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The post-translational regulation of the glucose-6-phosphatase complex by cAMP is a crucial determinant of endogenous glucose production and is controlled by the glucose-6-phosphate transporter.

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Running title: Regulation of G6Pase activity by cAMP involved G6PT

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Abstract

The excessive endogenous glucose production (EGP) induced by glucagon participates in the development of type 2 diabetes. To further understand this hormonal control, we studied the short-term regulation by cAMP of the glucose-6-phosphatase (G6Pase) enzyme, which catalyses the last reaction of EGP.

In gluconeogenic cell models, a one-hour treatment by the adenylate cyclase activator forskolin increased G6Pase activity and glucose production independently of any change in enzyme protein amount or G6P content. Using specific inhibitors or protein overexpression, we showed that the stimulation of G6Pase activity involved the protein kinase A (PKA). Results of site-directed mutagenesis, mass spectrometry analyses and *in vitro* phosphorylation experiments suggested that the PKA stimulation of G6Pase activity did not depend on a direct phosphorylation of the enzyme. However, the temperature dependent induction of both G6Pase activity and glucose release suggested a membrane-based mechanism. G6Pase is composed of a G6P transporter (G6PT) and a catalytic unit (G6PC). Surprisingly, we demonstrated that the increase in G6PT activity was required for the stimulation of G6Pase activity by forskolin.

Our data demonstrate the existence of a post-translational mechanism that regulates G6Pase activity and reveal the key role of G6PT in the hormonal regulation of G6Pase activity and of EGP.

The deregulation of endogenous glucose production (EGP) is a key stage in the development of type 2 diabetes. EGP is increased under basal conditions in diabetic patients and correlates with the importance of fasting hyperglycaemia^{1 2 3}. This EGP increase in the liver of diabetic patients is predominantly due to an increase in the gluconeogenesis rate compared with non-diabetic subjects, as glycogenolysis is largely unchanged¹. Endogenous glucose production depends on the activity of glucose-6-phosphatase (G6Pase), which catalyses the last biochemical reaction of both gluconeogenesis and glycogenolysis: the

hydrolysis of glucose-6-phosphate (G6P) in glucose and inorganic phosphate (Pi) ⁴. The G6Pase complex is composed of a glucose-6-phosphate translocase (G6PT) that transports G6P from the cytoplasm to the endoplasmic reticulum lumen and a glucose-6-phosphatase catalytic subunit (G6PC) ⁴. Whereas G6PT is ubiquitous, G6PC expression is restricted to the liver, kidney and small intestine, and does account for the gluconeogenic capacity of these three organs ⁵⁻⁷. Liver G6PC expression is increased in type 2 diabetes patients ⁸ and the adenovirus-mediated hepatic overexpression of *G6pc* in rats promotes deregulations in glucose homeostasis representative of the hallmarks of the human diabetic pathology ⁹. This has strongly suggested that hepatic glucose production (HGP) and G6PC may play a causal role in the pathogenesis of the disease. Consistent with these results, a liver specific deletion of the *G6pc* gene protects against the development of insulin resistance and obesity induced by a high-fat high-sucrose diet ¹⁰.

The short-term regulation of EGP allows the liver to rapidly and finely tune blood glucose concentration ¹¹. The short-term regulation of G6Pase activity and EGP are generally considered to be controlled by G6P concentration only, since the Km of G6Pase is approximately ten times higher than the mean G6P concentration *in vivo* ⁴. Contrary to this dogma, we provided numerous pieces of evidence over the past years that hormonal and nutrient signals may control G6Pase activity through short-term biochemical regulatory mechanisms ¹²⁻¹⁶. In addition, we demonstrated that glucagon increases the G6Pase flux *in vivo* and in perfused rat hepatocytes ¹⁷. Among the various hormonal signals controlling glucose homeostasis, glucagon currently appears as a leading causal factor for the development of type 2 diabetes ^{18,19}. Understanding how glucagon induces G6Pase activity in the liver should thus constitute a crucial step in the understanding of the etiology of the disease.

Experimental procedures

Chemicals, plasmids, adenovirus constructs and site-directed mutagenesis.

