Misfolded Proinsulin Affects Bystander Proinsulin in Neonatal Diabetes*

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It has previously been shown that misfolded mutant Akita proinsulin in the endoplasmic reticulum engages directly in protein complexes either with nonmutant proinsulin or with “hProCpepGFP” (human proinsulin bearing emerald-GFP within the C-peptide), impairing the trafficking of these “bystander” proinsulin molecules (Liu, M., Hodish, I., Rhodes, C. J., and Arvan, P. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 15841–15846). Herein, we generated transgenic mice, which, in addition to expressing endogenous proinsulin, exhibit β-cell-specific expression of hProCpepGFP via the Ins1 promoter. In these mice, hProCpepGFP protein levels are physiologically regulated, and hProCpepGFP is packaged and processed to CpepGFP that is co-stored in β-secretory granules. Visualization of CpepGFP fluorescence provides a quantifiable measure of pancreatic islet insulin content that can be followed in live animals in states of health and disease. We examined loss of pancreatic insulin in hProCpepGFP transgenic mice mated to Akita mice that develop neonatal diabetes because of the expression of misfolded proinsulin. Loss of bystander insulin in Akita animals is detected initially as a block in CpepGFP/insulin production with intracellular accumulation of the precursor, followed ultimately by loss of pancreatic β-cells. The data support that misfolded proinsulin perturbs bystander proinsulin in the endoplasmic reticulum, leading to β-cell failure.

During the progression of diabetes mellitus, the endocrine pancreas encounters difficulty in meeting insulin requirements (1); β-cell dysfunction is recognized as a major contributor to the disease (2–5). One element of β-cell dysfunction is ER stress (6–11) with ER accumulation of misfolded protein (12), especially proinsulin (13, 14). β-Cells ordinarily maintain a high level of proinsulin production with finite additional capacity before the biosynthetic apparatus is taxed to the point of ER stress (15). Chronically increased secretory demand, either in animal models or in humans, results in morphological depletion of β-secretory granules with a compensatory increase in apparent secretory pathway activity, including distention of the ER (16–18). These conditions may favor additional proinsulin misfolding (19).

The causality between misfolded proinsulin and β-cell failure is unequivocally established in congenital diabetes caused by preproinsulin coding sequence mutations, in which diabetes is inherited in an autosomal dominant manner (20–24). Insulin haploinsufficiency cannot account for the diabetes (25), yet despite three normal proinsulin alleles, both Akita and Munich mice each develop overt diabetes by expressing from a single allele a mutant proinsulin with replacement of one Cys residue that disrupts one of the three proinsulin disulfide bonds (26, 27). In addition to being retained in the ER, it has been suggested that misfolded proinsulin may impair normal insulin production via physical interactions between mutant and wild-type proinsulin gene products (26). Indeed, we have directly demonstrated protein complexes engaging misfolded mutant proinsulin with nonmutant proinsulin or with the fusion protein known as “hProCpepGFP” (28), a human proinsulin lacking any insulin misfolding mutations (but encoding the emerald green fluorescent protein bioengineered into the C-peptide domain (29)). Such a proinsulin allows us to differentiate between subpopulations of proinsulin(s) within the ER, enabling the study of “bystander” (dominant-negative) effects.

The hProCpepGFP fusion protein is distinct from rodent proinsulin by virtue of its (a) fluorescence, (b) molecular mass, (c) immunoreactivity with anti-GFP antibodies, and (d) processing to human insulin recognized by human-specific insulin antibodies. In rat insulinoma cell lines, constitutive expression of hProCpepGFP produces a translation product that undergoes folding with proper proinsulin disulfide bond formation, export from the ER, processing by prohormone convertases, packaging in secretory granules, and co-secretion of human insulin and CpepGFP along with endogenous insulin (28). In this report, we have now proceeded to generate transgenic mice

no assay; MEF, mouse embryonic fibroblast; hProCpepGFP, human proinsulin bearing emerald-GFP within the C-peptide.
in which hProCpepGFP expression is driven by the mouse Ins1 promoter exclusively in pancreatic β cells, allowing us to follow the behavior of this bystander proinsulin in the absence or presence of mutant Akita proinsulin.

