Overexpression of Parathyroid Hormone–Related Protein Inhibits Pancreatic β-Cell Death In Vivo and In Vitro

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Pancreatic β-cell survival is critical in the setting of diabetes as well as in islet transplantation. Transgenic mice overexpressing parathyroid hormone–related protein (PTHrP) targeted to β-cells using the rat insulin II promoter (RIP) display hyperinsulinemia, hypoglycemia, and islet hyperplasia, without a concomitant increase in β-cell proliferation rate or enlargement of individual β-cell size. Thus, the mechanism for increased β-cell mass is unknown. In this study, we demonstrated that β-cells of transgenic mice are resistant to the cytotoxic effects of streptozotocin (STZ) in vivo, as documented by a sixfold reduction in the rate of STZ-induced β-cell death in RIP-PTHrP mice relative to their normal siblings. The reduced cell death in transgenic mice is due neither to their increased islet mass nor to a decrease in their sensing of STZ, but rather results from PTHrP-induced resistance to β-cell death. This is also demonstrated in vitro by markedly reduced cell death rates observed in β-cells of transgenic mice compared with normal mice when cultured in the absence of serum and glucose or in the presence of STZ. Finally, we demonstrated that NH_2-terminal PTHrP inhibits β-cell death. These findings support the concept that PTHrP overexpression increases islet mass in transgenic mice through inhibition of β-cell death. ***Diabetes*** **51:3003–3013, 2002**

Failure of β-cell survival is critical to the etiology of type 1 (1–3) and type 2 (4,5) diabetes, as well as in the setting of islet transplantation (6,7). Despite an enormous increase in our understanding of islet differentiation and development, there is sparse information regarding the factors and pathways that regulate growth, survival, and death of islet cells. A number of approaches have been taken, including development of knockout and transgenic mouse models, to address these questions. Of the several β-cell–targeted transgenic mouse models generated to date, only a handful have displayed an increase in islet mass without displaying a concomitant negative effect on islet structure or function (8–12). The mechanisms through which the targeted proteins have brought about islet mass expansion are quite distinct. These mechanisms include accelerated replication of preexisting β-cells (9–12), an increase in β-cell size (hypertrophy) (9,11,12), a reduction in the rate of β-cell death (13,14), and an augmentation of islet neogenesis (11,12,15).

Parathyroid hormone–related protein (PTHrP), widely expressed in most tissues of the body in the fetus and adult, is also expressed in the four endocrine cell types of the islet and in pancreatic ductal cells (16). PTHrP receptors have been shown to be present in islets (17) and the β-cell line RINm5F (18). Transgenic mice overexpressing PTHrP as well as PTHrP-knockout mice have demonstrated critical roles for this peptide in the development and differentiation of many organs, including the skeleton (19,20), mammary gland (21), skin (22), teeth (23), vascular system (24), and others. To examine the consequences of PTHrP overexpression in pancreatic β-cells, we prepared transgenic mice in which the rat insulin II promoter (RIP) was used to drive expression of PTHrP in β-cells. The resultant RIP-PTHrP transgenic mice display dramatic islet hyperplasia with resulting insulin-mediated hypoglycemia (8). The enhanced islet mass in the RIP-PTHrP mice is not the result of a prenatal or developmental effect of PTHrP on the islet, since islet mass is normal at birth, but is rather a postnatal effect, first apparent at 3 months and accumulating with age (25). Importantly, the two- to threefold increase in islet mass observed in transgenic mice is not a result of an increase in the replication rate of β-cells in these mice, nor is it due to islet cell hypertrophy (25). Thus, the mechanism for the increase in islet mass in the adult RIP-PTHrP mouse is unknown. We postulate that by default it may be due to a slowing in β-cell death.

Due to the slow turnover rate of β-cells and the efficient clearing of apoptotic cells, measuring the rate of β-cell survival or death under normal physiological conditions is extremely difficult (26). Therefore, to examine the effect of PTHrP overexpression on β-cell death in vivo, we treated RIP-PTHrP mice and controls with the β-cell cytotoxic agent streptozotocin (STZ). As we reported previously, RIP-PTHrP mice are resistant to the diabetogenic effects of STZ, developing only delayed and mild hyperglycemia in

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HBSS, Hanks' buffered saline solution; IE, islet equivalents; PI, propidium iodide; PTHrP, parathyroid hormone–related protein; RIP, rat insulin II promoter; STZ, streptozotocin; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling.

TABLE 1
Sequences of the PCR primers

Gene	GenBank accession no.	Primers		Product size (bp)	Annealing temperature (°C)	Cycles (*n*)
		Sense	Antisense			
Glut-2	S77926	TTAGCAACTGGGTCTGCAAT	GCAGCGATTTCCTCAAAAG	170	56	25
BcL-2	M16506	AGCTGCACCTGACGCCCTT	GTTCAGGTACTCAGTCATCCAC	192	60	25
$BcLX_L$	AF088904	AGGTTCCTAAGCTTCGCAATTC	TGTTTAGCGATTCTCTTCCAGG	248	57	20
Bax	L22472	R&D Systems Catalog # RDP-43-025		420	60	20
Bad	L37296	R&D Systems Catalog # RDP-41-025		445	55	20

comparison to the immediate severe hyperglycemia observed in their normal littermates (25).

In the current study, we sought to directly examine RIP-PTHrP mice for evidence of resistance to β-cell death. We demonstrated that RIP-PTHrP mice are indeed markedly resistant to the cytotoxic effects of STZ, as evidenced by a sixfold reduction in STZ-induced β-cell death in transgenic mice compared with their normal littermates. Furthermore, these studies reveal that the resistance to STZ in transgenic mice is due neither to the overall increase in islet mass in these mice nor likely to a reduction in the transport of STZ into β-cells. Instead, PTHrP overexpression directly enhances β-cell survival, as observed by the resistance of β-cells from RIP-PTHrP mice to both STZ-induced and serum starvation–induced cell death in vitro. Levels of the anti-apoptotic proteins Bcl-2 and Bcl-X_L are similar in islets isolated from transgenic and normal mice. Finally, we demonstrated that inhibition of β-cell death is not a result only of chronic overexpression of PTHrP in β-cells, but can also be achieved by the exogenous addition of NH_2-terminal PTHrP peptide to cultures of normal islets, suggesting a role for this peptide in β-cell survival.

RESEARCH DESIGN AND METHODS

Transgenic mice. Two independent RIP-PTHrP transgenic mouse lines (1799 and 1807) having similar phenotypes were generated as described previously (8). In all of the experiments, neonatal and adult transgenic mice were compared with their corresponding normal littermates. The age of the mice varied with the experiments and is specified in each experiment. Genotyping of the mice was performed by tail DNA PCR as described (20). All studies were performed with the approval of, and in accordance with, guidelines established by the University of Pittsburgh School of Medicine Institutional Animal Care and Use Committee.

STZ treatment in vivo. Adult mice (3–6 months old) were injected intraperitoneally with STZ (Sigma, St. Louis, MO) at a single dose of 100 mg/kg. STZ was prepared fresh for each use in citrate buffer (10 mmol/l sodium citrate, 0.9% saline, pH 4.0–4.5) at a stock STZ concentration of 12.5 mg/ml. Initially, pancreata from normal mice were removed 6, 12, or 24 h after STZ treatment, fixed overnight in Bouin's fixative, and immunostained with terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) to determine the extent of cell death. Based on these pilot studies, the 12-h time point was chosen to compare cell death in normal and transgenic mouse pancreata.

Neonatal mice (7–10 days) were injected in preliminary experiments with 100 or 150 mg STZ/kg; blood glucose was measured at 1, 2, or 3 days after treatment or pancreata were removed 6 h after STZ injection. Subsequently, in neonatal mouse studies, STZ was administered intraperitoneally at a dose of 150 mg/kg. Glucose concentrations were measured 24 h after this dose, and pancreata were harvested 6 hours after STZ injections to compare β-cell death between normal and transgenic neonatal mice. Blood glucose was measured by a Precision Q.I.D. portable glucometer (Medisense, Bedford, MA).

