The FKHR gene was first identified from its disruption by the t(2;13) chromosomal translocation seen in the pediatric tumor alveolar rhabdomyosarcoma. It encodes for a member of the forkhead family of transcription factors. Recently, a homolog of FKHR in the nematode Caenorhabditis elegans was identified called DAF-16, which is a downstream target of two Akt homologs in an insulin-related signaling pathway. We have examined the possible role of Akt in the regulation of FKHR. We find that FKHR can bind in vitro to the insulin-responsive sequence (IRS) in the insulin-like growth factor-binding protein 1 promoter and can activate transcription from a reporter plasmid containing multiple copies of the IRS. Expression of active but not inactive Akt can suppress FKHR-mediated transcriptional activation. Akt can phosphorylate FKHR in vitro on three phosphoacceptor sites, at least a subset of which can also be phosphorylated by Akt in vivo. Importantly, mutation of these three sites to alanine residues enhances the transcriptional activity of FKHR and renders it resistant to inhibition by Akt. Expression of an Akt-resistant mutant of FKHR causes apoptosis in 293T cells in a manner dependent on DNA binding. These results suggest that FKHR may be a direct nuclear regulatory target for Akt in both metabolic and cell survival pathways.

Akt, also known as protein kinase B (PKB) and RAC kinase, is a serine/threonine protein kinase that plays central roles in signaling in response to mitogens and survival factors (1). The activation of Akt is triggered by upstream kinases, including phosphatidylinositol 3-kinase (2, 3) and Ca2+/calmodulin-dependent protein kinase kinase (4). Known physiological substrates for Akt include glycogen synthase kinase-3 (5), BAD (6, 7), and caspase-9 (8). However, none of these Akt substrates are known functions in the nucleus where a large portion of the FKHR gene encodes for a protein containing a DNA-binding motif shared with members of the HNF-3/forkhead family of transcription factors (12–14). Recently, the nematode Caenorhabditis elegans forkhead transcription factor DAF-16 was identified as a potential homolog of FKHR (15, 16). Genetic data in C. elegans suggest that DAF-16 is a downstream target of two Akt homologs in an insulin-related signaling pathway (17). We have investigated whether FKHR may be a direct downstream regulatory target of Akt in mammalian cells. Our data identify FKHR as a positive transcription regulator that can bind in vitro to the IGFBP-1 insulin-responsive sequence (IRS) and also induce apoptosis. We have identified three Akt phosphorylation sites in FKHR and have data suggesting that phosphorylation on all or a subset of these sites contributes to the down-regulation of FKHR’s ability to activate transcription and to induce apoptosis. Because its activity can be inhibited by Akt phosphorylation, FKHR is a potential Akt nuclear target in metabolic and survival pathways.

EXPERIMENTAL PROCEDURES

Plasmids

Two oligonucleotides (GCAAAAAACACTATTTTGAAGCAAACAC-AACTTTATTTGAAGCAAACACTATTTTTGAAGAA and TCGATTCA-AAAAAGTTTGTGTGTTCTCAAAATAGTGTGTGTGTTCCTCAAAAT-AGTTTGTGTGTTGCTAC) were annealed together and ligating into the KpnI and XhoI sites of pGL2-Promoter (Promega) to create 3’IRS-luc.

A cDNA construct containing the full-length open reading frame of wild-type FKHR (11) was assembled into the mammalian expression vector pcDNA3 (Invitrogen). An expression vector for FLAG-tagged FKHR was created by ligating a KpnI/XhoI cDNA fragment from pcDNA3-FKHR into pcDNA3-FLAG. pcDNA3 expression plasmids for FLAG-tagged FKHR H215R, FKHR T24A, FKHR S256A, FKHR S319A, FKHR(AAA), and FKHR(AAA) H215R were constructed by polymerase chain reaction mutagenesis and verified by sequencing.GST expression vectors for FKHR-N and FKHR-C were constructed by ligating NcoI and EcoRI-cut fragments, respectively, from pcDNA3-FKHR into pGEX-5X-2. pcDNA3 expression plasmids for FLAG-tagged FKHR H215R, FKHR T24A, FKHR S256A, FKHR S319A, FKHR(AAA), and FKHR(AAA) H215R were constructed by polymerase chain reaction mutagenesis and verified by sequencing. GST expression vectors for FKHR-N and FKHR-C were constructed by ligating NcoI and EcoRI-cut fragments, respectively, from pcDNA3-FKHR into pGEX-KG (18). FKHR-N and FKHR-C were predicted to include residues 1–237 and 291–416 (11), respectively. FKHR-N T24A, FKHR-C S256A, FKHR-C S319A, and FKHR-C(AA) were subcloned into pGEX-KG by ligation of the appropriate NcoI or EcoRI-cut fragments cut from pcDNA3 constructs.

