Original Article

Interaction of Glucokinase With the Liver Regulatory Protein Is Conferred by Leucine-Asparagine Motifs of the Enzyme

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The glucokinase regulatory protein (GRP) plays a pivotal role in the regulation of metabolic flux in liver by the glucose-phosphorylating enzyme glucokinase. Random peptide phage display library screening for binding partners of GRP allowed the identification of an asparagine-leucine consensus motif. Asparagine-leucine motifs of glucokinase located in the hinge region, as well as in the large domain, were changed by site-directed mutagenesis. The L58R/N204Y and the L309R/N313Y glucokinase mutants showed a significantly reduced interaction with GRP. The L355R/N350Y mutant had a fivefold-higher binding affinity for GRP than wild-type glucokinase. Imaging of glucokinase and GRP fluorescence fusion proteins revealed that the L58R/N204Y glucokinase mutant lacked glucose-dependent translocation by GRP, whereas the L355R/N350Y glucokinase mutant was trapped in the nucleus due to high affinity for GRP. The results indicate that the L58/N204 motif in the hinge region confers binding to GRP, while the L355/N350 motif may modulate the binding affinity for GRP. This latter motif is part of the α10 helix of glucokinase and accessible to GRP in the free and complex conformation.
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The glucose phosphorylating enzyme glucokinase regulates glycolytic flux at physiological millimolar glucose concentrations in liver and pancreatic β-cells (1–4). Glucokinase (hexokinase type IV; EC 2.7.1.2) has specific kinetic properties different from those of the other mammalian hexokinases (5). The enzyme shows a sigmoidal saturation curve and a low affinity for glucose. Furthermore glucokinase is not inhibited by its product glucose-6-phosphate. In addition to transcriptional mechanisms, hepatic glucokinase enzyme activity is modulated on the posttranslational level through an interaction with a regulatory protein (6–9). This glucokinase regulatory protein (GRP) is expressed mainly in hepatocytes, although there is also evidence for an expression in the hypothalamus (10). GRP inhibits glucokinase in a competitive manner with respect to the substrate glucose. Glucokinase inhibition by GRP is stimulated by fructose-6-phosphate and suppressed by fructose-1-phosphate. Both phosphate esters bind to a specific site of the GRP (11). Recent studies provide evidence for species-specific regulatory properties of fructose phosphates with respect to the inhibition of glucokinase by GRP (12). In mammalian hepatocytes, glucokinase is bound to the GRP and mainly localized in the nucleus at basal low-glucose concentrations. At increased glucose concentrations or in the presence of fructose, glucokinase rapidly translocates from the nucleus to the cytoplasm (13–19). Experiments with nonhepatic cellular models have clearly shown that GRP is required for the intracellular shuttling of glucokinase into the nucleus (20–22).

Previously, the amino acid residues V203 and N204, as well as the clusters formed by E52 and H141, K142, K143, and L144 in the glucokinase sequence, have been described to mediate the binding to GRP (23–25). These data implicated a model in which the tip of the small domain and the hinge region were involved in the interaction with GRP. Another study with glucokinase-hexokinase chimeras postulated a large interface in the glucokinase protein for GRP binding (22). Using a phage display library screening, we recently identified a binding motif within the GRP that is crucial for the interaction with the glucokinase protein (26). The aim of our present work was, therefore, using the same approach, to identify the corresponding glucokinase-binding motif for the interaction with the GRP. The experiments revealed an asparagine-leucine consensus–binding motif, which could be found in different areas of the glucokinase protein. Studies using glucokinase mutant proteins showed that this motif is of crucial importance for the interaction of glucokinase with the GRP and the cytoplasmic-nuclear distribution of glucokinase in hepatocytes.

RESEARCH DESIGN AND METHODS

Hybond N nylon membranes were obtained from Amersham (Braunschweig, Germany). The enhanced chemiluminescence detection system and autoradiography films were from Amersham Pharmacia Biotech (Freiburg, Germany). Restriction enzymes and modifying enzymes for the cloning procedures were from New England Biolabs (Beverly, MA) or Fermentas (Fermentas, St. Leo-Rot, Germany). Custom oligonucleotides were synthesized by Gibco (Karlsruhe, Germany) or MWG Biotech (Ebersberg, Germany). Media and supplements for culture of yeast were from Clontech (Palo Alto, CA). Columns for DNA purification were from Qiagen (Hilden, Germany). Collagenase H was from Roche (Penzberg, Germany) and collagen type I from rat tail from Sigma (Taufkirchen, Germany). All other reagents of analytical grade were from Merck (Darmstadt, Germany).

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ECFP, enhanced cyan fluorescent protein; ELISA, enzyme-linked immunosorbent assay; EYFP, enhanced yellow fluorescent protein; GRP, glucokinase regulatory protein; GST, glutathione S-transferase.

TABLE 1
Primers used for the preparation of the glucokinase mutants

Amino acid	Wild-type protein	Mutant protein	Mutagenesis oligonucleotide (localization in glucokinase cDNA sequence)
Single mutations			
L58R	Leu	Arg	5′-AGT GTG AAG ATG C**G**G CCC ACC TAC GTG-3′ (618–644)
N204Y	Asn	Tyr	5′-GTG GCA ATG GTG **T**AT GAC ACG GTG GCC-3′ (1056–1082)
L309R	Leu	Arg	5′-GTG CTG CTC AGG C**G**C GTG GAC GAA AAC-3′ (1371–1397)
N313Y	Asn	Tyr	5′-CTC GTG GAC GAA **T**AC CTG CTC TTC CAC-3′ (1383–1409)
N350Y	Asn	Tyr	5′-AAG CAG ATC TAC **T**AC ATC CTG AGC ACG-3′ (1494–1520)
L355R	Leu	Arg	5′-ATC CTG AGC ACG C**G**G GGG CTG CGA CCC-3′ (1509–1535)
Double mutations			
L58R/N204Y	Leu/Asn	Arg/Tyr	5′-AGT GTG AAG ATG C**G**G CCC ACC TAC GTG-3′ (618–644) 5′-GTG GCA ATG GTG **T**AT GAC ACG GTG GCC-3′ (1056–1082)
L309R/N313Y	Leu/Asn	Arg/Tyr	5′-GTG CTG CTC AGG C**G**C GTG GAC GAA **T**AC CTG CTC TTC CAC-3′ (1371–1409)
N350Y/L355R	Asn/Leu	Tyr/Arg	5′-AAG CAG ATC TAC **T**AC ATC CTG AGC ACG C**G**G GGG CTG CGA CCC-3′ (1494–1535)

Mutated nucleotides are bold and underlined.

