Title: cAMP opposes the glucose-mediated induction of the L-PK gene by preventing the recruitment of a complex containing ChREBP, HNF4α, and CBP.

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Short Title: Regulation of the L-PK gene by glucose and cAMP
ABSTRACT

Glucose and cAMP have opposing physiological effects. Glucose-mediated activation of the L-type pyruvate kinase (L-PK) gene is repressed by cAMP, making this an excellent model for studying the mechanism by which these contrary signals regulate gene expression. Using the 832/13 rat insulinoma cell line we demonstrate using RNA interference and chromatin immunoprecipitation that Carbohydrate Response Element Binding Protein (ChREBP), Hepatic Nuclear Factor 4α (HNF4α, and the coactivator CREB Binding Protein (CBP) are required for the glucose response of the L-PK gene, and are recruited to the promoter by glucose. The cAMP agonist forskolin blocked the glucose-mediated induction of the L-PK gene in a PKA-dependent manner, and blocked the recruitment of ChREBP, HNF4α, and CBP to the L-PK promoter, while simultaneously recruiting CBP to the cAMP-inducible gene, nuclear receptor subfamily 4, group A, member (NR4A2). Overexpression of CBP, but not ChREBP, reversed the cAMP repression of the L-PK gene. In addition, CBP augmented the glucose response of the L-PK promoter. We conclude that cAMP and glucose signaling converge on CBP, and that cAMP acts by disrupting the transcriptional complex assembled by glucose-derived signals.

**Keywords:** transcription, repression, promoter, signaling
INTRODUCTION

cAMP and glucose are opposing physiological signals. cAMP is elevated in cells experiencing metabolic fuel deprivation, whereas glucose represents metabolic fuel abundance (1). Cells respond to these contrary signals by altering gene expression patterns to adjust their metabolic phenotype. The L-type pyruvate kinase (L-PK; gene symbol: Pklr) gene encodes a key regulatory enzyme of glycolysis that catalyzes the terminal step in the oxidation of glucose to pyruvate with concomitant generation of ATP; therefore, its expression is tightly regulated. Glucose induces expression of the L-PK gene, while cAMP agonists, such as forskolin (an activator of adenylate cyclase), block this expression, making the L-PK gene an excellent model system for studying the molecular mechanisms of the reciprocal effects of glucose and cAMP on gene transcription (2).

The basic Helix-Loop-Helix Leucine Zipper (bHLH-LZ) transcription factor carbohydrate response element binding protein (ChREBP) is an important mediator of glucose action on induction of glycolytic (L-PK) and lipogenic enzyme genes (ACC, FAS) (3-9). The original model proposed by Uyeda and colleagues states that under low glucose conditions ChREBP is sequestered in the cytoplasm, presumably through PKA-mediated phosphorylation by a cAMP-generating signal, and that an increase in glucose metabolism promotes dephosphorylation and entry of ChREBP into the nucleus (3). Once in the nucleus, ChREBP mediates expression of target genes by binding to the carbohydrate response element (ChoRE) as a heterodimer with its partner Mlx (10,11). Signals that increase intracellular cAMP levels have been suggested to provide inhibitory phosphorylation of ChREBP, consequently abolishing its nuclear localization and DNA binding, and subsequently, transactivation of the L-PK gene. Recent studies, however, have shown that this model is most likely incomplete and that mechanisms in addition to the phosphorylation of ChREBP at sites 196 and 666 are necessary for controlling regulation of the L-PK gene (6, 12-14). Furthermore, it has been suggested recently that although glucose promotes a modest increase in the rate of nuclear ChREBP influx, cytoplasmic-nuclear shuttling is not the major mechanism of ChREBP regulation by glucose (13, 15), although this is still unresolved (16).
Several groups have shown that a second transcription factor, Hepatocyte Nuclear Factor 4 α (HNF4α), plays an important complementary role to ChREBP in the induction of L-PK gene expression (17-19). Both the ChREBP and the HNF4α binding sites (the ChoRE and L3, respectively) are required for maximal glucose-induced transactivation of the L-PK gene (17, 18).

While ChREBP and HNF4α have been implicated as contributing factors in the regulation of the L-PK gene by glucose, the mechanisms underlying both the glucose activation and cAMP-mediated repression of this gene are largely unknown. Therefore, two key questions regarding regulation of the L-PK gene that remain unanswered: 1) how does cAMP mediate repression of the glucose-stimulated induction? and 2) Is there a coactivator necessary to provide the maximum response to glucose?

CBP is a versatile transcriptional coactivator that plays a pivotal role in coordinating signaling events with the transcription machinery, thus permitting the appropriate level of gene activity to occur in response to diverse stimuli (20). HNF4α, a member of the nuclear hormone receptor superfamily, interacts with the coactivator CREB binding protein (CBP) (21), leading us to hypothesize that CBP may be the coactivator necessary to communicate multiple signals (e.g., glucose and cAMP) to the L-PK gene promoter.

