Glucokinase Regulatory Network in Pancreatic β-Cells and Liver

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The low-affinity glucose-phosphorylating enzyme glucokinase (GK) is the flux-limiting glucose sensor in liver and β-cells of the pancreas. Furthermore, GK is also expressed in various neuroendocrine cell types. This review describes the complex network of GK regulation, which shows fundamental differences in liver and pancreatic β-cells. Tissue-specific GK promoters determine a higher gene expression level and glucose phosphorylation capacity in liver than in pancreatic β-cells. The second hallmark of tissue-specific GK regulation is based on posttranslational mechanisms in which the high-affinity regulatory protein in the liver undergoes glucose- and fructose-dependent shuttling between cytoplasm and nucleus. In β-cells, GK resides outside the nucleus but has been reported to interact with insulin secretory granules. The unbound diffusible GK fraction likely determines the glucose sensor activity of insulin-producing cells. The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) has been identified as an activating binding partner of β-cell GK, increasing the V_{max} value of the enzyme, while the $S_{0.5}$ value for glucose remains unchanged. This effect is likely due to stabilization of a catalytically active enzyme conformation. The identification of chemical activators of GK paved the way to determining its crystal structure, revealing a catalytically less active super open conformation and a catalytically active closed conformation with a normal affinity for glucose. The glucose sensor function of GK in liver and β-cells results from the synergy of its regulatory properties with its transcriptionally and posttranslationally controlled levels. These factors have to be taken into account in designing pharmacotherapy for type 2 diabetes. *Diabetes* 55 (Suppl. 2):S55–S64, 2006

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EC_{50}, half-effective concentration; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; GK, glucokinase; GRP, glucokinase regulatory protein; PFK-2/FBPase-2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; $S_{0.5}$, substrate concentration for half-maximal activity.

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GLUCOKINASE AND ITS EXCEPTIONAL ROLE IN THE HEXOKINASE GENE FAMILY

The glucose-phosphorylating enzyme glucokinase (GK) (hexokinase type IV) has unique characteristics compared with the ubiquitously expressed hexokinase isoforms type I–III. The smaller 50-kDa size of the GK protein distinguishes it from the 100-kDa hexokinase isoforms (1). From a historical point of view, several kinetic preferences allowed this enzyme to act as a metabolic glucose sensor: *1*) its low affinity for glucose, in the physiological concentration range between 5 and 7 mmol/l, *2*) a cooperative behavior for glucose with a Hill coefficient (n_{Hill}) between 1.5 and 1.7, and *3*) a lack of feedback inhibition by glucose-6-phosphate within the physiological concentration range (2–4). A further characteristic of the GK enzyme is its exceptional sensitivity to sulfhydryl group oxidizing compounds, although the pattern of cysteine residues does not differ between GK and high-affinity hexokinase isoforms (5).

GK activity could be predominantly demonstrated in hepatocytes and pancreatic β-cells, both cell types that have to couple physiological glucose concentrations to metabolism. Pioneered by the work of Matschinsky's group (2–4), GK proved to be the flux-limiting glucose sensor in pancreatic β-cells within the scenario of metabolic stimulus-secretion coupling.

Sequence comparisons revealed a high homology between the 50-kDa mammalian GK and the 100-kDa mammalian hexokinases, which apparently developed by gene duplication of a so far unknown common ancestral 50-kDa hexokinase gene (1,6). Notably, the homology of GK to the 50-kDa yeast hexokinase is much lower, in the range of 30% (6). This evolution of hexokinase isoenzymes was of particular interest to solve the structure of GK. While crystallization of yeast hexokinase (Fig. 1*A*) and the 100-kDa hexokinases resulted in three-dimensional structures of enzymes in which two lobes form a substrate-binding cleft, GK crystallization failed for unknown reasons. A systematic replacement of residues in yeast hexokinase and mammalian hexokinase type I generated a model of GK that has served as a valuable tool to characterize the substrate-binding site and identify the locations of mutations leading to maturity-onset diabetes in the young (MODY)-2 (7,8). However, this model could not explain the cooperative behavior of the monomeric GK enzyme. Different models proposed a glucose-dependent conformational change of the GK protein either by "slow transition" through substrate binding or a "mnemonical" mechanism remembering a catalytically favorable state (9,10). Thus the coexistence of different GK conformations mainly prevented the formation of crystals suitable for high-resolution X-ray crystallography.

The identification of small molecule GK activators greatly powered the resolution of GK structure as it clamped the enzyme conformation through binding at an

