Ribozyme-mediated attenuation of pancreatic β-cell glucokinase expression in transgenic mice results in impaired glucose-induced insulin secretion

(antisense RNA/β-cell lines/diabetes/glucose phosphorylation/glucose sensing)

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ABSTRACT Phosphorylation of glucose to glucose 6-phosphate by glucokinase (GK; EC 2.7.1.2) serves as a glucose-sensing mechanism for regulating insulin secretion in β cells. Recent findings of heterozygous GK gene mutations in patients with maturity-onset diabetes of the young (MODY), a form of type II (non-insulin-dependent) diabetes characterized by autosomal dominant inheritance, have raised the possibility that a decrease in β-cell GK activity may impair the insulin secretory response of these cells to glucose. To generate an animal model for MODY we have expressed in transgenic mice a GK antisense RNA with a ribozyme element under control of the insulin promoter. Mice in two independent lineages had about 30% of the normal islet GK activity. Insulin release in response to glucose from in situ-perfused pancreas was impaired; however, the plasma glucose and insulin levels of the mice remained normal. These mice are likely to be predisposed to type II diabetes and may manifest increased susceptibility to genetic and environmental diabetogenic factors. They provide an animal model for studying the interaction of such factors with the reduced islet GK activity.

The mechanisms by which pancreatic β cells sense and respond to physiological changes in blood glucose have been the subject of extensive investigation. Glucose-induced insulin secretion requires the metabolism of glucose in β cells (1). The phosphorylation of glucose to glucose 6-phosphate, which determines the rate of glycolysis, has been proposed to constitute a key glucose-sensing mechanism for regulating insulin secretion (2). β cells and hepatocytes express a high-Km member of the hexokinase family, glucokinase (GK; EC 2.7.1.2), which is responsible for the majority of glucose phosphorylation activity in β cells (2). While in the liver transcription of the GK gene is induced by insulin (3, 4), GK expression in β cells is primarily regulated by glucose at the translational and post-translational levels (3, 5).

Recent DNA polymorphism studies have established a linkage between the GK locus and diabetes in patients with a non-insulin-dependent diabetes mellitus form termed maturity-onset diabetes of the young (MODY) (6, 7). This disease is characterized by an early age of onset and an autosomal dominant inheritance. Sequencing of the GK gene from MODY patients has detected a number of nonsense and missense mutations which are associated with regions of the enzyme molecule involved in glucose and ATP binding (6, 7). The molecular mechanism which makes these mutations dominant remains unknown. The inheritance pattern of the disease suggests that the patients’ β cells contain normal enzyme molecules encoded by the wild-type allele. The mutant proteins manifest drastically reduced activities (8, 9).

Since GK expression in β cells is not transcriptionally regulated, the wild-type allele cannot compensate for this reduction. The decreased GK activity may be sufficient to shift the threshold for glucose sensing, thereby resulting in impaired insulin secretion at physiological glucose levels. However, it remains unclear whether abnormal glucose uptake by the liver contributes to the disease.

Understanding of the human disease can benefit from an animal model, which will allow detailed biochemical and physiological studies. We sought to generate a mouse model for MODY by specifically reducing GK activity in β cells, without affecting its function in the liver. To this end a GK ribozyme was expressed in β cells in transgenic mice. Ribozymes are RNA molecules that possess catalytic RNA-cleavage activity (10, 11). By flanking the ribozyme catalytic element with two gene-specific fragments in antisense orientation the ribozyme activity can be targeted against a unique RNA sequence, thus reducing target mRNA levels and activity. The transgenic mice expressing the GK ribozyme manifest only a third of the normal islet GK activity. Insulin secretion in response to glucose is impaired; however, the mice remain euglycemic. These findings suggest that GK deficiency in the liver, in addition to that in the islets, may play a role in the induction of diabetes in MODY patients. These mice provide an animal model for studying the interaction of genetic and environmental diabetogenic factors with the reduced islet GK activity.

