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Research paper

New insights into the organisation and intracellular localisation of the two subunits of glucose-6-phosphatase

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ABSTRACT

Glucose-6 phosphatase (G6Pase), a key enzyme of glucose homeostasis, catalyses the hydrolysis of glucose-6 phosphate (G6P) to glucose and inorganic phosphate. A deficiency in G6Pase activity causes type 1 glycogen storage disease (GSD-1), mainly characterised by hypoglycaemia. Genetic analyses of the two forms of this rare disease have shown that the G6Pase system consists of two proteins, a catalytic subunit (G6PC) responsible for GSD-1a, and a G6P translocase (G6PT), responsible for GSD-1b. However, since their identification, few investigations concerning their structural relationship have been made.

In this study, we investigated the localisation and membrane organisation of the G6Pase complex. To this aim, we developed chimera proteins by adding a fluorescent protein to the C-terminal ends of both subunits. The G6PC and G6PT fluorescent chimeras were both addressed to perinuclear membranes as previously suggested, but also to vesicles throughout the cytoplasm. We demonstrated that both proteins strongly colocalised in perinuclear membranes. Then, we studied G6PT organisation in the membrane. We highlighted FRET between the labelled C and N termini of G6PT. The intramolecular FRET of this G6PT chimera was 27%. The coexpression of unlabelled G6PC did not modify this FRET intensity. Finally, the chimera constructs generated in this work enabled us for the first time to analyze the relationship between GSD-1 mutations and the intracellular localisation of both G6Pase subunits. We showed that GSD1 mutations did neither alter the G6PC or G6PT chimera localisation, nor the interaction between G6PT termini. In conclusion, our results provide novel information on the intracellular distribution and organisation of the G6Pase complex.

1. Introduction

Glucose-6 phosphatase (G6Pase) catalyses the last enzymatic step before the release of glucose into the blood: the hydrolysis of glucose-6 phosphate (G6P) into glucose and inorganic phosphate (Pi). G6Pase activity is restricted to the liver [1], the kidney cortex [2] and the small intestine [3] and confers on these three organs the capacity to release glucose into the systemic circulation. This enzyme plays a key role in glucose homeostasis since a deficiency in G6Pase activity leads to a severe metabolic disorder mainly characterised by hypoglycaemia in the post-absorptive state: glycogen storage disease type 1 (GSD-1) [4]. From human genetic studies, GSD-1 patients have been classified in two types based on mutations of the *G6Pase catalytic subunit (g6pc)* or the *G6P translocase (g6pt)*. GSD-1a results from mutations in *g6pc* and GSD-1b from mutations in *g6pt*. Both proteins are thought to be anchored in the endoplasmic reticulum (ER) and also expressed in nuclear membranes [5]. This localisation was suggested from biochemical and histochemical studies based on phosphatase enzymatic activity [6–8]. However, no recent data based on the detection of G6Pase proteins have confirmed this intracellular localisation.

Since G6PC and G6PT are sufficient to completely account for G6Pase activity [9,10], the current G6Pase substrate transport model proposes that the active site of G6PC is exposed to the ER lumen [11] and G6PT shuttles intracellular G6P across the ER

Abbreviations: CFP, cyan fluorescent protein; ER, endoplasmic reticulum; FRET, fluorescence resonance energy transfer; FLIM, fluorescence lifetime imaging; G6P, glucose-6 phosphate; G6Pase, glucose-6 phosphatase; G6PC, G6Pase catalytic subunit; G6PT, G6P translocase; GSD-1, glycogen storage disease; Pi, inorganic phosphate; YFP, yellow fluorescent protein.

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membrane into the lumen, where it is hydrolysed. This model, based on two proteins, takes into account the kinetic characteristics of the G6Pase system. Indeed, in intact microsomal membranes (which correspond to an intact G6Pase system), G6P is specifically hydrolysed at a moderate rate and with high affinity. In solubilised ER membranes, in which catalytic activity is not dependent on substrate transport, G6Pase hydrolyses G6P at a high rate and affinity but, it decreases its substrate specificity and is therefore able to hydrolyse other sugar-6 phosphate moieties [12]. Moreover, disruption of microsomal membranes of livers from GSD-1b patients permits the recovery of normal G6Pase activity, whereas intact GSD-1b liver microsomes exhibit very low G6Pase activity [13,14]. This set of data has suggested that G6PT is necessary for G6P transport in intact ER membranes and confers substrate specificity to the G6Pase system.

The importance of both proteins for optimal enzymatic activity has been confirmed in animal models. Using mice knocked out for *g6pc* (*g6pc*$^{-/-}$), Lei et al. have suggested that a loss of *g6pc* gene expression annuls both G6P transport and hydrolysis [15]. In *g6pc*$^{-/-}$ mice, a single administration of an adenovirus containing the cDNA encoding *g6pc* (AdCMV-G6PC) improved the majority of GSD-1a metabolic abnormalities. The AdCMV-G6PC infusion restored 19% of normal G6Pase activity in the liver and, interestingly, corrected G6P transport deficiency in hepatic microsomes of *g6pc*$^{-/-}$ mice [16]. The same authors examined microsomal G6P transport in transient expression studies in COS-1 cells and have shown that G6P transport is increased in cells transfected with either the *g6pc* or *g6pt* cDNA compared with mock-transfected cells. Moreover, G6P transport was more efficient in cells which co-expressed both genes [17]. These studies provide direct evidence that G6PT and G6PC are tightly inter-dependent for global G6Pase activity.

