Differential regulation of gene expression by insulin and IGF-1 receptors correlates with phosphorylation of a single amino acid residue in the forkhead transcription factor FKHR

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The transcription factor FKHR is inhibited by phosphorylation in response to insulin and IGF-1 through Akt kinase. Here we show that FKHR phosphorylation in hepatocytes conforms to a hierarchical pattern in which phosphorylation of the Akt site at S^{253}, in the forkhead DNA binding domain, is a prerequisite for the phosphorylation of two additional potential Akt sites, T^{24} and S^{316}. Using insulin receptor-deficient hepatocytes, we show that T^{24} fails to be phosphorylated by IGF-1 receptors, suggesting that this residue is targeted by a kinase specifically activated by insulin receptors. Lack of T^{24} phosphorylation is associated with the failure of IGF-1 to induce nuclear export of FKHR, and to inhibit expression of a reporter gene under the transcriptional control of the IGF binding protein-1 insulin response element. We propose that site-specific phosphorylation of FKHR is one of the mechanisms by which insulin and IGF-1 receptors exert different effects on gene expression.
Keywords: gene expression/hepatocyte/insulin/kinase/phosphorylation

Introduction

The mechanisms of insulin and IGF-1 action remain only partially understood (White, 1998). Following activation of the insulin receptor (IR) or IGF-1 receptor (IGF-1R) kinase, insulin receptor substrates (IRSs) are phosphorylated at signature Y-X-X-M motifs and activate phosphoinositol 3-kinase (PI 3-kinase). The lipid products of PI 3-kinase activate a cascade of PI (3,4,5)-phosphate (PIP_3)-dependent serine/threonine kinases such as PDK1 (Alessi *et al.*, 1997; Stephens *et al.*, 1998). Substrates of this kinase include PKC isoforms (Le Good *et al.*, 1998), the serum and glucocorticoid-inducible kinase SGK (J.Park *et al.*, 1999) and the product of the Akt proto-oncogene (Stokoe *et al.*, 1997). Because of the important role of Akt in insulin action (Kohn *et al.*, 1995, 1996), identification of its substrates has been a central endeavor for many laboratories. Based on similarity with the target Akt sequence on GSK-3 (Burgering and Coffer, 1995), a

consensus Akt kinase sequence, R-X-R-X-X-S/T, has been defined (Alessi *et al.*, 1996).

Genetic complementation studies in *Caenorhabditis elegans* have identified the Daf-16 gene as a target of the Akt kinase (Gottlieb and Ruvkun, 1994; Ogg *et al.*, 1997; Paradis and Ruvkun, 1998). The product of the Daf-16 gene belongs to the forkhead family of transcription factors, a group of ~100 proteins characterized by a highly conserved DNA binding domain, the 'forkhead' or 'winged-helix' domain (Lai *et al.*, 1993). A subgroup of forkhead proteins known as FKHR proteins are the closest mammalian homologs of the Daf-16 gene product. The family includes three expressed genes, FKHR, FKHRL1 and AFX, and two pseudogenes (Anderson *et al.*, 1998). Recent work in mammalian systems has confirmed that FKHR proteins are targets of the Akt kinase and are regulated by phosphorylation in an insulin- and IGF1-dependent manner (Biggs *et al.*, 1999; Brunet *et al.*, 1999; Guo *et al.*, 1999; Kops *et al.*, 1999; Nakae *et al.*, 1999; Rena *et al.*, 1999; Tang *et al.*, 1999).

Since FKHR proteins are negatively regulated by insulin and insulin is known to inhibit hepatic expression of several genes important for metabolic and growth control, such as phosphoenolpyruvate carboxykinase (PEPCK) (O'Brien *et al.*, 1990), apolipoprotein CIII (Li *et al.*, 1995), glucose-6-phosphatase (Lin *et al.*, 1997) and insulin-like growth factor binding protein-1 (IGFBP-1) (Orlowski *et al.*, 1991), we have focused our investigations on the mechanism by which insulin affects FKHR function in hepatocytes. We have shown that FKHR is the principal member of the FKHR family in murine hepatocytes and is phosphorylated on at least three sites in a PI 3-kinase-dependent manner (Nakae *et al.*, 1999). In this study, we show that FKHR phosphorylation in hepatocytes follows a hierarchical pattern in which S^{253} phosphorylation is required for the phosphorylation of T^{24} and S^{316}. While all three residues are phosphorylated in response to insulin in normal hepatocytes, T^{24} is not phosphorylated in IR-deficient hepatocytes that express IGF-1Rs. Phosphorylation of T^{24} affects nuclear localization of FKHR and its ability to stimulate transcription of a reporter gene under the control of the IGFBP-1 promoter. These data provide a potential explanation for the different effects of insulin and IGF-1 on gene expression.

Results and discussion

S^{253} is the gatekeeper site for FKHR phosphorylation in murine hepatocytes
We have shown that both endogenous and recombinant murine FKHR are rapidly phosphorylated in an insulin- and IGF1-dependent manner in hepatocytes (Nakae *et al.*, 1999). Site-directed mutagenesis of the potential Akt phosphorylation sites indicated that FKHR is phosphoryl-

ated on all three sites: T^{24}, S^{253} and S^{316}. However, while the mutation substituting S253A completely abolished the effect of insulin on FKHR phosphorylation, mutations T24A or S316A resulted in an ~30% decrease in insulin-induced phosphorylation (Nakae *et al*., 1999). These results suggested that S^{253} plays a gatekeeper role in insulin-induced phosphorylation of FKHR. We therefore tested a series of mutants in which S^{253} was replaced by an aspartic acid residue, in order to mimic the effect of serine phosphorylation. A partial list of the mutants employed in this study is shown in Figure 1A. Replacement of S253D restored insulin-induced phosphorylation of

