Activation of the Reg family genes by pancreatic-specific IGF-I gene deficiency and after streptozotocin-induced diabetes in mouse pancreas

Yarong Lu, André Ponton, Hiroshi Okamoto, Shin Takasawa, Pedro L. Herrera, and Jun-Li Liu

Fraser Laboratories for Diabetes Research, Department of Medicine, and 1McGill University and Genome Québec Innovation Centre, McGill University, Montreal, Quebec, Canada; 2Department of Advanced Biological Sciences for Regeneration and 3Department of Biochemistry, Tohoku University Graduate School of Medicine, Sendai Miyagi, Japan; and 4Department of Genetic Medicine and Development, University of Geneva Medical School, Geneva, Switzerland

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Early embryos result in a 50% decrease in the size of the exocrine pancreas without affecting the development of endocrine cells (19). Combined ablation of IGF-I and IGF-II genes results in an identical phenotype (19). Moreover, islet β-cell-specific inactivation of IGF-IR gene causes no change in β-cell mass despite hyperinsulinemia, glucose intolerance, and a decrease in glucose-stimulated insulin release, suggesting that IGF signaling is not essential for normal growth and development of the pancreatic islets (20, 52). Our own research of liver-specific IGF-I gene deficiency (in liver-specific IGF-I gene-deficient mice) suggests that IGF-I exerts an inhibitory effect on islet cell growth, albeit indirectly through controlling the release of growth hormone from the pituitary (53, 55).

More surprisingly, inactivation of pancreatic expression of IGF-I gene [in pancreatic-specific IGF-I gene-deficient (PID) mice] increases islet β-cell mass and renders mice resistant to the β-cell damage that occurred in both T1D and T2D models (26). These effects are unlikely to be caused directly by the deficiency of IGF-I, an undoubted growth factor. In an effort to reveal potential mediators of islet cell hypertrophy and/or hyperplasia in PID mice, we performed a DNA microarray analysis of pancreatic RNA prepared from control mice, PID mice, and both after streptozotocin-induced β-cell damage. As a result, multiple members of the Reg family genes were upregulated and seemed to promote islet cell growth, proliferation, and/or regeneration. Reg family proteins have been known for years; e.g., Reg1 [pancreatic thread protein (PTP), pancreatic stone protein (PSP)] stimulates islet cell proliferation/regeneration (45, 47); islet neogenesis-associated protein (INGAP; INGAP-related peptide (rp) Reg3β) promotes β-cell neogenesis from pancreatic ductal cells (35, 38). Consequently, five new members of the family (Reg2, -3α, -3β, -3γ, and -4) have been discovered (1, 22, 27, 56); three of them (Reg2, -3α, and -3β) exhibit elevated expression in our PID mice. Increased Reg2 and Reg3β levels have been reported in human and rodent T1D (4, 13). Transgenic Reg3β stimulates hypoglycemic growth (41). Although not yet directly known for promoting islet β-cell growth and regeneration, the mRNA levels of these previously uncharacterized Reg proteins exhibit significant increases under the two conditions of islet overgrowth and regeneration, much more than Reg1 and INGAP, the two known growth factors. In this report, we present subtype-specific quantification of mRNAs and some evidence on the localization of the Reg proteins.

Yarong Lu, André Ponton, Hiroshi Okamoto, Shin Takasawa, Pedro L. Herrera, and Jun-Li Liu. Activation of the Reg family genes by pancreatic-specific IGF-I gene deficiency and after streptozotocin-induced diabetes in mouse pancreas. Am J Physiol Endocrinol Metab 291: E50–E58, 2006. First published January 31, 2006; doi:10.1152/ajpendo.00596.2005.—We have recently reported that Pdx1-Cre-mediated whole pancreas inactivation of IGF-I gene [in pancreatic-specific IGF-I gene-deficient (PID) mice] increases islet β-cell mass and significant protection against both type 1 and type 2 diabetes. Because the phenotype is unlikely a direct consequence of IGF-I deficiency, the present study was designed to explore possible activation of proislet factors in PID mice by using a whole genome DNA microarray. As a result, multiple members of the Reg family genes (Reg2, -3α, and -3β, previously not known to promote islet cell growth) were significantly upregulated in the pancreas. This finding was subsequently confirmed by Northern blot and/or real-time PCR, which exhibited 2- to 8-fold increases in the levels of these mRNAs. Interestingly, these Reg family genes were also activated after streptozotocin-induced β-cell damage and diabetes (wild-type T1D mice) when islet cells were undergoing regeneration. Immunohistochemistry revealed increased Reg proteins in exocrine as well as endocrine pancreas and suggested their potential role in β-cell neogenesis in PID or T1D mice. Previously, other Reg proteins (Reg1 and islet neogenesis-associated protein) have been shown to promote islet cell replication and neogenesis. These uncharacterized Reg proteins may play a similar but more potent role, not only in normal islet cell growth in PID mice, but also in islet cell regeneration after T1D.
RESEARCH DESIGN AND METHODS