Production and Purification of GST Fusion Proteins

The expression and purification of GST fusion proteins was performed using DH5α bacteria as described previously (18).

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† The abbreviations used are: PKB, protein kinase B; HA, hemagglutinin; β-gal, β-galactosidase; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; HNF-3, hepatic nuclear factor 3; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; IGFBP-1, insulin-like growth factor-binding protein-1; IRS, insulin-responsive sequence; EMSA, electrophoretic mobility shift assay; MOPS, 4-morpholinopropanesulfonic acid; WT, wild-type.

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Transient Transfection Reporter Assay

293 cells grown in 12-well plates were transfected using Lipo-ffectAMINE (Life Technologies, Inc.) with 0.1 mg of pcDNA3 or FKHR expression plasmid, 0.1 mg of luciferase reporter, and 10 ng of pCMV-β-gal. Bluescript (Strategene) was included in all samples to adjust the total DNA transfected to 0.8 mg. Luciferase assays were performed as described previously (19).

EMSA

Binding reactions contained binding buffer (50 mM Tris (pH 7.5), 13 mM MgCl₂, 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol), 0.5 μg of poly(dI-dC), 0.5 μg of poly(dA-dT), 0.5 ng of ³²P-labeled oligonucleotide probe, and purified GST fusion protein (50 ng) or cellular lysate (2 μg). After 10-min incubation at 22 °C, reaction products were separated on a 4% polyacrylamide gel containing 0.5 × Tris borate-EDTA.

**Fig. 1. DNA binding and transcriptional activation by FKHR.** A, specific DNA binding by a bacterially expressed fragment of FKHR. GST fusion proteins were tested in EMSA with radiolabeled DNA probe containing the human IGFBP-1 insulin-responsive sequence (IRS WT) or either of two mutated versions (IRS mut1, IRS mut2). The sequences of the top strands of the probes used are shown above, with mutated bases in bold. The sequences in the IGFBP-1 IRS, which are similar to other IRSs, are underlined. The presence of multiple shifted bands in lane 3 is reproducible and may be due to protein degradation. B, DNA binding of recombinant epitope-tagged FKHR. 293 cells were transiently transfected with empty vector or FLAG-tagged FKHR plasmid. Whole cell lysates were prepared and used in EMSA with radiolabeled IRS WT probe. The arrowhead points to the DNA binding activity that exists in FLAG-FKHR-transfected, but not control, lysates. Monoclonal antibodies to FLAG (M2, Sigma) or HA (Babco) were included in binding reactions as indicated. An immunoblot of lysates using FLAG antibody is shown on the right. C, FKHR activates transcription from a reporter construct containing three copies of the IGFBP-1 IRS. 293 cells were transfected with 3×IRS-luc or reporter plasmid lacking the IRS sequence (pGL2-luc), pCMV-β-gal, and FLAG-tagged FKHR plasmid. The relative amounts of FKHR plasmid transfected is indicated, 1 signifying 10 ng. Cell lysates were prepared 24 h after transfection and luciferase activities measured, adjusted to β-gal activity. Values are expressed relative to control. An immunoblot of the same lysates using anti-FLAG M2 antibody is shown below.
**Immunoprecipitation/in Vitro Kinase Assay**

293 cells transfected with HA-tagged myr-Akt or Akt K179M plasmid were lysed and whole cell extracts prepared by resuspending in lysis buffer (50 mM Tris-HCl (pH 7.5), 0.1% Triton X-100, 1 mM EDTA, 50 mM NaF, 10 mM sodium β-glycerol phosphate, 5 mM sodium pyrophosphate, 1 mM Na₃VO₄, 0.1% 2-mercaptoethanol). Clarified extracts were incubated with anti-HA monoclonal antibody (Babco), and immunoprecipitates were incubated with 6.6 mM MOPS (pH 7.2), 8.3 mM β-glycerol phosphate (pH 7.0), 0.33 mM Na₃VO₄, 0.33 mM dithiothreitol, 10 µM of γ-[32P]ATP, 25 mM MgCl₂, 166 µM ATP, and 50 ng of GST fusion protein at 30 °C for 30 min.

