Human pancreatic β-cell glucokinase: subcellular localization and glucose repression signalling function in the yeast cell

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INTRODUCTION

GK (glucokinase), a member of the Hxk (hexokinase) family (Hxk IV) [1], plays an essential role in glucose phosphorylation in the liver and endocrine β-cells of the pancreas [2]. GK also acts as a glucose sensor, integrating blood glucose levels and insulin release in pancreatic β-cells [3] and regulating glucose metabolism in the liver [2]. Hepatic GK shows dynamic nuclear–cytoplasmic localization depending on the amount of glucose in the blood. The nuclear localization of hepatic GK is regulated by glucose: low levels of glucose cause an association of GK with mitochondria [8], whereas high glucose levels prevent GK translocation to this organelle. In β-cells, GK has also been localized on the secretory granules and, although it has been suggested that glucose causes dissociation of GK from this location [9], recent studies suggest that GK is an integral component of the granule and does not translocate during glucose stimulation [10].

GKβ (pancreatic β-cell glucokinase) and the yeast Hxk2 proteins have several structural and functional similarities. For example: (i) GKβ is a monomer without allosteric regulation by glucose 6-phosphate; (ii) yeast Hxk2, like GKβ, has a glucose-regulated subcellular distribution [11,12]; in higher glucose conditions Hxk2 shows a nuclear enrichment that is absent under low-glucose conditions; and (iii) both Hxk2 and GKβ proteins play a vital role in glucose signalling in the yeast and the pancreatic β-cell respectively. Current evidence suggests that the main role of Saccharomyces cerevisiae Hxk2 in the glucose signalling pathway is achieved by its interacting with both Mig1 (multicopy inhibitor of GAL gene expression 1) and Snf1 (sucrose non-fermenting 1) [13,14]. It has been proposed that Hxk2 inhibits the phosphorylation of the Mig1 repressor, when the cells are growing in high-glucose conditions, maintaining in this way the transcriptional repression of target genes [13,14]. Since GKβ plays a prominent role in pancreatic β-cell signalling (inactivation of one GK allele leads to maturity-onset diabetes of the young, whereas loss of both alleles is associated with permanent neonatal diabetes [15,16]), GKβ is considered as a promising drug target for diabetes therapy. Thus the study of the regulatory properties of GKβ is an important matter to be examined.

In the present study, we took advantage of the ability to manipulate the S. cerevisiae genetic system and the similarity between the yeast Hxk2 and pancreatic GKβ to study the role of GKβ in the yeast glucose signalling pathway and also to study the regulation of the different subcellular localization that this enzyme presents in yeast cells.
MATERIALS AND METHODS

Strains and growth medium

GFP (green fluorescent protein) and RFP (red fluorescent protein)—fluorescence experiments utilized yeast strain H250 (MATa Suc2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mig1-Δ2::LEU2) [17], W303-1A (MATa ura3-52 trp1-289 leu2-3,112 his3-Δ1 ade2-1 can1-100) [18], DBY1315 (MATa ura3-52 leu2-3,2-112 lys2-801 gal2) [19], DBY2052 (MATa hxk1::LEU2 hxk2-202 ura3-52 leu2-3,2-112 lys2-801 gal2) [19], Δsnf1 [20], Δsnf1 Δhxk1 Δhxk2 [20], Y03694 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 msn5Δ::kanMX4) (euroscarf) and FMY388 (MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0 MIG1::gfp), the last containing a GFP-tagged MIG1 ORF (open reading frame) at its chromosomal location. Yeast two-hybrid experiments employed strain Y187 (MATa ura3-52 his3-200 ade2-101 trp1-901 leu2-3,112 gal4Δ gal80Δ URA3::GAL1(GAL1)TATA-lacZ) [21].

Escherichia coli DH5α [F 808dlacZ ΔM15 recA1 endA1 gyrA96 thi-1 hsdR17(rK- mK+) supE44 relA1 deoR1 (lacZ ΔM15::Tn10)] was the host bacterial strain for the recombinant plasmid constructions.

