Karyopherin α2: a control step of glucose-sensitive gene expression in hepatic cells

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Glucose is required for an efficient expression of the glucose transporter GLUT2 and other genes. We have shown previously that the intracytoplasmic loop of GLUT2 can divert a signal, resulting in the stimulation of glucose-sensitive gene transcription. In the present study, by interaction with the GLUT2 loop, we have cloned the rat karyopherin α2, a receptor involved in nuclear import. The specificity of the binding was restricted to GLUT2, and not GLUT1 or GLUT4, and to karyopherin α2, not α1. When rendered irreversible by a cross-linking agent, this transitory interaction was detected *in vivo* in hepatocytes. A role for karyopherin α2 in the transcription of two glucose-sensitive genes was investigated by transfection of native and inactive green fluorescent protein–karyopherin α2 in GLUT2-expressing hepatoma cells. The amount of inactive karyopherin α2 receptor reduced, in a dose-dependent manner, the GLUT2 and liver pyruvate kinase mRNA levels by competition with endogenous active receptor. In contrast, the overexpression of karyopherin α2 did not significantly stimulate GLUT2 and liver pyruvate kinase mRNA accumulation in green fluorescent protein-sorted cells. The present study suggests that, in concert with glucose metabolism, karyopherin α2 transmits a signal to the nucleus to regulate glucose-sensitive gene expression. The transitory tethering of karyopherin α2 to GLUT2 at the plasma membrane might indicate that the receptor can load the cargo to be imported locally.

Key words: glucose signalling, GLUT2, liver, nutrient regulation.

INTRODUCTION

Glucose sensing is characterized by the detection of extracellular glucose levels, followed by the transduction of a signal to the nucleus. Glucose sensing in mammalian cells is tightly coupled to a sustained glucose metabolism. This generates ATP, the energy required for numerous steps of cellular life. In mammals, GLUT2 is a member of a family of proteins (GLUTs), which differ in tissue distribution and in the amino acid sequence of the intracytoplasmic domains [1]. The primary role of GLUT2 is to transport glucose inside the cell where it is metabolized to provide carbon and energy sources. The presence of the glucose transporter isoform GLUT2 is also required in both pancreatic β-cells and liver for triggering an adequate transcriptional response to glucose [2–8]. In yeast, evidence that several glucose signalling pathways are acting in concert with glucose metabolism have been reported [9,10]. Starting from plasma membrane sensors, Snf3p and Rgt2p, the glucose signalling pathway is mediated by protein–protein interactions, and leads to glucose-induced transcription of specific genes [9,10]. We studied previously the possible role of the large intracytoplasmic loop of GLUT2 in the transmission of transcriptional effects of glucose [11]. Functions have already been ascribed to specific domains of glucose transporters [12–14]. We showed that the large intracytoplasmic loop of GLUT2 located between transmembrane domains 6 and 7, when expressed as a fusion protein with the green fluorescent protein (GFP), was able to alter glucose sensing in the mhAT3F hepatoma cell line, without modifying glycogen synthesis [11]. This suggested that, in mammalian hepatoma cells, in concert with glucose metabolism, GLUT2 initiated a glucose-sensing pathway.

The aim of the present study was to identify the proteins that, through interaction with GLUT2, functioned as mediators for glucose sensing. We thus performed a two-hybrid screen [15] of a rat liver cDNA library to identify proteins interacting with the GLUT2 intracytoplasmic loop, and studied the physiological function of these proteins.

EXPERIMENTAL

Glucose transporter constructs

The coding regions of the intracytoplasmic loop between the transmembrane domains 6 and 7 from rat GLUT2 (amino acids 237–301) were amplified by PCR using a plasmid containing the GLUT2 full-length cDNA as a template (a gift from Dr B. Thorens, Institute of Pharmacology, Lausanne, Switzerland) [16]. The fragment was then inserted in-frame into the pLex9 vector as a fusion with the yeast LexA DNA-binding domain. Intracytoplasmic loops of GLUT1 (amino acids 206–271) and GLUT4 (amino acids 223–287) were similarly constructed in the pLex9 vector. The C-terminal domains of GLUT2 (amino acids 481–521), GLUT1 (amino acids 451–491) and GLUT4 (amino acids 467–509) were cloned into the pLex vector by Dr F. Giorgino (Endocrinology and Metabolic Diseases, Università degli studi di Bari, Italy) and used as controls for specificity.

Abbreviations used: DSS, disuccinyl suberate; GFP, green fluorescent protein; GST, glutathione S-transferase; LEF1, lymphoid-enhancer binding factor 1; L-PK, liver pyruvate kinase; NLS, nuclear localization sequence; RACE, rapid amplification of cDNA ends; SREBP, sterol-regulated-element-binding protein.

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Plasmid constructs were sequenced using the Prism kit (Perkin-Elmer, Paris, France).

Yeast two-hybrid screening

The yeast two-hybrid screening was performed as described previously [17]. The yeast strain L40 and yeast plasmids pLex, containing the LexA DNA-binding domain, and pGAD, containing the GAL4 activation domain, were from Dr A. Vojtek (Fred Hutchinson Cancer Research, Seattle, WA, U.S.A.) [17]. The rat liver cDNA library, constructed in the pGAD3S2X plasmid, was a gift from Dr M. Cognet-Vasseur (INSERM-U129, Paris, France). The coding region of the intracellular loop domain was used as bait. Positive cDNA clones were sequenced. Nucleotide sequence and amino acid sequence comparisons were performed using BLAST analysis. pLexRas and pGADRaf were employed as positive controls, whereas pLexLamin and pGADRaf were used as negative controls.

Rat karyopherin α2 cloning

A 5′-rapid amplification of cDNA ends (RACE) PCR was conducted according to the manufacturer's instructions (ClonTech, Palo Alto, CA, U.S.A.) to obtain the N-terminus domain of karyopherin α2. For this purpose, a rat liver Marathon-Ready cDNA library and the primer 5′-GGAAATGGCCCAGCAGGAGTCTGC-3′ starting from nt 736 of the positive clone of karyopherin α2 and one nested primer starting from nt 720 were employed. The recovered PCR fragment of 940 bp was sequenced and served as a template for PCR amplification of the N-terminal fragment of the cDNA, including the internal *Hin*dIII site. The C-terminus of the cDNA was excised (*Hin*dIII–*Xho*I) from the pGAD vector of the two-hybrid screen. The karyopherin α2 full-length cDNA was subcloned into pBSSK and sequenced.

