Phosphorylation of Serine 256 by Protein Kinase B Disrupts Transactivation by FKHR and Mediates Effects of Insulin on Insulin-like Growth Factor-binding Protein-1 Promoter Activity through a Conserved Insulin Response Sequence*

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Insulin inhibits the expression of multiple genes in the liver containing an insulin response sequence (IRS) (CAAAA(C/T)AA), and we have reported that protein kinase B (PKB) mediates this effect of insulin. Genetic studies in Caenorhabditis elegans indicate that daf-16, a forkhead/winged-helix transcription factor, is a major target of the insulin receptor-PKB signaling pathway. FKHR, a human homologue of daf-16, contains three PKB sites and is expressed in the liver. Reporter gene studies in HepG2 hepatoma cells show that FKHR stimulates insulin-like growth factor-binding protein-1 promoter activity through an IRS, and introduction of IRSs confers this effect on a heterologous promoter. Insulin disrupts IRS-dependent transactivation by FKHR, and phosphorylation of Ser-256 by PKB is necessary and sufficient to mediate this effect. Antisense studies indicate that FKHR contributes to basal promoter function and is required to mediate effects of insulin and PKB on promoter activity via an IRS. To our knowledge, these results provide the first report that FKHR stimulates promoter activity through an IRS and that phosphorylation of FKHR by PKB mediates effects of insulin on gene expression. Signaling to FKHR-related forkhead proteins via PKB may provide an evolutionarily conserved mechanism by which insulin and related factors regulate gene expression.

Insulin exerts important effects on gene expression in multiple tissues (1). In the liver, insulin suppresses the expression of a number of genes that contain a conserved insulin response sequence (IRS)1 (CAAAA(C/T)AA), including insulin-like growth factor-binding protein-1 (IGFBP-1), apolipoprotein CIII (apoCIII), phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (2–6). This observation suggests that insulin may regulate the expression of multiple hepatic genes through a common mechanism. Insulin rapidly suppresses the expression of IGFBP-1 and PEPCK at the transcriptional level, and this effect is not disrupted by pretreatment with cycloheximide (7, 8), indicating that it is mediated by post-translational modification of pre-existing factors, perhaps by their phosphorylation. Specific factors that mediate the inhibitory effects of insulin on hepatic gene expression through a conserved IRS remain to be identified.

Recent studies indicate that protein kinase B (PKB) functions downstream from phosphatidylinositol 3′-kinase (PI3K) in the insulin signaling pathway (9, 10) and that it plays an important role in mediating effects of insulin and related growth factors on glucose and amino acid transport, glycogen and protein synthesis, and cell survival (11–19). Following its activation, PKB is translocated to the nucleus where it may exert effects on gene expression (20, 21). Activated PKB increases the expression of leptin and fatty acid synthase in adipocytes (22, 29) and suppresses PEPCK mRNA levels in liver-derived cells stimulated by cAMP and glucocorticoids (24), mimicking the effects of insulin. Based on studies using pharmacological inhibitors and dominant negative and constitutively active forms of signaling peptides, we recently reported that PKB is both necessary and sufficient to mediate sequence-specific effects of insulin on basal promoter activity through an IRS (25). We now have sought to identify downstream mechanisms that mediate effects of insulin on gene expression through an IRS downstream from PKB.

