# Glucokinase Is an Integral Component of the Insulin Granules in Glucose-Responsive Insulin Secretory Cells and Does Not Translocate During Glucose Stimulation

**Catherine Arden,[1] Andrew Harbottle,[1] Simone Baltrusch,[2] Markus Tiedge,[2] and Loranne Agius[1]**

**The association of glucokinase with insulin secretory granules has been shown by cell microscopy techniques. We used MIN6 insulin-secretory cells and organelle fractionation to determine the effects of glucose on the subcellular distribution of glucokinase. After permeabilization with digitonin, 50% of total glucokinase remained bound intracellularly, while 30% was associated with the 13,000*g* particulate fraction. After density gradient fractionation of the organelles, immunoreactive glucokinase was distributed approximately equally between dense insulin granules and low-density organelles that cofractionate with mitochondria. Although MIN6 cells show glucose-responsive insulin secretion, glucokinase association with the granules and low-density organelles was not affected by glucose. Subfractionation of the insulin granule components by hypotonic lysis followed by sucrose gradient centrifugation showed that glucokinase colocalized with the granule membrane marker phogrin and not with insulin. PFK2 (6-phosphofructo-2-kinase-2/fructose-2,6-bisphosphatase)/FDPase-2, a glucokinase-binding protein, and glyceraldehyde phosphate dehydrogenase, which has been implicated in granule fusion, also colocalized with glucokinase after hypotonic lysis or detergent extaction of the granules. The results suggest that glucokinase is an integral component of the granule and does not translocate during glucose stimulation. *Diabetes* 53:2346–2352, 2004**

Glucokinase (hexokinase IV) is the major glucose phosphorylating enzyme in pancreatic β-cells (1), and because of its high flux control coefficient on glucose metabolism, it is regarded as the glucose sensor for insulin secretion (1–3). Its role in blood glucose homeostasis in humans is evident from mutations in the glucokinase gene, which cause either diabetes (maturity-onset diabetes of the young, type 2) or hypoglycemia (4).

From the [1]School of Clinical Medical Sciences-Diabetes, University of Newcastle upon Tyne, Newcastle upon Tyne, U.K.; and the [2]Institute of Clinical Biochemistry, Hannover Medical School, Hannover, Germany.

Address correspondence and reprint requests to Loranne Agius, School of Clinical Medical Sciences-Diabetes, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, U.K. E-mail: loranne.agius@ncl.ac.uk.

Received for publication 8 January 2004 and accepted in revised form 10 May 2004.

FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GKRP, glucokinase regulatory protein; PFK-2, 6-phosphofructo-2-kinase-2/fructose-2,6-bisphosphatase.



Glucokinase is also expressed in hepatocytes, where its catalytic activity is regulated by a 68-kDa inhibitory protein, glucokinase regulatory protein (GKRP) (5). Glucokinase is bound to GKRP within the nucleus in metabolically quiescent conditions and translocates to a free state in the cytoplasm in response to a rise in extracellular glucose (6–9). GKRP-null mice have decreased glucokinase protein but not mRNA, suggesting a role for GKRP in stabilization of glucokinase (10). Glucokinase has a very high flux control coefficient on hepatocyte glucose metabolism that is in large part explained by the reciprocal control exerted by GKRP (11).

The absence of GKRP from pancreatic β-cells (8,12) indicates that different mechanisms must account for the high flux control coefficient of glucokinase on glucose metabolism and insulin secretion in β-cells. Various studies have provided evidence for subcellular compartmentation of glucokinase in pancreatic β-cells. Glucokinase association with the mitochondria has been suggested from studies on pancreatic islet homogenates (13,14), or after the addition of recombinant β-propionyl-CoA carboxylase, a mitochondrial matrix protein and potential glucokinase-binding partner (15). Glucokinase is also associated with liver mitochondria through an interaction with the apoptotic protein BAD (16).

Other studies on pancreatic islets and insulin secretory cell lines using cell microscopy techniques have reported localization of glucokinase in a perinuclear zone (17) or colocalization with insulin granules based on immunoelectron (18,19) and immunofluorescent (18,20) microscopy as well as by expression of glucokinase yellow fluorescent protein (21,22). To date, evidence for the association of glucokinase with insulin granules is based entirely on cell microscopy techniques, which do not allow determination of the proportion of glucokinase associated with specific organelles and do not discriminate between enzyme that is an integral component of the organelle as opposed to weak binding to the organelle periphery.

The evidence for subcellular compartmentation of glucokinase in pancreatic β-cells raises the question of whether a localized compartment of glucokinase represents a storage pool of the enzyme that can be released in response to glucose, analogous to the nuclear store of glucokinase in hepatocytes. There are apparently conflicting reports on the effects of glucose on the compartmentation of glucokinase in β-cells. There was no evidence for glucose-induced translocation of glucokinase by immunofluorescence staining or by cell permeabilization (20).

