Insulin Stimulates Phosphorylation of the Forkhead Transcription Factor FKHR on Serine 253 through a Wortmannin-sensitive Pathway*

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In the nematode Caenorhabditis elegans, mutations of the insulin/insulin-like growth factor-1 receptor homologue Daf-2 gene cause developmental arrest at the dauer stage. The effect of Daf-2 mutations is counteracted by mutations in the Daf-16 gene, suggesting that Daf-16 is required for signaling by Daf-2. Daf-16 encodes a forkhead transcription factor. Based on sequence similarity, the FKHR genes are the likeliest mammalian Daf-16 homologues. FKHR proteins contain potential sites for phosphorylation by the serine/threonine kinase Akt. Because Akt is phosphorylated in response to insulin and has been implicated in a variety of insulin effects, we investigated whether insulin affects phosphorylation of FKHR. Insulin stimulated phosphorylation of endogenous FKHR and of a recombinant c-Myc/FKHR fusion protein transiently expressed in murine SV40-transformed hepatocytes. The effect of insulin was inhibited by wortmannin treatment, suggesting that PI 3-kinase is required for FKHR phosphorylation. Mutation of serine 253, located in a consensus Akt phosphorylation site at the carboxy-terminal end of the forkhead domain, abolished the effect of insulin on FKHR phosphorylation. In contrast, mutation of two additional Akt phosphorylation sites, at amino acids threonine 24 or serine 316, did not abolish insulin-induced phosphorylation. These data indicate that FKHR may represent a distal effector of insulin action.

Insulin promotes a wide range of metabolic and growth-promoting functions in multiple target cells (1). The diverse actions of insulin are mediated by its cell surface receptor. The insulin receptor is tyrosine-phosphorylated in response to insulin binding. Autophosphorylation activates the receptor as a kinase that is able to phosphorylate a host of intracellular protein substrates. The proximal effectors of insulin action include insulin receptor substrate proteins and several others (2). Insulin receptor substrate proteins serve an important function as “docking” molecules to promote the assembly of protein-protein complexes and the generation of intracellular signals. Among the distal mediators of insulin signaling, pathways based on the generation of 3-phosphoinositides through the lipid kinase activity of PI^3-kinase are thought to play a prominent role (3). For example, the serine/threonine kinase Akt and the atypical protein kinase C isoforms α and λ are among the kinases that are activated in a PI 3-kinase-dependent manner to regulate glucose transport, glycogen synthesis, cell survival, and gene expression in response to insulin (4–10).

The serine/threonine kinase Akt is an important target of PI 3-kinase. Recent studies showed that Akt is involved in insulin regulation of gene expression. It is not clear, however, whether transcription factors are direct targets for the kinase activity of Akt or whether Akt regulates gene expression by activating other kinases, such as mitogen-activated protein kinase, p70 S6 kinase, protein kinase C isoforms, and others (5–8).

Studies of the nematode Caenorhabditis elegans have provided insights into the conservation of signaling pathways relevant to mammalian metabolism and reproduction. Under optimal growth conditions, C. elegans grows rapidly to fertile adult hermaphrodites through four stages (L1–L4). However, when grown at high density or with high levels of pheromone, larvae enter a reversible arrest of development characterized by reduced metabolic activity, the dauer stage. Interestingly, mutations in the Daf-2, Age-1, Akt-1, and Akt-2 genes cause a constitutive dauer phenotype (11–15). The Daf-2 gene encodes the C. elegans homologue of the insulin/IGF-1 receptor (14); the Age-1 gene is the homologue of PI 3-kinase (13), and the two Akt genes are homologous to mammalian Akt (11). Thus, the dauer alleles define a signaling cascade homologous to the insulin and IGF-1 signaling cascade in mammals as important for metabolic regulation and reproduction in C. elegans. Interestingly, mutations of the Daf-16 gene in C. elegans prevent Daf-2, Age-1, and Akt mutants from entering the dauer stage. This observation suggests that Daf-16 is a negative regulator of the Daf-2/Age-1/Akt signaling pathway. Positional cloning experiments identified Daf-16 as a member of the HNF-3/forkhead family of transcription factors, with a unique forkhead domain of about 100 amino acids (15, 16). Interestingly, Daf-16 has four consensus Akt phosphorylation sites (17), raising the possibility that Daf-16 may be a direct substrate of Akt.

The closest mammalian homologues of Daf-16 are three members of the FKHR family: FKHR, FKHR-L1, and AFX (18). These genes were originally identified in chromosomal translocations associated with human rhabdomyosarcomas (hence the acronym forkhead in human rhabdomyosarcoma) as fusion proteins composed of the paired box and homeodomain regions of the transcription factors Pax-3 or Pax-7 and the transactivators.