TABLE 2
Sequences of GRP-binding peptides selected from a M13 phage display library

Amino acid sequence	Number of clones	
N M F G S L T S H V T A	4	
Q V N Q W S P L V N I R	3	
L G N Q G L S L T L R L	1	Consensus Motif N-$(X)_n$-L
Q L Y N N R S L F P A W	2	
N G N H R T L S A H A H	2	
V N P H H M Q L P K L H	1	
D E N L S L R P L F P K	1	
Q A E N K I D V H L P I	1	

The sequences of peptides displayed by GRP-binding phages were identified through a selection protocol consisting of three rounds of high-stringency panning from a 12-mer random peitide M13 phage display library. This procedure revealed the N-$(X)_n$-L consensus motif consisting of an asparagine (N) and leucine (L) residue that were separated by three to five amino acids.

Generation of the glucokinase mutants. Site-directed mutagenesis of β-cell glucokinase cDNA was performed as previously described (27). The sequences of the specific mismatch oligonucleotides for mutagenesis are shown in Table 1 and refer to human islet glucokinase (GenBank M90299) (28). All glucokinase mutations were verified by sequence analysis.

Expression and purification of recombinant proteins. The full coding sequences of rat liver GRP, wild-type glucokinase, and glucokinase mutants were subcloned in frame into the *Bam*HI and *Sal*I sites of the pGEX-6P-1 expression vector and expressed by the glutathione S-transferase (GST) Gene Fusion System in *E. coli* BL21 (Amersham Pharmacia Biotech, Freiburg, Germany). The GST-tag was cleaved by incubation with PreScission protease. Recombinant proteins were verified using PAGE and Western blot analysis as previously described (29). The eluted proteins were stabilized by 50% glycerol and stored at −20°C.

Immobilization of recombinant liver GRP. The recombinant rat liver GRP was immobilized by the GST-tag on Reacti-Bind glutathione-coated microplate strips (Pierce, Rockford, IL). Two-hundred microliters of the protein solution (200 ng/ml GRP in 0.1 mol/l $NaHCO_3$) were incubated overnight at 4°C with gentle agitation in the microplate wells. Thereafter, the supernatant was removed and the wells blocked with 300 μl blocking buffer (5 mg/ml BSA in 0.1 mol/l $NaHCO_3$) for 1 h at 4°C with gentle agitation.

Phage display screening. The selection of peptides able to bind to the GRP was performed with the Ph.D.-12 Phage Display Peptide Library (New England Biolabs) according to the manufacturer's instructions. Specifically bound phages were eluted with glycine buffer and the recovered phages amplified in ER2537 *E. coli* bacteria. The variable region was characterized by sequence analyses. The phage enzyme-linked immunosorbent assay (ELISA) experiments for quantification of binding affinity were performed as previously described (26). Samples of 10^{14} phages per well were analyzed for the interaction with the GRP.

Yeast two-hybrid analysis. Yeast two-hybrid analysis was performed with the Matchmaker GAL4 system 2 (Clontech) as described in the manufacturer's manual. The coding cDNA sequences of the β-cell wild-type and mutant glucokinase proteins were subcloned in frame (*Sma*I and *Bam*HI restriction sites) to the activation domain into pACT2. Rat liver GRP coding cDNA was subcloned in frame (*Nco*I and *Eco*RI restriction sites) to the binding domain into pAS2–1. All constructs were verified by sequence analyses. Yeast *S. cerevisiae* strain CG1945 or Y190 (Clontech) was transfected with the appropriate pAS2–1 and pACT2 plasmids by using the lithium acetate procedure and grown on SD agar plates without leucine and tryptophan. The quantitative chemiluminescent β-galactosidase assay of yeast two-hybrid interaction was performed as previously described (26).

Assay of glucokinase enzyme activity. Glucokinase enzyme activities were measured by an enzyme-coupled photometric assay (27,30,31). For each glucokinase mutant protein, three experiments were done in triplicate. Enzyme activities were expressed as units per milligram of purified glucokinase protein. One unit of enzyme activity was defined as 1 μmol glucose-6-phosphate formed from glucose and ATP per min at 37°C (30). The K_m values and Hill coefficients of glucokinase were calculated from Hill plots (32).

Western blot analyses. Cells were homogenized by sonication in PBS (pH 7.4), and insoluble material was pelleted by centrifugation. Cellular protein (10 μg) was fractionated by reducing 10% SDS-PAGE and electroblotted to polyvinylidine difluoride membranes. The membranes were stained by Ponceau to verify the transfer of comparable amounts of cellular protein. Glucokinase immunodetection was performed as previously described (29). For GRP, the blots were incubated with a GRP antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:500, followed by a 4-h incubation period with an anti-goat IgG peroxidase-labeled secondary antibody at a dilution of 1:20,000 at room temperature. The specific protein bands were visualized by chemiluminescence using the enhanced chemiluminescence detection system.

Construction of pECFP-C1, pEYFP-C1, and pEYFP-N1 fusion vectors. The cDNAs of β-cell glucokinase wild-type and mutant proteins were subcloned in frame (*Sma*I and *Bam*HI restriction sites) to both the enhanced cyan

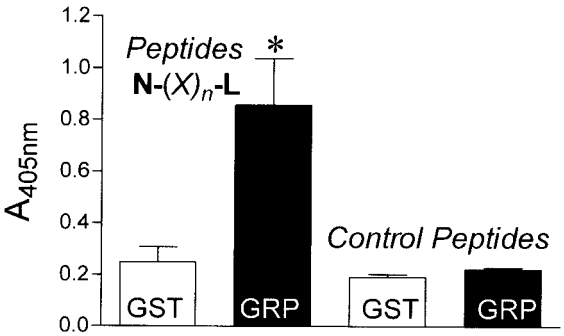

FIG. 1. Characterization of the GRP-binding affinity of consensus peptide–displaying phages by ELISA. Binding affinities of consensus peptide–displaying phages and control peptide phages to GST-GRP–coated (■, GRP) and GST-coated (□, GST) microplate wells were detected by ELISA. The A_{405} values are means ± SE from three independent experiments. *$P < 0.05$ vs. GST-coated wells (ANOVA/Bonferroni's test).

fluorescent protein (ECFP) into pECFP-C1 and the enhanced yellow fluorescent protein (EYFP) into pEYFP-C1 (Clontech). Rat liver GRP coding cDNA was subcloned in frame (*Bgl*II and *Eco*RI restriction sites) to the EYFP into pEYFP-N1. All constructs were verified by sequence analyses.

