Probing into the function of the gene product responsible for glycogen storage disease type Ib

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Abstract This study aimed at directly assessing glucose 6-phosphate (G6P) transport by intact rat liver microsomes. Tracer uptake from labeled G6P occurred with $T_{1/2}$ values that proved insensitive to unlabeled G6P or 100 μM vanadate, and could not be activated over background levels by intravesicular phosphate in the complete absence of G6P hydrolysis. [^{32}P]Phosphate efflux was similarly unaffected by G6P or phosphate in the incubation medium. We conclude that the gene product responsible for glycogen storage disease type Ib is functionally distinct from the bacterial hexose phosphate transporter, which operates as an obligatory phosphate:phosphate or G6P:phosphate exchanger. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Glucose 6-phosphatase system; Transport kinetics; Glucose 6-phosphate transport; Vectorial transport; Rat liver microsome

1. Introduction

Liver glucose 6-phosphatase (G6Pase) is tightly linked to the endoplasmic reticulum membrane, where it plays a crucial role in carbohydrate metabolism by controlling glucose production from glucose 6-phosphate (G6P) hydrolysis [1]. The absence of enzyme activity is associated with a class of metabolic disorders known as glycogen storage diseases type I (GSD-I): the most frequent form (GSD-Ia) is caused by a deficiency in the catalytic unit protein (p36), while a variant (GSD-Ib) would arise from impaired function of its associated putative G6P translocase (p46) [2].

The G6P transport function currently attributed to p46 mostly stems from its homology with the bacterial upper hexose phosphate transporter (UhpT) [1] and is mainly supported by circumstantial pieces of evidence [3–5]. In this respect, carriers related to the UhpT protein were also termed 'P_i-linked' because in each case P_i (inorganic phosphate) is accepted with relatively low affinity while some selected organic phosphate substrate is accepted with high affinity [6]. In contrast, all experiments aimed at testing G6P transport in intact microsomes have so far been performed in the absence of P_i on the trans side [3–5]. In the present study, we demonstrate that p46 does not function as a G6P translocase and appears functionally distinct from its bacterial UhpT counterpart.

2. Materials and methods

2.1. Materials

[U-^{14}C]G6P (317 mCi mmol^{-1}) was purchased from ICN Biomedicals, while [^{32}P]G6P (144 mCi mmol^{-1}) was prepared as previously described [7]. KH_2PO_4, G6P, mannose 6-phosphate, phlorhizin, chlorogenic acid, and sodium orthovanadate were from Sigma.

2.2. Microsome preparation

Liver microsomes were isolated from overnight-fasted male Sprague–Dawley rats (approx. 300 g body weight), as previously described [7], using buffer A (50 mM HEPES–Tris, 250 mM sucrose, pH 7.3) during the homogenization and resuspension steps. If necessary, microsomes were permeabilized by detergent treatment as reported in [8].

2.3. Enzyme and protein assays

G6Pase and mannose 6-phosphatase activities were measured at room temperature, as previously described [7], and the microsomal preparations routinely displayed more than 90% latency relative to mannose 6-phosphate. Vanadate inhibition of G6Pase activity was tested following a 3-min period of incubation in the presence of the indicated concentrations of inhibitor. Protein was assayed as reported in [9].

2.4. Uptake measurements

The kinetics of tracer uptake were followed at room temperature using a fast-sampling, rapid-filtration apparatus (FSRFA; US patent No. 5 330 717) developed in our laboratory [10]. Microsomes were preincubated for 3 min in buffer A supplemented or not with 0.1 mM or 5 mM vanadate, as indicated. When using P_i-loaded microsomes, the preincubation media also contained 25 mM KH_2PO_4. Uptake measurements were performed in 1 ml buffer A containing 10 μM [^{32}P]G6P or [U-^{14}C]G6P, and either unlabeled G6P or various inhibitors, as indicated. Influx experiments were started by injecting 40 μl of microsomes (approx. 0.8 mg protein) into the incubation chamber of the FSRFA. For efflux measurements, the microsomes and incubation media were mixed prior to their introduction into the chamber; following a 2-min period of incubation, efflux was initiated by injecting 40 μl of buffer A alone or buffer A plus unlabeled G6P or KH_2PO_4, as indicated. In both cases, the tracer content of the vesicles was followed in time by 18-point automatic sequential sampling and liquid scintillation counting, as previously described [7].