Recent genetic studies in Caenorhabditis elegans indicate that specific members of the forkhead/winged-helix family of transcription factors may be major targets of insulin receptor signaling downstream from PKB. Mutation of the insulin/IGF-I receptor homologue (daf-2), the catalytic subunit of PI3K (age-1), or PKB (akt-1 and akt-2) in C. elegans results in increased longevity and constitutive dauer formation (26–28), a stage of developmental arrest and reduced metabolic activity that enhances survival during periods of food deprivation and other environmental stresses (29). In each case, mutation of daf-16, which codes for a forkhead/winged-helix transcription factor, restores a normal life span and prevents entry into the dauer stage (28, 30, 31). These observations have suggested that daf-16 promotes entry into the dauer phase and enhanced longevity and that signaling via the insulin/IGF-I receptor-PI3K-PKB pathway may disrupt these effects of daf-16 (28, 30, 31).
Interestingly, analysis based on a consensus sequence for phosphorylation by PKB (Arg-Xaa-Arg-Xaa-Xaa-(Ser/Thr)-Hyd) (32) indicates that daf-16 contains four PKB phosphorylation sites (28), suggesting that it may be a direct target for signaling by PKB. Also, Ruvkun and co-workers (28) have noted that daf-16 can interact directly with an IRS in vitro. Several of these PKB sites are conserved in a group of closely related human forking proteins, including FKHR, FKHR1L, AFX1, and AF6q21 (33–36). FKHR is expressed in the liver (34) and has three of these conserved PKB sites, including Thr-24, Ser-256, and Ser-319. In separate studies, we have found that each of these sites in FKHR is phosphorylated by PKB in vitro and in cells (37). The DNA binding domains of FKHR and daf-16 also are highly conserved, suggesting that FKHR may interact with and regulate promoter activity through an IRS in vivo. As a first step in evaluating the role that FKHR and/or closely related forking proteins may play in mediating effects of insulin on hepatic gene expression, we asked whether FKHR may regulate the activity of the IGFBP-1 promoter through a conserved IRS and mediate effects of insulin on promoter activity downstream from PKB.

MATERIALS AND METHODS

Plasmid Construction and Mutagenesis—The Sau3A-HgaI fragment of the IGFBP-1 promoter, which extends 320 bp 5’ from the RNA cap site, was cloned into pGL2 (Promega) (BP1.Luc) and modified to create other reporter gene constructs using unique NheI and BamHI sites flanking the insulin response element, as previously reported (25). Oligonucleotides containing an array of three IRSs (5’-GAAGCTTCAACACTGCAAAACAAGTA-3’) (TFK81.IRS3) or an unrelated sequence of similar length containing two Gal4 sites (5’-GCTCCGGAG-GACAGTAGCCTGGAGGAGCATAGCTGGTCCAGCGG-3’) (TFK81). Gal4 were cloned into the KpnI-HindIII site in the polylinker region located immediately 5’ to the 81-bp thymidine kinase promoter in TK81.Luc (39).

The complete FKHR cDNA cloned into pCMV-5 (37) was used for initial functional studies. The FKHR cDNA containing 224 bp of 5’- and 133 bp of 3’-untranslated sequence in pFB-12A2 (34) was subcloned into the XbaI-Accl site in pμT-MAX (Promega) downstream from the CMV promoter for site-directed mutagenesis according to the manufacturer’s directions. Single-stranded DNA templates were prepared with helper phage R408 for mutagenesis using the following oligonucleotides. Thr-24–Ala(Asp), 5’-TGTTGTTGTCCATGGC-3’; Ser-256–Ala(Asp), 5’-CTGTGTTGTTGCTCATGGC-3’; Ser-319–Ala(Asp), 5’-ACTAATA-3’; Ser-319–Ala(Asp), 5’-ACTAATA-3’. All mutations were verified by dideoxy sequencing. The BamHI-NolI fragment containing 511 bp of the FKHR cDNA including 224 bp of 5’-untranslated sequence also was excised from pFB-12A2 and cloned into pcDNA3.1 (+) (Invitrogen) in the antisense orientation (FKHR.AS) for antisense studies.

Expression vectors for dominant negative forms of Raf (C4bRaf) and the 85-kDa regulatory subunit of PI3K (D) were transfected without the FKHR expression vector. The mean ± S.E. are indicated.

RESULTS

Effect of FKHR on IGFBP-1 promoter activity. HepG2 cells were transfected in triplicate with 10 μg of plasmid DNA including 3 μg of a luciferase reporter gene construct containing the native IGFBP-1 promoter (BP1.Luc), 2 μg of a CMV-driven β-galactosidase expression vector plus 0, 0.03, 0.1, 0.3, 1, 3, or 10 μg of a CMV-driven FKHR expression vector and appropriate amounts of empty vector. Cells were stabilized in serum-free medium for 18 h before lysates were prepared for analysis of luciferase and β-galactosidase activity levels. Levels of reporter gene activity are expressed relative to control dishes transfected without the FKHR expression vector. The mean ± S.E. are shown.

