Insulin secretion, insulin content and glucose phosphorylation in RINm5F insulinoma cells after transfection with human GLUT2 glucose-transporter cDNA

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The insulin-secretory response to glucose is defective in the RINm5F insulin-producing tumour cell line. Stable transfection with human low-affinity GLUT2 glucose-transporter cDNA revealed a significant improvement in stimulus–secretion coupling in these insulinoma cells. 3-*O*-Methylglucose uptake increased 10-fold in the concentration range 10–20 mM, whereas non-transfected control cells were unresponsive. Northern-blot analysis revealed a 7-fold increase in expression of the insulin gene in the GLUT2-transfected RINm5F cell clone T1. In contrast, glucokinase and GLUT1 glucose-transporter mRNA gene expression were not affected by transfection with GLUT2 glucose-transporter cDNA. The insulin content of transfected RINm5F cells was 7-fold higher after tissue culture at high

glucose concentrations than in non-transfected controls. GLUT2-transfected RINm5F cells also regained insulin-secretory responsiveness toward high glucose concentrations. Tissue culture for 72 h in 20 mM glucose induced glucokinase activity in the GLUT2-transfected RINm5F clone T1, raising the glucokinase/hexokinase phosphorylation ratio from 0.2 to 0.6. The experiments demonstrate that an increased glucose uptake via a low-affinity glucose transporter and an increased metabolic flux rate are important factors in the induction of insulin-gene expression and glucokinase activity and thus improved glucose-induced biosynthesis and secretion of insulin in RINm5F insulinoma cells.

INTRODUCTION

The ability of the pancreatic B-cell to release insulin in response to glucose stimulation is of central importance for blood glucose homeostasis. The high- K_m glucose transporter GLUT2 [1–4] and the low-affinity glucose-phosphorylating enzyme glucokinase [5–10] regulate the metabolic flux rate through glycolysis in these cells and thereby generate the signal for glucose-induced insulin secretion [5,6,11–13]. Under physiological conditions, glucokinase catalyses the rate-limiting step in glycolysis that initiates glucose-induced insulin secretion and is therefore called the ‘signal-recognition enzyme’ [12]. However, glucokinase requires the high- K_m glucose transporter GLUT2 for glucose uptake into the pancreatic B-cell in order to generate an increased metabolic flux through glycolysis in parallel with the increase in the physiological blood glucose concentration in the millimolar range [4].

Recent studies in humans and rodents support the concept that alterations in pancreatic B-cell glucokinase and GLUT2 glucose transporter are of pathogenic importance in the impaired signal recognition that occurs in non-insulin-dependent diabetes mellitus. Several investigators [14–17] have reported on a link between hereditary non-insulin-dependent maturity-onset diabetes of the young and mutations or polymorphisms in the glucokinase locus on human chromosome 7. On the other hand, alterations in expression of the high- K_m GLUT2 glucose-transporter gene also result in impaired glucose responsiveness in the pancreatic B-cell [18–20]. Using molecular-biology techniques, manipulations of these structures are possible in permanent insulin-producing cell lines. In the RINr insulin-secreting cell line transfected with the low- K_m glucose transporter GLUT1, insulin

mRNA levels and insulin biosynthesis were significantly increased [21], suggesting a permissive role for glucose transport in insulin biosynthesis. Hughes et al. [22] recently established a model of an ‘artificial β -cell’ derived from anterior pituitary cells. These cells exhibited a significant increase in insulin biosynthesis and secretion after transfection with the high- K_m glucose transporter GLUT2. However, the co-transfected insulin gene was under the control of the simian virus 40 early-gene promoter, thus regulation of natural insulin biosynthesis at the level of gene transcription cannot be commented upon. The RINm5F cell line [23,24] has been derived from a radiation-induced rat insulinoma and has been widely used for studying mechanisms of insulin secretion. This cell line is devoid of an insulin-secretory response to physiological glucose stimulation but has an intact insulin gene and expresses the glucokinase gene [25]. Therefore in the present study, we investigated the effect of stable GLUT2 glucose-transporter transfection of RINm5F insulinoma cells on the function of the insulin-secretory apparatus using insulin secretion and content as well as insulin mRNA levels as indicators.

