Transgenic Overexpression of Hepatocyte Growth Factor in the β-Cell Markedly Improves Islet Function and Islet Transplant Outcomes in Mice

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Recent advances in human islet transplantation have highlighted the need for expanding the pool of β-cells available for transplantation. We have developed three transgenic models in which growth factors (hepatocyte growth factor [HGF], placental lactogen, or parathyroid hormone-related protein) have been targeted to the β-cell using rat insulin promoter (RIP). Each displays an increase in islet size and islet number, and each displays insulin-mediated hypoglycemia. Of these three models, the RIP-HGF mouse displays the least impressive phenotype under basal conditions. In this study, we show that this mild basal phenotype is misleading and that RIP-HGF mice have a unique and salutary phenotype. Compared with normal islets, RIP-HGF islets contain more insulin per β-cell (50 ± 5 vs. 78 ± 9 ng/islet equivalent [IE] in normal vs. RIP-HGF islets, $P < 0.025$), secrete more insulin in response to glucose in vivo (0.66 ± 0.06 vs. 0.91 ± 0.10 ng/ml in normal vs. RIP-HGF mice, $P < 0.05$) and in vitro (at 22.2 mmol/l glucose: 640 ± 120.1 vs. 1,615 ± 196.9 pg · μg protein^{-1} · 30 min^{-1} in normal vs. RIP-HGF islets, $P < 0.01$), have two- to threefold higher GLUT2 and glucokinase steady-state mRNA levels, take up and metabolize glucose more effectively, and most importantly, function at least twice as effectively after transplantation. These findings indicate that HGF has surprisingly positive effects on β-cell mitogenesis, glucose sensing, β-cell markers of differentiation, and transplant survival. It appears to have a unique and unanticipated effective profile as an islet mass- and function-enhancing agent in vivo. ***Diabetes*** **50:2752–2762, 2001**

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3*O*MG, 3-*O*-methylglucose; BSA, bovine serum albumin; dNTP, deoxyribonucleoside triphosphates; GK, glucokinase; GLP-1, glucagon-like peptide-1; GSIS, glucose-stimulated insulin secretion; HBSS, Hank's buffered saline solution; HGF, hepatocyte growth factor; IE, islet equivalent; IPGTT, intraperitoneal glucose tolerance test; Kir6.2, K^+ inward rectifier 6.2; KRBB, Krebs-Ringer bicarbonate buffer; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PL, placental lactogen; PTHrP, parathyroid hormone-related protein; RIA, radioimmunoassay; RIP, rat insulin promoter; RT, reverse transcription; STZ, streptozotocin; SUR-1, sulfonylurea receptor-1.

Recent clinical studies have documented that human islet transplantation has the potential to replace pancreatic endocrine function in patients with type 1 diabetes (1,2). However, the current shortage of human islets is a barrier to the use of islet transplantation in clinical practice on a larger scale. To solve this problem, several approaches are being taken. These include the development of alternative species for islet harvesting and xenotransplantation (3,4); the development of continuous human β-cell and non–β-cell lines capable of processing, storing, and releasing insulin (5,6); the induction of endocrine differentiation of cell precursors (7–9); and the use of islet factors that can increase β-cell proliferation rates and/or function (10–16). Several groups have identified a number of islet factors that can induce pancreatic islet cells to expand in vitro and/or in vivo, including IGFs, glucagon-like peptide-1 (GLP-1), exendin, prolactin, hepatocyte growth factor (HGF), placental lactogen (PL), parathyroid hormone-related protein (PTHrP), and others (10–19).

Our group has developed a transgenic mouse model in which the expression of HGF has been targeted to the pancreatic β-cell using the rat insulin promoter (RIP)-II (15). Under basal conditions, RIP-HGF transgenic mice display a dramatic increase in islet size, islet number, and β-cell proliferation, associated with mild insulin-mediated hypoglycemia. However, nothing is known regarding the ability of islets from RIP-HGF transgenic mice to respond to hyperglycemia in vitro or in vivo or whether there are any differences between these transgenic mice and their corresponding normal littermates in terms of glucose tolerance or insulin secretory responses. In addition, available studies shed no light on the potential for HGF to enhance pancreatic islet transplant function or survival. The current study was designed to answer these questions.

HGF is a mesenchyme-derived factor that exhibits mitogenic, motogenic, morphogenic, and anti-apoptotic activities in a wide variety of normal cells and tissues (20,21). In addition, in vitro and in vivo studies have demonstrated that HGF is an insulinotropic factor for adult islet β-cells (11,15) and is able to induce the expression of insulin in exocrine cells that lack normal expression of this hormone (22,23). The receptor for HGF, c-Met, is a tyrosine kinase receptor that is present in islet β-cells (10). Furthermore, it has been determined that HGF is able to activate

phosphatidylinositol 3-kinase in fetal pancreatic islet cells in vitro (24). Ptasznik et al. (24) suggest that while HGF is a β-cell mitogen, it also causes β-cell dedifferentiation. However, no further approaches have been taken to define the intracellular signaling pathways implicated in HGF action on the islet. In addition, the relation between specific protein kinase activation and physiologically relevant insulin-secretory responses is quite inconclusive and often contradictory (25).

Another important observation in transgenic mice overexpressing HGF in the islet β-cell is their resistance to the diabetogenic effects of streptozotocin (STZ) (15). In addition, a recent report has shown that intraperitoneal injection of HGF has a favorable effect on amelioration of hyperglycemia in STZ-induced diabetic mice receiving a marginal mass of intrahepatic syngenic islets (26). In both cases, it has been postulated that HGF can induce a protective effect against death in islet cells, as observed in other cell types (27,28).

In the current study, we have attempted to address these unanswered questions. Specifically, we have examined the effects of HGF overexpression in β-cells of RIP-HGF transgenic mice on glucose tolerance in vivo and glucose-stimulated insulin secretion (GSIS) in vitro. Surprisingly, given that previous studies indicate that HGF drives the β-cell to dedifferentiate (24), the results indicate that islets overexpressing HGF display dramatically superior function regarding both glucose tolerance in vivo and GSIS in vitro. Moreover, the increase in GSIS and glucose tolerance induced by HGF correlates with increased glucose utilization, islet expression of GLUT2 and glucokinase (GK) mRNA, and glucose transport. Most importantly, HGF transgenic islets function far more efficiently than normal islets when transplanted into STZ-induced diabetic mice. These surprising results indicate that overexpression of HGF in the β-cell, with its resultant mitogenesis, pro-differentiation, and anti-apoptotic properties, may serve a particularly useful role in expanding the availability of β-cells both before and, more importantly, after islet transplantation.

RESEARCH DESIGN AND METHODS

Transgenic mice. The generation of RIP-HGF transgenic mice has been previously described in detail (15). Briefly, RIP-II was used to drive the expression of mHGF cDNA in islet β-cells. For each transgenic mouse offspring, genotyping was performed by tail DNA polymerase chain reaction (PCR) as described (29). Two different RIP-HGF transgenic mouse lines (51 and 54) with similar levels of HGF expression and phenotypes (15), continuously outbred onto a CD-1 background, were used in this study. RIP-HGF transgenic mice and their corresponding normal siblings were studied at age 3–4 months. In all of the experiments, transgenic mice were compared with their corresponding normal littermates. All studies were performed with the approval of and in accordance with guidelines established by the University of Pittsburgh Institutional Animal Care and Use Committee.

Intraperitoneal glucose tolerance test. Mice were fasted overnight (16–18 h), they were weighed, their fasting basal glucose was measured, and then they were injected intraperitoneally with 25% glucose in saline to a final amount of 2 g glucose/kg body wt. Blood samples were obtained from the snipped tail at 15, 30, 60, 90, and 120 min after glucose injection and analyzed for glucose levels by using a Precision Q.I.D. portable glucometer (Medisense, Bedford, MA). In a different set of mice, blood was obtained 30 min after glucose injection by retro-orbital bleeding as previously described (12), and plasma insulin concentrations were measured by radio immunoassay (RIA) using a kit from Linco Research (St. Louis, MO).

