Protein Kinase A Phosphorylates Hepatocyte Nuclear Factor-6 and Stimulates Glucose-6-phosphatase Catalytic Subunit Gene Transcription*

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Glucose-6-phosphatase is a multicomponent system that catalyzes the terminal step in gluconeogenesis. To examine the effect of the cAMP signal transduction pathway on expression of the gene encoding the mouse glucose-6-phosphatase catalytic subunit (G6Pase), the liver-derived HepG2 cell line was transiently co-transfected with a series of G6Pase-chloramphenicol acetyltransferase fusion genes and an expression vector encoding the catalytic subunit of cAMP-dependent protein kinase A (PKA). PKA markedly stimulated G6Pase-chloramphenicol acetyltransferase fusion gene expression, and mutational analysis of the G6Pase promoter revealed that multiple cis-acting elements were required for this response. One of these elements was mapped to the G6Pase promoter region between −114 and −99, and this sequence was shown to bind hepatocyte nuclear factor (HNF)-6. This HNF-6 binding site was able to confer a stimulatory effect of PKA on the expression of a heterologous fusion gene; a mutation that abolished HNF-6 binding also abolished the stimulatory effect of PKA. Further investigation revealed that PKA phosphorylated HNF-6 in vitro. Site-directed mutation of three consensus PKA phosphorylation sites in the HNF-6 carboxyl terminus markedly reduced this phosphorylation. These results suggest that the stimulatory effect of PKA on G6Pase fusion gene transcription in HepG2 cells may be mediated in part by the phosphorylation of HNF-6.

Glucose-6-phosphatase is a multicomponent system located in the endoplasmic reticulum that catalyzes the terminal step in gluconeogenesis and hepatic glycogenolysis (1, 2). The kinetics of glucose-6-phosphate hydrolysis by glucose-6-phosphatase are complex, and several models for the glucose-6-phosphatase system have been proposed (1, 2). One model has been proposed in which, in adults, in contrast to the fetus (3), the active site of glucose-6-phosphatase catalytic subunit (G6Pase)1 is contained within the lumen of the endoplasmic reticulum (1, 2). In this model the other components of the glucose-6-phosphatase system act as transport proteins to shuttle both substrate and product across the endoplasmic reticulum membrane (1, 2). These include a glucose-6-phosphate transporter and putative transporters for inorganic phosphate and glucose (1, 2). Inactivating mutations in the G6Pase and glucose-6-phosphate transporter genes gives rise to glycogen storage disease types 1a and 1b, respectively (4). Type 1 glycogen storage disease is characterized by severe hypoglycemia in the postabsorptive state, hyperlipidemia, hyperuricemia, and lactic acidemia (4–6). In addition, patients are prone to growth retardation, hepatic steatosis and cirrhosis, hepatic adenoma, and renal failure (4–7). Because of the wider tissue distribution of the glucose-6-phosphatase transporter, patients afflicted with glycogen storage disease type 1b also suffer from infectious complications as a result of functional deficiencies in neutrophils and monocytes, indicating an important role for this transporter in the immune response (4).

In contrast to glycogen storage disease type 1a, which is caused by decreased G6Pase activity, increased G6Pase activity contributes to the pathophysiology of diabetes. In both type 2 and poorly controlled type 1 diabetics, the ability of insulin to stimulate peripheral glucose utilization and to repress hepatic glucose production is reduced as a consequence of insulin resistance. Although the causes of insulin resistance are unclear (8), it is apparent that the elevation in hepatic glucose production is caused by an increased rate of gluconeogenesis rather than glycogenolysis in both type 1 (9) and type 2 diabetics (10). Several lines of evidence suggest that increased expression of key gluconeogenic enzymes including G6Pase contribute to this increase in hepatic glucose production. Thus, hepatic G6Pase expression is markedly elevated in various diabetic animal models (11–15), and overexpression of G6Pase in hepatocytes using recombinant adenovirus was associated with enhanced rates of gluconeogenesis as well as defects in glycogen metabolism (16). Furthermore, a moderate overexpression of G6Pase in rats, again using recombinant adenovirus, resulted in approximately a 1.6–3-fold increase in hepatic G6Pase enzymatic activity that was associated with glucose intolerance, hyperinsulinemia, decreased hepatic glycogen content, and increased peripheral triglyceride stores, changes similar to those found in early stage type 2 diabetic patients (17). These observations (16, 17) suggest that G6Pase is a major control point in the

