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Human and murine glycerol kinase: Influence of exon 18 alternative splicing on function

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Abstract

Glycerol kinase (GK) is a key enzyme in glycerol metabolism with two alternatively spliced forms—one with an 87 bp insertion corresponding to exon 18 (GK+EX18), and one lacking exon 18 (GK−EX18). We report the expression of GK±EX18 in various tissues and cell lines, as well as their enzymatic characteristics and subcellular localization. RT-PCR revealed differential expression in tissues and cell lines. Northern blot analysis revealed that both forms of the murine ortholog, *Gyk*, were highly expressed in murine heart and increased during embryonic development. K_m values for glycerol for GK±EX18 were not significantly different, although GK−EX18 had a higher V_{max} for glycerol. GK−EX18 had a lower K_m and V_{max} for ATP than GK+EX18. Immunofluorescence experiments showed that GK+EX18 co-localized to the mitochondria and the perinuclear region while GK−EX18 had a diffuse expression pattern. These data suggest specific and divergent roles for GK+EX18 and GK−EX18 in cellular metabolism and development.

Keywords: Glycerol kinase; Glycerol kinase deficiency; Glycerol metabolism; Isoforms; Differential expression; Subcellular localization; Enzyme kinetics; K_m; V_{max}; Apoptosis; Voltage-dependent anion channel

Glycerol kinase (GK) is an enzyme involved in fat and carbohydrate metabolism. GK phosphorylates glycerol to form glycerol 3-phosphate [1], which is important in the production of glycerides, glycerol lipids, and dihydroxyacetone phosphate (DHAP). DHAP is a critical intermediate in several metabolic pathways.

The GK gene family is made up of five genes, located on chromosomes 4, Xp, and Xq [2,3]. The Xp21.3 GK is responsible for the X-linked disorder, glycerol kinase deficiency [1], and is comprised of 21 exons spanning over 50 kb [2,4,5]. The other loci are intronless, one non-coding and three expressed pseudogenes [3]. There are two genes encoding brain- and testis-specific transcripts, and another encoding an intestine-specific transcript that are all on chromosome 4. Northern blot analysis of human GK reveals three transcripts with highest expression in the liver and kidney, and expression of only the smallest transcript in the testis [2]. GK genes were cloned from testis, brain, and liver cDNA libraries using expressed sequence tags (ESTs) [6] and positional cloning [7,8]. GK is alternatively spliced, with an 87 bp insertion corresponding to exon 18 of the Xp gene (GK+EX18) isolated from human fetal brain and testes [2]. The predominant form of GK found in the liver lacks exon 18, GK−EX18 [8].

The mouse glycerol kinase (*Gyk*) gene, syntenic to human Xp21.3 *GK*, is also alternatively spliced [9]. The alternatively spliced form including exon 18 was isolated from mouse brain RNA [9]. Targeted deletion of *Gyk* results in Gyk-deficient male mice that are normal at birth, then show postnatal growth retardation, hyperglycerolemia, elevated free fatty acids, and death by 3–4 days after birth [10]. We have shown that the affected male

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mice evidence hypoglycemia, and acidemia, and therefore a phenotype that is similar to but more severe than the metabolic phenotype observed in affected boys [11].

In this study, we composed a tissue expression profile of the two alternatively spliced forms of GK in human and mouse tissues as well as several cell lines by RT-PCR and Northern blot analysis. We showed that *GK−EX18* and *GK+EX18* are differentially expressed in human and mouse tissues, as well as various cell lines. In addition, kinetic studies were conducted and identified differences in kinetic parameters of the two forms (GK−EX18 and GK+EX18). Finally, immunofluorescence was performed and identified differences in subcellular localization indicate different functions for these alternatively spliced GK isoforms.

Experimental procedures

RT-PCR. Reverse transcriptase-polymerase chain reaction (RT-PCR) using primers to the human *GK* and murine *Gyk* sequence within exon 16 and the 3′-untranslated region (UTR) resulted in a 280 bp product for *GK+EX18* (*Gyk+EX18*) or a 193 bp product for *GK−EX18* (*Gyk−EX18*). RT-PCR was performed on poly(A) RNA from human adult brain, liver, kidney, testis, and human fetal brain and liver, and total RNA extracted from human placenta, mouse adult brain, heart, liver, kidney, brown and white fat, and newborn pup brain, heart, liver, kidney, and brown and white fat. Total RNA was also extracted from several human cell lines including, kidney (G-401 and G-402), lung (A549), adult hepatoblastoma (HepG2) and fetal liver (WRL-68), fibroblast, stem cells from fat with myotube characteristics (SS6-2), and lymphoblastoid cell lines from normal and GK-deleted patients. Total RNA from several non-human cell lines was also used, including African green monkey kidney (Cos7), rat liver (H_4IIE and McA-RH7777), and mouse liver (NMuLi).

