Proinsulin Disulfide Maturation and Misfolding in the Endoplasmic Reticulum*

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Upon nonreducing Tris-Tricine-urea-SDS-PAGE, newly synthesized proinsulin from pancreatic islets of normal rodents forms a band fast mobility representing the native disulfide isomer, which is efficiently secreted. In addition at least two slower migrating “isomer 1 and 2” bands are recovered, not discernible under reducing conditions, which represent minor species that exhibit less efficient secretion. Although rats and mice have two proinsulin genes, three distinct migrating species are also produced upon proinsulin expression from a single wild-type human proinsulin cDNA. The “Akita-type” proinsulin mutation, which causes dominant-negative diabetes mellitus due to point mutation C(A7)Y that leaves B7-cysteine without its disulfide pairing partner, is recovered as a form that near quantitatively co-migrates with the aberrant isomer 1 band of proinsulin. Anomalous migration is also demonstrated for several other mutations lacking a single cysteine. In islets from PERK−/− mice, which exhibit premature loss of pancreatic beta cells, hyper-synthesis of proinsulin increases the amount of non-native proinsulin isomers. Such findings appear consistent with an hypothesis that supranormal production of nonnative proinsulin may predispose to pancreatic beta cell toxicity.

Proinsulin is comprised sequentially of three regions, the amino-terminal B-chain, the proteolytically removable C-peptide, and the carboxyl-terminal A-chain. Proinsulin contains six cysteines that form three evolutionarily conserved disulfide bonds of the insulin/IGF-1 superfamily, including B7-A7 and B19-A20 interchain pairs and the A6-A11 intrachain pair. At 4.5 amino acids translated per second, it may require 25 s or less to synthesize nascent preproinsulin in the endoplasmic reticulum, and the three disulfide bonds are expected to form either co-translationally or shortly thereafter (1).

Recent evidence suggests that B-chain mutants have increased disulfide mispairing in the secretory pathway (2, 3), even for certain mutants that exert a generally favorable effect on the ultimate stability of the native state (4). Certainly, related members of the insulin/IGF-1 family are predisposed to forming nonnative disulfide bridges during progression through the protein folding pathway (5–7). Of the 15 potential disulfide isomers that might be formed, a few specific disulfide mispaired combinations, called swap I and swap II isofoms, may be preferred (8, 9), although these conclusions are based primarily on folding studies performed in vitro.

Recent hypotheses suggest that a subpopulation of wild-type proinsulin might also misfold in pancreatic beta cells (10). Proinsulin misfolding appears to be linked to the dominant-negative pathogenesis of diabetes mellitus in the “Akita” diabetic mouse, in which one allele of mouse proinsulin-II has the C(A7)Y mutation, which leaves B7-cysteine without its natural disulfide pairing partner (11, 12). However, recent studies have thus far been unable to discern any qualitative or quantitative differences in proinsulin folding either in the islets of Akita mice or in CHO1 cells expressing the Akita-type mutant proinsulin (10).

We have been recently engaged in the analysis of disulfide maturation of newly synthesized insulin precursor by nonreducing Tris-Tricine-urea-SDS-PAGE, as the method has excellent sensitivity to disulfide mispairing (4, 13). We now report distinct monomeric proinsulin structures produced in normal pancreatic islets or from the wild-type proinsulin cDNA, as well as a proinsulin cDNA expressing the Akita-type mutation. The results for the first time identify qualitative and quantitative differences in folding between wild-type and mutant proinsulin and also indicate a subpopulation of wild-type proinsulin molecules engaged in intramolecular disulfide mispairing.

EXPERIMENTAL PROCEDURES

Materials—Guinea pig anti-insulin was from Linco (St. Charles, MO); Polyclonal anti-BiP was from Affinity BioReagents. Zysorbin was from Zymed Laboratories Inc. The [35S]methionine/cysteine mixture was from ICN. Methionine/cysteine-deficient DMEM, dithiothreitol, and Ficoll 400 were from Sigma. MG115 was from Calbiochem. Isopropyl β-D-thiogalactopyranoside (IPTG) and proteinase inhibitor mixture were from Roche Applied Science.

Isolation and Labeling of Rat and Mouse Pancreatic Islets—Islets were isolated from male Sprague-Dawley 200–250-g rats. Isolated islets were washed and recovered overnight in RPMI 1640 with 11.1 mM glucose containing 10% fetal bovine serum plus 1% penicillin-streptomycin before experiments. Islets from 18-day-old PERK−/− and PERK−/− mice (14) were also isolated by pancreatic collagenase digestion, harvesting by gradient centrifugation, and finally recovered overnight in serum-containing medium.

