NF-κB Inhibits Glucocorticoid and cAMP-mediated Expression of the Phosphoenolpyruvate Carboxykinase Gene*

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Phosphoenolpyruvate carboxykinase (PEPCK)1 catalyzes a rate-controlling step in hepatic gluconeogenesis, and the transcription of this gene is regulated by several hormones (1, 2), including glucocorticoids, retinoic acid, and glucagon (via its second messenger, cAMP) (3–6). A detailed analysis of the PEPCK promoter has revealed that each hormone response is mediated by a set of DNA elements that comprise a complex hormone response unit. For instance, the glucocorticoid response unit (GRU), which is positioned between −455 and −86 relative to the transcription start site, is required for the stimulatory effect of glucocorticoids. The GRU includes two glucocorticoid receptor binding sites (GR1 and GR2), three accessory factor binding sites (gAF1, gAF2, and gAF3), and a cAMP response element (CRE) (7–9). Transcription factors that bind to these sites have been identified (Fig. 1). Hepatic nuclear factor 4 (HNF-4) and chicken ovalbumin upstream promoter transcription factor (COUP-TF) bind to gAF1, members of the hepatic nuclear factor 3 (HNF-3) family bind to gAF2, and COUP-TF binds to gAF3 to mediate the glucocorticoid response (9–11). Although a number of proteins bind the CRE, we showed that C/EBP-β acts as an accessory factor for the glucocorticoid response through this element (4).

Stimulation of PEPCK gene expression by cAMP, which is mediated by cAMP-dependent protein kinase A (PKA), also involves a cAMP response unit (CRU) that consists of several cis-acting elements (12, 13) (Fig. 1). The CRE at −90 to −82, a C/EBP-α binding site in the P3I region of the PEPCK promoter (between −246 and −238), and an AP-1 binding site extending from −260 to −250 are essential components of this CRU (14–17).

A variety of agents, including insulin, phorbol esters, compounds that elicit oxidative and cellular stress (such as H₂O₂ and sodium arsenite), and the cytokines TNF-α, IL-6, and IL-1 (18–23) repress PEPCK gene transcription. Although the hormone response units that confer stimulation of PEPCK gene transcription are well characterized, the mechanisms that lead to repression of PEPCK transcription are not fully understood.

An insulin response sequence (IRS) involved in both the insulin and phorbol ester responses is positioned between −413 and −407 relative to the transcription start site within the PEPCK gene promoter (24, 25) (Fig. 1). However, insulin still represses PEPCK gene expression when this IRS is deleted or mutated (24). This observation led to the suggestion that another element, acting more proximal to this IRS, is also involved in this response (designated by X in Fig. 1). Alternatively, insulin and the other negative regulators of PEPCK gene transcription could also work by disrupting protein-protein interactions necessary for communication between transcription factors and coactivators with the basal transcriptional machinery.

In insulin stimulates signaling pathways that lead to the acti-
The PEPCK gene promoter. The cis elements and associated trans factors required for the glucocorticoid-, cAMP-, or insulin-mediated responses of the PEPCK gene are shown. The central position of each element with respect to the transcription start site is shown above each site. Trans-acting factors involved in the GRU, CRU, or IRU are shown below their respective binding elements. An unidentified IRS binding protein binds to gAF2 to mediate the insulin response through this element. Because deletion of gAF2 only partially represses insulin-mediated effects on PEPCK gene expression, it is believed that another element/factor complex proximal to gAF2 is also involved in this response. This unknown factor is designated as X.

NF-κB serves as a transcription factor (31–33). Conversely, activators of protein kinase (31–33). In addition to its role in the immune response and inflammatory processes, NF-κB plays a role in the development and regeneration of the liver (36). The critical role of NF-κB in the liver is underscored by the observation that p65 knockout mice die before birth from massive degeneration of the liver due to apoptosis (37).

NF-κB is a ubiquitous transcription factor whose properties have been characterized primarily in cells of the immune system. In addition to its role in the immune response and inflammation, NF-κB is involved in cell cycle progression (35) and in liver development and regeneration (36). The critical role of NF-κB plays in hepatic development is underscored by the observation that p65 knockout mice die before birth from massive degeneration of the liver due to apoptosis (37).

