An mRNA Splice Variant of the AFX Gene with Altered Transcriptional Activity*

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Several studies indicate that FKHR and AFX, mammalian homologues of the Caenorhabditis elegans forkhead transcription factor DAF-16, function in the insulin signaling pathway. Here we describe the discovery of a novel AFX isoform, which we designated AFXc, in which the first 16 amino acids of the forkhead domain are not present. PCR analysis showed that this isoform is most abundant in the liver, kidney, and pancreas. In HepG2 cells, overexpressed AFXc induced reporter gene activity through the insulin-responsive sequences of the phosphoenolpyruvate carboxykinase (PEPCK), IGFBP-1, and G6Pase promoters. AFXc-mediated stimulation was repressed by insulin treatment, by bisperoxovanadate treatment, and by overexpression of constitutively active protein kinase B (PKB). Insulin treatment and PKB overexpression resulted in phosphorylation of AFXc. Furthermore, 5-aminoimidazole-4-carboxamide-1β-d-ribofuranoside (AICAR), an AMP-activated protein kinase activator, repressed AFXc-dependent reporter activation. Taken together, these findings suggest that AFXc is a downstream target of both the phosphatidylinositol 3-kinase/PKB insulin signaling pathway and an AMP-activated protein kinase-dependent pathway.

An important role of insulin in glucose homeostasis is regulating the transcription of genes critical in glucose metabolism (1). For example, insulin inhibits the expression of genes such as phosphoenolpyruvate carboxykinase (PEPCK) (2, 3), insulin-like growth factor-binding protein-1 (IGFBP-1) (4, 5), and the glucose-6-phosphatase catalytic subunit (G6Pase) (6). Insulin represses gene transcription through cis-acting elements known as insulin responsive sequences (IRs) in the regulatory region of the target genes (reviewed in Ref. 7). The PEPCK promoter contains one IRS (8), while in the IGFBP-1 promoter two copies of IRS are arranged as an inverted palindrome (9, 10). Three tandem copies of IRSs are present in the G6Pase promoter (6, 11).

Proteins involved in the insulin signaling pathway include the insulin receptor, phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB), and downstream transcription factors. Genetic studies suggest that an insulin-like signaling pathway exists in Caenorhabditis elegans. In the nematode, the pathway is composed of DAF-2, AGE-1, and AKT1/AKT2, which are considered the orthologs of mammalian insulin receptor (12), PI3K (13), and PKB (14), respectively. Together, the products of these genes negatively regulate the activity of DAF-16 (15), a forkhead transcription factor that binds IRSs (16). Because the major target of DAF-2/AGE-1 signaling in C. elegans is DAF-16 (14, 17), the orthologs of DAF-16 may represent distal effectors of insulin signaling in mammalian cells (12, 15, 18). The DAF-16 forkhead domain is most similar to those of human FKHR (67% identities) and AFX proteins (64% identities) (15, 16). Therefore, human forkhead factors FKHR and/or AFX may be downstream targets of the insulin-activated PI3K-PKB signaling pathway and also may be responsible for mediating insulin regulation of gene expression (12, 14–16).

AFX was originally identified on chromosome X as an oncogenic fusion protein in acute lymphoblastic leukemia (19, 20) and is involved in the regulation of the cell-cycle (21, 22), apoptosis (23), and tumorigenesis (24). The gene for AFX consists of three exons and is reported to encode a protein of 501 amino acids (25). It is expressed ubiquitously with high levels in placenta and skeletal muscle (19). AFX binds the IRS element from IGFBP-1 and induces a pronounced increase in the activity of a reporter gene under the control of the IGFBP-1 promoter. This transcriptional activation requires an intact IRS, and insulin treatment suppresses the activation through the PI3K-PKB signaling pathway (26). Indeed, AFX contains three putative PKB phosphorylation sites and can be phosphorylated by PKB both in vitro and in vivo (23, 26). Phosphorylation by PKB alters the nuclear import of AFX, shifting its localization from the nucleus to the cytoplasm and thereby inhibiting AFX transcriptional activity (27).

