Establishment of inducible cAMP early repressor transgenic fibroblasts in a porcine model of human type 1 diabetes mellitus

EUI-MAN JUNG, YU-KYUNG KIM, GEUN-SHIK LEE, SANG-HWAN HYUN, WOO-SUK HWANG and EUI-BAE JEUNG

1Laboratory of Veterinary Biochemistry and Molecular Biology, College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk 361-763; 2SooAm Biotech Research Foundation, Seoul 137-851; 3Laboratory of Veterinary Physiology, College of Veterinary Medicine, Kangwon National University, Chuncheon, Gangwon 200-701; 4Laboratory of Veterinary Embryology and Biotechnology, College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk 361-763, Republic of Korea

Received January 10, 2012; Accepted April 20, 2012

DOI: 10.3892/mmr.2012.895

Abstract. Diabetes mellitus is a metabolic disease caused by impaired insulin secretion from the pancreatic β cells and increased insulin resistance in peripheral tissues. Recently, the overexpression of inducible cyclic AMP (cAMP) early repressor (ICER) Iγ in rodent pancreatic β cells was found to induce insulin deficiency and glucagon overproduction similar to that found in human diabetes mellitus. ICER Iγ with only a DNA binding domain interrupts the transcriptional regulation of the cAMP responsive element-binding protein (CREB) target genes. Based on this information, we hypothesized that the overexpression of ICER Iγ, the most powerful competitor to CREB, could be useful for generating a pig model of diabetes. First, we evaluated the promoter activities of the human insulin gene for the β cell-specific overexpression of ICER Iγ in the pig pancreas. The maximum promoter activity region [-1,431 nucleotides (nt) to +1 nt, +1 = the transcriptional start site] of the insulin gene presented an activity level 3-fold higher than a promoterless construct. Second, ICER Iγ overexpression controlled by this promoter region significantly blocked the glucose-mediated insulin transcription, such as that regulated by the viral promoter in the pancreatic β cell line, MIN6. This suggests that the human insulin promoter may facilitate the overexpression of ICER Iγ in porcine pancreatic β cells. In addition, the overexpression of ICER Iγ in porcine β cells may induce human-like type 1 diabetes mellitus in pigs. In the present study, we generated transgenic fibroblasts containing ICER Iγ cDNA controlled by the human insulin promoter, as well as two screening markers, the green fluorescence protein and the neomycin resistance gene. These fibroblasts may provide a source for somatic cell nuclear transfer to generate a pig model that mimics human diabetes mellitus.

Introduction

Diabetes mellitus is a class of metabolic diseases in which an individual has high blood sugar either because the body does not produce enough insulin or because the pancreatic β cells are unresponsive to insulin. There are two main types of diabetes mellitus. Type 1 diabetes mellitus (also referred to as insulin-dependent diabetes mellitus or IDDM) is characterized by the failure to produce sufficient quantities of insulin. Individuals with this form of the disease require exogenous insulin for survival. Type 2 diabetes mellitus (also referred to as non-insulin-dependent diabetes mellitus or NIDDM) is caused by defects in insulin secretion along with insulin resistance (1,2).

The transcriptional regulation of the insulin genes is mediated by specific combinations of positive and negative factors through multiple cis-acting elements. The insulin genes are influenced by changes in glucose or cyclic AMP (cAMP) levels (3). The cAMP response element (CRE), one of the cis-acting elements, was first identified as an inducible enhancer of gene transcription (4). CRE is capable of activating gene transcription in response to increased cAMP levels. cAMP is known to be an important determinant of gene expression in pancreatic islet cells (5,6). Protein kinase A (PKA) plays a crucial role in the regulation of glycogen metabolism and activation of cAMP production. Additionally, this factor is involved in a number of diverse signaling pathways. High levels of cAMP activate PKA, which then phosphorylates the CRE-binding protein (CREB). CREB is known to stimulate insulin gene transcription by binding to the CRE in the promoter of the insulin gene (7,8).
The CREB gene also generates repressors to reduce the cAMP-induced transcription by alternative splicing (9). Inducible cAMP early repressor (ICER Iγ), one of the repressors, is generated from an alternative intronic promoter on the CREB gene. ICER Iγ that competes with CREB reduces the cAMP-induced transcription (10). It has been shown that the ICER Iγ overexpression in β cells increases the incidence of diabetes mellitus in a rodent model, as the overexpressed ICER Iγ competes with endogenous CRE-binding activators to block CRE-mediated transcription in pancreatic β cells (11,12). In addition, the overexpression of ICER Iγ leads to decreased numbers of β cells in transgenic mice (13,14).