protein kinase A; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; CRE, cAMP response element; CREB, cAMP-response element-binding protein; CRU, cAMP response unit.

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1 The abbreviations used are: G6Pase, glucose-6-phosphatase catalytic subunit; HNF, hepatocyte nuclear factor; PKA, catalytic subunit of cAMP-dependent protein kinase A; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; CRE, cAMP response element; CREB, cAMP-response element-binding protein; CRU, cAMP response unit.

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glucose-6-phosphatase system and represents a prime therapeutic target for the treatment of diabetes.

In the liver cAMP, glucocorticoids, glucose, fatty acids, lepin, and β₂ adrenergic receptor agonists all stimulate G6Pase gene expression (11, 14, 18–24), whereas insulin both inhibits basal G6Pase gene expression and overrides the stimulatory effects of cAMP, glucocorticoids, glucose, and fatty acids (11, 18–20, 22, 24). Multiple cis-acting elements in the G6Pase promoter are required for the full stimulatory effect of the cAMP signal transduction pathway on G6Pase gene expression (25–27). This paper shows that one of these elements is a binding site for hepatocyte nuclear factor (HNF-6) and demonstrates that HNF-6 is a substrate for the catalytic subunit of protein kinase A (PKA).

**Experimental Procedures**

Materials—[α-32P]dATP (>3000 Ci mmol⁻¹) and [γ-32P]dATP (>6000 Ci mmol⁻¹) were obtained from Amersham Pharmacia Biotech, and [3H]acetic acid sodium salt (>10 Ci mmol⁻¹) was obtained from ICN. BL21(DE3) pLyS and BL21-CodonPlus™(DE3)-RIL-competent Escherichia coli cells were obtained from Stratagene, and nickel-nitrilotriacetic acid agarose was from Qiagen. Purified bovine PKA was a generous gift from Drs. Jackie Corbin and Sharron Francis (28).

Plasmid Construction—The construction of a series of 5′ truncated G6Pase-CAT fusion genes has been described previously (29, 30). A site-directed mutant of the G6Pase HNF-6 motif was generated within the context of the −231 to +66 G6Pase promoter fragment using a previously described three-step PCR strategy (29, 31). The resulting construct was used as the template in a second PCR to create a site-directed mutant of the G6Pase HNF-6 motif within the context of the −129 to +66 G6Pase promoter fragment (Fig. 1). The heterologous XMB vector contains a minimal Xenopus 68-kDa albumin promoter ligated to the CAT reporter gene (32). Double-stranded complementary oligonucleotides representing the wild-type or mutated HNF-6 motif (Fig. 1B) were synthesized and digested with HindIII-compatible ends and ligated into HindIII-cleaved XMB in multiple (5–6) copies. The number of inserts was determined by restriction enzyme analysis and confirmed by DNA sequencing.