EXPERIMENTAL PROCEDURES

Materials—The rat insulin RIA (#RI-13K) and human insulin-specific RIA (#HI-14K) were from Linco-Millipore; the GFP enzyme-linked immunosorbent assay was from Pierce (#15186). Rabbit anti-GFP was from Immunology Consultants Labs (#RGFP-45A-Z). RPMI 1640 medium was from Invitrogen. Collagenase P and proteinase inhibitor mixture were from Roche Applied Science. Nitrocellulose membrane was from Bio-Rad; ECL solution was from Amersham Biosciences.

Expression of hProCpepGFP by Adenoviral Vector—The cDNA encoding hProCpepGFP has been described previously, and a replication-deficient adenoviral construct expressing hProCpepGFP has been similarly described (28). 293 cells and AtT20-PC2 cells were transfected for 24 h with adenovirus at 150 pfu per cell. Conditioned media from these cells were tested for insulin bioactivity using mouse embryonic fibroblasts overexpressing human insulin receptors (kindly shared by the laboratory of Dr. C. Roberts (Oregon Health Sciences University, Portland)).

Construction of the hProCpepGFP Transgene—XhoI restriction sites flanking the hProCpepGFP construct were created by PCR to be inserted downstream of the 8.3-kb mouse insulin promoter-I and upstream of the nontranslated human growth hormone gene that served as an exogenous enhancer (30) (provided by Drs. M. Hara and G. I. Bell, University of Chicago).

Generation and Use of Transgenic Mice—The linearized hProCpepGFP construct, driven by the mouse insulin promoter-I, was introduced into pronuclei of fertilized eggs from C57BL/6 females. Pronuclear injections were carried out in the University of Michigan Transgenic Animal Model Core. After birth of potential founder mice, the presence of the transgene was verified by PCR using GFP-specific primers (forward primer, 5′-AGG TCT ATA TCA CCG CCG ACA-3′; reverse primer, 5′-TGG TCC CAC ATA TGC ACA TG-3′) giving rise to a 400-bp product (Fig. 2A).

All animal experiments were performed in accordance with the rules and regulations of the University Committee on Use and Care of Animals at the University of Michigan. Animals were housed in a pathogen-free facility on a 12-h light/dark cycle and fed a standard rodent chow. Genotyping was performed by PCR using genomic DNA isolated from tail tips of 3- to 4-week-old mice. All biochemical and cell biological characterizations shown in this study employed animals derived from a single mouse lineage originating from founder #435.

Intraportal glucose tolerance tests were performed following fasting by injection of 1.0 mg of dextrose per g of body weight; in the experiments of Fig. 2D, plasma glucose was measured at 0, 60, and 120 min after injection. To examine organ-specific GFP expression, organs were dissected from euthanized mice and incubated for 2 h in 4% formaldehyde containing 10% sucrose before snap-freezing in liquid nitrogen and cryosectioned before fluorescence microscopy.

In some experiments, hProCpepGFP mice were mated to Akita mice (JAX Laboratories, Bar Harbor, ME). Presence of the Akita mutation was verified by absence of an Fnu4HI restriction site in the 280-bp PCR product of the Ins2 gene (forward primer, 5′-TGG TCA TGC CCT GGC CTG CT-3′; reverse primer, 5′-TGG TCC CAC ATA TGC ACA TG-3′). In other experiments, hProCpepGFP mice were mated to Ins2+/− mice (kindly provided by Dr. C. Polychronakos, McGill University, Montreal, Canada) for use in pancreatic imaging experiments (Fig. 5B).

