Université de Montréal

REGULATION OF THE EXPRESSION OF THE TWO COMPONENTS OF LIVER GLUCOSE-6-PHOSPHATASE

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SUMMARY

Glucose-6-phosphatase (G6Pase) plays an important role in glucose metabolism by catalyzing the terminal step of both glycogenolysis and gluconeogenesis. Although G6Pase is proposed to be a multifunctional and multicomponent system residing in the membrane of endoplasmic reticulum, until now neither the structure of its components nor the function of each protein has been totally understood. So far two components of the G6Pase system have been cloned, including the G6Pase catalytic subunit (p36) and the putative glucose-6-phosphate translocase (p46). Genetic deficiency of G6Pase leads to glycogen storage disease type-I (GSD-I), while mutations in p36 and p46 genes account for GSD-Ia and most of GSD-I non a respectively. Furthermore, diabetes mellitus is associated with increased G6Pase activity, which may contribute to the enhanced hepatic glucose production.

Previous studies have shown that p36 gene expression is under nutritional and hormonal regulation. In this work, the gene regulation of newly cloned p46 was investigated and compared with that of p36 gene. We found that under the conditions like increased glucose concentration, dietary phosphate deprivation or streptozotocin-induced diabetes, p36 and p46 genes were similarly up-regulated. However, the sensitivities of these two genes to different hormones or reagents were found to be quite different as shown in

HepG2 hepatoma cells. Insulin has dominant negative effects on both p36 and p46 gene expression, but compared to p36, p46 gene has a much lower sensitivity to insulin. Glucagon, cAMP and thapsigargin significantly increase p36 gene transcription but barely affect p46 gene, while glucocorticoids remarkably and sensitively induce both genes. Based on the distinct hormonal regulation of p36 and p46 gene expression, their possible roles in glucose metabolism were proposed.

We explored in two ways to study the yet unclear p46 function: (1) On the one hand, in order to study the p46 function in hepatic G6Pase system, we performed p46 overexpression in hepatocytes via recombinant adenovirus mediated gene transfer, which resulted in induced p36 transcription and increased G6Pase activity. In addition, overexpression of p46 led to significant metabolic impacts in primary hepatocytes, including decreased glycogen synthesis, increased glycogen degradation and decreased glycolysis; (2) On the other hand, we studied p46 gene transcription in leucocytes, where p36 is absent, and identified four different p46 transcripts, three of which are not present in liver. We hypothesize that mutated p46 gene might be responsible for neutropenia and neutrophil dysfunctions seen in GSD-Ib and Ic; p46 may bear other functions in leucocytes by differential mRNA splicing.

In conclusion, we characterized the gene regulation of newly cloned p46 gene, investigated effects of adenovirus mediated overexpression of p46 on glucose and glycogen metabolisms and discovered different transcripts of p46 gene in leucocytes.

Key works: glucose-6-phosphatase catalytic subunit; putative glucose-6-phosphate translocase; glucose; phosphate; hormones; gene regulation; overexpression

RÉSUMÉ

La glucose-6-phosphatase (G6Pase) catalyse l'hydrolyse du glucose-6-phosphate (G6P), qui est l'étape terminale aussi bien de la glycogénolyse que de la néoglucogénèse. La localisation dans la membrane du réticulum endoplasmique de la G6Pase est suggérée sur des bases biochimiques et génétiques et cette enzyme est constituée de plusieurs composantes. À ce jour, deux composantes du système G6Pase ont été clonées, une sous-unité catalytique de masse moléculaire 36 kDa (p36) et un transporteur putatif de G6P de 46 kDa (p46). Des études topologiques indiquent que p36 et p46 sont très hydrophobiques, avec 9 et 10 domaines transmembranaires, respectivement. Deux modèles différents ont été proposés pour décrire le système complexe de la G6Pase, nommés le modèle de transport du substrat et le modèle conformationnel. La distribution tissulaire de p36 est essentiellement dans les organes néoglucogéniques comme le foie, le cortex rénal et l'intestin grêle, tandis que l'expression de p46 est plus étendue, étant présente dans la majorité des tissus et dans plusieurs lignées cellulaires. La glycogènose de type I (GSD-I) est une maladie autosomale récessive causée par une déficience en G6Pase, caractérisée par une hypoglycémie sévère et une accumulation excessive de glycogène hépatique. Des mutations dans le gène de p36 sont trouvées essentiellement chez les patients GSD-Ia, tandis que des mutations dans le gène de p46 expliquent la majorité des cas de GSD-Ib, Ic et Id,

nouvellement qualifiés de "non a". Puisqu'une augmentation dans l'activité de la G6Pase est associée avec les deux types de diabète sucré et peut donc contribuer à l'augmentation de la production hépatique de glucose dans cette condition, p36 et p46 peuvent être considérés comme des gènes-candidats pour le diabète. La surexpression de p36 dans des hépatocytes et in vivo au moyen d'un adenovirus résulte en une augmentation de la néoglucogénèse et en une diminution du flux glycolytique et de la synthèse de glycogène, tandis que la surexpression de p36 dans les cellules d'insulinome INS-1 invalide la sécrétion d'insuline induite par le glucose.

Il est connu que l'activité de la G6Pase est augmentée dans le foie de rats à jeun ou diabétiques. Le clonage des gènes de la G6Pase (qui incluent maintenant les gènes de p36 et p46) et la disponibilité de sondes d'ADNc ont permis d'examiner si les changements d'activité de la G6Pase dans ces conditions était dûe à des altérations dans l'expression de ces gènes ou était la conséquence de modifications post-traductionnelles de l'enzyme. Il a été rapporté que dans les cellules FAO, le niveau d'ARNm de p36 était augmenté par l'AMP cylique et les glucocorticoïdes, tandis que l'insuline avait un effet dominant négatif de suppression de ce gène. Dans ces mêmes cellules, des concentrations élevées de glucose (25 mM) étaient associées avec une quantité accrue d'ARNm de p36 et cette observation fut ultérieurement confirmé dans des hépatocytes en culture primaire et *in* vivo. L'expression du gène de p36 est

donc règlé par des facteurs nutritionels et hormonaux. La régulation du gène de p46 nouvellement cloné, qui joue un rôle essentiel dans la G6Pase, n'a pas encore été exploré. Dans notre travail nous avons caractérisé l'expression de p46 en parallèle avec p36, dans le diabète expérimental, la déficience alimentaire en Pi, divers traitements hormonaux et différentes concentrations de glucose. Chez les rats rendus diabétiques par traitement à la streptozocine, nous avons trouvé une activité élevée de la G6Pase associée avec une augmentation de l'abondance de l'ARNm de p46 et une augmentation similaire de la protéine p46 dans le foie, le rein et l'intestin, outre la stimulation de l'expression du gène de p36 documenté auparavent.

Chez les rats nourris avec une diète déficiente en Pi, les niveaux relatifs d'ARNm de p36 et de p46 étaient augmentés ensemble dans le foie de concert avec une activité accrue de la G6Pase. Nous avons de plus étudié la régulation gènique de p36 et p46 dans les cellules HepG2, dont les concentrations de nutriments et d'hormones peuvent être aisément manipulés dans le milieu de culture. Nous avons trouvé que le glucose causait une augmentation dose-dépendante dans l'expression des gènes de p36 aussi bien que de p46 au niveau de l'ARNm et des protéines. Cependant, des études dose-réponse de différentes hormones et agents affectant l'expression des gènes de p36 et p46 ont révélé des sensibilités différentes de ces deux composantes du système G6Pase. Nous montrons dans les cellules HepG2 qu'alors que l'insuline, à des

concentrations physiologiques (0.01-10 nM), supprimait l'ARNm de p36, celle de p46 n'était affectée que de 20-30% et réduite au plus à 50% avec 1 μM d'insuline. De plus, l'AMP cyclique, le glucagon, ainsi que la thapsigargine (un inhibiteur de la Ca^{2+}-ATPase du RE) augmentaient l'ARNm de p36 aux concentrations 10-100 nM, sans affecter la transcription du gène de p46. Par contre, la dexamethasone (0.1-100 nM) augmentait similairement l'ARNm de p36 et de p46. Afin de caractériser ultérieurement l'impact métabolique d'une expression accrue de p46 et de comprendre la fonction de la protéine p46, nous avons surexprimé celle-ci au moyen d'un adenovirus recombinant dans des hépatocytes de rat en culture primaire. Les résultats montrent que la surexpression de p46 a pour conséquence d'induire l'ARNm de p36 et l'activité de la G6Pase. On observait également une diminution de la synthèse du glycogène et du flux glycolytique ainsi qu'une augmentation de la dégradation du glycogène. Puisque des mutations de p46 ont été trouvées chez des patients GSD-1 non a, qui ont par rapport aux patients GSD-1 a des symptômes additionnels comme une neutropénie et une dysfonction des neutrophiles et des monocytes, nous avons formulé l'hypothèse que p46 pourrait avoir d'autres fonctions que celle de contrôler p36, qui est absent des leucocytes. De plus, nous avons d'abord découvert dans une librairie d'ADNc de leucocytes humains et avons ensuite confirmé dans des échantillons sanguins la présence de quatre transcrits différents du gène de p46, dont trois ne sont pas présent dans le foie. Cette découverte supporte la possibilité que

d'autres produits du gène de p46, possédant des fonctions distinctes, puissent être formés par épissage alternatif.

En conclusion, nos résultats indiquent: (1) que dans le diabète insulinoprive, l'hyperglycémie, la déficience en insuline et l'augmentation de l'AMP cyclique due à des hormones contrerégulatrices non opposées peuvent contribuer de façon indépendante l'un de l'autre à une expression accrue des gènes de p36 et p46. La surexpression de p46 avec un adenovirus recombinant résulte en des changements métaboliques semblables à ceux d'une surexpression de p36, indicant que des dérégulations aussi bien de p36 que de p46 peuvent être impliquées dans l'activité accrue de la G6Pase, menant à une production hépatique de glucose plus forte qui peut exacerber l'hyperglycemie du diabète; (2) que la régulation hormonale distincte de p36 et p46 indique que celles qui affectent seulement p36 coïncide avec des modifications connues de la production hépatique de glucose, tandis que celles qui affectent p36 et p46 sont consistantes avec une stimulation de la synthèse de glycogène; (3) que p46 pourrait être une protéine multifonctionnelle avec des propriétés tissulaires spécifiques. Dans les tissus où p36 est présent, comme dans le foie, p46 pourrait founir le G6P nécessaire à son hydrolyse par p36. Dans d'autres tissus, qui ne possèdent pas p36, p46 a probablement d'autres fonctions qui sont déficientes dans les leucocytes des patients GSD-Ib.

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LIST OF ABBREVIATIONS

α-KG:	α-ketoglutarate
ACC:	acetyl coenzyme A carboxylase
ASO:	allele-specific oligonucleotide
BEAP:	N-bromoacetylethanolamine phosphate
CRE:	cAMP response element
CPT-cAMP:	8-(4-chlorophenylthio) adenosine 3':5'-cyclic monophosphate
CREB:	cAMP response element binding protein
ER:	endoplasmic reticulum
ESTs:	expressed sequence tags
FAS:	fatty acid synthase
FDG:	[^{18}F]-2-fluoro-2-deoxyglucose
F1P:	fructose-1-phosphate
Fru-2,6-P_2:	fructose-2,6-bisphosphate
HGP:	hepatic glucose production
HK:	hexokinase
HMP:	hexose-monophosphate
IDDM:	insulin-dependent diabetes mellitus
IGRP:	islet-specific glucose-6-phosphatase catalytic subunit-related protein
IRS:	insulin response sequence
GIRE:	glucose response element
GK:	glucokinase
G6P:	glucose-6-phosphate
G6Pase:	glucose-6-phosphatase
G6PT:	glucose-6-phosphate transporter
GLUT:	facilitative glucose transporter
GRE:	glucocorticoids response element
GSD-I:	glycogen storage disease type-I
L-PK:	L type pyruvate kinase
NIDDM:	non insulin-dependent diabetes mellitus
NPT2:	sodium/phosphate cotransporter type-2
OAA:	oxaloacetate
P36:	catalytic subunit of glucose-6-phosphatase
P46:	putative glucose-6-phophate translocase
PEPCK:	phosphoenolpyruvate carboxykinase
Pi:	inorganic phosphate
PPi:	pyrophosphate
PCR:	polymerase chain reaction
PET:	positron emission tomography
PKA:	protein kinase A
PRE:	phosphate response element

PTH:	parathyroid hormone
RT-PCR:	reverse transcription polymerase chain reaction
SSCP:	single stranded conformation polymorphism
X5P:	xylulose-5-phosphate

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1- LITERATURE REVIEW

1.1 Introduction

Glucose is a major source of energy for most mammalian cells; glucose homeostasis is thus crucial for whole body metabolism. The liver is the major site of glucose production, where glycogenolysis and gluconeogenesis take place. It plays an essential role to maintain the glucose concentration in the bloodstream, which is indispensable to life. The maintenance of euglycemia depends on a balance between the uptake and metabolism of glucose and the release of glucose via glycogenolysis and gluconeogenesis by the terminal step of glucose-6-phosphatase (G6Pase) (Nordlie et al., 1999). Deficiency of hepatic G6Pase activity is associated with a human genetic disease named glycogen storage disease type I (GSD-I) (Cori et al., 1952), while abnormally high liver G6Pase activity occurs in poorly controlled or untreated diabetes mellitus.

The G6Pase system has been extensively studied for several decades. Most of the earlier understandings of this system came from its pathological and kinetic studies. Two different models of the G6Pase system have been proposed based on experimental observations. Recently, using molecular biological techniques, the G6Pase system was characterized at the molecular

level. The genes of two components (a 36 kDa catalytic subunit and a 46 kDa putative G6P translocase) involved in the G6Pase system have been cloned, followed by gene regulation studies. Furthermore, gene manipulation and genetic engineering have also provided useful tools to reveal its physiological functions.

This literature review will cover recent developments in the G6Pase area, such as its rapid kinetics, topology, metabolic role and pathology and will here mainly focus on its molecular biology and regulation.

1.2 Characterization of the G6Pase system

1.2.1 Enzyme activities and kinetic properties

Glucose-6-phosphatase (EC3.1.3.9) catalyses the hydrolysis of glucose-6-phosphate (G6P) and inorganic pyrophosphate (PPi):

$$\text{Glucose-6-P} + H_2O \longrightarrow \text{Glucose} + \text{Pi}$$

$$\text{PPi} + H_2O \longrightarrow 2\,\text{Pi}$$

G6Pase also bears the ability to synthesize G6P via phosphotransferase activity, where carbamyl-P or PPi serves as phosphoryl donors (Foster et al, 1997) as shown in the following reactions:

$$\text{Carbamyl-P} + \text{Glucose} \longrightarrow \text{Glucose-6-P} + NH_3 + CO_2$$

PPi + Glucose ⟶ Glucose-6-P + Pi

Since G6Pase is located in the endoplasmic reticulum (ER) membrane, microsomes, *i.e.* heterogeneous vesicles enriched in ER membrane, were mostly used for kinetic studies of G6Pase. In intact liver microsomes, G6Pase hydrolyses G6P specifically with very low affinity for other substrates. The K_m for G6P is about 2-3 mmol/L at neutral pH. When microsomes are permeabilized by detergent, the specificity of G6Pase for G6P is lost, but the affinity is increased with a lowering of the K_m to 1 mmol/L and a doubling of the $V_{max,}$ hence the concept that part of the activity of G6Pase is latent in untreated microsomes (Sukalski et al., 1989). Different inhibitors of G6Pase have been reported, such as phlorizin (Soodsma et al., 1967) and vanadate (Singh et al., 1981). Recently, new compounds have been postulated to inhibit G6Pase activity by interacting with the putative G6P translocase, including 3-mercaptopicolinic acid (Foster et al., 1994), N-bromoacetylethanolamine phosphate (BAEP) (Foster et al., 1996) and chlorogenic acid and its derivatives (Hemmerle et al., 1997; Khan et al., 1998b; Arion et al., 1997), which provides a useful tool to study the properties of G6Pase system.

Part of the recent kinetic properties of G6Pase are summed up as follows: (a) Rapid kinetics of liver microsomal G6Pase provided evidence for tight-coupling between G6Pase transport and phosphohydrolase activity (Berteloot et al., 1991b); (b) A burst or hysteretic transition shows that G6P

hydrolysis in intact microsomes is more rapid at the beginning of the reaction than later on at steady state. During this very short presteady state (about 15 seconds), the enzyme seems to have no specificity for G6P, the specificity is gained only after hysteretic transition (Ajzannay et al., 1996); (c) Histone II-A can stimulate G6Pase and reveal mannose-6-phosphatase activities without permeablilization of liver microsomes (St-Denis et al., 1995b; Pederson et al., 1998a,b); (d) Hydrolysis of G6P results in 90% of glucose product formed ouside of the membrane, while only 10% of glucose is accumulated inside the lumen of microsomes (Berteloot, et al., 1995).

1.2.2 Two models

Currently, two models to describe the G6Pase system have been proposed.

The "substrate transport-catalytic unit" model was first developed by Arion and co-workers (Arion et al., 1975, 1976, 1980). In this model, the G6Pase system is predicted to be composed of a nonspecific catalytic subunit with its active site located in the ER lumen, together with at least four transmembrane spanning translocases (T1, T2α, T2β, T3) to transport the substrate (G6P via T1) and products (Pi via T2 and glucose via T3) respectively (Figure 1A). These associated translocases confer specificity to

this system by allowing selective substrates/products access to or egress from the sequestered catalytic subunit. G6P translocation through T1, but not G6P hydrolysis, is the rate-limiting step in the conversion of G6P to glucose.

The "combined conformational flexibility-substrate transport" model was first introduced by Schulze and co-workers (Schulze et al., 1980, 1986). In this model, G6Pase was depicted as a multifunctional enzyme in the ER membrane possessing both catalytic and substrate/product transport activities. By using a fast-sampling rapid-filtration apparatus (Berteloot et al., 1991a), our group demonstrated a presteady-state "burst" in the rate of G6P hydrolysis in untreated microsomes, which indicated a tight coupling between G6P transport and hydrolysis (Berteloot et al., 1991b). Accordingly, a revised version of this model was proposed (Berteloot et al., 1995) (Figure 1B). The catalytic site of the G6Pase is described to be located within a water-filled proteinaceous pore accessible to the substrate from the cytosolic side. G6Pase can exist in different conformational states that have distinct kinetic properties.

Until now, both hypotheses have received experimental support and neither of them can explain all the experimental data. As pointed out in a recent review (van de Werve et al., 2000), any model is nothing else but a working hypothesis, the future understanding of the nature of the G6Pase system will be probably based on the combination of the two models.

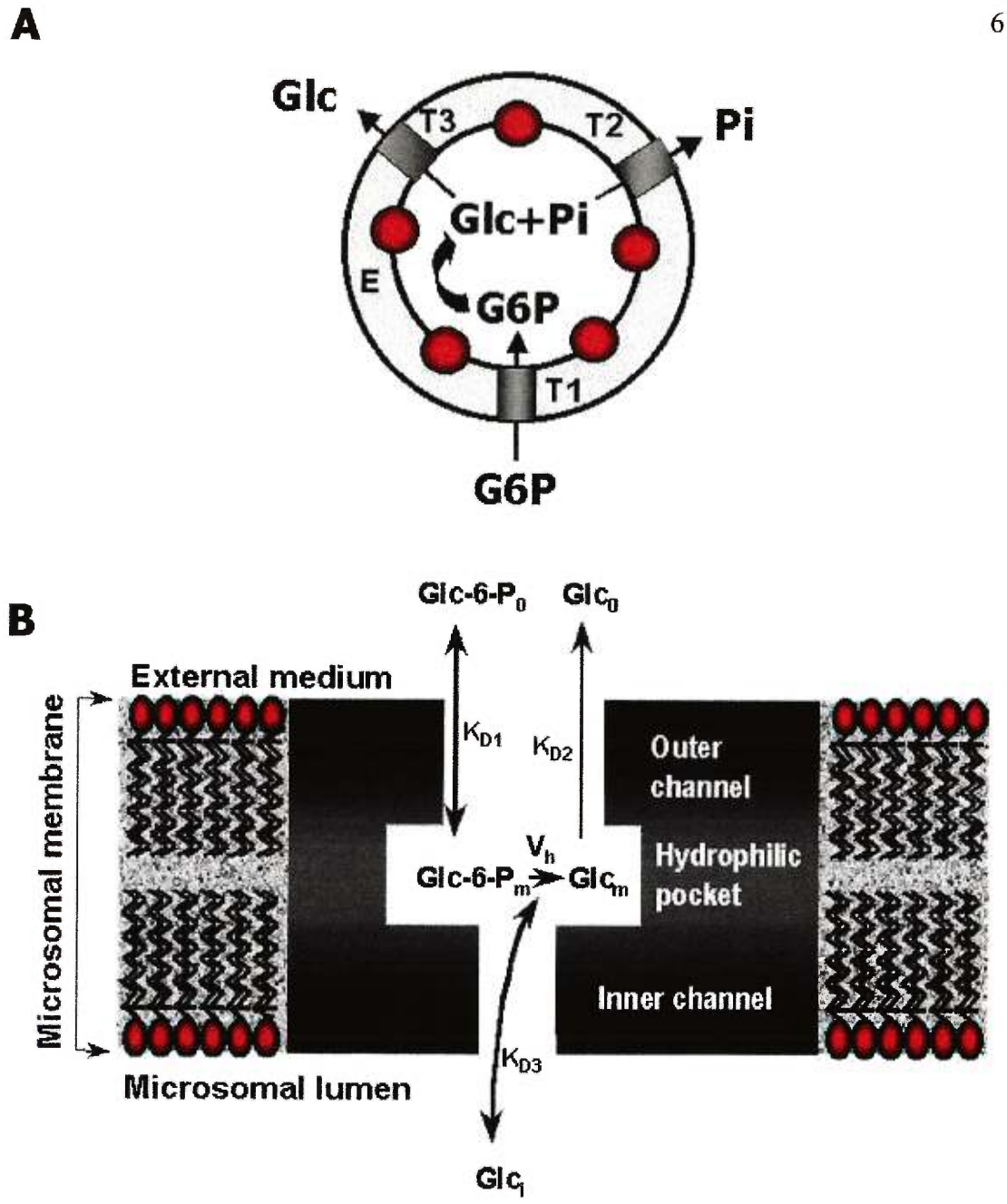


Figure 1: (A) Substrate transport-catalytic unit model. E represents catalytic subunit of the G6Pase; T1, T2 and T3 represent transporters of G6P, phosphate and glucose respectively. **(B) Modified version of the combined conformational flexibility-substrate transport model.** Microsomal glucose-6-phosphatase is depicted as a membrane protein with the catalytic site located in a hydrophilic pocket deep inside the protein where Glc-6-P hydrolysis occurs (V_h: rate of Glc-6-P hydrolysis). Exchange of substrate and products is realized by two channels with specific properties. An outer channel allows Glc-6-P uptake (K_{D1}) and Glc production (K_{D2}) outside the microsome. An inner channel transports Glc into the lumen of the microsome (K_{D3}). Glc and Glc-6-P represent their respective concentrations; the indexes o, m and i refer to the external medium, the pocket and the lumen of the microsome respectively.

For the convenience of writing, in this review we will use p36 and p46 to represent the two known components of the G6Pase system, *i.e.* catalytic subunit of G6Pase and putative G6P translocase respectively.

1.2.3 Localization and ontogeny

The catalytic subunit of the G6Pase (p36) is expressed mainly in two gluconeogenic organs, liver and kidney cortex (Mithieux, 1997). Its expression in small intestine was also recently reported (Rajas et al., 1999). Alternatively, the mRNA of a recently cloned pancreatic islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) was found to be highly expressed solely in pancreatic islets (Arden et al., 1999), of which the G6Pase activity was undetectable and its function is still unknown. Also in islets the reported G6Pase activity ranges from zero (Perales et al., 1991) to up to 10-fold higher than the hepatic level (Waddell et al., 1988); thus its actual level remains undefined. Whether p36 exists in a low amount in other tissues such as spleen and adrenal glands is still controversial (Shingu et al., 1996; Mithieux et al., 1996a). In contrast, the p46 transcript is more ubiquitously expressed than p36 mRNA (Lin et al., 1998b). As shown by Northern blot analysis, p46 transcript is clearly seen in liver, kidney, intestine, skeletal muscle, neutrophils/monocytes, brain and heart; it is also detectable in placenta, spleen, stomach, testis and uterus. With regard to the cellular localization, G6Pase is

associated with the ER membrane and the outer nuclear envelope (Kashnig et al., 1969; Nichols et al., 1984).

During development, the expression profiles of murine p36 and p46 differ in both liver and kidney (Pan et al., 1998c; Lin et al., 1998b). The transcription of p46 appears two days before that of p36 in murine liver during gestation. G6Pase activity increased markedly at birth, while G6P transport was not detectable then and peaked only 4 weeks after birth (Pan et al., 1998c). With the use of antibodies against p36 and p46 proteins, these observations were confirmed at the protein levels (Méchin et al., 2000), further indicating that G6Pase activity can occur before birth without detectable G6P transport, and the presence of p46 is not sufficient to elicit G6P uptake. It has been documented in our laboratory (Berteloot et al., 1991b) that the hydrolysis of G6P by G6Pase can produce glucose both inside and outside of liver microsomes when these vesicles are prepared from rats that are more than 1-week old. By comparing glucose production in liver microsomes from 1-day-old mice with those from older (1-2 weeks) or adult (10 weeks) animals, it was found that in 1-day-old microsomes, glucose was formed solely outside without glucose accumulation inside the vesicles (Annabi et al., 1997). This difference was further confirmed by light scattering experiments and kinetic studies (Puskas et al., 1999). Moreover, antibodies raised against the loop that contains the proposed catalytic site of the enzyme inhibit G6Pase activity in

fetal but not in adult liver microsomes. Based on these observations, it was hypothesized that the orientation of the catalytic site of p36 is different in fetal liver ER membrane from that in adult liver ER, and that a change (from outside to inside) in this orientation is likely to happen during fetal-to-neonatal transition (Puskas et al., 1999).

1.2.4 Topology

The cloning of cDNAs of p36 (Shelly et al., 1993) and p46 (Gerin et al., 1997) made it possible to deduce their amino acid compositions, which revealed that p36 and p46 are both highly hydrophobic proteins. Accordingly their topological models were proposed. The first topological model of p36 proposed by Chou's group suggested that it was a six transmembrane protein with both ends oriented towards the ER lumen (Lei et al., 1995b). However, based on the result of multiple sequence alignments that the active site of G6Pase shares a conserved phosphatase motif with that of the vanadate-containing choloperoxidase, a nine transmembrane helical model of the p36 was proposed (Hemrika et al., 1997a,b). Arg-83, His-119 and His-176 were predicted to reside on the same side of the ER membrane and to be involved in the active site of the enzyme. This new model was then confirmed by the expression of tagged-p36 (Pan et al., 1998a). By using a protease digestion assay, N- and C- termini of p36 were demonstrated to face the ER lumen and

cytoplasm respectively. Further experimental data suggested that His-176 could be the phosphoryl acceptor, while His-119 could provide protons to maintain the covalent bond of phosphoryl enzyme complex (E-P) during the acid-base exchange. The positively charged Arg-83, as well as Lys-76 and Arg-170 could be responsible for the positioning of the phosphoryl group in the E-P intermediate. Additionally, only one asparagine-linked glycosylation site (Asn-96) among three potential glycosylation sites of p36 is utilized, which is localized at a luminal hydrophilic loop of p36 according to the nine transmembrane model (Pan et al., 1998b).

Similarly, proteolytic digestion analysis indicated that p46 contained an even number of transmembrane helices with both N- and C- termini facing the cytoplasm. A protein glycosylation assay by creating glycosylation sites through mutations further confirmed a ten transmembrane helical model for p46 (Pan et al., 1999). Using a synthetic analogue of chlorogenic acid, a specific inhibitor of p46, as affinity label, Arion et al. (1998) suggested that two independent G6P binding sites were involved in the hydrolysis of G6P in intact microsomes. The high affinity site may account for the binding of G6P to p46, while the function of the low affinity site was unknown. Furthermore, by investigating the inhibitory effect of a specific sulfhydryl reagent, three thiol groups were demonstrated to be important for the G6Pase activity, of

which two are located in the p46 and the other one is located in the p36 (Clottes et al., 1998).

1.2.5 Glycogen storage disease type I

Glycogen storage disease type I (GSD-I), first known as von Gierke disease, is an autosomal recessive disease with an overall frequency of about 1/100,000 live births. This disease is characterized by hypoglycemia, hepatomegaly, kidney enlargement, growth retardation, lactic acidemia, hyperlipidemia and hyperuricemia, which are clearly related to defects in glucose metabolism (Nordlie et al., 1993a). According to the substrate transport model, GSD-I is classified into four subtypes: Ia, Ib, Ic and Id, corresponding to the deficiencies of the catalytic subunit, G6P transporter, Pi transporter and glucose transporter respectively. Clinically, GSD-Ia represents the majority of GSD (about 90%), while the rest 10% consists of GSD-Ib and very rare Ic cases (Nordlie et al., 1983). Only one GSD-Id case was reported (Chen et al., 1995). Up to now only the catalytic subunit p36 and the putative G6P translocase p46 have been cloned. Accordingly, mutations of p36 were discovered only in GSD-Ia patients, while mutations of p46 were discovered in GSD-Ib and Ic patients, therefore GSD-I might consist of only two major subtypes, classified as GSD-Ia and GSD-I non a (or GSD-Ib) (Veiga-da-Cunha et al., 1999, 2000). It has to be pointed out that besides the common symptoms

described above, GSD-I non a patients suffer additional infectious complications as a result of heritable neutropenia and functional deficiencies of neutrophils and monocytes, adding to the severity of the disease (Beaudet et al., 1980; Visser et al., 1998). Whether these additional symptoms are related to the broader tissue distribution of p46 than that of p36 and whether p46 is a multifunctional protein with tissue-specific properties are questions worth further investigation.

1.3 Molecular aspects of the G6Pase system

The cloning of the catalytic subunit (p36) and the putative G6P translocase (p46) genes of G6Pase are two major breakthroughs in the G6Pase research field, which have led to the investigation of the G6Pase system at the molecular level (Shelly et al., 1993; Gerin et al., 1997). In addition, a full length cDNA of the pancreatic islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) has been recently cloned (Arden et al., 1999). Up to date, numerous mutations of the p36 or p46 gene have been found in subtypes Ia or I non-a patients of GSD-I. In this section, gene structure and mutations of p36 and p46 genes will be discussed separately.

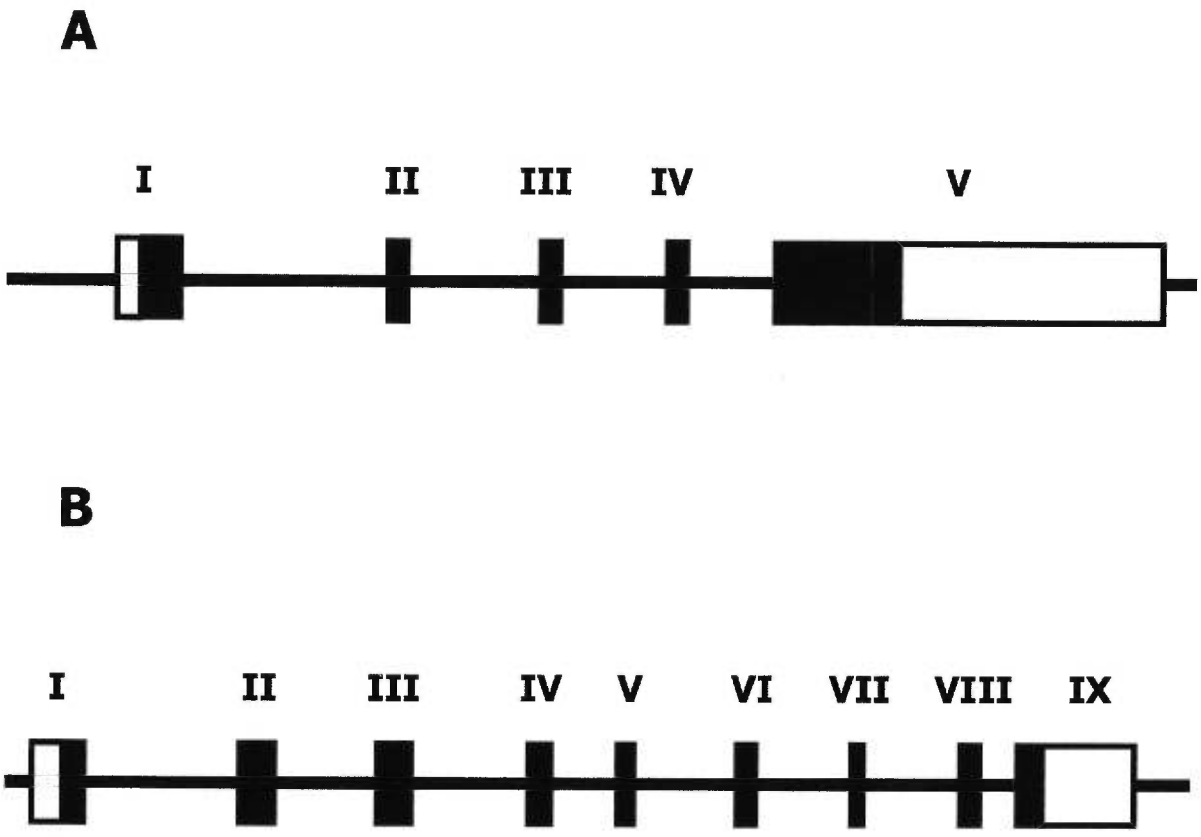


Figure 2: The structural organization of the human G6Pase catalytic subunit (p36) gene (A) and human putative G6P translocase (p46) gene (B). The exon coding regions are indicated by filled boxes and the untranslated regions by open boxes.

