Regulation by glucagon (cAMP) and insulin of the promoter of the human phosphoenolpyruvate carboxykinase gene (cytosolic) in cultured rat hepatocytes and in human hepatoblastoma cells

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A promoter fragment (–457 to +65) of the human cytosolic phosphoenolpyruvate carboxykinase gene, which by analogy to the rat promoter contains regulatory regions conferring glucagon (cAMP) and insulin responsiveness to the phosphoenolpyruvate carboxykinase gene, was cloned into a luciferase expression vector and transfected into cultured rat hepatocytes and human hepatoblastoma cells (HepG2) to study the regulation of the transgene by glucagon (cAMP) and insulin. A reporter gene that contained the rat promoter sequence from –493 to +33 was used for comparison. In cultured rat hepatocytes glucagon and its second messenger cAMP increased luciferase expression 4–6-fold over basal levels. Insulin reduced this effect by 40–70%.

Luciferase expression was also stimulated by the combination of dexamethasone and cAMP in HepG2 cells and this effect was inhibited by insulin. The phosphoinositide 3-kinase (PI 3-kinase) inhibitor, wortmannin, abolished this action of insulin in cultured rat hepatocytes. The results show that the promoter of the human phosphoenolpyruvate carboxykinase gene mediates the stimulatory action of glucagon and its second messenger cAMP. The inhibitory action of insulin was exerted through the PI 3-kinase pathway in cultured rat hepatocytes.

Key words: gluconeogenesis, HepG2 cells, liver, phosphoinositide 3-kinase.

INTRODUCTION

Phosphoenolpyruvate carboxykinase (GTP) (PCK; EC 4.1.1.32) converts oxaloacetate into phosphoenolpyruvate, which is a key regulatory step in the control of hepatic and kidney gluconeogenesis. In different species the gene is expressed in both the cytosol (*PCK1*) and the mitochondria (*PCK2*) to different extents. In human liver the cytosolic and the mitochondrial enzymes are expressed to almost the same amount, in rat liver the cytosolic form comprises 90% of the total and in birds the mitochondrial form prevails [1–6]. The mammalian human, rat and mouse cytosolic *PCK1* genes [7–9], and the human mitochondrial *PCK2* gene [10], have been isolated and characterized. The promoter of the rat *PCK1* gene has been identified and studied extensively (see [11,12] for recent reviews) while the promoter of the human *PCK1* gene has been isolated only recently [13]. In cultured rat hepatocytes transcription of the rat cytosolic *PCK* gene is stimulated by glucagon via the elevation of cAMP under the permissive action of glucocorticoids, an effect that is antagonized by insulin [14–17]. In rat hepatoma cells, which lack a functional glucagon receptor, transcription of the gene is stimulated by cAMP and also by glucocorticoids. Insulin antagonizes these actions (for review, see [17]). The high degree of sequence homology between the human and rat cytosolic *PCK* gene promoters suggests that the human promoter might be regulated by glucagon (cAMP) and insulin in the same way as the rat promoter [13]. The CRE (cAMP-responsive element), and the P3(I), P3(II) and P4 elements in the rat promoter, which are

required for its stimulation by cAMP [18], are highly conserved in the human promoter. An insulin-responsive region, which has been mapped between positions –416 and –407 in the rat promoter [19,20], is completely conserved in the human promoter corresponding to positions –395 to –386 [13]. However, cotransfection experiments with an expression plasmid encoding the catalytic subunit of the cAMP-dependent protein kinase, and a chloramphenicol acetyltransferase (*CAT*) reporter gene under the control of the human *PCK* gene promoter, revealed no clear stimulation of *CAT* expression in H4IIE rat hepatoma cells. No results from experiments using insulin as an antagonist are available [13].

The aim of the present study was to investigate the function of the human cytosolic *PCK* gene promoter by glucagon (cAMP) and insulin. The human *PCK* gene promoter responds to glucagon or cAMP in cultured rat hepatocytes, and to cAMP and/or dexamethasone in HepG2 cells. The stimulation was antagonized in either cell type by insulin.

