Glucose induces glucose 6-phosphatase hydrolytic subunit gene transcription in an insulinoma cell line (INS-1)

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Abstract Primer extension analysis and RNase protection assays revealed the identity of glucose 6-phosphatase gene transcripts in both the insulinoma cell line INS-1 and hepatic cells. In transient transfection assays of INS-1 cells, using constructs between the human glucose 6-phosphatase gene promoter and a luciferase reporter gene, the reporter gene activity was induced by dexamethasone and dibutyryl cAMP. Furthermore, the promoter was regulated by the glucose concentration in the medium. This effect was dependent on glucose metabolism. The data indicated that glucose 6-phosphatase gene transcription is regulated in a similar way in the insulinoma cell line and in liver.

Key words: Glucose 6-phosphatase; Glucose; Carbohydrate response element; Pancreas

1. Introduction

Glucose 6-phosphatase catalyses the hydrolysis of glucose 6-phosphate to glucose, which is the final step of gluconeogenesis and glycogen breakdown. The highest activities of the enzyme can be detected in liver and kidney. The liver enzyme is embedded in the membrane of the endoplasmic reticulum and consists of a multi-protein complex formed by transport proteins and the glucose-6-phosphatase catalytic subunit (G6Pase) ([1] for review). Hepatic G6Pase gene expression is induced by glucocorticoids and glucagon during starvation. In addition, it has been shown that in hepatic cells G6Pase expression is increased by high concentrations of glucose per se, i.e. the product of the enzymatic reaction [2,3]. This leads in hyperglycaemia to an increase of G6Pase activity, which could be of pathophysiological importance [2,3].

G6Pase activity can also be detected in pancreatic islets [4,5]. The amount of activity present in these cells is controversial [6–8]. This discrepancy might be caused by the low stability of islet G6Pase in vitro [6]. The function of the G6Pase enzyme in pancreatic β-cells is not clear, as islets are not a gluconeogenic tissue [9]. It has been suggested that changes of pancreatic G6Pase activity might modulate insulin release in response to glucose (e.g. [10]). G6Pase catalyses the opposite reaction to the central enzyme of pancreatic glucose sensing, glucokinase, resulting in an ATP-consuming substrate cycle [11,12]. In vitro, the overexpression of the catalytic subunit of G6Pase decreased glucose mediated insulin release [13]. Previous work has shown that G6Pase activity in pancreatic β-cells is increased by glucocorticoids in vivo [8]. Elevated glucose cycling has been reported in islets of *ob*/*ob* mice and animal models of type II diabetes [7,11,12,14]. The study of the regulation of G6Pase in pancreatic islets has been hampered by the cellular heterogeneity of the tissue and the loss of G6Pase expression in freshly isolated islets during their cultivation.

In the present paper we show that glucose has an influence on G6Pase gene transcription in a pancreatic cell line per se, like in liver. We performed our experiments with the highly differentiated insulinoma cell line INS-1, which has been well established for the analysis of regulation of gene expression by glucose [15–18]. INS-1 cells express only marginal amounts of glucose 6-phosphatase activity [13]. Therefore, we studied the regulation of a luciferase reporter gene under the control of the G6Pase gene promoter.

2. Materials and methods

2.1. Materials

Restriction endonucleases, modifying enzymes, luciferase assay reagent, RNase Protection kit and plasmids were purchased from Promega. Plasmid purification systems were from Qiagen. All radioisotopes and the sequencing kit were obtained from Amersham. N^6,2′-*O*-Dibutyryl cyclic AMP (db_2cAMP) was purchased from Boehringer Mannheim. All other reagents were purchased in analytical grade either from Sigma or Merck.