Expression vectors encoding the α and β forms of PKA were a generous gift from Dr. Richard Maurer (33). An empty vector control was generated by digesting the PKAβ plasmid with XhoI and HindIII to remove the open reading frame, filling in the 3′ end copies, and religating the Klenow fragment of E. coli DNA polymerase I, and then religating. A mammalian cell expression vector encoding the full-length form of PKA was generated by ligating a Klenow fragment of E. coli DNA polymerase I to a specific activity of 2.5 µCi/µmol. The labeled HNF-6 oligonucleotide (~7.5 fmol, ~30,000 cpn) was incubated with bacterial extract in a final reaction volume of 20 µl containing 20 µM HEPES, pH 7.5, 50 mM KCl, 1 mM MgCl₂, 0.5 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride at 4 °C. The soluble fraction was separated from the particulate fraction by centrifugation. Complementary oligonucleotides representing the mouse G6Pase HNF-6 motif (Fig. 1B) were synthesized with HindIII-compatible ends, gel purified, annealed, and then labeled with [α-32P]dATP by using the Klenow fragment of E. coli DNA polymerase I. The labeled HNF-6 oligonucleotide was incubated with bacterial extract for 1 h at 37 °C. After centrifugation, gels were dried and exposed to Kodak XAR5 film, and binding was analyzed by autoradiography.

**Cell Culture, Transient Transfection, CAT, and β-galactosidase Assays—**Human HepG2 hepatoma cells were grown and transfected in suspension using the calcium phosphate DNA co-precipitation method as described previously (29, 30). CAT and β-galactosidase assays were also performed as described previously (29, 30). The CAT activity directed by the various fusion gene constructs was corrected for the β-galactosidase activity in the same samples, and each construct was analyzed in duplicate in multiple transfections as specified in the figure legends.

**Gel Retardation Assays—IPTG was used to induce the expression of HNF-6 in the BL21(DE3) pLyS E. coli strain (Stratagene) transformed with the full-length HNF-6 pET15b plasmid described above.** Bacterial extracts were prepared by sonication in 20 mM HEPES, pH 7.5, 50 mM KCl, 1 mM MgCl₂, 0.5 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride at 4 °C. The soluble fraction was separated by centrifugation. Complementary oligonucleotides representing the mouse G6Pase HNF-6 motif were synthesized with HindIII-compatible ends, ligated into HindIII-cleaved XMB in multiple (5–6) copies. The number of inserts was determined by restriction enzyme analysis and confirmed by DNA sequencing.

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**HNF-6 Purification—**The full-length, wild-type and mutated, and carboxyl-terminal truncated forms of HNF-6 were expressed in the BL21-CodonPlus™(DE3)-RIL E. coli strain (Stratagene) transformed with the HNF-6 pET15b plasmids described above. Once bacterial cultures (1 liter) had reached an A₆₀₀ of ~0.6, protein expression was induced with IPTG (1 mM) by incubation for 4 h at 37 °C. After centrifugation bacterial pellets were stored at −20 °C overnight. The histidine-tagged HNF-6 protein was then partially purified using metal affinity chromatography. Briefly, bacterial pellets were thawed on ice for 15 min and then resuspended in 20 mM of lysis buffer, pH 8.0, containing 50 mM NaF, 500 mM NaCl, 20 mM imidazole, 1 mM benzamidine, 1 mM N-ethylmaleimide, 1 mM β-mercaptoethanol, and 1 mg/ml lysozyme. After incubation on ice for 30 min, bacteria were sonicated at 4 °C for 10 min with 30 s each with a 1-min incubation on ice between each round of sonication. Lyssates were then diluted with an additional 20 ml of lysis buffer, and particulate matter was removed by centrifugation. The supernatant was incubated with 5 ml of nickel-nitrirotiacetic acid agarose (Qiangen) for 1 h at 4 °C before the application of the slurry to a Ni-NTA column.
column. After washing twice with 50 ml of a buffer, pH 8.0, comprising 50 mM NaH₂PO₄, 500 mM NaCl, 60 mM imidazole, 10 mM β-mercaptoethanol, 0.5 mg/ml bovine serum albumin, and 1 mM phenylmethylsulfonyl fluoride, HNF-6 was eluted in a buffer, pH 7.2, containing 50 mM NaH₂PO₄, 500 mM NaCl, 250 mM imidazole, 10 mM β-mercaptoethanol, 0.5 mg/ml bovine serum albumin, and 1 mM phenylmethylsulfonyl fluoride. The purification of HNF-6 was analyzed via SDS-PAGE, and protein was visualized by Coomassie Brilliant Blue staining (35). Gels corresponding to phosphorylated HNF-6 were then cut out of the dried gel, were subsequently dried and exposed to Kodak XAR5 film. Bands were visualized via Coomassie Brilliant Blue staining (35). Gels of HNF-6 were visualized by Coomassie Brilliant Blue staining (35). Bands corresponding to phosphorylated HNF-6 were then cut out of the dried gels, and the incorporation of phosphate into HNF-6 was quantified by scintillation counting.