To determine whether FKHR may influence the expression of genes that are regulated by insulin through an IRS, we first performed transient transfection studies in HepG2 cells with a luciferase reporter gene construct containing the IGFBP-1 promoter (BP1.Luc) and a β-galactosidase expression vector driven by the CMV promoter. As shown in Fig. 1, co-transfection with an FKHR expression vector stimulates the IGFBP-1 promoter and luciferase activity in a dose-dependent fashion without altering levels of β-galactosidase expression, indicating that this effect is promoter-specific.

We next examined whether this effect of FKHR is mediated through an IRS. The IGFBP-1 promoter contains 2 IRSs located ~100 bp 5’ to the RNA cap site (IRSA (CAAAAACAA) and IRSB (TTATTTTG)), each is sufficient to mediate negative effects of insulin on promoter activity (3, 25). As shown in Table IA, mutation of IRSA and IRSB (BP1.mut) disrupts both the ability of insulin to inhibit and the ability of FKHR to stimulate promoter activity, whereas the presence of either IRSA

![FIG. 1. Effect of FKHR on IGFBP-1 promoter activity. HepG2 cells were transfected in triplicate with 10 μg of plasmid DNA including 3 μg of a luciferase reporter gene construct containing the native IGFBP-1 promoter (BP1.Luc), 2 μg of a CMV-driven β-galactosidase expression vector plus 0, 0.03, 0.1, 0.3, 1, 3, or 10 μg of a CMV-driven FKHR expression vector and appropriate amounts of empty vector. Cells were stabilized in serum-free medium for 18 h before lysates were prepared for analysis of luciferase and β-galactosidase activity levels. Levels of reporter gene activity are expressed relative to control dishes transfected without the FKHR expression vector. The mean ± S.E. are shown.](image-url)
Phosphorylation of FKHR Mediates Insulin Action

Table I

| Construct | Insulin | FKHR |
|-----------|---------|------|
|           | % inhibition | % stimulation |
| A. BP1.Luc | 44 ± 5 | 847 ± 66 |
| BPI.Luc mut | −7 ± 7 | 109 ± 21 |
| ΔIRS.A | 53 ± 5 | 758 ± 92 |
| ΔIRS.B | −1 ± 14 | 39 ± 8 |
| ΔIRS.B mut | 58 ± 6 | 365 ± 24 |
| ΔIRS.1 | −50 ± 14 | 20 ± 5 |
| ΔIRS.1M | 53 ± 4 | 839 ± 108 |
| −8 ± 5 | 112 ± 33 |
| B. TK81.Luc | −5 ± 8 | 12 ± 3 |
| Thym-Kin | 44 ± 11 | 618 ± 35 |
| Gal4 - Gal4 | −6 ± 11 | 37 ± 10 |

(ΔIRS.A) or IRSB (ΔIRS.B) alone is sufficient to mediate effects of insulin and FKHR on promoter function. Mutation of a single base pair within either IRSA (ΔIRS.Amut) or IRSB (ΔIRS.B mut) disrupts the effects of both insulin and FKHR on promoter activity, suggesting that both insulin and FKHR exert their effects in a sequence-specific fashion. Placing IRS3 3 bp from its native location to disrupt potential interactions with flanking sequences also mediates effects of insulin and FKHR on promoter activity, whereas placing a mutated sequence at this location (ΔIRS.1M) is not effective. As shown in Table I, introducing an array of IRSs (TK81.IRS3) immediately upstream from the thymidine kinase promoter also is sufficient to confer effects of both insulin and FKHR on promoter activity. In contrast, introducing another sequence of similar length containing two Gal4 binding sites at this location is not effective. Taken together, these results indicate that FKHR, like insulin, exerts effects on promoter activity through an IRS.

To determine whether the effects of insulin and FKHR on promoter activity are mediated through an IRS with similar sequence specificity, we created a series of reporter gene constructs where individual residues within a single IRS are mutated one at a time. As shown in Table II, point mutations which disrupt the ability of insulin to inhibit promoter activity also disrupt the ability of FKHR to stimulate promoter function. As shown in Fig. 2, this construct disrupts the ability of FKHR to stimulate promoter activity through an IRS-dependent mechanism.