In this report we demonstrate that glucose and cAMP converge on a complex containing CBP to regulate L-PK gene transcription. We present multiple lines of evidence that ChREBP, HNF4α, and CBP are co-resident on the L-PK gene promoter and are equally important for full activation of the L-PK gene by glucose. Additionally, we demonstrate that the cAMP-mediated repression of the L-PK gene involves disassembly of a complex of proteins containing these factors, and can be overcome by increasing CBP abundance.
MATERIALS AND METHODS

Cell Culture, RNA isolation and quantification by RT-PCR

832/13 cell culture conditions were as described previously (22). Total RNA was isolated from 832/13 cells using TRI-reagent (Molecular Research Center; Cincinnati, OH, USA) according to the manufacturer's protocol. iScript (Bio-Rad; Hercules, CA, USA) was used for first-strand synthesis of cDNA using 0.5 µg of RNA. 2.5 µl cDNA was combined with 12.5 µl 2x iTaq SYBR Green Supermix with ROX (BioRad), 0.5 µl each of forward and reverse primers, in a total reaction volume of 25 µl. Real-time RT-PCR was performed using the Applied Biosystems Prism 7300 sequence detection system and software. Relative mRNA levels for individual genes were normalized to cyclophilin. The forward and reverse primer sequences used for L-PK, NR4A2, and cyclophilin were as follows: L-PK (F) 5'-AACCTCCCCACTCAGCTACA, (R) 5'-TGCTCCACTTCTGTACCAG; NR4A2 (F) 5'-GGCTGGTCAAGATGGGTAGA, (R) 5'-AAATGCCCTTTCACGTTCTG; cyclophilin (F) 5'-TGGTGGCAAGTCCATCTACG, (R) 5'-AAAATGCCCAGCAAGTCAAAG.

Isolation of nuclear and cytosolic proteins, and immunoblotting

Nuclear and cytosolic fractions were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce; Rockford, IL, USA). The protein concentration was determined using the BCA assay (Pierce) with bovine serum albumin as the standard. Immunoblotting was performed as previously described (23). Antibodies used for quantification of CBP (Cat #: sc-369) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), ChREBP (Cat #: NB400-135) from Novus Biologicals (Littleton, CO, USA), HNF4α (Cat #: MAB10090) from Millipore (Temecula, CA, USA), GFP (Cat #: DB064) from Delta Biolabs (Gilroy, CA, USA), β Actin (Cat #: 4967) and Tubulin (Cat #: 2144) from Cell Signaling Technology (Danvers, MA, USA). Mlx antibody was a gift from Dr. Donald Ayer (University of Utah) (24).
Plasmids

The L-PK wild-type plasmid (pLPK-183), pL-PK* (constructed with the ChoRE replaced by a Gal4 DNA binding site), Gal4-DNA Binding Domain (DBD), Gal4-ChREBP, Gal4-S196A, Gal4-T666A or Gal4-S196A/T666A were described previously (5). The pG5-luc (5x Gal4) plasmid was from Promega (Madison, WI, USA). The pRc/RSV-CBP plasmid was a gift from Dr. Richard Goodman (Oregon Health & Science University) (25). RSV-PKA plasmid was a gift from Dr. William Walker (University of Pittsburgh) (26). Sequencing was conducted by the Genomics and Proteomics Core Laboratories of the University of Pittsburgh.

Transient transfection and luciferase assay

Transfections of 832/13 cells were performed using LipofectAMINE (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were transfected with DNA plasmids in serum-free media. Following an 18 h incubation, cells were treated for 6 h with 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin. Cells were lysed in Passive Lysis Buffer (Promega) and luciferase assays were performed with lysates using the Dual-luciferase Reporter Assay System (Promega) in a Luminoskan Ascent luminometer (Thermo Scientific; Waltham, MA, USA). Relative light units were normalized to protein content using a BCA protein assay (Pierce).

siRNA-mediated Suppression of Gene Expression.

The expression of ChREBP, HNF4α and CBP was decreased by transfecting pre-annealed duplexes (ChREBP: siRNA ID# 3 57311 and 190928, HNF4α: siRNA ID # 51661 and 199193, CBP: siRNA ID # 199670 and 57051) from Ambion (Austin, TX, USA) into 832/13 cells using Dharmafect reagent 1 (Dharmacon; Lafayette, CO, USA) according to the manufacturer’s suggested protocol. Suppression of the targeted genes was analyzed by immunoblotting.
**Chromatin Immunoprecipitation assay**