Islet isolation. Mouse islets were isolated as previously described (27), with some modifications. Briefly, 3 ml of 1.7 mg/ml Collagenase P (Roche Molecular Biochemicals, Indianapolis, IN) in Hanks' buffered saline solution (HBSS) was injected into the pancreatic duct. Subsequently, pancreata were removed and digested at 37°C for 17 min, before sieving through a 500-μm wire mesh to separate undigested tissue. The digested pancreas was pelleted and rinsed with HBSS, and islets were separated by density gradient in Histopaque (Sigma). After several washes with HBSS, islets were hand-picked under a microscope. Islets either were used immediately after isolation to obtain RNA or protein or were left in complete medium (RPMI medium with 10% fetal bovine serum, 5.5 mmol/l glucose, and 1% penicillin and streptomycin) for 1–2 days before islet cell cultures were prepared.

Primary islet cell culture. Cultures of single islet cells were prepared by handpicking 400 islet equivalents (IE) (1 IE = 200-μm-diameter islet) into microfuge tubes, rinsing with PBS, and trypsinizing with 400 μl trypsin-EDTA for 8–10 min at 37°C. Islet cells were dispersed by pipetting twice during digestion. One milliliter of complete medium was added to stop the trypsinization, and cells were pelleted by spinning at 2,000 rpm for 2 min before plating on 12-mm glass coverslips placed in 24-well plates. Initially, cells from ~100 IE were plated on each coverslip in a small volume of 50 μl complete medium and incubated at 37°C for 2 h, to allow cells to attach to the glass surface. Subsequently, 800 μl medium was added and cells were allowed to grow for 2–4 days. Thereafter, cells were either treated with 5 mmol/l STZ for 6 h or were kept in complete medium or RPMI without serum and glucose for 16–24 h before rinsing with PBS and finally fixing in freshly prepared 2% paraformaldehyde for 30 min at room temperature. Normal islet cultures were treated with 10^{-6} mol/l peptides (1-36, 38-94, or 107-139) during the period of serum starvation.

Immunostaining. Pancreata removed from transgenic and normal mice were fixed in Bouin's solution, embedded in paraffin, sectioned, and immunostained after deparaffinization and rehydration. Cell death was detected by enzymatic in situ labeling of DNA strand breaks using the TUNEL method. TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol. Proteinase K (Invitrogen Life Technologies, Rockville, MD) and the substrate NBT/BCIP (Vector Laboratories, Burlingame, CA) were obtained from the indicated manufacturers. After TUNEL, sections were stained with a mixture of antibodies against islet hormones, including rabbit anti–pancreatic polypeptide antibody (1:20 dilution), rabbit anti-glucagon antibody (1:50 dilution) (both from Biogenex, San Ramon, CA), and rabbit anti-somatostatin antibody (1:200 dilution) (Novocastra Laboratories, Newcastle, U.K.) overnight at 4°C, to identify islets in the section. Visualization of staining was achieved using antibody coupled to horseradish peroxidase enzyme and the substrate diaminobenzidine tetrahydrochloride (Biogenex). Costaining with insulin and propidium iodide (PI) (Sigma) was also used to detect β-cell death. Immunofluorescent staining of pancreatic sections or islet cell cultures was carried out with a guinea pig anti-porcine insulin antibody (Zymed, San Francisco, CA) at a 1:15 dilution for 30 min at room temperature, followed by a fluorescein isothiocyanate–conjugated rabbit anti–guinea pig IgG secondary antibody (Zymed) at a 1:100 dilution for 1 h at room temperature in the dark. Finally, samples were incubated for 10 min at 37°C with 2 μg/ml PI and 100 μg/ml RNase I (Ambion, Austin, TX) made in PBS, washed several times with water, and coverslipped using the Prolong antifade kit (Molecular Probes, Eugene, OR). Immunostaining for Glut-2 was done using an affinity-purified goat anti-human Glut-2 antibody at a 1:500 dilution (Santa Cruz Biotechnology, Santa Cruz, CA) as described (28).

RNA isolation, reverse transcription, and relative semiquantitative PCR. Total RNA was isolated from islets of mice aged 3 to 8 months, using Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Semiquantitative RT-PCR was performed as described in detail (28). The following pairs of murine gene-specific primers were used: Actin (Ambion), Glut-2, Bcl-2, Bcl-X_L, Bax, and Bad (Table 1). The size of the resulting product, the annealing temperature used, and the number of cycles previously determined to be in the nonsaturating part of the linear amplification portion of the PCR products are listed in Table 1 for each set of primers. Films were scanned and band densitometry was quantitated using the computer program ImageJ from the National Institutes of Health.

