# Protein Kinase B-α Inhibits Human Pyruvate Dehydrogenase Kinase-4 Gene Induction by Dexamethasone Through Inactivation of FOXO Transcription Factors

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**Starvation and diabetes increase pyruvate dehydrogenase kinase-4 (PDK4) expression, which conserves gluconeogenic substrates by inactivating the pyruvate dehydrogenase complex. Mechanisms that regulate PDK4 gene expression, previously established to be increased by glucocorticoids and decreased by insulin, were studied. Treatment of HepG2 cells with dexamethasone increases the relative abundance of PDK4 mRNA, and insulin blocks this effect. Dexamethasone also increases human PDK4 (hPDK4) promoter activity in HepG2 cells, and insulin partially inhibits this effect. Expression of constitutively active PKBα abrogates dexamethasone stimulation of hPDK4 promoter activity, while coexpression of constitutively active FOXO1a or FOXO3a, which are mutated to alanine at the three phosphorylation sites for protein kinase B (PKB), disrupts the ability of PKBα to inhibit promoter activity. A glucocorticoid response element for glucocorticoid receptor (GR) binding and three insulin response sequences (IRSs) that bind FOXO1a and FOXO3a are identified in the hPDK4 promoter. Mutation of the IRSs reduces the ability of glucocorticoids to stimulate PDK4 transcription. Transfection studies with E1A, which binds to and inactivates p300/CBP, suggest that interactions between p300/CBP and GR as well as FOXO factors are important for glucocorticoid-stimulated hPDK4 expression. Insulin suppresses the hPDK4 induction by glucocorticoids through inactivation of the FOXO factors. *Diabetes* 53:899–910, 2004**

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CA-PKB, constitutively active protein kinase B; cGRE, consensus glucocorticoid response element; EMSA, electrophoretic mobility shift assay; G6Pase, glucose-6-phosphatase; GR, glucocorticoid receptor; GRE, glucocorticoid response element; hPDK4, human pyruvate dehydrogenase kinase-4; IGFBP-1, insulin-like growth factor binding protein-1; IRS, insulin response sequence; mGRE2, mutant GRE2; PDC, pyruvate dehydrogenanse complex; PDK4, pyruvate dehydrogenase kinase-4; PEPCK, phosphoenolpyruvate carboxykinase; PDP, PDC phosphatase; PKB, protein kinase B; PPARα, peroxisome proliferator–activated receptor-α.

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In the well-fed state, the pyruvate dehydrogenanse complex (PDC) is relatively active and generates acetyl-CoA, the precursor for fatty acid synthesis and energy production by the Krebs cycle. In the starved state, PDC is relatively inactive, enabling the body to conserve three-carbon compounds for gluconeogenesis (1). Regulation of PDC activity is therefore important for control of glucose and lipid metabolism. PDC kinase (PDK) inactivates PDC by phosphorylating serine residues of the E1α component of the complex. PDC phosphatase (PDP) activates PDC by dephosphorylating E1α. PDC activity, therefore, depends on the extent of E1α phosphorylation, which is determined by PDK and PDP activities (2). Four isoenzymes of PDK have been identified in the human genome (3–5). In most tissues the activity state of PDC is significantly decreased in starvation and diabetes (6,7), while PDK4 expression is dramatically increased (8–12). The latter increases PDK activity, thereby contributing to the inactivation of PDC and the conservation of glucose and substrates (lactate, pyruvate, and alanine) that can be converted to glucose. This helps maintain euglycemia during starvation but exacerbates hyperglycemia in diabetes (2). A high-fat/low-carbohydrate diet also increases PDK4 protein and PDK activity in skeletal muscle (13,14). These findings suggest that the expression level of PDK4 is a major determinant of PDK activity. We have shown that glucocorticoids and peroxisome proliferator–activated receptor-α (PPARα) ligands induce PDK4 expression and that insulin inhibits these effects in a rat hepatoma cell line (15). None of these factors affect message stability, suggesting that hormonal regulation of PDK4 expression occurs at the transcriptional level (15).

Several important enzymes for glucose metabolism are regulated at the transcriptional level in response to glucocorticoids and insulin. Rates of transcription of phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase), and insulin-like growth factor binding protein-1 (IGFBP-1) are increased by cAMP and glucocorticoids, while insulin blocks these effects (16–18). The glucocorticoid response elements (GREs) have been identified in the promoters of PEPCK (19), G6Pase (20), and IGFBP-1 (20,21). Activation of protein kinase B (PKB) by insulin inhibits PEPCK and G6Pase induction by cAMP/dexamethasone (22,23). Basal and glucocorticoid-stimu-

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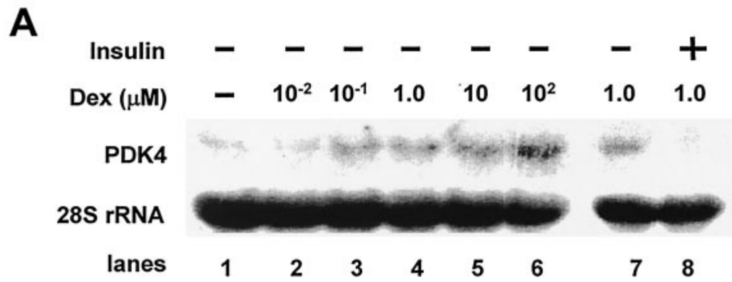


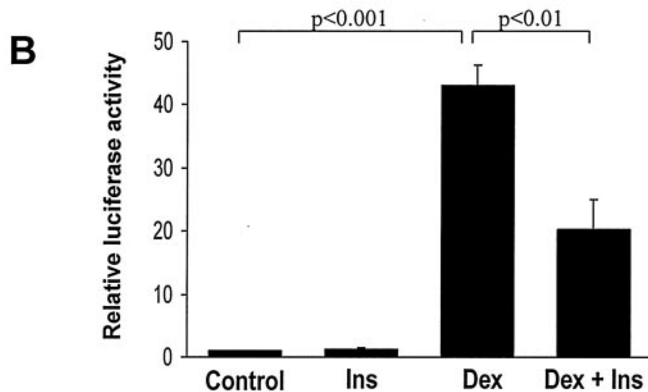


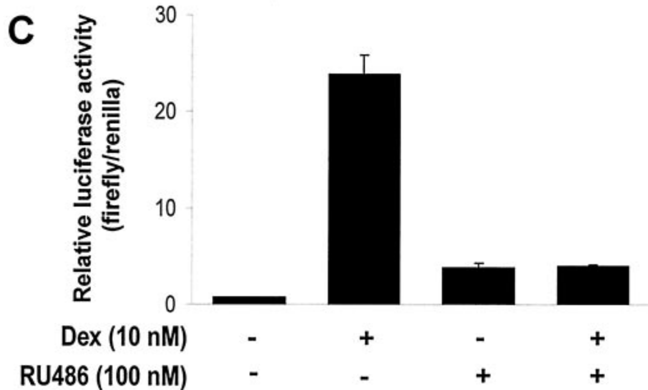


**FIG. 1.** ***A*: Dexamethasone increases and insulin decreases PDK4 expression. HepG2 cells were treated with dexamethsone and/or insulin (100 nmol/l) for 6 h. Total RNA (40 μg) was used for Northern blotting. *B*: GR increases the response to dexamethasone. 1759PDK4-luc (0.7 μg) was cotransfected with p6RGR (0.2 μg) and pRL-TK (0.1 μg). Cells were incubated with dexamethasone (1 μmol/l) and insulin (100 nmol/l). Luciferase activities are fold induction relative to vehicle. *C*: RU486 inhibits its induction by dexamethasone. Data are the ratio of reporter activity to renilla luciferase activity.**

lated expression of the IGFBP-1 gene is also repressed by PKB (17,23,24). Recently the FOXO (subclass of Forkhead Box factors) group, which consists of FOXO1a (FKHR), FOXO3a (FKHRL1), and FOXO4 (AFX1), has been identified as target transcription factors of PKB (25). (The nomenclature used for human FOXO factors is based on the recommendation of Kaestner et al. [26].) Phosphorylation of FOXO factors by PKB inhibits target gene expression by several mechanisms, including translocation of FOXO factors out of the nucleus (27–30), promotion of binding of FOXO factors to 14-3-3 proteins (31), alteration in the binding affinity of FOXO factors to DNA (32), and disruption of the interaction of FOXO factors with coactivators (33). A consensus binding sequence (TTGTTTAC) for FOXO factors has been identified (34), and highly related insulin response sequences (IRSs) have been shown to bind FOXO factors and mediate effects of insulin on the expression of the PEPCK (35–37), G6Pase (23,37–39), and IGFBP-1 (36,37,40,41) genes. These IRSs are also thought to function cooperatively with nearby GREs to enhance glucocorticoid stimulation of promoter activity (17), and interactions with the p300/CBP complex appear to be important for this effect (42).

