In Vivo Imaging of the Dynamics of Glucose Uptake in the Cytosol of COS-7 Cells by Fluorescent Nanosensors

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Glucose homeostasis is a function of glucose supply, transport across the plasma membrane, and metabolism. To monitor glucose dynamics in individual cells, a glucose nanosensor was developed by flanking the Escherichia coli periplasmic glucose/galactose-binding protein with two different green fluorescent protein variants. Upon binding of substrate the FLIPglu-170n sensor showed a concentration-dependent decrease in fluorescence resonance energy transfer between the attached chromophores with a binding affinity for glucose of 170 nM. Fluorescence resonance energy transfer measurements with different sugars indicated a broad selectivity for monosaccharides. An affinity mutant with a $K_d$ of ~600 μM was generated, which showed higher substrate specificity, and thus allowed specific monitoring of reversible glucose dynamics in COS-7 cells in the physiological range. At external glucose concentrations between 0.5 and 10 mM, reflecting typical blood levels, free cytosolic glucose concentrations remained at ~50% of external levels. The removal of glucose lead to reduced glucose levels in the cell, demonstrating reversibility and visualizing homeostasis. Glucose levels dropped even in the presence of the transport inhibitor cytochalasin B, indicating rapid metabolism. Consistently, the addition of 2-deoxyglucose, which is not recognized by the sensor, affects glucose uptake and metabolism rates. Within the physiological range, glucose utilization, i.e. hexokinase activity, was not limiting. Furthermore, the results show that in COS-7 cells, cytosolic glucose concentrations can vary over at least two orders of magnitude. The glucose nanosensor provides a novel tool with numerous scientific, medical, and environmental applications.

Tissues of multicellular organisms depend on continuous glucose supply. Typically, glucose utilization by a cell is a function of transport and metabolism. Most mammalian cells transport hexoses into or out of the cytosol down the concentration gradient by facilitated diffusion. In humans, transmembrane flux is mediated by a family of monosaccharide facilitators with 13 members differing in expression pattern, substrate specificity, and kinetic properties. Human GLUT1, one of the best-characterized family members, functions as a tetramer (1). Glucose transport is mediated by a fixed-site carrier mechanism, which simultaneously presents cooperative sugar import and export sites (2). Although transport by GLUT1 is intrinsically symmetric (3), kinetic properties for glucose import and export differ and depend, e.g. on redox status or ATP levels (4). Cytochalasin B as an inhibitor of GLUT-mediated transport interacts with the substrate efflux site (2), thus acting as a noncompetitive inhibitor for uptake (5) and competitive inhibitor for release (6, 7). Subsequent to uptake, phosphorylation by hexokinases takes place followed by the further glycolytic steps.

Mammals possess efficient sensing systems to maintain constant blood glucose levels. In pancreatic $\beta$-cells, glucose induces insulin secretion by a pathway involving glucose uptake by GLUT2 and metabolism (8, 9). Glucose sensing is thought to be mediated by specific hexokinases, e.g. by Hkx2 in the yeast model system (10) and by human glucokinase (8), which is able to replace sugar sensing functions of yeast Hkx2 (11). Additionally, GLUT2 serves as a sensor-controlling gene expression in response to external glucose supply (12). The hepatoportal glucose-sensing system appears to be critical for hypoglycemic detection and for eliciting sympathoadrenal responses (13) and also requires GLUT2 (14). Still, in healthy individuals, blood glucose can vary, potentially affecting glucose homeostasis of individual cells.

At least three rate constants determine glucose homeostasis: uptake, efflux, and phosphorylation. Phosphorylation is primarily a function of the cytosolic glucose, whereas facilitated diffusion depends on both cytosolic and external levels. Accordingly, steady-state levels of free cytosolic glucose will be reached when the rate of glucose uptake equals the sum of the rates of release and metabolism (15). External glucose supply will not become rate-limiting to glycolysis and will not affect glucose homeostasis until cytosolic glucose concentrations fall to levels close to or below hexokinase affinity. To be able to monitor homeostasis and perturbation, it would be useful to develop methods for directly measuring glucose levels in living cells with temporal and cellular or even subcellular resolution. Although NMR and Positron Emission Tomography methods are available, they are demanding, e.g. they require labeling with fluoroglucone (16, 17). Protein-based fluorescent nanosensors might provide novel tools for imaging of metabolites such as glucose (18, 19).