EXPERIMENTAL PROCEDURES

Animals and chemicals

Male Wistar rats, supplied by Harlan-Winkelmann (Borchen, Germany), with a body weight of 200–250 g and fed with a standard diet (Altromin), were used for preparation of hepatocytes. Chemicals of ‘pro analysis’ quality were purchased from

Abbreviations used: PCK, phosphoenolpyruvate carboxykinase (GTP); CPT-cAMP, chlorophenylthio-cAMP; PI 3-kinase, phosphoinositide 3-kinase; CRE, cAMP-responsive element; CAT, chloramphenicol acetyltransferase; MEM, minimum essential medium; IRS, insulin-responsive sequence.

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local suppliers. Collagenase A, chlorophenylthio-cAMP (CPT-cAMP), newborn calf serum and fetal calf serum were supplied by Boehringer Mannheim (Mannheim, Germany) and hormones and wortmannin by Sigma (Heidelberg, Germany). Culture medium M199, minimum essential medium (MEM) with Earle's salts, non-essential amino acids and trypsin were supplied by Life Technologies (Eggenstein, Germany) and Percoll by Pharmacia Biotech (Freiburg, Germany). HepG2 cells were provided kindly by Dr Thomas Kietzmann (Institut für Biochemie und Molekulare Zellbiologie, Göttingen, Germany).

Preparation and culture of primary rat hepatocytes

Hepatocytes were prepared by the collagenase perfusion technique and separated from non-parenchymal liver cells and debris by a Percoll density gradient. They were cultured for 48 h at a density of 4.5×10^4 cells/cm² in 1.5 ml of medium M199 on 60-mm-diameter culture dishes under a gas atmosphere of 16% O₂/5% CO₂/79% N₂. The medium was supplemented with 100 nM dexamethasone, 0.5 nM insulin and, for the first 5 h, with 4% newborn calf serum to support cell attachment. Cells were cultured further in 2.5 ml of serum-free M199 containing dexamethasone and insulin. The medium was changed after 24 h. After a total of 48 h of culture the cells were washed twice with insulin-free M199 with dexamethasone. Experiments were then started by applying fresh medium containing hormones at the concentrations given in the Figures.

Growth and culture of HepG2 cells

Human HepG2 hepatoblastoma cells were grown to confluency in tissue-culture bottles (175 cm²) in 25 ml of MEM containing 10% fetal calf serum. For subcultivation, trypsin-treated cells were diluted in MEM containing 10% fetal calf serum and plated again on 60-mm-diameter culture dishes at a density of 1.4×10^4 cells/cm². On the next day fresh serum-containing medium was applied and cells were transfected after another 4 h. Then, 24 h after transfection the cells were washed twice with serum-free medium and experiments were started by applying fresh medium containing hormones at the concentrations indicated in the Figures.

Plasmids

Plasmid pGL-rPCK-493/+33, which contains the rat promoter segment from -493 to +33 relative to the transcriptional start site of the rat *PCK* gene, was constructed by cloning a PCR-generated DNA fragment into the *Sma*I site of pUC19. The fragment was cut out again with *Bam*HI and *Kpn*I and inserted into the *Bgl*II- and *Kpn*I-digested luciferase expression vector pGL3 (Promega, Madison, WI, U.S.A.). The plasmid was kindly provided by Dr Jutta Bratke (Institut für Biochemie und Molekulare Zellbiologie, Göttingen, Germany). Plasmid pGL-hPCK-457/+65 containing a human promoter fragment from -457 to +65 relative to the transcriptional start site of the human *PCK* gene was generated by digestion of HPP-CAT (where HPP is human *PCK* promoter) [13] with *Bgl*II and subcloning of the resulting DNA fragment into the *Bgl*II site of pGL3. HPP-CAT was provided kindly by Dr R. O'Brien (Department of Molecular Physiology and Biophysics, Vanderbilt University Medical School, Nashville, TN, U.S.A.).

Mutation of a putative 'insulin-responsive element' in the human *PCK* gene promoter

The plasmid pGL-hPCK-457/+65mut was generated by a PCR approach using two different primer pairs carrying the mutations in the putative insulin-responsive sequence (IRS). pGL-hPCK-457/+65 was used as the template. One primer pair comprised sense primer RVprimer3 in the PGL3 vector (Promega) and the mutated antisense primer 5'-CAAAAGGGACCAGCTATAAGATGTCACCCC-3' (bases -415 to -386 in the human promoter, with the mutation underlined). The second primer pair comprised mutated sense primer 5'-GTCCCTTTTGCCAACCAGCAGCTCTTGTA-3' (bases -395 to -366, with the mutation underlined) and the antisense primer GLprimer2 in the PGL3 vector. The resulting two PCR fragments were linked together by the use of the primer pair RVprimer3 and GLprimer2. The resulting DNA fragment was digested with *Hind*III and *Mlu*I and subcloned into the *Hind*III/*Mlu*I-digested pGL3 vector. The TGGTG → GTCCC mutation from position -395 to -391 was confirmed by sequencing of the final plasmid.

