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Glucose-induced dissociation of glucokinase from its regulatory protein in the nucleus of hepatocytes prior to nuclear export

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ABSTRACT

The glucose phosphorylating enzyme glucokinase regulates glucose metabolism in the liver. Glucokinase activity is modulated by a liver-specific competitive inhibitor, the glucokinase regulatory protein (GRP), which mediates sequestration of glucokinase to the nucleus at low glucose concentrations. However, the mechanism of glucokinase nuclear export is not fully understood. In this study we investigated the dynamics of glucose-dependent interaction and translocation of glucokinase and GRP in primary hepatocytes using fluorescence resonance energy transfer, selective photoconversion and fluorescence recovery after photobleaching. The formation of the glucokinase:GRP complex in the nucleus of primary hepatocytes at 5 mmol/l glucose was significantly reduced after a 2 h incubation at 20 mmol/l glucose. The GRP was predominantly localized in the nucleus, but a mobile fraction moved between the nucleus and the cytoplasm. The glucose concentration only marginally affected GRP shuttling. In contrast, the nuclear export rate of glucokinase was significantly higher at 20 than at 5 mmol/l glucose. Thus, glucose was proven to be the driving-force for nuclear export of glucokinase in hepatocytes. Using the FLII12Pglu-700μ-δ6 glucose nanosensor it could be shown that in hepatocytes the kinetics of nuclear glucose influx, metabolism or efflux were significantly faster compared to insulin-secreting cells. The rapid equilibration kinetics of glucose flux into the nucleus facilitates dissociation of the glucokinase:GRP complex and also nuclear glucose metabolism by free glucokinase enzyme. In conclusion, we could show that a rise of glucose in the nucleus of hepatocytes releases active glucokinase from the glucokinase:GRP complex and promotes the subsequent nuclear export of glucokinase.

1. Introduction

Glucokinase regulates glycolytic flux at physiological millimolar glucose concentrations in the liver and pancreatic beta cells [1–4]. On the posttranslational level glucokinase activity is modulated by conformational changes of the enzyme and interaction with regulatory proteins [1–4]. Hepatocytes specifically express the glucokinase regulatory protein (GRP), which plays an important role as a competitive inhibitor of glucokinase [5–9].

The glucokinase:GRP interaction depends upon the conformation of the glucokinase protein, which in turn is crucially determined by the glucose concentration at the substrate binding site. The GRP binds glucokinase in the absence of glucose, when the enzyme resides in the super-opened conformation with low intrinsic activity [1,5,8,10–15]. Glucose causes a concentration-dependent multiple step transition of glucokinase to a closed conformation, in which the enzyme is active and not accessible for interaction with the GRP [10,14,16]. Several amino acids have been suggested to be crucial for interaction with GRP [11,15,17], but still required confirmation by co-crystallization of the glucokinase:GRP complex. Most of the postulated amino acids are located in three regions of the glucokinase protein. Recently, the crystal structure of the *Xenopus laevis* glucokinase:GRP complex has been generated successfully and revealed two binding domains [15]. One of these comprises Leu58, which had been postulated from a systematic random peptide phage display library screening to mediate GRP binding [11]. Naturally occurring activating and inactivating glucokinase mutations also displayed a reduced susceptibility to competitive inhibition by the GRP [13,18]. The formation of the glucokinase:GRP complex is stimulated by fructose-6-phosphate and suppressed by fructose-1-phosphate [1,6,8,19–21]. These fructose phosphates interact with the same binding site in the GRP [21,22]. However, species differences exist in the regulatory properties of the GRP [15,23–25].