Cell culture and transient transfection of COS-1 cells. COS-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 25 mmol/l glucose and 10% (vol/vol) FCS in a humidified atmosphere at 37°C and 5% CO_2. Cells were seeded at a density of 2×10^4 cells on three glass cover slips stored in a 35-mm dish, and transfection was performed the day thereafter with 1 μg plasmid DNA and 2 μl jetPEI (Qbiogene, Montreal, Canada) according to the manufacturer's instructions. Cells were cultured for 24 h, and thereafter coverslips were incubated in Dulbecco's modified Eagle's medium with either 5.5 or 25 mmol/l glucose for an additional 3 h.

Hepatocyte isolation, culture, and transient transfection. Primary hepatocytes were isolated from fed Wistar rats (150–200 g) using a modification of the collagenase perifusion method of Seglen (33) and Bader et al. (34). Isolated cells were suspended in Williams' medium E supplemented with 10 mmol/l glucose, 5% (vol/vol) FCS, 10^{-4} mmol/l dexamethasone, and 10^{-5} mmol/l insulin. Cell viability tested by trypan blue staining was at least 80%. Thereafter, hepatocytes were seeded at a density of 5×10^5 cells on three collagen type I–coated glass cover slips, stored in a 35-mm dish, and incubated in a humidified atmosphere at 37°C and 5% CO_2. After 3 h, the nonadherent cells were removed and attached cells transfected with 2 μg plasmid DNA and 6 μl jetPEI-Gal (Qbiogene) according to the manufacturer's instructions. Hepatocytes were cultured for 24 h, and thereafter cover slips were incubated in Williams' medium E with either 5 or 20 mmol/l glucose for an additional 3 h.

Fluorescence microscopy, image acquisition, and deconvolution. Cover slips were mounted in a custom-made chamber. The thermostatically controlled (37°C) chamber was filled with 200 μl of the appropriate medium and fixed on the stage of an Olympus IX81 inverted microscope (Olympus Optical, Tokyo, Japan) equipped with a PLAPO 60 × 1.4–NA oil-immersion objective (Olympus) and a YFP/CFP dual-band beamsplitter and emitter (AHF Analysentechnik, Tübingen, Germany). Alternate EYFP and ECFP excitation light with adjusted intensity was injected from the cell^R MT20 illumination system (Olympus BioSystems, Planegg, Germany) using HQ 500/20 and D 436/10 filters (AHF Analysentechnik). Images were taken with a charge-coupled device camera (6.45 × 6.45–μm pixels; Olympus BioSystems). System synchronization and multidimensional image acquisition during the experiment were controlled with cell^R hardware and software (Olympus BioSystems). EYFP/ECFP double-color images were removed from bleed-through artifacts by cell^R spectral unmixing. Deconvolution and image analysis were performed using AutoDeblur 9.3 WF software (Autoquant Imaging, Watervliet, NY).

Statistical analyses. Data are expressed as means ± SE. Statistical analyses were performed by ANOVA followed by Bonferroni's or Dunnett's test for multiple comparisons using the Prism analysis program (Graphpad, San Diego, CA).

RESULTS

Isolation of GRP-binding phages. To identify GRP-binding motif(s) within the glucokinase, a systematic screening of recombinant rat liver GRP was performed with a 12-mer random peptide M13 phage display library. A control experiment using immobilized GST protein was performed in order to exclude sequences that only bind to the GST-tag. The selected specific GRP-binding dodecapeptides showed a strong enrichment of the amino acid asparagine (Table 2). In comparison to the control experiment with scrambled peptides, the relative frequency of asparagine within the dodecapeptides increased fourfold (data not shown). Notably, the asparagine-containing peptide sequences also comprised a leucine residue separated from the asparagine by three to five amino acids in 15 clones (Table 2). This consensus sequence could not be detected in dodecapeptides isolated from the GST-control panning. Thereafter, the binding affinity of the isolated consensus peptide–displaying phages was verified through an ELISA-binding assay. Randomly amplified peptide phages of the original library were used as control. Peptides with the asparagine-leucine motif exhibited a high binding affinity to GRP that was significantly different from the control level of the GST (Fig. 1).

Localization of the GRP-binding motif in the glucokinase protein. The amino acid sequence of human glucokinase was screened for the residue asparagine alone and

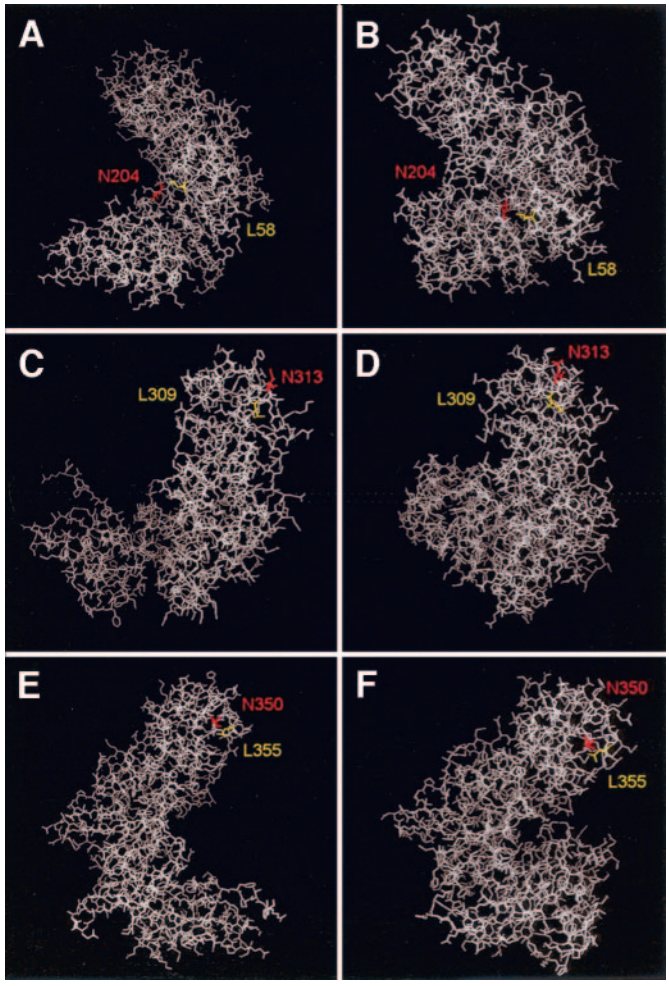

FIG. 2. Localization of asparagine-leucine motifs for GRP binding within the glucokinase protein. Glucokinase structure coordinates of the inactive "free" (*A*, *C*, and *E*) and active "complex" (*B*, *D*, and *F*) conformation of the enzyme protein were obtained from the RCSB Protein Data Bank (36). The proposed GRP-binding motif L58/N204 is depicted in *A* and *B*, the motif L309/N313 in *C* and *D*, and the motif N350/L355 in *E* and *F*. The structural glucokinase models were generated using the Vector NTI Suite program 3D Molecule Viewer (InforMax, Bethesda, MD).