MATERIALS AND METHODS

Plasmid Constructs. Two oligonucleotides were synthesized containing two 12-base fragments derived from mouse gene exon 3 sequence (12) that flank a hammerhead ribozyme catalytic element (10, 11). Annealing of the sense oligonucleotide 5'-GATCCCTTCCACTTTCTGTAGAGTCGGTAGGAGCAACAGCATCAGCGGTAC-3' and the antisense oligonucleotide 5'-CGGTGTAGCCTGTTTTCGTTCCCATCAGCAGAATGGAGAAG-3' created a fragment (GKRZ) with BamHI and Kpn I protruding ends, which was ligated downstream of a hybrid intron element (13) and upstream of the simian virus 40 late polyclonallylation site in pMLSIS.CAT (13). The combined 675-bp fragment was inserted into the Xba I and Sal I sites of pRIP-Tag (14) downstream of the rat insulin II gene promoter to form pRIP-GKRZ. A DNA fragment containing the neomycin-resistance gene under control of the pgk promoter (15)

Abbreviations: GK, glucokinase; MODY, maturity-onset diabetes of the young; U, unit(s).

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was inserted into the Sal I site of pRIP-GKRZ to form pRIP-GKRZ.neo.

Generation of Transgenic Mice. Linearized pRIP-GKRZ DNA was microinjected into C3HeB/FeJ mouse embryos, and transgenic mice were generated and bred according to established procedures (16).

RNA Analysis. Five million βTC6 cells (17) were electroporated with 30 μg of pRIP-GKRZ/neo DNA in 1 ml of Dulbecco’s modified Eagle’s medium (DMEM) at 800 μF and 250 V. RNA was extracted from G418-resistant and untransfected cells by using RNA Stat-60 (Tel-Test, Friendswood, TX). Five micrograms of RNA was reverse-transcribed, and 1% of the reaction volume was used in 35 cycles of PCR amplification with sense (5'-CTCTAGAATTGCTGCTGCGAGG-3') and antisense (5'-CACTGCATTCTAGTT- TGGTTTGTC-3') oligonucleotides flanking the intron. The amplified fragments were fractionated on a 1.25% agarose gel, visualized with ethidium bromide, and photographed under UV light. Poly(A)+ mRNA was selected with the Poly(A) Quik kit (Stratagene). Ten micrograms of mRNA was fractionated on a 1% agarose/0.22 M formaldehyde gel, blotted onto nitrocellulose filter, hybridized with a [32P]dCTP-labeled rat GK cDNA probe, and autoradiographed on an x-ray film. The blot was stripped and rehybridized with an α-tubulin probe to normalize for mRNA loading. The autoradiographs were scanned with an LKB Ultrascan XL densitometer.

Immunoblotting. Proteins were extracted from G418-resistant and untransfected cells as described (18). Twenty micrograms of protein was fractionated on a 12% polyacrylamide/SDS gel, electroblotted onto an Immobilon-P filter (Millipore), and probed as described (19) with a sheep anti-GK serum (19). The bound antibody was visualized with a horseradish peroxidase-conjugated second antibody and a chemiluminescent substrate (ECL, Amersham) by exposure to an x-ray film and quantitated by densitometry. Protein samples in separate lanes were stained with Coomassie blue, and the dried gel was quantitated by densitometry to normalize for protein loading.

Immunohistochemistry. Pancreas samples were sectioned and analyzed with a sheep anti-GK serum as described (19).

Glucose Phosphorylation. Islets were isolated from the pancreas by collagenase infusion through the bile duct (20). Islet homogenates from 1- to 3-month-old mice were incubated with glucose at various concentrations in the presence of ATP, glucose-6-phosphate dehydrogenase, and NAD, and the formation of NADH was monitored fluorimetrically as described (5).

Pancreas Perfusion. Anesthetized 4- to 7-month-old mice were cannulated through the aorta and portal vein and perfused with oxygenated Krebs-Ringer buffer containing glucose in an increasing concentration gradient. Samples were collected and assayed for glucose by a glucose analyzer and for insulin by RIA.

RESULTS

A synthetic DNA fragment was generated that consisted of two 12-bp fragments of mouse GK gene exon 3 sequence (12) in antisense orientation that flank a ribozyme catalytic domain (10, 11) (Fig. 1A). This region of the gene was chosen as target because it encodes the putative ATP-binding site of the protein (21). The ribozyme element was introduced to increase the efficiency of the antisense RNA molecules by providing them with the ability to cleave the target mRNA. The hybrid DNA fragment was placed downstream of the rat insulin II promoter and an intron element, and upstream of the simian virus 40 late polyadenylation site (Fig. 1B). Stable transfection of this construct, denoted RIP-GKRZ, into βTC cells resulted in a 45% reduction in GK mRNA.
levels; however, no cleavage products could be detected (Fig. 2B). Immunoblotting analysis revealed a 2- to 3-fold reduction in GK protein levels, compared with untransfected cells (Fig. 2C). GK activity was reduced from 1.80 ± 0.26 units (U)/g of protein in untransfected cells to 1.07 ± 0.07 U/g (1 U = 1 μmol of product per min). This reduction affected glucose-induced insulin release in these cells only marginally (data not shown), presumably because they contain much higher activities of the low-$K_m$ hexokinases (about 5 U/g of protein) (22), unlike normal islets, in which the predominant activity is that of GK (see Fig. 4).