1.3.1 Gene structure of p36 and mutations in GSD-Ia

The p36 cDNA was first cloned from albino deletion mutant mice, which are known to express markedly reduced G6Pase activity, by using differential screening of the liver cDNA library (Shelly et al., 1993). The mice p36 gene was shown to span approximately 10 kilobases on genome and consists of 5 exons : I(311 bp), II(110 bp), III(106 bp), IV(116 bp) and V(1615 bp). Taking advantage of the murine G6Pase cDNA sequence, Lei et al (1993) isolated a human p36 cDNA clone by RT-PCR. At the genomic level, the human p36 gene spans approximately 12.5 kilobases and consists of 5 exons : I(309 bp), II(110 bp), III(106 bp), IV(116 bp) and V(2451 bp), encoding a 357-amino acid polypeptide with a molecular weight about 36 kDa (Figure 2A). The human G6Pase gene was further localized on chromosome 17q21 as a single-copy gene (Lei et al., 1994). In addition, the p36 cDNA was also cloned from rat (Lange et al., 1994) and dog (Kishnani et al., 1997). Both the cDNA sequences of the coding region and deduced amino acid sequences of G6Pase from human, rodent and canine have high homology, while the homology in the 5′ or 3′ untranslated regions is lower. Recently, p36 cDNA clones were also isolated from two haplochromine cichild fishes, of which the amino acid has only 54.4% similarity to the human p36 protein, but the amino acids involved in the catalytic site are conserved (Nagl et al., 1999). Up to now, three transcription variants of the G6Pase gene have been reported: the first

one was discovered by different identities in the 3′ untranslated region (Lange et al., 1994); the second one skips exon 2 and encodes a truncated protein with 137 amino acids (Haber et al., 1995); the third one has a 492 base insertion in the 3′ untranslated region (Shingu et al., 1996).

The discovery of mutations of the p36 gene in GSD-Ia patients (Lei et al., 1993) confirmed that this disease is caused by the deficiency of p36. Since then, many mutations of this gene have been found worldwide in various ethnic groups, which established the molecular basis of GSD-Ia. These mutations include point mutations, insertions or deletions, which can exist as either homozygote or heterozygote. More than 40 different mutations of the p36 gene have been reported (reviewed by Chou et al., 1999), and transient expression analysis of part of these mutations has shown that G6Pase activity was either abolished or greatly reduced (Lei et al., 1993–1995). It is likely that other mutations untested by transient expression also have impaired activity. As a genetic disease, ethnic specific mutations have been identified. Two mutations, R83C and Q347X are prevalent in Caucasian patients, including French (Chevalier-Porst et al., 1996), Italian (Stroppiano et al., 1999) and North-American patients (Lei et al., 1995a). In Hispanics, the R83C and 130X are frequent mutations (Lei et al., 1995a). R83H is prevalent in Chinese patients (Lei et al., 1995a; Hwu et al., 1995; Lee et al., 1996), while the G727T splicing mutation is prevalent in Japanese patients (Kajihara et al., 1995;

Okubo et al., 1997; Karasawa et al., 1998). With the use of mutation analysis, GSD-IaSP (termed as a defect of a putative stabilizing protein of G6Pase) was demonstrated to be a defect in p36 as well (Lei et al., 1995c). Furthermore, knowing the molecular basis of the p36 gene and its prevalent mutations in different ethnic populations provides noninvasive and reliable diagnosis of GSD-Ia. Several methods such as restriction enzyme digestion, DNA sequencing, single stranded conformation polymorphism (SSCP), allele-specific oligonucleotide (ASO) probe and linkage analysis have been successfully used for carrier testing and prenatal diagnosis of the GSD-Ia (Matern et al., 1996; Qu et al., 1996; Parvari et al., 1996; Trioche et al., 1998; Wong, 1998).

1.3.2 Gene structure of p46 and mutations in GSD-I non-a

Although GSD-Ib, Ic and Id were predicted to due to deficiencies in G6P, Pi or glucose transporters, their molecular basis was only implied until the cDNA of a putative glucose 6-phosphate translocase (p46) was cloned by comparison of bacterial hexose 6-phosphate transporter sequences with human liver expressed sequence tags (ESTs) (Gerin et al., 1997). The human p46 gene was identified to have nine exons: I (317 bp), II (233 bp), III (244 bp), IV (159 bp), V (86 bp), VI (114 bp), VII (66 bp), VIII (139 bp), IX (723 bp) (Figure 2B). p46 gene encodes a 429 amino-acid polypeptide with a molecular weight

about 46 kDa, which was deduced to be a highly hydrophobic transmembrane protein. A similar sequence of the gene was published by three independent groups (Ihara et al., 1998; Marcolongo et al., 1998b; Gerin et al., 1999). Three *Alu* sequences were found at about positions -3400, -2800, -1800 in the p46 gene with respect to the ATG; multiple transcription starts mainly around position -200 and -100 were observed in human liver, kidney and leucocytes (Gerin et al., 1999). In addition, Lin et al. (1998b) isolated and characterized candidate murine and rat GSD-Ib cDNAs, which encode transmembrane proteins sharing 93-95% sequence homology with the human GSD-Ib protein.

Since mutations were first found in GSD-Ib patients (Gerin et al., 1997), this putative glucose 6-phosphate translocase gene was predicted to be the GSD-Ib gene. But interestingly, both GSD-Ib and GSD-Ic genes were located on chromosome 11q23 by independent studies (Annabi et al., 1998; Fenske et al., 1998), suggesting these two subtypes may have the same molecular base. Further mutation screenings in GSD-Ic and Id patients by the group of Van Schaftingen provide evidence that this putative p46 gene is causative for all GSD-I non-a, including GSD-Ib, Ic and Id (Veiga-da-Cunha et al., 1998, 1999). So far more than 40 different mutations in the p46 gene have been found in GSD-Ib patients (Gerin et al., 1997, 1999; Ihara et al., 1998; Marcolongo et al., 1998; Veiga-da-Cunha et al., 1998, 1999; Kure, 1998). Mutations in the same p46 gene, even the same mutations as in GSD-Ib, have

also been found in six GSD-Ic patients and the unique case of GSD-Id. Therefore, this putative p46 gene is most likely a GSD-I non-a gene, and the classification of GSD-I needs to be redefined. However, a recent study (Lin et al., 1999) reported that in one GSD-Ic patient, the p46 gene was intact, suggesting a distinct Ic locus. As previously discussed for GSD-Ia patients, the discovery of mutations in GSD-I non-a gene also provides the possibility of DNA-based diagnosis.

Compared to the p36 gene, which is expressed only in liver, kidney and small intestine, the transcript of the p46 gene was found in almost every tissue investigated (Lin et al., 1998b). A brain specific isoform of p46 (p46-β) containing exon 7 was reported (Middleditch et al., 1998), while exon 7 is not present in the liver isoform. The tissue distribution and transcriptional difference of p46 indicate that p46 may be a multifunctional protein, which can play different roles in different tissues. In gluconeogenic tissues such as liver, p46 can be a G6P transporter as demonstrated by transient expression of p46 in COS cells (Hiraiwa et al., 1999). In other tissues, the different transcripts of p46 may lead to other functions such as glucose phosphorylation, Ca^{2+} uptake and glycogen synthesis (van de Werve et al., 2000).

1.3.3 Gene structure of IGRP

The cDNAs of two alternatively spliced variants of the pancreatic islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) was recently cloned from a mouse βTC3 insulinoma cDNA library (Arden et al., 1999). The gene encoding IGRP was then localized to the proximal portion of mouse chromosome 2, which spans approximately 8 kilobases and consists of 5 exons: I (254), II (110), III (112), IV (116), V (1284) (Ebert et al., 1999). The longer cDNA variant of IGRP encodes a 355-amino acid protein with 50% overall identity to the liver p36 and exhibits a similar predicted transmembrane topology. The mRNA of IGRP is highly restricted in pancreatic islets and associated cell lines, particularly those of β-cell origin. Furthermore, the IGRP promoter is 150-fold more active than liver p36 promoter in the islet-derived HIT cells. Unlike liver p36, neither phosphatase nor phosphotransferase activity of IGRP could be measured in transfection system; therefore its physiological function remains open.

1.4 Regulation of the G6Pase system

1.4.1 Acute regulation

Regulation of G6Pase activity can happen in the short-term in addition to the long-term alteration of gene expression. Various metabolites and

molecules were identified as such regulators of G6Pase, which will be described in this section.

1.4.1.1 Fructose-1-phosphate and α-ketoglutarate

In liver, fructose-1-phosphate (F1P), the product of fructose phosphorylation, can inhibit G6Pase hydrolytic activity (Robbins et al., 1991). Interestingly, F1P also relieves the inhibition of the regulatory protein on glucokinase, favouring glucose phosphorylation to G6P (Van Schaftingen, 1989). It was thus suggested that F1P could increase hepatic G6P concentration and direct it into glycogen synthesis and glycolysis rather than glucose production. α-Ketoglutarate (α-KG) is a compound derived from glutamine metabolism. It was found that when complexed with Mg^{2+}, α-KG or oxaloacetate (OAA) could inhibit G6Pase activity in a dose-dependent manner (Mithieux et al., 1990). The former may play an important role in the regulation of hepatic glucose production since α-KG could significantly inhibit G6Pase at physiological concentrations of G6P (about 100 nM). This inhibition was only observed when intact microsomes were used. Disruption of microsomes with detergent abolished the inhibitory effect of α-KG or OAA, suggesting that inhibition was exerted on the G6P translocase rather than on the catalytic subunit of G6Pase. The same group further observed that liver α-KG concentration increased in the 72 h- and 96 h- fasted rats by three times

with regard to the 48 h-fasted rats, which might account for the 25-30% decrease of G6Pase activity observed during prolonged fasting. The decreased G6Pase activity may explain a 45% increase of G6P concentration, which may lead to a glycogen rebound seen in the liver of 72 h-fasted rats (Minassian et al., 1994).

1.4.1.2 Amino acids

Proline was first reported to stimulate glycogen synthesis more than could be accounted for by the increase in cell volume by this amino acid, which may be the possible mechanism for all the other glycogenic and gluconeogenic amino acids (Baquet et al., 1990). Although both L-proline and L-glutamine pass through α-KG at the initial stage of gluconeogenesis, L-proline can support hepatic glycogen formation with a concomitant increase in hepatic G6P while L-glutamine cannot (Bode et al., 1992). It was therefore suggested that an unknown L-proline derived metabolite might inhibit G6Pase (Bode et al., 1992). This metabolite may divert G6P produced from proline from glucose production to glycogen synthesis. It has been recently reported that proline perfusion may inhibit hepatic glucose production via α-KG-induced inhibition of G6Pase (Massillon et al., 1996b). Recently, chloride ion was reported to competitively inhibit both the synthetic and hydrolytic activities of G6Pase, with a more extensive inhibition effect on the former

(Pederson et al., 1998c). It was proposed that the biosynthetic activity of G6Pase might act in concert with glycogen synthase during amino acid-induced glycogenesis from glucose. As suggested by a previous study, the mechanism may involve amino acid-induced swelling of hepatocytes that results in a loss of chloride ions, thus leading to the deinhibition of glycogen synthase phosphatase (Meijer et al., 1992).

1.4.1.3 Fatty acids and phosphoinositides

Generally, both unsaturated fatty acids such as arachidonic acid and fatty acyl-CoA ester can inhibit G6Pase activity (Nordlie et al., 1967; Fulceri et al., 1995; Mithieux et al., 1993, 1996b). The previously reported inhibition of G6Pase by glycogen (Grant et al., 1989) and later proposed inhibition by a glycogen-associated compound (Liu et al., 1993) was recently demonstrated by fractionation to be due to glycogen-associated unsaturated fatty acids (Daniele et al., 1997). The inhibitory effects of fatty-acyl-CoA esters differ with the chain length, concentration and conditions of whether the microsomes are intact or disrupted. Normally, in a certain range of chain length (10-14 carbons) and concentration (1-20 μM), the higher the chain length and concentration of fatty-acyl-CoA, the stronger the inhibitory effect. The inhibitory effect of longer chain length fatty-acyl-CoA (≥16 carbons) is within the concentration of 1-2 μM. However, if the concentration is too high (about

10 μM), this inhibitory effect may even change into an activation effect due to a detergent-like release of activity. G6Pase is also inhibited in the presence of phosphoinositides in a dose-dependent manner (within a range of concertration 0.5-10 μM) with an order of efficiency as phosphatidylinositol (PI) $3,4,5P_3$ > $PI3,4P_2$ = $PI4,5P_2$ > PI3P = PI4P > PI, which may have special importance in the signaling pathway of insulin's inhibitory effect on hepatic glucose production (Mithieux et al., 1998). Since G6Pase is located in the ER membrane where lipid and phosphoinositides are present, their inhibitory effect might be of physiological importance for G6Pase regulation.

1.4.1.4 Hormones

Hormones may also exert acute regulations on G6Pase independent of alterations in gene expression. Data from both *in vitro* and *in vivo* studies have shown that changes in the intracellular G6P concentrations after hormone treatment (insulin and glucagon) or in the postprandial period (while insulin/glucagon is altered) did not correlate with the G6Pase flux, suggesting that the hormones could influence the enzyme activity directly (Christ et al., 1986; Newgard et al., 1984). Furthermore, within a short time (90 min) after refeeding fasted rats, G6Pase activity was reduced significantly without concomitant change in G6Pase protein abundance, which also indicated the existence of posttranslational regulation (Minassian et al., 1995). This

inhibition effect was observed only in the liver homogenate but not in the further isolated microsome fraction, indicating the dependence of a highly labile inhibitor or a metabolite, which was lost during microsome isolation. However, other studies have shown that inhibition of G6Pase activity by insulin only accounts for a small part of suppressed hepatic glucose production (Gardner et al., 1993; Barzilai et al., 1993), therefore other mechanism remains to be identified.

1.4.2 Gene regulation

With the availability of cDNA probes for the catalytic subunit (p36) and putative G6P translocase (p46) of G6Pase, it is now possible to determine whether the activity changes seen in different nutritional and endocrine states are the consequence of changes in the expression of these genes.

1.4.2.1 Glucose

Regulation of gene expression by nutrients in mammals is an important mechanism allowing them to adapt to the nutritional environment. It was repeatedly observed that glucose can up-regulate the expression of genes involved in glycolysis and lipogenesis in liver, adipose tissue and pancreas, such as L-pyruvate kinase (L-PK), aldolase B, fatty acid synthase (FAS),

acetyl coenzyme A carboxylase (ACC) and S14 (a gene that codes for a protein of unknown function but directly related to lipogenesis). The upregulation of genes encoding enzymes involved in the storage of energy is thus a reasonable response to the dietary change. When it was found that the gene expression of G6Pase, a gluconeogenic enzyme, was also upregulated by glucose, it seemed unexpected and paradoxical because glucose is a product and an inhibitor of the hydrolytic activity of G6Pase. One might expect that cells incubated in the absence or low concentration of glucose would be in a gluconeogenic mode and therefore have elevated G6Pase activity. However, this is not the case.

Glucose effect on G6Pase genes It was first observed by *in vitro* studies in Fao hepatoma cells that the mRNA abundance of p36 from cells incubated in 25 mM glucose was much higher than that from cells incubated without glucose (Lange et al., 1994). In primary hepatocytes, a maximal effect of glucose on p36 mRNA abundance was found at 11 mM (Argaud et al., 1997). This glucose effect was evidenced in streptozotocin-induced diabetic rats, where both p36 mRNA and G6Pase activity were enhanced in correlation with increased blood glucose concentration (Lin et al., 1994). It was further confirmed by *in vivo* studies with rats in which diabetes and hyperglycemia were induced by 90% pancreatectomy (Massillon et al., 1996). Compared to normal controls, pancreatectomized rats had a more than 5-fold increase in p36 mRNA and a 3-4-fold increase in p36 protein and equivalent increase in

glycemia. Restoration of normoglycemia in diabetic rats with insulin or the glycosuric agent phlorizin normalized p36 mRNA and protein within 8 h. If hyperglycemia was maintained in diabetic rats by glucose infusion, phlorizin failed to decrease p36 mRNA and protein. Consistently, hyperglycemic clamp in normal rats also increased p36 at both mRNA and protein levels (Massillon et al., 1998). These results provided evidence for a stimulatory effect of hyperglycemia *per se* on hepatic G6Pase gene expression *in vivo*, which indicates that both hormonal (hyperinsulinemia) and nutritional (hyperglycemia) factors may independently contribute to the physiologic regulation of G6Pase gene expression in diabetic liver. Septic (Ardawi et al., 1992), hemorrhagic (Maitra et al., 1994) and endotoxic (Maitra et al., 1999) shocks are characterized by an early phase of hyperglycemia and a late phase of hypoglycemia which are associated with a rapid increase of gluconeogenesis followed by a decrease of gluconeogenesis. Both mRNA and activity of G6Pase are first up-regulated and then down-regulated, corresponding well with changes in glycemia, providing additional evidence of glucose regulation of G6Pase gene expression. It has to be mentioned that, in contrast, hepatocytes from fetal rat liver showed a different response to glucose. The mRNA level of p36 was 67% lower in cells incubated in 5 mM glucose than without glucose (Chatelain et al., 1998), suggesting that glucose could have an inhibitory effect on p36 gene expression in fetal hepatocytes.

Glucose effect on other gluconeogenic genes The effect of glucose on the gene expression of other gluconeogenic enzymes was also investigated in primary hepatocytes. Unlike the significant stimulatory effect on G6Pase gene expression, glucose has little or negative effects on other gluconeogenic enzymes. In fact, the level of fructose-1,6-bisphosphatase mRNA only increased slightly after 48 h-incubation with high glucose, while mRNA level of phosphoenolpyruvate carboxykinase (PEPCK) was unaffected at 4 h but became undetectable after 24 h in primary hepatocyte culture (Argaud et al., 1997). PEPCK gene expression was also shown to be decreased by glucose in Fao cells (Kahn et al., 1989) and in normal rats with hyperglycaemic clamp (Massillon et al., 1998). The differential effect of glucose on gluconeogenic enzymes is of interest, which indicates these enzymes may play quite different roles in glucose homeostasis. The similar effect of glucose on terminal glycolytic enzyme L-PK (Decaux et al., 1989) and terminal gluconeogenic enzyme G6Pase is also interesting since these two enzymes catalyze opposite pathways in glucose metabolism. This counter-regulation may play a role in controlling glycogen storage in the liver by avoiding excessive storage during a postprandial period.

Effect of other metabolites on G6Pase genes Besides glucose, fructose-2,6-bisphosphate (Fru-2,6-P_2) also shows a stimulatory effect on p36 expression. Overexpression of double-mutant 6-phosphofructo-2-

kinase/fructose-2,6-bisphosphatase (6-PF-2-K/Fru-2,6-P_2ase), which possesses only kinase activity and cannot be down-regulated by cAMP phosphorylation, resulted in a 15-fold increase in Fru-2,6-P_2 with a concomitant increase in glycolytic flux, and led to a 6-fold increase in G6Pase mRNA level in Fao cells. Overexpression of wildtype 6-PF-2-K/Fru-2,6-P_2ase that decreased Fru-2,6-P_2 levels also decreased p36 mRNA (Argaud et al., 1997). From these results, we can conclude that high levels of Fru-2,6-P_2, which stimulates glycolytic flux, increase p36 gene expression, whereas low levels of Fru-2,6-P2, which stimulates gluconeogenic flux, reduce p36 gene expression. Similar to the glucose effect, the stimulatory effect of Fru-2,6-P_2 is also considered paradoxical because Fru-2,6-P_2 is a potent inhibitor of fructose-1,6-bisphosphatase which is a gluconeogenic enzyme as G6Pase. Additionally, high levels of lactate (10 mM) in the presence of glucose were shown to increase the mRNA level of p36 by 10-fold, accompanied by a modest, 1.5-fold increase in protein level (Guo & Lange, cited in van de Werve et al., 2000). Xylitol, which is directly converted into xylulose-5-phosphate (X5P), replicated the effect of hyperglycemia on G6Pase and PEPCK gene expression in rats (Massillon et al., 1998).

Glucose signaling pathway Since previous studies have shown that the glucose stimulatory effect on L-PK gene expression was dependent on glucokinase, this point was also verified in the case of G6Pase. It seems

glucose phosphorylation by glucokinase is also needed for its effect on G6Pase expression. The glucose effect observed in Fao hepatoma cells is much lesser than that in primary hepatocytes (3-fold vs. 20-fold), which is correlated with a much lower expression of glucokinase (GK) in Fao cells (1-fold vs. 5-fold). When GK was overexpressed in Fao cells with a recombinant adenovirus vector, the glucose stimulatory effect on the G6Pase transcription was increased to 21-fold, together with an increase in lactate production, which became then compatible with the effect in primary hepatocytes (Argaud et al., 1997). If GK was inhibited by a potent competitive inhibitor glucosamine, the stimulatory effect of glucose on G6Pase was abolished (Massillon et al., 1998). It thus indicates that glucose needs to be metabolized through phosphorylation at or beyond G6P to regulate gene expression. Based on previously studied glycolytic and lipogenic genes and similar studies of G6Pase, another evidence that glucose metabolism is needed for the glucose stimulation is that 2-deoxyglucose, which can be metabolized to 2-deoxyglucose-6-phosphate, has the same effect as glucose, while 3-O-methylglucose, which can not be phosphorylated, has no effect (Girard et al., 1997). Although G6P is a necessary step for glucose signaling, it is still unclear whether G6P itself or its metabolites act as the signal. Studies on FAS and ACC gene expressions favored the idea that G6P is the signal since its concentration correlates well with the intensity of gene induction by glucose (Girard et al., 1997), while studies on L-PK gene expression suggested that X5P, an intermediate of G6P

through non-oxidative branch of the pentose phosphate pathway is the signal, because in this case, L-PK gene expression was parallel with X5P concentration without change of G6P (Doiron et al., 1996). Further studies on G6Pase and PEPCK genes backed up the idea that X5P is the glucose signal (Massillon et al., 1998), indicating that flux via the pentose phosphate pathway is the common glucose signaling step in liver. Recently, a new alternative pathway of glucose signaling was proposed (Guillemain et al., 2000). Since transfection of the cDNA coding for the large intracytoplasmic loop of GLUT2 into hepatoma cells, which may block the protein interaction with endogenous GLUT2, inhibited glucose-induced accumulation of GLUT2 and L-PK mRNAs without affecting glycogen synthase, it was suggested that the plasma membrane glucose transporter GLUT2, via protein interaction between its loop and a yet unknown protein, may transduce a glucose signal from the plasma membrane to the nucleus in hepatic cells (Guillemain et al., 2000).

In summary, the glucose-signaling pathway involved in regulating glucose-sensitive gene expression in liver is shown in Figure 3. Firstly, glucose needs to be taken up into cells by GLUT2 and phosphorylated by GK into G6P; Then either G6P itself or X5P will activate protein kinase/phosphatase cascades, leading to a modification of the phosphorylation of glucose response

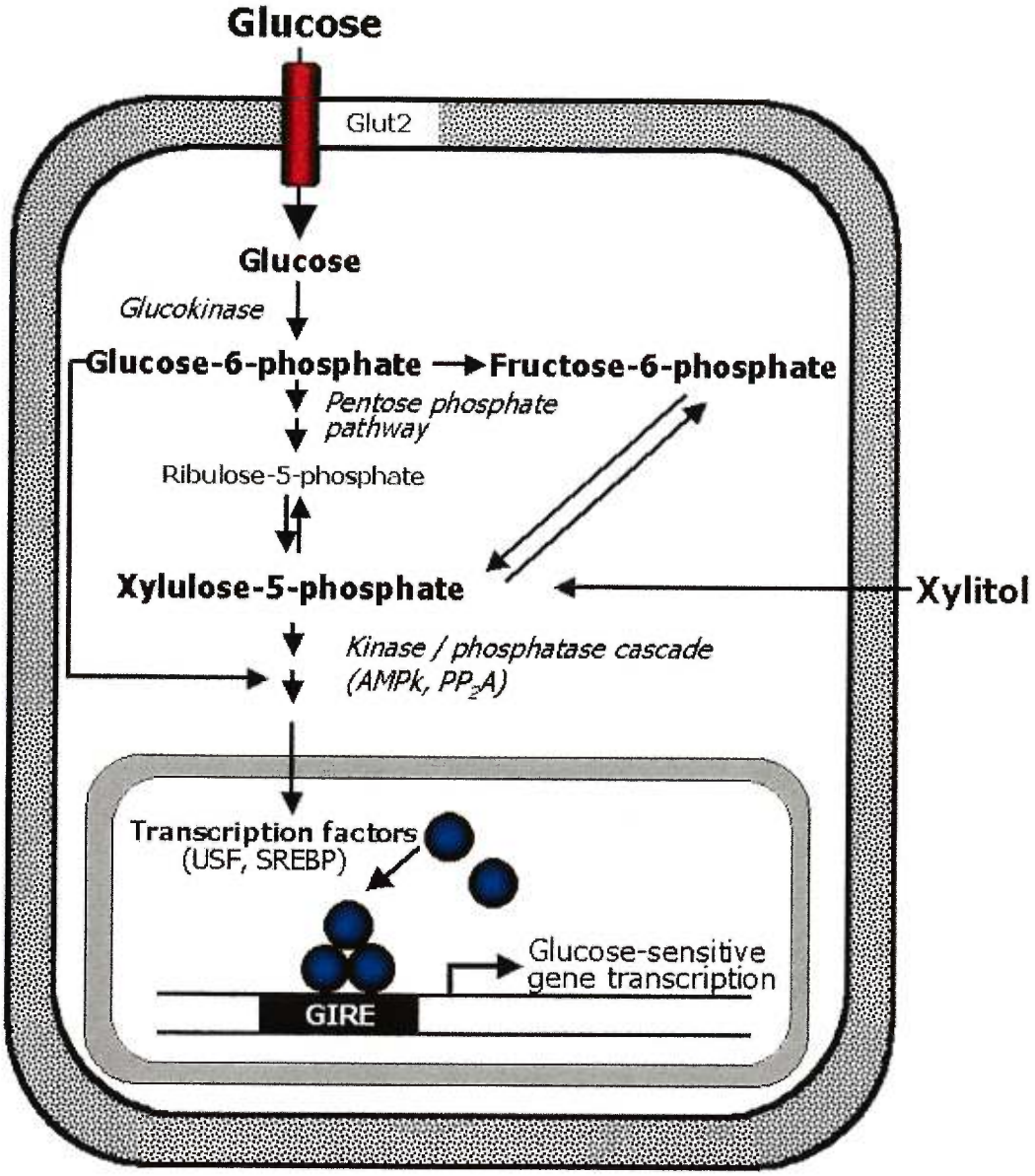


Figure 3: Scheme of the glucose signaling pathway to transcriptional regulation of glucose-sensitive gene. This figure shows that glucose goes into a hepatocyte through GLUT2. Among the metabolites of glucose metabolism, either glucose-6-phosphate or xylulose-5-phosphate could be the signal molecule to initiate kinase/phosphatase cascade, thus regulates the glucose-sensitive gene transcription. AMPK and PP_2A refer to AMP-activated protein kinase and protein phosphatase 2A, USF and SREBP refer to upstream stimulatory factor and sterol regulatory element binding protein, which are proposed to be involved in glucose signaling. GIRE represents glucose response element.

factors such as upstream stimulatory factor (USF); Finally, these nucleus transcriptional factors, by binding to the glucose response elements (GIREs) which are characterized by a CACGTG motif, will regulate downstream gene expression (Kahn, 1997). Additionally, GLUT2 may interact with another protein upon sensing the glucose concentration and initiate an alternative signaling pathway, resulting in the change of gene expression (not shown in figure).

1.4.2.2 Fatty acids

It was recently recognized that fatty acids, like other nutrients and hormones, can control gene expression (Gustafsson, 1998). Previous studies have shown that manipulations of dietary lipids influenced G6Pase activity (Garg et al., 1985). In order to investigate the effect of prolonged hyperlipidemia seen in diabetes on G6Pase gene expression, lipid infusion was conducted under well-controlled hormonal and metabolic conditions in rats. It was found that increased plasma fatty acid (0.2-1.6 mM), that is within the physiologic range, caused a rapid and significant increase in both p36 mRNA and protein, which indicates that hyperlipidemia may independently contribute to the enhanced hepatic glucose production in NIDDM (Massillon et al., 1997). Long chain fatty acids not only increased p36 mRNA transcription, but also stabilized p36 mRNA as shown by the effect of linoleate on p36 mRNA half-

life (Chatelain et al., 1998). It was suggested that the effect of lipids on gene transcription is mediated by peroxisome proliferator-activated receptors (PPARs). Clofibrate, a peroxisome proliferator, indeed induced the mRNA level of p36 (Chatelain et al., 1998). These results highlight the link between gluconeogenesis and fatty acid metabolism.

1.4.2.3 Calcium

In the liver, as in other organs, many cellular processes are activated by increased cytosolic free Ca^{2+} levels, such as respiration, gluconeogenesis, glycogenolysis and cell division. The changes in Ca^{2+} include both the influx of Ca^{2+} through plasma membrane and the efflux of Ca^{2+} from intracellular storage sites (mainly the endoplasmic reticulum), leading to the increase in cytosolic free Ca^{2+} levels. Hormones (including glucagon, catecholamines, vasopressin, angiotensin II, thyroid hormones) that stimulate gluconeogenesis were shown to evoke a redistribution of Ca^{2+}, while inhibition of Ca^{2+} flux blocks the stimulation of gluconeogenesis. Furthermore, increasing intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is sufficient to increase gluconeogenesis (Kraus-Friedmann et al., 1996). Glycogenolysis is also stimulated by increases in $[Ca^{2+}]_i$, because of the well known sensitivity of glycogen phosphorylase kinase to Ca^{2+}. G6Pase catalyses the common step of gluconeogenesis and glycogenolysis, and its activity is inhibited by calcium in

permeabilized hepatocytes (van de Werve, 1989), albeit in a complexed manner (Vidal et al., 1991). It was previously found that G6P could enhance Ca^{2+} uptake in liver microsomes (Benedetti et al., 1985). By utilizing a light scattering assay, G6P and Ca^{2+} were found to be mutually enhanced in microsomes from liver, brain and heart. This effect is independent of p36, which is not detectable in brain and heart (Chen et al., 1998). Elevated cytosolic calcium is also important in the pathogenesis of complications in diabetes mellitus. Both type I and type II diabetes are associated with a sustained elevation in basal levels of $[Ca^{2+}]_i$ resulted from the combination of increased calcium influx and decreased calcium efflux, which correlates well with hyperglycemia (Massry et al., 1997). In streptozotocin-treated rats (Makino et al., 1987) and Zucker diabetic rats (Zemel et al., 1990), the activity of Ca^{2+}-ATPase is reduced. The profound influence of Ca^{2+} signaling on gene expression has been recognized a long time ago. It is generally considered that genes coding for short-lived transcription factors are the immediate targets of Ca^{2+} signaling since Ca^{2+} signals are physically transient. Changes of these immediate early gene products will then alter expression of other genes (van Haasterren et al., 1999). The mechanisms of calcium signaling on gene regulation are shown in Figure 4.

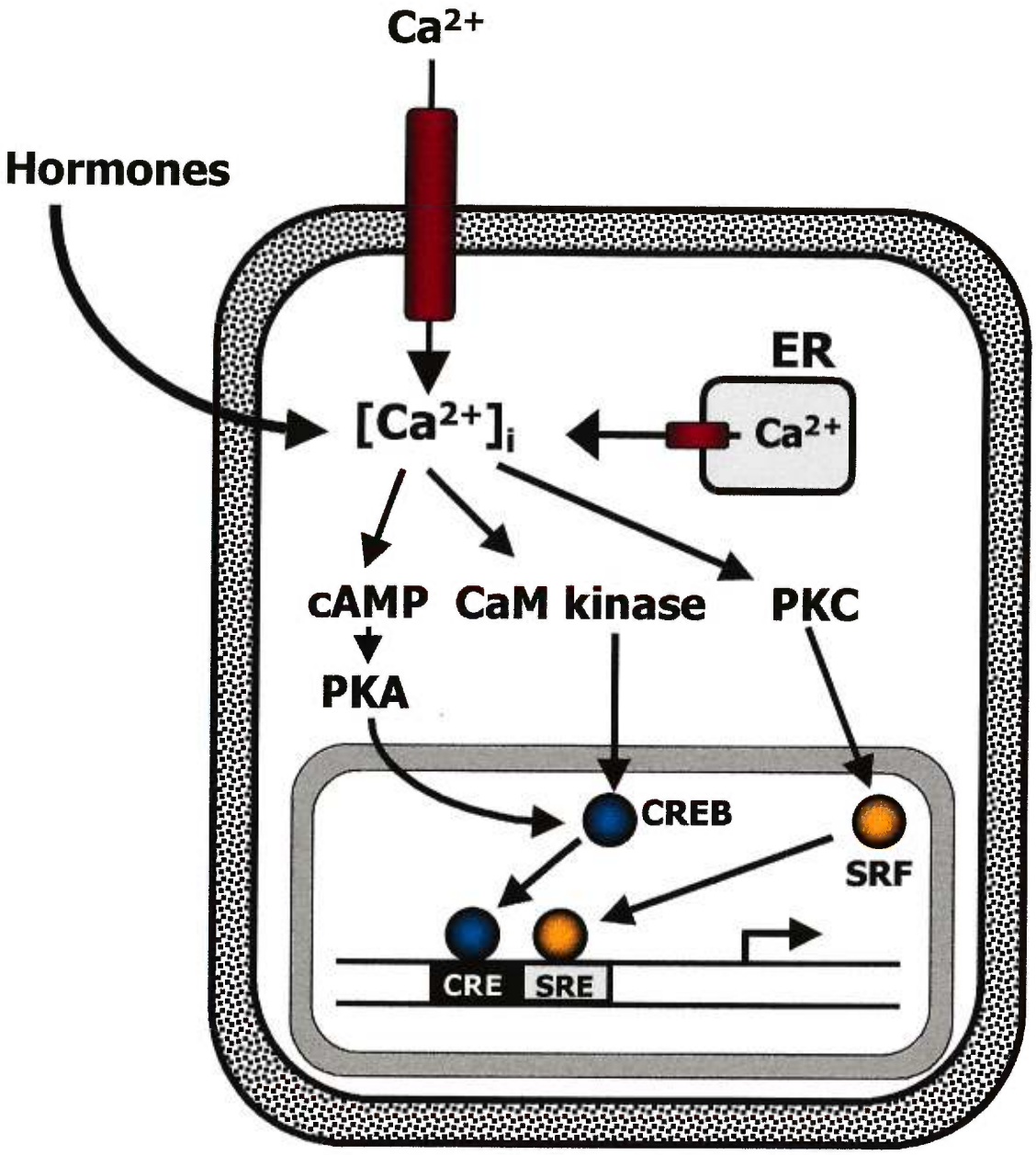


Figure 4: Scheme of the cacium signaling pathway of gene regulation. Hormones stimulate a transient elevation of cytosolic free [Ca^{2+}] by Ca^{2+} influx from the extracellular space or by Ca^{2+} release from intracellular space, which activates several different kinases and leads to the transcription of target genes. PKA, PKC and CaM/kinase represent protein kinase A, C and Ca^{2+}/calmodulin dependent protein kinase; CREB and SRF represent CRE binding protein and serum response factor; CRE and SRE represents Ca^{2+}/cAMP response element and serum response element.