Binding to the GRP leads to translocation of glucokinase to the nucleus in hepatocytes. In pancreatic beta cells, which do not express GRP,

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EYFP, enhanced yellow fluorescent protein; ECFP, enhanced cyan fluorescent protein; FRET, fluorescence resonance energy transfer; FRAP, fluorescence recovery after photobleaching; GRP, glucokinase regulatory protein; N/C-ratio, nuclear to cytoplasmic ratio

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glucokinase is predominantly localized in the extranuclear compartments [1,26]. Thus, nuclear compartmentation of glucokinase is a regulatory mechanism exclusive to the liver [1,26]. In response to an increase in the glucose concentration, glucokinase shuttles from the nucleus to the cytoplasm [5,11,27–30]. Likewise, small molecule glucokinase activators evoked an increase in cytoplasmic glucokinase in hepatocytes [12,30,31]. The mechanism of this glucokinase nuclear export, however, is not precisely understood with respect to the dynamics of glucokinase:GRP complex dissociation and glucokinase shuttling [1,8,19,26,28,29,32–36].

The molecular weight of both glucokinase and GRP is clearly above the reported barrier limit of the nuclear pore complexes. Thus, both proteins likely require nuclear import and export mechanisms [37]. In the present study we characterized different translocation characteristics of glucokinase and the GRP in primary hepatocytes using real-time fluorescence based techniques. The nuclear targeted glucose sensor FLII^{12}Pglu-700μ-δ6 (FLIPglu) [38,39] revealed rapid nuclear equilibration kinetics of glucose in hepatocytes. This rapid glucose equilibration appears to be the driving force for the glucokinase nuclear export, whereas the GRP serves as a nuclear retaining factor for glucokinase.

2. Materials and methods

2.1. Cell culture

MIN6 and COS cells were grown in DMEM supplemented with 25 mmol/l glucose, 10% (vol/vol) FCS, penicillin, and streptomycin in a humidified atmosphere at 37 °C and 5% CO_2. RINm5F, RINm5F-rGLUT2 [40] and INS1E cells were grown in RPMI 1640 supplemented with 10 mM glucose, 10% (vol/vol) FCS, penicillin, and streptomycin and for INS1E cells additionally with 50 μM/l 2-mercaptoethanol in a humidified atmosphere at 37 °C and 5% CO_2. Cells were transfected with the vector DNA using jetPEI (Qbiogene, Montreal, Canada). Isolation and culture of primary mouse and rat hepatocytes were performed as described [11,24]. Hepatocytes were either transfected as described [11,24] or transduced with adenovirus (FLIPglu or FLIPglu-NUC) at a MOI of 10.

2.2. Generation of plasmids and adenoviruses

FLII^{12}Pglu-700μ-δ6 (FLIPglu) cDNA (Addgene plasmid 17866) was subcloned into the pcDNA 3.1 vector [39]. Recombinant FLIPglu adenoviruses were generated as described previously [38]. A nuclear targeted version of FLIPglu was created by subcloning the coding cDNA as a SalI-NotI fragment into the pCMV/myc/NUC vector (Invitrogen, Carlsbad, CA, USA). Recombinant nuclear targeted FLIPglu adenoviruses were generated by subcloning the coding cDNA from the pCMV/myc/NUC as a SalI-XbaI fragment into the pShuttle-CMV vector. Generation of enhanced cyan fluorescent protein (ECFP)-GK, enhanced yellow fluorescent protein (EYFP)-GK and EYFP-GRP was described previously [11]. The EYFP in both vectors was replaced by Dendra2 using AgeI and BsrGI restriction sites. pTagRFP-GRP was generated by subcloning the coding cDNA of GRP as a BglII-EcoRI fragment into the pTagRFP-N vector.

