Glucose induces expression of rat pyruvate carboxylase through a carbohydrate response element in the distal gene promoter

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Pyruvate carboxylase is an enzyme of the so-called pyruvate cycling pathways, which have been proposed to contribute to glucose-stimulated insulin secretion in pancreatic β-cells. In the rat insulinoma cell line 832/13, transcripts from both the distal and proximal gene promoter for pyruvate carboxylase are up-regulated by glucose, with pyruvate carboxylase being expressed mainly from the distal gene promoter. At position −408 to −392 relative to the transcription start site, the distal gene promoter was found to contain a ChoRE (carbohydrate response element). Its deletion abolishes glucose responsiveness to a heterologous gene promoter. ChREBP (carbohydrate response element-binding protein) and its dimerization partner Mlx (Max-like protein X) bind to the ChoRE in vitro. ChREBP further binds to the distal promoter region at a high glucose concentration in situ. The E-box-binding transcription factors USF1/2 (upstream stimulatory factor 1/2) and E2A variant 2 [also known as E47 and TCF3 (transcription factor 3)] can also bind to the ChoRE. Overexpression of E2A diminishes the magnitude of the glucose response from the pyruvate carboxylase ChoRE. This illustrates that competition between ChREBP–Mlx and other factors binding to the ChoRE affects glucose responsiveness. We conclude that a ChoRE in the distal gene promoter contributes to the glucose-mediated expression of pyruvate carboxylase.

Key words: carbohydrate response element (ChoRE), carbohydrate response element-binding protein (ChREBP), E-box, insulinoma, Max-like protein X (Mlx), pyruvate carboxylase.

INTRODUCTION

In pancreatic β-cells, GSIS (glucose-stimulated insulin secretion) occurs by oxidation of glucose (reviewed in [1]). Pyruvate cycling, which refers to the conversion of pyruvate into oxaloacetate catalysed by PC (pyruvate carboxylase) followed by a series of mitochondrial and cytosolic reactions that generate cytosolic NADPH, is a second process that is correlated with GSIS [2–4]. Recent reports indicate that the concentration of PC in insulinoma cells and pancreatic β-cells directly affects GSIS [5,6].

The expression of PC is induced by glucose in pancreatic islets [7] and in insulinoma cell lines [8,9]. Glucose induces transcription of the PC gene in the rat insulinoma cell line RINm5F [8], but further elucidation of the mechanism for glucose regulation of PC has, to our knowledge, not been reported previously. The purpose of the present study was to identify the mechanism of glucose regulation of PC in insulin-secreting cells. Our testing was done with the glucose-responsive insulinoma cell line 832/13 [10], which is a popular cell line for studying pyruvate cycling [2,3,6] and glucose-regulated gene expression [11–15].

Rat PC is expressed from a distal and proximal gene promoter [16,17], with the distal promoter being reported to be the active promoter in pancreatic islets and insulinoma cells [9]. In the present paper we describe a functional ChoRE (carbohydrate response element) in the distal PC gene promoter. We show that the transcription factor ChREBP (carbohydrate response element-binding protein), which binds to ChoREs together with its dimerization partner Mlx (Max-like protein X), is required for glucose responsiveness. We further demonstrate that the magnitude of the glucose response is affected by competition between the glucose-responsive ChREBP–Mlx complex and various other proteins capable of binding to the ChoRE.

EXPERIMENTAL

Cell culture

Rat insulinoma cells 832/13 [10] were kindly provided by Dr Christopher Newgard (Duke University Medical Center, Durham, NC, U.S.A.). Cells were maintained as described previously [18]. Primary rat hepatocytes from male Wistar rats were isolated as described previously [19] under a protocol approved by the Louisiana State University Health Sciences Center (LSUHSC-NO, New Orleans, LA, U.S.A.) Institutional Animal Care and Use Committee and were provided by Dr Donald K. Scott (currently with University of Pittsburgh Medical Center, Pittsburgh, PA, U.S.A.).

Plasmids and adenoviral constructs

The sequence from −1146 to +20, relative to the transcription start site, of the distal gene promoter for rat PC was amplified by PCR from DNA isolated from 832/13 cells and was inserted between the NheI and HindIII sites of pGL3-Basic (Promega). The cloned sequence is identical with bases 15638950–15640115 of the rat genomic sequence (GenBank® accession number NW_047563.2) and is located at approx. 80 kb upstream of the PC coding region. The −408 to −392 sequence of the distal PC promoter was deleted by in vitro mutagenesis.

