Seven mutations in the human insulin gene linked to permanent neonatal/infancy-onset diabetes mellitus

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Permanent neonatal diabetes mellitus (PNDM) is a rare disorder usually presenting within 6 months of birth. Although several genes have been linked to this disorder, in almost half the cases documented in Italy, the genetic cause remains unknown. Because the Akita mouse bearing a mutation in the Ins2 gene exhibits PNDM associated with pancreatic β cell apoptosis, we sequenced the human insulin gene in PNDM subjects with unidentified mutations. We discovered 7 heterozygous mutations in 10 unrelated probands. In 8 of these patients, insulin secretion was detectable at diabetes onset, but rapidly declined over time. When these mutant proinsulins were expressed in HEK293 cells, we observed defects in insulin protein folding and secretion. In these experiments, expression of the mutant proinsulins was also associated with increased Grp78 protein expression and XBP1 mRNA splicing, 2 markers of endoplasmic reticulum stress, and with increased apoptosis. Similarly transfected INS-1E insulinoma cells had diminished viability compared with those expressing WT proinsulin. In conclusion, we find that mutations in the insulin gene that promote proinsulin misfolding may cause PNDM.

Introduction

Neonatal diabetes is a rare monogenic disorder associated with defects of pancreatic β cell mass and/or function. The disorder is distinguished by 2 clinical variants — transient and permanent — caused by specific genetic defects. Abnormalities of chromosome 6 or mutations of the ATP-sensitive potassium (KATP) channel KCNJ11 and ABCC8 genes are detected in most patients with transient neonatal diabetes mellitus (TNDM), in which diabetes usually presents in the first month of life but then remits with a variable time interval (1–4). In about 50% of patients with TNDM, diabetes relapses later in life (1–4). Permanent neonatal diabetes mellitus (PNDM) is more genetically heterogeneous and has been associated with mutations in 8 different genes: IPF-1, EIF2AK3, GCK, FOXP3, KCNJ11, PTF1A, ABCC8, and GLIS3 (4–11). Individuals with PNDM need insulin therapy, but those carrying mutations in KCNJ11 and ABCC8 can be successfully transferred to sulfonylureas (4, 12, 13). In most PNDM, diabetes manifests itself within the first 6 mo after birth, but delayed onset has been reported in subjects with mutations of EIF2AK3 (6, 14). PNDM can manifest itself as an isolated defect in glucose-stimulated insulin secretion (mutations of GCK, KCNJ11, and ABCC8; refs. 4, 7, 9) or as part of a syndrome involving other organ systems (4–6, 8–11, 15). Most patients (i.e., 40%–64%) with PNDM or infancy-onset diabetes bear dominant, activating, heterozygous mutations of KCNJ11, the pore-forming subunit of KATP channel (15), while activating mutations of its regulatory subunit SUR1 (ABCC8 gene) account for an additional 7% of cases (4). Other genetic defects leading to PNDM are much less common (5–8, 10, 11). We previously identified KCNJ11 mutations in 19 of 37 patients in an Italian cohort with diabetes onset by 7 mo of age (13, 16, 17), and we set about to search for the genetic causes of diabetes in the remaining subjects.

In one of these patients, kindred A, II-1, the C-peptide level at the time of diagnosis was surprisingly high, unlike the low C-peptide level in patients with PNDM caused by mutations of GCK, KCNJ11, and ABCC8 (4, 7, 9). High C-peptide immunoreactivity in the absence of apparent insulin resistance (18) has been identified in adult patients bearing point mutations in the proinsulin coding sequence leading to familial hyperproinsulinemia (19–21). Interestingly, a heterozygous mutation in the insulin-2 gene (Ins2) has previously been identified as the cause of early-onset diabetes

Nonstandard abbreviations used: Akita, Akita mouse introduction into human proinsulin; MDI, monogenic diabetes of infancy; PNDM, permanent neonatal diabetes mellitus; TNDM, transient neonatal diabetes mellitus.

