Novel insights into the regulation of the bound and diffusible glucokinase in MIN6 beta cells

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Running title: GK regulation in MIN6 beta cells

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GK, glucokinase; EYFP, enhanced yellow fluorescent protein; ECFP, enhanced cyan fluorescent protein; PS-CFP, photoswitchable cyan fluorescent protein; FRET, fluorescence resonance energy transfer
Abstract

A stable MIN6 beta cell clone overexpressing glucokinase as an ECFP fusion construct was generated for analysis of glucokinase regulation in these glucose-responsive insulin-secreting cells. A higher glucokinase enzyme activity accompanied by an improved glucose-induced insulin secretion indicated the integration of ECFP-GK into the functional pool of glucokinase protein in MIN6-ECFP-GK cells. Fluorescence recovery after photobleaching experiments of MIN6-ECFP-GK cells and photoactivation of a transiently transfected PS-CFP-GK construct in MIN6 cells indicate a higher motility of the diffusible glucokinase fraction at high glucose concentrations. In agreement with previous studies, we observed significant binding of ECFP-GK to insulin secretory granules. Using fluorescence lifetime imaging, we obtained evidence for an association between glucokinase and alpha-tubulin in MIN6-ECFP-GK cells. Furthermore, immunohistochemistry and fluorescence resonance energy transfer analysis by acceptor photobleaching showed distinct association between endogenous glucokinase and alpha-tubulin as well as beta-tubulin in MIN6 cells. Interestingly, glucokinase was also colocalized with kinesin, a motor protein involved in insulin secretory granule movement. Therefore, we suggest a role of a bound glucokinase protein fraction in the regulation of insulin granule movement along tubulin filaments.
The glucose phosphorylating enzyme glucokinase exhibits with its sigmoidal saturation curve and its low affinity for glucose specific kinetic properties within the mammalian hexokinase family (1). The enzyme is expressed in pancreatic beta cells, liver, endocrine cells of the gut, pituitary gland and brain and regulated in a tissue specific manner (2-8). In contrast to the high expression level of glucokinase in liver, a lower amount of glucokinase is sufficient in the beta cell to maintain its important role as a glucose sensor for metabolic stimulus-secretion coupling (9-11). Glucose mediates posttranslational glucokinase regulation in beta cells primarily by inducing a conformational change of the enzyme. The crystallographic characterization of the glucokinase structure demonstrated, that the specific transition between the “super open” and the “closed” conformation plays an important role in the regulation of the enzyme (12). The interaction with intracellular structures is another crucial element of glucokinase modulation in beta cells. Various studies provided evidence for glucokinase binding to proteins and cytoplasmic organelles (11; 13-24). The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase is an activating glucokinase binding partner in beta cells, which may act through stabilization of glucokinase (18; 22; 23). While it was shown by different experimental approaches that glucokinase binds to insulin secretory granules (14-16; 19-21), the identification of an association between glucokinase and mitochondria, as demonstrated in the liver (25; 26), remains inconclusive in beta cells (24; 27). Furthermore, actin filaments have been postulated as a glucokinase-binding anchor (28). Therefore further experimentation is required to develop an integrative concept of posttranslational glucokinase regulation in beta cells.

In the present study, we characterized an insulin-secreting MIN6 cell clone stably overexpressing an ECFP-GK fusion construct and analyzed glucokinase distribution and regulation by fluorescence microscopy techniques in MIN6 cells. The results show that glucokinase overexpression also as a fluorescent fusion construct improved the coupling between glucose metabolism and insulin secretion and indicate a glucose dependent metabolic channeling of glucokinase in MIN6 cells. Furthermore, we demonstrate that glucokinase associates with tubulin. Since glucokinase also binds to insulin granules, these data suggest that a bound glucokinase fraction may play a role in the regulation of insulin granule movement along tubulin filaments.

RESEARCH DESIGN AND METHODS

Materials. Restriction enzymes and modifying enzymes for the cloning procedures were from New England Biolabs (Beverly, MA) or Fermentas (St. Leo-Rot, Germany). The enhanced chemiluminescence (ECL) detection system and autoradiography films were from Amersham Pharmacia Biotech (Freiburg, Germany). All reagents of analytical grade were from Merck (Darmstadt, Germany). All tissue culture equipment was from Invitrogen (Karlsruhe, Germany) and Greiner-Bio One (Frickenhausen, Germany).

Cell culture and pseudoislet formation. MIN6 cells (passages 35 – 45) were grown in DMEM medium supplemented with 25 mmol/l glucose, 10% (v/v) fetal calf serum and penicillin and streptomycin in a humidified atmosphere at 37°C and 5% CO₂. MIN6 pseudoislets were generated by culturing 500,000 MIN6 cells in an uncoated 10 cm plastic dish. After formation, the pseudoislets were passaged weekly and grown for 4 – 5 weeks in DMEM medium supplemented with 25 mmol/l glucose, 10% (v/v) fetal calf serum and penicillin and streptomycin.