Unless otherwise specified, chemicals and reagents were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). The human cDNA of *G6pc* (Genbank number NM_000151) and

Slc37A4 (coding for G6PT, Genbank number Y15409) were cloned into the pcDNA3 plasmid (Life Technologies, Saint-Aubin, France) at EcoRI and Sall restriction sites to produce the pcDNA3-G6PC and pcDNA3-G6PT plasmids²⁰. The same human cDNA were inserted under a CMV promoter in an AAV5 backbone (Laboratoire de Thérapie Génique, U649, Nantes) to produce the AdCMV-G6PC and the AdCMV-G6PT constructs. The plasmid constructs coding for the catalytic subunit of the PKA and the pCMV-LUC were already described²¹. The *in silico* analysis of the G6PC sequence with KinasePhos, (kinasephos.mbc.nctu.edu.tw,²²) predicts the presence of a putative PKA phosphorylation sites on T145. Mutagenic oligonucleotides were designed to either mimic phosphorylation by PKA (T>D) or to avoid the phosphorylation by PKA (T>A) (described in Supplemental Table 1). Mutants of the G6PC protein were generated as already described²³.

Cell culture, adenoviral and forskolin treatments,

The human colon carcinoma Caco-2, human hepatoma HepG2 and rat kidney NRK cell lines were obtained from American Type Culture Collection (ATCC) and maintained and differentiated (for CaCo2 cells) as previously described^{23,24}. After confluence or differentiation, cells were treated with AdCMV-G6PC (300 pi/cell) for 3 h at 37 °C in DMEM containing 1 g/L D-glucose (Life Technologies, Saint-Aubin, France). After the treatment with recombinant adenovirus, the medium was replaced with complete medium containing 1 g/L D-glucose and the cells were further incubated for 48 h prior to experiments. Cells were incubated for 1h at 37 °C or 21 °C with 10⁻⁴M forskolin (FK) / 0.1% DMSO or with 0.1% DMSO alone in DMEM without D-glucose. When indicated, a 30 min pre-treatment with 20 µM H89 (inhibitor of PKA), or with 50 µM S4048 (inhibitor of G6PT) was applied. After incubations, culture medium was collected for measurement of glucose production. Cells were then recovered by trypsin and kept at -80°C until they were processed for G6Pase activity, intracellular glycogen, lactate and G6P levels assays.

Transient cell transfection

Transient transfections were performed as described previously²³. The CMV promoter activity and the G6Pase activity were assayed as already described^{20,24}.

Enzyme assays and metabolite determinations

Cells were homogenized in 10 mM HEPES, pH 7.3 by ultrasonication coupled with freeze-thaw cycles to disrupt cell membranes. G6Pase activity determination, glycogen, G6P and lactate content were assayed as previously described^{6,25}.

Immunoprecipitation assay

Frozen liver samples from C57BL6J or from L.G6PC^{-/-}²⁵ mice were lysed in a non-denaturing lysis buffer (50mM Tris pH 7.4, 300mM NaCl, 5mM EDTA, 1% Triton) supplemented with a protease inhibitor cocktail. Three mg of total proteins from whole cell extracts were pre-cleared with 15µl of a solution of protein A-sepharose (6 mg/ml PBS) supplemented with 0.05 % (w/v) BSA for 30 min at 4°C on a rotating wheel. Protein complexes were immunoprecipitated for 16-18h at 4°C while rotating with 1µg of G6PC antibody⁷ or P-CREB (Cell Signaling, Leiden, The Netherlands) in the presence of 15 µl of a solution of protein A-sepharose and 0.2% (w/v) BSA. The samples were centrifuged at 14,000 g for 30 sec at 4°C, and the pelleted beads were washed three times for 5 min at 4°C with washing buffer (50mM Tris pH 7.4, 300mM NaCl, 5mM EDTA, 0.1% Triton) supplemented with a protease inhibitor cocktail and once with PBS. Immunoprecipitated proteins were eluted from beads by denaturation and analysed by western-blotting. The specificity of G6PC protein purification was assessed using mass spectrometry-based analysis.

Western blotting analyses

Forty micrograms of the protein extract were analysed using rabbit polyclonal anti-G6PC antiserum (1:5,000) as previously described²⁰.