We examined the mechanisms responsible for the hPDK4 gene regulation by glucocorticoids and insulin. Dexamethasone activates the hPDK4 gene transcription through a GRE in a GR-dependent manner. PKB represses hPDK4 induction by dexamethasone through inactivation of FOXO factors, which bind to IRSs in the hPDK4 promoter. Studies with E1A suggest that interactions between p300/CBP and GR as well as FOXO factors are essential for activation of hPDK4 gene expression. Insulin inhibits induction of the hPDK4 by glucocorticoids through inactivation of FOXO factors. A report (43) that appeared while this article was in preparation showed that Foxo1 binds to an IRS in the mouse PDK4 promoter, which corresponds to the IRS3 identified in the human PDK4 promoter in this study.

## RESEARCH DESIGN AND METHODS

**Northern blot analysis.** HepG2 cells were grown in minimal essential media supplemented with 10% (vol/vol) fetal bovine serum, 1 mmol/l sodium

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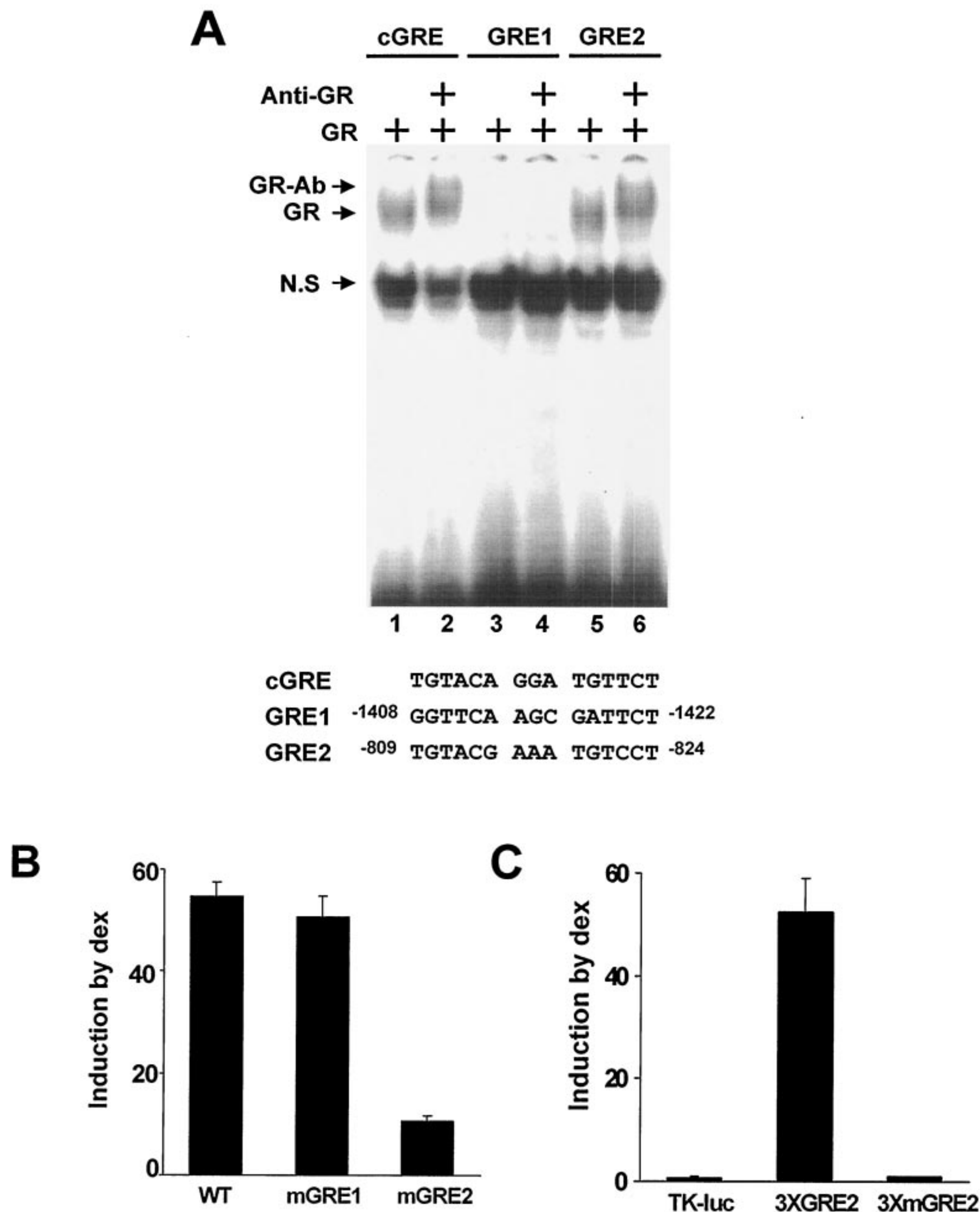


**FIG. 2. GRE in the hPDK4 gene. *A*: EMSA with GR (9.8 μg) and its antibody (2 μg). Consensus GRE and PDK4 GREs (antisense) sequences were used as $^{32}$P-labeled probes. *B*: GRE2 (−824/−809 bp) is a functional element. Mutant GRE1 and GRE2 constructs were used. Data are the ratio of reporter activity with versus without dexamethasone (10 nmol/l). *C*: Dexamethasone activates 3XGRE2-TK transcription. Transfections for *B* and *C* were as in Fig. 1*B*.**

pyruvate, and 1× antibiotic-antimycotic (GibcoBRL). When the cells reached 80% confluency, various concentrations of dexamethasone and insulin (100 nmol/l) were added to the medium. After 6 h of incubation, total RNA was extracted from cells using Ultraspec RNA isolation system following the manufacturer's instruction. Forty micrograms of total RNA were used for Northern blotting as previously described (10). The membrane was hybridized with $^{32}$P-labeled hPDK4 cDNA probe and 28S rRNA antisense oligonucleotide as loading control.

**Plasmid construction.** The hPDK4 promoter (−1,759/+222 bp and −848/+222 bp) was generated by PCR using human chromosome clone y61-d4 (y16d7) from UK HGMP Resource Centre as template (5) and constructed by cloning into the pGL3-basic (Promega). The *Bgl*II-*Hind*III HSV TK promoter fragment from pRL-TK vector (Promega) was inserted into pGL3-basic (TK-luc). Oligonucleotides with three copies of GRE2 and mutant GRE2 (mGRE2) were synthesized; 3XGRE2: 5′ CTAG AGGACATTTCGTACA AGGACATTTCG TACA AGGACATTTCGTACA 3′; 3XmGRE2: 5′ CTAG AGG**TG**ATTTCGT**TG**A AGG**TG**ATTTCGT**TG**A AGG**TG**ATTTCGT**TG**A 3′. Substituted sequences are indicated with bold letters. Annealed 3XGRE2 and 3XmGRE2 oligonucleotides with *Nhe*I-*Xho*I sites were cloned into the TK-luc polylinker. All constructs were confirmed by sequencing. Expression vectors for human GR (p6RGR) and human constitutively active PKBα/Akt1 were generously provided by Dr. David Crabb (Indiana University) and Dr. Richard Roth (Stanford University) (44), respectively. Expression vectors for constitutively active TSS-Ala FOXO1a and transcriptionally inactive FOXO1a (Helix3mut FOXO1a) have been described previously (23). Expression vectors for wild-type and constitutively active human FOXO3a were gifts from Dr. Michael Greenberg (Harvard Medical School) (28); E1A wild-type and mutant E1A Δ2-36 were gifts from Dr. David Livingstone (Dana-Farber Cancer Institute) (45).