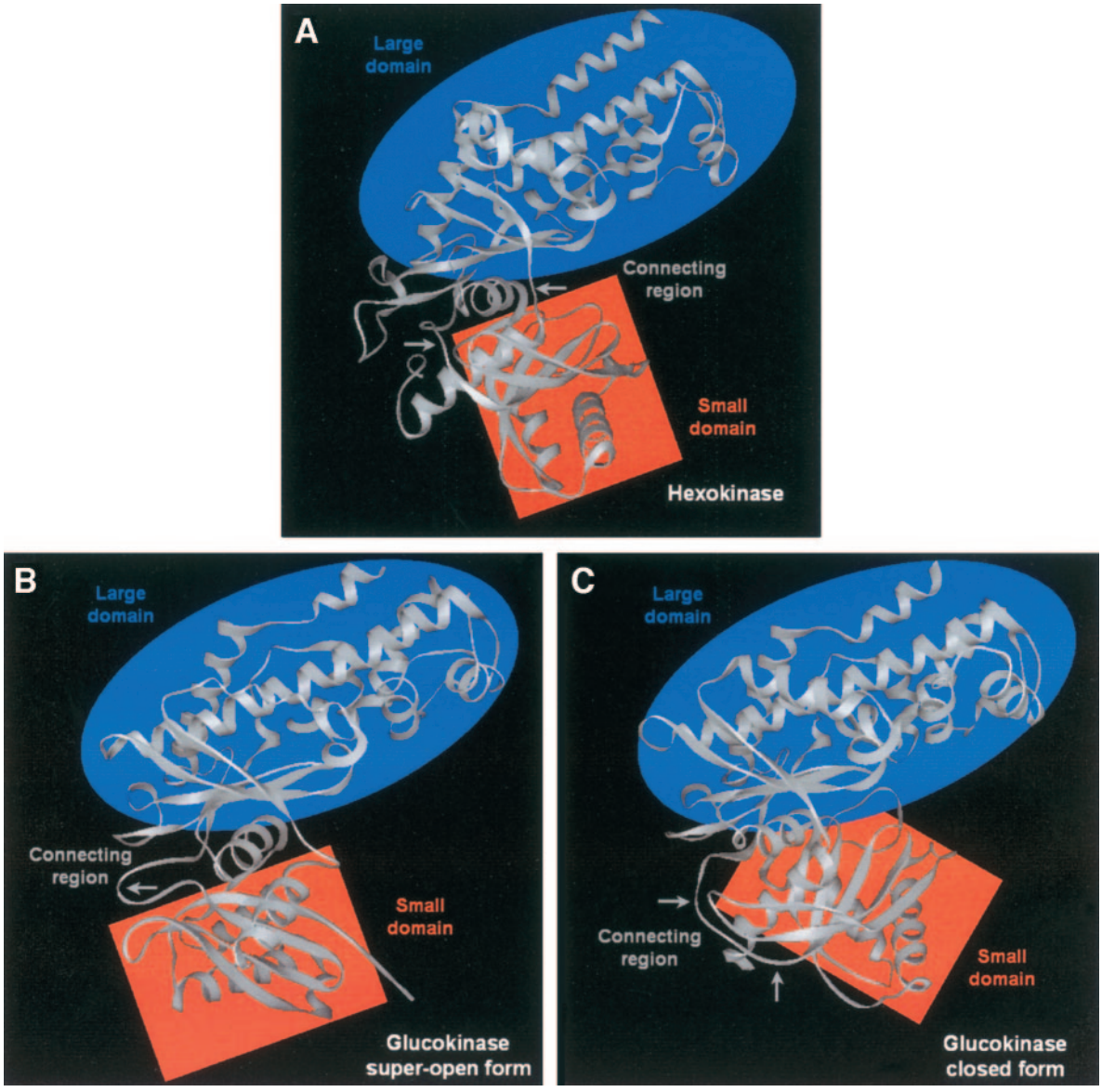

FIG. 1. Structures of GK and hexokinase. Shown are yeast hexokinase P II (*A*) and the "super-open" (*B*) and "closed" (*C*) conformation of human GK. In the models, the large domain is highlighted in blue and the small domain in red, and the connecting region is marked by gray arrows.

allosteric site in the hinge region between the large and small domains of the protein (11). Currently, Kamata et al. (12) could provide the proof for a so-called "super open" conformation of GK (Fig. 1*B*), which corresponds to the inactive form of the enzyme. In the active "closed conformation" (Fig. 1*C*), the glucose binding site is composed by residues of the large domain (Glu 256 and Glu 290), the small domain (Thr 168 and Lys 169), and the interconnecting region (Asn 204 and Asp 205) (12). In comparison to the structure of hexokinase I, only a few residues in connecting region I differed in GK, conferring greater flexibility (12,13). Crystallization of the "super open" conformation revealed a completely different spatial arrangement of the domains in comparison to the "open" conformation of hexokinase type I (12). The spatial arrangement of the amino acid residues in the cleft makes it difficult to bind glucose, thereby providing evidence that the "super open" form is catalytically inactive. Notably, the crystallization of the GK enzyme was possible in the absence of a chemical GK activator through deletion of the NH_2-terminal 15–amino acid residues. These results indicate that the GK enzyme undergoes a greater range of conformational changes than the 100-kDa hexokinase isoforms. Thus, positive cooperativity is explained by three different conformational changes: *1*) the thermodynamically favorable "super open" form in the absence of glucose, *2*) the slow transition from the "super open" form to the "closed" form after binding of glucose, and *3*) a fast "closed-open" transition after glucose phosphorylation. The fast "closed-open" transition is favored by high glucose concentration as a mechanism to maintain the catalytically more active "open" conformation (Fig. 2). This stabilization by glucose may also be achieved through binding of the competitive GK inhibitor mannoheptulose (14).

Chemical activators of GK actually prevent the slow cycle transition from the "open" form of the enzyme to the "super open" form in the presence of glucose (12). GK activators binding to the allosteric site of the connecting region between the two protein domains induce a decrease of the $S_{0.5}$ values for glucose in a concentration-dependent manner and a decrease of n_{Hill}, whereas the

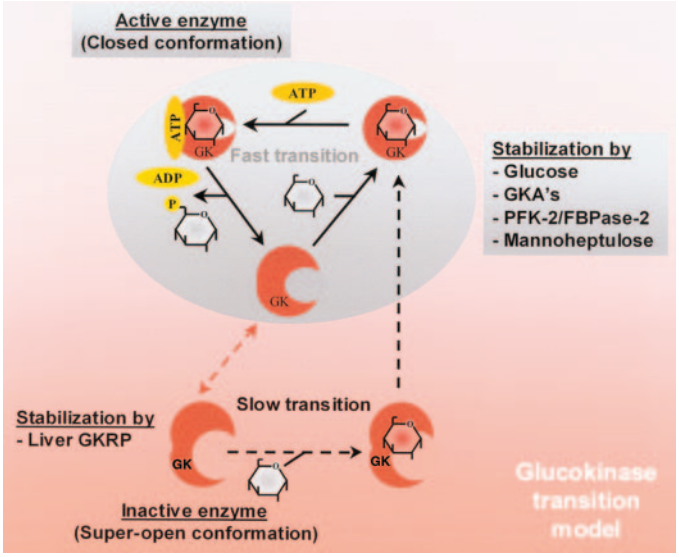

FIG. 2. Model of transition between the "closed" and "super-open" form of GK. Active GK in its "closed" conformation is demonstrated by a nearly filled red circle and the super-open, inactive conformation by a red open circle. After binding of glucose and ATP, GK phosphorylates glucose to glucose-6-phosphate and ADP and switches back from the "closed" conformation to an intermediate "open" conformation after release of the metabolites. Dependent on the present cellular metabolic conditions, GK remains in the circle of fast transition or switches into the "super-open" form with slow catalysis.