Islets were washed twice in methionine- and cysteine-deficient medium plus 1% bovine serum albumin, 50 μg/ml soybean inhibitor, and 10 mM HEPES, pH 7.35. The islets were then metabolically labeled with [35S]labeled amino acids in the same medium, and when a chase was employed (Fig. 1), it included complete medium plus 10% fetal bovine serum.

Proinsulin Mutagenesis—A human proinsulin cDNA was subcloned into the pcDNA3 vector. Mutant proinsulin cDNAs were created by three PCR reactions. Reaction 1 used forward and reverse primers to amplify a cDNA stretch encoding the signal peptide and the downstream site of the introduced mutation. Reaction 2 used another set of primers to amplify a cDNA encoding the mutation point as well as the
remaining carboxyl-terminal region of proinsulin. The products from reactions 1 and 2 were designed to share ~20-bp overlap in the region of the introduced mutation. Finally, a third PCR reaction used the primers at the 5' and 3' ends of the proinsulin coding sequence along with both gel-purified products from the first two PCR reactions as template, thereby generating the full-length mutant proinsulin cDNA. These were gel-purified and ligated into the pGEM T-vector (Promega). Mutations were confirmed by direct DNA sequencing, and the mutants subcloned into pcDNA3.

Transfection and Labeling of Cells in Culture—293T cells were cultured in high glucose DMEM plus 10% fetal bovine serum and 0.1% penicillin-streptomycin (Invitrogen). The cells were plated into 6-well plates 1 day before transfection. CLA14 cells, a recently described subclone of CHO cells that exhibit IPTG-induced expression of bioactive ATF6 that induces the synthesis of ER molecular chaperones (13), were treated with 15 μM IPTG beginning 1 day before transfection. 2 μg of plasmid DNA was transfected into each well of cells using Lipofectamine (Invitrogen). At 48 h after transfection, cells were pulse-labeled with 35S-labeled amino acids for the times indicated.

cloning was from Linco (catalog number 9015).

selected experiments, pretreatment of live cells before lysis with 10 mM N-ethyl maleimide to block free thiols had no effect on the fractional recovery of newly synthesized nonnative proinsulin isomers. A resolving gel containing 9.3% acrylamide, 0.6% bisacrylamide, 0.1% SDS, 12% glycerol, and 0.0025% Serva Blue in 50 mM Tris, pH 6.8.

Disulfide PAGE—In this paper, unless expressly stated, all media and subsequently employed buffers were devoid of reducing reagents. In selected experiments, pretreatment of live cells before lysis with 10 mM N-ethylmaleimide to block free thiols had no effect on the fractional recovery of newly synthesized nonnative proinsulin isomers. A resolving gel containing 9.3% acrylamide, 0.6% bisacrylamide, 0.1% SDS, 12% glycerol, and 0.0025% Serva Blue in 50 mM Tris, pH 6.8.

RESULTS AND DISCUSSION

Proinsulin Synthesis in Rat Pancreatic Islets—We examined by nonreducing Tris-Tricine-urea-SDS-PAGE (13) the immunoprecipitable proinsulin synthesized in rat pancreatic islets during a 5 min 35S amino acid labeling. The newly synthesized proinsulin existed as a major faster migrating species as well as two faintly detected slower migrating species (arrows highlighted by asterisk, Fig. 1). While proinsulin conversion intermediates also migrate more slowly than proinsulin under these gel conditions (15) 5-min is too short an interval for nascent proinsulin to have initiated proteolytic processing in immature secretory granules (16). After a 30-min chase, more slowly migrating bands appeared, consistent with conversion intermediates (bracket, Fig. 1). Under such short labeling conditions, none of the proinsulin forms were co-precipitated from cell lysates during immunoprecipitation with a polyclonal antibody against the ER molecular chaperone, BiP.

When radiolabeling was extended to 30 min, more 35S-proinsulin was recovered. In conjunction with this was the increased biosynthesis of the two slower migrating species (marked with arrows). During the 30-min labeling period, conversion intermediates (marked with bracket) were already apparent. These bands increased in abundance during a subsequent 30-min chase in conjunction with appearance of the labeled insulin band, but this was not observed for the anomalously slow migrating species of proinsulin. Rather, the slow migrating proinsulin was co-precipitated from cell lysates during immunoprecipitation with anti-BiP. Thus, proinsulin exists as a large native folded subpopulation plus minor isomeric subpopulations that have achieved different, nonnative conformations.