**Mammalian cell culture and transient expression assays.** Using METAFECTENE (Biontex, Germany), HepG2 cells with 50–60% confluency in 12-well plates were transfected with the hPDK4 promoter construct and pRL-TK and in some experiments with expression vectors for various proteins as indicated in the figure legends. Cells were subsequently incubated for 24 h with vehicle, dexamethasone (Biomol), RU486 (Sigma), and insulin (Calbiochem) (concentrations indicated in the figures or figure legends) 24 h after transfection. Cells were lysed in 250 μl of 1× Passive reporter lysis buffer

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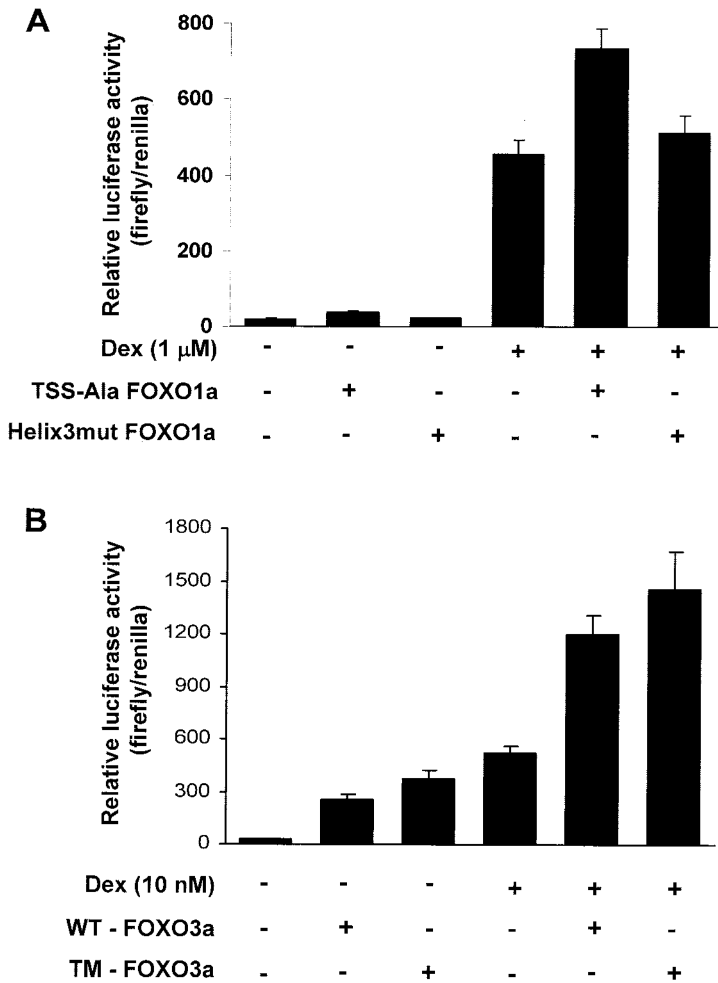


**FIG. 3. FOXO1a and FOXO3a transactivate PDK4 transcription.** ***A*****: 848PDK4-luc (0.6 μg), p6RGR (0.2 μg), and pRL-TK (0.1 μg) were cotransfected with TSS-Ala FOXO1a or helix3mut-FOXO1a (0.2 μg).** ***B*****: Transfection was conducted as in** ***A*** **with wild-type FOXO3a (WT-FOXO3a) or triple mutant FOXO3a (TM-FOXO3a).**

(Promega). Luciferase activities were measured using a Dual-luciferase reporter assay system (Promega). All transfection studies were performed in four independent experiments and repeated with plasmids prepared separately at least twice. Data are presented as means ± SE for four independent experiments. Sigma Plot, Version 3.02, was used for statistical analysis.

**In vitro transcription and translation of FOXO3a.** The *Hind*III-*Xba*I fragments of constitutively active FOXO3a cDNA were isolated from expression vectors generously provided by Dr. Michael Greenberg (28) and then cloned into pcDNA3 to use the T7 promoter (FOXO3a/pcDNA3). FOXO3a protein was in vitro translated from FOXO3a/pcDNA3 using TNT T7 coupled reticulocyte lysate system (Promega).

**Electrophoretic mobility shift assays.** The following oligonucleotides were synthesized for electrophoretic mobility shift assays (EMSAs) (all sequences are sense strands; core sequences for DNA binding are in bold). Consensus GRE: 5′ ACGT**TGTACA**GGA**TGTTCT** 3′; GRE1: 5′ ACGT**AGAATC**GCT**TGAACC** 3′; GRE2: 5′ ACGT**AGGACA**TTT**CGTACA** 3′; IRS1: 5′ ACGTTTAATT**TTGTTTTT**AATTA 3′; IRS2: 5′ ACGTTTTAGG**TTGTTTAT**TCCT 3′; IRS3: 5′ ACGTGCCTCCGAGTT**GTAAACAA**GGGCG 3′. Oligonucleotides were annealed and end labeled with [α-$^{32}$P]dATP (DuPontNEN) using Klenow enzyme. The labeled probe (200,000 cpm) was incubated with human recombinant GR (Panvera), human GST-FOXO1a (Upstate), and in vitro translated constitutively active FOXO3a in 10 mmol/l HEPES, pH 7.9, 60 mmol/l KCl, 1 mmol/l EDTA, 7% (vol/vol) glycerol, 1 mmol/l dithiothreitol, 2 μg poly (dI-dC), and 0.5% FBS for 20 min at room temperature. Unlabeled competitor oligonucleotides and 2 μg of each anti-GR (sc-1003X), anti-FOXO1a (sc-11350X), and anti-FOXO3a (sc-11351) (Santa Cruz Biotechnology) were added to the binding mixtures. DNA protein binding was analyzed on a nondenatured (4% wt/vol) acrylamide gel in 0.5× Tris-borate-EDTA buffer.

**Site-directed mutagenesis.** Mutant constructs of GRE1 (AT at −1418/−1419 bp to TG; AC at −1409/−1410 bp to TG) and GRE2 (AC at −821/−820 bp to TG; AC at −812/−811 bp to TG) were prepared by site-directed mutagenesis (Stratagene) using 1759PDK4-luc as template. Mutant constructs for IRS1 (GT at −736/−735 bp to CA), IRS2 (GT at −531/−530 bp to CA), and IRS3 (CA at −357/−356 bp to GT) were prepared using 848PDK4-luc as template. All mutant constructs were sequenced to confirm mutations. Three different sets of plasmids for transfection were prepared separately using Wizard plus miniprep kit (Promega).

