Insulin secretion and differential gene expression in glucose-responsive and -unresponsive MIN6 sublines

KOHTARO MINAMI,¹ HIDEKI YANO,¹ TAKASHI MIKI,¹ KAZUAKI NAGASHIMA,¹ CHANG-ZHENG WANG,¹ HIROKO TANAKA,¹ JUN-Ichi MIYAZAKI,² AND SUSUMU SEINO¹

¹Department of Molecular Medicine, Chiba University Graduate School of Medicine, Chiba 260–8670; and ²Department of Nutrition and Physiological Chemistry, Graduate School of Medicine, Osaka University, Suita 565–0871, Japan

Received 22 February 2000; accepted in final form 16 May 2000

Minami, Kohtaro, Hideki Yano, Takashi Miki, Kazuaki Nagashima, Chang-Zheng Wang, Hiroko Tanaka, Jun-ichi Miyazaki, and Susumu Seino. Insulin secretion and differential gene expression in glucose-responsive and -unresponsive MIN6 sublines. Am J Physiol Endocrinol Metab 279: E773–E781, 2000.—We have established two sublines derived from the insulin-secreting mouse pancreatic β-cell line MIN6, designated m9 and m14. m9 Cells exhibit glucose-induced insulin secretion in a concentration-dependent manner, whereas m14 cells respond poorly to glucose. In m14 cells, glucose consumption and lactate production are enhanced, and ATP production is largely through nonoxidative pathways. Moreover, lactate dehydrogenase activity is increased, and hexokinase replaces glucokinase as a glucose-phosphorylating enzyme. The ATP-sensitive K⁺ channel activity and voltage-dependent calcium channel activity in m14 cells are reduced, and the resting membrane potential is significantly higher than in m9 cells. Thus, in contrast to m9, a model for β-cells with normal insulin response, m14 is a model for β-cells with impaired glucose-induced insulin secretion. By mRNA differential display of these sublines, we found 10 genes to be expressed at markedly different levels. These newly established MIN6 cell sublines should be useful tools in the analysis of the genetic and molecular basis of impaired glucose-induced insulin secretion.

pancreatic β-cells; mRNA differential display; MIN6-m9; MIN6-m14

TYPE 2 DIABETES IS A MULTIFACTORIAL disorder characterized by both impaired insulin secretion from pancreatic β-cells and impaired insulin action at the target tissues, but it has not been determined which defect is primary (1). However, evidence has accumulated, suggesting that defective β-cell function itself is important in the pathogenesis of type 2 diabetes (23). For example, several types of type 2 diabetes have been found to be caused by mutations of genes expressed in β-cells that are distinct from the genes involved in insulin action. These include several subtypes of maturity-onset diabetes of the young (MODY 1–5). MODY 1–5 are caused by mutations in hepatocyte nuclear factor (HNF)-4α (39), glucokinase (36), HNF-1α (40), insulin promoter factor 1 (IPF-1) (29), and HNF-1β (4), respectively, all of which are related to pancreatic β-cell function, including insulin synthesis and secretion and β-cell development. In addition, maternally transmitted mitochondrial gene mutations are thought to cause diabetes due to impaired insulin secretion, possibly because of a defect in ATP production (35). Because insulin secretion is critical in the regulation of blood glucose concentrations, impaired glucose-induced insulin secretion due to alteration of the genes expressed in pancreatic β-cells that are involved in insulin synthesis and/or insulin secretion could well contribute to the development of type 2 diabetes.

One way to identify the genes associated with impaired glucose-induced insulin secretion would be to compare the genes expressed in the β-cells of normal subjects with those of type 2 diabetic patients who have impaired secretion, but this is not practical, because pancreatic islets from human subjects are difficult to obtain. Alternatively, islets from model animals such as the Goto-Kakizaki (GK) rat can be used for this purpose (5). Another approach is to compare gene expression between two different β-cell lines that exhibit different insulin secretory profiles, for example, between a cell line showing normal insulin response and one showing impaired response.

The MIN6 cell line is one of the few β-cell lines that retain insulin secretory response to glucose and other secretagogues (7, 19), and it has been used extensively in studies of the mechanisms of insulin secretion. During experiments, however, a sudden loss of glucose-induced insulin secretion from MIN6 cells during the course of the passages is sometimes noticed, possibly due to an outgrowth of cells with a poor response to glucose or a reduced expression of the genes responsible for glucose-induced insulin secretion. Accordingly, subcloning of MIN6 cells exhibiting different insulin-secretory responses to glucose might facilitate comparison of the physiological properties and the expression
of the genes associated with impaired insulin response to glucose.

