Glucokinase (GK) activity is essential for the physiological regulation of insulin secretion by glucose. Because the enzyme exerts nearly total control over glucose metabolism in the β-cell, even small changes in GK activity exert effects on glucose-stimulated insulin secretion and, consequently, the blood glucose concentration. Using quantitative imaging of multicolor fluorescent proteins fused to GK, we found that the association of GK with insulin granules is regulated by glucose in the β-cell. Glucose stimulation increased the rate of fluorescence recovery after photobleaching of GK to insulin granules, indicating that GK is released into the cytoplasm after glucose stimulation. Changes in fluorescence resonance energy transfer between two different fluorescent protein variants inserted on opposing ends of GK were observed after glucose stimulation and correlated with increased enzyme activity. Furthermore, glucose-stimulated changes in GK regulation were blocked by two inhibitors of insulin secretion. Insulin treatment restored GK regulation in inhibited cells and stimulated GK translocation and activation by itself. Together, these data support a model for post-translational regulation of GK whereby insulin regulates both the association of GK with secretory granules and the activity of the enzyme within the pancreatic β-cell.

Glucokinase (GK) plays an essential role in glucose-stimulated insulin secretion (GSIS) from pancreatic β-cells by determining the rate of metabolic flux in response to changes in the plasma glucose concentration (1). Small changes in GK activity have large effects on the rate of GSIS, as indicated by studies of both humans with maturity onset diabetes of the young, type 2 (MODY2) (2–4), and tissue-specific gene knock-out mice (5). It has long been known that glucose is an important regulator of GK activity in the β-cell, although the mechanisms through which glucose acts to regulate GK activity in the β-cell are not yet understood. Several studies have suggested that glucose influences GK activity by affecting either the rate of gene transcription or by altering the stability of the enzyme itself (1, 6, 7). However, these studies were not designed to explore the regulation of GK at the post-translational level, as was suggested by several studies that reported glucose-sensitive changes in GK activity independent of changes in GK protein levels (8–10). Thus, post-translational regulation of GK in the β-cell has not been clearly established, nor has a feasible mechanistic explanation for these data emerged.

One potential avenue for post-translational modulation of GK function has arisen from recent observations that GK is associated with insulin secretory granules (11, 12). Because agonist-stimulated changes in the cellular localization of proteins is an important mechanism for regulating protein function (13), and because regulation of GK in the liver is known to involve flux between the cytoplasm and the nucleus (14, 15), the prospect of GK regulation in the β-cell involving reversible association with secretory granules presented an attractive hypothesis. GK association with a variety of bound and cytoplasmic compartments has been described in recent years (12, 16–18); however, these studies have not established a clear role for different GK-associated compartments in the response of the β-cell to glucose. Indeed, it is not known whether translocation between compartments even occurs. Thus, although evidence exists for the multicompartment localization of GK in β-cells, the significance of this compartmentalization, the mechanisms involved, and functional impact of alterations in GK localization remain controversial, if not wholly uncertain.

One potential explanation for the lack of consensus in examining GK localization arises from the low resolution methods employed in these studies, namely biochemical fractionation techniques and immunofluorescent microscopy. Low resolution techniques, such as immunolabeling (12) and biochemical fractionation (18), are of limited value in studying the association of GK with secretory granules because these techniques require the prospect of GK regulation in the β-cell to glucose. Indeed, it is not known whether translocation between compartments even occurs. Thus, although evidence exists for the multicompartment localization of GK in β-cells, the significance of this compartmentalization, the mechanisms involved, and functional impact of alterations in GK localization remain controversial, if not wholly uncertain.

Recent developments in fluorescent protein-based techniques, along with improvements in live cell imaging technology, now enable measurements of a wide variety of biochemical processes in living cells. One such technique that has been
successfully used to monitor changes in protein localization and mobility in living cells is fluorescence recovery after photobleaching (FRAP) (19–22). In FRAP, fluorophores in a region of the cell are irreversibly photobleached, which markedly reduces the signal in that region. As photobleached fluorophores diffuse away from the region, they are replaced by unbleached fluorophores that diffuse in from outside the photobleached region. Thus, the fluorescence signal recovers with time. If the photobleached fluorophores are bound to stationary intracellular components, then the fluorescence signal will not recover. Therefore, FRAP can be used to measure not only diffusion, but also binding sites within the cell. Another technique, fluorescence resonance energy transfer (FRET), has been used to expand the spatial resolution limit to the <100-Å range, thereby permitting detection of changes in protein conformation and protein-protein interactions (23–26). These two techniques allow effective quantification of subtle changes in protein localization and structure that are often masked by the signal to noise limitations, finite collection ranges, and the physical resolution limits of current microscope systems. Not surprisingly, these high resolution techniques, when used in conjunction with more traditional approaches, have led to major advances in our understanding of protein motion and localization in the context of their natural cellular environment.