## RESULTS

**Regulation of hPDK4 expression by dexamethason and insulin.** Treatment of HepG2 cells with dexamethasone increased PDK4 mRNA message in a concentration-dependent manner (Fig. 1*A*, lanes 1–6). Insulin inhibited the effect of dexamethasone (Fig. 1*A*, lanes 7 and 8), as observed with 7,800 C1 cells (15). To study hPDK4 gene regulation at the transcriptional level, a luciferase reporter plasmid containing the hPDK4 promoter sequence and its 5′ untranslated region extending from −1759 to +222 bp (1759PDK4-luc) was cotransfected with the human GR expression vector, p6RGR, into HepG2 cells (Fig. 1*B*). As expected from previous findings that HepG2 cells express very low levels of GR (46), dexamethasone had little effect on PDK4 promoter activity without cotransfected GR (data not shown). However, when GR is coexpressed, dexamethasone increased hPDK4 promoter activity over 40-fold ($P < 0.001$ compared with cells not treated with dexamethasone). Insulin inhibited hPDK4 expression induced by dexamethasone by >40% ($P < 0.05$, dexamethasone-treated vs. dexamethasone plus insulin-treated cells). To validate that transcriptional activation by dexamethasone is GR-dependent, transfected cells were treated with RU486 (GR antagonist) in the absence or presence of

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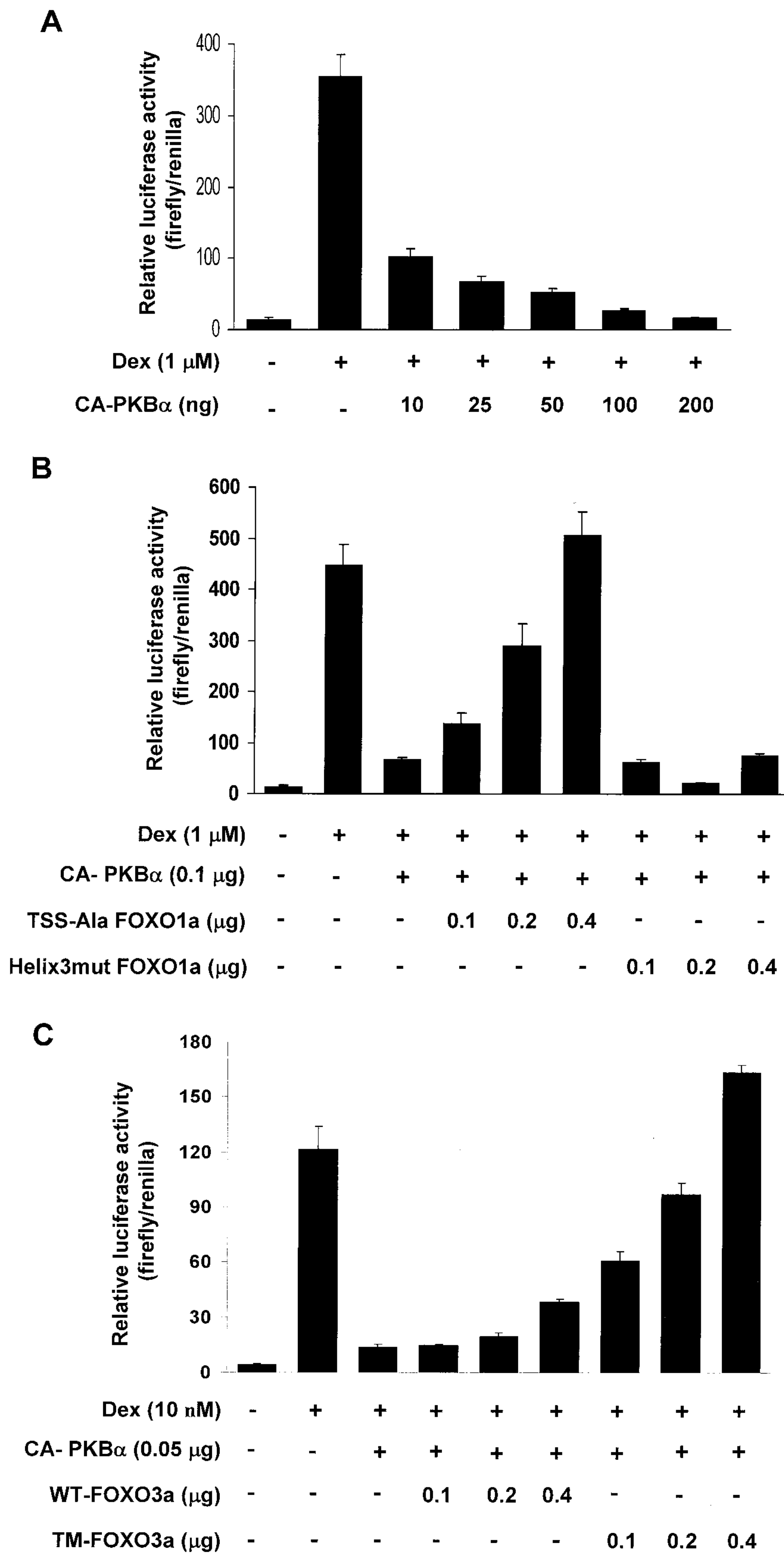


**FIG. 4.** ***A*: Constitutively active PKBα abrogates dexamethasone induction of hPDK4. 848PDK4-luc (0.6 μg), p6RGR (0.2 μg), and pRL-TK (0.1 μg) were cotransfected with CA-PKBα. FOXO1a (*B*) and FOXO3a (*C*) prevent CA-PKBα inhibition of hPDK4 expression. 848PDK4-luc (0.5 μg), p6RGR (0.2 μg), and pRL-TK (0.1 μg) were cotransfected with CA-PKBα, TSS-Ala FOXO1a, helix3mut-FOXO1a, WT-FOXO3a, and TM-FOXO3a.**

dexamethasone. Although RU486 induced some activation of 1759PDK4-luc expression, as expected from its effects on GR (46), RU486 completely inhibited dexamethasone-stimulated gene expression (Fig. 1*C*). These findings suggest that glucocorticoids regulate expression of the hPDK4 gene via activation of the GR.

**GR binds to the region −824/−809 bp within the hPDK4 promoter.** Computer analysis using MatInspector

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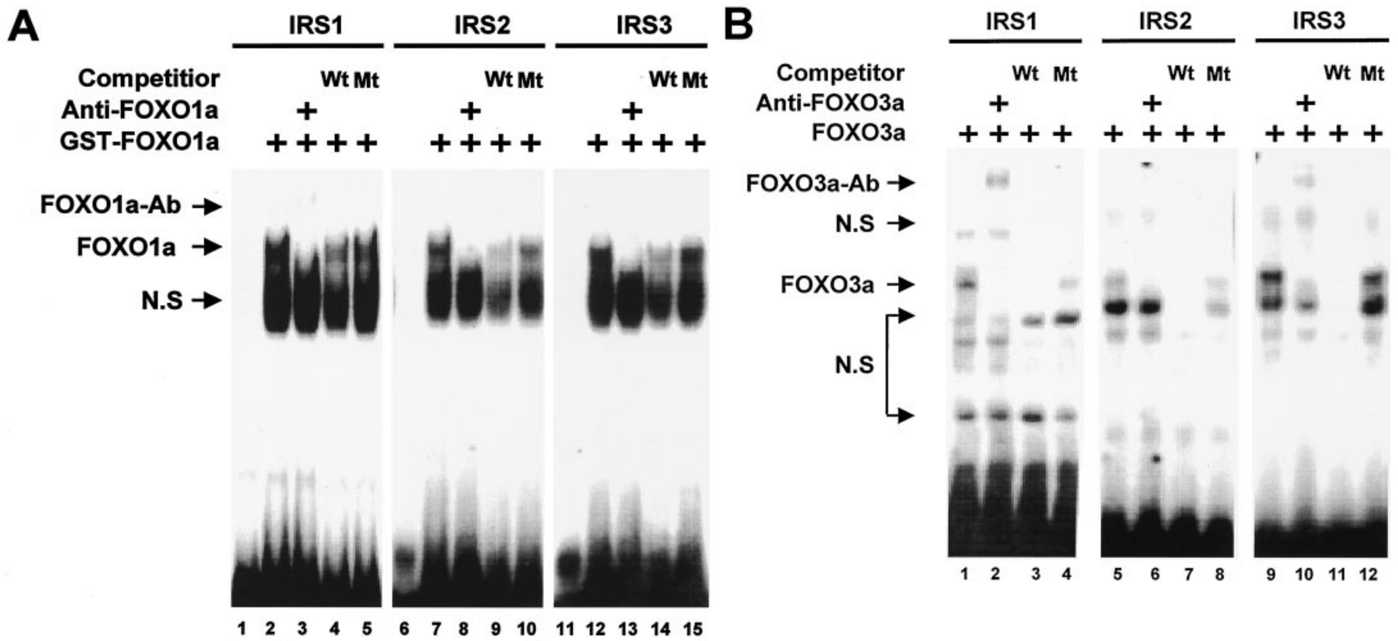