Stable overexpression of ECFP-GK and ECFP in MIN6 cells. The pECFP-C1 (Clontech Mountain View, CA) vector was used for expression of the fluorescence
protein ECFP and the previously generated pECFP-C1-GK vector (29) for expression of a fusion construct comprising ECFP and glucokinase. MIN6 cells were transfected with the vector DNA by the use of jetPEI™ (Qbiogene, Montreal, Canada) as described in the manufacturer’s manual. Positive clones were selected through resistance against G418 (1100 µg/ml) and characterized further for ECFP-GK and ECFP expression by fluorescence microscopy and Western blot analyses. Cell viability was determined using a microtitre plate-based MTT assay as described (30). In the present study we used the MIN6-ECFP-GK cell clone K3 and the MIN6-ECFP cell clone K8. For fluorescence recovery after photobleaching experiments cells were seeded at a density of 7 x 10^4 cells on 35 mm collagen coated glass bottom dishes (MatTek, Ashland, MA) and were cultured for 48 h. Thereafter cells were incubated for 3 h in DMEM medium without phenol red at the indicated glucose concentration.

Assay of glucokinase enzyme activity and Western blot analyses. For glucokinase activity measurements and glucokinase and ECFP Western blot analyses cells were homogenized in phosphate buffered saline (pH 7.4) and insoluble material was pelleted by centrifugation. The protein concentration was quantified by a Bio-Rad protein assay. Glucokinase enzyme activity was measured in soluble fractions by an enzyme-coupled photometric assay as described previously (31). For Western blot analyses 40 µg cellular protein was fractionated by reducing 10% SDS-PAGE and electroblotted to polyvinylidene difluoride (PVDF) membranes. The membranes were stained by Ponceau to verify the transfer of comparable amounts of cellular protein. Nonspecific binding sites of the membranes were blocked by non-fat dry milk overnight at 4°C. Glucokinase immunodetection was performed as described (11). For ECFP the blots were incubated with the BD Living Color A.v. antibody recognizing wild-type GFP and its enhanced color variants (Clontech Mountain View, CA) at a dilution of 1:5,000, followed by a 2-h incubation period with an anti mouse IgG peroxidase-labeled secondary antibody at a dilution of 1:10,000 at room temperature.

Insulin secretion. MIN6-ECFP-GK and MIN6-ECFP pseudoislets were washed in Krebs Ringer buffer. Thereafter 100 pseudoislets were hand selected and perfused in a specially designed chamber (32). Perfusion was performed in a closed system at 37°C with 95% O2 and 5% CO2 in bicarbonate-buffered Krebs Ringer solution supplemented with 0.1 % albumin and without glucose or with 20 mmol/l glucose or 40 mmol/l KCl as indicated (33). Insulin was measured radioimmunologically with rat insulin as a standard. Insulin content was determined in the soluble fraction of homogenized pseudoislets.

Transient transfection of MIN6 cells. The vectors pEYFP-N1, pEYFP-Mito and pEYFP-Tub (Clontech Mountain View, CA) were used for ubiquitous and cell organelle localized expression of the fluorescence protein EYFP, respectively. The vectors pEYFP-phogrin (34) were used for localization of insulin granules. The pPS-CFP2-C vector (Evrogen, Moscow, Russia) was used for expression of the photoswitchable cyan fluorescent protein PS-CFP. The cDNA of beta cell GK wild-type was subcloned in frame (Smal and BamHI restriction sites) to the photoswitchable cyan fluorescent protein (PS-CFP) into pPS-CFP2-C for expression of a fusion construct comprising PS-CFP and glucokinase. Cells were seeded at a density of 7 x 10^4 cells on 35 mm collagen coated glass bottom dishes (MatTek, Ashland, MA). After 48 h transfection was performed with 2 µg plasmid DNA and 4 µl jetPEI™ (Qbiogene, Montreal, Canada) according to manufacturer’s instructions. Cells were cultured for 48 h in DMEM medium with 25 mmol/l glucose and thereafter for 3 h in DMEM medium without phenol red at the indicated glucose concentration.

Immunostaining. MIN6 cells were seeded on collagen type I glass cover slips and grown for 48 h in medium with 25 mmol/l glucose.
Thereafter cells were fixed overnight with 4% paraformaldehyde in PBS at 4°C and immunostained as previously described (35) with rabbit glucokinase antibody (11) diluted 1:500 in PBS, guinea pig polyclonal insulin (ab-7842) antibody diluted 1:100 in PBS (Abcam, Cambridge, UK), goat polyclonal actin antibody (sc-1615) diluted 1:100 in PBS, goat polyclonal beta-tubulin antibody (sc-7396) diluted 1:50 in PBS, goat polyclonal gamma-tubulin antibody (sc-9935) diluted 1:100 in PBS (Santa Cruz, Santa Cruz, CA), mouse monoclonal kinesin antibody (K-1005) diluted 1:1000 in PBS, mouse monoclonal alpha-tubulin antibody (T-6199) diluted 1:500 in PBS (Sigma, St. Louis, MO) respectively. Thereafter cells were incubated with the appropriate secondary antibody, Cy2 or Cy3 donkey anti-rabbit antibody, Cy5 donkey anti-goat antibody, Cy5 donkey anti-mouse antibody, Cy5 donkey anti-guinea pig antibody, all diluted 1:200 in PBS (Jackson Immuno Research, West Grove, PA). Finally, cover slips were washed three times with PBS and fixed onto slides. With the exception of the samples used for acceptor photobleaching experiments ProLong antifade reagent was added to the mounting medium (Molecular Probes Invitrogen Detection Technologies, Eugene, OR).