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‡ The abbreviations used are: PI, phosphatidylinositol; PCR, polymerase chain reaction; IGF, insulin-like growth factor; DMEM, Dulbecco’s modified Eagle’s medium; WT, wild type.
FKHR Is the Principle Member of the FKHR Family in Murine SV40-transformed Hepatocytes—Insulin affects the liver-specific expression of several genes important for metabolic regulation. To test which member of the FKHR family was the likeliest candidate as an effector of insulin action in liver, we performed Northern analysis on mRNA from murine SV40-transformed hepatocytes (21). The results are shown in Fig. 1A, and indicate that FKHR is ~10-fold more abundant than FKHRL1. AFX mRNA was not detected in the same blot (not shown).

Cloning of Mouse FKHR cDNA—Using available partial sequence information (GenBank™ accession number AA254887), cDNA clones encoding the 3' end of murine FKHR were amplified from a Marathon cDNA library. The 5' end was obtained from mouse genomic DNA. Murine FKHR shares 92% identity at the amino acid level with human FKHR (data not shown). The forkhead DNA-binding domain is identical in human and mouse FKHR and is highly conserved among mouse FKHR and the other two members of the FKHR family, sharing 82 and 86% identity with human FKHRL1 and AFX cDNA, respectively (18). The open reading frame encodes a 652-amino acid polypeptide, with a predicted molecular mass of 69,497 kDa. Inspection of the amino acid sequence reveals three consensus phosphorylation sites for the Akt kinase (RXRXX[S/T]) around threonine 24 (PKRQSTCW), serine 253 (PRRAASM), and serine 316 (FRPRTSSN).

RESULTS

FKHR Is the Principle Member of the FKHR Family in Murine SV40-transformed Hepatocytes

Mouse FKHR cDNA—Using available partial sequence information (GenBank™ accession number AA254887), cDNA clones encoding the 3' end of murine FKHR were amplified from a Marathon cDNA library. The 5' end was obtained from mouse genomic DNA. Murine FKHR shares 92% identity at the amino acid level with human FKHR (data not shown). The forkhead DNA-binding domain is identical in human and mouse FKHR and is highly conserved among mouse FKHR and the other two members of the FKHR family, sharing 82 and 86% identity with human FKHRL1 and AFX cDNA, respectively (18). The open reading frame encodes a 652-amino acid polypeptide, with a predicted molecular mass of 69,497 kDa. Inspection of the amino acid sequence reveals three consensus phosphorylation sites for the Akt kinase (RXRXX[S/T]) around threonine 24 (PKRQSTCW), serine 253 (PRRAASM), and serine 316 (FRPRTSSN).
FKHR without affecting the amount of $^{32}$P in FKHR recombinant protein caused an increase in the basal phosphorylation of FKHR. Wortmannin (50 nM) abolished phosphorylation of FKHR in response to insulin (Fig. 2, lanes 2, WT FKHR; lanes 3 and 4, S316A mutant; lanes 5 and 6, S253A mutant; lanes 7 and 8, T24A mutant; lanes 9 and 10, untransfected cells). The upper panel shows the results of $^{32}$P labeling experiments; the lower panel shows the results of $^{35}$S labeling experiments.

**Fig. 2.** Phosphorylation of transfected mouse FKHR in response to insulin is inhibited by wortmannin. SV40-transformed mouse hepatocytes (WT cells) were transiently transfected with a plasmid encoding wild type mouse FKHR (pCMV5-e-Myc mFKHR WT). 48 h after transfection, cells were labeled with $^{32}$P orthophosphate as indicated in Fig. 1C (upper panel) or with $^{35}$S methionine (lower panel). Wortmannin (50 nM) or wortmannin (100 nM) was added 30 min prior to the addition of insulin. Insulin stimulation was carried out for 15 min. Thereafter, cells were lysed and immunoprecipitated with a monoclonal anti-c-Myc antibody as indicated under “Experimental Procedures.” The experiment was repeated three times with identical results.

In Vivo Phosphorylation of FKHR in Response to Insulin and IGF-1 in SV40-transformed Hepatocytes—To test the hypothesis that insulin stimulates FKHR phosphorylation, SV40-transformed hepatocytes from normal mice (WT) or from insulin receptor-deficient mice (-/-) were labeled with $^{32}$P orthophosphate, stimulated with insulin for 5–15 min, and FKHR was immunoprecipitated using an antibody against the human protein (20). In WT cells, insulin rapidly induced a 2-fold increase in $^{32}$P content of FKHR. This increase was associated with an electrophoretic shift of the band corresponding to FKHR on the gel. In -/- cells, which lack insulin receptors, phosphorylation of FKHR in response to insulin was slower and increased by about 50% over basal after 15 min of stimulation (Fig. 1B). The response observed in -/- cells is presumably due to IGF-1 receptors. As a control, phosphorylation of insulin receptors in response to insulin is shown in Fig. 1C. These data indicate that FKHR is phosphorylated in an insulin- and IGF-1 receptor-dependent manner in mouse hepatocytes. Based on the Northern blot shown in Fig. 1A, we conclude that FKHR is the main immunoreactive species recognized by the antibody.

