) form Myc-Akt expression vectors (Upstate Biotechnology). Cell extracts were immunoprecipitated using anti-Myc antibody (PL14, Medical Biological Laboratory). The kinase reaction was performed using CA or DN form Myc-Akt, 2 µg of purified GST fusion protein as a substrate, 5 µCi of [γ-32P]ATP, and 20 µM ATP in a kinase reaction buffer (20 mM HEPES, pH 7.9, 20 mM β-glycerophosphate, 10 mM MgCl2, 1 mM DTT, 50 µM Na3VO4) for 1 h at 30 °C. The reaction products were resolved by SDS-PAGE and analyzed with a bio-imaging analyzer.

In vitro kinase assay for GST pull-down assay was performed with [35S]Methionine-labeled FKHR wt and 3A mutant, 0.7 mM ATP, and activated Akt (Upstate Biotechnology) in a kinase reaction buffer. After each reaction mixture was incubated for 2 h at 30 °C, GST pull-down
In vitro translated HNF-4 was synthesized in the TNT-coupled reticulocyte lysate system (Promega) with T7 RNA polymerase. GST-FKHR or GST was expressed in Escherichia coli strain BL21, purified using glutathione-Sepharose beads, eluted with an elution buffer (50 mM Tris-HCl, pH 8.0, 20 mM reduced glutathione), dialyzed to reaction buffer (22), and concentrated using Aquacide II (Calbiochem). The C3P double-stranded oligonucleotides were prepared as described previously (22). Four microliters of sequentially serum-free DMEM for 4 h and then stimulated with insulin medium (DMEM) contained 10% fetal bovine serum for 24 h and subsequently added end-labeled oligonucleotide (0.14 ng, 11,000 cpm) and incubated at 4 °C for 30 min. The binding reaction was carried out as described previously (22). The reaction mixtures were directly loaded onto 5% non-denaturing polyacrylamide gels made in 1×TBE (90 mM Tris-HCl, pH 8.0, 89 mM boric acid, 2 mM EDTA). After electrophoresis was performed at 130 V for 3 h at 4 °C, the gels were dried and analyzed with a bio-imaging analyzer.

Immunofluorescence—HepG2 cells were plated onto glass coverslips at 20% confluence and transfected using FuGENE-6 regents (Roche Molecular Biochemicals). Cells were incubated in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum for 24 h and sequentially serum-free DMEM for 4 h and then stimulated with insulin (100 ng) for 45 min. Cells were fixed and permeabilized as described previously (18). After blocking with 1% bovine serum albumin, 0.1% Triton X-100 in phosphate-buffered saline for 30 min, cells were incubated with rat anti-HA monoclonal antibody (1:250 dilution; 3F10, Roche Molecular Biochemicals) and anti-FLAG monoclonal antibody (1:500 dilution; M2) and then stained with Cy3-conjugated anti-rat and Cy3-conjugated anti-mouse secondary antibodies (1:1000 dilution each; Amersham Biosciences).

Antibodies—Anti-HNF-4 rabbit polyclonal antibody was generated against a GST-HNF-4(133–366). Anti-FKHR rabbit polyclonal antibody was generated against a GST-FKHR-(541–632).

RESULTS

In Vivo and in Vitro Binding of HNF-4 to FKHR—GST pull-down assay was conducted to test whether FKHR could interact with HNF-4. 293T whole cell extracts, transiently expressed HA-HNF-4, were incubated with GST or GST-FKHR fusion protein. As seen in Fig. 1A, HA-HNF-4 associated with GST-FKHR but failed to bind to GST protein. Furthermore, communoprecipitation assay was carried out to confirm the interaction between HNF-4 and FKHR in HepG2. FKHR was immunoprecipitated with anti-FKHR antibody from HepG2 cells, and the immune complexes were subsequently resolved by SDS-PAGE, followed by Western blotting with antibodies against FKHR or HNF-4. The FKHR-precipitated complexes included HNF-4 (Fig. 1B), demonstrating the in vivo interaction between FKHR and HNF-4.

