Ins1 Gene Up-Regulated in a β-Cell Line Derived from Ins2 Knockout Mice

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The authors have derived a new β-cell line (βIns2−/−lacZ) from Ins2−/− mice that carry the lacZ reporter gene under control of the Ins2 promoter. βIns2−/−lacZ cells stained positively using anti-insulin antibody, expressed β-cell–specific genes encoding the transcription factor PDX-1, glucokinase, and Glut-2, retained glucose-responsiveness for insulin secretion, and expressed the lacZ gene. Analysis of Ins1 expression by reverse transcriptase–polymerase chain reaction (RT-PCR) showed that Ins1 transcripts were significantly raised to compensate for the lack of Ins2 transcripts in βIns2−/−lacZ cells, as compared to those found in βTC1 cells expressing both Ins1/Ins2. Thus, transcriptional up-regulation of the remaining functional insulin gene in Ins2−/− mice could potentially contribute to the β-cell adaptation exhibited by these mutants, in addition to the increase in β-cell mass that we previously reported. We have also shown that lacZ expression, as analyzed by determining β-galactosidase activity, was up-regulated by incubating βIns2−/−lacZ cells with GLP-1 and/or IBMX, 2 known stimulators of insulin gene expression. These cells thus represent a new tool for testing of molecules capable of stimulating Ins2 promoter activity.

Keywords β-Cell Line; Compensatory Responses; Diabetes; Glucose Homeostasis; Insulin Genes; Knockout Mice

Insulin resistance and β-cell dysfunction are the two major characteristic features of type 2 diabetes. It is now well admitted that overt diabetes does not develop as long as the β-cells can secrete increasing amounts of insulin to overcome insulin resistance [1]. Several groups have extensively investigated the molecular mechanisms that regulate insulin gene expression as well as insulin secretion during the past several years [2, 3]. Besides, a few studies in rodents also showed that the β-cell mass could significantly increase during pregnancy [4] or after glucose infusion for a short period, revealing potential plasticity of the β-cell compartment [5, 6]. Moreover, the study of knockout mice for insulin receptor substrate (IRS)-1, an intracellular mediator of insulin signaling, provided convincing evidence that insulin resistance could be overcome if β-cell mass were increased [7]. This conclusion was strongly supported by the observation that insulin resistance in IRS-2–knockout mice, which fail to increase their β-cell mass, leads to overt diabetes [8].

In recent years, transgenic and gene-targeting approaches in the mouse were extensively applied to examine the effects of manipulating the expression of genes encoding key players in β-cell development and/or function, such as the transcription factor PDX-1 [9, 10] or proteins involved in glucose-regulated insulin secretion, such as glucokinase (GK) [11] or the glucose transporter Glut-2 [12]. We previously generated knockout mice for the Ins1 and Ins2 genes [13]. The single Ins1−/− or Ins2−/− mutants were viable, fertile, and did not show any major metabolic alteration. Total pancreatic insulin content in Ins1−/− or Ins2−/− mice was comparable to that found in control animals, indicating that compensatory up-regulation take place in these mutants. Analysis of the
transcripts of the remaining functional insulin gene revealed a dramatic increase of Ins1 transcripts in total pancreatic RNA from Ins2−/− mice. Interestingly, morphometric analysis of the pancreases showed that β-cell mass in both kinds of mutants was significantly augmented, particularly in Ins2−/− mutants. Thus, absence of either one of the 2 insulin genes was partly compensated at the tissue level by a specific increase of the β-cell compartment [13].

It was, however, difficult to determine whether some compensatory up-regulation of Ins1 could also take place in individual β-cells in Ins2−/− mice. We have addressed this issue here using a β-cell line derived from Ins2−/− mutants. Because Ins2−/− mice carry lacZ reporter gene under the control of the Ins2 promoter, such β-cells also represent an interesting tool for the testing of drugs that could up-regulate Ins2 promoter activity.

MATERIALS AND METHODS

Animals

Generation of Ins2−/− mice has been described previously [14]. These mutant mice were crossed with Rip1-Tag2 mice [15] (kindly provided by D. Hanahan) and an Ins2−/−, Rip1-Tag2 mouse line was established. The offspring were genotyped by polymerase chain reaction (PCR) using specific primers.