Transfection protocol

The plasmids pGL-hPCK-457/+65, pGL-hPCK-457/+65mut and pGL-rPCK-493/+33 were transfected into rat hepatocytes and human HepG2 hepatoblastoma cells by the calcium phosphate DNA-precipitation method [21]. Plasmid DNA (2 µg) was precipitated in 150 µl of 2× concentrated Hepes (1× concentrated is made of 25 mM Hepes, pH 7.05, 140 mM NaCl and 0.75 mM Na₂HPO₄; buffer A) with 125 mM CaCl₂, and added to 1.5 ml of a freshly isolated hepatocyte cell suspension on a 60-cm-diameter culture dish. For the transfection of HepG2 cells 2.5 µg of plasmid DNA in 150 µl of buffer A were used and the precipitate was added to the cells on a 60-mm-diameter culture dish. In both protocols the cells and the plasmid DNA precipitate were mixed thoroughly. After transfection, medium was changed and incubation continued as described above. The protocol for the transfection of primary hepatocytes has been used widely in different applications and with a variety of plasmids. A promoter-less control luciferase transgene is not expressed. The non-stimulated *PCK* gene promoter/luciferase transgene is expressed at basal levels. Its expression can be stimulated by glucagon or CPT-cAMP maximally between 24 and 48 h of culture. In the present study a 48 h culture period was employed, because after that time the hepatocytes had completely recovered from the isolation procedure. After 48 h of culture the expression of the transgene was stimulated with glucagon or CPT-cAMP for 8 h. During this stimulation period the expression of the luciferase gene increased linearly with time [22–24].

Other assays and data analysis

The Promega luciferase bioluminescence assay was performed as described by the supplier. Luminescence was measured with a LB953 luminometer from Berthold (Wildbad, Germany). The relative light units were normalized to protein content of the cell extracts. Protein concentration was determined by the Bradford method using a commercially available dye reagent (Bio-Rad, München, Germany).

The results were calculated from a number of different cell cultures, each run in triplicate, as indicated in the Figure legends. They are expressed as means ± S.E.M. Significance of differences between the means with glucagon alone and in the presence of

insulin was calculated by the use of Student's *t* test applied to paired values. Significant inhibition by insulin was defined by the *P* values as indicated in the Figure legends.

RESULTS AND DISCUSSION

Stimulation by glucagon (cAMP) of the human and rat *PCK* gene promoters in cultured rat hepatocytes: inhibition of the stimulation by insulin

In cultured rat hepatocytes the expression of the luciferase transgene under the control of the human *PCK* gene promoter was stimulated 4-fold over the basal non-stimulated expression by 5 nM glucagon. Half-maximal stimulatory glucagon concentrations were reached at 0.11 nM. In the presence of 100 nM insulin the maximal stimulation by 5 nM glucagon was only 2.7-fold over non-stimulated values, which corresponds to a 43% inhibition of the maximal stimulation observed in the absence of insulin. Again, half-maximal stimulation was reached at 0.11 nM glucagon. Insulin alone did not affect basal, non-stimulated luciferase expression. Thus insulin inhibited the maximal stimulation but did not shift the half-maximal stimulatory concentrations of glucagon (Figure 1A). When the cAMP analogue CPT-cAMP was used instead of glucagon, the expression of the luciferase transgene was stimulated by 10 μ M CPT-cAMP 4.7-fold over the basal non-stimulated expression. In the presence of 100 nM insulin this stimulation was only 2.5-fold, which corresponds to a 58% inhibition. Half-maximal stimulation was reached at 1.2 μ M CPT-cAMP in the absence of insulin and at 1 μ M in the presence of 100 nM insulin. Therefore the maximal stimulation was inhibited by insulin. The half-maximal stimulatory concentration of cAMP was unaffected. Again insulin did not affect *PCK* gene promoter-driven basal, non-stimulated luciferase expression (Figure 1B).