Subsequent studies with this antisense construct revealed that it reduces the activity of the IGFBP-1 promoter activity in a dose-dependent fashion without decreasing the level of β-galactosidase expressed by a CMV-driven vector, indicating that this effect is promoter-specific (data not shown). As shown in Fig. 4B, the ability of FKHR antisense RNA to inhibit activity of the IGFBP-1 promoter (BP1.Luc) is abolished when both IRSA and IRSB are mutated (BP1.mut), whereas the presence of either IRSA (ΔIRS.A) or IRSB (ΔIRS.B) alone is sufficient to render the IGFBP-1 promoter responsive to inhibition by FKHR antisense RNA. Similarly, introducing an array of IRSs 5’ to the TK promoter is sufficient to confer a negative effect of FKHR antisense RNA on the activity of this heterologous promoter. These results support the hypothesis that endogenous FKHR and/or closely related proteins contribute to basal promoter activity through an IRS-dependent mechanism.

Next we used this antisense construct to determine whether endogenous FKHR may contribute to the regulation of IGFBP-1 promoter activity in HepG2 cells, we next performed studies with a CMV-driven vector that expresses 244 bp of the 5’-untranslated region and the first 287 bp of the FKHR coding region in an antisense orientation. As shown in Fig. 4A, this construct disrupts the ability of FKHR to stimulate promoter activity through an IRS but does not interfere with the ability of HNF-3β to stimulate promoter function through a consensus HNF-3 binding sequence placed at the same location. This result indicates that this antisense construct disrupts IRS-dependent transactivation by FKHR selectively.

Table I

| Construct | Luciferase activity |
|-----------|-------------------|
|           | Insulin | FKHR |
| A. BP1.Luc | 44 ± 5 | 847 ± 66 |
| BPI.Luc mut | −7 ± 7 | 109 ± 21 |
| ΔIRS.A | 53 ± 5 | 758 ± 92 |
| ΔIRS.B | −1 ± 14 | 39 ± 8 |
| ΔIRS.B mut | 58 ± 6 | 365 ± 24 |
| ΔIRS.1 | −50 ± 14 | 20 ± 5 |
| ΔIRS.1M | 53 ± 4 | 839 ± 108 |
| −8 ± 5 | 112 ± 33 |
| B. TK81.Luc | −5 ± 8 | 12 ± 3 |
| Thym-Kin | 44 ± 11 | 618 ± 35 |
| Gal4 - Gal4 | −6 ± 11 | 37 ± 10 |
The effects of insulin and FKHR on promoter activity was examined for the IRS (D) point mutation (D) IRS.A) alone or an array of IRSs introduced upstream (D) IRS.B) with/without 100 nM insulin for 18 h prior to lysis and analysis of luciferase activity.

| Construct | Luciferase activity |
|-----------|---------------------|
| ΔIRS.1    | GCTAGATGCAAAAACCTTGTCGAC |
| ΔIRS.1m1  | GCTAGATGCAAAAACCTTGTCGAC |
| ΔIRS.1m2  | GCTAGATGCAAAAACCTTGTCGAC |
| ΔIRS.1m3  | GCTAGATGCAAAAACCTTGTCGAC |
| ΔIRS.1m4  | GCTAGATGCAAAAACCTTGTCGAC |
| ΔIRS.1m5  | GCTAGATGCAAAAACCTTGTCGAC |
| ΔIRS.1m6  | GCTAGATGCAAAAACCTTGTCGAC |
| ΔIRS.1m7  | GCTAGATGCAAAAACCTTGTCGAC |
| ΔIRS.1m8  | GCTAGATGCAAAAACCTTGTCGAC |
| ΔIRS.1M   | GCTAGATGCAAAAACCTTGTCGAC |

TABLE II

Sequence-specific effects of insulin and FKHR

HepG2 cells were transfected with 10 μg of DNA/dish including 3 μg of a reporter gene construct containing a single IRS (ΔIRS.1) with/without mutation of individual base pairs (ΔIRS.1m1-ΔIRS.1m8) or multiple residues (ΔIRS.1M) with/without 1 μg of FKHR expression vector and appropriate amounts of empty vector. Cells were refed with serum-free medium with/without 100 nM insulin for 18 h prior to lysis and analysis of luciferase activity.