However, immunoelectron microscopy showed increased glucokinase staining of insulin granules after long-term culture (5 days) at high glucose (19), whereas Rizzo et al. (21) reported glucose-induced translocation of glucokinase from the granules to the cytoplasm. These techniques did not allow quantification of the proportion of glucokinase associated with the granules.

In this study, we investigated the association of glucokinase with subcellular organelles in MIN6 glucose-responsive insulin secretory cells and demonstrated that half of the bound glucokinase is associated with insulin granules and that the remainder cofractionates with a mitochondria-rich fraction. We show that glucokinase is an integral component of the insulin secretory granule and that it does not translocate in response to glucose.

## RESEARCH DESIGN AND METHODS

**Cell culture and incubation conditions.** MIN6 cells (p20–29) were cultured in Dulbecco's modified Eagle's medium containing 25 mmol/l glucose, 50 μmol/l β-mercaptoethanol, and 15% (vol/vol) fetal bovine serum (FBS) (23). Using a process similar to that for MIN6 cells, βTC-tet cells were cultured in medium, but the serum supplement was 2.5% FBS and 15% horse serum. Once confluent, tetracycline (1 μg/ml) was added to induce growth arrest, and the cells were cultured for 16–20 days (24). INS 1E cells were cultured in RPMI-1640 media containing 50 μmol/l β-mercaptoethanol, 1 mmol/l sodium pyruvate, and 5% FBS (25). For determination of glucose phosphorylation, the cells were cultured in 24-well plates and incubated in medium containing [2-$^3$H]glucose and either 5 or 25 mmol/l glucose as described previously (20).

**Insulin secretion.** MIN6 and INS 1E cells were cultured in 24-well plates and washed with Krebs-Ringer buffer (26). They were preincubated for 30 min at 37°C in Krebs Ringer buffer containing 5 mmol/l glucose, followed by a 2-h incubation at the indicated glucose concentration for determination of insulin secretion (26). Insulin was determined in the medium using a rat insulin enzyme-linked immunosorbent assay (Mercodia, Uppsala, Sweden).

**Cell permeabilization and homogenization.** Free and bound glucokinase activity and immunoreactivity in MIN6 cells were determined after incubation in medium with the glucose concentrations indicated. Digitonin permeabilization was carried out as previously described (20). For cell homogenization, the monolayers were scraped in 150 mmol/l KCl, 3 mmol/l Hepes, and 1 mmol/l dithiothreitol (pH 7.2) and passed six times through 27-gauge and then 23-gauge needles. Half of the homogenate was centrifuged at 13,000*g* (15 min) to generate supernatant and pellet fractions.

**Preparation of insulin granules.** MIN6 organelles were fractionated on a Percoll gradient based on a method described by Hutton and Peshivaria (27). Briefly, cells from six flasks (75 cm$^2$) were scraped in buffer containing 275 mmol/l sucrose, 10 mmol/l MES, 1 mmol/l EGTA, 0.2 mmol/l benzamidine, 0.2 mol/l PMSF, 10 μg/ml leupeptin, 10 μg/ml antipain, and 10 μg/ml pepstatin and passed six times through 27-gauge and then 23-gauge needles. The homogenate was precleared at 1,700*g* and the supernatant layered on 27% (wt/vol) Percoll in extraction buffer and centrifuged at 35,000*g* (50 min). The top 10 ml were discarded, and 1-ml fractions were collected and washed twice with extraction buffer at 13,000*g* (15 min) to remove the Percoll. Pellets were suspended in extraction buffer for determination of enzyme activity, immunoreactivity, and protein.

**Granule lysis.** For hypotonic lysis, fractions containing the insulin granules were pooled, sedimented at 13,000*g*, and lysed in 5 mmol/l sodium carbonate (28) for 20 min. The lysate was layered on a sucrose gradient containing 250 μl 90% sucrose, 500 μl 35% sucrose/2.5 mmol/l sodium carbonate, and 500 μl 5% sucrose/2.5 mmol/l sodium carbonate and centrifuged at 140,000*g* (16 h), and 1 ml fractions were removed for immunoblotting. For detergent lysis, the granules were suspended in HSE buffer (20 mmol/l Hepes, 150 mmol/l NaCl, and 5 mmol/l EDTA, pH 7.4) without or with 1% (wt/vol) Triton-X100 (29) for 30 min. The lysate was centrifuged at 13,000*g*, and protein in the supernatant and pellet was determined by immunoblotting.