Mass spectrometry-based analyses

Immunoprecipitated proteins and microsomal preparations were incubated 30 min in a solution containing 0.2% of deoxycholate. Proteins (entire immunoprecipitated preparation and 50-100 µg of microsomal proteins) were then solubilized in Laemmli buffer (10 mM Tris pH 8.1 mM EDTA, 5% β-mercaptoethanol, 5% SDS, 10% Glycerol, 0.01% Bromophenol blue), boiled for 5 min and electrophoresed for 1 h at 5mA/gel then overnight at 9 mA/gel.(total of 2 162 VH) into a 12% polyacrylamide gel. After fixation for 2h in 50% v/v ethanol, 3% v/v phosphoric acid, followed by three 1 min water washes, proteins were stained with colloidal Coomassie Blue (0.12% Brilliant blue G250, 10% Ammonium sulfate, 10% phosphoric acid, 20% Methanol). Stained protein bands were excised prior to destaining, in-gel reduction and alkylation of proteins, which were performed using a MassPREP Station (Waters, Manchester, UK). Briefly, destaining was done by three washes in a mixture containing 25 mM NH₄HCO₃:CH₃CN (1:1, v:v) followed by 30 min of dehydration in acetonitrile at 60°C for 5 min. Cysteine residues were reduced by 50 µL of 10 mM dithiothreitol, 25mM NH₄HCO₃ at 57°C for 30 min and alkylated by 50 µL of 55 mM iodoacetamide, 25mM NH₄HCO₃ for 30 min. After washing with 50 µL of 25 mM NH₄HCO₃, dehydration was done with acetonitrile during 15 min. Proteins were cleaved in-gel using 40 µL of 12.5 ng/µL of modified porcine trypsin (Promega, Madison, WI, USA) in 25 mM NH₄HCO₃ at 37°C for 4 hours. Tryptic peptides were first extracted using a 60% acetonitrile solution containing 0.5% formic acid, then secondly with a 100% acetonitrile solution before nanoLC-MS/MS (nanoflow liquid chromatography coupled to tandem mass spectrometry) analysis on two different analytical systems.

NanoLC-MS/MS analyses were performed using a nanoACQUITY UPLC system (Waters, Milford, MA, USA) system coupled to a maXis Q-TOF mass spectrometer equipped with a nano-electrospray source (System 1; Bruker Daltonics, Bremen, Germany). Both instruments were controlled by Hystar 3.2 (Bruker Daltonics). The solvent system consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in ACN (solvent B). Peptides were injected and first trapped during 3 min on a precolumn (Symmetry C18, 20 mm x 0.18 mm, 5 µm particle size, Waters) at a flow rate of 5 µL/min with 99% A, then eluted at 45°C on a separation column (ACQUITY UPLC® BEH130 C18, 200mm x 75 µm, 1.7 µm particle size, Waters) at a flow rate

of 400 nL/min using a 9 min linear gradient from 1 to 35% B. The mass spectrometer was operating in positive mode, with the following settings: source temperature was set to 200°C while dry gas flow was 4 L/min and nano-electrospray voltage was optimized to -4500 V. External mass calibration of the TOF was achieved before each set of analyses using Tuning Mix, which contained calibration peptides in the 322-2722 m/z range (Agilent Technologies, Palo Alto, USA). Online correction of TOF calibration during analyses was then performed using methylstearate ($[M+H]^+$ 299.2945 m/z) and hexakis(2,2,3,3,4-tetrafluoropropoxy)phosphazine ($[M+H]^+$ 922.0098 m/z) as lock-masses which were spiked into the dopant solvent and thus delivered with the nebulizer gas.

NanoLC-MS/MS analyses were also performed using a nanoACQUITY UPLC system coupled with a SYNAPT HDMS Q-TOF mass spectrometer equipped with a nano-electrospray source (System 2; Waters). Both instruments were controlled by MassLynx v4.1 (SCN 566, Waters). The solvent system was identical to that used in the first system (see above). Trapping of peptides was done as described for the first system (see above), as well as separation except that a 35 min linear gradient from 1 to 40% B was used here and followed by 5 min at 65% B. The mass spectrometer was operating in positive mode with the following settings: source temperature was set to 80°C, cone gas flow was 30l/h, cone voltage was 40V and the nanoelectrospray voltage was optimized to 3.5kV. Mass calibration of the TOF was achieved using phosphoric acid (H_3PO_4) on the 50-2000 m/z range. Online correction of this calibration was done using product ions derived from the [Glu1]-fibrinopeptide B (GFP) as lock-mass compounds. The ion $(M+2H)^{2+}$ at m/z 785.8426 was used to calibrate MS data and the fragment ion $(M+H)^+$ at m/z 684.3469 to calibrate MS/MS data.