The NF-κB/Rel family of transcription factors includes the subunits p50, p52, p65, c-Rel, and RelB. These proteins share an N-terminal region of homology, known as the rel homology domain, that is important for DNA binding and dimerization. Two transactivation domains are found in the C-terminal region of p65, c-Rel, and RelB, but these are lacking in p50 and p52 (31, 38). In the cytoplasm, NF-κB binds to the inhibitory protein, IκB, to form an inactive NF-κB/IκB complex (39). Most agents that activate NF-κB do so by stimulating the phosphorylation and subsequent degradation of IκB, thus allowing NF-κB to translocate to the nucleus and regulate transcription (40, 41).

NF-κB regulates transcription by several different mechanisms. In most cases, NF-κB activates transcription by binding to an NF-κB element and interacting with the basal transcription machinery (38, 42). However, NF-κB represses transcription in the presence of a corepressor, such as the Drosophila melanogaster ventral organizer with Groucho (43–45). NF-κB also represses transcription by interacting directly with transcription factors, such as GR, or by competing with other factors for binding to coactivators, such as p300, CBP, or SRC-1 (46–51).

We now have evidence that the p65 subunit of NF-κB represses PEPCK gene transcription, in a DNA-independent fashion, by disrupting the coactivation function of CBP. These data suggest that the NF-κB signaling pathway contributes to the repression of hormone-activated PEPCK gene expression.
ysulfonamide, 20 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM Na$_2$P$_4$O$_7$, 0.4 mM Na$_3$MoO$_4$, 0.125 µM okadaic acid, 1 µM meprozin, 1 µM pepstatin, and 1 µM leupeptin). After homogenization in a tight-fitting Dounce homogenizer, cell lysates were centrifuged at 2000 × g for 10 min, and nuclear pellets were resuspended in buffer B (buffer A containing 42 mM NaCl and 10% glycerol). The resuspended nuclei were exposed to consistent agitation for 30 min and centrifuged at 13,000 × g for 20 min. The supernatant was used for DNA-binding reactions. Protein amounts were determined using the Bio-Rad reagent. Oligonucleotides corresponding to the NF-κB binding site were annealed and end-labeled using polynucleotide kinase in the presence of [γ-$^32$P]dATP, 5′-TCAGAGGGGACTTTCCGAGAGG-3′ and 5′-CTCGCCGAgACCTCCCAAGCGGCA-3′. DNA-binding reactions were performed in a buffer containing 10 mM Tris·HCl, pH 7.6, 50 mM NaCl, 0.5 mM dithiothreitol, 10% glycerol, 0.2% Nonidet P-40, and 3 µg of poly(dI·dC) using 5 µg of nuclear protein and the labeled NF-κB probe (approximately 60,000–75,000 counts/min per reaction). For supershift assays, reactions were performed in the presence or absence of 1 µg of antibody specific for p65, c-Rel, RelB, p50, or p52. Samples were incubated at room temperature for 25 min and analyzed by electrophoresis on a 6% nondenaturing polyacrylamide gel in TAE buffer (7 mM Tris, pH 7.6, 3 mM sodium acetate, 1 mM EDTA).

Site-directed Mutagenesis—The plasmid pML32 contains the PEPCk promoter sequence from −467 to +69, relative to the transcription start site, ligated to the CAT reporter gene, as described previously (3). The mutation of pML2 to make the construct pML32 (mMLB) was performed using the site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the oligonucleotides 5′-CCCTTGCCCAACAGCCTCAATACCGCCGAGAC-3′ and 5′-TCCTTGCGGAGATCTTGTCGGCAA-3′. The mMLB mutants were also constructed using the QuikChange kit. The following oligonucleotides were used to construct these mutants: ΔRHD, 5′-CCCTTGCCGCTATGTTGGCCACATGTTGTCTCCG-3′ and 5′-GAGCTCCCGGTCGGCAGGCCGCCAG-3′; ΔTA, 5′-CTGAAAGGCTGGATAGAAGGTCGAC-3′ and 5′-GTCACCCCTTTA-TGCTGGTTAAGC-3′; S276A, 5′-CTGGCCGAGGCCCCTGGCCAGGCGGAGA-GCT-3′ and 5′-GAGCTCCCGGTCGGCAGCCAGGGCCGCCG-3′. The DNA sequence of each mutant was verified.