The full-length coding region of karyopherin α2 (amino acids 1–529) and several domains of the protein were subcloned into the pGAD3S2X yeast expression vector. Several domains were obtained by PCR: the N-terminal domain alone (amino acids 1–95); the N-terminus and the first five armadillo repeats (amino acids 1–285); the 10 armadillo repeats (amino acids 96–482); the last five armadillo repeats and the C-terminal domain (amino acids 286–529); and the C-terminal domain alone (amino acids 482–529).

The full-length and truncated karyopherin α2 were also cloned in-frame into the pEGFP vector (ClonTech) as a fusion protein with GFP. A truncated karyopherin α2 (amino acids 96–482), deleted of its importin β-binding domain and C-terminus, was constructed by PCR, subcloned into the pEGFP vector and sequenced.

Protein–protein interaction using two-hybrid system

Interactions between the GLUT2 loop and different domains of karyopherin α2 were tested in the two-hybrid system using the β-galactosidase filter assay.

In vitro binding assays

Karyopherin α2 (amino acids 24–529) and full-length karyopherin α1 were inserted in-frame and fused with the glutathione S-transferase (GST) gene in the pGEX vector (Amersham Biosciences, Orsay, France). The recombinant proteins were obtained by overexpression in *Escherichia coli* BL21 according to the manufacturer's recommendations. GST fusion proteins bound to glutathione beads (10–20 μg) were mixed with 40 μg of liver or skeletal muscle plasma membranes prepared as described previously [18]. Incubations were performed in a TNE buffer [10 mM Tris/HCl (pH 7.6), 120 mM NaCl, 1 mM EDTA, 1 mM PMSF and 5 μg/ml leupeptin] for 2 h at 4 °C on a rotating wheel. The beads were then washed four times with 1 ml of TNE buffer. The fusion proteins were eluted with reduced glutathione. A quarter of the volume of the eluate was loaded on to an SDS/polyacrylamide gel and the proteins immunoblotted.

Radiolabelled proteins were generated using an *in vitro* transcription/translation system (TNT; Promega, Charbonniéres, France) and [^{35}S]methionine (Amersham Biosciences). Various plasmids containing coding sequences were used as templates: lymphoid-enhancer binding factor 1 (LEF1) and karyopherin α1 plasmids were from Dr M. Prieve (Department University of California, Irvine, CA, U.S.A.) and the sterol-regulated-element-binding protein (SREBP) plasmid was from F. Foufelle (INSERM 432, Paris, France). ^{35}S-labelled translation products were incubated for 2 h at 4 °C with GST–karyopherin α2 or α1. The beads were washed extensively and the complexes recovered after elution were analysed by SDS/PAGE. Gels were dried and the proteins were visualized by autoradiography. The radioactivity in the eluate or on the beads was quantified according to the manufacturer's instructions.

Cross-linking experiments

Hepatocytes from adult fed rats were isolated as described previously [18]. Freshly isolated cells were distributed (2×10^6 cells/vial), gassed for 3 s with 5% CO_2 and then incubated for 2 h in medium 199 (Life Technologies, Cergy-Pontoise, France) in the absence or presence of 20 mmol/l glucose, with increasing amounts (0, 1, 2 and 3 mM) of disuccinyl suberate (DSS; Pierce, Bezons, France) dissolved in DMSO. Subsequently, the cells were pelleted and washed twice in 2 ml of cold PBS [137 mM NaCl, 1.3 mM KCl, 16.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (pH 7.5)] to eliminate the unbound cross-linker. Cells were lysed in Reporter lysis buffer (Promega). Proteins were quantified and subjected to Western blotting. Duplicate blots were immunoblotted with either an anti-GLUT2 antibody and/or an anti-(karyopherin α) antibody as indicated below.

Western-blot analysis and antibodies

Proteins were separated by SDS/PAGE [10% (w/v) gels] and transferred on to a nitrocellulose membrane. After overnight incubation in 5% (w/v) non-fat milk in Tris-buffered saline [TBS; 20 mM Tris/HCl (pH 7.6) and 137 mM NaCl], the blots were washed in TBS containing 0.1% (v/v) Triton X-100 and incubated with the appropriate antibodies. A monoclonal anti-GFP antibody (ClonTech), a rat anti-GLUT2 antibody (East-Acre, Southbridge, MA, U.S.A.), a mouse anti-GLUT2 antibody (from Dr B. Thorens, Institute of Pharmacology, Lausanne, Switzerland) and an anti-(*Xenopus* karyopherin α) antibody (a kind gift from Dr M. Lohka, Department of Biological Sciences, University of Calgary, Canada) were used. By Western blotting, the anti-(karyopherin α) antibody recognized the several isoforms of endogenous mouse and rat karyopherin α (60 kDa) and, more importantly, it recognized both the full-length (87 kDa) and truncated (80 kDa) rat karyopherin α2–GFP fusion protein when transfected in mhAT3F cells (results not shown). The anti-GFP antibody recognized a non-specific 50 kDa band in mhAT3F cells as well as the GFP fusion proteins of the expected size. The detection of immune complexes was performed using enhanced chemiluminescence (ECL®; Amersham Biosciences).

Cell culture

The mhAT3F hepatoma cell line was derived from transgenic mice expressing the Simian virus 40 large T and small t antigens under the control of the anti-thrombin III promoter [19]. The mhAT3F cells were generously given by Dr B. Antoine (INSERM 129, Paris, France). Cells were grown in Dulbecco's modified Eagle's medium/Ham F12 glutamax (Life Technologies) supplemented with 100 units/ml penicillin, 10 μg/ml streptomycin, 0.1 μM insulin (NovoNordisk, Boulogne-Billancourt, France), 1 μM dexamethasone (Merck, Riom, France), 1 μM tri-iodothyronine (Sigma, St Quentin Fallavier, France) and 5% (v/v) fetal calf serum (Jacques Boy, Reims, France).