FKHR to 78% of control levels (Figures 1B and 3A). ^{32}P incorporation into double mutants in which either T^{24} or S^{316} was replaced by alanine along with S253D was still increased in response to insulin. In contrast, phosphorylation of the triple mutant S253D/T24A/S316A was comparable to that of the S253A mutant, and was not increased by insulin (Figure 1C). These data raised the possibility that phosphorylation of Ser^{253} is required, but not sufficient for the effect of insulin on FKHR phosphorylation. The proposed model of ordered phosphorylation of FKHR is reminiscent of the hierarchical phosphorylation of $p70^{s6k}$ by Akt (Alessi, 1998) and of the translational regulator 4E-BP1 by mTOR (Gingras *et al*., 1999).
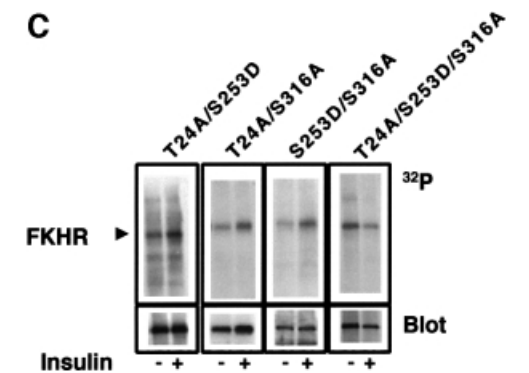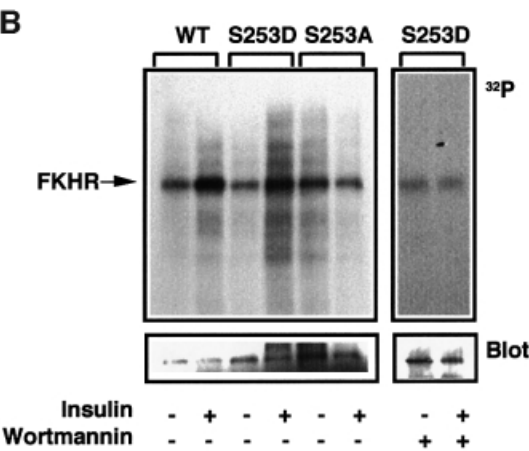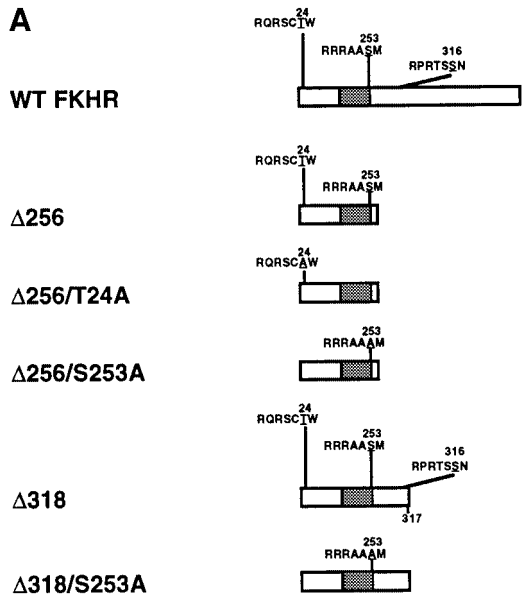
A domain between amino acids 256 and 317 is required for the hierarchical phosphorylation of FKHR

To determine further the structural requirements for FKHR phosphorylation, we constructed two truncated mutants: Δ256, which includes the entire forkhead domain and lacks the transactivation domain; and Δ318, which includes all potential Akt phosphorylation sites (Figure 1A). These truncations remove the putative nuclear exclusion sequence described by Biggs *et al*. (1999). Nevertheless, they do not interfere with subcellular localization of the truncated proteins (data not shown), probably because the small size of the truncated proteins (~30 kDa) makes them diffuse across the nuclear pore independently of the shuttle mechanism required by larger proteins (Gorlich and Mattaj, 1996). Both truncated mutants were phosphorylated in response to insulin (Figure 2). Mutation of S^{253} inhibited insulin-induced phosphorylation of the Δ318, but not of the Δ256 mutant, indicating that S^{253} phosphorylation is not required for FKHR phosphorylation on T^{24} (the only additional site remaining in this construct) if residues downstream of amino acid 255 are removed. This is confirmed by the observation that a Δ256 mutant in which T^{24} is replaced by alanine (Δ256/T24A) was not phosphorylated in response to insulin, suggesting that in the Δ256 truncated protein T^{24} is the only site of insulin-dependent phosphorylation (Figure 2). These data indicate that removal of amino acids downstream of residue 256, but not downstream of residue 318, abolishes the requirement for S^{253} phosphorylation as preliminary to the phosphorylation of either T^{24} or S^{316}. Thus, the domain comprised between amino acids 256 and 317 plays an inhibitory role in the phosphorylation of T^{24} and S^{316}. The