Materials—Radioisotopes (γ-$^32$P]dATP and [3H]sodium acetate) were obtained from Amersham Pharmacia Biotech and ICN, respectively. Insulin was purchased from Collaborative Bioproducts. 8CPT-cAMP was purchased from Roche Molecular Biochemicals. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma. Antibodies specific to p50 or p65 were purchased from Santa Cruz Biotechnology. Dexamethasone and H$_2$O$_2$ were purchased from Sigma, IL-6 was purchased from Promega, and TNF-α from RD Research.

RESULTS

The ability of insulin and phorbol esters (PMA) to repress PEPCk mRNA expression has been extensively documented (18–20, 25, 52). Given the central role of PEPCk in maintaining gluconeogenesis, several investigators postulated that the hypoglycemia commonly associated with endotoxemia could result from decreased expression of this hepatic enzyme. In rats, endotoxin treatment is believed to result in the release of the proinflammatory cytokines, IL-6, IL-1, and TNF-α, all of which reduce PEPCk mRNA in rat hepatocytes and H4IE hepatoma cells (21–23). Interestingly, each of the agents that repress PEPCk gene expression promotes the nuclear localization and activation of NF-κB (28, 30–33). In view of these results, we decided to determine whether the different NF-κB subunits have an effect on PEPCk gene transcription.

p65 Represses PEPCk Gene Transcription—Transient transfection experiments were conducted to determine if the p65 or p50 subunits of NF-κB repress PEPCk gene expression. We chose to focus on these subunits, because they are expressed and activated in the cell lines used in our experiments (see below). Experiments designed to examine PEPCk gene expression were performed by co-transfecting pML32 (a plasmid with the wild-type −467 to +69 sequence of the PEPCk promoter located upstream of a CAT reporter gene) with either the catalytic subunit of PKA or the glucocorticoid receptor (GR), to enhance the expression of the gene. HepG2 cells were transfected with the PKA expression vector, which results in a 10- to 12-fold induction of PEPCk gene expression in response to this effector. The expression of p65 effectively repressed the PKA response in a concentration-dependent manner, whereas p50 expression had no effect (Fig. 2A). The same experiments were performed to examine the effects of p65 or p50 on glucocorticoid-mediated induction of pML32. H4IE hepatoma cells were used for these experiments, because they give a more robust response to glucocorticoids than do HepG2 cells. Increasing amounts of p65 also repressed the glucocorticoid response, whereas p50 had a small effect (Fig. 2B). We were not able to compare the level of protein expression of p65 and p50 due to the low transfection efficiency of H4IE and HepG2 cells. We were able to show by Western blot in COS-1 cells, however, that the p50 expression vector does express protein (data not shown). It is possible that p50 is expressed at a lower level than p65 and that higher p50 protein levels would further repress PEPCk gene transcription. We chose not to transfect higher amounts of the p50 expression vector, however, because higher amounts of the empty expression vector had a repressive effect on PEPCk gene expression. For subsequent studies, we chose to examine the role of p65 on PEPCk gene expression.

To confirm that the effects of p65 on PEPCk gene expression were not due to a direct repressive effect on transcription, or to a direct inhibition of CAT activity, p65 was co-transfected with a reporter plasmid that has six HIV long terminal repeat κB elements positioned upstream of a minimal thymidine kinase promoter and the CAT reporter gene (referred to as 6xκB/CAT). A 2- to 3-fold stimulation of transcription was observed, whereas no stimulation (or inhibition) of the parent vector TK-CAT was seen (Fig. 2C). We were unable to use p50 for these experiments, because this protein lacks a transactivation domain and so does not activate 6xκB/CAT.