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in the Akita mouse (22), in which a Cys→Tyr substitution at proinsulin position 96 (C96Y; encoding residue 7 of the insulin A-chain) disrupts one of the 2 disulfide bonds connecting the insulin B- and A-chains. As a consequence, the Akita proinsulin accumulates in the ER, inducing ER stress and apoptosis of pancreatic β cells (22–25). We hypothesized that certain mutations of the human insulin gene (INS) might also give rise to neonatal diabetes mellitus. In this report we describe 7 proinsulin mutations, which we believe to be novel, associated with PNDM or infancy-onset diabetes mellitus that are likely to promote proinsulin misfolding, ER stress, and β cell failure.

**Results**

**Mutational analysis.** We identified 7 heterozygous mutations of the INS gene in 10 unrelated probands with PNDM or infancy-onset diabetes. As would be expected for a defective gene specific to β cells, all affected neonates had only diabetes and no other clinical features. We adopted a nomenclature describing the mutations according to their position in the mature insulin chains: L<sup>B6</sup>P (that is, a Leu→Pro substitution at residue 6 of the insulin B-chain), L<sup>B11</sup>Y, L<sup>B15</sup>P, L<sup>B15</sup>Y<sup>B16</sup>delinsH (that is, replacement of Leu and Tyr by His at residues 15–16 of the B-chain), C<sup>A6</sup>Y, and Y<sup>A19</sup>X. The proinsulin mutation located in the dibasic doublet between C-peptide and A-chain (K<sup>64</sup>–R<sup>65</sup>, based on the N-terminal Phe residue of the insulin B-chain as residue 1) is denoted as R65C. In all but 2 families, the mutation originated de novo, and neonatal diabetes was observed only in subjects carrying the INS gene mutation and not in unaffected parents. Mutation R65C was identified as a spontaneous mutation as well as in 2 familial cases (Figure 1). Mutation C<sup>A6</sup>Y was also detected in a Danish patient (diabetes onset, age 3 mo); however, DNA samples from 2 children of the proband, who presented with diabetes at 6.5 and 2.5 mo postpartum, were not available for analysis. All mutations found in patients with PNDM or infancy-onset diabetes were different from those previously described to lead to familial hyperinsulinemia or hyperproinsulinemia (19–21, 26–32). Notably, all affected residues — including...
Clinical characteristics of patients

<table>
<thead>
<tr>
<th>Family subject</th>
<th>Kindred A</th>
<th>Kindred B</th>
<th>Kindred C</th>
<th>Kindred D</th>
<th>Kindred E</th>
<th>Kindred F</th>
<th>Kindred G</th>
<th>Kindred H</th>
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<tr>
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<tr>
<td>Mutation</td>
<td>R65C</td>
<td>R65C</td>
<td>R65C</td>
<td>R65C</td>
<td>L11V</td>
<td>Y81H</td>
<td>L81I</td>
<td>P</td>
<td>C44Y</td>
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<tr>
<td>Amino acid</td>
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<tr>
<td>Lysine</td>
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<td>Phenylalanine</td>
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<td></td>
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<tr>
<td>Glucose (mg/dl)</td>
<td>234</td>
<td>NA</td>
<td>612</td>
<td>521</td>
<td>594</td>
<td>610</td>
<td>&gt;1,000</td>
<td>504</td>
<td>306</td>
</tr>
<tr>
<td>C-peptide (ng/ml)</td>
<td>0.8</td>
<td>0.85</td>
<td>0.5</td>
<td>0.57</td>
<td>0.7</td>
<td>0.97</td>
<td>0.14</td>
<td>3.2</td>
<td>0.8</td>
</tr>
<tr>
<td>Age 12 yr</td>
<td>1.41</td>
<td>0.67</td>
<td>0.6</td>
<td>0.37</td>
<td>1</td>
<td>1</td>
<td>0.6</td>
<td>1.14</td>
<td>0.8</td>
</tr>
<tr>
<td>Age 41 yr</td>
<td>16 mo</td>
<td>37 yr</td>
<td>21 yr</td>
<td>28 mo</td>
<td>13 yr</td>
<td>28 mo</td>
<td>30 mo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-peptide (ng/ml)</td>
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<td>0.84</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</table>

HG, hyperglycemia; KA, ketoacidosis; NA, not available; ND, not detectable. 