**Enzyme activity determination.** Glucokinase and low-$K_m$ hexokinase activity were determined as previously described (30) except for assays on the insulin granules, where a radiochemical assay was used (31). Glutamate dehydrogenase (32) and aryl sulfatase (33) were determined as previously described. Protein was determined using Biorad reagent, and enzyme activities were expressed as milliunits per milligrams of cell protein, where 1 mU is the amount converting 1 nmol of substrate per minute.

**Immunoblotting.** Samples containing 20 μg protein were resolved by SDS-PAGE on either 10% (glucokinase, phogrin, glyceraldehyde-3-phosphate dehydrogenase [GAPDH], cathepsin D, and PFK-2 [6-phosphofructo-2-kinase-2/fructose-2,6-bisphosphatase]) or 15% (insulin) polyacrylamide gels. After electrotransfer of protein to nitrocellulose membranes, these were incubated for 90 min with primary antibody: glucokinase (H-88; Santa Cruz Biotech, Santa Cruz, CA), insulin (Chemicon, Temecula, CA), GAPDH (Hytest; Biotrend, Köln, Germany), cathepsin D (Santa Cruz Biotech, Santa Cruz, CA), phogrin (gift from Dr. John Hutton) (34), and PFK-2 (chicken polyclonal IgY against recombinant fructose 2,6-bisphosphatase domain) (35). After washing, membranes were incubated for 1 h with the appropriate peroxidase-conjugated anti-IgG (Jackson Immunoresearch Laboratories). Immunoreactive bands were visualized using an enhanced chemiluminescence kit (Amersham Biosciences) and determined using the Alpha Imager Program (Alpha Immunotech).

**Immunostaining.** Cells cultured on coverslips coated with 1% gelatin were fixed and immunostained as previously described (20). Confocal images were visualized and captured using a Leica TCS-SP2-UV microscope with a 63×, NA 1.3 objective.

## RESULTS

**Colocalization of glucokinase and insulin immunostaining at both low and high glucose.** Colocalization between glucokinase and insulin granules has been reported previously in intact islets (18) and MIN6 cells (20) as well as in glucose-starved βTC cells (21). The latter study reported glucose-induced translocation of granule-associated glucokinase to the cytoplasm. In the present study, colocalization of glucokinase with insulin granules was observed in MIN6 cells incubated at either 5 or 25 mmol/l glucose, with no apparent difference between low and high glucose (Fig. 1). Similarly, colocalization between glucokinase and insulin granules was observed in INS1E and βTC-tet cells incubated at both 5 or 25 mmol/l glucose (data not shown). Glucokinase catalytic activity was detectable spectrometrically in both the supernatant and pellet fractions of MIN6 cells but only in the supernatant of INS1E and βTC-tet cells (Table 1), possibly due to the low granule content or high pellet NADPH oxidase activity, respectively. However, glucokinase immunoreactivity was detectable in the pellet fraction of all three cell lines (data not shown). Glucose-responsive insulin secretion in MIN6 and INS1E was in agreement with previously reported values (26,36). Although the ratio of glucose phosphorylation at high versus low glucose was similar for all three cell lines (Table 1), the activity of low-$K_m$ hexokinase was lowest in INS1E and highest in βTC-tet. MIN6 rather than INS1E was used for isolation of granules (see below) because of their high granule content.

**Effects of glucose on free and bound glucokinase activity and immunoreactivity.** Because immunofluorescence staining is not sufficiently sensitive to detect adaptive changes in glucokinase distribution in β-cells, we used digitonin permeabilization and cell homogenization to test for changes in distribution of glucokinase activity and immunoreactivity. MIN6 cells are conventionally cultured in medium containing 25 mmol/l glucose (23). In these conditions, the digitonin method released 45% of catalytic activity and 60% of immunoreactive glucokinase, while homogenization of the cells released 70% of immunoreactive and catalytically active glucokinase (Fig. 2*A–D*, $t = 0$). This indicates that the bound fraction, as determined by the digitonin method, includes enzymes that are either weakly bound to organelles or associated with subcellular structures that are disrupted by homogenization.