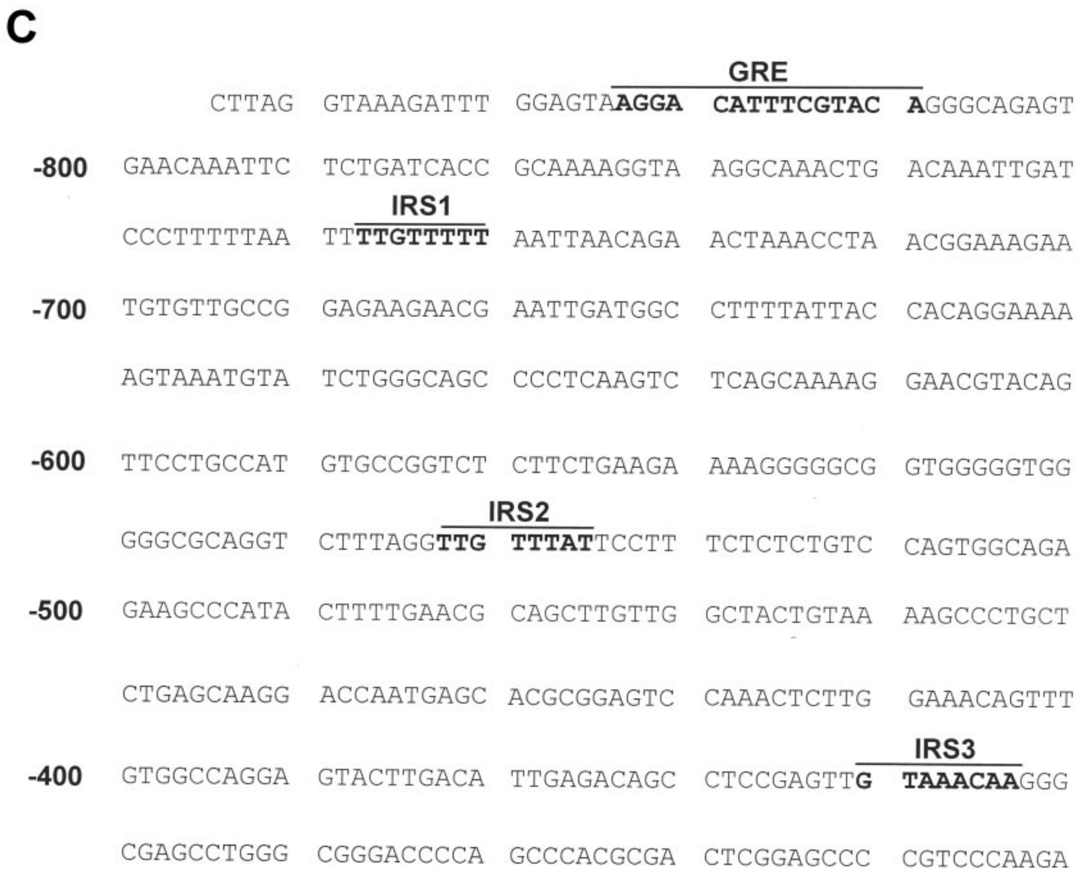


FIG. 5. FOXO1a (*A*) and FOXO3a (*B*) bind to regions −738/−731 bp (IRS1), −533/−526 bp (IRS2), and −361/−354 bp (IRS3) in the hPDK4 promoter. GST-FOXO1a (0.67 μg) (*A*) and FOXO3a (4 μl) (*B*) with appropriate antibodies (2 μg) and $^{32}$P-labeled probes containing IRS sequences were used for EMSA. Unlabeled oligonucleotides (200-fold molar excess) were used for competition. Core sequences for FOXO binding are in bold; mutated sequences in small letters. N.S., nonspecific binding. *C*: hPDK4 promoter sequences.

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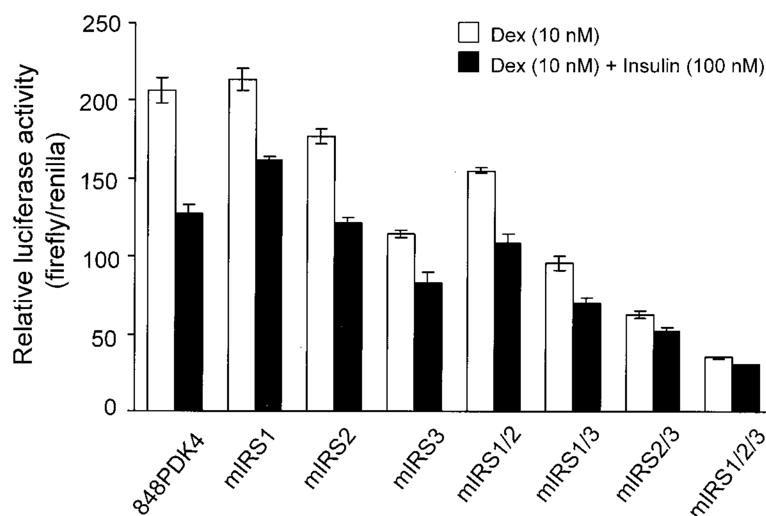


**FIG. 6. IRSs are required for dexamethasone induction and insulin inhibition. Transfection with 848PDK4-luc and corresponding IRS mutant constructs was as in Fig. 1*B*.**

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program (www.genomatix.de) revealed two potential GRE sequences in the hPDK4 promoter: the −1422/−1408 bp region (GRE1) and the −824/−809 bp region (GRE2). To determine whether GR binds to these elements, EMSA was carried out with purified GR and its antibody (Fig. 2*A*). GR binds to a consensus GRE (cGRE) as well as to the putative GRE2 sequence but not to the putative GRE1 (Fig. 2*A*, lanes 1, 3, and 5). Anti-GR antibody supershifts the bands formed with cGRE and GRE2, confirming that GR binds to the GRE2 (Fig. 2*A*, lanes 2 and 6). Furthermore, mutation of the GRE2 element in 1759PDK4-luc virtually abrogated induction of PDK4 promoter activity by dexamethasone, whereas mutation of the GRE1 element was without effect (Fig. 2*B*). Three copies of GRE2 sequences (−824/−809 bp, 3XGRE) and mutant GRE2 (3XmGRE) were cloned into TK-pGL3-basic (see RESEARCH DESIGN AND METHODS) in order to study whether GRE2 element per se activates transcription in response to dexamethasone. 3XGRE2 was strongly activated by dexamethasone (~60-fold), but no induction was observed with TK-luc or 3XmGRE2 (Fig. 2*C*). These findings suggest that glucocorticoids induce PDK4 gene expression through GRE2 in a GR-dependent manner.

