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Biochemistry

A mutant human proinsulin is secreted from islets of Langerhans in increased amounts via an unregulated pathway

(prohormone sorting/hyperproinsulinemia/transgenic mice/insulin biosynthesis)

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ABSTRACT **A coding mutation in the human insulin gene (His-B10 → Asp) is associated with familial hyperproinsulinemia. To model this syndrome, we have produced transgenic mice that express high levels of the mutant prohormone in their islets of Langerhans. Strain 24-6 mice, containing about 100 copies of the mutant gene, were normoglycemic but had marked increases of serum human proinsulin immunoreactive components. Biosynthetic studies on isolated islets revealed that ≈65% of the proinsulin synthesized in these mice was the human mutant form. Unlike the normal endogenous mouse proinsulin, which was almost exclusively handled via a regulated secretory pathway, up to 15% of the human [Asp^{10}]proinsulin was rapidly secreted after synthesis via an unregulated or constitutive pathway, and ≈20% was degraded within the islet cells. The secreted human [Asp^{10}]proinsulin was not processed proteolytically. However, the processing of the normal mouse and human mutant proinsulins within the islets from transgenic mice was not significantly impaired. These findings suggest that the hyperproinsulinemia of the patients is the result of the continuous secretion of unprocessed mutant prohormone from the islets via this alternative unregulated pathway.**

In earlier studies we defined a mutation at position B10 (His → Asp) in the coding region of an insulin allele that cosegregated with affected members of a kindred with hyperproinsulinemia (1, 2). The predicted amino acid substitution does not involve the dibasic processing sites for conversion of proinsulin to insulin, as has been found in two other families with hyperproinsulinemia (3, 4). However, replacement of the histidine residue at position B10 with aspartic acid would be expected to inhibit the association of this proinsulin into hexamers in the presence of zinc, inasmuch as coordination of zinc by this residue stabilizes insulin hexamers (5). The isoelectric point of the mutant (pro)insulin is reduced, while its receptor binding affinity and biological potency are increased by about 4.5-fold (6). Although none of these changes in the properties of the [Asp^{10}]proinsulin would necessarily be expected to alter its proteolytic processing to insulin, the clinical phenotype of this mutation is one of greatly increased circulating proinsulin levels with mild carbohydrate intolerance (1).

To explore the mechanism leading to the hypersecretion of proinsulin in these patients, we have introduced the mutant human insulin gene into mouse embryos to create transgenic animals in which the abnormal proinsulin is expressed in the islets of Langerhans along with the two normal mouse proinsulins. The results indicate that the mutant proinsulin is selectively secreted in increased amounts via an unregulated pathway, bypassing proteolytic conversion and leading to hyperproinsulinemia.

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METHODS

Production of Transgenic Mice. A 12.7-kilobase (kb) *Hin*dIII DNA fragment containing the human [Asp^{B10}]insulin gene was isolated from recombinant clone λMD41 by electrophoretic elution from an agarose gel (7). Fertilized eggs were obtained from mating (C57BL/6 × SJL) F_1 mice and were microinjected as described by Brinster *et al.* (8). About 200 copies of the gene were microinjected per nucleus. Progeny were analyzed for the presence of the human insulin gene by dot-blot hybridization on DNA from tail biopsy samples with a nick-translated 1100-base-pair (bp) *Bgl* II–*Rsa* I fragment of the human insulin gene as probe (9). To confirm that the human gene was intact, transgenic mouse DNA was digested with *Hin*dIII, *Xho* I, and *Pvu* II, analyzed by Southern blot hybridization, and compared with the restriction enzyme map of λMD41.

Insulin Biosynthesis. Islets of Langerhans were isolated as described elsewhere (10) from pooled pancreata of three or four control or transgenic mice and were labeled in groups of 50–100 in 500 μl of medium (11) containing 5 mg of glucose per ml and 50 μCi (1 Ci = 37 GBq) each of [^{35}S]methionine (1463 Ci/mmol), [^{3}H]leucine (186 Ci/mmol), and [^{3}H]phenylalanine (134 Ci/mmol) (Amersham). After a 3-hr preincubation in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum, islets were labeled for 30 min at 37°C in 95% air/5% CO_2. Islets were then washed and chase-incubated for up to 2 hr in medium containing unlabeled methionine, phenylalanine, and leucine. Medium was separated from islets, centrifuged, and then acidified to 3 M with glacial acetic acid. Islets were extracted with acidic ethanol containing 0.5 mg of bovine serum albumin as described (12). Extracts of islets dissolved in 3 M acetic acid or acidified medium samples were then chromatographed over 1 × 50 cm columns of Bio-Gel P-30 eluted with 3 M acetic acid containing 50 μg of bovine serum albumin per ml, and the fractions were assayed in a liquid scintillation counter.