The PI3K-PKB pathway is an important but not unique route by which the activity of forkhead transcription factors are regulated (28, 29). A PI3K inhibitor or a dominant-negative PKB mutant decreases but does not abolish insulin-induced phosphorylation of AFX (26), suggesting the existence of an alternate signaling pathway. A candidate for this alternative pathway is the mammalian AMP-activated protein kinase (AMPK) cascade. AMPK influences many metabolic processes that become dysregulated in the diabetic state (30). However, the mechanism by which AMPK modulates transcriptional activity is unknown due to the fact that the transcription factor(s) involved remains unidentified.

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† The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF384029 (the coding region of AFX).
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1 The abbreviations used are: PEPCK, phosphoenolpyruvate carboxykinase; IGFBP, insulin-like growth factor-binding protein; IRS(s), insulin-responsive sequence(s); PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; G6Pase, glucose-6-phosphatase; AMPK, AMP-activated protein kinase; hPv, potassium bisperoxo(1,10-phenanthroline)oxovanadate; AICAR, 5-aminoimidazole-4-carboxamide-1β-d-ribofuranoside; SEAP, secreted alkaline phosphatase; PEPCK p, PEPCK promoter; myr, myristate.
In this study, we investigated the involvement of AFX in the mammalian insulin signaling pathway. We discovered a novel AFX isoform, AFXζ, characterized its tissue distribution, DNA binding ability, and transcriptional activity. Reporter gene assays demonstrated that AFXζ is a potent transcription activator with properties distinct from those of the previously described isoform, AFXα, and that AFXζ is regulated by the insulin signaling pathway and by an agent known to affect AMPK activity. AFXζ may represent an integration point of the insulin signaling and AMPK pathways, allowing these pathways to regulate downstream gene expression cooperatively.

**EXPERIMENTAL PROCEDURES**

**Oligonucleotides—**Oligonucleotides for AFX amplification were based on the sequence in GenBank™ accession number X93996. All primers were synthesized by Life Technologies, Inc. The sequences added to facilitate cloning are represented in lowercase: 5′-GAA-GACTGGCAGGAATGTGCCTCCTGG-3′; 3′-5′-GAA-ACTTGGCTTCACTGAAC-3′ and 5′-GCCGCTGGCTCCAC-ATCTGGAAGCAGG-3′; 5′-gaaggcactATGGATCCGGGAATGAG-3′; 3′-2′-gaaggcactTACCGGCTGCTGCAAGG-3′; 5′-3′-CTTGTCG-GAGGCTTCACTGAAC-3′; and 3′-5′-GCAGGTCTGACGTGCTTCTT-3′.

Cloning of AFX cDNA—AFX cDNA was amplified using primers 5′-1 and 3′-1 from Marathon-Ready human liver and heart cDNA libraries (CLONTECH). Nested primers 5′-2 and 3′-2 were used to perform a second round PCR. Conditions for the PCR reactions were: 94 °C for 1 min; 94 °C for 15 s, 68 °C for 2 min, repeat for 30 cycles; and 68 °C for 3 min and dwell at 15 °C. The PCR products were digested with Sall and cloned into the Xhol site of pCDNA6/His A (Invitrogen, Carlsbad, CA) and separately into the SarI site of pET-30a (Novagen, Madison, WI). The sequences of the constructs were confirmed using dRhodamine Terminator Cycle Sequencing Kits on an ABI 377 machine (Applied Biosystems).

**Genomic PCR and Multiple Tissue cDNA PCR of AFX—**Human genomic DNA from four individuals were kindly provided by Dr. Chuck Yang Yu (Columbus, Ohio). Human MTC cDNA panels I and II were purchased from CLONTECH. The Expand High Fidelity PCR System (Roche Molecular Biochemicals) was used to perform PCR amplifications with primers 5′-3 and 3′-3. PCR products were separated on 2% agarose gels. DNA was stained with Vistra Green and was visualized on the Storm FluorImager system (Molecular Dynamics, Sunnyvale, CA). The sequences of genomic PCR products were analyzed using programs in the GCG package.

**Expression and Purification of His-tagged AFX—**His-tagged AFXζ was expressed in Escherichia coli BL21 strain after induction by 0.4 mM isopropyl-1-thio-β-D-galactopyranoside at 30 °C for 4 h. The bacterial fusion protein was purified using TALON metal affinity columns (CLONTECH) according to the manufacturer’s instructions.