In the present study, we have established two sublines of MIN6 cells that exhibit different insulin-secretory properties and metabolic features. In addition, we have compared gene expression between the two sublines by use of mRNA differential display and found 10 genes expressed at markedly different levels. These two sublines of MIN6 cells should be useful for clarifying the molecular and genetic basis of impaired glucose-induced insulin secretion.

MATERIALS AND METHODS

Subcloning of MIN6 cells. MIN6 cells were cultured in DMEM containing 25 mM glucose, 1% heat-inactivated FCS, 50 μM 2-mercaptoethanol, 100 μg/ml streptomycin sulfa- 
tate, and 60.5 μg/ml penicillin G under a humidified condition of 5% CO2-95% air at 37°C. Subcloning of MIN6 cells was performed by the limiting dilution method. Briefly, the cells were diluted and cultured in a petri dish to form separate colonies originating from a single cell. Each colony was then picked up and further cultured in a new dish to obtain a cloned cell. The cells were then screened by an index of glucose-stimulated insulin secretion. The criterion was a significant increase in insulin secretion by 25 mM glucose compared with that by 3 mM glucose.

Measurement of insulin secretion. Cells (1 × 10⁵ cells/well, 48-well plate) were seeded and precultured in DMEM for 2 days. The cells were preincubated for 30 min in HEPES-balanced Krebs-Ringer bicarbonate buffer (KRH: 119 mM NaCl, 4.74 mM KCl, 2.54 mM CaCl₂, 1.19 mM MgCl₂, 1.19 mM KH₂PO₄, 25 mM NaHCO₃, and 10 mM HEPES, pH 7.4) containing 0.5% BSA (7) with 5 mM glucose and then were incubated for 2 h with various concentrations of glucose or α-ketoisocaproate (KIC). Released insulin was measured by ELISA (Mitsui Pharmaceuticals, Tokyo, Japan). The amount of insulin secretion was normalized by the cellular protein content, rather than by cell number or DNA content, because the protein contents of m9 and m14 cells were practically identical (m9: 0.21 ± 0.029, m14: 0.20 ± 0.010 μg cellular protein/10⁵ cells, means ± SE; n = 5–7).

Measurement of glucose and lactate concentrations in culture media. Cells were cultured at a density of 1 × 10⁵ cells/well in a 48-well plate. After 1, 3, and 5 days of culture, aliquots of the medium were removed and deproteinized by the addition of 0.3 M perchloric acid (PCA). The centrifuged supernatant was used for measurement of lactate and glu- cose with appropriate kits (Roche Diagnostics, Mannheim, Germany).

Measurement of cellular ATP content and respiratory chain-driven ATP synthesis. Cells were incubated for 2 h in the presence or absence of an uncoupler of oxidative phosphorylation in mitochondria, carbonyl cyanide m-chlorophene- 
hydrazone (CCCP) (Sigma, St. Louis, MO), with or without glucose. The cells then were washed twice with ice-cold KRH, solubilized with 100 μl of cell culture lysis reagent (Promega, Madison, WI), and the lysates were collected. The amount of ATP was measured with an ATP bioluminescence assay kit (Roche Diagnostics) according to the manufactur- 
er's instructions.

Respiratory chain-driven ATP synthesis in digitonin-permealized cells was measured as previously described (32). Briefly, detached cells were suspended in Ham's F-10 me- dium and kept at room temperature for 30 min. Cells were washed and resuspended in 150 mM KCl, 25 mM Tris-HCl, 2 mM EDTA, 10 mM KH₂PO₄, 1 mM ADP, 0.1% BSA, 20 μg/ml digitonin, 10 mM malate, and 10 mM glutamate (pH 7.4). Reactions were continued for 10 min at 37°C, were termi- 
nated by adding 0.5 M (final concentration) PCA, and were chilled on ice for 15 min. The cells were then harvested, and the amount of ATP was measured as described above. To eliminate non-mitochondria-derived ATP production, ATP production in the presence of 10 μM CCCP was subtracted from that in the absence of CCCP.