Clinical characteristics. All patients were the product of uneventful pregnancies; their clinical features at presentation of diabetes are shown in Table 1. In 3 subjects (kindreds E, G, and H), failure to thrive or intercurrent infection resulted in further analysis leading to a diagnosis of diabetes. In the case of patient II-1 of kindred A (mutation R65C), glycosuria was checked because the mother had been diagnosed with insulin-dependent diabetes at 1 yr of age and was found positive in the diaper at 76 d postpartum. Six patients (kindreds B, C, D, F, I, and PN-30) were referred to a local hospital for severe hyperglycemia with ketosis.

C-peptide, randomly determined 1–56 d after discovery of diabetes and during the course of insulin therapy, was detected in 8 probands (data from 2 probands and 2 affected parents were not available). In 2 of these 8 probands, C-peptide appeared unexpectedly high (kindred A, II-1, and kindred H; Table 1). Furthermore, sequential assay of C-peptide in 2 patients on insulin therapy (kindred A, II-1, and kindred F) and in one on sulfonylurea for 60 d followed by mixed insulin-sulfonylurea therapy (kindred H) revealed declining values over the ensuing months after initial diagnosis. At the termination of the present study, C-peptide was undetectable in all but 3 patients. Basal pancreatic glucagon level was found to be in the normal to high range in 2 subjects (kindred A, I-1, 21.3 ng/dl; kindred D, 16.5 ng/dl), and glucagon secretion, as determined by an insulin tolerance test, increased approximately 50% as a result of insulin-induced hypoglycemia in both patients (kindred A, I-1, 30 ng/dl at 38 mg/dl plasma glucose; kindred D, 22 ng/dl at 34 mg/dl plasma glucose).

Clinical and metabolic features of PNDM or infancy-onset diabetes caused by INS gene mutations compared with those from other causes. In all but 1 patient with an INS gene mutation (kindred F; Table 1), birth weight was near normal (median, 3,055 ± 227 g). This finding was in sharp contrast with the birth weight of patients with KCNJ11 mutations (median, 2,455 ± 370 g; P < 0.00021, INS versus KCNJ11). At the time of diagnosis, C-peptide was barely detectable in 4 of 10 KCNJ11 mutants of the Italian cohort (refs. 13, 16, and F. Barbetti, unpublished observations), whereas in patients for whom C-peptide data were available, measurable or even high C-peptide levels were found in all 8 individuals with INS mutations. The diagnosis of diabetes in patients with INS mutations associated with PNDM/MDI are shown in yellow.

Figure 2
Amino acid sequences of WT and mutant proinsulins. The amino acid sequence and disulfide bonds (s=s) of WT proinsulin are shown. Amino acid substitutions associated with PNDM/MDI are shown in yellow.
occurred at 37–1,460 d postpartum (median, 88 ± 412.4 d), which is about 5 wk later than onset in patients with KCNJ11 mutations (range, 1–220 d; median, 53 ± 58.3 d; \( P < 0.07 \)). With exclusion of 1 case (kindred C, I-1, 1,460 d), the comparison reached statistical significance (median, 87 ± 94.7 d; \( P < 0.00038 \)). Indeed, individuals with INS gene mutation can present with diabetes well beyond 6 mo after birth (F. Barbetti, unpublished observations). This feature is also found in patients with EIF2AK3 mutations (Wolcott-Rallison syndrome), which disrupt the normal ER stress response and cause dysregulated secretory protein synthesis in pancreatic \( \beta \) cells (6, 14). Finally, because most INS gene mutations occur spontaneously, these patients cannot be distinguished on clinical grounds from those with type 1 diabetes (besides the absence of type 1 diabetes autoantibodies). For this reason we propose here to replace the term PNDM with monogenic diabetes of infancy (MDI), a broad definition that may include any form of diabetes—permanent or transient—with onset during the first years of life caused by a single gene defect.