Cells were cultured in 17 mM glucose (high glucose) except when otherwise indicated. Cells were also grown in glucose-free medium for 24 h. Under these conditions, no glucose was added to the culture medium, although some glucose was present in the fetal calf serum (low glucose).

Transfection experiments

The mhAT3F cells were plated and stably transfected using the calcium phosphate DNA precipitation method [20]. Full-length and truncated karyopherin α2 (amino acids 96–482) in the pEGFP vectors and the void vectors were transiently transfected using Lipofectin (Life Technologies) as recommended by the manufacturer.

Fluorescence analysis

The mhAT3F cells were grown on Permanox four-chamber slides (Lab-Tek, Falcon) in the presence of 17 mM glucose. Cells were washed three times with PBS, fixed for 30 min in 4% paraformaldehyde, permeabilized in 0.2% (v/v) Triton X-100 and quenched for 10 min in 1% (w/v) BSA. The detection of GLUT2 was performed using a primary antibody against mouse GLUT2 and a tetramethylrhodamine β-isothiocyanate ('TRITC')-conjugated secondary antibody. Nuclei were stained using Hoescht 33258 (0.5 μg/ml) for 5 min. After extensive washes in PBS, the coverslips were mounted in Mowiol (Hoescht, Romainville, France). Fluorescence microscopy was performed using a Zeiss LSM510 confocal laser-scanning microscope with an Apochromat lens (63× objective) or an Olympus fluorescence microscope.

Northern-blot analysis

Total RNAs were purified from rat tissues and confluent mhAT3F cells after culture for 24 h under different experimental conditions and Northern-blot analysis was performed as described previously [18]. Blots were hybridized with radiolabelled cDNA probes. Quantification of specific mRNA was performed by computer-based-scan analysis.

Quantitative reverse transcription-PCR

mhAT3F cells, transiently transfected with GFP, karyopherin α2–GFP or truncated karyopherin α2–GFP, were cultured for 24 h in a medium containing low or high glucose concentrations. Transfected cells were sorted from the non-transfected cells using the fluorescent properties of GFP (excitation at 488 nm, and emission at 510 nm) using a Beckman Coulter Altra HSS with an argon laser and Expo32 software. From a single culture dish, total RNAs were extracted from both transfected- and non-transfected-sorted fractions by the RNeasy kit (Qiagen, Courtaboeuf, France). Total RNA was extracted from at least 2.5×10^5 cells and the yield varied from 1–1.5 μg/1×10^5 cells. A reverse transcription was performed. The mRNA levels of GLUT2, liver pyruvate kinase (L-PK) and L19 ribosomal protein were determined using real-time PCR (Light Cycler system; Roche, Meylan, France). The forward and reverse primers respectively were: 5′-GTCCAGAAAGCCCCAGATACC-3′ and 5′-GTGACATCCTCAGTTCCTCTTAG-3′ for GLUT2; 5′-AGAACCATGAAGGCGTGAA-3′ and 5′-CACAAACGACAGGCTTGC-3′ for L-PK; and 5′-AAGATCGATCGCCACATGTATCA-3′ and 5′-TGCGTGCTTCCTTGGTCTTAGA-3′ for L19. The ribosomal L19 mRNA, representing a control for the RNA extraction, was unaffected by culturing under high or low glucose conditions. The non-transfected-sorted fractions were used as the internal control for the glucose response (results not shown). Results were expressed as the ratio of the levels of GLUT2/L19 and L-PK/L19 mRNA in GFP–karyopherin-transfected cells as compared with GFP-transfected cells cultured in high glucose medium, which were taken as 100%.

Statistical analysis

Results are expressed as means ± S.E.M. Statistical analysis was performed by using the Student's t test for unpaired data.

RESULTS

Identification of proteins interacting with the GLUT2 intracellular loop

To identify proteins that bind directly to the intracytoplasmic loop of the GLUT2 glucose transporter, we performed a yeast two-hybrid screen of a liver cDNA library fused with the yeast GAL4 activation domain, using the GLUT2 loop as bait. Among the 5.4×10^6 independent clones analysed, 19 gave a strong positive signal for β-galactosidase activity in yeast expressing the GLUT2 loop, whereas a negative signal was obtained in yeast expressing unrelated bait, such as lamin. Positive clones were sequenced, and classified as five distinct proteins; one was investigated in the present study. BLAST analysis of this clone provided evidence that it coded for a protein similar to karyopherin α2, starting from the amino acid 23 of the mouse karyopherin α2.

The N-terminus of the rat karyopherin α2 cDNA was obtained using the 5′-RACE procedure. Upon sequencing of the recovered fragment, an ATG was observed that could be the initiating methionine, as it conformed to the consensus Kozak sequence for translational initiation. The fragment was also similar to the first 23 amino acids of the mouse karyopherin α2, called pendulin (Protein Information Resource database accession number S57345). The cloned protein was the rat variant of a mouse karyopherin α2 (GenBank® accession number AJ130946), since BLAST analysis showed that only six amino acids out of 529 were not conserved between our sequence and mouse karyopherin α2.