Yeast cells were grown in the following media: YEPD, high-glucose (2 % glucose, 2 % peptone and 1 % yeast extract); YEPE, low-glucose (0.05 % glucose, 3 % ethanol, 2 % peptone and 1 % yeast extract); and synthetic medium containing the appropriate carbon source and lacking appropriate supplements to maintain yeast strains expressing the GKβ−−−− gene or a mitochondrial fluorescence signal. A 1 μl aliquot of DAPI (4,6-diamidino-2-phenylindole; 2.5 μg/ml in 80 % glycerol) was added, and a cover slide was placed over the microscope slide. GFP, RFP and DAPI localization in live cultures was monitored by direct fluorescence using a Leica DM5000B microscope. To avoid the non-linear range of fluorescent signals, cells highly overexpressing GFP-tagged fusion protein were excluded from further analyses. The localization of proteins was monitored by visual inspection of the images. At least 100 cells were scored in each of at least three independent experiments. The distribution of fluorescence was scored in the following way: N indicated a nuclear fluorescence signal; C indicated a cytoplasmic fluorescence signal without nuclear or mitochondrial fluorescence signals; and M indicated a mitochondrial fluorescence signal.

To stain cells with MitoTracker® Red 580, yeast were immobilized on poly-L-lysine-coated slides. Then they were incubated with 0.5 μM MitoTracker® diluted in DMSO and glycerol 80 % (v/v) for 20 min at room temperature (22 °C). Finally, MitoTracker® solution was aspirated and a glass cover slide was immediately placed over the microscope slide. Images were processed in Adobe Photoshop CS.

Plasmids

The expression plasmid YEpl532-HXK2, YEpl532-HXK2/ gfp and pWS-GST/GKβ were constructed as indicated previously [13,22].

Plasmids YEpl532-GKα and pWS93-GKα carried a 1398 bp DNA fragment with the complete coding region of the HXK2 gene. Plasmid YEpl532-GKβ was generated by cloning a 1398 bp DNA fragment, synthesized by PCR using the plasmid pWS-GST/GKβ [22] as the template and the primer pair OL1 (sense: 5′-ATGGGAGATCC-3′) and OL2 (antisense: 5′-GGACATGG-3′) into the BamHI-EcoRI site of YEpl532-HXK2. The resulting plasmids expressed GKα or GKβ from the HXK2 promoter as fusion proteins with GFP.

For two-hybrid analysis, plasmids pGBK7-MIG1, pGBK7-MIG1S311A and pGBK7-HXK2 were constructed as indicated previously [13,14,23]. Plasmids pGBK7-GKβ and pGADT7- GKα carried a 1398 bp DNA fragment with the complete coding region of the GKβ gene. To make plasmid pGBK7-GKβ an EcoRI-Sall fragment obtained from pWS93-GKβ carrying the complete GKβ gene was subcloned into the EcoRI-Sall sites of pGBK7. To make plasmid pGADT7-GKα, an EcoRI-Sall fragment containing the complete coding region of the GKβ gene obtained from pWS93-GKβ plasmid was subcloned into a previously EcoRI-Xhol cleaved pGADT7 vector. The DNA sequence of all PCR-generated constructs was verified by sequencing and all of the clones used were verified by sequencing analysis of fusion points.

Fluorescence microscopy

Yeast strains expressing the GKβ−−−−, GKβ−−−−, Hxk2−−−−, Mig1–GFP or Su9 (F,F, ATPase subunit 9)–RFP fusion proteins were grown to early-exponential phase (OD600 of less than 0.8) in synthetic high-glucose medium (SD-ura). Half of the culture was shifted to synthetic low-glucose medium (SE-ura) for 1 h. The medium contained the appropriate carbon source and lacked the appropriate supplements to maintain the selection of plasmids. Cells (25 μl) were loaded on to poly-L-lysine-coated slides, and the remaining suspension was immediately withdrawn by aspiration. A 1 μl aliquot of DAPI (4,6-diamidino-2-phenylindole; 2.5 μg/ml in 80 % glycerol) was added, and a cover slide was placed over the microscope slide. GFP, RFP and DAPI localization in live cultures was monitored by direct fluorescence using a Leica DM5000B microscope. To avoid the non-linear range of fluorescent signals, cells highly overexpressing GFP-tagged fusion protein were excluded from further analyses. The localization of proteins was monitored by visual inspection of the images. At least 100 cells were scored in each of at least three independent experiments. The distribution of fluorescence was scored in the following way: N indicated a nuclear fluorescence signal; C indicated a cytoplasmic fluorescence signal without nuclear or mitochondrial fluorescence signals; and M indicated a mitochondrial fluorescence signal.