Fluorescence microscopy. A cellR/ Olympus IX81 inverted microscope system, as described previously (29) was used, additionally equipped with a Cellcubator (Olympus) enabling full control of temperature, CO₂ and humidity. Glass bottom dishes and slides were fixed on the microscope stage and images were obtained with a PlanApo 100 x 1.45 NA oil-immersion objective. D 436/10-455DCLP-D480/40, HQ 500/20-530DCLP-D560/40, HQ480/20-Q495LP-HQ510/20, HQ620/60-Q660LP-HQ700/75 filter sets were used for ECFP, EYFP, Cy2 and Cy5, respectively (AHF Analysentechnik, Tübingen, Germany). Deconvolution was performed using AutoDeblur 9.3 WF software (MediaCybernetics, Silver Spring, MD). Colocalization was calculated with Image J 1.32 (W. Rasband, National Institutes of Health) using the plug-in module Colocalization Finder (C. Laumonerie, Strasbourg).

Fluorescence recovery after photobleaching and fluorescence distribution after photoconversion measurements were taken with an Olympus Fluoview1000 confocal inverted microscope using the multi-line argon laser (458nm, 488nm) to excite ECFP and green PS-CFP, and an UPLSAPO 60 x 1.35 NA oil-immersion objective. Emitted light of ECFP was detected at 475 – 575 nm wavelength range and of green PS-CFP at 510 – 610 nm. Scanning was performed with 2 μs/pixel sampling speed and 512 x 512 or 128 x128 image format. Bleaching and photoconversion was done during imaging with simultaneous scanner using tornado mode (5 % and 2 % intensity, respectively) or ROI mode (20 %) with a 405 nm laser diode (25 mW). Quantification of image intensities was done with Olympus FV10-ASW 1.4 software. Living cells were maintained at 37°C using a thermostatically controlled chamber. For fluorescence lifetime imaging the system was equipped with a TimeHarp 200 TCSPC board (PicoQuant, Berlin, Germany). ECFP was excited with a 440 nm Laser (40 MHz, <80 ps, 1-3 mW) and detected using a HQ 465/30 emission filter. Time resolved data from a 3 min acquisition period were analyzed with PicoQuant MicroTime 200 software and fluorescence lifetime was calculated from the TCSPC histogram with a tail-fit using a bi-exponential decay. Acceptor photobleaching experiments were performed with an Olympus Fluoview1000 confocal inverted microscope and an UPLSAPO 60 x 1.35 NA oil-immersion objective. Prebleach Cy3 and Cy5 images were collected simultaneously following excitation with the krypton argon laser (561nm, 1.5 % intensity). To photobleach Cy5 a rectangular region of interest was irradiated for 5 sec with the helium neon laser (633 nm, 100 % intensity). Immediately thereafter postbleach Cy3 and Cy5 images were collected with the same settings as the prebleach images. Fluorescence resonance energy transfer
(FRET) efficiency was calculated in a Cy3 present region inside the photobleached region after background correction as 100 X [(Cy3 Postbleach – Cy3 Prebleach)/Cy3Postbleach].

Statistical analyses. Data are expressed as means ± SEM. Statistical analyses were performed by ANOVA followed by Bonferroni’s test for multiple comparison or Student’s t test using the Prism analysis program (Graphpad Inc., San Diego, CA).

RESULTS

Stable overexpression of an ECFP-GK fusion construct in glucose responsive MIN6 cells. After transfection of the fluorescence protein ECFP and an ECFP-GK fusion construct into MIN6 cells, 10 overexpressing clones each were selected by antibiotic resistance and fluorescence microscopy (data not shown). The stable cell clones MIN6-ECFP-GK (K3) and MIN6-ECFP (K8) were chosen for further experiments. Western blot analyses revealed a comparable expression level of the ECFP fluorescence protein and the ECFP-GK fusion construct (Fig. 1A) as well as the specificity of the ECFP-GK fusion construct (Fig. 1B). The ECFP-GK was exclusively detectable in the cytoplasm, whereas the fluorescence protein ECFP was uniformly distributed in the whole cell. The MIN6-ECFP-GK cells showed a 14-fold increase in glucokinase enzyme activity in comparison to MIN6-ECFP cells (Fig. 1C) indicating an unrestricted kinetic activity of the ECFP tagged glucokinase. The $V_{\text{max}}$ value in MIN6-ECFP-GK cells was with 34 ± 2 units/mg protein (Fig. 1C) significantly higher than in MIN6 control cells with 2.4 ± 0.5 units/mg protein (data not shown) and in MIN6-ECFP cells with 2.5 ± 0.5 units/mg protein (Fig. 1C). The $S_{0.5}$ was comparable with 6.7 ± 0.4 mmol/l in MIN6-ECFP cells and 7.1 ± 0.3 mmol/l in MIN6-ECFP-GK cells. MIN6-ECFP-GK and MIN6-ECFP cells both cultured in medium supplemented with the antibiotic G418 showed a comparable cell growth (data not shown).