**Cell Death Assays**

Morphologic Assay—293T cells were transfected with 0.1 µg of pcMV-β-gal, 0.5 µg of Bluescript (Stratagene), and 0.5 µg of pcDNA3 or FLAG-tagged FKHR expression vector. 24 h after transfection, at least 300 transfected cells per sample were analyzed for apoptosis as described previously (20).

**Gel Fragmentation Assay**—293T cells grown on 10-cm plates were transfected with 4 µg of Bluescript and 4 µg of pcDNA3 or FLAG-tagged FKHR expression plasmid. 48 h after transfection, cells were lysed and DNA precipitated as described previously (21).

**RESULTS AND DISCUSSION**

The IRS in the IGFBP-1 promoter has been shown previously to be bound specifically in vitro by HNF-3, a forkhead family member (22, 23). We performed in vitro DNA binding assays to determine whether FKHR might be able to recognize the IGFBP-1 IRS also. In EMSAs, we found that when expressed as a glutathione S-transferase (GST) fusion protein, an N-terminal fragment of FKHR containing the forkhead DNA-binding motif could bind to a probe containing the IGFBP-1 IRS (Fig. 1A). In contrast, probes containing either of two mutant versions of the IRS (Fig. 1A) were not bound by FKHR. Significantly, these two IRS mutations have been shown to eliminate the response of the IGFBP-1 promoter to insulin in transient transfection assays (23). We found that mutation of histidine 215 in FKHR to arginine (H215R) significantly reduced DNA binding activity (Fig. 1A). This histidine residue is conserved in all forkhead family members and is involved in making contacts with DNA (24). In order to examine whether recombinant full-length FKHR expressed in mammalian cells could also bind to the IGFBP-1 IRS, we performed EMSA with radiolabeled IGFBP-1 IRS probe using extracts from transfected 293 cells. Extracts from cells transfected with FLAG-FKHR were found to contain a novel supershifted complex not found when using control extracts (Fig. 1B). Inclusion of a FLAG antibody but not an unrelated antibody in the reactions significantly disrupted this complex, indicating that it contained FLAG-FKHR. This complex bound to the IRS specifically because it was not seen when either of the two mutant probes was used (data not shown). Consistent with a critical role of histidine 215 for DNA binding, lysates from cell transfected with FLAG-FKHR H215R contained no detectable binding activity for the IRS probe (Fig. 1B).

Next, we wished to test whether FKHR might be able to activate transcription through the IGFBP-1 IRS. A C-terminal region of FKHR has been found previously to activate transcription when fused to a heterologous DNA-binding domain (25, 26). We constructed a luciferase reporter construct (3×IRS-luc), which contained three tandem copies of the IGFBP-1 IRS placed upstream of a basal promoter and the firefly luciferase gene. Transfection of epitope-tagged FKHR in 293 cells resulted in transcriptional enhancement in a dose-dependent manner with 3×IRS-luc, whereas no enhancement was observed with a reporter plasmid lacking IRS elements (Fig. 1C). In contrast, FKHR H215R was unable to elicit any significant activation of 3×IRS-luc. The H215R mutation, however, did not impair the ability of FKHR to transactivate when fused to the yeast GAL4 DNA-binding domain (data not shown). Collectively, these results suggest that FKHR can activate transcription directly through the IRS from the IGFBP-1 promoter.

Since expression of active Akt is known to be sufficient to mimic insulin's inhibitory effects on transcription through the IGFBP-1 IRS (27), we tested whether FKHR might be able to be negatively regulated by Akt. Cotransfection of plasmid for wild-type, but not kinase-dead Akt, containing a point mutation in its catalytic domain (K179M), resulted in a significant repression of FKHR-mediated transcriptional activation (Fig. 2). A constitutively active mutant of Akt containing a myristoylated amino terminus (myr-Akt) was also a potent inhibitor (data not shown). Thus, Akt can suppress transcriptional activation by FKHR, and this effect requires the catalytic activity of Akt.