HPLC. Fractions containing proinsulin or insulin from gel chromatography were combined, evaporated to dryness, and redissolved in 50% acetic acid for injection. Chromatography was carried out as described (13) on a 4.6 × 250 mm Vydac C_{18} column. Authentic standards of the rat insulins and of synthetic [Asp^{B10}]insulin (provided by P. Katsoyannis, Mount Sinai School of Medicine) were used to determine elution volumes. Recovery of insulin standards after concentration from gel chromatography buffers as described above was 1.25-fold greater for [Asp^{B10}]insulin than for the two mouse insulins.

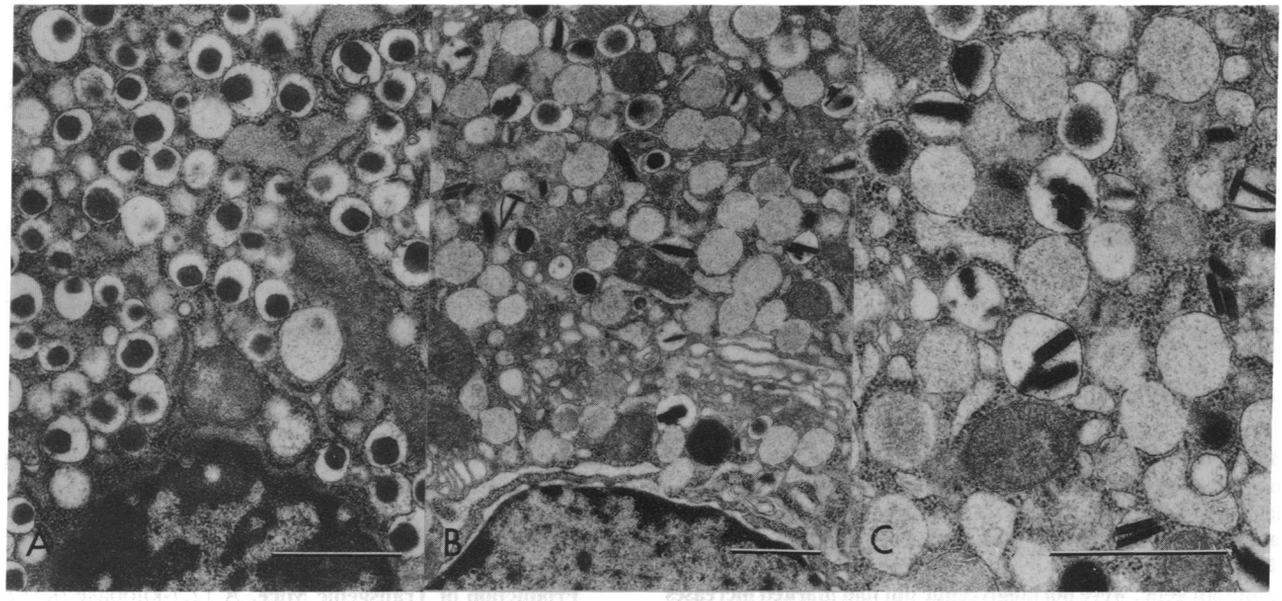

FIG. 1. Electron micrographs of thin sections of control (*A*) and transgenic (*B* and *C*) beta cells. Fixation was in buffered glutaraldehyde/paraformaldehyde (Karnovsky's fluid). [×17,100 (*A*), ×11,400 (*B*), and ×22,800 (*C*); bars = 1 μm.)

Immunoassays. Human proinsulin and C-peptide levels in serum and column fractions were assayed as described by Cohen *et al.* (14) and Polonsky *et al.* (15), respectively.