Pancreatic Islet Preparation—For islet isolation, pancreata were digested in 2 mg/ml collagenase-P in Hanks’ balanced salt solution containing calcium and magnesium in a shaking water bath for 30 min at 37 °C. After washing in ice-cold calcium and magnesium-free Hanks’ balanced salt solution, islets were hand picked and incubated for overnight recovery in RPMI 1640 medium containing 11 mM glucose, 10% fetal bovine serum, and 1% penicillin-streptomycin.

Fluorescence Imaging of Primary β-Cells—Isolated islets were dissociated with trypsin to produce dispersed β-cells that were incubated for 48 h on coverslips in RPMI 1640 medium containing 11 mM glucose and 10% fetal bovine serum. The cells were first fixed in 4% formaldehyde, permeabilized with 0.1% Nonidet P-40, incubated in blocking solution, and immunolabeled with guinea pig anti-insulin antibody and a Cy3-conjugated donkey-anti-guinea pig secondary antibody. A mouse monoclonal antibody anti-ICA512 was kindly provided by Dr. M. Solimena (University of Dresden) and was used as previously described (31) in conjunction with a goat anti-mouse secondary antibody conjugate. Samples were imaged on an Olympus FV-500 confocal microscope in the Morphology and Image Analysis Core (MIAC) laboratory of the University of Michigan Diabetes Research and Training Center.

Total Internal Reflection Fluorescence Microscopy—Isolated islets were distributed on coverslips and incubated for 48 h in RPMI 1640 plus 10% fetal bovine serum and 11 mM glucose. Samples were then examined by prismsless (through-the-objective) total internal reflection fluorescence microscopy, performed by directing an argon ion laser (488 line) through a custom side port to a side-facing dichroic mirror Q495LPw/AR (Chroma Technology) and an HQ500 LP emission filter (Chroma Technology) on an Olympus IX70 (inverted) microscope. Such illumination detects intracellular fluorescent organelles within ~200 nm of the underlying plasma membrane (32). Digital images were captured on a cooled charge-coupled device camera (SensiCam QE, Cooke Corp.).

SDS-PAGE and Western Blotting—Freshly isolated islets were divided into two equal groups incubated for 48 h in recovery medium containing either 2.8 mM or 11 mM glucose. For Western blotting, ~100 islets were boiled in SDS gel sample buffer containing 100 mM dithiothreitol. Proteins were resolved by SDS 12%-PAGE for analysis of both hProCpepGFP and proinsulin protein abundance. Gels were electrotransferred to nitrocellulose, and blotted with rabbit anti-GFP antibody and a secondary anti-rabbit horseradish peroxidase conjugate to detect hProCpepGFP, or guinea pig antibody.
anti-insulin and appropriate secondary antibody to detect proinsulin. Western blotting for β-tubulin was performed in parallel as a sample loading control. The blots were developed with enhanced chemiluminescence.

Stimulation of Insulin and CpepGFP Secretion with Secretagogues—Before stimulation, pancreatic islets were washed and incubated for 3 h in KRHB containing 2.8 mM glucose (129 mM NaCl, 5 mM NaHCO₃, 4.8 mM KCl, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 1.2 mM MgSO₄, and 10 mM Hepes, pH 7.4, plus 0.2% RIA grade bovine serum albumin). Stimulation of secretion was performed for 3 h in KRHB containing 16.7 mM glucose, 1 mM tolbutamide, 1 mM isobutylmethylxanthine, and 1 μM phorbol 12-myristate 13-acetate (33). Unstimulated and stimulated media were collected for comparative analysis.

Live Mouse Pancreatic Imaging—Mice were anesthetized with 70–80 μl of ketamine (0.42%) plus xylazine (0.15%) and placed on a warm sterile stage. The surgical site was shaved with depilatory cream and scrubbed using chlorhexidine gluconate and alcohol. A small longitudinal incision was used to expose the tail and a portion of the body of the pancreas, maintaining splenic vascular connections to avoid trauma to the spleen. Using a Nikon AZ100 microscope, digital fluorescence images of the pancreatic surface were captured on a Photometrics CoolSnapEZ digital camera (Roper). Immediately after imaging, the incision was closed, and the mouse was individually placed in a warm cage on a padded surface until fully recovered. Animal survival from the imaging procedure was 100%. Re-imaging of the mouse pancreas followed the same procedure. Where indicated, mice were treated with streptozotocin (200 mg/kg body weight) after a 6-h fast.