| Insulin | % inhibition | % stimulation |
|---------|--------------|---------------|
| Insulin | 40 ± 5       | 709 ± 118     |
| FKHR    | 15 ± 4       | 108 ± 27      |
| ΔIRS.1M | 41 ± 6       | 277 ± 84      |
| ΔIRS.1M | −9 ± 5       | 26 ± 16       |
| ΔIRS.1M | 4 ± 2        | 7 ± 5         |
| ΔIRS.1M | 9 ± 4        | 93 ± 20       |
| ΔIRS.1M | 9 ± 4        | 15 ± 102      |
| ΔIRS.1M | 50 ± 5       | 407 ± 10      |
| ΔIRS.1M | 50 ± 3       | 644 ± 31      |
| ΔIRS.1M | −6 ± 8       | 23 ± 10       |

Fig. 2. Sequence-specific effects of insulin and FKHR on promoter activity. The effects of insulin and FKHR on promoter activity were measured in transient transfection studies in HepG2 cells with reporter gene constructs containing a single IRS (ΔIRS.1) with/without point mutation (ΔIRS.1m1–ΔIRS.1m8) or more extensive mutation of the IRS (ΔIRS.1M) (Table II). The effects of insulin (percent inhibition) and FKHR (percent stimulation) on promoter activity for each construct and the linear regression line are shown. The relationship between the effect of insulin and FKHR on promoter activity was examined for significance by Pearson correlation analysis.

essential and sufficient to mediate this effect of insulin (25). As shown in Fig. 4C, expression of FKHR antisense RNA reduces the activity of the native IGFBP-1 promoter (BP1.Luc), and there is no additional reduction in promoter function by insulin or PKB in the presence of FKHR antisense RNA. Studies with reporter gene constructs containing either IRSA (ΔIRS.A) or IRSB (ΔIRS.B) alone or an array of IRs introduced upstream from the thymidine kinase promoter (TK.IRS3) yielded similar results (Fig. 4C). In each case, FKHR antisense RNA reduces promoter activity and there is no additional inhibitory effect of insulin or PKB on promoter function in combination with FKHR antisense RNA.

We next performed studies to determine whether FKHR antisense RNA disrupts the ability of insulin and PKB to inhibit promoter activity through an IRS selectively or also disrupts other effects of insulin and PKB in HepG2 cells. As shown in Fig. 4D, FKHR antisense RNA, insulin, and PKB each reduce promoter activity to a similar extent in a reporter gene construct containing a single IRS (ΔIRS.1), and there is no additional effect of insulin or PKB on promoter activity in combination with FKHR antisense RNA, consistent with results obtained with other constructs containing an IRS (Fig. 4C). In contrast, we observed that insulin and PKB both stimulate promoter activity when the IRS is replaced by an HNF-3 binding site (ΔIRS.HNF-3) and that FKHR antisense RNA does not disrupt this effect of insulin or PKB (Fig. 4D, right panel). This result confirms that FKHR antisense RNA disrupts the ability of insulin and PKB to suppress promoter activity through an IRS without disrupting other effects of insulin and PKB on promoter activity in HepG2 cells.

Taken together, these results indicate that FKHR, or a closely related factor, is required for the ability of insulin and PKB to suppress promoter activity through an IRS and suggest the possibility that insulin and PKB may inhibit promoter activity.
activity largely by disrupting IRS-dependent transactivation by FKHR. Based on these findings and previous studies indicating that PKB is necessary and sufficient for insulin to inhibit promoter activity through an IRS (25), we next examined whether insulin inhibits FKHR-stimulated promoter activity and whether PKB mediates this effect of insulin.