1.4.2.4 Phosphate

Cytosolic inorganic phosphate (Pi) plays an important role in cellular energy metabolism and glucose homeostasis such as regulating hepatic glucose output (Berner et al., 1988). Clinical studies have shown that hypophosphatemia is associated with glucose intolerance in both the hyperglycemic and euglycemic states (DeFronzo et al., 1980), as well as insulin insensitivity (Paula et al., 1998). Interestingly, based on the studies of renal Pi reabsorption, it was found that deprivation of dietary Pi can induced gene expression of Na/Pi cotransporter type II (NPT2) at both mRNA and protein levels, resulting in increased Na/Pi cotransport in the renal brush membrane (Werner et al., 1994; Levi et al, 1994; Tenenhouse et al., 1995). Inversely, a high Pi diet decreases NPT2 gene expression (Tenenhouse et al., 1994; Levi et al., 1994). Besides the NPT2 gene, other genes, such as the genes of renal vitamin D-24-hydroxylase (Wu et al., 1996), dietary Pi-regulated RNA-1 (Custer et al., 1997) and parathyroid hormone (PTH) (Kilav et al., 1995; Hernandez et al., 1996; Marks et al., 1997), are also known to be regulated by dietary Pi. The mechanism of the regulation of PTH by Pi may be due to the posttranscriptional change of mRNA stability by the binding of parathyroid proteins to the PTH mRNA (Moallem et al., 1998). Recently, a phosphate response element (PRE) with a CACGTG motif was identified in the promoter of the NPT2 gene and the corresponding binding transcriptional

factor (mouse transcriptional factor μE3) was also isolated (Kido et al., 1999), which further indicates the important role of Pi in gene regulation. On the one hand, rat Na/Pi cotransporter-I was demonstrated to be upregulated by insulin and glucose, and downregulated by cAMP and glucagon (Li et al., 1996); on the other hand, many enzymes involved in glucose metabolism are regulated by Pi, which indicates a tight link between phosphate homeostasis and glucose homeostasis. Based on the foregoing reports and the importance of phosphate transport in the G6Pase system, the effect of Pi deprivation on G6Pase components was investigated (Xie et al., 1999). It was found that a low-Pi diet increased both p36 and p46 mRNA levels and p36 protein in rat liver, with a concomitant increase in G6Pase activity but without changes in the kinetic parameters of microsomal phosphate transport. Further investigations have shown that a low-Pi diet increased the liver cAMP level, altered the activities of the key enzymes in gluconeogenesis and hepatic glucose production (Xie et al., 2000).

1.4.2.5 Hormones

Hormonal signaling pathway The expression of genes coding for G6Pase is also under hormonal regulation. Various hormones can affect the gene expression, such as insulin, glucagon and glucocorticoids. Different signaling pathways are linked to these hormonal actions. Insulin and glucagon

are secreted by the pancreatic β- and α- cells respectively upon physiological changes such as fasting and feeding. Insulin binding to its receptor on the plasma membrane results in autophosphorylation of the receptor and initiation of intracellular signal transduction cascades. The inhibitory effect of insulin on G6Pase might be mediated by the activation of PI3-Kinase, then through the PI3-kinase cascade to eventually modify transcriptional factors and alter the gene expression (Dickens et al., 1998). The binding of glucagon to its receptor on the cell membrane activates a coupled G-protein (Gs), which, in turn, stimulates the activity of the membrane-associated adenylate cyclase. This converts ATP to cAMP, which activates the cAMP dependent protein kinase (PKA). PKA then migrates into the nucleus and phosphorylates transcriptional activators such as the cAMP response element binding protein (CREB). The interaction between CREB and cAMP response element (CRE) thus activates gene transcription. Glucocorticoids are secreted by the adrenal cortex and are transported via the blood stream in a complex with transport proteins, then are taken up into the target cells by an yet unknown mechanism. In the cytosol, the effect of glucocorticoids is mediated by the glucocorticoid receptor (GR). In the absence of glucocorticoids, GR is trapped by heat shock proteins (HSP). Once glucocorticoids bind to the GR, it will dissociate from HSP and translocate into the nucleus where it binds to glucocorticoid response elements (GRE) on the promoter region of the target gene to activate gene expression.

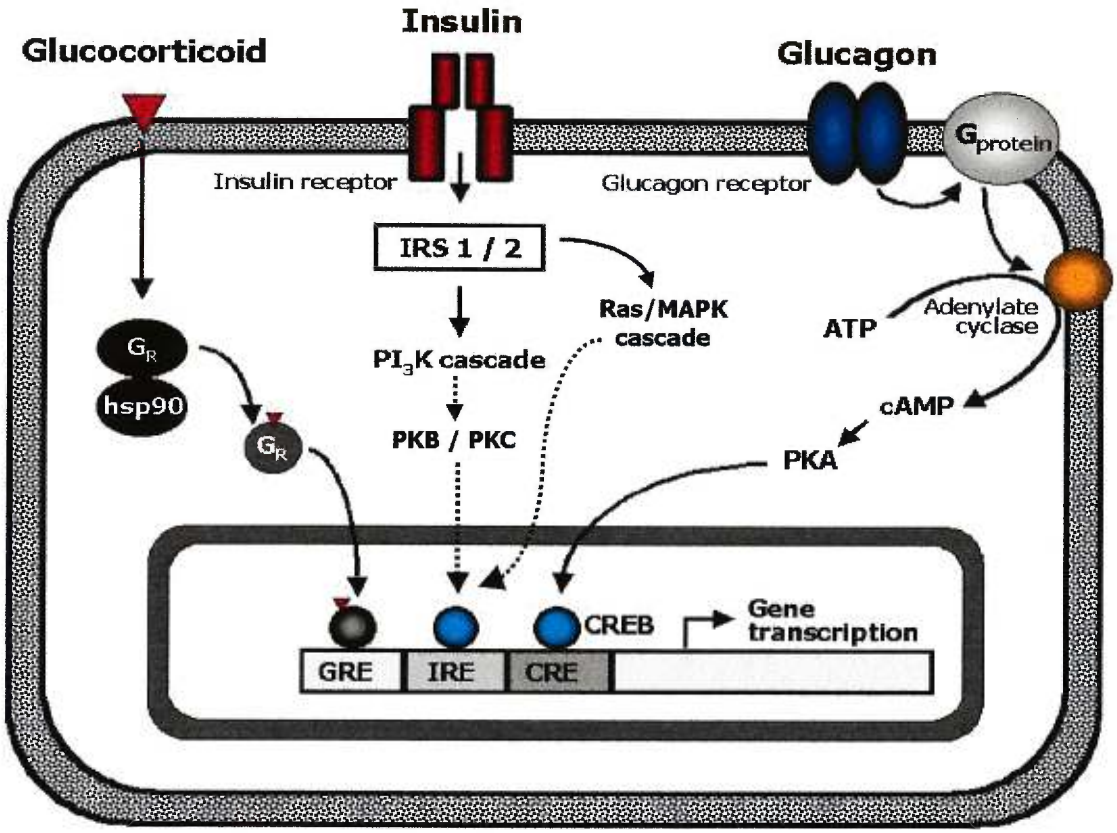


Figure 5: Scheme of the hormonal signaling pathway of gene regulation. The signaling pathways of insulin, glucagon/cAMP and glucocorticoid are integrated in this figure, as explained in the text.

Alternatively, it can also interact with other transcriptional factors by cross talk and affect gene expression indirectly. The hormonal pathway involved in G6Pase gene expression is shown in Figure 5.

Effect of hormones on G6Pase genes In Fao cells, 1 μM dexamethasone (a synthetic glucocorticoid) can largely increase p36 mRNA levels (about 10-fold) with or without the presence of glucose. Insulin (1 μM) completely blocks the stimulatory effect of dexamethasone, indicating its dominant negative role in the regulation of G6Pase gene expression (Lange et al., 1994). These observations agree well with reports on the hormonal regulation of PEPCK gene expression (Wynshaw-Boris et al., 1986) and indicate that both G6Pase and PEPCK genes are regulated reciprocally by dexamethasone and insulin. More detailed time-course studies of hormonal regulation of the p36 gene (Argaud et al., 1996) have shown: (1) CPT-cAMP (100 μM) increased p36 mRNA 4-fold after 3 h, but a longer exposure (24 h) to this cyclic nucleotide decreased mRNA levels to 50% of control values, while the peak effect was found at 6 h; (2) Compared to the cAMP effect, the time course of 1 μM dexamethasone on gene expression was relatively slower. Actually, the stimulatory response of dexamethasone in Fao cells is biphasic. At 3 h there was a modest increase (1.6-fold) in mRNA, then this effect diminished and long-term stimulation began, leading to a 3.3-fold increase of p36 mRNA at 48 h; (3) The inhibitory effect of insulin in Fao cells was rapid;

1 μM insulin decreased the p36 mRNA level by 50% at 2 h. A dose-response study shows that the negative effect of insulin was already seen at very low concentration (100 pM) of insulin; (4) In primary hepatocytes, the insulin inhibitory effect is similar to that seen in Fao cells, while the difference is that only the combination of cAMP and dexamethasone shows significant stimulatory effect. If they are added alone to the cells, the effect is marginal. Hormonal regulation of G6Pase was also investigated *in vivo*. In a partial-hepatectomy model, a model of hepatic regeneration characterized by an increased glucagon to insulin ratio, p36 gene expression was upregulated rapidly at the mRNA level with (Haber et al., 1995) or without changes in p36 protein (Zakko et al., 1998). Alternatively, in primary hepatocyte cultures from 20-day-old rat fetus, Bt_2cAMP induced an increase in p36 mRNA level in a dose-dependent manner with a 24 h delay compared with that in adult hepatocyte cultures. Dexamethasone had no effect on p36 mRNA in fetal hepatocytes, either alone or accompanied by cAMP, while the antagonistic effect of insulin was also observed in fetal hepatocytes to overcome the effect of Bt2cAMP (Chatelain et al., 1998). Recently, an unexpected result from a clinical study was reported, indicating that therapeutic insulin administration was associated with increased level of hepatic G6Pase activity and p36 protein expression in preterm infants (Burchell et al., 2000). G6Pase is known to be down-regulated by insulin in adults. It is therefore necessary to determine whether during the first five days preterm infants have very different basal

levels of a variety of hormones or a counter-regulatory response from those of well term infants or adults. This may provide useful information to understand the property of G6Pase during the neonatal period, which is different afterwards.

G6Pase in kidney Since kidney is one of the two major gluconeogenic organs, the gene expression of kidney G6Pase has also been studied. In rat renal tubule suspensions, p36 mRNA level was increased 2-3-fold by 10^{-8} to 10^{-5} M dexamethasone, whereas the most effective concentration of insulin, 10^{-9} M, induced only a 40% decrease (Simonnet et al., 1999). These results show that kidney p36 is more sensitive to glucocorticoids than to insulin, which is different from liver p36. The time courses of p36 mRNA and activity during the transition between fed and fasted states are also not identical in liver and kidney. In liver, p36 mRNA and activity increased during the first 48 h of fasting, then decreased during 72 h and 96 h of prolonged fasting. Upon refeeding p36 mRNA recovered to the fed level after 24 h. In kidney, p36 mRNA and activity increased throughout a 96 h-fast continuously, and it needed 48 h-refeeding to return to the fed level (Minassian et al., 1996). Therefore it seems that the mRNA of p36 increased more rapidly in the liver than in the kidney during fasting and also decreased more rapidly during refeeding, suggesting the existence of different regulatory mechanisms in liver and kidney which may explain their different contributions to gluconeogenesis.

During prolonged fasting p36 may play an essential role to trigger the shift from a principally hepatic to a hepatic and renal gluconeogenesis when kidney can account for up to 45% of total glucose production (Owen et al., 1969). It is postulated that decreased glucocorticoids during prolonged fasting might contribute to the decreased p36 rather than insulin and glucagon, which do not vary within 24 to 96 h fasting.

G6Pase and diabetes Increased G6Pase activity and expression is associated with rodent models of both type I and type II diabetes. In streptozotocin-induced diabetic rats, G6Pase mRNA and activity are increased to the same extent in kidney and liver (Mithieux et al., 1996a) and are also increased in small intestine of diabetic rats (Rajas et al., 1999). However, another group reported that G6Pase mRNA was increased 2-3-fold in liver but not in the kidney of streptozotocin-induced diabetic rats, and this increase could be corrected by insulin treatment (Shingu et al., 1996). In 90% pancreatectomized rats, p36 mRNA increased more than 5-fold and its protein level increased 3-4-fold in liver (Massillon et al., 1996). In other animal models of diabetes, such as the ob/ob mice (Khan et al., 1995,1998a), BB rats (Haber et al., 1995), Zucker diabetic fatty (ZDF) rats (Trinh et al., 1998) and non-obese diabetic (NOD) mice (Mosseri et al., 2000), p36 was also found to be increased in its mRNA, protein or activity. The fact that both G6Pase expression and activity are increased in many animal models of diabetes type I

and II thus makes it a very attractive candidate gene for diabetes. In fact, changes in gene expression are likely to play a major role in the pathophysiology of NIDDM as suggested by the recent identification of mutations in glucokinase gene (Vionnet et al., 1992) and in transcription factors HNF-1 and HNF-4 genes (Yamagata et al., 1996a,b), which lead to a form of NIDDM called maturity-onset diabetes of the young (MODY). It is unlikely that the mutations in the coding region of G6Pase genes are the cause of diabetes because those mutations will down-regulate the gene instead of up-regulation. Linkages between G6Pase gene mutations and diabetes have not yet been established. One recent screening of the promoter region of p36 in 154 NIDDM patients did not find any mutations (Yoshiuchi et al., 1998), but it could not be excluded that mutations in other regulatory regions of G6Pase gene or even other regulatory proteins of G6Pase may be the primary genetic lesion. A gradual rise in G6Pase expression may induce symptoms such as glucose intolerance of NIDDM, which is supported by the result obtained from overexpression of p36 *in vivo* (Trinh et al., 1998). Alternatively, upregulation of G6Pase could be secondary to metabolic alterations during the development of diabetes, which is supported by the fact that G6Pase gene expression is induced by increased concentration of glucose within the pathophysiological range (Massillon et al., 1996; Argaud et al., 1997).

1.4.3 Promoter studies

The cloning of promoter regions of p36 gene in human (Schmoll et al., 1996), rat (Argaud et al., 1996) and mouse (Streeper et al., 1997) have made it possible to study the molecular basis of the control of G6Pase expression. The promoter hallmarks, a TATAAA-box located at position –31 and a CCAAT-box located at –207, have been defined (Argaud et al., 1996). In this paper, cis-acting elements were first proposed as follows: (1) For the negative insulin response sequence (IRS), no exact match was found except a 9/10 bases similarity to the consensus sequence of IRS (TGGTGTTTTG) at position –1449. Since the most conserved base of this consensus sequence is not conserved in G6Pase, it is likely that the action of insulin on G6Pase gene expression is mediated through a novel response element; (2) A 7/8 match to the consensus sequence of the cAMP response element (CRE) was found at position –164; (3) Glucocorticoid response element (GRE) half sites were found at position –1552, possessing a GC pair (TGTTCT) which is required for glucocorticoid receptor binding (Scheidereit et al., 1984; Beato et al., 1989). Besides the hormonal response elements, some putative binding sites of transcriptional factors were identified, such as AP-I, HNF-5 and LF-A.

CRE It was then reported that the DNA element essential for optimal liver-specific expression of p36 gene was contained within nucleotides –234 to +3 (Lin et al., 1997). A first CRE between nucleotides –136 to –129 was identified, and transient expression assays in HepG2 cells indicated that this CRE can mediate transcriptional activation of the G6Pase gene by cAMP. Electromobility shift assays have shown that transcription factors HNF-1α, HNF-3γ, CRE binding protein (CREB) and CCAAT/enhancer binding protein (C/EBP) can bind to the promoter region of p36 gene, which may play an important role for p36 gene expression (Lin et al., 1997). It was also suggested that sequences from –508 to –246 and –245 to –235 contain negative regulatory elements that suppress p36 gene expression. A second cAMP response element was identified at the p36 gene promoter from nucleotides –161 to –152 by expressing a luciferase reporter gene under the p36 gene promoter in H4IIE hepatoma cells (Schmoll et al., 1999). This CRE is involved in the induction of p36 gene transcription by both cAMP and glucocorticoid, suggesting an interaction between cAMP signaling and the glucocorticoid response on this CRE. However, a more complicated concept has been proposed that mutiple cis-acting elements through a tissue-specific mechanism are required for the full stimulatory effect of cAMP on p36 gene expression. It was demonstrated that HNF-1 binding site in the p36 promoter is critical and may act as an accessory factor for the stimulation of p36 gene transcription by the cAMP pathway in a kidney-derived cell line but not in HepG2 cells

(Streeper et al., 2000a). Recent studies have also shown that with deletions of the above-mentioned two CREs, the G6Pase promoter was still induced by PKA. A region between –114 and –99 on the G6Pase promoter was proposed to mediate a cAMP-PKA signal by binding with HNF-6 (Streeper, et al., 2000b).

IRS Using a chloramphenicol acetyltransferase (CAT) reporter gene expression in hepatoma cells, a multicomponent IRS of the mouse p36 gene was identified from the nucleotides –271 to –159, including two regions A and B (Streeper et al., 1997). It is likely that region B (from –198 to –159) contains an IRS whereas region A (from –271 to –199) acts as an accessory element that enhances the action of a single IRS located in region B. Three copies of the sequence T(G/A)TTT(T/G)(G/T) were found in region B, while this motif also exits in PEPCK and other gene promoters. This indicates that insulin may exert its inhibitory effect coordinately on PEPCK and G6Pase through the same IRS to repress gluconeogenesis. The existence of this multicomponents IRS was recently confirmed in human p36 gene (Ayala et al., 1999). It was found that HNF-1 is an accessory factor which binds to region A of the IRS (Streeper et al., 1998), while transcriptional factor FKHR binds to region B of the IRS of p36 gene promoter (Ayala et al., 1999).

GRE The glucocorticoid response element (GRE) in the p36 promoter was demonstrated (Lin et al., 1998a). Maximal stimulation of the G6Pase promoter activity by glucocorticoids requires binding of the glucocorticoid receptor to its cognate site at nucleotides –178 to –164. Interestingly, as in region A of the IRS, binding of HNF-1α at nucleotides –226 to –212 is absolutely required for the glucocorticoid-stimulated transcription, which shows that HNF-1α is also acting as an accessory factor for GRE (Lin et al., 1998a). Thus, it appears that HNF-1 can enhance both positive and negative effects of hormones on the expression of p36 gene.

GIRE Although the p36 promoter does not contain an obvious consensus sequence for the glucose response element (GIRE) as involved in the regulation of the L-PK gene by glucose, the cis-active element(s) mediating the glucose effect was (were) localized between nucleotides –161 and +4 (Schmoll et al., 1999).

1.5 Genetic manipulation of G6Pase components

The development of molecular biology provides elegant techniques such as transgenic mice, recombinant adenovirus and stable transfection to manipulate genes of interest at the DNA level and these were also applied in the G6Pase field.

Overexpression of p36 by recombinant adenovirus was first performed in INS-1 cells (a well differentiated insulinoma cell line) to study its effect on glucose metabolism and insulin secretion (Trinh et al., 1997). In INS-1 cells, overexpressed p36 was verified to be normally glycosylated and correctly sorted to the ER. Overexpression resulted in a 10-fold increase in G6Pase activity, which led to a 4.2-fold activated glucose cycling and 30% impaired insulin secretion proportional to the decreased glucose usage. Similar results were obtained by means of stable transfection, which is time-consuming compared to the adenovirus technique. Several stable transformants with various degrees of G6Pase activities were established by transfecting a mouse β-cell line (MIN6) with the cDNA of rat G6Pase catalytic subunit (Lizuka et al., 2000). In those p36 overexpressing clones, glucose-stimulated insulin secretion, as well as intracellular ATP content and glucose usage, was reduced proportionally to the increased activity of G6Pase. These results indicate that overexpression of p36 alone in INS-1 and MIN6 cells already had a significant impact on glucose metabolism and insulin secretion. Although the phenomena observed in p36-overexpressed cells resembled those seen in obesity and NIDDM (Khan et al., 1989, 1990; Trinh et al., 1997), in the case of MIN6 cells only high overexpression (24-fold) can abolish the glucose induced insulin secretion, while the effect of moderate overexpression (3-7-fold, also within the range of increased liver G6Pase activity in diabetes) was marginal. In INS-

1 cells, 10-fold overexpression of p36 did not lead to severe alteration of β cell function and metabolism as seen in diabetes. It suggests that altered G6Pase in diabetes may be partially responsible for the glucose insensitivity while other genes may be involved in the etiology of β cell dysfunction. It must be pointed out that the presence of G6Pase in islets of Langerhans is still uncertain (Tokuyama et al., 1995). In addition, as previously explained, another islet-specific G6Pase catalytic subunit related protein (IGRP) was recently reported; it is therefore not sure that the overexpression of liver form of p36 could reflect the native situation in pancreas.

Since liver is the major tissue where gluconeogenesis takes place and where G6Pase localizes, a recombinant adenovirus was also used to overexpress p36 in both cultured primary hepatocytes (Seoane et al., 1997) and *in vivo* (Trinh et al., 1998). Overexpression of p36 by adenovirus in primary hepatocytes significantly decreased glycogen content and G6P levels without changes in UDP-glucose level. Since the binding of G6P to glycogen synthase alters its conformation in a way that facilitates the covalent dephosphorylation and activation of the enzyme, lowered G6P level will deactivate glycogen synthase (glycogen synthase a/b ratio decreases, while total is unchanged), which are responsible for the decreased glycogen synthesis. Lactate production and [3-^{3}H] glucose usage were also significantly decreased. Conversely glucose production from gluconeogenic substrates such as pyruvate and

gluconeogenic amino acids was more effective, which means glycolysis was impaired while gluconeogenesis was enhanced by overexpression of p36. Further studies have shown that overexpression of p36 neither altered the translocation of glucokinase nor increased control strength of glucokinase on both glycolysis and glycogen synthesis (Aiston et al., 1999). It was thus proposed that the main regulatory function of G6Pase is to buffer the G6P concentration in the hepatocytes. Since under such conditions GK was not altered, the decreased ratio of glucokinase: G6Pase could play an important role to determine the metabolic fate of glucose in liver cells. From the foregoing results, the authors concluded that overexpression of the G6Pase catalytic subunit alone in hepatocytes is sufficient to markedly reduce glucose utilization and storage and to augment glucose production, in agreement with the result obtained in INS-1 cells (Trinh et al., 1997). This is against the idea proposed by the substrate-transport model that G6P transport by the putative translocase is the rate-limiting step (Arion et al., 1980) in the conversion of G6P to glucose and Pi. Furthermore, one-time systemic infusion of recombinant adenovirus containing p36 cDNA into normal rats caused a 1.6-3 fold increase in G6Pase activity in the liver, which was sufficient to significantly alter the fuel homeostasis. Within a week, rats that received p36-virus exhibited glucose intolerance, hyperinsulinemia, decreased hepatic glycogen content, increased peripheral lipid storage and tendency to hyperglycemia (Trinh et al., 1998), all of which are part of the NIDDM

syndrome. The result that overexpression of p36 *in vivo* was able to perturb both glucose and lipid homeostasis indicates that impaired G6Pase may play an important role in the development of diabetes. In order to address whether G6Pase precedes and contributes to the development of NIDDM, ZDF rats at various developmental stages were used to investigate G6Pase expression. It was found that induction of G6Pase activity did not precede but was secondary to the appearance of hyperglycemia and insulin resistance, which indicates that it is not the primary cause of the disease and its major role may be to exacerbate hyperglycemia in this case (An et al., 1999).

Besides overexpression of p36 which mimics the diabetic state, a knockout animal model is also an attractive tool for the study of the G6Pase system and related glycogen storage disease. The p36 knockout mice were generated by replacing exon 3 and the flanking introns of the p36 gene with a neomycin cassette (Lei et al., 1996). This animal model manifests essentially the same pathophysiology as human GSD-Ia patients, including hypoglycemia, growth retardation, hepatomegaly, kidney enlargement, hyperlipidemia and hyperuricemia. Kinetic studies have shown that the knockout of the p36 gene destroys both G6Pase activity and G6P transport, providing direct evidence for a tight coupling between G6P transport and catalysis, which is in agreement with rapid kinetic studies of G6Pase (Berteloot et al., 1991b; St-Denis et al., 1995a). Recently, this p36 knockout mice model has been used to attempt gene

therapy for future applications to human GSD-Ia (Zingone et al., 2000). A single administration of recombinant adenovirus containing murine p36 cDNA into p36 knockout mice resulted in 19% restoration of G6Pase activity during postinfusion day 7-14. Survival rate of these rats after weaning increased to 100% compared to previous 15% and their growth rate also increased greatly. Moreover, adenovirus-mediated gene transfer essentially corrected the abnormal plasma glucose, cholesterol, triglyceride profile and partially alleviated liver and kidney enlargement and glycogen deposition.

2- METHODOLOGY

2.1 Microsome preparation and enzyme activity

2.1.1 Microsome preparation

Both normal Wistar rats and streptozocin induced diabetic rats were sacrificed by decapitation after overnight fasting. Rat livers were homogenized in 50 mM Tris-Hepes buffer individually and processed as follows at 4 °C: Firstly, rat liver was homogenized at 1200 rpm with a YAMATO LH-21 homogeniser; Secondly, the resultant homogenate was centrifuged at 1000g for 10 min, the first supernatant was further centrifuged at 12,000g for 10 min and the second supernatant was then ultra-centrifuged at 35,000g for 60 min; Thirdly, the final pellet was resuspended in ice-cold 50 mM Tris-Hepes buffer with 250 mM sucrose at pH 7.3. This microsome fraction was at a protein concentration of about 7 mg/ml and kept at –80 °C for enzyme activity measurement, as well as Western blot analysis.

2.1.2 G6Pase activity measurement

The G6Pase hydrolytic activity is based on the reaction:

$$[U\text{-}^{14}C]\text{Glucose-6-phosphate} \xrightarrow{\text{G6Pase}} [U\text{-}^{14}C]\text{Glucose} + Pi$$

The reaction is initiated at 30 °C by adding 10 µl microsomes (at a protein concentration of 7 mg/ml) into 90 µl Tris-Hepes buffer, pH 7.3, at different G6P concentrations with a constant [U-^{14}C]G6P. Usually two final G6P concentrations (0.2 mM and 5 mM) are used, representing concentrations close to K_m and V_{max}. The reaction can last 2-5 min and is stopped by adding 0.5 ml 0.3M $ZnSO_4$ and mixed. Then 0.5 ml saturated $Ba(OH)_2$ is added followed by mixing to form a Zn/Ba precipitate that retains the unhydrolyzed [U-^{14}C]G6P. The mixture is centrifuged for 1 min at 16,000g and 0.5 ml of the supernatant containing the [U-^{14}C]glucose is counted and G6Pase activity is calculated according to the formula below:

$$\frac{(cpm-cpm_0)}{time(2 \text{ or } 5 \text{ min})} \times \frac{(\text{total volume } 1100ul)}{(\text{counted volume } 500 \text{ ul})} \times \frac{1}{(100\%/\text{nmols})} \times \frac{1}{(\text{protein mg})}$$

One unit is defined as the amount of enzyme which forms 1 micromole of glucose per minute in the conditions of the assay.

2.2 Yeast two-hybrid system

2.2.1 Principle

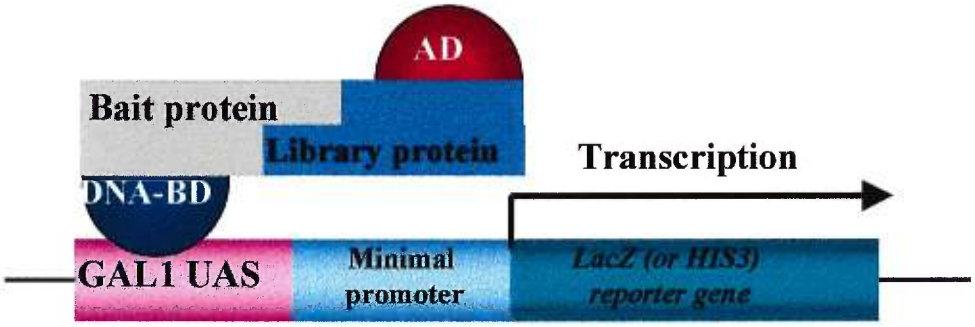
The yeast two-hybrid assay is based on the fact that many eukaryotic transcription factors are composed of physically separable, functionally independent domains. The yeast GAL4 protein is such a regulator that contains a DNA-binding domain (DNA-BD) and an activation domain (AD). The

function of BD is to bind to an upstream activation site (UAS), while AD directs the RNA polymerase II complex to transcribe the gene downstream of the UAS. The yeast two-hybrid system is composed of two different cloning vectors that are used to generate fusions of either BD or AD to genes encoding proteins that potentially interact with each other. These two recombinant plasmids are coexpressed in yeast and targeted to the yeast nucleus. Only the interaction of the two hybrid proteins can restore the transcriptional activation function of GAL4 by bringing BD and AD into close physical proximity in the promoter region. With the use of lacZ or HIS3 as downstream reporter gene, the protein-protein interaction can be easily detected. A schematic overview is shown in Figures 6 and 7.

Our previous idea was to use p36 as a bait protein to screen two libraries (human liver cDNA library and human leucocyte cDNA library), in order to look for interacting protein(s) with p36. During the time of this work p46 was cloned, so accordingly we decided to use both p36 and p46 as bait proteins to screen libraries and also to try to study the interaction between p36 and p46 by using the yeast two-hybrid system.

The DNA-BD/protein x (bait) hybrid binds to the GAL1 UAS but cannot activate transcription without the activation domain (AD)

In the absence of bait protein, the AD/library fusion protein cannot bind to the GAL1 UAS and thus does not activate transcription



Interaction between the bait and library proteins *in vivo* activates transcription of the reporter gene.

Figure 6: Schematic diagram of the GAL4-based Two-Hybrid systems. The DNA-BD is amino acids 1-147 of the yeast GAL4 protein, which binds to the GAL1 UAS upstream of the reporter gene. The lacZ and HIS3 reporter genes are separate constructs integrated in the yeast genome. The AD is amino acids 768-881 of the GAL4 protein and has transcriptional activation function.