Bacterial periplasmic-binding proteins (PBP) constitute a...
large family of diverse ion or metabolite binding systems. PBPs were successfully employed as binding moieties for biosensors by liganding with fluorophores (20, 21). Although these biosensors are suitable for in vitro applications, liganded proteins are not suitable for in vivo imaging. As shown recently, the substrate-induced FRET change between two GFP variants attached to the Escherichia coli periplasmic maltose-binding protein (MBP) can be used to visualize maltose uptake into living yeast in real time (22). Although PBPs have little sequence homology and extensive sequence length variation, they share high tertiary structure similarity (23). Each ellipsoidal binding protein consists of two globular domains. The binding site is located in the cleft between the domains, and upon binding, the two domains engulf the substrate and undergo a hinge-twist motion (24). PBPs can be divided into two types based on different topological arrangements of the central β-sheets and position of the termini (23). MBP belongs to type II binding proteins with termini being located at the distal ends of the lobes relative to the hinge region. A comparison of the crystal structures of bound and unbound states shows that the hinge-twist motion brings the termini closer together. Consistently, in the case of FLIPmal (22), the decrease in distance leads to increasing FRET between attached chromophores (Fig. 1A). In GGBP (a type I PBP), termini are located at the proximal ends of the two lobes (25, 26). Thus, because of the different chromophore positions, the substrate-induced hinge-twist motion is predicted to move the attached chromophores further apart, causing a decrease in FRET. To demonstrate that type I PBPs can also be used as binding moieties, a FRET-based glucose sensor was developed by flanking GGBP with two GFP variants. To perform in vivo imaging at physiological concentrations, affinity mutants were generated. The FLIPglu-600µ mutant was used to determine free steady-state glucose concentrations in the cytosol of COS-7 cells at different levels of extracellular supply. The rapid decrease of cytosolic glucose levels in the presence of the transport inhibitor cytochalasin B indicates rapid glucose consumption, consistent with the high affinity of hexokinases in COS-7 cells. Consistently, the addition of 2-deoxyglucose alters the rates of glucose accumulation and consumption in living cells.

EXPERIMENTAL PROCEDURES

FLIP Constructs and Plasmids—A cassette was constructed using an enhanced cyan fluorescent protein PCR product following by a linker and an enhanced yellow fluorescent protein PCR product (Clontech). A mgIβ PCR product encoding mature GGBP (positions 70–927 relative to ATG) was fused between the two GFP genes. The chimeric gene was inserted into pRSET (Invitrogen) or pcDNA3.1 (Invitrogen) and transferred to E. coli BL21(DE3)Gold (Stratagene) and COS-7 cells. The mutations F16A and D236A were generated using QuikChange (Stratagene). FLIPglu-170n. GGBP binds its substrates by liganding with fluorophores (20, 21). Although these biosensors are suitable for in vitro applications, liganded proteins are not suitable for in vivo imaging. As shown recently, the substrate-induced FRET change between two GFP variants attached to the Escherichia coli periplasmic maltose-binding protein (MBP) can be used to visualize maltose uptake into living yeast in real time (22). Although PBPs have little sequence homology and extensive sequence length variation, they share high tertiary structure similarity (23). Each ellipsoidal binding protein consists of two globular domains. The binding site is located in the cleft between the domains, and upon binding, the two domains engulf the substrate and undergo a hinge-twist motion (24). PBPs can be divided into two types based on different topological arrangements of the central β-sheets and position of the termini (23). MBP belongs to type II binding proteins with termini being located at the distal ends of the lobes relative to the hinge region. A comparison of the crystal structures of bound and unbound states shows that the hinge-twist motion brings the termini closer together. Consistently, in the case of FLIPmal (22), the decrease in distance leads to increasing FRET between attached chromophores (Fig. 1A). In GGBP (a type I PBP), termini are located at the proximal ends of the two lobes (25, 26). Thus, because of the different chromophore positions, the substrate-induced hinge-twist motion is predicted to move the attached chromophores further apart, causing a decrease in FRET. To demonstrate that type I PBPs can also be used as binding moieties, a FRET-based glucose sensor was developed by flanking GGBP with two GFP variants. To perform in vivo imaging at physiological concentrations, affinity mutants were generated. The FLIPglu-600µ mutant was used to determine free steady-state glucose concentrations in the cytosol of COS-7 cells at different levels of extracellular supply. The rapid decrease of cytosolic glucose levels in the presence of the transport inhibitor cytochalasin B indicates rapid glucose consumption, consistent with the high affinity of hexokinases in COS-7 cells. Consistently, the addition of 2-deoxyglucose alters the rates of glucose accumulation and consumption in living cells.