Islet isolation. Mouse islets were isolated as previously described by Ricordi and Rastellini (30), with some modifications. Briefly, the pancreas was injected through the pancreatic duct with 3 ml of 1.7-mg/ml Collagenase P (Roche Molecular Biochemicals, Indianapolis, IN) in Hanks' buffered saline solution (HBSS), removed, incubated at 37°C for 17 min, and then passed through a 500-μm wire mesh. The digested pancreas was rinsed with HBSS, and islets were separated by density gradient in Histopaque (Sigma, St. Louis, MO). After several washes with HBSS, islets were handpicked under a microscope.

Glucose-stimulated insulin secretion. Insulin release from normal and transgenic isolated islets was measured as described by Martin et al. (31) with some modifications. Briefly, Krebs-Ringer bicarbonate buffer (KRBB) supplemented with 10 mmol/l HEPES was prepared and continuously bubbled with a mixture of O_2/CO_2 (95/5%) to a final pH of 7.4. Isolated islets were then preincubated in KRBB with 3% bovine serum albumin (BSA) and 5.5 mmol/l glucose for 1 h at 37°C in a 5%-CO_2 incubator. After washing the islets once in KRBB plus 1% BSA and 2.8 mmol/l glucose, groups of 10 islets of similar size from normal and transgenic mice were incubated in 1 ml of fresh KRBB plus 1% BSA and different glucose concentrations (2.8, 5.5, 8.3, 11.1, 16.6, and 22.2 mmol/l) for 30 min at 37°C in the 5%-CO_2 incubator. These experiments were performed in triplicate at each glucose concentration tested. After incubation, buffer was removed and frozen at −20°C until insulin determination by RIA (Linco). Islets were then washed three times with phosphate-buffered saline (PBS) and digested overnight in 1 ml of 0.1 N NaOH at 37°C. After neutralization with 0.1 N HCl, protein was measured by the Bradford method. Results are expressed as the insulin released per microgram of protein.

Glucose utilization in isolated islets. Isolated islets were incubated in KRBB containing 1% BSA and 2.8 mmol/l glucose for 90 min at 37°C in a 5%-CO_2 incubator to stabilize and bring the islets to baseline metabolic rates. Glucose utilization was measured in triplicate at each glucose concentration tested in batches of 50 islets from RIP-HGF transgenic mice and normal littermates. Islets were incubated in 100 μl KRBB containing 5.5 or 22.2 mmol/l glucose containing 2 μCi of [5-^{3}H]glucose (15.3 Ci/mmol) (NEN Life Science Products, Boston, MA) at 37°C for 90 min. Glucose metabolism was stopped by adding 50 μl of 5% trichloroacetic acid to the tubes. The tubes were then placed in a 20-ml scintillation vial containing 500 μl of distilled H_2O and incubated at 50°C overnight to allow the [^{3}H]$H_2$0 in the reaction mixture to equilibrate with the water in the scintillation vial. After removing the tube with the islets and adding scintillation liquid, the vials were counted in a β-counter. The radioactivity recovered from blank tubes without islets was <0.01%. The recovery of [^{3}H]H_2O, calculated in each experiment by adding known amounts of [^{3}H]H_2O (1 mCi/g) (NEN Life Science Products) to KRBB followed by equilibration with H_2O, was 60–80%. Results are expressed as picomoles of glucose used per hour per microgram of protein.

RNA isolation and reverse transcription. After isolation, islets were aliquoted and stored at −70°C until RNA was isolated. Total RNA was isolated using Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. First-strand cDNA was synthesized by reverse transcription (RT) of 1–2 μg total islet RNA using the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA). Briefly, after total RNA was heated for 2 min at 70°C, RT was performed in a final volume of 20 μl containing 1× reaction buffer, 1 μmol/l oligo dT, 20 units recombinant RNase inhibitor, 0.5 mmol/l deoxyribonucleoside triphosphates (dNTPs), and 200 units Moloney Murine Leukemia Virus reverse transcriptase. Tubes were incubated for 5 min at 4°C, 60 min at 42°C, and 5 min at 95°C and then brought to 4°C. Finally, the cDNA was diluted up to 50 μl.

Relative semi-quantitative PCR. PCR was performed with 5 μl cDNA in a final volume of 25 μl containg 1× *Taq* buffer (Promega, Madison, WI), 2.5 mmol/l $MgCl_2$ (Promega), 200 μmol/l dNTPs (Promega), 0.5 μCi (α-^{32}P) deoxycytidine triphosphate (3,000 Ci/mmol) (Amersham Pharmacia Biotech), 4 nmol/l actin primer pair (Ambion, Austin, TX), 16 nmol/l actin competimers (Ambion) that allow actin RNA to be used as an internal control, 1.25 units of *Taq* DNA polymerase (Promega), and 400 nmol/l of one of the following pairs of murine gene-specific primers: HGF, insulin, glucagon, GLUT2 GK, sulfonylurea receptor-1 (SUR-1), and K^+ inward rectifier 6.2 (Kir6.2) (Table 1). Tubes were placed in a thermal cycler (MJ Research, Waltham, MA), and the following program was applied: 3 min at 94°C for denaturation followed by a number of cycles previously demonstrated to be nonsaturating on the linear amplification portion of the PCR products (Table 1) at 94°C for 30 s, corresponding annealing temperature for 1 min (Table 1), and 72°C for 1 min. The PCR products were separated on a 6% polyacrylamide gel in Tris-borate EDTA buffer, and the gel was dried and developed using X-ray film. Films were scanned, and band densitometry was quantitated using the computer program ImageJ from the National Institutes of Health.

GK and GLUT2 immunohistochemistry. Pancreata from RIP-HGF transgenic mice and normal littermates were removed, fixed in Bouin's solution, embedded in paraffin, and sectioned. After deparaffinization and rehydration, 5-μm sections were immunostained for GLUT2 (affinity-purified goat anti–human GLUT2 antibody, 1:500 dilution) and GK (affinity-purified goat anti–

TABLE 1
Sequences of the PCR primers

Gene	Gene Bank Acc. no.	Primers		Product size (bp)	Annealing temp. °C	Cycles
		Sense	Antisense			
HGF	M29145	CGGGGTAAAGACCTACAGG	CCCATTGCAGGTCATGC	146	58	25
Insulin (I, II)	X04725 X04724	CCCAGGCTTTTGTCAAACAGCA	CTCCAGTGCCAAGGTCTGAA	179–185	60	15
Glucagon	Z46845	CCATTTACTTTGTGGCTGG	GGTGTTCATCAACCACTGC	236	58	20
GLUT2	S77926	TTAGCAACTGGGTCTGCAAT	GCAGCGATTTCCTCAAAAG	170	56	25
GK	L38990	AAGGGAACAACATCGTAGGA	CATTGGCGGTCTTCATAGTA	130	56	25
SUR-1	AF037306-AF037308	CCAAGGGAAGATTCAAATTCAA	GTCCTGTAGGATGATGGACAGG	261	58	25
Kir6.2	AF037313	GGGCATTATCCCTGAGGAATAT	GGGCATTATCCCTGAGGAATAT	223	57	20

human GK antibody, 1:1,000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA). After blocking with 3% normal donkey serum, slides were incubated with these antibodies for 1 h at room temperature. Sections were then washed with PBS and incubated with the corresponding biotinylated secondary antibody for 30 min at room temperature. Visualization of the staining was achieved by using the avidin-biotin immunoperoxidase complex system. Sections were counterstained using hematoxylin.