Northern blot analysis. Probes were made using PCR and double labeling with [^{32}P]dATP and [^{32}P]dCTP. Primers to exon 18 of *Gyk* were used to label an 87 bp probe to identify transcripts specific for *Gyk+EX18*. To compare the expression of *Gyk−EX18*, primers to exon 17 were used to label an 87 bp probe. Murine blots containing poly(A)$^+$ RNA at different stages of embryonic development (7, 11, 15, and 17 days post coitum) and multiple tissue northern (MTN) (Clontech, Palo Alto, CA) were probed with 87 bp probes specific for exon 18 and exon 17. Blots were incubated in ULTRAhyb (Ambion, Austin, TX) at 42 °C for 1 h, and then in the same solution containing the labeled probe at 42 °C overnight. Blots were washed and exposed on film for one week. A β-actin probe was used as a control for RNA loading.

Expression constructs. To determine enzyme activity, both forms of GK (GK−EX18 and GK+EX18) were subcloned into pcDNA3.1/Zeo (−) (Invitrogen, Carlsbad, CA) at the *Xba*I and *Bam*HI sites. For studying the localization of GK−EX18 and GK+EX18, the full-length cDNAs were subcloned into pShuttle at *Bam*HI and *Kpn*I sites. For easier detection, three FLAG tags (DYKDDDDK) were added to the N-terminus.

Fractionation and kinetic studies. Cos7 cells were transiently transfected with 2 μg of plasmid containing *GK−EX18* or *GK+EX18* using SuperFect (Qiagen, Valencia, CA). Cells were harvested after 48 h, and protein extracts were obtained by sonication. For the fractionation studies, the whole lysate was centrifuged at 600*g* for 10 min (nuclear fraction). The nuclear fraction was resuspended in lysis buffer, and GK activity was assayed in the nuclear fraction and supernatant (cytosolic fraction). GK activity was measured radiochemically [12,13]. For the kinetic studies, whole cell lysate was used to assay GK activity. The concentrations for glycerol and ATP were varied independently (1–8 μM glycerol and 3–30 μM ATP) in the presence of saturating concentrations of the other substrate (2.5 mM ATP and 75 μM glycerol). Michaelis–Menten constants, K_m and V_{max}, were derived by Hanes–Woolf analysis.

Immunofluorescence staining and photomicroscopy. Cos7 cells were transiently transfected in 12-well plates using 900 ng DNA and 2.5 μl Superfect (Qiagen), and stained 48 h later. The mitochondria were stained using the MitoTracker vital stain (Molecular Probes, Eugene, OR). The MitoTracker dye was added to the cells and incubated at 37 °C for 15 min prior to rinsing with TBS. Cells were rinsed in TBS and then fixed in 3% paraformaldehyde for 10 min. Normal goat serum was used to block non-specific sites, and cells were incubated in mouse anti-FLAG antibody (Stratagene, La Jolla, CA) at a concentration of 1:200, and the anti-LaminA antibody (nuclear envelope marker) (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 1:200 overnight at 4 °C, and then incubated with the secondary antibodies (goat anti-mouse IgG-FITC and goat anti-rabbit IgG-Texas red) (Santa Cruz Biotechnology) at a concentration of 1:200 overnight at 4 °C. Slides were thoroughly rinsed in TBS, then mounted with Vectastain with DAPI (Vector Labs, Burlingame, CA), and viewed with a fluorescence microscope. An anti-porin antibody (Molecular Probes) was used to stain the nuclear fraction of Cos7 cell lysate to determine if the mitochondria were associated with the nucleus; the antibody was used at a concentration of 1:100. G402 cells were plated in a 12-well plate and stained after 48 h. Immunostaining was performed with a goat anti-GK antibody (US Biological, Swamscott, MA) at a 1:500 dilution and a secondary antibody, Cy3-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove PA), at a 1:200 dilution. The mitochondria were stained with the mouse anti-porin IgG monoclonal antibody (Molecular Probes Invitrogen Detection Technologies, Eugene, OR) at a 1:500 dilution and secondary antibody, FITC-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories), at a 1:200 dilution.

Results

RT-PCR was performed using primers specific to the region around exon 18. Human adult liver and kidney, as well as mouse adult liver, mouse newborn liver, and mouse newborn brown fat express GK without exon 18 only (GK−EX18/Gyk−EX18) (Figs. 1A and 2). Human testis and the kidney cell line, G-402, only expressed GK with exon 18 (*GK+EX18*) (Figs. 1A and B). All other human and murine tissues and cell lines that were tested expressed both forms (Figs. 1 and 2). All of the non-human cell lines (Cos7, H_4IIE, NMuLi, and McA-RH7777) (data not shown), and the human lymphoblastoid cell line derived from a GK-deleted patient (Fig. 1B) did not have any PCR products. Interestingly, the adult mouse white and brown fat, and newborn white fat also did not show PCR products (Fig. 2).