The amino acid sequence of FKHR contains three putative consensus Akt phosphorylation sites (Thr24, Ser256, and Ser319) (Fig. 3A), all of which are conserved in the C. elegans DAF-16 transcription factor (17). In order to test whether any of these sites might be phosphorylated in vitro by Akt, we performed kinase assays using immunoprecipitated HA-tagged myr-Akt or Akt K179M from cell lysates. Because of difficulties we had in expressing full-length FKHR in bacteria, we expressed and purified two smaller fragments of FKHR as GST fusion proteins to test as potential substrates. One fragment included Thr24 (FKHR-N) and the other contained Ser256 and Ser319 (FKHR-C). Both GST-FKHR-N and GST-FKHR-C were phosphorylated by immunoprecipitated myr-Akt but not inactive Akt (Fig. 3A). In contrast, no phosphorylation was seen when GST was used as a substrate (Fig. 3A). Next, versions of each FKHR protein containing alanine substitutions at phosphorylation sites were compared as substrates for myr-Akt. Mutation of Thr24 in GST-FKHR-N was found to prevent its phosphorylation (Fig. 3B, top). Also, mutation of Ser256 and Ser319 each individually led to a partial reduction in phosphorylation.
of GST-FKHR-C, while mutation of both eliminated any detectable phosphorylation (Fig. 3B, bottom). These results support the notion that Thr24, Ser256, and Ser319 all can serve as Akt phosphorylation sites in vitro.

We observed FKHR expression by Western blot using lysates prepared from 293 cells transfected with myr-Akt-HA or Akt K179M-HA or mock-transfected cells were incubated with monoclonal anti-Akt antibody, and immunoprecipitates were tested in kinase reactions using GST, GST-FKHR-N, or GST-FKHR-C as substrates. Above, the residues surrounding the three putative consensus Akt phosphorylation sites are listed, with the Akt consensus residues (29) depicted in bold. B, site-specific in vitro phosphorylation by Akt. Anti-HA immunoprecipitates from mock-transfected or myr-Akt-HA-transfected cells were incubated with wild-type and mutant versions of GST-FKHR-N (top) or GST-FKHR-C (bottom). C, in vivo phosphorylation of FKHR by Akt. Top, 293 cells transfected with empty vector, FLAG-FKHR, and GST-Akt or GST-Akt K179M were lysed and extracts were separated on a 7.5% polyacrylamide gel that was blotted with anti-FLAG M2 or anti-GST antibody (Santa Cruz). The asterisk denotes the faster migrating band seen in the absence of wild-type Akt. The bracket indicates the position of the shifted bands seen in the presence of Akt. The arrowhead indicates the positions of GST-Akt WT and GST-Akt K179M. Middle, alternatively, lysates prepared above from 293 cells transfected with FLAG-tagged wild-type FKHR and GST-Akt were incubated with anti-FLAG M2 antibody, and immunoprecipitates were treated with λ phosphatase (PPase) (New England Biolabs) where indicated. Reactions were separated on a 7.5% polyacrylamide gel and blotted with anti-FLAG M2 antibody. Bottom, 293 cells transfected with FLAG-tagged wild-type FKHR or FKHR(AAA) and GST-Akt where indicated were lysed, and extracts were separated on a 7.5% polyacrylamide gel and blotted with anti-FLAG M2 antibody. D, transcription inhibition by Akt is dependent on intact phosphoacceptor sites. 293 cells were transfected with IRS-luc, pCMV-β-gal, and FLAG-tagged wild-type FKHR or the indicated point mutants, along with myr-Akt-HA or empty vector. Luciferase activity was determined 24 h after transfection as described in Fig. 1C. The results shown are representative of two independent experiments. An immunoblot of lysates using FLAG or HA antibody is shown below.
wild-type FKHR, suggesting that Thr\textsuperscript{24}, Ser\textsuperscript{256}, and/or Ser\textsuperscript{319} might be phosphorylated by endogenous kinases (Fig. 3C, bottom). Whereas wild-type Akt caused a shift in migration of bands for wild-type FKHR, the single band seen for FKHR(AAA) remained unchanged. Thus, overexpression of Akt can alter the phosphorylation state of FKHR \textit{in vivo}, and this can be visualized as a mobility shift by SDS-PAGE. These data support the idea that Akt can phosphorylate FKHR on at least a subset of the \textit{in vitro} phosphorylation sites (Thr\textsuperscript{24}, Ser\textsuperscript{256}, and Ser\textsuperscript{319}) \textit{in vivo}.