Here, we have utilized FRAP, FRET, and biochemical fractionation to explore post-transcriptional regulation of GK in the β-cell. We report that, in the absence of glucose, GK is associated with insulin granules, as determined by FRAP assays and biochemical fractionation. Glucose leads to a release of GK from this bound state into the cytoplasm. Furthermore, the release of GK from the granule-bound state is accompanied by increased GK activity and a conformational change, as detected using FRET. We also found that either the inhibition of insulin secretion or insulin receptor function blocked glucose-stimulated GK translocation and conformational changes. Moreover, insulin was able to stimulate GK translocation and activation in the absence of glucose. Together, these results point to the regulation of GK localization and activity by insulin secreted from β-cells in response to glucose. These data provide an important advance to our understanding of GSIS and its association with hexokinase activity in our lysates.

EXPERIMENTAL PROCEDURES

Materials—Fluorescent protein expression vectors were obtained from BD CLONTECH. Fluorescently tagged secondary antibodies were obtained from Molecular Probes. Chemicals were from Sigma-Aldrich unless otherwise noted. Restriction enzymes were obtained from New England Biolabs. PCR primers were purchased from Integrated DNA Technologies. DNA isolation reagents were obtained from Qiagen. Optical filters were obtained from Chroma. Peroxidase-conjugated secondary antibodies were obtained from Jackson Immunoresearch. Cell culture reagents were obtained from Media and Reagents Core of the Diabetes Research and Training Center at Vanderbilt University.

Generation of Constructs—A cDNA encoding the rat β-cell GK isoform was inserted into the pEGFP-N3 vector. A pEYFP-N3 vector was made by inserting YFP from the pEYFP-N1 vector into the same position as the GFP in the N3 vector. β-Cell GK cDNA was then inserted into this vector to make GK-YFP. GFP-GK-YFP was made by transfer of GFP sequences from pECFP-N1 into the GK-YFP plasmid. The C-peptide GFP construct was made by inserting GFP into the murine proinsulin II cDNA at the native Xmal site in the C-peptide region of the gene. This construct was generated in two parts by splicing the N-terminal portion of the gene into pECFP-N3 (generated as described above for the pEYFP-N3 vector) and inserting the C-terminal portion into the pEYFP-C1 construct. The full-length construct was made by insertion of the BarG1, Xbal fragment of the C-terminal portion into the vector containing the N-terminal fragment. The dsRed C-peptide construct was made by removal of the fluorescent protein from the C-peptide GFP construct and insertion of dsRed cDNA. Details of the assembly and the plasmid maps are available upon request.

constructions were verified by sequencing reactions performed by the Vanderbilt-Ingram Cancer Center DNA Sequencing Shared Resource. Plasmid DNAs were introduced into βTC3 cells by 10 50-μg square wave pulses of 300 V at 500-ms intervals with a BTX ECM830 electro- 5 μg of each construct was used per 20% of a 90% confluent T-75 flask of βTC3 cells suspended in Dulbecco’s PBS (BioWhittaker) in a 2-mm gapped cuvette.