the identified asparagine-leucine–binding motif. Within the whole glucokinase molecule consisting of 465 amino acids, the hydrophilic residue asparagine appeared 12 times; 4 of these occurrences (N83/L88, N283/L288, N350/L355, and L386/N391) were part of an asparagine-leucine motif. In addition, asparagine and leucine were neighboring amino acids (L164-L165-N166) or appeared in a complex structure (N179-N180/L184-L185). Because these motifs were not detected by our systematic phage display approach, we excluded these sequences from further analyses. In the glucokinase model, the asparagine N313 was surrounded by several leucines (L301, L304, L306, L307, L309, L314, and L315) and could therefore form a possible GRP-binding structure. Both the three-dimensional model created on the basis of the crystal structure of yeast hexokinase B (35) and the recently described crystal structures of the "complex" (active) and "free" (inactive) glucokinase conformations (36) proved to be helpful for the localization of the putative GRP-binding motifs. From the possible asparagine-leucine motifs in the glucokinase protein sequence, only the amino acids N350 and L355 appeared in the "free" (Fig. 2*E*), as well as in the "complex" tertiary structure as a putative binding anchor of GRP (Fig. 2*F*). Interestingly, the leucine of this motif is part of a postulated glucokinase nuclear export signal (22). A second proposed nuclear export signal contained leucine L309 (22), which is separated by three amino acids from the asparagine N313. The L309/N313 motif was presented for interaction with GRP in the "free" (Fig. 2*C*) rather than the "complex" (Fig. 2*D*) conformation of glucokinase. This asparagine-leucine motif, as well as the N350/L355 sequence, is located in the COOH-terminal lobe of the glucokinase protein.

Screening the asparagines in the tertiary structure of the glucokinase enzyme, the N204 residue also yielded a remarkable spatial vicinity to the L58 residue in the catalytic cleft in the "free" conformation of the enzyme (Fig. 2*A*). The asparagine N204 is localized in the hinge region, and in previous studies this residue has been implicated in the binding of the GRP (25). N204 is also part of the glucose-binding side of glucokinase, and therefore the putative binding anchor L58/N204 is lost in the active conformation (Fig. 2*B*).

To elucidate which putative asparagine-leucine glucokinase–binding sequences are involved in the interaction with the GRP, a series of six single and three double mutations were generated by site-directed mutagenesis. In the mutant glucokinase proteins, the aliphatic nonpolar amino acid leucine (L) was systematically replaced by the strongly basic residue arginine (R) and the hydrophilic amide asparagine (N) by the hydrophobic aromatic amino acid tyrosine (Y).

Yeast two-hybrid interactions of GRP with wild-type and mutant glucokinase protein. To study the interaction of the glucokinase mutant proteins with the GRP, the glucokinase cDNA and the mutated glucokinase cDNAs were subcloned into vectors of the GAL4 system. Protein expression of wild-type and mutant glucokinase was confirmed by Western blot analyses (data not shown). Control experiments excluded nonspecific interactions of the proteins with the truncated activation and binding domains of the vectors (data not shown). Experiments were performed in the yeast host strain Y190, and protein interactions were quantified by the β-galactosidase reporter enzyme. The wild-type glucokinase showed a strong binding to the GRP with a 10-fold increase of the reporter gene activity compared with background activity by the truncated binding domain interaction (Fig. 3*A*).

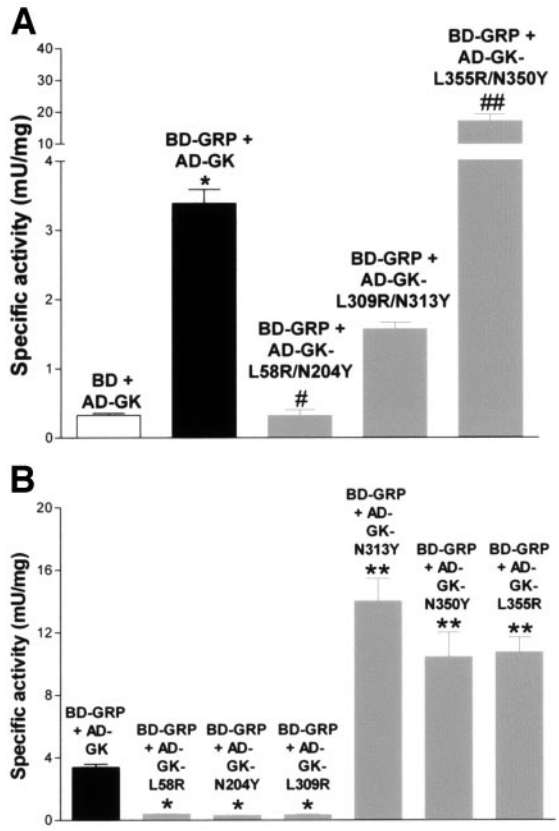

FIG. 3. Yeast two-hybrid interactions of wild-type (WT) and mutant glucokinase proteins with the GRP. Full-length cDNAs of human β-cell glucokinase wild-type and mutant proteins (L58R, N204Y, L309R, N313Y, N350Y, L355R, L58R/N204Y, L309R/N313Y, and N350Y/L355R) were cloned as fusion proteins together with the activation domain (AD) of the GAL4 yeast two-hybrid system. The rat liver GRP was fused to the binding domain (BD). Yeast two-hybrid interactions were quantified by a chemiluminescent β-galactosidase reporter gene assay from yeast extracts. Shown are means ± SE from five independent experiments. Double-mutant proteins are shown in *A* (P* < 0.05 vs. background activity of wild-type glucokinase fused to the activation domain and a binding domain without fusion partner; #*P* < 0.05, ##*P* < 0.01 vs. interaction between GRP and wild-type glucokinase). Single-mutant glucokinase proteins are shown in *B* (**P* < 0.05, ***P* < 0.01 vs. binding activity between GRP and wild-type glucokinase [ANOVA/Bonferroni's test]).**

The double mutation L58R/N204Y (Fig. 3*A*) and the single mutations L58R and N204Y (Fig. 3*B*) in the glucokinase protein significantly decreased the binding affinity to the GRP by a factor of 10, 7, and 12, respectively. The double mutation L309R/N313Y of the glucokinase protein showed only a twofold decrease of reporter gene activity in comparison to the wild-type glucokinase protein (Fig. 3*A*). Experiments performed with glucokinase mutants where only amino acid L309 or amino acid N313 was changed resulted in opposite effects. While a significant decrease to the background values was observed with the L309R mutant, the single mutation N313Y resulted in a fourfold increase of reporter gene activity (Fig. 3*B*). In contrast, the N350Y/L355R glucokinase mutant showed a strongly increased binding to the GRP, with a fivefold-higher reporter gene activity than wild-type glucokinase (Fig. 3*A*). The two corresponding single mutations N350Y and L355R also increased the reporter gene activity by a factor of three in both cases (Fig. 3*B*).