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Yeast two-hybrid analysis

The yeast two-hybrid analysis [24] employed yeast vectors pGADT7 and pGBDKT7 and host strain Y187 (described above), in accordance with the Matchmaker two-hybrid system 3 from Clontech. Transformed yeasts were grown in high-glucose (SD−−−−Leu,Trp) medium. Assays for β-galactosidase activity followed protocols as described previously [25]. Expression levels of the GAD (Gal4-activating domain) and GBD (Gal4-binding domain) fusion proteins were controlled by Western blot analysis. Experiments were performed a minimum of three times.
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Figure 1 Glucose regulates the subcellular localization of both Hxk2–GFP and GKβ–GFP

(A) Yeast strain DBY1315 [WT (wild-type)] expressing Hxk2–GFP, GKβ–GFP or GKα–GFP, from plasmids YEp352-HXK2/gfp, YEp352-GKβ/gfp or YEp352-GKα/gfp respectively, was grown on high-glucose synthetic medium (H-Glc) until a D600 of 1.0 was reached and then transferred to low-glucose synthetic medium (L-Glc) for 60 min. Then, cells were stained with DAPI and imaged for GFP and DAPI fluorescence. (B) Cells growing under low-glucose conditions and expressing GKβ–GFP were stained with Mitotracker® Red 580. Cells growing under low-glucose conditions and expressing both GKβ–GFP and Su9–RFP were stained with DAPI and imaged for DAPI, GFP and RFP fluorescence. (C) The localization of fluorescent reporter proteins was determined in at least 100 cells in three independent experiments. N indicates a nuclear fluorescence signal; C indicates a cytoplasmic fluorescence signal without nuclear or mitochondrial fluorescence signals; and M indicates a mitochondrial fluorescence signal. Values are means ± S.D. for at least three independent experiments.

ChIP (chromatin immunoprecipitation) assay

ChIP assays were performed essentially as described previously [26]. Cells were harvested and disrupted by vortex mixing in the presence of glass beads, and the lysate was sonicated to generate DNA fragments that ranged in size from 200 to 400 bp. To immunoprecipitate HA (haemagglutinin)-tagged proteins, we incubated the extract overnight at 4°C with anti-HA antibodies (Santa Cruz Biotechnology). To immunoprecipitate Hxk2 protein, we incubated the extract overnight at 4°C with anti-Hxk2 antibodies [11]. The sequence primers for PCR to amplify the SUC2 (sucrose fermentation 2) promoter region containing the MIG1 element were 5′-TTA TTACTCTGAACAGGA-3′ (sense) and 5′-AAGTCGTCGAACAGGA-3′ (antisense).

RESULTS

Subcellular localization of human GKα in yeast cells

It has been previously described that human GKα complements the glucose signalling defect of S. cerevisiae Δhxk2 mutants, indicating that GKα may substitute for Hxk2 in its role in regulating glucose signalling in yeast [22]. To gain insight into the molecular mechanism by which GKα regulates this process in yeast, the glucose-dependent subcellular location of GKα was determined in S. cerevisiae cells.

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with the mitochondria and the fluorescence signals completely overlapped.

Since liver and β-cell GK isoforms differ only in the first 15 N-terminal residues, it was important to determine whether the observed location of GKβ was also shared by the liver isoform. As can be seen in Figures 1(A) and 1(C), in cells grown overnight in high-glucose conditions, a fraction of GKβ–GFP was enriched in the nucleus, and in cells grown overnight on low-glucose medium GKβ–GFP was found mainly associated to mitochondria (Figures 1A–1C). Therefore our results suggest that, in yeast, the subcellular localization of β-cell and liver GK isoforms was similar and did not depend on their N-terminus.