Animal procedures. On a hybrid C57BL/6 × DBA background, mice homozygous for IGF-I/Ins2 carrying the Pdx1-Cre transgene (L/L+; PID mice) were crossed with L/L− mice (lacking the transgene; control mice), and the resulting offspring, females only, were used in experiments. This was based on our previous report that, although both male and female PID mice showed similar extents of islet enlargement, female PID mice exhibited more complete resistance to streptozocin-induced diabetes. The protection in male mice was only partial (26). Genotypes were determined using a double-PCR strategy, as described before (26). The animals were maintained in 12:12-h dark-light cycles at room temperature with free access to food and water. At the desired age, the mice were anesthetized with a cocktail of ketamine-xylazine-acepromazine and killed by cervical dislocation. For streptozocin-induced islet cell damage and diabetes, PID mice and control littersmates, 3 mo old, were injected daily for 5 days with streptozotocin (80 mg/kg ip; Calbiochem), prepared fresh as a 0.8% solution in 0.1 M sodium citrate, pH 4.5 (26). This dose of streptozotocin causes a significant loss of β-cells and 90% reduction in serum insulin levels. When the control mice became diabetic by 15 days, they were killed for the purpose of performing pancreatic RNA analysis and histology. All animal handling procedures were approved by the McGill University Animal Care Committee.

RNA isolation and Northern blots. Total RNA was isolated by acid guanidinium isothiocyanate-phenol-chloroform extraction (5). To minimize RNA degradation, control, PID, control-T1D, and PID-T1D mice were anesthetized, and their pancreases were dissected and homogenized immediately. After centrifugation, the aqueous phase was mixed with 1 vol of isopropanol and passed through Ribopure glass fiber filter cartridges (Ambion no. 1924) for purification. The RNA quality was confirmed using Agilent’s Bioanalyzer. For Northern blot analysis, 10 μg of total RNA was subjected to electrophoresis and transferred to positively charged nylon membranes (Roche Applied Science, Penzberg, Germany). Antisense RNA probes were transcribed from mouse Reg1, Reg2, Reg3x, and Reg3β cDNAs (27, 48) and from the pTRI-β-actin-mouse vector (Ambion, Austin, TX), by use of a digoxigenin RNA labeling mix (Roche). The membranes were hybridized to probes overnight at 68°C in DIG Easy Hybridization solution (Roche) and washed according to the manufacturer’s instructions. To detect the chemiluminescence signals, the membranes were incubated with alkaline phosphatase-conjugated antibody against digoxigenin (Roche) for 30 min. The substrate CSPD (Roche) was applied for 10 min before the membranes were exposed to BioMax light films (Kodak) for 10 min. The intensity of the hybridization signals on the autoradiographs was quantified using a FluorChem 8900 imaging system (Alpha Innotech).

Oligonucleotide microarray analysis. Two samples of pancreatic RNA from each of the four groups of mice (control, PID, control-T1D, and PID-T1D) were used for microarray analysis, each pooled from three individual animals to minimize variability. From each sample, 5 μg of total RNA were used for the microarray experiment. Affymetrix GeneChip one-cycle target labeling and control reagents kit was used according to the GeneChip Analysis Technical Manual, Rev. 5 (Affymetrix, 2004). The target cRNAs derived from each sample were hybridized to the Affymetrix GeneChip Mouse Genome 430 2.0 array. The chips were stained and washed using the GeneChip Fluidics Station 450 and visualized on an Affymetrix GeneChip Scanner 3000. The image files were processed using the GeneChip Operating Software, which calculates “signal” values and provides “detection” calls that are “present,” “marginal,” or “absent” for each probe set. Only genes with a detection call present in both control and PID duplicates were subsequently used for comparison. The result was processed using the Robust Multichip Average system, which involves a probe-specific background correction, a probe-level multichip quantile normalization, and a robust probe set summary of the log-normalized probe-level data (16).