Fig. 1. Insulin-dependent phosphorylation of wild-type and mutant FKHR constructs in SV40-transformed murine hepatocytes. (**A**) Deletion constructs utilized in the present study. The consensus Akt sites are indicated in the single-letter amino acid code. The phosphorylated or mutant residue is underlined. The forkhead domain is represented by the shaded box. (**B**) Replacement of S253D partially restores insulin-dependent phosphorylation of FKHR. Normal mouse hepatocytes were transiently transfected with wild-type and mutant FKHR constructs, and insulin-induced phosphorylation was studied using ^{32}P labeling. In some experiments, cells were treated with wortmannin prior to the addition of insulin (right hand panel). In the upper panel, following immunoprecipitation with anti-*c*-myc antibody and SDS–PAGE, proteins were transferred to nylon membranes for autoradiography. The filters were then re-probed with an anti-FKHR antiserum to normalize the protein content of each lane (lower panel). (**C**) T^{24} and S^{316} are phosphorylated in S253D mutant FKHR. Experiments were performed as indicated above, except that double and triple mutants were used, as shown at the top of each panel.
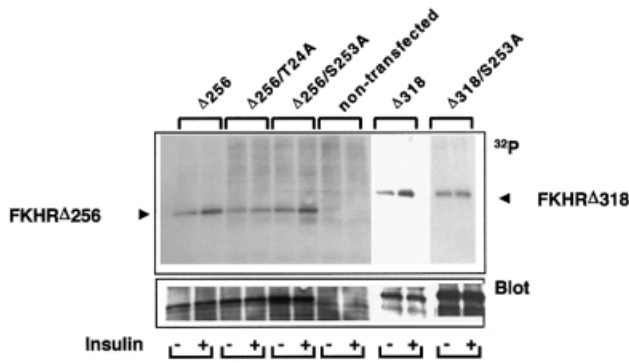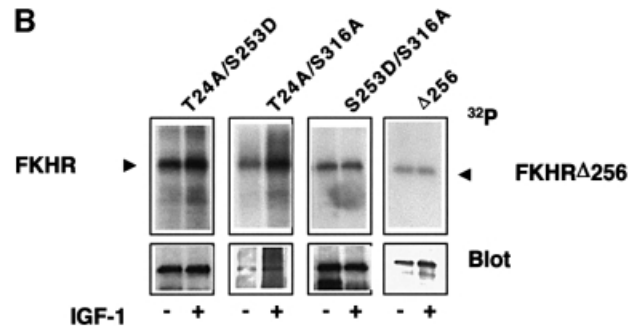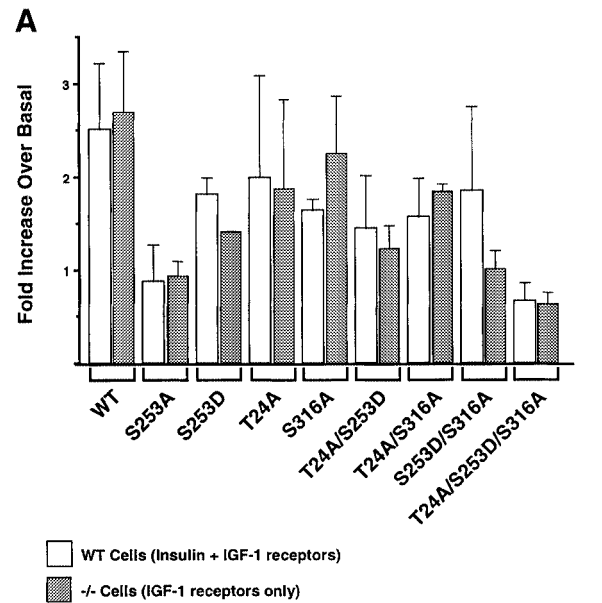
Fig. 2. The C-terminal half of FKHR contains negative regulatory elements for FKHR phosphorylation by insulin. Two truncated mutants were employed for these experiments, one encompassing amino acids 1–255 (Δ256) and one encompassing amino acids 1–317 (Δ318). Additional mutations were introduced as indicated at the top of each lane (see also Figure 1A). Phosphorylation experiments and quantification of the data were carried out as indicated in the legend to Figure 1.

Fig. 3. (**A**) Different patterns of FKHR phosphorylation are mediated by insulin and IGF-1. Wild-type and mutant FKHR as indicated in Figure 1A were transfected into SV40-transformed hepatocytes derived from either normal mice (empty bars) or IR-deficient mice (full bars). Each experiment was repeated a minimum of three times. The data are expressed as the mean ± SD of the fold increase over basal phosphorylation levels in the absence of insulin (control hepatocytes) or IGF-1 (IR-deficient hepatocytes), and were quantitated using scanning densitometry and the NIH Image 1.60 software. The difference between the effect of insulin to phosphorylate the S253D/S316A mutant in WT and IR-deficient hepatocytes is statistically significant ($P < 0.05$ analyzed by one-factor ANOVA). (**B**) T^{24} is not phosphorylated in response to IGF-1 treatment in IR-deficient hepatocytes. The mutants employed for these experiments are indicated at the top of each lane. A representative experiment is shown.

boundaries of the inhibitory domain are inferred from the observation that phosphorylation of the Δ318 mutant is inhibited by the S253A mutation, whereas phosphorylation of the Δ256 mutant is not. This region contains phosphorylation sites by proline-directed kinases, raising the possibility that basal phosphorylation of these sites would prevent S^{316} and T^{24} phosphorylation. This hypothesis would explain our previous paradoxical observation that rapamycin treatment increases FKHR phosphorylation (Nakae *et al*., 1999). In this model, a rapamycin-sensitive kinase would phosphorylate the inhibitory domain of FKHR and prevent random phosphorylation of the 'Akt' sites at T^{24} and S^{316}. Inhibition of the rapamycin-sensitive kinase would result in a relaxed specificity of FKHR phosphorylation, and would cause a broader inhibition of FKHR in response to various kinases resulting in the inhibitory effect of rapamycin on gene expression (Abraham, 1996).