RESULTS

Several transgenic mice containing variable numbers of copies of the human [Asp^{B10}]insulin gene were obtained, and one of these, mouse 24-6, exhibited marked hyperproinsulinemia in serum. Mouse 24-6 contained ≈100 copies of the human gene (based on hybridization intensity in the dot-blot assay), and it was not a mosaic because its offspring in outcrosses exhibited the expected proportion of affected individuals. The sera from mouse 24-6 and its progeny were normoglycemic (175 mg of glucose per dl of serum) but contained ≈435 pmol of human proinsulin (normal range, 5–12 pmol/liter) and about 300 pmol of human C-peptide (normal range, 300–600 pmol/liter). Fractionation of the serum over Bio-Gel P-30 columns in 3 M acetic acid showed that about 60% of the C-peptide immunoreactivity was made up of material corresponding in size to human C-peptide, while the remainder represented cross-reacting proinsulin (data not shown). These results suggested that some processing of the mutant proinsulin to insulin and C-peptide was occurring.

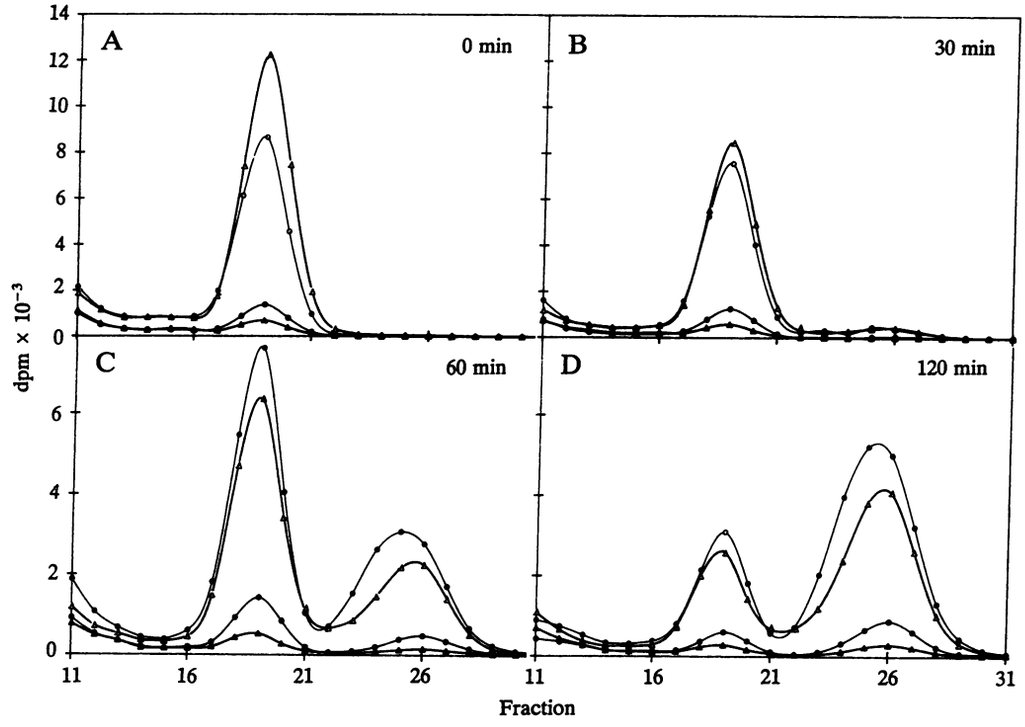

FIG. 2. Bio-Gel P-30 chromatography of labeled proteins from control and transgenic islets showing progressive conversion of proinsulin (fractions 17–21) to insulin (fractions 22–29) during a 2-hr chase. ○, ^{3}H-labeled control islets; ●, ^{35}S-labeled control islets; △, ^{3}H-labeled transgenic islets; ▲, ^{35}S-labeled transgenic islets.

Comparison of electron micrographs of transgenic and normal mouse islets (Fig. 1) revealed the presence of abundant numbers of large, pale, and homogeneous-appearing storage granules in transgenic mice, while the majority of granules in control islets displayed the typical central crystalline insulin inclusions surrounded by a clear space (11). However, HPLC analysis showed that the bulk of the stored material was insulin rather than proinsulin in both control and transgenic islets (data not shown). Hence, the large numbers of pale granules in the transgenic islets are not proinsulin-rich immature granules.