RESULTS

An Element Located between −129 and −85 in the G6Pase Promoter Contributes to the Full Stimulatory Effect of the cAMP Signal Transduction Pathway on G6Pase-CAT Fusion Gene Expression—To examine the molecular mechanisms by which the cAMP signal transduction pathway stimulates mouse G6Pase gene expression, the liver-derived HepG2 cell line was transiently co-transfected with a series of G6Pase-CAT fusion genes and an expression vector encoding PKA. PKA markedly stimulated G6Pase-CAT fusion gene expression, and mutational analysis of the G6Pase promoter revealed that multiple regions were required for this response (Fig. 1A; Ref. 27). This strategy led to the identification of a cAMP response element (CRE) in the mouse G6Pase promoter located between −162 and −155 (27). However, even when this element was deleted, PKA still induced G6Pase-CAT fusion gene expression ~4–5-fold (Fig. 1A; Ref. 27). Further deletion of the G6Pase promoter sequence between −129 and −85 resulted in an additional reduction in the stimulatory effect of PKA on G6Pase-CAT fusion gene expression (Fig. 1A). These results indicate that the cAMP signal transduction pathway stimulates the expression of G6Pase through a complex cAMP response unit (CRU; Ref. 36) and that an element located between −129 and −85 in the G6Pase promoter contributes to the full stimulatory effect of PKA on G6Pase-CAT fusion gene expression.

An HNF-6 Binding Site Located between −110 and −101 in the G6Pase Promoter Contributes to the Full Stimulatory Effect of PKA on G6Pase-CAT Fusion Gene Expression—Examination of the mouse G6Pase promoter sequence between −129 and −85 for known transcription factor binding sites revealed a putative binding site for HNF-6 located between nucleotides −110 and −101 (Fig. 1B; Ref. 37). The sequence of this HNF-6 motif is highly conserved among the mouse, rat, and human G6Pase genes (Fig. 1B); this seems to be the only HNF-6 motif in the mouse G6Pase promoter. Samadani and Costa (38) have previously shown that HNF-6 is expressed in HepG2 cells. To determine whether this putative HNF-6 binding site was required for the stimulatory effect of PKA on G6Pase-CAT fusion gene expression, this motif was mutated by site-directed mutagenesis in the context of the −129 to +66 G6Pase promoter fragment. The fusion gene containing this mutation, designated −129 HNF-6 SDM, was transiently co-transfected into HepG2 cells in combination with an expression vector encoding PKA. Mutation of the G6Pase HNF-6 motif resulted in a decreased stimulation of G6Pase-CAT fusion gene expression by PKA. The HNF-6 motif was mutated as shown in B. After transfection, cells were incubated for 18–20 h in serum-free medium. The cells were then harvested, and both CAT and β-galactosidase activity were assayed as described previously (29, 30). Results are presented as the ratio of CAT activity, corrected for β-galactosidase activity in the cell lysate, in PKA-transfected versus empty vector-transfected cells (expressed as fold induction and represent the mean ± S.E. of 5–18 (A) or 3 (C) experiments in which each construct was assayed in duplicate. SDM, site-directed mutant. B, comparison of the mouse G6Pase promoter sequence between −114 and −99 with the equivalent sequence from the rat and human G6Pase promoters. The putative HNF-6 binding motif is boxed. The consensus HNF-6 sequence is taken from Ref. 37.
by PKA compared with the wild-type –129 G6Pase-CAT fusion gene construct (Fig. 1C) that was equivalent to that seen when the entire HNF-6 site was deleted, as is the case with the –85 G6Pase-CAT fusion gene (Fig. 1C). This result suggests that HNF-6 could be the factor that is mediating the stimulatory effect of PKA on G6Pase-CAT fusion gene expression through this region.