Insulin and IGF-1 receptors differ in their ability to phosphorylate T^{24}
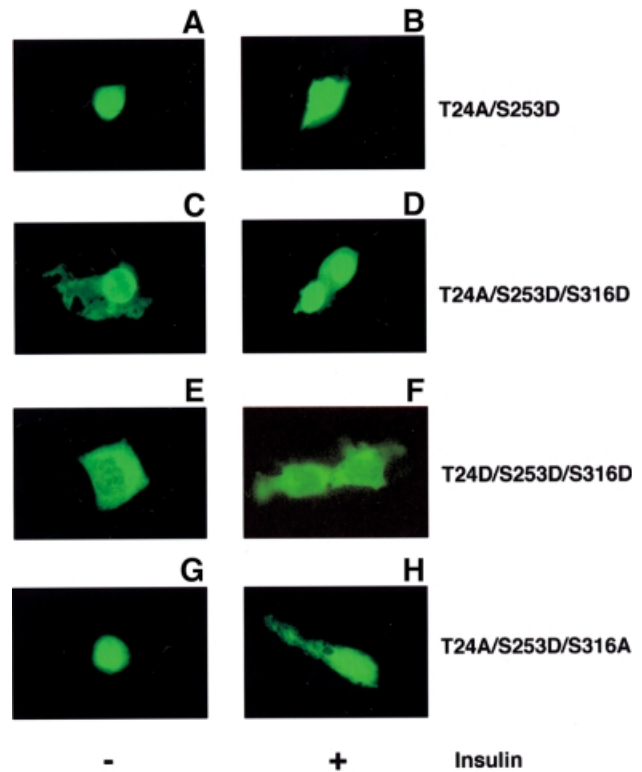
We have previously shown that hepatocytes lacking IRs are refractory to the effects of insulin to stimulate glycogen synthesis (B.C.Park *et al*., 1999) and suppress glucose production (Rother *et al*., 1998), despite the presence of IGF-1Rs. In contrast, these cells maintain the ability to grow in response to IGF-1, suggesting that IGF-1Rs have a greater ability to generate growth-promoting signals than metabolic signals (Rother *et al*., 1998). Since FKHR has the potential to regulate genes important for glucose metabolism, an IR-specific effect, we hypothesized that the difference in metabolic regulation by IRs and IGF-1Rs in liver can be accounted for by different patterns of FKHR phosphorylation. To address this question, we transfected wild-type and mutant FKHRs in IR-deficient cells and performed phosphorylation experiments in response to IGF-1. As summarized in Figure 3A, the pattern of FKHR phosphorylation induced by IGF-1R is similar, but not identical to that induced by IRs. An S253D/S316A double mutant, in which T^{24} is the sole phosphorylation site, fails to undergo IGF-1-dependent phosphorylation, as does a Δ256 truncated mutant, in

which T^{24} is also the sole phosphorylation site (Figure 3A, second set of bars from the right, and Figure 3B). These data suggest that the T^{24} residue is targeted by an IR-specific kinase.

To begin to characterize the nature of the T^{24} kinase, we studied the phosphorylation of an S253D mutant in the presence of wortmannin. Insulin-dependent phosphorylation of the S253D mutant is inhibited by wortmannin, suggesting that the S^{316} and T^{24} kinases are also PIP$_3$ dependent, like Akt (Figure 1A). Nevertheless, it is unlikely that Akt is the T^{24} kinase, since both IR and IGF-1R activate Akt to a similar extent in hepatocytes (B.C.Park *et al*., 1999). Interestingly, it has been shown that the FKHR family member AFX is not phosphorylated on T^{28}, the residue homologous to T^{24} in FKHR, in NIH 3T3 cells over-expressing IRs (Kops *et al*., 1999).

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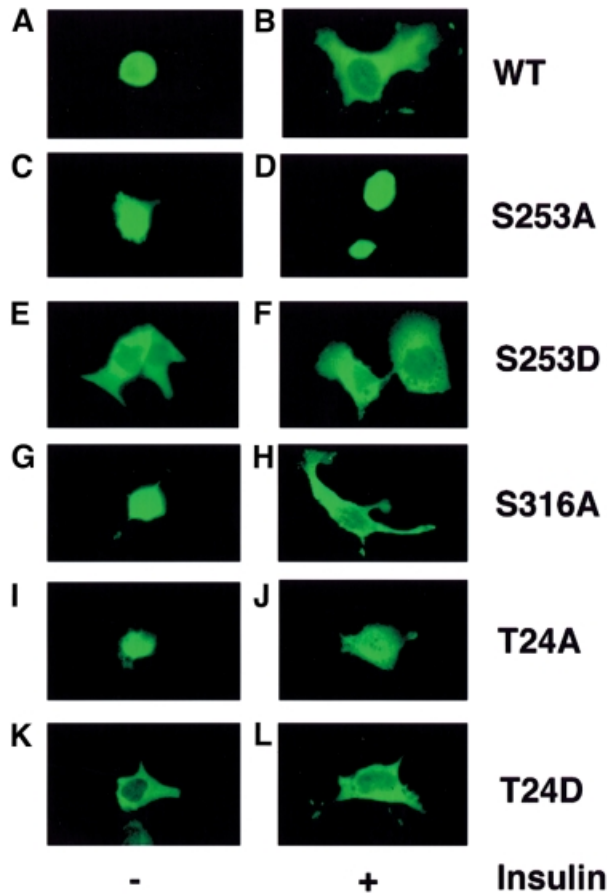
Fig. 4. Subcellular localization of phosphorylation site mutant FKHRs in insulin-treated SV40-transformed hepatocytes. Following transient transfection, hepatocytes were seeded into 4-well slide culture chambers and incubated in serum-free medium for 4 h prior to the addition of insulin. Myc epitope-tagged FKHR was visualized with anti-myc monoclonal antibody and FITC-conjugated anti-mouse IgG. (**A** and **B**) Wild-type FKHR; (**C** and **D**) S253A mutant; (**E** and **F**) S253D mutant; (**G** and **H**) S316A mutant; (**I** and **J**) T24A mutant; (**K** and **L**) T24D mutant.

Fig. 5. Role of T^{24} in subcellular localization of FKHR. Experiments were performed as indicated in the legend to Figure 4. (**A** and **B**) T24A/S253D double mutant; (**C** and **D**) T24A/S253D/S316D triple mutant; (**E** and **F**) T24D/S253D/S316D triple mutant; (**G** and **H**) T24A/S253D/S316A triple mutant.