2.2. RNA analysis

Total RNA from rat pancreas, INS-1 cells and liver were isolated by the guanidine thiocyanate method [19]. For RT-PCR 1 μg total RNA was transcribed using an oligo-dT primer. G6Pase cDNA was amplified by PCR using specific primers designed according to the rat G6Pase sequence [20]. The sequences of the oligonucleotides are available upon request. The PCR products were resolved by agarose gel electrophoresis and transferred onto a nylon membrane (Amersham) by passive capillary diffusion. Southern blotting was carried out as described in [21]. After prehybridisation the filter was hybridised with a full-length cDNA probe of G6Pase, which was labelled with [α-^{32}P]dCTP using the 'Prime-a-gene system' (Promega) according to the manufacturer's instructions. Primer extension analysis was performed using α-^{32}P-labelled oligonucleotide, which hybridises to bp 109–132 of the rat G6Pase mRNA [20]. 500 000 cpm of the primer were hybridised overnight to 10 μg (liver) or 40 μg (INS-1, pancreas) of total RNA in 100 μl 40 mM PIPES (pH 6.4), 1 mM EDTA, 400 mM NaCl, 80% (v/v) formamide at 38°C. Following precipitation the reverse transcription was carried out as described [21] in a volume of 20 μl. RNA was hydrolysed by adding 16 μl 500 mM NaOH and incubating for 2 min at 90°C. Subsequently samples were neutralised with 8 μl 1 M HCl. Products were precipitated and analysed on a

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Abbreviations: G6Pase, glucose 6-phosphatase catalytic subunit; db_2cAMP, N^6,2′-*O*-dibutyryl cyclic AMP; SAPK2, stress-activated protein kinase 2

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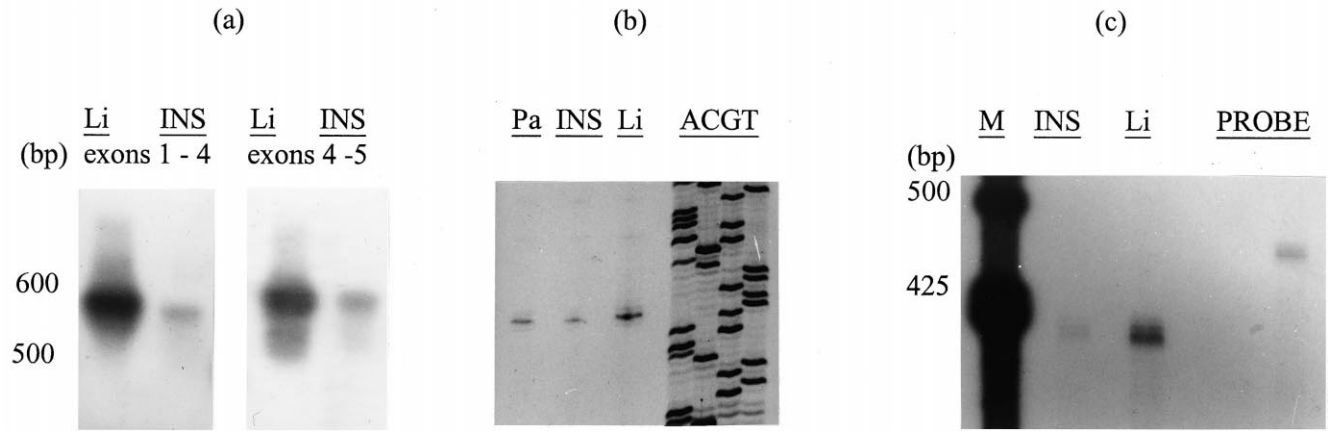

Fig. 1. Characterisation of G6Pase expression in INS-1 cells. Detection of G6Pase expression in INS-1 cells (INS) and liver (Li) RT-PCR (a) using exon-specific primers as indicated and subsequent Southern blotting. Mapping of the 5′-end of G6Pase mRNA in INS-1 cells by primer extension analysis (b). 10 μg of total liver RNA (Li); 40 μg of total INS-1 cell RNA (INS); 40 μg of total pancreas RNA (Pa). Standard DNA sequencing reactions were used as size markers. RNase protection assays (c) performed with a probe complementary to the rat liver sequence between nucleotides 1 to 400. DNA size marker (M); 30 μg of total INS-1 cell RNA (INS); 5 μg of total liver RNA (Li); undigested probe (PROBE).

sequencing gel, together with a sequencing reaction as a size marker. For the RNase protection assay the sequence of the rat G6Pase cDNA from nucleotide 1 to nucleotide 400 [20] were amplified by PCR. The product was cloned into the *Eco*RI/*Hin*dIII site of pGEM7 using nested restriction sites within the primer sequences. The template was linearised with *Eco*RI and in vitro transcription was performed in the presence of [α-^{32}P]CTP using SP6 polymerase. 500 000 cpm of the riboprobe were hybridised overnight at 50°C with 5 μg (liver) or 30 μg (INS-1) of total RNA in 20 μl 40 mM PIPES (pH 6.4), 1 mM EDTA, 400 mM NaCl, 80% (v/v) formamide. The probe contained 50 nucleotides vector sequence, which did not hybridise with G6Pase cDNA. RNase digestion was performed with 10 U RNase ONE (Promega) at RT for 60 min using the RNase protection kit (Promega). Samples were analysed on an 8% polyacrylamide gel. The undigested probe and the protected fragments were sized by comparison to ϕX174 DNA standards (Promega) end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase.