**HNF-6 Binds to the G6Pase HNF-6 Motif**—To provide evidence that HNF-6 is indeed the factor that is mediating the effect of PKA through the –129 to –85 promoter sequence, HNF-6 binding to this region was analyzed using the gel retardation assay. IPTG was used to induce the expression of a histidine-tagged form of mouse HNF-6 in bacteria, and then a soluble extract from these cells was incubated with a labeled double-stranded oligonucleotide representing the G6Pase HNF-6 motif (Fig. 2A). No protein binding was detected in the gel retardation assay using lysate from non-IPTG-treated bacteria (data not shown); however, a single protein-DNA complex was detected using lysate from IPTG-treated cells (Fig. 2A). Competition experiments, in which a 100-fold molar excess of a unlabeled oligonucleotide was added to the labeled probe, were used to correlate protein binding with the PKA response. An oligonucleotide representing the wild-type G6Pase HNF-6 binding site competed effectively with the labeled probe for protein binding (Fig. 2A). By contrast, an oligonucleotide that contains a mutation identical to that in the –129 HNF-6 SDM construct (Fig. 1) failed to compete (Fig. 2A). Thus, the binding of HNF-6 to the G6Pase promoter (Fig. 2A) correlates with the stimulatory effect of PKA on G6Pase-CAT fusion gene expression (Fig. 1).

**The G6Pase HNF-6 Motif Can Confer a Direct Stimulatory Effect of PKA on the Expression of a Heterologous Fusion Gene**—To determine whether the G6Pase HNF-6 motif was sufficient to mediate a direct stimulatory effect of PKA on gene transcription, six copies of a double-stranded oligonucleotide representing the wild-type G6Pase promoter sequence between –114 and –99 (Fig. 1B) were ligated into the heterologous XMB expression vector (32). Transient co-transfection of the resulting construct, designated HNF-6 WT XMB, into HepG2 cells with the expression vector encoding PKA resulted in an approximately 6-fold stimulation of reporter gene expression (Fig. 2B). Similarly, three copies of a larger double-stranded oligonucleotide representing the wild-type G6Pase promoter sequence between –114 and –77 conferred a 4.12 ± 0.19-fold induction (n = 4) of reporter gene expression by PKA when ligated into the heterologous XMB expression vector (data not shown). To verify that the stimulatory effect of PKA was mediated through the HNF-6 binding site, five copies of a double-stranded oligonucleotide containing the same mutation (Fig. 1B) that abolished HNF-6 binding in gel retardation assays (Fig. 2A) were ligated into the XMB vector. No basal reporter gene expression was detected when the resulting construct, designated HNF-6 MUT XMB, was transiently transfected into HepG2 cells. Furthermore, co-transfection with the expression vector encoding PKA failed to induce reporter gene expression (Fig. 2B). These results support a model in which HNF-6 is a target of PKA signaling.