Glucose transport in isolated islets. 3-*O*-methylglucose (3*O*MG) transport was measured according to the method of Johnson et al. (32). Briefly, isolated islets were washed twice with calcium- and magnesium-free HBSS. After centrifugation, 500 islets were incubated in calcium- and magnesium-free HBSS containing 3 mmol/l EGTA at 37°C for 15 min to disperse the islet cells. Next, cells were resuspended in 150 μl PBS containing 2 mmol/l [^{14}C]urea (0.5 μCi/μmol) (57 mCi/mmol; NEN Life Science Products) and incubated at 37°C for 20 min. Glucose uptake was measured by placing 50 μl of the cell suspension loaded with urea on top of 50 μl PBS containing 2 mmol/l [^{14}C]urea (0.5 μCi/μmol) and 5.5 or 22.2 mmol/l [^{3}H]3*O*MG (5 μCi/μmol) (81.5 Ci/mmol, NEN Life Science Products). This mixture was layered over 150 μl dibutyl phthalate-dinonyl phthalate (4:1) suspended on top of 50 μl of 1 mol/l D-glucose, 10 mmol/l EDTA, and 0.1% SDS. To perform the uptake, tubes were incubated for 15 s at 15°C and then spun for 30 s to terminate the uptake. A total of 25 μl from the upper and 25 μl from the lower layers were counted in a β-counter. The uptake was measured in triplicate in each experiment performed at the concentrations mentioned above. Uptake of L-[^{3}H]glucose was measured by the same procedure. L-glucose transport represented 20–27% of D-glucose transport for the islet cells obtained from RIP-HGF transgenic mice or from normal littermate islets.

Islet transplantation and follow-up. RIP-HGF transgenic mice and their normal siblings were used as islet donors for transplantation under the kidney capsule of STZ-induced diabetic, severe combined immunodeficient (SCID) mice (BALB/cByJ) (Jackson Laboratory, Bar Harbor, ME). SCID mice (aged 3–4 months) were rendered diabetic by injecting 200–250 mg/kg body wt of STZ i.p. Diabetes was confirmed by the presence of hyperglycemia (>300 mg/dl), polyuria, and weight loss. Random, nonfasted blood glucose was measured from the snipped tail by the glucometer. Islets were isolated as described above and maintained overnight in RPMI medium with 5 mmol/l glucose, 1% penicillin, and streptomycin plus 10% fetal bovine serum. The following day, 250 or 500 IE (1 IE = one 125-μm diameter islet) (Table 2) was transplanted beneath the kidney capsule of anesthetized (45 mg/kg Ketamine and 5 mg/kg Xylazine, i.p.) SCID mice by loading them into PE50 tubing (Becton Dickinson, Sparks, MD) adapted to a 1-ml syringe. Two aliquots of 50 IE each, from normal or transgenic islets used for each transplant were extracted using the acid/ethanol method, as previously described, to determine insulin content (15). To determine whether β-cell composition of the isolated normal and transgenic islets was comparable, 50 IE of each were embedded in paraffin, sectioned, and stained for insulin, and nuclei were stained using Hoechst-33258 dye (Sigma, St. Louis). β-cells (insulin-positive cells) and total islet cells (Hoechst-positive cells) were counted, and the percent of β-cells per islet was calculated.

To ensure that the mass of islet cells in RIP-HGF and normal islets was equivalent, in a separate experiment 50 IE aliquots were isolated from RIP-HGF (n = 8) and normal (n = 8) mice and assayed for protein content (Bradford method) and DNA content (Hoechst-33258 fluorescence). These analyses (see RESULTS) demonstrate that 50 IE from normal and transgenic islets contained equivalent amounts of protein and DNA.

Blood glucose levels were measured at days 1, 2, 3, and 7 and then weekly up to day 56 after transplantation. An intraperitoneal glucose tolerance test (IPGTT) was performed in transplanted and nondiabetic sham-operated SCID mice 49 days after transplantation and after a 16-h fast, as described above. At day 56, mice transplanted with 500 normal IE or 250 RIP-HGF transgenic IE underwent unilateral nephrectomy to confirm an increase in blood glucose levels after graft removal.

Statistical analysis. Data are expressed as the mean ± SE. Analysis of variance or unpaired two-tailed Student's t test were used to determine statistical significance. Statistical significance was considered at $P < 0.05$.

RESULTS

Glucose tolerance in RIP-HGF transgenic mice. Although fasting blood glucose levels were significantly lower in RIP-HGF transgenic mice than in normal littermates (15), glucose tolerance had not previously been assessed in these mice. Glucose tolerance was therefore assessed in RIP-HGF transgenic mice and their corresponding normal littermates using a standard IPGTT (Fig. 1). Importantly, blood glucose levels remained significantly lower in RIP-HGF transgenic mice than in normal littermates at every time point studied after intraperitoneal glucose injection (Fig. 1*A*). Plasma insulin levels at 30 min during the IPGTT as well as insulin/glucose ratios were significantly increased in RIP-HGF transgenic mice compared with those obtained in their normal littermates (Fig. 1*B*). Thus, RIP-HGF islets display more exuberant insulin responses to glucose in vivo than those of their normal littermates, and this results in superior glucose tolerance.

GSIS in islets isolated from RIP-HGF transgenic mice. Previous studies have not examined insulin secretory responses in vitro to glucose in isolated RIP-HGF islets. Therefore, to determine whether the enhanced glucose tolerance observed in RIP-HGF transgenic mice in vivo could be the result of an increase in insulin release per islet cell or the result of increased β-cell mass, we studied GSIS in islets isolated from RIP-HGF transgenic mice and from normal littermates (Fig. 2). In these studies, insulin secretion is corrected for islet protein as described in RESEARCH DESIGN AND METHODS and in the legend to Fig. 2.

TABLE 2
Islet number and insulin content in normal and RIP-HGF transgenic mouse islets used for the transplants in STZ-induced SCID diabetic mice

	n	IE	Islet number per 250 IE	Islet insulin (ng/IE)	β-cells (%)
Normal	8	250	204 ± 7	50 ± 5	91 ± 2
RIP-HGF Transgenic	8	250	189 ± 8*	78 ± 9†	93 ± 1

Data are means ± SE. *P = 0.16, †P = 0.024.