The PEPCk Gene Promoter Contains a κB Element—As mentioned above, NF-κB can mediate transcription in a DNA-binding-dependent or -independent manner. Therefore, the PEPCk promoter was examined for potential κB elements, which consist of the consensus sequence GGGRRNNYYY (38). A site resembling this consensus κB element (GGGGAAATCC) was identified at position +40 to +49 relative to the transcription start site in the PEPCk promoter. Electrophoretic mobility shift assays (EMSAs) were performed using oligonucleotides that correspond to this site, or to a consensus κB site, and nuclear extracts isolated from H4IE cells (Fig. 3A). DNA binding activity by NF-κB was detected in cells incubated in serum-free DMEM. This is consistent with other reports that describe the presence of NF-κB in the nucleus of untreated hepatoma cells (53). As expected, nuclear extracts obtained after the treatment of H4IE cells with a combination of dexamethasone and cAMP (Dex/cAMP) provided a reduced formation of the NF-κB/DNA complex, as compared with untreated cells. However, DNA binding by the NF-κB in nuclear extracts was increased in response to treatment of cells with insulin, hydrogen peroxide, and PMA (all of which are inhibitors of PEPCk gene transcription), even in cells that had been treated with Dex/ cAMP, showing that these compounds overcome the repressive effect of Dex/cAMP on DNA binding. The consensus sequence oligonucleotide bound one protein complex, whereas the oligonucleotide corresponding to the PEPCk sequence bound two protein complexes (Fig. 3A). Supershift analyses using antibodies specific for the p50 or p65 subunits of NF-κB revealed that the consensus oligonucleotide bound to a p50/p65 heterodimer. The c-Rel, RelB, and p52 subunits were not part of this complex (Fig. 3B). With regard to the PEPCk κB element, supershift assays revealed that the faster migrating band consisted of the p65/p50 heterodimer. Binding of the slower migrating band was inconsistent, but consisted of p50 and an unidentified protein (referred to as “X”). Supershift analysis revealed that
p65 represses PKA and glucocorticoid-mediated PEPCK gene transcription. A, experiments were designed to test the effects of p65 and p50 in the response of the PEPCK gene to PKA. The PKA catalytic subunit was used in these experiments, because it mimics cAMP-dependent PEPCK gene expression. HepG2 hepatoma cells were transiently co-transfected with 5 μg of pFL32, RSV-neo (a plasmid used to normalize for the amount of DNA used per transfection), and 2.5 μg of a PKA catalytic subunit expression plasmid. Cells were harvested after 18 h, and CAT activity was measured. Activation of CAT activity by PKA was arbitrarily set at 100%. The effect of NF-κB on PEPCK gene transcription was examined by co-transfecting 2.5 or 5.0 μg of an expression vector encoding either p65 (top) or p50 (bottom). CAT activity was calculated, and the results represent the mean ± S.E. of five experiments. Addition of 5.0 μg of the p65 expression vector significantly repressed PKA-mediated PEPCK gene expression (p < 0.05, Student’s t test) as compared with the no addition control.

B, experiments were performed to examine the effects of p65 and p50 on the glucocorticoid response. H4IIE hepatoma cells were co-transfected with 10 μg of pFL32 and 5 μg of a GR expression plasmid, and the amount of DNA used per transfection was normalized with the RSV-neo plasmid. Dexamethasone (500 nM) was added to cells to stimulate the glucocorticoid response, which was arbitrarily set at 100%. Either p65 or p50 expression plasmids (2.5 or 5.0 μg) were co-transfected to examine the effect of NF-κB on the glucocorticoid response. Cells were harvested after 18 h, and CAT activity was measured. Results represent the mean ± S.E. of seven experiments. Addition of 2.5 or 5.0 μg of the p65 expression vector significantly repressed glucocorticoid-mediated PEPCK gene expression (p < 0.05, Student’s t test) as compared with the no addition control.

C, H4IIE cells were transiently transfected with the reporter plasmid 6xκB/CAT, in combination with 2.5 or 5.0 μg of the p65 expression vector, to test whether p65 has a general repressive effect on transcription. Because the 6xκB/CAT reporter plasmid is derived from the TK-CAT parent vector (a reporter plasmid with the thymidine kinase promoter upstream of CAT), the effect of p65 on TK-CAT expression was also examined (C). Cells were incubated for 18 h in serum-free DMEM and subsequently harvested for assay of CAT activity. Results represent the mean ± S.E. of three to eight experiments. Addition of 5.0 μg of the p65 expression vector significantly activated CAT activity (p < 0.001, Student’s t test) as compared with the no addition control.
protein X is not p65, c-Rel, RelB, or p52 (data not shown).

p65 Repression of PEPCK Gene Transcription Does Not Require DNA Binding—The kB element at +40 was mutated from GGGAAATCC to GCTCAAATCC in pPL32 (referred to as pPL32 (mkB)) to determine if p65 represses PEPCK transcription by binding to this element. An oligonucleotide corresponding to this mutation did not bind p65, p50, or the slower migrating complex (Fig. 3C). Mutation of the kB binding site to pPL32 (mB), in the context of pPL32, did not abrogate p65-mediated repression of PEPCK gene transcription (Fig. 4A), suggesting that NF-kB represses PEPCK gene transcription in a manner independent of binding to this DNA element. The effect of several PEPCK gene transcription repressors (insulin, PMA, IL-6, and TNF-α) on pPL32 and pPL32 (mB) was examined to further confirm that the kB element of the PEPCK promoter is not important for repression of transcription of this gene. Each agent repressed expression from pPL32 and pPL32 (mB) to the same extent, thus the kB element does not play a role in the repression of PEPCK gene transcription by these agents (Fig. 4B). These data also reveal that the binding of protein X to the PEPCK gene promoter is not required for the repressive effects of different agents on PEPCK gene expression.