For comparison, a reporter gene consisting of the promoter of the rat *PCK* gene directing the expression of the luciferase reporter gene was transfected into cultured rat hepatocytes. Glucagon, at 5 nM, resulted in a 6.2-fold increase in luciferase expression over the basal, non-stimulated expression. Half-maximal stimulation was reached at 0.26 nM glucagon. In the presence of insulin the maximal stimulation was only 3.5-fold, which corresponds to a 51% inhibition. Half-maximal stimulation was achieved at 0.3 nM glucagon. Basal, non-stimulated expression of the luciferase gene was not affected by insulin (Figure 1C). CPT-cAMP, at 10 μ M, stimulated luciferase expression 5-fold over the basal, non-stimulated expression. Half-maximal stimulation was achieved at 0.29 μ M CPT-cAMP. In the presence of 100 nM insulin the maximal stimulation by 10 μ M CPT-cAMP was only 3.7-fold, which corresponds to a 32% inhibition. The half-maximal stimulation was reached at 0.44 μ M CPT-cAMP. Basal expression was not affected by insulin (Figure 1D).

Thus the maximal activation of the human and rat *PCK* gene promoters by glucagon or CPT-cAMP was inhibited by insulin. Insulin did not change the half-maximal stimulatory concentrations of glucagon or cAMP, which were in the same range for both promoters.

In the rat cytosolic *PCK* gene the CRE1, CRE2, P3 and P4 promoter elements mediate cAMP responsiveness [11]. Computer analysis revealed that these regions are highly conserved in the human promoter, thus it should also be regulated by glucagon or its second messenger, cAMP. However, functional analysis has only been carried out by overexpressing the catalytic subunit of protein kinase A along with a human *PCK* promoter-driven *CAT* reporter gene [13]. The results presented in this paper demonstrate that the human promoter is stimulated by glucagon and cAMP in cultured rat hepatocytes. Therefore, the stimulation

Figure 1 Concentration dependency of the glucagon- or cAMP-stimulated increase in human and rat *PCK* gene promoter activity in cultured rat hepatocytes: inhibition by insulin

Cells were transfected either with plasmid pGL-hPCK—457/+65 (A, B) or with plasmid pGL-rPCK—493/+33 (C, D), in which the luciferase gene is under the control of either a human or a rat *PCK* gene promoter fragment. After 48 h of culture the expression of the transgene was stimulated for 8 h with glucagon (A, C) or CPT-cAMP (B, D) at the concentrations indicated. Insulin at 100 nM was added where indicated. Then cells were harvested and luciferase activity was measured in cell lysates by the use of a luminometer. Relative luciferase activity was calculated as the increase over non-stimulated activity in the absence of glucagon or CPT-cAMP, set to 1. Values are the means $\pm$ S.E.M. from three to ten separate cell-culture experiments, each run in triplicate. Statistics: Student's *t* test for paired values; values in the presence of insulin are different from values in the absence of insulin with **P* $\leq$ 0.05 and ***P* $\leq$ 0.01.

in response to glucagon is very likely to be mediated by the glucagon-stimulated increase in intracellular cAMP concentrations.

Insulin, the physiological antagonist of glucagon action, decreases the glucagon-stimulated increase in cAMP by the activation of phosphodiesterase, thereby attenuating the stimulation by glucagon of *PCK* gene expression [14,15,22]. In the present study insulin inhibited the stimulation by glucagon or cAMP of luciferase expression from the human as well as from the rat cytosolic *PCK* gene promoter, indicating that insulin was antagonistic through both promoters. However, because insulin is also inhibitory when the non-hydrolysable cAMP analogue CPT-cAMP is used, the effect must occur downstream from cAMP hydrolysis, as has been suggested previously [22,25].