2.3. Reporter gene constructs

The construction of the plasmids pGL3-1.2 and pGL3 (−161/+4) has been described previously [22] and were created by cloning the −1227/+57 bp or the −161/+4 bp fragments of the human G6Pase gene promoter into the *Xho*I/*Sac*I site of the promoterless vector pGL3 basic, which contains the luciferase reporter gene.

2.4. Cell culture and transfections

INS-1 cells were cultured as in [23] in the presence of 11 mM glucose. 36 h before transfection 1×10^6 cells were seeded in 35 mm dishes. 4 h before transfection the cells were fed with fresh medium. Transfections were performed by the calcium phosphate/DNA co-precipitation method as described [22] using 10 μg reporter gene construct and 2 μg pSV-β-galactosidase. 18 h after transfection cells were glycerol-shocked (15%, 2 min) and incubated for 4 h in DMEM without serum (5.5 mM glucose). Subsequently, the medium was changed to DMEM without serum for 20 h in the presence or absence of db_2cAMP (500 μM), dexamethasone (1 μM) and the carbohydrates indicated in the figures. Cell extract luciferase activity was determined using a Luciferase Assay Reagent (Promega) and normalised by β-galactosidase activity [22]. Statistical analysis was performed using the InStat program.

3. Results

In RNA isolated from INS-1 cells a G6Pase transcript was detectable by RT-PCR. The RT-PCR products obtained from INS-1 cell RNA, possessed a similar size to those obtained from liver RNA and hybridised to a full-length G6Pase cDNA probe (Fig. 1a). Similar results were obtained with pancreatic RNA (data not shown). To characterise the 5′-end of the G6Pase transcript in INS-1 cells RNase protection and primer extension assays were performed. Primer extension analysis (Fig. 1b) revealed that the transcription initiation site of the G6Pase cDNA in INS-1 cells, pancreas and liver is identical and corresponded to the start site of the rat G6Pase mRNA reported by [20]. This was further confirmed by RNase protection assays (Fig. 1c) of RNA of INS-1 cells and liver using a probe which hybridised to exons 1 and 2 of the hepatic transcript. With this probe products of identical sizes were obtained from INS-1 and hepatic RNA. These results strongly indicate that the same promoter regulates G6Pase gene transcription in INS-1 cells and liver.

Following transfection with the G6Pase promoter/luciferase reporter gene construct pGL3-1.2 low basal luciferase activity could be detected in INS-1 cells (Fig. 2). Incubation with the protein kinase A activator db_2cAMP and the synthetic glucocorticoid dexamethasone increased luciferase activity in INS-1

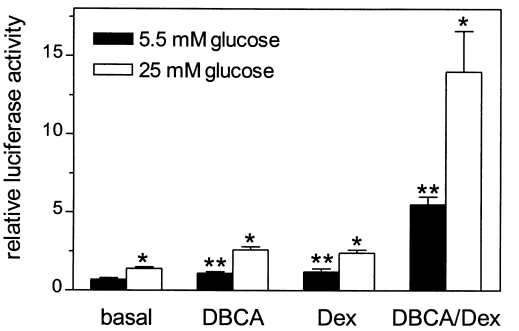

Fig. 2. Regulation of the G6Pase promoter by dexamethasone, db_2cAMP and glucose in INS-1 cells. INS-1 cells were transfected with the plasmid pGL3-1.2 and incubated in the presence or absence of db_2cAMP (DBCA) dexamethasone (Dex) with the indicated D-glucose concentration. The normalised luciferase activity of the promoterless pGL3 basic vector was set as 1. This background expression was not affected by the glucose concentration or the hormone mimetica. All values represent the mean ± S.E.M. ($n=3$). *$P<0.05$ versus respective incubation with 5.5 mM glucose; **$P<0.05$ versus incubation without mediator.