EXPERIMENTAL

Materials

Restriction and DNA-modifying enzymes were obtained from Boehringer-Mannheim, Stratagene Inc. (La Jolla, CA, U.S.A.) and Pharmacia LKB Biotechnologies (Milwaukee, WI, U.S.A.). pRC/RSV plasmid was purchased from Invitrogen Corporation, San Diego, CA, U.S.A. The SP6/T7 Transcription Kit, Random Primed DNA Labeling Kit and digoxigenin (DIG) Nucleic Acid Detection Kit were from Boehringer-Mannheim, silica gel matrix was from Diagen, Düsseldorf, Germany, and Hybond N hybrid-

ization transfer membranes were obtained from Amersham, Braunschweig, Germany. ^{125}I -labelled insulin was purchased from Behringwerke, Frankfurt, Germany and 3-*O*-methyl-D-[1- ^3H]glucose from Amersham, Braunschweig, Germany. Geneticin (G 418) was from GIBCO Life Technologies, Gaithersburg, NJ, U.S.A. Glucose-6-phosphate dehydrogenase was from Sigma, St. Louis, MO, U.S.A., and ATP (disodium salt), NADP (disodium salt) and 6-phosphogluconate dehydrogenase were from Boehringer-Mannheim. All other reagents of analytical grade were from Merck, Darmstadt, Germany.

Plasmid construction for stable expression of human GLUT2 glucose transporter

The pBluescript SK plasmid containing the full-length GLUT2 glucose-transporter cDNA [3] was digested with *KpnI*–*NotI*. The cDNA fragment of approx. 2.5 kb was purified from agarose gel by binding on silica gel matrix and cohesively ligated to the eukaryotic expression vector pRC/RSV, the multiple cloning site of which was digested with *XbaI*–*NotI*. The resulting 7.5 kb plasmid/cDNA fragment was made blunt-ended by treatment with T4 DNA polymerase and gel-purified by binding on silica gel matrix. Then the blunt ends were ligated and a portion of the ligation mixture was used to transform competent *INF α F'* *Escherichia coli*.

RINm5F insulinoma culture

The insulin-producing tumour cell line RINm5F [23] was grown in RPMI 1640 medium, supplemented with 10 % (v/v) fetal calf serum, 100 units/ml penicillin and 10 $\mu\text{g}/\text{ml}$ streptomycin. All experiments were performed with cells between passage 43 and 47. To test natural antibiotic resistance, the cells were incubated with different concentrations of the antibiotic geneticin before transfection. Geneticin concentrations above 50 $\mu\text{g}/\text{ml}$ of medium completely inhibited the growth of the cells, therefore we used concentrations of 250 $\mu\text{g}/\text{ml}$ for selection of positively transfected clones. The pRC/RSV-GLUT2 plasmid contained a Rous Sarcoma virus long terminal repeat promoter for transcription initiation of the human GLUT2 glucose-transporter mRNA and an aminoglycoside-resistance gene, which was expressed from the simian virus 40 early promoter. RINm5F insulinoma cells were transfected by electroporation. After 24 h, geneticin was added to the medium, and positive cell clones were selected by geneticin resistance. After further purification, these clones were tested for expression of GLUT2 glucose transporter by Northern-blot analysis and glucose-transport measurement. The cell clone with the highest stable expression of human GLUT2 was analysed in detail.

Glucose transport

RINm5F cells were plated at a density of 5×10^5 cells/35 mm plastic dish and grown to confluency within 6 days. The culture medium was removed and the cells were washed twice with Dulbecco's PBS. Then the cells were incubated twice for 15 min each at 37 °C with Krebs–Ringer medium, buffered with bicarbonate and Hepes at pH 7.0 supplemented with 0.1 % (w/v) BSA. 3-*O*-Methylglucose-uptake measurements were performed in the same medium supplemented with 3-*O*-methyl-D-[1- ^3H] glucose (4 $\mu\text{Ci}/\text{ml}$) for a period of 20 s and stopped by the addition of an excess of ice-cold medium containing 100 mM

glucose [26]. Cells were washed seven times with this medium and thereafter lysed by addition of 0.5 % SDS. The radioactivity in the lysate was counted, and the results were expressed per mg of protein, which was determined by the BCA protein assay (Pierce, Rockford, IL, U.S.A.). The K_m values were calculated by a double-reciprocal Michaelis–Menten plot (see Figure 4). The K_m values were calculated by using a least-squares linear-regression algorithm (Inplot 4 Scientific Software; GraphPad Corp., San Diego, CA, U.S.A.).