832/13 cells were cultured in 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin. Following a 6 h incubation, chromatin immunoprecipitation assays were performed by following the Upstate (Temecula, CA, USA) Biotechnology ChIP assay kit protocol, as described previously (6). The ChoRE containing portion of the L-PK promoter and a fragment of the coding region 5kb downstream of the L-PK transcriptional start site were targeted for amplification. Forward and reverse primers used to amplify the ChoRE- containing region of the L-PK gene promoter were as follows: 5’-GGATGCCCAATATAAGCT CA-3’ and 5’-CCATGCTGCTACGTTGCTTA-3’ (upstream and downstream, respectively). Primers used to amplify the CRE of the NR4A2 promoter were as follows: 5’-TTGCTTTGTACCAAATGCCC -3’ and 5’-TTGTAGTAAACCGACCCGC-3’ (upstream and downstream, respectively). Antibodies used for immunoprecipitation of HNF4α (Cat # sc-8987), CBP and IgG (Cat #: sc-2027) were from Santa Cruz Biotechnology and ChREBP from Novus Biologicals.

**Sequential Chromatin Immunoprecipitation Assay**

GFP-ChREBP was transduced into 832/13 cells, incubated for 24 hours then cultured in 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin for 6 h. Sequential chromatin immunoprecipitation was performed as described previously (27). GFP-ChREBP was immunoprecipitated with anti-GFP antibody, followed by elution with GFP peptide and then eluate was subjected to second immunoprecipitation with either CBP or HNF4α antibodies. GFP antibody and GFP blocking peptide (Cat #: DB064P) were from Delta Biolabs.
Statistical analyses

A one-way ANOVA was performed to detect statistical differences (p values < 0.05). A Tukey post hoc test was used to determine statistical differences within the ANOVA. All data are reported as means ± SEM.
RESULTS

cAMP agonists block the glucose-mediated induction of the L-PK gene. Glucose increases the expression of the L-PK gene while cAMP agonists block this induction (2). To further investigate a mechanism for the glucose-stimulated induction and cAMP-mediated repression of the L-PK gene, we used the INS-1-derived 832/13 β-cell line, which has been selected for its fine-tuned sensitivity to glucose (22). 832/13 cells treated with 20 mM glucose for 6 h had an approximate 3-fold induction of L-PK promoter activity, which has been previously reported (6). Here we now show that this induction was completely blocked by co-treatment with 10 μM forskolin, an activator of adenylate cyclase (Fig. 1A). In addition, transfection of a constitutively-active PKA mimicked the effect of forskolin, suggesting a cAMP-PKA pathway requirement for the repression of this gene (Fig. 1B). Similar to the effects seen with promoter activity, the 3-fold increase in L-PK mRNA levels induced by glucose was also blunted by forskolin; whereas the Nuclear Receptor subfamily 4, group A, member 2 (NR4A2 or Nurr1) control gene was induced approximately 5-fold under the same conditions, demonstrating specific and coordinate regulation of gene expression by cAMP (Fig. 1C). Because forskolin completely inhibited L-PK gene promoter activity (Fig. 1A) but did not appreciably affect mRNA stability in either the unstimulated (data not shown) or glucose-induced state (Fig. 1D), we conclude that the cAMP-mediated repression of the L-PK gene occurs predominantly at the transcriptional level.

Depletion of either ChREBP or HNF4α blunts expression of the L-PK gene. Whether or not ChREBP and its neighboring factor HNF4α, are equally required for glucose-mediated expression of the L-PK gene in β cells is undetermined. To address this issue, we suppressed endogenous ChREBP levels using two different siRNA duplexes. This resulted in an 80% decrease of ChREBP abundance (Fig. 2A), which was sufficient to decrease the expression of the L-PK gene by 84% and 62%, for duplex 1 and 2, respectively (Fig 2B).
Additionally, siRNA-directed suppression of HNF4α abundance by 73% and 51%, for duplex 1 and 2, respectively (Fig. 2C), revealed that glucose was unable to induce the L-PK gene without this factor (Fig. 2D), similar to that seen in the data above with siChREBP.

We therefore conclude that an siRNA-mediated decrease in the abundance of HNF4α or ChREBP is equally sufficient to blunt the expression of the glucose-responsive L-PK gene.