Fig. 3. NF-κB binds to the PEPCK kB element. Nuclear extracts were prepared from H4IE hepatoma cells treated with Dex/cAMP for 4 h and with stimulators of NF-κB DNA binding activity (10 nM insulin, 1 mM H2O2, or 1 μM PMA) to determine if these agents could overcome the inhibitory effects Dex/cAMP have on NF-κB DNA binding activity. Control cells were incubated in serum-free DMEM. Nuclear extracts were prepared as described under “Experimental Procedures.” A DNA probe corresponding to a consensus kB element, or to the putative PEPCK kB element, was incubated with 5 μg of nuclear extracts for 20 min. Binding reactions were analyzed on a 6% native polyacrylamide gel (A). Antibodies specific for the p65, c-Rel, RelB, p50, and p52 subunits of NF-κB were used in supershift assays to verify that the DNA binding activity observed in gel shift assays was due to NF-κB (B). Nuclear extracts were incubated with 1 μg of a specific antibody for 20 min before gel shift assays were performed, as described in A. Binding reactions were analyzed on a 6% native polyacrylamide gel. Nuclear extracts from H4IE cells treated with serum-free DMEM or 10 nM insulin were also used in gel shift reactions to determine if an oligonucleotide corresponding to a mutation of the PEPCK kB element (mutated from GGGAAATCC to GCTCAAATCC) bound to proteins (C). No DNA binding was observed in this case.
DNase footprinting experiments were performed, using the −467 to +69 region of the PEPCK promoter and purified NF-κB, in an effort to locate other potential NF-κB elements in the PEPCK gene promoter. NF-κB only binds to the +40 region (data not shown). Therefore, p65 represses PEPCK gene transcription by a mechanism that does not appear to involve direct DNA binding.

**Mutation of p65 Abrogates Repression of PEPCK Gene Transcription**—The p65 protein (Fig. 5A) consists of an N-terminal DNA binding and dimerization domain, referred to as a rel homology domain (RHD), and two C-terminal transactivation domains (TA1 and TA2) (38, 54). The RHD binds directly to GR, and deletion of the RHD creates a p65 mutant that no longer represses GR-mediated transcription from a construct that has two GREs located upstream of a luciferase reporter (2xGREtkluc) (54). Although the two transactivation domains of p65 do not interact with GR, deletion of these domains also blocks GR-mediated transcription from the 2xGREtkluc reporter construct (54). Therefore, two p65 mutants (∆RHD, with a deletion of amino acids 22–248 and ∆TA, with a deletion of amino acids 350–551) were constructed to determine if either of these domains is involved in PEPCK gene expression. Both ∆RHD and ∆TA blunted the glucocorticoid response, although each was less effective than the wild type p65 (Fig. 5B, top). This could be indicative of an interaction of p65 with GR, but it is also possible that the RHD and TA domains of p65 interact with another transcription factor or coactivator necessary for the PEPCK glucocorticoid response. The absence of either the RHD or TA domains did not affect the ability of p65 to repress the PKA response (Fig. 5B, bottom).

Several groups have shown that, in addition to an interaction with GR, p65 interacts with CBP both in vitro and in vivo (48, 55). Furthermore, this interaction is important for the transcriptional activation of several genes, including the interleukin-6, E-selectin, and VCAM-1 genes (55, 56). The region of p65 important for an interaction with CBP has been defined. The N-terminal region of p65, when phosphorylated on serine 276, interacts with CBP and the mutation of this residue to alanine significantly reduces binding of p65 to CBP (48). The S276A mutant of p65, therefore, was constructed, and its effect on PEPCK gene expression was examined. The S276A mutant of p65 was not an effective inhibitor of either the glucocorticoid or PKA responses of the PEPCK gene. This suggests that the binding of p65 to CBP is involved in the repressive effects of p65 on these responses (Fig. 5B, top and bottom).