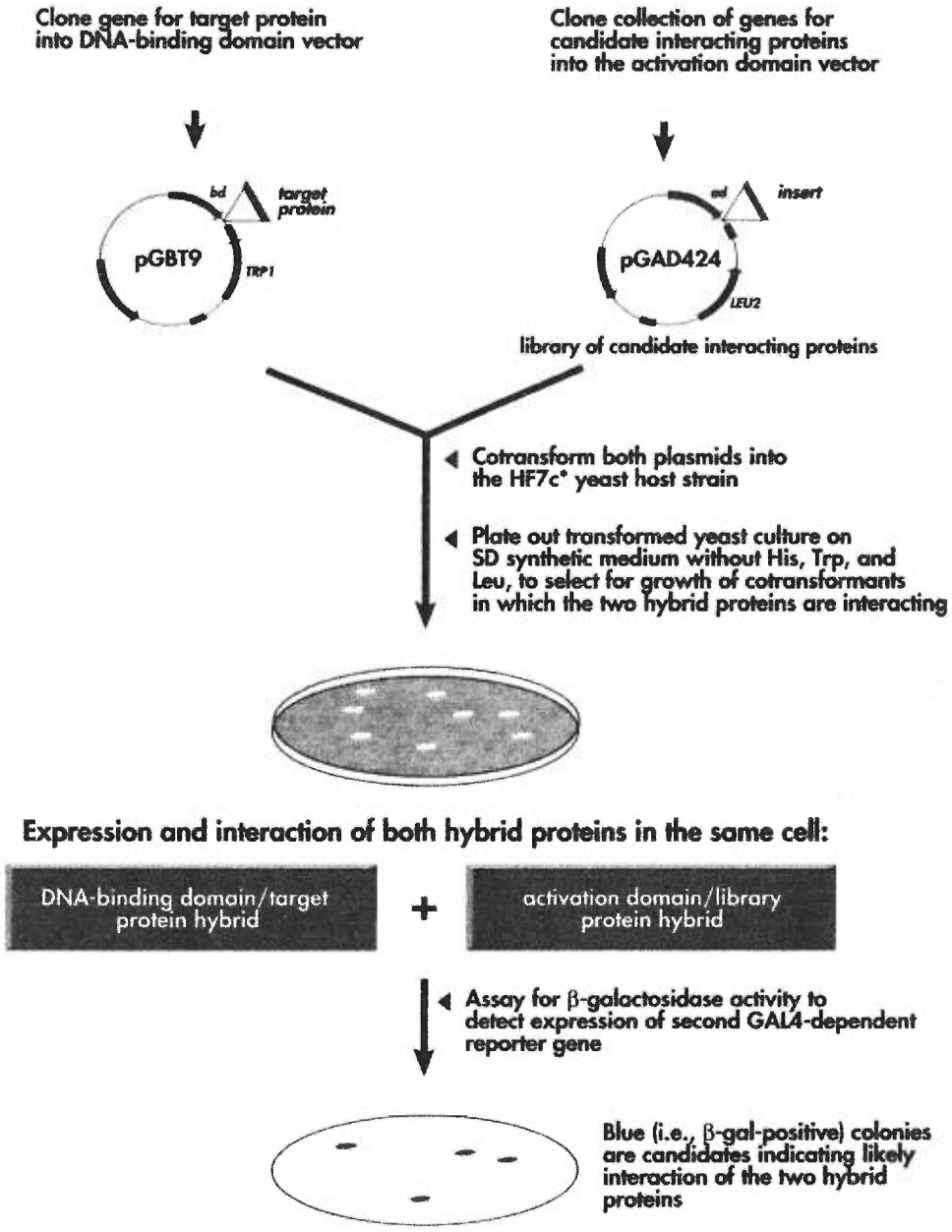


Figure 2: Screening GAL4 activation domain fusion libraries for proteins that interact with a target protein.

2.2.2 Methods

2.2.2.1 Library titering and amplification

It is important to know the titer of the library before library amplification. To do the titering, 1 μl of library was diluted to 10^3 or 10^6 fold first. Then different dilutions were spread onto separate LB/amp plates and the plates were incubated overnight at 37 °C. By counting the number of colonies we can calculate the titer of library according to the dilution times. The titers of human liver cDNA library and human leucocyte cDNA library used in our experiments at the time were 1.6×10^9 cfu/ml and 1.4×10^9 cfu/ml respectively. For library amplification, ten μl of the library (in *E.coli*) was plated at 20,000 colonies/150-mm plate on LB-Amp agar to obtain at least two to three times the number of independent colonies of the library. The plates were further incubated at 37 °C overnight and the colonies were scraped into 1 liter of LB-Amp broth, and then incubated at 37°C for 2 hours with shaking at 200 rpm. The plasmid DNA of the library was isolated using a QIAGEN Plasmid Mega Kit.

2.2.2.2 Plasmid constructs for the yeast two-hybrid system

The following plasmids were constructed with recombinant DNA techniques according to Molecular Cloning (Sambrook et al., 1989).

(1) pGBT9-p36Δ1 : PCR fragment (pNIH as template, primers L1/L2) cut by EcoRI + Sal I was inserted into pGBT9. This subclone has the full length cDNA of G6Pase gene.

(2) pGBT9-p36Δ6 : PCR fragment (pNIH as template, primers H1/H4) cut by EcoRI+Sal I was inserted into pGBT9. This subclone has the first 448 nucleotides (1-448) of G6Pase gene.

(3) pGBT9-p36Δ10: PCR fragment (pNIH as template, primers H5/H6) cut by EcoRI+Sal I was inserted into pGBT9. This subclone has the 692 nucleotides (298-990) of G6Pase gene.

(4) pGAD424-p36Δ1 : EcoRI + SalI fragment of pGBT9- G6PΔ1 was inserted into pGAD424 (AD vector of two-hybrid system).

(5) pGAD424-p36Δ6: EcoRI + SalI fragment of pGBT9- G6PΔ6 was inserted into pGAD424 (AD vector of two-hybrid system).

(6) pGAD424-p36Δ10 : EcoRI + SalI fragment of pGBT9- G6PΔ10 was inserted into pGAD424 (AD vector of two-hybrid system).

(7) PGBT9-p46 : PCR fragment (pcDNAI-p46 as template, primers L3/L4) cut by EcoRI+Sal I was inserted into pGBT9.

The corresponding fragments of Δ6 and Δ10 on p36 cDNA are shown in Figure 8, while Δ1 represents the full- length cDNA of p36.

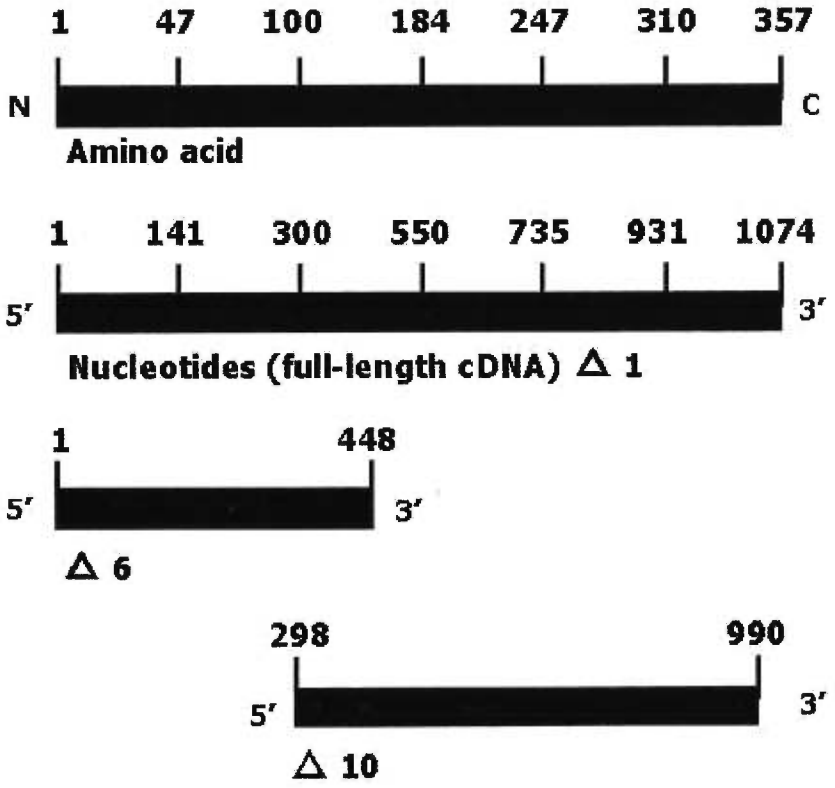


Figure 8: Scheme of cDNA fragments of p36 gene used in the yeast two-hybrid system.

Primers used for plasmid construction are as follows (from 5'to 3'):

P1 (L1): GCGGAATTCATGGAGGAAGGAATG

P2 (L2): GCAGGTCGACGTTACAACGACTTCTTG

P3 (H1): GAAGAATTCATGGAGGAAGGAATG

P4 (H4): GCAGGTCGACGTTACCGAAATCTGTAGGT

P5 (H5): GAAGAATTCCCCCTGATAAAGCAG

P6 (H6): GCAGGTCGACGTTAACTCTTGCAGAAGGA

P7 (L3): GCAGAATTCGGCTGGTAGGGCCTGCTC

P8 (L4): GCAGTCGACTGGACTCTCTTCACTCAG

All of the above plasmids were verified by restriction endonuclease analysis. pGBT9-p36-Δ1, -Δ6 and -Δ10 were also verified by partial sequencing. The sequencing primers were: Primer 1: 5'-TCATCGGAAGAGAGTAG-3'; Primer 2 : 5'-TACCACTACAATGGATG-3'. The autonomous reporter gene activation and yeast cell toxicity were also checked and none of the above construct was toxic or able to activate reporter gene transcription.

2.2.2.3 Cotransformation

In order to perform cotransformation, competent yeast cells were prepared by the LiAc method. This method typically results in a transformation efficiency of 10^5 per μg of a single type of DNA or 10^4 per μg of two types of DNA. To screen the library, cotransformation could be done either

simultaneously or sequentially with the PEG/LiAc method according to the protocol. pGBT9-p36Δ1, Δ6, Δ10 and pGBT-p46 were used to screen the human liver cDNA library and human leucocyte cDNA library. In addition, the following combinations were used to detect interactions between two known proteins:

(1) pGBT9-p36Δ1 + pGAD424-p36Δ1

(2) pGBT9-p36Δ6 + pGAD424-p36Δ6

(3) pGBT9-p36Δ10 + pGAD424-p36Δ10

(4) pGBT9-p36Δ1 + pGAD424-p36Δ6

(5) pGBT9-p36Δ1 + pGAD424-p36Δ10

(6) pGBT9-p36Δ6 + pGAD424-p36Δ1

(7) pGBT9-p36Δ6 + pGAD424-p36Δ10

(8) pGBT9-p36Δ10 + pGAD424-p36Δ1

(9) pGBT9-p36Δ10 + pGAD424-p36Δ6

(10)pGBT9-p46 + pGAD424-p36Δ1

(11)pGBT9-p46 + pGAD424-p36Δ6

(12)pGBT9-p46 + pGAD424-p36Δ10

2.2.2.4 Detection

Cotransformed colonies were detected by colony lift β-galactosidase filter assay. Briefly, VWR 410 grade filters were presoaked in a buffer

containing X-gal, substrate of β-galactosidase. Colonies were lifted onto another dry filter and cells were permeabilized by freeze/thaw with liquid nitrogen. Then the filters with the cells were put on the presoaked filters with the cells facing up. After incubation at 30 °C, positive colonies would turn blue within 30 minutes to 8 hours.

2.3 Construction of recombinant virus AdCMV-p46 and overexpression of p46

2.3.1 Introduction

Recombinant virus mediated gene transfer is a valuable tool to investigate metabolic regulatory mechanisms and to understand the function of newly discovered proteins. Among different viral vectors, adenovirus is of particular interest for metabolic engineering because it mediates high-efficiency (>90%) transfer of genes into mammalian cells, including primary cells with low replicative activity such as hepatocytes and cells from the islets of Langerhans. This allows rapid and efficient modulation of the activity of specific proteins within metabolic pathways. Other advantages of the adenovirus include its capacity of expressing relatively large (up to 7 kb) DNA inserts and its abilities to propagate high-titer viral stock, to infect an extremely

broad range of mammalian cells and also to transfer foreign genes *in vivo* (such as liver) by infusion (Becher et al., 1994; Antinozzi et al., 1999).

Because of all these advantages of recombinant adenovirus, this technique has been used to study the metabolic impact and functions of the catalytic subunit of G6Pase (p36) (Seoane et al., 1997). Here we used the same technique to overexpress the putative G6P translocase (p46) to see its metabolic impacts. Figure 9 is a scheme showing the general strategy of preparing recombinant adenovirus.

2.3.2 Methods

2.3.2.1 Construction of plasmid pACCMV-p46

The plasmid pcDNA1.1-p46 containing the full length cDNA of p46 provided by Dr. Van Schaftingen was cut by restriction enzymes Hind III and Xho I and filled in at 5' with Klenow. The recombinant adenovirus vector pACCMV.pLpA, which consists of Ad5 sequence, was cut by EcoR I and Sal I and filled in with Klenow. Then these two blunt end fragments were ligated into p46 expression plasmid (pACCMV-p46), which will be used for recombinant adenovirus construction. The plasmid construction is shown in Figure 10.

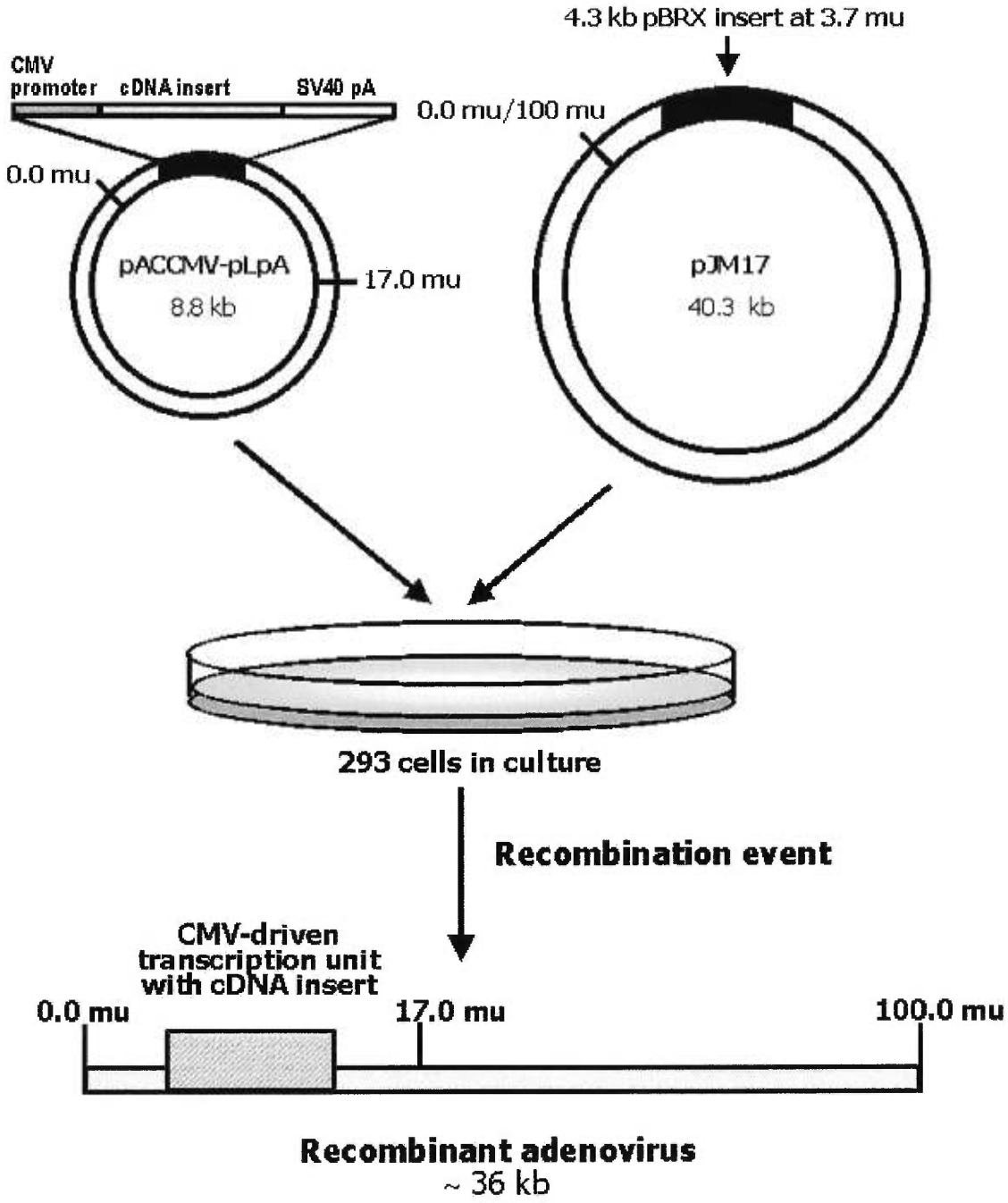


Figure 9: General strategy for preparing recombinant adenovirus by homologous recombination. The cDNA of interest is cloned into the pACCMVpLpA vector. The plasmids pAC and pJM17 are purified and co-transfected into 293 cells, where homologous recombination occurs. Since 293 cells were originally produced by adenovirus transformation, the missing E1 gene function of pJM17 is provided in trans. The final product is a recombinant adenovirus that is replication defective but fully infectious.

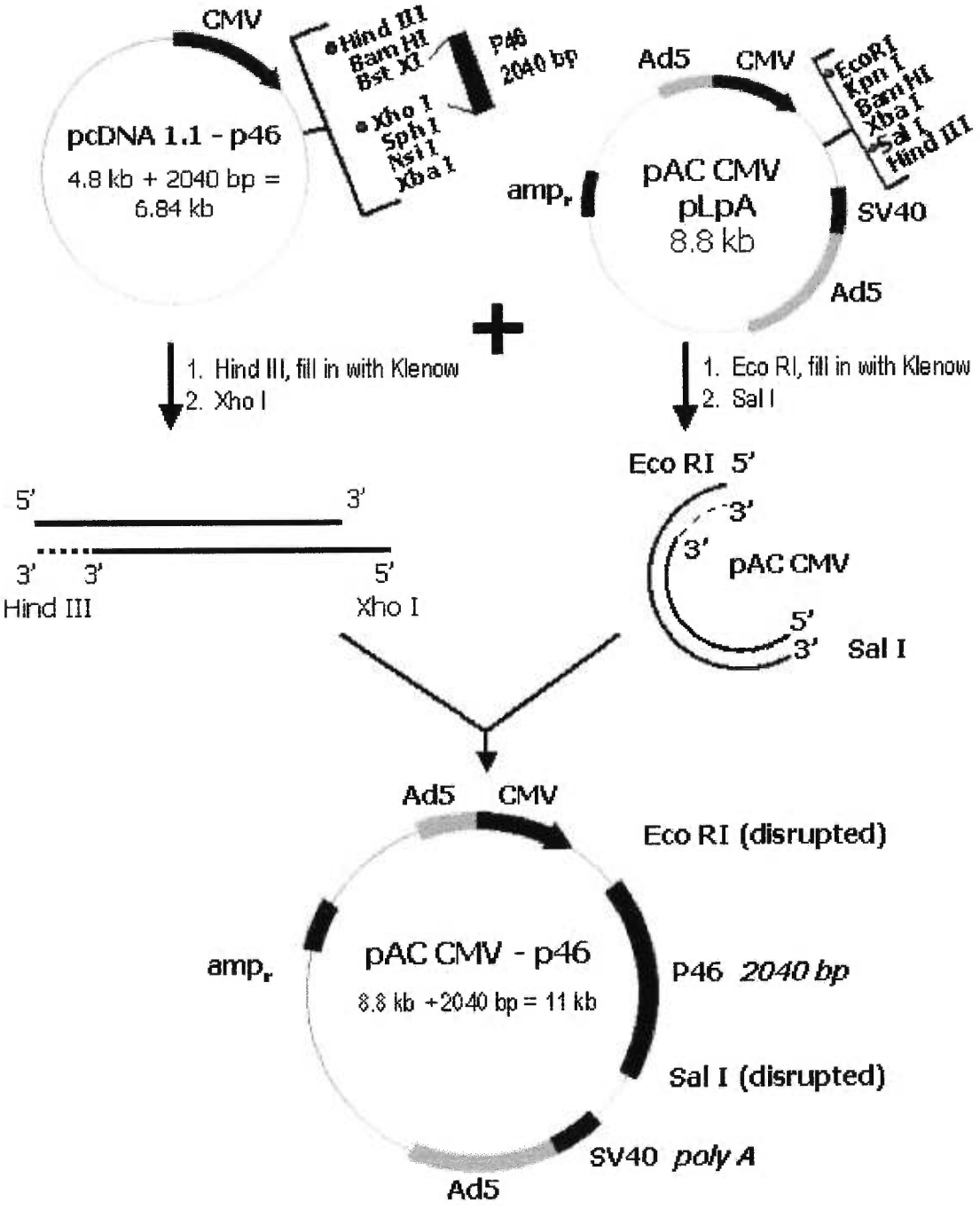


Figure 10: Construction of the plasmid pAC CMV - p46

2.3.2.2 Amplification and purification of plasmid

The plasmid pJM17 was derived from Ad5 containing insertions and deletions in the E3 gene. Since it is a large plasmid (40 kb), the preparation of pJM17 poses particular problems for its propensity of shearing during handling. Usually, to amplify pJM17, single clones were picked up from freshly transformed plates and amplified in small volume of LB (15 ml) because large-scale amplification has high risk for shearing. After overnight incubation at 37 °C with shaking, plasmids are isolated and purified by using QIAGEN miniprep kit. The quality of the purified pJM17 DNA is determined by digestion with Hind III and the DNA with minimum of shearing will reslove as a distinct ladder of 7-8 bands between 2 and 8 kb on a 0.8% agarose gel.

2.3.2.3 Co-transfection of 293 cells

The E1-transformed 293 cell line is maintained in 60-mm plates at 37 °C in an atmosphere of 5% CO_2 in high-glucose (4.5 g/liter) DMEM containing 10% fetal bovine serum and 100 U/ml penicillin/streptomycin. Five ug total DNA was transfected into 40-80% confluent 293 cells with SuperFect Transfection Reagent (QIAGEN). The ratio of DNA was JM17: pACCMV-p46 was 3:2. After transfection, cells were maintained by changing media

every 4-5 days. When the recombinant event occurs in the co-transfected 293 cells, the resulting recombinant virus completes the life cycle in the permissive host, resulting in cell lysis and the formation of plaques of dead cells. Usually the complete cell lysis occurs after 2-4 weeks. In this case, the monolayer of 293 cells was completely lysed in 12-20 days. The cell lysate was collected and centrifuged in order to get rid of the pellet of cell debris. The viral supernate was stored at –20 °C. The recombinant adenovirus (AdCMV-p46) with the full-length cDNA of p46 was verified by Southern blot and Western blot analysis.

2.3.2.4 Treatment of hepatocytes with adenovirus

Primary hepatocytes were treated with AdCMV-p46 and control virus AdCMV-βgal for 2 h in serum-free DMEM, then washed off with PBS and further cultured in DMEM medium with 25 mM glucose, 0.2% BSA, 10 nM dexamethasone and 1 nM insulin.

2.3.2.5 G6Pase activity measurement

After 24 h of further incubation, cells were washed with PBS and harvested by scraping into 50 mM Tris-Hepes, 250 mM sucrose buffer, then immediately frozen in liquid nitrogen and stored at -80 °C. In order to measure

the G6Pase hydrolytic activity, frozen cells were thawed on ice for permeabilization and then centrifuged for 1 min at 16,000g. The resultant pellet was resuspended in 50mM Tris-Hepes, 250 mM sucrose buffer. Both the supernate and the pellet were used for activity measurement.

2.4 Intracellular localization of p36

2.4.1 Introduction

Caveolae are often seen as small caves or flask-shaped membrane micro-invaginations (approximately 50-100 nm) associated with the plasma membrane of a wide variety of cells. They are enriched in glycosphingolipids, cholesterol, sphingomyelin, and lipid-anchored membrane proteins and are characterized by a light buoyant density and resistance to solubilization by Triton X-100 at 4 °C. The chief structural proteins of caveolae are the caveolins. Up to now, three members of caveolin family have been found, termed caveolin-1, 2 and 3. Proteins associated with caveolae include G protein-coupled receptors, G proteins and adenylyl cyclase, molecules involved in the regulation of intracellular calcium homeostasis, the endothelial isoform of nitric oxide synthase, multiple components of the tyrosine kinase-mitogen-activated protein kinase, and numerous lipid signaling molecules (Okamoto et al, 1998; Engelman et al, 1998). Particularly, the insulin receptor

is restricted to caveolae and colocalized with caveolin; insulin signaling is caveolae-dependent (Gustavsson et al, 1999).

More recent work has indicated that caveolae are not statically attached to the cytoplasmic surface of the plasma membrane but rather are moving to other intracellular organelles. Since intracellular membranes are continuous systems, we hypothesized that as an ER-associated protein, G6Pase might also be associated with caveolae and be directly regulated by interacting with those signal transduction molecules in caveolae.

2.4.2 Methods

2.4.2.1 Construction of plasmid

The pcDNA3.1/His vector (Invitrogen, CA) is a mammalian expression vector that can add N-terminal fusion tag to the expressed protein for detection (by using anti-tag antibody) and purification (by using polyhistidine-binding resin) purposes. Here an EcoR I-Sal I fragment containing full length p36 cDNA was inserted in frame into the pcDNA3.1/HisC vector at the EcoR I-Xho I sites to obtain tagged-p36 expressing plasmid pcDNA3.1/His-p36 as shown in Figure 11.

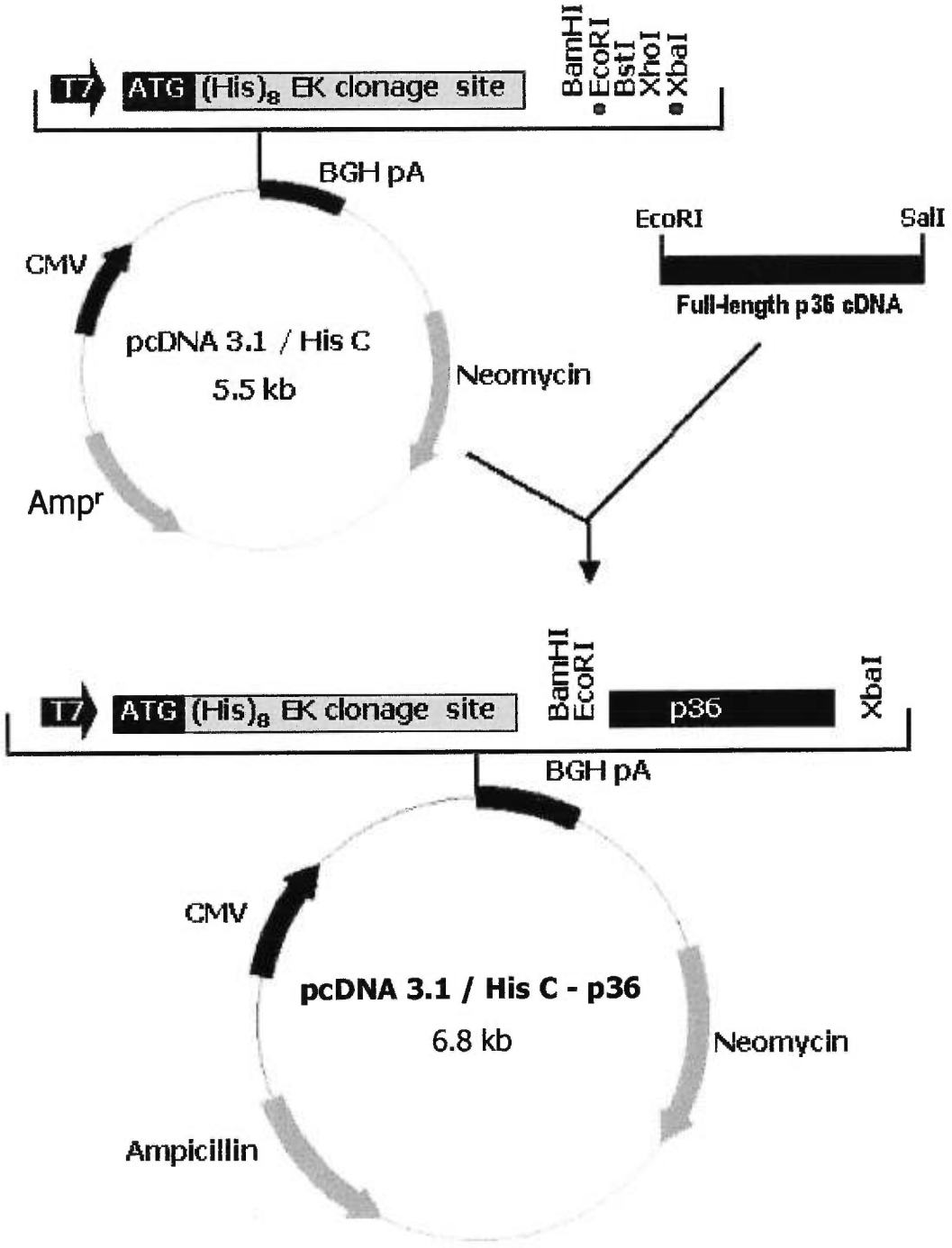


Figure 11: Construction of the plasmid pcDNA 3.1 / His-p36

2.4.2.2 Transfection of COS-7 cells

COS-7 cells are cultured in DMEM with 25 mM glucose and 5% CO_2 at 37 °C. One day before transfection cells were plated in 100-mm plates without antibiotics in the media. Four μg DNA of pcDNA3.1/His-p36 was mixed with appropriate amount of LIPOFECTAMINE PLUS reagent (Life Technologies Inc.). Mock transfection was performed with the same amount of pcDNA3.1 vector DNA. Transfection lasted 6 h in serum-free medium and was then replaced by fresh media containing 10% FBS. After 24-48 h incubation at 37 °C in 5% CO2, cells were collected by scraping into 1 ml lysis buffer, which contains 50 mM Tris.Cl (pH 8.0); 150 mM NaCl; 0.02% sodium azide; 100 μg/ml PMSF; 1 μg/ml aprotinin; 1% Triton 100.

2.4.2.3 Isolation of caveolae fraction

Cell lysate was loaded atop a 5-20% sucrose density gradient and subjected to centrifugation for 4 h at 100,000 rpm. After centrifugation, gradient fractions were collected, of which caveolae should be at fractions 4 and 5.

2.4.2.4 Detection of expressed tagged-p36 by Western blotting

To detect the recombinant protein, cell lysates are thawed on ice and an aliquot containing 20 μg of protein is boiled in 4X loading buffer to denature the protein. Samples are loaded on a 10% SDS-PAGE gel and the migration is performed at 100 V for about 1.5 h. Then the gel is transferred onto a nitrocellulose membrane eletrophoretically at 100 V for 1 h and the resulting membrane is first saturated with 5% milk for 1 h and then undergoes two rounds of immunodetection. The membrane is first incubated with 1:5000 diluted Anti-Xpress antibody overnight with agitation at 4 °C followed by three washes with TBST and is secondly detected with 1:30,000 diluted alkaline phosphatase conjugated anti-mouse IgG antibody followed by three washes with TBST. BCIP/NBT is used as the colour substrate of alkaline phosphatase. In parallel, anti-caveolin-1 antibody was used to verify the correct fractionation since caveolin-1 is the marker protein of caveolae (this experiment was done by Dr. Annabi, Montréal).

2.5 Detection of different transcripts of the putative G6P translocase in human leukocytes

2.5.1 Introduction

Glycogen storage disease type I (GSD-I) was classified into four subtypes as Ia, Ib, Ic and Id, according to the possible deficiencies of four putative proteins of the glucose-6-phosphatase (G6Pase) system. GSD-Ib and Ic are not due to mutations of the G6Pase catalytic subunit, as in GSD-Ia (Lei et al., 1995; Veiga-da-Cunha et al., 1998), but patients often have neutropenia and neutrophil dysfunction in addition to impaired liver G6Pase (Lange et al., 1980; Gitzelmann et al., 1993). Up to now, different mutations of the G6Pase catalytic subunit (p36) have been found in GSD-Ia patients from various sources, while other types of GSD-I are not associated with defects of this catalytic subunit. Recently, a putative glucose-6-phosphate translocase (p46) cDNA was cloned and two mutations of this gene at the genomic level were identified in two GSD-Ib patients (Gerin et al., 1997). The p46 gene was further localized on chromosome 11q23 (Veiga-da-Cunha et al., 1998), in agreement with the previous work of chromosome localization of GSD-Ib on chromosome 11q23 (Annabi et al., 1998) and GSD-Ic on chromosome 11q23.3-24.2 (Fenske et al., 1998). Furthermore, the p46 gene was characterized to have nine exons, spanning approximately 4kb at the genomic level (Marcolongo et al., 1998). Since the discovery of the p46 cDNA, more mutations, including point mutations, deletions and insertions, have been found by different groups. All these mutations strongly suggest that this p46 gene is tightly linked to the G6Pase system and GSD-Ib and Ic diseases. However, p46

might be more that a G6P transporter, but till now, functional test data of this gene were not available.

Most clinical cases of GSD-Ib and Ic indicated that neutropenia and recurrent infections were associated with these two subtypes of GSD-I. Since G6P transport in neutrophils is not related to G6P hydrolysis (G6Pase mRNA, protein and activity being undetectable in these cells, unpublished observations from our laboratory), the mutations in the p46 gene have to be explained by another impairment than that of the G6Pase system. We therefore decided to investigate the transcription of this p46 gene in human leukocytes.

2.5.2 Materials and methods

2.5.2.1 Materials

The human leukocyte Matchmaker cDNA library was purchased from CLONTECH Inc. (Palo Alto, CA). The mRNA source of the library was a pool of 6 h and 24 h PHA-stimulated peripheral blood leukocytes from four adult Caucasian males. Taq DNA polymerase and all the restriction enzymes were from Pharmacia Biotech (Baie d'Urfé, QC).

2.5.2.2 Amplification of human leukocyte cDNA library

As described in 2.2.2.1.

2.5.2.3 PCR and subcloning

Two primers were designed according to the published sequence of a putative G6P translocase (Gerin et al., 1997). The forward primer, P1, (5′-GCAGAATTCGGCTGGTAGGGCCTGCTC-3′), was complementary to the sequence before the ATG (position 143-160); the reverse primer, P2, (5′-GCAGTCGACTGGACTCTCTTCACTCAG-3′), was complementary to the sequence between position 1452-1469, including the stop codon [the position number corresponds to the previously published sequence of the putative glucose-6-phosphate translocase (Gerin et al., 1997)]. Ten ng of library DNA were denatured at 94°C for 10 min, then PCR was carried out as follows: 94°C, 1 min; 50°C, 45 sec; 72°C, 1 min 30 sec for 30 cycles followed by elongation at 72°C for 5 min. The products were separated on a 1% agarose gel. DNA bands were cut off and purified using the GLASSMAX DNA Isolation Matrix System (GIBCO BRL). The isolated DNA was then ligated into the pCR2.1-TOPO cloning vector (Invitrogen) with blue/white screening.