**Overexpression of ChREBP is not sufficient to prevent cAMP-mediated repression of the L-PK gene.** Because a decrease in ChREBP nuclear abundance clearly impaired the expression of the L-PK gene (Fig. 2A and 2B), we hypothesized that simply restoring ChREBP abundance may be sufficient to rescue the forskolin-mediated repression of this gene. To test this hypothesis, we overexpressed either wild-type ChREBP or a mutant ChREBP (S196A / S626A / T666A), wherein these three previously defined PKA phosphoacceptor sites have been removed to increase the probability of nuclear localization and DNA binding (3,15). Adenovirus-mediated overexpression of wild-type and the triple phospho-mutant ChREBP in the 832/13 rat insulinoma cell line led to a five- and eight-fold increase in ChREBP mRNA levels, respectively (data not shown). This translated to quantitative increases in the nuclear abundance of wild-type and phosphoacceptor mutant ChREBP proteins, as compared to barely detectable endogenous nuclear ChREBP protein levels (Fig. 3A). Despite these large increases in the nuclear abundance of wild-type or phosphoacceptor mutant ChREBP, this maneuver was unable to overcome the repression induced by forskolin (Fig. 3B). Because restoring the abundance of ChREBP was insufficient to overcome cAMP-mediated repression, even when the ostensibly repressive PKA sites have been removed, we conclude that mechanisms other than absolute protein quantity or shuttling exist to communicate the induction by glucose and repression by cAMP to the L-PK gene.

**Glucose-dependent recruitment of ChREBP and HNF4α to the L-PK gene promoter is decreased by cAMP.** We next sought to determine whether ChREBP occupancy on the L-PK gene promoter was impacted by glucose and forskolin treatment. A chromatin immunoprecipitation assay using ChREBP
antisera revealed that raising the glucose concentration from 2 to 20 mM induced a 3.8-fold increased recovery of the portion of the L-PK gene promoter containing the ChoRE (Fig. 4), consistent with previous reports (6). However, when 832/13 cells are treated concurrently with 20 mM glucose and 10 μM forskolin, there was an 80% decrease in recovery of the same promoter fragments. Similarly, HNF4α recruitment to a region of the L-PK gene promoter containing the L3 element was increased 2.4-fold in the presence of glucose (Fig. 4); exposure to forskolin decreased recovery of this promoter region by 72%. A region that does not contain a ChoRE served as a negative control for transcription factor binding (data not shown). We conclude that cAMP agonists decrease the occupancy of ChREBP and HNF4α on their respective elements in the L-PK promoter despite the presence of a stimulatory concentration of glucose.

cAMP decreases wild-type and phospho-mutant ChREBP transactivation of the L-PK gene promoter. We examined ChREBP transactivation capacity using glucose and cAMP using the L-PK gene promoter, wherein the 17 nucleotides of the ChoRE were replaced with the 17 bases of the Gal4 DNA binding sequence. In this experiment, there is a 3.7-fold activation of Gal4-ChREBP fusion construct by glucose, whereas forskolin repressed transactivation of the wild-type factor by 68%. Note that mutation of the PKA sites in ChREBP was unable to prevent the forskolin repression (Fig. 5). We conclude that in the context of the L-PK gene promoter, repression by cAMP is mediated at least in part by factors other than, but potentially associated with, ChREBP.

siRNA-mediated Suppression of CBP Prevents Glucose-mediated Induction of the L-PK gene. It has been shown that CBP associates with HNF4α, and that overexpression of CBP augments HNFα transactivation (21,28); however, whether CBP contributes to the glucose-mediated induction of the L-PK gene has never been tested directly. Therefore, we used siRNA-directed suppression of CBP to examine its potential role in the glucose-induction of the L-PK gene. Indeed, when CBP abundance is decreased by 70% using siRNA duplex transfection (Fig. 6A), the ability of glucose to induce the expression of the L-
PK gene was lost (Fig. 6B). To our knowledge, this is the first report demonstrating an absolute requirement for CBP to mediate the glucose response to this or any other glucose-responsive gene.

**The glucose-mediated recruitment of CBP to the L-PK gene promoter is blocked by cAMP.** Because CBP is required for the glucose-mediated expression of the L-PK gene (Fig. 6B), we next investigated whether glucose recruits CBP directly to the L-PK gene promoter and, in addition, if the cAMP-mediated deactivation of the L-PK gene was associated with loss of this coactivator molecule. Using chromatin immunoprecipitation, we observed a 2.7-fold increase in CBP occupancy on the ChoRE containing region of the L-PK gene promoter in response to increased (2 mM vs. 20 mM) glucose stimulation. Furthermore, forskolin treatment abolished this interaction (Fig. 7A). Concomitant with loss of CBP on the L-PK gene promoter, in the presence of the cAMP signal we readily detected CBP association with the NR4A2 gene promoter (Fig. 7B). Taken together with the effects of siRNA-directed suppression of CBP, we conclude that the forskolin-mediated repression of L-PK gene transcription occurs, at least in part, due to a loss of CBP occupancy on the L-PK gene promoter.