**Functional studies.** Figure 2 shows the location of the mutated residues. Two mutations each affect 1 of the 3 highly conserved insulin disulfide bonds, similar to that reported in the Akita mouse: C\( ^{A6} \)Y perturbs C\( ^{A6} - C^{A11} \), while Y\( ^{A19} \)A disrupts C\( ^{B19} - C^{A20} \) by generating a premature stop codon that eliminates C\( ^{A20} \) as well as N\( ^{A21} \) at the C terminus. Another mutation is an in-frame substitution in which \( \beta \)-chain Leu15 and Tyr16 are replaced by a single His residue: L\( ^{B15} \)Y\( ^{B16} \)delinsH. In 3 additional cases, proinsulin mutations were located in invariant residues of the \( \beta \)-chain (L\( ^{B6} \)P, L\( ^{B6} \)V, and L\( ^{B11} \)P; Supplemental Figure 1). Of these, L\( ^{B6} \)P and L\( ^{B11} \)P are located in the so-called “hydrophobic core” on the contact surface buried within insulin dimers. The mutations predicted to most dramatically affect proinsulin secondary structure included L\( ^{B15} \)Y\( ^{B16} \)delinsH (by causing significant \( \alpha \)-helix disruption; Figure 3) and R65C (by abolishing...
ing the C-peptide/A-chain cleavage site while creating an unpaired Cys residue with potential to form inappropriate disulfide bonds).

In 3 independent experiments, all proinsulin mutants except L\textsubscript{B6}V were expressed as recombinant proteins in HEK293 cells and examined for \textsuperscript{35}S-protein expression and secretion at 1 h after synthesis (Figure 4A). After immunoprecipitation from cells and analysis by nonreducing Tris-tricine-urea-SDS-PAGE, WT proinsulin and proinsulin-R65L (a hyperproinsulinemia-inducing mutation associated with mild fasting hyperglycemia in an adult patient; ref. 19) were well expressed, with similar fractions of fast-migrating native proinsulin disulfide isomer and slower-migrating nonnative forms (34). Within 1 h of synthesis, both WT proinsulin and the R65L mutant underwent efficient secretion to the culture media. However, all MDI-causing mutations and the Akita mutation introduced into human proinsulin (hAkita; mutation C\textsuperscript{A}(Y) exhibited substantially decreased recovery with an increased fraction of nonnative proinsulin disulfide isomers and a profound decrease in secretion (Figure 4A). In a separate set of experiments, proinsulin mutants that were not secreted appeared to be degraded intracellularly (data not shown). As an independent assay, proinsulin released into serum-free culture media was detected by RIA (Figure 4B), confirming defective secretion for all MDI-producing proinsulin mutants. These data establish that MDI-producing proinsulin mutations perturb proinsulin structure and lead to defective secretion.

Next, WT proinsulin, the R65L mutant, the hAkita mutation, and all new MDI-associated mutations except L\textsubscript{B6}V were expressed as recombinant human proinsulins in INS-1E rat insulinoma cells, which are capable of processing proinsulin into insulin and C-peptide. The recombinant proinsulins were readily detected by immunofluorescence with a human-specific anti-proinsulin antibody at 48 h after transfection (Figure 5). However, at 96 h after transfection, immunofluorescence was still detectable in 61% ± 2.8% of INS-1E cells transfected with WT proinsulin (n = 4 transfections) and 56% ± 4.0% of cells transfected with the R65L mutation (n = 3 transfections), but almost none was detected in cells transfected with the hAkita mutation or with R65C, C\textsuperscript{A}(Y), L\textsuperscript{B15}Y\textsuperscript{B16}delinsH, and Y\textsuperscript{A19}X (mean, 3 transfections each). An intermediate percentage of viable INS-1E cells at 96 h was observed for mutations L\textsuperscript{B6}P (36% ± 2.2%; n = 2 transfections) and L\textsuperscript{B11}P (17.7% ± 4.8%; n = 5 transfections).

The same constructs tested by immunofluorescence were used to evaluate XBP1 gene splicing, a marker of ER stress. Tunicamycin (5 \textmu g/ml for 4 h), a general inducer of ER stress, was used as positive control. As seen in Figure 6A, no difference was detected between untransfected cells and those transfected with WT proinsulin or the R65L mutation. In contrast, recombinant human proinsulins containing MDI-associated mutations (including L\textsuperscript{B6}P; data not shown) and the hAkita mutation induced increases of the shorter, splice-activated form of XBP1 from 30% (R65C and Y\textsuperscript{A19}X) to 400% (C\textsuperscript{A}(Y)) (Figure 6B). We then tested protein
expression of chaperonin Grp78, also known as BiP, as an additional marker of ER stress. As shown in Figure 7, Grp78 protein amounts increased in 293 cells 9 and 16 h after transfection with C\(^{A6Y}\) and L\(^{B11YB16delsinsH}\) mutant proinsulins compared with WT proinsulin; higher levels of Grp78 were observed at 24 h after transfection for mutations L\(^{B6P}\) and L\(^{B11P}\).