In both systems, spectra were acquired by automatic switching between MS and MS/MS modes. This was done in the mass range of 50-2200 m/z (MS, 0.5 sec; MS/MS, 3 sec) for system 1 and 250-1500 m/z (MS, 0.5 sec) and 50-2000 m/z (MS/MS, 0.7sec) for system 2. The most abundant peptide ions (at least the 3 most intense with an absolute intensity threshold of 1500 for system 1, and only the 3 most intense with a threshold of 60 counts/sec for system 2), preferably with a charge of 2 to 4, were selected from each MS spectrum for

further isolation and CID (Collision Induced Dissociation) fragmentation using argon as collision gas. Ions were excluded after acquisition of one MS/MS spectrum and exclusion was released after 0.6 min. NanoLC-MS/MS raw data generated on system 1 and 2 were respectively converted into « .mgf » peaklists with DataAnalysis 4.0 (BrukerDaltonics, Bremen Germany) and « .pkl » peaklists with PLGS 2.3 (Waters).

Peaklists were searched using a local Mascot server (version 2. 2. 0, MatrixScience, London, UK) against a combined target-decoy protein database containing protein sequences of *Mus musculus* (taxonomy 10090) derived from SwissProt (created 2015-05-10, 33682 entries) and common contaminants such as human keratins and trypsin. The database was created using an in-house database generation toolbox ²⁶. Database searches were performed using the following settings: semi-trypsin was specified as enzyme and up to one missed cleavage by trypsin and five variable modifications (oxidation of methionine, carbamidomethylation of cysteine, acetylation of protein N-termini, and phosphorylation of serine/threonine and tyrosine) were considered. Mass tolerances on precursor and fragment ions were set to 20 ppm and 0.2 Da, respectively for system 1 and 50 ppm and 0.2 Da for system 2. Only peptides allowing protein identification with false discovery rate below 1% were retained.

In vitro phosphorylation

In vitro phosphorylation was performed either before or after immunoprecipitation of G6PC or P-CREB as a positive control. Lysates of mouse hepatocytes were treated with 2µl of the catalytic subunit of PKA (New England Biolabs, Evry, France) per µg of proteins in the presence of 200µM ATP and 300µCi/µmol d'AT³²P (Amersham, Les Ulis, France) as recommended by the supplier (New England Biolabs). Proteins were then separated by electrophoresis. After electrophoresis, gels were dried and analyzed on a PhosphorImager SI (Molecular Dynamics).

Statistical analysis

Comparisons were performed using Student's *t* test between two groups or type 1 or type 2

ANOVA analyses and *Bonferroni* post hoc test in multiple groups. Results are presented as means \pm SD. A *p* value of <0.05 was considered to be statistically significant.

RESULTS

Cyclic AMP stimulates G6Pase activity independently of G6PC protein change

To investigate the short-term regulation of G6Pase activity by glucagon/cAMP, we chose the human colon carcinoma cell line Caco-2 as a first experimental model. Indeed, these cells are known to spontaneously differentiate into enterocyte-like cells when cultured after post-confluence, a time at which they also exhibit glycogen accumulation²⁷. Moreover, whereas *G6pc* is expressed in these cells²⁴, basal endogenous enzymatic activity was almost negligible (Fig. 1A). In line with this result, G6PC protein was not detectable by western blotting, either in control or forskolin-treated cells (Fig. 1A). Interestingly, the overexpression of G6PC promoted by AdCMV-G6PC in G6PC-transduced cells increased G6PC protein levels and G6Pase activity by about 17-fold (Fig. 1A).

Forskolin treatment, which activates adenylate cyclase, promoted a 70% stimulation of G6Pase activity in cells overexpressing G6PC independently of G6PC protein levels (Fig. 1A). A cytomegalovirus (CMV) viral promoter controls the *G6pc* gene expression in the AdCMV-G6PC construct. To check for any putative effect of forskolin on CMV promoter activity, Caco-2 cells were transfected with a pCMV-LUC construct, and treated or not with forskolin as above. No effect on luciferase activity was noted after treatment by forskolin (Fig. 1B), ruling out the hypothesis that an enhancement in *G6pc* gene expression may account for the increase in G6Pase activity induced by forskolin. Differentiated CaCo2 cells transduced with the AdCMV-G6PC construct were thus a suitable model to study the regulation of G6Pase activity independently of its transcriptional regulation.