Northern-blot analysis

Total RNA was isolated by a combined water-saturated phenol/chloroform/3-methylbutan-1-ol extraction as described by Chomczynski and Sacchi [27]. Then 20 $\mu\text{g}/\text{lane}$ was subjected to electrophoresis on denaturing formamide/formaldehyde/1 % agarose gels and transferred to Nylon membranes. Under conditions described previously [9], non-radioactive hybridization was performed using 11-DIG-UTP-labelled antisense cRNA probes [28] coding for rat GLUT1 glucose transporter [29], human GLUT2 glucose transporter [3], rat liver glucokinase [30] and rat preproinsulin I [31]. The DIG-labelled hybrids were detected by an enzyme-linked immunoassay using an anti-DIG–alkaline phosphatase antibody conjugate. The subsequent enzyme-catalysed chemiluminescence detection with the substrate AMPPD [3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphonyloxy)phenyl-1,2-dioxane] visualized the hybrids on a light-sensitive film for quantification by densitometry. Ribosomal bands were used as control markers for gel loading.

Insulin secretion and content

Cells were seeded at a density of $1 \times 10^5/\text{well}$ in 24-well culture dishes and grown for 72 h in RPMI 1640 medium with 10 mM glucose. They were then washed twice with glucose-free medium and preincubated twice with glucose-free medium for 15 min at 37 °C. Insulin secretion during a 90 min incubation period was measured in medium containing glucose concentrations of 0.2 or 20 mM. After this time period, the incubation medium was removed and gently centrifuged to remove floating cells. The cell number was determined after trypsinization in a Neubauer counting chamber. For the measurement of cellular insulin content, the cells were lysed in acetic acid. Insulin was determined radioimmunologically with a rat insulin standard. $(\text{NH}_4)_2\text{SO}_4$ was used to precipitate the antibody-bound insulin.

Glucose phosphorylation

Glucokinase and hexokinase activities were measured in cytoplasmic fractions by a photometric assay using glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, ATP and NADPH [32]. Glucokinase activity was determined by subtracting the hexokinase activity measured at 1 mM glucose from the activity measured at 100 mM glucose. Enzyme activities were expressed as munits/mg of protein. One unit of enzyme activity is defined as 1 μmol of glucose 6-phosphate formed from glucose and ATP per min at 37 °C.

Data analysis

The experimental data are expressed as means $\pm$ S.E.M. Statistical analyses were performed using Student's *t* test.

RESULTS

Northern-blot analysis

The transfection of RINm5F insulinoma cells with a pRC/RSV-GLUT2 plasmid provided three geneticin-positive clones. All three clones (T1, T2 and T3) expressed GLUT2 glucose-transporter mRNA (Figure 1). Insulin mRNA levels were 4–6 times higher than in non-transfected control RINm5F cells (Figure 1). For all further studies, the T1 clone was selected, because it expressed the highest GLUT2 glucose-transporter mRNA levels together with high insulin mRNA levels (Figure 1). GLUT2 mRNA levels in clone T1 (Figures 1 and 2) were comparable with those in normal liver (results not shown). Non-transfected RINm5F cells preferentially expressed the low- K_m GLUT1 glucose-transporter mRNA, whereas GLUT2 mRNA was virtually undetectable (Figure 2). The human GLUT2 mRNA levels in the transfected cell clone remained stable during the whole experimental period (more than 6 months). Interestingly, the expression of the high- K_m glucose-transporter GLUT2 did not affect expression of glucokinase (2.8 kb and 4.4 kb mRNA species) and GLUT1 genes to any significant extent, but insulin mRNA levels increased dramatically by a factor of more than seven (from 761 to 5844 arbitrary units) (Figure 2). In general, GLUT1 and GLUT2 gene expression correlated well with the kinetic properties of glucose uptake in the transfected cell clone.