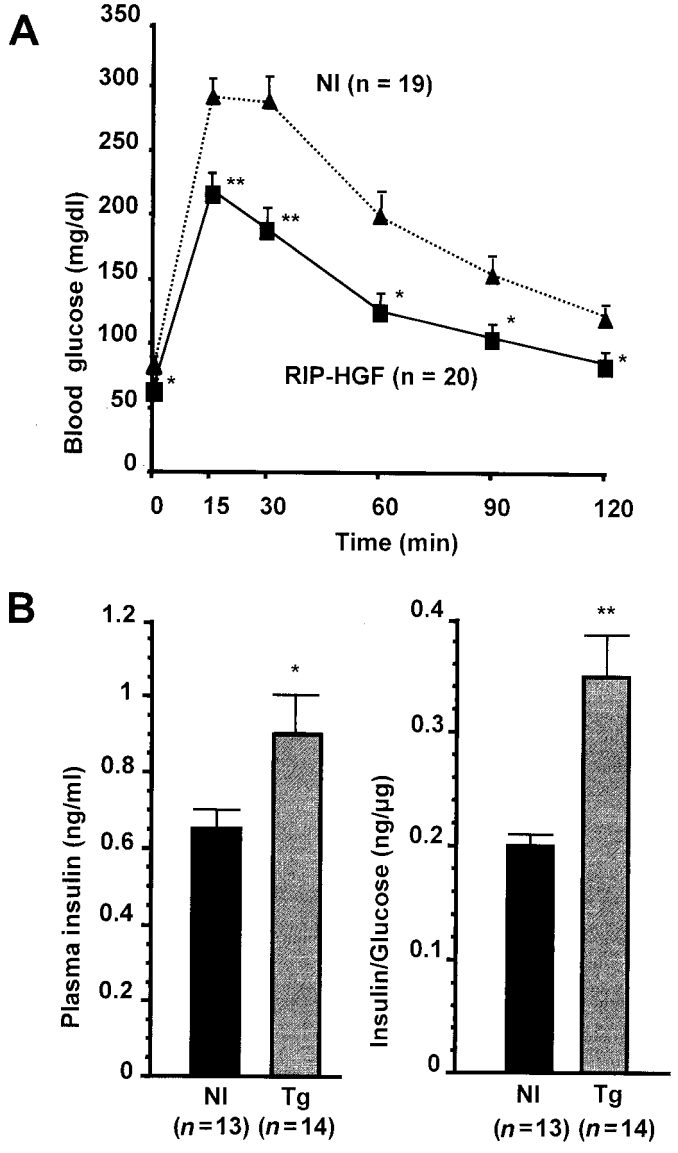

FIG. 1. IPGTT performed in RIP-HGF transgenic mice and their corresponding normal littermates (*A*). After fasting overnight, mice were injected intraperitoneally with glucose (2 g/kg body wt), and blood glucose levels were measured at the time points indicated in the figures. *B*: Plasma insulin concentration in RIP-HGF transgenic mice and normal littermates. Mice were injected with glucose (2 g/kg body wt), and blood was obtained 30 min later by retro-orbital bleeding. Results are means ± SE. The number of mice (*n*) is indicated in the panels. **P* < 0.05, ***P* < 0.01.

Interestingly and in striking contrast to RIP-PTHrP and RIP-PL1 islets we previously described (12,14), islets isolated from RIP-HGF transgenic mice secreted far more insulin than normal islets at every glucose concentration tested (Fig. 2*A*), including the lowest glucose concentration tested (2.8 mmol/l). In addition, when we incubated RIP-HGF and normal islets with 2.8 mmol/l glucose for 30 min immediately after exposure to 22 mmol/l glucose, insulin release was dramatically reduced. This result indicates that insulin release remains regulated by glucose. When glucose was absent in the buffer used in these experiments, the quantity of insulin released by RIP-HGF mouse islets was not significantly different from the amount of insulin released by normal islets (Fig. 2*A*). The 2- to 2.5-fold increase in GSIS in RIP-HGF transgenic islets was constant throughout the different glucose concentrations tested. Reanalysis of the insulin release dose-response curves expressed as percentage of maximal GSIS (Fig. 2*B*) indicates that glucose sensitivity in the islets from RIP-HGF transgenic mice was not different when compared with that in normal islets.

Analysis of islet-specific mRNA expression in RIP-HGF transgenic mice. We analyzed the mRNA expression of a panel of islet-specific genes by semi-quantitative RT-PCR using mouse β-actin as a control housekeeping mRNA. As shown in Fig. 3*A*, overexpression of mHGF mRNA in the islets from RIP-HGF transgenic mice results in an increase in insulin mRNA expression, as previously reported using RNase protection assay (15). However,

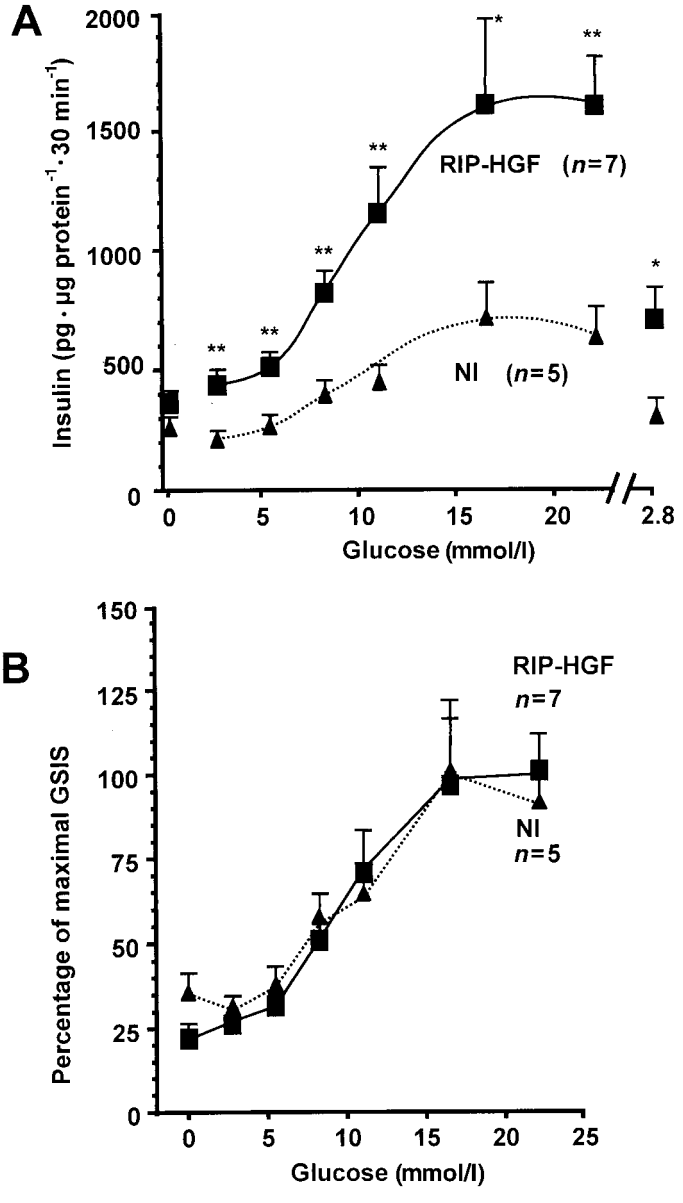

FIG. 2. GSIS in RIP-HGF transgenic mice and their corresponding normal littermates (*A*). Groups of 10 islets were incubated with different glucose concentrations as indicated in the panels, and insulin was measured by RIA. Experiments were performed in triplicate. The number of mice (*n*) from which islets were isolated for each separate experiment is indicated in the figures. *B*: Data from panel *A* are presented as percentage of maximal GSIS for RIP-HGF and normal mice. Results are means ± SE. **P* < 0.05, ***P* < 0.01.