The mechanisms underlying diabetes mellitus are complex and not entirely understood. Therefore, the mechanisms have been widely studied using in vitro and in vivo methods. The majority of in vivo experiments have been carried out in rodents, although some studies have been performed on larger animals (15,16). However, the phenotypic characteristics of this disease in rodent models do not thoroughly reflect the mechanisms of human diabetes. Thus, in the present study, we produced porcine transgenic fibroblasts that were genetically modified to induce type 1 diabetes mellitus-like symptoms in a porcine model. This was accomplished by overexpressing ICER Iγ in porcine β cells. These cells may provide a source for somatic cell nuclear transfer methods used to generate an animal model of diabetes.

Materials and methods

Cell culturing. Unless otherwise indicated, all cells were grown at 37°C and all cell culture materials were obtained from Invitrogen (Carlsbad, CA, USA). Porcine fibroblasts were obtained from miniature pig fetuses (Yucatan pigs, Optifarm Solution Inc., Gyeonggi-do, Korea) on the 30th day of pregnancy, and the mouse β cell line, MIN6 (ATCC, Manassas, VA), was cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS; Welgene, VA), was cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS; Welgene, Daejeon, South Korea), 50 U/ml penicillin and 50 µg/ml streptomycin in a humidified 5% CO₂ atmosphere.

Genomic DNA extraction and PCR. Genomic DNA from the cells was isolated with a G-DEX™ IIc Genomic DNA Extraction kit (iNtRON Biotechnology, Seoul, South Korea). Genomic DNA (1 µg) was amplified in a 20-µl PCR reaction containing 1 U LA-Taq polymerase (Takara, Otsu, Shiga, Japan) for long-range PCR, or 1 U i-Start Taq polymerase (iNtRON) for the other PCR reaction, 2 mM dNTPs (Takara) and 10 pmol of each specific primer. The detail of all primers are described in Table I. The PCR reactions were denatured at 95°C for 30 sec, annealed at 58°C for 30 sec, and extended at 72°C for 1 or 2 min. The PCR products were subjected to cloning processes and/or separated on a 0.7% agarose gel, stained with ethidium bromide and photographed under UV illumination. The image was scanned using GelDoc EQ (Bio-Rad, Hercules, CA, USA).

RNA preparation and semi-quantitative PCR. Total RNA from MIN6 cells or pig pancreas was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The concentration of the total RNA was determined by measuring the absorbance at 260 nm. First-strand cDNA was prepared by subjecting total RNA (1 µg) to reverse transcription using mMLV reverse transcriptase (Invitrogen) and random primers (9-mers; Takara). To determine the optimal conditions for logarithmic phase PCR amplification for target cDNA, aliquots of total cDNA (1 µg) were amplified using different numbers of cycles. The cytochrome c oxidase subunit 1 (1A) gene was amplified as the internal control to rule out the possibility of RNA degradation and to control for variations in mRNA concentrations. A linear relationship between PCR product band visibility and the number of amplification cycles was observed for the target mRNA. The 1A and insulin target genes were quantified using 28 and 30 cycles, respectively. The PCR reactions were denatured at 95°C for 30 sec, annealed at 58°C for 30 sec, and extended at 72°C for 30 sec. The PCR products were on a 2.3% agarose gel, stained with ethidium bromide and photographed under UV illumination. The image was scanned and the band density was analyzed using GelDoc EQ and its software (Bio-Rad).

