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Glucose-6-phosphate isomerase deficiency results in mTOR activation, failed translocation of lipin 1α to the nucleus and hypersensitivity to glucose: Implications for the inherited glycolytic disease

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ABSTRACT

Inherited glucose-6-phosphate isomerase (GPI) deficiency is the second most frequent glycolytic erythroenzymopathy in humans. Patients present with non-spherocytic anemia of variable severity and with neuromuscular dysfunction. We previously described Chinese hamster (CHO) cell lines with mutations in GPI and loss of GPI activity. This resulted in a temperature sensitivity and severe reduction in the synthesis of glycerolipids due to a reduction in phosphatidate phosphatase (PAP). In the current article we attempt to describe the nature of this pleiotropic effect. We cloned and sequenced the CHO lipin 1 cDNA, a gene that codes for PAP activity. Overexpression of lipin 1 in the GPI-deficient cell line, GroD1 resulted in increased PAP activity, however it failed to restore glycerolipid biosynthesis. Fluorescence microscopy showed a failure of GPI-deficient cells to localize lipin 1α to the nucleus. We also found that glucose-6-phosphate levels in GroD1 cells were 10-fold over normal. Lowering glucose levels in the growth medium partially restored glycerolipid biosynthesis and nuclear localization of lipin 1α. Western blot analysis of the elements within the mTOR pathway, which influences lipin 1 activity, was consistent with an abnormal activation of this system. Combined, these data suggest that GPI deficiency results in an accumulation of glucose-6-phosphate, and possibly other glucose-derived metabolites, leading to activation of mTOR and sequestration of lipin 1 to the cytosol, preventing its proper functioning. These results shed light on the mechanism underlying the pathologies associated with inherited GPI deficiency and the variability in the severity of the symptoms observed in these patients.

1. Introduction

Glucose-6-phosphate isomerase (GPI, EC 5.3.1.9) is a cytosolic, non rate-limiting enzyme in glycolysis. It catalyzes the reversible isomerization of glucose-6-phosphate to fructose-6-phosphate. Mutations in GPI are the second most frequent cause of inherited glycolytic enzymopathy in humans [1]. This autosomal recessive disorder is characterized by a non-spherocytic anemia of variable severity which can present with neuromuscular dysfunctions defined by muscle weakness and mental retardation [2]. Patients and mice with the same GPI mutations can have different outcomes in the severity of the anemia or the neuromuscular dysfunction [2–4]. Unfortunately, the mechanism by which GPI deficiency causes these symptoms is not well understood [1,2]. Hence, the current therapeutics for these patients involve splenectomy and blood transfusions reserved for the most severe hemolytic cases [1,2].

We have recently isolated three independent CHO (Chinese hamster ovary) derived cell lines, each of which presented a different point mutation in GPI [5]. These cell lines displayed low GPI activity, were all deficient in the synthesis of glycerolipids, and had decreased phosphatidate phosphatase (PAP, EC 3.1.3.4) activity (Fig. 1). They also presented a temperature sensitive phenotype; these cells were unable to grow at 40 °C [5]. Expression of wild-type GPI in these cells recovered glycerolipid biosynthesis and PAP activity and corrected the temperature dependent phenotype, demonstrating a dependence of PAP activity and glycerolipid biosynthesis on GPI activity through an, as yet, unknown mechanism.

PAP is crucial for animal cell glycerolipid biosynthesis, catalyzing the dephosphorylation of phosphatidic acid (PA) to form diacylglycerol (DAG), which is required for the de novo synthesis of phosphatidylethanolamine (PE), phosphatidylcholine (PC) and triglycerides (TG)

Abbreviations: GPI, glucose-6-phosphate isomerase; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; PAP, phosphatidate phosphatase; PA, phosphatidic acid; DAG, diacylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; TG, triglycerides; PPAR, peroxisome proliferator-activated receptor; CHO, Chinese hamster ovary; mTOR, mammalian target of rapamycin; $^{32}P_i$, [^{32}P]orthophosphate; GFP, green fluorescent protein; FBS, fetal bovine serum; PDK1, phosphoinositide-dependent kinase-1; S6K, S6 Kinase; AMPK, adenosine monophosphate activated protein kinase; PI3K, phosphoinositide 3-kinase; GSK-3β, glycogen synthase kinase 3 beta; PTEN, phosphatase and tensin homolog; NHSA, non-spherocytic hemolytic anemia