These findings are consistent with the possibility that the T^{24} kinase is tissue specific. Other studies have found that T^{24} (or its corresponding residue in FKHRL1 and AFX) can be phosphorylated by Akt *in vitro*, or *in vivo* by over-expressed Akt (Biggs *et al*., 1999; Brunet *et al*., 1999; Guo *et al*., 1999; Rena *et al*., 1999; Tang *et al*., 1999). Thus, it is possible that the phosphorylation of T^{24} by Akt is cell-type specific, and depends on the extent of Akt activation and/or nuclear translocation in different cells. Further work will be required to identify the T^{24} kinase. However, since our studies were conducted in a physiological target cell of insulin action and without manipulating Akt expression, they may reflect the physiological regulation of FKHR function by insulin. A potential candidate for the T^{24} kinase is the serum- and gluco- corticoid-inducible kinase SGK, which is activated in response to insulin, undergoes nuclear translocation and has been shown to target sequences similar to the Akt consensus sequence surrounding T^{24} (J.Park *et al*., 1999). Nevertheless, this kinase is also activated by IGF-1, an observation that is not consistent with our predictions.
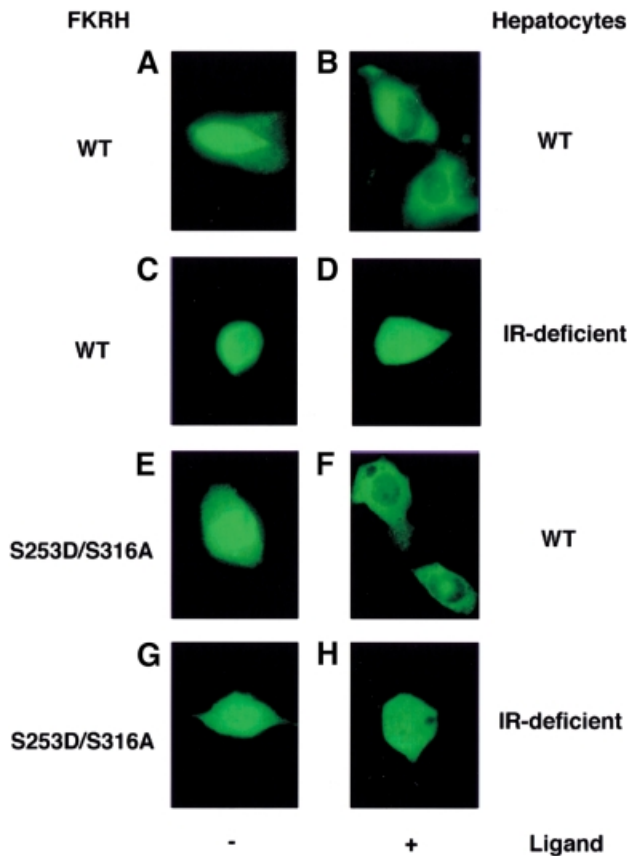
Subcellular localization of FKHR mutants in insulin-treated cells

Akt-mediated phosphorylation leads to nuclear exclusion of FKHR (Biggs *et al*., 1999; Brunet *et al*., 1999). We asked whether insulin-induced phosphorylation would affect the subcellular localization of FKHR in transiently transfected hepatocytes. Following transfection, hepatocytes were treated with insulin (in control cells) or IGF-1 (in IR-deficient cells, which possess IGF-1Rs), and a fluoroscein isothiocyanate (FITC)-conjugated anti-cMyc antibody was used to study the subcellular distribution of the various FKHR mutants using immunofluorescence. Under basal conditions, wild-type FKHR localizes to the nucleus. Insulin causes a rapid redistribution of FKHR to the cytoplasm (Figure 4A and B). Mutations of the phosphorylation sites had different effects on subcellular localization of FKHR. The S253A mutant was constitutively localized to the nucleus, and did not translocate in response to insulin (Figure 4C and D). The S253D mutant localized to both nucleus and cytoplasm, suggesting that S^{253} phosphorylation relieves a constraint that keeps FKHR in the nucleus (Figure 4E and F). Indeed, S^{253} lies next to the putative <u>KSPRRR</u>AAS^{253} nuclear localization sequence (Boulikas, 1993; Görlich and Mattaj, 1996).

The S316A mutant localized to the nucleus in the absence of insulin and translocated to the cytoplasm following insulin stimulation, albeit more slowly than wild-type FKHR (Figure 4G and H). The T24A mutant localized to the nucleus in the absence of insulin and was impaired in its ability to translocate to the cytoplasm
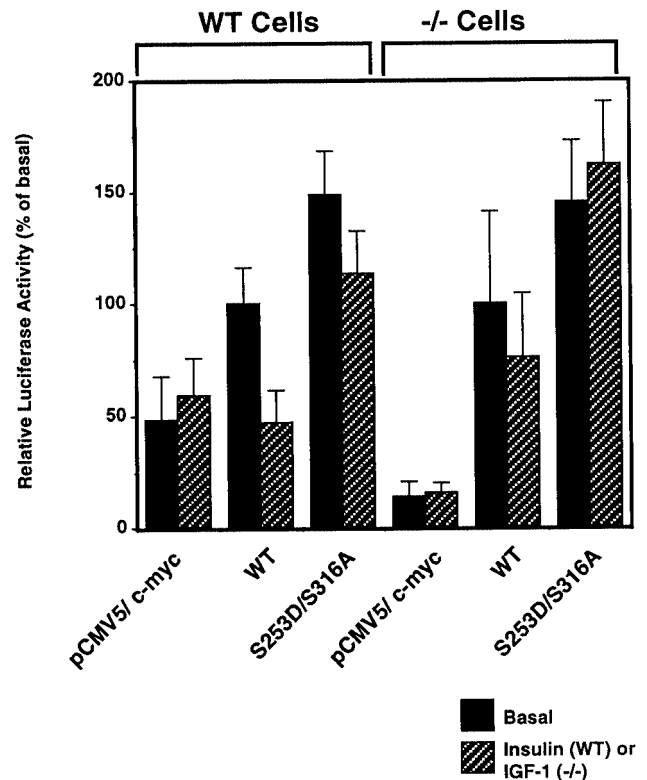
Fig. 6. Subcellular localization of an S253D/S316A double mutant FKHR in control and IR-deficient hepatocytes. Experiments were performed as indicated in the legend to Figure 4, except that IR-deficient hepatocytes were treated with IGF-1 to induce nuclear export of FKHR. (**A** and **B**) Nuclear exclusion of wild-type FKHR in insulin-treated SV40-transformed hepatocytes from normal mice; (**C** and **D**) nuclear exclusion of wild-type FKHR in IGF-1-treated IR-deficient hepatocytes; (**E** and **F**) nuclear exclusion of S253D/ S316A FKHR in insulin-treated control hepatocytes; (**G** and **H**) nuclear exclusion of S253D/S316A FKHR in IGF-1-treated IR-deficient hepatocytes.