The nuclear export of human GKβ in the yeast cell is Mig1-dependent

We have recently described that, under low-glucose conditions, Hxk2 exits the nucleus using a pathway in which Mig1 is involved [13,14]. In order to determine whether Mig1 is also affecting the exit from the nucleus of GKβ–GFP under the same conditions, we determined the subcellular distribution of GKβ–GFP in Δmig1 mutant cells (Figure 2A). The results indicated that GKβ–GFP fusion protein was enriched in the nucleus in both high- and low-glucose-grown cells, suggesting that in the absence of Mig1, GKβ–GFP was not able to exit the nucleus and reach the mitochondria. The introduction in these cells of a plasmid expressing MIG1 under its own promoter (YEp351-MIG1) allowed GKβ–GFP to exit the nucleus under low-glucose conditions (Figure 2A), as in wild-type cells (Figure 1A), suggesting that in low-glucose conditions, Mig1 is necessary to export the GKβ–GFP fusion from the nucleus to the mitochondria.

We also studied the subcellular distribution of GKβ–GFP in a Δhxk1 Δhxk2 double-mutant strain (Figure 2B). The results demonstrated that, in the absence of Hxk1 and Hxk2, GKβ–GFP was targeted to the mitochondria at both high- and low-glucose concentrations. Since, in the absence of Hxk1 and Hxk2, the protein kinase Snf1 is constitutively activated, we repeated the experiment in a triple Δhxk1 Δhxk2 Δsnf1 mutant (Figure 2C). In this case, no mitochondrial distribution of GKβ–GFP was observed under any growth conditions (similar results were obtained with a single Δsnf1 mutant; Figure 2C). These results suggested that Snf1 played a major role in regulating the exit of GKβ–GFP from the nucleus. When Snf1 was activated, either by growing the cells under low-glucose conditions or by deleting negative regulators such as Hxk1 and Hxk2, GKβ–GFP exited the nucleus and presented a mitochondrial distribution.

It is known that Snf1 is involved in the phosphorylation of Mig1 under low-glucose conditions, leading to its exit from the nucleus to the cytoplasm [28]. Since GKβ–GFP followed the same pattern of distribution as Mig1 and, as we have described above, GKβ–GFP did not exit the nucleus in the absence of Mig1, even under conditions where Snf1 is activated (low-glucose), we suggest that Mig1 could be involved in the exit of GKβ–GFP from the nucleus under low-glucose conditions. In agreement with this suggestion, we observed that GKβ–GFP fusion protein was enriched in the nucleus, in both high- and low-glucose-grown cells, in the absence of Msn5 (multicopy suppressor of SNF1 mutation 5) (Figure 3A), a member of the importin family of nuclear transport proteins which is required to export Mig1 from the nucleus in low-glucose conditions [29]. This result suggests that, in the absence of Msn5, GKβ–GFP was not able to exit the nucleus and reach the mitochondria. Thus, taken together, these results demonstrated that Mig1 and GKβ–GFP could form part of a nuclear complex whose export to the cytoplasm was dependent on both the phosphorylation of Mig1 by Snf1 and the exportin Msn5. The close relationship between Mig1 and GKβ–GFP was confirmed when we observed that a Mig1–GFP protein fusion was dragged to the mitochondrial network under low-glucose conditions only if GKβ was expressed in the same cells (Figure 3B). These results suggested that Mig1 and GKβ may form a complex in which Mig1 confers the determinants for nuclear export and GKβ confers the determinants for mitochondrial localization under low-glucose conditions.
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The experiments described above strongly suggest that Mig1 and GKβ may form part of an in vivo complex. To confirm the possible physical interaction between Mig1 and GKβ, we have used a yeast two-hybrid assay. Plasmid pGADT7-GKβ (expressing a fusion of the GAD to GKβ) was co-transformed with a plasmid expressing a fusion of the GBD with Hxk2, Mig1, Mig1S311A or Untagged GKβ, into an appropriate reporter strain. The interaction between the selected proteins was monitored by measurement of the β-galactosidase activity. As shown in Figure 4, the GAD–GKβ fusion protein produced a strong interaction with GBD–Mig1. However, this interaction was absent in a GBD–Mig1S311A mutant, indicating that the Ser311 residue of Mig1, previously identified as essential for the Hxk2 interaction [30], was also required for interaction with GKβ. No interaction was observed between GAD–GKβ and GBD–Hxk2 or GBD–GKα, indicating that these proteins did not form dimers. Similar levels of all protein fusions were detected in all of the cases (results not shown).