Newborn Pancreatic Imaging—Newborn animals were euthanized with isoflurane inhalation and pancreas excised. Using identical equipment and fluorescence exposures as those employed in adult animals, surface fluorescence of hProCpepGFP-derived peptides was obtained from the intact neonatal pancreas. For fluorescence imaging of intact newborn and adult pancreas, when the anterior and posterior surfaces of the pancreas were flipped over on the glass slide, few if any of the same islets or anatomical landmarks were visible, indicating that fluorescence in this system is derived only from islets on the pancreatic surface facing the excitation beam.

Image Analysis—For quantification of the merged confocal signals and protein bands, we used ImageJ 1.37c software (National Institutes of Health). For evaluation islets area and GFP intensity we used the NIS Elements-2.35 software (Nikon).

RESULTS

Generating hProCpepGFP Transgenic Animals—Endoproteolytic processing of hProCpepGFP in regulated secretory cell lines results in production of a protein that reacts with human insulin-specific antibodies and, upon SDS-PAGE, co-migrates with authentic insulin (28). To confirm production of bioactive human insulin, hProCpepGFP was expressed either in AtT20-PC2 cells where the protein could be endoproteolytically processed in secretory granules, or in 293 cells where it could not. Serum-free conditioned media collected over a 24-h period was briefly incubated with MEFs overexpressing human insulin receptors. The MEFs were lysed in the presence of phosphatase inhibitors, and proteins were resolved by SDS-PAGE with electrophoresis to nitrocellulose, followed by immunoblotting with antibodies against total insulin receptor (lower panel) or phospho-receptor (reflecting receptor activation, upper panel). Control AtT20-PC2 cells secrete no detectable bioactive insulin, 293 cells produce both unprocessed human proinsulin and hProCpepGFP, but these proteins exhibit negligible bioactivity on insulin receptors, and AtT20-PC2 cells process proinsulin and hProCpepGFP into bioactive human insulin. B, schematic representation of the hProCpepGFP transgene, incorporated into the vector used by Hara et al. (30), used to generate transgenic mice for the present studies.

We therefore proceeded to generate transgenic founder mice expressing hProCpepGFP driven by the mouse Ins1 promoter (Fig. 1B), which were identified as positive by PCR (Fig. 2A). From these founders, the transgene was transmitted to progeny with expected Mendelian ratios, creating transgenic mouse lines. None of the transgenic animals from any of these lines exhibited a pathological phenotype. The line with the most robust transgene expression as measured by fluorescence intensity (see below) is the one we have most characterized. Transgenic mouse body weight (Fig. 2B) and random glucose levels (Fig. 2C) were found to be comparable to that of age- and gender-matched C57BL/6 controls (Fig. 2D). Thirty minutes after glucose injection, plasma insulin levels in both groups (using a rat insulin RIA that cross-reacts with insu-
Ins1-hProCpepGFP Mice Express the Transgene Specifically in the Endocrine Pancreas—To corroborate tissue specificity of the 8.3-kb Ins-1 promoter, organs of nontransgenic controls and transgenic hProCpepGFP mice were removed, and thick frozen sections (7 μm) were prepared for epifluorescence microscopy from heart, lung, liver, spleen, pancreas, stomach, small intestine, large intestine, kidney, muscle, testicles, abdominal fat, thymus, bone marrow, and brain. (Within the brain, special examination of the hypothalamus (not shown) revealed no detectable GFP-expressing neurons in the arcuate nucleus, ventromedial hypothalamic nucleus, dorsal hypothalamic nucleus, lateral hypothalamic, or preoptic area.) Indeed, fluorescence derived from hProCpepGFP was identified exclusively in pancreatic islets (Fig. 3) (with weak green fluorescence detected in renal tubules for which we cannot exclude renal tubular clearance of CpepGFP as is seen for C-peptide (34) and circulating GFP (35), although the intensity of this fluorescence was only marginally greater than that obtained by renal tubular autofluorescence (36) in control tissue sections from C57BL/6 mice (not shown)).