2.5.2.4 Total RNA extraction and mRNA isolation

Total RNA was extracted from 10 ml human heparin blood of healthy volunteers with TRIzol LS Regent (GIBCO BRL). The Oligotex™ mRNA Midi Kit (QIAGEN) was then used for mRNA isolation from 100 μg total RNA.

2.5.2.5 Nested RT-PCR

A Titan™ One Tube RT-PCR System (Boehringer Mannheim) was used for RT-PCR. The reverse transcription condition was at 50°C for 30 min, then the template was denatured at 94°C for 2 min, followed by 40 cycles of amplification at 94°C (30 sec), 60°C (30 sec), 68°C (1 min), and elongated at 68°C for 7 min. The complete p46 coding region was amplified with outer primers, P1 and P2. Nested PCR was performed with inner primers specific for each transcripts, P3: (5'-GACAGTCAGCCCTTGTAG-3') and P4: (5'-GCTCCCAATACCAGGATCC-3') to detect α; P5: (5'-GACTCCCCCAAGGATGTTGC-3') and P4 to detect β; P6: (5'-GTGAGTGCTTCCAAAGAG-3') and P7: (5'-GTAGGTGTCCAGCCTAC-3') to detect γ; P8: (5'-GCAAAGGTGAGCGGGAGAAG-3') and P9: (5'-CAGTCCCGCCTGTGCCCTG-3') to detect δ. PCR condition was first at 94°C for 5 min, then 30 cycles at 94°C (30 sec), 60°C (30 sec), 72°C (30 sec) and followed by 72°C for 10 min.

3- ARTICLES

3.1 Article 1

Up-regulation of liver glucose-6-phosphatase in rats fed with a Pi-deficient diet. Wensheng Xie, Yazhou Li, Marie-claire Méchin and Gérald van de Werve, Biochem. J. (1999) 343: 393-396

My contribution to this article was the characterization of the effect of Pi deprivation on the gene expression of both p36 and p46 at the transcriptional level, including total RNA extraction from rat livers and Northern blot analysis of p36 and p46 mRNA abundance.

Up-regulation of liver glucose-6-phosphatase in rats fed with a phosphate-deficient diet

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Key words: Phosphate deficiency, glucose-6-phosphatase, rat liver microsomes

Running title: Up-regulation of liver glucose-6-phosphatase by a Pi-deficient diet

Abbreviations used: G6P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; p36, G6Pase catalytic subunit; p46, putative G6P translocase protein; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; NBT, nitro blue tetrazolium; EQU, tracer accumulation inside microsomes at transport equilibrium stage.

SYNOPSIS

Because phosphate (P_i) deprivation markedly affects the Na P_i cotransporter in kidney and has been related to insulin resistance and glucose intolerance, the effect of a P_i-deficient diet on the liver microsomal glucose-6-phosphatase (G6Pase) system was investigated. Rats were fed with a control diet ($+P_i$) or a diet deficient in phosphate ($-P_i$) during two days and sacrificed on the morning of the third day, after an overnight fast (fasted) or not (fed). Kinetic parameters of P_i transport ($t_{½}$ and equilibration) into liver microsomes were not changed by the different nutritional conditions. In contrast, it was found that G6Pase activity was significantly increased in the ($-P_i$) groups. This was due to an increase in the V_{max} of the enzyme, without change in the K_m for G6P. There was no correlation between liver microsomal glycogen content and G6Pase activity, but both protein abundance and mRNA of liver 36kDa catalytic subunit of G6Pase (p36) were increased. The mRNA of the putative G6P translocase protein (p46) was changed in parallel with that of the catalytic subunit, but the p46 immunoreactive protein was unchanged. These findings indicate that dietary P_i-deficiency causes increased G6Pase activity by upregulation of the expression of the 36 kDa catalytic subunit gene.

INTRODUCTION

The liver glucose-6-phosphatase (G6Pase) is a multiprotein complex, which resides in the endoplasmic reticulum membrane and plays an important role in the regulation of blood glucose concentration [1]. Until now, two components have been cloned: a 36 kDa catalytic subunit (p36) [2] and more recently a 46 kDa protein (p46) [3], that has been proposed to be a G6P transporter. P36 gene expression has now been well studied. Positive effectors that increase p36 mRNA include glucose, fatty acids, cyclic AMP and glucocorticoids, whereas insulin decreases p36 mRNA [4]. Accordingly, in fasting and diabetes, when the insulin/glucagon ratio is low, p36 mRNA, protein and activity are high [5]. Hormonal control of p46 has not yet been reported. Inorganic phosphate imbalance leads to a number of disorders [6,7]. Hypophosphatemia in several diseases (hypophosphatemic rickets, adult-onset hypophosphatemic osteomalacia, renal P_i leak) is associated with insulin resistance and glucose intolerance [8], but this association depends on the severity of hypophosphatemia [9]. A P_i-deficient diet causes upregulation of the expression of the rat kidney NaP_i-2 cotransporter [10-12], while a potential effect of P_i deprivation on the liver endoplasmic reticulum G6Pase complex, which includes a P_i transport system, has not been investigated.

Our results show that a P_i-deficient diet upregulates rat liver microsomal G6Pase activity by increased expression of the p36 catalytic subunit gene, but

without significant change in the abundance of the putative G6P translocase p46 protein.

EXPERIMENTAL

Materials

[^{32}P]KH_2PO_4 and [U-^{14}C]glucose-6-phosphate were from ICN Biomedicals (Montreal, QC) and [α-^{32}P]dCTP was from Pharmacia Biotech (Baie d'Urfé, QC). Bovine serum albumin, glucose-6-phosphate and amyloglucosidase were from Sigma (St Louis, MI). Hexokinase and glucose-6-phosphate dehydrogenase were purchased from Boehringer (Laval, QC). Molecular markers were purchased from Bio-Rad (Mississauga, ONT). The rabbit polyclonal antiserum against the recombinant G6Pase catalytic subunit (antibody against p36) was a kind gift of Dr. J.Y. Chou (N.I.H., Bethesda). The rabbit antiserum against N-terminal of p46 (GYGYYRTVIFSSAMFGGY) was produced in our laboratory. The BCIP/NBT stock solutions and the anti-rabbit IgG-AP conjugate were from the Promega Co (Madison, WI). TRIZOL LS Reagent was from Gibco BRL (Burlington, ONT).

Animals

Male Sprague-Dawley rats (300-350 g body weight) were used as the liver donor. Rats were fed for 48 hours with either a P_i-deficient diet (No. 86128) (-P_i) or a control (No. 86129) (+P_i) diet (Teklad, Madison, WI) containing

0.03% (w/w) or 1% (w/w) phosphate, respectively. All other components are the same in both diets. The fasted group had food removed 18 hours before sacrifice by decapitation while the fed group had constant access to food. According to these conditions, rats were classified into 4 groups: 1. Fed ($-P_i$); 2. Fed ($+P_i$); 3. Fasted ($-P_i$); 4. Fasted ($+P_i$). There were 5 rats in groups 1 to 3, and 6 rats in group 4.

Preparation of rat liver microsomes and G6Pase assay

Rat liver microsomes were isolated individually as described previously [13], except that the buffer was 250mM sucrose, 50mM Hepes-Tris at pH 7.3. G6Pase activity was assayed as in [14], before and after detergent (Chapso 0.8%) treatment.

Glycogen

Glycogen was asssayed in liver microsomes with the amyloglucosidase assay [15].

Phosphate transport

Zero-trans phosphate transport across the microsomal membrane was measured as described before [13].

Western blot analysis

Rat liver microsomes (50μg) were subjected to a 12% SDS-PAGE at 100V for about 1 hour in the Laemmli buffer. Then proteins were electro-transferred to a nitrocellulose membrane at 100V for 1 hour. The membrane was saturated for 30min in 100mM TBS, pH7.5, containing 10% milk. Membranes were

incubated in the primary antibody solution (diluted to 1/500 for anti-p36 or 1/200 for anti-p46). The membrane was washed and incubated in the alkaline phosphatase conjugated anti-rabbit IgG solution (diluted as specified) for 1 hour. The membrane was washed again and detected in BCIP/NBT substrate system. The membrane was scanned and the quantification of each specific band was analyzed by using a Dual Light™ Transilluminator.

Northern blot analysis

Total liver RNA was individually extracted with TRIZOL LS Reagent. The same amount of RNA was fractionated by 1% agarose-formaldehyde gel electrophoresis, then transferred to a nylon membrane by a capillary technique in 20×SSC solution. The membrane was rinsed in 2×SSC solution, dried and immobilised under UV light. The hybridization was performed with [α-^{32}P]dCTP labelled probe (full length cDNA of p36 or p46) overnight at 68°C. The membrane was washed properly and exposed to a film for a certain time. The intensities of the mRNA bands were determined using a densitometer (Dual Light™ Transilluminator).

Data analysis

Phosphate transport kinetic analysis was done according to Equation 1:

$$A = EQU\,(1-e^{-kt}) + A_o \qquad \text{(Equation 1)}$$

in which A represents the tracer accumulation into microsomes at time t, EQU is the tracer accumulation at equilibrium, k is the rate constant and A_o the radioactive background.

The parameter $t_{1/2}$, the time at which 50% of a process has been completed, was calculated using Equation 2:

$$t_{1/2} = Ln2 / k \qquad \text{(Equation 2)}$$

Statistical analysis was performed according to Students' t test. Differences were considered significant when the p value was less than 0.05.

RESULTS AND DISCUSSION

Pi transport

Renal Na P_i cotransport is markedly upregulated by dietary phosphate deprivation [10-12], but it is not known whether liver microsomal phosphate transport is also affected by this diet. Liver microsomes were prepared individually from each rat in the 4 experimental groups. Table I shows that the $t_{1/2}$ of tracer [^{32}P]KH_2PO_4 (10 μM) uptake into microsomes was about 23 seconds and did not change with the nutritional conditions (fasted or fed, + P_i or - P_i). The transport process reached equilibrium after about 2 min, as we documented before [13], and the tracer accumulation at that time (EQU) was about 9 pmol/mg. Similarly to the $t_{1/2}$, EQU values were not statistically different for the four experimental groups. These results show that the transport rate and the phosphate accessible space of liver microsomes were unaffected either by fasting or by a (-P_i) diet.

G6Pase activity

The reported association of phosphate deprivation and glucose intolerance [8,9] lead us to further investigate the liver G6Pase system in our experimental conditions, since G6Pase is responsible for hepatic glucose production and the maintenance of euglycemia. Fig. 1 shows that the activity of G6Pase, measured in liver microsomes with 0.2mM G6P before and after detergent treatment, was increased in both fed and fasted groups of ($-P_i$) rats compared to controls ($+P_i$). It can be seen that this diet-induced increase in activity was additive with the well-documented proper effect of fasting [16] as well as with that resulting from detergent treatment. The increase in G6Pase activity due to the ($-P_i$) diet was caused by an increase in V_{max} rather than a change in the K_m of G6Pase for G6P (Table II). Also shown in Table II, is that fasting increased significantly the V_{max} of G6Pase and that the K_m of G6Pase for G6P decreased in the presence of detergent, as has been shown before. From these results, it could be deduced that the ($-P_i$) diet upregulates G6Pase activity in liver.

It has been reported that glycogen-bound fatty acids regulate G6Pase activity and might be responsible for changes in this enzyme activity in the fasting-refeeding period [17]. We thus explored the possibility that increased G6Pase activity was due to a lower liver microsomal glycogen concentration in the ($-P_i$) group. This was however not the case. As shown in Fig. 2, the microsome glycogen content was decreased to a large extent by fasting, but there was no obvious correlation between glycogen content and G6Pase activity.

We further verified if upregulation of G6Pase activity by dietary P_i deprivation was caused by changes in the protein amount of G6Pase p36 catalytic subunit and/or of p46 putative G6P transporter.

Western analysis of p36 and p46 G6Pase protein

The abundance of immunoreactive microsomal protein was measured with a polyclonal antibody against the catalytic subunit of G6Pase. The visualized band was at a position corresponding to a M*r* of about 36kDa (Fig. 3A). The corresponding density scan results are shown in Fig. 3C. One can see that the effect of the ($-P_i$) diet on G6Pase activity was accompanied by a parallel increase in p36 catalytic subunit abundance and that, as for G6Pase activity, there was an additive effect of ($-P_i$) diet and fasting on this parameter.

The 46kDa component of G6Pase is a putative glucose-6-phosphate transporter, which may also be a regulator of the G6Pase catalytic subunit. It was thus possible that a ($-P_i$) diet has some effect on this protein, resulting in the change in G6Pase activity. An antibody against the N-terminal part of p46 was used to detect p46 protein abundance. The scan results of the specific band are shown in Fig. 3B. It is apparent that unlike p36, the p46 protein associated with microsomes was not significantly changed in the present experimental conditions (Fig. 3C).

Northern blot analysis of p36 and p46 G6Pase mRNA

In order to know if the increased p36 protein was due to increased synthesis or decreased degradation, the p36 mRNA abundance was measured. Northern

blots of p36 mRNA and p46 mRNA were performed with the full length cDNA of p36 or p46 as a probe, respectively. Fig. 4A shows a representative blot for p36 in the four experimental groups and Fig. 4C shows the quantification of the bands. It can be seen that fasting increases p36 mRNA, a result consistent with the corresponding Western blot and that has been reported previously [16]. Also in agreement with the immunoreactivity, p36 mRNA increased significantly in the ($-P_i$) diet groups as compared to the ($+P_i$) diet groups, showing that alimentary P_i deficiency affects p36 gene expression. The liver p46 mRNA changes were parallel to those of p36. Fig. 4 shows indeed that p46 mRNA abundance was increased with fasting, as documented before [18], as well as with the ($-P_i$) diet in the fed and fasted groups. The fact that these changes in mRNA were not associated with changes in protein (see Fig. 3), suggests either increased degradation of p46 or less translation of p46 mRNA. Interestingly, a low phosphate diet induces a post-transcriptional effect on parathyroid hormone gene expression that is related to the binding of parathyroid proteins to the 3'-untranslated region of parathyroid hormone mRNA; this low phosphate diet results in decreased stability of the transcript [19].

CONCLUSIONS

Contrary to effects with kidney, where dietary P_i deprivation markedly induces overexpression of the NaP_i-2 cotransporter, this diet does not affect

liver microsomal P_i transport. The present results show for the first time that P_i deprivation *in vivo* causes microsomal liver G6Pase upregulation. In our experimental conditions, an increase in the p36 catalytic subunit alone is sufficient to enhance G6Pase activity. This could in turn favor increased hepatic glucose production and be partially causal in the association found between hypophosphatemia and glucose intolerance.

We do not know how P_i deficiency leads to increased gene expression. Studies using cultured cells, in which the extracellular parameters can be controlled better than *in vivo*, could give us that information. It will also be interesting to see if other enzymes, besides G6Pase, involved in glucose production by the liver are also affected by P_i deprivation. The candidate genes to be affected by Pi-deficiency are glucokinase, pyruvate kinase (that are expected to be down-regulated) and phosphoenolpyruvate carboxykinase (that is expected to be upregulated). This work is in progress.

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FIGURE LEGENDS

Figure 1. Effect of fasting and a P_i-deficient diet on microsomal G6Pase activity

G6Pase activity was measured with 0.2 mM glucose-6-phosphate at 30°C for 2min. Results are mean values ± S.E.M. for each group (n = 5-6). Bars headed by the same letters are statistically different from each other by : *a* p< 0.001; *b* p< 0.02; *c* p< 0.01; *d* p< 0.01 (unpaired Student's *t* test).

Figure 2. Comparison of microsomal glycogen content and G6Pase activity

Glycogen content was measured in microsomes as described in the Methods section. G6Pase activity was measured at 0.2 mM G6P in intact microsomes at 30 °C for 2 min.

Figure 3. Immunoblot analysis of p36 and p46

(A, B) One representative visualization of specific bands of p36 and p46 respectively in the four experimental groups. (C) Quantification of specific bands for each group. The fed (+Pi) group was taken as control (100%). Results are mean values ± S.E.M. for each group (n = 5-6). Bars headed by the same letters are statistically different from each other by : *a* p< 0.02 ; *b* p< 0.02 (unpaired Student's *t* test).

Figure 4. Northern blot of mRNA of p36 and p46

(A, B) One representative visualization of p36 and p46 mRNA levels respectively in the four experimental groups. (C) Quantification of p36 mRNA and p46 mRNA of each group. The fed (+Pi) group was taken as control (100%). Results are mean values ± S.E.M. (n = 3-4). Bars headed by the same letters are statistically different from each other by : *a* p< 0.05; *b* p < 0.05; *c* p< 0.05; *d* p< 0.05 (Unpaired Student's *t* test).

Table I Kinetic parameters for phosphate transport in microsomes. Effect of fasting and of a P_i-deficient diet.

Groups of rats	$t_{1/2}$ (s)	EQU (pmol/mg protein)
Fed ($-P_i$)	23 ± 1.4	8.7 ± 0.3
Fed ($+P_i$)	22 ± 1.3	10.0 ± 0.4
Fasted ($-P_i$)	24 ± 0.9	9.0 ± 1.1
Fasted ($+P_i$)	22 ± 1.7	10.0 ± 1.7

Phosphate transport in intact microsomes was measured with tracer (10 μM) [^{32}P]KH_2PO_4 for different times up to 2 min at which equilibrium of uptake was reached. Results are means ± S.E.M for each group (n = 4-5). $t_{½}$ and EQU values are not statistically different from each other respectively in the four groups.

Table II Kinetic parameters for G6P hydrolysis in microsomes: Effect of fasting and of a P_i-deficient diet.

	V_{max} (munits/mg)		K_m (mM)	
Group of rats	Intact	Detergent-treated	Intact	Detergent-treated
Fed (-P_i)	176 ± 8 [a]	330 ± 10 [c]	2.66 ± 0.14	2.15 ± 0.19
Fed (+P_i)	132 ± 4 [a]	194 ± 8 [c]	2.68 ± 0.13	2.13 ± 0.08
Fasted (-P_i)	214 ± 3 [b]	381 ± 11 [d]	2.90 ± 0.10	2.26 ± 0.15
Fasted (+P_i)	158 ± 5 [b]	321 ± 9 [d]	2.70 ± 0.11	2.35 ± 0.47

The G6Pase activity of intact or detergent-treated microsomes was measured with 0.2, 0.5, 1, 2 and 5 mM G6P for 2 min at 30°C. V_{max} and K_m were calculated by non-linear regression of Michaelis-Menten kinetics. Results are mean values ± S.E.M for each group (n = 5-6). Values in each vertical column, headed by the same letters, are statistically different from each other by $p < 0.02$ (unpaired Student's *t* test).

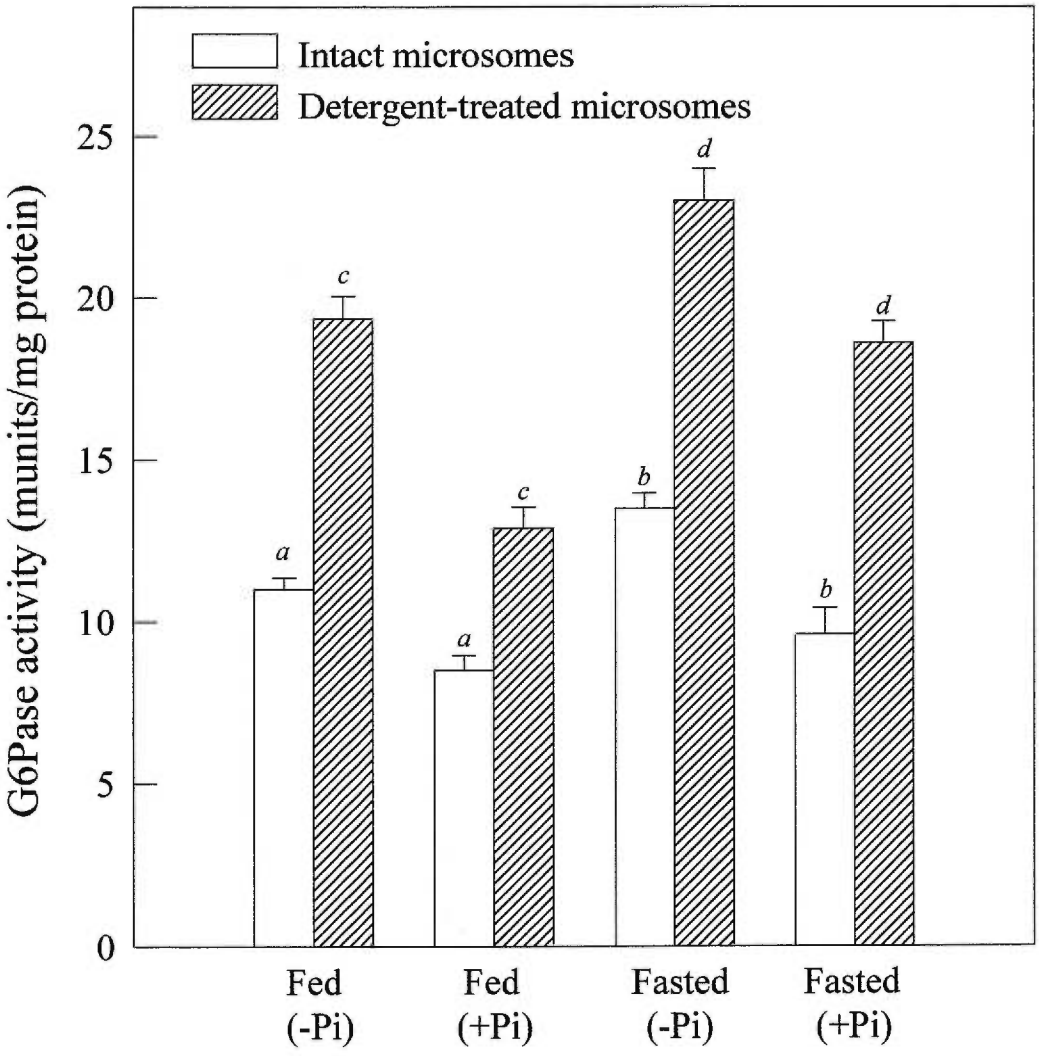

Intact microsomes
Detergent-treated microsomes
G6Pase activity (munits/mg protein)
0
5
10
15
20
25
a
c
a
c
b
d
b
d
Fed (-Pi)
Fed (+Pi)
Fasted (-Pi)
Fasted (+Pi)

Figure 1

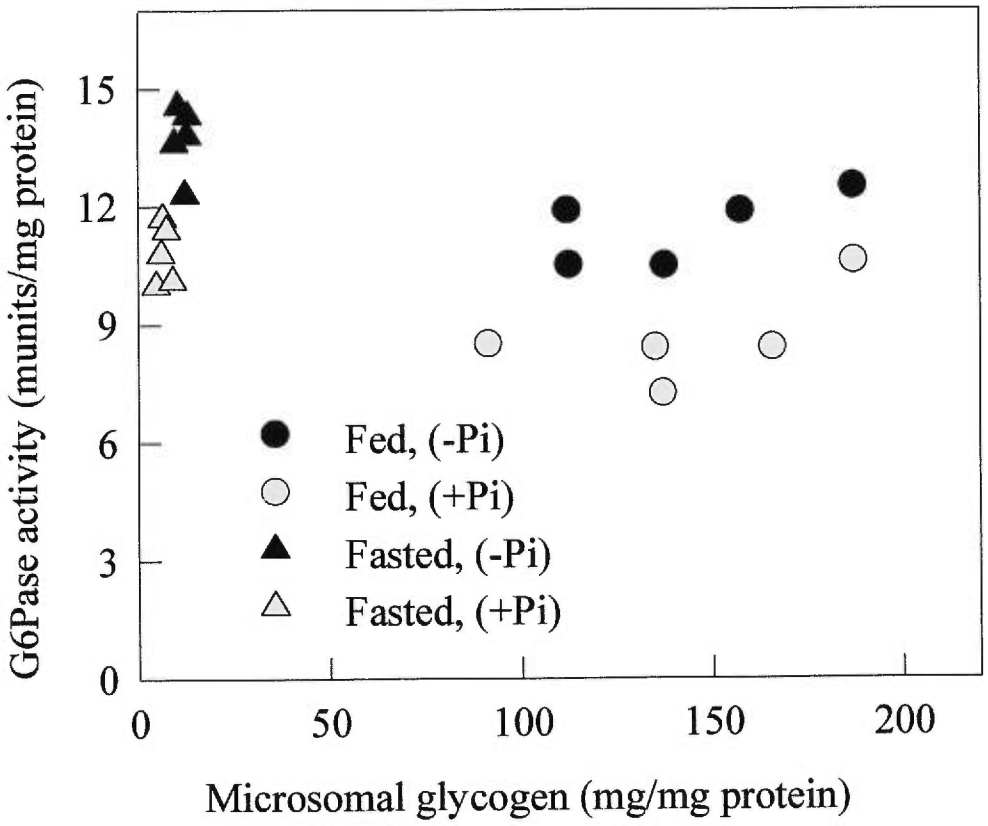
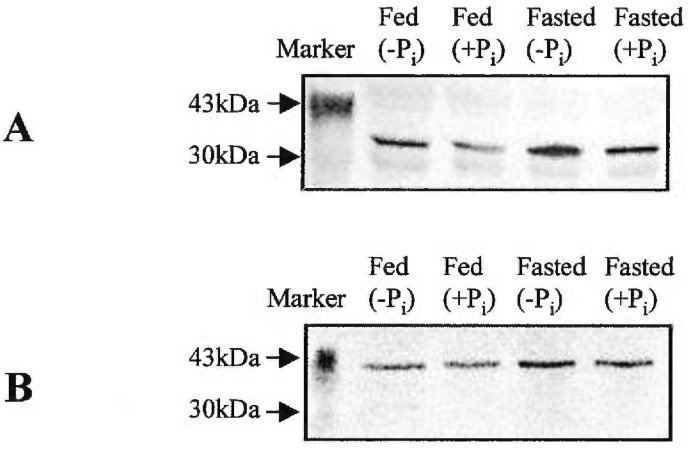

G6Pase activity (munits/mg protein)
15
12
9
6
3
0
0
50
100
150
200
Microsomal glycogen (mg/mg protein)
Fed, (-Pi)
Fed, (+Pi)
Fasted, (-Pi)
Fasted, (+Pi)

Figure 2


A
Marker
Fed (-Pi)
Fed (+Pi)
Fasted (-Pi)
Fasted (+Pi)
43kDa
30kDa
B
Marker
Fed (-Pi)
Fed (+Pi)
Fasted (-Pi)
Fasted (+Pi)
43kDa
30kDa

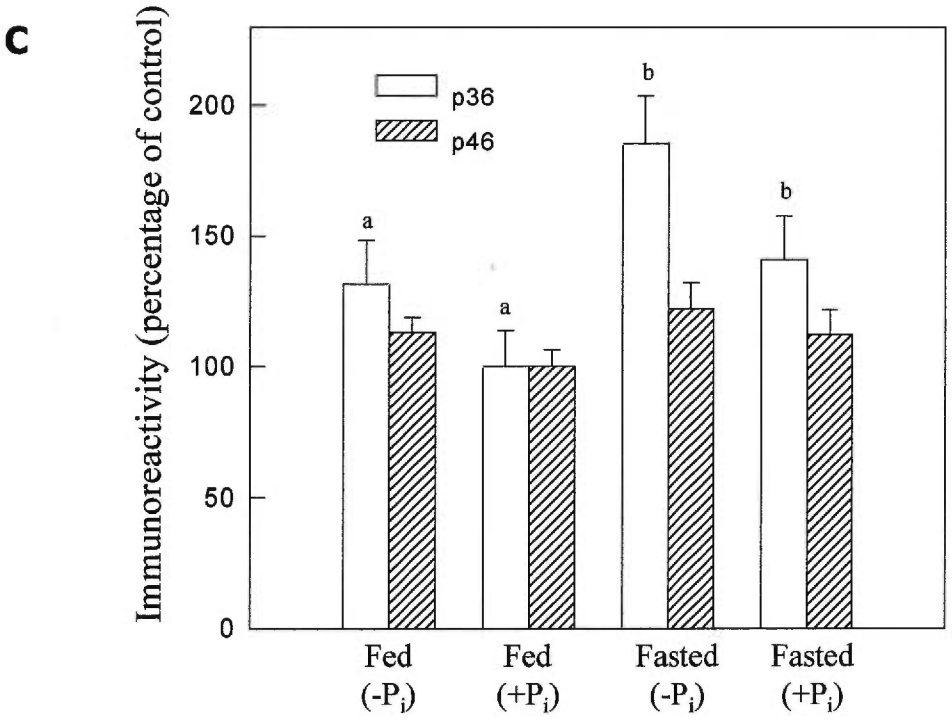

C
Immunoreactivity (percentage of control)
p36
p46
200
150
100
50
0
a
a
b
b
Fed (-Pi)
Fed (+Pi)
Fasted (-Pi)
Fasted (+Pi)

Figure 3

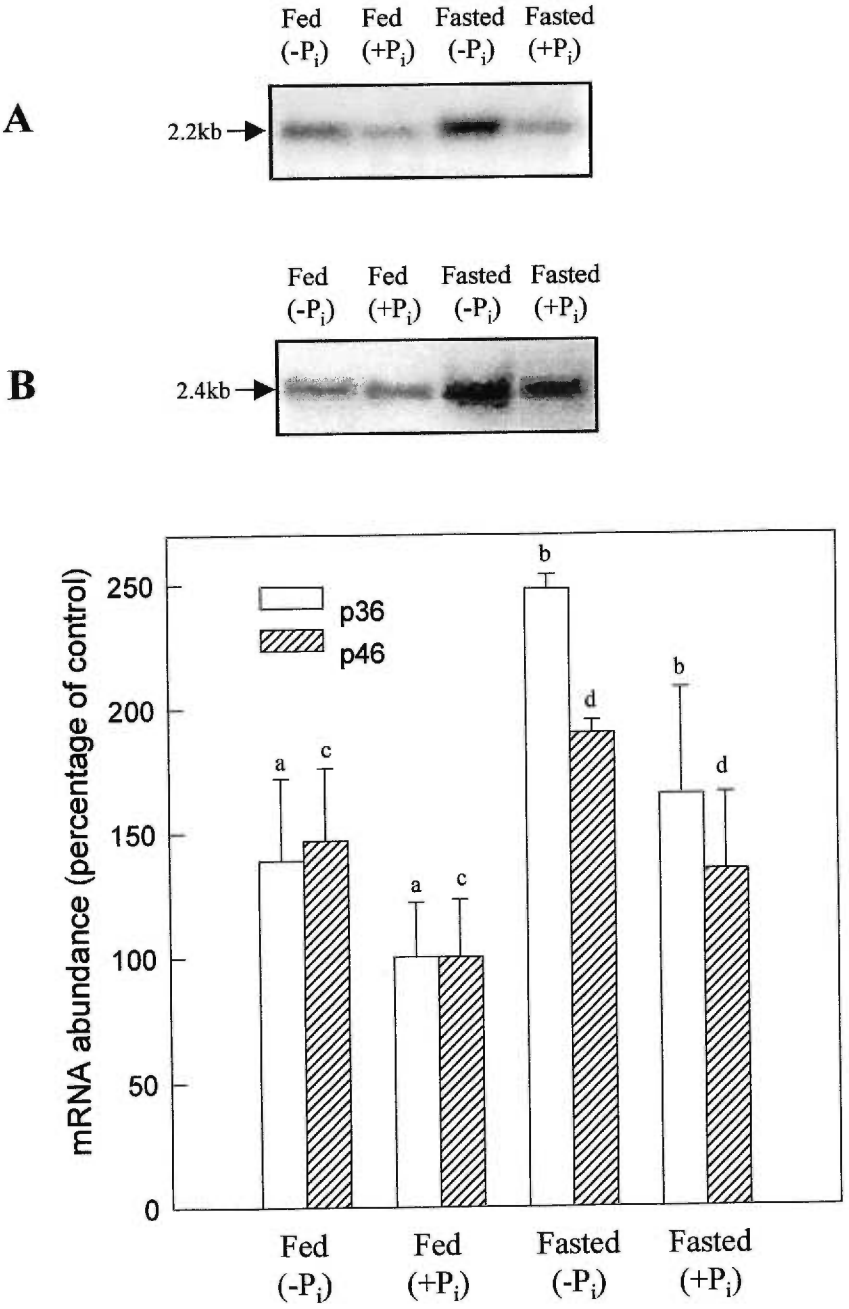

A
Fed (-Pi)
Fed (+Pi)
Fasted (-Pi)
Fasted (+Pi)
2.2kb
B
Fed (-Pi)
Fed (+Pi)
Fasted (-Pi)
Fasted (+Pi)
2.4kb
C
mRNA abundance (percentage of control)
250
200
150
100
50
0
p36
p46
a
c
a
c
b
d
b
d
Fed (-Pi)
Fed (+Pi)
Fasted (-Pi)
Fasted (+Pi)

Figure 4

3.2 Article 2

Diabetes affects similarly the catalytic subunit and putative glucose-6-phosphate translocase of glucose-6-phosphatase. Yazhou Li, Marie-Claire Méchin, and Gérald van de Werve, J. Biol. Chem. (1999) 274:33866-33868

Diabetes Affects Similarly the Catalytic Subunit and Putative Glucose 6-Phosphate Translocase of Glucose 6-Phosphatase*

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Running title: Glucose 6-phosphatase components in diabetes

[1] Abbreviations used in the manuscript are: G6Pase, glucose 6-phosphatase; G6P, glucose 6-phosphate; p36, catalytic subunit of G6Pase; p46, putative G6P translocase; MIX, methyl isobutylxanthine.