V_{max} value was only increased by a few compounds (11,12,15,16).

GK STABILITY AND SULFHYDRYL SENSITIVITY

It is essential for the glucose sensor function of GK that the enzyme preserves its catalytic properties under conditions of cellular stress in particular, since changes in the metabolic redox state favor the generation of oxygen free radicals. Pancreatic β-cells differ from other cell types such as the liver in that they show a low enzymatic anti-oxidative defense status (17).

In comparison to high-affinity hexokinases, GK always proved to be a sensitive enzyme prone to loss of catalytic function after cell disruption and preparation of subcellular fractions. Two strategies proved to be effective to preserve the GK protein function: the substrate glucose itself and dithiol reagents such as dithiothreitol (5,18–20). This supports the assumption that the extraordinary structural flexibility brings cysteine residues in close vicinity for the oxidative formation of disulfide bridges (5,21). That the most significant conformational change of the GK protein occurs during the transition between the "super open" conformation and the "open" conformation likely explains the high susceptibility to sulfhydryl oxidation in the absence of glucose or at low glucose concentrations (5,21,22).

Interestingly, the pattern of cysteine residues is highly conserved between mammalian GK and the low-affinity 100-kDa hexokinases forming a ring structure around the catalytic cleft (5,21). The sulfhydryl group oxidizing pyrimidine derivative alloxan shows a structural similarity to glucose and inhibits the GK enzyme with half-effective concentration (EC_{50}) values in the range between 2 and 5 μmol/l, whereas the sensitivity of the HK isoenzymes is at least two magnitudes lower (5,23). This indicates a significantly higher affinity of alloxan to the substrate-binding site of GK compared with glucose. On the other hand, glucose efficiently protected GK against inhibition by alloxan. Of note, only 60% of GK activity could be maximally protected against alloxan, indicating that a portion of the GK protein exists in a conformation with a high affinity for alloxan but a low affinity for glucose (5). From the different conformations of GK, it is likely that alloxan preferentially binds the enzyme in its "super open" conformation where the affinity of glucose is low from the spatial arrangement of the side groups forming the substrate binding pocket (12) in agreement with the observation that liver GK protein undergoes an activity decay in the absence of reducing agents (22).

In conclusion, GK pays a high price for its cooperative kinetics based on the high conformational flexibility, namely the sensitivity of the sulfhydryl groups, which may under some conditions hamper the enzyme function. Thus, GK requires posttranslational regulatory mechanisms, in particular, the interaction with regulatory proteins conferring stabilization of distinct GK conformations preserving the glucose sensor function.

GK REGULATION IN LIVER: THE PREDOMINANCE OF THE REGULATORY PROTEIN

In the mammalian GK gene, transcription is driven by tissue-specific promoters in liver and pancreatic β-cells (14,24,25). There is experimental evidence that the β-cell promoter is also active in GK-expressing cells of the gut, hypothalamus, and anterior pituitary gland (14,26). The liver-specific promoter is mainly affected by insulin and glucagon, which explains the extraordinary transcriptional regulation by the nutritional state (25). It could be demonstrated that in conditions of carbohydrate availability, the SREBP-1c pathway is involved in upregulation of GK mRNA expression (27,28). Overall, the activity level of the GK promoter results in GK protein levels and concomitantly high glucose phosphorylation activities, which are 10- to 20-fold higher than in pancreatic β-cells (29). GK protein levels are also regulated by the nutritional status, however, to a much lower extent than on the level of GK mRNA transcription (30). Thus, on the cellular level, the liver GK represents a high-capacity glucose-phosphorylating system in comparison to the low capacity system in β-cells and neuroendocrine cells (14). The GK pool in liver likely comprises a functional reserve operative during short-term regulation of liver metabolism, as during feeding.

The discovery of a specific glucokinase regulatory protein (GRP) by Van Schaftingen et al. (31) more than 15 years ago was a landmark in our understanding of GK regulation by fructose phosphates and glucose in the liver. The 62-kDa protein acts as a competitive inhibitor of GK and is therefore the key example of a GK-binding protein with a regulatory function (Fig. 3*A*). The GRP has binding sites for fructose phosphates, thereby lowering (fructose-1-phosphate) or reinforcing (fructose-6-phosphate) its interaction with GK (31). On the other hand, glucose also releases GK from the GK-GRP complex through conformational changes of the enzyme protein (32,33).

GRP is not merely an inactivating binding partner of GK but also provides a regulated translocation of the GK protein between the cytoplasm and the nucleus (34–37). At low glucose concentrations or in the presence of fructose-6-phosphate, GK is sequestered in the nucleus of hepatocytes, while high glucose concentrations or fructose-1-phosphate provoke a rapid release of GK from GRP and a translocation to the cytoplasm (Fig. 3*A*). It should be