Due to the low transfection efficiency of hepatoma cells, the expression of each construct was examined in COS-1 cells. These cells were transiently transfected with 10 μg of each of the p65 expression vectors (encoding native p65, ∆RHD, ∆TA, and S276A). Each protein was expressed at approximately the same level, as assessed by Western blot analysis (data not shown). This result is in agreement with similar studies reported in 293 cells (54).

**Transcriptional Activation by S276A Is Not Potentiated by CBP**—As noted above, several groups have reported that p65 interacts with CBP, and this interaction is important for p65-mediated transcriptional activity (48, 55, 56). We sought to demonstrate the same effect in hepatoma cells, because we

**PEPCK gene expression to determine whether the κB element plays a role in p65-mediated repression** (B). These cells were treated with serum-free DMEM (control), 500 nM dexamethasone, or dexamethasone in the presence of 10 nM insulin, 1 μM PMA, 160 ng/ml IL-6, or 1 ng/ml TNF-α. After an 18-h incubation, cells were harvested and CAT assays were performed. Results represent the mean ± S.E. of three experiments. No significant differences were observed in the repression of expression from the pPL32 and pPL32 (mxB) reporter genes.
found that the S276A mutant of p65 does not significantly repress the glucocorticoid or PKA responses of the PEPCK gene (Fig. 5B). We initially tried to immunoprecipitate p65 from H4IIE cells to determine if there is an increased association of p65 with CBP after treatment of these cells with either insulin, PMA, or TNF-α. We did see an increased association of p65 with CBP after treatment with these different agents, however, the results were difficult to repeat consistently due to the difficulty in immunoprecipitating sufficient amounts of CBP from H4IIE cells (data not shown). Therefore, we tried another approach to show that CBP and p65 have a functional interaction in hepatoma cells. H4IIE cells were co-transfected with a reporter plasmid that has five consensus kB elements positioned upstream of a luciferase reporter gene (Stratagene) and 5.0 mg of either an expression vector encoding p65 or S276A (Fig. 6). Luciferase activity was stimulated 3- and 1.4-fold by p65 and S276A, respectively. Co-transfection of an expression vector that encodes CBP potentiated p65-mediated transcription but did not affect S276A-mediated transcription. These data are consistent with experiments performed in Jurkat cells (48), and they support previous results, which show that the Ser-276 residue of p65 is an important site for its interaction with CBP.

**CBP Potentiates the PKA and Glucocorticoid Responses of the PEPCK Gene**—As shown in Fig. 1, many of the factors important for cAMP- and glucocorticoid-mediated stimulation of PEPCK gene transcription have been identified and the role of putative coactivators in these responses is being investigated. The results described above suggest that CBP is involved in the regulation of PEPCK gene transcription by both PKA and glucocorticoids. To test this possibility, an increasing amount of an expression vector encoding CBP was co-transfected with pPL32 or pPL32luc (a PEPCK promoter/luciferase construct) to examine the effect CBP has on the PKA or glucocorticoid re-

**FIG. 5. Structure of p65 mutants and their effect on PEPCK gene transcription.** p65 consists of an N-terminal DNA binding and dimerization domain (rel homology domain or RHD) and two C-terminal transactivation domains, as illustrated in A. The RHD (amino acids 22–248) or transactivation domains (amino acids 350–551) were deleted using the QuikChange mutagenesis kit (Stratagene). These p65 mutants are referred to as ΔRHD and ΔTA, respectively. Serine 276 was mutated to an alanine (referred to as S276A) using the same method. H4IIE or HepG2 cells were transiently transfected with pPL32 to examine the glucocorticoid or PKA responses, respectively, of the PEPCK gene, as described in Fig. 2. Expression vectors encoding ΔRHD, ΔTA, or S276A (5 μg each) were co-transfected to determine if these constructs also repress pPL32. The effect of the p65 mutants was compared with 5 μg of the co-transfected p65 expression vector. CAT assays were performed, and the results, illustrated in B, reflect the mean ± S.E. of seven experiments for the glucocorticoid response, and the mean ± S.E. of five experiments for the PKA response. S276A did not significantly repress either the glucocorticoid or PKA responses of the PEPCK gene, as determined by the Student’s t test.