RESULTS

Construction of a Glucose Sensor—Mature GGBP was flanked with two GFP variants by attaching a cyan fluorescent protein (CFP) and a yellow fluorescent protein (YFP) to the N and C termini, respectively. Because of the relative positions of the termini to the hinge region of GGBP, the substrate-induced hinge-twist motion is predicted to move the chromophores further apart and cause a decrease in FRET. To demonstrate that type I PBPs can also be used as binding moieties, a FRET-based glucose sensor was developed by flanking GGBP with two GFP variants. To perform in vivo imaging at physiological concentrations, affinity mutants were generated. The FLIPglu-600µ mutant was used to determine free steady-state glucose concentrations in the cytosol of COS-7 cells at different levels of extracellular supply. The rapid decrease of cytosolic glucose levels in the presence of the transport inhibitor cytochalasin B indicates rapid glucose consumption, consistent with the high affinity of hexokinases in COS-7 cells. Consistently, the addition of 2-deoxyglucose alters the rates of glucose accumulation and consumption in living cells.
hydrogen-bonding and stacking forces among aromatic amino acid residues in the binding site (29). Mutation of phenylalanine-16 of GGBP into alanine produced FLIPglu-600\(\mu\), which had a binding constant of 0.59 mM for glucose, thus providing a range for glucose quantification at least between 0.07 and 5.3 mM. Furthermore, the higher maximum ratio change of 0.29 in FLIPglu-600\(\mu\) permits more sensitive measurements than FLIPglu-170n (Fig. 1C and Table 1). The Hill coefficient was determined as 0.99, consistent with the formation of a 1:1 FLIPglu-600\(\mu\)/glucose complex. The affinity for galactose with a \(K_d\) of 0.85 mM was also reduced as compared with FLIPglu-170n (Table 1). Spectra at three different glucose concentrations (zero, \(K_d\), saturation) showed the same isosbestic point and high overall FRET efficiency as FLIPglu-170n. Apart from glucose and galactose, none of the other sugars tested induced a significant decrease in ratio at 1 and 10 mM concentrations (Fig. 2B). Only ribose, xylose, lactose, and melezitose led to decreased ratios at non-physiological concentrations of 100 mM (30–50% of the ratio change at saturating glucose concentrations). Thus, in addition to its decreased binding affinity and its increased sensitivity as compared with FLIPglu-170n, FLIPglu-600\(\mu\) displays higher substrate selectivity than FLIPglu-170n. Because glucose is expected to be present in significantly higher concentrations as compared with other monosaccharides, FLIPglu-600\(\mu\) is thus suitable for glucose monitoring in living cells.

**In Vivo Characterization of FLIPglu-600\(\mu\)—For in vivo characterization, FLIPglu-600\(\mu\) was expressed in the cytosol of cultured Green African monkey kidney-derived COS-7 cells. FRET was imaged microscopically by excitation at 436 nm and high speed switching between emission filters for YFP and CFP. Emission intensity images were recorded using a digital camera, and ratio images were calculated on a pixel-by-pixel basis. To quantify the ratios obtained by microscopic imaging, data were integrated over entire cells. Merged YFP-CFP emission intensity images showed FLIPglu-600\(\mu\) expression in the cytosol but neither in the polyploid nuclei nor in lysosomes (Fig. 3A). Overall, the nanosensor appeared to be equally distributed within the cytosol with lower intensities at the periphery, but probably because of its large molecular mass, it is excluded from entry into the nucleus as would normally be expected for GFP distribution (30). However, emission intensities were uneven levels roughly corresponding to local differences in cell thickness, e.g. adjacent to the nucleus. The emission ratio intensity was increased both at the cell periphery and in regions around the nucleus. Further optimization of the imaging technology will be required to resolve potential local differences in glucose levels.