The increase of cell viability by 25 % at 25 mmol/l glucose in comparison to 3 mmol/l glucose in MIN6-ECFP-GK cells was also observed in MIN6-ECFP cells (22 %). Thus, 25 mmol/l glucose usually used for the culture of MIN6 cells provided optimal growth conditions.

Insulin secretion in MIN6-ECFP-GK pseudoislets in comparison to MIN6-ECFP pseudoislets. Both MIN6-ECFP-GK and MIN6-ECFP cells were able to form islet like structures in the same way as observed with non-transfected MIN6 cells. MIN6-ECFP-GK pseudoislets showed a comparable glucokinase enzyme activity as MIN6-ECFP-GK cells (data not shown). MIN6-ECFP-GK and MIN6-ECFP pseudoislets (Fig. 2) as well as MIN6-ECFP-GK and MIN6-ECFP cells (data not shown) are responsive to glucose and KCl. Notably, MIN6-ECFP-GK pseudoislets showed a significantly higher rate of insulin secretion than MIN6-ECFP pseudoislets in response to a 20 min perfusion phase with 20 mmol/l glucose, while insulin secretion in response to a 20 min perfusion phase with 40 mmol/l KCl, a non nutritional stimulus, was virtually identical in both cell types (Fig. 2A). On average MIN6-ECFP-GK pseudoislets showed a 50 % higher secretory response to glucose in comparison to MIN6-ECFP pseudoislets (Fig. 2B). The insulin content was not significantly different in MIN6-ECFP-GK and MIN6-ECFP cells (data not shown).

Interaction of glucokinase with subcellular structures in MIN6 cells. MIN6-ECFP-GK cells and MIN6-ECFP were additionally transfected with an EYFP-alpha-tubulin construct for localization of tubulin filaments, an EYFP-cytochrome-C-oxidase construct for localization of mitochondria or an EYFP-phogrin construct for localization of insulin granules. While ECFP-GK showed distinct colocalization with tubulin (Fig. 3A, 20.5 %) and insulin granules (Fig. 3B, 12 %), there was only little colocalization with mitochondria (Fig. 3C, 6.5 %). The fluorescence protein ECFP alone exhibited no colocalization with tubulin, insulin granules or mitochondria (data not shown).
Binding of ECFP-GK to insulin granules, tubulin filaments and mitochondria was further analyzed with the EYFP tagged constructs through lifetime FRET measurements. By interaction of the target proteins, the ECFP and EYFP tags get close to each other and the resulting energy transfer efficiency is detectable by a reduction in fluorescence lifetime of ECFP. To exclude interference through movement of insulin granules and mitochondria, the lifetime measurement was performed in the whole cytoplasm. Each TCSPC histogram was tail-fit with a bi-exponential decay for calculation of the average lifetime and the corresponding single lifetimes. The average reduced weighted chi-squared residual value for EYFP transfected cells was 1.031 ± 0.010, for EYFP-alpha-tubulin transfected cells 1.029 ± 0.016, for EYFP-phogrin transfected cells 1.021 ± 0.027 and for EYFP-cytochrome-C-oxidase transfected cells 1.025 ± 0.021. The ECFP lifetime is presented in this study for simplicity as a single value. The obtained ECFP lifetime value of control cells with 2.55 ± 0.03 is in agreement with previous reports (36-38). Because of a residual dimerization tendency of ECFP and EYFP the obtained lifetime values have to be estimated in relation to EYFP transfected control cells. Both the average lifetime (Fig. 4A) and the ECFP-GK specific lifetime (Fig. 4B) were significantly decreased in MIN6-ECFP-GK cells transfected with EYFP-phogrin and EYFP-alpha-tubulin in comparison to control cells indicating interaction of glucokinase with insulin granules and tubulin. The second lifetime (Fig. 4C) resulted from the autofluorescence of MIN6 cells and was comparable in all MIN6-ECFP-GK cells irrespective from the transfected construct.