We tested the specificity of the karyopherin α2–GLUT2 interaction (Table 1). The intracellular loop and C-terminus of GLUT2 and those of two other facilitative glucose transporters, GLUT1 and GLUT4, were assayed for their interactions with karyopherin α2. A very high amino acid similarity existed between the GLUT2 and GLUT1 loops (73%). Nevertheless, intracellular loops, as well as C-terminal domains, of GLUT1 and GLUT4 glucose transporters were unable to interact with karyopherin α2 in the yeast two-hybrid system. Furthermore, karyopherin α2 did not interact with the C-terminal domain of GLUT2. In contrast, clone 4, another protein identified during the screening of the library, interacted with both the GLUT2

Table 1 Specific interaction of karyopherin α2 with the GLUT2 intracytoplasmic loop, but not the intracytoplasmic domains of GLUT1 and GLUT4: comparison with clone 4 obtained during the same library screen

The intensity of the blue coloration obtained after the β-galactosidase assay is indicated by the number of +. The Ras/Raf interaction was considered as the positive control (+++) and the lamin/Raf interaction as the negative control (−). d, growth deficient co-transfected yeasts; C-term, C-terminus. Results are from a representative experiment performed twice with similar results.

	pLex GLUT2 loop	pLex GLUT2 C-term	pLex GLUT1 loop	pLex GLUT1 C-term	pLex GLUT4 loop	pLex GLUT4 C-term
pGAD karyopherin α2	+++	−	−	−	−	−
pGAD clone 4	+++	−	++	d	−	−

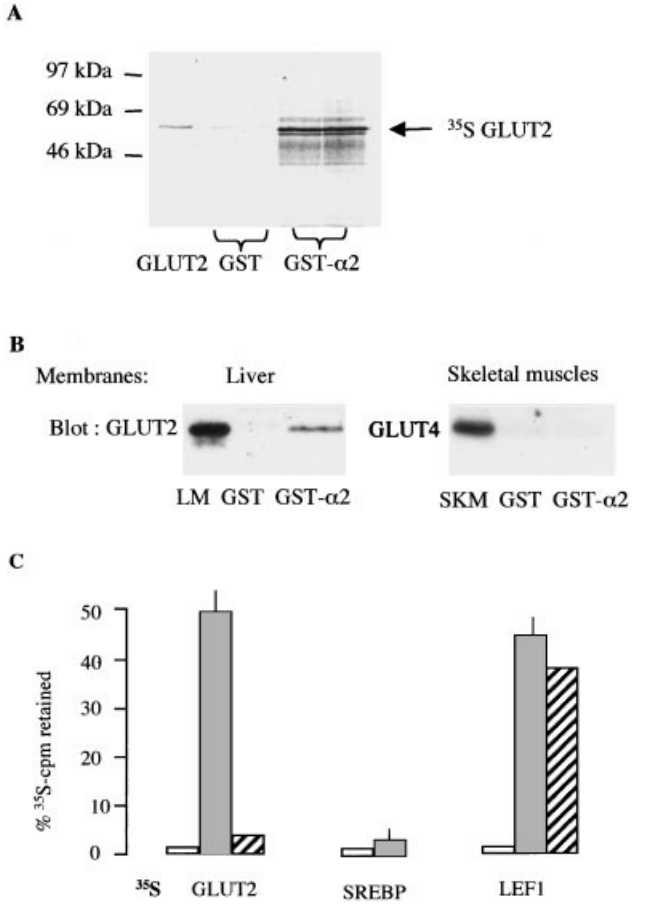

Figure 1 *In vitro* binding assay of immobilized GST–karyopherin α2 fusion protein

(**A**) Physical interaction between GST–karyopherin α2 and GLUT2. GLUT2 lane, 2 μl of [^{35}S]GLUT2 produced by reticulocyte lysate. GST lanes, no retention of [^{35}S]GLUT2 on immobilized GST; GST-α2 lanes, [^{35}S]GLUT2 retained on immobilized GST–karyopherin α2 (representative of five experiments). Molecular-mass markers (in kDa) are indicated on the left. (**B**) Binding of native GLUT2 protein in its membrane environment to the GST–karyopherin α2, as determined by Western blotting using an anti-GLUT2 antibody. Samples were loaded as follows: LM lane, 2.5 μg of crude liver plasma membrane; GST and GST-α2 lanes, 40 μg of the liver membrane incubated with 10 μg of GST or GST–karyopherin α2 respectively. SKM lane, 25 μg of crude skeletal muscle; GST and GST-α2 lanes, 40 μg of skeletal muscle membrane incubated overnight with immobilized GST or GST–karyopherin α2 respectively. Western-blot analysis of the proteins retained by the immobilized GST–karyopherin α2 fusion protein was revealed by an anti-GLUT4 antibody. A quarter of the respective eluate was loaded on the gels. (**C**) Quantification and specificity of binding of GLUT2, SREBP and LEF1 to immobilized GST alone (open bars), GST–karyopherin α2 (closed bars) and GST–karyopherin α1 (hatched bars). [^{35}S]GLUT2, [^{35}S]SREBP (negative control) and [^{35}S]LEF1 (positive control) were produced by reticulocyte lysate. The results are expressed as the percentage of the ^{35}S-labelled protein retained on immobilized GST, GST–karyopherin α2 or GST–karyopherin α1. The experiments with karyopherin α2 and α1 were performed independently four and two times respectively.

and GLUT1 loops. Thus the interaction of the intracellular loop of GLUT2 with karyopherin α2 was specific for this isoform of glucose transporter.

Karyopherin α2 binds to GLUT2 *in vitro* and *in vivo*

Using an *in vitro* transcription/translation system, the complete [^{35}S]GLUT2 protein was produced and loaded on to immobilized GST–karyopherin α2. As shown in Figure 1(A), immobilized GST–karyopherin α2, but not GST alone, retained [^{35}S]GLUT2. The interaction of karyopherin α2 with the complete GLUT2 protein was thus confirmed using this *in vitro* binding assay. We quantified the binding of GLUT2 to GST–karyopherin α1 and GST–karyopherin α2 (Figure 1C). Binding of 50% of the radiolabelled [^{35}S]GLUT2 to GST–karyopherin α2 was detected, a value nearly identical with the positive control [^{35}S]LEF1 (45%) [21]. The transcription factor SREBP1-c, involved in nutrient regulation of gene expression, was used as a negative control [22]. In addition, the binding of [^{35}S]GLUT2 to GST–karyopherin α1 was almost undetectable, whereas 37% of the LEF1 was able to bind to the α1 isoform of the receptor. A competition for the binding to GST–karyopherin α2 was observed between GLUT2 and LEF1 (results not shown). We concluded that the binding of GLUT2 was specific for karyopherin α2 compared with α1 and quantitatively relevant.