Glucose-6 phosphatase activation by cAMP modulates metabolic fluxes

We next evaluated whether the increase in G6Pase activity induced either by AdCMV-G6PC or forskolin affected glucose production and glycogenolysis in differentiated Caco-2

cells. Interestingly, forskolin treatment was required for both control and G6PC-transduced cells to produce glucose from glycogen (Fig. 1C, E). The stimulation of glucose efflux by forskolin was 5 times higher in G6PC-transduced cells compared with control cells (Fig. 1C) and was parallel to a proportional decrease in lactate production (Fig. 1D). Importantly, the cell content of G6P was comparable under the four conditions studied (Fig. 1F). This strongly suggests that the enhanced glucose production upon forskolin treatment was independent of an increase in G6P concentration.

We then measured the effect of forskolin treatment on G6Pase activity and glucose production in two other cell types. The latter are exclusively gluconeogenic since they do not store glycogen. As in differentiated CaCo2 cells, basal endogenous enzymatic activity was negligible in both HepG2 (from hepatic origin) and NRK (from renal origin) cells, while G6Pase activity was increased by about 300-fold in HepG2 and 20-fold in NRK cells transduced with AdCMV-G6PC (Fig. S1A and B). Interestingly, forskolin treatment increased G6Pase activity in both cell types (Fig. S1A and B). The short-term regulation of G6Pase activity by cAMP is thus a crucial determinant of both glycogenolysis and gluconeogenesis.

Protein kinase A is involved in the activation of G6Pase by cAMP but does not phosphorylate G6PC

We then assessed the role of PKA in the induction of G6Pase activity by cAMP. In differentiated Caco-2 cells, the induction of G6Pase activity by forskolin was totally inhibited by the specific PKA inhibitor H89 (Fig. 2A). In parallel, H89 decreased by 66.7 ± 0.1 % the production of glucose induced by forskolin in differentiated CaCo-2 cells. Accordingly, in CaCo2 and HepG2 cells overexpressing G6PC, the overexpression of the catalytic subunit of PKA increased G6Pase activity by about 40% and 20% respectively (Fig. 2B and C). We thus tested whether PKA directly phosphorylates G6PC. *In-silico* analysis of the G6PC protein sequence predicted T145 as a PKA target site (Fig 2D). The mutation of T145 either in aspartate (D), which mimicked a constitutive phosphorylation, or in alanine (A), which mimicked a non-phosphorylated protein ²⁸, had no effect on G6Pase activity in HepG2 cells

(Fig 2E). In addition, PKA stimulated the G6Pase activity conferred either by the wild-type G6PC (Fig 2C) or by the T145A-mutated G6PC (Fig 2F), suggesting that T145 was probably not a target of PKA.

We then studied whether G6PC might be phosphorylated on another amino acid residue. We first performed an immunoprecipitation of G6PC to purify the protein. Hepatic G6PC has proved difficult to purify, because of its extreme lability and transmembrane topology, especially as the purity of the enzyme increases²⁹. Using non-denaturing conditions, we successfully immunoprecipitated G6PC from liver extracts but with a very low yield (Figure 3A). This low yield of purification was consistent with the low expression of G6PC, which represents approximately only 0.1% of total liver microsomal proteins⁴.

Using mass spectrometry, we confirmed that our protocol allowed us to specifically purify the G6PC protein. Indeed, after immunoprecipitation, the complexity of the liver lysates was strongly reduced (Figure 3B). From the analysis of the immunoprecipitated lysate, we identified 7 of the 17 theoretical tryptic peptides from G6PC, thus covering 30% of the protein sequence (Figure 3C and Supplemental Table 2). Even by analysing the pooled protein bands from three lysates, we were not able to detect other peptides. Notably, the putatively phosphorylated peptide was not detected. This is probably due to the low abundance of G6PC peptides extracted from gel bands, to the fact that the phosphorylated fraction is probably even less abundant, and also to the particular structure of the expected phosphorylated peptide that bears several tryptic cleavage sites (K/K/K/P/TYGFR), thus implying a probable decreased MS signal due to trypsin miscleavages.