Real-time and RT-PCR. One microgram of pancreatic RNA from each mouse was transcribed into first-strand cDNA using M-MLV Reverse Transcriptase and oligo(dt)12–18 primers (Invitrogen). Real-time PCR was performed using ABI 7500 System (Applied Biosystems) with ABI certified Reg3β [pancreatitis-associated protein (PAP)], Reg2, β-actin TaqMan primers and Universal PCR Master Mix, following the manufacturer’s instructions. Five individual samples were amplified from each group in duplicates. The results of each sample were normalized using β-actin. Fold change was determined on the basis of comparative cycle threshold values for all duplicates and converted into signal log ratio. RT-PCR was performed using Reg3x (forward sequence: GTT ACC TTT GTC CTT ACA AAC; reverse sequence: TAT CTC CAC TCC CAT CCA CC; length: 420; melting temperature (Tm): 58°C) (26) and GAPDH (forward sequence: TCA ACA GGA CCC TTC ATA GC; reverse sequence: AAT CTC CCT ACT TCT GAT AAC C; length: 474; Tm: 56°C) primers (20). Expression levels were corrected by β-actin (forward sequence: AAA GAC TAC CTC ATG AAG ATC C; reverse sequence: TTA CTC CAC TCC CAT CCA CC; length: 420; Tm: 58°C) expression. The protection in male mice was only partial (26).

Immunohistochemistry. The pancreata from 3.5-mo-old control, PID, control-T1D, and PID-T1D mice were fixed, embedded in paraffin, and cut into 5-μm sections. The use of diaminobenzidine substrate, a mouse monoclonal antibody against recombinant rat Reg1 protein, of 144 amino acids without the signal sequence (Dr. Okamoto) (44) resulted in a brown immunoreactive signal with a hematoxylin counterstain (blue) of the cell nuclei. To ensure specific staining to Reg proteins, control slides were stained without the primary antibody, which revealed no specific signals. Because of high homologies within the Reg family proteins, especially a 76% sequence identity between Reg1 and Reg2, this antibody would likely cross-react with other Reg proteins. Because it has not been fully tested for subtype specificity, we have termed its staining as Reg1 immunoactivity (Reg1-IR) in this report. Images of all pancreatic tissues were captured with a Retiga 1300 digital camera (Q Imaging, Burnaby, BC, Canada) at magnifications of ×400 (oil) using Northern Eclipse software version 6.0 (Empix Imaging, Mississauga, ON, Canada).

Triple-labeled immunofluorescence for Reg, insulin, and amylase. To further determine which type of pancreatic cells produce Reg proteins under various conditions, we have performed a colocalization study using immunofluorescence. Paraffin sections of pancreatic tissues from four groups of mice were dewaxed, rehydrated, and blocked with 5% normal donkey serum (NDS; Jackson Immuno Research, West Grove, PA). They were first incubated with rabbit anti-amylase (1:100 in 1% NDS; Sigma) at 4°C overnight followed by rhodamine red-X-conjugated donkey anti-rabbit IgG (1:100; Jackson) for 2 h at room temperature. After the signal was confirmed under a microscope, the sections were blocked again with NDS and incubated with guinea pig anti-insulin (1:100 in 1% NDS; Biomedics) at 4°C overnight followed by incubation with 7-amino-4-methylcoumarin-3-acetic acid (AMCA)-conjugated donkey anti-guinea pig IgG for 2 h at room temperature. After signal confirmation, the sections were again blocked with NDS and incubated with mouse anti-Reg1 (1:500 in 1% NDS; H. Okamoto) at 4°C overnight followed by FITC-conjugated donkey anti-mouse IgG for 2 h at room temperature (26). Sections were washed with PBS after each step, mounted, and stored at 4°C at the end. The images were analyzed using an Axioskop 2 plus microscope (Carl Zeiss), a Retiga 1300 digital camera, and Northern Eclipse software (Empix Imaging).