The same two-hybrid results were obtained for all

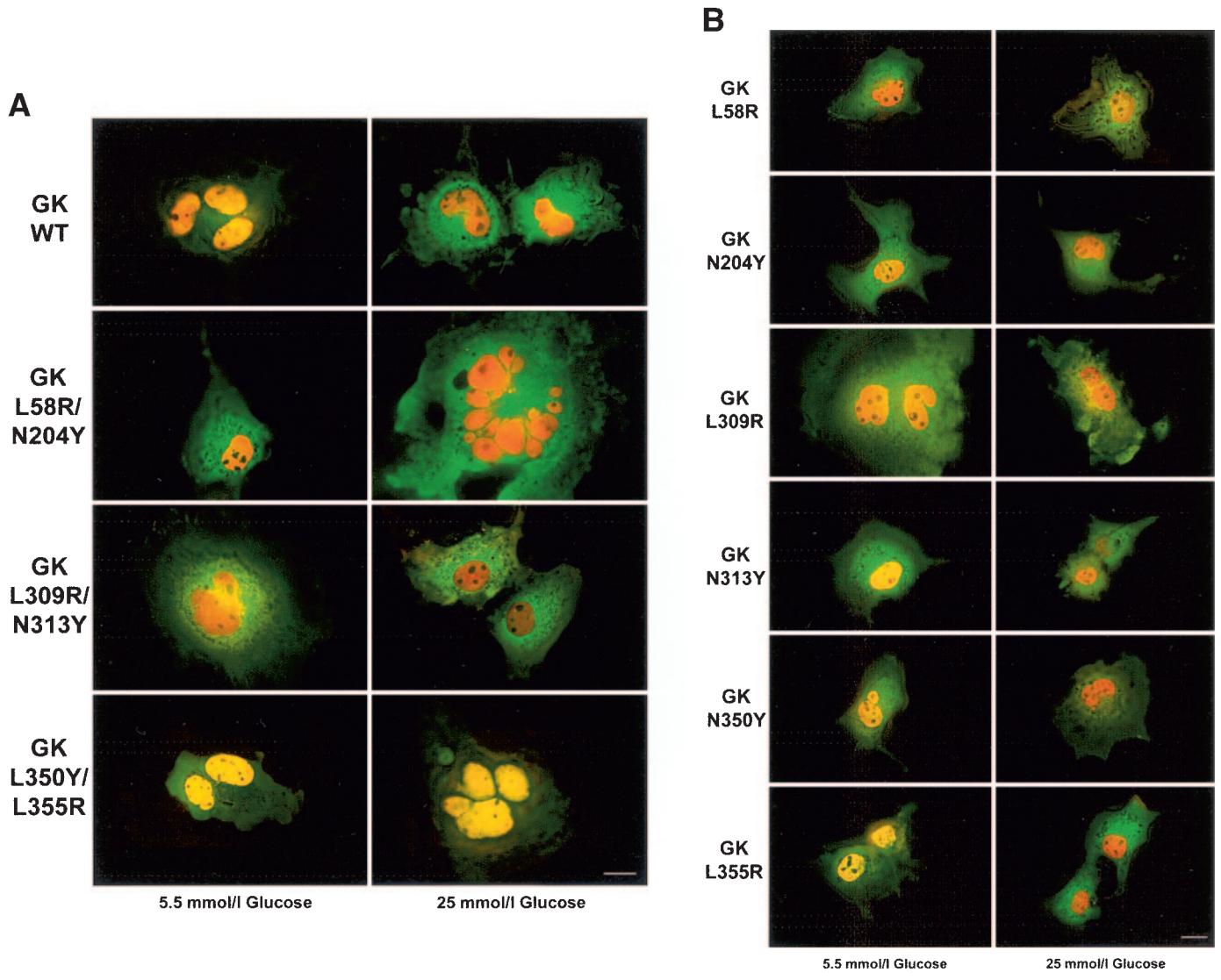

FIG. 4. Effects of 5.5 or 25 mmol/l glucose on the localization of EYFP-GRP, wild-type (WT) ECFP-glucokinase (GK), and mutant ECFP-glucokinase in COS-1 cells. *A*: COS-1 cells were transfected with EYFP-GRP and wild-type or mutant ECFP-glucokinase (L58R/N204Y, L309R/N313Y, and N350Y/L355R). *B*: COS-1 cells were transfected with EYFP-GRP and mutant ECFP-glucokinase (L58R, N204Y, L309R, N313Y, N350Y, and L355R). Fluorescence images were taken after a 3-h incubation period at 5.5 or 25 mmol/l glucose. In the merged images, EYFP is depicted in red and ECFP in green. The depicted fluorescence images were obtained from z-stacks after deconvolution and are representative of four cells each in five independent experiments. Scale bar, 20 μm.

mutants when the glucokinase-GRP interactions were evaluated semiquantitatively through activation of the HIS3 reporter gene in the yeast strain CG1945 and growth selection on SD agar plates lacking leucine, tryptophan, and histidine for 5 days after plating (data not shown).

Kinetic properties of recombinant wild-type and mutant glucokinase proteins. All glucokinase proteins exhibited a molecular weight of ~52 kDa, which was comparable to that of the wild-type glucokinase protein (data not shown). Sufficient amounts of glucokinase protein for kinetic analysis could be obtained for the glucokinase mutants L58R, N204Y, N313Y, N350Y, L58R/N204Y, and N350Y/L355R. Recombinant wild-type human glucokinase protein showed a V_{max} value of 35.0 ± 0.5 units/mg protein, an $S_{0.5}$ value of 6.2 ± 0.3 mmol/l, and a Hill coefficient of 1.6 ± 0.1 for glucose. In enzyme activity studies, the double-mutant glucokinase protein L58R/N204Y, as well as the single-mutant proteins L58R and N204Y, showed a complete loss of glucokinase activity. All other glucokinase mutations showed only slight differences in comparison to the wild-type protein. The N313Y mutation resulted in a significant increase of the V_{max} value to 40.1 ± 0.6 units/mg protein and an $S_{0.5}$ value of 9.3 ± 0.3 mmol/l for glucose, while the Hill coefficient (1.6 ± 0.1) was not affected. The single-mutation N350Y decreased the V_{max} for the substrate glucose (26.0 ± 0.3 units/mg protein), while both the $S_{0.5}$ value (6.5 ± 0.2 mmol/l) and the Hill coefficient (1.4 ± 0.1) were not different from wild-type glucokinase. The double-mutation N350Y/L355R also showed a significant decrease of the V_{max} value with 29.0 ± 0.4 units/mg protein but showed an increase in the $S_{0.5}$ value with 7.3 ± 0.3 mmol/l. The Hill coefficient (1.6 ± 0.1) was not changed in comparison to the wild-type protein.