Abbreviations used: ACC, acetyl CoA carboxylase; ChIP, chromatin immunoprecipitation; ChoRE, carbohydrate response element; ChREBP, carbohydrate response element-binding protein; DN, dominant-negative; EMSA, electrophoretic mobility-shift assay; G6Pase, catalytic subunit of glucose-6-phosphatase; GK, glucokinase; GSIS, glucose-stimulated insulin secretion; HA, haemagglutinin; L-PK, liver-type pyruvate kinase; Mlx, Max-like protein X; NeuroD1, neurogenic differentiation factor 1; PC, pyruvate carboxylase; Pol II, RNA polymerase II; QRT-PCR, quantitative reverse transcription–PCR; RLU, relative light unit; RT, reverse transcription; USF, upstream stimulatory factor.

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One or two copies of the −412 to −388 sequence of the distal rat PC promoter were cloned into the pTA-Luc vector (Clontech Laboratories) between the SacI and NheI sites. One or two copies of the homologous human sequence were likewise cloned into pTA-Luc. Expression plasmids for mouse ChREBP and Mix-γ, with and without FLAG and HA (haemagglutinin) tags, as well as the empty expression plasmid CMV4 were kindly provided by Dr Howard Towle (Department of Biochemistry, University of Minnesota, Minneapolis, MN, U.S.A.). Similar expression plasmids for rat ChREBP and rat Mix-β were constructed. The rat ChREBP and rat Mix-β sequences were derived by RT (reverse transcription)–PCR of RNA from rat hepatocytes. The rat ChREBP expression plasmid corresponds to bases 34–2652 of GenBank® accession number AB074517, with the exception of the presence of T, G, T, T and G at positions 159, 897, 1439, 1910 and 2022 respectively, inserted between the BglIII and HindIII sites of CMV4. The rat Mix-β expression plasmid corresponds to bases 6–740 of GenBank® accession number DQ350893 inserted between the BglIII and Sall sites of CMV4. Expression plasmids for rat NeuroD1 (neuronal differentiation factor 1, also known as BET2) and the rat E2A [also known as E12/E47 and TCF3 (transcription factor 3)] splice variants were created by RT–PCR of RNA from 832/13 cells and insertion of fragments into CMV4 between the BglIII and HindIII sites for NeuroD1, and between the BglIII and Sall sites for the E2A variants. The insert for rat NeuroD1 is identical with bases 88–1161 of GenBank® accession number NM_019218. The inserts for E2A variant 1 (also known as E12 [20]) and E2A variant 2 (also known as E47 [20]) are identical with bases 74–2023 of GenBank® accession number NM_132524 and bases 74–2014 of GenBank® accession number NM_001235237 respectively, with the exception of the presence of A and T at positions 246 and 919. Two additional splice variants, termed E2A variant 1Q and E2A variant 2Q, with an extra CAG codon inserted between bases 1228 and 1229 of GenBank® accession numbers NM_133524 and NM_001035237 respectively, were also cloned. Analogous to the description in [21], an expression plasmid for rat NeuroD1 was constructed as bases 88–789 of GenBank® accession number NM_019218, followed by a TAG stop codon, inserted between the BglIII and HindIII sites of CMV4. Adenoviral constructs for expression of wild-type mouse ChREBP [22] and DN-ChREBP [23] were kindly provided by Dr Howard Towle. Expression of proteins from expression plasmids and adenoviruses were confirmed by Western blotting (results not shown).

Transfection
The 832/13 cells were transfected with Lipofectamine™ 2000 (Invitrogen) in 12-well plates for determination of luciferase activities as described previously [18]. Each well contained transfection mixtures consisting of 1 μg of a firefly luciferase reporter, 0.25 μg of pRL-TK plasmid (Promega), as a control for transfection efficiency, and, in some experiments, 1 μg of expression plasmids or the empty vector CMV4. When two expression plasmids were co-transfected, 0.5 μg of each was used. Transfected cells were treated with 2 mM or 20 mM glucose for 20 h. RLU (relative light units) were calculated as the ratio of firefly and Renilla luciferase activities. Experiments were designed as generalized randomized complete block designs. As described previously [18], ANOVA of logarithm-transformed data was conducted. Post-hoc contrasts of activities at 2 and 20 mM glucose were tested by the least-significant difference procedure. Post-hoc contrasts comparing the glucose responses (expression at 20 mM relative to the expression at 2 mM glucose) were tested with Bonferroni adjustments for multiple comparisons. Means ± S.E.M. were calculated and retransformed back to linear scale for presentation. Each figure summarizes the results of four experiments with each treatment performed in duplicate. For extraction of protein for EMSAs (electrophoretic mobility-shift assays), 832/13 cells were seeded in six-well plates at a concentration of 2 × 10^4 cells/well and transfected with Lipofectamine™ 2000 the following day. For each well, 2.5 μg of expression plasmid or the empty vector CMV4 was used. When two expression plasmids were co-transfected, 1.25 μg of each was used. After incubation for 3 h with transfection mixtures, the cells were incubated for 20 h in the normal maintenance medium {consisting of a base medium [RPMI 1640 with 10% (v/v) fetal bovine serum, 10 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol] supplemented with 11 mM glucose} before being harvested.