Vector construction. All restriction enzymes were obtained from Takara. Various regions of the human insulin promoter [from nucleotides (nt) -1,873 to +1 nt, +1 = the transcriptional start site] were prepared by long-range PCR using human genomic DNA (Clontech Mountain View, CA, USA) as the template and specific primers containing restriction enzyme sites (MluI at the 5’ end or XhoI at the 3’ end). Amplified fragments were digested with MluI and XhoI and ligated into the promoterless luciferase expression plasmid, pGL3-Basic (Promega, Madison, WI, USA). The ICER Iγ expression cassette plasmid was produced in several steps. ICER Iγ cDNA was prepared by PCR using cDNA from pig pancreas as the template. Amplified fragments were inserted into pcDNA3.1 (Invitrogen) or ligated into the recombinant pGL3 construct containing the human insulin promoter region (-1,431 nt to +1 nt). For the selection cassettes, the enhanced green fluorescent protein (EGFP) gene was amplified from pIRE2-EGFP (Clontech), digested with EcoRV and BamHI, and inserted into the pIREs-Neo plasmid (Clontech). Both the EGFP and neomycin resistance gene (Neo) were further amplified by PCR, digested with SalI, and ligated into the recombinant pGL3 vector encoding porcine ICER Iγ cDNA controlled by the human insulin promoter. Finally, the sequences of the targeting vector were confirmed by nucleotide sequencing (Genotech Co. Ltd., Daejeon, Korea).

Transient transfection and reporter gene assay. Transient transfection was carried out using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. To control for different transfection efficiencies of the various luciferase constructs, the Rous sarcoma virus (RSV)-lacZ plasmid was co-transfected into the MIN6 cells with luciferase constructs containing the insulin promoter (17,18). Briefly, 3x10⁴ cells were seeded in 6-well tissue culture plates 1 day prior to transfection. In total, 4 µg of the constructs containing the insulin promoter and 0.5 µg of RSV-lacZ plasmid were co-transfected into the cells under serum-free DMEM. After incubating for 4 h, the medium was replaced with DMEM containing 10% FBS and glucose (0.1 mM or 4 mM) for an additional 48 h. Cellular lysates were assayed for luciferase activity.
regulated insulin transcription following treatment - γ constructs were finally introduced into the mouse in a promoterless pGL3-basic vector. These insulin promoter DNA. The fragments were linked to the luciferase (Luc) gene promoter region from -1,873 nt to +1 nt using human genomic containing different deletions of the upstream human insulin expression, we generated a series of promoter variants which would generate the maximum activity for tissue-specific To determine the promoter region of the human insulin gene Human insulin promoter activities in the mouse Results Data analysis. Data are presented as the means ± standard error of the mean (SEM). A statistical analysis was performed using the Student’s t-test, with two-pair comparisons. P<0.05 was considered to indicate a statistically significant difference. Results Human insulin promoter activities in the mouse β cell lines. To determine the promoter region of the human insulin gene which would generate the maximum activity for tissue-specific expression, we generated a series of promoter variants containing different deletions of the upstream human insulin promoter region from -1,873 nt to +1 nt using human genomic DNA. The fragments were linked to the luciferase (Luc) gene in a promoterless pGL3-basic vector. These insulin promoter constructs were finally introduced into the mouse β cell line, MIN6, and the relative promoter activities were measured. As shown in Fig. 1, the promoter region (-1432 nt to +1 nt) resulted in a level of activity 3-fold higher than that of the promoterless construct. Notably, the region from -1,431 nt to -482 nt may contain certain enhancing elements, while a different region (-1,872 nt to -1,431 nt) may possess inhibitory factor-binding sites. Thus, the region containing -1,431 nt to +1 nt was considered the best candidate for a β cell-specific expression promoter. Effect of porcine ICER Iγ overexpression on insulin production in mouse β cells. The overexpression of ICER Iγ has previously been found to significantly repress insulin expression in a rodent model (12,13); however, it is unclear whether porcine ICER Iγ can inhibit insulin transcription. We therefore isolated porcine ICER Iγ cDNA that was sub-cloned into overexpression vectors regulated by the cytomegalovirus (CMV) promoter as a positive control or by the human insulin promoter (-1,432 nt to +1 nt). The production of insulin mRNA was evaluated after transiently transfecting the mouse β cell line, MIN6, with the two constructs (Fig. 2). Wild-type MIN6 cells showed significantly upregulated insulin transcription following treatment with 4 mM glucose. This increased level of transcription was significantly attenuated by the overexpression of porcine ICER Iγ under the control of the CMV or insulin promoter. Thus, our results indicate that porcine ICRE Iγ overexpression effectively inhibits insulin production in the β cells.