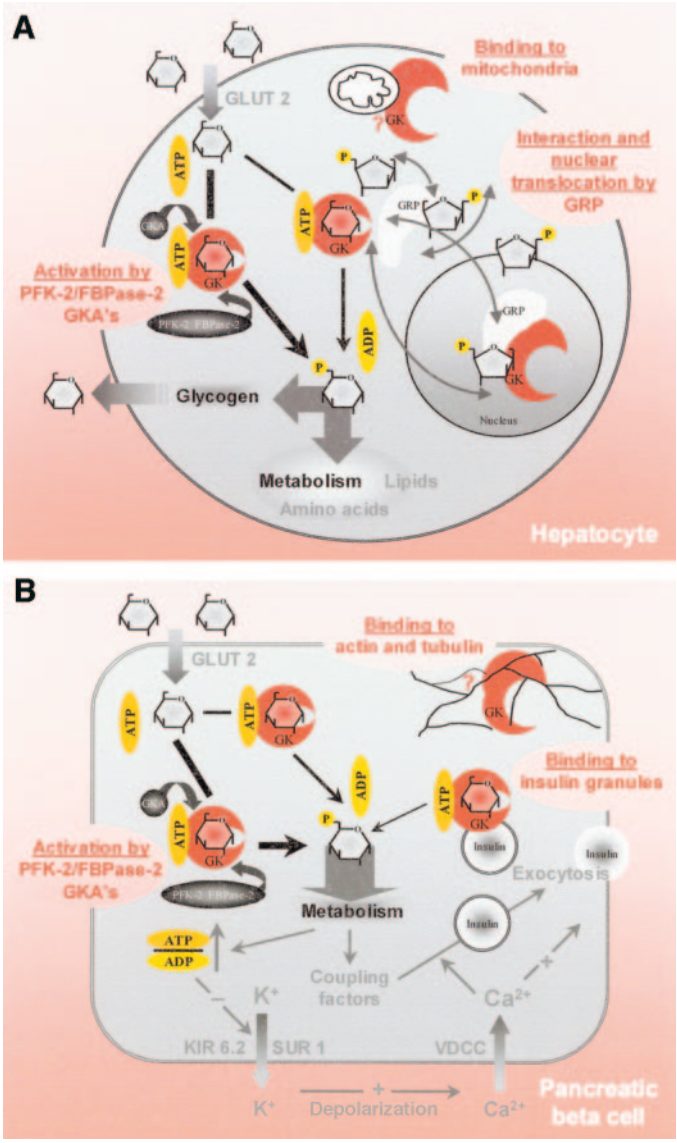

FIG. 3. Model of posttranslational GK regulation in liver (*A*) and pancreatic β-cells (*B*). *A*: GK enzyme activity is mainly regulated by the liver-specific GRP on the posttranslational level. Inhibition of GK through GRP is suppressed by fructose-1-phosphate and stimulated by fructose-6-phosphate. GKAs and the bifunctional enzyme PFK-2/FBPase-2 activate GK enzyme activity. *B*: Metabolic stimulus-secretion coupling in pancreatic β-cells crucially depends on the GK enzyme activity status. Activation of GK through PFK-2/FBPase-2 and GKAs increases oxidative glucose metabolism.

emphasized at this point that GRP is expressed in hepatocytes at an approximately two- to threefold excess in comparison to the GK protein. The translocation of GK by the GRP could be also demonstrated in COS-1 cells, which express neither GK nor GRP. The fluorescent protein itself was uniformly distributed in the cells as shown for enhanced yellow fluorescent protein (EYFP) (Fig. 4*A*). In contrast, the EYFP-GK fusion construct was exclusively located in the cytoplasm (Fig. 4*B*) comparable to the GK localization in pancreatic β-cells and irrespective of the glucose concentration. Transfection of EYFP-GRP alone in COS-1 cells resulted in a predominantly nuclear, but also cytoplasmic, distribution (Fig. 4*C*). Cotransfection of both GK as enhanced cyan fluorescent protein (ECFP) fusion construct (Fig. 4*D*) and GRP as EYFP fusion construct (Fig. 4*E*) led to a translocation of GK to the nucleus at low glucose concentrations.

Thus, there is general consensus that translocation of GK to the nucleus at low glucose concentrations requires GRP as an anchor protein conferring transport to the nuclear pore. On the other hand, it is not clear whether the translocation of GK from the nucleus to the cytoplasm requires GRP. The GK protein itself contains a motif that could serve as a nuclear export signal, but GK export seems to be insensitive to leptomycin B, an inhibitor of the classic nuclear export machinery (37).

Overexpression of GK or GRP demonstrated that both proteins affect the flux control coefficients on glycolysis and glycogen synthesis, supporting the hypothesis that the interaction of both proteins is an important regulatory element of hepatic glucose metabolism (38). Notably, the nucleus cannot be regarded as an organelle compartment that separates GK from cellular changes of key metabolites such as glucose and fructose phosphates because the nuclear pore is clearly permissive for compounds of small molecular size, and the GRP is able to pass this barrier in both directions (39). Furthermore, it must be taken into account that GK may interact in the cytoplasm with other important binding partners (Fig. 3*A*). We recently demonstrated that the bifunctional regulatory enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) binds to and activates GK (40–42). The interaction with PFK-2/FBPase-2 potentially stabilizes the GK enzyme protein in the cytoplasm as a counterpart to the GRP, thereby preserving its glucose sensor function. It was also demonstrated that PFK-2/FBPase-2 increases GK protein expression by posttranscriptional mechanisms. Recent studies indicate that GK integrated in a multienzyme complex including the pro-apoptotic protein BAD and also together with GRP associates with mitochondria in hepatocytes (43,44). Interestingly, it was shown that PFK-2/FBPase-2 did not bind to mitochondria, which substantiates its stabilizing role only for the freely diffusible cytoplasmic GK fraction (43).

A homozygous knockout of GRP resulted in reduced GK protein expression in hepatocytes, although transcriptional regulation of GK was not affected in these mice (45,46). This indicates that GRP apparently plays an important role for the stabilization of the enzyme protein and it also explains the observed glucose intolerance after glucose refeeding, since a functional GRP-bound GK pool was missing in the nucleus in starved animals (45).

Molecular mechanisms of GK-GRP interaction: binding motifs of GRP. Because the interaction of GK with GRP is of crucial importance for GK compartmentalization in hepatocytes and the stability of the GK enzyme, our group followed a systematic peptide phage display approach to identify binding epitopes conferring interaction of both proteins (40,47). Through phage display screening, the -LSA-XX-VAG- consensus motif could be identified as a strong binding partner of GK (40). This motif showed a homology to the human GRP motif 185-SVGL SAPVAGQMD-197, providing evidence that this epitope of GRP is of crucial importance for interaction with GK (40). Van Schaftingen and coworkers (48) could demonstrate by mutational analyses that the S179 residue of the GRP in vicinity to this consensus motif affects the affinity to GK. However, GRP mutants distant from the consensus motif also significantly decreased the affinity to GK, as shown for the K514A mutant (48,49).