QRT–PCR (quantitative RT–PCR)
Sequences of primers and probes are listed in the 5′→3′ direction. Total RNA was isolated from 832/13 cells or primary rat hepatocytes using TRI Reagent® (Molecular Research Center). One-step QRT–PCR was performed using Taqman one-step RT–PCR master mix (Applied Biosystems). Taqman TAMRA probes (Applied Biosystems) for detecting rat PC, rat ChREBP and rat Mix mRNA contained 6-carboxyfluorescein at the 5′ end; the sequences were CCATAAOGCCACACCGAGCT, TCTCCA-GGCTCGAAACAGCTGCTCTG and ACCATGGCTCCATCGTGCACAGCA respectively. Sequences of forward and reverse primers were: PC, CCCTGTTGCCACATTGTT and TCGAGAAGGATGTCTCTGAAA; ChREBP, GACCCTC-TCTCAGGGGATAACA and GGAAAGTGGCTGGGATCC; and Mix, GGGAATTCTTAAAGAGGAGCTGA and TGGGAGCCAAATGGAATAC. Taqman MGB probes (Applied Biosystems) for detecting L-PK (liver-type pyruvate kinase) mRNA and PC mRNA with different exon 1 forms [1A, 1B, 1B(−)], 1C and 1D] spliced to exon 2 contained 6-carboxyfluorescein at the 5′ end; the sequences were TACAGAAATTCGCC-CCAGAG, TATGAGACGATGCTGAAGT, CCAGAACAG-TCATGAGACGATGCTGAAGT and TGCGAGCCAAATGGAATAC. Forward and reverse primers for L-PK were CGACGGGCGTCATCTCCTTAG and GATACCCACCGCTGCTACTTAG. The common reverse primer for PC mRNA with different exon 1 forms was CCCCCCGACAATCTTTTGA. Forward primers were TGTCACCCCTTTCAGGGAGAGAACAG, GGCCCTGCTTGATAGAAGTATTTG, GCGAGTCACCTAGGTGTTGG, ATCTGCTTTGAGAGGGTAGT and GCGCCAC-ACGCCCTTGGA respectively. 18S RNA levels were measured with predeveloped Taqman assay reagents (Applied Biosystems). Standards for each assay were dilutions of a sample of total RNA isolated from 832/13 cells. The amount of each mRNA form was normalized to the amount of 18S rRNA. The glucose response was calculated as the normalized amount of an mRNA form at 20 mM glucose divided by the normalized amount at 2 mM glucose.

Antibodies
Antibodies against the FLAG epitope (Octa probe; sc-807), Mix (sc-14705), E2A (sc-349), NeuroD1 (sc-1084), USP (upstream stimulatory factor) 1 (sc-8983 X) and USP2 (sc-861 X), as well as non-specific rabbit IgG (sc-2027) were from Santa Cruz Biotechnologies. Antibodies against the HA epitope (H6908) and γ- tubulin (T5192) were from Sigma–Aldrich. Antibodies against ChREBP (NB400–135) and Pol II (RNA polymerase II) (05–623) were from Novus and Millipore respectively.
Western blot analysis

Whole-cell lysates were made in Laemmli sample buffer [62.5 mM Tris/HCl, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.00125% Bromophenol Blue with 1× protease inhibitors (04693159001 from Roche)]. Electrophoresis of whole-cell lysates was performed on NuPAGE Bis-Tris 4–12% gels (Invitrogen) followed by transfer on to Invitrolon PVDF membranes (Invitrogen). Primary antibodies were against ChREBP and γ-tubulin. The secondary antibody was horseradish peroxidase-conjugated anti-(rabbit-IgG) antibody (GE Healthcare). Luminescence was generated with the Amershams ECL (enhanced chemiluminescence) advance Western blotting detection kit (GE Healthcare) and recorded on a VersaDoc Imaging System (Bio-Rad Laboratories). Images of ChREBP are contrast-enhanced.