Fig. 7. Expression of an IGFBP-1/luciferase reporter gene in hepatocytes co-transfected with wild-type and S253D/S316A mutant FKHR. Control and IR-deficient hepatocytes were transfected with wild-type or mutant FKHR in addition to the IGFBP-1/luciferase reporter gene (p925GL3) (Ooi *et al.*, 1992). Plasmid pRSV-β-galactosidase was used as an internal control of transfection efficiency (Ooi *et al.*, 1992). After transfection, β-galactosidase and luciferase activity were measured in the same sample as described in Materials and methods. The data represent the mean ± SD from five independent experiments.

following insulin treatment (Figure 4I and J). The T24D mutant was largely cytosolic (although some nuclear staining was still observed) following insulin treatment (Figure 4K and L). These data indicate that S^{253} phosphorylation is required, but is not sufficient to effect nuclear export of FKHR.

### Role of T^{24} in subcellular localization

Since we have demonstrated an IR-specific pattern of T^{24} phosphorylation, we wanted to address more closely the role of this residue in the nuclear export of FKHR. To this end, we studied the T24A mutation in the context of S253D and S253D/S316D mutations. We hypothesized that T^{24} phosphorylation is required for FKHR export following S^{253} phosphorylation. Consistent with this hypothesis, double mutants T24A/S253D (Figure 5A and B) and triple mutants T24A/S253D/S316D (Figure 5C and D) were constitutively nuclear, indicating that failure to phosphorylate T^{24} has a dominant inhibitory effect on FKHR nuclear export. The fact that a small fraction (~10%) of the triple mutant T24A/S253D/S316D is cytosolic could be explained by increased protein degradation, or by an

additional pathway for nuclear exclusion (Figure 5C and D). As controls, we show that the triple mutant T24D/S253D/S316D was constitutively cytosolic (Figure 5E and F), whereas phosphorylation-defective triple mutant T24A/S253D/S316A was constitutively nuclear (Figure 5G and H).

We further reasoned that, if T^{24} plays a role in FKHR nuclear export following phosphorylation of S^{253} and is phosphorylated by an IR-stimulated kinase that fails to be activated by IGF-1Rs, the following predictions should be met: (i) IGF-1 should be less potent than insulin in stimulating FKHR translocation in IR-deficient cells; and (ii) the subcellular localization of a double mutant FKHR in which T^{24} is the sole remaining phosphorylation site should differ in control and IR-deficient hepatocytes. Figure 6 shows the results of these experiments. While insulin treatment resulted in the rapid translocation of FKHR in >70% of control hepatocytes (Figure 6A and B), IGF-1 treatment of IR-deficient hepatocytes resulted in FKHR translocation in ~10% of transfected IR-deficient cells (Figure 6C and D), despite the fact that FKHR phosphorylation was similar to that elicited by insulin (Figure 3A). Moreover, in control hepatocytes, insulin stimulated nuclear export of an S253D/S316A double mutant FKHR that can only be phosphorylated on
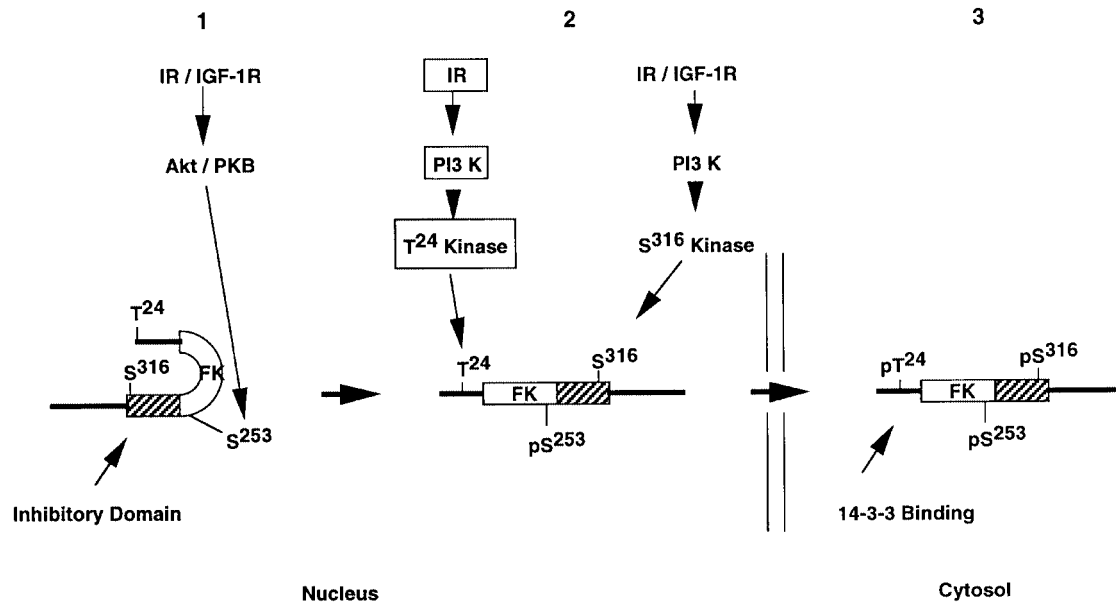
Fig. 8. Proposed model for multisite regulation of FKHR function. Under conditions in which insulin signaling is inactive, FKHR localizes to the nucleus. Following insulin stimulation, Akt translocates to the nucleus and phosphorylates S^{253} in the forkhead domain of FKHR. This primes FKHR for further phosphorylation of T^{24} and S^{316}. T^{24} phosphorylation is effected by an IR-specific kinase and is required for FKHR nuclear export. S^{316} phosphorylation is effected by both IR and IGF-1R-stimulated kinases, and is not a requisite for FKHR nuclear export. Once FKHR is phosphorylated on T^{24} and exported to the cytoplasm, pT^{24} has the potential to bind 14-3-3 and cause cytoplasmic retention of FKHR (Brunet *et al.*, 1999). The T^{24} and S^{316} kinases are also PIP_3 dependent, like Akt. Nevertheless, it is unlikely that the T^{24} kinase is Akt, because Akt is activated to the same extent in control and IR-deficient hepatocytes (B.C.Park *et al.*, 1999), and T^{24} fails to be phosphorylated in IR-deficient hepatocytes.