Derivation of the βIns2−/−lacZ Cell Line

β-Cell lines from Ins2−/−, Rip1-Tag2 mice were isolated as previously described [16]. Briefly, 10-week-old Ins2−/−, Rip1-Tag2 mice were killed by cervical dislocation, and the pancreases were perfused through the bile duct with 2 mg/mL collagenase (Sigma, St. Louis, MO) dissolved in Hank’s buffered saline solution (HBSS, Life Technologies, Gaithersburg, MD). The pancreases were incubated for 20 minutes at 37°C with gentle agitation. The digested material was washed 3 times in HBSS containing 10% fetal bovine serum (FBS; Techgen, Les Ullis, France) and then recovered in RPMI with 10% FBS. Islets were hand-picked under a dark-field microscope and cultured in the same medium in an incubator with 5% CO2 at 37°C. Islets were then placed individually in 24-well plates and cultured in high-glucose medium containing 25 mM Hepes (Life Technologies), 15% horse serum (Life Technologies), 2.5% FBS, 200 U/mL penicillin, and 200 μg/mL streptomycin. After approximately 2 months in culture, islets that clearly expanded were removed, trypsinized, and plated onto 96-well plates to recover β-cell lines. At confluency, cells were usually split at a 1:2 or 1:3 ratio. βTC1 cells (obtained from D. Hanahan) used in some experiments were cultured in the same medium.

Immunocytochemistry and Histochemistry

For insulin detection, cells were fixed in 4% paraformaldehyde, incubated first with guinea pig polyclonal anti-insulin antibody and then with peroxidase-coupled goat anti–guinea pig serum as described [14]. The peroxidase activity was revealed using diaminobenzidine. To detect β-galactosidase (β-gal) activity, cells were fixed in 0.25% glutaraldehyde and then stained using 5-bromo-4-chloro-3-indolyl β-d-galactoside (X-gal) as described [13].

Reverse Transcriptase (RT)-PCR Analysis of Gene Expression

The primers and probes used to analyze insulin 1/insulin 2, glucagon, somatostatin, pancreatic polypeptide (PP), and β-actin transcripts have been specified previously [13]. The primer sets and oligonucleotide probes used to analyze the transcripts for PDX-1, GK, Glut-2, and TATA-binding protein (TBP) were as follows: 5′-TCGGCTGGGATCAGGGAGCA-3′/5′-GGTCCGCTGTGTGAAGTACAT-3′ and 5′-[32P]-GACC TTCTCCGAATGGAA CC-3′ for PDX-1; 5′-CACCCAACG GCAGAAATCACC-3′/5′-CATTGTGGAAGTC-5′ and 5′- [32P]-GGGCAATGGAATCCAGGCA-3′ for GK; 5′-GA GCCAAGACCGGTCTCA-3′/5′-GTGAAAGCCAGGAC CACCCC-3′ and 5′-[32P]-GCCCTCTGCTTCCAGTACAT-3′ for Glut-2; 5′-AAAGAGGCCACCGAATCTG-3′/5′-TACT GAACCTGCTTGGGTC-3′ and 5′-[32P]-GCCCTCTGCTTCCAGTACAT-3′ for TBP. The following PCR reactions were used: 48°C, 45 minutes; 94°C, 2 minutes, 94°C, 30 seconds; 60°C, 1 minute, 68°C, 2 minutes for 25 cycles; 72°C 10 minutes. The PCR products were run on agarose gels, transferred onto Hybond membranes (Amersham, Saclay, France), and hybridized using [32P]-labeled oligonucleotide probes. For insulin 1/insulin 2, a unique primer pair was used for RT-PCR, and the PCR products were digested with MspI before Southern blot analysis. Hybridization using a single [32P]-labeled oligonucleotide probe revealed a fragment of 71 bp for insulin 1 and another of 112 bp for insulin 2. Quantification of PCR products was performed using a Phosphorimager equipped with ImageQuant software (Molecular Dynamics) and was expressed in relation to the internal control.

Total Cellular Insulin Content and Insulin Secretion

Total cellular insulin content was obtained by radioimmunoassay (RIA) kit (ICN, Irvine, CA) using acid/alkohol extracts as described [16]. Insulin secretion assays were performed with cells cultured in 48-well plates when they reached 70% to 80% confluency as described [15]. Briefly, cells were preincubated for 1 hour in Krebs-Ringer buffer, then incubated
for 2 hours in Krebs-Ringer buffer containing different glucose concentrations as indicated. The secretion medium was recovered, centrifuged for 10 minutes at 1000 rpm, and supernatants were stored at −80°C until used for determining insulin levels by RIA.