As an early apoptosis marker, we assessed surface staining of annexin V. In 3 independent flow cytometry experiments at 24 h after transfection of HEK293 cells, we detected a 4-fold increase of annexin V positivity in cells expressing proinsulin L\(^{B11YB16delsinsH}\) compared with cells expressing WT proinsulin (\(P = 0.015\); Figure 8A). However, using this technique, we were unable to observe effects with other MDI mutations or even the hAkita mutation. As a late apoptosis marker, we evaluated by flow cytometry the percentage of propidium iodide–positive cells at 24, 48, and 72 h after transfection (Figure 8B). With this assay, we detected increased numbers of apoptotic 293 cells transfected with the L\(^{B11YB16delsinsH}\) and the C\(^{A6Y}\) mutations at 24 h after transfection compared with WT proinsulin (\(P < 0.003\) and \(P < 0.01\), respectively), but for the remaining MDI mutations we did not find any change at all points tested. Taken together, these data suggest that at least some of the proinsulin mutants, in addition to being defective in insulin production (loss of function), might also compromise β cell viability (toxic gain of function).

**Discussion**

Previously, abnormal circulating proinsulin had been reported in subjects with elevated plasma levels of immunoreactive insulin but normal insulin sensitivity (19, 21, 27, 28, 30). Such patients with familial hyperproinsulinemia are diagnosed as adults with mild diabetes, impaired glucose tolerance (19–21, 27, 28, 30, 32), or even episodic hyperglycemia (26, 31). Mutations of the human INS gene causing familial hyperinsulinemia or familial hyperproinsulinemia encode defects either in insulin receptor binding (F\(^{B24S},\) Y\(^{A3L}\); Insulin Chicago, Los Angeles, and Wakayama; refs. 27, 28, 30) or in the dibasic C-peptide/A-chain cleavage site (R65H, R65L, R65P; Proinsulin Tokyo and Kyoto; refs. 19–21, 31, 32), with the exception of the H\(^{P10G}\)D mutant (Proinsulin Providence) that may affect insulin packaging (29). However, in familial hyperproinsulinemia, the abnormal INS gene product is efficiently secreted. In addition, no INS coding sequence polymorphisms have been found associated with type 2 diabetes (35, 36).

In this study we describe what we believe to be new heterozygous mutations within the proinsulin coding sequence causing early-onset, insulin-requiring diabetes (2–48 mo postpartum). In the Italian patient cohort with PNDM, mutations of the INS gene were the second leading cause (9 of 37, or 24.3%) following activating mutations of KCNJ11 (19 of 37, or 51.3%). Spontaneous mutations in a small gene are unlikely to be a chance observation (9), yet in 7 patients the mutations arose de novo. We also describe 2 familial cases with vertical transmission of insulin-requiring diabetes. In 2 patients (kindred A, II-1, and kindred H) in which hyperglycemia was detected before severe diabetes symptoms, C-peptide was found at normal to high levels, but was decreased or undetectable within 2–12 mo of diagnosis. The INS gene mutations described herein are highly reminiscent of the diabetes phenotype observed in male Akita mice, in which a heterozygous mutation of the Ins2 gene (C\(^{A7Y}\), disrupting an interchain disulfide bond of insulin) causes a doubling of plasma glucose within 4 wk of birth (37) with a rapid decrease of pancreatic β cells (22). A proposed molecular pathogenesis of diabetes in the Akita mutant is (a) proteotoxic proinsulin misfolding, (b) engorgement of the β cell ER with activation of ER stress response pathways, and (c) generation of downstream...
Insulin cDNA was kindly provided by H. Lou (NIH, Bethesda, Maryland, USA). Full-length insulin cDNA was amplified by PCR in 3 independent probands, as well as Y<sup>B15</sup>Y′, like the Akita mutant (Figure 4), revealed the presence of improper disulfide bonds with little or no secretion, consistent with entrapment within the ER. The direct demonstration of cell death induced by expression of at least 2 of the mutants (Figure 8) strongly supports a proteotoxic, apoptotic mechanism.