**Fig. 1. Substrate-induced FRET changes.** A, CFP and YFP were fused to N and C termini of PBPs. In MBP, the termini are located on the distal ends of the lobes relative to the hinge region. Upon the binding of maltose, chromophores come closer together and FRET increases. B, GGBP termini are positioned on the proximal ends. Thus, glucose binding moves chromophores further apart; hence, FRET decreases. C, using non-linear regression on glucose titration curves of purified FLIPglu-170n (red) and FLIPglu-600\(\mu\) (blue), binding affinities were determined as 170 nM and 589 \(\mu\)M, respectively. D, spectra at three different substrate concentrations (zero (black), \(K_d\) (red), saturation (blue)) share an isosbestic point at 503 nm. Because of close overall chromophore distance, CFP emission is low compared with YFP emission.

**Table 1**

Properties of the nanosensors

| Nanosensor           | \(K_d\) nanosensor\(\mu\) | \(K_d\) binding protein\(\mu\) | Range for quantification\(\mu\) | \(\Delta r_{\text{max}}\)\(\mu\) |
|----------------------|----------------------------|-------------------------------|--------------------------------|---------------------|
| FLIPglu-170n (WT)    | Glucose 0.17                | Glucose 0.21                  | 0.019–1.53                     | 0.23                |
| FLIPglu-600\(\mu\) (P16A) | Galactose 0.51             | Galactose 0.48                | 65.4–5301                      | 0.29                |
| FLIPglu-control (D236A) | Galactose > 100 mM      |                               |                                |                     |

\(a\) \(K_d\) of the nanosensors determined by FRET.

\(b\) \(K_d\) of the binding protein alone (28).

\(c\) Range of concentration for which a nanosensor can be used. The range for quantification was defined as the range between 10 and 90% saturation.

\(d\) \(\Delta r_{\text{max}}\) in vitro maximum change in ratio between absence and saturation of the binding protein.
To keep the initial glucose concentration low, growth medium was replaced by glucose-free solution immediately before measurements. Within <30 s after the addition of 10 mM external glucose, a decrease in ratio was observed, thus visualizing glucose import by FLIPglu-600μ directly (Fig. 3B). More rapid measurements will be required to resolve the rate of uptake. During continuous external supply, the ratio remained constant. The addition of glucose to a concentration of 20 mM did not further decrease the ratio, demonstrating that the sensor was already saturated at 10 mM external glucose (Fig. 3B). To demonstrate reversibility, external glucose was removed by perfusion with glucose-free medium after the addition of 10 mM glucose and glucose detection inside the cytosol (Fig. 3C and supplemental movie). After washing out glucose, the emission intensity ratio increased within <30 s after a lag period, showing that cytosolic glucose concentration decreased rapidly when external glucose was removed. Thus, glucose binding in vivo is reversible. Consistent with the increased distance between the fluorescent moieties, glucose led to a decrease in YFP and a corresponding increase in CFP emission intensity (Fig. 3D). The sugar-independent slow decrease in fluorescence intensity was attributed to photobleaching induced by the frequent measurements as previously observed for Ca^{2+} measurements using the Cameleon (31). Consistent with the in vitro data, neither the addition of 10 mM maltose, trehalose, arabinose, nor ribose affected the ratio, demonstrating that the decrease in ratio of FLIPglu-600μ emission was the result of glucose concentration changes (Fig. 3E).

**Generation of a Control Sensor**—Because analyte detection by the sensors depends on substrate-induced conformational changes, FLIPglu-600μ conformation might respond to changes in other parameters, e.g. pH or ionic strength. To exclude artifacts attributed to indirect effects, a control sensor (FLIPglu-control) was generated by mutagenesis of aspartate 236 to alanine in GGBP, a side chain involved in the binding of glucose by hydrogen bonding (29). FLIPglu-control did not show a decrease in ratio up to 100 mM glucose (data not shown). As in the case of FLIPglu-600μ, merged YFP-CFP emission intensity images showed unequal intensity in the cytosol and exclusion from nuclei (Fig. 4, A and B). External supply of 10 mM external glucose did not lead to a change in ratio of FLIPglu-control-expressing cells. Furthermore, cells expressing FLIPglu-170n did not show any response to changes in external glucose concentrations, indicating that cytosolic sugar concentrations never dropped to values below 1.5 μM, i.e. fully saturated FLIPglu-170n (Fig. 4C). Taken together, these controls demonstrate that the ratio changes observed by FLIPglu-600μ were specific for glucose.