To rule out the possibility that the interaction between karyopherin α2 and GLUT2 could only occur with recombinant molecules, we tested the interaction of karyopherin α2 with GLUT2 protein in their natural environment: the liver plasma membranes. Crude liver plasma membranes, enriched in GLUT2, were incubated with immobilized GST–karyopherin α2 or with GST alone. As shown in Figure 1(B), immobilized karyopherin α2 interacted specifically with a protein from liver plasma membrane that was detected by immunoblotting with an anti-GLUT2 antibody. The detergent solubilization of liver plasma membrane did not modify the binding of GLUT2 to karyopherin α2 (results not shown). Interaction with karyopherin α2 thus seemed specific, and compatible with the structure of GLUT2 when inserted into the liver plasma membranes. When crude skeletal muscle plasma membranes, devoid of GLUT2, but enriched in GLUT4, were loaded on to GST or GST–karyopherin α2, no specific binding of GLUT4 was observed on both types of GST beads (Figure 1B). This confirmed the specificity of the binding of karyopherin α2 to GLUT2 and restrained the putative function to GLUT2-expressing tissues or cells.

The interaction between endogenous GLUT2 and karyopherin α2 was analysed further *in vivo* in freshly isolated rat hepatocytes. The interaction was not detected by co-immunoprecipitation in intact hepatocytes in the absence of overexpression of the two proteins either due to the efficiency of the antibodies or to the transitory nature of the interaction. Cross-linking agents were used to stabilize the transitory interaction between the two

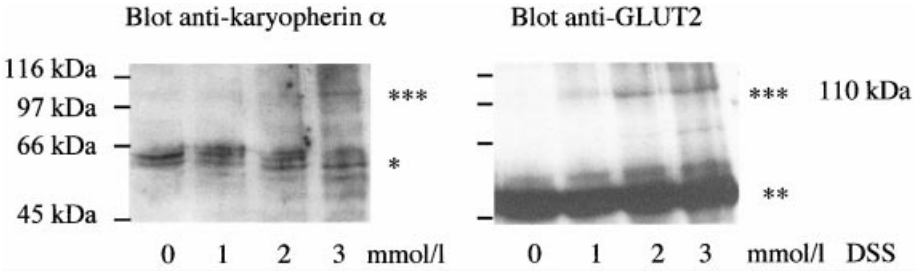

Figure 2 ***In vivo*** **interaction between GLUT2 and karyopherin α in isolated rat hepatocytes by cross-linking assays**

Hepatocytes were incubated in the presence of 20 mM glucose and increasing concentrations of DSS (1, 2 and 3 mM). A negative control was obtained in the absence of DSS (0 mM). Total protein (10 μg) was loaded on duplicate gels, transferred on to nitrocellulose, and blots were probed with anti-(karyopherin α) and anti-GLUT2 antibodies. Each blot was stripped and reprobed with the other antibody to check for the exact superimposition of the signals. Karyopherin α (*), GLUT2 (**) and the GLUT2–karyopherin α complex (***) are shown. Molecular-mass markers (in kDa) are indicated on the left.

Table 2 Identification of the domain of karyopherin α2 interacting with the GLUT2 loop using the two-hybrid system

The intensity of the blue coloration obtained after the β-galactosidase assay is indicated by the number of +. The Ras/Raf interaction was considered as the positive control (+++) and the lamin/Raf interaction as the negative control (−). All the controls, pLex lamin co-transfected in yeast with the various pGAD karyopherin domains, were negative for the β-galactosidase filter assay. Results are a representative experiment performed two or three times with similar results. Domains are indicated in parentheses.

Amino acid residues of karyopherin α2	β-galactosidase activity
1–529 (full-length)	+++
1–95 (N-terminus)	−
1–285 (five N-terminus armadillos)	−
96–482 (ten armadillos)	+++
286–529 (five C-terminus armadillos)	+
482–529 (C-terminus)	−

proteins thus allowing the complex to be easily detected. In hepatocytes, the cross-linking agent DSS was able to covalently link endogenous GLUT2 (50 kDa) with several proteins, as revealed by the anti-GLUT2 antibody (Figure 2). One of the complexes migrated at an apparent molecular mass of 110 kDa and can be observed even in the presence of low DSS concentrations. When the blot was incubated with an anti-(karyopherin α) antibody, the karyopherin α was detected as several bands at approx. 60 kDa (Figure 2). In the presence of DSS, a protein complex of 110 kDa was immunodetected with the anti-(karyopherin α) antibody (Figure 2). The complex was only detected at 3 mM DSS, due to the low specificity of the anti-(karyopherin α) antibody, which was raised against *Xenopus* isoforms. The signal was strictly superimposable with the one observed with the anti-GLUT2 antibody. The 110 kDa complex was composed of proteins recognized by anti-(karyopherin α) (60 kDa) and anti-GLUT2 (50 kDa) antibodies, providing experimental evidence for an *in vivo* interaction between the two endogenous proteins.

The domain of karyopherin α2 that interacts with the GLUT2 loop was identified using deletion mutants (Table 2). The 10 armadillo repeats of karyopherin α2 were found to be sufficient to interact with GLUT2, as tested by using the two-hybrid system. This domain constituted the truncated karyopherin α2 in the following experiments and was still able to bind to the GLUT2 loop in yeast. The N-terminal domain of karyopherin α2 did not interact with GLUT2, thus preserving the possibility of binding to the protein import machinery through importin β [23].

We then investigated whether karyopherin α2 and GLUT2 mRNA were expressed in the same tissues. A single 2.2 kb karyopherin α2 mRNA species not only appeared in all tissues expressing GLUT2, including liver, pancreas, intestine and kidney (results not shown), but also in tissues that did not express GLUT2, such as heart and placenta. The level of expression of rat karyopherin α was also highly variable among the different tissues, as reported for mouse tissues [24].

Effect of the overexpression of the karyopherin α2 in mhAT3F

Hepatoma mhAT3F cells were transfected with GFP–karyopherin α2 constructs, and analysed for the production and location of fusion proteins. The fusion proteins showed the expected molecular masses of 87 kDa for GFP–karyopherin α2 and 80 kDa for the GFP–truncated karyopherin α2 (amino acids 96–482), as determined by Western blotting with antibodies against the GFP and karyopherin α (results not shown).