T^{24} (Figure 6E and F). In contrast, IGF-1 failed to stimulate nuclear export of the same mutant transfected in IR-deficient hepatocytes (Figure 6G and H). These data provide a functional correlation for the observed difference in the phosphorylation patterns of FKHR in control and IR-deficient hepatocytes. Further evidence for a functional role of T^{24} phosphorylation derives from the studies of Brunet *et al.* (1999) in which the sequence surrounding T^{24} in FKHRL1 was shown to bind the scaffolding protein 14-3-3, providing a mechanism for cytoplasmic sequestration of FKHR.

T^{24} phosphorylation affects inhibition of gene expression by insulin

To establish a correlation between T^{24} phosphorylation and the effect of FKHR on gene expression, we performed reporter gene assays in hepatocytes transiently transfected with wild-type or mutant FKHR and the luciferase gene under the transcriptional control of an insulin response element derived from the rat IGFBP-1 promoter (Orlowski *et al.*, 1991). Transfection of a wild-type FKHR cDNA increased IGFBP-1/luciferase activity ~2-fold in control cells and ~6-fold in IR-deficient cells (Figure 7). Insulin inhibited IGFBP-1/luciferase expression ~50% in control cells ($P <0.05$), whereas IGF-1 inhibited the same constructs ~20% in IR-deficient cells ($P = $ n.s.). Moreover, insulin inhibited gene expression induced by an S253D/S316A double mutant by 25% in control hepatocytes ($P <0.01$), whereas IGF-1 not only failed to inhibit reporter gene expression, but also paradoxically stimulated luciferase activity in IR-deficient hepatocytes ($P <0.01$ for the increase) (Figure 7). The partial inhibition of insulin-dependent suppression of the S253D/S316A con-

struct compared with the wild-type FKHR construct can be attributed to the effect of the S^{316} mutation.

Conclusions

The identification of T^{24} on FKHR as the target of an IR-specific kinase provides preliminary evidence of an IR-specific pathway to control gene expression *vis-à-vis* the pathway shared in common with the IGF-1R. Our model is depicted in Figure 8. Both insulin and IGF-1, acting through the wortmannin-sensitive kinase Akt, phosphorylate FKHR on S^{253}, priming the molecule for phosphorylation of two additional sites, T^{24} and S^{316}. While S^{316} is phosphorylated by both IRs and IGF-1Rs, T^{24} is targeted by an IR-specific kinase. Nuclear exclusion of FKHR requires the action of the T^{24} kinase, thereby conferring specificity of insulin responsiveness onto selected target genes. The proposed model of multistep regulation of FKHR through phosphorylation is reminiscent of the regulation of the transcription factor Pho4 in yeast. This protein is phosphorylated on five different sites by different proline-directed kinases to regulate three aspects of Pho4 function: nuclear export, transactivation and nuclear import (Komeili and O'Shea, 1999). The model is also consistent with the emerging notion that Akt kinase activity is necessary, but not sufficient for insulin action. This conclusion is supported by a recent study showing that insulin inhibition of PEPCK gene expression requires a downstream effector of PI 3-kinase distinct from Akt, the atypical PKCλ and Rac (Kotani *et al.*, 1999). A similar model is also borne out from our studies on the regulation of glycogen synthesis in hepatocytes. We have shown that IRs are more potent than IGF-1Rs in stimulating glycogen synthesis, despite

the fact that they are equipotent in stimulating Akt. The greater potency of IRs to stimulate glycogen synthesis correlates with their ability to activate specifically as yet unidentified rapamycin-sensitive kinase(s) (B.C.Park *et al.*, 1999). The identification of the kinases involved in FKHR phosphorylation will be crucial to our understanding of the mechanism of insulin action.

Materials and methods

Antibodies
Anti-*c*-myc antibody (clone 9E10) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FKHR antisera were raised by immunizing rabbits with a peptide corresponding to amino acids 18–33 of the mouse FKHR sequence (PRQRSCTWPLPRPEFN).

Cell culture and labeling with [^{32}P]orthophosphate
SV40-transformed mouse hepatocytes used in these studies have been described in previous publications (Rother *et al.*, 1998; B.C.Park *et al.*, 1999). Briefly, cells were passaged at 33°C in alpha-modified Eagle's medium (AMEM) supplemented with 4% fetal calf serum (FCS), 2 mM glutamine and 10 nM dexamethasone. For [^{32}P]orthophosphate labeling experiments, cells were sequentially incubated in serum-free Dulbecco's modified Eagle's medium (DMEM) for 8 h, serum- and phosphate-free DMEM for 1 h, and DMEM supplemented with 0.05 mCi/ml [^{32}P]orthophosphate (New England Nuclear, Boston, MA) for 4 h. Insulin (100 nM) or IGF-1 (200 ng/ml) were added for 15 min. At the end of the incubation, cells were solubilized and processed for immunoprecipitation with anti-*c*-myc antibody (9E10) as described (Nakae *et al.*, 1999). Transient transfections were carried out with lipofectamine (Gibco-BRL) according to the manufacturer's instructions using 10 µg of plasmid DNA. Immune complexes were resolved on SDS–PAGE, transferred to nylon membranes and analyzed by autoradiography. To normalize the amount of ^{32}P incorporated into each band for the amount of protein on the gel, the blots were probed with anti-FKHR antiserum followed by detection of the immune complexes with horseradish peroxidase-conjugated anti-rabbit IgG (ECL; Amersham, Buckinghamshire, UK) and densitometric analysis with the NIH Image 1.60 software (Lauro *et al.*, 1998).