Phosphorylation of FKHR Mediates Insulin Action

Fig. 4. Effects of FKHR antisense RNA on promoter activity and regulation. A, FKHR antisense RNA disrupts transactivation by FKHR but not HNF-3β. HepG2 cells were transfected with 15 μg of plasmid DNA including 1 μg of ΔIRS.1, ΔIRS.1M, or ΔIRS.HNF-3 reporter gene constructs with/without 3 μg of a CMV-driven FKHR or HNF-3β expression vector and/or 10 μg of the FKHR antisense vector (FKHR.AS) as indicated. Cells were refed with serum-free medium 18 h before analysis of luciferase activity. B,IRS-dependent effect of FKHR antisense RNA on promoter activity. HepG2 cells were transfected with 15 μg of plasmid DNA/dish including 3 μg of reporter gene constructs (BP1.Luc, BP1.mut, ΔIRS.A, ΔIRS.B, ΔIRS.1, ΔIRS.M, TK, and TK.IRS3) and 10 μg of the FKHR.AS vector. C, effects on FKHR antisense RNA, insulin, and PKB on promoter activity through an IRS. Cells were transfected with 16 μg of DNA/dish including 3 μg of reporter gene constructs containing a functional IRS (BP1.Luc, ΔIRS.A, ΔIRS.B, and TK.IRS3) with/without 10 μg of FKHR antisense vector and/or 1 μg of constitutively active PKB expression vector (Myr-PKB). Cells were treated with serum-free medium with/without 100 nM insulin for 18 h before lysis and analysis of luciferase activity. D, effects of FKHR antisense RNA, insulin, and PKB on promoter activity in the presence of an IRS versus HNF-3 binding site. Cells were transfected with 16 μg of plasmid DNA/dish, including 1 μg of ΔIRS.1 or ΔIRS.HNF-3 reporter gene construct with/without 10 μg of FKHR antisense and/or 1 μg of PKB (Myr-PKB) expression vectors. Cells were treated with serum-free medium with/without 100 nM insulin for 18 h before lysis and analysis of luciferase activity.
and co-transfection with a dominant negative form of the 85-kDa regulatory subunit of PI3K (Δp85) block this effect of insulin completely, indicating that insulin suppresses FKHR-stimulated promoter activity through a PI3K-dependent mechanism. Rapamycin, which prevents the activation of p70 S6 kinase downstream from PI3K (45), does not disrupt this effect of insulin. In contrast, expression of a kinase-deficient, dominant negative form of PKB (Lys-179-PKB) blocks the effect of insulin, whereas constitutively active PKB (Myr-PKB) inhibits FKHR-stimulated promoter activity, mimicking the effect of insulin. Together, these results indicate that PKB is necessary and sufficient to mediate the effect of insulin on FKHR-stimulated promoter activity.

FKHR contains three consensus PKB phosphorylation sites (Thr-24, Ser-256, and Ser-319), and we have found that PKB phosphorylates each of these sites in vitro and in cells (37). To determine whether the phosphorylation of these sites is required for insulin or PKB to disrupt transactivation by FKHR, we first mutated Thr-24, Ser-256, and Ser-319 to alanine individually (Thr-24–Ala, Ser-256–Ala, and Ser-319–Ala FKHR) and together ((Thr/Ser/Ser)-Ala FKHR). Mutation of these residues to alanine does not disrupt the ability of FKHR to stimulate promoter activity (Fig. 5B). However, overexpression of (Thr/Ser/Ser)-Ala and Ser-256–Ala (but not Thr-24–Ala or Ser-319–Ala) FKHR completely abolishes the ability of insulin and PKB to inhibit FKHR-stimulated promoter activity (Fig. 5B). Similar studies with the TK.IRS3 construct confirm that overexpression of (Thr/Ser/Ser)-Ala and Ser-256–Ala (but not Thr-24–Ala or Ser-319–Ala) FKHR completely abolishes the ability of insulin and PKB to inhibit promoter function (data not shown). These findings indicate that phosphorylation of Ser-256 is required for the ability of insulin and PKB to disrupt IRS-dependent transactivation by FKHR.

To determine whether the introduction of a negative charge at these sites is sufficient to disrupt the ability of FKHR to stimulate promoter activity, we next mutated Thr-24, Ser-256, or Ser-319 to aspartate. As shown in Fig. 5C, mutation of Ser-256 to aspartate (Ser-256–Asp) disrupts IRS-dependent transactivation by FKHR. In contrast, Thr-24–Asp and Ser-319–Asp mutations do not disrupt the ability of FKHR to stimulate promoter activity through an IRS-dependent mechanism (ΔIRS.1 versus ΔIRS.1M). Similar studies with the TK.IRS3 construct confirm that mutation of Ser-256 (but not Thr-24 or Ser-319) to aspartate disrupts transactivation by FKHR (not shown). Taken together, these results indicate that phosphorylation of Ser-256 by PKB and the introduction of a negative charge at this site is necessary and sufficient to disrupt IRS-dependent transactivation by FKHR.
Phosphorylation of FKHR Mediates Insulin Action