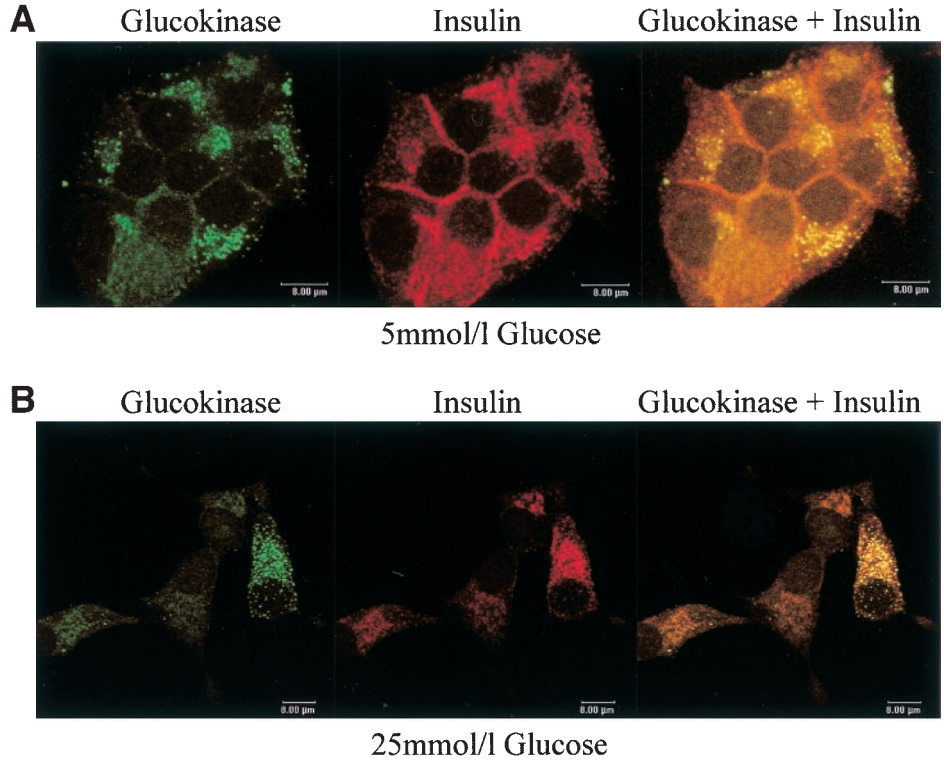


**FIG. 1. Colocalization of glucokinase and insulin granules. MIN6 cells were incubated for 3 h with 5 (*A*) or 25 (*B*) mmol/l glucose. They were immunostained for glucokinase (fluorescein isothiocyanate green) and insulin (Texas red). Yellow staining indicates colocalization.**

Incubation of the cells in glucose-free medium for 1 or 3 h caused a decrease in glucokinase activity and immunoreactivity associated with the bound fraction by the digitonin technique (Fig. 2*A* and *C*). Qualitatively similar but smaller changes were observed by cell homogenization (Fig. 2*B* and *D*). Incubation of the cells for 2 h in glucose-free medium followed by 1 h with either 5 or 25 mmol/l glucose was associated with an increase in bound glucokinase activity and a decrease in free activity, indicating reversal of the changes caused by the glucose-free medium (Fig. 2*E* and *F*). These experiments on MIN6 and others on INS1E (data not shown) did not provide evidence for dissociation of bound glucokinase by glucose.

**Association of glucokinase immunoreactivity with subcellular organelles.** Glucokinase immunoreactivity associated with the bound fraction after either digitonin permeabilization or cell homogenization (Fig. 2) may represent glucokinase binding to more than one type of organelle. To investigate this further, cell homogenates were fractionated on a Percoll gradient to separate the insulin granules from other organelles, and the fractions were immunoblotted for glucokinase, insulin, and phogrin (a marker for the insulin granule membrane) (34). Marker enzymes for mitochondria (glutamate dehydrogenase) and lysosomes (arylsuphatase) were also determined. There were two main peaks of glucokinase immunoreactivity that coincided with the major peak of insulin immunoreactivity (fractions 2–7 from the bottom of the gradient) corresponding to the dense insulin granules and the mitochondrial peak (fractions 17–22) based on colocalization with glutamate dehydrogenase (Fig. 3). Glucokinase activity was detectable radiochemically in the insulin granule fractions ($118 \pm 33$ pmol $\cdot$ min$^{-1}$ $\cdot$ mg protein$^{-1}$, $n = 6$). There was negligible activity of glutamate dehydrogenase in the insulin granule fractions, but there was immunoreactivity to insulin and phogrin in the mitochondria-rich fractions. The ratio of insulin to phogrin immunoreactivity was higher in the dense insulin granule fractions compared with the low-density mitochondria-rich fractions, suggesting that the latter most likely contain either imma-

TABLE 1
Glucokinase activity and glucose responsiveness of MIN6, INS 1E, and βTC-tet cells

| | MIN6 | INS 1E | βTC-tet |
|---|---|---|---|
| Glucokinase activity (mU/mg protein) | | | |
| Supernatant | 2.5 ± 0.3 | 1.8 ± 0.3 | 0.1 ± 0.04 |
| Pellet | 0.7 ± 0.2 | ND | ND |
| Glucose phosphorylation (nmol · 3 h$^{-1}$ · mg$^{-1}$) | | | |
| 5 mmol/l glucose | 104 ± 12 | 143 ± 12 | 137 ± 20 |
| 25 mmol/l glucose | 268 ± 26* | 354 ± 14* | 275 ± 30* |
| Insulin secretion (μg · 2 h$^{-1}$ · mg protein$^{-1}$) | | | |
| 5 mmol/l glucose | 435 ± 52 | 267 ± 46 | NM |
| 25 mmol/l glucose | 1,370 ± 167* | 1,005 ± 105* | NM |

Data are means ±SE, $n = 4$–16. Glucokinase was determined spectrometrically in supernatant and pellet fractions of cell homogenates. Glucose phosphorylation and insulin secretion were determined at 5 and 25 mmol/l glucose. *$P < 0.001$ relative to 5 mmol/l glucose. ND, not detectable; NM, not measured.