Assay of enzyme activities. The activity of lactate dehydrogenase (LDH) was determined as follows. Briefly, cell ex- traction were incubated in a glycyglycine buffer (pH 10.0) with 1 mM lactate, 5 mM NAD, 50 mM glutamate, and 10 unit/ml glutamate-pyruvate transaminase at 25°C. Velocity of NADH formation was monitored by reading absorbances at 340 nm. Enzyme activity was expressed as nanomoles of NADH per minute per milligram of protein. For determination of glucose-phosphorylating activity, dis- rupted cells were centrifuged, and supernatants were incub- ated in a triethanolamine buffer (pH 7.4) containing 0.5 mM NAD, 80 μM ATP, 1 mM glucose-6-phosphate dehydroge- 
nase, and 0.5 or 50 mM glucose at 30°C. Velocity of NADH formation was monitored by reading absorbances at 340 nm. Enzyme activity was expressed as nanomoles of NADH per minute per milligram of protein.

Electrophysiological analyses. Whole cell recordings of ATP-sensitive K⁺ current were performed as described previ- ously (11). The extracellular solution contained 135 mM NaCl, 5 mM KCl, 5 mM CaCl₂, 2 mM MgSO₄, 5 mM HEPES, and 3 mM glucose (pH 7.4). The pipette solution contained 107 mM KCl, 11 mM EGTA, 2 mM MgSO₄, 1 mM CaCl₂, and 11 mM HEPES (pH 7.2). The membrane potentials of the cells were measured by the perforated patch-clamp method in the current clamp mode (2). The extracellular solution contained 125 mM NaCl, 5 mM KCl, 1.3 mM KH₂PO₄, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 2.8 mM glucose (pH 7.4). The pipette solution contained 130 mM potassium aspartate, 10 mM KCl, 10 mM EGTA, 10 mM MOPS (pH 7.2), and 100 μg/ml nysta- tin.

Whole cell Ba²⁺ currents through the voltage-dependent calcium channels (VDCCs) were recorded as described (11). Briefly, Ba²⁺ was used as a charged carrier for measurement of VDCC currents. The extracellular solution contained 40 mM Ba(OH)₂, 20 mM 4-aminopyridine, 90 mM tetraethylam- 
monium monohydroxide, 10 mM tetraethylammonium chloride, 140 mM methanesulfonate, and 10 mM MOPS (pH 7.4). The pipette solution contained 10 mM CsCl, 130 mM cesium aspartate, 10 mM EGTA, 5 mM Mg-ATP, and 10 mM MOPS (pH 7.2). Cells were maintained at a holding potential of −60 mV, and square pulses of 400-ms duration at potentials between −40 and +70 mV in steps of 10 mV were applied every 4 s. Recordings were performed with the use of the EPC-7 amplifier (List Electronics, Darmstadt, Germany).

Measurement of intracellular calcium concentration. Cells were loaded with 2 μM fura 2 acetoxymethyl ester (Dojindo, Kumamoto, Japan) for 50 min in the incubation buffer con- taining 154 mM NaCl, 6.2 mM KCl, 3.3 mM CaCl₂, 1.5 mM KH₂PO₄, 1.6 mM MgSO₄, 12.4 mM NaHCO₃, 20 mM HEPES, and 2.8 mM glucose (pH 7.4) and then were mounted on the stage of the microscope. The perfusion rate was 1 mm/min at 37°C. Intracellular calcium concentration ([Ca²⁺]i) was measured by a dual-excitation wavelength method (340/380 nm) as described (11). Fluorescence emission at 510 nm was monitored, and the ratio calculation was digitized every 10 s by a computerized image processor (Argus-50/CA; Hamamatsu Photonics, Hamamatsu, Japan).
**RESULTS**

**Subcloning of cells from MIN6 cells.** We subcloned a total of 42 cell lines from MIN6 cells and screened them by an index of glucose-induced insulin secretion. Among these, two cell lines, designated m9 and m14, were selected as a good responder and a poor responder, respectively, to glucose. The difference in morphology between these two cell lines is that m14 cells are generally round, whereas m9 cells have an irregular shape (Fig. 1). Both cell lines were established at 18 passages.

**mRNA differential display.** mRNA differential display was performed with a commercial kit (Takara, Otsu, Japan) according to the manufacturer’s instructions. Differentially expressed bands of interest were extracted from the gel and reamplified by PCR and were then cloned into pGEM-T Easy Vectors (Promega). The obtained clones were then subjected to RNA blot analysis. Sequencing was performed with the ABI PRISM dye terminator cycle sequencing FS ready reaction kit and DNA sequencer model 377 (Applied Biosystems, Foster City, CA). The sequences were compared with those in the database at the National Center for Biotechnology Information by means of the BLAST network service.