FIG. 6. Comparison of forkhead DNA binding domains for FKHR, FKHRL1, AFX1, AF6q21, daf-16, HNF-3β, and HNF-3γ. The positions of α helices (Helix 1, Helix 2, and Helix 3), β strands (S1, S2, and S3), and wing domains (W1 and W2) and the basic region within the forkhead/winged-helix DNA binding domain are indicated. Residues with greater than 50% homology among FKHR-related proteins (FKHR, FKHRL1, AFX1, AF6q21, and daf-16) are shaded. Serine 256 in FKHR is identified by a star. The percentage of amino acids that are homologous relative to FKHR is shown.

DISCUSSION

In the present study, we sought to determine whether FKHR, a member of the forkhead/winged-helix family of transcription factors, may contribute to the regulation of gene expression by insulin and provide a target for mediating effects of insulin and PKB on gene expression through a conserved IRS. To date, 80 members of the forkhead family have been identified, and many have been found to play an important role in development and in the determination of tissue-specific gene expression (46, 47). Genetic studies in C. elegans have suggested that daf-16 is a major target for signaling by the insulin/IGF-I receptor-PI3K-PKB pathway, based on the effects of mutations on the development and survival of intact organisms (28, 30, 31). A preliminary report by Paradis and Ruvkun, together with Nasrin and Alexander-Bridges (28) also indicates that daf-16 may interact directly with an IRS in vitro. In the present study, we utilized reporter gene constructs in a mammalian cell culture model to demonstrate that FKHR, a human forkhead homologue of daf-16, may contribute to the regulation of promoter activity through an IRS in vitro. The results of these studies demonstrate that FKHR stimulates promoter activity in a highly sequence-specific fashion through an IRS and that phosphorylation of Ser-256 by PKB is necessary and sufficient for insulin to disrupt IRS-dependent transactivation by FKHR. To our knowledge, these findings provide the first direct evidence that FKHR-like forkhead proteins stimulate promoter activity through a conserved IRS and that phosphorylation of FKHR or closely related proteins may mediate sequence-specific effects of insulin on gene expression downstream from PKB.

Analysis based on a known consensus sequence for phosphorylation by PKB (32) suggested that FKHR contains three PKB phosphorylation sites (Thr-24, Ser-256, and Ser-319) and we have found in separate studies that PKB phosphorylates each of these sites in vitro and in cells (37). In the present study, we used pharmacological inhibitors and expression vectors for dominant negative and constitutively active forms of signaling peptides to determine that insulin inhibits FKHR-stimulated promoter activity through a mechanism mediated by PKB. Based on studies where Thr-24, Ser-256, and Ser-319 were mutated to alanine, a neutral amino acid that is not susceptible to phosphorylation by kinases, or to aspartate, which has a negative charge, we showed that phosphorylation of Ser-256 by PKB is necessary and sufficient for insulin to disrupt IRS-dependent transactivation by FKHR. It remains to be determined whether the phosphorylation of Thr-24 or Ser-319 affects other functions of FKHR.

As shown in Fig. 6, Ser-256 is located in the basic region of the DNA binding domain of FKHR. X-ray crystallography performed with HNF-3-γ indicates that this region of the forkhead/winged-helix DNA binding motif forms a random coil within the minor groove of target sites where it may interact with phosphatase residues and stabilize DNA/protein interactions (48). It is interesting to speculate that the phosphorylation of Ser-256 and the introduction of a negative charge at this site might reduce the stability of this interaction and disrupt binding. However, in vivo footprinting and gel shift studies with nuclear extracts have so far failed to detect a nucleoprotein complex involving an IRS whose formation is disrupted by insulin treatment (2, 3, 49), suggesting that other mechanisms also must be considered. Another possibility is that phosphorylation of Ser-256 might disrupt interactions between FKHR and a co-activating factor required for transactivation or induce the recruitment of a co-repressor. Preliminary immunocytochemical studies in this laboratory indicate that phosphorylation by PKB also might result in the redistribution of FKHR from the nucleus to the cytoplasm within cells (2). Additional studies are in progress to examine the specific mechanism(s) by which the phosphorylation of Ser-256 might disrupt IRS-dependent transactivation by FKHR.