Key Words: diabetes; glucose; insulin; cyclic AMP; glucose 6-phosphatase; putative glucose 6-phosphate translocase.

SUMMARY

The effect of streptozocin diabetes on the expression of the catalytic subunit (p36) and the putative glucose 6-phosphate translocase (p46) of the glucose 6-phosphatase system (G6Pase) was investigated in rats. In addition to the documented effect of diabetes to increase p36 mRNA and protein in liver and kidney, a ~2-fold increase in the mRNA abundance of p46 was found in liver, kidney and intestine, and a similar increase in p46 protein level in liver. In HepG2 cells, glucose caused a dose-dependent (1-25 mM) increase (up to 5-fold) in p36 and p46 mRNA and a lesser increase in p46 protein, whereas insulin (1 μM) suppressed p36 mRNA, reduced p46 mRNA level by half and decreased p46 protein by about 33 %. Cyclic AMP (100 μM) increased p36 and p46 mRNA by >2 and 1.5-fold respectively, but not p46 protein. These data suggest that insulin deficiency and hyperglycemia might each be responsible for upregulation of G6Pase in diabetes. It is concluded that enhanced hepatic glucose output in insulin-dependent diabetes probably involves dysregulation of both the catalytic subunit and the putative glucose 6-phosphate translocase of the liver G6Pase system.

INTRODUCTION

A number of previously reported glucose 6-phosphatase (EC 3.1.3.9) activity changes have been shown to be associated with long-term regulation in the 36 kDa catalytic subunit (p36) mRNA level. Insulin deficiency achieved by acute fasting or experimental diabetes in the rat increases G6Pase activity (1), and p36 mRNA (2), regardless of glycemia (3, 4). Studies in Fao hepatoma cells showed that insulin suppressed p36 mRNA levels and that cyclic AMP (cAMP) counteracted this effect (5), whereas cAMP alone increased p36 mRNA by a factor of four (6). Liu *et al.* (2) observed that blood glucose concentrations correlated with G6Pase mRNA levels, suggesting that G6Pase transcription might be controlled by glucose or a glucose-derived metabolite. This presumption was later verified by direct demonstration of glucose (7) and fructose 2,6-bisphosphate (8) stimulation of p36 expression.

A major breakthrough in the G6Pase field has been the finding of a human cDNA sequence encoding a 46 kDa protein (p46) homologous to bacterial phosphate ester transporters (9). Mutations of the p46 gene were found in glycogen storage disease type 1b (GSD-1b) patients, which have impaired G6Pase activity but normal p36 catalytic subunit (9). It was therefore proposed that p46 was the putative G6P translocase of the G6Pase system. Until now, there has been no report on the regulation of the expression of p46 and its role in the control of G6Pase activity is unknown. Here we show that p46 mRNA and protein are induced by glucose and are repressed by insulin, in parallel to p36 and that p36

and p46 mRNA are increased by cAMP. These results may explain the present finding that both the catalytic subunit and the putative G6P translocase are impaired in insulin-dependent diabetes.

EXPERIMENTAL PROCEDURES

Animals-Male Wistar rats (200-250 g) (Charles River, Canada) were given one intravenous administration of streptozocin (75 mg/kg) (Upjohn, Ontario), dissolved in citrate, pH 4.0 (n = 6) or vehicle alone (n = 7). The diabetic state was assessed by the presence of hyperglycemia after a 24-hour fasting period, by glucosuria and by a lesser weight gain. The two groups were sacrificed by decapitation after an overnight fast, one week after the treatment. Blood was obtained as well as tissue (liver, heart, hindlimb skeletal muscle, kidney, small intestine and brain) samples, that were quickly freeze-clamped in liquid nitrogen and kept at –80 °C for subsequent analysis.

Preparation of rat liver microsomes and G6Pase assay-Rat liver microsomes were isolated individually from each rat as described previously (10), and resuspended at a protein concentration of about 7 mg/ml in 50 mM Tris-Hepes, pH 7.3, 250 mM sucrose. G6Pase activity was assayed with 5 mM [U-^{14}C]G6P after detergent (Chapso 0.8%) treatment as described in (10).

Cell culture-HepG2 cells were maintained in Dulbecco's Modified Eagle Medium containing 10% bovine fetal serum. To investigate the effect of glucose, cells were kept in the presence of the indicated glucose concentrations for 48 h at

37 °C. Insulin (1 μM) or cAMP [0.5-100 μM, in the presence of 50 μM methyl isobutylxanthine (MIX) to inhibit cAMP phosphodiesterases], were added to 80-90 % confluent HepG2 cells in serum-free DMEM for the indicated times, in the presence of 5 mM glucose. Cells were harvested by scraping after different times of glucose or hormone treatment and kept at –80 °C for subsequent analysis.

Immunoblotting analysis- The rabbit polyclonal antiserum against the recombinant G6Pase catalytic subunit (antibody against p36) was a kind gift of Dr. J.Y. Chou (N.I.H., Bethesda). The rabbit antiserum against the N-terminus of p46 (GYGYYRTVIFSSAMFGGY) was produced in our laboratory as explained in Xie *et al.*, 1999 (11) and according to the methods described in detail by Mechin *et al.*, 1996 (12). Microsomal fractions isolated from different tissues or HepG2 cell homogenates (50 μg protein) were subjected to a 12 % SDS-PAGE at 100 V for about 1 h in Laemmli buffer (13). Proteins were then electro-transferred to a nitrocellulose membrane at 100 V for 1 h. The membrane was saturated for 1 h in 100 mM TBS, pH 7.5, containing 10% dried milk (w/v) and further incubated overnight in the primary antibody solution (diluted to 1/500 for anti-p36 or 1/200 for anti-p46). After washing, the membrane was incubated in the alkaline phosphatase conjugated anti-rabbit IgG solution (diluted to 1/2000) for 2 h, washed again and detected in BCIP/NBT substrate system (Promega). The membrane was scanned and the quantification of each specific band was analyzed by using a Dual Light™ Transilluminator.

Northern Blot Analysis-Total RNA was isolated from either frozen tissues of the two groups of rats or from HepG2 cells with Trizol LS reagent (Gibco-BRL) following the protocol of the manufacturer. About 20 μg of total RNA was electrophoresed on a 1% formaldehyde-denatured agarose gel in 1 × MOPS running buffer. RNA was transfered to a nylon membrane (Bio-Rad) by capillary action overnight. The membrane was then hybridized with [^{32}P]-labelled G6Pase catalytic subunit (p36) (14) or putative G6P translocase (p46) (9) probes overnight at 68 °C in ExpressHyb™ Hybridization solution (Clontech). After hybridization, membranes were washed five times with different proportions of SSC/SDS buffer and exposed to a X-ray film for the indicated times at –80 °C. Both p36 and p46 probes were full length human cDNA fragments labelled by a random primer method with Ready-To-Go DNA labelling beads (Amersham Pharmacia Biotech). Probes were then purified with MicroSpin™ G-50 columns (Amersham Pharmacia Biotech). Quantification was performed with a scanning densitometer (Dual light™ Transilluminator) and equal amount of loading was verified by the 28S ribosomal RNA signal obtained by ethidium bromide staining.

Analytical Procedures-Glucose was assayed with a blood glucose monitor (Accu-Chek® Advantage^{MD}, Boehringer Mannheim, Canada) and protein with a Bio-Rad kit based on the method of Bradford (15), with bovine serum albumin as standard. Statistical analysis was performed according to Students' t test. Differences were considered significant when the p value was less than 0.05.

RESULTS AND DISCUSSION

Characteristics of diabetic rats-Table I shows the effect of streptozocin or vehicle treatment on body weight, fasting blood glucose, glucosuria and liver G6Pase activity of the two groups of rats. As expected, diabetic animals were markedly hyperglycemic, had glucosuria and gained less weight after treatment than the corresponding controls. G6Pase activity measured in microsomes isolated from livers of the rats was increased by about 2-fold in the diabetic group, as documented before (1, 2).

Effect of streptozocin-diabetes on p36 and p46 mRNA and protein in different tissues–The catalytic subunit of G6Pase, p36, is known to be expressed mainly in gluconeogenic tissues such as liver and kidney (16), and has been also found recently in small intestine (17). It is apparent from the Northern blots shown in Fig. 1A that the tissue distribution of p46 in rats is parallel to that of p36 (16, 17), with higher mRNA abundance of p46 in liver and kidney compared to intestine and no signal in skeletal muscle, as well as in brain and heart (results not shown). Using a more sensitive Northern blotting analysis with poly(A)$^+$ RNAs, Lin *et al.* (18) found p46 mRNA in almost all rat tissues investigated.

Insulin-deficient streptozocin diabetes increased the p46 transcript by 2-3-fold in liver, kidney and intestine and, as reported previously, also increased liver p36 mRNA (Fig. 1A) (2). Fig. 1B further shows by immunoblotting analysis that p36 protein is increased in diabetic liver and kidney compared to control as shown before (2, 16) and that p46 protein is increased in liver in parallel with p36. Both

proteins were undetectable either in control or in diabetic skeletal muscle. These results indicate that in the G6Pase system, p46, in addition to p36, could play a role in glucose production by liver and their dysregulation in diabetes. They also show that p46 mRNA is up-regulated similarly to p36 mRNA by diabetes in tissues (liver, kidney and intestine) where p36 is present and where p36 mRNA is also enhanced (2, 17, 19).

Effect of glucose, insulin and cAMP on the mRNA and protein levels of p36 and p46 in HepG2 cells–The relative contributions in diabetes of insulin deficiency, hyperglycemia and cAMP (resulting from counterregulatory hormones unopposed by the lack of insulin) on p36 and p46 were further assessed in HepG2 cells. A dose-dependent increase by glucose (1-25 mM) of both p36 and p46 mRNA was observed in these cells (Fig. 2A). While a maximal level of p46 mRNA was obtained at 15 mM glucose, the p36 mRNA level still increased at higher (25 mM) glucose concentrations (Fig. 2A). Interestingly, these changes already occurred at physiological (5-10 mM) concentrations of glucose and were further exacerbated at higher (15-25 mM) concentrations, corresponding to those found in the circulation of the diabetic rats (see Table I). Accordingly, p46 protein levels were also increased by glucose in a dose-dependent (1-25 mM) matter (Fig. 2B), but the effect was smaller than that on the corresponding mRNA.

P46 mRNA was decreased by half after 24 h exposure to insulin (1 μM) and increased by 2-fold after 6 h in the presence of cAMP (100 μM, in the presence

of 50 μM MIX). P36 mRNA was suppressed by insulin and increased by cAMP (Fig. 3A), as previously documented (5, 6). A lower concentration of cAMP (0.5 μM, plus 50 μM MIX) caused a 1.5-fold increase in p36- and p46 mRNA (results not shown). Fig. 3B shows that the p46 protein level was decreased by insulin (1 μM, 24 h) to a lesser extent than mRNA and was unaffected by cAMP. The fact that the regulation of p46 expression is parallel to that of p36 may suggest that similar response elements for insulin and cAMP respectively (20, 21) are involved in the control of the two genes. The dichotomy between changes in p46 mRNA and p46 protein with cAMP suggests either increased degradation of p46 or less translation of p46 mRNA in that condition.

Taken together, these results indicate that several parameters (insulin deficiency, hyperglycemia and increased intracellular cAMP consequent to unopposed counterregulatory hormones) contribute independently from each other to elevated p36 and p46 in insulin-dependent diabetes and may exacerbate hyperglycemia by increased hepatic glucose output. Consistent with this idea, a potent inhibitor of p46, chlorogenic acid (22), is currently being studied as a pharmaceutical tool to inhibit hepatic glucose production in diabetes (23). Overexpression of p36 in primary hepatocytes with a recombinant adenovirus (24) resulted in increased flux through G6Pase, suggesting that an increase in the catalytic subunit alone is sufficient to activate G6Pase in the intact cell. The mechanism(s) by which overexpression of p46 might affect p36 catalytic activity is however not known

and deserves further studies.

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FOOTNOTES

* This work was supported by Medical Research Council of Canada Grants ME-10783 and MT-10804.

LEGENDS TO FIGURES

FIG. 1. **Effect of diabetes on p36 and p46 mRNA and protein of different tissues.** *A*, Northern analysis of p36 and p46 on total RNAs extracted from the indicated tissues sampled from control and diabetic rats, as described in the Experimental Procedures section. *B*, immunoblotting analysis of p36 and p46 in microsomal fractions isolated from the indicated tissues, as described in the Experimental Procedures section. Analysis was performed several times for all tissues with similar results. A representative experiment is shown.

FIG. 2. **Effect of glucose concentration on p36 and p46 mRNA and protein in HepG2 cells.** *A*, Northern analysis of p36 and p46 on total RNAs extracted from HepG2 cells incubated with the indicated concentrations of glucose, as described in the Experimental Procedures section. *B*, immunoblotting analysis of p36 and p46 in homogenates of HepG2 cells incubated with the indicated concentrations of glucose, as described in the Experimental Procedures section. All values (n = 3) were statistically different from each other ($p < 0.02$) for p36 and p46 mRNA, except at 15 and 25 mM glucose for the latter. Immunoblotting analysis was performed several times for all conditions with similar results. A representative experiment is shown.

FIG. 3. **Effect of cyclic AMP and insulin on p36 and p46 mRNA and protein in HepG2 cells.** *A*, Northern analysis of p36 and p46 on total RNAs extracted from HepG2 cells incubated with the indicated concentrations of insulin and cAMP, as described in the Experimental Procedures section. *B*, immunoblotting analysis of p46 in homogenates of HepG2 cells incubated with the indicated concentrations of insulin and cAMP, as described in the Experimental Procedures section. Analysis was performed several times for all conditions with similar results. A representative experiment is shown.

TABLE I

Characteristics of control and diabetic rats

Plasma and urine samples were taken after an overnight fast for the assay of glucose as described in the Experimental Procedures section. Body weight was measured before sacrifice. The activities of glucose 6-phosphatase ([U-^{14}C]glucose production at 5 mM glucose 6-phosphate) were measured in microsomes isolated from rat liver and pretreated with 0.8 % chapso, as described in the Experimental Procedures section. Values are means ± S.E. (n = 6 or 7).

Group	*n*	Weight difference[a]	Plasma glucose	Glucosuria	G6Pase activity
		g	mM		*milliunits/mg protein*
Control	7	16.1 ± 3.5	3.4 ± 0.08	negative	98 ± 8
Diabetic	6	-7.2 ± 8.0[b]	20.1 ± 3.3[b]	positive	168 ± 19[b]

[a]This is the weight difference from treatment to sacrifice

[b] $p < 0.01$ *versus* control.

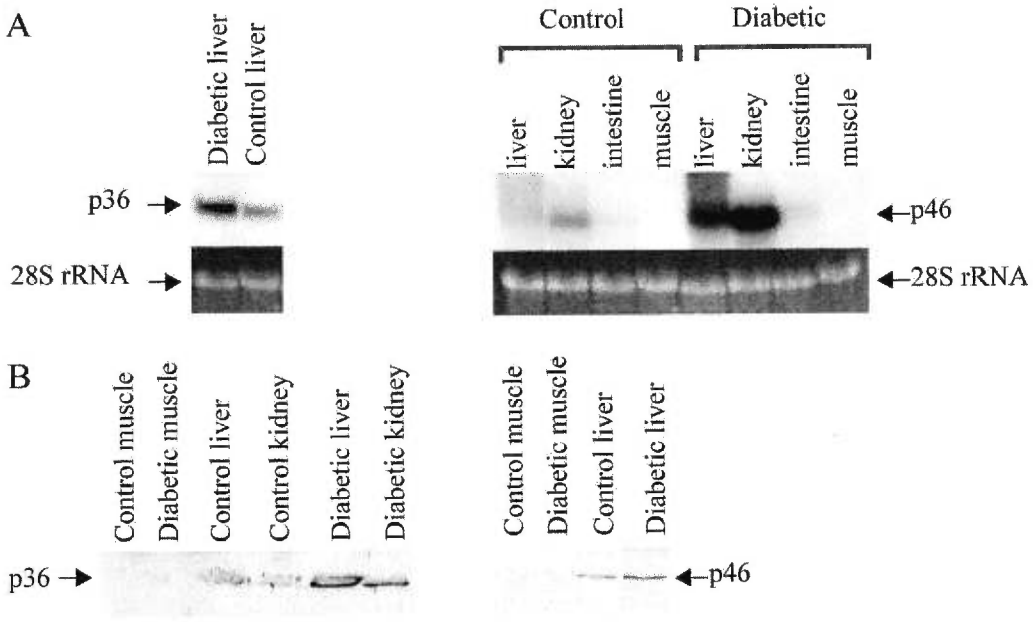
A
Diabetic liver
Control liver
p36
28S rRNA
Control
Diabetic
liver
kidney
intestine
muscle
liver
kidney
intestine
muscle
p46
28S rRNA
B
Control muscle
Diabetic muscle
Control liver
Control kidney
Diabetic liver
Diabetic kidney
p36
Control muscle
Diabetic muscle
Control liver
Diabetic liver
p46

Figure 1

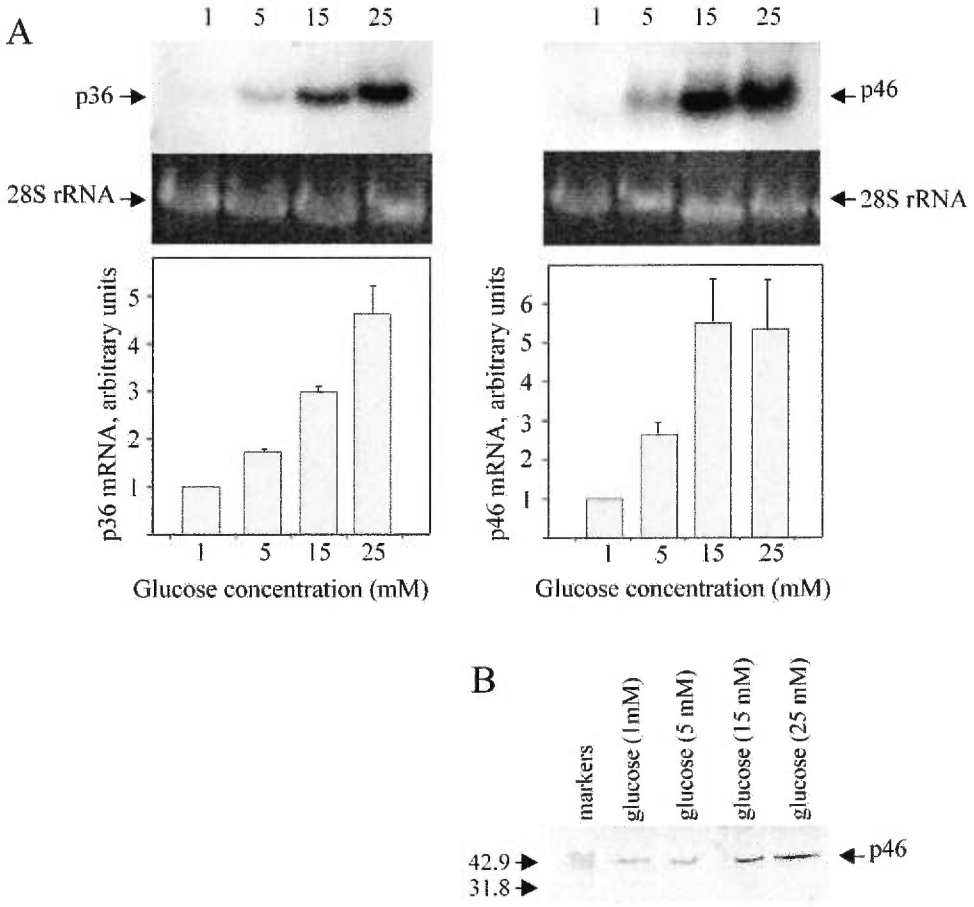
A
1 5 15 25
p36
28S rRNA
p36 mRNA, arbitrary units
Glucose concentration (mM)
1 5 15 25
p46
28S rRNA
p46 mRNA, arbitrary units
Glucose concentration (mM)
B
markers
glucose (1mM)
glucose (5 mM)
glucose (15 mM)
glucose (25 mM)
42.9
31.8
p46

Figure 2

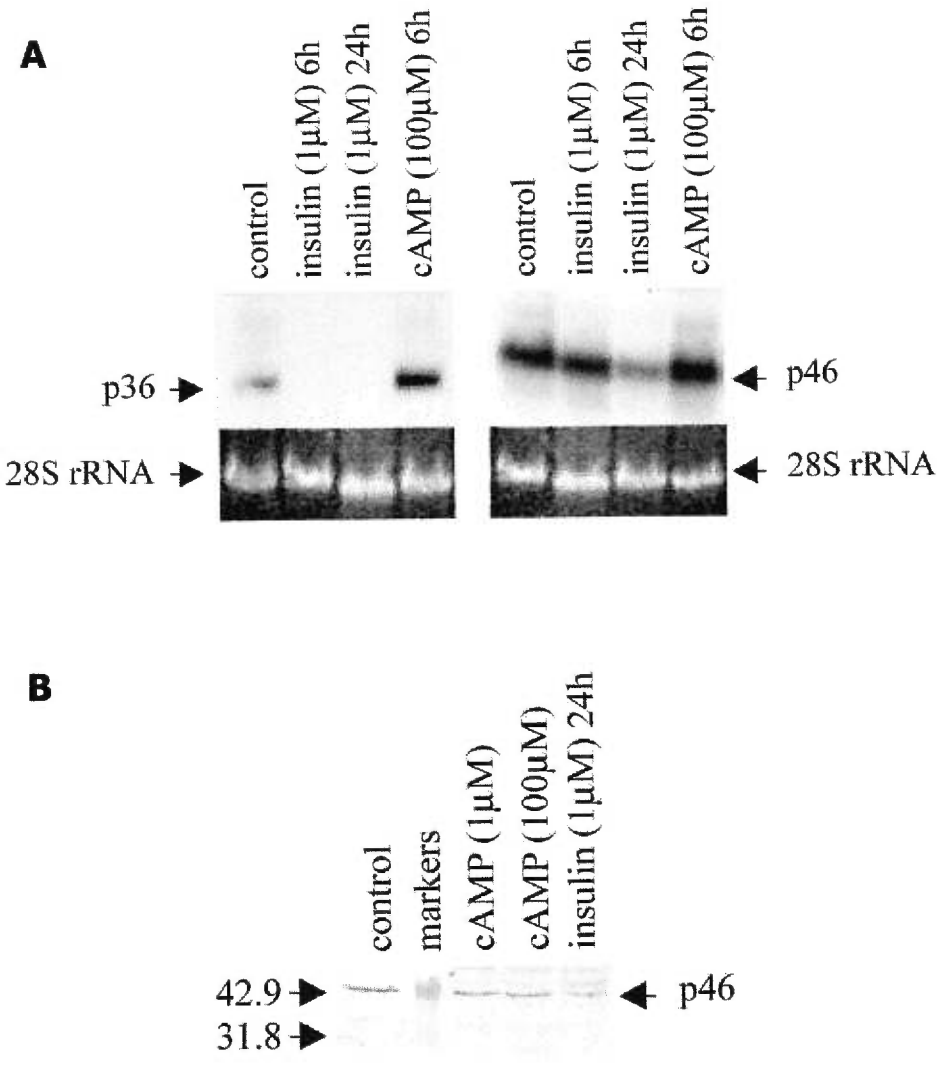
A
control
insulin (1μM) 6h
insulin (1μM) 24h
cAMP (100μM) 6h
control
insulin (1μM) 6h
insulin (1μM) 24h
cAMP (100μM) 6h
p36
p46
28S rRNA
28S rRNA
B
control
markers
cAMP (1μM)
cAMP (100μM)
insulin (1μM) 24h
42.9
31.8
p46

Figure 3

3.3 Article 3

Distinct hormone stimulation and counteraction by insulin of the expression of the two components of glucose 6-phosphatase in HepG2 cells. Yazhou Li and Gérald van de Werve, Biochem. Biophys. Res. Commun. (2000) 272:41-44

Distinct Hormone Stimulation and Counteraction by Insulin of the Expression of the Two Components of Glucose 6-phosphatase in HepG2 Cells

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Running title: Hormonal regulation of G6Pase gene expression

[1] Abbreviations used in the manuscript are: G6Pase, glucose 6-phosphatase; G6P, glucose 6-phosphate; p36, catalytic subunit of G6Pase; p46, putative G6P translocase; cAMP, cyclic AMP; MIX, methyl isobutylxanthine; $[Ca^{2+}]_i$, cytosolic free calcium

ABSTRACT

We found recently (J. Biol. Chem., 274, 33866-33869, 1999) that the expression of the catalytic subunit (p36) and putative glucose 6-phosphate translocase (p46) of the liver glucose-6 phosphatase system was stimulated by cyclic AMP and glucose and repressed by insulin. We now further show in HepG2 cells that whereas insulin (0.01-10 nM) suppressed p36 mRNA, it only reduced p46 mRNA by half at 1μM. Cyclic AMP (0.01-100 μM) caused a 2.7-fold increase in p36 mRNA but barely increased p46 mRNA. In contrast, dexamethasone (0.1-100 nM) increased both p36 and p46 mRNA by more than threefold. The effects of cyclic AMP and dexamethasone were counteracted by 1μM insulin. The endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (1-100nM) increased p36 mRNA by two fold but not p46 mRNA. It thus appears that the hormonal changes which affect p36 alone concur with known modifications in glucose production; those that affect both p36 and p46 are rather consistent with glucose storage.

Key Words: glucose 6-phosphatase; catalytic subunit; putative glucose 6-phosphate translocase; gene expression; insulin; glucagon; dexamethasone; cyclic AMP; calcium.

INTRODUCTION

Liver glucose production is ultimately realized by glucose 6-phosphatase (G6Pase) (EC 3.1.3.9) (reviewed in refs.1 and 2). Insulin deficiency, the presence of unopposed counterregulatory hormones and hyperglycemia may each contribute in the streptozotocin-treated diabetic rat to enhanced hepatic glucose formation, through an up-regulation of G6Pase that affects both the 36-kDa catalytic subunit (p36) (3,4) and the putative glucose 6-phosphate (G6P) translocase, a 46-kDa protein (p46) (5). Glucose and cyclic AMP (cAMP) indeed increased both p36 and p46 mRNA but the p46 protein level was unaffected by cAMP (5). Insulin severely represses the expression of p36 (6) and reduces that of p46 (5). Taken together, these properties indicate that both p36 and p46 are regulated in parallel, however the comparative sensitivity of p36 and p46 to hormones remains to be defined. In this study we evaluated the differential response of p36 and p46 mRNA to physiological concentrations of insulin and cAMP, but also to dexamethasone and to changes in cytosolic free calcium ($[Ca^{2+}]_i$) induced by the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (7) in HepG2 cells. The results indicate that p36 is sensitive to cAMP and thapsigargin stimulation and insulin inhibition whereas p46 is virtually not, but that dexamethasone has similar effects on both p36 and p46 gene expression. The possible role of p36 and p46 in the G6Pase system is discussed in the light of this differential expression of the two genes.

MATERIALS AND METHODS

Materials. Bovine serum albumin and fetal bovine serum and Dulbecco's Modified Eagle Medium (DMEM), as well as insulin (lyophilized) were purchased from Life Technologies Inc. (Birlington, ON, Canada). Glucagon was from Eli Lilly Inc. (Toronto, ON, Canada). Adenosine 3': 5'-cycle monophosphate (cAMP), methyl isobutylxanthine (MIX), dexamethasone and thapsigargin were from Sigma (Oakville, ON, Canada). Insulin, glucagon, cAMP and MIX were dissolved in 0.9% NaCl, while dexamethasone and thapsigargin were dissolved in dimethyl sulfoxide (DMSO).

Cell culture. HepG2 cells were maintained in DMEM containing 10% bovine fetal serum. Before treatment, cells were adapted in serum-free DMEM (but containing 0.1% BSA) overnight. Insulin, glucagon, cAMP (in the presence of 50μM MIX to inhibit cAMP phosphodiesterases), dexamethasone or the Ca^{2+}-ATPase inhibitor thapsigargin were added to 80-90 % confluent HepG2 cells in serum-free DMEM at the indicated concentrations and times, in the presence of 5mM glucose. Control cells were treated with the same final concentration (0.01%) of DMSO in the cases of dexamethasone and thapsigargin. Cells were harvested by scraping after different times of treatment and kept at –80 °C for subsequent analysis. With thapsigargin, cells were exposed for 10 min with the inhibitor and further incubated without it for the indicated time.

Northern Blot Analysis. Total RNA was isolated from HepG2 cells with Trizol LS reagent (Life Technologies Inc.) following the protocol of the manufacturer. Fifteen μg of total RNA was electrophoresed on a 1% formaldehyde-denatured agarose gel in 1 × MOPS running buffer. RNA was transfered to a nylon membrane (Bio-Rad, Mississauga, ON, Canada) by capillary action overnight. The membrane was then hybridized with [^{32}P]-labelled p36 (8) or p46 (9) probes overnight at 68 °C in ExpressHyb™ Hybridization solution (CLONTECH, Palo Alto, CA, USA). After hybridization, membranes were washed five times with different proportions of SSC/SDS buffer and exposed to an X-ray film at –80 °C. Both p36 and p46 probes were full length human cDNA fragments labelled by a random primer method with Ready-To-Go DNA labelling beads (Amersham Pharmacia Biotech, Baie d' Urfe, QC, Canada). Probes were then purified with MicroSpin™ G-50 columns (Amersham Pharmacia Biotech, Baie d' Urfe, QC, Canada). Quantification was performed with a scanning densitometer (Dual light™ Transilluminator) and equal amount of loading was verified by the 18S and 28S ribosomal RNA signal obtained by ethidium bromide staining.

Statistic Analysis. The results were presented as the mean ± S.D. of three or four independent experiments.

RESULTS

Dose response of p36 and p46 mRNA to insulin. Fig. 1 shows that after 24 h insulin caused a dose-dependent decrease in the G6Pase catalytic subunit p36 mRNA in HepG2 cells, that was half maximal at about 0.03 nM. At 10 nM insulin, p36 mRNA was not detectable anymore. Similar results were reported in FAO cells (6,10). In contrast to p36, p46 mRNA was not reduced more than by 60% at the highest (0.1-10 μM) insulin concentrations investigated. Changes in p46 protein that occur at 1 μM insulin were found before to be parallel to those of the corresponding mRNA (5).

Dose response of p36 and p46 mRNA to cAMP and counteraction by insulin. The effect of increasing concentrations of cAMP (in the presence of 50 μM MIX) on p36 and p46 transcripts was examined in HepG2 cells after 6 h (Fig. 2), because longer incubation times reversed this effect in FAO cells (6). It can be seen that cAMP produced a 2.7-fold increase in the p36 mRNA level with a maximal effect at 1 μM, whereas the p46 mRNA was only slightly (1.25-fold) affected. This latter result is in agreement with our previous observations that cAMP did not increase the p46 protein (5). Consistently, glucagon (1 μM) increased p36 and p46 to similar extends as with an optimal concentration of cAMP (Fig. 2). Insulin (1 μM) counteracted the cAMP-induced increase in p36 mRNA but did not decrease the p46 mRNA more than when added alone (see Fig. 1).

Dose response of p36 and p46 mRNA to dexamethasone and counteraction by insulin. Dexamethasone (0.1-100 nM) increased in 24 h the level of both p36 and p46 message by a factor between 2- and 3-fold with similar sensitivity (Fig. 3). This stimulatory effect was inhibited by 1 μM insulin on p36 as well as on p46 mRNA (Fig. 3), in agreement with the property of insulin to inhibit glucocorticoid receptor activity (11).

Dose response of p36 and p46 mRNA to thapsigargin in HepG2 cells. In order to evaluate the effect of increased $[Ca^{2+}]_i$ on the p36 and p46 message, independent of inositol trisphosphate, we used an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, thapsigargin (7). Because prolonged application of this drug resulted in cell death, we exposed the HepG2 cultures for 10 min to increasing concentrations of thapsigargin and further cultured them up to 6 h before harvesting and mRNA analysis. It has been emphasized before that a $[Ca^{2+}]_i$ perturbation of a few minutes is sufficient for full induction of transcripts (12). Fig. 4 shows that p36 mRNA was increased up to 2-fold by increasing thapsigargin concentrations (1-100 nM) whereas p46 mRNA was unchanged in the same conditions.

DISCUSSION

Insulin, cAMP and calcium affect differently p36 and p46 mRNA. The negative effect of insulin on p36 mRNA, which is partly attributable to a decreased transcription rate (6), was found to be within its physiological concentration range (0.05-0.1nM), indicating that the expression of G6Pase must be very sensitive to down-regulation by insulin. This is not the case for p46, the mRNA of which was only reduced by 20-30% by normal insulin concentrations. Suppression of hepatic glucose production after a meal can thus appropriately be achieved by increased insulin secretion that occurs in that situation followed by decreased G6Pase activity. Conversely, glucagon and cAMP, that stimulate glycogenolysis and gluconeogenesis, increase p36 message and enhance glucose release by the liver without significantly affecting p46. Therefore both inhibition and stimulation of G6Pase seem to be principally the consequence of changes in the catalytic subunit of G6Pase. A similar conclusion can be drawn from the effect of thapsigargin. The elevation in $[Ca^{2+}]_i$ expected to occur following inhibition of the endoplasmic reticulum Ca^{2+}-ATPase in HepG2 cells by thapsigargin increased by up to two fold the p36 mRNA without affecting p46 mRNA. Although cAMP and calcium increase p36 mRNA to the same extend, calcium is a less potent signal than cAMP in inducing glucose production because the former increases fructose-2,6-bisphosphate and flux through glycolysis whereas the latter lowers

fructose-2,6-bisphosphate and inhibits glycolysis, thereby directing G6P towards glucose formation.