**FIG. 6. Effect of CBP on p65- and S276A-mediated transcription.** H4IIE cells were transiently transfected, as described in Fig. 2, to examine the effect of CBP on NF-κB activation, which was assessed by transfection of a reporter plasmid containing five consensus kB elements positioned upstream of a luciferase reporter gene (Stratagene). Cells were co-transfected with 5.0 μg of expression vectors encoding either p65 or S276A to stimulate reporter activity. Luciferase activity in the absence of co-transfected p65 or S276A was arbitrarily set at 100%. To examine the effect of CBP on NF-κB activity, cells were co-transfected with 5.0 or 10.0 μg of a CBP expression vector. Cells were harvested and luciferase assays were performed. The results represent the mean of five experiments ± S.E. Addition of 5.0 μg of the CBP expression vector significantly enhanced p65-mediated transcription ($p < 0.05$ Student’s t test) as compared with the no addition control.
Transcription can be regulated by the competitive binding of specific transcription factors to a transcriptional coactivator, such as CBP or p300. For instance, p65 and STAT 2 compete for binding to p300 to mediate transcription of the HIV gene in response to TNF-α and interferon-α, respectively (26). Also, p65 and the p53 tumor suppressor gene compete for binding to limiting amounts of CBP in COS cells (57). Experiments were performed to address the possibility that p65 represses PEPCK gene transcription by competing with the binding of other factors to CBP. If so, the expression of CBP should relieve repression mediated by p65. It was difficult to perform these experiments in HepG2 cells, because even small amounts of transfected CBP enhanced the PKA response (Fig. 7A). However, this was not a problem with the glucocorticoid response in H4IIE cells. Because CBP potentiates the glucocorticoid response, an amount of the CBP-encoding plasmid that does not significantly enhance the glucocorticoid response (2.5 µg) was co-transfected with pPL32. This same amount of the expression vector encoding p65, in combination with RSV-neo (to normalize for the amount of DNA per transfection), were co-transfected to determine the effect of CBP on PEPCK gene expression, as described for the PKA response above. Luciferase assays were performed, and the results illustrated represent the mean ± S.E. of three experiments, because even small amounts of transfected CBP enhanced the glucocorticoid response. The total amount of transfected DNA was normalized in each case by the addition of the control plasmid, RSV-neo. The response to 500 nM dexamethasone was set arbitrarily to 100%, and the results reflect the mean ± S.E. of three experiments. Addition of 2.5 µg of a CBP expression vector significantly blocked p65-mediated repression of the PEPCK gene (p < 0.05, Student's t test).

**DISCUSSION**

The complex process of glucose homeostasis involves the coordinate regulation of a number of key metabolic enzymes. One of these, PEPCK, catalyzes a rate-controlling reaction in hepatic gluconeogenesis and, as such, plays a central role in the maintenance of plasma glucose levels. The activity of PEPCK is not affected by allosteric or post-translational modification but is directly related to protein abundance, which is controlled at the level of gene expression through the action of a number of hormones (1, 2, 14). In normal, fasted animals, a decrease of the plasma glucose signal leads to the release of glucagon and glucocorticoids, which stimulate PEPCK gene transcription. In the fed state the elevated plasma glucose stimulates insulin secretion, which results in repression of PEPCK gene transcription, as does glucose itself (24, 58).

A variety of other agents, in addition to insulin and glucose, repress PEPCK gene transcription (18–23). A number of
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changes in glucose metabolism occur during infection, neoplastic growth, or tissue damage. Hypoglycemia is a major complication in the septic shock syndrome and is believed to result from the repression of PEPCK transcription by the concerted actions of TNF-α, IL-1, and IL-6 on the liver (21–23). Phorbol esters, okadaic acid, and agents that cause oxidative and cellular stress also repress PEPCK gene transcription (19, 20, 52).

Interestingly, the above-mentioned transcriptional repressors of the PEPCK gene are all activators of NF-κB.

The observation that NF-κB has antagonistic effects on glucocorticoid- and cAMP-mediated gene transcription is well established (50, 51, 54). For instance, p65 represses GR-mediated activation of a CAT reporter gene linked to either the murine mammary tumor virus promoter or to a consensus GRE (54). This p65-mediated repression could occur because of a physical interaction between p65 and GR or from the competition of p65 and GR for limiting amounts of coactivators such as CBP and SRC-1 (54). It has also been suggested that CREB and p65 compete for limiting amounts of CBP, because the expression, by transfection, of increasing amounts of CREB blocks transcription mediated by a construct in which the Gα4 DNA binding domain is fused to the transactivation domains of NF-κB (49). Glucocorticoids also blunt NF-κB responses by stimulating the transcription of the NF-κB inhibitor, IκB, thus limiting nuclear translocation of NF-κB (59). cAMP, acting through PKA, also inhibits nuclear translocation of p65 in certain cell lines by an unknown mechanism (34, 60).