EMSA

Nuclear extracts of 832/13 cells were isolated using the NE-PER kit (Pierce Chemicals). Protease inhibitors were included in reagents. Protein concentrations of nuclear extracts were determined using the BCA (bicinchoninic acid) Protein Assay kit (Pierce Chemicals). Double-stranded probes representing ChoRE-containing segments of rat gene promoters for L-PK, ACC (acetyl CoA carboxylase) and PC (carboxylic acid) had the sense sequences 5′-GG-GCCACAGGGGCATTCAAGTACCAGCAGA-3′, 5′-CTCTCCATATGCTGAGCACATGGCAGA-3′, 5′-AGAGACAGGTGCTACTCT-AGAGTGAATGA-3′ and 5′-GTGCTCATTGGAAAAAGTCTTACG and GCCGCCCATGTGACCGGAGAACA, and for a segment of exon 13 of the PC gene, CCAGACAGCATTTGTAAGACCTCACCACGGCGAGAATA and GGGCATCCTCGGACAGGT. Experiments were set-up and analysed as split-plot designs as previously described [14,18] with ANOVA conducted with logarithm-transformed data. Post-hoc tests for comparisons of means were conducted with Bonferroni adjustments for multiple comparisons. Means ± S.E.M. were calculated and retransformed back to linear scale for presentation.

RESULTS

Glucose-dependent expression of PC mRNA in 832/13 cells

PC mRNA is up-regulated by glucose in the INS-1 cell line, from which 832/13 cells originate [9]. We first verified that PC mRNA is also up-regulated by glucose in 832/13 cells. There was minimal up-regulation by 6 h, but by 12 h there was a robust, statistically significant glucose response (Figure 1A). In a separate set of experiments, we explored how glucose affects the individual splice variants of PC mRNA. Transcription from the distal promoter results in variants in which exon 1C or exon 1D is spliced to exon 2, whereas transcription from the proximal promoter gives variants in which exon 1A, exon 1B, or a shortened form of exon 1B, which we term exon 1Bc (−), is spliced to exon 2 [17]. For comparison, we also determined the glucose response of total PC mRNA and of L-PK mRNA, which is a well-known glucose-regulated transcript in 832/13 cells [14,15]. All transcripts, except the PC transcripts from the distal promoter at the 6 h time point, were significantly (P < 0.01 by two-sample t tests) up-regulated by glucose (Figure 1B). The transcripts from the proximal PC promoter were up-regulated to a higher degree than the more modestly regulated transcripts from the distal promoter. Total PC mRNA was also modestly regulated by glucose, suggesting that the distal promoter contributes more transcripts to the total pool of PC mRNA than the proximal promoter.

Underscoring the pre-eminence of the distal promoter relative to the proximal promoter, we observed a more pronounced recruitment of Pol II to the distal compared with the proximal promoter by ChIP assays at both the 6 and 24 h time points (Figures 1C and 1D). There was almost as little binding of Pol II to the proximal promoter as to a coding region of the PC gene (exon 13), where the binding could be attributed to actively transcribing Pol II. As control targets in the ChIP experiments, we used the L-PK promoter as an example of an active, glucose-responsive gene promoter in 832/13 cells [14,15] and the hepatic GK promoter as an example of an inactive, glucose-unresponsive promoter [14,24].

The stability of total PC mRNA relative to 18S rRNA was assessed by treating cells with the transcriptional inhibitor actinomycin D. The stability was not influenced by the glucose

Glucose induction of pyruvate carboxylase

The 832/13 cells were seeded at a concentration of 40 × 10⁶ cells in a 75 cm² tissue culture flask and incubated in base medium supplemented with 5 mM glucose overnight. Cells were exposed to base medium supplemented with 2 or 20 mM glucose for 6, 12 or 24 h. ChIP was conducted with 2 μg of antibody for each precipitation as described previously [14,18] except for the absence of glass beads during sonication. Target gene regions were quantified by real-time PCR with iTag SYBR Green Supermix with ROX (Bio-Rad Laboratories). Unbound DNA from the 2 μm

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Glucose induces expression of PC mRNA in 832/13 cells