The cellular location of the fusion proteins was studied by confocal fluorescence microscopy. Fluorescence of GFP did not show any specific location in the mhAT3F cells [11]. GFP–karyopherin α2 was spread throughout the cytoplasm and was more concentrated in the nuclei of three transfected cells, regardless of the level of expression obtained in the transiently transfected cells (Figure 3). It must be noticed that we were unable to obtain any stable clones of mhAT3F cells expressing GFP–karyopherin α2, possibly due to a cytotoxic effect of a high level of karyopherin α2 expression.

As expected, GFP–truncated karyopherin α2 remained located in the cytoplasm (Figure 3), immobilized by the deletion of the domain that binds to the import machinery. Two stable clones of mhAT3F expressing GFP–truncated karyopherin α2 were established (Figure 3). One clone expressed a 6-fold higher level of GFP–truncated karyopherin α2 than the other (Figure 3), as determined by Western blotting (results not shown). The protein ratio of GFP–truncated karyopherin α2 compared with endogenous karyopherin α2 cannot be established, due to the broad specificity of the antibody for all the karyopherin α isoforms.

As expected, the location of GLUT2 protein was at the plasma membrane, but an intracytoplasmic staining was also detected (Figure 3). This pattern of expression was observed in wild-type cells, cells expressing GFP–karyopherin α2 and cells expressing

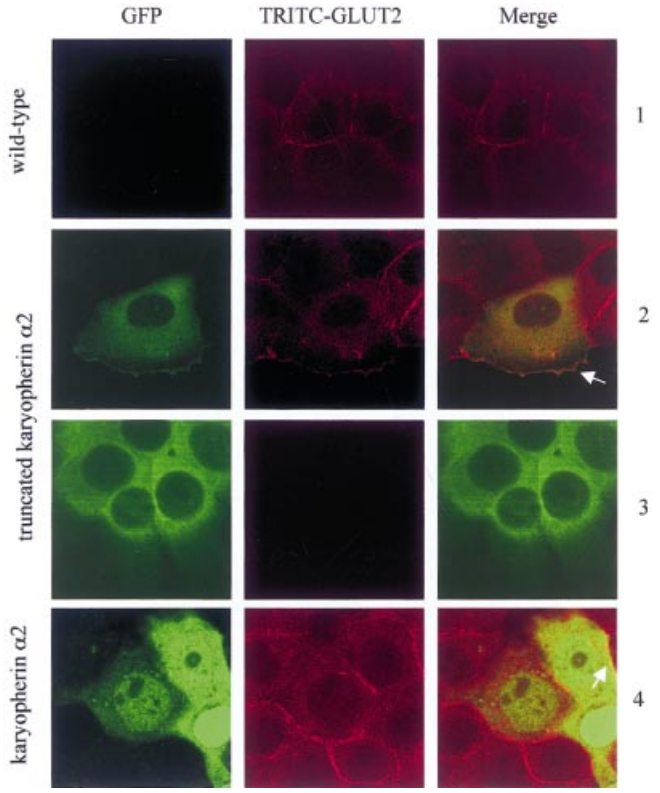

Figure 3 Location of the GFP–karyopherin α2 and GFP–truncated karyopherin α2 fusion proteins and GLUT2 in mhAT3F cells by confocal microscopy

(**1**) Wild-type mhAT3F cells. (**2**) A clone of mhAT3F cells stably expressing a low level of GFP–truncated karyopherin α2. (**3**) A clone of mhAT3F cells stably expressing a high level of GFP–truncated karyopherin α2. (**4**) mhAT3F cells transiently transfected with GFP–karyopherin α2. GLUT2 protein in mhAT3F cells was detected by using a mouse anti-GLUT2 antibody and revealed by a tetramethylrhodamine β-isothiocyanate-coupled secondary antibody. Expression of full-length and truncated karyopherin α2 was directly visualized by the GFP tag. Merged images were obtained by the simultaneous opening of the green and red channels; a yellow signal is representative of a co-localization of the two proteins and indicated by arrows. The mean size of the mhAT3F cell nucleus was 14 ± 0.3 μm ($n = 18$).

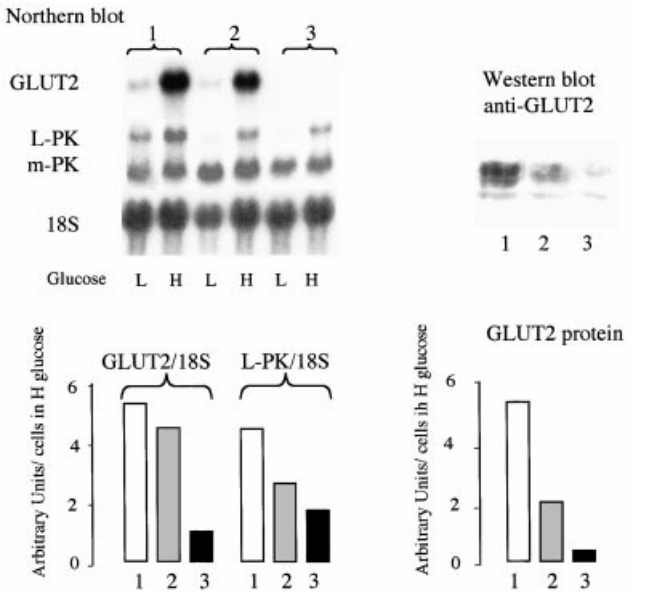

Figure 4 Effect of the overexpression of truncated karyopherin-α2 on gene expression

Northern- and Western-blot analyses of GLUT2 and L-PK mRNA accumulation and GLUT2 levels respectively in mhAT3F cells stably transfected with GFP alone (1), low (2) and high (3) expression of GFP–truncated karyopherin α2. For the Northern-blot analysis, cells were cultured in the presence of low (L) or high (H) glucose concentrations, and Northern blots were hybridized to GLUT2, L-PK and 18S probes. The signals from three independent blots were quantified by scanning densitometry and presented in arbitrary units. Only the values obtained in high glucose are shown. For the Western-blot analysis, cells were cultured in high glucose and GLUT2 was determined using an anti-GLUT2 antibody. The signals from two blots were quantified by scanning densitometry and presented in arbitrary units.

low levels of GFP–truncated karyopherin α2. In hepatoma cells expressing high levels of GFP–truncated karyopherin α2, the GLUT2 signal disappeared both at the plasma membrane and in the cytoplasm (Figure 3). Importantly, some co-localization can be seen at the plasma membrane and inside the cells between GLUT2 and immobilized truncated karyopherin α2 signals and GLUT2 and GFP–karyopherin α2 signals (Figure 3). The localization of the karyopherin α2 in the vicinity of GLUT2 suggested that the karyopherin α2 could load the cargo at the plasma membrane.