**Constitutively active FOXO1a and FOXO3a increase both hPDK4 basal expression and induction by dexamethasone.** Because FOXO factors have been shown to contribute to the ability of glucocorticoids to stimulate the expression of other insulin-regulated genes, we asked whether they also contribute to regulation of hPDK4 expression. 848PDK4-luc reporter construct were cotransfected with constitutively active TSS-Ala FOXO1a, which is mutated to alanine at the three phosphorylation sites for PKB, or with transcriptionally inactive helix3mut-FOXO1a, which is mutated to arginine at histidine 215, resulting in loss of DNA binding activity (23,41). TSS-Ala FOXO1a increased not only hPDK4 basal expression but also induction by dexamethasone by about twofold, while helix3mut-FOXO1a had no effect (Fig. 3*A*). Greater amounts of FOXO1a did not induce further increase in promoter activity, suggesting HepG2 cells may express enough FOXO1a as shown previously (47). FOXO3a was also examined to determine whether it activates hPDK4 gene transcription since FOXO3a is also expressed in HepG2 cells (36). Wild-type FOXO3a (WT-FOXO3a) and constitutively active triple mutant FOXO3a (TM-FOXO3a), which is mutated to alanine at the three phosphorylation sites for PKB (28), activated hPDK4 basal expression up to 13-fold (Fig. 3*B*). This activation is comparable to the ~20-fold induction of hPDK4 by dexamethasone. FOXO3a also increased hPDK4 induction by dexamethasone ~2.7-fold. These findings suggest that FOXO1a and FOXO3a are important for basal as well as dexamethasone-induced hPDK4 expression.

**Constitutively active PKBα blocks the induction of hPDK4 by dexamethasone; constitutively active FOXO1a and FOXO3a prevent PKB inhibition.** To determine whether insulin might repress hPDK4 expression through PKB, constitutively active PKBα (CA-PKB) was cotransfected with 848PDK4-luc (Fig. 4*A*). CA-PKBα completely inhibited dexamethasone-stimulated hPDK4 induction in a concentration-dependent manner. As shown in Fig. 3, FOXO factors appear to cooperate with GR to activate hPDK4 gene transcription. Therefore, PKB may inhibit the dexamethasone-stimulated hPDK4 induction through inactivation of endogenous FOXO factors. Therefore exogenously introduced TSS-Ala FOXO1a and TM-FOXO3a, which cannot be phosphorylated by PKB, may prevent hPDK4 repression by PKB. As predicted, TSS-Ala FOXO1a completely prevented PDK4 repression by PKB in a dose-dependent manner ($P < 0.001$ at the highest tested concentration), whereas helix3mut FOXO1a had no effect (Fig. 4*B*). Likewise, TM-FOXO3a completely prevented hPDK4 repression by PKB. WT-FOXO3a was less effective than TM-FOXO3a (Fig. 4*C*), in agreement with the previous finding that PKB phosphorylates and inactivates FOXO3a (28). These results suggest that FOXO factors cooperate with GR to activate hPDK4 gene in response to glucocorticoids and that the ability of PKB to repress hPDK4 induction by dexamethasone may require phosphorylation of FOXO factors.

**FOXO1a and FOXO3a bind to the regions −738/−731 bp (IRS1), −533/−526 bp (IRS2), and −361/−354 bp (IRS3) in the hPDK4 promoter.** Computer analysis shows that the hPDK4 promoter has three potential IRSs for FOXO binding. To determine whether FOXO1a and FOXO3a bind to these elements, the three potential IRSs were employed for EMSA with purified GST-FOXO1a or in vitro translated FOXO3a. Probes containing IRS1, IRS2, and IRS3 formed DNA-protein complexes with FOXO1a

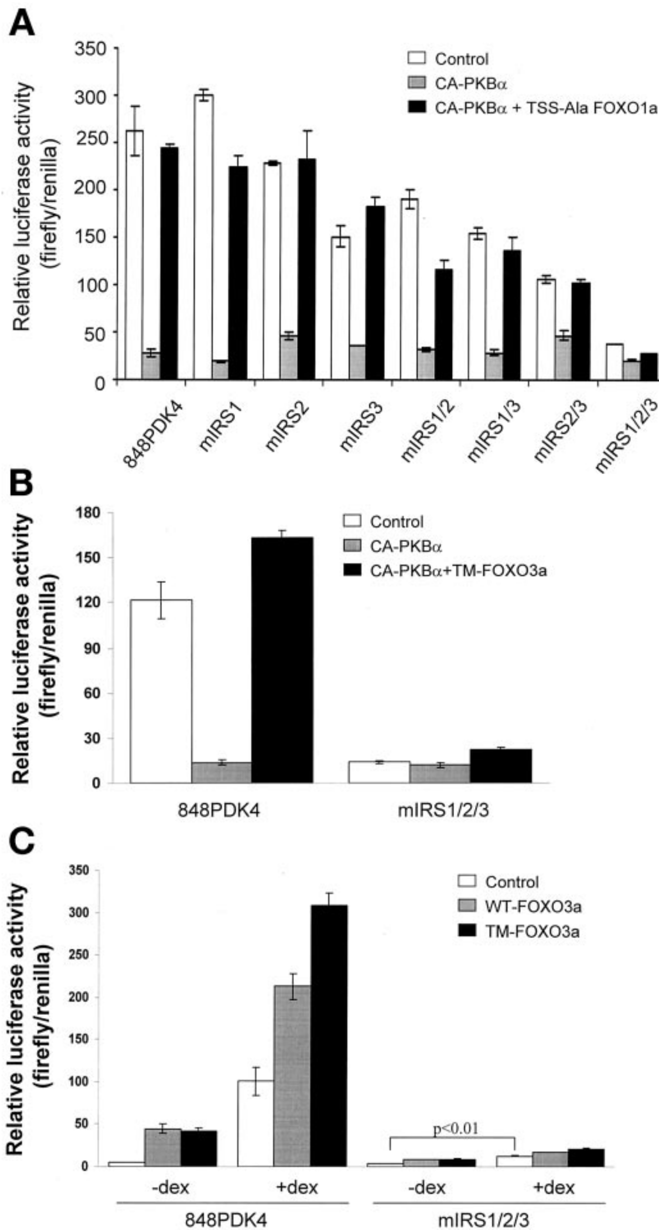


**FIG. 7. FOXO1a (*A*) and FOXO3a (*B*) do not prevent PKB inhibition after mutation of the three IRSs. 848PDK4-luc (0.5 μg) and mutant constructs were cotransfected with p6RGR (0.2 μg), pRL-TK (0.1 μg), CA-PKBα (50 ng), and TSS-Ala FOXO1a or TM-FOXO3a (0.4 μg). *C*: Mutation of the three IRSs abolishes transactivation of PDK4 promoter activity by WT-FOXO3a and TM-FOXO3a in the basal and the dexamethasone-induced states.**

and FOXO3a that were either supershifted or disappeared with appropriate antibodies (Fig. 5*A* and *B*), suggesting that FOXO1a and FOXO3a bind to these regions. Excess unlabelled wild-type probes (Wt) inhibited the DNA-protein interaction, but mutant oligonucleotides (Mt) did not, suggesting TG sequences are important for FOXO binding (Fig. 5*A* and *B*). Figure 5*C* shows the relationship of the GRE and three IRSs in the hPDK4 promoter.

**IRSs are necessary for glucocorticoid-stimulated hPDK4 expression and insulin represses hPDK4 induction through IRSs.** To further investigate whether IRSs are physiologically functional, reporter constructs with mutated IRSs were prepared and transfected into HepG2 cells. Mutation of the IRSs decreased the hPDK4 induction by dexamethasone, suggesting that FOXO binding to IRSs is required for full activation of the hPDK4 gene by dexamethasone (Fig. 6). Although IRS1 mutation alone did not cause a decrease in hPDK4 induction by dexamethasone, the combinational mutations with either IRS2 or IRS3 caused a greater decrease than the IRS2 or IRS3 mutations alone. While mutation of IRS2 alone resulted in an ~10% decrease in the dexamethasone response, mutation of IRS3 alone caused an ~50% decrease, suggesting IRS3 contributes the most to the dexamethasone-stimulated hPDK4 induction. The relative contributions of these elements appeared to increase in the following order: IRS1, IRS2, IRS3. The combinational mutations of IRSs caused further decreases, suggesting that IRSs cooperate to activate hPDK4 gene by dexamethasone. Mutations of all three IRSs (mIRS1/2/3) caused 80% decrease in hPDK4 induction by dexamethasone and 35% decrease in basal expression (data not shown). Similarly, CA-PKBα also caused an ~40% decrease in the hPDK4 basal expression, which was prevented by constitutively active FOXO factors (data not shown), suggesting FOXO binding to IRSs also contributes to the basal expression of hPDK4 gene. Insulin was no longer effective against basal expression (data not shown) as well as dexamethasone-stimulated hPDK4 induction after mutation of all three IRSs, suggesting insulin represses hPDK4 expression through the IRSs.