Hence, from the present results it may be argued that the human promoter, like the rat promoter, responds to cAMP, the signal that represents increased blood glucagon concentrations. The result is an increased rate of *PCK* gene expression and hepatic gluconeogenesis during fasting periods. The increase in insulin concentrations after a carbohydrate-rich meal attenuates hepatic *PCK* gene expression and gluconeogenesis [26]. Thus the human *PCK* gene promoter may contribute to the glucostat function of the liver in humans. Because no antagonism by insulin was observed in human and rat hepatoma cells after stimulation of human *PCK* promoter/*CAT* reporter gene expression with the catalytic subunit of cAMP-dependent protein kinase [13], primary cultured hepatocytes may serve as a more adequate model system to investigate the regulation of the human *PCK* gene promoter by glucagon and insulin.

Stimulation by CPT-cAMP and dexamethasone of the human and rat *PCK* gene promoters in HepG2 cells: inhibition of the stimulation by insulin

In HepG2 cells luciferase expression under the control of the human *PCK* gene promoter was not significantly stimulated by CPT-cAMP alone (1.4-fold) but was increased 2.0-fold over basal non-stimulated expression by dexamethasone alone. The combination of cAMP and dexamethasone stimulated luciferase expression 2.8-fold over basal non-stimulated values. Insulin inhibited the dexamethasone-stimulated luciferase expression by 59 % and the cAMP/dexamethasone-stimulated expression by 76 % (Figure 2A). Luciferase expression under the control of the rat *PCK* gene promoter was hardly stimulated by dexamethasone alone (1.4-fold) and was stimulated 2.6-fold over basal by CPT-cAMP alone. Dexamethasone and cAMP in combination stimulated luciferase expression by 4.3-fold. This stimulation was inhibited by 48 % in the presence of insulin (Figure 2B). Basal non-stimulated luciferase expression from both promoters was not affected by insulin.

Stimulation by glucagon (cAMP) of the human *PCK* gene promoter containing a mutation in a putative IRS in cultured rat hepatocytes and HepG2 cells: inhibition of the stimulation by insulin

The rat *PCK* gene promoter contains two different *cis*-acting elements that mediate the inhibitory action of insulin on *PCK* gene transcription. One element is located between -437 and -402 and the other between -271 and +69 [12]. Attempts to more precisely define the IRS by using *PCK* gene promoter/*CAT* fusion genes with progressively shortened promoter fragments yielded inconsistent results in transient-transfection experiments. By the use of H4IIE cells stably transfected with *PCK* gene promoter/*CAT* transgenes an IRS was located between -416

Figure 2 Stimulation of the human and rat *PCK* gene promoters by CPT-cAMP and dexamethasone in human HepG2 cells: inhibition by insulin

Cells were transfected either with plasmid pGL-hPCK -457/+65 (A) or with plasmid pGL-rPCK -493/+33 (B) and incubated for 24 h with 100 μ M CPT-cAMP and 500 nM dexamethasone (Dex). Insulin was added at a concentration of 100 nM (black bars). Cells were processed as described in Figure 1. Relative luciferase activity was calculated as the increase over non-stimulated basal activity in the absence of CPT-cAMP and dexamethasone, which was set to 1. Values are the means $\pm$ S.E.M. from three to six separate cell-culture experiments, each run in triplicate. Statistics: Student's *t* test for paired values; values in the presence of insulin are different from values in the absence of insulin with ** $P \leq 0.01$ and * $P \leq 0.05$.

and -407 in the rat *PCK* gene promoter. Mutational analysis in the context of a heterologous thymidine kinase promoter was used to confirm that this element mediates the insulin inhibition of the induction of a *PCK* promoter/*CAT* transgene by dexamethasone and cAMP. However, mutation of this element in the context of the otherwise wild-type rat *PCK* gene promoter did not abrogate insulin responsiveness [19,20]. The rat IRS is perfectly conserved in the human *PCK* gene promoter between -395 and -386 [13]; thus it was of interest to see whether this element behaved like the respective sequence in the rat promoter, i.e. mutation of the IRS in the context of the otherwise wild-type human promoter should not abrogate insulin responsiveness. By the use of a PCR approach the sequence TGGTG between -395 and -391 in the human promoter was converted into GTCCC. A reporter gene consisting of the mutated *PCK* promoter directing the expression of the luciferase gene was transfected into cultured rat hepatocytes. In the absence of insulin luciferase expression was stimulated by glucagon 3.9-fold over basal non-stimulated expression. In the presence of 100 nM insulin glucagon stimulated expression by only 2.6-fold, which corresponds to a 47 % inhibition. In the absence and presence of insulin, half-maximal expression was reached at 0.09 and 0.12 nM glucagon, respectively (Figure 3A). Thus, as with the wild-type human *PCK* gene promoter, the mutation of the putative insulin-responsive region did not affect the inhibition by insulin of glucagon stimulation. When CPT-cAMP was used instead of glucagon the expression of the mutated promoter was stimulated maximally 3.2-fold over basal expression. In the presence of insulin this stimulation was only 2.2-fold, which corresponds to a 45 % inhibition of the maximal response. Half-maximal stimulation was reached at 0.7 μ M CPT-cAMP in the absence of insulin and at 1.25 μ M CPT-cAMP in the presence of insulin (Figure 3B). Therefore, as with the wild-type human *PCK* gene promoter, a mutation of the putative insulin-responsive region did not affect the inhibition by insulin of stimulation by cAMP.