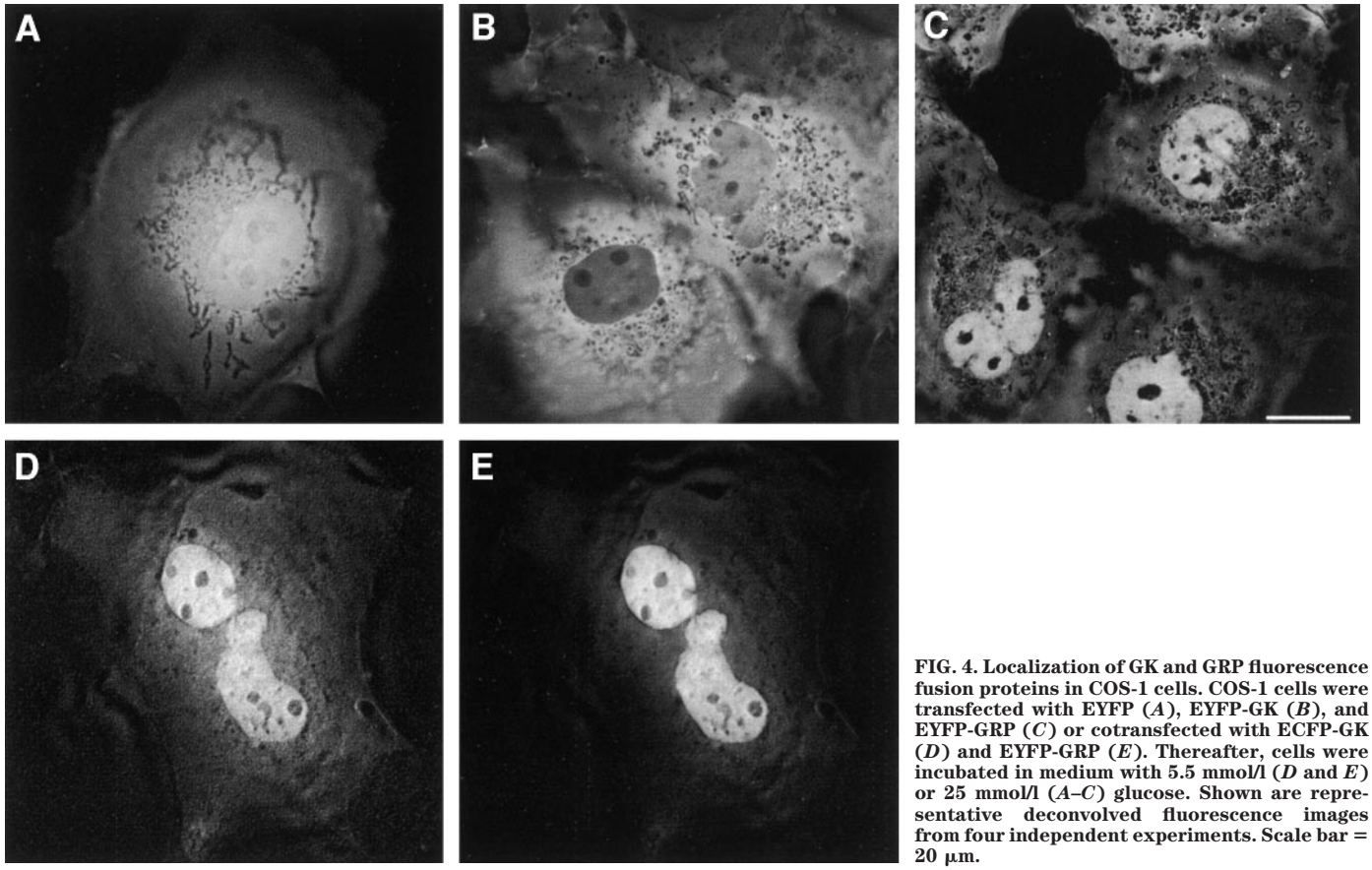

FIG. 4. Localization of GK and GRP fluorescence fusion proteins in COS-1 cells. COS-1 cells were transfected with EYFP (*A*), EYFP-GK (*B*), and EYFP-GRP (*C*) or cotransfected with ECFP-GK (*D*) and EYFP-GRP (*E*). Thereafter, cells were incubated in medium with 5.5 mmol/l (*D* and *E*) or 25 mmol/l (*A–C*) glucose. Shown are representative deconvolved fluorescence images from four independent experiments. Scale bar = 20 μm.

Molecular mechanisms of GK-GRP interaction: binding motifs of GK. In contrast to the GRP protein, the crystal structure of GK allows a distinct spatial localization of binding motifs. Mutational analyses indicate that the interaction of GK and GRP comprises different spatial binding motifs of the GK protein covering the large and small domain of the enzyme and not for a unique specific binding epitope (49,50).

Using the peptide phage display library screening, we found an enrichment of peptides, which all shared asparagine-leucine motifs (47). Analysis of the GK protein sequence localized the four asparagine-leucine motifs N83/L88, N283/L288, N350/L355, and L386/N391 as a potential binding anchor for GRP. A closer inspection of the three-dimensional structure of GK also revealed a spatial vicinity of the amino acids asparagine and leucine for L164-L165-N166 and N179-N180/L184-L185 (47). Mutation studies already showed that the N166 and N180 residues participate in the interaction with GRP (49,50). Also, asparagine N313 proved to be of potential interest for binding to GRP, because this residue is spatially surrounded by leucine residues, and L309 has been postulated as a nuclear export signal of GK (37). Another asparagine-leucine motif could be identified on the surface of the hinge region comprising the L58 and N204 residues. N204 is not only part of the substrate binding site of GK, but also proved to be important for the interaction with GRP (7,12,49,51,52). These data provide evidence that the GK protein interacts with GRP at multiple sites in which the common asparagine-leucine motif confers the binding to GRP.

Systematic mutation analyses of GK and the use of fluorescent fusion proteins verified the physiological relevance of the asparagine-leucine motifs for interaction with GRP (47). The N204Y, L58R, and L58R/N204Y GK mutants showed a significant decrease of GRP binding affinity and an exclusively cytoplasmic localization. Notably, a spatial vicinity of N204 and L58 in the hinge region exists only in the "super open" form of GK (12), which supports the assumption that interaction with GRP is favored at this particular conformation of the enzyme.