Next, we examined whether the three Akt phosphorylation sites were important for FKHR activity \textit{in vivo}. We found that proteins containing alanine substitutions at the three phosphoacceptor sites singly (T24A, S256A, and S319A) or together (AAA) possessed greater activity than wild-type protein (Fig. 3D). Thus, phosphorylation on each of these three sites may contribute to the inhibition of transcriptional activation by FKHR. When myr-Akt was coexpressed, we found that single mutations of each of the three potential Akt phosphorylation sites resulted in partial resistance to Akt inhibition, while mutation of all three sites together produced a mutant form of FKHR that was relatively insensitive to inhibition (Fig. 3D). These data support a model whereby phosphorylation on Thr\textsuperscript{24}, Ser\textsuperscript{256}, and Ser\textsuperscript{319} each contributes to the inhibitory effects of Akt.

In addition to its roles in glucose transport and metabolism, Akt is a critical mediator of cell survival signals elicited by serum and growth factors including insulin. Since Akt serves...
as an inhibitor of apoptosis, and FKHR is negatively regulated by Akt, we tested whether FKHR might have proapoptotic activity. We transfected 293T cells with wild-type FKHR or FKHR(DDD) and examined transfected cells 48 h later for apoptotic morphology. Transfection of FKHR(DDD) induced features of apoptosis as identified by rounded cells and membrane blebbing (Fig. 4A). FKHR(DDD)-transfected cells also displayed chromatin condensation and nuclear fragmentation, which are also typical features of apoptotic cells (data not shown). Furthermore, FKHR(DDD) caused DNA fragmentation and activated caspase-3 as assessed in a substrate cleavage assay (Fig. 4A, data not shown). In contrast, wild-type FKHR caused only a slight but reproducible level of apoptosis in morphological assays and caused relatively insignificant levels of DNA fragmentation, despite having higher levels of expression than FKHR(DDD) (Fig. 4A). Since the apoptotic activities of wild-type FKHR and FKHR(DDD) correlate with their relative abilities to activate transcription (Fig. 3D), apoptosis induced by FKHR may involve its ability to activate gene transcription. Consistent with this idea, introduction of an H215R mutation, which disrupts DNA binding (Fig. 1A), in FKHR(DDD) significantly reduced its ability to induce apoptosis as assessed by both morphology and DNA fragmentation (Fig. 4B). As expected, this mutation resulted in a protein that was impaired in its ability to activate transcription from the 3×IRES-luc reporter (data not shown). Thus, the induction of apoptosis by FKHR(DDD) is likely to be dependent, at least in part, on its ability to function as a transcription factor. We postulate that the residual apoptotic activity seen with this mutant protein may be due to residual DNA binding activity and/or synergistic interactions with endogenous forkhead proteins.

Our data suggest that FKHR is a direct target of the protein kinase Akt. The C. elegans AKT/DAF-16 pathway is regulated by an insulin-like receptor and is thought to be involved in reproductive metabolism, dauer state entry, and longevity (15, 16). Given our biochemical data suggesting a role for Akt in the direct phosphorylation and inhibition of FKHR, a similar relationship between AKT-1/2 and DAF-16 would be predicted to exist. Because of its abilities to bind in vitro to the IGFBP-1 IRS and activate transcription from a reporter containing multiple IRSs, FKHR is a candidate mediator of IGFBP-1 expression. Further experiments are necessary to determine whether endogenous FKHR may be an important target of insulin in regulating hepatic IGFBP-1 gene transcription. We also find that FKHR possesses proapoptotic activity, especially when it exists in the more active “dephosphorylated” state. The ability of FKHR to induce apoptosis appears to depend on its transcriptional activity. It will be of considerable interest to determine whether endogenous FKHR may be involved in promoting apoptosis in response to known apoptotic stimuli. If so, FKHR may represent an important regulatory target for anti-apoptotic signals that utilize Akt. While this manuscript was in preparation, it was reported that another member of the forkhead family, which is related to FKHR, is also negatively regulated by Akt (28). It will be of interest to determine whether FKHR and other related family members may possess redundant or nonredundant roles in vivo.

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