Statistical analysis. Data are expressed as means ± SE. Student’s t-test and one-way analysis of variance followed by posttest comparisons were performed using InStat software version 3 (GraphPad Software, San Diego, CA).
RESULTS

Increased expression of Reg family genes in the pancreas of PID mice: DNA microarray analysis. We performed a DNA microarray analysis of pancreatic RNA prepared from control mice, PID mice, and both after streptozotocin-induced β-cell damage. Of the 39,000 transcripts included in the mouse MOE430-2 gene chip, the expression of about 8,000 were detectable. Among the four groups of mice, e.g., control, PID, control after being made T1D, and PID after being made T1D, 430 transcripts exhibited specific alterations greater than 1.5-fold, as illustrated in Fig. 1. The results are partially highlighted in Table 1, with genes grouped into three clusters: Reg family, islet and endocrine, and immunity-related genes. Compared with control littermates, PID mice exhibited altered levels of expression in 72 transcripts, highlighted in column 1 in Fig. 1. Among them, the mean levels of Reg family genes Reg2, Reg3α, and Reg3β (PAP) were increased 2.55-, 2.03-, and 3.02-fold, respectively. The expression levels of the other 3 Reg family genes, Reg3γ, INGAP-rp (Reg3δ), and Reg4 were not altered. Reg1 was not included in the probe collection. As a confirmation of gene knockout and test of validity, the IGF-I gene expression was decreased. Additionally, the expression of many genes related to immune responses, such as immunoglobulin joining chain and heavy-chain 6, was inhibited in PID mice.

Increased expression of Reg family genes in the pancreas of PID mice: Northern blots, real-time PCR, and RT-PCR. Generated from small sample numbers, the microarray results need to be reconfirmed with large samples and a more accurate quantification. We performed Northern blots, real-time PCR, and/or conventional RT-PCR to different molecular targets, shown in Fig. 2. With the use of Northern blots and digoxigenin-labeled riboprobes transcribed from full-length cDNA templates, Reg1 mRNA level was slightly elevated in PID mice vs. control littersmates. Those of Reg2, -3α, and -3β were increased significantly, 2.5-, 2.0-, and 2.7-fold, respectively. Similar results in Reg2 and Reg3β were obtained using real-time PCR and in Reg3α using RT-PCR. Lastly, INGAP-rp (Reg3δ) mRNA level was unaffected in PID mice, determined using RT-PCR alone. Thus, revealed by microarray analysis and confirmed using Northern and other techniques, the mRNA levels of Reg2, -3α, and -3β in the pancreas were more than doubled in PID mice. These changes seemed to be age dependent, because they were not seen in younger mice aged 1 mo (data not shown). Changes in Reg1 and INGAP-rp (Reg3δ) were insignificant. Two other Reg genes, Reg3γ and Reg4, were not tested.

Increased expression of Reg family genes in control mice after streptozotocin-induced diabetes: DNA microarray analysis. The level of Reg1 is increased during islet cell regeneration after pancreatectomy or streptozotocin-induced T1D (3, 45). As part of the DNA microarray analysis, we have compared control untreated mice and 15 days after streptozotocin-induced diabetes (when the β-cells were mostly destroyed and islet regeneration was activated). The status of β-cell damage and hyperglycemia has been described in our previous report (26). Among the detectable transcripts, the expression of 264 transcripts in T1D group exhibited alterations greater than 1.5-fold vs. untreated control mice, as shown in Fig. 1. Among them, the levels of Reg family genes Reg2, -3α, -3β, and -3γ were increased 5.94-, 4.35-, 12.12-, and 3.21-fold, respectively. Similarly, INGAP-rp (Reg3δ) level was only slightly elevated and Reg4 level was unaffected, whereas Reg1 probe was not included. As a confirmation and test of validity, the expression of β-cell-specific genes such as insulin I and II and islet amyloid polypeptide was ablated together with β-cells. Changes in immune-related genes were more limited and the directions reversed than in PID mice (result 1), including 2.36- and 2.31-fold increases, respectively, in the transcripts of chemoattractant 13 and interferon-γ-induced GTPase.