**In Vitro Translation of AFX—**AFXα and AFXζ proteins were translated in vitro from pCDNA6/His-AFXα and pCDNA6/His-AFXζ templates, respectively. The TNT T7 quick coupled transcription/translation system (Promega, Madison, WI) was utilized according to the manufacturer’s protocol. One microgram of the expression construct was used in each reaction.

**Electrophoretic Mobility Shift Assay—**The DNA binding abilities of in vitro translated AFXα, AFXζ, and purified bacterial fusion protein His-tagged AFXζ were tested in a gel shift assay system (Promega). IRS elements from PECK, IGFBP1, and G6Pase promoters were used as probes. Briefly, oligonucleotides for the sense and antisense strand of each IRS were annealed, and double-stranded fragments were end-labeled using T4 polynucleotide kinase. The manufacturer’s protocol was followed except that [γ-32P]ATP (NEN Life Science Products, Boston, MA) was used in substitution of the suggested [γ-32P]ATP to label the DNA fragment. About 2 × 10^6 cpm probe was used in each binding reaction. For competition experiments, a 50 molar excess of each corresponding unlabeled oligonucleotide was added to the reaction. One hundred nanograms of in vitro translated AFX protein or 200 ng of purified His-tagged AFXζ was used in each binding reaction. The DNA-protein complexes were separated on 6% DNA retardation gel (Invitrogen). Gels were dried prior to autoradiography.

**Generation of Reporter Constructs—**Double-stranded DNA fragments containing each of the IRS elements from the promoters of PECK, IGFBP-1, and G6Pase were cloned into the Nhel/BgIII site of the pSEAP2-promoter vector (CLONTECH). Dissected fragments of the PECK and IGFBP-1 IRS elements, referred to as PECK-a and PECK-b, and IGFBP-1a and IGFBP-1b, respectively, were cloned into the same site of the pSEAP2-promoter. In addition, a 501-bp fragment corresponding to the human PECK 5′ regulatory sequence (−458 + 43 relative to the transcription start site, abbreviated as PECK p) was cloned into the BglII/EcoRI site of the same vector.

**Cell Culture, Transient Transfection, and Reporter System Analysis—**The human hepatoma cell line HepG2 was cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Life Technologies). Transient transfection experiments were performed in Dulbecco’s modified Eagle’s medium supplemented with 10% dialyzed fetal bovine serum (HyClone Laboratories) in 24-well plates. HepG2 cells were transfected using LipofectAMINE 2000 (Life Technologies) with 0.2 μg (unless otherwise specified) of appropriate SEAP reporter construct CMV-β, and either pcdNA6/His-AFXα or -AFXζ expression plasmid. Each set of reporter assays includes a transfection in which no AFX expression construct was added. In experiments where PKB effects were tested, 0.2 μg of myr-Akt1 in pUSEamp (+) (Upstate Biotechnologies, Lake Placid, NY), which expresses constitutively active PKB, was included. For experiments in which the cells were treated with soluble agents, the medium was changed 24 h after the transfection and was replaced with medium containing appropriate concentrations of bpV (potassium bisperoxo(1,10-phenanthroline) oxovanadate, Alexis Biochemicals, San Diego, CA), porcine insulin, or AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribosanofuranose, Sigma) for 15–18 h. Secreted alkaline phosphatase (SEAP) and β-galactosidase activities were measured using PEP-Tag and Galacto-Star systems (Tropix Inc., Bedford, MA), respectively.

**Cellular Fractionation and Western Blotting—**HepG2 cells were grown in 100-mm dishes and were transfected with 4 μg of pCDNA6/His-AFXα and/or myr-Akt1 or were treated for 12 h with insulin (100 nM). Cells were washed with phosphate-buffered saline, were removed gently from the plate by scraping, and were pelleted by centrifugation. Cells were lysed for 1 min on ice in 0.2 ml of mild extraction buffer (10 mM HEPES, pH 8, 1.5 mM MgCl2, 10 mM KCl, 5 mM dithiothreitol, 0.5% Nonidet P-40, complete protease inhibitor mixture (Boehringer), and 1 mM sodium orthovanadate.) The lysate was transferred to a microcentrifuge tube and was spun for 3 min on setting 3 in an Eppendorf 5415C Microfuge. The resulting supernatant was collected and the cytosolic fraction, and the residual nuclear pellet was resuspended in 0.1 ml of nuclear extraction buffer (10 mM HEPES, pH 8, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, complete protease inhibitor mixture, and 1 mM sodium orthovanadate) and placed on ice for 15 min. The tubes subsequently were spun for 10 min in a microcentrifuge at maximum speed, and the resulting nuclear extract was transferred to a new tube for storage. Protein concentrations were determined using the DC Protein Assay (Bio-Rad, Hercules, CA).