To further assess the biosynthesis, processing, and secretion of the normal and mutant proinsulins, we isolated islets from transgenic and normal mice and studied the incorporation of [^{35}S]methionine, [^{3}H]phenylalanine, and [^{3}H]leucine into proinsulin and insulin in biosynthetic experiments. Methionine does not occur in human [Asp^{10}]proinsulin or in mouse proinsulin I, but it is present in mouse proinsulin II (16). Thus, [^{35}S]methionine incorporation provides a means for identifying the biosynthesis of endogenous mouse insulin II. The results of a pulse–chase experiment are shown in Fig. 2. Analysis of the ratios of newly synthesized proinsulin and insulin in the transgenic vs. normal animals during the 2-hr chase period indicated that the total conversion of proinsulin to insulin was not significantly affected (Fig. 3), even though the mutant proinsulin initially was about 65% of the proinsulin-related material synthesized. This is most evident in Fig. 2*A*, which shows that incorporation of tritiated leucine and phenylalanine into proinsulin was increased in the transgenic animals, while incorporation of [^{35}S]methionine (present only in mouse proinsulin II) was reduced by about half.

To validate the estimates based on amino acid incorporation data, we further analyzed the proinsulin and insulin column fractions by HPLC. The results of some of these analyses are shown in Fig. 4. Under the conditions described, the two mouse proinsulins were well separated. However, an oxidized (methionine sulfoxide-containing) form of mouse proinsulin II comigrated with proinsulin I (Fig. 4*A*). Its contribution to the ^{3}H radioactivity of this peak could be determined from the amount of ^{35}S radioactivity in peak A in comparison with peak B (nonoxidized mouse proinsulin II). The mutant [Asp^{10}]proinsulin was eluted just after the peak containing mouse proinsulin II (peak B). The relative amounts of mouse proinsulin II and human proinsulin in peak B from the transgenic islets were calculated on the basis of the ^{35}S content of the peak (Fig. 4*A*). Analysis by HPLC of labeled material from the 2-hr insulin fractions from transgenic islets gave four peaks, while the corresponding fractions from control islets gave only three (Fig. 4*B*). Peaks A and C comigrated with the oxidized and nonoxidized forms, respectively, of a rat insulin II standard, and peak B (without ^{35}S) corresponded to a rat insulin I standard. Peak D comigrated with synthetic [Asp^{B10}]insulin. The ratio of synthesis of the two mouse proinsulins was 25% I:75% II, and ≈65% of the proinsulin component in transgenic islets was the mutant human prohormone. The ratio of the two mouse proinsulins was unchanged in the transgenic islets relative to that observed in normal mouse islets. In the 2-hr insulin fraction, HPLC analysis showed that the ratio of the mouse insulins was 30% I:70% II, and the mutant human insulin was 54% of the total (after appropriate correction for recovery as described in *Methods*).

The amount of radioactivity attributable to human proinsulin/insulin declined in the transgenic islets during the chase period, and the reason for this became evident when the release of newly synthesized proinsulin and insulin into the medium was analyzed. These data (summarized in Fig. 5) showed a marked increase in the secretion of the mutant human proinsulin from the transgenic islets during the chase period. Analysis of the secreted proinsulin fractions by HPLC revealed that the increased ^{3}H radioactivity was due almost

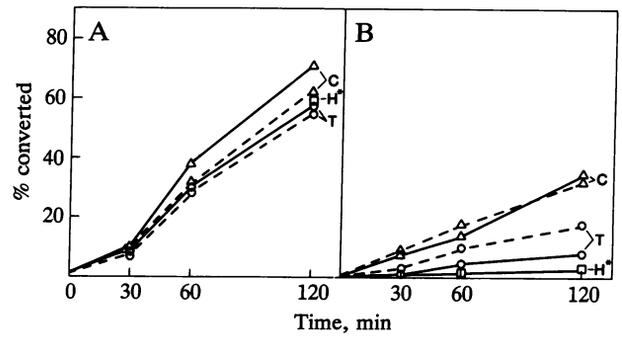

FIG. 3. Graphs showing the percent conversion of proinsulin to insulin in islets and medium combined (*A*) and in the medium alone (*B*) in the experiment shown in Fig. 2. C, control proinsulin; H*, human [Asp^{10}]proinsulin; T, total transgenic proinsulin. --- Mouse proinsulin II based on [^{35}S]methionine incorporation.

entirely to intact human [Asp^{10}]proinsulin (Fig. 6). Up to 15% of the total pool of labeled proinsulin was secreted from the transgenic islets, while only about 2% of the total mouse proinsulin was secreted from control islets during a 2-hr chase. The secretion of the mouse proinsulins from the transgenic mouse islets, however, is only slightly increased (Fig. 5).