Of the patients described herein, 9 of 10 showed normal weight at birth, a finding clearly different from the low birth weight in patients bearing KCNJ11 mutations (15), which suggests that β cell insufficiency may occur primarily after birth. Pancreatic β cell proliferation is low in humans after birth (40). A postpartum cell insufficiency may occur primarily after birth. Pancreatic β cell deficiency in male Akita mice stands in striking contrast with the finding that Ins<sup>1–/–</sup>Ins<sup>2–/–</sup> mice, which lack all insulin and die of neonatal diabetes, actually show hyperplastic pancreatic islets (38). The translational studies described herein support the hypothesis that all of the new INS gene mutations reported in patients with PNDM/MDI cause proinsulin misfolding. Two mutants described in this report, C<sup>M</sup>V and Y<sup>A19</sup>X, directly lack 1 of the 3 stabilizing disulfide bonds of proinsulin (39), while the mutants L<sup>B6</sup>P, L<sup>B6</sup>V, L<sup>B15</sup>P, and L<sup>B15</sup>V<sub>B16</sub>delinsH involve less obvious structural distortions. The clinical phenotype of the patient carrying the Y<sup>A19</sup>X mutation (diabetes onset at age 88 d) seems to indicate that the mutant protein is translated and that the mRNA decay usually associated with a premature stop codon is not involved. Further evidence for proinsulin misfolding comes from an increased ER stress response (Figure 6). Moreover, examination of R6SC, found in 3 independent probands, as well as Y<sup>A19</sup>X and C<sup>M</sup>V, like the Akita mutant (Figure 4), revealed the presence of improper disulfide bonds with little or no secretion, consistent with entrapment within the ER. The direct demonstration of cell death induced by expression of at least 2 of the mutants (Figure 8) strongly supports a proteotoxic, apoptotic mechanism.

mutation presented with diabetic ketoacidosis at 86 and 134 d after birth. However, this result indicates that such rare patients with INS gene mutants are likely to be hidden within the larger population of those diagnosed with type 1 diabetes. Finally, we advocate the concept of substituting the term MDI for PNDM (41), reflecting our awareness that onset of hyperglycemia in monogenic forms of diabetes extends beyond the neonatal period (refs. 6, 9, 15, and the present study).

**Methods**

Subjects. The Early Onset Diabetes Study Group of the Italian Society of Pediatric Endocrinology and Diabetes (SIEDP) has been studying 37 patients who had diabetes onset within the first 220 d of life and are negative for type 1 diabetes autoantibodies. Of these probands, we detected activating heterozygous mutations of KCNJ11 in 19 subjects (refs. 13, 16, 17, and C. Colombo and F. Barbetti, unpublished observations) and a homzygous inactivating mutation of GCK in a single case (7). We screened the remaining 17 patients for mutations in the coding region of the INS gene and genotyped the parents of each proband with an INS mutation. The INS gene was also examined in 2 additional patients from Denmark with infancy-onset diabetes. Written informed consent was obtained from all participants or their parents. The study was approved by the ethics committee of the Early Onset Diabetes Study Group of the SIEDP.

Clinical studies. We compared the clinical features of the patients carrying mutations of the INS gene with those of patients from our case series carrying a KCNJ11 mutation. An insulin tolerance test was performed by injecting 0.1 IU/kg BW i.v. of regular insulin. Blood samples were withdrawn at −15, 0, 15, 30, and 45 min relative to the time of insulin injection for determination of plasma glucose, pancreatic glucagon (Linco), growth hormone, and cortisol.