For Western blotting, 20 μg of cellular extract was subjected to SDS-PAGE with subsequent blotting to nitrocellulose. AFXζ and phospho-AFXζ proteins were detected using an AFX antibody (Santa Cruz Biotechnology sc5224) or a phospho-AFX (Ser-193) antibody (NEB 9471) according to the manufacturers’ recommendations.

**RESULTS**

Full-length AFX cDNA Sequence—We amplified AFX cDNAs by PCR from a human liver cDNA library using a primer set based on GenBank™ sequence X93996 (25). In all cloned ampiclons, a single A nucleotide was found to be absent 20 nucleotides downstream of the previously designated initiation codon (Fig. 1B). This same “deletion” was also identified in PCR products using human heart cDNA library as DNA template. Using primers 5′-3 and 3′-3, we amplified the identical sequence from four independent human genomic DNA samples. Further confirmation for the absence of this A nucleotide comes from GenBank™ sequence Y11284, the gene sequence of human AFX exon 1. Correction of the sequence leads to slight changes of the predicted amino acid sequence of the protein N terminus as the initiating codon is shifted upstream (Fig. 1B, sequence 2) to the most likely initiation codon as predicted by the ATGpr program (31). The coding region of human AFX thus consists of 653 amino acids (Fig. 1C). The predicted forkhead domain is located at amino acids 97–184 of the revised sequence.

A Novel Isoform of Human AFX—Further analyses of the cloned AFX cDNAs revealed the presence of a shorter splice variant that encodes a shorter protein lacking amino acids 209–458.
AFXζ, a Novel Forkhead Transcription Factor Splice Form

58–112 (Fig. 1C); we designate this alternate form as AFXζ and will refer to the longer form as AFXα. As shown in Fig. 1A, the termini of the 165-bp cDNA region that is not present in AFXζ conform to the consensus sequences of splice junctions, suggesting that this deletion represents a cryptic intron. The amino acids previously identified as PKB phosphorylation sites in AFXα are retained in AFXζ as Thr-32, Ser-142, and Ser-207, but the Myb DNA binding homology domain of AFXα is absent in AFXζ.

Tissue Expression Pattern of AFXζ mRNA—To investigate the tissue distribution of the AFXζ alternate splice form, PCR reactions were performed on first-strand cDNAs from different tissues using primer set 5–3 and 3–3 (Fig. 1D) that generates a 424-bp product for AFXα and a 259-bp product for AFXζ. Among PCR amplifications from 16 different tissues, the 424-bp fragment representing AFXα was ubiquitous, while the 259-bp fragment from AFXζ was more tissue-specific. The greatest expression of AFXζ was observed in liver, kidney, and pancreas RNA (lanes 6, 8 and 9). It was also readily detectable in heart and placenta (lanes 2 and 4). Lung, skeletal muscle, spleen, thymus, and small intestine expressed AFXζ at lower levels (lanes 5, 7, 10, 11, and 15). AFXζ transcripts were not detected in RNA samples from brain, prostate, testis, ovary, colon, and leukocyte (lanes 3, 12, 13, 14, 16, and 17).

DNA Binding of AFXζ—Because part of the forkhead DNA binding domain (amino acids 97–184 in AFXα, Fig. 1C) is absent in AFXζ, we examined whether AFXζ protein can bind DNA fragments that harbor IRSs. In electrophoretic mobility shift assays, a His-tagged AFXζ bacterial fusion protein bound the IRS elements from the PEPCK (Fig. 2B, lane 2), IGFBP1 (lane 5) and G6Pase (lane 8) promoters. A single DNA-protein complex was detected in each experiment. Corresponding unlabeled DNA fragments competed for binding of the probes (lanes 3, 6, and 9). In vitro translated proteins for AFXζ (Fig. 2C) and AFXα (Fig. 2D) were also used as the protein sources for gel shift assays, and a variety of shifted bands were observed. Multiple DNA-protein complexes have been shown previously in the binding of GST-AFXζ and Trx-AFXα to an IRS within the IGFBP-1 promoter (26, 32). Therefore, AFXζ protein binds IRS fragments even though it lacks the first 16 amino acids of the forkhead domain as identified in AFXα.