Northern blot analysis using a *Gyk* exon 18 probe with a mouse MTN showed strong expression of three transcripts (similar to humans) in the heart and kidney with less expression in the brain (Fig. 3A). A probe to exon 17 showed strong expression of *Gyk* in the kidney and liver, moderate expression in the heart, and low levels of expression in the brain. There was strong

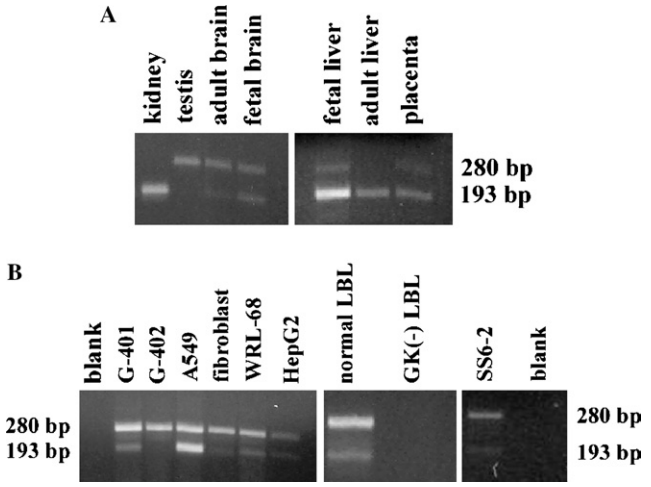

Fig. 1. The two alternatively spliced *GK* isoforms are differentially expressed in human tissues and human cell lines. RT-PCR using primers in exon 16 and the 3′UTR, which give rise to a 280 bp product corresponding to *GK+EX18* or a 193 bp product corresponding to *GK−EX18*. (A) Human adult and fetal tissues, (B) various human cell lines.

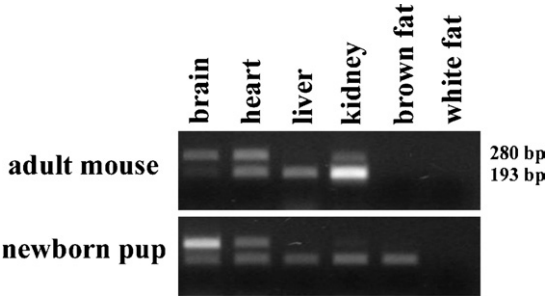

Fig. 2. The two alternatively spliced *Gyk* are differentially expressed in adult and newborn mice. RT-PCR using primers specific for murine *Gyk* gives rise to a 280 bp product corresponding to *Gyk+EX18* or a 193 bp product corresponding to *Gyk−EX18*.

expression in the testis of only the smallest transcript (similar to humans) (Fig. 3B). The murine embryo blot showed the three transcripts with increasing intensity as development progressed (7, 11, 15, and 17 dpc) (Fig. 3B). The smallest transcript was faint even at 17 dpc. A β-actin probe was used to determine RNA loading (Fig. 3C).

The K_m and V_{max} of GK−EX18 and GK+EX18 were calculated for glycerol and ATP. Table 1 shows the K_m and V_{max} values for glycerol and ATP for both alternatively spliced forms. The K_m values for glycerol and ATP were 2.2 and 6.4 μM for GK−EX18, and 2.6 μM and 12.2 μM for GK+EX18, respectively. The V_{max} values for glycerol and ATP were 16,540 μU/mg and 1914 μU/mg for GK−EX18, and 11,752 μU/mg and 3167 μU/mg for GK+EX18, respectively. There was no significant difference ($p > 0.05$) between GK−EX18 and GK+EX18 with respect to K_m for glycerol, however there was a significant difference ($p < 0.05$) between the