In contrast to proinsulin, almost no newly synthesized insulin was found in the medium during the 120-min chase (Fig.

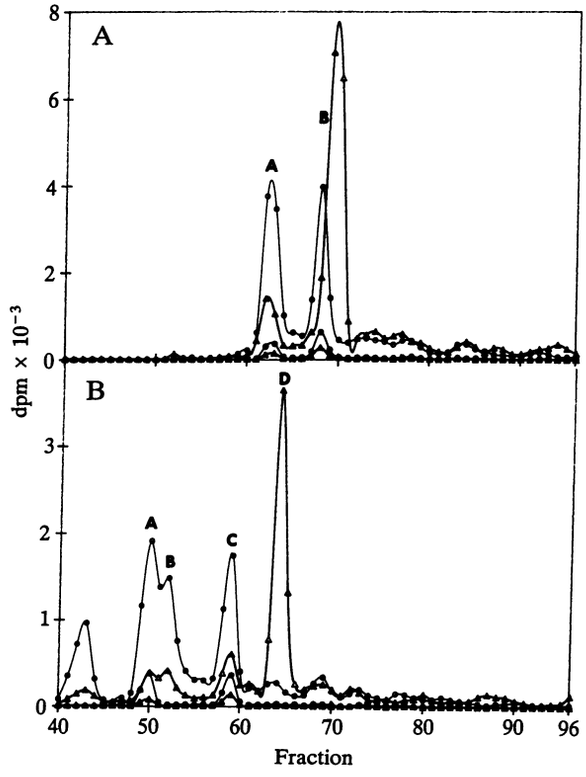

FIG. 4. HPLC analysis of labeled islet proteins. (*A*) Elution profile of the combined zero-time proinsulin fractions (tubes 17–21 in Fig. 2*A*). Peak A represents a mixture of mouse proinsulin I and oxidized (methionine sulfoxide-containing) proinsulin II, while peak B contains mouse proinsulin II; immediately following it is human [Asp^{10}]proinsulin. (*B*) Profile for the 2-hr insulin fractions (tubes 22–28 in Fig. 2*D*). Peak A marks the elution position of an oxidized standard of rat insulin II; peak B, of a rat insulin I standard; peak C, of a rat insulin II standard; and peak D, of a human [Asp^{B10}]insulin standard. (Rat and mouse insulins I and II have identical structure.) ○, Tritiated control islets; ●, ^{35}S-labeled control islets; △, tritiated transgenic islets; ▲, ^{35}S-labeled transgenic islets.

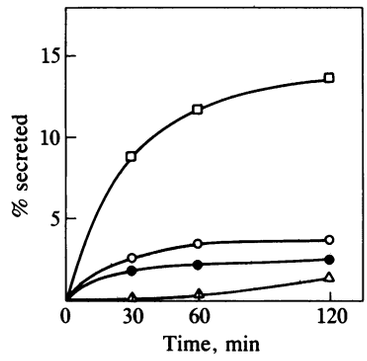

FIG. 5. Secretion of labeled proinsulin or insulin during a 2-hr chase incubation of ^{3}H- and ^{35}S-labeled control or transgenic islets, calculated from P-30 chromatography of media. □, Human [Asp^{10}]-proinsulin from transgenic islets; ○, mouse proinsulin I and II from transgenic islets; ●, mouse proinsulin I and II from control islets; △, total insulin from either control or transgenic islets.

5). It only began to appear at 120 min, consistent with other data indicating that the secretion of newly synthesized insulin occurs mainly in the interval between 2 and 4 hr after biosynthesis, when glucose-stimulated release of new granules begins (17, 18). However, the early release of proinsulin from transgenic islets was not affected by the glucose concentration of the medium (data not shown), indicating that it most likely occurs via a nonregulated or "constitutive" pathway (19).