Subcellular localization of wild-type and mutant glucokinase proteins in COS-1 cells and primary hepatocytes. To investigate the subcellular localization of the mutant glucokinase proteins compared with wild type,

TABLE 3
Nuclear-to-cytoplasmic glucokinase ratio in COS-1 cells transfected with EYFP-GRP and ECFP-GK wild-type and mutant proteins

Glucokinase	Glucose (mmol/l) 5.5	25
Wild type	2.5 ± 0.5	0.7 ± 0.1
Single mutant		
L58R	0.8 ± 0.1*	1.0 ± 0.1
N204Y	1.0 ± 0.1*	0.9 ± 0.1
L309R	1.0 ± 0.3*	0.8 ± 0.1
L313Y	1.9 ± 0.3	0.8 ± 0.1
N350Y	1.7 ± 0.2	1.0 ± 0.1
L355R	2.5 ± 0.2	0.7 ± 0.1
Double mutant		
L584R/N204Y	0.8 ± 0.1*	0.8 ± 0.1
L309R/N313Y	0.7 ± 0.1*	0.7 ± 0.1
L350Y/L355R	3.1 ± 0.2	2.0 ± 0.2*

COS-1 cells were transfected with EYFP-GRP and wild-type or mutant ECFP-GK. The nuclear-to-cytoplasmatic flucorescence intensity ratio was calculated from 20 nuclear and 20 cytoplasmic pixel measurements (cell^R software; Olympus BioSystems) of each image. Nuclear-to-cytoplasmic glucokinase ratios were calculated from four cells each in five independent experiments. *$P < 0.01$ vs. wild-type glucokinase at the same glucose concentration (ANOVA/Dunnett's multiple comparison test).

fusion proteins were generated with EYFP and ECFP. For live-cell imaging of the fluorescence proteins, COS-1 cells were used as an established cellular model, which neither expressed glucokinase nor GRP (20). Protein expression in transfected COS-1 cells was confirmed by Western blot analysis (data not shown). While transient transfection of EYFP resulted in a uniform cellular distribution of the fluorescence protein, the EYFP-glucokinase construct was exclusively located in the cytoplasm. None of the glucokinase mutants showed a difference in comparison to the cytoplasmic distribution of wild-type glucokinase. EYFP-GRP was predominantly localized in the nucleus of COS-1 cells, although a weak expression was also detectable in the cytoplasm (data not shown).

In COS-1 cells cotransfected with ECFP-glucokinase and EYFP-GRP, the glucokinase protein was localized in the nucleus together with GRP at 5.5 mmol/l glucose (Fig. 4*A*). At 25 mmol/l glucose, glucokinase was predominantly detectable in the cytoplasm, while GRP was located in the nucleus (Fig. 4*A*). In contrast, the mutant glucokinase proteins L58R/N204Y and L309R/N313Y were located in the cytoplasm at both 5.5 and 25 mmol/l glucose (Fig. 4*A*). The ECFP-N350Y/L355R glucokinase mutant showed a distinct fluorescence in the nucleus at low and high glucose concentrations (Fig. 4*A*). This was demonstrated by a high nuclear-to-cytoplasmic glucokinase ratio of 2.0 at 25 mmol/l glucose compared with 0.7 for the wild type (Table 3). The single-mutant glucokinase proteins L58R, N204Y, and L309R showed a cytoplasmic localization irrespective of the glucose concentration (Fig. 4*B* and Table 3). The subcellular localization of the glucokinase mutants N313Y, N350Y, and L355R was comparable to that of the wild-type glucokinase protein (Fig. 4*B* and Table 3).

Primary rat hepatocytes were transfected with EYFP constructs for further analyses of the glucokinase mutant proteins. The protein expression level of the endogenous GRP and the wild-type or mutant glucokinase was confirmed by Western blot analysis (data not shown). Wild-type EYFP-glucokinase was predominantly in the hepatocyte nucleus at 5 mmol/l glucose, as demonstrated by a high nuclear-to-cytoplasmic glucokinase ratio of 2.3, whereas a uniform cellular distribution was detectable at 20 mmol/l glucose with a nuclear-to-cytoplasmic glucokinase ratio of 1.4 (Fig. 5). The glucokinase double-mutant L58R/N204Y was found exclusively in the cytoplasm at both high and low glucose concentrations (Fig. 5). The nuclear-to-cytoplasmic glucokinase ratio was significantly lower for the mutant L58R/N204Y and the mutant L309R/N313Y when compared with the wild-type protein (Fig. 5). In contrast to L58R/N204Y, the L309R/N313Y glucokinase mutant was clearly visible in the nucleus at 5 mmol/l glucose (Fig. 5). The N350Y/L355R glucokinase mutant showed a significantly increased nuclear-to-cytoplasmic glucokinase ratio both at 5 and 20 mmol/l glucose (Fig. 5).