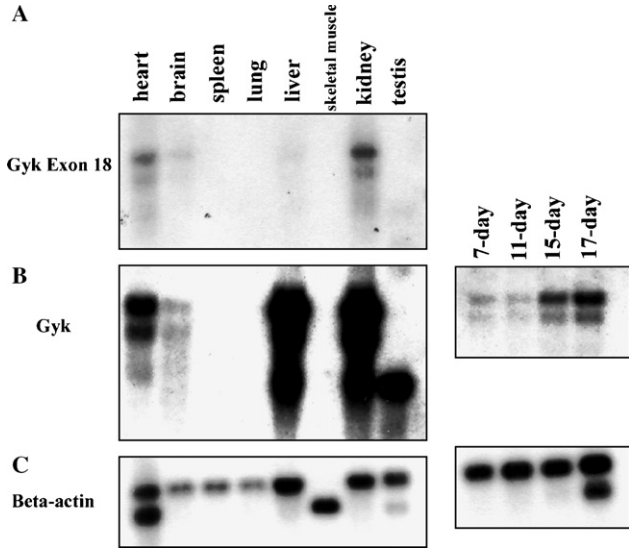

Fig. 3. Northern blot analysis of mouse tissues probed with exon 18. (A) A mouse MTN blot probed with exon 18. (B) The MTN and the whole embryo Northern blots probed with exon 17. (C) The MTN and the whole embryo Northern blots probed with the β-actin control.

two for the K_m of ATP and the V_{max} for glycerol and ATP. Although the K_m for glycerol for GK−EX18 and GK+EX18 were similar, GK−EX18 had a higher V_{max}. GK−EX18 had a lower K_m and V_{max} for ATP than GK+EX18.

GK activity was determined for the nuclear (600*g*) and cytosolic (supernatant) fractions. The activity for GK−EX18 and GK+EX18 in the nuclear fraction was very similar. GK+EX18 had about 92.5% activity of GK−EX18 (data not shown). The cytosolic fraction, however, showed that GK−EX18 had a much higher activity than GK+EX18: GK+EX18 had about 12.3% activity of GK−EX18 (data not shown).

To investigate further the subcellular localization of GK+EX18 and GK−EX18, immunofluorescence staining was performed. Flag-tagged GK−EX18 and GK+EX18 constructs were transiently transfected into Cos7 cells and anti-FLAG antibodies were used to localize the GK proteins. Anti-LaminA antibody (a known nuclear envelope marker) and MitoTracker Dye (mitochondrial vital stain) were used to determine the subcellular localization of the GK proteins. Fig. 4 shows GK-Flag (A and E), anti-LaminA (B and F), and DAPI, a chromatin dye, (C and G) staining as well as a merged image of the three colors (D and H). LaminA staining was observed to surround and enclose the DAPI chromatin dye and was expressed around each nucleus in the multi-nucleated cells (Figs. 4B, C, F, and G). When GK−EX18 was transfected, the GK-Flag staining revealed a diffuse expression pattern with a slightly stronger halo around the nucleus (Figs. 4A and D). GK+EX18 showed a more localized staining which was outside of the nucleus in the perinuclear region

Table 1
Kinetic characterization of glycerol kinase in whole cell extracts of transfected Cos7 cell homogenates

	Glycerol		ATP	
	K_m (μM)	V_{max} (μU/mg protein)	K_m (μM)	V_{max} (μU/mg protein)
GK−EX18	2.2 ± 0.28	16,540 ± 1013*	6.4 ± 1.61*	1,914 ± 183*
GK+EX18	2.6 ± 0.58	11,752 ± 1247*	12.2 ± 1.49*	3,167 ± 486*

Cos7 cells were transfected with 2 μg of plasmids containing GK−EX18 and GK+EX18, and harvested 48 h later for assay. The K_m and V_{max} for glycerol and ATP were derived by Hanes–Woolf analysis.
Asterisks indicate comparisons: $*p < 0.05$.