Concerning their protein characteristics, neither proteins of the G6Pase system have been purified to homogeneity, but their individual structures within the membrane have been predicted using proteolytic digestion of tagged proteins [18,19]. We have reported that [^{32}P]G6PC migrates at an apparent molecular mass of 37 kDa in SDS-PAGE under denaturing conditions. This is consistent with its molecular mass determined by immunoblotting [20]. Using radiation inactivation analysis, Ness et al. suggested that the molecular weight of functional G6Pase ranged around 75–100 kDa, in both intact and solubilised ER membrane [21]. These observations have suggested that the catalytic subunit (37 kDa) could be associated with another polypeptide through disulphide bonds, and that this association could be maintained under some denaturing conditions. This has raised the hypothesis that G6PT might be able to interact with G6PC, resulting in the formation of a complex.

The first aim of the present study was to document the intracellular localisation of G6Pase subunits. The proteins of the G6Pase complex were thus singly labelled on the C or N terminus with cyan fluorescent protein (CFP) for G6PC or yellow fluorescent protein (YFP) for G6PT. We also took advantage of these constructs to test the hypothesis of a direct interaction between both subunits of the G6Pase enzymatic system. To this purpose, we used fluorescence resonance energy transfer (FRET) with CFP as the donor and YFP as the acceptor. The second aim of this work was to test a putative direct interaction between the G6PT cytoplasmic termini. We thus developed a G6PT protein doubly labelled on the C and N termini with CFP and YFP, respectively. Using this chimera protein, we obtained further information on the structural conformation of G6PT within the membrane. Finally, we studied whether the presence of GSD-1 mutations affected the localisation of both G6Pase subunits and/or the interaction between the C and N termini of the G6PT protein.

2. Materials and methods

2.1. Generation of constructs

We used pSVK3-G6PC and pSVK3-G6PT constructs which contained the coding sequence for human *g6pc* (Genbank number NM 000151) and *g6pt* (Genbank number Y15409) cloned at *EcoRI* and *Sal I* restriction sites (Bady and Mithieux unpublished results). Chimera proteins G6PC-CFP and G6PT-YFP with fluorophores at the C-terminus end were generated introducing *g6pc* and *g6pt* cDNA into pECFP-N1 and pEYFP-N1 vectors (Clontech) at *EcoRI* and *Sal I* restriction sites. Chimera proteins CFP-G6PC and YFP-G6PT with fluorophores at the N-terminus end were generated introducing *g6pc* and *g6pt* cDNA into pECFP-C1 and pEYFP-C1 vectors (Clontech) at *EcoRI* and *Sal I* restriction sites. Sequences encoding stop codons avoiding correct labelling were deleted by mutagenesis (QuikChange, site-directed mutagenesis kit, STRATAGENE). The resulting chimera contained no additional amino acids between the G6PC/G6PT and the CFP/YFP sequences (See Supplemental Fig. 1 for amino acid sequences of the chimera proteins).

In the case of the *CFP-G6PT-YFP* chimera protein, the DNA fragment encoding G6PT-YFP fusion protein was obtained after digestion of pG6PT-YFP plasmid at *XhoI/HpaI* restriction sites. For an easy cloning strategy, this cDNA was inserted to *XhoI/HpaI* in pECFP-Nuc vector. This cloning strategy induced the removal of the nuclear localisation signal of the simian virus large T-antigen contained in the pECFP-Nuc vector and produced a chimera protein containing a linker of 13 amino acids between the CFP and G6PT sequences (See Supplemental Fig. 1).

The sequences of all plasmid constructions were controlled by sequencing (GenomExpress).

2.2. Cell culture and cDNA transfection

HeLa cells (ECACC 85060701), HepG2 cells (ECACC 8511430) and NRK cells (ECACC 86032002) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 1 g/L or 5 g/L D-glucose, respectively, and supplemented with 10% foetal bovine serum (Invitrogen), 5 mM glutamine, streptomycin (1 μg/μL) and penicillin (1 U/mL) at 37 °C in a humidified 5% CO_2/95% air atmosphere. For transient transfection, 1 day before transfection, 100,000 cells were plated out in two-well cell culture plates (Labteck, NUNC). The complete medium was refreshed 1 h prior to transfection. Cells were transfected using the Exgen 500 reagent (Euromedex) with 200 ng of each construct. After 48 h of incubation, the transfection efficiency was about 60% (data not shown).

2.3. Immunoblotting and immunohistochemistry

Whole proteins extracts were prepared from cells homogenised in 10 mM HEPES, pH 7.3 by ultrasonication coupled with freeze–thaw cycles to disrupt cell membranes. Proteins (40 μg) were separated by SDS-10% polyacrylamide electrophoresis gel and then transferred to Immobilon-P membrane (Millipore). Analysis of G6PC expression was performed as previously described using an antibody raised against the C-terminal part of the protein [22].

HepG2 hepatoma cells were fixed with 2% paraformaldehyde in PBS and permeabilised with 0.1% saponin in PBS. The cells were incubated for 1 h at room temperature with a G6PC antibody (used at 1:1000) as previously described [22]. The cells were rinsed and then incubated with goat anti-rabbit coupled to Alexa-546 (used at 1:1000, Invitrogen) for 1 h at room temperature.

2.4. Cell microscopy and FRET measurement

Cells were observed using an inverted two-photon laser scanning microscope Axiovert 200M (LSM510 NLO META, Carl Zeiss)