Glucose transport

As expected from mRNA levels of human GLUT2 glucose transporter in transfected RINm5F cells, 3-*O*-methylglucose uptake was 3-fold higher at a concentration of 2 mM (90 ± 15 compared with 250 ± 48 nmol/min per mg of protein) and 9-fold higher at a concentration of 20 mM (87 ± 9 compared with 783 ± 129 nmol/min per mg of protein) than in non-transfected controls ($P < 0.05$) (Figure 3). In non-transfected RINm5F cells, 3-*O*-methylglucose uptake reached a maximum at a concentration of around 2 mM and showed no further increase between

Figure 1 Densitometric analysis of GLUT2 and insulin mRNA levels in non-transfected and GLUT2-transfected insulinoma RINm5F cells

Total RNA (10 μ g) isolated from GLUT2-glucose-transporter-transfected RINm5F cell clones (T1, T2, T3) and non-transfected control cells was subjected to Northern-blot hybridization. The blot was probed with human GLUT2 glucose transporter [3] and rat preproinsulin I [31] antisense cRNAs labelled with 11-DIG UTP. GLUT2 glucose-transporter and insulin mRNA levels were quantified by densitometric analysis of the autoradiograms and expressed as arbitrary units. n.d., not detectable.

concentrations of 10 and 20 mM (Figure 3). In contrast, in the GLUT2-transfected RINm5F cell clone, transport of 3-*O*-methylglucose was increased by 300 % at concentrations above 10 mM (497 ± 95 compared with 783 ± 129 nmol/min per mg of protein). It should be noted that no difference was found between the total protein content of the cells and the kinetics of cell growth in the transfected and control groups (results not shown). The K_m values for 3-*O*-methylglucose transport, obtained by double-reciprocal Michaelis-Menten transformation (Figure 4), were 0.5 mM for non-transfected control cells and 12 mM for GLUT2-transfected cells. These values are in agreement with previous observations [1,22] and reflect the kinetic properties of the high- K_m GLUT2 glucose transporter.

Insulin secretion and content

Normal non-transfected RINm5F cells did not release insulin in the millimolar concentration range in response to the physiological stimulus, glucose (Table 1). In contrast, 90 min

Figure 2 Northern-blot analysis of non-transfected and GLUT2-transfected insulinoma RINm5F cells

Total RNA (20 μ g) from non-transfected and GLUT2-transfected cells was loaded in the left and right lanes respectively. mRNA levels were related to ribosomal bands and visualized in an ethidium bromide-stained agarose gel. Blots were probed with antisense cRNAs coding for (a) human GLUT2 glucose transporter [3], (c) rat GLUT1 glucose transporter [29], (d) rat glucokinase [30] and (b) rat preproinsulin I [31] by non-radioactive hybridization. A representative blot of four independent experiments is shown.

Figure 3 Concentration-dependent uptake of 3-*O*-methylglucose by non-transfected and GLUT2-transfected insulinoma RINm5F cells

Incubations were performed at 37 °C in medium containing 3-*O*-methyl- α -[1- 3 H]glucose (4 μ Ci/ml) for a period of 20 s. Uptake in non-transfected cells (●) and GLUT2-transfected cells (■) was expressed as nmol/mg of cell lysate protein. Data are means $\pm$ S.E.M. for three experiments.

Figure 4 Kinetic characteristics of 3-*O*-methylglucose uptake by (a) non-transfected and (b) GLUT2-transfected RINm5F insulinoma cells

Rates of 3-*O*-methylglucose uptake are plotted using the Michaelis–Menten transformation. K_m values were calculated by using a least-squares linear-regression algorithm and are 0.52 mM and 12.6 mM for control and GLUT2-transfected cells respectively.

Table 1 Effects of GLUT2 transfection on glucose-induced insulin secretion in RINm5F insulinoma cells

Cells were incubated for 90 min in Krebs–Ringer medium containing various glucose concentrations. Insulin was measured in centrifuged supernatants by radioimmunoassay and expressed per number of cells. Data are means $\pm$ S.E.M. for eight experiments, * $P < 0.01$ compared with control cells.

Glucose concentration (mM)	Insulin secretion (ng/90 min per 5×10^6 cells)	
	Control	GLUT2-transfected cells
0	7.9 ± 1.2	15.9 ± 1.8
2	6.6 ± 0.6	$16.0 \pm 1.4^*$
20	6.6 ± 0.9	$32.6 \pm 3.2^*$

Table 2 Effects of GLUT2 transfection on insulin content in RINm5F insulinoma cells

Cells were incubated for 90 min in Krebs–Ringer medium containing various glucose concentrations. Insulin was measured by radioimmunoassay after cell lysis with acetic acid and expressed per number of cells. Data are means $\pm$ S.E.M. for eight experiments, * $P < 0.01$ compared with control cells.