**Cytosolic Glucose Concentrations at Different External Levels**—To quantify cytosolic glucose concentrations, cells expressing FLIPglu-600μ were perfused with increasing concentrations of external glucose in a step protocol (Fig. 5A). After a
constant ratio was reached for each concentration, glucose was removed by perfusion with glucose-free solution. Again, frequent exposures over long observation times lead to photobleaching as indicated by a continuous decrease in baseline. Perfusion with 0.1 mM glucose had no effect on the ratio, whereas stepwise increments of external levels between 0.5 and 10 mM led to reversible and increasing ratio changes (Fig. 5A). The ratio changes (Δ ratio) followed a hyperbolic curve, reflecting saturation of FLIPglu-600/H9262 (Fig. 5B). As an internal calibration, it was necessary to determine both the minimum ratio change in the absence of glucose (Δrmin) and maximum ratio change (Δrmax) at glucose saturation using non-linear regression. Δrmin, Δrmax, and the Kd as determined in vitro were used to calculate cytosolic glucose concentrations (see Equations 3 and 4). Internal glucose levels increased with external concentrations but remained at ~50% relative to the external medium (Fig. 5C). Suggesting significant metabolism of glucose. Assuming symmetric transport, the ratio of the maximum glucose transport rate to the glucose consumption rate in COS-7 cells was determined as ~10 using non-linear regression (27). The lack of response to 0.1 mM glucose is thus consistent with the detection limit of FLIPglu-600μ (Table I) and indicates that in the absence of external glucose supply, cytosolic levels drop to very low concentrations, potentially in the Kd range of 50 μM for hexokinases present in COS-7 cells (32, 33). Additional affinity mutants will be required to resolve cytosolic concentrations below 65 μM. Taken together, the results show that in COS-7 cells, cytosolic glucose concentrations can vary over at least two orders of magnitude.

Glucose Consumption in Preloaded Cells—As an independent confirmation, glucose transport inhibitors were used to test whether FLIPglu-600μ actually responds to changes in glucose levels and to determine the relative role of metabolism and release of glucose under conditions where cytosolic concentrations drop. FLIPglu-600μ-expressing cells did not respond to glucose supply in the presence of cytochalasin B, an inhibitor of GLUT-mediated glucose transport (5–7), demonstrating that glucose uptake was impaired (Fig. 6). The inhibition was reversible because cells that had been washed for 9 min with inhibitor-free medium were able to take up glucose again as indicated by a decrease in ratio (Fig. 6). Subsequently, the same cells were loaded with 5 mM external glucose. After reaching a constant ratio, cytochalasin B was added again. Despite the block in transport, the emission ratio increased rapidly, reflecting rapid glucose utilization. The rapid metabolism determined by the nanosensor is consistent with the consumption rates of ~1 fmol glucose cell−1 min−1 (34). At least at the time resolution of the experiments shown, the rates of uptake and metabolism were comparable.

Glucose Dynamics in the Presence of 2-Deoxyglucose—Glucose analogs allow manipulation of the rates of uptake and metabolism. 2-Deoxyglucose (DG), which is transported into the cell by GLUT transporters, is phosphorylated by hexokinase in the cytosol (35) and thus acts as competitive inhibitor of glucose transport and phosphorylation. The reaction product 2-deoxyglucose-phosphate in turn inhibits phosphoglucose isomerase. Thus, in the presence of DG, glucose 6-phosphate accumulates and inhibits hexokinase (36). To determine whether the nanosensor discriminates DG, purified FLIPglu-600μ was incubated with DG and 3-O-methylglucose. Neither analog induced a significant decrease in ratio at 1 and 10 mM concentrations (Fig. 7A). Only at non-physiologically levels of 100 mM, ratio changes for DG and 3-O-methylglucose were 50 and 30% of the ratio change at saturating glucose concentrations, respectively.