**RNA blot analysis.** Ten micrograms of total RNA from m9 and m14 cells were subjected to formaldehyde-agarose gel electrophoresis. The RNA was transferred to a nylon membrane and ultraviolet cross-linked. The membrane was hybridized with α-[32P]dCTP-labeled probes corresponding to cDNA of mouse glucokinase (GenBank Acc. No. L38990, nt 98–652), mouse hexokinase (J05277, nt 1464–1903), hamster sulfurylurea receptor 1 (SUR1; L40623, nt 3126–4049), mouse Kir6.2 (U73626, nt 753–1427), mouse GLUT-1 (M23384, nt 473–885), mouse GLUT-2 (X15684, nt 297–955), mouse NADH dehydrogenase subunit 1 (J01420, nt 2761–3061), mouse cytochrome c oxidase subunit 2 (J01420, nt 7016–7500), or mouse β-actin (X03672, nt 571–1170). Megaprime random primer labeling kit (Amersham-Pharmacia Biotech, Uppsala, Sweden) was used for labeling the probes. The cDNAs obtained by mRNA differential display were similarly labeled and were used as probes for hybridizations. Blots were exposed to Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY) at –70°C.

**Statistical analysis.** Statistical significance of difference between the two groups was determined by unpaired Student’s t-test. Differences were considered significant at P < 0.05.

**Insulin secretory properties.** Insulin secretion from m9 cells was stimulated by glucose in a concentration-dependent manner (Fig. 2A) comparable to that from the original MIN6 cells (7). The cells also secreted insulin by KIC, a nonglucose insulin secretagogue, in a concentration-dependent manner (Fig. 2B). Moreover, as in normal islets, the stimulatory effects of glibenclamide and acetylcholine on insulin secretion were observed in m9 cells (data not shown). In contrast, insulin secretion from m14 cells responded poorly to increments of both glucose (Fig. 2A) and KIC (Fig. 2B). However, in the presence of 30 mM KCl, m14 cells secreted almost the same amount of insulin as m9 cells (Fig. 2C). Figure 2D shows the effect of 3-isobutyl-1-methylxanthine (IBMX), a cAMP phosphodiesterase inhibitor, on glucose-induced insulin secretion in m9 and m14 cells. High glucose-induced insulin secretion was potentiated by 100 μM IBMX similarly (two- to threefold increases vs. that in the absence of IBMX) in both sublines. Furthermore, forskolin, an activator of adenylate cyclase, and 8-bromoadenosine 3′,5′-cyclic monophosphate, a membrane-permeable analog of cAMP, had effects similar to those of IBMX (data not shown). These properties of insulin secretion in both cell lines were retained through at least 40 passages.

**Metabolic features.** Although glucose consumption by m14 cells was much greater than that by m9 cells (Fig. 3A), the increment of cellular ATP content after stimulation of 25 mM glucose was not significantly different between the sublines (Fig. 3B). The ATP content of m9 cells was greatly reduced by 10 μM CCCP, an uncoupler of oxidative phosphorylation in mitochondria, but the compound did not affect ATP production in m14 cells (Fig. 3B). In accordance with this observation, a nonoxidative glucose metabolite lactate in m14 media was significantly higher than in m9 cell media (Fig. 3A). Cellular LDH activity was also significantly higher in m14 cells (Table 1).

To examine the functional capacity of the mitochondrial oxidative phosphorylation system, ATP synthesis driven by the respiratory chain was measured in digitonin-permeabilized m9 and m14 cells. When stimulated with malate and glutamate, CCCP-
sensitive ATP production (ATP production without CCCP minus the production with 10 μM CCCP) in m14 cells was not reduced but rather was enhanced compared with that in m9 cells (Fig. 3C). The expression of mitochondrial genes of m9 and m14 cells, as assessed by NADH dehydrogenase subunit 1 and cytochrome c oxidase subunit 2 mRNA, was not different (Fig. 4A).

Fig. 3. Metabolic features of m9 and m14 cells. A: changes in lactate and glucose concentrations in the culture media. Cells were seeded onto a 48-well plate at day 0. At days 1, 3, and 5, aliquots of each medium were removed, and the concentrations of lactate and glucose were determined. Data are means ± SE (n = 6). B: cellular ATP contents. Cells were precultured and preincubated as described above. Incubation was performed in the presence or absence of 10 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP), with or without 25 mM glucose for 2 h. Cells were lysed, and ATP contents were determined by a bioluminescence method. C: respiratory chain-driven ATP synthesis. Digitonin-permeabilized cells were incubated with 20 mM malate and 20 mM glutamate for 10 min. ATP production in the presence of 10 μM CCCP was subtracted. Data are means ± SE (n = 7). *P < 0.05, **P < 0.01. NS, difference is not significant.
Table 1. Enzyme activities in MIN6 sublines