Also the L309R/N313Y GK mutant showed a lower nuclear/cytoplasmic GK ratio in hepatocytes than wild-type GK. To our surprise, the N313Y mutation of GK revealed a higher binding affinity to GRP than the wild-type protein and the L309R GK mutant showed a loss of GRP binding in yeast two-hybrid studies (47). The L309 residue is part of a postulated nuclear export signal of GK as demonstrated by studies upon mutant GK-GFP fusion proteins in HeLa cells (37). The relevance of this postulated GK nuclear export signal is not fully clarified, since nuclear export of GK could not be inhibited by leptomycin B (53). Thus, the L309R GK mutant indicated a block of nuclear import by the GRP but cannot provide additional evidence for a functional nuclear export motif within the GK protein.

Mutation of N350Y and L355R, either alone or in combination, significantly increased the affinity to GRP in comparison to the wild-type GK with a synergistic effect of the double mutation. In the three-dimensional structure of GK, the N350/L355 motif is accessible at the surface in the "closed" as well as in the "super-open" conformation of GK. Both residues are located in the α10 helix of GK, a structure that is specific for this enzyme and shares a low homology with the other isoforms of the hexokinase family (7,54).

The conformation of the α10 helix of GK deserves

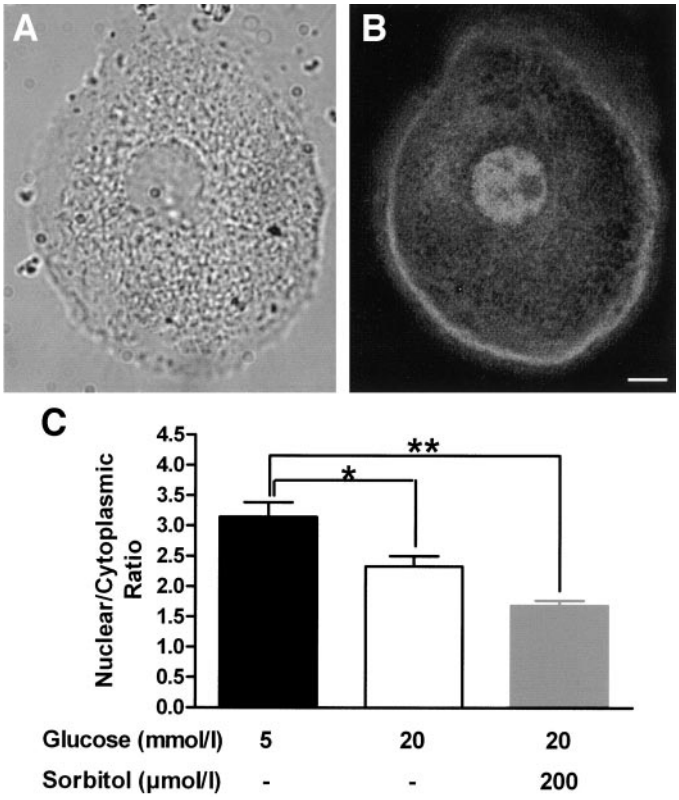

FIG. 5. Subcellular distribution of N350Y/L355R mutant EYFP-GK in primary rat hepatocytes. Rat hepatocytes were transfected with N350Y/L355R mutant EYFP-GK. Transmitted light image (*A*) and deconvolved fluorescence image (*B*) for a representative hepatocyte incubated for 3 h at 20 mmol/l glucose plus 200 μmol/l sorbitol are shown. Scale bar = 20 μm. The nuclear/cytoplasmatic fluorescence intensity ratio (*C*) was calculated from five fluorescence images each of cells incubated at 5 mmol/l glucose (■), 20 mmol/l glucose (□), or 20 mmol/l glucose plus 200 μmol/l sorbitol (▨) for 3 h. Shown are means ± SE from three independent experiments. **P* < 0.05, ***P* < 0.01 compared with 5 mmol/l glucose.

specific attention for the interaction with GRP. While in the closed form, it has a conformation similar to yeast hexokinase II, and the helical structure becomes clearly exposed in the super-open conformation of GK (12). Thus, it is likely that the GK α10 helix may facilitate GRP recognition and may modulate the binding strength of the GK-GRP complex through stabilization. Notably, the N350Y/L355R GK mutant showed a significantly higher nuclear/cytoplasmic GK ratio in primary hepatocytes than wild-type GK, irrespective of the glucose concentration (47). Importantly, in cells expressing the N350Y/L355R GK mutant, the glucose-dependent decrease in the nuclear/cytoplasmic GK ratio as a key characteristic of the wild-type protein was preserved (Fig. 5*C*). Incubation of N350Y/L355R GK mutant transfected hepatocytes with 20 mmol/l glucose plus 200 μmol/l sorbitol (Fig. 5*A* and *B*) resulted in a significantly lower nuclear/cytoplasmic GK ratio compared with incubation at 5 mmol/l glucose (Fig. 5*C*). These data are also in agreement with the reported effect of glucose plus sorbitol on GK wild-type protein (53,55). In conclusion, the L58/N204 and the L355/N350 leucine-asparagine motifs could be clearly identified as functional elements of the GK protein, which confer interaction with the GRP and the nuclear shuttling of GK (47).

Because the interaction between GK and GRP depends on the conformation of GK and is apparently favored in the "super open" conformation, this raises the question whether small molecule GK activators affect this process. GK activators stabilize the enzyme conformation in the "open" form, thereby decreasing the $S_{0.5}$ for the substrate glucose (11,12,16). However, GK activators differ in their potency to block or reverse the interaction between GK and GRP. Whereas the affinity of GK for GRP was not affected by GKA1 (15), RO-28-1675 was able to reverse the inhibitory action of GRP (11). In perspective, GK activators offer the opportunity to design molecules that modulate GK-GRP binding affinity in a highly selective manner. Because GRP does not play a functional role for GK regulation in pancreatic β-cells, these compounds would preferentially activate liver GK and may reverse the imbalance between glycolysis, glycogen synthesis, and hepatic glucose production in type 2 diabetes (56,57).

