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The ATP-stimulated translocation promoter (ASTP) activity of glycerol kinase plays central role in adipogenesis

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ABSTRACT

Glycerol kinase (GK) is a multifunctional enzyme located at the interface of carbohydrate and fat metabolism. It contributes to both central carbon metabolism and adipogenesis; specifically, through its role as the ATP-stimulated translocation promoter (ASTP). GK overexpression leads to increased ASTP activity and increased fat storage in H4IIE cells. We performed metabolic flux analysis in human *GK*-overexpressing H4IIE cells and found that overexpressing cells had significantly altered fluxes through central carbon and lipid metabolism including increased flux through the pentose phosphate pathway and increased production of lipids. We also observed an equal contribution of glycerol to carbohydrate metabolism in all cell lines, suggesting that GK's alternate functions rather than its enzymatic function are important for these processes. To further elucidate the contributions of the enzymatic (phosphorylation) and alternative (ASTP) functions of GK in adipogenesis, we performed experiments on mammalian GK and *E. coli* GK. We determined that the ASTP function of GK (which is absent in *E. coli* GK) plays a greater role than the enzymatic activity in these processes. These studies further emphasize GK's diverse functionality and provides fundamental insights into the multiple protein functions of glycerol kinase.

1. Introduction

There are many proteins, which are known to display additional functional activities outside of their regular enzymatic functions. These "moonlighting" proteins have at least two different functions within a single polypeptide chain and can exhibit unrelated functional activities within or outside of the cell [1–6]. Moonlighting proteins consist of a wide array of proteins that are not limited to enzymes, chaperones, and transcription factors [2]. There are several enzymes in central carbon metabolism alone, which are known to have additional moonlighting functions outside of their regular metabolic (enzymatic) functions [7]. For example, in glycolysis, 7 of the 10 glycolytic enzymes exhibit various moonlighting activities [7]. These alternate functions can result in an absence of genotype-phenotype correlations for a metabolic single gene disorder if a particular gene of interest encodes an enzyme whose moonlighting activities are not yet known. Since one or more of these moonlighting activities may contribute to the phenotype of the disorder, this could result in a phenotype that cannot be explained by the loss of the metabolic activity of the enzyme.

Glycerol kinase (GK) is an important enzyme found at the interface of both carbohydrate and fat metabolism, it catalyzes the conversion of glycerol to glycerol 3-phosphate [8]. GK is expressed in several tissues,

Abbreviations: ASTP, ATP stimulated translocation promoter; GK, glycerol kinase; GKD, glycerol kinase deficiency; VDAC, voltage-dependent anion channel; *Gyk*, murine ortholog of glycerol kinase; KO, Knock out; MFA, metabolic flux analysis; PPP, pentose phosphate pathway; *glpK*, *E. coli* glycerol kinase; T3P, triose-3-phosphate; WT, wild type; WT-EV, empty vector wild type a; hGK1 and hGK2, human *GK*-overexpressing H4IIE cells; GA3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; AcoA, Acetyl CoA; oxPPP, oxidative pentose phosphate pathway; G6PDH, glucose-6-phosphate dehydrogenase

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including muscle and brain [9,10], though its activity is highest in the liver and kidneys [9,11]. Mutations or deletions of the gene encoding GK causes glycerol kinase deficiency (GKD), an X-linked, single gene inborn error of metabolism that is characterized by hyperglycerolemia and glyceroluria [12] GKD is an example of a disease in which loss of enzymatic activity does not explain the complex phenotypes observed. Previous studies have shown no genotype-phenotype correlation between individuals with the same mutation, such that one individual may be symptomatic while a second individual is asymptomatic [13,14]. Possible explanations for this could be the role of modifier genes, altered fluxes through different (related) metabolic pathways, or the effects of GK's other protein activities (moonlighting functions).

Aside from its enzymatic role, GK is also a moonlighting enzyme [7]. In rat liver, GK was found to act as the ATP-stimulated translocation promoter (ASTP), which is responsible for enhancing the nuclear binding of the activated glucocorticoid-receptor complex [15]. In addition, studies have shown GK to bind with histones [16], interact with porin or the voltage-dependent anion channel (VDAC) on the outer surface of the outer mitochondrial membrane [17], and to possibly play a role in apoptosis [18,19]. All of these roles show GK to be a multifaceted enzyme.

Recent studies have also shown an emerging role for GK in adipogenesis. Gaudet and colleagues [20] first identified the N288D mutation in GK in a study of patients with hyperglycerolemia, obesity and risk for type 2 diabetes mellitus, thus identifying a possible link between GK and adipogenesis. This missense mutation in exon 10 was found to result in substitution of a highly conserved, small polar asparagine for a negatively charged aspartic acid [20]. Affected males and obligate female carriers showed increased risk for obesity, insulin resistance and type II diabetes mellitus [20]. Later studies showed that GK containing the N288D mutation has only residual phosphorylation activity [8], suggesting that it may instead be one of the moonlighting functions of GK involved in the observed phenotype.