cells approximately two-fold. Dexamethasone and db_2cAMP acted synergistically. Elevating the D-glucose concentration from 5.5 mM to 25 mM activated the G6Pase promoter approximately two-fold in INS-1 cells. This effect was additive to those of dexamethasone or db_2cAMP. In HeLa cells, MCF7 breast cancer cells and the embryonic kidney cell line 293 no expression of the reporter gene was observed after transfection of pGL-1.2 and glucose, dexamethasone and db_2cAMP failed to induce reporter gene expression (data not shown). This demonstrated that expression and regulation of the reporter gene construct is tissue-specific.

Several sugars were used to determine whether they were able to mimic the activating effect of D-glucose on the G6Pase promoter in INS-1 cells (Fig. 3). The failure of L-glucose, which cannot be taken up into the cells, to increase the promoter activity, excluded osmotic effects. 3-*O*-Methylglucose, which is not phosphorylated, and 2-deoxyglucose, which is phosphorylated into 2-deoxyglucose 6-phosphate, but cannot be degraded by the Embden-Meyerhoff pathway [16], were not able to increase the gene promoter activity. In addition, neither fructose nor inositol had any effect. Mannose, however, was able to partly replace D-glucose. The glucokinase inhibitor mannoheptulose [24] prevented the augmentation of the G6Pase promoter activity by 25 mM glucose.

The inhibitor of the stress-activated protein kinase 2 (SAPK2), SB203580, caused a general stimulation of luciferase expression, but did not block the glucose effect (Fig. 4). To constrict the region in the promoter, which mediated the glucose effect, transient transfections were carried out with the plasmid pGL (−161/+4). Using this plasmid glucose led to a two-fold induction of reporter gene expression, from 2.5 ± 0.9 relative luciferase activity to 5.1 ± 0.3 relative luciferase activity (mean ± S.E.M., $n = 4$) in the presence of dexamethasone and db_2cAMP (expression of the promoterless vector pGL3 was set as 1).

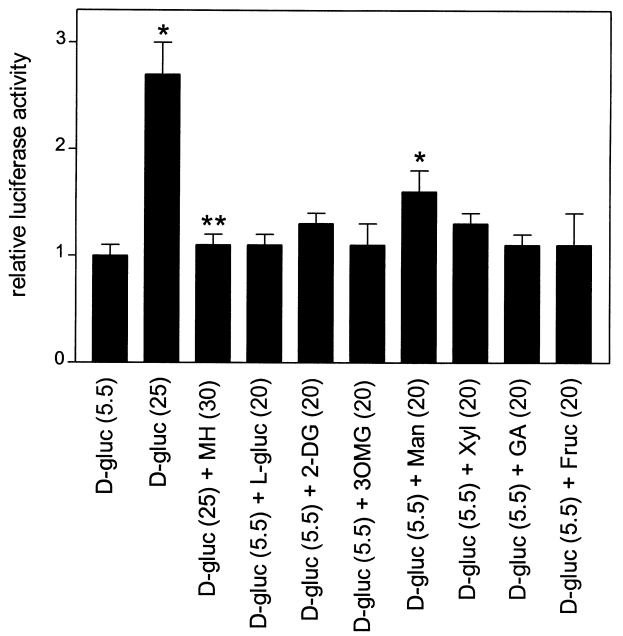

Fig. 3. Effect of various carbohydrates on G6Pase promoter activity in INS-1 cells. After transfection with the reporter gene vector pGL3-1.2 INS-1 cells were incubated in the presence of dexamethasone and db_2cAMP and the indicated carbohydrates. D-GLUC (5.5): 5.5 mM D-glucose; D-gluc (25): 25 mM D-glucose; MH (30): 30 mM mannoheptulose; L-gluc (20): 20 mM L-glucose; 2-DG (20): 20 mM 2-deoxyglucose; 3-OMG (20), 20 mM 3-*O*-methylglucose; Man (20): 20 mM mannose; Xyl (20): 20 mM xylitol; GA (20): 20 mM glucosamine; Fruc (20): 20 mM fructose. The results are expressed as fold stimulation compared to normalised luciferase activity measured in the presence of 5.5 mM D-glucose, which was set as 1.0. All values represent the mean ± S.E.M. ($n = 3$). *$P < 0.05$ versus 5.5 mM D-glucose, **$P < 0.05$ versus 25 mM D-glucose.