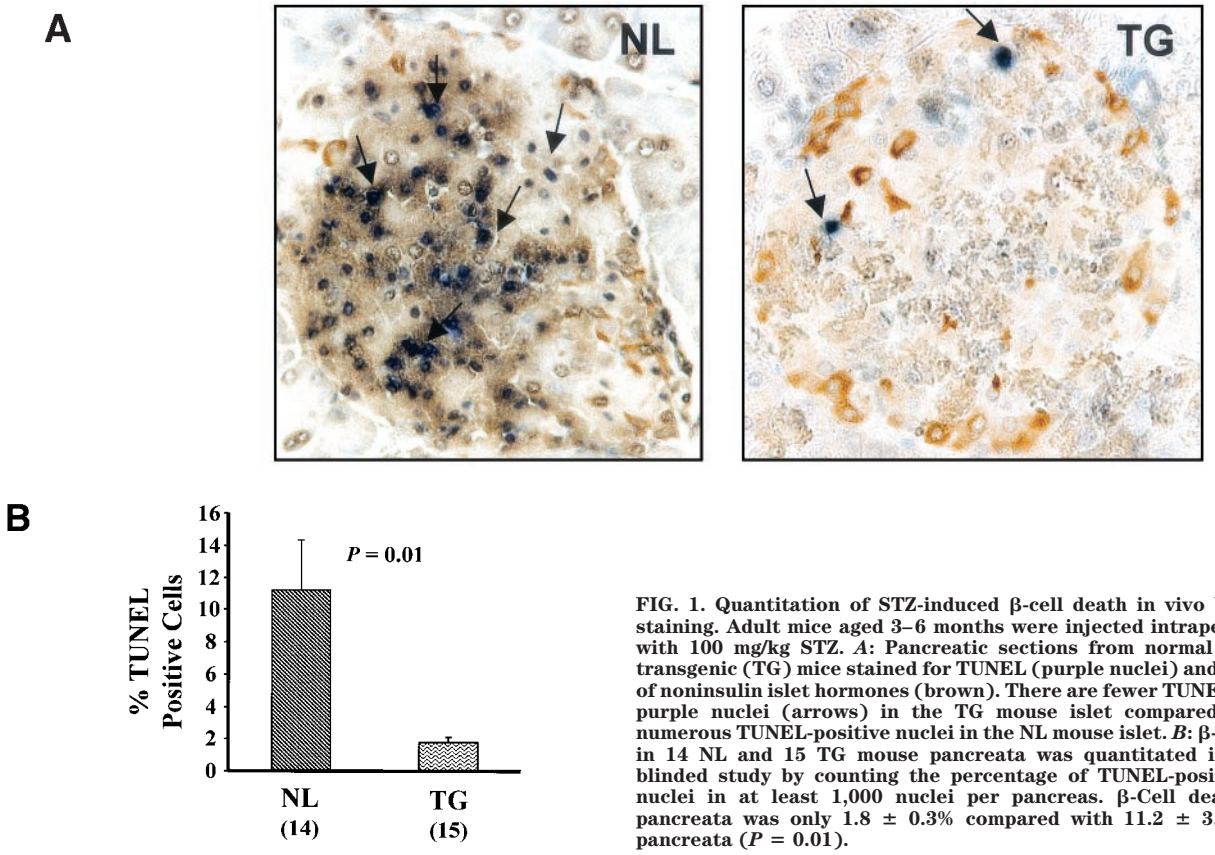

FIG. 1. Quantitation of STZ-induced β-cell death in vivo by TUNEL staining. Adult mice aged 3–6 months were injected intraperitoneally with 100 mg/kg STZ. *A*: Pancreatic sections from normal (NL) and transgenic (TG) mice stained for TUNEL (purple nuclei) and a cocktail of noninsulin islet hormones (brown). There are fewer TUNEL-positive purple nuclei (arrows) in the TG mouse islet compared with the numerous TUNEL-positive nuclei in the NL mouse islet. *B*: β-Cell death in 14 NL and 15 TG mouse pancreata was quantitated in a single blinded study by counting the percentage of TUNEL-positive β-cell nuclei in at least 1,000 nuclei per pancreas. β-Cell death in TG pancreata was only 1.8 ± 0.3% compared with 11.2 ± 3.1% in NL pancreata (P = 0.01).

Western analysis. Whole islet extracts were made in freshly prepared lysis buffer (5% SDS, 80 mmol/l Tris-HCl, pH 6.8, 5 mmol/l EDTA, 0.5 mmol/l phenylmethylsulfonyl fluoride) at a ratio of 20 μl/100 IE. Islets were sonicated, the supernatant containing the cell lysate was separated by centrifugation, and protein concentrations were measured using the MicroBCA assay (Pierce). Forty micrograms of protein from each sample was added to loading buffer and analyzed using 10% SDS-polyacrylamide gels. Proteins were transferred from the gels to Immobilon-P membrane (Millipore, Bedford, MA) using standard techniques. Blots were incubated with primary antibodies against Bcl-2 (polyclonal rabbit anti-rat/mouse Bcl-2 antibody at 1:2,000 dilution) (PharMingen International, San Diego, CA), Bcl-X_L (mouse monoclonal antibody at 1:200 dilution) (Santa Cruz Biotechnology), Glut-2 (at a 1:200 dilution), and actin (rabbit polyclonal antibody at 1:400 dilution) (Sigma). For Bcl-2 and actin, the same secondary antibody, peroxidase-conjugated donkey anti-rabbit IgG, was used at a 1:8,000 dilution (Jackson ImmunoResearch Laboratories, West Grove, PA); for Glut-2, a peroxidase-conjugated donkey anti-goat IgG was used at a 1:8,000 dilution (Santa Cruz Biotechnology); and for Bcl-X_L, peroxidase-linked sheep anti-mouse IgG was used at a 1:5,000 dilution (Amersham, Piscataway, NJ). Chemiluminescence was detected using the enhanced chemiluminescence system (Amersham).

Statistical analysis. Data are expressed as means ± SE. Unpaired two-tailed Student's t test was used to determine statistical significance. Differences were considered significant at $P < 0.05$.

RESULTS

Adult RIP-PTHrP mice are resistant to the cytotoxic effects of STZ in vivo. To examine whether β-cells of RIP-PTHrP mice are resistant to cell death, we studied β-cell responses to in vivo injection of STZ, a β-cell–specific cytotoxic agent. In pilot studies, normal mice injected with STZ at a single dose of 100 mg/kg were killed 6, 12, or 24 h postinjection. Pancreatic sections from these mice were stained for TUNEL to determine the extent of β-cell death. Minimal TUNEL staining was observed at the 6-h time point in normal mice, indicating that 6 h is too early to assess cell death following STZ administration at that dose. Markedly increased numbers of TUNEL-positive nuclei were observed by 12 h, and by 24 h there was a massive loss of β-cells (data not shown), making analysis of cell survival at this late time point impossible to assess. The 12-h time point was therefore chosen for subsequent measurement and comparison of β-cell death between normal and transgenic mouse pancreata.

Figure 1*A* shows TUNEL staining of a representative section from normal and transgenic mouse pancreata following STZ administration. Numerous TUNEL-positive nuclei, representing dying cells, were seen in the islets of normal mouse pancreata. In contrast, only a few TUNEL-positive nuclei were observed in the RIP-PTHrP mouse pancreata. This was confirmed by blinded quantitation of the percentage of TUNEL-positive β-cell nuclei from 14 normal and 15 transgenic pancreata. As shown in Fig. 1*B*, direct quantitation showed a sixfold decrease in β-cell death in transgenic (1.8 ± 0.3%) versus normal (11.2 ± 3.1%) mouse pancreata following exposure to STZ.

Cell death was also quantitated by costaining pancreatic sections from these mice with insulin and PI. Condensed, bright-red pyknotic nuclei representing dead β-cells were more prevalent in the islets of normal mice relative to their transgenic siblings (Fig. 2*A*). Quantitation of the percentage of condensed β-cell nuclei in a subset of five normal and five transgenic mouse pancreata again revealed markedly (6.4-fold) lower β-cell death in transgenic mice (2.4 ± 0.7%) compared with normal siblings (15.4 ± 4.2%) following STZ administration (Fig. 2*B*). These two studies independently demonstrate that compared with their normal littermates, adult RIP-PTHrP mice are markedly resistant to the cytotoxic effects of STZ.