The RIP-GKRZ construct was microinjected into mouse embryos, and 7 transgenic mouse lineages were generated. Lineages 2 and 4 expressed the transgene, as judged by PCR analysis of islet cDNA. Immunoblotting analysis of isolated islets revealed a 2-fold reduction in islet GK protein, compared with normal islets (data not shown), an effect commensurate to that observed in the transfected cells. Immunohistochemical analysis of pancreas sections with a GK antiserum revealed a reduced staining intensity in transgenic islets, compared with normal controls (Fig. 3).

Glucose phosphorylation activity at various glucose concentrations was assayed by a fluorimetric method in islets isolated from the transgenic mice. GK activity in RIP-GKRZ islets was reduced by 70%, compared with normal islets, while the activity of the ubiquitous low-$K_m$ hexokinases remained essentially unaffected (Fig. 4). This resulted in a decrease in the ratio of GK to hexokinase activity from 3.5 to 1.3.

Although no data on islet GK activity are available from MODY patients, it is assumed that the wild-type GK allele produces half of the normal activity. Therefore islet GK activity in the RIP-GKRZ mice is likely to be as low or lower than that of MODY patients. Nevertheless, the RIP-GKRZ mice maintained normal fasting plasma glucose levels (124 ± 8 mg/dl) and manifested normal glucose tolerance (data not shown). Fasting plasma insulin levels (0.65 ± 0.12 ng/ml) were similar to those of normal controls (0.64 ± 0.22 ng/ml). The oldest mice analyzed to date were 1 year old. In contrast,
analysis of glucose-induced insulin secretion from \textit{in situ}-perfused pancreas (Fig. 5) revealed a markedly reduced response of the transgenic pancreas, compared with that of normal controls, in the glucose concentration range of 75–200 mg/dl.

**DISCUSSION**

These results demonstrate the ability of a hybrid antisense-ribozyme RNA to effect cell-specific changes in gene expression \textit{in vivo}. Expression of the RIP-GKRZ gene resulted in a significant reduction of approximately 70% in both GK protein level and activity. The sequence specificity of this approach is demonstrated by a lack of effect on expression of the related hexokinase genes. Quantitation of GK mRNA in transfected β cells suggests that the attenuation of expression may occur in part through degradation of the target GK transcript. Additional attenuation may be exerted through reduced translational activity of the GK mRNA, presumably by the formation of double-stranded RNA hybrids with the GKRZ transcripts, as has been observed in other antisense RNA experiments (23). Translation of GK mRNA may be particularly sensitive to inhibition in β cells, since GK expression in these cells is regulated mainly at translational and post-translational levels (3, 5). The incomplete inhibition of expression obtained with the antisense approach may represent an advantage for studying its consequences \textit{in vivo}, since it mimics the situation in MODY. In addition, total shut-off of expression may be lethal.

In spite of the considerable reduction in β-cell GK activity, below the level that gives rise to diabetes in MODY patients, the RIP-GKRZ mice had normal blood glucose and insulin levels. As demonstrated by the perfusion results (Fig. 5), the insulin secretory response to glucose alone is greatly reduced in the transgenic mice, compared with normal controls, in a manner similar to that observed in MODY patients (24). It is possible that in the mouse other insulin secretagogues can compensate for the reduction in glucose-induced secretion. Alternatively, these results may indicate that an impaired liver function, in addition to that of β cells, is required for the induction of overt diabetes by GK deficiency. Such liver impairments have been documented in MODY patients (25).

The finding that partial attenuation of expression of the normal GK protein is sufficient to impair the sensitivity of β cells to glucose supports the interpretation of the dominance of the GK mutations in MODY as a gene-dosage effect, rather than a gain-of-function negative dominant effect of the mutant protein.

The reduced β-cell GK activity in the RIP-GKRZ mice may represent a predisposition to diabetes. This may develop into overt disease in certain physiological conditions, such as those caused by age, sex, weight, diet, and genetic background differences. Thus, these mice provide an experimental system for studying the effect of such factors on the development of type II diabetes.

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