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Effect of lysine to alanine mutations on the phosphate activation and BPTES inhibition of glutaminase

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ABSTRACT

The *GLS1* gene encodes a mitochondrial glutaminase that is highly expressed in brain, kidney, small intestine and many transformed cells. Recent studies have identified multiple lysine residues in glutaminase that are sites of N-acetylation. Interestingly, these sites are located within either a loop segment that regulates access of glutamine to the active site or the dimer:dimer interface that participates in the phosphate-dependent oligomerization and activation of the enzyme. These two segments also contain the binding sites for bis-2[5-phenylacetamido-1,2,4-thiadiazol-2-yl]ethylsulfide (BPTES), a highly specific and potent uncompetitive inhibitor of this glutaminase. BPTES is also the lead compound for development of novel cancer chemotherapeutic agents. To provide a preliminary assessment of the potential effects of N-acetylation, the corresponding lysine to alanine mutations were constructed in the $hGAC_{\Delta 1}$ plasmid. The wild type and mutated proteins were purified by Ni^{+}-affinity chromatography and their phosphate activation and BPTES inhibition profiles were analyzed. Two of the alanine substitutions in the loop segment ($K_{311}A$ and $K_{328}A$) and the one in the dimer:dimer interface ($K_{396}A$) form enzymes that require greater concentrations of phosphate to produce half-maximal activation and exhibit greater sensitivity to BPTES inhibition. By contrast, the $K_{320}A$ mutation results in a glutaminase that exhibits near maximal activity in the absence of phosphate and is not inhibited by BPTES. Thus, lysine N-acetylation may contribute to the acute regulation of glutaminase activity in various tissues and alter the efficacy of BPTES-type inhibitors.

1. Introduction

A unique catalytic property of the mitochondrial glutaminase encoded by the *GLS1* gene is its potent activation by phosphate and other polyvalent anions (Curthoys et al., 1976). The K_M for glutamine decreases in the presence of increasing phosphate concentration and phosphate activation correlates with the association of inactive dimers to form active tetramers (Godfrey et al., 1977; Morehouse and Curthoys, 1981) and larger oligomers (Ferreira et al., 2013). BPTES, bis-2[5-phenylacetamido-1,2,4-thiadiazol-2-yl]ethylsulfide, functions as a very specific and potent uncompetitive inhibitor (K_I of 0.2 μM) with respect to glutamine (Hartwick and Curthoys, 2011). BPTES blocks the allosteric activation caused by phosphate binding, promotes the formation of an inactive tetramer, and prevents the formation of larger phosphate-induced oligomers (Robinson et al., 2007). Therefore, BPTES is a potent inhibitor of this isoform of glutaminase that binds to an allosteric site and prevents a conformational change that is required for activity.

The *GLS1* gene contains 19 exons and uses alternative splicing and different polyadenylation sites to form multiple mRNAs (Porter et al., 2002). The kidney-type glutaminase (KGA) mRNA (Shapiro et al., 1991) is derived from exons 1–14 and 16–19 and is terminated at alternative polyadenylation signals to produce a more abundant 4.7-kb and a less abundant 3.4-kb mRNA that encode the same 74-kDa precursor (Fig. 1). The first 16 amino acids of the KGA sequence form an amphipathic α-helix that functions as a mitochondrial targeting sequence (Shapiro et al., 1991). Following translocation into mitochondria, the matrix processing protease generates the mature 66-kDa subunit by removal of the N-terminal 72-amino acids (Srinivasan et al., 1995). A variant of the KGA cDNA, termed GAC, was initially cloned from a human carcinoma cDNA library (Elgadi et al., 1999). The GAC mRNA is derived from exons 1–15 and contains a unique C-terminal coding sequence and 3′-UTR. The shorter GAC precursor protein is also translocated into the mitochondria and similarly processed to produce a 58-kDa subunit. The central core region of either *GLS1* gene product forms a highly conserved structure that is characteristic of all crystallized forms of glutaminase (Brown et al., 2008; Cassago et al., 2012; DeLaBarre et al., 2011; Thangavelu et al., 2012).

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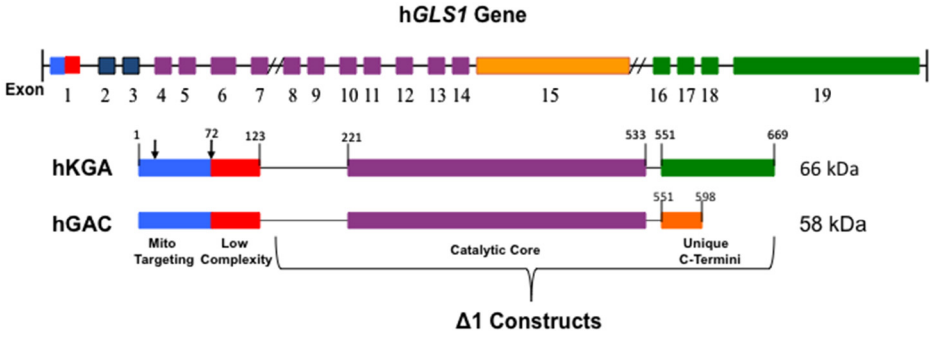

Fig. 1. Structures of the human *GLS1* gene and the hKGA and hGAC isoforms of human glutaminase. The h*GLS1* gene encodes 19 exons that are alternatively spliced to encode the two isoforms that contain unique C-termini. The initial precursors of both hKGA and hGAC are cleaved at two sites (arrows) by the matrix processing protease following translocation into the mitochondria. The Δ1 constructs lack the sequence encoded by exon 1.