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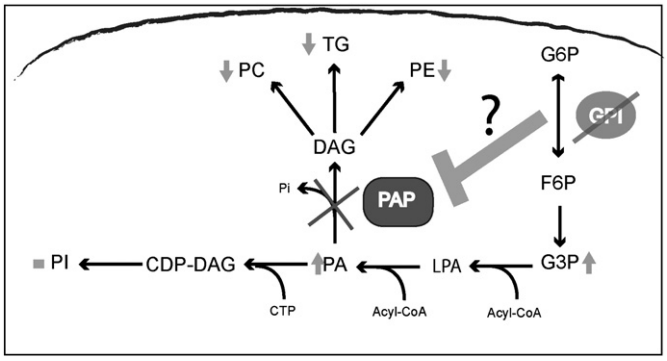

Fig. 1. Schematic diagram of pathways involving PAP and GPI in mammalian cells. GPI catalyzes the reversible conversion of G6P to F6P in the second step of glycolysis. We previously described three GPI-deficient mutant cell lines that also presented deficiencies in PAP activity [5]. This activity is required for the dephosphorylation of PA to supply DAG in the de novo biosynthesis of PC, PE and TG. As a result, the mutant cell lines presented a severe reduction in the synthesis of PC, PE and TG, accompanied by an accumulation of PA and G3P with no changes in the synthesis of PI (synthesized independently of PAP). The exact nature of the relationship between GPI and PAP is not yet understood. Abbreviations: GPI, glucose-6-phosphate isomerase; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; PAP, phosphatidate phosphatase; G3P, glycerol-3-phosphate; PA, phosphatidic acid; LPA, lysophosphatidic acid; DAG, diacylglycerol; CDP-DAG, CDP-diacylglycerol; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TG, triglycerides; P_i, inorganic phosphate.

(Fig. 1). This activity is encoded for, in mammals, by the lipin genes (*Lpin 1*, *Lpin 2*, and *Lpin 3*) [6,7] which share high sequence identity and belong to the HAD superfamily of phosphatases. All lipins contain a nuclear localization signal (NLS), which targets certain isoforms to the nucleus [8]. It is thought that these proteins have dual functionality [9]; in the cytoplasm they act as phosphatases involved in glycerolipid biosynthesis [7], while in the nucleus they are co-transcriptional factors, binding to members of the PPAR family as well as other nuclear receptors such as hepatocyte nuclear factor 4α, estrogen receptor α and the glucocorticoid receptor [9].

Of the lipins, lipin 1 is the most studied. Lipin 1 is expressed as two alternative splice isoforms, lipin 1α and lipin 1β. Lipin 1α localizes mainly to the nucleus, while lipin 1β, which has an extra 33–37 amino acid insert and is targeted almost exclusively to the cytoplasm [10,11]. PAP/lipin 1 localization and activity are regulated through phosphorylation, with at least 19 phosphorylation sites identified by mass spectrometry [10]. It is likely that hyperphosphorylation, due to mTOR signaling, results in lipin 1α not localizing to the nucleus and lipin 1β not associating with membranes, where its substrate is located and synthesis of glycerolipids occurs [12]. Phosphorylation of lipin 1 has been shown to occur in a rapamycin-inhibitable manner [12,13], implicating the mTOR complex in the regulation of lipin 1 [10,12,13].

Similar to GPI deficiencies, inborn errors in lipin genes result in pathologies in humans and mice. For example, a point mutation in lipin 1 causes a severe lipodystrophy and neuropathies due to a demyelization in the homozygous mutant mice [14,15] as well as myopathies in children due to an alteration in the phospholipid composition of the skeletal muscle fiber membranes [16]. Mutations in the lipin 2 gene are the cause of severe anemia in Majeed Syndrome [17].