hProCpepGFP Distribution in the Secretory Pathway—To explore the distribution of hProCpepGFP in pancreatic β-cells, isolated islets were dissociated into single cells in media containing 11 mM glucose. After 48 h, samples were fixed, permeabilized, and processed for immunofluorescence with anti-insulin. Only hProCpepGFP-expressing cells (i.e., green cells) labeled positively with anti-insulin. Within β-cells, especially those exhibiting adequate spreading on coverslips, GFP fluorescence exhibited a predominantly punctate distribution largely overlapping with the distribution of insulin immunofluorescence (Fig. 4A), with a fraction proximate to the cell surface. Total internal fluorescence microscopy was employed to look for fluorescent subplasmalemmal secretory pathway organelles; GFP-positive granules were readily identified.
In addition to an intracellular distribution, the subplasmalemmal distribution of GFP-positive organelles also largely overlapped with the immunofluorescence distribution of the granule membrane protein ICA512 (Fig. 4C, upper row). The data indicate that the distribution of green fluorescence comes from hProCpepGFP-derived peptides residing in the secretory pathway, largely within \( \text{H}9252 \)-granules, comparable to that of insulin.

**Regulation of hProCpepGFP Protein Levels**—To investigate glucose-dependent regulation of the expressed hProCpepGFP protein, isolated islets from transgenic mice were incubated in either low (2.8 mM) or high (11 mM) glucose-containing medium for 48 h. Islet lysates were resolved by reducing SDS-PAGE, electrotransferred to nitrocellulose, and immunoblotted with anti-GFP to follow hProCpepGFP, anti-insulin to follow endogenous proinsulin, and anti-\( \text{H}9252 \)-tubulin as a loading control. The \( \text{H}11011 \)-40-kDa hProCpepGFP band (Fig. 5A) was increased (\( \approx 5 \)-fold) upon incubation in high glucose versus low glucose. Similarly in these islets, endogenous mouse proinsulin was up-regulated (\( \approx 10 \)-fold, Fig. 5A). These data indicate that the overall glucose-dependent regulation of hProCpepGFP protein levels (the sum of transcriptional, translational, and protein half-life effects) exhibits behavior similar to that of endogenous mouse proinsulins.