**HNF-6 Is Phosphorylated by PKA in Vitro**—Rousseau and co-workers (39) have previously noted that rat HNF-6 contains five potential PKA phosphorylation sites, but only three of these strongly match the consensus PKA phosphorylation sequence (40). All five sites are perfectly conserved among human, rat, and mouse HNF-6. Fig. 3 shows that PKA can phosphorylate a histidine-tagged form of HNF-6 in a time- and concentration-dependent manner in vitro. The kinetics of the phosphorylation were markedly affected by the concentration of magnesium in the reaction (Fig. 3, compare A and B). In the presence of low magnesium (0.1 mM), PKA phosphorylates HNF-6 with an apparent $K_m$ of ~0.25 μM (Fig. 3A). However, in the presence of high magnesium (5 mM), we were not able to calculate an apparent $K_m$, because we were unable to add sufficient HNF-6 to the phosphorylation reaction to reach $V_{max}$ (Fig. 3B). In the presence of either low (0.1 mM) or high (5 mM) concentrations of magnesium, the time course of HNF-6 phosphorylation was similar with maximal phosphorylation by ~20 min (Fig. 3, C and D). However, the maximal incorporation of phosphate into HNF-6 was much greater when phosphorylation reactions contained 5 mM magnesium. A maximum stoichiometry of ~1.75 mol of phosphate/mol of HNF-6 was calculated in the presence of 5 mM magnesium. In contrast, in
the presence of 0.1 mM magnesium, a maximum stoichiometry of ~0.3 mol of phosphate/mol of HNF-6 was obtained. Such magnesium-dependent variations in the kinetics and stoichiometry of phosphorylation by PKA have been reported for other PKA substrates (41–43).

The three consensus PKA phosphorylation sites in HNF-6 are located in the carboxyl-terminal region of the protein (39). Therefore, an expression vector was constructed that encoded a histidine-tagged carboxyl-terminal truncated form of HNF-6 in which these three putative PKA phosphorylation sites were deleted. The phosphorylation of this truncated form of HNF-6 by PKA was markedly reduced compared with the nontruncated form of the protein (Fig. 4). This result suggests that HNF-6 is phosphorylated in vitro by PKA predominantly on one or more of the carboxyl-terminal sites that match the consensus PKA phosphorylation sequence. Further support for this conclusion was obtained by constructing an expression vector that encoded a histidine-tagged full-length form of HNF-6 in which these three putative PKA serine phosphorylation sites were changed to alanine residues by site-directed mutagenesis (Fig. 4). The phosphorylation of this mutated form of HNF-6 by PKA was markedly reduced compared with the wild-type form of the protein (Fig. 4).

**Fig. 3. HNF-6 is phosphorylated by PKA in vitro.** The ability of PKA to phosphorylate a partially purified 6x histidine-tagged HNF-6 fusion protein was assessed as described under "Experimental Procedures." Phosphorylation reactions were analyzed by SDS-PAGE, and phosphate incorporation was quantitated by scintillation counting. A and B show the relationship between HNF-6 concentration and 32P incorporation in the presence of 0.1 mM or 5 mM magnesium acetate, respectively. Phosphorylation reactions were performed for 5 (A) or 1 min (B) at room temperature. Under these conditions the rate of 32P incorporation into HNF-6 was linear at all HNF-6 concentrations tested. C and D show the relationship between time and 32P incorporation in the presence of 0.1 mM or 5 mM magnesium acetate, respectively. Phosphorylation reactions were performed using 1 μM HNF-6. Each panel shows the mean data ± S.E. from three experiments with a representative autoradiograph shown as an inset. Plots without error bars indicate that the S.E. values were smaller than the plot symbol.
To explore the functional consequence of mutating these three serine residues on PKA-stimulated G6Pase-CAT fusion gene expression, expression vectors encoding full-length wild-type and mutated HNF-6 were constructed. Fig. 5 shows that the co-transfection of the wild-type −129 G6Pase-CAT fusion gene construct with either of these expression vectors stimulated reporter gene expression to the same extent as that achieved by co-transfection with the expression vector encoding the catalytic subunit of PKA. Because phosphorylation by PKA has little effect on HNF-6 binding to DNA (data not shown), we hypothesize that it increases the transactivating potential of HNF-6 but that this effect is only apparent under conditions in which the concentration of HNF-6 is limiting.