To determine the domain of HNF-4 involved in the interaction with FKHR, each of the HA-tagged HNF-4 N-, M-, and C-fragments expressed in 293T was incubated with GST or GST-FKHR fusion protein (Fig. 1C). FKHR associated with the N-region but neither with the M- nor C-region of HNF-4 (Fig. 1D). The N-terminal fragment contains several functional domains, such as AF1, a DNA binding domain, and a part of AF2. To define which domains of HNF-4 could associate with FKHR, it was expressed using in vitro transcription/translation and tested for the interaction with GST-HNF-4 subregions using GST pull-down assay. As shown in Fig. 1E, FKHR interacted with the NM fragment of HNF-4 containing the DNA binding domain.

Mechanism of the Repression of HNF-4-mediated Transactivation by FKHR—To investigate the functional significance of this association, a luciferase reporter assay using the eight copies of the HNF-4 binding element was conducted. HNF-4 enhanced promoter activity, but FKHR did not alter it (Fig. 2A). When FKHR wt was cotransfected along with HNF-4, luciferase activity was strongly decreased in a dose-dependent manner. The FKHR 3A mutant, in which three putative Akt phosphorylation sites (Thr-24, Ser-253, and Ser-316) were replaced by a nonphosphorylatable alanine residue, resulting in the predominant localization in the nucleus, repressed the reporter activity more dramatically than FKHR wt. These data prompted us to propose the hypothesis that FKHR may inhibit the binding of HNF-4 to the cognate DNA and repress the transactivation. To determine whether FKHR could inhibit the transactivation by an HNF-4 tethered to DNA by a heterologous DNA binding domain, the luciferase reporter assay using Gal4-HNF-4 fusion was conducted. The transactivation by Gal4-HNF-4 fusion was not repressed by FKHR (Fig. 2B). EMSA indicated that GST-FKHR prevented HNF-4 from binding to its recognition motif in a dose-dependent manner, whereas a control GST protein did not (Fig. 2C). These data suggested that the transcriptional reduction of HNF-4 results from the inhibitory effect of FKHR on HNF-4 binding to target DNA elements.

Effects of Insulin on the Repression of HNF-4 Transactivation by FKHR—We tested a possible role of insulin in the repression of HNF-4-mediated transactivation activity by FKHR. Whereas insulin did not alter the HNF-4-mediated transactivation activity, insulin restored that activity, in the presence of cotransfected FKHR wt, to 80% of the HNF-4-mediated transactivation activity, but not FKHR 3A mutant (Fig. 3A). As a step toward understanding the molecular mechanism of this restoration, we first investigated the effect of Akt phosphorylation on HNF-4 because of being a putative Akt phosphorylation site (RXRXXS/T) in the NM region of HNF-4 (RDRIST: 125–130 amino acids). To determine whether HNF-4 is a substrate of Akt in vitro, GST-full-length HNF-4 or GST-HNF4 NM region was bacterially expressed for this assay. The CA, but not DN, form of Akt immunoprecipitated from 293T cells effectively phosphorylated GST FKHR as a positive control. However, CA-Akt failed to phosphorylate GST-full-length HNF-4 or GST-HNF4 NM region in an in vitro kinase assay (Fig. 3B), suggesting that insulin stimulation via the PI3K/Akt pathway induced the phosphorylation of FKHR, but not that of HNF-4. We next investigated the subcellular localization of FKHR in the presence of HNF-4. We coexpressed HNF-4 and FKHR, respectively, in HepG2 cells in the absence or presence of insulin. Whereas HNF-4 was exclusively in the nucleus when exposed to insulin, FKHR was found to be predominantly in the cytoplasm (Fig. 3C).