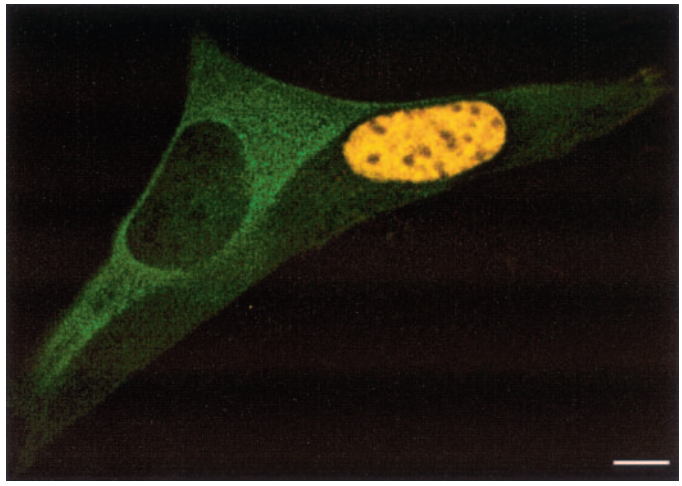

FIG. 6. Localization of EYFP-GK and EYFP-GRP in MIN6 cells. MIN6 cells were transfected with ECFP-GK and EYFP-GRP. Fluorescence images were taken after a 3-h incubation period at 3 mmol/l glucose. The deconvolved fluorescence image shows an only ECFP-GK–transfected cell (*left*) together with an ECFP-GK/EYFP-GRP–cotransfected cell (*right*). In the merged image, EYFP is depicted in red and ECFP in green. Scale bar = 5 μm.

GLUCOKINASE REGULATION IN PANCREATIC β-CELLS

In comparison to the liver, pancreatic β-cells show three fundamental differences, which determine the regulatory principles in this cell type.

First, the β-cell–specific promoter does not show a dramatic nutritional regulation by insulin and glucagon (25,29), and GK protein levels were only marginally increased by the nutritional state (29,57). From GK mutations in maturity-onset diabetes of the young, it is well known that the functional reserve of GK is low in β-cells tolerating only a decrease of ~50% of enzyme activity (2,58,59). GK expression is indispensable for stimulus-secretion coupling in β-cells, as impressively demonstrated by lethal hyperglycemia in homozygous GK knockout mice (60,61).

Second, the GRP of liver does not play a role for GK compartmentalization in β-cells. There is so far no clear experimental evidence that GRP is expressed in insulin-producing cells, either on the level of mRNA or on the level of protein. In insulin-producing MIN6 cells, a transfected ECFP-GK fusion construct is exclusively localized in the cytoplasm (Fig. 6, *left cell*) unless an EYFP-GRP fusion construct is expressed as well in the cell (Fig. 6, *right cell*). In the situation of ECFP-GK and EYFP-GRP cotransfection, both proteins reside mainly in the nucleus.

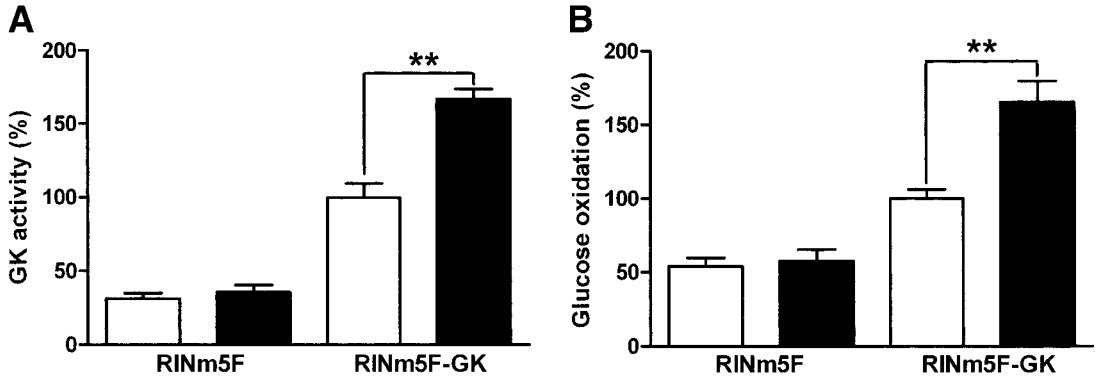

FIG. 7. Effects of adenoviral rat liver PFK-2/FBPase-2 overexpression on GK enzyme activity and glucose oxidation rate in RINm5F and RINm5F-GK cells. Cells were transduced with wild-type AdWT (□) or liver PFK-2/FBPase-2 AdPFK2L (■) adenoviral constructs (MOI 10). *A*: GK activities were measured in cell extracts after sonication. *B*: Glucose oxidation was measured in the presence of 2 mmol/l glucose. Data are expressed in percent of AdWT-transduced RINm5F-GK cells. Data are means ± SE from four independent experiments. **$P < 0.01$ compared with AdWT-transduced RINm5F-GK cells (ANOVA/Bonferroni's test).

Third, in contrast to the liver, glucose proved to be the key regulator of GK in β-cells. Because GK protein levels show only marginal variations under physiological conditions, GK is regulated on the posttranslational level (2,3,14,57,62–66) (Fig. 3*B*).

Compartmentalization of GK in insulin-producing cells. Selective permeabilization of GK-overexpressing RINm5F cells by digitonin revealed that GK exists in two different states: a freely diffusible portion with a high intrinsic activity and a portion bound to intracellular structures with a low intrinsic activity (57). In these RINm5F-GK model cells, GK was released from the bound state at high glucose concentration, providing evidence that compartmentalization participates in GK regulation in insulin-producing cells.

By the use of cell imaging techniques and subcellular fractioning of primary β-cells and insulin-producing cell lines, it could be demonstrated that GK binds to insulin secretory granules (42,67–70). This explains the punctate pattern of GK and a costaining with insulin in β-cells (69). There are conflicting data whether the interaction of GK with insulin granules is regulated by glucose. Rizzo et al. (69) showed that high glucose released GK from insulin granules to the cytoplasm in βTC3 cells. In MIN6 cells, however, GK association with components of the granule membrane of dense insulin granules and low-density organelles was not affected by glucose (42). In βTC3 cells, the interaction between GK and insulin granules proved to be dependent on the generation of nitric oxide (NO), and GK was also associated with neuronal NO synthase, thereby regulating NO synthesis in insulin-secreting cells (70). There are so far no experimental data available explaining the physiological function of GK interaction with insulin granules.