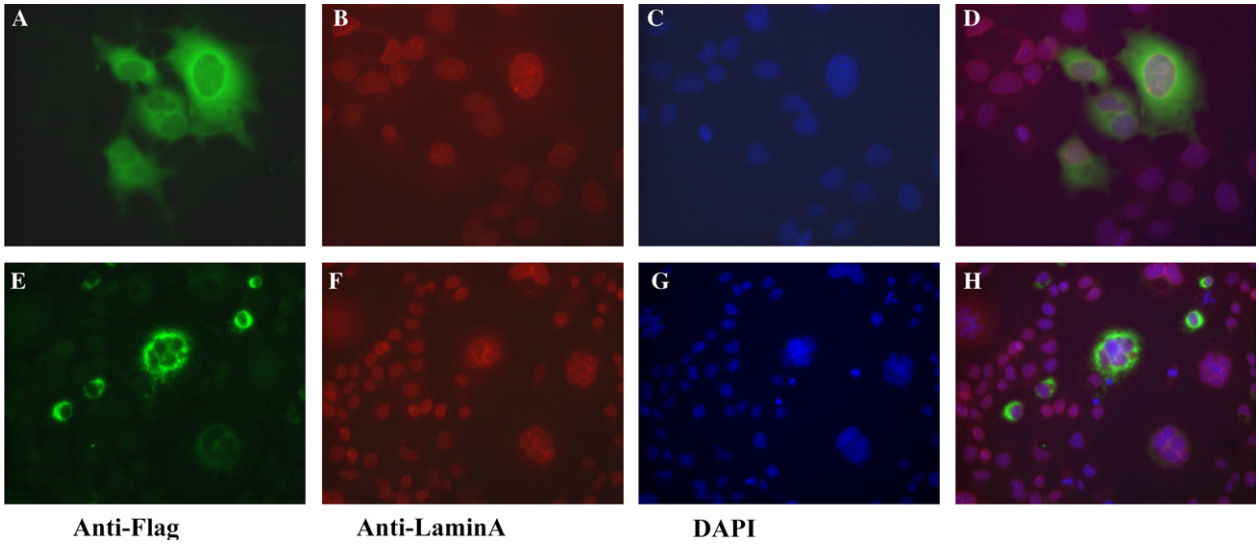

Fig. 4. Fluorescence microscopy (40×) of transiently transfected (Flag-tagged GK±EX18) Cos7 cells stained with anti-Flag and anti-LaminA (nuclear envelope marker) antibodies. GK−EX18 (A–D) shows staining of anti-Flag-FITC (A), anti-LaminA (B), DAPI (C), and the merged images of the three colors (D). GK+EX18 (E–H) shows similar corresponding panels.

but not within the nucleus (Figs. 4E and H). Fig. 5 confirms that GK−EX18 has a more diffuse expression pattern; and is expressed in the nucleus, the cytoplasm, and the mitochondria (Figs. 5A–H). GK+EX18 appears to co-localize primarily to the mitochondria: however, it does not seem to localize to every mitochondria (Figs. 5I–P). It does not appear to be expressed in the nucleus.

Immunostaining for porin (the outer mitochondrial membrane voltage-dependent anion channel, VDAC a known mitochondrial protein) was carried out on the nuclear fraction of Cos7 cell lysate. The mitochondria were associated with the nuclear fraction in these cells as determined by the halo of porin staining around the nuclei (Fig. 6). Staining of G402 cells, which express only GK+EX18 (Fig. 1B), with an antibody against GK (Fig. 7A) and porin (Fig. 7B) confirms that GK+EX18 is localized to the mitochondria (Fig. 7C).

Discussion

Alternative splicing is a mechanism that allows diversity in function and regulation of gene expression [14]. The GK isoforms we have studied here are derived from the Xp21 gene. In addition to the 87 bp insertion/deletion of exon 18, another alternatively spliced form has been identified—an 18 bp insertion between exons 8 and 9. This is referred to as exon 8A and was initially isolated from a fetal brain library, but could not be mapped in an Xp-derived YAC clone [2]. It was later confirmed by RT-PCR studies done in patient cell lines [5,15]. Our studies have focused on the presence or absence of the 87 bp exon 18 to determine possible differences in the function of these two isoforms. We showed that two isoforms of GK (GK±EX18, both lacking exon 8A) are differentially expressed in a variety of tissues and cell lines, and selected kinetic properties (K_m for ATP and V_{max} for glycerol and ATP) are statistically different. In addition, the two GK isoforms have different subcellular localizations. These differences suggest specific and divergent functions for these two isoforms.

Expression studies showed that GK−EX18 and GK+EX18 are differentially expressed in a wide range of tissues and cell types. It appears that most cells (particularly the immortalized cell lines) express both forms