<table>
<thead>
<tr>
<th></th>
<th>m9</th>
<th>m14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate dehydrogenase activity</td>
<td>79.4 ± 27.6</td>
<td>348 ± 94.2**</td>
</tr>
<tr>
<td>Rate of glucose phosphorylation at 0.5 mM glucose</td>
<td>2.07 ± 0.19</td>
<td>7.87 ± 0.33**</td>
</tr>
<tr>
<td>50 mM glucose</td>
<td>11.1 ± 2.75</td>
<td>7.90 ± 0.76</td>
</tr>
</tbody>
</table>

For lactate dehydrogenase activity, cell extracts were incubated in a glycylglycine buffer (pH 10.0) with 1 mM lactate, 5 mM NAD, 50 mM glutamate, 10 unit/ml glutamate-pyruvate transaminase at 25°C. Data are means ± SE (n = 3). For glucose-phosphorylating activity, disrupted cells were centrifuged, and supernatants were incubated in a triethanolamine buffer (pH 7.4) containing 0.5 mM NAD, 5 mM ATP, 1 unit/ml glucose-6-phosphate dehydrogenase, and 0.5 or 50 mM glucose at 30°C. Enzyme activity was expressed as nmol NADH·min⁻¹·mg protein⁻¹. Data are means ± SE (n = 4). *P < 0.05, **P < 0.01; m9 vs. m14.

RNA blot analysis revealed the expression of high Michaelis-Menten constant ($K_m$) glucose-phosphorylating enzyme glucokinase mRNA to be markedly decreased in m14 cells compared with m9 cells (Fig. 4B, top), whereas low $K_m$ enzyme hexokinase mRNA was markedly increased in m14 cells (Fig. 4B, bottom). In the presence of 50 mM glucose (representing the activity of glucokinase plus hexokinase), the difference in glucose-phosphorylating activity was not significant between m9 and m14 cells, whereas in the presence of 0.5 mM glucose (representing hexokinase activity), the activity was significantly higher in m14 cells (Table 1). Glucokinase activity (glucose-phosphorylating activity at 50 mM glucose minus the activity at 0.5 mM glucose) was extremely low in m14 cells (Table 1).

In addition, we also examined the mRNA levels of glucose transport proteins GLUT-1 and GLUT-2. GLUT-1 mRNA expression was detected in both m9 and m14 cells, whereas GLUT-2 expression was not detected in either of the cell lines (Fig. 4D). To confirm the level of GLUT-2 mRNA expression in these cell lines, we performed RT-PCR. GLUT-2 PCR products from m9 and m14 cDNAs were only faintly detected after 30 cycles of amplification, whereas GLUT-1 PCR products were clearly detected after 25 cycles of amplification (data not shown). There was no significant difference in the expression of GLUT-1 and GLUT-2 mRNA between m9 and m14 cells.

Electrophysiology and $[Ca^{2+}]_i$. Because insulin secretion is triggered by an elevation of $[Ca^{2+}]_i$, through activation of VDCCs resulting from membrane depolarization by closure of ATP-sensitive K⁺ ($K_{ATP}$) channels, we next examined the electrophysiological features and $[Ca^{2+}]_i$ of the two cell lines. As shown in Fig. 5A, the normalized peak $K_{ATP}$ channel conductance of m14 cells was significantly lower than that of m9 cells, indicating that the number of functional $K_{ATP}$ channels on the plasma membrane is less in m14 cells. However, mRNA levels of Kir6.2 and SUR1, the components of the β-cell $K_{ATP}$ channels (6), were not significantly different in m9 and m14 cells (Fig. 4C). The resting membrane potential, which is determined primarily by $K_{ATP}$ channels in β-cells (18), was significantly higher in m14 than in m9 cells (Fig. 5B).