(A) 832/13 cells were grown in medium (RPMI 1640 medium with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol) containing 2 or 20 mM glucose. Prior to the experiment, cells were grown overnight in the presence of 2 mM glucose. Total PC mRNA was determined by a QRT-PCR assay using an amplicon encompassing the splice site between exons 12 and 13. The PC mRNA levels were normalized relative to 18S rRNA. Results are means ± S.E.M. for four experiments. * P < 0.05; ***P < 0.001 for difference between levels at 2 and 20 mM glucose as determined by two-sample t test. (B) 832/13 cells were treated in four experiments as in (A). The amount of each PC splice form and L-PK mRNA were quantified by highly specific QRT-PCR assays and normalized to 18S rRNA. The glucose response is calculated as the normalized concentration of an mRNA species at 20 mM glucose divided by the normalized concentration at 2 mM glucose. (C and D) ChiP experiments were conducted with 832/13 cells incubated at 2 or 20 mM glucose for (C) 6 h and (D) 24 h. Immunoprecipitations were conducted with an antibody against Pol II or with non-specific antibody (control IgG). The segments −36 to +67 of the hepatic GK gene promoter, −39 to +46 of the L-PK gene promoter, −71 to +19 of the distal PC gene promoter (PC dist), −77 to +11 of the proximal PC gene promoter (PC prox), and a segment of exon 13 of the PC gene (PC coding) were targeted for amplification. *P < 0.05; **P < 0.01; ***P < 0.001, amount of target promoter precipitated by the Pol II antibody compared with the control IgG at a given glucose concentration. (E) 832/13 cells were pre-incubated for 24 h in the presence of 2 or 20 mM glucose. At time point 0 h, medium with 10 μg/ml actinomycin D in addition to 2 or 20 mM glucose was added. PC mRNA levels were normalized relative to 18S rRNA levels. Normalized levels were set to 100 at time point 0 h. Results are means ± S.E.M. from four experiments.

![Figure 1 Glucose induces expression of PC mRNA in 832/13 cells](image)

An evolutionarily conserved ChoRE in the distal PC gene promoter is necessary and sufficient for a glucose response

To test whether the distal PC gene promoter was glucose-responsive, we cloned the promoter sequence from −1146 to +20, relative to the transcription start site at +1, into the pGL3-Basic luciferase vector. The sequence at positions −408 to −392 (Figure 2A) has sequence similarity to a ChoRE, which is a cis-regulatory element consisting of two motifs related to the E-box motif 5′-CACGTG-3′ separated by five base pairs [25]. To test involvement of this putative ChoRE in glucose responsiveness, it was deleted from the −1146 to +20 promoter fragment. Results are shown in Figure 2(B). Whereas, the background promoter activity of pGL3-Basic was down-regulated by glucose, insertion of the −1146 to +20 fragment resulted in a modest, but highly

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<th>PC mRNA splice form</th>
<th>1A2</th>
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(Glucose responses)
Figure 2  Glucose responsiveness of the distal PC gene promoter depends on a ChoRE

(A) Sequences of a ChoRE-like element in the PC gene promoters from rats, cows and humans are listed. The numbering for the bovine and human sequences is based on an assumption of the same transcription start site as for the rat promoter. (B) 832/13 cells were transfected with pGL3-Basic-based luciferase reporters. The full −1146 to +20 PC promoter fragment and a PC promoter fragment in which the ChoRE-like sequence was deleted were compared. (C) 832/13 cells were transfected with pTA-Luc containing one or two copies of the ChoRE-like sequence from the rat PC gene promoter, or two copies of the ChoRE from the rat G6Pase gene promoter. (D) 832/13 cells were transfected with pTA-Luc containing one or two copies of the homologous human sequence. Vectors without inserts are used as controls (No insert). *P < 0.05; **P < 0.001 for differences in expression levels at 2 and 20 mM glucose.

The bovine PC promoter P3, which is homologous with the distal rat promoter, contains a similar ChoRE sequence to that in rats (Figure 2A; [26]). To test whether a similar element exists in the human genome, we performed a BLAST search with the −1146 to +20 rat promoter sequence. We identified a homologous region (bases 2348543–2349253 of GenBank® accession number NW_001838025.2) located approx. 86 kb upstream of the human PC precursor. The sequence homologous with the rat −413 to −387 sequence is listed in Figure 2(A).

One or two copies of the human sequence at −412 to −388 were inserted into pTA-Luc. The human sequence also mediated the glucose-responsiveness of the promoter in pTA-Luc (Figure 2D).

A ChREBP–Mix complex binds to the PC ChoRE in vitro

The transcription factor ChREBP and its dimerization partner Mix mediate glucose responsiveness from other ChoREs in hepatocytes and insulinoma cells [13–15,18,22,27,28]. We therefore tested whether ChREBP–Mix plays the same role for the PC ChoRE.

In the EMSA shown in Figure 3(A), we identified a faint complex (Figure 3A, C-I in lanes 2 and 6) that increased in intensity upon co-expression of FLAG–ChREBP and HA–Mix (Figure 3A, lane 5) and that became supershifted with antibodies against ChREBP and Mix (Figure 3A, lanes 7 and 8). Complex
C-I thus contained both ChREBP and Mlx. Co-expression of FLAG–ChREBP and HA–Mlx generated additional bands (Figure 3A, labelled ‘a’ and ‘b’ in lane 5). Expression of HA–Mlx (Figure 3A, lanes 3 and 5) further intensified the band labelled ‘c’. From EMSAs with antibodies against USF1 and USF2 (results not shown), we further concluded that the intense band, labelled complex U-I, contained both USF1 and USF2, whereas the slightly faster migrating complex, labelled U-II, contained USF1.