The profound decrease observed in GLUT2 expression prompted us to test if the karyopherin α2 was involved in the transmission of the signal that regulates the expression of glucose-sensitive genes, by using the two clones of mhAT3F cells overexpressing the truncated karyopherin α2. The mRNA levels of two glucose-sensitive genes, GLUT2 and L-PK, were measured by Northern blotting of total RNAs extracted from mhAT3F cells cultured in low- and high-glucose medium (Figure 4). Glucose was able to significantly stimulate the expression of GLUT2 and L-PK mRNA in control cells. There was a marked attenuation of the accumulation of GLUT2 and L-PK mRNA in cells expressing the truncated karyopherin α2 in the presence of high glucose. A reduction was also observed at a low glucose concentration. This effect was specific for glucose-sensitive genes, because muscular pyruvate kinase ('m-PK') mRNA remained unaffected. Furthermore, the GLUT2 protein was barely detectable in cells transfected with truncated karyopherin α2 when compared with control cells (Figure 3). When quantified by Western blotting, there was a 2- and 5-fold reduction in GLUT2 protein levels respectively, in the two clones (Figure 4). The GFP–truncated karyopherin α2 acted as a dominant-negative mutant by blocking the signal mediating the expression of GLUT2 and L-PK in hepatoma cells, suggesting that karyopherin α2 regulated the expression of glucose-sensitive genes.

To compare the effect of full-length and truncated karyopherin α2 on the glucose transcriptional response, we used transiently transfected cells (Figure 5). All the GFP-expressing cells were sorted regardless of the level of expression of the transfected gene, and the efficiency of transfection was approx. $11 \pm 2\%$ for both karyopherins. As expected, the overexpression of truncated karyopherin α2 decreased the GLUT2 and L-PK mRNA levels when compared with the 100% values obtained in GFP-transfected cells cultured in high-glucose medium. On the contrary, GLUT2 and L-PK mRNA levels were not statistically different in mhAT3F cells expressing GFP–karyopherin α2 when compared with those transfected with GFP alone, regardless of the glucose concentrations. Statistical differences were observed by comparing the mRNA levels of the two genes measured in karyopherin α2- and truncated karyopherin α2-transfected cells. The expression of glucose-sensitive genes was thus regulated by

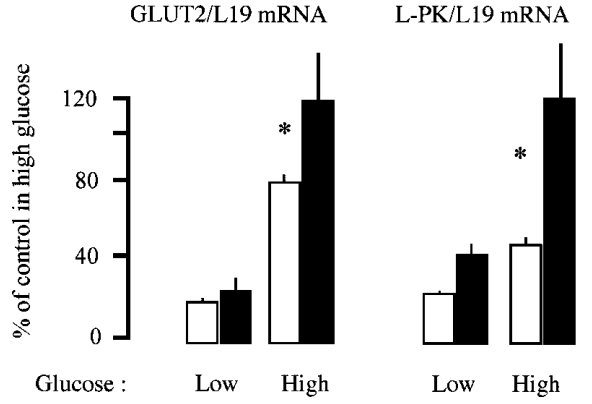

Figure 5 Effect of the overexpression of full-length and truncated karyopherin-α2 on gene expression

GLUT2 and L-PK mRNA accumulation in mhAT3F cells transiently transfected with GFP–karyopherin α2 (closed bars) and GFP–truncated karyopherin α2 (open bars). Fluorescent cells were sorted and total RNA was extracted. Specific mRNAs (GLUT2, L-PK and L19) were measured by real-time PCR (Light Cycler) using appropriate primers. The results were normalized for mRNA extraction using L19 mRNA as a constant. We ascribed a 100% value to the GLUT2/L19 and L-PK/L19 mRNA measured in cells transfected with GFP alone and cultured in high-glucose medium as controls. In low-glucose medium, the GLUT2/L19 was 27 ± 3% and L-PK/L19 was 39 ± 3% of control, these values were similar to those obtained in non-transfected cells. The values obtained in GFP–karyopherin-α2-transfected cells were statistically different from those obtained in GFP–truncated karyopherin α2-transfected cells (*$P < 0.05$; $n = 4$).

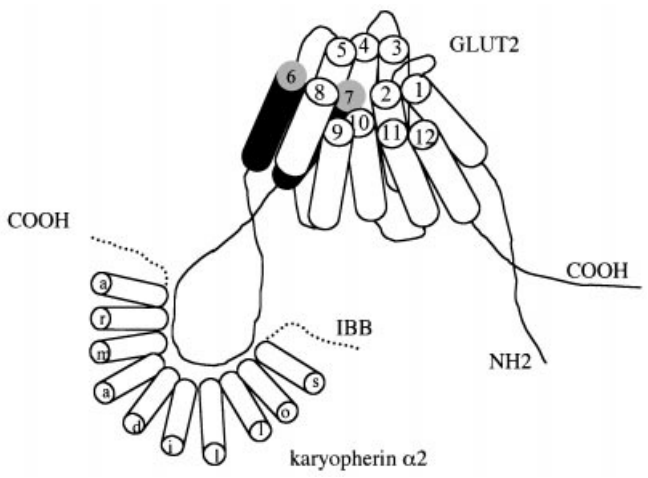

Figure 6 Schematic representation of the interaction between karyopherin α2 and GLUT2

The large loop between transmembrane domains 6 and 7 (in black) of GLUT2 interacts with the armadillo domain of karyopherin α2. The importin β binding (IBB) domain of karyopherin α2 is not involved in the interaction.

a nucleocytoplasmic import pathway mediated by karyopherin α2. The effect of karyopherin α2 is probably limited by the amount of cargo.