$[Ca^{2+}]_i$ was elevated dramatically in response to 25 mM glucose in m9 cells, whereas no significant change in $[Ca^{2+}]_i$ was observed in m14 cells (Fig. 5C). Furthermore, the current-voltage relationships of VDCCs in m9 and m14 cells are shown in Fig. 5D. The VDCC inward currents in m14 cells are significantly less than those in m9 cells.

mRNA differential display. To investigate differences in gene expression between m9 and m14 cells, we performed mRNA differential display of these cell lines. By use of 216 combinations of anchored and arbitrary primers, 98 unique bands were detected by differential RT-PCR. Among these differentially displayed bands, RNA blot analysis showed 10 genes to be differentially expressed between the two cell lines (Fig. 6). As shown in Table 2, the sequences of five clones represent known genes. Clones g-1, g-2, and g-3 were underexpressed in m14 cells and represent stanniocalcin (STC), delta-like protein precursor (DLK)/preadipocyte factor 1 (Pref-1), and KIAA0480, respectively. Clones p-1 and p-2 were overexpressed in m14 cells and represent Na⁺/H⁺ exchanger 3 kinase A regulatory protein (E3KARP) and CCK-B receptor, respectively. Four matched only expressed sequence tag (EST) sequences, and one did not match any sequences in the GenBank database.

RNA blot analysis of the unknown genes. To determine tissue expression of the unknown genes that are differentially expressed between m9 and m14 cells, RNA blot analysis of mouse tissues was performed (Fig. 7). Clone g-4 was expressed in ovary, brain, kidney, and testis. Clone g-5 was expressed in ovary and testis.
testis. Expression of clone p-3 was observed in ovary, lung, kidney, intestine, and testis. Clone p-4 was expressed exclusively in brain. No hybridizing signals were detected in clone p-5 (data not shown).

**DISCUSSION**

In the present study we have established and characterized two sublines derived from MIN6 cells. One, designated m9, has features similar to those observed in the original MIN6 cells (7, 19) and retains glucose-induced insulin secretion after repetitive passages. The other, designated m14, has impaired glucose-induced insulin secretion. Thus both a glucose-responsive and a glucose-unresponsive cell line have been cloned from a single β-cell line, facilitating comparison of the physiological properties and the expression of the genes associated with impaired glucose-induced insulin secretion.

In m14 cells, KIC-induced insulin secretion was also impaired, and cellular ATP was generated largely through nonoxidative pathways, metabolic features similar to those of mitochondrial DNA-depleted β-cell lines (12, 28, 32). However, because the mitochondrial gene expression of m14 cells was not different from that of m9 cells (Fig. 4A) and direct activation of mitochondrial ATP production in digitonin-permeabilized cells was not reduced in m14 cells (Fig. 3C), it is not likely that the loss of KIC-induced insulin secretion or the increase in nonoxidative ATP production found in m14 cells is the result of mitochondrial dysfunction.

In accordance with the enhancement of nonoxidative ATP production, glucose consumption and lactate production were markedly enhanced in m14 cells (Fig. 3A). Glucose enters pancreatic β-cells through specific transport proteins and is metabolized mainly in the glycolytic pathway, generating pyruvate as a substrate of the tricarboxylic acid cycle in mitochondria. In a nonoxidative state, pyruvate is converted to lactate by LDH, and lactate is then released from the cells. In general, however, because β-cells express little LDH activity, most glucose-derived pyruvate enters the oxidative pathway (24, 26). In contrast, m14 cells exhibited significantly higher LDH activity than m9 cells (Table 1), which could lead to a diversion of pyruvate from mitochondrial oxidation. This might cause the acceleration of glucose metabolism in glycolysis due to compensation for reduced mitochondrial ATP generation.

Interestingly, it has been reported that low LDH activity is important in β-cell glucose sensing through unidentified mechanisms and that stable overexpression of LDH attenuates glucose-induced insulin secretion in MIN6 cells (42). Thus the impaired glucose-induced insulin secretion in m14 cells could be due in part to enhanced LDH activity.

Glucokinase is a rate-limiting enzyme in β-cell glycolysis and is thought to be the glucose sensor for glucose-induced insulin secretion (20), because it has a $K_m$ higher than the physiological concentration of glucose. Like normal β-cells (17) and the original MIN6 cells (8), m9 cells express glucokinase predominantly, whereas m14 cells express hexokinase, a low-$K_m$ isozyme of the glucose-phosphorylating enzyme, in replacement of glucokinase (Fig. 4B). In accordance with the results of RNA blotting, glucokinase activity (glu-
cose-phosphorylating activity in the presence of 50 mM glucose minus that in the presence of 0.5 mM glucose) in m14 cells is close to zero (Table 1), and this could account for the impaired glucose sensitivity in m14 cells (Fig. 2A).