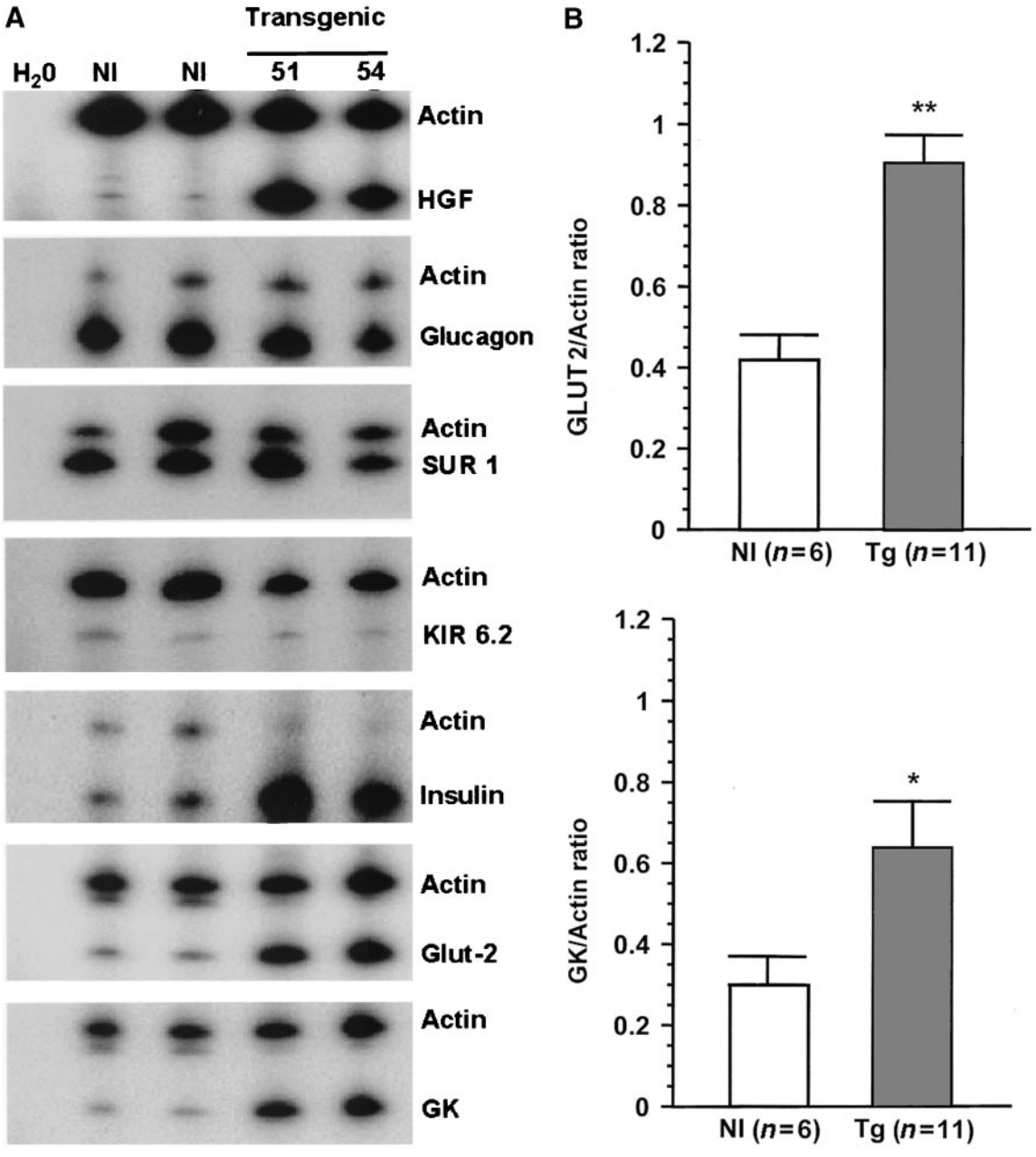

FIG. 3. Expression of islet-specific mRNAs in normal and RIP-HGF transgenic mouse islets. *A*: Relative semiquantitative RT-PCR was performed with 1–2 μg islet RNA from normal littermates (NI) and RIP-HGF (lines 51 and 54) transgenic mouse islets. Mouse β-actin was used as internal control. See Table 1 for primer sequences, number of cycles, and annealing temperatures used in these experiments. *B*: Densitometric analysis of multiple autoradiograms generated with the GLUT2 and GK RT-PCRs of RNA samples from several normal (NI) and RIP-HGF transgenic (Tg) mice (*n*). Y-axis represents arbitrary units. Results are mean ± SE. *$P < 0.05$, **$P < 0.01$.

mRNA expression for another islet hormone, glucagon, was similar in isolated islets from RIP-HGF transgenic mice and normal littermates. Similarly, the levels of mRNA expression of the ATP-sensitive potassium channel components, SUR1 and Kir6.2, were similar in RIP-HGF and normal islets (Fig. 3*A*). Interestingly and in contrast to glucagon, SUR-1, and Kir6.2, mRNA expression of GLUT2 and GK (the two key components in the β-cell glucose sensor system) were far higher in RIP-HGF transgenic mouse islets than in normal islets (Fig. 3*A*). This was confirmed in multiple experiments (Fig. 3*B*) revealing a statistically significant twofold increase in the levels of both GLUT2 as well as GK mRNA in islets from RIP-HGF transgenic mice compared with the corresponding levels in normal littermate islets. Thus, HGF overexpression results in the unanticipated and specific upregulation of three mRNAs, insulin, GLUT2, and GK, each of which is critical in the glucose-stimulated insulin secretory response.

GLUT2 and GK immunostaining. Immunohistochemical analysis of normal and transgenic islets was therefore performed using antibodies against GLUT2 and GK, as shown in Fig. 4. Quantitative differences in the intensity of staining and the distribution pattern of these two peptides were not observed between RIP-HGF transgenic and normal littermate islet cells. Because immunohistochemistry

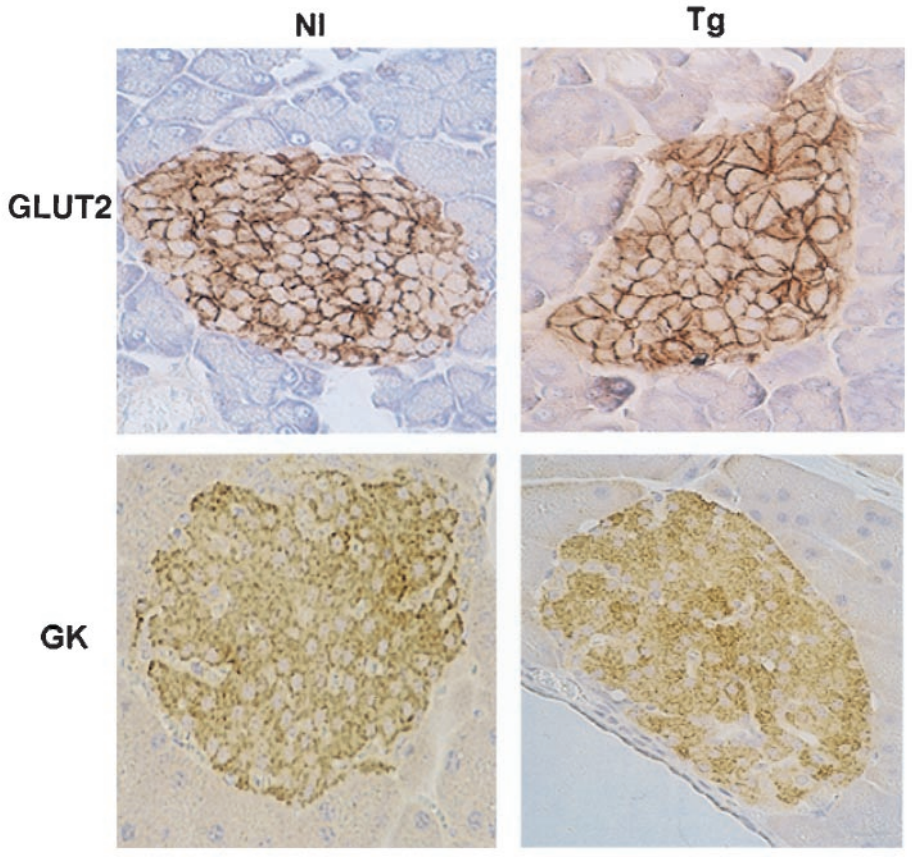

FIG. 4. Immunohistochemical detection of GLUT2 (upper panels) and GK (lower panels) in pancreatic islets of RIP-HGF transgenic mice (right panels) and normal siblings (left panels). The intensity and pattern of staining of these proteins in sections from transgenic mice are very similar to that from normal littermates. Total absence of staining was observed when primary antibodies were omitted (not shown).

is not able to detect subtle changes in the expression of proteins such as GK and GLUT2, we explored these two proteins at a functional level, as described below.

Glucose utilization. To assess the metabolic consequences of HGF overexpression in RIP-HGF islets, glucose utilization was measured in islets isolated from normal and RIP-HGF transgenic mice at 5.5 and 22.2 mmol/l glucose. As shown in Fig. 5, glucose utilization was markedly increased in islets from RIP-HGF transgenic mice compared with the values obtained with normal islets at 5.5 and 22.2 mmol/l glucose.