Glucose concentration (mM)	Insulin content (ng/ 5×10^6 cells)	
	Control	GLUT2-transfected cells
0	41.2 ± 4.7	105.9 ± 7.3
2	45.7 ± 7.4	$212.6 \pm 25.3^*$
20	59.4 ± 8.9	$419.0 \pm 58.6^*$

incubations of GLUT2-transfected RINm5F cells showed a 2-fold increase in insulin release when the glucose concentration was raised from 2 to 20 mM (Table 1). GLUT-2-transfected RINm5F cells also had a 2.5 times higher insulin content than non-transfected control cells (Table 2). After a 90 min incubation with 2 and 20 mM glucose, the insulin content was 5-fold and 7-

Table 3 Effects of GLUT2 transfection on glucose-phosphorylating enzyme activities in RINm5F insulinoma cells

RINm5F cells were incubated for 72 h in RPMI 1640 medium containing 0.2 or 20 mM glucose. The incubation medium was changed daily to prevent a decrease in the glucose concentration. Glucose phosphorylation was measured by a photometric assay in cytoplasmic fractions and expressed in munits/mg of protein. Data are means $\pm$ S.E.M. of four experiments, * $P < 0.01$ compared with control cells.

RINm5F cells	Glucose concentration (mM)	Enzyme activity (munits/mg of protein)	
		Glucokinase	Hexokinase
Control	0.2	0.88 ± 0.15	2.36 ± 0.09
	20	1.08 ± 0.11	2.21 ± 0.12
GLUT2-transfected	0.2	$0.48 \pm 0.08^*$	2.31 ± 0.12
	20	$1.66 \pm 0.19^*$	2.63 ± 0.08

fold higher respectively than without glucose (Table 2). Whereas increasing the glucose concentration in the incubation medium from 0 or 2 mM to 20 mM did not significantly affect the insulin content of control cells, the insulin content of GLUT2-transfected RINm5F cells was 2 and 4 times higher respectively (Table 2).

Glucose phosphorylation

Glucose phosphorylation in RINm5F tumour cells is achieved by both high-affinity hexokinase and low-affinity glucokinase enzyme activity. Long-term (72 h) tissue culture at high glucose concentrations (20 mM) significantly increased glucokinase activities in GLUT2-transfected RINm5F cells from 0.48 munit/mg of protein to 1.66 munits/mg of protein (Table 3). In contrast, non-transfected control cells, which preferentially expressed the high-affinity GLUT1 glucose transporter, showed no significant change in glucokinase activity with glucose concentration. Hexokinase activities were not affected by increasing glucose concentrations in either non-transfected or GLUT2-transfected cells. Thus glucose appears to be a positive modulator of the glucokinase/hexokinase phosphorylation ratio, raising it from 0.2 to 0.6 after long-term glucose exposure.

DISCUSSION

RINm5F insulin-producing tumour cells are an excellent model for modulation of the glucose-recognition apparatus, comprising the low-affinity glucose-transporter GLUT2 and the low-affinity glucose-phosphorylating enzyme glucokinase. In contrast with normal pancreatic B-cells [1], RINm5F insulin-producing tumour cells preferentially express the high-affinity glucose-transporter GLUT1 (Figure 2), and therefore glucose-uptake kinetics were shifted toward low glucose concentrations with no additional glucose uptake occurring between 10 and 20 mM glucose (Figure 3). Transfection of GLUT2 cDNA under the control of a viral promoter resulted in coexpression of GLUT2 and GLUT1 glucose transporters. The K_m value for 3-*O*-methylglucose uptake increased from 0.5 to 12 mM and thus enabled the cells to increase 3-*O*-methylglucose uptake in the range 2–20 mM (Figure 3). The higher rate of glucose uptake in the presence of millimolar glucose concentrations lead to an increased metabolic flux rate resulting in an increased expression of the insulin gene. This is evident from the significantly higher amounts of insulin mRNA detected in all three GLUT2-expressing RINm5F insulinoma clones than in non-transfected cells (Figure 1). These results are consistent with previous reports [33], which provided evidence

for the presence of glucose-responsive elements within the 5' flanking region of the rat insulin gene.