Higher Transcriptional Activity of AFXζ—To test the transcriptional activity of AFXα and AFXζ, cellular assay systems were developed. Reporter constructs were generated by inserting the fragments spanning the IRS elements of the PEPCK, IGFBP-1, and G6Pase promoters (Fig. 2A) or an expanded DNA fragment of the PEPCK promoter (−458−+43) regulatory sequence into the pSEAP2-promoter vector. First, we determined the transcriptional role of AFXζ on the expression of a reporter driven by the PEPCK IRS (−425−−399) and the full-length promoter (−458−+393 of PEPCK (PEPCK p). Fig. 3A shows that in HepG2 cells, AFXζ activated both PEPCK IRS and PEPCK p-enhanced reporter transcription in a dose-dependent manner and to a similar extent, suggesting that transactivation at this single IRS accounts for the entire stimulation.

We next compared the ability of AFXα and AFXζ to activate reporters containing previously characterized IRSs in HepG2 cells. As shown in Fig. 3B, AFXα expression activated the IGFBP-1 reporter −4-fold. This result is consistent with previous observations that AFXα induces a 6-fold increase of reporter chloramphenicol acetyltransferase activity when under the control of the IGFBP-1 promoter (26). AFXζ failed to activate the reporters containing the IRS elements from PEPCK and G6Pase promoters or the reporter containing the extended PEPCK promoter region (−458−+43). On the other hand, AFXζ not only stimulated IGFBP-1 reporter transcription to a high level (−7-fold, Fig. 3B), AFXζ also increased transcription of the IRS containing PEPCK and G6Pase and extended the PEPCK promoter constructs to 7–9-fold. Therefore, AFXζ appeared to have a broader and more potent ability to activate transcription when compared with AFXα.

The PEPCK IRS overlaps with the consensus binding sequences for HNF3 and C/EBP; the IGFBP-1 IRS also overlaps with an HNF3 site (33–36). To investigate in more detail the sequence elements mediating transcriptional activation by both AFXα and AFXζ, a number of reporter constructs were generated by inserting dissected PEPCK and IGFBP-1 IRS
fragments (Fig. 2A) into the pSEAP2-promoter vector. These reporter constructs were cotransfected into HepG2 cells with either the AFXα or the AFXζ expression plasmids. Again, AFXζ activated all reporter constructs tested to a degree greater than that achieved with AFXα (Fig. 3B). It is noteworthy that AFXζ activated both PEPCk-a (IRS and HNF-3 sites) and PEPCk-b (IRS and C/EBP sites) 4-fold, while the effect on PEPCk (all three sites) appeared to be higher (8-fold). This suggested that AFXζ, unlike AFXα, can act cooperatively with factors binding at both the HNF3 and C/EBP sites. In the case of the IGFBP-1 promoter, AFXα appeared to require both IRS sites to mediate transcriptional activation. These observations suggested that AFXζ may be less stringent or have altered DNA binding affinity compared with AFXα.

Repressive Effect of Insulin on the Transcriptional Activity of AFXζ—We tested whether the transactivational properties of AFXζ respond to insulin treatment. In HepG2 cells, AFXζ activated the reporter transcription under the control of PEPCk promoter (Fig. 3). When the cells were treated with increasing concentrations of insulin, the induced PEPCk p-SEAP activity decreased to 50% compared with the untreated cells (Fig. 4A). Insulin also inhibited the AFXζ-induced PEPCk IRS-SEAP activity in a dose-dependent manner (Fig. 4B), decreasing reporter activity to the basal level at 200 nM insulin. Additionally, we observed that insulin repressed PEPCk IRS-SEAP in cells in which exogenous AFXζ had not been introduced (data not shown). Because reverse transcription-PCR analysis showed that the AFXζ mRNA splice variant is expressed in HepG2 cells (data not shown), this insulin-dependent inhibition may be exerted through endogenous AFXζ. Our data suggest that insulin suppresses PEPCk expression and that AFXζ can mediate this effect through IRS.