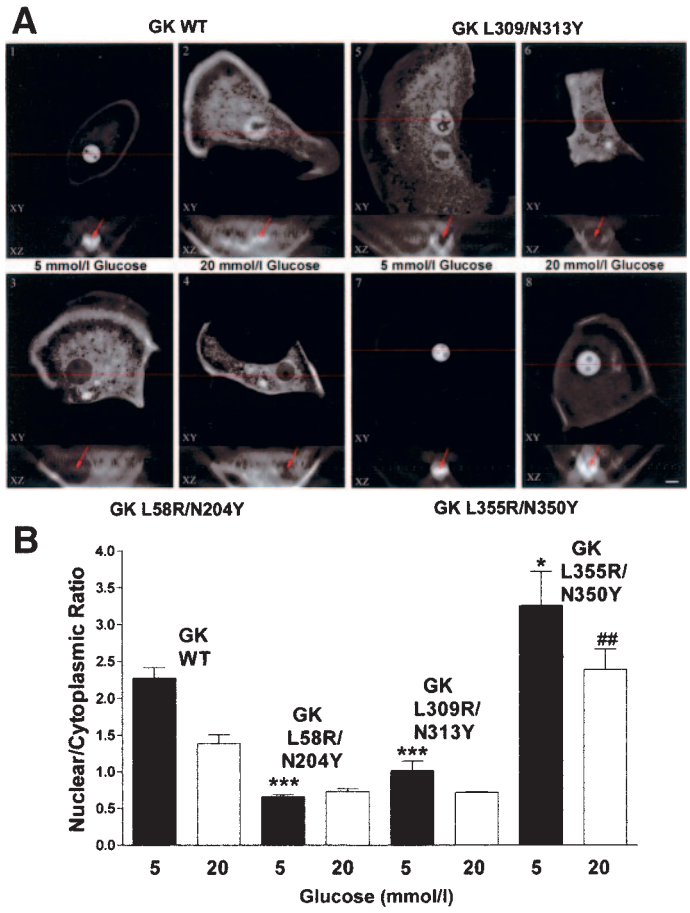

FIG. 5. Different subcellular distribution of wild-type (WT) EYFP-glucokinase (GK), L58R/N204Y, L309R/N313Y, and N350Y/L355R mutant EYFP-glucokinase in primary rat hepatocytes. *A*: Rat hepatocytes were transfected with EYFP-glucokinase wild-type or mutant EYFP-glucokinase (L58R/N204Y, L309R/N313Y, and N350Y/L355R). Fluorescence images were taken after incubation with 5 (images 1, 3, 5, and 7) or 20 (images 2, 4, 6, 8) mmol/l glucose for 3 h. Each panel shows an xz image of the line marked in the xy image. The nuclear region in the xz image is marked by a red arrow. The depicted fluorescence images were obtained from z-stacks after deconvolution and are representative of three independent experiments. Scale bar, 20 μm. *B*: The nuclear-to-cytoplasmatic fluorescence intensity ratio was calculated from 20 nuclear and 20 cytoplasmic pixel measurements (cell^R software; Olympus BioSystems) of each image. Shown are means ± SE of three independent experiments. *$P < 0.05$, ***$P < 0.001$ vs. wild-type glucokinase at 5 mmol/l glucose (■); ##$P < 0.01$ vs. wild-type glucokinase at 20 mmol/l glucose (□).

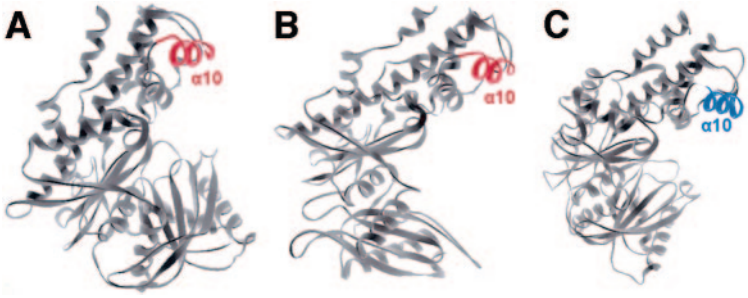

FIG. 6. Structures of human glucokinase and yeast hexokinase P II. In the model of glucokinase, the α10 helix is marked in red in the "complex" (*A*) and "free" (*B*) conformation. In the model of yeast hexokinase P II, the α10 helix is marked in blue (*C*). Glucokinase and yeast hexokinase P II structure coordinates were obtained from the Research Collaboratory for Structural Bioinformatics Data Bank (36,37). The structural models were generated using the Vector NTI Suite program 3D Molecule Viewer (InforMax).

DISCUSSION

Posttranslational regulation of glucokinase in liver is crucially dependent upon the interaction with the regulatory protein (GRP) (15). It was therefore the aim of this study to identify potential binding motifs of GRP within the glucokinase protein by a systematic peptide phage display methodology. Using this strategy, we obtained peptides with an asparagine-leucine motif, which showed a high affinity to the GRP in continuative ELISAs. At first, we analyzed the glucokinase structure for localization of asparagine-leucine motifs as a possible binding anchor for GRP. An asparagine-leucine motif exists four times (N83/L88, N283/L288, N350/L355, and L386/N391) in the glucokinase protein, of which only the amino acids N350 and L355 are presented in a sterically accessible localization (36). In addition, a sequence alignment for the hexokinase family revealed that the asparagine N350 exists exclusively in the glucokinase sequence (37), whereas N83, N283, and N391 also could be found in high-affinity hexokinase isoenzymes. The analysis of the tertiary structure of glucokinase showed that a spatial vicinity of the amino acids asparagine and leucine was obvious for L164-L165-N166 and N179-N180/L184-L185, in which only the latter group completely corresponded to the characteristics of the consensus epitope. Previous studies of mutated glucokinase proteins provided evidence for a participation of asparagine N166 and N180 in the binding to GRP (23,25). Both amino acid residues are located between the smaller domain and the hinge region of glucokinase, which essentially determines the cooperativity of the enzyme. But the mutation of the residues N166 to arginine and N180 to aspartic acid was less effective with respect to the interaction properties to the GRP. Furthermore, the asparagine N313 was studied because this residue is surrounded by several leucines with L309 as part of a postulated glucokinase nuclear export signal (22). Finally, the analysis of the tertiary glucokinase structure revealed a close spatial vicinity of L58 and N204 on the surface of the glucokinase hinge region. The glucokinase residue N204, which is highly conserved within the hexokinase family, is part of the glucose-binding site and also mediates the binding to GRP (21,25,35,36,38).

In agreement with previous studies, the glucokinase mutant N204Y showed a loss of GRP-binding affinity and of intrinsic glucokinase activity (21,25). Comparable results also were obtained for the glucokinase mutants L58R and L58R/N204Y. Notably, the L58R/N204Y glucokinase double mutant was exclusively expressed in the cytoplasm irrespective of the glucose concentration in both GRP-overexpressing COS-1 cells and primary hepatocytes. These results correspond to the loss of GRP binding determined in the yeast two-hybrid–binding assay. While N204 has been described to play an important role for glucose binding, the amino acid L58 thus far has not been considered to participate in the formation of the catalytic pocket of glucokinase. Interestingly, the spatial vicinity of N204 and L58 is apparent only in the super-open conformation, but not the closed conformation, of glucokinase (36). Through replacement of L58 by the basic amino acid residue arginine, we potentially induced a change in the glucokinase conformation that may significantly affect the transition between the active and inactive state of the enzyme. Therefore, both the intrinsic glucose phosphorylation activity and the GRP-binding affinity were nearly completely abolished in the L58R glucokinase mutant. However, these data also indicate that the conformation of the glucokinase hinge region in the super-open form and, in particular, the L58/N204 motif are of crucial importance for the interaction with GRP.