In addition to Gaudet's patient studies, studies by our group and others have also suggested that glycerol kinase is important in adipogenesis. A *Gyk* (murine ortholog) knockout (KO) mouse was created by Huq et al. [21]. The *Gyk* KO male mice are born in normal numbers and size, but become growth retarded by day of life (dol) 2 and ultimately die on dol 3–4 [8,21,22]. Studies from our own group of the *Gyk* KO mouse have shown altered expression of several genes involved in adipocyte differentiation in the KO mouse [23]. Additionally, metabolic flux analysis (MFA) studies involving H4IIE rat hepatoma cells overexpressing human *GK* showed significantly altered fluxes through central carbon metabolism, including a substantially higher flux through the pentose phosphate pathway (PPP) [24], and increased lipid synthesis [25].

The objective of our study was to elucidate the role of the enzymatic (phosphorylation) and alternative (ASTP) functions of GK in adipogenesis. We hypothesized that the increased flux through PPP may lead to increased lipid synthesis and that both the phosphorylation and ASTP functions of GK were involved in adipogenesis and lipogenesis. In order to address these hypotheses, for this current work, we performed stable isotope labeling and mass isotopomer analysis, in the *GK*-overexpressing H4IIE cells, to evaluate the metabolic fluxes through not only carbohydrate but also lipid metabolism. In addition, we performed experiments to differentiate the contributions made by the glycerol phosphorylation activity of GK and the moonlighting (ASTP) activity of GK. Our results indicated that *GK*-overexpressing cells showed significantly altered fluxes through both central carbon and lipid metabolism. Through analysis of triose-3-phosphate isotopomers, we were able to corroborate the results of our previous experiment [24] and also found evidence towards the compartmentation of glycerol pools within the cell. Our MFA results also suggested that the metabolic changes seen may not be due to an increase in the enzymatic function, but may instead be due to one of the moonlighting functions of GK, its ASTP activity. We subsequently verified this through studies of ASTP in a tissue culture model: H4IIE cells overexpressing human or *E. coli* glycerol kinase (*glpK*). Our results indicated that *E. coli* glycerol kinase lacks ASTP activity and that the phosphorylation activity does not play a role in adipogenesis or lipogenesis. Overall our work confirms that GK is required for normal cellular adipogenesis and lipogenesis and this will further elucidate the complexities of GKD.

2. Methods

2.1. H4IIE cell lines and stable transfection of human and E. coli GK

H4IIE, a rat hepatoma cell line (American Type Culture Collection), and *GK*-overexpressing cell lines derived from the H4IIE cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 in minimal essential medium (MEM) with L-glutamine (Mediatech), supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin–streptomycin–neomycin (Invitrogen).

Human *GK* cDNA (NCBI accession NM_000167), was inserted into the pCMV-Tag2 vector (Stratagene) [24]. This vector or the empty pCMV-Tag2 vector were stably transfected into H4IIE cells to create the human *GK*-overexpressing cell lines hGK1 and hGK2, along with the wild type containing empty vector (WT-EV).

To stably transfect *E. coli* glycerol kinase (*glpK*) into the H4IIE cells, the cDNA encoding amino acids 1–502 of *glpK* (NCBI accession NC_000913) was inserted into the *Hin*dIII site of the pCMV-Tag2 vector. This vector was stably transfected into the H4IIE cells by using Lipofectamine 2000 transfection reagent as per the manufacturer's protocol (Invitrogen), to create the *glpK*-overexpressing cell line, eGK. Stable cells were selected with and maintained in media containing 500 μg ml^{-1} G418 (Mediatech).

2.2. ^{13}C Labeling experiment

The ^{13}C labeling experiment was performed by growing H4IIE cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 g L^{-1} of 1-^{13}C glucose and 5.86 mg L^{-1} of U—^{13}C glycerol. The cells were plated on 10-cm plates at an initial density of 12.0×10^6 cells $plate^{-1}$, were grown on the aforementioned ^{13}C glucose-glycerol mixture for 7 days (passage 1), then re-plated at an initial density of 12.0×10^6 cells $plate^{-1}$ in fresh media containing the same ^{13}C glucose-glycerol mixture, and grown again for 7 days (passage 2). Cells were grown on the ^{13}C glucose-glycerol mixture for two full passages to wash out any natural carbon present in the biomass prior to the labeling experiment, as well as to achieve isotopic steady state. During both passages, the medium was replaced and extracellular fluxes were measured every 3 days. Cells were harvested at the end of both passages; and protein, lipid, and glycerol fractions were extracted from each sample.

2.3. Protein extraction, hydrolysis, and derivatization

Whole cell pellets were split into three fractions. As previously described [24], the fraction used for protein extraction was lyophilized and hydrolyzed for 12 h at 130°C in hydrolysis tubes (Pierce Endogen), containing 6 N hydrochloric acid. Before hydrolysis, the hydrolysis tube was evacuated, flushed with nitrogen to remove residual oxygen, and re-evacuated. Residual acid in the hydrolysate was evaporated by flushing with nitrogen at 70°C for 1 h. The hydrolysate was *tert*-butyldimethylsilyl (t-BDMS)-derivatized with 50 μl *N,N*-dimethylformamide (Pierce Endogen) and 50 μl *N*-methyl-N-(*tert*-butyldimethylsilyl) trifluoroacetamide (Pierce Endogen), for 1 h at 70°C, and injected into the gas chromatograph–mass spectrometer (GC–MS) immediately.

2.4. Fatty acid extraction and methylation

The cell pellet fraction used for lipid extraction was performed as