Although up to about 15% of newly synthesized [Asp^{10}]-proinsulin was secreted, this fraction did not fully account for the decline in radioactivity of the mutant proteins within the islets. Comparison of the levels of incorporation of [^{35}S]methionine vs. [^{3}H]leucine and [^{3}H]phenylalanine throughout the chase in both islets and medium from the transgenic animals revealed a progressive decline in the total amount of human material present, whereas no such decrease occurred in the mouse insulin-related components (data not shown). Since HPLC analysis did not reveal any change in the ratio of the two mouse (pro)insulins, except for the slightly slower rate of conversion of mouse proinsulin II relative to mouse I or human proinsulin throughout the experiment (Fig. 3), it appears that a significant fraction of the human material was selectively degraded. We estimate this fraction to be about 20% of the total (Table 1).

DISCUSSION

The results we have obtained with transgenic mice in these experiments are consistent with earlier reports of tissue-

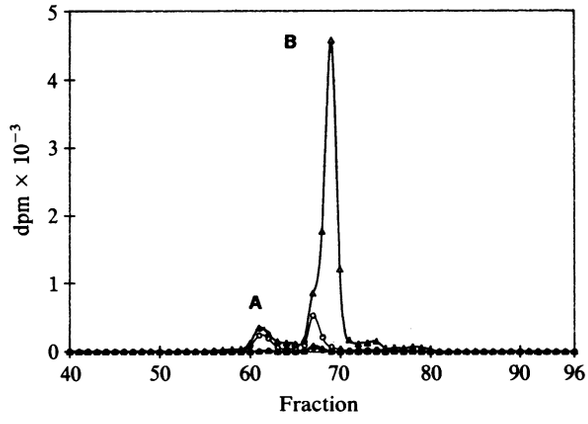

FIG. 6. HPLC profile of secreted proinsulin fraction during a 2-hr chase incubation of control (○, ●) or transgenic (△, ▲) mouse islets. ○, ^{3}H radioactivity; ●, ^{35}S radioactivity. (See Fig. 4*A* for identification of peaks.)

Table 1. Fates of human [Asp^{B10}]proinsulin and mouse proinsulin in a 2-hr postlabeling period

Fate	Proinsulins	
	Human Asp-B10	Mouse I and II
Percent secreted	14	2.5–3.8
Percent degraded	≈20	0
Percent stored	≈66	96–98

specific expression of insulin and C-peptide in the islets of Langerhans of transgenic mice harboring intact human insulin genes (20, 21). The relatively high levels of human insulin expression achieved in the case of strain 24-6 mice (65% of total proinsulin) make these animals excellent phenocopies of the hyperproinsulinemic syndrome reported by Gruppuso *et al.* (1). The mice have significantly increased levels of human proinsulin in serum, in the range of 65 microunits of insulin immunoreactive equivalents per ml. These values are near those reported (1) for the hyperproinsulinemic family members (45–72 microunits/ml). On the assumption that [Asp^{10}]-proinsulin has a biological potency of about 15% of that of insulin (i.e., about 5 times the value for normal human proinsulin), this serum level is equivalent to ≈9–10 microunits of insulin-like bioactivity per ml—a not excessive value. Converting the human C-peptide levels in the mouse serum into insulin equivalents based on the assumption that the serum ratio of C-peptide to insulin is normal (≈10:1) yields an estimate of only 2–3 microunits of additional insulin equivalents per ml—a value that may reflect some degree of secretory suppression by the increased proinsulin. The less abundant mouse insulins would only contribute an additional 1–2 microunits/ml. Thus, the total circulating insulin-like activity in the transgenic mice would be ≈12–15 microunits/ml, a near-normal blood value that is consistent with their normal glucose levels. It is probable that rapid metabolism of the superactive [Asp^{B10}]insulin through receptor-mediated endocytosis might lower its relative serum level and, thus, could explain the failure to detect an abnormal insulin in the circulation in the propositus (1).