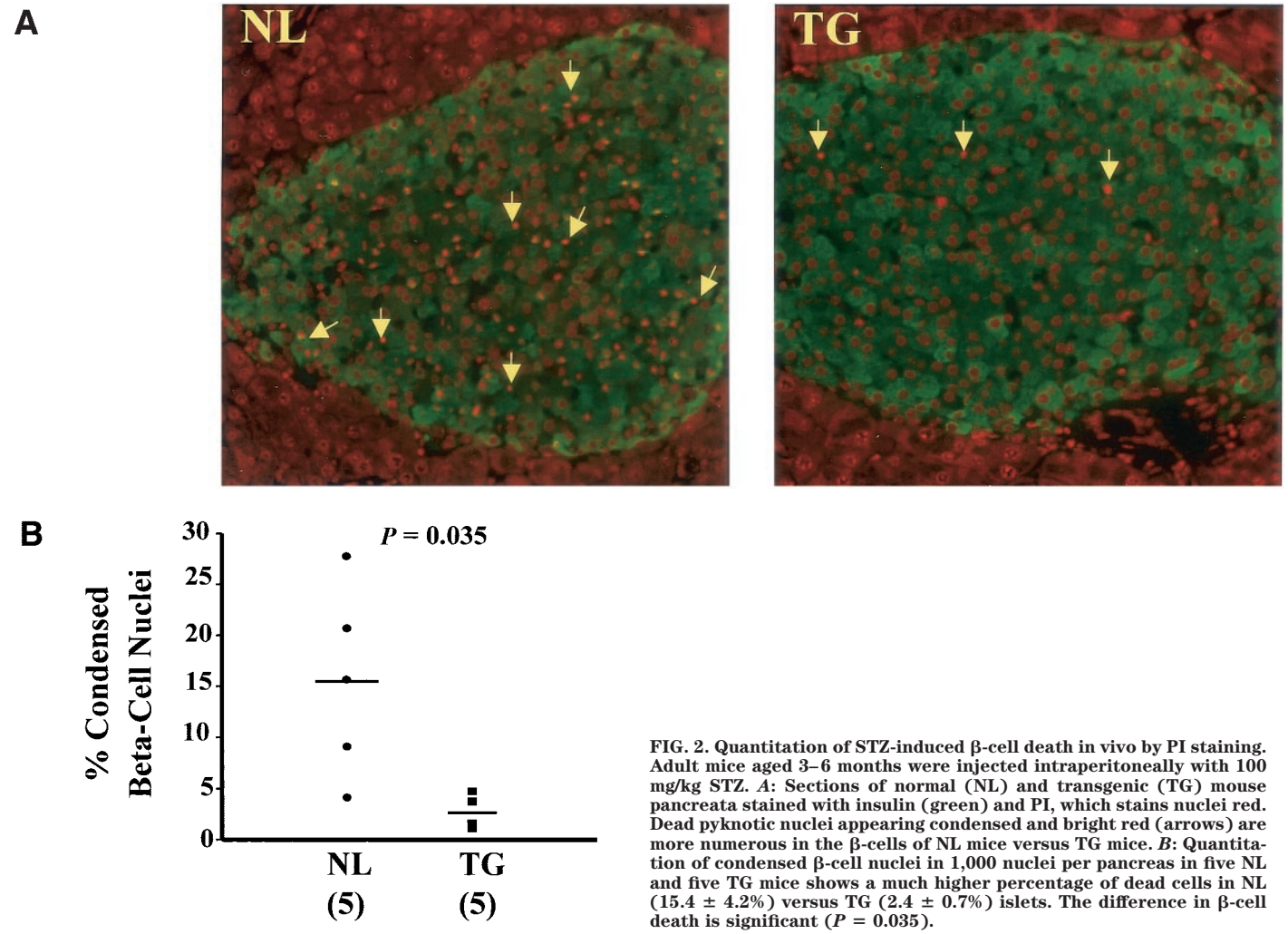

FIG. 2. Quantitation of STZ-induced β-cell death in vivo by PI staining. Adult mice aged 3–6 months were injected intraperitoneally with 100 mg/kg STZ. *A*: Sections of normal (NL) and transgenic (TG) mouse pancreata stained with insulin (green) and PI, which stains nuclei red. Dead pyknotic nuclei appearing condensed and bright red (arrows) are more numerous in the β-cells of NL mice versus TG mice. *B*: Quantitation of condensed β-cell nuclei in 1,000 nuclei per pancreas in five NL and five TG mice shows a much higher percentage of dead cells in NL (15.4 ± 4.2%) versus TG (2.4 ± 0.7%) islets. The difference in β-cell death is significant ($P = 0.035$).

Neonatal RIP-PTHrP mice are resistant to STZ in vivo. There are several possible explanations as to why the RIP-PTHrP mice might be resistant to the cytotoxic and diabetogenic (25) effects of STZ. One explanation for the apparent resistance to STZ in adult RIP-PTHrP mice could be that they have a two- to fourfold increase in overall β-cell mass and β-cell number (8,25). Thus, RIP-PTHrP mice could in theory lose the same number of β-cells as their normal littermates and yet show a decreased percentage of cell death due to their initial advantage of an amplified β-cell mass. A twofold increase in islet mass could also result in sufficient β-cells surviving after STZ treatment to keep RIP-PTHrP mice from becoming hyperglycemic. In this scenario, individual RIP-PTHrP β-cells need not be STZ resistant. Therefore, to directly determine the effect of STZ on normal and RIP-PTHrP mice matched for islet mass and β-cell number, we took advantage of our previous findings (25). We have shown earlier that neonatal RIP-PTHrP mice at 1 week of age have islet mass and plasma glucose values very similar to those of their normal littermates (25). Therefore, litters aged 7–10 days were injected with 150 mg/kg STZ. As shown in Fig. 3*A*, blood glucose levels obtained 24 h after STZ injection were strikingly higher in normal mice (178.8 ± 17.6 mg/dl) compared with transgenic mice (90.5 ± 12.2 mg/dl; $P = 0.0003$), in spite of their islet mass being identical at that age. To directly examine and compare the cytotoxic effects of STZ in islet mass–matched normal and RIP-PTHrP neonatal mice, β-cell death was quantitated by measuring the percentage of condensed nuclei in pancreatic sections costained with insulin and PI. As observed in older animals, neonatal transgenic mice had a significantly ($P = 0.004$) lower rate of β-cell death (3 ± 0.3%) compared with normal siblings (4.3 ± 0.3%) following treatment with STZ (Fig. 3*B*). These studies confirm that individual β-cells of neonatal RIP-PTHrP mice are indeed resistant to the diabetogenic and cytotoxic effects of STZ, in a setting in which islet mass is equivalent to that of normal mice.

RIP-PTHrP mice do not have reduced Glut-2 expression. Another possible explanation for the resistance to STZ in RIP-PTHrP mice could be that they have a reduced ability to transport STZ from the extracellular to the intracellular compartment compared with β-cells from normal mice. It is well established that STZ is transported into β-cells through the Glut-2 transporter (29), which is also responsible for the transport of glucose. Thus, a decrease in the activity of Glut-2 would lead to diminished sensitivity to STZ as well as to glucose. A priori, this is an unlikely explanation for STZ resistance in RIP-PTHrP mice, since we have previously shown by islet perifusion studies that transgenic and normal islets have identical

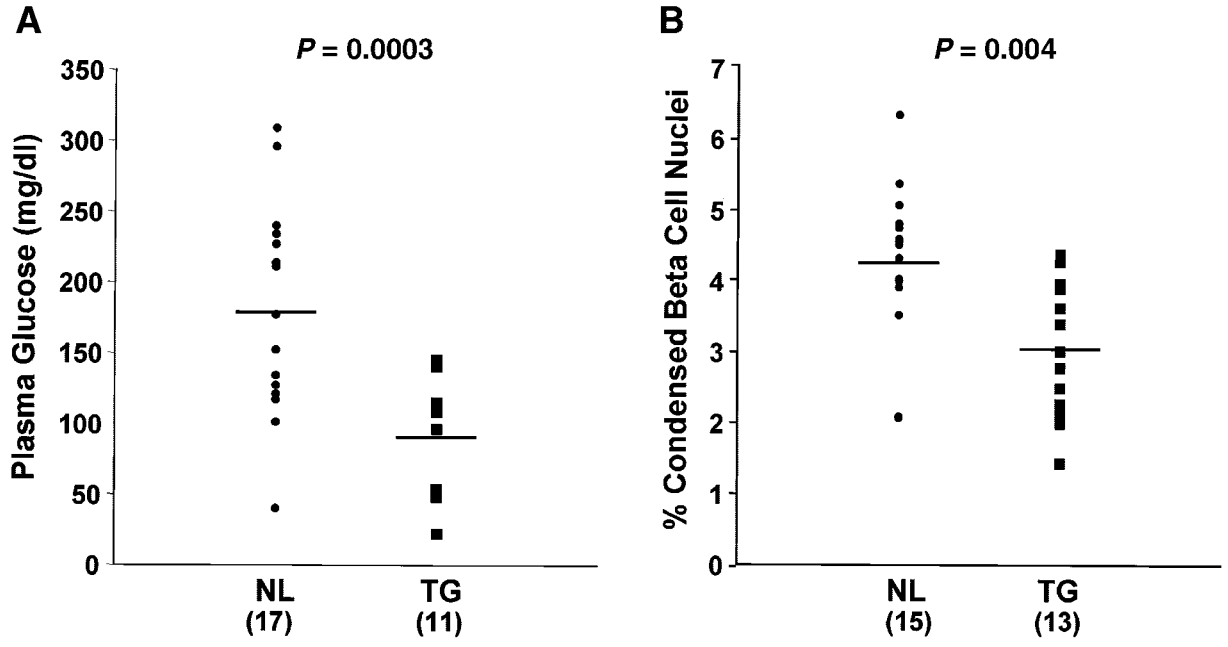

FIG. 3. Blood glucose (*A*) and percentage of β-cell death (*B*) in STZ-treated neonatal mice. Mice aged 7–10 days were injected intraperitoneally with 150 mg/kg STZ. *A*: Mice were killed 24 h later, and blood glucose was measured from 17 normal (NL) and 11 transgenic (TG) mice. The difference in the blood glucose levels of NL mice (178.8 ± 17.6 mg/dl) versus TG mice (90.5 ± 12.2 mg/dl) was highly significant ($P = 0.0003$). *B*: Pancreata removed from mice 6 h after STZ injection were stained for insulin and PI, and the percentage of condensed β-cell nuclei were counted in ~500–1,000 nuclei per pancreas from 15 NL and 13 TG mice. The number of dead β-cells in NL mice (4.3 ± 0.3%) was significantly higher ($P = 0.004$) than in TG mice (3 ± 0.3%).