The induction of insulin-gene transcription by glucose was accompanied by an elevated intracellular insulin content and an improved insulin-secretory response to glucose stimulation in the GLUT2-transfected cells. The insulin-secretory response of GLUT2-transfected RINm5F cells increased 2-fold when the glucose concentration in the medium was increased from 2 to 20 mM, whereas non-transfected RINm5F cells did not respond to increasing glucose concentrations. In comparison, dispersed rat islet cells pre-exposed to 16.7 mM glucose exhibited a 2-fold higher insulin-secretory response when the glucose concentration was increased from 2.8 to 22 mM [34]. Hughes et al. [22] demonstrated a similar stimulatory effect of glucose on insulin biosynthesis and secretion in GLUT2-transfected AT20 anterior pituitary cells, previously transfected with human proinsulin cDNA.

These observations are in agreement with studies in isolated pancreatic islets [35], which revealed that insulin biosynthesis is tightly controlled by glucose at the translational level through stimulation of elongation rates of nascent preproinsulin and initiation of protein synthesis. The improved glucose-transport kinetics after transfection with the human GLUT2 glucose transporter also resulted in a 3-fold increase in glucokinase activity during long-term incubation with 20 mM glucose, whereas hexokinase activity was not affected. Glucose appears to be an induction factor for glucokinase activity at the level of translation or posttranslational modification, as glucokinase mRNA levels remained unchanged after incubation with high glucose concentrations. The increase in the glucokinase/hexokinase ratio from 0.2 to 0.6 demonstrates a shift in the glucose-phosphorylation kinetics towards higher glucose concentrations within the millimolar range. These results are in agreement with previous studies that suggested a positive effect of glucose metabolism on the regulation of glucokinase activity independent of glucokinase gene transcription in pancreatic B-cells [36–38].

The phosphorylation by glucokinase, rather than glucose transport, is rate-limiting and represents the flux-generating step in glycolysis and thus the signal-generating step for initiation of glucose-induced insulin secretion [5,6,12,13], as recently confirmed by Tal et al. [39]. Only when there is virtually no GLUT-2 expression, as is the case in our RINm5F control cells (Figure 2) and in various diabetic animal models [4], is glucose transport at physiological glucose concentrations likely to become a rate-limiting factor. In principle, any type of glucose transporter can increase glucose-transport capacity, as has been shown by Shibasaki et al. [21], who overexpressed the high-affinity GLUT1 glucose transporter in RINr cells and observed an increase in insulin gene expression and insulin biosynthesis in these cells. The aim of the present study was to make the RINm5F cell glucose-sensitive in the physiological glucose concentration range by increasing glucose-transport capacity by expression of the low-affinity-type GLUT2 glucose transporter. This is the most efficient way to increase glucose-transport capacity at physiological glucose concentrations and thereby improve glucose responsiveness. It is clear that GLUT2 gene expression can be reduced to a considerable extent in islet cells without causing deterioration of glucose responsiveness, although at present, no data are available to indicate the exact amount of GLUT2 expression required for unaffected glucose responsiveness in the millimolar concentration range.

High hexokinase activities in RINm5F insulinoma cells apparently limit the glycolytic flux rate at high glucose concentrations relative to that in normal pancreatic B-cells. Hexokinase

activity in these tumour cells [25] therefore needs to be decreased to achieve glucose-phosphorylation kinetics that can completely mirror the typical postprandial glucose concentration changes. The present study shows that the RINm5F insulinoma cell represents an excellent model for transfection experiments and provides evidence for the importance of the GLUT2 glucose transporter and glucokinase, the two major components of the glucose-recognition apparatus of the pancreatic B-cell in stimulus-secretion coupling. The concept of metabolic gene regulation in pancreatic B-cells by products generated during glucose metabolism may also be relevant for diabetes therapy. RINm5F tumour cells can be transplanted into diabetic rats and can thus provide a model system for studying the effects of manipulations of the glucose-recognition apparatus on glucose-induced insulin biosynthesis and secretion in pancreatic B-cell-derived insulinoma cells.

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