In hepatocytes, the L309R/N313Y glucokinase mutant showed a significantly lower presence in the nucleus than the wild-type glucokinase at both low and high glucose concentrations, but interestingly, the nuclear-to-cytoplasmic glucokinase ratio was higher than in the case of mutant L58R/N204Y at 5 mmol/l glucose. Thus, glucokinase translocation in hepatocytes reflects the higher GRP-binding affinity of the L309R/N313Y glucokinase mutant in the yeast two-hybrid–binding assay when compared with mutant L58R/N204Y. Surprisingly, the single-mutation N313Y resulted in a protein with a higher binding affinity to GRP than that of wild-type glucokinase. A significantly higher intrinsic activity than the wild-type protein was also observed with this mutant. However, in comparison with naturally occurring glucokinase mutations inducing a hypoglycemic syndrome, the increase of enzyme activity of the N313Y mutant was less impressive (39,40). The L309R glucokinase mutant showed a loss of GRP binding in yeast two-hybrid studies and of nuclear localization in COS-1 cells. Notably, the L309 residue is part of a leucine-rich region from glucokinase amino acids 300–310, which was postulated to represent a nuclear export signal for glucokinase (22). A L306A/L307A/L309A mutant glucokinase fragment comprising amino acids 299–359 showed a higher nuclear-to-cytoplasmic glucokinase ratio compared with the wild-type sequence after transfection as a green fluorescent protein fusion protein in HeLa cells (22). However, the existence of a glucokinase nuclear export signal remains open because nuclear export of glucokinase was not inhibited by leptomycin B (41). Our present data obtained with the L309R glucokinase mutant holoenzyme indicate a block of nuclear import by the GRP but do not allow us to conclude that this residue has a functional role in nuclear export.

The GRP-binding affinity of glucokinase mutants N350Y and L355R increased by a factor of 3 and that of the double-mutant N350Y/L355R by a factor of 5 in comparison to the wild-type glucokinase. Thus, the mutation of the whole asparagine-leucine motif resulted in a significantly

higher glucokinase-GRP interaction than the single changes of asparagine and leucine, indicating a synergistic role of these two residues in the modulation of the GRP-binding affinity. Interestingly, the N350/L355 motif is accessible in both the closed and super-open conformation of glucokinase, but presentation of these residues appears different. N350 and L355 are located in the α10 helix of glucokinase, a structure that has a low homology with the other enzymes of the hexokinase family (35,37). In addition, asparagine N350 exists exclusively in the glucokinase sequence.

While the conformation of the α10 helix in the closed form (Fig. 6*A*) appears similar to the corresponding conformation of yeast hexokinase II (Fig. 6*C*), the helical structure formed by the amino acid residues 344–355 is clearly exposed in the super-open conformation of glucokinase (Fig. 6*B*). Therefore, the glucokinase α10 helix may play an important role for GRP recognition and modulation of glucokinase-GRP binding. While in the super-open conformation, the glucokinase-GRP complex is formed through an interaction with the glucokinase hinge region, the α10 helix may be relevant for complex stabilization. This assumption is supported by the fact that the N350Y/L355R glucokinase mutant showed a significantly higher nuclear-to-cytoplasmic glucokinase ratio in primary hepatocytes than wild-type glucokinase at both 5 and 20 mmol/l glucose. The nuclear-to-cytoplasmic glucokinase ratio of the wild-type protein at both glucose concentrations was comparable with glucokinase distribution data obtained from liver samples of starved and 2-h–refed rats, as well as with data upon primary hepatocytes at high and low glucose (42–44). A nuclear accumulation of glucokinase, which was comparable to that of the N350Y/L355R mutant protein, has been described only after an excessive overexpression of GRP in hepatocytes (45). Interestingly, a lack of glucokinase translocation in response to high glucose concentrations has recently been reported for Zucker diabetic fatty rats, indicating a functional role in the pathogenesis of type 2 diabetes (46). Mutations within the α10 helix may be the underlying mechanism for this defect. While in COS-1 cells the cellular distribution of the glucokinase single mutants N350Y and L355R was comparable to that of wild-type glucokinase, the double mutation showed an additive effect with strong nuclear localization both at high and low glucose concentrations. This argues in favor of a functional role of the asparagine-leucine motif for the conformation of the α10 helix that results in a high binding affinity for GRP, a concomitant nuclear translocation of glucokinase, and ultimately a reduced glucose phosphorylation activity in the cytoplasmic compartment.

The functional relevance of the glucokinase-GRP interaction and the metabolic impact of the shuttling of glucokinase between the cytoplasmic and nuclear compartment has been convincingly demonstrated in GRP knockout mice with impaired glucose tolerance (47) and by glucose infusion in rats (19). In the present study, we identified the L58/N204 and the L355/N350 leucine-asparagine motifs as functional elements of the glucokinase protein that confer interaction with the GRP and the nuclear shuttling of glucokinase. In particular, the L355/N350 motif may modulate the strength of binding to the GRP. Thus, the glucokinase mutant proteins generated in the present study may be useful tools to analyze the molecular aspects and the dynamics of glucokinase translocation in hepatocytes by live-cell–imaging techniques. Glucokinase mutants with different binding affinities for GRP will help to clarify the mechanism of nuclear export in response to changes of the glucose and fructose concentrations, an issue that is currently a matter of investigation in various experimental approaches (20,22,43,46).

Recently, different small chemical compounds have been described that are able to activate glucokinase (36,48–50). While only some of these glucokinase activators increase the V_{max} of glucokinase (36,48,50), all of them decrease the $S_{0.5}$ of the enzyme for the substrate glucose. The effect on the glucokinase-GRP interaction has been analyzed for two different glucokinase activators. The affinity of glucokinase for GRP was not affected by GKA1 (49), whereas RO-28-1675 reversed the inhibitory action of GRP (48). The future development of compounds, which modulate the glucokinase-GRP–binding affinity, will open the perspective of a selective activation of liver glucokinase, as there is so far only marginal evidence for a physiological role of GRP in pancreatic β-cells (10,29). The activation of glucose metabolism in liver represents a promising therapeutic concept to counteract the imbalance between glycolysis, glycogen synthesis, and hepatic glucose production in type 2 diabetes. Perspectively, the described glucokinase mutant proteins may help to elucidate the molecular mechanisms of glucokinase activators within the process of GRP binding and glucokinase translocation.

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