Repressive Effect of Bisperoxovanadate and PKB on the Transcriptional Activity of AFXζ—We also investigated the effect of bisperoxovanadate (bpV), an inhibitor of phosphatases (37) that can deactivate components of the insulin signaling pathway by dephosphorylation (38). Similar to the effect seen with insulin, bpV reduced the AFXζ-induced PEPCk p-SEAP activity in a dose-dependent manner (Fig. 5A). The addition of bpV repressed reporter gene activity at concentrations as low as 0.5
MuM. More significantly, the reporter activity was diminished by 5 \mu M bpV to below the basal level. We observed toxicity of bpV in HepG2 cells above 5 \mu M as evidenced by the reduction of the control \beta-galactosidase activity. The data suggest that the suppressive effect of insulin on the transcriptional activity of AFX\zeta may be mediated through a phosphatase-sensitive pathway.

To assess the role of the PI3K/PKB insulin signaling pathway in the suppression of AFX\zeta transactivation, a plasmid encoding constitutively active PKB was cotransfected with an AFX\zeta expression construct and the PEPCK p-SEAP reporter construct. Expression of constitutively active PKB has been shown to mimic the effect of insulin by inhibiting the transcriptional activity of AFX\zeta in a manner consistent with the described behavior of AFX\zeta (27). No clear evidence of phosphorylation on Ser-142 of AFX\zeta was seen upon AICAR stimulation.

Repressive Effect of AICAR on the Transcriptional Activity of AFX\zeta—As shown above, the treatment of HepG2 cells with bisperoxovanadate (Fig. 5A) inhibited the reporter activity of PEPCK IRS to a greater extent than with maximally effective concentrations of insulin (Fig. 4) or with the overexpression of constitutively active PKB (Fig. 5B). This observation suggested to us that bisperoxovanadate may act through mechanisms in addition to the PI3K/PKB pathway. Although the activities of forkhead proteins are thought to be regulated by the insulin signaling pathway through the effect of PI3K and PKB, it is possible that additional pathways are involved (14, 28, 29, 40). AMPK acts at the transcriptional level to regulate gene expression in mammals (41–43), but the transcription factor(s) mediating the effect remains unidentified. Recently, AICAR, an activator of AMPK, has been shown to mimic the effect of insulin in regulating the expression of PEPCK and G6Pase (44).

We used AICAR to treat HepG2 cells that had been cotransfected with an AFX\zeta expression construct and PEPCK IRS-or
were transiently transfected pcDNA6/His-AFX (GenBank™ accession NP-061259), which also contains 505 amino acids. During the process of cloning AFX from the human liver and heart libraries, we discovered the novel isoform, AFX\textsubscript{\textgamma}, gene and named it AFX\textsubscript{\textgamma} and we discovered a novel splice variant of the forkhead factor AFX (GenBank™ accession X93996, (25)), which recognizes phosphorylation of Ser-142 on AFX\textsubscript{\textgamma}, and was subsequently stripped and reprobed with an AFX (C-terminal) antibody (lower panel). B, a prominent phospho-AFX\textsubscript{\textgamma} (Ser-142) band appears in the cytosol, but not the nucleus, of insulin and PKB-treated AFX\textsubscript{\textgamma} transfectants. Cytosolic and nuclear fractions from similarly treated cells were subjected to cellular fractionation experiments indicated that phosphorylation of Ser-142 appears in the nuclear fraction of HepG2 cells.

**PEPCK p-SEAP reporter constructs.** As shown in Fig. 7, increasing concentrations of AICAR progressively inhibited AFX\textsubscript{\textgamma}-induced SEAP activity. At 200 \textmu M, AICAR reduced the transcriptional activity of AFX\textsubscript{\textgamma} on the PEPCK promoter to 48% of untreated cells (Fig. 7A). A similar reduction by AICAR treatment was also observed when PEPCK IRS-SEAP was used as the reporter(Fig. 7B). At 200 \textmu M AICAR, the induced SEAP activity was decreased to 20% of the stimulation ascribed to transfected AFX\textsubscript{\textgamma}. The data show that AICAR mimics the insulin effect in repressing AFX\textsubscript{\textgamma}-induced gene expression and that this effect can be mediated through IRSs. These findings support the hypothesis that AMPK mediates effects on transcription through modulation of forkhead transcription factors.