When the biosynthesis of insulin was examined in islets isolated from the pancreases of control or transgenic mice, we were surprised to find no significant inhibition of conversion of the [Asp^{10}]proinsulin to insulin, nor did its presence affect the conversion of the endogenous mouse proinsulin. Instead, a significant fraction of the mutant human proinsulin (5–6 times normal) was rapidly secreted from the islets within the first 1–2 hr after biosynthesis. The time course of this process and its lack of regulation by glucose (data not shown) are consistent with the conclusion that the mutant proinsulin enters an unregulated pathway similar to that described in AtT 20 cells (19). Normally, in islets, proinsulin is almost exclusively targeted to the regulated granule pathway, where it is converted to insulin, stored, and secreted in response to glucose and other signals. In studies on rat islets, Rhodes and Halban (22) found that only 0.5% of rat proinsulin enters an unregulated pathway, and in the experiments with control mice reported here, the values were also low—in the range of 2–3%. An additional important feature of the unregulated pathway in AtT 20 cells is the absence of any proteolytic processing of hormone precursors (19), and this was also observed with the secreted mutant proinsulin in this study.

We propose that the partial diversion of [Asp^{10}]proinsulin to the alternative (constitutive) pathway also occurs in the affected family members described by Gruppuso *et al.* (1) and that this may account for the increased levels of the prohormone found in these patients. However, it must be emphasized that the total fraction of mutant proinsulin entering the unregulated pathway is at most only 15%. As shown in Table 1, a fraction (≈20%) is degraded by unidentified mechanisms,

but the majority (≈66%) enters the regulated pathway, where it is converted and later is secreted normally in response to glucose (data not shown). These results suggest that the replacement of histidine by aspartic acid at position 10 causes some of the mutant proinsulin to enter other exit pathways that are not available to normal proinsulin molecules. The most reasonable explanation would be that the Golgi sorting of [Asp^{10}]proinsulin is less efficient, but our data do not provide any indication as to whether this might be due to decreased binding of the mutant prohormone to a "receptor" that normally transfers proinsulin into nascent secretory vesicles in the trans Golgi, or whether it might be due to enhanced binding of the mutant form to receptor(s) that divert secretory proteins into unregulated and/or degradative (?lysosomal) pathways.

It is tempting to speculate that the change in aggregation properties of the mutant proinsulin may somehow account for its aberrant sorting behavior. [Asp^{B10}]Insulin is capable of forming dimers (23), although it does not hexamerize in the presence of zinc or crystallize (D.F.S., unpublished data). Further evidence that [Asp^{B10}]insulin does not crystallize normally is provided by electron micrographs of the β cells in the transgenic mice (Fig. 1). It is not known at present whether sufficient zinc is present in the Golgi to promote the self-association of proinsulin into hexamers or whether this might even be necessary for correct sorting—e.g., guinea pig (pro)insulin does not self-associate (24), and yet it appears to be converted and secreted normally (J. Michael and D.F.S., unpublished data). The altered properties of guinea pig insulin are partly due to the substitution of histidine by asparagine at position B10, but it also differs at a number of other positions from other mammalian insulins and has markedly reduced biological potency. An alternative explanation for the altered sorting of the [Asp^{10}]proinsulin might be that it binds to nascent and/or recycling insulin receptors on their passage through the *trans* Golgi (25, 26) and is either transported to the cell surface or to sites of intracellular degradation. Thus, it may be significant that the increased fraction of mutant proinsulin released is more or less proportional to the increment in its biological potency relative to mouse proinsulin.

The altered sorting behavior of the mutant proinsulin described here accounts rather well for the clinical picture in the patients with this genetic syndrome. However, the impairment of glucose tolerance in the propositus (1) is difficult to explain in view of the clear indications that conversion to proinsulin is not impaired and that some secretion of converted material occurs in the mouse model. Since [Asp^{B10}]insulin has 5-fold greater biological potency than normal insulin, it should certainly be effective in regulating blood glucose, unless most of it is removed from the portal blood by hepatic insulin receptor-mediated uptake and degradation (27, 28). An alternative explanation for the impaired glucose tolerance of the propositus could be the presence of additional genetic factors predisposing to diabetes (e.g., both maternal grandparents had noninsulin dependent diabetes), while the abnormal proinsulin gene was derived via the paternal grandfather who was not diabetic (1).

Recently, Gross *et al.*[‖] reported that expression of rat [Asp^{10}]preproinsulin II in AtT20 cells leads to significantly increased secretion of the mutant proinsulin via the constitutive pathway. The findings reported here show that the abnormal sorting properties conferred by this mutation are not confined to the AtT20 line with its very prominent constitutive pathway (19). Further study of this interesting mutation should help to clarify our concepts of the mechanisms of protein sorting and of secretory regulation in neuroendocrine cells.

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