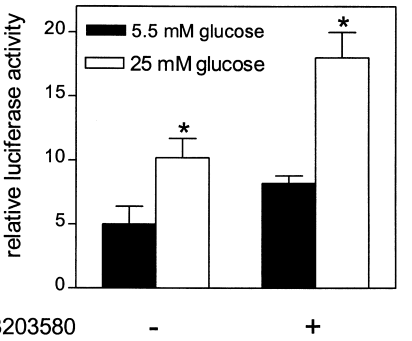

Fig. 4. The SAPK2 inhibitor SB203580 does not prevent the regulation of the G6Pase promoter by glucose. INS-1 cells transfected with the plasmid pGL3-1.2 were incubated with a combination of dexamethasone and db_2cAMP in the presence or absence of the SB203580 (10 μM) as indicated. The normalised luciferase activity of the promoterless pGL3 basic vector was set as 1. All values represent the mean ± S.E.M. ($n = 3$). *$P < 0.05$ versus respective incubation with 5.5 mM glucose.

4. Discussion

The data strongly suggest that INS-1 cells and liver cells express the same transcript of the G6Pase gene using the identical promoter. Our results do not, however, exclude the additional presence of another isoform of G6Pase in pancreas and INS-1 cells, which has recently been reported [25]. Incubation with the protein kinase A activator db_2cAMP and the synthetic glucocorticoid dexamethasone increased luciferase activity in INS-1 cells. These dexamethasone results are consistent with previous work showing that G6Pase activity in pancreatic β-cells is increased by dexamethasone in vivo [8]. The expression in INS-1 cells differed slightly from that observed after transfection of H4IIE hepatoma cells. In H4IIE cells a higher basal expression was observed and dexamethasone was a more potent activator than in INS-1 cells [22].

We found the transcription of the G6Pase gene in INS-1 cells to be regulated by glucose. This indicates that the recently described induction of hepatic gene expression by glucose [2,3] is also caused by stimulation of gene transcription. Several sugars were used to determine whether they were able to mimic the activation of D-glucose on the promoter. Our data show that the glucose effect depends on phosphorylation and glycolytic metabolism of glucose. Mannose, which can be phosphorylated by β-cell glucokinase [24] and metabolised by INS-1 cells [17], could mimic D-glucose to some extent. The molecular mechanism by which glucose metabolism stimulates G6Pase gene transcription is unclear. The *cis*-active element(s) mediating the glucose effect have to be localised between nucleotides −161 and +4. The human G6Pase promoter does not contain an obvious consensus sequence for the carbohydrate responsive element, which is involved in the regulation of the L-PK gene by glucose [26]. The regulation of the L-PK gene transcription is mediated by intermediates of the pentose

pathway [27], because inositol [27] and 2-deoxyglucose [16] are also active. Both carbohydrates could not replace glucose as an activator of the G6Pase promoter. This led to the conclusion that the glucose effects on L-PK and G6Pase expression are mediated by different mechanisms. The induction by glucose was additive to the effects of dexamethasone and db_2cAMP, suggesting that the glucocorticoid receptor or protein kinase A are not involved in the regulation by glucose. SAPK2 has been shown to be involved in the regulation of insulin gene transcription by glucose [28]. However, inhibition of SAPK2 activation failed to prevent the glucose effect on G6Pase expression.

It has been suggested that the induction of hepatic G6Pase activity by glucose serves as a mechanism by which the liver avoids excessive glucose storage during the postprandial phase [2,3]. There have been no reports suggesting a similar induction of G6Pase by glucose in β-cells in vivo so far. Long-term exposure of pancreatic β-cells to high glucose concentrations results in the induction of key glycolytic and lipogenic enzymes and promotes glycogen deposition [15–18]. These effects were studied using INS-1 cells as an in vitro model. It might be that a possible induction of G6Pase in islets could have a similar function to that in liver, i.e. protection against high glucose load. In vivo an induction of G6Pase by glucose may be able to modulate the glucose mediated insulin secretion due to an increased glucose cycling.

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