When both FLAG–ChREBP and HA–Mlx–β are expressed, band C-I can, at least partially, be supershifted with antibodies against FLAG, HA, ChREBP and Mlx (Figure 3B, lanes 2–5). This confirms that FLAG–ChREBP and HA–Mlx–β overexpression led to complex formation with these proteins. Intensities of complexes ‘a’ and ‘b’ were diminished upon both anti-ChREBP antibody and anti-Mlx antibody treatment (compare lanes 1, 4 and 5 of Figure 3B), suggesting that the complexes contained the ChREBP and Mlx epitopes. In two of three EMSAs with a clearly discernible ‘c’ complex, the intensity of the ‘c’ complex was diminished upon anti-HA antibody and anti-Mlx antibody treatment (Figure 3B, lanes 3 and 4), but not upon anti-FLAG antibody and anti-ChREBP antibody treatment (Figure 3B, lanes 2 and 5). This suggests that complex ‘c’ contained HA–Mlx, but not FLAG–ChREBP.
Figure 4  Binding of transcription factors ChREBP, USF1 and USF2 to the distal PC gene promoter in situ

ChIP assays with an anti-ChREBP antibody or non-specific antibody (control IgG) were conducted with 832/13 cells incubated at 2 or 20 mM glucose for (A) 6 h or (B) 24 h. ChIP assays with an anti-USF1 antibody, an anti-USF2 antibody or a non-specific antibody were conducted with 832/13 cells incubated at 2 or 20 mM glucose for (C) 6 h or (D) 24 h. For ChIP assays, the segments $-146$ to $-26$ of the hepatic GK gene promoter, $-204$ to $-58$ of the L-PK gene promoter, and $-503$ to $-339$ of the distal PC promoter were targeted for amplification. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, amount of target promoter precipitated by the antibody of interest compared with the control IgG at a given glucose concentration. ##$P < 0.01$; ###$P < 0.001$, amount of target promoter precipitated by the antibody of interest at 2 mM compared with 20 mM glucose.

Figure 3(C) illustrates that complex C-I was also generated with ChoRE-containing promoter segments from G6Pase, L-PK and ACC, and that co-expression of FLAG–ChREBP and HA–Mlx led to more intense bands. On the basis of results in [23], the C-I complex was probably a tetrameric complex containing two ChREBP–Mlx heterodimers with each heterodimer binding to one of the two E-box-like elements of the ChoRE. Furthermore, complex ‘a’ had clear affinity for all four ChoREs, whereas complexes ‘b’ and ‘c’ have a preferential affinity for the PC ChoRE.

USF1 and USF2 bind to the distal PC gene promoter in a glucose-independent manner, whereas ChREBP binds in a glucose-dependent manner

Glucose-dependent binding of transcription factors ChREBP, USF1 and USF2 to the distal PC gene promoter in 832/13 cells in situ was tested by ChIP assays. Figure 4(A) shows that there was no significant binding of ChREBP to the PC promoter at a time point (6 h) when there is, as shown in Figure 1(B), only marginal glucose induction of transcripts from the distal PC promoter. However, at a time point (24 h) when there is robust glucose induction, there was a clear binding of ChREBP to the PC promoter at 20 mM glucose and insignificant binding at 2 mM glucose (Figure 4B). A similar clear binding of ChREBP to the PC promoter has been observed after 12 h at 20 mM glucose (results not shown). Both USF1 and USF2 bound to the PC promoter in situ, whereas only USF2 bound to the L-PK promoter (Figures 4C and 4D). Neither after 6 h nor after 24 h is binding of USF1 or USF2 dependent on the glucose concentration. Binding of USF2 at 2 mM glucose to the GK promoter at 6 h ($P < 0.05$) is probably a statistical type I error.

ChREBP is required for glucose responsiveness

We tested whether ChREBP–Mlx has a functional role in the glucose response from the PC ChoRE. We utilized adenovirally expressed DN-ChREBP, as this virus can lead to a complete block of glucose induction of L-PK mRNA in 832/13 cells [29]. Transfection experiments with pTA-Luc containing two
Figure 5 The glucose response from ChoREs depends on ChREBP

(A–C) At 24 h before transfections, 832/13 cells were infected with an adenovirus for expression of DN-ChREBP, an adenovirus for expression of wild-type ChREBP (wt ChREBP), or left uninfected. The cells were transfected with the pTA-Luc luciferase reporter containing (A) two copies of the L-PK ChoRE, (B) the pTA-Luc reporter containing two copies of the PC ChoRE or (C) pGL3-Basic containing the −1146 to +20 fragment of the distal PC gene promoter. Differences in the glucose response compared with uninfected cells at the 0.1% (###) significance level are shown.