**FOXO factors exert their function through the IRSs.** The above data suggest that insulin exerts its function through IRSs for FOXO binding. Therefore, we examined whether FOXO factors exert their function through IRSs. As this would predict, TSS-Ala FOXO1a completely prevented PKB inhibition with most of the mutant IRS constructs but not with mIRS1/2/3, whose expression is relatively very low and therefore cannot be affected by PKB (Fig. 7*A* and *B*). Mutation of all three IRSs virtually abolished transactivation of hPDK4 promoter activity by WT-FOXO3a and TM-FOXO3a in the basal and dexamethasone-induced states (Fig. 7*C*). These results suggest that FOXO factors exert their function through the three IRSs. Although mIRS1/2/3 contains an intact GRE, its promoter activity was increased only fourfold by dexamethasone. This result suggests again that FOXO binding to IRSs is important for hPDK4 induction by glucocorticoids.

**The p300/CBP that interact with GR and FOXO factors is required for hPDK4 gene activation by dexamethasone.** Interactions with p300/CBP are important for the ability of GR to activate its target genes (48). Recent evidence indicates that FOXO factors also interact with p300/CBP to activate transcription (42). Therefore, we examined the role of p300/CBP in promoting hPDK4 expression. E1A and mutant E1A were cotransfected with the hPDK4 promoter. E1A binds to p300/CBP and represses histone acetyltransferase activity of p300/CBP, leading to gene repression (45). Wild-type E1A completely inhibited hPDK4 induction by dexamethasone. E1A Δ2–36, a modified form in which the p300/CBP interacting domain is deleted (45), was not inhibitory, suggesting that the recruitment of p300/CBP to the hPDK4 gene is essential for transcriptional activation by dexamethasone (Fig. 8*A*). Next we tested whether interaction between FOXO factors and p300/CBP contributes to activate transcription of the

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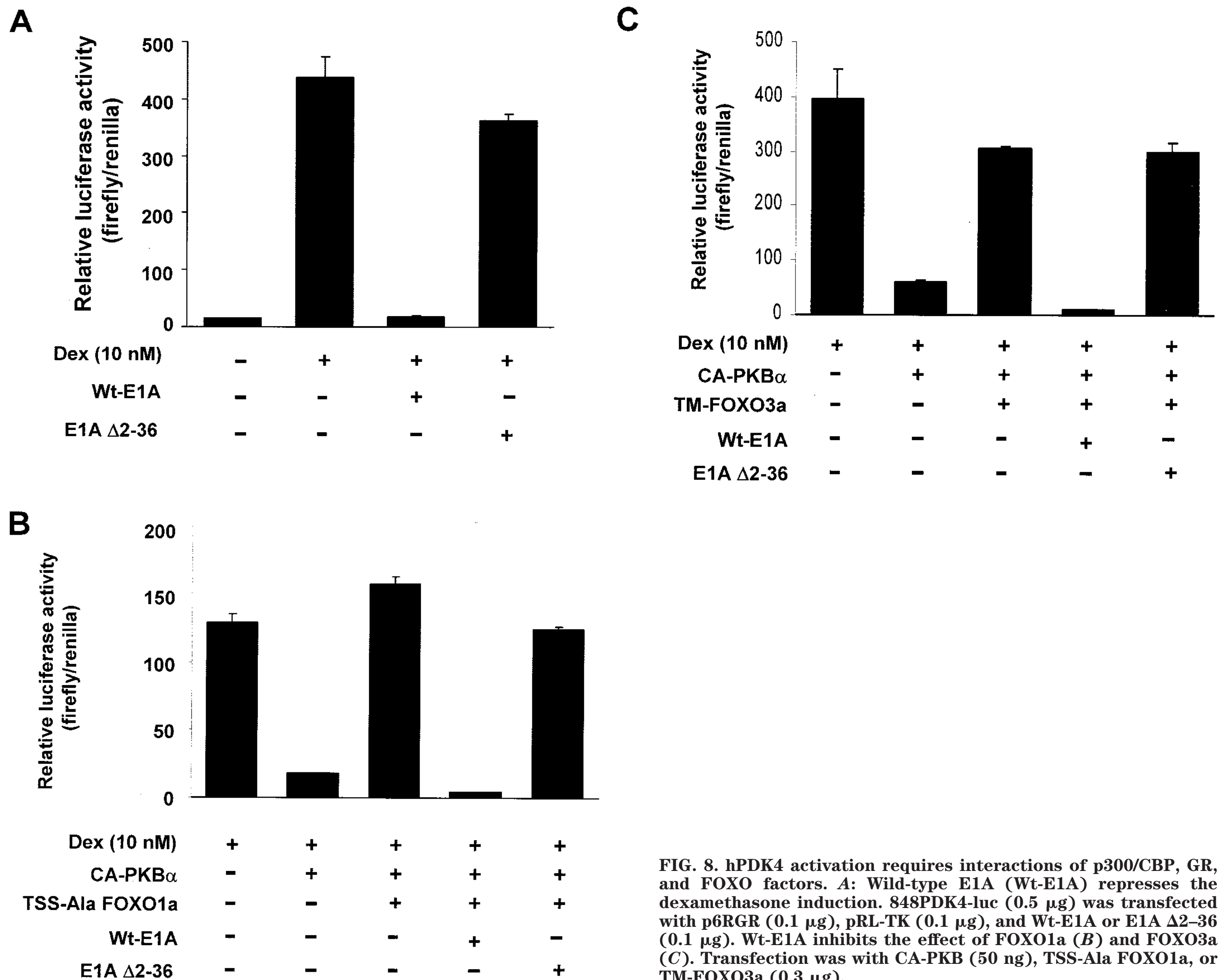


**FIG. 8. hPDK4 activation requires interactions of p300/CBP, GR, and FOXO factors. *A*: Wild-type E1A (Wt-E1A) represses the dexamethasone induction. 848PDK4-luc (0.5 μg) was transfected with p6RGR (0.1 μg), pRL-TK (0.1 μg), and Wt-E1A or E1A Δ2–36 (0.1 μg). Wt-E1A inhibits the effect of FOXO1a (*B*) and FOXO3a (*C*). Transfection was with CA-PKB (50 ng), TSS-Ala FOXO1a, or TM-FOXO3a (0.3 μg).**

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hPDK4 gene. Wild-type E1A completely blocked the effects of constitutively active FOXO1a and FOXO3a on PKB inhibition (Fig. 8*B* and *C*), but E1A Δ2–36 was totally ineffective. These data suggest that interactions between p300/CBP and GR as well as FOXO factors are important for glucocorticoid-stimulated hPDK4 expression.

## DISCUSSION

We studied the mechanism responsible for hPDK4 gene regulation by glucocorticoids and insulin. Dexamethasone increased the PDK4 mRNA message level in HepG2 cells, although it was not as effective as in rat 7800 C1 cells (15). Transfection studies suggest that this difference between rat and human cells is due to the low expression level of GR in HepG2 cells. Indeed, dexamethasone caused marked activation of an exogenous hPDK4 promoter in HepG2 cells only after coexpression of GR. RU486, a GR antagonist, abolished the effect of dexamethasone, and EMSA studies showed that GR binds to the −824/−809 bp (GRE) region within the hPDK4 promoter. Mutation of the GRE virtually repressed the induction of hPDK4 expression by dexamethasone. Therefore, we conclude that dexamethasone activates PDK4 gene expression through a GRE in a GR-dependent manner.