**DISCUSSION**

In this study we note a corrected sequence for the forkhead transcription factor AFX (GenBank™ accession X83996, (25)), and we discovered a novel splice variant of the AFX gene and named it AFX\textsubscript{\textgamma}. The revised sequence for human AFX protein, which we designated AFX\textsubscript{\textgamma}, contains 505 amino acids and shares 87.7% identity with the mouse AFX protein (GenBank™ accession NP-061259), which also contains 505 amino acids. During the process of cloning AFX from the human liver and heart libraries, we discovered the novel isoform, AFX\textsubscript{\textgamma}, in which 55 amino acids, including the first 16 amino acids of the forkhead domain (Fig. 1C), are absent. The three characterized PKB phosphorylation sites in AFX\textsubscript{\textgamma} (26) are conserved in AFX\textsubscript{\textgamma} as Thr-32, Ser-142, and Ser-207.

A recent structural analysis of AFX\textsubscript{\textalpha} shows that it exhibits the forkhead winged-helix fold that is characterized by three major \alpha-helices and only helix 3 binds DNA (45, 46). AFX\textsubscript{\textalpha} and AFX\textsubscript{\textgamma} showed similar in vitro DNA binding activities (Fig. 2), indicating that the differences between the isoforms do not grossly disrupt the structure of the forkhead protein domain even though most of AFX\textsubscript{\textalpha} helix 1 is replaced by other amino acids in AFX\textsubscript{\textgamma}. However, the sequence of the predicted AFX\textsubscript{\textgamma} helix 1 mirrors the alignment of hydrophobic (Leu-54) and anionic (Glu-53 and Asp-60) amino acids that were shown to participate in the protein core and the protein-solvent interface, respectively (45). Thus, AFX\textsubscript{\textgamma} conserves key structural aspects of helix 1, and this conservation evidently translates into similar DNA binding in the gel shift assays. However, because Pro-52 limits the length of helix 1, we predict that this helix is truncated by one turn in AFX\textsubscript{\textgamma}. Our gel shift data do not address the question of whether AFX\textsubscript{\textgamma} and AFX\textsubscript{\textalpha} show subtle qualitative or quantitative differences in DNA binding because of the changes within helix 1.

An unexpected phenomenon observed in this study is that AFX\textsubscript{\textgamma} appears to activate the reporter transcription to higher levels than AFX\textsubscript{\textalpha} (Fig. 3). As others have noted for similar forkhead factors, insulin repressed the transcriptional activity of AFX\textsubscript{\textgamma} (Fig. 4). This inhibition appears to be mediated through the PI3K-PKB pathway, as treatment with bpV, an inhibitor of protein phosphatases, or overexpression of constitutively active PKB both suppressed the AFX\textsubscript{\textgamma}-stimulated reporter activity (Fig. 5). Because it is a potent transcriptional activator of genes crucial to glucose metabolism as well as a downstream target of activated PKB, AFX\textsubscript{\textgamma}, like FKHR, may mediate gene regulation by insulin. Moreover, as shown in Fig. 6A, both insulin-stimulation and co-expression of constitutively activated PKB phosphorylated AFX\textsubscript{\textgamma} on Ser-142. Furthermore, cellular fractionation experiments indicated that phosphoryl-
activated AFXζ accretes selectively in the cytosolic fraction (Fig. 6B). These results suggest that removal of amino acids 58–112 of AFXζ does not affect its capacity to be phosphorylated and that AFXζ can mediate downstream effects of the insulin signaling pathway.

Our findings for AFXζ are consistent with the model recently advanced by Brownawell et al. (27) for AFXα regulation by insulin. The model suggests that insulin stimulation of the PI3K/PKB pathway results in phosphorylation of AFXα with subsequent cytoplasmic retention leading to a nuclear depletion of the transcription factor.