Glucose uptake in islet cells from RIP-HGF transgenic mice and normal littermates. To explore whether the increased GLUT2 mRNA expression in RIP-HGF transgenic islets might be associated with an elevation of glucose entry into islet cells, we performed 3*O*MG uptake studies in isolated dispersed islet cells (Fig. 6). We used the same two glucose concentrations employed for the glucose utilization studies in Fig. 5 (5.5 and 22.2 mmol/l). Glucose transport in transgenic islet cells at 5.5 mmol/l glucose was not significantly different from that observed in normal islet cells. However, glucose uptake was significantly increased in RIP-HGF transgenic islet cells at 22.2 mmol/l glucose (Fig. 6), suggesting that an increase in the expression of the low-affinity glucose transporter, GLUT2, is likely to be responsible for the increase in glucose uptake and the insulin release observed at this glucose concentration in RIP-HGF transgenic mouse islets.

Islet transplantation. The data presented above suggest that RIP-HGF islets are functionally superior to normal islets and may therefore function in a transplant setting more effectively than normal islets. To directly address

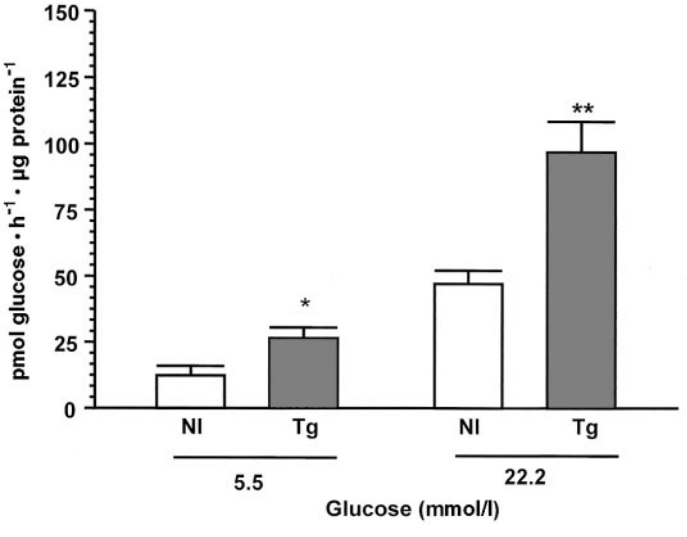

FIG. 5. Glucose utilization in RIP-HGF (■) and normal mouse islets (□). Groups of 50 islets were incubated in KRB containing 5.5 or 22.2 mmol/l glucose plus 2 µCi of [5-^{3}H]glucose at 37°C for 90 min. Glucose usage was determined by measuring the formation of [^{3}H]H_2O. Results are means ± SE of triplicate determinations in five different experiments, each performed with one RIP-HGF and 1–2 normal mice. *$P < 0.05$, **$P < 0.01$.

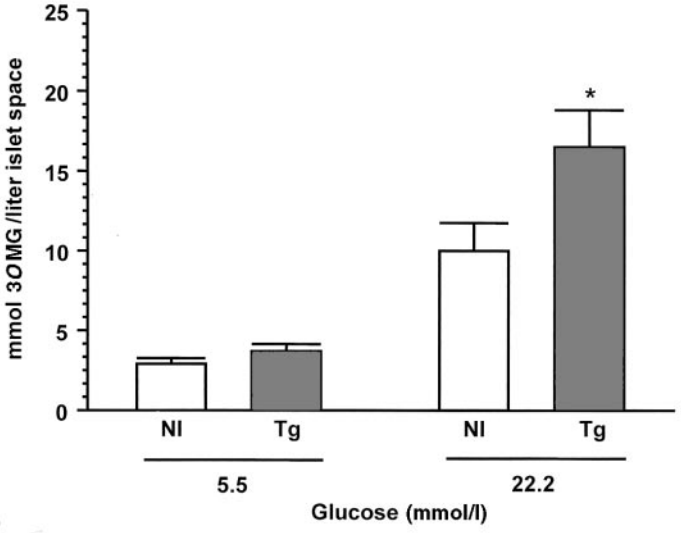

FIG. 6. Glucose uptake in isolated islet cells from RIP-HGF and normal mice. Results are means ± SE of triplicate determinations in five different experiments, each performed with 2–3 RIP-HGF and normal mice. The transport measurement was performed in isolated dispersed cells from 500 islets. *$P < 0.05$.

this hypothesis, we next compared the performance of islets from normal and transgenic mice transplanted under the kidney capsule of diabetic SCID mice. First, we determined the minimal number of IE from normal mice required to normalize blood glucose levels in STZ-induced diabetic SCID mice during the period of time studied (8 weeks). As indicated in RESEARCH DESIGN AND METHODS, 1 IE is defined as being an islet of 125 μm in diameter. After transplantation of 500 IE from normal mice to SCID mice, blood glucose concentration immediately and precipitously dropped to the normal range, and the mice remained euglycemic throughout the 56-day period of the study (Fig. 7). After removal of the kidney containing the islet graft, blood glucose levels returned to original pretransplant values. These results indicate that 500 IE is sufficient to normalize blood glucose in a diabetic SCID mouse. In contrast, 250 IE from normal mice were clearly insufficient to sustain a blood glucose value <300 mg/dl (Fig. 7).

Based on these observations, we next compared the performance of 250 IE isolated from RIP-HGF transgenic mice and normal littermates in SCID diabetic mice. Because of the increased average islet size in RIP-HGF mice (15), we wanted to ensure that the number and size of islets transplanted were comparable for transgenic and normal islets. Thus, the 250 IE from normal and transgenic mice were selected by handpicking, such that 250 normal IE corresponded to 204 ± 7 islets ($n = 8$), while 250 RIP-HGF IE corresponded to 189 ± 8 islets ($n = 7$). Thus, the total number and overall mass of the two types of islets were equivalent (data shown in Table 2). Interestingly and as anticipated, the insulin content in the transgenic islets was significantly increased when compared with the content extracted in the islets from the normal littermates, as previously reported (15). Insulin content was 50 ± 5 ng/IE ($n = 8$) in islets isolated from normal littermates and 78 ± 9 ng/IE ($n = 7$, $P < 0.025$) in RIP-HGF transgenic islets (Table 2). As shown in Fig. 7, in dramatic contrast to results obtained with 250 normal IE, 250 RIP-HGF IE were able to immediately reduce blood glucose concentrations in diabetic SCID mice. Moreover, this return to normal glucose level was sustained for the complete 8 weeks of the study. These levels were significantly lower ($P < 0.01$) than the glucose levels observed in the mice transplanted with 250 IE from normal mice. At day 56, 100% of the animals transplanted with 250 transgenic IE displayed glucose concentrations <200 mg/dl, whereas none of the diabetic SCID mice transplanted with 250 normal IE were <200 mg/dl. After removal of the kidney containing the islet graft, blood glucose levels returned to pretransplant diabetic levels, confirming that the transplant was the source of insulin.