The combination of 100 μ M CPT-cAMP and 500 nM dexamethasone stimulated the expression from the mutated human *PCK* gene promoter/luciferase transgene in HepG2 cells by 2.6-

Figure 3 Concentration dependency of the glucagon- or cAMP-stimulated increase in activity of the mutated human *PCK* gene promoter in cultured rat hepatocytes: inhibition by insulin

Cells were transfected with plasmid pGL-hPCK—457/+65mut, which has a TGGTG → GTCCC conversion in the putative insulin-responsive region located between —395 and —391 in the human *PCK* gene promoter. Cells were processed as described in Figure 1. Expression of the luciferase transgene was stimulated with either glucagon (A) or CPT-cAMP (B) at the indicated concentrations in the absence or presence of 100 nM insulin. Relative luciferase activity was calculated as the increase over non-stimulated activity in the absence of glucagon or CPT-cAMP, which was set to 1. Values are the means $\pm$ S.E.M. from three or four separate cell-culture experiments, each run in triplicate. Statistics: Student's *t* test for paired values; values in the presence of insulin are different from values in the absence of insulin with $*P \leq 0.05$.

Figure 4 Stimulation of the mutated human *PCK* gene promoter by CPT-cAMP and dexamethasone in human HepG2 cells: inhibition by insulin

HepG2 cells were transfected with the plasmid pGL-hPCK—457/+65mut, described in Figure 3. Cells were incubated for 24 h with 100 μ M CPT-cAMP and 500 nM dexamethasone (Dex) in the absence (white bars) or presence (black bars) of 100 nM insulin. The expression of luciferase was measured in cell lysates using a luminometer. Relative luciferase activity was calculated as the increase over basal non-stimulated activity in the absence of CPT-cAMP and dexamethasone, set to 1. Values are the means $\pm$ S.E.M. from three separate cell-culture experiments, each run in triplicate. Statistics: Student's *t* test for paired values; values in the presence of insulin are different from values in the absence of insulin with $*P \leq 0.05$.

fold over the non-stimulated value. This increase of expression was prevented by 100 nM insulin (Figure 4). Hence, as in the rat promoter, the IRS in the human *PCK* gene promoter is not sufficient on its own to confer full insulin responsiveness to the promoter either in cultured rat hepatocytes or in HepG2 human hepatoblastoma cells. These results validate findings from previous studies with the rat *PCK* gene promoter, which showed that the deletion of the distal IRS between —437 and —402 alone did not abrogate insulin responsiveness. Insulin repression of the *PCK* gene promoter also requires the proximal IRS located between —271 and +69 [19,20].

Experiments to identify insulin responsiveness of the rat *PCK* gene promoter have been carried out in the rat hepatoma cell line H4IIE [19,20]. Therefore, these cells were also used to define the regulation by insulin of the human promoter. However, very low expression of a transfected luciferase transgene under the control of the human wild-type or mutated promoter was observed (results not shown), which corroborated the previous findings that only retinoic acid weakly stimulated the activity of the human promoter in H4IIE rat hepatoma cells; dexamethasone and the co-transfected catalytic subunit of protein kinase A as well as insulin were without effect [13]. Hence, obviously the functionality of the human *PCK* gene promoter depends on the proper cellular background. Therefore, constructs used in the present study could not be tested in H4IIE cells. Up to now no comprehensive results on the cell-specific regulation of *PCK* gene expression in rat hepatocytes and hepatoma cells are available. Because the rat *PCK* gene promoter carrying mutations in the insulin-responsive regions has never been investigated in cultured rat hepatocytes it will be a goal of future studies to define the cell-specific determinants of the regulation of *PCK* gene expression by comparing the stimulation by glucagon (cAMP) as well as the inhibition by insulin of the wild-type and mutant rat and human promoters in primary rat hepatocytes and in hepatoma cell lines.