The \( \text{Ins}1 \) gene product is up-regulated in animals that have genetic loss of other insulin alleles (25, 37). We therefore mated \( \text{Ins}1 \)-hProCpepGFP transgenic mice to \( \text{Ins}2^{-/-} \) animals. Fig. 5B shows in situ surface pancreatic fluorescence from hProCpepGFP transgenic males at 3–4 months of age in \( \text{Ins}2^{-/-} \) and \( \text{Ins}2^{-/-} \) genotypes. Neither \( \text{Ins}2^{+/+} \) nor \( \text{Ins}2^{-/-} \) genotypes caused hyperglycemia by random blood glucose check, but loss of one \( \text{Ins}2 \) allele resulted in a compensatory increase in \( \text{Ins}1 \)-hProCpepGFP-derived islet fluorescence (as well as endogenous \( \text{Ins}1 \)) (25, 37), and \( \text{Ins}2^{-/-} \) mice showed an even greater increase (Fig. 5B, with islet fluorescence intensity per pancreatic area quantified from these images in the bar graph in Fig. 5C). The data clearly indicate that \( \text{Ins}1 \)-hProCpepGFP transgenic mice up-regulate hProCpepGFP-derived products in response to physiological demand. In islets isolated from \( \text{Ins}1 \)-hProCpepGFP, \( \text{Ins}2^{-/-} \) animals, stimulation for 2 h with a secretagogue mixture, including 16.7 mM glucose (see “Experimental Procedures”) induced secretion of 21.6% of total human insulin and 25.5% of rodent insulin (Fig. 5D), indicating that endogenous mouse...
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hProCpepGFP transgenic mice were incubated for 3 h in 2.8 mM glucose (basal secretion) followed by another 3 h in the presence of the secretagogue mixture. Secretion to the media was measured by a rodent insulin RIA. In both C57BL/6 control islets and islets from transgenic hProCpepGFP mice, insulin secretion was similarly stimulated (Fig. 6A). In parallel, a GFP-specific enzyme-linked immunosorbent assay was employed to examine the secretion of CpepGFP (and possibly small amounts of hProCpepGFP precursor that remain unprocessed/partially processed in secretory granules). Whereas there was no GFP-specific immunoreactivity released from C57BL/6 control islets, islets from hProCpepGFP transgenic animals stimulated with the secretagogue mixture exhibited a parallel stimulated secretion of GFP-specific immunoreactivity (Fig. 6B). We were also able to demonstrate stimulated release of GFP-containing peptides from transgenic mouse islets with high glucose alone, but the enzyme-linked immunosorbent assay signal was less robust. Altogether, the data in Figs. 5 and 6 indicate that human insulin and CpepGFP are stored like mouse insulin in pancreatic β-secretory granules, and secreted in parallel.

Transgenic hProCpepGFP × Akita Mice Get Diabetes—Evidence suggests that proinsulin misfolding in β-cells may impair the trafficking of bystander proinsulin in the ER, thereby inhibiting insulin production. To follow the fate of bystander proinsulin, we mated hProCpepGFP transgenic animals with Akita mice that bear a proinsulin-C(A7)Y point mutation in a single allele of Ins2. This mutation is identical to one of the newly identified preproinsulin mutations responsible for permanent neonatal human diabetes (20–24).

As with other mice bearing the Akita mutation (26, 38–40), transgenic animals developed similar male-predominant diabetes (Fig. 7). To investigate effects on bystander proinsulin, we evaluated the steady-state levels of hProCpepGFP precursor and CpepGFP product in islets by Western blotting. In a 5-week-old hProCpepGFP transgenic male control animal (random blood glucose, 147 mg/dl), the major GFP-immunoreactive band was a ~30-kDa species (Fig. 8A, lane 2) that co-migrated with authentic CpepGFP standard (lane 4), indicating a predominance of secretory granule-specific processing (consistent with Figs. 4 and 6). However, in 5-week-old diabetic (random blood glucose, 350–400 mg/dl) transgenic Akita islets, a decrease in mature CpepGFP was accompanied by an increase in the steady-state level of the hProCpepGFP precu-
sor (Fig. 8A, lane 3). Importantly, in pre-diabetic males (random blood glucose, <250 mg/dl), the intra-islet hProCpepGFP: CpepGFP ratio was already increased 3-fold (Fig. 8B). After diabetes onset, this ratio climbed still further (not shown). Dispersed β-cells from such islets exhibited a change in intracellular green fluorescence that no longer showed a distribution in subplasmalemmal organelles overlapping with the ICA512 granule membrane marker (Fig. 4C, lower row). These results strongly suggest that in Akita islet β-cells, the intracellular transport of bystander hProCpepGFP is impaired, blocking insulin synthesis and causing further hProCpepGFP accumulation in the ER (28), which can only add to ER stress. Indeed, in the islets isolated from such animals, steady-state BiP levels appeared elevated (supplemental Fig. S1).