Binding of endogenous glucokinase to subcellular structures was analyzed by immunohistochemistry and FRET in MIN6 cells. Glucokinase exhibited colocalization with insulin (Fig. 5A). Furthermore glucokinase showed distinct colocalization with alpha-tubulin (Fig. 5C) and beta-tubulin (Fig. 5D), but only minor colocalization with actin (Fig. 5B) and gamma-tubulin (Fig. 5E). Interestingly, glucokinase was colocalized with kinesin (Fig. 5F), a molecular motor protein involved in insulin granule movement in beta cells. Acceptor photobleaching was used to measure FRET between glucokinase/Cy3 and alpha-tubulin/Cy5 or beta-tubulin/Cy5. By interaction of the target proteins, the Cy3 and Cy5 tags are close to each other, then photobleaching of Cy5 fluorescence will result in enhanced Cy3 fluorescence, because of the dequenching of Cy3 following the removal of Cy5. FRET efficiencies measured in cells only immunolabeled with glucokinase/Cy3 or immunolabeled with glucokinase/Cy3 and Cy5 alone were below zero (data not shown). In MIN6 cells immunolabeled with glucokinase/Cy3 and alpha-tubulin/Cy5 (Fig. 6A, C) the FRET efficiency was 10.8 ± 0.4 % indicating direct association between glucokinase and alpha-tubulin. The amount of FRET observed in cells immunolabeled with glucokinase/Cy3 and beta-tubulin/Cy5 (Fig. 6B, D) was 5.0 ± 0.7 %.

Effects of glucose and mannoheptulose on the distribution and motility of glucokinase in MIN6 cells. The cytoplasmic ECFP-GK distribution was not significantly different in MIN6-ECFP-GK cells incubated for 3 h at 3 mmol/l or 25 mmol/l glucose (data not shown). Cytoplasmic motility of ECFP-GK was analyzed through fluorescence recovery after photobleaching. Bleaching was performed for 1.1 sec with a 405 nm laser in a small circle region, which were randomly placed in the cytoplasm. Recovery of ECFP-GK was measured in the region of photobleaching. MIN6-ECFP-GK cells showed with t½ ~ 0.6 sec a significantly faster ECFP-GK recovery after 3 h incubation at 25 mmol/l glucose than after 3 h incubation at 3 mmol/l (t½ ~ 1 sec) (Fig. 7). Notably, addition of 10 mmol/l mannoheptulose during the incubation period also increased the recovery of ECFP-GK compared to 3 mmol/l glucose alone (Fig. 7).
Furthermore, glucokinase motility was analyzed by photoconversion of a PS-CFP (39) fusion construct, transiently transfected in MIN6 cells (Fig. 8). Using an ECFP filter-set cyan PS-CFP-GK was detectable exclusively in the cytoplasm and cyan PS-CFP uniformly inside the whole cell, while no fluorescence was detectable after excitation with 488 nm laser light. Cells with a comparable cyan fluorescence intensity level were used for the experiments. Photoconversion was performed through irradiation with a 405 nm laser for 1.1 sec in a small circle region, which were randomly placed in the cytoplasm. Fluorescence intensity of green PS-CFP and PS-CFP-GK was tracked in the region of photoconversion and a region 7 µm and 15 µm distant from that region by excitation with 488 nm laser light. Distribution of photoswitched PS-CFP was comparable in MIN6 cells incubated for 3 h at 25 mmol/l (Fig. 8C) or at 3 mmol/l glucose (data not shown). PS-CFP appeared in the 15 µm distant region with t½ ~ 0.7 sec and equilibration of fluorescence intensity between all three regions appeared at ~ 25 sec. The cellular mean fluorescence intensity of green PS-CFP was at 25 sec 2822 ± 186. In contrast, PS-CFP-GK accumulated with t½ ~ 4 sec in the 15 µm distant region and the mean fluorescence intensity of green PS-CFP-GK was only 209 ± 202 in MIN6 cells incubated for 3 h at 25 mmol/l (Fig. 8A) and 1625 ± 194 in MIN6 cells incubated for 3 h at 3 mmol/l (Fig. 8B). Furthermore, PS-CFP-GK fluorescence intensity did not fully equilibrate inside the cell at 25 sec and was highest in the region of photoconversion. Interestingly, PS-CFP-GK fluorescence intensity was at 25 sec comparable in the region 7 µm and 15 µm distant from the region of photoconversion in MIN6 cells incubated for 3 h at 25 mmol/l (Fig.7A), but not in cells incubated for 3 h at 3 mmol/l (Fig. 8B).

Loss of green PS-CFP-GK in the region of photoconversion was further monitored by a faster sampling interval and photoconversion period of 188 msec (Fig. 8E) and compared to those of PS-CFP (Fig. 8D). Green PS-CFP left the region of photoconversion with t½ ~ 0.3 sec independent of the glucose concentration (Fig. 8D). In contrast, green PS-CFP-GK fluorescence in MIN6 cells decreased with t½ ~ 0.4 sec faster after 3 h incubation at 25 mmol/l glucose or after 3 h incubation at 3 mmol/l glucose plus 10 mmol/l mannoheptulose than after 3 h incubation at 3 mmol/l glucose (t½ ~ 0.6 sec) (Fig. 8E).