The major isoform of glucose transporters expressed in rodent pancreatic β-cells is GLUT-2 (30), and this high-\(K_m\) glucose transporter has been a candidate glucose sensor for insulin secretion in β-cells (34). However, it is now generally accepted that the most important β-cell glucose sensor is not GLUT-2 but glucokinase (20). In support of this, the overexpression of the GLUT-1 isoform in MIN6 cells has been found not to affect glucose-induced insulin secretion, whereas overexpression of hexokinase does alter glucose sensitivity (8). Moreover, in the present study, we found that the GLUT-2 mRNA level is very low in both m9 and m14 cells (Fig. 4D); nevertheless, the glucose sensitivity of m9 cells is comparable to that of the original MIN6 cells predominantly expressing the GLUT-2 isoform (7, 19). These findings also suggest that GLUT-2 is not a major glucose sensor in pancreatic β-cells and indicate that the loss of GLUT-2 expression cannot account for the impaired glucose-induced insulin secretion found in m14 cells.

Electrophysiological experiments show that the number of functional K\(\text{ATP}\) channels of m14 cells is less than in m9 cells (Fig. 5A) and that the function of VDCCs also is impaired in m14 cells (Fig. 5D). In pancreatic β-cells, K\(\text{ATP}\) channels play a crucial role in glucose-induced insulin secretion by coupling cell metabolism to electrical activity. Closure of K\(\text{ATP}\) channels depolarizes the plasma membrane and leads to activation of the VDCCs, triggering exocytosis of insulin-containing granules (25). Dysfunction of these channels, therefore, could be responsible for the impaired glucose-induced insulin secretion in m14 cells. The loss of KIC-induced insulin secretion and the loss of glucose-induced insulin secretion, despite the comparable amount of ATP production with m9 cells (even though the pathway of ATP synthesis is different), could be explained, at least in part, by abnormalities in K\(\text{ATP}\) channels or VDCCs. Furthermore, it has recently been reported that mitochondria-derived glutamate potentiates glucose-induced insulin secretion distinct from that due to ATP (16). In m14 cells, because mito-

---

**Table 2. Summary of differentially expressed genes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Best Match in GenBank</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>g-1</td>
<td>stanniocalcin</td>
<td>AF099098</td>
</tr>
<tr>
<td>g-2</td>
<td>DLK/Pref-1</td>
<td>Q09163</td>
</tr>
<tr>
<td>g-3</td>
<td>KIAA0480</td>
<td>AB007949</td>
</tr>
<tr>
<td>g-4</td>
<td>EST</td>
<td>AA285653</td>
</tr>
<tr>
<td>g-5</td>
<td>EST</td>
<td>AU018846</td>
</tr>
<tr>
<td>p-1</td>
<td>E3KARP</td>
<td>AF004900</td>
</tr>
<tr>
<td>p-2</td>
<td>CCK-B receptor</td>
<td>D12817</td>
</tr>
<tr>
<td>p-3</td>
<td>EST</td>
<td>AU021144</td>
</tr>
<tr>
<td>p-4</td>
<td>EST</td>
<td>D28695</td>
</tr>
<tr>
<td>p-5</td>
<td>no match</td>
<td></td>
</tr>
</tbody>
</table>

Sequencing was performed as described in MATERIALS AND METHODS. The sequences were compared with those in the database at the National Center for Biotechnology Information using the BLAST network service. DLK, delta-like protein precursor; Pref-1, preadipocyte factor 1; E3KARP, Na\(+\)/H\(+\) exchanger 3 kinase A regulatory protein.

---

**Fig. 6.** Expression of the genes identified by mRNA differential display. Total RNA (10 \(\mu\)g) isolated from the cells was electrophoresed (m9 on left lane, m14 on right lane) and transferred onto a nylon membrane. A: the genes strongly expressed in m9 cells; B: the genes expressed exclusively in m14 cells. Numbers on the right indicate sizes of transcripts (kb). β-actin was hybridized to confirm equal loading (bottom, each box).

**Fig. 7.** Tissue expression of the unknown genes that are differentially expressed between m9 and m14 cells. Total RNA (10 \(\mu\)g) isolated from mouse tissues was blotted and hybridized. Lane 1, ovary; lane 2, brain; lane 3, heart; lane 4, lung; lane 5, liver; lane 6, kidney; lane 7, skeletal muscle; lane 8, intestine; lane 9, testis; lane 10, pancreas. Numbers on the right indicate sizes of transcripts (kb).
chondrial metabolism might be impaired due to enhanced LDH activity, sufficient glutamate may not be generated, and this could also reduce glucose-induced insulin secretion.