In the present study we describe the cloning of lipin 1α and lipin 1β from the wild-type CHO cell line. We examined the subcellular distribution and effect of expression of these isozymes in the parent and GPI-deficient CHO strains. We found that GPI-deficient cells mislocalized lipin 1α to the cytosol, presented an abnormal, activated mTOR signaling pathway and were hypersensitive to glucose. Altogether, the data presented here serves to explain the dependency of PAP activity on GPI and sheds light on the mechanism of the pathophysiological state of GPI-deficient cells and the variability in the degree of severity of the symptoms in GPI-deficient patients.

2. Material and methods

2.1. Materials

^{32}P-inorganic phosphate ($^{32}P_i$) was obtained from Perkin Elmer/New England Nuclear. Lipids were purchased from Avanti Polar Lipids. Silica gel G and silica gel 60 thin-layer chromatography plates (EMD), Ham's F12 and DMEM medium (Cellgro or Gibco), fetal bovine serum (HyClone) and tissue culture dishes were obtained from Fisher Scientific. Antibodies were purchased from Cell Signaling with the exception of anti-actin (catalog no. MS-1295, Thermo Scientific). All other reagents, unless otherwise specified, were purchased from Sigma-Aldrich.

2.2. Cell lines and cell culture conditions

If not specified otherwise, cells were maintained in Ham's F12 medium supplemented with 10% fetal bovine serum, 1 mM glutamine, penicillin G (100 U/ml) and streptomycin (75 U/ml). Cells were cultured at 33 °C or 40 °C using 5% CO_2. Cell lines used were ZR-82 [18], a peroxisome-deficient strain derived from wild-type CHO-K1; GroD1, a GPI mutant and glycerolipid deficient cell line derived from ZR-82 [5]; and, GroD1$^{t(GPI)}$ a stable GroD1 cell population expressing wild-type hamster GPI [5].

2.3. Cloning and sequencing of hamster lipin 1

Total RNA was isolated from cells with the RNeasy kit (Qiagen) and first strand synthesis of RNA was performed with SuperScript III reverse transcriptase (Invitrogen). Hamster lipin 1 was cloned from CHO-K1, ZR-82 and GroD1 cells from total cDNA using primers designed for regions of high homology among known lipin cDNA's at either end of the cDNA; forward 5′ ATG AAT TAC GTG GGG CAG 3' and reverse primer 5′ CCA GGG TCC CCA CAA CCT ATC CTT TAA T 3'. Once the PCR product was obtained, the initial sequences were obtained with primers used to generate the PCR product. As new sequences for hamster lipin 1 were obtained, new primers were designed to sequence the entire cDNA. Digestion of the β isoform from the PCR product was performed with Ale I (New England Biolabs) to allow sequencing of lipin 1α. CHO-K1 lipin 1α and lipin 1β were cloned into the pSC vector using a blunt PCR cloning kit from Stratagene. cDNA sequences for hamster lipin 1β beta and lipin 1α were submitted to the NHI GenBank sequence database, accession numbers GU474204 and GU474205 respectively.

2.4. Expression of hamster lipin 1

For stable mammalian expression, lipin 1 was cloned using the forward primer 5′ GTT GTT GAA TTC CAC CAA TGA ATT ACG TGG GGC AGT T 3′ and reverse primer 5′ GTT GTT GAA TTC AGT GGT GAT GGT GAT GAT GAG CTG AGG CTG AAT TGT ACG T 3′ and inserted as an EcoRI fragment into the pBABEpuro vector [19] to generate pBABE(Lpin1α)puro and pBABE(Lpin1β)puro vectors. HEK293T cells were co-transfected with helper virus pCL-10A1 (Imgenex) and pBABEpuro vectors, using Fugene 6 (Roche) to produce supernatant rich in non-replicative retroviral particles. Cells were infected for 3 h with virus containing supernatant in the presence of 10 μl/ml polybrene. Medium was changed to Ham's F12 containing 10% FBS and 6 μg/ml puromycin 24 h post infection.

2.5. Phospholipid biosynthesis

For phospholipid biosynthesis, short-term labeling with $^{32}P_i$ was used. Cells were plated into six well tissue culture plates (2.5×10^5 cell/well) and allowed to attach overnight at 33 °C. The next day, vials were placed at 40 °C for 2 h, medium was then changed to growth medium containing $^{32}P_i$ (20–50 μCi/ml) and incubated for 2.5 to 3 h at 40 °C.