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Abbreviations: FSRFA, fast-sampling, rapid-filtration apparatus; G6P, glucose 6-phosphate; G6Pase, glucose 6-phosphatase; GSD, glycogen storage disease; P_i, inorganic phosphate; p36, catalytic unit of G6Pase; p46, GSD-Ib gene product, the putative G6P transport protein; S.E.M., standard error of the mean; S.E.R., standard error of regression; UhpT, upper hexose phosphate transporter

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2.5. Data analysis

Three to five uptake series were run under each of the reported experimental conditions. Influx and efflux data were analyzed by Enzfitter or P. Fit software (both published and distributed by Biosoft) according to the first-order rate equation

$$U = U_0 + U_\infty(1-e^{-kt}) \quad (1)$$

or the single exponential decay equation

$$U = U_0 e^{-kt} + U_\infty \quad (2)$$

respectively. Note that the parameters U, U_0, and U_∞ stand for the intravesicular amounts of tracer at time (t), at time zero, and at equilibrium, respectively, whereas k is the first-order rate constant of the uptake process from which the $T_{1/2}$ values (i.e. the time at which 50% of the process has been completed) reported in this paper have been calculated ($T_{1/2} = \ln 2/k$). Note that the errors associated with the kinetic parameter values represent standard error of regression (S.E.R.), which result from weighted or unweighted non-linear regression analysis of the data.

3. Results and discussion

3.1. Tracer uptake from [^{32}P]G6P

Tracer uptake from [^{32}P]G6P was completely suppressed following detergent treatment and upon incubation with 5 mM of either chlorogenic acid or phlorhizin (Fig. 1A), two specific inhibitors of the putative T1 G6P translocase [11,12]. Our microsomal preparations thus appear fully functional with regard to the expected role of p46. However, as depicted in Fig. 1B, over the first 2-min period of incubation, tracer uptake from [^{32}P]G6P occurred with $T_{1/2}$ values that proved insensitive to unlabeled G6P (see Table 1), which acts as a competitive inhibitor of tracer G6P transport and hydrolysis. This result does not prove that p46 does not act as a glucose 6-phosphate translocase, provided that [^{32}P]P_i rather than [^{32}P]G6P is the main labeled species to be found in the intramicrosomal space. In this regard, the mean $T_{1/2} \pm$ S.E.M. (standard error of the mean; value of ^{32}P uptake (17.1 ± 0.5 s, see Table 1) closely matches that previously reported for [^{32}P]P_i efflux (22 ± 3 s, see [13]), in accordance with the demonstration that the rates of tracer accumulation observed in such studies should reflect those of P_i efflux [13]. Moreover, using [^{14}C]G6P as the labeled substrate, it was shown that the steady-state level of tracer molecules is directly proportional to G6Pase activity [9], and that G6P at best represents a tiny fraction of the intramicrosomal glucose pool independently of whether a substrate transport or conformational model is considered [13]. The lower steady-state values of ^{32}P uptake, observed in Fig. 1B, at increasing concentrations of unlabeled G6P clearly conform to these conclusions. Therefore, these data challenge the contention of Lei et al. [4] and Hiraiwa et al. [5] that tracer uptake from labeled G6P characterizes G6P transport.

Table 1
$T_{1/2} \pm$ S.E.R. values of [^{32}P]P_i equilibration

Experimental conditions	Influx	Efflux
10 μM G6P (control)	16.4 ± 1.1	–
1 mM G6P	16.4 ± 2.8	16.9 ± 3.7
5 mM G6P	16.9 ± 3.4	16.6 ± 2.7
10 mM G6P	18.5 ± 4.3	17.4 ± 2.1
20 mM P_i	–	18.4 ± 1.6

Experimental conditions and analyses were as described in the legend of Fig. 1.