**Increasing CBP abundance enhances glucose-mediated activation and abrogates the repression of the L-PK gene by cAMP.** Since CBP is recruited to the L-PK gene promoter in a glucose-dependent manner and its occupancy is lost under conditions where cAMP is elevated (Fig. 7A), we hypothesized that CBP levels may be a limiting factor in regulation of this gene, as has been suggested for other genes regulated at the transcriptional level (29). Therefore, we examined L-PK gene promoter activity following CBP overexpression. Glucose-induced L-PK promoter activity 3.7-fold; when CBP was overexpressed activity was amplified to 5.7-fold (Fig. 8). We note that basal promoter activity was not enhanced by simply increasing CBP abundance (data not shown); however, augmenting CBP levels potentiated the glucose-mediated induction of promoter activity. In addition, increasing CBP abundance was sufficient to overcome the forskolin-mediated repression of the L-PK gene (Fig. 8). Taken together with the above findings, there is a clear dependence on CBP to regulate expression of the L-PK gene by both glucose and cAMP.
ChREBP, HNF4α, and CBP co-occupy the LPK gene promoter in a glucose-dependent manner, and this complex is disrupted by cAMP. Because CBP is required for glucose to induce the expression of the L-PK gene (Fig. 6B) and glucose recruits CBP to the L-PK gene promoter (Fig. 7A), we next examined whether CBP, ChREBP and HNF4α are present together in this context. To test this hypothesis, sequential ChIP (SeqChIP) analysis was performed. This assay provides information regarding co-occupancy of multiple proteins within a specific genomic region (30). In the first SeqChIP experiment, there was a 2.6-fold increase in promoter fragments recovered by immunoprecipitating overexpressed GFP-tagged ChREBP (Fig. 9A), followed by eluting with GFP peptide and a subsequent second IP of the eluate using CBP antisera (Fig. 9B). There was a 4.1-fold increase in CBP SeqChIP signal at 20 mM glucose. When forskolin was added, there was a 12.8-fold decrease in CBP SeqChIP signal in the context of the ChoRE of L-PK gene promoter (Fig. 9B). In the next experiment, we first immunoprecipitated GFP-tagged ChREBP (Fig. 9C), followed by a second IP with antisera against HNF4α (Fig. 9D). There was an approximate 1.6-fold increase in HNF4α SeqChIP signal, while forskolin diminished this recovery by 3-fold. We conclude that forskolin disrupts the ability of glucose to promote a complex containing CBP, ChREBP, and HNF4α on the L-PK gene promoter, thus terminating transcription of this gene.
DISCUSSION

The L-type pyruvate kinase (L-PK) gene is an excellent model to study the reciprocal effects of cAMP and glucose, as glucose increases and cAMP decreases the expression of this gene (2). While the promoter elements that confer regulation by these opposing stimuli has been characterized (17,18, 28), less is known about the assembly and disassembly of transcription factors and other regulatory molecules in response to glucose or cAMP-derived signals. The glucose-responsive transcription factor ChREBP is a key regulator of the L-PK gene (3-16). However, confusion exists regarding the mechanism of transcriptional regulation by ChREBP. The original model for ChREBP-mediated regulation of the L-PK gene states that glucose promotes induction of this gene by opposing cAMP-stimulated phosphorylation (3); however recent studies show that this model is incomplete and warrants revision (6, 12-15).

Therefore, in the current study, we examined the factors involved in both the activation of the L-PK gene in response to glucose and the repression by cAMP. The following key findings emerged: 1) suppression of the glucose-stimulated L-PK gene by cAMP is likely to be mediated via a PKA-dependent pathway (Fig. 1B); 2) siRNA-mediated suppression of ChREBP, HNF4α, or CBP completely prevented glucose-stimulated induction of the L-PK gene (Fig. 2B, 2D and 6B); 3) glucose-stimulated wild-type or phospho-mutant ChREBP transactivation capacity is repressed by cAMP in the context of the L-PK gene promoter (Fig. 5); 4) decreased expression of the L-PK gene by cAMP coincides with decreased CBP, HNF4α, and ChREBP association with the L-PK gene promoter (Fig. 4 and 7A); 5) overexpression of either wild-type or phospho-mutant ChREBP is neither sufficient to activate the L-PK gene under low glucose conditions nor can they override the cAMP-mediated repression of the gene (Fig. 3B); 6) augmenting CBP abundance enhances the glucose-stimulated induction and is sufficient to overcome repression of the L-PK gene promoter activity by cAMP (Fig. 8).

Forskolin mediates its repressive effects on glucose-induced L-PK gene expression by manipulating ChREBP at many levels. In addition to the cAMP-dependent decreased recruitment of ChREBP to the L-PK gene promoter (Fig. 4), forskolin treatment resulted in a significant nuclear exclusion of ChREBP.
(unpublished results). When ChREBP was bound to the promoter via Gal4 fusion constructs, forskolin decreased ChREBP transactivation, both in the presence or absence of the PKA-phosphoacceptor sites (Fig. 5). We note that when similar experiments were performed with the Gal4-ChREBP chimeras using a multimerized Gal4 DNA binding site promoter, mutation of the PKA phosphoacceptor sites protected ChREBP from repression of transactivation by forskolin (data not shown). Clearly promoter context is an important determinant of the mechanism by which cAMP regulates ChREBP. Because ChREBP was sensitive to PKA-mediated phosphorylation in a heterologous promoter context, but not in the context of the L-PK gene promoter, we speculate that when a complex assembles on the L-PK gene promoter in response to elevated glucose concentrations, it masks the PKA sites in ChREBP. However, when a cAMP-derived signal releases CBP from the complex and certain proteins are recycled off the promoter (e.g., CBP to NR4A2), this potentially reveals the PKA sites in ChREBP, allowing phosphorylation and shuttling from the nucleus to the cytoplasm, as per previous models (3).