Using an antibody raised against phosphorylated S and T, we also failed to detect a phosphorylation of these amino acids on the G6PC protein after immunoprecipitation (Fig 3D). We then performed an *in-vitro* PKA phosphorylation assay in mouse hepatocytes and then purified G6PC or the canonical PKA target CREB (cAMP Response Element Binding Protein) by immunoprecipitation. Using this method, no phosphorylation was detected on the G6PC protein, whereas the three bands characteristic of the phosphorylated form of CREB³⁰ were

detected (Fig 3E). From these experiments, we concluded that G6PC was not directly phosphorylated by PKA.

The activation of G6Pase and glucose production by cAMP depends on membrane-based mechanisms.

The activation of G6Pase activity by glucagon in hepatocytes is temperature-dependent, suggesting the involvement of membrane and/or vesicles movements in this regulation¹⁷. Interestingly, the cAMP/PKA pathway controls the formation of vesicles, Golgi structure and trafficking between the endoplasmic reticulum and Golgi apparatus (see³¹ as a review). We thus characterized the effect of temperature on the G6Pase activity and glucose efflux induced by forskolin in Caco-2 cells. The effect of forskolin and AdCMV-G6PC on both the G6Pase activity and glucose release (previously observed at 37°C) was completely abolished at 21°C in Caco-2 cells (Fig. 4A and B), and also in both other gluconeogenic cell models (Fig. S1C and D). These findings suggest that the effect of cAMP on G6Pase activity and metabolic fluxes depended on membrane-based mechanisms.

The G6PT activity is involved in the effects of cAMP on G6Pase activity and glucose efflux

The specific enzymatic properties of G6Pase depend on the integrity of the ER membrane³². The G6Pase enzymatic complex is composed of two subunits localized in the ER membrane, G6PT, which transports the substrate inside the ER lumen and G6PC, which catalyses glucose production. Both subunits have been proposed to interact in an enzymatic complex embedded in the ER membrane to account for G6Pase activity^{20,33}. The steady state G6Pase kinetics depend on conformational changes of the enzymatic complex (G6PC and G6PT) within the membrane³³, which are induced right after the initial hydrolysis of the substrate^{34,35}. We thus hypothesized that the temperature-dependent induction of G6Pase activity by cAMP/PKA depended on G6P transport, *i.e.* on G6PT activity. We investigated the role of G6PT subunit using G6PC-transduced Caco-2 cells treated with the specific G6PT

inhibitor S4048. We used a dose of S4048, which had no effect on the basal G6Pase activity conferred by overexpression of AdCMV-G6PC (Figure 4C). Under these experimental conditions, the inhibition of G6PT activity by S4048 suppressed the activation effect of forskolin on G6Pase activity and decreased by half its effects on glucose production (Figure 4C and D). On the contrary, increasing G6PT activity by AdCMV-G6PT stimulated G6Pase activity to the same level as forskolin treatment (Fig 4E). Interestingly, forskolin treatment had no additional effect on the induction of G6Pase activity by AdCMV-G6PT (Fig 4F). Finally, the induction of G6Pase activity by both forskolin and AdCMV-G6PT was completely inhibited in the presence of S4048 (Fig 4F). This strongly suggests that forskolin increased G6Pase activity by stimulating G6PT activity.

Discussion

Type 2 diabetes affects 350 million people worldwide and is nowadays considered as an epidemic disease. Among the deregulations leading to hyperglycaemia, excessive hepatic glucose production is considered as a main triggering or worsening feature. G6Pase is the mandatory enzyme in HGP and an increase in G6Pase activity is involved in the increased HGP during type 2 diabetes^{8,9}. On the contrary, the inhibition of liver G6Pase activity reduces hyperglycaemia in *ob/ob* mice³⁶ and the absence of G6Pase activity in the liver protects against the development of obesity and type 2 diabetes¹⁰. Since the apparent Km of G6Pase is higher than the intracellular concentration of G6P, it has long been considered that this enzyme is mainly regulated by the delivery of G6P from the cell metabolism. Instead, we highlight here the key role of the short-term regulation of G6Pase activity in the hormonal regulation of glucose release from gluconeogenic cells. Our data also show that cAMP stimulated G6Pase activity and glucose production through the stimulation of G6PT activity *via* a membrane-based mechanism.