Although the regulation of p36 and p46 mRNA tends to be affected in parallel, the dose-response of the respective messages to insulin, cAMP and thapsigargin are quite different for these two components of the G6Pase system. Hormonal changes compatible with increased hepatic glucose production essentially modify the expression of the catalytic subunit of G6Pase, p36, without significant effect on that of p46.

Dexamethasone affects similarly p36 and p46. Effectors such as glucose (5) and the synthetic glucocorticoid dexamethasone (this work), that enhance glycogen synthesis, increase both p36 and p46 mRNA. Glucocorticoids stimulate gluconeogenesis as well as glycogen synthesis, and have only a marginal effect on glucose production (13,14). Taken together, these properties suggest that p46 favours carbon flux through G6P towards glycogen, even if G6Pase activity is concomitantly increased. Such a mechanism would be compatible with the proposition of Aiston et al. (15) that G6Pase acts as a buffer for G6P. G6P hydrolysis might serve either for glucose export by the liver (increased p36 alone) or to feed a glucose/G6P substrate cycle to keep intracellular G6P available for glycogen synthesis (increased p46 and p36). This cycle is indeed increased in diabetes (16) and could be a metabolic adaptation whereby hyperglycemia appropriately induces both components of the liver G6Pase system (5). Similarly, hyperglycemia partially compensates

the down-regulation of synthase phosphatase induced by insulin deficiency in diabetes (17).

ACKNOWLEDGMENTS

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LEGENDS TO FIGURES

FIG. 1. **Dose response of p36 and p46 mRNA to insulin in HepG2 cells.** Northern analysis of p36 (○) and p46 (Δ) on total RNAs extracted from HepG2 cells incubated for 24 h with the indicated concentrations of insulin, as described in the Materials and Methods section. Densitometric scanning values are presented as mean ± S.D. (n =3). Representative blots are shown on top, in which all controls represent samples without any treatment and are considered as one arbitrary unit for quantitative analysis.

FIG. 2. **Dose response of p36 and p46 mRNA to cAMP and to 1 μM glucagon and antagonism by insulin in HepG2 cells.** *A.* Northern analysis of p36 (○) and p46 (Δ) on total RNAs extracted from HepG2 cells incubated for 6 h with the indicated concentrations of cAMP and 50μM MIX with (●, ▲) or without (○, Δ) 1μM insulin. *B.* Northern analysis of p36 and p46 on total RNAs extracted from HepG2 cells incubated for 6 h with 1 μM glucagon. Densitometric scanning values are presented as mean ± S.D. (n =3). Representative blots are shown on top, in which all controls represent samples without any treatment and are considered as one arbitrary unit for quantitative analysis.

FIG. 3. **Dose response of p36 and p46 mRNA to dexamethasone and antagonism by insulin in HepG2 cells.** Northern analysis of p36 (○) and p46 (Δ) on total RNAs extracted from HepG2 cells incubated for 24 h with the indicated concentrations of dexamethasone with (●, ▲) or without (○, Δ) 1μM insulin. Densitometric scanning values are presented as mean ± S.D. (n =3 or 4). Representative blots are shown on top, in which all controls represent samples without any treatment and are considered as one arbitrary unit for quantitative analysis.

FIG. 4. **Dose response of p36 and p46 mRNA to thapsigargin in HepG2 cells.** Northern analysis of p36 (○) and p46 (Δ) on total RNAs extracted from HepG2 cells incubated with the indicated concentrations of thapsigargin for 10 min and further cultured for 6 h. Densitometric scanning values are presented as mean ± S.D. (n =3). Representative blots are shown on top, in which all controls represent samples without any treatment and are considered as one arbitrary unit for quantitative analysis.

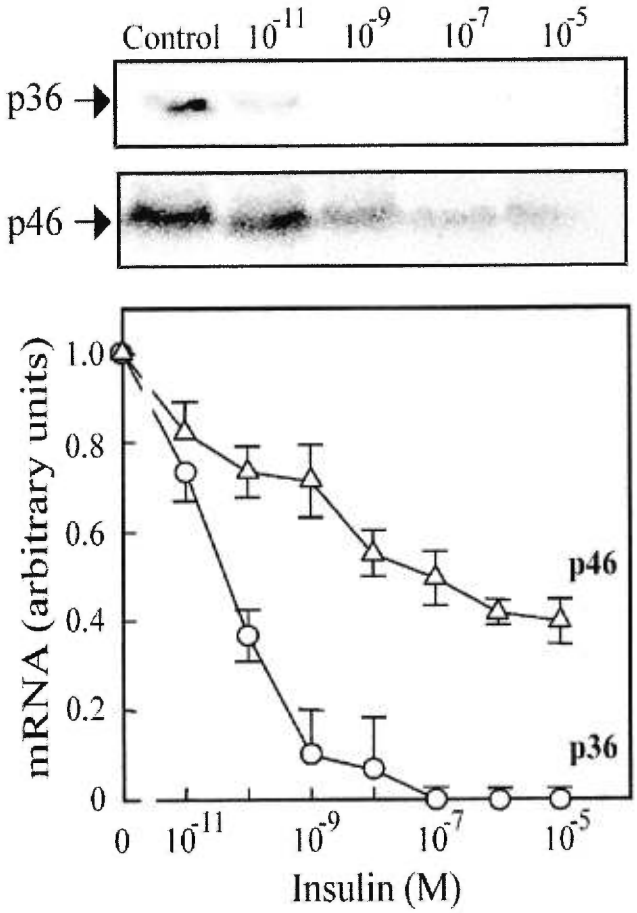
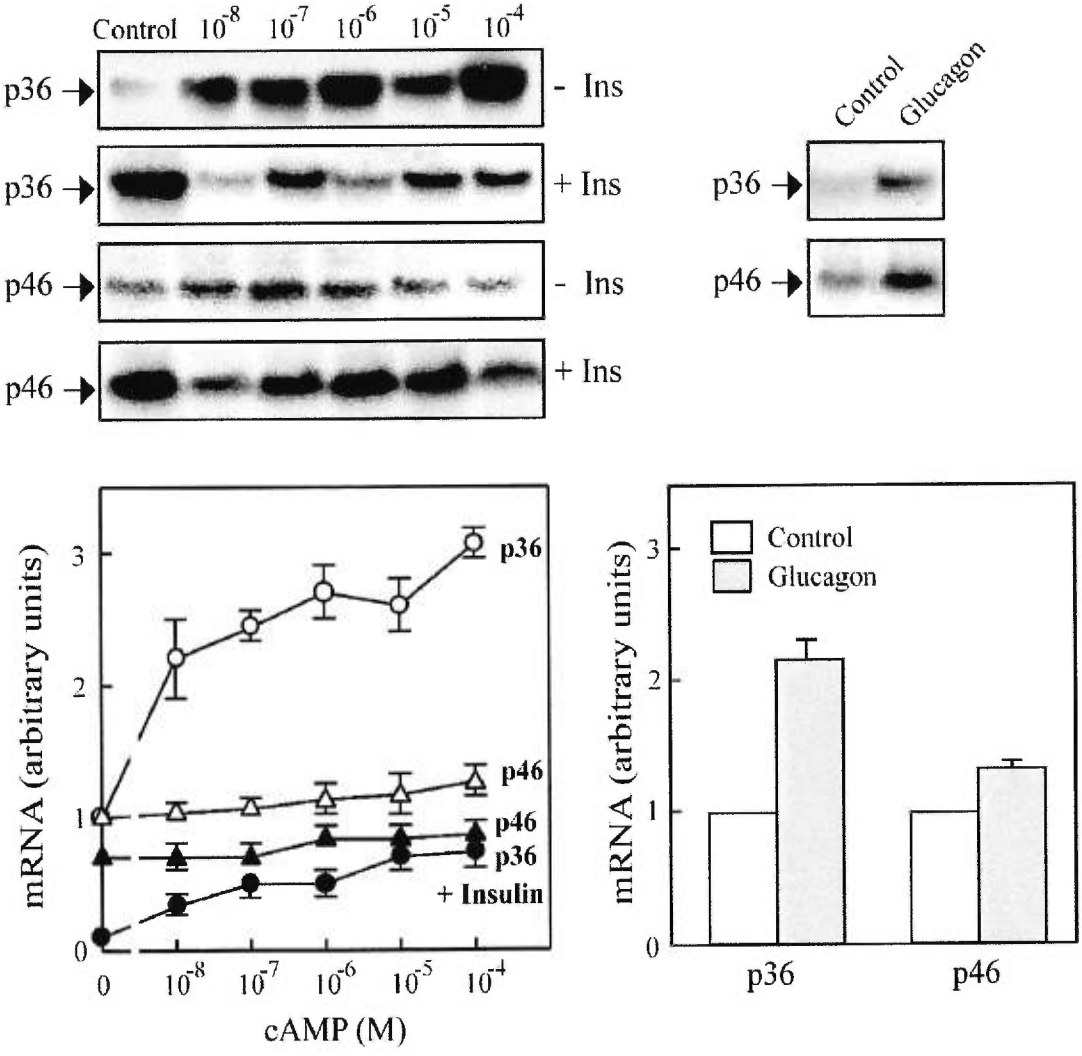

Control
10^{-11}
10^{-9}
10^{-7}
10^{-5}
p36
p46
mRNA (arbitrary units)
1.0
0.8
0.6
0.4
0.2
0
p46
p36
0
10^{-11}
10^{-9}
10^{-7}
10^{-5}
Insulin (M)

Figure 1


Control
10-8
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p46
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p46
+ Ins
Control
Glucagon
p36
p46
mRNA (arbitrary units)
p36
p46
p46
p36
+ Insulin
cAMP (M)
Control
Glucagon
mRNA (arbitrary units)
p36
p46

Figure 2

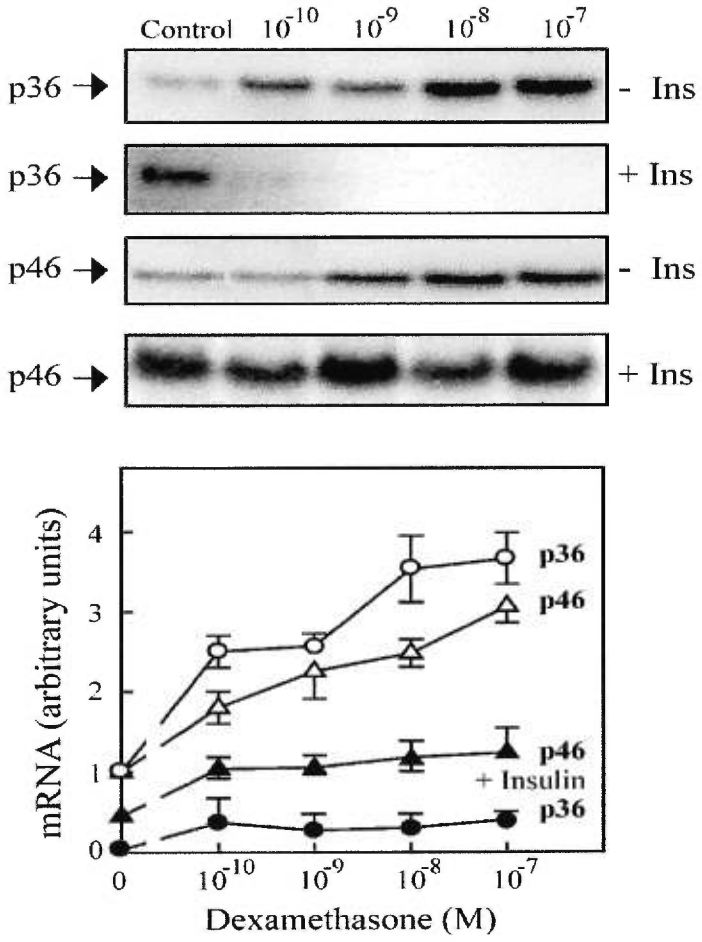
Control
10-10
10-9
10-8
10-7
p36
- Ins
p36
+ Ins
p46
- Ins
p46
+ Ins
mRNA (arbitrary units)
0
1
2
3
4
p36
p46
p46
+ Insulin
p36
0
10-10
10-9
10-8
10-7
Dexamethasone (M)

Figure 3

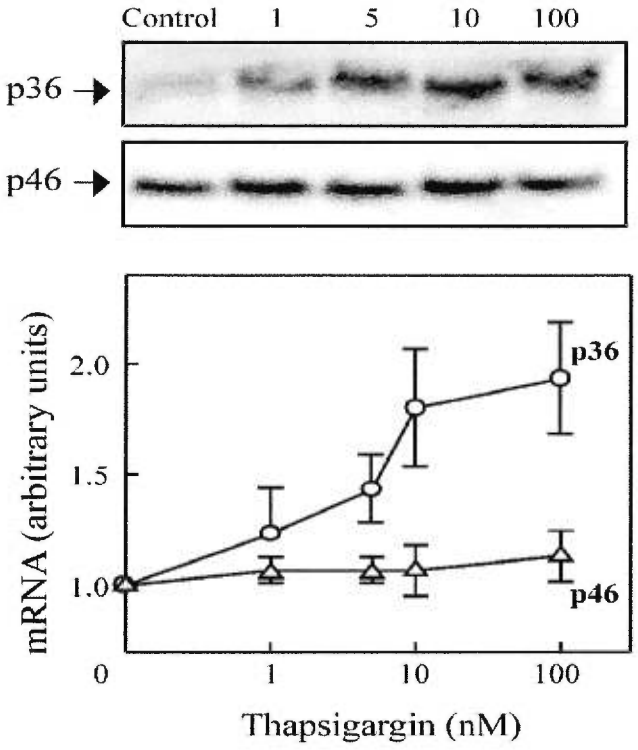
Control
1
5
10
100
p36
p46
mRNA (arbitrary units)
2.0
1.5
1.0
0
1
10
100
p36
p46
Thapsigargin (nM)

Figure 4

4- OTHER RESULTS

4.1 Looking for interacting proteins of G6Pase components by the yeast two-hybrid system

Unfortunately, all the combinations tested by the two-hybrid system ended up with negative results except combinations $_{\Delta}10/_{\Delta}6$ and p46/$_{\Delta}6$, which turned to blue much later (48 h) than should be expected, and therefore were usually determined as false positives. There are two possibilities: the first one is that p36 and p46 do not interact with each other, which seems unlikely because if p46 is the auxiliary protein to confer the G6P to p36, there should be some kind of contact between the two. However, this contact could be indirect and undetectable by the yeast two-hybrid system if another unknown protein lies between p36 and p46. The second possibility is that the yeast two-hybrid system may not be an appropriate tool to detect interaction between membrane proteins; however in literature there are a few examples of successful detection of interaction between membrane proteins by this method, such as the interaction between $\alpha 1$ and $\beta 1$ subunits of the chicken Na, K-ATPase (Colonna et al., 1997). In our case, we do not know if p36 or p46, as highly hydrophobic transmembrane proteins, were expressed in the correct natural conformation in the yeast. Additionally, since our constructs $_{\Delta}6$ and $_{\Delta}10$ were based on the former six transmembrane topology model of p36, there still

might be a chance to detect the interaction if the constructs are designed based on the new model of p36 (Pan et al., 1998a). Constructs containing the extracellular and intracellular loops of p36 or p46 with minimal length of amino acids may be used as bait in the two-hybrid system because it may be expressed as soluble protein but still keep the conformation for interaction with another protein. Alternatively, to detect interaction between p36 and p46, other methods could be applied such as cross-linking and coimmunoprecipitation.

4.2 Adenovirus-mediated overexpression of p46 in primary hepatocytes and HepG2 cells

4.2.1 Overexpression of p46 induces the transcription of p36 gene

Total RNA extracted from primary hepatocytes or from HepG2 cells, which were treated with control β-galactosidase or p46 recombinant adenovirus, was used for Northern blot analysis. As shown in Figure 12A, 24 hours after the virus treatment, p46 mRNA level was markedly increased compared to the control, although the chosen picture here is a film after an exposure time of only 1 h. Western blot analysis done in Dallas (collaboration with Dr. Newgard, data not shown) confirmed that p46 protein was also greatly increased, indicating that p46 was successfully overexpressed. Surprisingly, p36 mRNA level was induced concomitantly with the overexpression of p46 in

either primary hepatocytes or hepG2 cells, as shown in Figure 12B and 13. This increase of p36 mRNA is significant and repeatable; p36 mRNA abundance increased 4.5 and 2 times in primary hepatocytes and in HepG2 cells, respectively. However, Western blot analysis done in Dallas (data not shown) indicates the p36 protein level was unchanged. That increased transcription of p36 did not result in increased p36 protein is probably because of decreased mRNA stability or decreased protein translation, which has to be verified. In addition, transfection of HepG2 cells with plasmid DNA of pACCMV-p46 also resulted in enhanced p36 mRNA signal as shown in Northern blot (Figure 14). Transfection with 10 μg DNA resulted in 70-80% increase of p36 mRNA at the glucose concentrations of 15 and 25 mM.

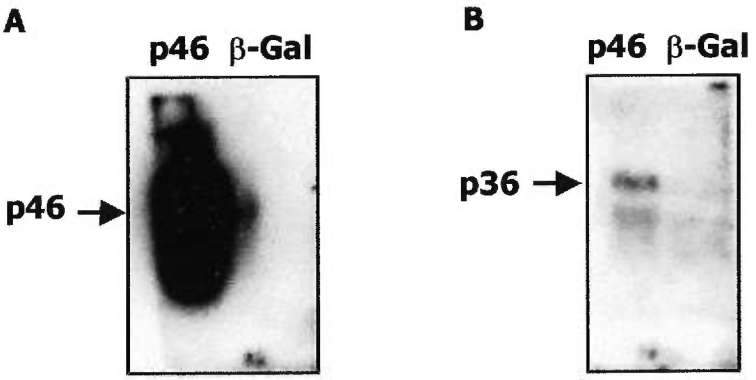

Figure 12: Overexpression of p46 in primary hepatocytes induced p36 gene transcription. Northern blot analysis of p46 (A) and p36 (B) genes on total RNAs extracted from primary rat hepatocytes were infected by either control virus AdCMV-βGal or AdCMV-p46.

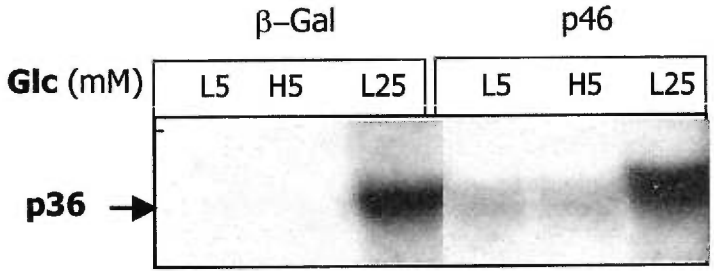

Figure 13: Overexpression of p46 in HepG2 cells induced p36 gene transcription. Northern blot analysis of p36 gene on total RNAs extracted from HepG2 cells were infected by either control virus AdCMV-βGal or AdCMV-p46 in 5 or 25 mM glucose. L and H represent low and high dose of virus.

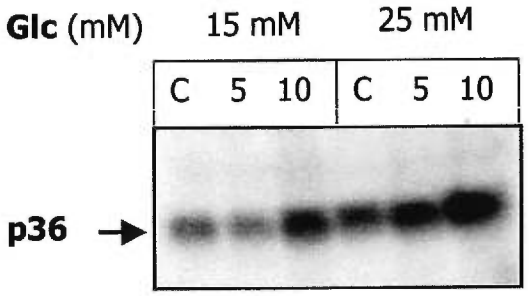

Figure 14: Transient transfection of pACCMV-p46 in HepG2 cells induced p36 gene transcription. 5 or 10 μg DNA of pACCMV-p46 or pACCMV were transfected into HepG2 cells at the glucose concentration of 15 and 25 mM. After 48 h, HepG2 cells were harvested by scraping and total RNAs were extracted for Northern blot analysis.

4.2.2 Overexpression of p46 increases the G6Pase activity

We next measured the phosphohydrolytic activity of G6Pase in frozen hepatocytes with the method validated by van de Werve (1989). The result indicated that the G6Pase activity was retained in the pellet while the activity in the supernate was not measurable. As shown in Figure 15, p46 overexpression induced a significant increase in G6Pase activity. This increase of activity is similar either with or without CHAPSO treatment, which is about 51% and 57% respectively. Detergent treatment increased the G6Pase activity only slightly in either control virus treated hepatocytes or AdCMV-p46 treated hepatocytes, suggesting that after freeze/thawing of the hepatocytes, the G6Pase system exists in a state that allows almost free access of G6P to the catalytic site of the enzyme.

Furthermore, the metabolic impact of overexpression of p46 was investigated by measuring the glycogen content and lactate level. The results show that p46 overexpression inhibits glycogen synthesis in hepatocytes from fasted rats, activates glycogenolysis in hepatocytes from fed rats and inhibits glycolytic flux. In addition, co-overexpression of p46 and p36 results in additive effects on both glycogen and glucose metabolisms.

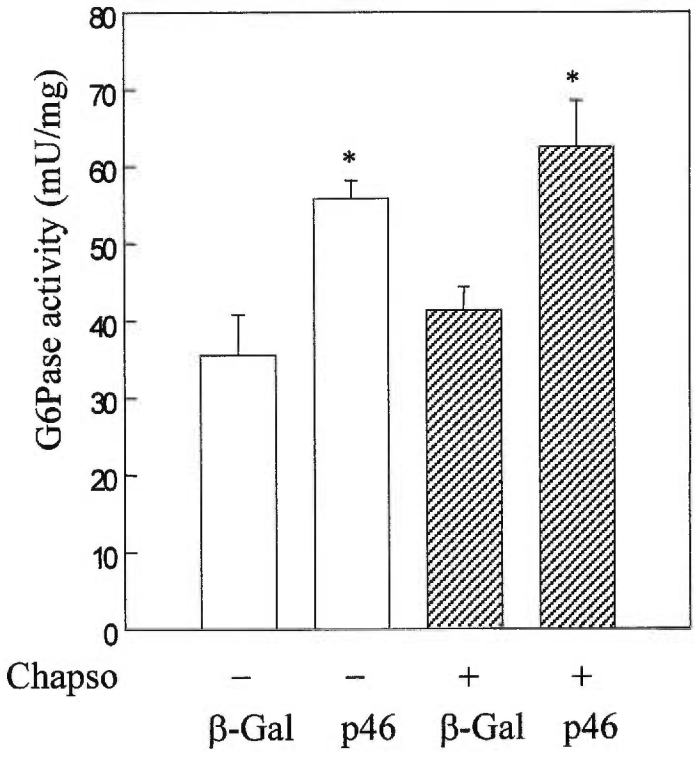

Figure 15: Effect of overexpression of p46 on G6Pase activity in primary rat hepatocytes. The G6Pase activity was measured with 5 mM G6P for 2 min at 30 °C either with or without 0.8% CHAPSO treatment. *, statistical significance at $p < 0.001$ for comparison with control virus treatment ($n = 4$).

4.3 Tagged-p36 protein is not present in the caveolae fraction

The recombinant plasmid that can express an N-terminal tagged p36 fusion protein was constructed and transfected into COS-7 cells. Immunodetection of tagged-p36 with anti-tag antibody has shown a very specific band between molecular weight markers 32 kDa and 43 kDa (Figure 16), which indicates that the tagged-p36 protein was successfully expressed in COS cells and could be easily detected. We next investigated the subcellular localization of this tagged-p36 in order to see if it is present in the caveolae fraction. As shown in Figure 17, after ultracentrifugation on sucrose gradient, caveolae should be in the fractions 4, 5 and 6 as indicated by the presence of its marker protein caveolin-1. However, tagged-p36 is not present in these fractions but detected in fractions 11 and 12, which are the fractions corresponding to microsomes. These results indicated that the subcellular localization of p36 is restricted in the endoplasmic reticulum. Due to the uncertain function of p46 and its proposed involvement in glycogen synthesis, it is interesting to construct a tagged-p46 plasmid to study its subcellular localization. Furthermore, this tagged-protein expression system could be used to purify p36 and p46 protein in mammalian cells by its N-terminal polyhistidine. By taking advantage of the availability of anti-tag antibody, we could perform co-immunoprecipitation of p36 and p46 to describe their interaction.

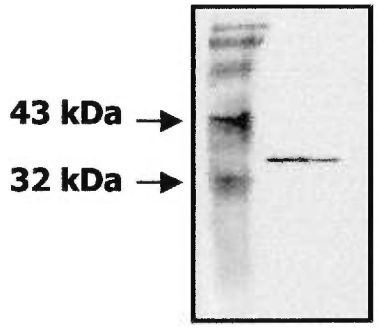

Figure 16: Expression of tagged-p36 protein in COS-7 cells. COS-7 cells were transfected with pcDNA3.1/His-p36. Anti-Xpress antibody was used in Western blot analysis to detect tagged-p36 protein in cell lysate.

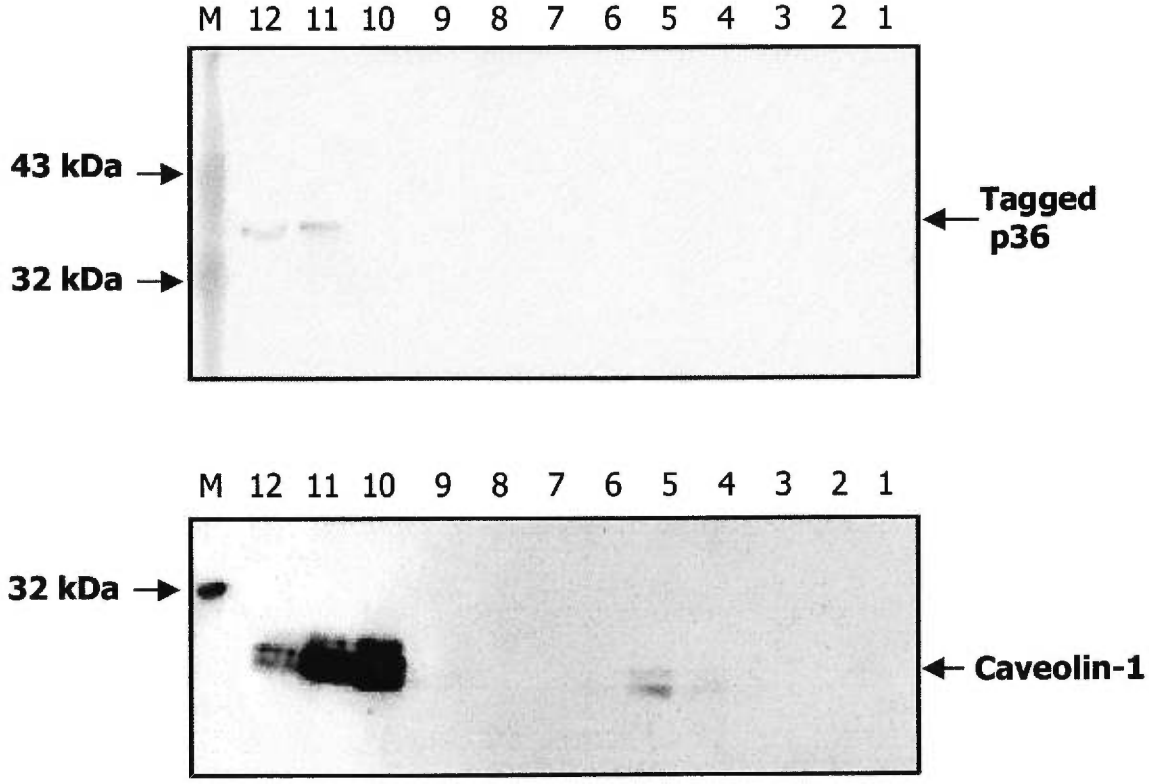

Figure 17: Tagged-p36 protein is not present in the caveolae fraction of COS-7 cells. After transfection, COS-7 cells were lysed and fractionated by ultracentrifugation on sucrose gradient. Twelve fractions were then collected; caveolae is in fractions 4, 5 and 6, while microsomes are in fractions 11 and 12.

4.4 Four different transcripts of the putative G6P translocase originating from differential mRNA splicing in human leukocytes

4.4.1 Identification of differential splicing variants

The products of PCR from the leukocyte library appeared as a series of bands between 1.3-1.6kb on 1% agarose gel (not shown). Since they were not clearly separated on the gel, all these bands were cut together and purified, then cloned into pCR2.1-TOPO vector. Four different types of recombinant plasmids were found after restriction enzyme digestion analysis. This showed that the insertion of PCR fragments had 4 different sizes between 1.3-1.6kb, when the recombinant plamids were cut by EcoR I. With the unique BstE II cutting site inside the p46 cDNA at position 863, we further confirmed by EcoR I +BstE II digestion that there were 4 different types (Figure 18) and we named them α, β, γ and δ. Of 51 randomly picked colonies, 37/51 is α type, 2/51 is β type, 4/51 is γ type and 7/51 is δ type (1/51 is a false positive clone). Several representatives of each type were then chosen for DNA sequencing (Bio S&T Inc.), as shown in Figure 19. The sequence of p46-α was found to be identical to the previously published one from the human liver EST cDNA sequence; p46-β has a 66 nt insertion at position 1153, which is identical to the most recently published sequence of the p46 isoform from human brain (Middleditch et al., 1998); p46-γ has a 321 nt insertion at position 550 and also

a 86 nt deletion between positions 953 and 1038; p46-δ has a 97 nt insertion at position 1038. Deduced amino acid sequences alignment is shown in Figure 20.

4.4.2 Presence of different transcripts in human blood

The presence of four different transcripts of p46 gene was found by nested RT-PCR from mRNA of human blood sample obtained from healthy volunteers. This corresponds to the transcripts found by PCR from the human leukocyte library reported above. As shown in Figure 21, α, β, γ and δ specific fragments were amplified with their specific primers. The result indicates that the transcription of this putative G6PT gene (p46) in human leukocytes is indeed more complicated than its transcription observed in the liver. In the liver, with the same primers used above, only the α type transcript was found (data not shown).

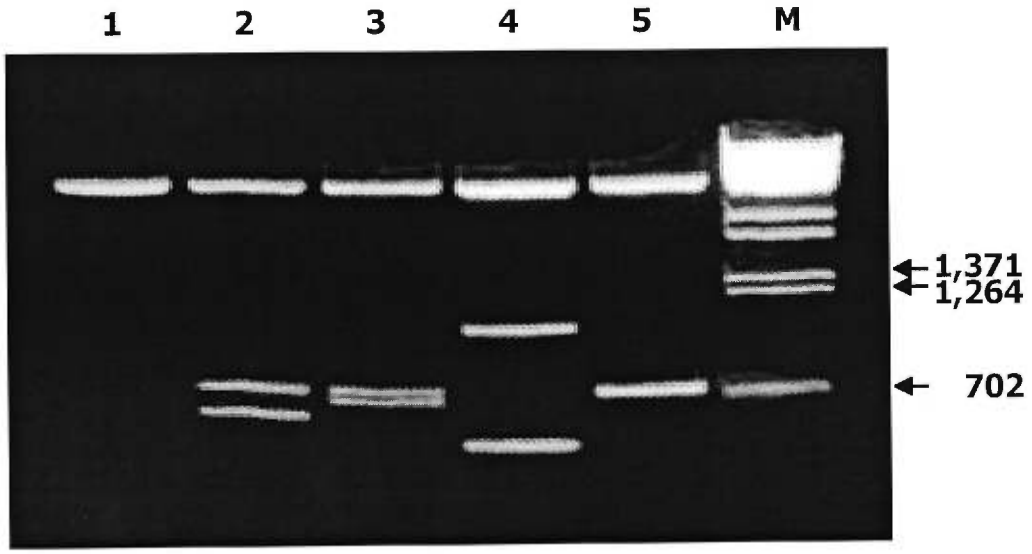

Figure 18: Identification of four different G6PT subclones by restriction enzyme analysis. Plasmids were cut by EcoR I +BstE II, electrophoresis was carried out on a 1.5% agarose gel. Lane 1, pCR2.1-TOPO vector; Lane 2, pCR-2.1-G6PTα; Lane 3, pCR-2.1-G6PTβ; Lane 4, pCR-2.1-G6PTγ; Lane 5, pCR-2.1-G6PTδ; M, lambda DNA-BstE II fragments marker.