A sequence that shares high homology with a prospective IRS was identified in the human *PCK* gene promoter between —295 and —283 by computer analysis [13]. This element has not been functionally investigated and is not conserved in the rat promoter. The role of this element, on its own or in co-operation with the upstream sequence, was not explored. It also was not within the scope of the present study to identify functionally and structurally the insulin-responsive regions in the human *PCK* gene promoter, which will be investigated in a separate study. However, the preliminary results shown here suggest that the human and rat promoters are very similar.

Involvement of phosphoinositide 3-kinase (PI 3-kinase) in insulin inhibition of the human and rat *PCK* gene promoters in cultured rat hepatocytes

Studies in cultured rat hepatocytes and in rat hepatoma cells showed that stimulation of the expression of the rat *PCK* gene by glucagon or cAMP was inhibited by insulin through the PI 3-

Figure 5 Stimulation of the human and rat *PCK* gene promoters by CPT-cAMP and glucagon in cultured rat hepatocytes

Abrogation of insulin inhibition by wortmannin. Cells were transfected with either plasmid pGL-hPCK — 457/+ 65 (A) or plasmid pGL-rPCK — 493/+ 33 (B) and maintained for 8 h with 1 μ M CPT-cAMP or 1 nM glucagon (Ggn). Insulin was added at a concentration of 100 nM (black bars). Wortmannin (Wort), a PI 3-kinase inhibitor, was included at a concentration of 250 nM where indicated. Cells were processed as described in Figure 1. Relative luciferase activity was calculated as the increase over non-stimulated basal activity in the absence of CPT-cAMP or glucagon, set to 1. Values are the means $\pm$ S.E.M. from three or four separate cell-culture experiments, each run in triplicate. Statistics: Student's *t* test for paired values; values in the presence of insulin are different from values in the absence of insulin with $^{**}P \leq 0.01$.

kinase pathway [27–30]. The luciferase expression vector controlled by the human promoter was transfected into rat hepatocytes to investigate whether PI 3-kinase was also required for the insulin inhibition of the human *PCK* gene promoter. Inhibition of the glucagon (cAMP)-dependent activation by insulin was studied in the absence and presence of wortmannin, a PI 3-kinase inhibitor. The rat promoter was investigated for comparison. CPT-cAMP or glucagon stimulated luciferase expression from the reporter construct by 2.7- and 3.8-fold over basal non-stimulated expression. Insulin inhibited this stimulation by 71 and 51%, respectively. Wortmannin alone had no significant effect on the stimulation of luciferase expression by cAMP or glucagon but it abrogated the inhibition by insulin (Figure 5A). When the luciferase gene was driven by the rat *PCK* gene promoter, CPT-cAMP or glucagon stimulated luciferase expression 4.5- or 5.1-fold over basal non-stimulated expression. Insulin inhibited this stimulation by 46 and 47%, respectively, and this effect was abolished by wortmannin. The stimulation of luciferase expression by CPT-cAMP was increased further by 1.7-fold in the presence of wortmannin alone (Figure 5B). The data indicate that in cultured rat hepatocytes the glucagon (cAMP)-stimulated activation of the human and rat *PCK* gene promoters is inhibited by insulin through activation of the PI 3-kinase pathway.

Transcription of the rat *PCK* gene is inhibited by insulin in both primary cultured rat hepatocytes and rat hepatoma cells [12,15], and this appears to require an intact PI 3-kinase pathway [27–30]. The data presented here show that the human *PCK* gene promoter is also inhibited by insulin through activation of the PI 3-kinase pathway in primary cultured rat hepatocytes. The downstream PI 3-kinase target that ultimately mediates the transcriptional inactivation of the *PCK* gene promoter has not been identified. However, it must be at a site downstream of cAMP, because insulin inhibition of the CPT-cAMP stimulation of the rat and human promoters was abrogated by wortmannin.

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