Phosphorylation of FKHR in Response to Insulin Is Inhibited by Wortmannin—To investigate further whether phosphorylation of FKHR is mediated by a PI 3-kinase-dependent pathway, we performed phosphorylation experiments in the presence of the selective inhibitors wortmannin, which blocks the activity of PI 3-kinase (24, 25), and rapamycin, which blocks the activity of p70 S6 kinase (26). For these experiments, epitope-tagged FKHR was transiently transfected in WT cells using plasmid pCMV5-e-Myc. In 10 experiments, insulin increased $^{32}$P content of the recombinant protein ~2-fold (Fig. 2, lanes 1 and 2), consistent with the effect on the endogenous protein. The insulin-dependent increase in FKHR phosphorylation was abolished by treatment of cells with wortmannin (Fig. 2, lanes 5 and 6). In contrast, rapamycin caused an increase in the basal phosphorylation of FKHR without affecting the amount of $^{32}$P in FKHR recovered from insulin-treated cells (Fig. 2, lanes 3 and 4). Consistent with the $^{32}$P loading experiments, $^{35}$S labeling experiments revealed that wortmannin, but not rapamycin, inhibited the insulin-induced shift in molecular mass of FKHR (Fig. 2, lower panel). These data indicate that FKHR is phosphorylated by insulin receptors through a PI 3-kinase-dependent pathway.

Identification of the Site of Insulin-dependent Phosphorylation—To investigate which one of the consensus Akt phosphorylation sites is phosphorylated in vivo by insulin, WT hepatocytes were transfected with expression vectors for wild type and mutant FKHR (pCMV5-e-Myc WT, T24A, S253A, and S316A). Mutation of Ser253 completely abolished the effect of insulin on FKHR phosphorylation (Fig. 3, lanes 5 and 6), whereas mutations of T24A and S316A decreased insulin-induced FKHR phosphorylation by ~30% but did not abolish the effect of insulin (Fig. 3, lanes 3, 4, 7, and 8). However, all three mutants inhibited the mobility shift induced by insulin stimulation of WT FKHR.

**DISCUSSION**

The present studies support the identification of FKHR as a target of insulin-stimulated, PI-dependent kinases (possibly Akt) and as a distal effector of the insulin receptor signaling cascade. Site-directed mutagenesis data are consistent with a model in which Ser253 is required for the effect of insulin on FKHR phosphorylation, whereas Thr24 and Ser316 are not. The slight decrease of insulin-induced phosphorylation in the T24A and S316A mutants, in addition to the inhibition of the insulin-induced mobility shift, indicate that these sites may be phosphorylated in vivo but that they are not necessary for the effect of insulin. Thus, Ser253 appears to act as a gatekeeper site for insulin-induced FKHR phosphorylation in SV40-transformed hepatocytes. Recently, it has been reported that phosphorylation of the related molecule FKHRL1 in response to IGF-1 in 293 and Jurkat cells occurs at three Akt sites: Thr24, Ser253, and Ser316, corresponding to Thr24, Ser253, and Ser316 of FKHR (27). However, the S253A mutant FKHRL1 still retained the ability to be phosphorylated in response to IGF-1, unlike the corresponding mutant of FKHR, which lost its ability to be phosphorylated in response to insulin. Further experiments will be required to determine whether this difference is simply a reflection of the experimental design or indicates a potential mechanism of signaling diversity among different members of the FKHR family.
FKHR phosphorylation is exquisitely sensitive to wortman- nin, consistent with a role of PI-dependent kinases in this process (11, 27). Interestingly, basal phosphorylation of FKHR is increased in the presence of rapamycin. At present, we cannot provide an explanation for this finding. However, the insulin-dependent mobility shift is not affected by rapamycin treatment, suggesting that the insulin-dependent kinases that regulate FKHR phosphorylation are not rapamycin-inhibitable. Because FKHR has the potential to participate in cell cycle control and oncogenesis and because phosphorylation of FKHR presumably inhibits its transcriptional activity, the observation of increased FKHR phosphorylation in the presence of rapamycin is consistent with antiproliferative effect of rapamycin (26).

Our observations raise two important questions: how does insulin-induced phosphorylation affect the function of FKHR and what are the insulin-regulated target genes of FKHR? Based on the data of Brunet et al. (27), the primary hypothesis to be tested is whether insulin affects the intracellular distribution of FKHR, in a similar fashion to the effect of IGF-1 on FKHR1. In preliminary experiments, we have been able to show that phosphorylation of serine 253 affects FKHR binding to DNA in gel shift assays, consistent with the possibility that insulin may regulate FKHR function at different levels.

With respect to the question of which genes are subject to regulation by FKHR in an insulin-dependent manner, we have shown that FKHR mediates insulin inhibition of IGFBP-1 transcription in a phosphorylation-dependent manner (28). The identification of the exact role of FKHR phosphorylation in insulin and IGF-1 signaling will provide important new insight into the mechanism by which insulin and IGF-1 regulate gene expression.

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