Increased expression of Reg family genes in control mice after streptozotocin-induced diabetes: Northern blots, real-time PCR, and RT-PCR. The microarray result was reconfirmed using various techniques available, as shown in Fig. 2. With the use of Northern blot hybridization, the level of pancreatic Reg1 mRNA (not included in the microarray chip) was increased 1.5-fold. Those of Reg2, -3α, and -3β were increased more significantly, 3.4-, 2.7-, and 3.6-fold, respectively. Similar results were obtained using real-time PCR for Reg2 and -3β and conventional RT-PCR for Reg3α. Lastly, INGAP-rp (Reg3δ) mRNA level was increased only 1.5-fold.
TID mice with the use of RT-PCR. Thus, in the pancreas of control mice after being made diabetic, there were 2- to 8-fold significant elevations in the levels of Reg2, -3α, and -3β mRNAs. Changes in Reg1 and INPAP-rp (Reg3β) were significant but less dramatic. Two other Reg genes, Reg3γ and Reg4, were not tested.

Levels of Reg family mRNAs were not increased further in PID mice after being made diabetic. As described above and illustrated in Fig. 2, the levels of Reg2, -3α, and -3β mRNAs were significantly elevated in PID mice (second group) vs. control littermates (first group) and were increased even further in control mice after being made diabetic (third group). To explore a compounded effect of PID and T1D, in Northern blot analysis we had included a fourth group, e.g., PID mice after being made diabetic (PID-T1D). As we have reported previously (26), PID mice only exhibit marginal hyperglycemia and islet damage after streptozotocin treatment. The levels of Reg family mRNAs in PID-T1D group exhibited levels of increases similar to the PID alone (second group) but less than the T1D (third group). Consistent with the fact that PID mice are protected from streptozotocin and have only a very mild form of diabetes, the PID-TID mice did not exhibit uniformed increases in Reg family gene expression after streptozotocin treatment in contrast to control T1D mice (in Fig. 1, column 3, and Table 1). Another comparison we have made is in Table 1, data column 4: PID-TID vs. control T1D mice. Fifteen days after an identical treatment with streptozotocin, PID-TID mice exhibited slightly smaller increases in the expression of Reg family genes but preserved (increased) expression of β-cell specific genes, again consistent with the mild diabetes.

Cell-specific alterations in Reg immunoreactivities. The results described above represent specific changes in mRNA level only, and the RNA was isolated from the whole pancreas. To reveal changes at the protein level, especially with cell-specific resolution, we have performed immunohistochemistry using a specific antibody against Reg proteins (anti-Reg1) provided by Dr. H. Okamoto. As shown in Fig. 3, top left, in normal control mice Reg1-1R was detectable at scattered exocrine cells (brown pigmentation indicated by blue arrows) as reported, but not within the endocrine islets (black arrows) (27, 56). In PID mice (Fig. 3, top right) brown Reg1-IR was clearly visible in some islets in addition to exocrine cells. In control T1D mice (Fig. 3, bottom left) with significant atrophy after the onset of diabetes, many islet cells were positively stained for Reg1-IR. A drastic increase in the staining was also observed in patches of exocrine cells, some being adjacent to the islets.

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Table 1. Changes in gene expression profiles in PID, TID, and PID-TID mice revealed by oligonucleotide microarray analysis

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Gene Title</th>
<th>Gene Symbol</th>
<th>PID vs. Control</th>
<th>TID vs. Control</th>
<th>PID-TID vs. PID</th>
<th>PID-TID vs. TID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1448290_at</td>
<td>Pancreatitis-associated protein (Reg3β)</td>
<td>Pap</td>
<td>3.02</td>
<td>12.12</td>
<td>1.83</td>
<td>-2.19</td>
</tr>
<tr>
<td>1416139_at</td>
<td>Regenerating islet-derived 2</td>
<td>Reg2</td>
<td>2.55</td>
<td>5.94</td>
<td>-1.53</td>
<td>-3.56</td>
</tr>
<tr>
<td>1449495_at</td>
<td>Regenerating islet-derived 3 alpha</td>
<td>Reg3α</td>
<td>2.03</td>
<td>4.35</td>
<td>-1.25</td>
<td>-2.67</td>
</tr>
<tr>
<td>1448872_at</td>
<td>Regenerating islet-derived 3 gamma</td>
<td>Reg3γ</td>
<td>-1.01</td>
<td>3.21</td>
<td>1.56</td>
<td>-2.08</td>
</tr>
<tr>
<td>1424009_at</td>
<td>Islet neogenesis associated protein-related protein (Reg3β)</td>
<td>Ingap-rp</td>
<td>1.42</td>
<td>1.30</td>
<td>-1.24</td>
<td>-1.13</td>
</tr>
<tr>
<td>1418931_at</td>
<td>Regenerating islet-derived 4</td>
<td>Reg4</td>
<td>1.10</td>
<td>1.04</td>
<td>1.04</td>
<td>1.02</td>
</tr>
</tbody>
</table>