<table>
<thead>
<tr>
<th>Name</th>
<th>Restriction enzyme</th>
<th>Direction</th>
<th>Sequences (5' to -3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human insulin promoter (-1,873)</td>
<td>MluI</td>
<td>Forward</td>
<td>AGC CGT CTC AAG GAG GCA CCC A</td>
</tr>
<tr>
<td>Human insulin promoter (-1,432)</td>
<td>MluI</td>
<td>Forward</td>
<td>AGC CGT GTA CCC CAG GGG CTC AG</td>
</tr>
<tr>
<td>Human insulin promoter (-482)</td>
<td>MluI</td>
<td>Forward</td>
<td>AGC CGT CTC GGC ACC GGG CC</td>
</tr>
<tr>
<td>Human insulin promoter (+1)</td>
<td>XhoI</td>
<td>Reverse</td>
<td>CTC GAG GGC CAG CAC GGC CAG</td>
</tr>
<tr>
<td>ICER Iγ cDNA</td>
<td>Ncol</td>
<td>Forward</td>
<td>CCA TGG ATG GCT GTA ACT GGA GAT GAA ACA G</td>
</tr>
<tr>
<td>ICER Iγ cDNA</td>
<td>XbaI</td>
<td>Reverse</td>
<td>TCT AGA CTA ATC TGT TTT AGG AGA GCA AAT GTC</td>
</tr>
<tr>
<td>EGFP cDNA</td>
<td>EcoRV</td>
<td>Forward</td>
<td>GAT ATC CAC AAC CAT GGT GAG CAA GGG CGA</td>
</tr>
<tr>
<td>EGFP cDNA</td>
<td>BamHI</td>
<td>Reverse</td>
<td>GGA TCC TTA CTT GTA CAG CTC GTC CAT GCC</td>
</tr>
<tr>
<td>Confirming primer a</td>
<td></td>
<td>Forward</td>
<td>GCA GGA CAG GCT GCA TCA GA</td>
</tr>
<tr>
<td>Confirming primer b</td>
<td></td>
<td>Reverse</td>
<td>GCA ACC CGA CTC TCC AGA CA</td>
</tr>
<tr>
<td>Confirming primer c</td>
<td></td>
<td>Forward</td>
<td>CAT GAA GCA CGA CGA CTT CT</td>
</tr>
<tr>
<td>Confirming primer d</td>
<td></td>
<td>Reverse</td>
<td>CCT AGG AAT GCT CGT CAA GA</td>
</tr>
<tr>
<td>1A</td>
<td></td>
<td>Forward</td>
<td>CCA GGA TTT GGA ATT ATT TC</td>
</tr>
<tr>
<td>1A</td>
<td></td>
<td>Reverse</td>
<td>GAA AAT AAA GCC TAA GGC TC</td>
</tr>
<tr>
<td>Insulin</td>
<td></td>
<td>Forward</td>
<td>CCC TGT TGG TGC ACT TCC TA</td>
</tr>
<tr>
<td>Insulin</td>
<td></td>
<td>Reverse</td>
<td>CAC TTG TGG GTC CTC CAC TT</td>
</tr>
</tbody>
</table>