**Determination of β-Gal Activity**

Cells were cultured in 48-well plates in high-glucose medium and incubated for 48 hours in the presence of GLP-1 (Sigma) and/or 3-isobutyl-1-methylxanthine (IBMX) (Sigma) at the indicated concentrations. Cells were then washed with phosphate-buffered saline (PBS) solution and lysed in a commercial lysis buffer (Boehringer, Mannheim, Germany). β-Gal activity in the protein extracts was determined by colorimetric assay using o-nitrophenyl β-D-galactopyranoside (ONPG) (Sigma) as substrate. Blank values obtained with lysates from βTC1 cells were subtracted.

**RESULTS AND DISCUSSION**

**β-Cell Line Derived From Ins2−/− Mice**

In previous studies, β-cell lines were isolated from islets of transgenic mice expressing SV40 large T antigen gene under the control of rat Ins1 gene promoter (Rip1-Tag2 mice) [15, 16]. Therefore Ins2−/− mice were crossed with Rip1-Tag2 mice and an Ins2−/−, Rip1-Tag2 mouse line was established. Hyperplastic islets from pancreases of Ins2−/−, Rip1-Tag2 mice were isolated prior to tumor formation, i.e., at 8 to 10 weeks. After a 2-month culture in high-glucose medium (22 mM), some islets clearly expanded as a result of efficient proliferation of β-cells transformed by SV40 T antigen, and individually trypsinized. One of the β-cell lines recovered from cultured cells used in this study was designated βIns2−/−lacZ. These cells showed a doubling time of 10 days and grew in islets-like clusters. The cells were not further subcloned to avoid clonal effects and used up to passage 19 in the experiments described here.

We first confirmed that βIns2−/−lacZ cells were β-cells. βIns2−/−lacZ cells were stained using an anti-insulin antibody (Figure 1A). Ins2−/− mice carry the lacZ reporter gene under control of the Ins2 promoter and the presence of β-gal in βIns2−/−lacZ cells was detected by X-gal staining (Figure 1B). Because the explanted islets of origin also contained α, δ, and PP cells, which produce glucagon, somatostatin, and pancreatic polypeptide, respectively, we checked for the expression of the corresponding genes for these hormones by RT-PCR, using total RNA from βIns2−/−lacZ cells. No transcript was found for somatostatin and pancreatic polypeptide. Few glucagon transcripts were detected (not shown), as is frequently observed when deriving a β-cell line using Rip1-Tag2 construct [17].

We also analyzed the expression of genes encoding PDX-1, GK, and Glut-2. PDX-1 is a transcription factor that is essential for pancreas development and in the regulation of islet gene expression in mature β-cells [18]. It was shown that PDX-1 expression in the liver upon adenoviral vector-mediated gene transfer in the mouse resulted in transdifferentiation of a small fraction of hepatocytes into insulin-secreting cells [19]. GK is the high-Km hexokinase present in both β-cells as well as in hepatocytes and catalyzes the initial conversion of glucose to glucose-6-phosphate. Finally, Glut-2 is a glucose transporter also present in β-cells and hepatocytes [20]. The role of GK and Glut-2 in glucose sensing was fully confirmed by global and tissue-specific disruption of the corresponding genes in the mouse. Knockout mice for GK or Glut-2 presented defective insulin secretion and developed diabetes [11, 12]. As presented in Figure 2, transcripts for βIns2−/−lacZ cells continue to synthesize insulin and express important β-cell–specific marker genes. Moreover, Ins2 promoter drives expression of lacZ gene in these cells.

**Glucose-Induced Insulin Secretion From βIns2−/−lacZ Cells**

We examined the ability of βIns2−/−lacZ cells to secrete insulin in response to glucose because this property is frequently lost when deriving a β-cell line [17]. We first measured total cellular insulin content in acid-alcohol extracts.

**FIGURE 1**

Immunocytochemical detection of insulin (A) and histochemical analysis of lacZ expression (B) in βIns2−/−lacZ cells. (A) Cells were fixed and stained using an anti-insulin antibody. (B) Cells were fixed and β-gal activity was visualized by X-gal staining. Magnification: 10×.
FIGURE 2
RT-PCR analysis of β-cell–specific gene expression. Transcripts for PDX-1 (A), Glut-2 (B), and GK (C) were amplified using total RNA from βIns2−/−lacZ cells and analyzed by Southern blotting. TBP mRNA was coamplified as control. The results of 2 separate samples are presented. The sizes of various PCR products correspond to 275 bp (PDX-1), 150 bp (Glut-2), 162 bp (GK), and 233 bp (TBP).

FIGURE 3
Glucose-induced insulin secretion in βIns2−/−lacZ cells. Insulin release was measured in the absence or in the presence of 0.5 mM IBMX. The incubation time was 2 hours. Values are means ± SE from triplicate assays.