We previously analyzed the activity of the endogenous enzyme *in vivo* or in perfused hepatocytes^{12,17}. In these former experimental protocols, we could not demonstrate that hepatic G6Pase activity was stimulated by cAMP independently of the induction of the

endogenous gene expression. In the current work, using an exogenous G6PC cDNA construct (under the control of a viral promoter insensitive to forskolin), we unequivocally demonstrate the existence of a post-translational regulation of G6Pase activity by cAMP. Indeed, G6Pase activation was independent of any variation in either the cellular G6P content or the G6PC protein amount and had profound repercussions in the cell glucose release.

Our results infer that G6Pase activation by cAMP exerts a crucial regulatory action on glucose metabolism within the cells. The stimulation of G6Pase activity was concomitant with both an increase in glucose production and a decrease in lactate efflux, the two processes being consistent from a quantitative viewpoint. Gluconeogenesis is regulated by cAMP at other enzymatic levels such as the fructose-1,6 bisphosphatase/phosphofrutokinase 1 cycle. These canonical enzymatic regulations of gluconeogenesis by cAMP do probably not account for the observed changes in glucose metabolism here, as the increase in G6Pase activity and glucose production were totally suppressed at 21 °C. It is noteworthy that these temperature-dependent modulations of glucose metabolism in Caco-2 cells in the presence of G6PC are perfectly consistent with the cAMP-regulated glucose efflux taking place under more physiological conditions, such as in perfused rat hepatocytes ¹⁷.

The analysis of the molecular mechanisms leading to the stimulation of G6Pase activity by cAMP strongly suggested the role of PKA. However, the results of site-specific mutagenesis, *in vitro* phosphorylation and peptides analysis by mass spectrometry strongly suggested that direct phosphorylation of G6PC by PKA is not the regulatory mechanism of G6Pase activity. Instead, using the G6PT inhibitor S4048, we showed that the induction of G6Pase activity by cAMP depends on G6P transport. The transporter G6PT is not sufficiently rapid to allow equilibration of G6P across the microsomal membrane ³⁷. Thus, G6P transport is a limiting step in the regulation of G6Pase activity and glucose production. Consistent with this result, the adenoviral overexpression of G6PT mimicked the stimulation of G6Pase activity induced by cAMP.

The induction of G6Pase activity by cAMP did not take place at 21 °C, a temperature known to dramatically inhibit membrane fluidity. As a consequence, conformational changes

of transmembrane proteins are markedly hindered. Rapid conformational changes are crucially involved in the modulation of G6Pase activity within the ER membrane, e.g. during the first seconds of the hydrolytic reaction ^{34,38}. These conformational changes are followed by an activation of G6PT highlighted by an increase in substrate specificity ³⁴. Our recent results suggest that G6PT exhibits a pore-like structure in the ER membrane ²⁰. A conformational transition of G6PT, which might be hindered to take place at low temperature, may thus be involved in the cAMP-dependent activation of this protein. Consistent with our *in vitro* results, the intravenous infusion of S4048 in fed rats strongly inhibits the increase in glucose production induced by glucagon ³⁹. Interestingly, membrane-based mechanisms are also involved in the regulation of G6Pase activity by insulin ^{14,16}. Moreover, the inhibition of G6Pase activity by insulin is lost when G6PC has free access to G6P in detergent-treated microsomes, suggesting that the short-term regulation of G6Pase activity by insulin is also dependent on G6PT ¹⁴.

In conclusion, we report in this work the existence of a post-translational mechanism that regulates G6Pase activity under the control of cAMP. This mechanism is causal in the stimulation of glucose release from the cell independently of any increase of the G6P concentration. In addition, we show that it involves G6PT, the transporter subunit of the enzymatic complex. Thus, this work shed important new light into the biochemical mechanisms involved in the hormonal control of G6Pase and of EGP. Inhibiting G6PT activity redirects G6P toward glycogen storage and has proven to be efficient to decrease hyperglycaemia in *db/db* mice ⁴⁰. Therefore, understanding the mechanism linking the G6P transport and the hormonal short-term regulations of glucose production is a promising challenge in the understanding of the mechanisms underlying EGP and its deregulations in type 2 diabetes.

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Conflict of interest No potential conflicts of interest relevant to this article were reported.