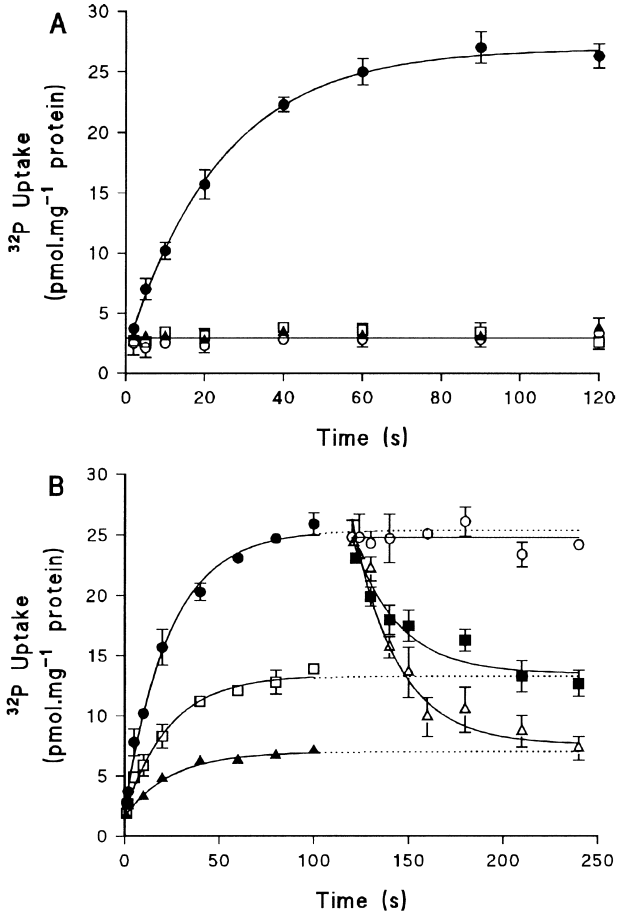

Fig. 1. Tracer uptake from [^{32}P]G6P, and test of G6P:$^{32}P_i$ exchange activity. In A, the uptake media consisted of 1 ml buffer A containing 10 μM [^{32}P]G6P (●), and either 0.8% 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate (○), 5 mM phlorhizin (▲), or 5 mM chlorogenic acid (□). In B, influx experiments were performed as described in (A) in the absence (●) or presence of either 1 mM (□) or 5 mM (▲) unlabeled G6P. For efflux, microsomes were incubated for 2 min in the control medium, and then challenged by buffer addition alone (○) or in combination with unlabeled G6P to get either 1 (■) or 5 (△) mM G6P in the final mixture. The dotted lines represent the extrapolated plateau values under influx conditions. In A and B, the solid lines shown for influx and efflux are the best-fit curves to Eqs. 1 and 2 in the text, respectively, with $T_{1/2}$ values as reported in Table 1. Missing error bars were smaller than the symbol size.

3.2. Tests of G6P:$^{32}P_i$ and P_i:$^{32}P_i$ exchange activities

The rationale behind the following series of experiments is that the existence in the microsomal membranes of G6P:P_i and/or P_i:P_i exchange activities should lead to increased rates of [^{32}P]P_i efflux in the presence of unlabeled G6P and/or P_i in the trans compartment. Note that the low concentration of 10 μM [^{32}P]G6P used in these studies was chosen to maximize the sensitivity of the assays. As depicted in Fig. 1B, over the last 2-min period of incubation, microsomes actively loaded with [^{32}P]P_i by 2 min incubation with 10 μM [^{32}P]G6P were challenged by the addition of either various concentrations of unlabeled G6P or 20 mM P_i (not shown) to the incubation media. Compared to buffer addition alone, unlabeled G6P (and P_i) initiated [^{32}P]P_i efflux through competitive inhibition of [^{32}P]G6P hydrolysis, as inferred from the similar steady-state values observed under symmetrical influx and efflux conditions [9]. However, the $T_{1/2}$ values of [^{32}P]P_i efflux proved to