The structure of human glutaminase ($hGAC_{71–598}$) bound to BPTES was recently determined (DeLaBarre et al., 2011). The hGAC forms a highly symmetrical tetramer containing two molecules of BPTES that are positioned at the dimer:dimer interfaces (PDB:3UO9). The N-terminal region (residues 71–135) contains an amino sequence of low complexity. This region and the unique C-terminal segment (residues 547–598) are not evident in the X-ray crystallographic structure, suggesting that they are highly flexible or disordered. Residues 137–224 form small helical domains of unknown function that are positioned on the sides of the tetramer opposite from the dimer:dimer interfaces. The catalytic core of the hGAC (residues 224–546) forms a compact globular structure that is composed of two domains. One domain is entirely α-helical and the other contains both α-helices and β-sheets. The two domains form a pocket, which contains the active site serine residue. A co-crystallized glutamate molecule was tightly bound within this grove and appropriately positioned adjacent to the active site serine. Each BPTES interacts in a highly symmetrical fashion with residues in a loop sequence (residues 309–334) and α-helix-13 (residues 386–399), which forms the interface between two dimers. The loop sequence is normally unstructured, but it adopts a specific conformation upon binding of BPTES. The reported structure provides a detailed molecular model of how BPTES promotes the formation of an inactive tetramer. Additional structures suggest that the loop segment plays an important role in mediating the conformational changes that are essential for glutaminase activity (Cassago et al., 2012; Thangavelu et al., 2012).

Recent studies indicate that many of the enzymes of carbohydrate metabolism, the TCA cycle, β-oxidation, and amino acid oxidation are regulated by acetylation of specific lysine residues (Choudhary et al., 2009; Zhao et al., 2010). Previous mass spectrometric screens identified K_{311} (Choudhary et al., 2009), K_{320} (Weinert et al., 2013), K_{328} (Wisniewski et al., 2010), and K_{396} (Still et al., 2013) as sites of lysine acetylation in hGAC. All of these sites occur within the loop sequence or the α-helix-13 that bind BPTES. Mutations of residues within these segments have pronounced effects on oligomerization and activation of human glutaminase. For example, a K_{320}A mutant of GAC forms extended oligomers in the absence of phosphate and exhibits a 400-fold increase in catalytic efficiency (Ferreira et al., 2013). In addition, K396 forms a salt bridge with D386 in the complementary α-helix that contributes to the dimer:dimer association (DeLaBarre et al., 2011). Thus, acetylation of this lysine residue would prevent this interaction and reduce formation of active tetramers. Thus, N-acetylation of specific residues in glutaminase is very likely to regulate its activity. To provide a preliminary assessment of the potential effects of N-acetylation, the corresponding lysine to alanine mutations were expressed and their phosphate activation and BPTES inhibition profiles were analyzed.

2. Materials and methods

2.1. Cloning

The $hGAC_{Δ1}$ sequence, which lacks the sequence encoded by exon 1 of the GLS1 gene (Kenny et al., 2003), was cloned into the pET-15b plasmid. The QuikChange Lightning Site-Directed Mutagenesis Kit was used to introduce specific lysine to alanine mutations. The mutated PCR products were transformed into XL10 Gold competent cells and plated on LB media containing 100 μg/ml ampicillin. Plasmid DNA was purified using Wizard Plus SV Minipreps DNA Purification System and sequenced to establish that the DNA contained the correct mutation. This DNA was then transformed into BL-21(DE3)-pRARE cells and plated on LB/agar plates containing 100 μg/ml ampicillin and 50 μg/ml chloramphenicol.

2.2. Protein expression

Single colonies from the LB/agar plates were grown overnight in 5 ml 2xYT medium containing 100 μg/ml ampicillin and 50 μg/ml chloramphenicol. A 1 ml aliquot of the overnight culture was inoculated into 500 ml of medium containing the same antibiotics. The cell cultures were grown on a 37 °C shaker for 4–5 hours to reach an $O.D._{600\,nm}$ of 0.8–1.0. The cultures where then cooled on ice before adding 2.5 ml of 100 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cultures were then incubated overnight on an 18 °C shaker to induce expression of $hGAC_{Δ1}$. The cells were then pelleted by centrifugation at 5000 × *g* for 15 min at 4 °C.

2.3. Protein purification

The cells were resuspended in 20 ml of lysis buffer containing 300 mM potassium chloride, 10 mM potassium phosphate, 10 mM Tris chloride, 10 mM imidazole, 10% glycerol and Protease Inhibitor Cocktail (Roche Diagnostics), pH 8.0. The K_{320}A protein was solubilized by sonicating the resuspended cells for seven 1-min cycles at 50% duty with an output setting of 7. The remaining cell samples (wild type $hGAC_{Δ1}$, K_{311}A, K_{328}A, K_{396}A) were disrupted by passing through a micro fluidizer 5 times with a pressure of 100 psi. Cell debris was removed by ultracentrifugation at 128,000 × *g* for 30 min at 4 °C. The supernatant was then filtered through 0.22 μm syringe filter. A 5-ml HiTrap Chelating HP nickel column was used to purify the His_6-tagged $hGAC_{Δ1}$. The column was charged with 0.1 M nickel sulfate and equilibrated with lysis buffer. The protein sample was loaded on the column at a flow rate of 3 ml/min and the flow through was collected. The column was then washed with 24 ml lysis buffer and the wash was collected as a separate sample. The column was