**DISCUSSION**

Multiple promoter elements are required for the full stimulatory effect of the cAMP signal transduction pathway on G6Pase gene transcription in hepatoma cells that together comprise a CRU (27, 36). The large induction of G6Pase-CAT fusion gene expression obtained by using the PKA co-transfection technique was critical for the delineation of such a multiple component CRU (Fig. 1A; Ref. 27). Thus, in contrast, using cAMP analogs both Chou and co-workers (25) and Burchell and co-workers (26) reported the involvement of single elements in the cAMP response. Lin et al. (25) found that a region of the human G6Pase promoter encompassing the sequence between −136 and −134 was required for the stimulatory effect of cAMP on G6Pase fusion gene expression in HepG2 cells, whereas Schmoll et al. (26) found that the sequence located between −161 and −152 was critical for the combined stimulatory effects of cAMP and glucocorticoids in H4IIE hepatoma cells. The reason for these disparate results with the human promoter is unclear, but our data indicate that both of the equivalent regions in the mouse G6Pase promoter contribute to the induction of G6Pase-CAT fusion gene expression by PKA (Fig. 1A; Ref. 27). However, even with both of these regions deleted, mouse G6Pase-CAT fusion gene expression was still induced by ∼5-fold in response to PKA (Fig. 1A). Further truncation of the G6Pase promoter sequence between −129 and −85 resulted in a reduction in this stimulatory effect of PKA on G6Pase-CAT fusion gene expression (Fig. 1A). The data presented in this paper suggest that the stimulatory effect of PKA through the −129 to −85 region of the G6Pase promoter is mediated by the phosphorylation of HNF-6. Mutation of this HNF-6 motif in the context of an otherwise intact CRU has little effect on the induction of G6Pase-CAT fusion gene expression by PKA (data not shown). In contrast, mutation of the CRE, located between −162 and −155, in the context of an otherwise intact CRU almost abolishes the induction of G6Pase-CAT fusion gene expression by PKA in HepG2 cells (data not shown) and LLC-PK cells (27). These results are consistent with the 5′ deletion analysis (Fig. 1A) that shows a much greater contribution of this CRE than the HNF-6 motif to the PKA response. Whether the relative contribution of the HNF-6 motif to the induction of G6Pase gene transcription by PKA increases under certain metabolic conditions and whether HNF-6 is important for the induction of other hepatic genes by PKA remains to be determined.

HNF-6 is a member of the ONECUT family of transcription factors that is characterized by a bipartite DNA binding domain consisting of a single cut domain and an atypical homeodomain (39). Classical homeodomains are 60 amino acids long and contain a conserved tryptophan and histidine at positions 48 and 50 of the homeodomain as opposed to the homeodomain in the ONECUT transcription factor family, in which the amino acid residues located at positions 48 and 50 are phenylalanine and methionine, respectively (37). The cut domain has been shown to be required for HNF-6 binding to DNA in all target proteins.
genes examined, whereas for a subset of HNF-6 target genes, the homeodomain seems to be dispensable for DNA binding (37). Both the cut domain and the homeodomain of HNF-6 are also involved in transcriptional activation by HNF-6 (37, 44). Activation of HNF-6 target gene transcription on promoters that do not require the HNF-6 homeodomain for DNA binding involves the recruitment of the CREB-binding protein (44). The interaction of CREB-binding protein with rat HNF-6 requires an LXXLL motif (where L is a leucine residue and X is any amino acid) in the cut domain and the amino acid residues located at positions 48 and 50 (phenylalanine and methionine, respectively) of the homeodomain (44). The LXXLL motif has previously been shown to be important for the interaction of other transcription factors with CREB-binding protein (45). In contrast, activation of gene transcription by HNF-6 on target genes that require the homeodomain for DNA binding involves the recruitment of the coactivator p300/CREB-binding protein-associated factor through an unidentified domain (44).