**DISCUSSION**

Glucokinase plays a key role in metabolic stimulus-secretion coupling in pancreatic beta cells (5; 6; 40). In the present study glucokinase regulation was analyzed in insulin-secreting MIN6 cells with special emphasis on glucokinase compartmentalization. In previous studies stable glucokinase overexpression in rat insulinoma cells has been successfully used to analyze glucose metabolism (11; 41) and fluorescence tagged glucokinase proofed to be a useful tool for glucokinase localization in short-term experiments (19; 20; 29; 42). By combination of both techniques we now generated a stable MIN6 cell clone expressing a fusion construct of ECFP and glucokinase. The exclusive cytoplasmic localization of ECFP-GK in these cells corresponds to the distribution of endogenous glucokinase in MIN6 cells (16).

As a control in our experiments we used a stable MIN6 cell clone with a matching expression level of the fluorescence protein ECFP alone.

In contrast to the monolayer MIN6 cell culture, primary beta cells are growing in a network inside the islet. MIN6 cells are able to form islet like structures (43; 44), called pseudoislets. We successfully generated such pseudoislets from stably transfected MIN6-ECFP-GK and MIN6-ECFP cells. While glucokinase enzyme activity in MIN6-ECFP cells and pseudoislets was comparable to control cells (21), MIN6-ECFP-GK cells and pseudoislets showed a significantly higher glucokinase enzyme activity similar to that in primary beta cells (11; 45). Furthermore MIN6-ECFP-GK pseudoislets showed a significantly better
insulin secretory response to glucose stimulation as compared to MIN6-ECFP pseudoislets, confirming on the one hand the integration of ECFP-GK into the functional pool of glucokinase protein and on the other hand the beneficial effect of glucokinase overexpression on glucose-induced insulin secretion in MIN6 cells.

To date, there are controversial reports on glucose toxicity in insulin-secreting cells. An increase in oxidative stress has been reported to aggravate glucose toxicity in INS1 and RIN1046-38 cells after overexpression of glucokinase (46), while in other studies the viability of RINm5F cells was found not to be affected by glucokinase overexpression (35). Moreover, experimental evidence has been provided that during apoptosis glucokinase expression decreases in cells treated with high glucose (24). However, in the present study both MIN6-ECFP-GK and MIN6-ECFP cells showed highest viability at 25 mmol/l glucose.

In insulin-producing beta cells glucokinase occurs in a diffusible and a bound fraction in the cytoplasm and different binding partners have been elucidated and proposed. Recent studies indicate that glucokinase together with the pro-apoptotic protein BAD (25) and also the glucokinase regulatory protein (26) associate with mitochondria in hepatocytes. A role of mitochondria as a glucokinase binding anchor in beta cells has also been considered but not confirmed experimentally (27). The conclusion by Kim and colleagues (24) that exposure to a chronically high glucose concentration decreases interaction between glucokinase and mitochondria remains speculative, since only colocalization but not interaction was demonstrated. Furthermore changes in EGFP-GK expression and distribution shown after 4 days may have simply been a result of the transient transfection method. In analogy to the applied MitoTracker probe (24) we used an EYFP-cytochrome-C-oxidase construct to label mitochondria. With this approach we discovered only little colocalization between ECFP-GK and mitochondria in MIN6-ECFP-GK cells. Furthermore, lifetime FRET measurements did not reveal binding between ECFP-GK and mitochondria using the EYFP-cytochrome-C-oxidase construct and exclude tight association of glucokinase with the mitochondria matrix. Therefore the involvement of mitochondria in glucokinase regulation remains unresolved.

Association of glucokinase with insulin secretory granules has already been intensely analyzed. It has been demonstrated that glucokinase shows a tight binding to the outer structure of the granules (21) and that interaction is regulated by insulin (19) and mediated by nitric oxide synthase (20). In contrast, studies dealing with glucokinase binding to actin and other filamentous structures in beta cells are scarce (28). Our present data reveal tubulin as a new glucokinase binding site. We showed an interaction between ECFP-GK and alpha-tubulin through lifetime FRET measurements. Identification of glucokinase binding to insulin granules in the same experimental approach can be regarded as a validation for our results. Furthermore, we demonstrated colocalization between endogenous glucokinase and both, alpha- and beta-tubulin, which are heterodimerized to microtubules in cells. The observed minor colocalization between glucokinase and gamma-tubulin might be due to a low expression level of this isoform in MIN6 cells. Colocalization between glucokinase and tubulin might represent a primary glucokinase binding site or may be caused by close vicinity of glucokinase attached to granules and the tubulin filaments. However, using acceptor photobleaching based FRET measurements we elucidated interaction between endogenous glucokinase and alpha-tubulin as well as beta-tubulin indicating at least in part a direct association. As with microtubules, actin is involved in cellular morphology, cell movement and intracellular transport. While a portion of glucokinase appears to be colocalized with actin filaments in the cytoplasm of cultured rat hepatocytes (28), we did not observe colocalization between
glucokinase and actin in MIN6 cells. Consistent with previous studies actin was mainly localized in MIN6 cells nearby the plasma membrane (47). It has been proposed that actin blocks the attachment of insulin granules to the plasma membrane (47). Interestingly, we detected in the present study a colocalization between glucokinase and kinesin, a motor protein, which moves cargoes along microtubules. It has been clearly shown by Varadi and colleagues, that insulin granule transport along tubulin filaments is mediated by kinesin I (48; 49). Therefore our results support the hypothesis, that tubulin and insulin granule bound glucokinase has an independent role in the regulation of insulin granule movement along tubulin filaments.