Microscopy and Spectroscopy—Cells were grown in Dulbecco’s modified Eagle’s medium containing 15% horse serum and 2.5% fetal bovine serum with high glucose and switched to 5 mM glucose medium 24 h prior to experimentation. Cells were starved for 2–3 h in glucose-free DMEM plus 0.1% bovine serum albumin (27) prior to experimentation. For FRET and fluorescence staining, cells were grown on coverslips and fixed for 30 min with fresh 4% paraformaldehyde in PBS at 4 °C. Cells were permeabilized with 0.1% Triton X-100, blocked with 3% bovine serum albumin, and stained with rabbit anti-GK (28) and/or guinea pig anti-insulin (Linco) where indicated. Anti-rabbit ALEXA-488 and anti-guinea pig ALEXA-546 were applied for the detection of GK and insulin immunostaining where appropriate. Visualization was with a Zeiss LSM510 microscope (63× 1.4 numeric aperture) using the 457-, 488-, and 514-nm argon lines and a 543-nm helium-neon laser to excite CFP, GFP/ALEXA-488, YFP, and dsRed/ALEXA-546, respectively. Emitted light was passed through bandpass filters for collection of CFP (470–510 nm), GFP/Alexa-488 (505–530 nm), and YFP (530–550 nm). Long-pass filters were used for collection of Alexa-546 (580 nm) and dsRed (590 nm). Examination of FRET and localization of the FRET ratio for granules was performed by selecting regions that were positive for dsRed fluorescence and calculating the ratio of YFP/CFP intensities in these regions using NIH Image software. Regions that contained no dsRed fluorescence were determined to be cytoplasmic areas. Pretreatment ratios from whole cells were normalized to 1.0 for comparison purposes in Fig. 4. Living cells were maintained at 32 °C using the Bioptechs Delta T open dish system. Identical results were obtained at 37 °C, although maintaining focus on single granules became more problematic because of increased granule motion and focal drift. Fluorescence spectra of CFP-GK-YFP was obtained from cells lysates (1.5% Triton X-100, PBS, 30 min) using a spectrofluorimeter. Spectra were normalized to cells not expressing the construct.

Digestion—For immunofluorescent staining, cells were grown on coverslips and fixed for 30 min with fresh 4% paraformaldehyde in PBS. Cells were permeabilized with 0.1% Triton X-100, PBS, 30 min) using a spectrofluorimeter. Spectra were normalized to cells not expressing the construct.

RESULTS

Colocalization between GK and insulin granules in βTC3 cells was detected using an affinity-purified anti-GK antibody (28) in an immunofluorescent staining experiment (Fig. 1A). This finding agrees with previous investigations into the distribution of GK in primary β-cells and insulinoma cell lines (11, 12). In addition, similar results were obtained using MIN6 cells and INS1 cells (data not shown), thereby supporting the notion...
that the association of GK with insulin granules is a phenomenon that is not limited to certain cell lines. To more directly assess the interaction of GK with insulin granules, GK-YFP was made and expressed in cells along with a CFP construct that was targeted to insulin granules by insertion of CFP into the connecting peptide segment of the murine proinsulin II cDNA (C-peptide CFP) (30). As shown in Fig. 1B, the distribution of these constructs was identical to the distribution of endogenous GK and insulin granules, indicating that this system was appropriate for monitoring the association of GK with insulin granules in living cells. Moreover, a GFP-tagged hexokinase II (HK-GFP) variant (15) did not localize to granules in insulin granules in living cells. Furthermore, a GK construct that was targeted to granules by insertion of CFP into the connecting peptide segment of the murine proinsulin II cDNA (C-peptide CFP) (30). As shown in Fig. 1B, the distribution of these constructs was identical to the distribution of endogenous GK and insulin granules, indicating that this system was appropriate for monitoring the association of GK with insulin granules in living cells. Moreover, a GFP-tagged hexokinase II (HK-GFP) variant (15) did not localize to granules (Fig. 1C). This suggests that granule association occurs only with GK and is not a general property of all hexokinase isoforms.

Quantification of the association between GK and insulin granules is a complex problem because the “bound” and “free” compartments are not readily distinguished from each other. Indeed, the bound compartment in this case involves granules that are, themselves, mobile within the free cytoplasmic compartment, thus making it difficult to measure changes in GK distribution. To be able to quantify changes in GK distribution in β-cells, we used the highly sensitive FRAP technique to measure the release of GK-YFP from CFP-labeled insulin granules into the cytoplasmic compartment. As shown in Fig. 2A, a region containing several CFP-labeled granules was photobleached with a high intensity pulse of 514-nm laser to destroy the fluorescence from GK-YFP (31) and the recovery of GK-YFP to the CFP-labeled granules was measured. The analysis was performed only on granules that remained in place during the course of the experiment, as indicated by the CFP fluorescence. Recovery of the GK-YFP to the photobleached granules occurred over several seconds and was considerably slower than recovery in the cytoplasm (Fig. 2A, red dotted line), which occurs with a t1/2 of ~100 ms (32). Although GK-YFP fluorescence recovered to granules after stimulation with at least 1 mM glucose for 30 min, it did not recover to granules in cells cultured with less than 1 mM glucose (Fig. 2B). At high concentrations of glucose (>10 mM), it is expected that the total amount of GK-YFP associated with the granules would decrease. However, recovery of GK-YFP to granules at higher concentrations was still much slower that recovery to the cytoplasm (Fig. 2A), indicating that recovery at these concentrations cannot be explained by simple diffusion of unbound GK into the field.