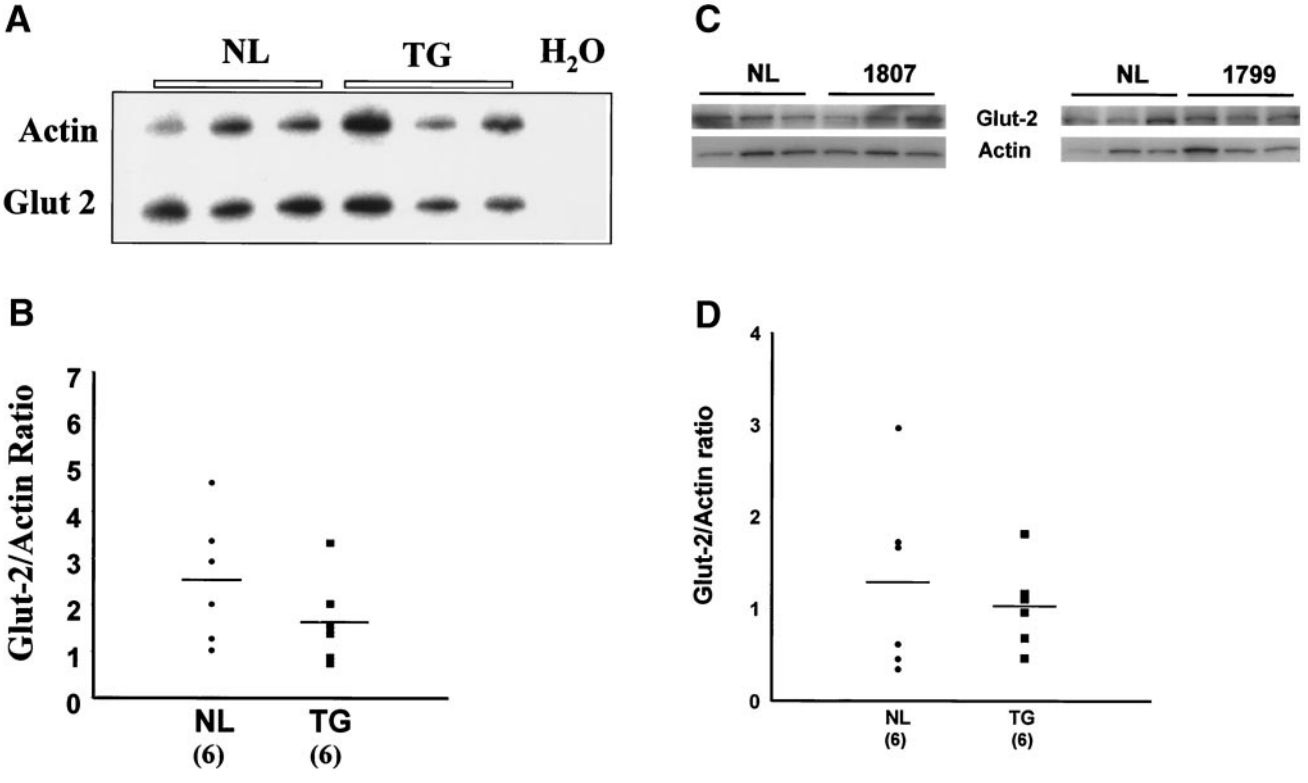

FIG. 4. Quantitation of Glut-2 mRNA and protein from islets of normal (NL) and transgenic (TG) mice. *A*: Semiquantitative RT-PCR on islets from three NL and three TG mice using Glut-2 primers together with actin primers as an internal control. H_2O represents the negative control with no RNA. *B*: Densitometric scanning of RT-PCR of six NL and six TG samples indicates no significant difference ($P = 0.22$) in the ratio of Glut-2 to actin between these mice. *C*: Western blot analysis on islet extracts from six NL and six TG mice (three each from two independent TG mouse lines, 1799 and 1807) using Glut-2 antibody together with actin antibody as an internal control. *D*: Densitometric scanning and quantitation of Glut-2-to-actin ratio in six NL and six TG samples indicates no significant difference in Glut-2 protein between these mice.

insulin secretory responses to glucose in vitro (8), suggesting that the Glut-2 function in these islets is equivalent.

To directly compare levels of Glut-2 expression, Glut-2 mRNA was quantitated in islets of normal and transgenic mouse pancreata using semiquantitative RT-PCR (Fig. 4*A*). As shown in Fig. 4*B*, no significant difference was observed in the level of Glut-2 mRNA when islets from six normal and six transgenic mice were examined by semiquantitative RT-PCR followed by densitometric scanning.

This result was further confirmed at the protein level by immunohistochemical staining and quantitatively by Western blot analysis using a Glut-2 antibody. Immunohistochemistry revealed a similar pattern and intensity of staining in normal and transgenic mouse pancreata (data not shown). Western blot analysis indicated similar levels of Glut-2 protein in islets of normal and transgenic mice (Fig. 4*C* and *D*). The identical responses of normal and transgenic mouse islets to glucose in vitro, as well as similar levels of mRNA and protein, indicate that the resistance to STZ in RIP-PTHrP mice is unlikely to be due to reduced transport of STZ.

β-Cells of RIP-PTHrP transgenic mice are resistant to cell death in vitro. Studies described thus far strongly suggest that overexpression of PTHrP in β-cells sustains their survival when challenged by STZ in vivo. To unequivocally demonstrate the ability of PTHrP overexpression to inhibit β-cell death, we examined the effect on β-cell survival in vitro of two independent inducers of cell death: STZ and nutrient (serum/glucose) deprivation. Serum starvation and glucose deprivation are well known inducers of cell death in β-cells (30). β-Cells from normal and transgenic mice were cultured either under conditions of glucose and serum deprivation or in the presence of STZ in vitro and examined for cell death by co-staining for insulin and PI. Many pyknotic β-cell nuclei were observed in cell cultures of normal islets deprived of serum and glucose (Fig. 5*A*, panel III) compared with occasional pyknotic nuclei seen in cell cultures of transgenic islets (Fig. 5*A*, panel IV). Blinded quantitation of 7–11 islet cell cultures revealed 20 ± 4.9% pyknotic nuclei in β-cells from normal mice compared with only 3.9 ± 1.1% pyknotic nuclei in β-cells from transgenic mice grown in the absence of serum and glucose (Fig. 5*B*). In contrast, the numbers of pyknotic nuclei in normal (1.9 ± 0.6%) and RIP-PTHrP (1.1 ± 0.4%) β-cells were low and indistinguishable when grown in serum- and glucose-replete medium (Fig. 5*B*). Quantitation of pyknotic β-cell nuclei in STZ-treated cultures also showed a significant reduction in the percentage of cell death in cultures of transgenic islets (6.32 ± 0.68%) compared with normal islets (10.91 ± 1.44%), as shown in Fig. 5*C*. These studies clearly indicate that β-cells of RIP-PTHrP mice are resistant to cell death induced by both STZ and serum and glucose deprivation in vitro, and that this protective effect is independent of islet mass.