Analyses of MIN6-ECFP-GK cells after incubation at low and high glucose did not provide evidence for glucose-dependent differences in the localization of ECFP-GK. Furthermore, we studied glucokinase distribution and motility by fluorescence recovery after photobleaching, a technique, which was previously used to analyze glucokinase binding to insulin granules (19). Recovery of ECFP-GK after photobleaching occurs with $t_{1/2} \sim 0.6$ sec in MIN6 cells cultured for 3 h at 25 mmol/l glucose. Notably, our calculated recovery $t_{1/2}$ is dependent upon cell volume, culture conditions during the experiment, as well as bleaching setup and limited by the sampling interval of 1.1 sec. However, within the same experimental setup, but after incubation at 3 mmol/l glucose the recovery process was with $t_{1/2} \sim 1$ sec significantly slower. Small molecules like glucokinase with 55 kDa show unhindered diffusion in the cytoplasm. In contrast, molecules or complexes larger than ~ 200 kDa are hindered in their diffusion, presumably due to the cytoskeletal network of cells (50). While glucokinase in a complex with one or two other proteins is highly diffusible, diffusion of glucokinase may be impeded, when imbedded in a larger complex. Possibly, the decreased motility at low glucose is mediated by different binding of glucokinase in its open and closed conformation to cytoplasmic proteins in a metabolic channeling process. This hypothesis is supported by the fact, that mannoheptulose, a competitive inhibitor of glucokinase and stabilizer of the closed glucokinase conformation (45), is able to accelerate the glucokinase recovery process at 3 mmol/l glucose. However, to better analyze this aspect of glucokinase regulation we generated a glucokinase fusion construct with the newly developed monomeric photoconvertible protein PS-CFP (39). After irradiation with ~ 400 nm light distribution of the converted protein pool can be monitored by its green fluorescence. In contrast to PS-CFP alone, the PS-CFP-GK construct showed after 25 sec no full equilibration between the region of photoconversion and other regions in the cytoplasm, indicating, at least in some of the randomly chosen regions for photoconversion, a bound PS-CFP-GK fraction. Furthermore, the amount of photoconverted PS-CFP-GK was higher and equilibration was slightly faster in MIN6 cells incubated for 3 h at 25 mmol/l glucose in comparison to cells incubated at 3 mmol/l glucose. The decrease of photoconverted PS-CFP in the region of photoconversion was independent from the glucose concentration or the presence of mannoheptulose. Interestingly, photoconverted PS-CFP-GK decreased slower at 3 mmol/l glucose than at 25 mmol/l glucose or at 3 mmol/l glucose in the presence of mannoheptulose, providing further support for a role of glucose in regulating glucokinase motility.

At present, most of the molecular mechanisms underlying regulation of the diffusible and bound glucokinase fraction in pancreatic beta cells are unknown. Through elucidation of tubulin as a glucokinase binding anchor, we highlight a potential role of glucokinase in regulation of insulin granule movement along tubulin filaments. Whether glucokinase binding to alpha-tubulin is modified by alteration of microtubule polymerization and through glucose mediated changes in the motility of diffusible glucokinase has to be elucidated.
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in future studies. Prospectively, glucokinase fusion constructs with photoconvertible proteins as well as FRET measurements may help to further elucidate glucokinase binding, distribution and motility in beta cells in dependence on glucose and other compounds such as the new small chemical glucokinase activators (8).
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FIGURE LEGENDS