Functional studies. Insulin cDNA was kindly provided by H. Lou (NIH, Bethesda, Maryland, USA). Full-length insulin cDNA was amplified by PCR using the following primers containing HindIII or EcoRI restriction sites: Ins-Hind, 5′-CTCGTTGGAAGCTTGTCCTTCTGCCATGGCCCTG-3′; Ins-Eco, 5′-CTCTGTTGAGAATTCCTCCATCTCTCGTGAGCAGG-3′. Amplification product was digested with EcoRI and HindIII, run on 1% agarose gel, purified using Qiagen. Whole-exome sequencing of all INS gene mutants are likely to be hidden within the larger population of those diagnosed with type 1 diabetes. Finally, we advocate the concept of substituting the term MDI for PNDM (41), reflecting our awareness that onset of hyperglycemia in monogenic forms of diabetes extends beyond the neonatal period (refs. 6, 9, 15, and the present study).

Of the patients described herein, 9 of 10 showed normal weight at birth, a finding clearly different from the low birth weight in patients bearing KCNJ11 mutations (15), which suggests that β cell insufficiency may occur primarily after birth. Pancreatic β cell proliferation is low in humans after birth (40). A postpartum cell insufficiency may occur primarily after birth. Pancreatic β cell deficiency in male Akita mice stands in striking contrast with the finding that Ins<sup>1–/–</sup>Ins<sup>2–/–</sup> mice, which lack all insulin and die of neonatal diabetes, actually show hyperplastic pancreatic islets (38). The translational studies described herein support the hypothesis that all of the new INS gene mutations reported in patients with PNDM/MDI cause proinsulin misfolding. Two mutants described in this report, C<sup>M</sup>V and Y<sup>A19</sup>X, directly lack 1 of the 3 stabilizing disulfide bonds of proinsulin (39), while the mutants L<sup>B6</sup>P, L<sup>B6</sup>V, L<sup>B15</sup>P, and L<sup>B15</sup>V<sub>B16</sub>delinsH involve less obvious structural distortions. The clinical phenotype of the patient carrying the Y<sup>A19</sup>X mutation (diabetes onset at age 88 d) seems to indicate that the mutant protein is translated and that the mRNA decay usually associated with a premature stop codon is not involved. Further evidence for proinsulin misfolding comes from an increased ER stress response (Figure 6). Moreover, examination of R6SC, found in 3 independent probands, as well as Y<sup>A19</sup>X and C<sup>M</sup>V, like the Akita mutant (Figure 4), revealed the presence of improper disulfide bonds with little or no secretion, consistent with entrapment within the ER. The direct demonstration of cell death induced by expression of at least 2 of the mutants (Figure 8) strongly supports a proteotoxic, apoptotic mechanism.

Of the patients described herein, 9 of 10 showed normal weight at birth, a finding clearly different from the low birth weight in patients bearing KCNJ11 mutations (15), which suggests that β cell insufficiency may occur primarily after birth. Pancreatic β cell proliferation is low in humans after birth (40). A postpartum decline in measurable C-peptide, taken together with other results presented herein, support the hypothesis that postnatal failure to maintain β cell mass due to proteotoxic proinsulin misfolding is a primary cause of PNDM in these patients with INS gene mutations. We are not able at this stage of our study to explain why the parents of familial cases with the R6SC mutation were diagnosed with diabetes at 1 and 4 yr of age, while 2 probands with the same
Expression of WT proinsulin and each new proinsulin mutant (with the exception of L\(^{40}\)W) plus control proinsulin-R65L and b\(\alpha\)kita mutants were visualized at 48 and 96 h after transfection by immunofluorescence using monoclonal antibody to human proinsulin (1:300 dilution; Abcam) and Alexa Fluor 594 goat anti-mouse (1:500 dilution; Invitrogen). Monoclonal antibody to human proinsulin has no cross-reactivity with either rat proinsulin 1 or rat proinsulin 2. Transfected cells were visualized by immunofluorescence after the following procedure: cells were washed with PBS, fixed in 4% paraformaldehyde for 10 min, washed quickly in PBS, permeabilized in PBS plus 0.2% Triton X-100 for 15 min, blocked in PBS plus 10% FBS for 30 min, incubated with primary antibody for 1 h at 37°C, washed 3 times with PBS plus 0.5% Tween, incubated with secondary antibody for 1 h at 37°C, washed 3 times with PBS plus 0.5% Tween, and mounted with 50% glycerol. Insulin-expressing cells were visualized with a Nikon TE2000-E Eclipse fluorescence microscope and counted along 2 diameters of each well.