Author contributions

M.S. conducted and designed experiments on glucose production and G6Pase activity, performed data analyses and wrote the manuscript. J.C. conducted and designed experiments on G6PC phosphorylation, performed data analyses and wrote the manuscript. Z.C. contributed to the data. F.B. and F.D. performed MS analyses and interpretation and wrote the manuscript. G.M. contributed to the discussion and interpretation of data, supervised the study and edited the manuscript. A.G-S. contributed to the discussion and interpretation of data, supervised the study and wrote and edited the manuscript. A.G-S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure Legends

Figure 1: Cyclic AMP induces G6Pase activity independently of G6PC protein levels and modulates metabolic fluxes.

G6PC and TUBULIN (TUB) protein levels (top A), G6Pase activity (bottom A), glucose production (C), lactate production (D), glycogenolysis (E) and G6P content (F) were measured in differentiated CaCo2 cells either untreated (C) or treated with forskolin (C+F), transduced with G6PC (Ad) or transduced with G6PC and treated with forskolin (Ad + F). a: $p < 0,01$ vs C; b, b': $p < 0,05$ or $p < 0,001$ vs Ad. c: $p < 0,001$ vs C+F. B: Reporter activity of the CMV promoter used to overexpress G6PC depending on the forskolin treatment.

Figure 2: G6Pase activation by cAMP depends on PKA.

A: G6Pase activity measured in differentiated CaCo2 cells transduced with G6PC, untreated (Ad), treated with forskolin (Ad+F) or treated with forskolin and H89 (Ad+F+H89). G6Pase activity measured in CaCo2 (B) and HepG2 (C, E, F) transfected with pcDNA3-G6PC (G6PC) or the mutated constructs (T145A/D) with the catalytic unit of PKA (+ PKA). In these experiments, the extent of overexpression ranged from 100 to 130 fold. *: $p < 0,05$ vs Ad or G6PC conditions. D: Scheme of the predicted 2D structure of the mouse G6PC protein (modified from ⁴¹), including the potential PKA phosphorylation site indicated by a grey square.

Figure 3: The direct phosphorylation of G6PC is not involved in the induction of G6Pase activity by cAMP/PKA.

A: Immunoprecipitation (IP) of G6PC proteins followed by an immunoblot (IB) against G6PC performed in the liver of WT and L.G6pc^{-/-}. B: 1D gel electrophoresis of proteins obtained after immunoprecipitation of G6PC from WT mouse liver microsomes compared to whole

microsome proteins. Dotted squares represent bands where Immunoglobulins (Igg) or G6PC were detected by MS. C: Scheme of the predicted 2D structure of the mouse G6PC protein. Grey circles highlight sequences identified by MS. D: Immunoprecipitation (IP) of G6PC proteins followed by an immunoblot (IB) either against phosphorylated Ser/Thr residues or against G6PC performed in the liver of WT and L.G6pc^{-/-}. E: 1D electrophoresis of proteins obtained after immunoprecipitation of G6PC or CREB from WT hepatocytes treated with the catalytic unit of PKA in the presence of ³²P. The picture represents ³²P incorporation.

Figure 4: Cyclic AMP induces G6Pase activity via membrane-based mechanisms involving G6PT.

G6Pase activity (A) and glucose production (B) measured in differentiated CaCo2 cells cultured for 1H at 21°C, untreated (C) or treated with forskolin (C+F), transduced with G6PC (Ad) or transduced with G6PC and treated with forskolin (Ad + F). G6Pase activity (C) and glucose production (D) measured in differentiated CaCo2 cells cultured in the absence (plain bars) or presence (hatched bars) of S4048 at 37°C. (E) G6Pase activity measured in differentiated CaCo2 cells transduced with AdCMV-G6PC (AdG6PC), AdCMV-G6PT (AdG6PT) or both (AdG6PC+AdG6PT) and treated with forskolin (+ F) b: p<0,05 vs AdG6PC. c: p<0,05 vs Ad+F, d: p<0,05 vs AdG6PC + G6PT. (F) G6Pase activity measured in differentiated CaCo2 cells transduced with both AdCMV-G6PC and AdCMV-G6PT (AdG6PC+AdG6PT) and treated with forskolin (+ F) or with forskolin and S4048 (+ F + S4048). d: p<0,05 vs AdG6PC + G6PT.