Translocation of the GK-YFP was also examined using a digitonin permeabilization assay (Fig. 2C). In this case, βTC3 cells were incubated with increasing glucose concentrations prior to treatment with digitonin and separation of the cell into both soluble (cytoplasmic) and membrane-bound (pellet) compartments by centrifugation. Under low glucose conditions, GK was detected only in the membrane-bound fraction. Increasing glucose concentrations resulted in a gradual redistribution of GK to the soluble compartment, corresponding with the dose-response data obtained from FRAP measurements. Together, these observations indicate that GK colocalizes with insulin granules in β-cells and that the enzyme translocates from the bound to the free compartment in response to changes in the glucose concentration.

Based on these observations, we hypothesized that GK translocation in the β-cell may constitute a potential mechanism for acute regulation of GK activity. To test this possibility, we measured GK activity under the conditions that resulted either in the tight association of GK with granules (glucose starvation) or reduced granule binding and release to the cytoplasm (10 mM glucose). Stimulation of cells with 10 mM glucose for 1 h resulted in increasing the cellular GK activity 40% when compared with untreated cells (Fig. 3C) (29) (p < 0.05, by t test) when compared with unstimulated cells. This increase in GK activity is consistent with cytoplasmic GK having a higher activity than granule-bound GK. However, the GK activity assay was not sensitive enough to examine the relative activity of the bound and free compartments, although increased GK activity occurs under conditions where translocation to the cytoplasm has been observed. Therefore, we developed a fluo-
Residence-based probe for examining different GK conformational and/or activity states.

Changes in protein activity often reflect alterations in their conformational state and such conformational changes can be directly measured by examination of FRET between fluorescent proteins of different colors (23). To make use of this fact, we assembled a GK fusion protein that was tagged on both the C- and N-terminal ends by attaching CFP as a donor chromophore to the N terminus of GK-YFP. Analysis of the emission spectra of extracts from cells expressing CFP-GK-YFP revealed that glucose stimulation was accompanied by a shift in the emission spectra (Fig. 3A). The relative FRET ratio of this probe was calculated from samples treated identically to those where GK activation was observed (Fig. 3, B and C), suggesting that the changes to the FRET signal from the CFP-GK-YFP construct reports changes in GK conformation that correlate with increased activity (Fig. 3D). To assess the changes in FRET spectra in single living cells, the CFP-GK-YFP construct was introduced into cells along with a C-peptide construct containing the dsRed fluorescent protein. As shown in Fig. 3 (E and F), granule-bound CFP-GK-YFP displayed a strong FRET signal, whereas cytoplasmic CFP-GK-YFP displayed a weak FRET signal. These results are consistent with GK having a lower activity when associated with insulin granules and a higher activity when released into the cytoplasm.

Because the quantitative imaging data we obtained supports the post-translational regulation of GK by glucose, we next focused on a mechanism that would satisfactorily explain the effect of glucose on GK activity and localization. The simplest potential mechanism for glucose-induced regulation of GK would be the induction of GK translocation and activation upon the binding of glucose to GK. Because glucose enters β-cells through facilitated diffusion, this hypothesis predicts that regulation of GK would occur concurrent with the addition of the glucose bolus. However, we did not find evidence of changes in GK localization (as determined by our FRAP assay) until 20 min after stimulation with glucose (Fig. 4A). Because regulation of GK occurs much later than glucose stimulation, it is not adequately explained by simple association between GK and glucose. Moreover, the glucose concentration-response profile of this process (see above) was not consistent with reported affinities for glucose-GK interactions ($K_m = 8 \text{ mM}$ (Ref. 1)).

Recent studies have shown a role for insulin signaling in β-cells in maintaining normal GSIS (33, 34). Indeed, islets from mice that lack insulin receptors exhibit impaired GSIS despite maintaining a normal response to arginine (33). Furthermore, insulin receptor signaling stimulates a broad range of intracellular signaling pathways in the β-cell, which modulate a wide variety of cellular functions. Because evidence for the regulation of GSIS by an insulin autocrine/paracrine feedback loop has recently emerged, we performed experiments to test the hypothesis that GSIS and the subsequent autostimulation of insulin receptors on the β-cell is the signal that leads to both GK translocation and activation.