EGFP, enhanced green fluorescent protein.
which was further confirmed by EGFP expression observed with fluorescence microscopy (data not shown). Integration of the targeting vector into the genomic DNA was determined by PCR-based methods using primer sets specific for the vector. Genomic DNA from positive colonies was amplified with the primers a and b (amplicon size, 600 bp; Fig. 2B) or with the primers c and d (product size, 1 kb; Fig. 2C). In total, 62 positive colonies were obtained after 32 rounds of transfection trials (Table II). This indicated that our system was associated with a relatively high transgenic efficiency (91.9%; 57 out of 62 colonies), although it produced a low number of positive colonies (1.9 colonies per transfection). Thus, our method involving dual screenings and liposome-mediated gene deliveries can reduce the number of false positive colonies during gene modification in pig fibroblasts. Finally, aliquots of the colonies confirmed to be positive (0.6x10^6 cells) were frozen for somatic cell nuclear transfer.

Discussion

In the present study, we describe the generation of a pig fibroblast cell line for producing a porcine model of diabetes...
mellitus. This established pig fibroblast cell line contained the porcine ICER Iγ gene under the control of the human insulin promoter, resulting in the downregulation of endogenous insulin production and decreased numbers of β cells, similar to previous studies (13,14,19). Two selection markers, the EGFP and Neo r genes, were useful for screening the transgenic fibroblasts and for visual identification of transgenic piglets.

β cells in the pancreatic islets play an essential role in serum glucose homeostasis in vertebrates. These cells are capable of acting as a sensor of systemic glucose levels during the feeding state of the organism, and the transcription of genes associated with metabolism is induced by changing levels of the products of glucose catabolism; this represents specific regulation of the β cells in response to glucose concentrations in the body (20,21). The insulin promoter immediately regulates insulin gene transcription via glucose and the human promoter enables strongly regulated insulin production (22,23). Based on this evidence, we focused on the insulin promoter as a β cell-specific regulator. The current insulin promoter highly upregulated ICER Iγ gene expression in the mouse β cell line in the presence of high glucose levels, and this upregulation effectively blocked insulin production by competing with CREB for the endogenous insulin promoter.

Components of metabolic syndrome, such as obesity, lipid abnormalities, glucose intolerance and hypertension, have long been a topic of great interest in biomedical research. One of the typical consequences of metabolic syndrome is diabetes mellitus (24-26). There are approximately 160,000 diabetics worldwide reported by the World Health Organization (WHO), and it is expected that the number of diabetic patients will double within the next 20 years (27). To further our understanding of this disease, rodent models have been extensively used due to their small size, ease of handling and short generation interval. Well-known rodent models of diabetes mellitus have been established by alloxane and a uric acid derivative resulting in insulin deficiency and hyperglycemia (28,29), Additionally, streptozotocin and a nitrosurea derivative from *Streptomyces achromogenes* selectively destroy pancreatic β cells, thereby inducing diabetes (30-32). The bio-breeding

![Figure 3. Schematic structure of the targeting vector and PCR-based confirmation of transgenic porcine fibroblasts. (A) The targeting vector contained expression and selection cassettes. The expression cassette included the porcine inducible cyclic AMP early repressor (ICER) Iγ gene controlled by the human insulin promoter (-1,431 nt to +1 nt). The selection cassette contained the green fluorescent proteins (EGFP) and neomycin-resistance (Neo r) genes linked by internal ribosomal entry site (IRES) sequences. ICER Iγ, porcine ICER Iγ cDNA; poly A, poly A tail signaling sequences; pCMV, cytomegalovirus promoter. Integration of the targeting vectors into the genomic DNA of the fibroblasts was confirmed with specific primer sets, indicated by arrows (a to d). (B) The PCR products with primer a and b confirmed the chromosomal insertion of the expression cassette into the positively selected colony. (C) Transgenic fibroblasts containing the selection cassette were identified with primers c and d. M, molecular marker; Ne, negative control without template; Co, a positively selected colony by the selection markers, EGFP and Neo r.](image-url)

References

BioGreen 21 Program (No. PJ008323), Rural Development Administration, Republic of Korea.

Acknowledgements

This study was supported by a grant from the Next-Generation BioGreen 21 Program (No. PJ008323), Rural Development Administration, Republic of Korea.