**XP1 splicing.** HEK293T cells were transfected with WT or mutant INS cDNA. At 2 d after transfection, RNA was extracted and retrotranscribed, and 50 ng was used for PCR amplification of XP1. Amplified fragments were resolved by agarose gel electrophoresis and scanning densitometry.

Grp78 protein expression. HEK293T cells’ cytoplasmic proteins were extracted in lyso buffer (containing 0.5% Triton X, 1 mM DTT, 1 mM PMSF, 4 mg/ml aprotinin, and 2 \(\mu\)g/ml pepstatin) at 9, 16, and 24 h after transfection with WT or mutant INS cDNA, after which they were loaded on SDS-PAGE gels at 10% and electrophoresed. Filters were decorated with anti-Grp78 antibody (catalog no. PA1-41051, 1:1,000 dilution; Affinity Bioreagents) followed by a peroxidase-conjugated goat anti-rabbit secondary antibody for enhanced chemiluminescence. Anti-human \(\beta\)-tubulin (Santa Cruz Biotechnology Inc.) was used at 1:1,000 dilution with the same secondary antibody.

**Bio synthesis of WT proinsulin and mutations R65L, R65C, L\(^{14}\)P, L\(^{14}\)IP, L\(^{14}\)Y, L\(^{14}\)W, delinsH, C\(^{27}\)RM, Y\(^{32}\)YX, and b\(\alpha\)kita.** Transfected HEK293T cells were preincubated in Met/Cys-deficient DMEM, pulse-labeled with \(^{35}\)S-amino acids, and then chased in complete media. Transfected cells were lysed in 0.1% SDS, 1% Triton X-100, 0.2% deoxycholate, 100 mM NaCl, 10 mM EDTA, and 25 mM Tris, pH 7.4, plus a proteinase inhibitor cocktail. Lysed cells and chase media were subjected to immunoprecipitation with guinea pig anti-insulin, and samples were analyzed by Tris-tricine-urea-SDS-PAGE as described previously (33). To quantify proinsulin secretion, at 40 h after transfection cells were washed and then further incubated for 16 h in serum-free DMEM containing 25.5 mM glucose plus 0.2% bovine serum albumin (RIA-grade). The incubation media were collected, and aliquots were taken to be measured total immunoreactivity of insulin-containing peptides (i.e., proinsulin) using an anti-rat insulin RIA (Millipore) that reacts with WT and mutant proinsulins.

**Apoptosis assays.** HEK293 cells were seeded in a 24-well plate (150,000 cells/well) 24 h before transfection. Cells at 50%–60% confluency were transiently transfected with 800 ng plasmid containing WT proinsulin, a proinsulin mutant (with the exception of L\(^{40}\)W), human familial hyper(pro)insulinemia R65L, or b\(\alpha\)kita. To assay annexin V surface staining, adherent cells at 24 and 48 h after transfection were recovered by trypsinization and collected with complete DMEM. Each culture medium was collected and added to the corresponding sample in order to count detached cells. The annexin V assay (Calbiochem) was performed according to the manufacturer’s protocol. The number of apoptotic cells was measured by FACS Calibur flow cytometer (Becton Dickinson). In the propidium iodide assay, cells were similarly recovered by trypsinization, fixed with 70% cold ethanol, treated with RNase, and stained with 20 ng/ml propidium iodide. The number of apoptotic cells was measured by flow cytometry as described above. The percentage of apoptosis in untransfected, control HEK293 cells (4%), mean of 5 experiments) was subtracted from the percentage of apoptotic cells observed in transfections with WT or mutant (pro)insulins.

**Modeling.** Computer modeling of the structures of human WT and mutant insulins were generated by homology to bovine insulin as a template using Modeller software version 8.1 (http://www.salilab.org/modeller/). Ramachandran plots of the models indicated that there were no residues in disallowed regions.

**Statistics.** Values are expressed as mean ± SEM. A 2-tailed Student’s \(t\) test was used for all statistical analysis. \(P\) values less than 0.03 were considered significant.

**Note added in proof.** Since submission of this report, 4 papers have been published describing INS gene mutations associated with PNDM (42–45).

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