2.3. Glucokinase enzyme activity and western blot analyses

MIN6 cells and hepatocytes were cultured as indicated. The nuclear fraction was isolated by a stepwise fractionation. Briefly, cells were lysed in 0.5 ml buffer containing 20 mmol/l HEPES (pH 7.9), 10 mmol/l KCl, 0.2% NP40, 10% glycerol, 1 mmol/l EDTA, 1 mmol/l DTT, 1 μg/ml pepstatin, 1 mmol/l PMSF, and protease inhibitors for 10 min on ice. The cytoplasmic fraction was collected as supernatant after centrifugation at 13,000 rpm for 30 s. The pellet was re-suspended in 100 μl buffer containing 20 mmol/l HEPES, 10 mmol/l KCl, 350 mmol/l NaCl, 20% glycerol, 1 mmol/l EDTA, 1 mmol/l DTT, 1 μg/ml pepstatin, 1 mM PMSF, 1 mmol/l vanadate and protease inhibitors for 20 min on ice. The nuclear fraction was collected as supernatant after centrifugation at 13,000 rpm for 30 s. The protein concentration was quantified by the Bradford protein assay. Glucokinase enzyme activity was measured by an enzyme-coupled photometric assay as described [11]. A total of 40 μg protein was fractionated by reducing 10% SDS-PAGE and electroblotted to polyvinylidine difluoride membranes. Western blotting was performed as described [38] with antibodies against glucokinase (sc-7908, diluted 1:500, or sc-1980, diluted 1:500 Santa Cruz Biotechnology, Santa Cruz, CA, USA).

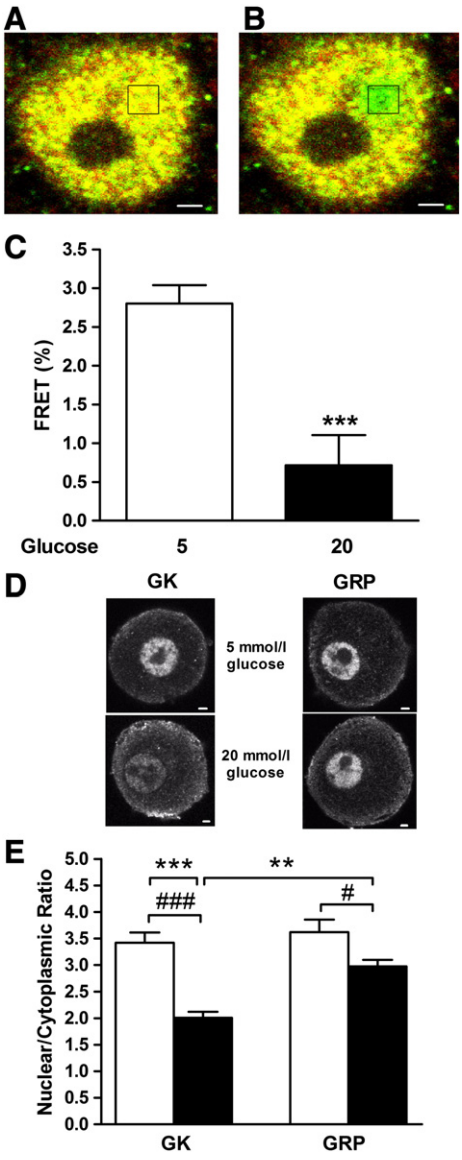

Fig. 1. Direct interaction between glucokinase and GRP and localization of both proteins in rat hepatocytes. Primary rat hepatocytes grown on glass coverslips were incubated for 2 h with 5 mmol/l glucose (white bars) or 20 mmol/l glucose (black bars). Finally, the cells were fixed and immunostained for glucokinase and GRP. The appropriate secondary antibody was labeled with Cy3 for glucokinase and Cy5 for GRP. FRET was measured in the nucleus as an increase in Cy3 fluorescence after Cy5 photobleaching. Exemplarily shown are merged images before (A) and after (B) photobleaching. Scale bar 2 μm. (C) Data are expressed as means ± SEM from three individual hepatocyte preparations with 30 cells in total. $^{***}p < 0.001$ (Student's *t* test). (D) Representative images for glucokinase and GRP localization at 5 mmol/l glucose and 20 mmol/l glucose are shown. Scale bar 2 μm. (E) The nuclear/cytoplasmic ratio was calculated for glucokinase and GRP. Data are expressed as means ± SEM from three individual hepatocyte preparations with 12 cells in total. $^{**}p < 0.01$; $^{***}p < 0.001$ (ANOVA/Bonferroni's test); $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$ compared to 5 mmol/l glucose (Student's *t* test).