Site-directed mutagenesis
Some of the constructs used in this study (wild type, T24A, S253A and S316A) have been described previously (Nakae *et al.*, 1999). Overlap extension PCR was used to generate the S253D, S253D/S316A, T24A/S253D/S316D, Δ256, Δ256/S253A, Δ318 and Δ318/S253A expression vectors. The 5′ fragment contained a unique *Bgl*II restriction site at the 5′ end, and a mutagenic oligonucleotide at the 3′ end; the 3′ fragment contained a unique *Age*I restriction site at the 3′ end, and the mutagenic oligonucleotide at the 5′ end. Mutant nucleotides are underlined. Following amplification of each individual fragment, a second PCR was carried out to generate a single fragment containing the mutation and straddling the two unique restriction sites at the 5′ and 3′ ends, respectively. The resulting PCR fragment was used to replace the wild-type sequence in a pCMV5-cMyc expression vector (Nakae *et al.*, 1999). To construct the S253D mutant, the following primers were employed: 1, 5′-GACCTCATCACCAAGGCCATC-3′, corresponding to nucleotides 490–510; 2, 5′-GGCCCATCATTACATTTTGGCCCAGGAC-3′, corresponding to nucleotides 1489–1462; 3, 5′-GTTGTCCATGTCCGCAGCTCT-3′, corresponding to nucleotides 768–748; and 4, 5′-AGAGCTGCGGACATGGACAAC-3′, corresponding to nucleotides 748–768. To generate the S253D/S316A mutant, the S253D mutant was used as a PCR template and the same primers 1 and 2 were employed; primer 3, 5′-ACTAGCATTTGCGCTGGTTCG-3′, corresponding to nucleotides 957–937; primer 4, 5′-CGAACCAGCGCAAATGCTAGT-3′, corresponding to nucleotides 937–957. To generate the T24A/S253D/S316D mutant, the S253D mutant was used as a template of PCR; primer 3, 5′-ACTAGCATTGTCGCTGGTTCG-3′, corresponding to nucleotides 957–937; primer 4, 5′-CGAACCAGCGACAATGCTAGT-3′, corresponding to nucleotides 937–957. This PCR fragment containing the S253D/S316D mutations was treated with *Bgl*II and *Age*I and ligated into the T24A mutant FKHR expression vector. To generate the Δ256 mutant, the same primers 1 and 2 used in the construction of the S253D mutant were employed; primer 3, 5′-TTTACT-GTTCTAGTCCATGGA-3′, corresponding to nucleotides 777–757;

primer 4, 5′-TCCATGGACTAGAACAGTAAA-3′, corresponding to nucleotides 757–777. For the construction of the Δ256/S253A mutant, the Δ256 mutant was used as a template for PCR; primer 3, 5′-CTAGTCCATGGCCGCAGCTCT-3′, corresponding to nucleotides 768–748; primer 4, 5′-AGAGCTGCGGCCATGGACTAG-3′, corresponding to nucleotides 748–768. To construct the Δ318 mutant, primer 3, 5′-GATGGTACTCTAATTTGAGCT-3′, corresponding to nucleotides 969–949, and primer 4, 5′-AGCTCAAATTAGAGTACCATC-3′, corresponding to nucleotides 949–969, were employed. To construct the Δ318/S253A mutant, the Δ318 mutant was used as a template and the same primers 3 and 4 used for the S253A mutant were employed. To construct the Δ256/T24A, T24A/S253D, T24A/S316A and T24A/S253D/S316A mutants, PCR products derived from constructs Δ256, S253D, S316A and S253D/S316A were treated with *Bgl*II and *Age*I and ligated into *Bgl*II- and *Age*I-digested T24A mutant.

Immunofluorescence
SV40-transformed mouse hepatocytes were transiently transfected using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's protocol. After 24 h, cells were seeded into 4-well slide culture chambers (Lab Tek). Cells were incubated in serum-free medium for 4 h and then stimulated with insulin (100 nM) or IGF-1 (200 ng/ml) for 0–60 min. Cells were fixed using a solution of 2% paraformaldehyde in 150 mM NaCl and 2 mM MgCl$_2$ and permeabilized in 0.5% Triton X-100. Myc epitope-tagged FKHR was visualized with anti-myc monoclonal antibody (9E10) (Santa Cruz Biotechnology) and FITC-conjugated AffiniPure donkey anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.).

Luciferase and β-galactosidase assays
The day prior to transfection, cells were plated onto 6-cm tissue culture dishes in 2 ml of AMEM containing 4% FCS, 2 mM glutamine and 10 nM dexamethasone. Transfections were carried out on cells at a 70–80% stage of confluence using 1.5 µg of plasmid DNA and 2 µg of IGFBP-1/luciferase reporter gene (p925GL3) (Ooi *et al.*, 1992). Plasmid pRSV-β-galactosidase (20 ng) was used as an internal control of transfection efficiency (Ooi *et al.*, 1992). After transfection, cells were incubated in complete AMEM for 36 h. Thereafter, medium was replaced with serum-free AMEM containing 0.1% bovine serum albumin with or without insulin (100 nM) or IGF-1 (200 ng/ml) and incubation was continued for 16 h. For luciferase and β-galactosidase assay, cells were washed twice with ice-cold phosphate-buffered saline, lysed in 360 µl of buffer containing 25 mM glycylglycine, 15 mM MgSO$_4$, 4 mM EGTA, 1 mM dithiothreitol (DTT), 1% Triton X-100, and centrifuged. Supernatant (0.1 ml) was added to the assay buffer (25 mM glycylglycine, 15 mM MgSO$_4$, 4 mM EGTA, 15 mM potassium phosphate pH 7.8, 1 mM DTT, 2 mM ATP), and assayed immediately after addition of D-luciferine (Sigma) using a Lumat LB 9507 luminometer (EG&G Berthold, Germany). β-galactosidase activity in cell lysates was assayed according to the manufacturer's instructions (Tropix, Bedford, MA). Assays were performed in triplicate. Luciferase activity (relative light units) was normalized for β-galactosidase activity (relative light units) in the same sample.

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