Ins1 Gene is Up-Regulated in βIns2−/−lacZ Cells
To investigate possible up-regulation of the Ins1 gene in the absence of Ins2 transcripts in βIns2−/−lacZ cells, we compared insulin transcripts produced in βIns2−/−lacZ cells with those in βTC1 cells. This latter cell line was derived from Rip1-Tag2 mice in which both the Ins1 and Ins2 genes are functional. Insulin gene expression was analyzed by RT-PCR using total cellular RNA. In βTC1 cells, Ins1 transcripts represent the minority of insulin transcripts (Figure 4B). In βIns2−/−lacZ cells, the amounts of Ins1 transcripts were significantly higher than those found in βTC1 cells (Figure 4A). Quantitative analysis of RT-PCR products for insulin as well as β-actin transcripts showed that Ins1 transcripts in βIns2−/−lacZ cells represent 55% to 88% of total insulin transcripts present in βTC1 cells. These results indicate that the Ins1 gene is up-regulated in βIns2−/−lacZ cells to compensate for the absence of Ins2 transcripts. It can be inferred from this study that Ins1 gene up-regulation could also potentially take place in individual β-cells in vivo in Ins2−/− mice, in the compensating responses exhibited by these mutants to maintain normal glucose homeostasis [14].
Ins1 UP-REGULATED IN Ins2 KNOCKOUT MICE β-CELL LINE

FIGURE 4
RT-PCR analysis of insulin gene expression in βIns2−/−lacZ (A) and βTC1 (B) cells. Transcripts for Insulin 1 (Ins1)/Insulin 2 (Ins2) were amplified using total RNA from βIns2−/−lacZ or βTC1 cells and analyzed by Southern blotting. β-Actin mRNA was coamplified as control. The results of 2 separate samples are presented. The sizes of various PCR products correspond to 76 bp (Insulin 1), 112 bp (Insulin 2), and 243 bp (β-actin).

FIGURE 5
Analysis of β-gal activity in βIns2−/−lacZ cells cultured under various conditions. β-Gal activity was determined in βIns2−/−lacZ cells cultured in high-glucose medium (22 mM) supplemented or not for 48 hours with 10−7 M GLP-1 and/or 0.5 mM IBMX. A blank value obtained with βTC1 cells was subtracted. Values are means ± SE from triplicate and quadruplicate assays.

Induction of the Ins2-lacZ Gene in βIns2−/−lacZ Cells

Regulation of insulin gene expression has been examined in many studies using different β-cell lines transfected with reporter genes under control of rodent or human insulin promoter. In some of these studies, several-fold induction of insulin promoter activity by glucose was reported [21, 22]. Because βIns2−/−lacZ cells express the lacZ gene inserted at the Ins2 locus, these cells represent an interesting tool for testing or screening of molecules that can up-regulate Ins2 promoter activity, because lacZ expression can be easily monitored by measuring β-gal activity in cellular extracts. We could not examine the effect of glucose on Ins2-lacZ expression, because only a moderate decrease, if any, of β-gal activity was observed when βIns2−/−lacZ cells grown in high glucose were transferred to low-glucose medium (not shown). We could, however, test the effect of GLP-1 and IBMX on Ins2-lacZ gene expression in βIns2−/−lacZ cells. GLP-1 is a hormone secreted by the gut during digestion and has been shown to stimulate insulin gene transcription under hyperglycemic conditions [23]. IBMX has also been reported to stimulate insulin gene expression [24].

As shown in Figure 5, incubation of βIns2−/−lacZ cells cultured in high-glucose medium with 10−7 M GLP-1 for 2 days resulted in 97% increase in β-gal activity as compared to βIns2−/−lacZ cells cultured without GLP-1. A similar increase in β-gal activity was also observed for βIns2−/−lacZ cells incubated with 0.5 mM IBMX. The β-gal activity in βIns2−/−lacZ cells cultured in the presence of both GLP-1 and IBMX was increased up to 157% as compared to untreated cells. These results demonstrate that the Ins2 promoter can be induced in βIns2−/−lacZ cells.

In conclusion, the advantage of such a model βIns2−/−lacZ cell line is twofold: (1) to understand the transcriptional network governing the expression of the Ins2 promoter, and (2) to study the mechanisms involved in the up-regulation of Ins1 promoter activity. These cells also represent an interesting new tool for the screening of molecules that could stimulate Ins2 promoter activity under high-glucose conditions, and therefore would have potential therapeutic interest for type 2 diabetes, because the Ins2 gene in the mouse is homologous to the human insulin gene.

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