We have thus shown that the expression of two glucose-sensitive genes is dependent on the level of karyopherin α2. A titration of the glucose effect was observed depending on the presence of active karyopherin α2.

DISCUSSION

In a previous report [11], we were able to inhibit the transcriptional effect of glucose by expression of the intracytoplasmic loop of GLUT2, although no modification of glucose incorporation into glycogen was observed. We postulated that the GLUT2 loop is likely to interact with protein(s) involved in an important step in the regulation of transcriptional activity by glucose.

We have focused our investigation on one of the proteins cloned by the interaction with the GLUT2 loop, due to its binding specificity and also because of its known function that might be relevant to a role in a signalling cascade. In the present study, we report a transitory interaction of the GLUT2 loop with an isoform of the import receptor karyopherin α2. This interaction is restricted to tissues that express GLUT2, since karyopherin α2 did not interact with the ubiquitous GLUT1 or with the insulin-sensitive GLUT4. Furthermore, GLUT2 only interacted in a quantitatively relevant manner both *in vitro* and *in vivo* with the α2 isoform of karyopherin and not the α1 isoform.

The karyopherin α proteins have an important physiological role in cells, as demonstrated by the fact that they are highly conserved among species from yeast to human [24]. In mouse, the karyopherin α protein family is composed of at least five members [24]. The karyopherins are soluble receptors for proteins carrying nuclear localization sequences (NLSs), and participate in the nuclear import machinery in co-operation with several other partners [25]. The interaction between karyopherin α2 and its successive partners is transitory and dependent upon the affinity for the substrates. Each karyopherin recognizes a set of proteins with an NLS domain either directly or indirectly through adapter molecules [26]. In the present study, we cloned the rat homologue of the mouse karyopherin α2 (or α-P1) isoform. The structure of karyopherin α is characterized by a central motif of 10 armadillo repeats, an amino acid motif of 42 amino acids originally described in the *Drosophila* segment polarity gene product [27]. We identified the central part of the karyopherin α2 as the functional domain interacting with GLUT2 (Figure 6). This domain is known to be involved in the binding of protein cargoes equipped for nuclear import [28]. The N-terminus of the protein is devoted to the binding of importin β, a protein of the nuclear import machinery [23]. This domain was not involved in binding to the GLUT2 loop and might thus be preserved to allow a nuclear transport capacity.

The GLUT2 loop sequence is involved in the binding to karyopherin α2, but does not constitute a functional NLS [29], since native GLUT2 has never been observed in the nucleus. Nevertheless, the GLUT2 loop contains over 20% of basic residues, a sufficient feature to target the free GFP–GLUT2 loop into the nucleus [11].

Several hypotheses might be drawn to explain the role of the interaction between GLUT2 and karyopherin α2. First, karyopherin α2 might target GLUT2 into the nucleus. This is quite unexpected since, as mentioned above, GLUT2 has never been observed in the nucleus. Second, karyopherin α2 might import domains of GLUT2 into the nucleus, followed by a ligand-mediated proteolytic cleavage of GLUT2 loop that would precede the stimulation of glucose-sensitive gene expression in the nucleus. A similar mechanism was observed for the factor Notch [30]. Third, karyopherin α2 might be tethered to GLUT2 at the plasma membrane to receive a local cargo to be imported into the nucleus. Whatever its cargo is, the amount of active receptor might also control the expression of glucose-sensitive genes.

To test the hypothesis that the receptor participates in glucose sensing, we expressed full-length and truncated karyopherin α2

in hepatoma cells. Increasing active karyopherin $\alpha 2$ in the cells did not interfere with the sensing pathway. This suggested that the amount of karyopherin $\alpha 2$ was not limiting this specific pathway, rather it might be limited by the amount of cargo. The truncated receptor was unable to reach the nucleus, due to deletion of the binding domain for the nuclear import machinery, while preserving the cargo binding domain. Since the truncated receptor indeed inhibited the expression of two glucose-sensitive genes, we suspected that it was sequestered in the cytoplasm as a complex with its cognate cargo and constituted a dominant-negative mutant. Competition with the endogenous receptor might have occurred in the cytoplasm, resulting in a loss of function. We conclude that a decrease in active karyopherin $\alpha 2$ negatively regulated the expression of GLUT2 and L-PK genes.

Several proteins are imported into the nucleus by karyopherin $\alpha 2$. Among them, a transcription activator, LEF-1, was identified in mammalian cells [21], but, so far, it appears not to be relevant in the glucose transcriptional effect. In *Arabidopsis*, pleiotropic regulatory locus 1 ('PRL1'), identified as a novel suppressor of glucose signalling, was cloned by its interaction with an isoform of karyopherin α present in plants [31]. By analogy, in mammalian cells, karyopherin $\alpha 2$ might participate in the glucose-sensing pathway through its role in the nuclear import of cargo proteins. This import mechanism might help to confer the specificity of glucose regulation in GLUT2-expressing cells, as opposed to tissues expressing other GLUT isoforms.

Nuclear import of proteins is a process that depends on sustained energy production. In digitonin-permeabilized cells, it requires ATP, GTP and a regenerating system [32]. In intact hepatocytes, an adequate ATP provider for this import process might be glucose metabolism acting in concert with the signalling pathway, suggesting a need for co-operation between the two processes.

In conclusion, in the present study, we have defined new roles for both karyopherin $\alpha 2$ and GLUT2 in the regulation of gene expression. We report that GLUT2 interacted with karyopherin $\alpha 2$, and that an appropriate amount of import receptor protein is required for the expression of two glucose-sensitive genes. Since the function of karyopherin $\alpha 2$ is to import cargo proteins into the nucleus, we propose that the karyopherin $\alpha 2$ can load cargoes at the plasma membrane. Then, the cargo directly or indirectly regulates the expression of glucose-sensitive genes, in concert with glucose metabolism. The identity of the protein(s) associated with karyopherin $\alpha 2$ and imported into the nucleus is currently under investigation in our laboratory.

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