Taking all of these results together, we suggest that GKβ forms a similar complex with Mig1 as Hxk2 does, and that this complex is involved in the glucose-regulated expression of the SUC2 gene. This complex exits the nucleus under conditions of Snf1 activation, the presence of Mig1 and Msn5 being necessary for nuclear export.

**DISCUSSION**

In the present study, we have taken advantage of the facility to manipulate the *S. cerevisiae* genetic system to determine the mechanisms by which GKβ regulates glucose signalling in yeast cells and to examine the glucose-dependent localization of the GKβ in yeasts.

Reports suggest that Hxk2, the yeast orthologue of GKβ, has a dual function in yeast cells, first initiating the intracellular metabolism of glucose by its enzymatic activity and secondly, signalling glucose repression by its interaction with nuclear Mig1
that GK is glucose-responsive growth in high-glucose conditions, and both are able to regulate S. cerevisiae. A representative ChIP assay from three independent experiments is shown. The migration of standard markers is indicated on the left-hand side. A representative ChIP assay from three independent experiments is shown.

repressor, regulating its phosphorylation status [14]. Since Hxk2 protein mutations without phosphorylating activity but retaining activity in glucose repression signalizing have not been achieved [33,34], the idea that the sugar kinase activity and the sugar signalling properties are mediated through separate domains of Hxk2 is still controversial. In the present study, we demonstrate that recombinant human GKβ is able to signal glucose repression in S. cerevisiae; both GKβ and Hxk2 have a nuclear location during growth in high-glucose conditions, and both are able to regulate the glucose-responsive SUC2 gene in S. cerevisiae. We also report that GKβ is physically associated with the Mig1 protein repressor and ChIP assays confirmed that both GKβ and Hxk2 interacted with Mig1 in a cluster with DNA fragments containing the Mig1 site of the SUC2 promoter. Therefore our results suggest that GKβ and Hxk2 use similar mechanisms to regulate glucose signalling in yeast.

The other important finding of the present study regards the subcellular location of GKβ. We report that GKβ moves between the nucleus and mitochondria in the yeast cell in response to the glucose concentration in the medium: under high-glucose conditions, GKβ is enriched in the nucleus and resides there in association with Mig1; however, under low-glucose conditions, GKβ exits the nucleus and eventually reaches the mitochondria, either because it has uncharacterized mitochondrial determinants (GKβ), lacks the hydrophobic N-terminal sequence present in Hxks I and II that allows their binding to the mitochondria [35], or because it interacts with another protein that has these mitochondrial localization determinants, as in the case of the mammalian pro-apoptotic factor BAD (Bcl-2/Bcl-X, antagonist, causing cell death) which is involved in the mitochondrial targeting of GK in both liver and pancreatic β-cells [6]. For GKβ to reach this subcellular location, first Mig1 must be present in the cell and Snf1 must be active. Then, binding of Mig1 to GKβ allows the Mig1–GKβ complex to exit the nucleus by a mechanism dependent on the exportin Msn5. Finally, the complex is located in the mitochondria network, a location that has never been reported for either Mig1 or Hxk2.

Since the subcellular distribution of GKa in mammalian pancreatic β-cells is also regulated by glucose: low levels of glucose causes an association of GK with mitochondria, whereas high-glucose levels prevents GK translocation to this organelle [8], the yeast system offers a possibility to study the nature of the determinants present in GKa or the identification of interactive proteins that target GKa to the mitochondria under low-glucose conditions.

The role of Mig1 in regulating GKa exit from the nucleus could be compared with the role of the GKR in mammalian cells [4,5]. However, these two proteins present different domain structures and only 17% similarity (assessed by Clustal analysis), suggesting that the relationship between the two proteins is only at the level of their interaction with GK.

Taken together, the results of the present study indicate that despite the strong phylogenetic difference between the human GKa and the yeast Hxk2 proteins, the mechanism of glucose signalling is maintained, suggesting that the function of the regulatory domain of these proteins has been conserved throughout evolution.

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