Exogenously added NH_2-terminal PTHrP inhibits β-cell death. The results thus far clearly demonstrate that PTHrP overexpression in β-cells makes them resistant to cell death. To determine whether the protective effect of PTHrP requires chronic transgenic expression of PTHrP or whether short-term exposure to PTHrP peptides per se may have a protective effect on β-cells, we starved normal islet cell cultures of serum and glucose in the presence or absence of PTHrP peptides. We have previously demonstrated that PTHrP is posttranslationally processed to give rise to several peptides, including an NH_2-terminal peptide (1-36), a midregion peptide (38-94), and a COOH-terminal peptide (107-139) (31). As shown in Fig. 6, islet cultures treated with the NH_2-terminal PTHrP peptide showed a statistically significant 40% reduction in β-cell death compared with control cultures. Neither the midregion nor the COOH-terminal peptides had any significant effect on β-cell death. These results suggest that NH_2-terminal PTHrP may be responsible at least in part for the protective effects displayed by overexpression of the PTHrP transgene (1–141) in the RIP-PTHrP mice.

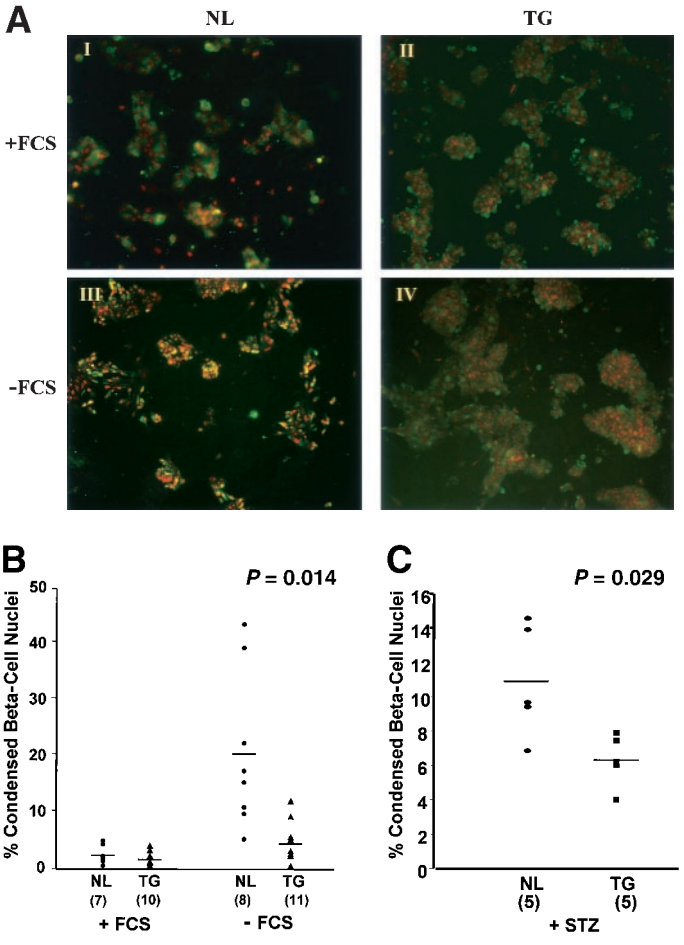

FIG. 5. Quantitation of β-cell death in primary islet cell cultures. *A*: Insulin and PI staining of primary islet cell cultures. Primary islet cell cultures from adult (>6 months old) normal (NL) (I and III) and transgenic (TG) (II and IV) mice were kept overnight in either complete medium (+FCS) (I and II) or medium without serum and glucose (–FCS) (III and IV) and subsequently costained with insulin and PI. Numerous condensed brightly stained nuclei (indicating dead cells) are visible in NL mouse cultures grown without serum (III) versus very few or no dead cells in TG mouse cultures (IV). *B*: Quantitation of β-cell death in 7–11 cultures of NL or TG islet cells grown in complete medium (+FCS) or medium without serum and glucose (–FCS). There are very few condensed β-cell nuclei in either NL or TG cultures grown in complete medium (+FCS). However, there is a substantial increase in β-cell death in cultures from NL mice (20 ± 4.9%) grown in serum and glucose free medium (–FCS) compared with cultures from TG mice (3.9 ± 1.1%), which have significantly reduced β-cell death ($P = 0.014$). *C*: Quantitation of β-cell death in five cultures each of NL or TG islet cells grown in medium with 5 mmol/l STZ for 6 h. There was a significant ($P = 0.029$) reduction in β-cell death in TG versus NL islet cell cultures.

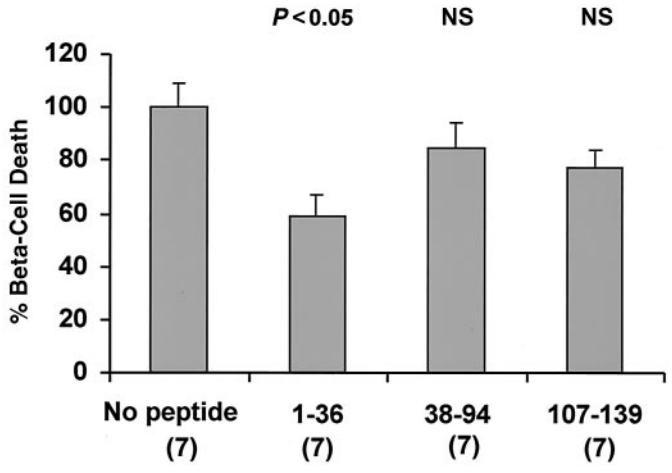

FIG. 6. Effect of PTHrP peptides on β-cell death. Normal islet cell cultures were deprived of serum and glucose for 16–18 h with simultaneous addition of 10^{-6} mol/l of 1-36, 38-94, or 107-139 PTHrP peptides. The control included islet cultures with no peptides added. Percentage of condensed β-cell nuclei after costaining with insulin and PI was quantitated from seven individual cell cultures for each treatment, counting at least 400 to 1,600 cells per culture. Keeping cell death in the control, no peptide added, cultures as 100%, there was a significant reduction in cell death with addition of the NH_2-terminal (1-36) PTHrP peptide, but not the 38-94 or 107-139 peptides.

Islets from RIP-PTHrP mice display increased Bcl-2 mRNA expression but no change in Bcl-2 protein. PTHrP is known to inhibit or delay cell death in many other cell types (32–37), including chondrocytes (32,33) and breast cancer cells (37), where it has been shown to specifically upregulate the expression of the anti-apoptotic gene Bcl-2 (37,38). To examine whether PTHrP might alter expression of members of the Bcl gene family in β-cells, mRNA expression of four major members of the Bcl family was measured by semiquantitative RT-PCR from freshly isolated islets. Steady-state Bcl-2 mRNA was about threefold higher in islets from both lines of RIP-PTHrP transgenic mice compared with normal mice (Fig. 7*A*), and the increase was significant for each RIP-PTHrP mouse line when 5–6 individual samples were quantitated by densitometric scanning (Fig. 7*B*). Importantly, the increase in Bcl-2 mRNA appeared to be specific, since there was no change in the mRNA levels of three other members of the Bcl family, the anti-apoptotic gene Bcl-X_L and the pro-apoptotic members Bad and Bax (Fig. 7*C* and *D*).

Bcl-2 and Bcl-X_L proteins were quantitated by Western blot analysis in islets isolated from normal and transgenic mice. Surprisingly, and in contrast to the Bcl-2 mRNA findings, there was no significant difference in the level of Bcl-2 protein in the islets of normal versus transgenic mice (Fig. 8*A* and *B*). The levels of Bcl-X_L protein were also similar between normal and transgenic mouse islets (Fig. 8*A* and *C*).