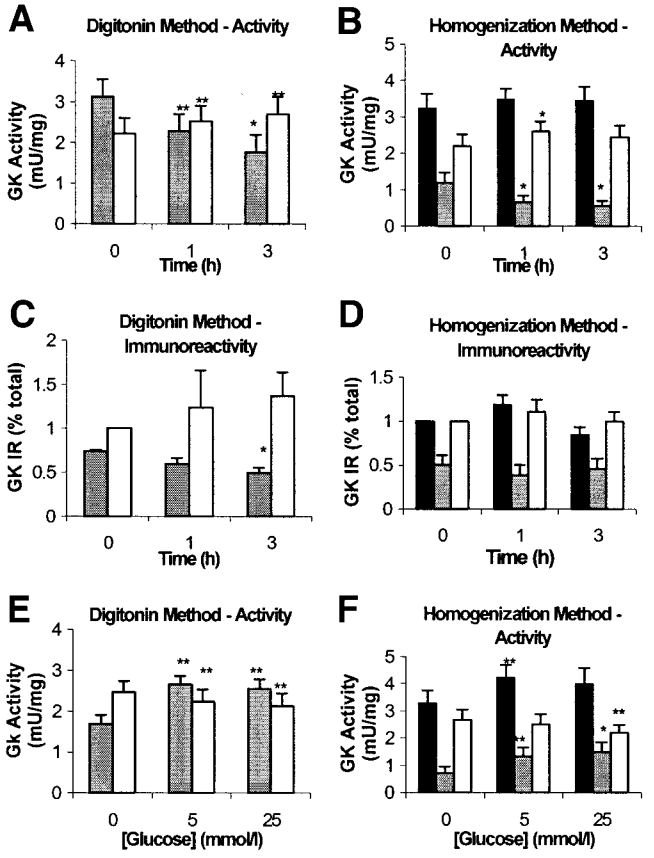


**FIG. 2. Effects of glucose withdrawal and re-addition on glucokinase distribution. *A–D*: MIN6 cells precultured with 25 mmol/l glucose were incubated for 1 or 3 h in glucose-free medium and either permeabilized with digitonin for determination of free (open bars) and bound (shaded bars) glucokinase (GK) activity (*A*) and immunoreactivity (IR) (*C*) or homogenized for determination of glucokinase activity (*B*) and immunoreactivity (*D*) in homogenates (solid bars), pellet (shaded bars), and supernatant (open bars). *E–F*: MIN6 were incubated for 2 h in glucose-free medium followed by 1 h with 0, 5, or 25 mmol/l glucose, and glucokinase activity was determined by digitonin permeabilization (*E*) and cell homogenization (*F*). Glucokinase activity is expressed as milliunits per milligram and immunoreactivity as percentage of total. Results are means ± SE, $n = 6–10$. *$P < 0.05$, **$P < 0.01$ vs. $t = 0$ (*A–D*) or glucose-free medium (*E– F*).**