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PID, pancreatic-specific IGF-I gene deficient; T1D, 15 days after streptozotocin-induced diabetes. Control and PID mice were 3-mo-old female littermates. Of the 8,000 detectable transcripts, results of 3 gene clusters were illustrated and expressed as fold increases (or decreases when negative). The Robust Multichip Average method was used for comparison between groups. Values were fold changes vs. control mice taken from the mean of 2 RNA samples. The designations of Reg3β and Reg3γ marked in parentheses are based on Refs. 1, 27, and 40.
In the fourth group of PID-T1D mice (Fig. 3, bottom right), Reg1-IR in exocrine cells was obviously higher than in control and PID mice (Fig. 3, top left and top right), although less than in control-T1D mice (Fig. 3, bottom left). Their islets were mostly protected from streptozotocin-induced damage and exhibited low levels of Reg1-IR (Fig. 3, bottom right). Presently, the specificity of available antibodies against various subtypes of Reg proteins has been unsatisfactory for this purpose (data not shown).

![Increased Reg1 immunoreactivity (Reg1-IR) in mouse pancreas of PID and control mice after streptozotocin-induced type 1 diabetes (T1D, 15 days after initial injection). Control or PID mice were 3-mo-old female littermates.](image-url)
Evidence for islet neogenesis and regeneration involving Reg proteins. Increased β-cell mass in PID mice indicates islet hypertrophy, hyperplasia, and/or increased islet neogenesis (26). In control T1D mice, islet neogenesis is expected for the mice to recover from diabetes (3). In adult rodents, pancreatic acinar or ductal cells are putative progenitors of new islets (3, 38). To provide evidence that Reg family proteins are involved in islet neogenesis in both scenarios, we performed a triple-labeled immunofluorescence on pancreatic sections. As shown in Fig. 4, as positive control for the three-color scheme in control pancreas (top), an insulin-stained islet (blue) and the surrounding amylase-labeled acinar cells (red) were clearly segregated. Reg1-IR (green) normally produced in exocrine cells was invisible in this field. In PID mice (Fig. 4, middle), we have detected several clusters of cells that were positively labeled by all three antibodies. It seems that they were newly formed islets, from amylase-producing acinar cells (red), induced by Reg proteins (green) that started to synthesize insulin (blue), albeit at a low level. This phenomenon was not observed in control mice. The “new” islets resemble those in hamsters and mice in response to INGAP treatment (see Figs. 1B and 7A in Ref. 38) and exocrine cell transdifferentiation in rats (21). In T1D mice (Fig. 4, bottom), we detected scattered “new β-cells” that were presumably formed from Reg1-IR-producing acinar cells (white arrows) and serve as β-cell precursors. Although still dispersed as single cells among exocrine tissues, these cells produced noticeable amounts of Reg1-IR and insulin concurrently, whereas the mice were undergoing acute diabetes 15 days after streptozotocin. The only other member of the Reg family that is known to stimulate β-cell neogenesis is INGAP (38). On the basis of previous reports, our result provides further evidence that the expression of the Reg genes is associated with islet neogenesis (3, 38), which may potentially contribute to increased β-cell mass in PID mice.

DISCUSSION

This study was designed in order to understand the unexpected phenotype of PID mice; i.e., as an undisputed growth factor, IGF-I gene deficiency causes increased islet cell mass rather than growth defect. The islet cells are further protected from damage caused in both T1D and T2D models (26). These beneficial outcomes are likely caused by indirect activation of proislet factors rather than a direct IGF-I deficiency. Indeed, using a high throughput microarray analysis, we have hereby rediscovered Reg family proteins: 1) The mRNA levels of Reg2, -3α, and -3β in the pancreas were significantly increased in PID mice, which were associated with islet enlargement and protection against damage; 2) those mRNA levels were again increased in control mice after being made diabetic, when islet cells were expected to undergo active regeneration, and minor elevations were also detected on Reg1 and INGAP-rp (Reg3β) mRNA levels in diabetic pancreas; and 3) interestingly, the
most significant changes occurred in new members of the Reg family that are not yet known to play a significant role in islet cells, rather than Reg1 and INGAP, which stimulate islet cell replication and/or regeneration. Our results suggest that these uncharacterized Reg proteins are involved in promoting islet cell growth, protection, and/or regeneration.