DISCUSSION

The results presented in this study clearly indicate that overexpression of PTHrP in pancreatic β-cells protects them from STZ-induced cell death in vivo and in vitro, as well as from cell death induced by nutrient deprivation in vitro. Our previous studies have shown that RIP-PTHrP mice display a progressive, life-long increase in overall islet mass, and an increase in the size and number of individual islets (8,25). This results from an increased number of β-cells, but occurs without a concomitant increase in the rate of β-cell proliferation or individual cell size (25). Taken together, our previous and present results provide compelling, although indirect, evidence that PTHrP overexpression enhances islet mass in transgenic mice through a subtle inhibition or deceleration of the normal rate of β-cell death in vivo.

The purpose of this study was to directly document whether a reduction in the rate of cell death actually occurs in RIP-PTHrP islets. We used the β-cell–specific cytotoxic and diabetogenic agent STZ to induce β-cell death in normal and transgenic littermates in vivo so that we could measure and compare β-cell death in these mice. We observed a sixfold reduction in β-cell death following STZ administration in transgenic mice compared with normal mice. It could be argued that the twofold increase in islet mass in adult RIP-PTHrP mice (8) provides sufficient extra mass to "buffer" STZ-induced β-cell death. To address this issue, we demonstrated that β-cells of neonatal transgenic mice, which have an islet mass equivalent to that of normal mice, are also resistant to the cytotoxic effects of STZ. This confirms that the resistance to STZ-induced cytotoxicity is not purely a result of enhanced islet mass.

Another potential explanation for the higher tolerance of RIP-PTHrP mice to the cytotoxic effects of STZ could be a decrease in the transport of STZ into PTHrP-overexpressing β-cells. Previous glucose-stimulated insulin secretion (GSIS) data (8), as well as similar Glut-2 mRNA and protein levels in islets from transgenic and normal mice in the current study, imply that Glut-2 levels and activity are probably similar in the β-cells of these mice. Collectively, these findings support the idea that PTHrP overexpression directly induces resistance within β-cells to STZ-mediated cell death in vivo.

To unequivocally and directly demonstrate the ability of PTHrP overexpression to inhibit β-cell death, islet cell cultures from normal and transgenic mice were examined in vitro for their ability to survive under conditions of STZ cytotoxicity or nutrient deprivation. The rationale for using a second, different, cell death–inducing stimulus—serum and glucose deprivation—in addition to STZ was twofold. First, if a subtle but undetectable decrease in the sensitivity and transport of STZ into β-cells of RIP-PTHrP mice were the cause of their resistance to STZ-induced cell death, then using nutrient deprivation as a stimulus would bypass that problem. Second, nutrient deprivation induces a more physiologic form of cell death, widely believed to occur through the mitochondrial apoptotic pathway (39). Thus, if β-cells of RIP-PTHrP mice were shown to be resistant to this form of cell death, a more general protective effect of PTHrP overexpression on β-cell survival would be indicated. As hypothesized, β-cells from RIP-PTHrP mice demonstrated strikingly and significantly reduced cell death compared with islet cultures from normal littermates under conditions of both STZ treatment and serum starvation. These studies indicate that overexpression of PTHrP has the ability to inhibit β-cell death mediated via at least two different stimuli, STZ induction in vivo and in vitro and nutrient-deprivation in isolated islet cell cultures in vitro.

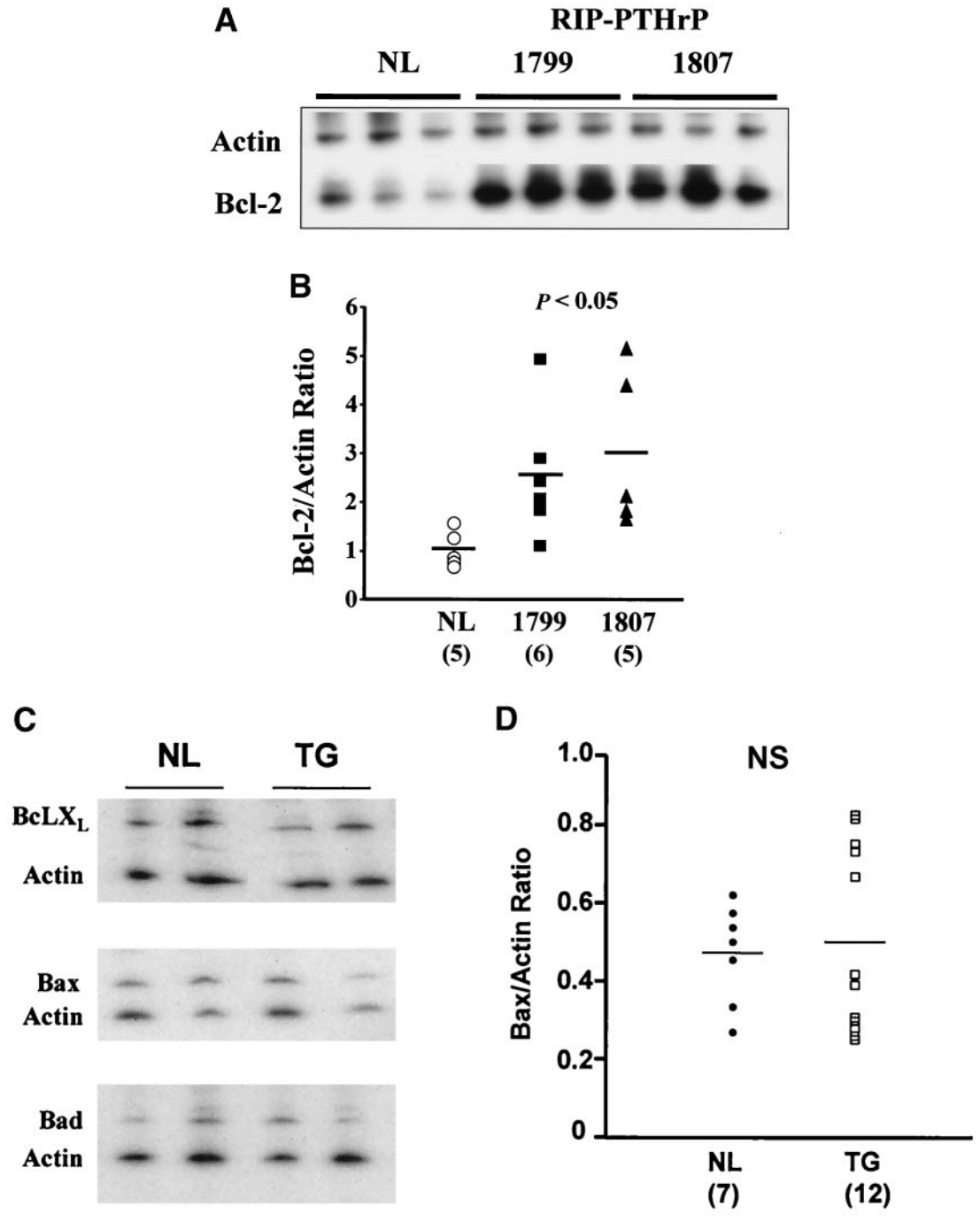

FIG. 7. Quantitation of mRNA of members of the Bcl family. Semiquantitative RT-PCR was used to compare levels of mRNA of members of the Bcl family in islets of normal (NL) and two independent lines (1799, 1807) of RIP-PTHrP transgenic (TG) mice. *A*: RT-PCR from three each of NL, 1799, and 1807 samples using Bcl-2 primers and actin as an internal control. *B*: Quantitation of Bcl-2–to–actin ratios from five to six samples each of the three lines of mice. There was a significant increase in the Bcl-2 mRNA in both lines of TG mice compared with NL. *C*: RT-PCR on two samples each of NL and TG mouse islets using primers for Bcl-X_L, Bax, and Bad in conjunction with actin primers as an internal standard. *D*: Quantitation of Bax-to-actin ratio by densitometric scanning on 7–12 samples of NL and TG mouse islets exhibited no difference in the level of Bax mRNA between the two. A similar quantitation carried out for Bcl-X_L, and Bad indicated no difference in mRNA levels between NL and TG islets (data not shown).