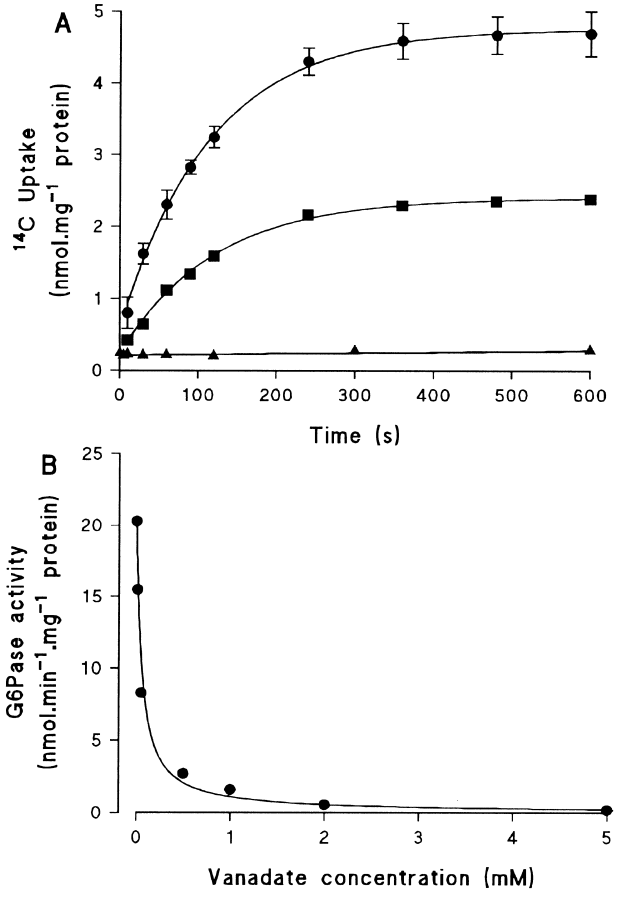

Fig. 2. Effect of vanadate on the kinetics of radiotracer accumulation from [U-^{14}C]G6P (A) and on G6Pase activity (B). In A, the uptake medium consisted of 1 ml buffer A containing 1 mM [U-^{14}C]G6P and either 0 (●), 0.1 (■), or 5 (▲) mM orthovanadate. The best-fit curves corresponding to Eq. 1 in the text are shown for control ($U_\infty = 4.18 \pm 0.01$ nmol mg^{-1} protein, $k = 0.0087 \pm 0.0004$ s^{-1}) and 0.1 mM vanadate ($U_\infty = 2.17 \pm 0.01$ nmol mg^{-1} protein, $k = 0.0084 \pm 0.0004$ s^{-1}). In B, G6Pase activity was assayed using symmetrical conditions of incubation and the indicated concentrations of orthovanadate. The line shown is the best-fit curve when assuming competitive inhibition ($I_{50} = 67 \pm 15$ μM). In A and B, missing error bars were smaller than the symbol size.

be insensitive to outside G6P and P_i, and similar to those given above for ^{32}P uptake (mean $T_{1/2} \pm$ S.E.M. value of 17.4 ± 0.4 s, see Table 1). It can be concluded then that microsomal G6P transport appears functionally distinct from UhpT, which operates as an obligatory G6P:phosphate or phosphate:phosphate exchanger [6]. In this regard, a recent study from our lab also failed to demonstrate $^{32}P_i$:P_i exchange activity when measured under zero-trans entry conditions in the absence of G6P hydrolysis [13].

3.3. Tests of [^{14}C]G6P transport and [^{14}C]G6P:P_i exchange activity

The most serious limitation to studies aimed at evaluating the kinetics of G6P transport in intact microsomes is the ever-present G6Pase activity, which does not allow us to directly assess the relative contributions of intravesicular substrate and product pools to the dynamics of the uptake process [13]. Accordingly, complete and specific inhibition of p36 activity would be desirable to directly assess the putative transport functions associated with p46. In this respect, a similar K_i value of approx. 20 μM was recently reported for orthovanadate inhibition of G6Pase activity in intact and detergent-disrupted microsomes [14], thus supporting the concept that p36 rather than p46 is the target of vanadate action. In line with this view, vanadate was shown to inhibit the steady-state level of intramicrosomal tracer accumulation from labeled G6P without a measurable effect on $T_{1/2}$ [9], and to offer no protection against *N*-ethylmaleimide alkylation of sulfhydryl residues that qualify to represent important thiol groups on p46 [15]. Furthermore, vanadate did not inhibit glucose or P_i transport when measured under zero-trans influx conditions [13] or from efflux studies in which microsomes were actively loaded with [^{14}C]glucose or [^{32}P]P_i by prior incubation with [^{14}C]G6P [9] or [^{32}P]G6P [13], respectively.

The experiments depicted in Fig. 2 corroborate, at a G6P concentration of 1 mM and room temperature, that G6P hydrolysis rather than transport is the target for vanadate inhibition of tracer uptake. Indeed, 100 μM vanadate did not affect the rate of tracer uptake ($T_{1/2} = 80 \pm 4$ or 83 ± 4 s in the absence or presence of vanadate, respectively; see Fig. 2A), while inhibiting steady-state tracer accumulation and G6P hydrolysis by $52 \pm 4\%$ (Fig. 2A) and $60 \pm 13\%$ (Fig. 2B), respectively. Fig. 2 further demonstrates that a concentration of vanadate as high as 5 mM is needed to completely inhibit tracer uptake and G6P hydrolysis under symmetrical conditions.