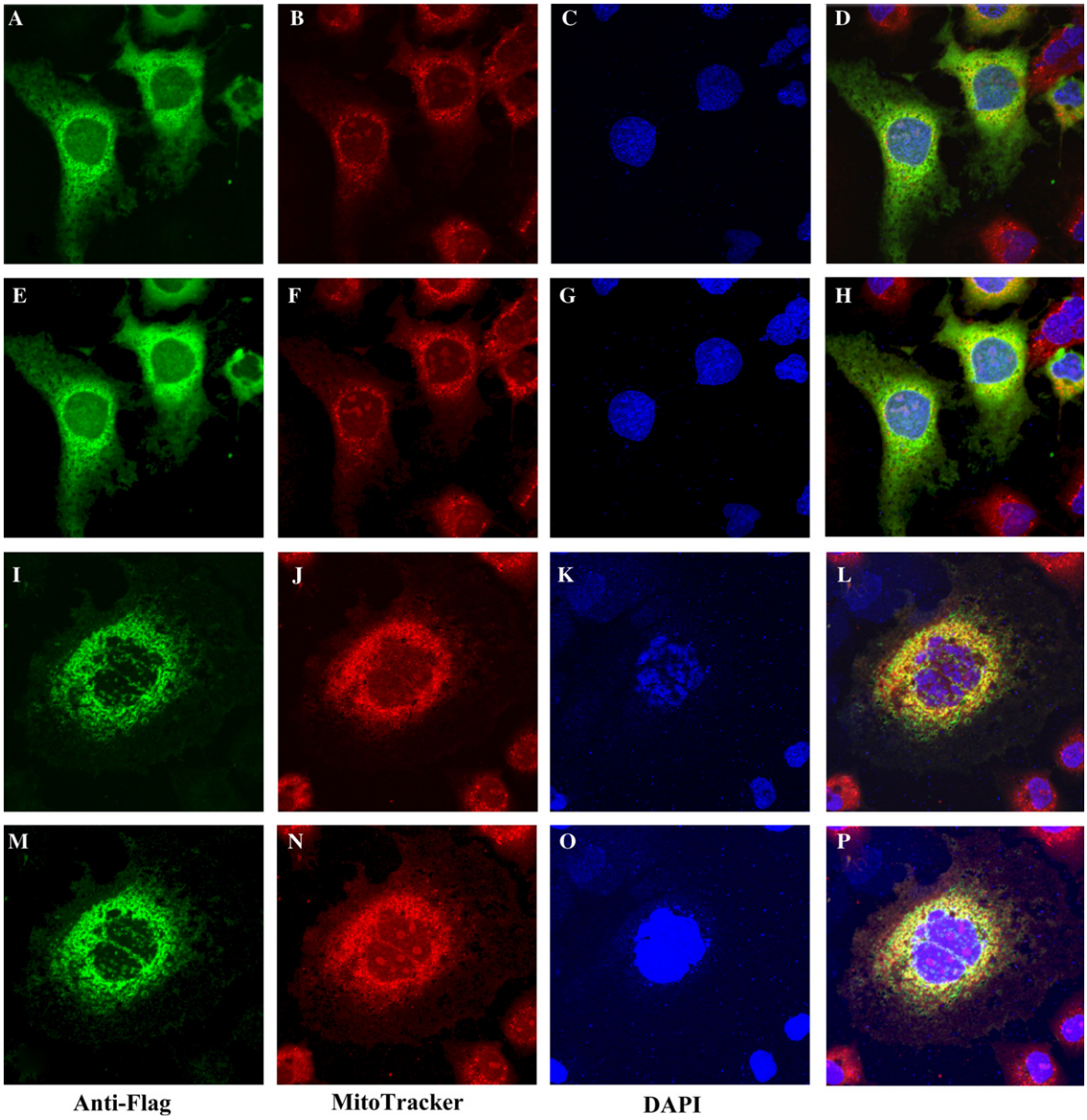

Fig. 5. Immunofluorescent microscopy of transiently transfected (Flag-tagged GK±EX18) Cos7 cells stained with the mitochondrial vital dye, MitoTracker, and anti-Flag antibody. GK−EX18 (A–H) and GK+EX18 (I–P) transfected cells were stained with anti-Flag antibody conjugated to FITC (A, E, I, M). Cells were also stained with MitoTracker vital stain (B, F, J, and N) and DAPI (chromatin dye) (C, G, K, and O). The merged images of the three stains (D, H, L, and P) show co-localization. Images were obtained with a confocal microscope (63×), and A–D and I–L are 0.7 μm sections through the cell, while E–H and M–P are overlay projections of each of the sections taken.

to varying degrees. GK+EX18 appears to be expressed in cells requiring a large amount of energy, like the immortalized cell lines, most fetal tissue, and the brain. Interestingly, human fetal liver expresses both forms, but the adult liver only expresses GK−EX18, suggesting that GK+EX18 has a specific function during fetal development.

GK is a highly conserved gene, and previous expression data have reported similar expression patterns between human and mouse [9]. Our Northern blot analysis reveals that *Gyk* is expressed at relatively high levels in the mouse heart, which differs from the pattern of human GK expression [2]. In the developing embryo, *Gyk* expression increases between 7 and 17 days post coitum, again indicating an important role during early development.