Pancreatic β-cells can now be added to the growing list of cell types protected from cell death induced by a variety of different stimuli through PTHrP overexpression (32–37). Interestingly, it should be noted that the anti–cell death effect of PTHrP is cell-type specific. Whereas PTHrP is protective in some cell types, it has been shown to induce cell death in other cell types (40). The cellular and signaling mechanisms through which PTHrP inhibits death in these various cell types remain unclear. In some cases, nuclear localization of PTHrP, mediated by a nuclear localization signal, is required for anti-apoptotic action (32,36,37), whereas in other cases it appears to act through an autocrine/paracrine mechanism (34,35). Protection of β-cell death by the exogenous addition of the NH_2-terminal

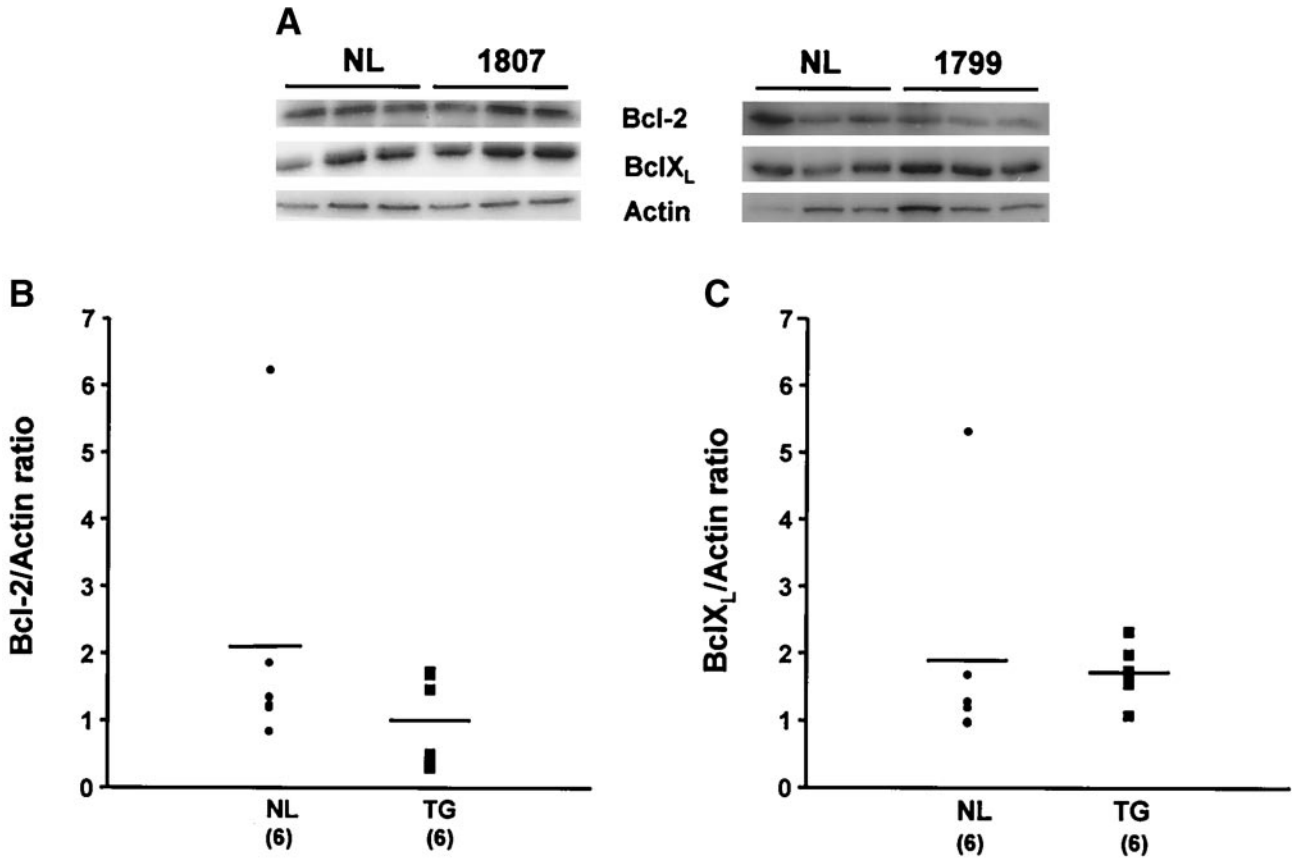

FIG. 8. Quantitation of Bcl-2 and Bcl-X_L protein. *A*: Western analysis of Bcl-2 and Bcl-X_L proteins, with actin as an internal standard, in islets from NL and TG mice (1799 and 1807). *B*: No significant differences are detected in the expression of Bcl-2 or Bcl-X_L between NL and TG mouse islets as observed by the Bcl-to-actin ratios for each after densitometric scanning.

fragment of PTHrP suggests that this region of PTHrP is important in regulating β-cell survival and that it probably acts through an autocrine/paracrine mechanism.

To begin to address the cellular mechanism through which PTHrP might inhibit β-cell death, expression of members of the Bcl family of genes was examined in islets isolated from normal and transgenic mice, initially at the mRNA and subsequently at the protein level. The rationale for considering the Bcl family of genes was as follows. First, this pathway is the major pathway involved in apoptosis mediated by serum deprivation (39). Second, the Bcl gene family is also involved in other pathways of cell death, including necrosis (41), which may be the pathway of cell death mediated by STZ (42). Third, inhibition of apoptosis by PTHrP in breast cancer cells and chondrocytes is thought to be mediated through upregulation of Bcl-2 mRNA and protein (37,38). In the current study, semiquantitative RT-PCR demonstrated an increase in Bcl-2 mRNA in both lines of transgenic mice. This upregulation of Bcl-2 mRNA appeared to be specific, since there was no change in the level of Bcl-X_L, Bad, or Bax mRNA. However, no change was seen in Bcl-2 at the protein level in RIP-PTHrP islets. Although these studies leave the increase in Bcl-2 mRNA unexplained, one must reasonably conclude that upregulation of Bcl-2 protein is not critical for PTHrP-mediated cell survival in β-cells. This is in contrast to the anti-apoptotic action of PTHrP in chondrocytes and breast cancer cells, in which Bcl-2 protein upregulation has been observed (37,38). However, these results are in accord with those of Allison et al. (43), who demonstrated that transgenic mice overexpressing Bcl-2 in pancreatic β-cells are not resistant to high doses of STZ in vivo, unlike the RIP-PTHrP mice. Thus, cellular pathways other than Bcl-2 probably mediate the anti-apoptotic effect of PTHrP overexpression in β-cells.

One such potential mechanism through which PTHrP may inhibit β-cell death is through the regulation of L-type calcium channels. Addition of NH_2-terminal PTHrP peptide has been shown to inhibit cell death in cerebellar granule neurons by downregulating the activity of this channel (34,35). Given the neuroendocrine nature of pancreatic β-cells, the electrophysiological similarities they share with neuronal cells, and the fact that NH_2-terminal PTHrP inhibits cell death in an autocrine/paracrine manner in both neuronal and pancreatic β-cells, regulation of β-cell death and survival through the voltage-sensitive calcium channels is an attractive possibility.

In summary, we have clearly shown that overexpression of PTHrP in β-cells has a protective effect on their survival. Dissecting the signaling pathways and molecular mechanisms through which PTHrP overexpression inhibits β-cell death will likely provide important information from the perspective of both PTHrP biology and understanding β-cell death and survival. Whether the protective effect of PTHrP overexpression on β-cells will prove applicable to islets in the setting of islet transplantation or type 1 diabetes is an interesting question to be addressed in the future. The recognition that PTHrP overexpression en-

hances β-cell survival may provide potential therapeutic targets for pharmaceutical agents aimed at improving the survival of β-cells in diabetes.

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