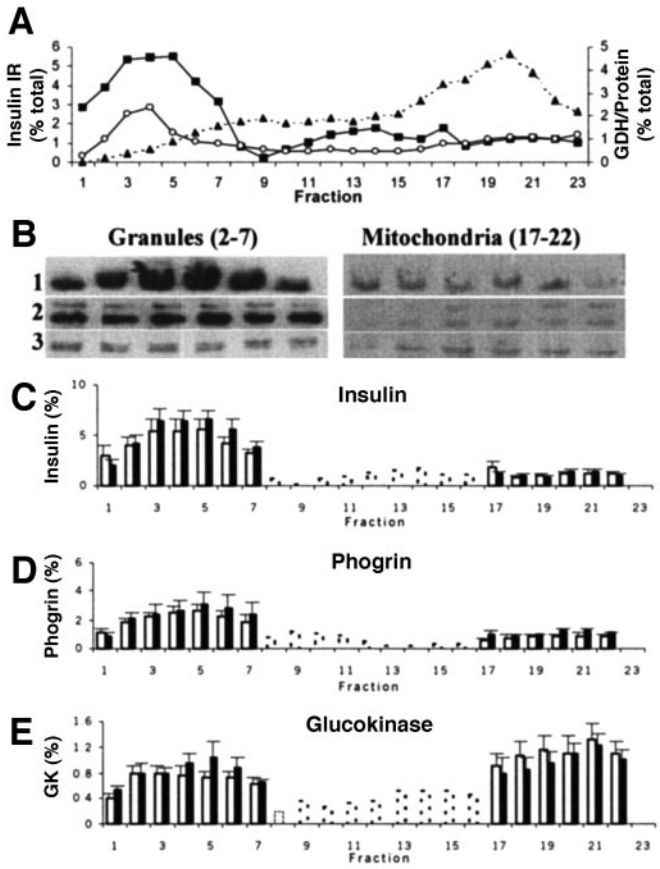


**FIG. 3. Effects of glucose on the association of glucokinase (GK) immunoreactivity (IR) with insulin granules and mitochondria after Percoll gradient fractionation. *A*: MIN6 cells cultured with 25 mmol/l glucose were homogenized and the organelles fractionated on a Percoll gradient (RESEARCH DESIGN AND METHODS). Protein (○), glutamate dehydrogenase (GDH) (▲), and immunoreactive insulin (■) in the fractions were expressed as percentage of total loaded on the gradient. *B*: Representative immunoblot for insulin (ref. 1), phogrin (ref. 2), and glucokinase (ref. 3). *C–E*: MIN6 cells were incubated in glucose-free medium for 2 h followed by 1 h of incubation without (□) or with (■) 25 mmol/l glucose. They were fractionated as in *A*. Immunoreactive insulin (*C*), phogrin (*D*), and glucokinase (*E*) in the fractions were expressed as percentage of total. Results are means ± SE, $n = 4–10$.**

ture granules or remnant vesicles that have released their insulin. The ratio of glucokinase to phogrin immunoreactivity was fourfold higher in the low-density fractions (Table 2). Fractionation of INS-1E and βTC-tet organelles confirmed the association of glucokinase immunoreactivity with insulin granules in these cell lines (data not shown).

**Glucose does not cause translocation of glucokinase immunoreactivity from either the insulin granules or the low-density fractions.** To test for adaptive changes of glucokinase association with organelles, MIN6 was incubated in either glucose-free medium or medium containing 25 mmol/l glucose for 1 h before isolation of the organelles. Immunoreactive glucokinase associated with both the granules and the low-density fractions was not released by high compared with low glucose (Fig. 3, Table 2).

**Colocalization of GAPDH and PFK-2 with glucokinase immunoreactivity.** Percoll fractions were immunoblotted for PFK-2, a glucokinase-binding protein (35), and for GAPDH, which has been implicated in granule movement and membrane fusion (37). Both the insulin granules and low-density organelles showed immunoreactivity to PFK-2 and GAPDH (Table 2). When compared with recovery of phogrin, recovery of GAPDH was similar in the insulin granules and low-density fractions, whereas the recovery of PFK-2 relative to that of phogrin was twofold higher in the latter fractions, similar to the higher glucokinase-to-phogrin ratio (Table 2).

**Glucokinase is not released with core protein during granule lysis.** To determine whether the glucokinase immunoreactivity recovered with the insulin granules is associated with the core or the periphery of the granule, insulin granules were lysed hypotonically and the lysate fractionated on a sucrose gradient. The fractions were immunoblotted for insulin, phogrin, glucokinase, GAPDH, PFK-2, and the lysosomal marker cathepsin-D, since the lysosomal peak overlaps with the insulin granule peak (Fig. 4). There was negligible overlap in distribution of phogrin and insulin immunoreactivity, confirming total release of the insulin following hypotonic lysis. Glucokinase was recovered with phogrin and not with the insulin,

TABLE 2
Effects of glucose on the distribution of immunoreactivity to glucokinase, PFK-2, and GAPDH

| | Glucose-free medium | | 25 mmol/l glucose | |
|---|---|---|---|---|
| | Granules | Mitochondria | Granules | Mitochondria |
| Phogrin | 14.4 ± 2.2 (1.0) | 4.7 ± 1.0 (1.0) | 16.1 ± 4.5 (1.0) | 5.6 ± 0.4 (1.0) |
| Insulin | 30.6 ± 5.5 (2.1) | 7.1 ± 1.5 (1.5) | 34.9 ± 5.0 (2.2) | 6.8 ± 0.9 (1.2) |
| Glucokinase | 4.9 ± 1.7 (0.3) | 6.6 ± 1.3 (1.4) | 5.8 ± 0.9 (0.4) | 5.9 ± 0.9 (1.1) |
| PFK-2 | 17.8 ± 5.5 (1.2) | 10.3 ± 2.1 (2.2) | 17.4 ± 2.8 (1.1) | 12.5 ± 3.4 (2.2) |
| GAPDH | 4.6 ± 1.0 (0.3) | 2.7 ± 1.0 (0.6) | 4.0 ± 0.4 (0.3) | 2.3 ± 0.7 (0.4) |