Differences in enzyme activity are a criteria to determine alternative functions. The K_m for glycerol was not significantly different between GK−EX18 and GK+EX18, indicating that both isoforms have similar affinities for glycerol. However, the K_m for ATP as well as the V_{max} for both glycerol and ATP were significantly different between the two isoforms. GK−EX18 had a lower K_m and V_{max} for ATP, but a higher V_{max} for glycerol. The biological significance of these differences

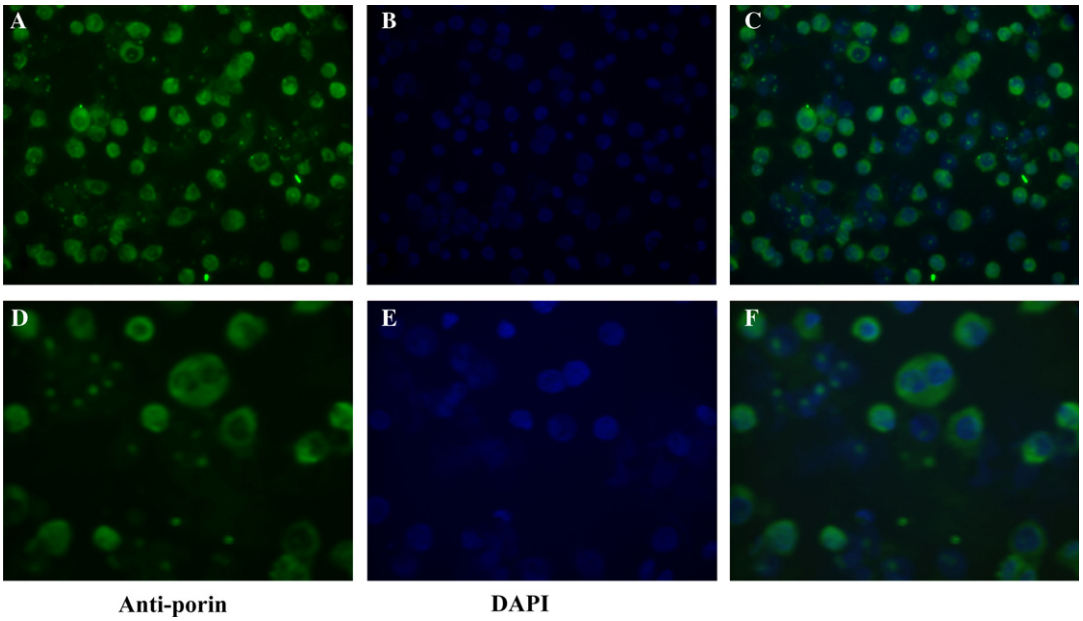

Fig. 6. Immunofluorescence staining for porin (mitochondrial protein) in the nuclear fraction of Cos7 cell lysate. Immunostaining with anti-porin antibody-FITC (A,D) on the nuclear fraction and stained with DAPI (B,E). The merged images (C,F) show that the mitochondria (porin) are perinuclear and associate with the nucleus. Images were taken at 20× (A–C) and 40× (D–F).

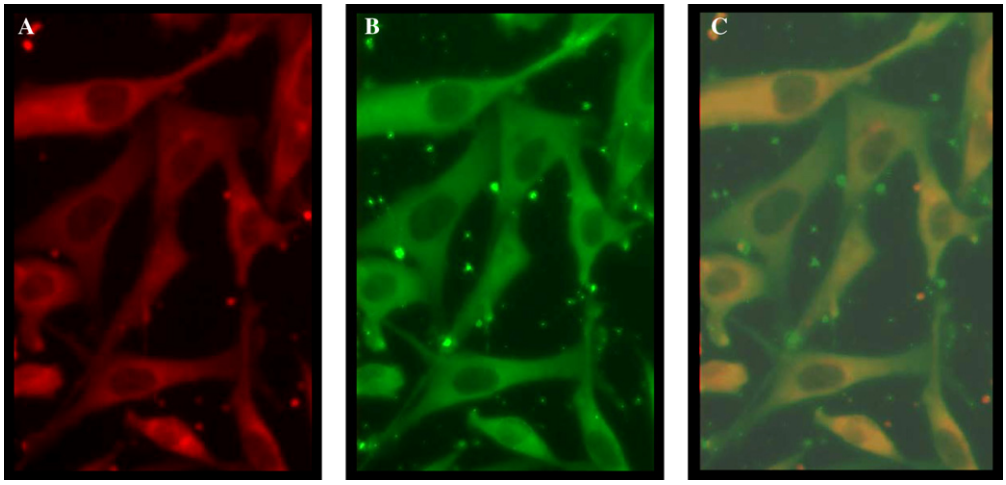

Fig. 7. Immunofluorescence staining for GK and porin (mitochondrial protein) in G402 cells. Immunostaining of G402 cells with anti-GK antibody (A), anti-porin antibody-FITC (B), and merged (C).