PCR analysis showed that AFXα is ubiquitously expressed and that AFXζ is more restricted in its expression but that the two isoforms are coexpressed in multiple tissues (Fig. 1D). It is possible that each AFX protein functions on a distinct subset of target genes. For example, in HepG2 cells, while AFXζ activates IRSs in the IGFBP-1, PEPCK, and G6Pase promoters, AFXα did not activate elements within the latter two promoters (Fig. 3). Another possibility is that the two forms may antagonize each other (47) in their transcriptional activities as ers (Fig. 3). Another possibility is that the two forms may antagonize each other (47) in their transcriptional activities as

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The tissue-specific expression pattern of AFX isoforms may also lead to functional consequences (49, 50). In all tissues tested, AFXζ mRNA is expressed at a lower level than that of AFXα, but the strongest expression of AFXζ is in the liver, kidney, and pancreas (Fig. 1D). The pancreas and the liver are crucial to glucose metabolism, and both are severely affected during the progression of diabetes. In comparison, AFXζ is expressed at high levels in placenta and skeletal muscle (19). The localization of both AFX isoforms in tissues important to glucose metabolism, coupled with their different transactivating properties on key metabolic genes, suggests to us combinatorial possibilities for the transcriptional control of glucose homeostasis by the AFX isoforms.

We speculate that the AFX splice forms might fulfill different functions based on the fact that AFXζ lacks the Myb DNA-binding homologous region, which is present between amino acids 91–125 in AFXα (25). The myb gene family includes the proto-oncogene c-myb and two structurally related genes, A- and B-myb (51). B-myb is regulated in a cell cycle-dependent manner (52) and is important for the differentiation of neuroblastoma cells (53). It is possible that the Myb homologous region in AFXζ plays an important role in its involvement in cell cycle (21, 22), apoptosis (23), and in tumorigenesis (24). Considering both the possible role of the Myb region and the differential transcriptional activities of AFX proteins, we reason that the two isoforms of AFX may display distinct functions: AFXζ may regulate cell-cycle, apoptosis, and tumorigenesis, whereas AFXζ is a distal effector of the insulin signaling pathway.

Treatment of HepG2 cells with bisperoxovanadate (Fig. 5A), a broad spectrum inhibitor of phosphotyrosine phosphatase (37), inhibited the reporter activity under the control of PEPCK promoter to a greater extent than with maximally effective concentrations of insulin (Fig. 4) or over-expression of constitutively active PKB (Fig. 5B). These observations suggest that

peroxovanadate may act by mechanisms other than inhibiting the dephosphorylation of insulin receptor, PI3K, PKB, and AFXζ. Our findings support the emerging notion that the PI3K-PKB pathway is necessary but not unique in regulating forkhead factors (26, 54, 55). Moreover, Brunet et al. reported that PKB does not effectively catalyze the phosphorylation of FKHR1 at Ser-315 in vitro (40). Peptide maps of mouse FKHR1 show phosphorylated residues apart from the PKB-mediated phosphorylation sites (56). Taken together, it is possible that other route(s) distinct from the PI3K/PKB insulin signaling pathway may mediate phosphorylation of forkhead factors (14, 28, 29, 40).

AMPK is a component of a highly conserved protein kinase cascade (57), and it influences many metabolic processes that become altered in the diabetic state (reviewed in Ref. 30). Application of AICAR is one of the most specific methods for activating AMPK in intact cells (58). Recently, Lochhead et al. (44) showed that treatment of hepatoma cells with AICAR mimics the effect of insulin to repress the expression of the two key gluconeogenic genes, PEPCK and G6Pase. They hypothesized that AMPK and insulin may lie on distinct pathways that join at a point upstream of the two promoters (44). We speculate that the FKHR subgroup of forkhead factors may be a converging point linking the insulin signaling pathway and AMPK pathway in coordinately regulating the expression of important genes involved in gluconeogenesis. Indeed, we demonstrated that treating HepG2 cells with AICAR caused a dose-dependent inhibition of AFXζ-mediated PEPCK IRS and promoter activity (Fig. 7). Therefore, AMPK appeared to be involved in the regulation of the transcriptional activity of AFXζ, possibly by phosphorylating AFXζ at sites other than Ser-142. Winder et al. hypothesized that activation of AMPK may partially correct the metabolic perturbations in type 2 diabetes resulting from defects in the insulin signaling cascade (30). Our data suggested that AFXζ lies at the crossroads of insulin and AMPK signaling, and therefore may have profound impact in the treatment of type 2 diabetes.

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