Additionally, the glucose-dependent increase in HNF4α recruitment to the L-PK gene promoter is blocked by cAMP treatment (Fig 4), but without a cAMP-mediated change in HNF4α nuclear abundance (unpublished observations). Phosphorylation of the putative PKA sites in HNF4α does not mediate the repressive effects of cAMP on transactivation of the L-PK gene (28). Therefore, the exclusion of HNF4α from the L-PK gene promoter by cAMP cannot be explained by HNF4α phosphorylation-dependent events. We hypothesize that the exclusion is due to a decreased stability of the complex containing ChREBP, HNF4α, and CBP.

Since coordinated regulation of gene transcription requires assembly of multi-regulatory complexes on specific gene promoters concomitant with disassembly on other promoters and because coactivator molecules are often limiting (29, 31), they provide an attractive target upon which to finely tune the control of gene expression in response to multiple signals. We have shown that loss of CBP occupancy on the L-PK gene promoter in response to forskolin coincides with loss of L-PK mRNA levels concomitant
with gain of CBP occupancy on the NR4A2 gene promoter and a corresponding increased expression of this gene (Fig. 1C, 7A and B). This demonstrates a selective and coordinated regulation of gene transcription in response to a change in signals, as has been suggested for other systems (32, 33). Because of the absolute requirements for ChREBP and HNF4α (Fig. 2B and D) and in addition, the recruitment of the co-activator CBP, we propose a heretofore undescribed mechanism of both the glucose-mediated induction of the L-PK gene and its repression by cAMP. In this model, CBP is the limiting factor in the regulation of the L-PK gene. Initially, a rise in glucose metabolism promotes the association of ChREBP, HNF4α, and CBP with the L-PK gene promoter, increasing transcription of the gene. However, when cAMP levels become elevated, this multi-regulatory complex is disrupted, CBP is recruited to other gene promoters (e.g., NR4A2) and unbound ChREBP is potentially shuttled to the cytoplasm. Based on an earlier report demonstrating association of HNF4α with CBP via GST pulldown analysis (21) and our current results above, we suspect that recruitment of CBP to the L-PK gene promoter likely stabilizes the complex assembled in response to glucose and that cAMP represses the gene by interfering with these protein-protein interactions. Additionally, putative PKA site(s) in CBP may be responsible for disruption of the complex at some genes (including L-PK) concomitant with assembly at others (e.g., NR4A2).

Moreover, siRNA-mediated suppression of CBP diminishes the glucose-induction of L-PK mRNA, while enhancing CBP both potentiates the glucose-mediated induction of promoter activity and prevents cAMP-mediated repression of such activity; we interpret these findings to indicate that CBP is a bona fide coactivator of the L-PK gene. We’ve clearly shown via Sequential ChIP (Fig. 9) that ChREBP, CBP and HNF4 co-reside as a complex on the L-PK promoter in a glucose-dependent manner, and that cAMP-mediated repression of the L-PK gene disrupts this complex assembly; however, whether CBP binds to ChREBP or HNF4α, or both, has yet to be elucidated.

We have thus demonstrated that a complex requiring ChREBP, HNF4α, and CBP is necessary for the activation of the L-PK gene in response to glucose. In addition, we report here for the first time that the
coactivator CBP is recruited to the L-PK gene promoter in response to glucose; further, decreasing the association of this coactivator with the L-PK gene promoter, either by siRNA technology or by increasing cAMP levels, blocks glucose-induced expression of the L-PK gene. Thus, a complex composed of ChREBP, HNFα, and CBP is a focal point for the opposing physiological signals, glucose and cAMP.