To examine whether the FKHR phosphorylation by Akt affects its binding affinity with HNF-4, we performed the GST pull-down assay using FKHR mutants (3A, 3D, and S253D). HNF-4 bound to unphosphorylated FKHR wt with the same affinity as the FKHR 3A mutant (Fig. 3D, left). However, the binding affinity of FKHR wt phosphorylated by Akt with HNF-4 significantly decreased in comparison to that of FKHR 3A mutant (Fig. 3D, right). Next, we tested FKHR mutants, in which Thr-24/Ser-253/Ser-253 was replaced by an aspartic acid residue, to mimic the effect of phosphorylation. The FKHR mutants (3D and S253D) interacted with HNF-4 weaker than FKHR wt (Fig. 3, E and F). Taken together, these findings suggest that insulin stimulation promotes the dissociation of HNF-4 from FKHR, resulting in its subsequent retention into the cytoplasm, and reverses the inhibitory effects of FKHR on the transactivation by HNF-4 through facilitating the access to the target elements.

DISCUSSION

FKHR binds to specific DNA elements and activates its transcription in the nucleus. Once cells are exposed to extracellular signals, such as insulin and insulin-like growth factor 1 (IGF1), PI3K-regulated kinase Akt phosphorylates FKHR, thereby in-
ducng the exit of FKHR from the nucleus and the repression of FKHR-dependent transcription (12–15, 23–26). It has been shown that FKHR is able to function as a DNA binding-independent transcriptional cofactor of nuclear receptors such as estrogen, retinoid, and thyroid hormone receptors in addition to the original function as a DNA binding transcription factor (16, 17). In this report, FKHR was identified as a cofactor for HNF-4. Our data showed that FKHR associated with the DNA binding domain of HNF-4 and inhibited the access of HNF-4 to its binding element, leading to repress HNF-4 mediated transcription. Moreover, one striking finding is that insulin stimulation reversed the repression by FKHR wt, but not by FKHR 3A mutant, suggesting that the reverse regulation might result from insulin dependent shuttling of FKHR from the nucleus to the cytoplasm. It has been reported that the localization of FKHR is altered through a phosphorylation by various kinases, such as CK1, DYRA1A, and SGK in addition to Akt (23, 24, 27–29). These observations raise the possibility that HNF-4 may be a “downstream target” of diverse signals mediated by the above kinases via FKHR.

Recent reports have shown that the DNA binding activity of HNF-4 is modulated by diet in vivo. Namely, the HNF-4 DNA binding activity in nuclear extracts of fasted rat liver was markedly reduced in comparison with that of refed rat liver in EMSA, at least resulting from the phosphorylation of HNF-4 by PKA (9). We found that the HNF-4 DNA binding activity was decreased by the interaction of HNF-4 with FKHR. It is known that the physiological effects of fasting are mediated by the activation of CAMP-dependent PKA, coincident with the inactivation of Akt by the decrease in insulin secretion. Therefore, because of not only the phosphorylation of HNF-4 by PKA but also the reinforcement interaction of HNF-4 with FKHR resulting from the translocation of FKHR to the nucleus, the DNA binding activity of HNF-4 to the target DNA elements might be decreased in the fasted states.

The primary target tissues for insulin are muscle, adipose tissue, and liver. Tissue-specific knock-out of the insulin receptor in muscle failed to produce diabetes, but disruption of the gene in the liver exhibited a diabetic phenotype, suggesting that insulin actions in the liver play central roles in glucose homeostasis (30, 31). Insulin decreases transcription of the genes encoding gluconeogenic enzymes and increases transcription of those encoding glycolytic enzymes in the liver. A number of studies have established that FKHR activates the transcription of gluconeogenic enzymes in the absence of insulin and inhibits the transcription in the presence of insulin. On the other hand, HNF-4 positively regulates the genes involved in glucose transport and glycolysis (4, 6). Here, we provided a novel mechanism that insulin reversed the repression of HNF-4 transcriptional activity by FKHR. One might expect that in the fasted state, FKHR as an inhibitor of HNF-4 represses glycolysis, whereas FKHR as a DNA binding transcription factor enhanced gluconeogenesis, leading to glucose secretion. In contrast, in the fed state, because FKHR phosphorylated by insulin translocates into the cytoplasm following the dissociation from HNF-4, HNF-4 that is released from FKHR promotes the glucose transport and glycolysis, leading to glucose uptake. The identification of an insulin-regulated transcriptional inhibitor, FKHR, might assist in clarifying a molecular mechanism of glucose homeostasis.

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