remains unclear. Seltzer and McCabe [16] examined the kinetic properties of soluble and particulate-associated glycerol kinase in bovine adrenals. They reported the K_m for glycerol as 6.3 and 4.0 μM for the soluble and mitochondrial fractions, respectively [16]. The soluble fraction is about three times higher, and the mitochondrial fraction is about twice as high when compared to our glycerol K_m values for GK±EX18. The V_{max} for glycerol were 426.5 and 25.5 μU/mg protein for the soluble mitochondrial fractions, respectively. The V_{max} for glycerol in the soluble fraction is approximately 39- and 28-fold lower, and for the mitochondrial fraction, 649- and 461-fold lower than the V_{max} values for GK−EX18 and GK+EX18, respectively. The K_m and V_{max} values for ATP in the soluble and mitochondrial fractions were 12.8 and 5.3 μM, and 288 and 20.4 μU/mg protein, respectively. The ATP K_m for GK−EX18 was similar to the mitochondrial fraction value, and ATP K_m for GK+EX18 was similar to the soluble fraction value. This is surprising since GK−EX18 appeared to be distributed diffusely, including in the cytoplasm according to our immunofluorescence microscopy results. In addition, GK+EX18 appeared to localize to the mitochondria. These disparities could be a result of the different systems studied. Seltzer and McCabe measured the kinetics of endogenous GK in adrenal tissues, whereas

we studied GK transiently transfected into a cell line, Cos7. In addition, Seltzer and McCabe did not distinguish between GK±EX18 and we did not look at differential expression of the two isoforms in adrenals.

Immunofluorescence studies showed that GK+EX18 localized to the perinuclear region which contained the mitochondria both in Cos7 and G402 cells. It was almost absent in the nucleus. We cannot rule out that GK+EX18 could in fact be localizing to the endoplasmic reticulum (ER) and not the mitochondria since it has been shown that overexpressed proteins may be mistargeted to the ER [17]. The co-localization of GK+EX18 with porin is strong evidence for mitochondrial compartmentalization. We hypothesize that if GK+EX18 primarily localizes to the mitochondria, then it is for a specific function because we know from previous studies looking at GK activity in mitochondrial fractions [16] and competition assays with antibodies to porin [18] that GK appears to bind to the outer membrane of the mitochondria. GK−EX18 had a more diffuse expression pattern and was seen in the cytoplasm and nucleus as well as the mitochondria.

Interestingly, protein prediction programs (http://us.expasy.org/) indicate that exons 18 and 19 encode a transmembrane hydrophobic domain, and exon 17 contains a nuclear localization signal (NLS) [3]. The hydrophobic transmembrane domain is similar to that which is found in Bax, and targets Bax to the mitochondrial membrane and is required for its pro-apoptotic activity [19]. This suggests that exon 18 allows GK to bind to the mitochondrial membrane more efficiently or perhaps independently of porin. It was previously shown that GK is both cytosolic and mitochondrial bound [1], and that it binds via porin (VDAC). VDAC is a small abundant protein, which is capable of forming voltage-gated pores in planar lipid bilayers [18]. It is one component of the permeability transition pore complex (PTPC), a polyprotein complex formed in the contact site between the inner and outer membranes, and involved in apoptosis [20]. VDAC and the adenine nucleotide translocator (ANT) form the VDAC–ANT complex, which can bind cytosolic proteins such as hexokinase (HK) and GK [21]. HK has been shown to use internal ATP via ANT, suggesting that HK was functionally coupled to the ANT [22]. This suggests that mitochondrial bound GK is involved in metabolic channeling and regulation of energy metabolism [23]. It is thought that functional coupling of kinases to the ANT regulates oxidative phosphorylation by converting intra-mitochondrial ATP into ADP, and increases the free energy (ΔG) in the ATP system [24]. It has been shown that HK and Bax (a pro-apoptotic gene) compete for the same binding site on VDAC [25] and when Bax is bound to VDAC, cytochrome *c* is released from the mitochondria, which results in apoptosis [26]. Binding of activated HK inhibits apoptosis and prevents the PTPC from opening. Mitochondrial bound GK may have a pro-apoptotic role [27].

In addition to phosphorylating glycerol, GK has an alternative function in the cell. The rat liver ATP-stimulated glucocorticoid receptor translocation promoter (ASTP) was cloned [28], and its deduced amino acid sequence was 99% identical with murine Gyk and 95% identical with the human GK sequences [9]. ASTP or GK translocates the activated glucocorticoid–glucocorticoid receptor complexes to nuclei in the presence of ATP [29]. Exon 17 of GK contains a NLS [3] and GK−EX18 is expressed in the nucleus, suggesting a possible role in ASTP function. Additional research will be required to determine whether ASTP activity is associated with a single GK isoform.

In conclusion, we have shown that two alternatively spliced forms of GK are differently expressed in a number of cell types, have different enzymatic characteristics, and show different subcellular localizations. Further studies are necessary to determine the precise differences in function of the two isoforms and their specific roles in cellular metabolism, transcriptional activation, and apoptosis.

Acknowledgments

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