Pancreatic Insulin Content in Live Mice—Based on the foregoing studies (Figs. 4, 6, and 8), visualization of CpepGFP fluorescence should provide a potentially quantifiable marker of pancreatic islet insulin content that can be followed in vivo, serially, as a function of age. With this in mind, under an approved animal protocol, live hProCpepGFP transgenic mice were anesthetized and, under sterile conditions, a small left flank incision and single suture was used to briefly externalize the pancreatic tail and body while maintaining intact the pancreatic blood supply and connective tissue attachments to the spleen. The living pancreas was placed rapidly between two glass coverslips with the entire animal positioned on the stage of a dissecting microscope for rapid epifluorescence imaging of the pancreatic surface. A representative image from an 8-week-old animal is shown in Fig. 9A (left). Mean islet area per pancreatic field (a measure that can be used to follow both islet hypo/hyperplasia plus hypo/hypertrophy (Fig. 9B, left)) and mean islet fluorescence intensity (a measure proportional to islet insulin content (Fig. 9B, right)) were quantified from digital images. The pancreas was then returned to the abdominal cavity, the incision closed, and all mice recovered uneventfully and gained weight normally between procedures (not shown). The procedure was repeated at 2-week intervals, identifying the same fields and many of the same islets. At 10 weeks of age (Fig. 9A, middle), the mean islet area was largely unaltered from that seen at 8 weeks (Fig. 9B, left) and fluorescence intensity was, if anything, increased (Fig. 9B, right). However, if, after imaging at 10 weeks, animals were rendered diabetic by treatment with high dose streptozotocin, then by 12 weeks such animals exhibited random glucose levels that were >500 mg/dl by glucometer, and islet fluorescence intensity became undetectable (Fig. 9A, right).

We then used a similar procedure to examine hProCpepGFP x Akita animals. For this experiment, newborn hProCpepGFP x Akita males were euthanized (as yet, we have not attempted survival surgery in newborns), and the entire unfixed pancreas was examined for surface epifluorescence as in adult live animals. As the newborn stage, Akita males are normoglycemic, and by epifluorescence, pancreatic CpepGFP/insulin levels appeared normal or perhaps even supranormal (Fig. 10A; an externally added fluorescence marker (inset) was used as an internal standard for fluorescence imaging). By contrast, in a
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2-month-old male hProCpepGFP x Akita diabetic male (random blood glucose: 537 mg/dl), despite the increase in background pancreatic autofluorescence, islet fluorescence intensity was undetectable (Fig. 10B). Unlike normal male adult pancreata, male hProCpepGFP x Akita diabetic mice exhibited few detectable islets by hematoxylin and eosin staining (Fig. 10, C and D) and with this, little detectable endogenous insulin by immunofluorescence (Fig. 10, E and F). Consistent with previous histological studies of Akita diabetic males demonstrating islet atrophy (41), altogether the data in Fig. 10 indicate that the profound depletion of pancreatic CpepGFP and insulin reflects a loss of pancreatic β-cells. Thus, although misfolded Akita proinsulin initially perturbs post-translational trafficking of bystander proinsulin in β-cells, it culminates ultimately in virtually complete loss of β-cells, which is thought to be mediated by β-cell apoptosis (42).

DISCUSSION

In the present study we have studied a tagged subpopulation of insulin precursor protein in vivo, serving as bystander proinsulin molecules whose processing and storage as CpepGFP human insulin (Figs. 5D and 8)) whose distribution matches that of mouse insulin (Figs. 4A, 5D, and 6) and the granule membrane protein ICA512 (Fig. 4C). Furthermore, overall regulation of hProCpepGFP levels in islet β-cells appears similar to that reported for the endogenous Ins1 gene product (25, 37) (Fig. 5). Thus, hProCpepGFP fulfills the criteria as a reporter of bystander proinsulin and insulin in healthy and diabetic animals. It is with such a reporter that we have previously shown selective protein complex formation engaging both misfolded Akita mutant and nonmutant proinsulin molecules (28), whereas the present study represents the first in vivo demonstration of the pathophysiological effects of misfolded proinsulin on nonmutant proinsulin bystanders.