Data are means ± SE (recovery as a ratio to phogrin), $n = 4–10$. MIN6 were incubated for 2 h in glucose-free medium followed by 1 h without or with 25 mmol/l glucose. The cells were homogenized and the organelles fractionated on a Percoll gradient, as in Fig. 3. Immunoreactivity in the percoll fractions corresponding to the insulin granules or the mitochondria is expressed as percentage of total immunoreactivity loaded on the gradient.

suggesting that it is not associated with the core components. Likewise, GAPDH and PFK-2 cofractionated with glucokinase and phogrin, whereas cathepsin D did not colocalize with these enzymes or with phogrin, confirming that immunoreactivity to these proteins could not be accounted for by lysosomal contamination.

Granule-associated glucokinase characteristically appears as a double band within the 50-kDa region by immunoblotting. Following sonication and/or treatment of the granules with detergent and subsequent centrifugation, the upper glucokinase band is released more easily into the supernatant, while the lower band remains associated with the pellet fraction (Fig. 5). This is suggestive of different molecular forms of glucokinase that may differ in their association with the insulin granules.

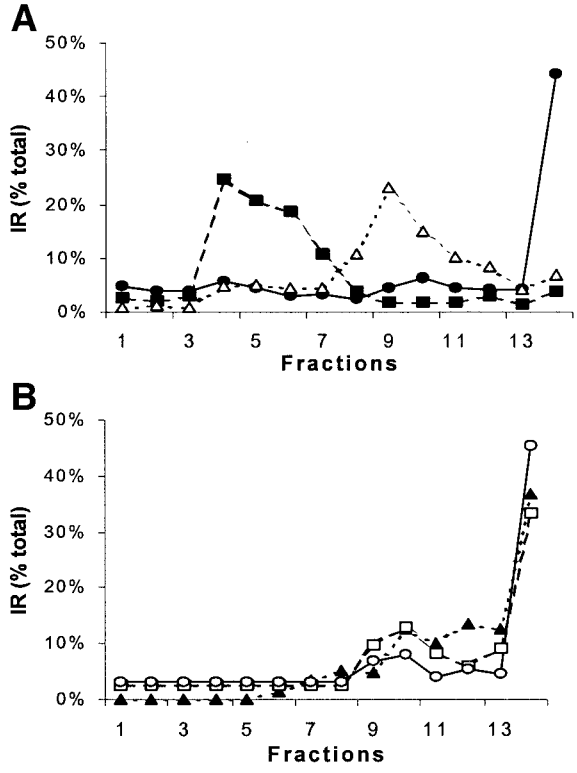


**FIG. 4. Distribution of granule proteins after hypotonic lysis and sucrose gradient centrifugation. Granule fractions 3–6 were pooled, lysed hypotonically, and fractionated on a sucrose gradient. *A*: Glucokinase (●), insulin (■), and cathepsin D (△). *B*: Phogrin (□), GAPDH (○), and PFK-2 (▲). Immunoreactivity (IR) is expressed as percentage of total. Results are representative of six experiments.**

## DISCUSSION

In this study, we confirmed colocalization of glucokinase with insulin granules by fluorescence microscopy in MIN6 cells cultured at either low or high glucose in agreement with previous studies using cell microscopy techniques (18–21). Since techniques based on fluorescence microscopy do not allow quantitative determination of the proportion of total cellular glucokinase associated with a particular organelle type and also do not distinguish between enzyme that is an integral component of the organelle as opposed to weakly bound to the periphery, we used subcellular fractionation to determine the association of glucokinase with different organelles

We show in this study that ~30–50% of the glucokinase is associated with the particulate fraction after homogenization of MIN6 cells. After fractionation of the organelles, the glucokinase is distributed primarily into two fractions, one of which corresponds to the dense insulin granules, while the other cofractionates with mitochondria and low-density phogrin-containing organelles with a low insulin content. Although the insulin granule peak overlaps, in part, with the lysosomes, we could exclude the possibility that granule-associated glucokinase included immunoreactive protein entrapped in lysosomes because after hypotonic lysis and fractionation on a sucrose gradient, glucokinase did not colocalize with a lysosomal marker. The glucokinase immunoreactivity associated with the low-density vesicles could represent remnant granules after insulin secretion with partial loss of membrane components, as suggested by the increase in the glucokinase-to-phogrin ratio or the association of glucokinase with mitochondria.