Consistent with previous findings regarding the regulation of PEPCK and G6Pase (33), insulin inhibits the ability of dexamethasone to stimulate hPDK4 gene expression. Therefore we focused our studies on the mechanism responsible for the insulin inhibition of glucocorticoid-stimulated hPDK4 expression. Wortmanin, a phosphatidylinositol 3-kinase (PI3K) inhibitor, blocks insulin inhibition of PDK4 induction by dexamethasone in 7800 C1 cells (B.H. and R.A.H., unpublished studies), suggesting that insulin inhibits PDK4 expression through the PI3K/PKB pathway. Consistent with this finding, expression of constitutively active PKBα completely repressed hPDK4 induction by dexamethasone. We next examined the role of FOXO factors (FOXO1a and FOXO3a), downstream targets of PKB, for involvement in the hPDK4 gene regulation. FOXO factors exert their function as transcriptional activators, while insulin and PKB inhibit their function by phosphorylation. Both FOXO1a and FOXO3a activate basal hPDK4 expression regardless of increased amounts of FOXO factors. Although FOXO1a, FOXO3a, and FOXO4

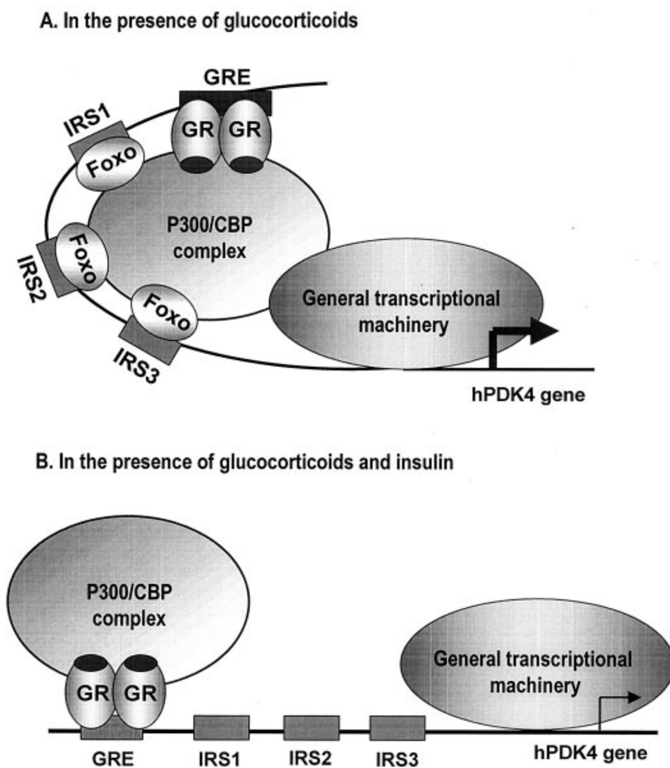


**FIG. 9. A model for the hPDK4 gene regulation.**

are equally effective in activating basal IGFBP-1 expression (42), FOXO3a caused a greater increase in basal hPDK4 expression than FOXO1a (13-fold vs. 2-fold). FOXO3a might have a distinct effect on basal hPDK4 expression. Different levels of FOXO protein expression might also be a factor, particularly since FOXO1a and FOXO3a expression are driven by the cytomegalovirus (CMV) and SV-40 promoters, respectively. FOXO1a and FOXO3a lead to further increases in hPDK4 expression by dexamethasone. Moreover, TSS-Ala FOXO1a and TM-FOXO3a, which cannot be phosphorylated by PKB, completely prevented PKB inhibition. In accord with this observation, we identified three related IRSs that bind FOXO1a and FOXO3a in the 5′ promoter of the hPDK4 gene. Mutation of IRSs disrupts the effects of FOXO factors and impairs the ability of glucocorticoids to stimulate PDK4 promoter activity. Similarly, Yeagley et al. (17) reported that mutation of the IRSs in the PEPCK and IGFBP-1 genes causes 33–67% decrease in induction of these genes by dexamethasone. Mutations in all three IRSs (mIRS1/2/3) caused an 80% decrease in induction of PDK4 gene by dexamethasone. Furthermore, the mIRS1/2/3 promoter activity was increased only fourfold by dexamethasone, despite the presence of an intact GRE, which is capable of activating a target gene by itself, as shown in experiments with 3XGRE-TK-luc. This suggests that FOXO binding to three IRSs are required for full transcriptional activation of hPDK4 gene by glucocorticoids. Several accessory factors, including an IRS for FOXO binding, are required for full activation of PEPCK gene expression by glucocorticoids (49). Likewise, hepatocyte nuclear factor 1 (HNF-1) is necessary for glucocorticoid-stimulated G6Pase (20) and IGFBP-1 (50) expression. The combination of the GRE and three IRSs in the hPDK4 promoter may be important for the ability of insulin to suppress glucocorticoid-stimulated expression.

Next, we asked the question why FOXO factors play an important role in glucocoticoid-stimulated hPDK4 expression. It appears that FOXO factors cooperate with GR through an interaction with p300/CBP as shown for IGFBP-1 gene regulation (42). The viral oncogene E1A, which binds to p300/CBP and represses histone acetyltransferase activity of p300/CBP, completely abolished the dexamethasone effect as well as the effects of TSS-Ala FOXO1a and TM-FOXO3a on PKB inhibition. Based on our findings, we propose a model for hPDK4 gene regulation by glucocorticoids and insulin (Fig. 9). The GR activated by glucocorticoid binding translocates into the nucleus where it forms a homodimer and binds to the GRE. The p300/CBP coactivator complex interacts cooperatively with GR and FOXO factors (Fig. 9*A*). The p300/CBP may have multiple surfaces that enable cooperative interactions with FOXO factors. The p300/CBP coactivator complex may promote the rate of formation of a stable transcriptional initiation complex and thereby enhance the rate of PDK4 transcription. GR may be primarily responsible for the recruitment of p300/CBP to the hPDK4 gene because mutation of the GRE virtually abolished the PDK4 induction by dexamethasone. The relative contributions of the three IRSs (IRS1$<$ IRS2$<$ IRS3) to the glucocorticoid response appeared to correlate with the distance from the GRE. The IRS that is located furthest from the GRE (IRS3) may play the most important role in mediating the interaction between p300/CBP complex and the general transcription machinery. Insulin inhibition of FOXO bindings to the three IRSs may interfere with the interaction between p300/CBP complex and the general transcriptional machinery. Therefore the rate of formation of a stable transcriptional initiation complex may be significantly decreased, resulting in the suppression of the glucocorticoid response (Fig. 9*B*). Insulin signaling via PKB causes phosphorylation of FOXO factors that can cause their translocation out of the nucleus (36,37,41,42, 47), promotion of the binding of 14-3-3 proteins (31), and a decrease in DNA binding activity (32). Phosphorylation of FOXO1a by PKB also disrupts the interaction of FOXO1a with PPAR-$\gamma$ coactivator 1$\alpha$ (PGC-1$\alpha$), leading to repression of the induction of PEPCK and G6Pase gene expression by PGC-1$\alpha$ (33). Therefore, it may also be possible that the interactions between p300/CBP and FOXO factors are disrupted by phosphorylation.

Taken together, our findings suggest that in the absence of insulin and glucocorticoids, FOXO factors contribute to hPDK4 basal expression. In starved and diabetic states, elevated levels of glucocorticoids activate GR and thereby recruit a p300/CBP complex to the hPDK4 gene. FOXO factors as well as GR may cooperatively interact with the p300/CBP complex to make a stable transcription initiation complex, leading to active hPDK4 gene expression. Insulin promotes inactivation of FOXO factors and thereby reduces glucocorticoid-stimulated PDK4 gene activation.

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