To demonstrate that the transplanted islets were equivalent in islet cell mass, in a separate experiment we compared 50 IE, handpicked exactly as in the transplant experiments, and measured DNA and protein content. For protein content, the RIP-HGF islets contained 0.26 ± 0.03 μg protein/IE, and the normal islets contained 0.22 ± 0.02 μg protein/IE ($P = 0.3$). For DNA content, the RIP-HGF islets contained 33 ± 3 ng DNA/IE, and the normal islets contained 32 ± 1 ng DNA/IE ($P = 0.8$). Thus, 1 IE from both normal and transgenic islets is identical in terms of both protein and DNA content. To confirm that these islets were representative of those transplanted, we also measured insulin content in these islets. The extracts from normal mice contain 51 ± 8 ng insulin/IE, and RIP-HGF islets contain 80 ± 2 ng/IE. These values are almost exactly the same as those observed in the transplant experiments above and as previously reported (15). Thus, one can be confident that the SCID mice receiving 250 IE

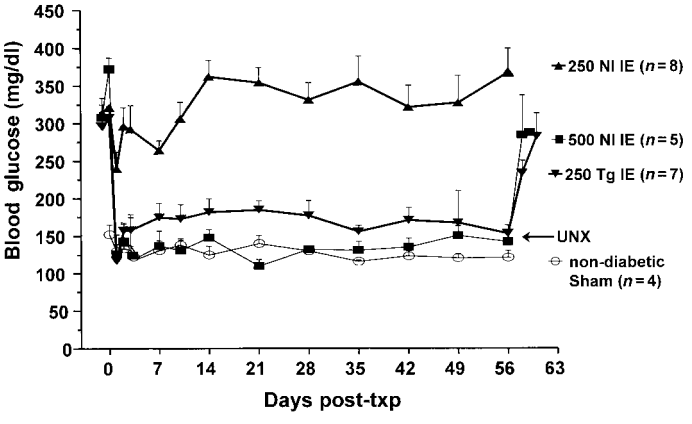

FIG. 7. Comparison of nonfasting blood glucose levels in STZ-induced diabetic SCID mice after transplantation of islets from RIP-HGF transgenic (Tg) mice and normal littermates (Nl). Transgenic islets (250 IE) or normal islets (250 or 500 IE) were transplanted under the kidney capsule of STZ-induced diabetic SCID mice. Nonfasting blood glucose levels were also measured in control nondiabetic sham-operated SCID mice ($n = 4$). Random, nonfasted glucose was measured from the snipped tail with a portable glucometer. Results are means ± SE of five transplants with 500 IE from Nl, eight transplants with 250 IE from Nl, and seven transplants with 250 IE from Tg. Blood glucose after the transplant of either 500 IE from Nl or 250 IE from Tg was significantly lower ($P < 0.01$) than the blood glucose values obtained after the transplant of 250 IE Nl at every time point measured before uninephrectomy (UNX). Blood glucose values were not significantly different after the transplant of either 500 IE from Nl or 250 IE from Tg, except at day 21 posttransplant ($P < 0.025$). UNX was performed in SCID mice transplanted with 250 IE from Tg and 500 IE from Nl mice at day 56 posttransplant.

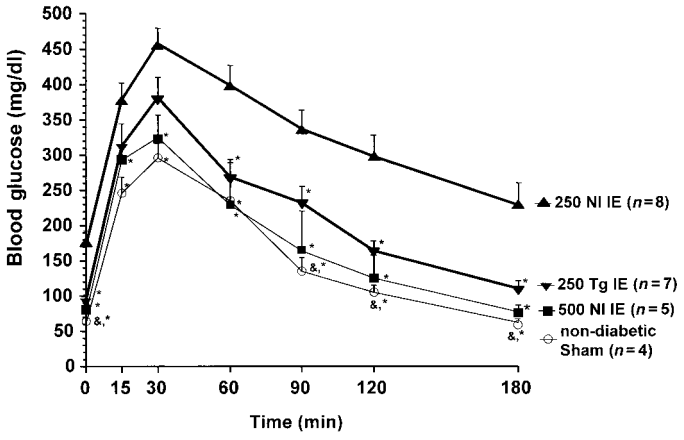

FIG. 8. IPGTT performed in nondiabetic sham-operated SCID mice (n = 4) and STZ-induced diabetic SCID mice transplanted with 250 IE from normal (n = 8), 250 IE from RIP-HGF (n = 7), and 500 IE from normal mice (n = 5). At day 49, after fasting overnight, mice were injected intraperitoneally with glucose (2 g/kg body wt). Blood glucose levels were measured from the snipped tail at the time points indicated in the figures with a portable glucometer. Results are means ± SE. *P < 0.05 compared with blood glucose values obtained in STZ-induced diabetic SCID mice transplanted with 250 IE from normal littermates, &P < 0.05 compared with values obtained in STZ-induced diabetic SCID mice transplanted with 250 IE from RIP-HGF mice. Results obtained from 250 IE from Tg and 500 IE from Nl were not significantly different at any time point studied.

of normal or transgenic islets received the equivalent mass of islet cells.

To determine whether the β-cell content of the transgenic and normal islets used for transplantation were comparable, islets used in each transplant were analyzed histomorphometrically, as described in RESEARCH DESIGN AND METHODS. These studies demonstrated that the RIP-HGF islets were composed of 93 ± 1% insulin-positive cells per islet, and the normal islets were composed of 91 ± 2% insulin-positive cells/islet (Table 2). Thus, islet mass, islet cell number, and β-cell content were equivalent in animals receiving 250 normal compared with 250 transgenic IE.

To further determine the function of the transplanted transgenic HGF islets, IPGTT experiments were performed at day 49 and compared with control, nondiabetic, sham-operated SCID mice and with diabetic SCID mice transplanted with normal islets. As shown in Fig. 8, basal fasting blood glucose levels were significantly lower in SCID mice transplanted with 250 RIP-HGF transgenic IE than in SCID mice transplanted with 250 IE from normal littermates. Importantly, after intraperitoneal glucose injection, glucose tolerance was markedly superior in SCID mice transplanted with 250 RIP-HGF IE than that of SCID mice transplanted with 250 normal IE.

Taken together, these results indicate that only half as many HGF transgenic islets as normal islets are required to produce a sustained reduction in blood glucose and long-term improved glucose tolerance in diabetic mice.

DISCUSSION

The results presented herein are surprising because previous studies have suggested that exposure to HGF increases mitogenesis but decreases differentiation markers in β-cells (24). In addition, of the three transgenic models we initially studied (RIP-PTHrP, RIP-PL1, and RIP-HGF) (12–15), the RIP-HGF mice had the least impressive phenotype under basal conditions. The results in the current study indicate that overexpression of HGF in the β-cells of transgenic mice enhances glucose tolerance in vivo, increases GSIS in vitro, improves glucose sensing in vitro, and markedly improves the performance of renal subcapsular islet grafts in vivo in diabetic mice. Until now, these effects have not been described in other transgenic mouse islets. All together, these data suggest that gene transfer of HGF may be particularly effective for improving islet survival and function after transplant.

We first assessed in vivo glucose tolerance in RIP-HGF mice and normal littermates. RIP-HGF transgenic mice displayed superior glucose tolerance and more robust insulin secretion than their normal littermates. This increase in glucose tolerance and insulin secretion might simply be the result of the increase in islet size and number observed in RIP-HGF transgenic mice. However, this is not likely the explanation, because two other transgenic mouse lines that also show a similar increase in islet size and number (RIP-PTHrP and RIP-PL1 transgenic mice) do not display this superior glucose tolerance compared with normal littermates (33). These findings suggest that HGF overexpression specifically upregulates insulin secretion in response to glucose, independent of islet size.

To further define whether the improvement in glucose tolerance in RIP-HGF transgenic mice was the result of an increase in insulin secretion from RIP-HGF islets, we performed in vitro GSIS experiments. Importantly, these experiments were designed such that islet mass was similar in the two groups; the studies were performed using matched islet size. It has been previously reported with perifusion studies that RIP-PTHrP and RIP-PL1 transgenic islets secrete an equivalent amount of insulin as normal islets in response to glucose (12,14). Similar results were obtained when GSIS experiments with static incubations were performed in these two transgenic mouse models (33). In marked contrast to the RIP-PL1 and RIP-PTHrP islets (12,14), islets from RIP-HGF transgenic mice secreted dramatically more insulin than normal islets even at the lowest glucose concentration used in the same kind of GSIS experiments. Analysis of these data suggests several conclusions: *1*) the increase in insulin secretion by RIP-HGF islets compared with that of normal islets is independent of islet number or β-cell mass; *2*) despite the superficially similar phenotypes in the three transgenic mouse models (increased islet size and number and hypoglycemia), the physiological responses of these models to glucose in vivo and in vitro are quite distinct; *3*) among the three growth factors overexpressed in the islet, HGF appears to have the most salutary effect on glucose tolerance and insulin secretion; *4*) simply having an increased β-cell mass does not mandate superior glucose tolerance. This latter point has been repeatedly observed. For example, transgenic mice with increased expression of IGF-II in the β-cells and displaying increased islet mass exhibit hyperinsulinemia and increased GSIS but poor glucose tolerance (17).