(D) Western blots of whole-cell lysates from 832/13 cells grown for 24 h at 2 or 20 mM glucose. Antibodies against ChREBP and γ-tubulin were used. Lysates were from four experiments, I, II, III and IV, and each lane contained 50 μg of protein. The molecular mass in kDa is indicated on the right-hand side. (E and F) 832/13 cells were pre-incubated in medium (RPMI 1640 medium with 10% (v/v) fetal bovine serum, 10 mM Hepes, 2 mM l-glutamine, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol) containing 2, 11 or 20 mM glucose for 24 h. Cells were subsequently transfected with (E) the pTA-Luc reporter containing two copies of the PC ChoRE or (F) pGL3-Basic containing the −1146 to +20 fragment of the distal PC gene promoter. Differences in the glucose response compared with cells pre-incubated at 2 mM glucose at the 0.1% (###) significance level are shown.

We conclude that ChREBP is required for the glucose response.

Overexpression of E2A reduces the magnitude of the glucose response from the PC ChoRE

In insulinoma cells, another glucose-responsive transcription factor capable of binding to E-boxes, which is present in e.g.
the insulin gene promoters, is NeuroD1 with its dimerization partner E2A [30–32]. We hypothesized that NeuroD1–E2A could contribute to the glucose-responsiveness of the PC ChoRE. Overexpression of NeuroD1 and DN-NeuroD1 had little effect on the glucose responsiveness of pTA-Luc containing two PC ChoREs (Figure 6A). However, E2A overexpression increased luciferase expression at 2 mM glucose more than at 20 mM glucose resulting in a significantly diminished glucose response. Overexpression of all four types of E2A occurring in 832/13 cells decreases the magnitude of the glucose response (Figure 6B).

We conducted an EMSA with the PC ChoRE probe and with nuclear extracts from cells transfected with expression plasmids for NeuroD1, E2A variant 2 (E47) or the empty expression plasmid CMVS4. Overexpression of E2A led to formation of complexes E-I and E-II (Figure 6C, lane 2), with E-I overlapping the ChREBP–Mlx-containing complex C-I (Figure 6C). E-I and E-II could be supershifted with an anti-E2A antibody (Figure 6C, lane 5), but not an anti-NeuroD1 antibody (Figure 6C, lane 6). Weaker supershifted bands generated with antibodies against ChREBP and Mlx (Figure 6C, lanes 7 and 8) were interpreted as supershifts of the C-I complex. The results suggest that E2A dimers rather than the E2A–NeuroD1 heterodimers bind to the PC ChoRE. When E2A was not overexpressed, E2A antibodies had no effect on the EMSA band pattern (results not shown) suggesting that the endogenous level of nuclear E2A dimers is very low. Accordingly, the PC promoter region showed as little...
E2A binding in ChIP assays as the inactive hepatic GK promoter region (results not shown).

We conclude that whereas endogenous levels of E2A may have minimal effect on the PC ChoRE, overexpression of E2A generates E2A-containing complexes that bind to the ChoRE and reduce glucose responsiveness.

Overexpression of ChREBP and Mix has diverse effects on glucose responsiveness depending on the type of ChoRE

Despite the role of ChREBP–Mix in mediating glucose responsiveness from ChoREs, overexpression of ChREBP and Mix can, depending upon the type of ChoRE, result in a reduced glucose response. Thus overexpression of rat Mix-β diminished the magnitude of the glucose response of pTA-Luc containing two copies of the PC ChoRE (Figure 7A). For pTA-Luc containing two copies of the ACC ChoRE or the L-PK ChoRE, co-overexpression of rat ChREBP and rat Mix-β caused a decrease and increase respectively in the glucose response (Figures 7B and 7C). These divergent effects depending upon the type of ChoRE are not particular to the expression plasmids for rat ChREBP and Mix-γ that we have generated, as similar results were obtained with expression plasmids for mouse ChREBP and Mix-γ (results not shown).

A possible explanation for the divergent effects that depend solely on the sequence of the ChoREs is generation of transcription factor complexes which interact with the ChoREs with different affinities. In addition to the C-I complex, at least three complexes generated upon ChREBP and/or Mix overexpression could also bind to the ChoREs with varying affinities depending on the type of ChoRE (Figure 3C). If these complexes are less glucose-responsive than the C-I complex and able to effectively compete with C-I for binding to a particular ChoRE, then they would diminish the magnitude of the glucose response analogous to the effect of E2A overexpression.