Interestingly, cAMP-mediated potentiation of glucose-induced insulin secretion appears to be retained in glucose-unresponsive m14 cells (Fig. 2D). As described above, the function of K_{ATP} channels and VDCCs is impaired in m14 cells, and [Ca^{2+}]_i remains unchanged after stimulation by high glucose (Fig. 5C). These findings suggest that the cAMP-mediated potentiation pathway is regulated differentially from K_{ATP} channel-mediated glucose-induced insulin secretion, consistent with previous findings with high KCl-exposed pancreatic islets (38).

Although pancreatic β-cell lines derived from rodents have been extensively used to investigate the mechanism of glucose-induced insulin secretion, their phenotypic instability is a common problem (13). Thus attempts were made to isolate cell lines exhibiting stable responsiveness to glucose, and the cloning of βTC and INS-1 cell-derived sublines have been reported (3, 13). These cell lines retain correct glucose responsiveness after prolonged propagation. cAMP-mediated potentiation of glucose-induced insulin secretion is observed in both clonal cell lines (3, 13), and glucokinase is the predominant glucose-phosphorylating enzyme in clonal βTC cells (13). These observations are consistent with ours in m9 cells, although GLUT-2 mRNA expression is apparent in the βTC cells (13). This further indicates that glucokinase, but not GLUT-2, is critical for glucose sensing in β-cells.

As a model of type 2 diabetes, GK rats have been intensively investigated (10, 15, 33). Pancreatic islets of GK rats are poorly responsive to glucose, whereas high K^{+}-stimulated insulin secretion is not reduced (10), and both glucose usage and lactate production are accelerated (15). Although these properties resemble those of m14 cells, K_{ATP} channel function itself is not altered in GK rat islets, and VDCC activity is rather enhanced (10, 33). Moreover, alterations in glucokinase have not been reported in GK rats, further suggesting that the physiological properties of m14 cells are different from those of GK rat islets.

Impaired glucose-induced insulin secretion may well be caused by alterations in the genes expressed in pancreatic β-cells that are involved in insulin synthesis and/or insulin secretion. As an approach to identify genes associated with impaired glucose-induced insulin secretion, we performed mRNA differential display using m9 and m14 cells. An advantage of this strategy is that the number of differentially expressed genes between the glucose-responsive (m9) and -unresponsive (m14) insulin-secreting cell lines should be minimal because of their derivation from a single cell line. We found 10 genes to be quite differentially expressed between these cell lines. Among them, STC and DLK/Pref-1 are particularly interesting, because they should participate in the regulation of calcium mobilization (22) and β-cell differentiation (21), respectively. STC is a calcium-regulating hormone originally discovered in bony fish (37), the high expression of which in differentiated brain neurons in humans and in mice was recently demonstrated (22, 41). Zhang et al. (41) have suggested that the molecule may regulate transmembrane calcium fluxes and contribute to protection against hypercalcemia in terminally differentiated neurons. The absence of STC gene expression, therefore, could be associated with dysfunction of calcium regulation in m14 cells. DLK is a transmembrane protein of the EGF-like family of homeotic proteins (14), and Pref-1 is a variant derived from the same gene (27). In the developing pancreas, fetal antigen-1 (FA-1), the cleaved form of Pref-1, is expressed in most epithelial cells at an early stage, but its expression later becomes restricted to the β-cells (31). Moreover, it is reported that FA-1 is colocalized with insulin in the insulin secretory granules of β-cells in the adult human pancreas (9). Pref-1/FA-1, therefore, may have a role as an autocrine growth factor in β-cells and may regulate their differentiation and proliferation (9, 21).

The reduced expression of the DLK/Pref-1 gene in m14 cells may thus affect their differentiation and proliferation. Among the five unknown clones, the expression of clones g-5 and p-4 is restricted to a few tissues, suggesting their unique roles. Whether or not alteration of these genes occurs independently in the development of impaired glucose-induced insulin secretion or whether there is a major gene responsible for the complex abnormalities remains to be determined.

This study was supported by a Grant-in-Aid for Creative Basic Research (10NP0201) from the Ministry of Education, Science, Sports and Culture, Japan, by a Scientific Research Grant from the Ministry of Health and Welfare, Japan, by a grant from Takeda Chemical Industries, by the Yamanouchi Foundation for Research on Metabolic Disorders, by the Suzukem Memorial Foundation, and by Mitsui Pharmaceuticals.

K. Minami is a visiting research fellow from the Institute of Biological Science, Mitsui Pharmaceuticals; C.-Z. Wang is supported by a Postdoctoral Fellowship for Foreign Researchers from the Japan Society for the Promotion of Science.

REFERENCES