Of the three consensus PKA phosphorylation sites in HNF-6, one is located in the vicinity of the LXXLL motif in the cut domain (serine residue 309), and the other two are located in the homeodomain (serine residues 411 and 440). As described above, both the cut domain and the homeodomain are involved in DNA binding and in recruitment of coactivators, and thus the phosphorylation of HNF-6 by PKA could potentially have affected either or both parameters. Because phosphorylation by PKA has little effect on HNF-6 binding to DNA (data not shown), we hypothesize that it increases the transactivation potential of HNF-6. However, this putative effect of PKA-dependent phosphorylation on HNF-6 transcription potential is only apparent under conditions in which the concentration of HNF-6 is limiting (Fig. 5). Thus, overexpression of either wild-type or mutant HNF-6 stimulates basal G6Pase-CAT fusion gene expression to the same extent as that achieved by co-transfection with the expression vector encoding PKA (Fig. 5). It may be possible to prove that PKA-dependent phosphorylation increases the transactivation potential of HNF-6 by analyzing the effect of mutating the three PKA phosphorylation sites in the context of an HNF-6 molecule in which the basal activation domains (37, 44) have been mutated but only if these same domains are not also required for the PKA response. These observations are somewhat related to those recently reported by Quinn and co-workers (46, 47), who investigated the relative contributions of different domains in CREB to transcription initiation. Of particular note is the observation that the constitutive activation domain in CREB mediates recruitment of the polymerase complex, whereas the kinase-inducible domain mediates later PKA-stimulated steps in transcription initiation. In the case of HNF-6 we hypothesize that when the HNF-6 binding site in the G6Pase promoter is fully occupied, the basal activation domains in HNF-6 are sufficient to mediate a maximal rate of transcription initiation. Interestingly, protein kinase C and casein kinase II phosphorylate the Cut/CCAAT displacement protein, a member of the superclass of cut homeodomain proteins, on residues located in the cut domain and alter the binding of the Cut/CCAAT displacement protein to DNA (48, 49).

Because overexpression of HNF-6 stimulates G6Pase fusion gene expression (Fig. 5), this raises the possibility that hormones/metabolites could regulate G6Pase gene expression indirectly through an action on HNF-6 gene expression. There is circumstantial evidence that HNF-6 expression may be regulated by cAMP/PKA. Thus a CRE is present in the HNF-6 promoter (50) and growth hormone, which activates PKA in liver (51), stimulates HNF-6 expression (52). However, whether the CRE in the HNF-6 promoter contributes to this stimulation is unknown (50). The available data show that the effect of growth hormone on HNF-6 expression is mediated at least in part through increases in signal transducer and activator of transcription-5 and HNF-6 binding (53) and a decrease in CCAAT/enhancer-binding protein-α binding (54) to the HNF-6 promoter. The relative roles of growth hormone-stimulated PKA and Janus Kinase/signal transducer and activator of transcription activation in mediating these changes remain to be determined.

The phosphorylation of HNF-6 by PKA varied with changes in the magnesium ion concentration (Fig. 3). Other substrates are also differentially phosphorylated by PKA when the magnesium ion concentration is altered (41, 43). Thus, Singh et al. (41, 43) demonstrated that PKA phosphorylates a single site in the α subunit of phosphorylase kinase at low magnesium ion concentrations; however, at high magnesium ion concentrations, PKA phosphorylates the α subunit of phosphorylase kinase on three additional sites. Furthermore, Berglund et al. (42) showed that the optimal phosphorylation of 1-type pyruvate kinase by PKA was found within a very narrow range of magnesium ion concentration. However, when the magnesium ion concentration was increased above 10 mM or decreased below 4 mM, PKA was less active (42).

In summary, the data presented in this manuscript demonstrate that an HNF-6 site located between −110 and −101 in the G6Pase promoter may contribute to the full stimulatory effect of PKA on G6Pase-CAT fusion gene expression. In addition, HNF-6 was shown to be phosphorylated by PKA in vitro. Taken together, these results suggest that the stimulatory effect of PKA on G6Pase gene transcription may be mediated in part by the phosphorylation of HNF-6.

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