To analyze the effect of DG on glucose uptake and metabolic rates, cells expressing FLIPglu-600μ were perfused with glucose in the presence and absence of DG (Fig. 7B). In the absence of DG, rates for glucose uptake (ratio change 0.14/min) and for decrease of cytosolic glucose levels after washing with glucose-free solution (ratio change 0.13/min) were similar. During initial perfusion in the presence of DG, the rate for glucose uptake (ratio change 0.08/min) decreased significantly, reflecting inhibition of glucose transport by DG. Repeated perfusion with DG lead to increased rates of glucose uptake (ratio change 0.16/min). Increased uptake rates during the second perfusion with DG suggested that inhibition of glucose consumption exceeds the effect on transport activity. The rates of glucose consump-
In Vivo Imaging of Glucose Dynamics

A nanosensor for fluorescent imaging of metabolites in vitro, ex vivo, and in vivo was developed. The FLIPglu nanosensor was built on the basis of a mutated GGBP periplasmic glucose-binding protein. Conformational changes upon substrate binding are transformed into FRET changes between two GFP chromophores attached to N and C termini of the GGBP. FLIPglu-600µ covers at least a range of between 65 and 5300 µM glucose. Imaging of COS-7 cells expressing FLIPglu-600µ permitted dynamic real-time measurements of cytosolic glucose concentrations. The use of ultrahigh and ultralow affinity mutants together with pharmacological studies demonstrated the suitability and specificity of the nanosensor.

Minimally Invasive Metabolite Detection—Compartmentalization of metabolic reactions and thus transport within and between cells can only be understood if their subcellular distribution is established by non-destructive dynamic monitoring techniques. Most of the data on metabolite levels are derived from methods that require fixation or fractionation of tissue (37, 38) and which are static. Furthermore, currently available methods often have low resolution and are prone to artifacts, e.g. contamination from other cell types or compartments. Although NMR and Positron Emission Tomography methods permit measurement of glucose metabolism in living organisms even deep into the tissues, they are demanding, requiring the use of tracers, and do not provide cellular or subcellular resolution (16, 17). Thus, little is known about the dynamic changes in concentration of metabolites such as sugars and amino acids/neurotransmitters at the site of transport, e.g. cytosolic glucose levels in different cell types or neurotransmitter levels in the synaptic cleft relative to the cytosol of adjacent neurons and glial cells, but also regarding the distribution of different sugars between cellular compartments. The availability of FLIPglu and FLIPmal (22) nanosensors may therefore contribute to a better understanding of metabolism and compartmentalization.

Glucose Concentration in COS-7 Cells—All mammalian cells depend on continuous glucose and energy supply. By means of efficient sensing systems, blood glucose levels are maintained within the physiological range. Genetic disorders, in which control of glucose levels is disturbed, lead to severe symptoms (9, 39). Here, FLIPglu-660µ was used to measure glucose homeostasis in the cytosol of individual COS-7 cells. COS-7 cells express the glucose transporter GLUT1 (40). Pharmacological studies indicate that active transport via sodium/glucose co-transporter does not contribute significantly to glucose uptake (41). Hexokinase rather than glucokinase is used for glucose phosphorylation (32).

The import of externally supplied glucose into the cytosol was visualized and quantified. The removal of external glucose led to an increase in ratio, reflecting decreasing cytosolic glucose levels and reversibility of glucose binding to FLIPglu-600µ. The addition of other sugars not able to interact with the nanosensor did not change the ratio, indicating that the observed responses were the result of changes in free glucose levels in the cell. Gradual addition of increasing external concentrations showed that glucose was detected in a concentration-dependent manner. The ratio changes followed a hyperbolic curve, reflecting unpaired binding properties of the nanosensor in vivo. The saturation for uptake as estimated by modeling (data not shown) is consistent with the properties of GLUT1 (9). Controls using FLIPglu-control and saturated FLIPglu-170n ruled out that parameters other than glucose affected the response. Between 0.5 and 10 mM external supply, steady-state glucose concentrations in the cytosol were always ~50% of the external level, indicating rapid glucose utilization. The decrease of cytosolic glucose levels during inhibition of export by cytochalasin B confirmed that consumption of glucose occurs rapidly. The rapid rate of glucose metabolism is also supported by the effect of DG on glucose homeostasis. However, internal levels did not fall near the Km of hexokinase (50 µM) (33). Thus, the external supply within the physiological range did not limit glucose utilization. Similar results were obtained for NMR-based glucose measurements in the brain (11). As a next step, it would be interesting to study glucose levels in tissues or organs using transgenic animals, fungi, or plants.