To assess the importance of insulin signaling in glucose-stimulated GK regulation, we pretreated cells with nifedipine,
an agent that blocks insulin secretion (35) through inhibition of L-type calcium channels, prior to glucose stimulation. As shown in Fig. 4B, nifedipine inhibited glucose-stimulated FRAP recovery of GK-YFP to CFP-labeled granules. In addition, we found that simply stimulating inhibited cells with insulin restored GK-YFP recovery. A second drug, diazoxide, that inhibits insulin secretion by opening ATP-sensitive potassium channels (36), also inhibited glucose-stimulated GK-YFP recovery to insulin granules (Fig. 4C). The addition of insulin restored GK-YFP recovery in diazoxide-treated cells. These findings are consistent with a role of insulin secretion in glucose-stimulated regulation of GK. Because insulin stimulation restored GK-YFP dynamics, it is likely that insulin stimulation was required for glucose-stimulated GK regulation. Finally, insulin alone, in the absence of glucose, also stimulated GK-YFP FRAP recovery (Fig. 4D).

Next, we tested whether insulin receptor signaling was important to the redistribution of endogenous GK using our subcellular fractionation-based assay. As shown in Fig. 4E, treatment with an inhibitor specific to the insulin receptor family of tyrosine kinases, AG1024 (37), blocked glucose-stimulated GK translocation. Similarly, insulin also stimulated translocation...
of GK in the absence of glucose. GK translocation was observed with as little as 1 nM insulin. Taken together, these results suggested that insulin stimulation of β-cells mediated the glucose-stimulated effects on GK regulation. We further tested this model by examining whether glucose-stimulated changes in our FRET assay also required insulin secretion (Fig. 4F). Inhibition of insulin secretion with either nifedipine or diazoxide was also found to inhibit glucose-stimulated FRET changes in cells expressing CFP-GK-YFP. In addition, stimulation of the inhibited cells with insulin restored changes in CFP-GK-YFP FRET. Insulin alone also induced changes in FRET in the absence of glucose. Once again, changes in CFP-GK-YFP FRET and GK translocation correlated with increased GK activity in response to 5 min of insulin stimulation (Fig. 4G). GK activation by insulin was similar in magnitude to GK activation by glucose. These findings, together with our observations using FRAP and biochemical fractionation, support a role for insulin secretion and subsequent autocrine/paracrine activation of insulin receptors in mediating the effects of glucose stimulation on GK localization and activity.

**DISCUSSION**

We have used highly sensitive fluorescence methods to demonstrate the regulation of GK activity and localization by glucose. The findings we have obtained provide direct evidence that GK associates with insulin granules and further extend the initial observations by Toyoda et al. (11) that were based on the immunocytochemical staining methods in rat islets. Consistent with previous reports (11, 12), we also detected colocalization between GK and insulin in the majority of granules. Although the vast majority of insulin granules exhibited GK staining, some did not. However, this is readily explained by the limitations of the technique, which include a limited 8-bit collection range, subtle drift in focus, and damage from the fixative. Together, these factors may have diminished our ability, in some instances, to detect GK localization to insulin granules.

Our use of quantitative fluorescent imaging strategies enabled us to further characterize the nature of GK-granule association. We were able to demonstrate recovery of GK-YFP to CFP-labeled granules conditional to glucose exposure, indicating measurable association of GK with the granule. It is unlikely that the presence of the fluorescent protein tag in the granule or on GK contributed to these measurements for several reasons, especially because the distribution of GK-YFP and C-peptide CFP clearly reflected the distribution of endogenous GK and insulin granules. Moreover, GK-GFP fusion
proteins have been used as reliable indicators for GK translocation in hepatocytes (15), whereas similar strategies for introducing GFP into secretory granules have been successful as well (38–40). In addition, our FRAP findings were inconsistent with changes to granule motion (Fig. 2A), and cytoplasmic diffusion (Fig. 2A). Finally, our high resolution measurements using live cell imaging correspond exactly to low resolution measurements made of endogenous GK in cellular extracts (Fig. 2, B and C). Taken together, these results demonstrate the conditional association of GK with insulin granules.