DISCUSSION

We have studied the glucose regulation of PC in the 832/13 rat insulinoma cell line. As in the parental cell line, INS-1, expression of transcripts derived from the distal gene promoter are induced by glucose in 832/13 cells (Figures 1A and 1B; [9]). Surprisingly, expression of PC transcripts from the proximal promoter was also strongly up-regulated by glucose (Figure 1B). As the distal promoter is clearly the most active of the PC gene promoters in 832/13 cells (Figures 1C and 1D), the transcripts from the proximal promoter are considered of minor importance in this cell line.

Responsiveness of the distal promoter to glucose depends on an evolutionarily conserved ChoRE (Figure 2). On the basis of the identification of intense bands in EMSAs as complexes with USF1 and USF2, it was suggested previously that PC may not be regulated by ChREBP [33]. However, we identified a fainter band (complex C-I) as a complex with ChREBP and Mix (Figure 3A). Similar complexes were seen with other well-known ChoREs (Figure 3C), suggesting that C-I is the glucose-responsive complex consisting of two ChREBP–Mix heterodimers [23]. Furthermore, ChREBP binds to the distal PC promoter at a high glucose concentration in situ (Figure 4B), even when the total concentration of ChREBP is down-regulated by glucose in 832/13 cells (Figure 5D). A role for ChREBP–Mix in glucose responsiveness was further established by observation of a reduced glucose response upon expression of DN-ChREBP (Figures 5B and 5C) and upon a lowering of the cellular ChREBP level by pre-incubation at a high glucose concentration (Figures 5E and 5F). The mechanism by which a high glucose concentration reduces the ChREBP concentration in 832/13 cells is currently not understood. However, it must be a cell line- or cell type-specific phenomenon, as glucose increases the ChREBP concentration in hepatocytes [34].

The glucose induction of transcripts from the distal PC promoter is delayed and of lesser magnitude than that of the well-known glucose-responsive L-PK gene (Figure 1B). High transcript stability, as suggested by the results shown in Figure 1(E), can lead to slow kinetics of transcript
up-regulation [35]. Furthermore, there was a delayed and less pronounced binding of ChREBP to the distal PC promoter compared with the L-PK promoter (Figures 4A and 4B). Part of the glucose responsiveness of ChREBP is due to increased nuclear localization at a high glucose concentration [13,36,37]. With the more pronounced binding of USF1 and USF2 to the PC ChoRE compared with the L-PK ChoRE (Figures 3C, 4C and 4D), it is possible that it simply takes longer for ChREBP to reach a sufficiently high nuclear concentration to effectively compete for binding to the PC ChoRE. Alternatively, binding of ChREBP to the PC ChoRE may require slower-occurring post-translational modifications in response to glucose than those required for binding to the L-PK ChoRE.

Overexpression of Mix led to a reduction in glucose responsiveness from the PC ChoRE (Figure 7A). A probable explanation is formation of protein complexes, such as the complex 'c' shown in Figure 3, which compete with the glucose-responsive ChREBP–Mix complex for access to the ChoRE. Likewise, overexpression of E2A led to the appearance of E2A-containing complexes binding to the ChoRE (Figure 6C) and a diminished glucose response (Figure 6B).

In summary, we have identified a functional ChoRE in the distal PC gene promoter to which the glucose-responsive transcription factor ChREBP binds in a glucose-dependent manner. We have further shown that other E-box-binding transcription factors can bind to the ChoRE and affect the magnitude of the glucose response.

Appropriate control of PC expression is important for GSIS. Thus knockdown of PC to a residual PC activity of less than 50% significantly reduces GSIS in insulin-secreting cells [5,6] whereas overexpression of PC, with a coincident doubling of the PC activity, increases GSIS [5]. In the diabetic state of rodents and humans, the level of islet PC is diminished compared with non-diabetic controls [38–40]. Despite hyperglycaemia, glucose induction of PC expression is obviously not sufficient to maintain normal PC levels in the diabetic state. The causes are unknown. Glucose induction of PC might become diminished in diabetes. This could happen if ChREBP is down-regulated by the high glucose concentration, as in 832/13 cells, or if competitors capable of binding to the PC ChoRE are up-regulated. Alternatively, down-regulatory signals elicited by the diabetic state may be dominant over the glucose induction. A better understanding of the effects of acute and chronic high glucose levels on expression of PC and other enzymes involved in glucose metabolism and GSIS may ultimately lead to novel approaches to combat diabetes.

REFERENCES


AUTHOR CONTRIBUTION

Rebecca Buckley performed the EMSA experiments; Ray Scioneaux performed the cotransfection experiments with luciferase reporters and the E2A and NeuroD1 expression plasmids; and Kim Pedersen supervised the project and performed the other experimental work.

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