Using this saturating concentration of inhibitor, Fig. 3 allows us to establish that similar levels of tracer uptake are observed in vanadate- and detergent-treated microsomes during the whole 1-h period of these studies. Therefore, tracer uptake in vanadate-treated vesicles represents non-specific binding of [^{14}C]G6P to the microsomal membranes, which amounts at 60 min of incubation to approx. 6% of the total water space of 5 μl mg^{-1} protein [13] accessible to substrate transport. As also shown in Fig. 3, the rate of tracer uptake was not increased in P_i-loaded microsomes under experimental conditions aimed at minimizing any potential competition between outside [^{14}C]G6P and P_i for transport (both [^{14}C]G6P and P_i were present at a final concentration of 1 mM in the uptake media). Therefore, in the complete absence of G6Pase

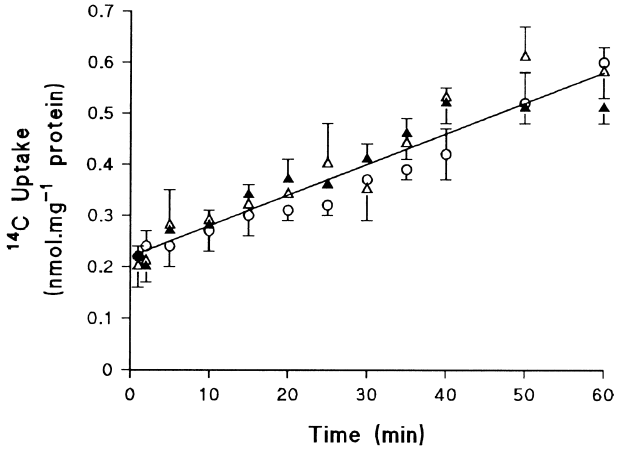

Fig. 3. Tests of [^{14}C]G6P transport and [^{14}C]G6P:P_i exchange activities. Rat liver microsomes were first preincubated in buffer A containing either 0.8% 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate (○), 5 mM vanadate (▲), or 5 mM vanadate plus 25 mM KH_2PO_4 (△). Uptake conditions were as described in the legend of Fig. 2A.

activity, p46 does not exhibit [^{14}C]G6P:P_i exchange activity and does not function as a G6P translocase. We thus challenge the contention of Fulceri et al. [3] that the swelling phase observed upon G6P addition to rat liver microsomes characterizes G6P transport rather than intravesicular accumulation of glucose and P_i produced by G6P hydrolysis. Indeed, this conclusion mostly rests on the observation that similar results were obtained in the absence or presence of 100 μM vanadate, which was claimed, in contrast to the results depicted in Fig. 2, to inhibit up to 94% of enzyme activity at a G6P concentration of 25 mM.

4. Conclusions

The present study demonstrates that p46 does not function as a G6P translocase and appears functionally distinct from its bacterial UhpT counterpart, which was shown to catalyze the electroneutral exchange of internal and external P_i in addition to heterologous exchange of P_i and G6P [6]. This conclusion fits in with our recent suggestion that a pore-like structure takes on the P_i transport function usually attributed to the putative T2 phosphate translocase in intact microsomes [13]. It also proves consistent with the redundant observation that approx. 90% of the total glucose or P_i produced during the course of the enzyme reaction in intact microsomes does not cycle through the intravesicular space [13,16], which invalidates a major postulate of the substrate transport-catalytic unit concept [12]. In this respect, then the GSD-Ib gene product cannot be viewed as a G6P transport protein in the classical sense advocated by this theory. From the demonstration that proper functioning of the G6Pase system does require the simultaneous presence of p36 and p46 in the endoplasmic reticulum membrane [4,5], we therefore suggest that p46 might instead represent a G6P sensor protein modulating the previously characterized hysteretic transition that brings the G6Pase system into a less competent catalytic form for the profit of higher substrate specificity [7]. In support of this view, uncoupling between the p36 and p46 proteins has been readily documented in the past in detergent-permeabilized microsomes [11] and in intact preparations following histone II-A treatment [17,18].

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