There are at least two possible reasons why RIP-HGF islets may show superior GSIS: they may have a higher content of insulin, and/or they may have improved glucose sensing. Both possibilities appear to be operative. Favor-

ing the first possibility are previous and present observations indicating that RIP-HGF islets, even when corrected for islet size, protein content, or cell number, display increased insulin mRNA expression and insulin peptide content (15). In addition, as observed when the GSIS results are expressed as percentage of maximal GSIS, normal islets and transgenic islets seem to sense glucose in a similar way. On the other hand, the significant increase in glucose utilization at 5.5 and 22.2 mmol/l glucose suggests that these transgenic islets have increased glucose metabolism that also contributes in part to the increase in GSIS. Similar patterns of GSIS and glucose utilization have been observed in RIP–IGF-II transgenic mice (17).

Importantly, RIP-HGF transgenic islets do not secrete significantly higher amounts of insulin when glucose is absent in the incubation buffer. Insulin secretion appears almost fully suppressible at low glucose concentrations. This ability to downregulate insulin secretion may contribute to the relatively mild basal hypoglycemia observed in these mice (15). Normal or near-normal suppression of insulin secretion in response to hypoglycemia would be an important attribute to any bioengineered, transplanted islets.

In an initial approach to examine the expression of islet genes implicated in the unexpected salutary glucose metabolism/sensing in the RIP-HGF transgenic islets, we found that increased expression of HGF resulted in significant increases in insulin, GLUT2, and GK mRNA expression. Whereas this has been previously shown in in vitro experiments using exogenous HGF, alone or in combination with activin A in rat pancreatic AR42J exocrine cells (22,23), it has not previously been described in mature β-cells in vivo. To our knowledge, this is the first time that HGF has been observed to enhance in vivo the β-cell expression of GLUT2 and GK, two key regulators of insulin secretion. This increased expression of GLUT2 and GK at the mRNA level was not observed by immunohistochemistry but was corroborated by the elevated glucose utilization observed in RIP-HGF transgenic islets, suggesting that the enhanced GSIS in these islets is the result of an increased insulin content and likely also the result of accelerated fuel metabolism resulting in increased insulin secretion. Moreover, glucose transport is modestly but significantly increased in RIP-HGF transgenic islets at 22.2 mmol/l glucose. It would be expected that GLUT2, the low-affinity, high-capacity glucose transporter, would be the primary glucose transporter at this glucose concentration. A recent report has shown that the absence of GLUT2 in mouse islets induces hyperglycemia, hypoinsulinemia, glycosuria, and early death (34). The islets of these mice display glucose utilization similar to normal islets at the lowest glucose concentrations reported (1.6 and 6 mmol/l), but no further increase was observed at 20 mmol/l glucose in GLUT2-null islets (35). In addition, insulinoma cell lines completely lacking GLUT2 and GK expression do not exhibit GSIS (36–38). However, stable transfection of glucose unresponsive insulinoma cells with GLUT2 cDNA confers GSIS and increased insulin content in these cells (36–38). Furthermore, transfection with GLUT1 did not permit glucose sensing, although glucose metabolism was similar in GLUT2- and GLUT1-transfected cells (36). On the other hand, it has been reported that differences in GLUT2 gene expression between α- and β-cells correlate with differences in glucose transport but not in glucose utilization (39). Further studies will be required to define the exact mechanisms through which GLUT2 and GK contribute to increased GSIS in the RIP-HGF transgenic islets.

One of the main obstacles to islet transplantation is the scarcity of islet tissue. Recently, the successful transplantation of human islets in several patients with type 1 diabetes has been reported (1,2). Each diabetic patient in this study received islets from two to four donors. The characteristics of the RIP-HGF islets (increased β-cell proliferation, insulin content, GK and GLUT2 mRNA, and superior GSIS) make HGF an attractive candidate for enhancing islet function before and after transplantation. In our transplant studies, as predicted, we have observed that RIP-HGF transgenic islets do indeed improve islet graft performance and induce euglycemia. Furthermore, whereas 250 IE derived from normal mice were inadequate to correct diabetes, 250 RIP-HGF IE fully and promptly reversed diabetes in the STZ-induced diabetic SCID mouse. Significantly, this correction of hyperglycemia was maintained continuously for 8 weeks and reversed when the kidney containing the transplant was removed. Moreover, fasting blood glucose levels at day 49 posttransplant in mice with 250 RIP-HGF IE were significantly lower than in those transplanted with 250 normal IE and similar to those transplanted with 500 normal IE or nondiabetic sham-operated SCID mice. In addition, glucose tolerance was significantly improved in SCID mice transplanted with 250 RIP-HGF IE and was similar to the glucose tolerance observed in the diabetic SCID mice transplanted with 500 normal IE.

These favorable actions of RIP-HGF transgenic islet grafts could result from several mechanisms. First, the ~50% increase in insulin content and the improved insulin secretion of RIP-HGF islets could account for the initial drop in blood glucose values observed at day 1 posttransplant. This rapid reduction in blood glucose could favor the performance of the graft in absence of toxicity induced by hyperglycemia. Second, HGF overexpression in transgenic islets increases β-cell proliferation in vivo (15). In vitro, exogenous HGF or transduction with adenoviruses containing mHGF cDNA was able to increase DNA synthesis in mouse and human islets (10,11,40). Finally, because HGF has been suggested to have anti-apoptotic effects (27,28), these islets could be less vulnerable to the cell death that occurs in the immediate posttransplantation period (41). As noted earlier, we have observed in previous studies that RIP-HGF islets are more resistant to the diabetogenic effects of STZ (15). In addition, Nakano et al. (26) recently demonstrated that intraperitoneal injection of HGF ameliorates hyperglycemia in STZ-induced diabetic mice receiving a marginal mass of intrahepatic islet grafts. The authors suggest that HGF could have a protective effect on the grafted islets from the toxic effect induced by continuous hyperglycemia. Taken together, these results suggest that HGF may very likely have a unique profile as an agent that could be beneficial for the survival and function of islet grafts.

One might reasonably ask what advantage gene transfer

of islet growth factors may have on improving transplant survival compared with simply adding growth factors such as HGF to human islet cultures awaiting transplant. Two considerations are germane here. First, the Edmonton group has repeatedly emphasized the importance of brief (a few hours) cold ischemia time. Thus, in future transplant protocols there may be little time to expose islets to growth factors before transplant. Second, as the current study suggests, continued local production of growth-stimulatory, insulin secretion–enhancing factors by β-cells after transplant is likely to be important but not achievable using conventional culture techniques pretransplant.

In summary, these studies indicate that elevated expression of HGF in the islet leads to an increase in GSIS, glucose utilization, and glucose transport, likely induced at least in part by overexpression of GLUT2 and GK, the two key regulatory molecules in glucose sensing. Finally, HGF-overexpressing islets function after transplantation in a manner superior to normal islets both at early as well as late time points. These findings suggest that gene therapy strategies employing delivery of HGF, either alone or in combination with other molecules, may likely enhance islet transplant efficiency and duration and reduce the numbers of normal islets required for islet transplantation.

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