β-Cell mass from morphometric analysis has been the parameter considered most critical to understanding pancreatic deficits that develop during diabetes progression (1), whereas in older studies, extraction of the pancreas to measure total insulin content has been used to follow the size of the insulin storage pool. Work on methods for noninvasively measuring β-cell mass has been actively pursued (44–46). Herein allows the monitoring of pancreatic insulin content. This tool can be used to investigate physical interactions between misfolded and bystander proinsulins (28) and to serve as a visual marker of pancreatic insulin content in states of health and disease.

Importantly, although a precursor of bioactive human insulin (Fig. 1), hProCpepGFP expression in transgenic mouse islets is not an overexpression system and does not perturb the development, growth, or metabolism in these animals (Fig. 2) even as it is used as a reporter of secretory granule insulin content (Figs. 4, 5D, 6, and 8A). We are confident that insulin overexpression is not occurring in this model, because transgene expression, which is limited to islet β-cells (Figs. 3 and 4), is driven by the weak Ins1 promoter (43) similar to that first employed by Hara et al. (30). Further, total insulin level in the transgenic mouse islets is similar to that in control islets. Moreover, expression of the hProCpepGFP transgene does not prevent male Akita mice from developing diabetes (Fig. 7). Yet, within the islet β-cells of Ins1-hProCpepGFP mice, the hProCpepGFP protein is primarily stored as endoproteolytically processed products (CpepGFP and...
we present the Ins1-hProCpepGFP transgenic mice as a valuable new in vivo tool to specifically follow pancreatic insulin content (which is the sum of β-cell mass, gene expression, and protein production and turnover); indeed, our methodology for serial pancreatic imaging (Fig. 9) does not alter normal animal growth and metabolism. Note that, because CpepGFP is a secretable/expendable product, any possible GFP photobleaching that might occur during imaging is irrelevant in sequential analysis (because there is sufficient time for complete secretory granule turnover during the intervals between imaging sessions).

With these facts in mind, we are struck by the findings in Akita male mice, in which major pathophysiological changes are detected by the hProCpepGFP reporter. In the neonatal pancreas of Akita males, hProCpepGFP-derived epifluorescence is normal (or possibly increased) compared with wild-type animals (Fig. 10). Yet in conjunction with severe diabetes in adult males, a great decrease in the steady-state level of CpepGFP is noted, reflecting diminished insulin content. The increase in the hProCpepGFP precursor/mature CpepGFP (Fig. 8) correlates with a change in intracellular distribution of the protein that no longer resides in secretory granules (Fig. 4C, bottom panels) and undoubtedly reflects hProCpepGFP within the ER (28). The islets of Akita males go on to develop signs of ER stress (supplemental Fig. S1), and finally pancreatic insulin content becomes unmeasurably low when the animals have developed full-blown diabetes, and histological analysis shows islet loss (Fig. 10 and Ref. 41), consistent with the proposed apoptotic death of pancreatic β-cells (42).

Earlier studies showed that in the presence of Akita proinsulin, other secretory protein cargo, including those making intramolecular disulfide bonds are initially unperturbed (28), although subsequently as β-cells become sicker, global secretory defects become manifest (47). The simplest interpretation of the data in the present study is that misfolded proinsulin initially results in adverse consequences on the trafficking of bystander proinsulin, which leads to development of severe diabetes and a state of vast β-cell demise.

One area that must now be pursued is a more precise molecular understanding of the earliest dominant-negative effects on bystander proinsulin. We and others have reported direct physical interactions in complexes involving misfolded proinsulin and bystander proinsulin (26, 28), which may be analogous to the propagative misfolding of islet amyloid polypeptide that can lead to β-cell cytotoxicity (12). Along with bystander proinsulin, resident proteins of the ER are likely also to be engaged in abnormally increased protein complexes as well (26, 28, 47). Such engagement may trigger ER stress (48), including adverse downstream consequences of the ER stress response (49). Understanding the mechanisms of aberrant protein complex formation may lead to developing a means (such as by use of small molecules (50)) to prevent propagative misfolding. Such an approach may be critical to the development of therapies to minimize β-cell proteotoxicity in diabetes.

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