Reg family proteins have been known for years. In rats, a 90% pancreatectomy causes a drastic increase in islet β-cell regeneration, which was thought to be driven by novel growth factors (54). In 1988, Terazono et al. (45) isolated a novel cDNA from these regenerating islets and named it Reg, or regeneration-associated gene; it is now called Reg1 (PTP, PSP). Ever since then, Reg-related genes have been shown to constitute a multigene family in humans and rodents. Independently, by using a partial pancreatic duct obstruction (cellulo-phant wrapping), Rosenberg et al. (37) have shown islet neogenesis from ductal epithelium, which functioned to reverse a diabetic state. A soluble tissue fraction, ilotropin, was later proven effective, too (39). A major constituent of the extract was characterized, its cDNA was cloned, and it was named islet neogenesis-associated protein (INGAP) (35). With homology with Reg3 proteins, INGAP is expressed in acinar but not in islet cells of mice and hamsters (35). On the basis of its genomic organization and protein structure, the hamster INGAP has the greatest homology to mouse INGAP-rp (also named Reg36; 72% amino acid identity) (43). Four other members of the Reg family (Reg3α, -3β, -3γ, and -4) have been discovered since then. All 7 subtypes of Reg proteins are encoded by independent genes, but in a highly similar configuration (1, 18, 27, 56).

Reg1 is involved in cell cycle progression and normal islet growth and could potentially be used to prevent T1D (31, 32). Disruption of Reg1 gene through homologous recombination causes significant reductions in islet cell mass and the rate of DNA synthesis in isolated islet cells (47). Conversely, islet-specific Reg1 transgenic mice exhibit increased DNA synthesis in islet cells. When crossed onto a nonobese diabetic (NOD) background, Reg1 transgene causes a significant delay in the development of the diabetes (47). In another study (12), injection of Reg1 into NOD mice also postpones the onset of diabetes. In isolated rat islets, Reg1 significantly increased [³H]thymidine incorporation into the cell nuclei (50). As for its intracellular mechanism, Reg1 has been reported to cause phosphorylation of ATF-2 via a PI3K-dependent pathway (42). As a specific transcription factor, ATF-2 activates cyclin D1 promoter and increases cyclin D1 protein level in islet cells. As a Ser/Thr kinase, cyclin D1 promotes cell cycle progression by inactivating retinoblastoma protein (51). To support its role in neogenesis, Reg1 has been found in acinoductular cells that are also positive for cytokeratin 19, trypsin, and chromogranin A and supposedly a source of islet neogenesis (46). Most recently, Reg1 and Reg2 proteins are indicated in the early response of the pancreas to obesity-induced T2D in mice. At 2 wk after a high-fat diet, well before onset of obesity and insulin resistance, there are two- to threefold increases in Reg1 and Reg2 protein levels and a 15-fold elevation in Reg2 mRNA levels (34). Hamster INGAP is mitogenic for pancreatic duct but not β-cell-derived cells. Because ductal cell proliferation seems to be a prerequisite for islet neogenesis, it is postulated that this protein could be involved in islet neogenesis (35). Indeed, a pentadecapeptide fragment of INGAP increases β-cell mass and reverses diabetes in mice (38). Although a receptor is still elusive, INGAP-induced transformation from duct cells to islets is dependent on PI3K-mediated activations of Akt1 and ERK1/2, which in turn cause activation of islet-specific transcription factors including neurogenin-3 and Pdx1 (17). Thus Reg1 and INGAP are known growth factors to the islet cells.