Fig. 1. Glucokinase enzyme activity and protein content of MIN6-ECFP-GK and MIN6-ECFP cells. For Western blot analyses, 30 µg of MIN6-ECFP-GK (lane 1) and MIN6-ECFP (lane 2) cellular protein were analyzed by immunoblotting using an antibody against glucokinase (A) or ECFP (B). Shown are representative blots from 3 independent experiments. Glucokinase enzyme activities (C) were measured spectrophotometrically in cell extracts of MIN6-ECFP-GK cells (black triangle) and MIN6-ECFP cells (black circles) after sonication. Shown are means ± SEM in mU/mg cellular protein from 5 independent experiments. * P < 0.05; ***p<0.001 compared with MIN6 ECFP cells (Student's t-test).
Fig. 2. Insulin secretion from MIN6-ECFP-GK and MIN6-ECFP pseudoislets. Perifusion data are depicted as a kinetic profile (A) and a median value for a 20 min stimulation with either 20 mmol/l glucose or 40 mmol/l KCl in comparison with a control perifusion phase without glucose (B). Shown are means ± SEM of insulin secretion rates expressed in ng insulin/µg cellular DNA from four independent experiments. *p<0.05; ***p<0.001; compared with the control perifusion phase at 0 mmol/l glucose (ANOVA/ Bonferroni’s test).
Fig. 3. Analysis of colocalization of ECFP-GK in MIN6-ECFP-GK cells with tubulin and cytoplasmic organelles. MIN6-ECFP-GK cells were additionally transfected with EYFP labeled constructs for localization of tubulin (A), mitochondria (B) and insulin granules (C) and cultured in medium with 25 mmol/l glucose. In the merged images EYFP is depicted in red and ECFP in green. Yellow color indicates colocalization. The depicted fluorescence images were obtained from z-stacks after deconvolution and are representative of 3 independent experiments. Scale bar, 10 µm.
Fig. 4. Analysis of interaction of ECFP-GK in MIN6-ECFP-GK cells with tubulin and cytoplasmic organelles. MIN6-ECFP-GK cells were additionally transfected with EYFP labeled constructs for localization of tubulin, mitochondria and insulin granules and cultured in medium with 25 mmol/l glucose. Thereafter fluorescence lifetime was measured with an Olympus Fluoview1000 confocal microscope and a PicoQuant TimeHarp 200 equipment. Shown are means ± SEM of the average lifetime (A) and single lifetimes from four individual experiments. Lifetime 1 (B) displays the ECFP-GK specific fluorescence lifetime and lifetime 2 (C) the MIN6 autofluorescence lifetime. *p<0.05; ** P < 0.01; ***p<0.001; compared with EYFP transfected cells (ANOVA/ Bonferroni’s test).
Fig. 5. Colocalization of endogenous glucokinase with the cytoskeleton and organelles in MIN6 cells. MIN6 cells were seeded on glass cover slips and grown for 48 h in medium with 25 mmol/l glucose. Finally, cells were fixed and immunostained for insulin (A), actin (B), alpha-tubulin (C), beta-tubulin (D), gamma-tubulin (E) and kinesin (F). Additionally cells were immunostained for glucokinase. In the merged images glucokinase is depicted in green and insulin, actin, alpha-tubulin, beta-tubulin, gamma-tubulin and kinesin in red. Yellow color indicates colocalization. The depicted fluorescence images were obtained from z-stacks after deconvolution and are representative for three independent experiments. Scale bar, 10 µm.
Fig. 6. Imaging FRET efficiencies of glucokinase and tubulin by acceptor photobleaching. MIN6 cells were seeded on glass cover slips and grown for 48 h in medium with 25 mmol/l glucose. Finally, cells were fixed and immunostained for alpha-tubulin (A, C) or beta-tubulin (B, D) and glucokinase. The appropriate secondary antibody was labeled with Cy3 for glucokinase and Cy5 for alpha-tubulin and beta-tubulin. Shown are merged images before (A, B) and after (C, D) photobleaching. Glucokinase is depicted in green and tubulin in red. FRET was measured as an increase in Cy3 fluorescence following Cy5 photobleaching. Shown are means ± SEM of 15 cells in 3 independent experiments.
Fig. 7. Effects of glucose and mannoheptulose on the fluorescence recovery of ECFP-GK after photobleaching in MIN6-ECFP-GK cells. Photobleaching and fluorescence recovery measurements were performed in a circle region randomly in the cytoplasm. The bleaching period is indicated by a grey background. MIN6-ECFP-GK cells were cultured in medium with 3 mmol/l glucose (white circle, dashed line), 25 mmol/l glucose (black circle, solid line), 3 mmol/l glucose plus 10 mmol/l mannoheptulose (white square, dashed line) or 25 mmol/l glucose plus 10 mmol/l mannoheptulose (black square, solid line). Shown are means ± SEM of 2 – 4 independent experiments. * $P < 0.05$ compared with cells cultured at 25 mmol/l glucose (Student’s $t$-test).
Fig. 8. Effects of glucose and mannoheptulose on the distribution of PS-CFP-GK and PS-CFP after photoconversion in MIN6 cells. Cells were cultured in medium with 3 mmol/l glucose (B) or 25 mmol/l glucose (A, C) and photoconversion of cyan PS-CFP-GK (A, B) and PS-CFP (C) was performed for 1.1 sec in a circle region. The activation period is indicated by a grey background. Distribution of green PS-CFP (C) and PS-CFP-GK (A, B) was measured in the region of photoconversion (black circle), a region 7 µm (white circle) and 15 µm (black square) distant from the region of photoconversion. Cells were cultured in medium with 3 mmol/l glucose (white circle), 25 mmol/l glucose (black circle), 3 mmol/l glucose plus 10 mmol/l mannoheptulose (white square) and photoconversion of cyan PS-CFP (D) and PS-CFP-GK (E) was performed for 188 msec in a circle region. The activation period is indicated by a grey background. Reduction of green PS-CFP (D) and PS-CFP-GK (E) was measured with a fast sampling interval of 188 msec in the region of photoconversion. Shown are means ± SEM of 3 independent experiments.