Improvement of Glucose Imaging—Because PBPs are relatively small proteins, the expected change in distance upon substrate binding between the N and C termini to which the GFP variants were grafted is expected to be small, e.g. 1 nm for MBP (42, 43). Nevertheless, both MBP- and GGBP-based nanosensors show FRET changes upon binding, potentially indicating that other parameters, e.g. changes in the relative orientation of the chromophore dipoles, are involved (22). Because of the structural differences, FLIPmal displays lower overall FRET efficiency as compared with FLIPglu (Supplemental Fig. 1, A and B). Consistent with the position of the termini in the two PBPs, the binding of substrate increases FRET efficiency for FLIPmal and decreases for FLIPglu. FRET efficiency strongly depends on chromophore distance when it is close to the Förster distance (R0) (43). Thus, to explain the substrate-induced FRET change for both nanosensors, we have to assume that the chromophore distance in FLIPglu is lower than R0, whereas in FLIPmal distance is higher than R0. Upon substrate binding, the chromophore distance moves toward R0 in both nanosensors but in opposite directions (Supplemental Fig. 1C).

In vivo imaging of glucose concentrations requires that the nanosensors are specific. Unlike low affinity FLIPglu-600µ, high affinity FLIPglu-170n showed a decrease in ratio at concentrations of 1 and 10 mM when incubated with sugars other than glucose. Although the sugars used for analysis of substrate specificity were more than 99% pure, it cannot be excluded that contaminations with glucose are responsible for the observed ratio changes. Accordingly, the decrease in ratio of high affinity FLIPglu-170n at concentrations of 1 and 10 mM might not necessarily reflect low substrate selectivity. Still, the
recognition of sugars other than glucose inducing a decrease in ratio cannot be excluded. The addition of the same sugars to low affinity FLIPglu-600 μM did not lead to decreased ratios. Thus, in contrast to FLIPglu-170n, the binding of the tested sugars to FLIPglu-600 μM can be excluded, indicating that FLIPglu-600 μM can be used to specifically monitor glucose concentrations in living cells. Pseudocolored images of COS-7 cells expressing FLIPglu-170n, FLIPglu-600 μM, and FLIP-control showed uneven ratio distribution. Differences in fluorescence of CFP and YFP emission in the absence of external glucose seem to roughly correspond to local differences in cell thickness. Further optimization of the imaging system, e.g., confocal imaging or FLIM (44), will be required to resolve potential gradients within the cytosol. Such gradients may be expected due to the patterns of the subcellular distribution of hexokinases and glucokinases, which are bound to mitochondria or actin filaments depending on the physiological status of the cells (32, 45). Optical sectioning by confocal laser microscopy using a blue laser might solve this problem. An extended set of binding mutants would be useful to cover a wider range of glucose concentrations for determining glucose levels in different tissues. Furthermore, targeting sequences attached to FLIPglu can be used to direct them selectively to different subcellular compartments to measure glucose concentrations at a subcellular level.

CONCLUSIONS

This study together with a recent report on the development of chemically modified PBPs (20) indicates that many of the periplasmic binding proteins can be developed into nanosensors for a wide range of analytes. This assumption is supported by the fact that GGBP and MBP are basically unrelated at the primary sequence level (11.6% identity), and although they share similar tertiary structure, the proteins fall into two different structural families and/or types (23) in which the termini are located at different relative positions. In summary, the nanosensors were used to determine cytosolic glucose levels in living cells. The results suggest that cytosolic concentration can vary by several orders of magnitude depending on external supply. Because of the high affinity of hexokinase and a maximum transport to a maximum phosphorylation ratio of 10, external glucose supply was not limiting at physiological blood glucose levels. Interestingly, cytosolic levels responded rapidly to modulation of the external supply. Thus, FLIPglu-600 μM may provide a tool that might help toward a better understanding of the mechanisms of glucose sensing and metabolic disorders associated with glucose homeostasis in all classes of organisms.

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