Previous studies reporting colocalization of glucokinase with insulin granules did not address whether glucokinase is an integral component of the insulin granule or is loosely bound to the granule periphery. In this study, hypotonic lysis of insulin granules followed by sucrose

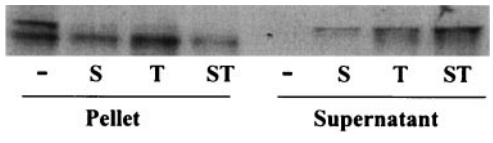


**FIG. 5. Detergent extraction of granule-associated glucokinase. Granule fractions were pooled and suspended in HSE buffer without (-) or with 1% Triton-X100 (T) and were either untreated or sonicated (S). Immunoreactive glucokinase was determined in the pellet and supernatant. Results are representative of two experiments.**

gradient fractionation enabled separation of insulin from the granule membrane marker phogrin. Because immunoreactive glucokinase distributed with phogrin and not insulin, it can be inferred that glucokinase is associated with the outer structure rather than the granule core. Like phogrin, glucokinase remained associated with the granules after detergent extraction, suggesting that it is an integral component of the granules. The double band of granule-associated glucokinase, as shown by immunoblotting, suggests that there may be different molecular forms of glucokinase associated with the granules, possibly representing either cross-linking of disulphide bonds (38) or nitrosylation of cysteine residues (22). The faster migrating form was more resistant to detergent extraction.

Two other proteins that colocalized with glucokinase and phogrin are GAPDH and PFK-2. The latter protein catalyzes the formation and degradation of fructose 2,6-bisphosphate and is a binding partner for glucokinase (35). Since a higher fraction of PFK-2 than glucokinase was recovered with both the insulin granules and the low-density organelles, it raises a question as to whether PFK2 acts as the binding protein or molecular scaffold for glucokinase or whether it has a role in stabilizing glucokinase, as was recently shown in insulin secretory cells (39).

We tested for association of GAPDH with the insulin granules because a previous study on hamster insulinoma tumor insulinoma lysates showed that a cytoplasmic protein with immunoreactivity to GAPDH catalyzed fusion of insulin granules with plasma membrane vesicles in vitro (37). In this study, we demonstrated the association of GAPDH with insulin granules. This raises a question as to whether the GAPDH isoform that catalyzes fusion is also present in the granules in addition to the cytoplasm and is causing granule fusion with the membrane. The association of glucokinase with phogrin and GAPDH therefore suggests a role for glucokinase in glucose-induced granule movement or membrane fusion.

Two possible roles can be envisaged for granule-associated glucokinase. It either represents a store of "kinetically inactive" enzyme that is released to the cytoplasm during glucose-induced insulin secretion, or it has a direct kinetic function when associated with the granule that determines glucose-mediated changes in granule maturation, movement, or membrane fusion during insulin secretion. The attraction of the former hypothesis is its analogy to the nuclear compartmentation of glucokinase in the hepatocyte. Rizzo and colleagues (21,22) reported that glucokinase translocates from the granules to the cytoplasm in response to glucose or insulin based on a decline in glucokinase immunoreactivity that is associated with the digitonin bound fraction after the addition of glucose and also based on differences in the rate of granule fluorescence recovery after photobleaching (FRAP) in cells expressing a glucokinase—yellow fluorescent protein fusion protein. Using similar digitonin permeablization or cell homogenization, we found no evidence for either glucose-induced translocation (present study) or insulin-induced translocation (L.A. and C.A., unpublished observations). To enable direct analysis of the effect of glucose on granule-associated glucokinase, we used Percoll gradient fractionation to separate the granules from other organelles and found no evidence for adaptive changes in the glucokinase content of the granules. A tentative explanation for the apparently conflicting results between the present study and the previous study (21) is that in the present study we determined a glucokinase fraction that is "integral" to the granule and is retained after density gradient fractionation, whereas the FRAP method used previously measures a diffusible glucokinase fraction in the vicinity of the granules. Differences in rates of diffusion may reflect changes in macromolecular crowding around the granule in response to glucose or insulin.

In summary, we provide direct evidence for the association of glucokinase with insulin granules based on fractionation of organelles and show that following hypotonic lysis, glucokinase cofractionates with phogrin, GAPDH, and PFK2 but not insulin. We suggest that glucokinase is an integral component of the granule, that it does not translocate in response to glucose, and that it may be involved in glucose-mediated changes in granule movement or membrane fusion.

## ACKNOWLEDGMENTS

Funding of a PhD studentship to C.A. by the Development Trust at the University of Newcastle is gratefully acknowledged. We also thank the Wellcome Trust, Diabetes U.K. (grant BDA RD01/0002364), and the Medical Research Council (grant G0100348) for equipment grant support.

We thank Drs. Jun-ichi Miyazaki, Claus Wollheim, and Shimon Efrat for providing MIN6, INS 1E, and βTC-tet cells, respectively, and Dr. John Hutton for the kind gift of the phogrin antibody.

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