It is important to note that this PKB phosphorylation site is conserved in several closely related forkhead family members, including FKHRL1, AFX1, AF6q21, and daf-16 where the serine residue is replaced by a threonine (Fig. 6). Because FKHRL1, AFX1, and AF6q21, like FKHR, are expressed in the liver (34–36), it is possible that they also may be expressed in HepG2 cells and contribute to the regulation of IGFBP-1 promoter activity. In this study, we used an antisense construct to examine the role that endogenous FKHR may play in the regulation of promoter activity. Although our FKHR antisense construct did not disrupt the ability of HNF-3-β to stimulate promoter activity through an HNF-3 binding site, it is possible that it might disrupt the expression of more closely related proteins, including FKHRL1, AFX1, or AF6q21. The observation that this PKB phosphorylation site is conserved in these proteins suggests the possibility that they also may contribute to the regulation of hepatic gene expression by insulin down-

2 J. Wu, K. Colley, and T. Unterman, unpublished observations.
stream from PKB, together with FKHR. Because FKHR, FKHR1L1, AFX1, and AF6q21 are expressed in many tissues (34–36), it is reasonable to speculate that they also may play an important role in mediating the effects of insulin and other growth factors on gene expression in other settings.

The observation that this PKB phosphorylation site is present in daf-16 (Fig. 6) supports the concept that signaling through the insulin/IGF-1 receptor/Pi3K/PKB pathway may disrupt transactivation by daf-16 in C. elegans, consistent with results of genetic studies indicating that signaling via PKB disrupts effects of daf-16 on longevity and dauer formation (28, 30, 31). At the same time, the fact that this phosphorylation site is absent in HNF-3 proteins (Fig. 6) is consistent with results in the present study indicating that PKB no longer inhibits promoter activity when an IRS is replaced by a consensus HNF-3 binding site (Fig. 4D). Several other studies have also indicated that interactions with HNF-3 proteins are insufficient to mediate inhibitory effects of insulin on promoter activity through an IRS (50–52). If insulin and/or PKB do alter the transcriptional activity of HNF-3 members of the forkhead family, it is likely that they do so by a different mechanism.

Another feature, which distinguishes FKHR-related proteins from other members of the forkhead family, is the insertion of five additional amino acids at the N-terminal region of helix 3 within the DNA binding domain (Fig. 6). Crystallographic studies indicate that helix 3 of the forkhead/winged-helix DNA binding motif is presented to the major groove of target sequences where it is thought to play a critical role in sequence-specific interactions (48). Sequences flanking helix 3 also have been found to be important in determining sequence specificity for forkhead proteins (53). It is interesting to speculate that the insertion of these additional amino acids at the N-terminal end of helix 3 may cause FKHR-related members of the forkhead family to interact with a distinct set of related target sequences.

In this context, it is interesting to note similarities in the roles played by daf-16 in C. elegans and genes known to be regulated through an IRS in mammals. In C. elegans, daf-16 function is required for wild-type organisms to enter the dauer phase and enhance survival in response to nutrient deprivation (29). Similarly, several genes that are regulated by insulin in the liver through an IRS are important in the adaptation to nutritional restriction in mammals. The abundance of hepatic IGFBP-1 mRNA and circulating levels of IGFBP-1 are increased 10-fold in short-term fasting (54–56) where high IGFBP-1 mRNA and circulating levels of IGFBP-1 are increased 10-fold in short-term fasting (54–56) where high IGFBP-1 mRNA and circulating levels of IGFBP-1 are elevated in patients with Type 2 diabetes (57–60). It is interesting to speculate that FKHR-related proteins (65–67). Another feature, which distinguishes FKHR-related proteins from other members of the forkhead family, is the insertion of five additional amino acids at the N-terminal region of helix 3 within the DNA binding domain (Fig. 6). Crystallographic studies indicate that helix 3 of the forkhead/winged-helix DNA binding motif is presented to the major groove of target sequences where it is thought to play a critical role in sequence-specific interactions (48). Sequences flanking helix 3 also have been found to be important in determining sequence specificity for forkhead proteins (53). It is interesting to speculate that the insertion of these additional amino acids at the N-terminal end of helix 3 may cause FKHR-related members of the forkhead family to interact with a distinct set of related target sequences.

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