In certain circumstances, PKA can also activate NF-κB. For instance, phosphorylation of the Ser-276 residue in p65 by PKA activates transcription from an NF-κB-dependent reporter construct in Jurkat T cells (48). Several groups have shown that CREB functionally interacts with the phosphorylated Ser-276 residue in p65 (48, 55). Our results also demonstrate the importance of this site for the function of p65 in H4IIE hepatoma cells (Fig. 6). The S276A mutation abrogates the repressive effect of p65 on PEPCK gene expression (Fig. 5B). These results suggest that the wild type p65 in H4IIE cells is phosphorylated on this site, perhaps through PKA, which is constitutively active in these hepatoma cells (4, 61). Interestingly, the sequence surrounding Ser-276 is a potential consensus site for several other kinases, including protein kinase B (PKB). We are currently exploring the possibility that insulin and the other repressors of PEPCK gene expression stimulate phosphorylation of this site in hepatoma cells.

NF-κB may play a role in the tonic regulation of both the basal and hormone-stimulated activity of the PEPCK gene. As shown in Fig. 3, NF-κB is present in the nuclei of hepatoma cells incubated in serum-free DMEM. The amount/activity of this nuclear factor decreases when the cells are incubated in either dexamethasone or cAMP. Thus, the low basal activity of the PEPCK gene promoter could be caused by the presence of NF-κB, and part of the increase following hormone treatment could be due to the absence of the repressing effect of NF-κB. Insulin, phorbol esters, and cytokines could exert their significant repressive effects on PEPCK gene transcription by promoting the entry of NF-κB into the nucleus and thereby reinstating tonic inhibition. The data also suggest that CBP plays a central role in the function of both the GRU and CRU in that p65 interferes with the action of these regulatory domains. GR is known to bind to p65, which could interfere with the binding of the former to CBP. In the case of the PKA response of the PEPCK gene, it is probable that p65 competes with CREB for binding to CBP, because CREB phosphorylated on Ser-133, and p65 phosphorylated on Ser-276, interact with the same region of CBP (48, 62). This hypothesis is supported by our observation that the S276A mutant of p65 does not repress the PKA response of the PEPCK gene.

The effect of insulin on the PEPCK gene, like that of glucocorticoids, retinoic acid, and cAMP, appears to be mediated by a multicomponent insulin response unit (IRU) (2). αFA2, an accessory factor binding site in the GRU, is a component of the IRU. This DNA segment (the distal IRS) mediates the repression of a reporter gene when placed in the context of a heterologous promoter (24). Members of the HNF-3/forkhead receptor (FKHR) family of transcription factors, which have in common a winged helix motif of 100 amino acids for monomeric recognition of specific DNA sites (63), are candidate factors for mediating the insulin response, because they bind to the PEPCK IRS (11, 64, 65). These proteins have gained recent attention, because genetic studies in Caenorhabditis elegans indicate that the forkhead transcription factor, daf-16, is the target of a signaling pathway that includes the insulin/insulin growth factor-1 receptor homologue (daf-2), the catalytic subunit of PI3K homologue (age-1), and PKB homologues (akt-1 and akt-2) (66–68). PI3K is involved in the action of insulin on PEPCK gene transcription (27), and conflicting views about the role of protein kinase B (akt) have been reported (69–71). We are currently investigating the role of these proteins in insulin-mediated repression of PEPCK gene expression. However, because an insulin response persists when this IRS is mutated or deleted in the context of the otherwise wild type PEPCK gene promoter (39), we postulate that another, more proximal, IRS is a second component of the IRU (2).

The studies reported here show that the p65 subunit of NF-κB is increased in the nuclei of H4IIE cells after insulin treatment. This protein is insulinomimetic in that it prevents the induction of PEPCK gene transcription by dexamethasone and cAMP. p65 could also be the final pathway to the PEPCK gene in the actions of phorbol esters, oxidation stress, and certain cytokines, all of which increase the activity of p65 and prevent induction of the PEPCK gene. The specificity of these effects is now under investigation.

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