Utilizing FRET to gather structural information is a classic application of fluorescence spectroscopy. Here, the use of FRET provided evidence that suggests the existence of different conformations of GK with different enzyme activities. FRET has been previously used to measure conformational changes in a wide variety of constructs that sense a variety of cellular parameters, including intracellular Ca$^{2+}$ concentration (41, 42), nitric oxide production (43), protease activity (44–47), and, most recently, kinase activity (48–50). The success of these previous studies suggests that labeling with fluorescent proteins does not generally disrupt the folding of the target protein and is therefore an effective strategy for distinguishing protein conformations within a living cell. Our FRET-based strategy allowed us to follow the different conformations of GK that were tightly correlated with differences in GK activity (Figs. 3 (B and C) and 4 (F and G)). In addition, our finding that the cytoplasmic GK correlated with the more active GK conformation fits with our finding that glucose stimulation increased cellular GK activity along with redistribution of GK to the cytoplasmic compartment. Because changes in GK activity were observed soon after stimulation with glucose (1 h) and insulin (5 min), increased GK activity cannot be explained by transcriptional activation of GK, which has been reported in response to longer periods of stimulation with glucose (8–10, 29). Taken together, these results support a novel model in which the post-translational regulation of GK results in changes in the conformation of GK and increased GK activity.

It is important to note that the enzymatic activity of granule-bound GK alone can support GSIS from the β-cell. This is based on observation that, under glucose-starved conditions, the measured GK activity was at least 50% of the total activity measured under high glucose conditions. Under these low glucose conditions, virtually all of the endogenous GK was found associated with granules as determined by subcellular fractionation (Fig. 2C) and immunocytochemistry (Fig. 1A). This distinguishes regulation of GK in β-cells from regulation of GK in hepatocytes, where GK activity can drop nearly to zero (14). Furthermore, these observations highlight the fact that GK activity in β-cells is modulated over a narrow range, because even subtle changes in GK activity have a profound effect on GSIS (2). Thus, changes in GK conformation and activation state that correlate with GK association and dissociation from the granule provide a novel mechanism for rapidly altering the rate of insulin secretion in response to changes in glucose concentration.

Three independent techniques; FRET, FRAP, and biochemical fractionation, were used in this study to address the role of insulin secretion in mediating the effects of glucose on GK regulation in β-cells. In all cases, data obtained from these three techniques indicated that regulation of GK by glucose was linked to insulin signaling. First, insulin alone effectively substituted for the absence of glucose. Second, blockade of either insulin secretion or insulin receptor function impaired the ability of glucose to regulate GK. Taken together, it is reasonable to conclude that the effects of glucose on GK are best explained by an autocrine/paracrine feedback loop.

model is appealing because it also explains the time delay between the glucose bolus and the effects on GK and may have additional implications within intact pancreatic islets. Insulin from neighboring β-cells could play a role in coordinating the glucose responsiveness of the islet. Furthermore, such insulin-dependent paracrine signaling could also account for the enhanced GSIS from whole islets over isolated β-cells (51).

Although autocrine feedback stimulation of β-cells by insulin is sufficient to explain our observations, it is certainly conceivable that glucose may have additional effects on GK regulation unrelated to insulin secretion. However, until all of the regulatory mechanisms of GK have been identified, it will be difficult to define the precise contributions of insulin and glucose in regulating GK. Moreover, the physiological implications of glucose- and insulin-mediated regulation of GK are also uncertain and difficult to assess until more details are known. This is particularly true because insulin can act through multiple types of insulin-sensitive receptors at the concentrations utilized in our experiments. In the context of a whole pancreas, it is likely that multiple factors influence post-translational regulation of GK and much work is needed to elucidate the role that these factors play in regulating GK localization and activity and, ultimately, GSIS.

Our results indicate the existence of a novel mechanism for linking insulin signaling and glucose metabolism in β-cells. Besides the recently demonstrated effect of insulin on GK gene transcription (52), these results also indicate that insulin also modulates GK activity at a post-translational level. Knowledge of this functional association between insulin signaling and GK is important in light of the growing realization that insulin signaling in the β-cell, and the defects brought about by insulin resistance, may play a causative role in the pathogenesis of type 2 diabetes mellitus (33, 53). Indeed, because mutations in GK are known to cause three different glycemic disorders, including MODY2 (1), it is reasonable to consider that defects in either the cytoplasmic localization or activation of this enzyme brought on by impaired insulin signaling in β-cells may contribute to the diminished GSIS that characterizes type 2 diabetes. The dual effects of insulin on GK in the β-cell, both post-translational and transcriptional, may be important in enabling both rapid and long-lasting changes in the activity of this enzyme. Clearly, further studies using the methods and strategies described herein will provide additional details into the molecular interactions involved that affect these dynamics within pancreatic β-cells.

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Regulation of β-Cell GK Localization and Activity by Glucose

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