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REFERENCES


FIGURE LEGENDS

Fig 1. cAMP represses L-PK promoter activity and mRNA levels, but does not decrease mRNA stability in 832/13 cells. A. 832/13 cells were transiently transfected with 2 μg L-PK promoter driven-luciferase construct (pLPK-183) for 18 h and luciferase activity was measured following an additional 6 h incubation with 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin *P < 0.01 vs. 20 mM glucose. B. 832/13 cells were co-transfected with 1 μg pLPK-183 and either control pUC19 (1 μg), constitutively active RSV-PKA Cα (1 μg), or wild-type RSV-PKA plasmids (1 μg). Cells were treated with 2 mM or 20 mM glucose, with 10 μM forskolin added to control samples only. Following a 6 h incubation, luciferase activity was measured. *P < 0.05 vs. 20 mM glucose. C. 832/13 cells were treated for 6 h with 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin. Following extraction of total RNA, relative levels of L-PK and NR4A2 mRNA were analyzed by real-time RT-PCR. *P < 0.001 vs. 20 mM glucose, ‡ P < 0.005 vs. 20 mM glucose. D. 832/13 cells were treated with 20 mM glucose for 6 h, followed by treatment with or without 10 μM forskolin in the presence of the transcriptional inhibitor Actinomycin D. The decay of mRNA was determined by quantifying the amount of specific mRNA for the L-PK gene at 2, 4 and 6 h time points. Data shown are means ± SEM from three independent experiments.
Fig 2. Decreasing the abundance of ChREBP or HNF4α inhibits expression of the L-PK gene.

832/13 cells were transfected with either a scrambled control siRNA duplex (siScramble) or siRNA duplexes targeted to a 21 base pair region of the rat ChREBP or HNF4α genes. After 48 h of duplex exposure, nuclear extracts were harvested for immunoblotting using antibodies directed against either (A) ChREBP or (C) HNF4α, with the loading control, tubulin. The immunoblots are a representative of 2 independent experiments. 832/13 cells were transfected with above duplexes for 48 h, followed by a 6 hour culture with 2 mM or 20 mM glucose, in the presence or absence of 10 μM forskolin. L-PK mRNA levels were analyzed by RT-PCR (B and D). Values represent the means ± SEM from three independent experiments. *P < 0.05 vs. siScramble for mRNA experiments.

Fig 3. Overexpression of either wild-type or phospho-mutant ChREBP is not sufficient to prevent cAMP-mediated repression of the L-PK gene. A. 832/13 cells were transduced with GFP-tagged wild-type and phospho-mutant ChREBP (S196A/ S626A/ T666A) adenoviruses (14). Following a 24 h incubation, nuclear extracts were prepared for immunoblotting with antibodies against either ChREBP, GFP or β Actin as a loading control. B. After a 24 h exposure to adenovirus, cells were additionally treated with 2 mM or 20 mM glucose for 6 h in the presence or absence of 10 μM forskolin. Relative mRNA levels of L-PK were analyzed via RT-PCR. Data are means ± SEM. *P < 0.05 vs. 20 mM glucose for each respective group.

Fig 4. Recruitment of ChREBP and HNF4α to the L-PK gene promoter is decreased in the presence of cAMP. 832/13 cells were cultured in 2 mM or 20 mM glucose for 6 h in the presence or absence of 10 μM forskolin. Chromatin immunoprecipitation experiments were conducted with an antibody against ChREBP, HNF4α or control IgG. The gene promoter for L-PK was targeted for amplification. Data are expressed as means ± SEM from three independent experiments. *P < 0.05 vs. 20 mM glucose, ‡ P < 0.05 vs. 20 mM glucose.
Fig 5. **cAMP-mediated decrease in ChREBP transactivation of the L-PK gene promoter.** 832/13 cells were co-transfected for 18 h with the pL-PK* plasmid (wherein the ChORE of the L-PK gene is replaced with the Gal DNA binding site) (1 µg), and with plasmids (1 µg) encoding GAL4-DBD, wild-type GAL4-ChREBP, or the indicated GAL4-ChREBP phosphorylation mutants. Cells were treated for 6 h with 2 mM or 20 mM glucose in the presence or absence of 10 µM forskolin. Results are shown as relative luciferase reporter activity for three independent experiments. Values are means ± SEM. *P < 0.05 vs. 20 mM glucose for each respective group.

Fig 6. **siRNA-mediated suppression of CBP inhibits glucose-mediated induction of the L-PK gene.**

A. 832/13 cells were transfected with either siScramble or siRNA duplexes targeted to rat CBP mRNA for 48 h. Nuclear extracts were immunoblotted for CBP and β Actin as a loading control. The immunoblot is a representative of 2 independent experiments. B. 832/13 cells were transfected with the above duplexes for 48 h, followed by a 6 h culture with 2 mM or 20 mM glucose, in the presence or absence of 10 µM forskolin. L-PK mRNA levels were quantified by RT-PCR. Values represent the means ± SEM from three independent experiments. *P < 0.05 vs. siScramble.

Fig 7. **Glucose-dependent recruitment of CBP to the L-PK gene promoter is blocked by cAMP.** 832/13 cells were cultured in 2 mM or 20 mM glucose in the presence or absence of 10 µM forskolin for 6 h. Relative promoter occupancy was determined by chromatin immunoprecipitation assay, using antibodies directed against ChREBP and CBP (A), or CBP alone (B), with IgG antisera serving as a control. The gene promoters for L-PK (A) and NR4A2 (B) were targeted for amplification. Data are means ± SEM from three independent experiments. *P < 0.1 vs. 20 mM glucose, ‡P < 0.05 vs. 20 mM glucose.