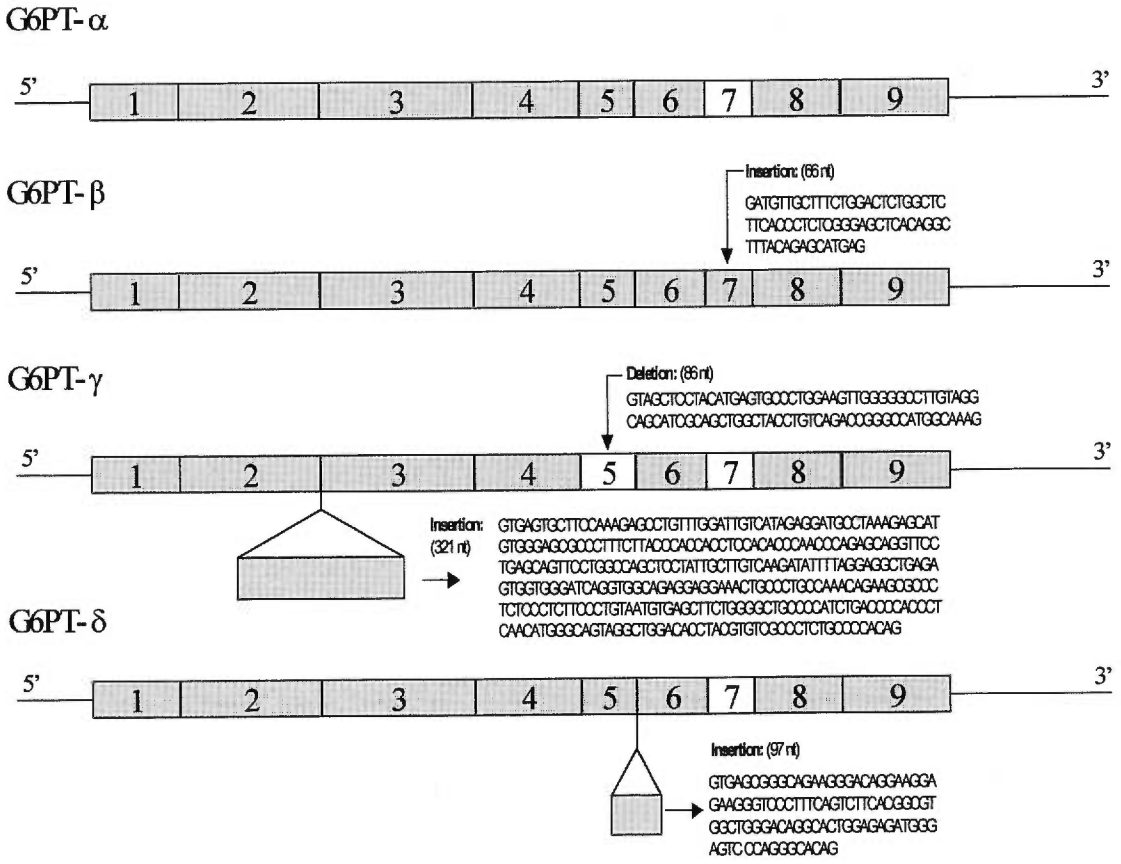

Figure 19: Schematic representation of four different G6PT transcripts : α,β,γ,δ. Positions and relative lengths of exons 1-9 are indicated by boxes 1-9. Shaded boxes represent the transcripts while empty boxes are skipped. Where there is a difference (insertion or deletion), the sequences are indicated.

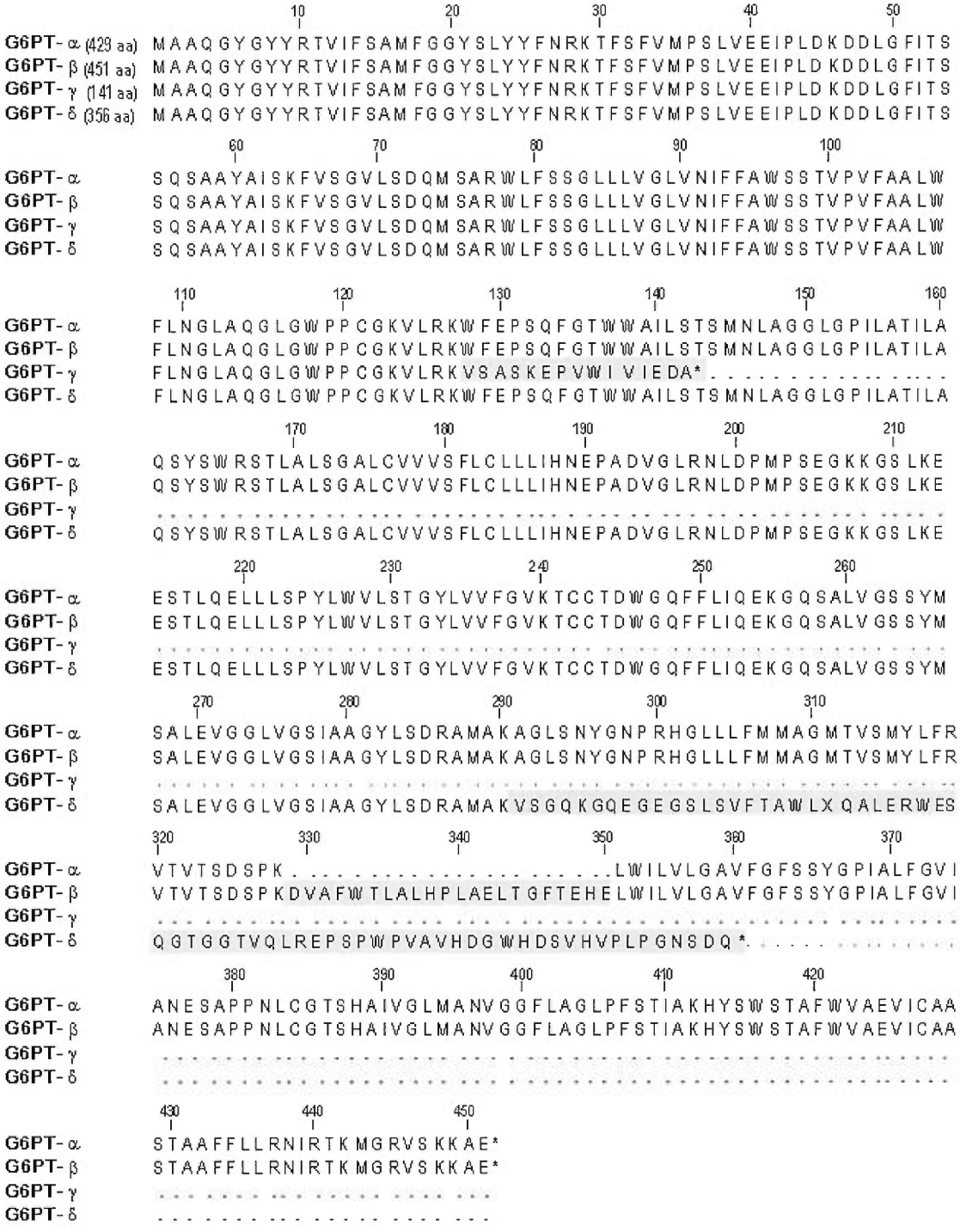

Figure 20: Alignment of protein sequences deduced from the DNA sequences of four different transcripts. Sequences that differ are underlined, stop codons are represented by stars, G6PT (p46) represents putative G6P translocase.

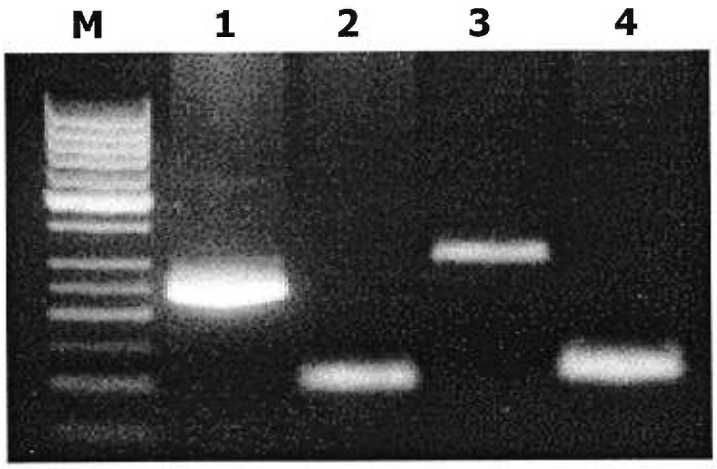

Figure 21: Detection of different p46 transcripts in human blood samples by nested RT-PCR. Electrophoresis was carried out on a 2.0% agarose gel. M, 50bp DNA ladder; Lane 1, 241bp fragment specific for transcript α; Lane 2, 101bp fragment specific for transcript β; Lane 3, 300bp fragment specific for transcript γ; Lane 4, 113bp fragment specific for transcript δ.

4.4.3 Discussion

Four transcripts of the putative G6P translocase (named p46$_{\alpha\text{-}\delta}$), two that correspond to previous published forms in liver (p46-α) (Gerin et al., 1997) and brain (p46-β) (Middleditch et al., 1998) and two new transcripts (p46-γ and δ) were identified from a human leukocyte cDNA library and were further confirmed by nested RT-PCR from human blood samples. Compared to the cDNA sequence of p46-α, p46-β has a 66nt insertion between exon 6 and exon 8, which comes to be exon 7. The protein therefore is 451 aa, after adding 22 aa to 429 aa. This transcript is also found in human brain and is claimed to be a brain isoform (Middleditch et al., 1998). The presence of the p46-β in human leukocytes thus indicates that this isoform is not restricted to the brain. A different splicing pathway (p46-γ) in which intron 2 (321 nt) is retained while exon 5 (86 nt) is skipped has been identified. This insertion of intron 2 introduces a stop codon soon after the insertion, coding a shorter protein (142 aa) than the previously found p46-α (429 aa). P46-δ has a 97 nt insertion between exon 5 and exon 6, which comes to be the first 97nt of intron 5. This transcript encodes a 356 aa protein. As we know, p46 protein is a highly hydrophobic protein and has been reported to be a membrane protein with 12 helical transmembrane domains (Gerin et al., 1997). However, both p46-γ and δ lose the C-terminal KK signal, which is supposed to target the proteins to the endoplasmic reticulum membrane (Jackson et al., 1993); these two transcripts

might thus exist in the cytosol. However, both membrane-bound and soluble forms could be responsible for leukocyte dysfunction in GSD-Ib.

It remains to be elucidated whether these transcripts are precursors of mature mRNA. However, it will be hard to explain why an exon is deleted (in the case of γ) and only part of the intron is transcribed (in the case of δ). Therefore it is more plausible that the existence of transcription variants in leukocytes is due to differential mRNA splicing. Alternative splicing of the primary transcript of p46 gene may predict the expression of four different p46 isoforms or even different functions. Since we know that both the G6Pase activity and the catalytic subunit are undetectable in leukocytes, it is likely that p46 may play other roles than just being a G6P transporter in the G6Pase system. Recently, the group of Van Schaftingen discovered mutations of p46 gene in both GSD-Ib and Ic patients (Veiga-da-Cunha et al., 1998), which suggests that GSD-Ib and Ic could originate from the defect of the same gene. We predict that differential mRNA splicing of p46 may result in multiple functional proteins. These functions may be linked to glucose phosphorylation (Bashan et al., 1993) and Ca^{++} uptake by the endoplasmic reticulum (Kilpatrick et al., 1990), which are impaired in GSD-Ib leukocyte; or related with phosphate transport, which is deficient in liver microsomes from GSD-Ic patients (Nordlie et al., 1993). The relative abundance of the four p46 transcripts reported here was variable in blood samples; in some samples, p46-β was barely detectable, in agreement with a former study (Gerin et al., 1999).

This is not unexpected, as the regulation of p46 gene expression is likely to be under hormonal and nutritional control. It is also worth looking for the presence of different p46 transcripts in other tissues, which can provide more information about their tissue-specific property and whether this property is related to different functions. Further studies on the multiple functions of p46 in leukocytes may help us to find an explanation for neutropenia and neutrophil dysfunction in GSD-Ib and Ic patients.

5- DISCUSSION

5.1 G6Pase in diabetes

We found that in streptozotocin-induced diabetic rats, a rodent model of insulin deficiency, p46 is induced in parallel to p36 at both the mRNA and protein levels in liver, kidney and intestine, where both p36 and p46 localize and express in high abundance. These observations indicated: firstly, that although the tissue distribution of p46 is broader than that of p36 as shown by previous studies (Lin et al., 1998), it mainly presents in gluconeogenic tissues and its mRNA abundance is similar to that of p36 based on our Northern blot analysis; secondly, p46 expression is induced to an extent similar to that of p36 in streptozotocin-induced diabetic rats, suggesting p46, as with p36, may play a role in the pathology of diabetes and contribute to uncontrolled hepatic glucose production. As reviewed in Part 1, enhanced G6Pase activity is associated with both type I and type II diabetic rodent models; it would therefore be interesting to examine whether p46 is also induced in type II diabetes. It is likely to be the case in type II diabetes because: (1) both insulin resistance seen in type II diabetes and insulin deficiency seen in type I diabetes are often associated with hyperglycemia; (2) hyperglycemia *per se* induces the gene expression of p36 (Massillon et al., et al, 1996, 1998); (3) in HepG2 cells, glucose induces p46 gene expression as well as that of p36 in the physiological

and pathophysiological glucose range. Certainly this point has to be verified by doing the experiments. Furthermore, on the one hand, treatment with an inhibitor of G6P translocase (chlorogenic acid derivative) reduced plasma glucose levels in both mice and rats, and also improved glucose tolerance in ob/ob mice and their control littermates (Herling et al., 1998; Parker et al., 1998); on the other hand, we found overexpression of p46 in primary hepatocytes with an adenovirus altered glucose metabolism, resulting in reduced glycogen content and lactate production, which also indicates p46 may play a deleterious role in diabetes.

As the terminal enzyme catalyzing the glucose formation in both glycogenolysis and gluconeogenesis, G6Pase (now including both p36 and p46) is considered a candidate gene for NIDDM and obesity. It is thus interesting to know whether increased G6Pase expression is primary or secondary to the development of diabetes. Overexpression of p36 in rats suggests that increased expression of p36 is able to induce the development of glucose intolerance and hyperinsulinemia, resulting in turn in increased lipid storage in peripheral tissues (Trinh et al., 1999); thus enhanced G6Pase activity could be the primary factor. However, studies on hepatic G6Pase activity at various developmental stages in ZDF rats have shown that induction of G6Pase does not precede the appearance of metabolic abnormalities such as hyperglycemia and hyperlipidemia. Therefore induction of G6Pase activity is

secondary to those abnormalities and its role may be to exacerbate hyperglycemia (An et al., 1999). We have to keep in mind that under *in vivo* conditions, G6Pase activity needs both p36 and p46. In order to understand how p36 and p46 contribute to the development of diabetes, it is thus necessary to look at the profiles of gene expression of both p36 and p46 at the mRNA and protein levels, as well as their correlation with functional G6Pase activity, during the development of both types of diabetes. In type I diabetes, insulin deficiency will cause the increase of counter-regulatory hormones such as glucagon and catecholamines, resulting in increased gene expression and activity of G6Pase, which will enhance hepatic glucose production, leading to hyperglycemia. In type II diabetes, increased G6Pase activity could be secondary to the appearance of diabetic symptoms as seen in ZDF rats, but the possibility of genetic defects in either p36 or p46 gene can not be ruled out until linkage between gene mutations of both genes and NIDDM is established. Indeed, a mutation in PEPCK gene that causes a loss of transcriptional repression of PEPCK by insulin led to unrestrained gluconeogenesis (Rosella et al., 1993) and expression of this mutated PEPCK gene in transgenic mice resulted in development of glucose intolerance and increased weight gain (Rosella et al, 1995).

5.2 Nutritional and hormonal regulation of p36 and p46

Compared to the whole animal models, cultured cells allow us to study the effects of glucose regulation and hormonal regulation on gene expression separately. It is easy to manipulate effectors' concentrations and time of treatment, thus providing a system that can be finely tuned *in vitro* to study the gene regulation. HepG2 cells were used in this work to study the gene regulation of both p36 and p46 by glucose and various hormones such as insulin, glucagon and glucocorticoid. The effect of intracellular Ca^{2+} on G6Pase gene expression was also addressed.

5.2.1 Glucose

As previously reported in Fao hepatoma cells and primary hepatocytes (Lange et al., 1994; Argaud et al., 1997), glucose also increased gene expression of p36 in HepG2 cells in a glucose concentration range of 1-25 mM. Interestingly, p46 expression was concomitantly induced at both mRNA and protein levels and reached the maximal expression at a glucose concentration of 15 mM in the culture medium. The stimulatory effect of glucose on p36 was seen in the presence of insulin in primary hepatocytes (Argaud et al., 1997) or was found to be independent of insulin *in vivo* (Massillon et al., 1996). In our work, the glucose effect was observed in serum free conditions, further demonstrating that glucose *per se* positively regulates p36 and p46 gene expression. Glucose phosphorylation via glucokinase is

important for the glucose stimulatory effect on the p36 gene as well as other glucose-sensitive genes. Indeed, overexpression of GK in Fao cells significantly increased the glucose effect (Argaud et al., 1997) while inhibition of GK abolished it (Massillon et al., 1998). It was also proposed that xylulose-5-phosphate is the metabolite downstream of G6P that mediates the glucose signal for p36 gene expression (Massillon et al., 1998). Obviously this point needs to be verified in the case of p46 gene expression to know if it is through the same glucose signaling pathway. Although it seems paradoxical that hyperglycemia increases G6Pase expression which may favor further release of glucose by the liver, this observation could be understood as a balance of regulation among different metabolic pathways. For example, during the postprandial period, the induction of both components of G6Pase by hyperglycemia will balance the negative effect of insulin to avoid complete suppression of the gene. This may help prevent excessive hepatic storage of glucose and prepare for the following transition to the fasted state when increased glucose output is needed.

5.2.2 Insulin

In either Fao cells (Argaud et al., 1996) or HepG2 cells (this work), insulin can inhibit p36 gene expression rapidly and efficiently as observed by dose response or time course studies. This meets the physiologic needs to

immediately decrease hepatic glucose production after absorption and is also in agreement with decreased G6Pase activity and glycemia in diabetes by insulin treatment. From our dose response curve, we can see that p36 gene transcription is very sensitive to insulin; the negative effect of insulin is observed at a concentrations as low as 0.01 nM. In contrast, the sensitivity of p46 to insulin is much lower, which indicates that insulin regulation of G6Pase activity is exerted mainly on p36 rather than on p46. When insulin is added in combination with other positive effectors of p36 or p46 such as cAMP, glucagon and dexamethasone, the effect of insulin is dominant negative. This dominant effect of insulin over other hormones enables the body to acutely inhibit postprandial hepatic glucose production (HGP), thus preventing hyperglycemia from occuring. Recently mechanisms by which insulin may inhibit HGP were proposed as follows (Guignot & Mithieux, 1999): (1) Insulin itself is able to suppress HGP by solely decreasing hepatic G6P levels through its stimulating effect on glycolysis. (2) Under hyperglycemic condition, the effect of insulin to suppress HGP involves both the increasing of GK flux and the inhibition of G6Pase activity. This inhibition of G6Pase thus plays a crucial role in the suppression of HGP, which could be lost in genetically obese (fa/fa) rats due to insulin resistance (van de Werve et al., 1987). In addition, insulin may also exert its effect at the posttranscriptional level to destabilize mRNA, as seen for PEPCK (Christ et al., 1993). Furthermore, identification of several insulin response sequences in the promoter region of p36 gene revealed the

molecular basis of p36 gene regulation by insulin. Further studies on p46 gene promoter will help to explain the lesser sensitivity of p46 to insulin, as well as how this regulation contributes to G6Pase activity.

5.2.3 cAMP

When cultured hepatoma cells were treated with cAMP in the presence of MIX (in this work) or with a cAMP analog (Lange et al., 1994), G6Pase activity was increased with elevated p36 mRNA and protein levels, while p46 protein was not changed although a slight increase in mRNA was observed. It seems therefore that increased intracellular cAMP induces G6Pase by affecting mainly p36. The effect of cAMP is rapid, reaching a maximum at 6 h and then declining, probably due to the degradation of cAMP and corresponding changes in the signaling pathway. Glucagon, which uses cAMP as the second messenger, has similar effects on p36 in HepG2 cells.

5.2.4 Glucocorticoids

In contrast to cAMP, which barely affects p46, dexamethasone treatment of cultured HepG2 cells induced the gene expression of both p36 and p46 to a similar extent. Compared to Fao cells, HepG2 cells are more sensitive to dexamethasone. A minimal effect on G6Pase gene expression was seen at

the concentration of 0.1 nM while in Fao cells 1 μM was needed to see an effect. Consistently, rats treated with the glucocorticoid analogue triamcinolone have increased p46 protein levels in liver microsomes compared to untreated rats (Méchin, personal communication), further indicating that p46 gene expression is significantly altered by glucocorticoids. *In vivo* studies of dexamethasone treatment of normal, adrenalectomised and hypophysectomised rats have shown that dexamethasone increased G6Pase activity in both intact and detergent disrupted liver microsomes with a decreased latency, which suggests that dexamethasone increased the G6P transport activity relatively more than G6P hydrolytic activity (Voice et al., 1997). In 2S FAZA hepatoma cells, dexamethasone also induced G6Pase activity in both intact and disrupted microsomes. However, in this case the increase in G6P transport is to a lesser extent than that in G6P hydrolysis as reflected by an increased latency (Garland, 1988). Nevertheless, the consistence between induced p46 gene expression and increased G6Pase activity by dexamethasone implies an essential role of p46 for G6Pase activity in intact vesicles. Further studies are needed to address how decreased p46 expression may alter the G6Pase activity and its latency. Since glucocorticoids are known to activate both gluconeogenesis and glycogenesis while dexamethasone treatment did not significantly alter blood glucose, its role may be mainly glycogenic under this condition. Indeed, liver glycogen levels were increased when rats were unable to counter-regulate normally against the administation of excess glucocorticoid

due to adrenolectomy or hypophysectomy (Voice et al., 1997). The possible roles of p46 in glycogen synthesis will be discussed in the section of p46 functions.

5.3 p46 functions

5.3.1 p36-related function of p46 — is p46 a G6P transporter?

Although the function of p46 is still unclear and it will obviously remain an intriguing topic in G6Pase field, there are a few reports that directly or indirectly tackled this subject and provided us experimental evidence to conceive p46 functions. Using p36 knock-out mice, Lei et al have previously shown that the G6Pase activity is absolutely required for G6P transport into the lumen of liver microsomes; without p36, G6P uptake is undetectable (Lei et al., 1996). This result suggests that p36 itself is involved in G6P transport. Furthermore, when the p36 gene was delivered into the liver of p36 knock-out mice, it was observed that the G6P transport rate was highly correlated to the restored G6Pase activity during a period of 70 days (Zingone et al., 2000), which again indicates that G6P transport activity is p36-dependent and G6P transport and hydrolysis are highly-coupled events. One can speculate that there are two distinct sites on p36, one for hydrolysis and the other one for substrate binding, while p46 could act as a regulatory protein of p36 which is

required for both substrate binding and hydrolysis. In COS-1 cells (a cell line expressing endogenous p46 but not p36), a very low level of G6P uptake was detected in microsomes isolated from mock-transfected control cells. When COS-1 cells were transfected with p46 cDNA or p36 cDNA alone, G6P uptake was increased to a small extent compared to the mock cells. However, G6P uptake was markedly increased in microsomes from COS-1 cells cotransfected with both p36 and p46 (Hiraiwa et al., 1999). In this COS-1 transient transfection system, similar amounts of G6Pase hydrolytic activity were detected in disrupted microsome fractions isolated from cells transfected either with p36 cDNA alone or with both p36 and p46 cDNAs, which indicates that p36 bears the hydrolytic activity. In intact microsomes from cells transfected with p36 cDNA alone, the latency of G6Pase activity is normal (about 50%), as in liver microsomes, but G6Pase activity in intact microsomes isolated from p36 and p46 co-transfected cells is almost the same as seen after detergent disruption, which means transfection of p46 in COS-1 cells caused the disappearance of latency. It was explained that the p46 is the rate-limiting step of G6P hydrolysis and the disappearance of latency is the consequence of reaching the optimal p36/p46 ratio, which determines the G6Pase activity *in vivo* (Hiraiwa et al., 1999). However, on the one hand, p36 overexpression alone via recombinant adenovirus is sufficient to increase G6Pase activity in intact microsomes, providing evidence against p46 being the rate-limiting step (Trinh et al., 1997, 1998). On the other hand, in the fasted state and

streptozotocin-induced diabetic rats, G6Pase activity is increased only in disrupted microsomes but not in intact microsomes, while both p36 and p46 gene expression were found to be induced in these conditions, again suggesting that p46 is not rate-limiting. From all these results we can conclude that both G6P uptake and phosphohydrolase activity are dependent on the presence of the two components (p36 and p46) of the G6Pase system. Functional p46 is necessary for G6P transport, but current data do not allow us to conclude that p46 is a G6P transporter. Recently, the relationship between G6Pase activity and [^{18}F]-2-fluoro-2-deoxyglucose (FDG) release kinetics was studied in two cell culture systems by using positron emission tomography (PET) imaging technique (Caraco et al., 2000). It was found that the release of FDG from these cells correlated with p46 mRNA abundance, provided that p36 is present. That is to say that adequate levels of both p36 and p46 are required to compose a functional G6Pase *in vivo* to facilitate glucose production. Moreover, this functional G6Pase complex can be conveniently quantified by measuring FDG-PET. However in this study, G6Pase activities were measured only in disrupted microsomes. Therfore it will be interesting and necessary to measure also the activities in intact microsomes, which is a direct way to address how G6P transport is correlated with p46 expression and G6Pase activity. Based on the observation that during development the expression of p36 and p46 genes appears much earlier than that of G6P transport activity, it was proposed that additional factor(s) might be involved in modulating G6P transport. Since the

gene expression profile of glucokinase meets the developmental appearance of G6P transport function, and also because of its important role for maintaining the cytoplasmic G6P level, glucokinase was proposed to be such a candidate (Zingone et al., 2000). It is not impossible that another unknown protein is required to facilitate G6P transport, since in COS cells transfection with both p36 and p46 cDNAs only restored the G6P uptake ability to 10% of that of liver (Hiraiwa et al., 1999). Alternatively, a 55 kDa protein was identified in human liver microsomes as a G6P transporter by photoaffinity labelling with G6P translocase inhibitor chlorogenic acid analogue as probe (Kramer et al., 1999). However, direct evidence is lacking to link this 55 kDa protein to the cloned putative G6P translocase, since the calculated molecular weight from the sequence of the cloned cDNA is 46 kDa. With the use of the antiserum against the N-terminal peptide of this putative G6P translocase, a specific band around 46 kDa was detected by Western blot analysis in both rat liver microsomes and human hepatoma cell line HepG2 (Articles 1 & 2; Méchin et al., 2000).

5.3.2 Other functions of p46

5.3.2.1 Pi transporter

GSD-Ic is clinically diagnosed on the basis of deficiency in pyrophosphate/Pi transport activity in liver microsomes (Nordlie et al., 1983). As in GSD-Ib patients, mutations of the p46 gene were also discovered in most GSD-Ic patients (Veiga-da-Cunha et al., 1998, 1999; Janecke et al., 1999). One can speculate that p46 may have the ability to transport both G6P and Pi by different sites on the p46 protein, as is indeed the case for the bacterial G6P transporter. However, since the same mutations were found in both GSD-Ib and Ic patients, it is difficult to explain how the same mutations can result in different phenotypes. One argument is that neutropenia and neutrophil dysfunctions are present in those GSD-Ic patients with p46 mutations as seen in GSD-Ib patients, while another GSD-Ic patient has no neutropenia and also no mutation of p46 gene was reported (Lin et al., 1999). This suggests real GSD-Ic is not caused by mutations in p46 and a distinct GSD-Ic locus must exist. Therefore those previously diagnosed GSD-Ic patients with p46 mutations are probably a misclassification and should be reclassified as GSD-Ib patients, while GSD-Ic still exists. Interestingly, another argument is that mutations in the p46 gene are not necessarily associated with the appearance of neutrophil impairment because some GSD-Ib and Ic patients with p46 mutations were found to have no evidence of neutropenia and neutrophil dysfunction while other GSD patients with the same mutations have the symptoms (Galli et al., 1999). So at this stage the question of whether defects in an independent Pi transporter is the cause of GSD-Ic remains open.

5.3.2.2 G6P sensor/receptor

The role of G6P is essential in regulating several glucose metabolic pathways. In glycolysis, G6P increases fructose 2,6-bisphosphate which allosterically activates phosphofructokinase-1; increased levels of G6P are also associated with glucokinase translocation. G6P can stimulate glycogen synthesis by acting as an allosteric activator of glycogen synthase and increasing its dephosphorylation by synthase phosphatase. Although G6P hydrolysis is the terminal step of glucose production in both gluconeogenesis and glycogenolysis, the flux control coefficient of G6Pase on gluconeogenesis is remarkably low (Groen et al., 1986). Based on the finding that overexpression of the G6Pase (p36) has no effect on glucokinase translocation and that it also does not increase the control strength of glucokinase, it was proposed that the main regulatory function of G6Pase is to buffer G6P concentration in hepatocytes (Aiston et al., 1999). It was also suggested that the buffering role of G6Pase would be determined not only by the catalytic subunit p36 but also by G6P transport into the endoplasmic reticulum. We observed that either glucose or glucocorticoids can increase the expression of both p36 and p46, while increased glucose and glucocorticoids favor glycogen synthesis instead of glucose production. Therefore we propose that p46 may act as a G6P sensor and under these conditions direct G6P towards glycogen. It

is well known that the intracellular G6P pool is not homogenous but rather compartmentalized (Kalant et al., 1987). Although overexpression of either GK or HK-1 by adenovirus increased G6P level, only overexpression of GK was glycogenic (Seoane et al., 1996). This is consistent with their different compartmentation in that HK remains associated with mitochondria, while GK and glycogen synthase undergo a translocation from the nucleus or perinuclear region to a peri-plasma membrane region. As a membrane-associated protein, p46 could be involved in this movement and could probably control glycogen synthesis by interacting with protein phosphatase type-1 (PP-1) or its glycogen targeting subunits. Through binding to G6P, p46 could transfer the information of increased concentration of G6P produced by GK to raise a signal that activates glycogen synthase, thus acting as a G6P sensor. Obviously, the possible role of p46 in glycogen synthesis has to be demonstrated by experimental data.

The cDNA of p46 was cloned by comparison of liver ESTs with the DNA sequences of bacterial transporters for monophosphate ester, such as the hexose phosphate transporter (UhpT) and receptor (UhpC) (Gerin et al., 1997). By multiple sequence alignments, a consensus involving 14 sequences related to p46 was identified (Méchin & van de Werve, 2000). Based on the comparison of the consensus with specific residues of UhpC and UhpT, it was

proposed that p46 could have both G6P transporter and receptor function. Actually, the homology of p46 to UhpC is higher than to UhpT.

5.3.2.3 Other roles of p46 in non-gluconeogenic tissues

Compared to GSD-Ia patients, GSD-Ib and Ic patients have an additional deficiency in their immune system as neutropenia and neutrophil dysfunctions, resulting in recurrent bacterial infections (Gitzelmann et al., 1993). A reduction in glucose metabolism that is due to a defect in intracellular glucose phosphorylation was observed in leucocytes of GSD-Ib patients (Potashnik et al., 1990; Bashan et al., 1993). Compared to normal controls, increased extracellular glucose failed to elevate intracellular G6P level, thereby limiting the activity of G6P dehydrogenase to generate NAPDH in the hexose-monophosphate (HMP) shunt. For this reason, a glucose stimulated respiration burst catalyzed by the NADPH oxidase was absent in GSD-Ib patients (Verhoeven et al., 1999). Since mutations in p46 gene are the genetic defects in these patients, p46 must somehow participate in glucose phosphorylation. This function of p46 is independent of p36 because we found p36 was undetectable in leucocytes by Northern blot and Western blot analyses. In addition, we found that two distinct isoforms of p46 (this work), as well as the brain isoform (Middleditch et al., 1998), were present in leucocytes but not in liver, indicating p46 gene may undergo tissue-specific mRNA splicing to obtain

different functions. It is possible that mutated p46 could not bind G6P produced by hexokinase, and thus fails to transfer it to the correct intracellular space, which results in the inhibition of hexokinase by trapped G6P and unavailability of G6P to HMP shunt. To this end, it will be interesting to study the intracellular localization of p46 by *in situ* immunoblotting with p46 antibody or by means of expressing tagged-p46 or green florescence protein-p46 fusion protein.

Furthermore, the respiratory burst abnormality in neutrophils and monocytes of GSD-Ib patients is associated with impaired calcium mobilization which is probably due to defective calcium stores in the endoplasmic reticulum (Kilpatrick et al., 1990). Interestingly, G6P and Ca^{2+} sequestration are found to be mutually enhanced in microsomes (Chen et al., 1998). Therefore impaired p46 could either induce a decrease in G6P levels with a consequent reduced calcium store or directly interfere with Ca^{2+} sequestration by interacting with Ca^{2+}-ATPase. We observed that inhibition of endoplasmic reticulum Ca^{2+}-ATPase by thapsigargin induced the p36 gene transcription without changes in p46, while others have shown that rats treated with an inhibitor of G6P translocase upregulated p36 gene expression (Simon et al., 2000).

5.4 Conclusions

In conclusion, our results indicated: (1) In insulin-dependent diabetes, hyperglycemia, insulin deficiency and increased cAMP due to unopposed counterregulatory hormones may contribute independently from each other to elevated p36 and p46 gene expression. (2) Overexpression of p46 via recombinant adenovirus induced p36 transcription and resulted in similar metabolic consequences as seen in overexpression of p36, which include decreased glycogenesis and glycolysis as well as increased glycogen degradation. This indicates that dysregulations of both p36 and p46 may be involved in increased G6Pase activity, which may lead to enhanced hepatic glucose production and exacerbated hyperglycemia in diabetes. (3) Distinct hormonal regulations of p36 and p46 genes infer that hormonal changes which affect p36 alone concur with known modifications in glucose production, while those affecting both p36 and p46 are rather consistent with glucose storage. (4) p46 may be a multifunctional protein with a tissue-specific property. In those tissues where p36 is present such as in liver, p46 may confer G6P to p36 for G6P hydrolysis. In other tissues when p36 is absent, p46 probably bears other functions that could explain neutropenia and neutrophil dysfunctions in GSD-Ib patients.

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