Although Reg family genes were activated under two different conditions in this study, the implications in PID mice and control mice after being made diabetic are quite different. 1) PID mice have 2.3-fold increased β-cell mass, and the islets are resistant to both T1D and T2D. Increased islet density and islet cell size indicate increased neogenesis and hypertrophy in PID mice (26). Concurrent increases in Reg2 and Reg3β levels strongly suggest that Reg proteins are involved. Increased islet neogenesis was strongly suggested by our triple-labeled experiment (Fig. 4, middle row), where insulin-producing clusters (new islets) were captured producing Reg1-IR proteins and amylyase as well. This type of neogenesis, i.e., transdifferentiation from exocrine to endocrine, has been proposed recently (21). Although it might be one possibility, our evidence does not necessarily contradict to the notion that acinar cells undergo an intermediate, ductal-like dedifferentiation before becoming new β-cells (49). 2) In control mice, 15 days after streptozotocin treatment, most islet β-cells have been destroyed (26). To restore β-cell mass and recover from T1D, β-cells would be increased through active neogenesis, which is much more prominent than replication. Even greater increases in the levels of Reg2 and Reg3β, under this condition and in both islets and exocrine cells, again suggest that Reg proteins are involved in islet neogenesis. Except for Reg1, similar change in T1D has never been reported for other Reg proteins (30). Furthermore, this observation is consistent with two recent reports of T1D models. Reg3β (PAP) is overexpressed in islets of human T1D (13); the level of mouse Reg2 gene expression (but not of Reg1 and Reg3β) is significantly increased in the pancreas of diabetes-prone NOD and in cyclophosphamide (causing T1D)-treated mice (4). It is important to note that, although Reg2 is closely related to Reg1 and Reg3β resembles INGAP (Reg36), they are definitely distinct proteins coded by independent genes and potentially more potent islet growth factors. Reg2 has a unique 7-aa insertion (QVAEDFD) near its NH₂ terminus, although an overall 76% sequence identity to Reg1 (48), and has only 40% identity to INGAP or the 15-aa hamster INGAP peptide (30). Reg3β has a 5-aa insertion (PNGGG) near its COOH terminus and only 40% identity to Reg1 (1), along with a 52% identity to INGAP and a 67% identity to the 15-aa INGAP peptide (1, 38). Their significant upregulation in our systems is in contrast to the minor or no increases in the levels of Reg1 and INGAP.

Insufficient islet β-cell mass is a key element in the development of autoimmunity-induced T1D and in T2D associated with insulin resistance. Various growth factors such as epidermal and hepatocyte growth factors, PTH-related protein, prolactin, placental lactogen, and glucagon-like peptide-1 stimulate islet cell growth (7, 11, 28, 36). β-cell mass can also be supplemented by islet transplantation, where growth factors have been found to promote the survival of transplanted cells in rodents (2, 6, 36). Many intracellular signaling molecules [such as p8, Cdk4, T-antigen, Akt1/PKB, insulin receptor substrate (IRS)-1, IRS-2, Bcl-2 and Bcl-x(L)] and islet-specific
transcriptional factors (such as PAX-6, nkd 6.1, neurogenin-3, and Pdx1) also affect islet cell replication, islet cell differentiation (and thus islet neogenesis), and cell apoptosis (11). Unfortunately, after decades of research, very few of these factors have been proven potent and specific enough for clinical applications. Novel growth factors, including members of Reg family, are still highly desirable in combating diabetes. Toward this goal, the receptors for Reg proteins and signal mechanisms are still unclear, and questions remain in their intracellular processing, potential secretion, and regulation; except for nutrients, cytokines, and glucocorticoids, what else regulates their endogenous production? The most intriguing question from our demonstration of increased levels of Reg2, -3α, and -3β mRNAs in PID mice of increased islet growth and protection is whether they are the underlying mechanism and have more potent effects than Reg1 or INGAP. To provide direct evidence, we are working on their overexpression or knocking down in islet cells or transgenic mice.

Results from present study would imply that IGF-I produced from the pancreas normally inhibits Reg protein production, a prediction that so far has not been supported by experimental evidence. In isolated rat islets, Reg1 mRNA level is significantly increased after a 2-day incubation with insulin, growth hormone, and platelet-derived growth factor but unaffected by IGF-I treatment (10). In a preliminary experiment, a general and robust overexpression of IGF-I gene in metallothionein (MT)-IGF mice (29) caused no significant change in pancreatic Reg1 or Reg2 mRNA levels but 5- to 25-fold drastic elevations in the levels of Reg3α and Reg3β (Y. Lu et al., unpublished observations). Although this result cannot be directly reconciled with our data obtained from the PID mice, it clearly suggested that the expression of some Reg family genes is affected by IGF-I and provides an interesting clue for future investigation. Finally, the changes in immunity-related genes reflected from the DNA microarray (Table 1) are interesting. Whether they can be confirmed using more quantitative methods such as Northern blotting and what it might mean for islet function remains unknown. Toward this goal, the receptors for Reg proteins and signal mechanisms are still highly desirable in combating diabetes.

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