Fig 8. **Increasing CBP abundance enhances glucose-mediated activation and abrogates the repression of the L-PK gene by cAMP.** 832/13 cells were co-transfected for 18 h with 1µg pLPK-183 (the wild-type L-PK promoter driving expression of luciferase) and either control pUC19 (1 µg) or
decreasing concentrations of pRc/RSV-CBP (1 μg, 500 ng, 250 ng pUC19, or 125 ng) along with various concentrations of pUC19 to maintain 1 μg total transfected plasmid. Cells were treated with 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin. Luciferase activity was then measured following a 6 h incubation. Data are means ± SEM from three independent experiments. *P < 0.05 vs vector control for each group.

Fig 9. CBP, ChREBP and HNF4α become co-resident on the L-PK gene promoter in a glucose-dependent manner and this complex is disrupted by cAMP. 832/13 cells were transduced with GFP-ChREBP adenovirus, cultured for 24 h, followed by treatment with 2 mM or 20 mM glucose in the presence or absence of 10 μM forskolin for 6 h. Subsequently, GFP-ChREBP was immunoprecipitated with either anti-IgG or anti-GFP antibodies (A). CBP was then immunoprecipitated from the resulting eluate using anti-CBP with anti-IgG antisera used as a control (B). In a separate experiment performed under the same conditions, GFP-ChREBP was immunoprecipitated using IgG control or GFP antibodies (C), followed by a second immunoprecipitation of the eluate with either IgG or HNF4α antibodies (D). For all immunoprecipitates, the ChoRE-containing region of the L-PK gene promoter was targeted for amplification via real-time RT-PCR (A-D). Shown are means ± SEM from three independent experiments. *P < 0.05 vs 20 mM glucose.
Figure 1A

<table>
<thead>
<tr>
<th>Glucose (mM)</th>
<th>2</th>
<th>20</th>
<th>2</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forskolin (10 μM)</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 2C

![Image of gel electrophoresis]

- **HNF4α**
  - siScramble
  - siHNF4α #1
  - siHNF4α #2
  - 52kDa

- **Tubulin**
  - 50kDa
Figure 2D

Relative L-PK mRNA levels

<table>
<thead>
<tr>
<th>Glucose (mM)</th>
<th>2</th>
<th>20</th>
<th>2</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forskolin (10 μM)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

- siScramble
- siHNF4α #1
- siHNF4α #2
Figure 3A

<table>
<thead>
<tr>
<th>Virus</th>
<th>GFP</th>
<th>ChREBP</th>
<th>ChREBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChREBP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β Actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(S196A/S626A/T666A)

95kDa
42kDa
Figure 3B

Virus: GFP  ChREBP  ChREBP (S196A/S626A/T666A)
Figure 4

Relative L-PK promoter occupancy

Glucose (mM) 2 20 2 20
Forskolin (10 μM) - + + +

- IgG
- ChREBP
- HNF4α
Figure 6A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CBP</th>
<th>β Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>siScramble</td>
<td></td>
<td></td>
</tr>
<tr>
<td>siCBP #1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>siCBP #2</td>
<td>265 kDa</td>
<td>42 kDa</td>
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</table>
Figure 6B

Relative L-PK mRNA levels

<table>
<thead>
<tr>
<th>Glucose (mM)</th>
<th>2</th>
<th>20</th>
<th>2</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forskolin (10 μM)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

- siScramble
- siCBP #1
- siCBP #2
Figure 7A

Relative L-PK promoter occupancy

Glucose (mM) 2 20 2 20
Forskolin (10 μM) - + +

Legend:
- IgG
- ChREBP
- CBP

* Indicates significant difference
Figure 8

Fold Luciferase Activity
20 mM over 2 mM glucose

Vector Control
CBP

DMSO
Forskolin (10 μM)
CBP
Figure 9A

**L-PK SeqChIP Signal**

<table>
<thead>
<tr>
<th>Glucose (mM)</th>
<th>2</th>
<th>20</th>
<th>2</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forskolin (10 μM)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 9B

![Graph showing L-PK SeqChIP Signal with IgG and CBP bars for different glucose and forskolin conditions.]

- Glucose (mM): 2, 20, 2, 20
- Forskolin (10 μM): - (none), + (present)

*Note: The asterisk (*) indicates a statistically significant difference.
Figure 9D

- **L-PK SeqChIP Signal**

  - **Glucose (mM)**: 2, 20, 2, 20
  - **Forskolin (10 μM)**: -, +, +

- **IgG**
- **HNF4α**