Recent studies indicate that, in β-cells, GK is associated with mitochondria (71). The interaction with this organelle proved to prevent apoptosis of MIN6N8 cells and primary β-cells induced by exposure to high glucose concentrations. In this scenario, GK reduced the oligomerization of the pro-apoptotic protein Bax, concomitant release of cytochrome C from mitochondria, and induction of apoptosis (71). It remains to be clarified whether a substantial portion of GK binds to mitochondria, since high-resolution fluorescence microscopy with fluorescent GK proteins did not show enrichment at the mitochondrial organelle but, interestingly, showed binding to matrix proteins (S.B., unpublished data). The expression level of GK protein seems to be a crucial factor affecting the stoichiometry of GK distribution in insulin-producing cells.

PFK-2/FBPase-2: a physiological activator of GK. Through screening of a random peptide phage display library for interaction partners of GK, we could identify, in addition to the consensus sequence of GRP, a second binding motif, -SLKVWT-. Analysis of protein databases revealed that this motif corresponds to the phosphatase domain of the bifunctional regulatory enzyme PFK-2/FBPase-2, a key regulator of glucose metabolism (40,72,73). PFK-2/FBPase-2 is expressed in liver as well as in insulin-producing cells (40,73). In liver-specific PFK-2/FBPase-2, the isoform is regulated by the cAMP-dependent protein kinase A, whereas insulin-producing cells expressed the brain isoform (41). From yeast two-hybrid studies, it became clear that the binding affinity of PFK-2/FBPase-2 was significantly lower than GRP (40). Furthermore, it could be demonstrated that GK interacts with both the liver and islet isoform of PFK-2/FBPase-2 (41).

In contrast to GRP, the interaction of PFK-2/FBPase-2 resulted in an activation of GK (41). This could be demonstrated through overexpression of PFK-2/FBPase-2 in insulin-producing cells, with both the liver and the islet isoform. The activation of GK was also not dependent on the cellular levels of fructose-2,6-bisphosphate (41).

Transient adenoviral PFK-2/FBPase-2 transduction of GK-expressing RINm5F cells resulted in a significant increase of GK activity (Fig. 7*A*). Importantly, PFK-2/FBPase-2 increased the V_{max} values of GK but did not affect the affinity for glucose or the positive cooperativity (41). PFK-2/FBPase-2 also amplified the increase of intrinsic GK activity by glucose, indicating that the interaction probably affects the conformation of the enzyme (41). The increase of intrinsic GK activity in PFK-2/FBPase-2–overexpressing RINm5F-GK cells led to a corresponding increase of the glucose oxidation rate (Fig. 7*B*).

Our data argue in favor of a model in which the GK activity state is regulated through a binding to organelles or the cellular matrix and a stimulatory interaction of a freely diffusible enzyme with PFK-2/FBPase-2 or other so far unknown proteins. It could be demonstrated that the enzyme propionyl-CoA carboxylase and a liver phosphoprotein phosphatase were potential binding partners of GK (74,75). However, propionyl-CoA carboxylase is a mitochondrial enzyme, a localization that does not correspond

to the physiological distribution of GK. The phosphoprotein phosphatase was identified in liver, but there is so far no evidence that GK is regulated by enzyme interconversion (25,74). In β-cells, the activation of GK by PFK-2/FBPase-2 closely mirrors the physiological upregulation of intrinsic activity by glucose or the nutritional state (41).

The conformation of the GK protein may determine the affinity either to the bound or diffusible fraction. It is likely that the catalytically inactive "super-open" conformation may interact with the matrix-bound fraction and interaction, while PFK-2/FBPase-2 stabilizes the fast "closed-open" transition. In addition, the GK protein will become less susceptible to oxidative attack of the sulfhydryl groups. This model implicates glucose as the key regulator of GK enzyme conformation and thus also of its subcellular localization in insulin-producing cells. Chemical activators of GK act through prevention of the shift to the catalytically unfavorable "super open" conformation, thus favoring diffusible fraction of GK with high intrinsic activity (11,12). It is not clear at the moment how GK activators interfere with the binding of GK to PFK-2/FBPase-2. The molecular size of the chemical activators versus the whole PFK-2/FBPase-2 enzyme argues in favor of a different spatial complex formation with GK. The specific binding of chemical activators within the hinge region of the GK protein clamps the enzyme at a conformation that is controlled by the glucose concentration. However, both GK activators and PFK-2/FBPase-2 retain the glucose sensor function of the enzyme and therefore the physiological metabolic stimulus-secretion coupling in β-cells.

CONCLUDING REMARKS AND PERSPECTIVE

The metabolic glucose sensor function of GK is based on a complex regulatory network that acts in a tissue-specific manner. Key principles of GK regulation comprise the transcriptional control, which determines the GK protein levels and the glucose phosphorylation capacity as well as compartmentalization through interaction with proteins. It is important to note that the high expression level of GK in liver in combination with a liver-specific inhibitory GRP ensures a high capacity of glucose phosphorylation and a high reserve pool of GK in both nuclear and cytoplasmic compartments. In β-cells, and probably also in other nonliver cell types expressing GK, glucose phosphorylation capacity is much lower with shuttling mechanisms for the enzyme between matrix proteins, insulin granules, and the cytoplasmic compartment. Here, the β-cell must sustain a critical level of GK activity through stabilization of the enzyme, which is emphasized by the functional role of PFK-2/FBPase-2 as an activating binding partner. The identification of the GK structure provided newer insights into our understanding of GK regulation because it demonstrated that the specific transition between the "super open," "open," and "closed" conformations is a basic element of enzyme regulation, which likely interferes with the tissue-specific compartmentalization of the enzyme. Furthermore, the knowledge of GK structure proved to be essential to clarify the mechanism of GK activators and mutations leading to a decrease or activation of enzyme activity, now summarized as "glucokinase disease" (3,14). It will be a challenge for future studies to develop an integrative concept of GK regulation in different tissues, including brain, pituitary gland, and endocrine cells of the gut. The use of GK mutants with defined kinetic properties and innovative GK activating compounds will allow the investigation of compartmentalization and conformational changes of the enzyme under conditions of live cell imaging. Prospectively, the development of chemical GK activators and a detailed analysis of their tissue-specific action will pave the way to an optimized new class of drugs for the therapy of type 2 diabetes.

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