Recombinant Proinsulin Synthesis in Heterologous Cells—Rat pancreatic beta cells synthesize both proinsulin-1 and proinsulin-II gene products. To determine whether the minor, slower migrating proinsulin species were unique to rat islets, we examined heterologous cells transfected to express human proinsulin, which has a slightly different sequence than that of rat proinsulins. Fig. 2A shows full-length gels derived from insulin immunoprecipitation of untransfected and transfected CLA14 cells and their bathing media; these cells were IPTG-induced to overexpress ATF6 and ER molecular chaperones, which tends to increase recombinant secretory protein expression (13). The first two lanes of Fig. 2A indicate the spectrum of nonspecific radiolabeled bands recovered from these cells and medium. After transfection nonreducing Tris-Tricine-urea-SDS-PAGE analysis revealed three newly synthesized proinsulin species (although the band spacing is slightly different than that from rat islets), with the fastest band predominating and already beginning to be secreted during a 50-min chase. These bands collapsed into a single species of slower mobility under reducing conditions, strongly indicating that the minor, slower migrating proinsulin bands are derived from a common translation product. To confirm this point we performed a two-dimensional gel analysis (Fig. 2B). 293T cells were transfected with the proinsulin cDNA and three specific insulin-immunoprecipitable bands were recovered by nonreducing SDS-PAGE (lanes 2 and 3) over and above background from cells transfected with empty vector (lane 1). Individual species were excised from the nonreducing gel. Original samples from mock-transfected or transfected cells (that had not previously been subjected to electrophoresis) were compared under reducing conditions (lanes 6 and 7) against each of the nonreduced excised bands that were now re-run in a second, reduced, dimension. A standard of chemically purified iodinated proinsulin was run as a control under nonreduced (lane 5) or reduced (lane 11) conditions. Thus the minor bands represent isomers that co-migrate upon reduction (Fig. 2B).

When exploring the fate of heterologously expressed proinsulin in 293T cells, we found that these distinct forms do not appear to be handled identically in the secretory pathway. Specifically by 4 h of chase, essentially all of the major, faster migrating proinsulin went on to be secreted (Fig. 2C), while the slower migrating species that were recovered intracellularly at 1 h were not fully recovered at the 4 h chase time, being only partially secreted (see below).

Two exposures of the same gel are shown in Fig. 2D, to enhance visibility of more poorly recovered proinsulin forms. Wild-type proinsulin, as before, was recovered primarily as the well secreted native isomer and two more slowly migrating bands that are arbitrarily named isomer 1 and isomer 2, re-
Disulfide Mispairing in Proinsulin

It has previously been reported that the misfolding of the Akita mutant of mouse proinsulin-II, C(A7)Y, cannot be distinguished from the folding of normal mouse proinsulin-II when expressed either in pancreatic islets or in CHO cells (10). We therefore introduced the C(A7)Y mutation into the human proinsulin cDNA and examined the recombinant product in 293T cells; a representative experiment (in duplicate) is shown in Fig. 3A. A decreased amount of the radiolabeled hAkita mutant was recovered by nonreducing Tris-Tricine-urea-SDS-PAGE. Moreover, the recovered hAkita mutant was quite obviously distinct from that of wild-type proinsulin, exhibiting no band comparable with the native proinsulin species, with most of the population co-migrating with the isomer 1 band of proinsulin. Virtually none of the hAkita mutant was secreted at any chase time (data not shown), unlike the (albeit impaired) secretion of the isomer 1 band of wild-type proinsulin.

En route to the manufacture of all six single Cys mutants of proinsulin, we have examined the C(B19)A point mutant, which leaves A20-cysteine unpaired. Once again, the mutant proinsulin nearly quantitatively failed to form a band comparable with the native proinsulin isomer and was not secreted. Instead, the C(B19)A proinsulin largely disappeared intracellularly after a 5-h chase in a manner that was partially inhibited by the presence of the proteasome inhibitor MG115 (Fig. 3B). Such a finding suggests that at least some of the nonnative proinsulin isomers undergo disposal by ER-association degra-

was very efficiently secreted over 2.5 h, two slower migrating forms of proinsulin were arbitrarily designated isomer 1 and isomer 2 bands, and these were secreted with decreased efficiency. The C(A11)S mutant was comprised of forms that co-migrated primarily with native proinsulin and isomer 1, while the C(A20)A mutant yielded a nonsecreted product that co-migrated with the isomer 2 band of proinsulin.

Expression in islet beta cells of proinsulin lacking one of the 6 critical Cys residues (Cys-A7) is responsible for dominant-negative pathogenesis in the Akita diabetic mouse (10–12, 17–19). It was therefore of interest to examine such proinsulin mutants in relation to the electrophoretic mobility of wild-type proinsulin isomers. We began by creating point mutants in the least important and most important proinsulin disulfide bonds (Fig. 2D). It has already been reported that mutation of both A6 and A11 cysteines does not prevent formation of a band with normal gel mobility nor the ability of the protein to be secreted (13, 20). Expression of the C(A11)S proinsulin mutant yielded two bands: the faster (minor) form essentially co-migrated with native proinsulin and was secreted with high efficiency, and a band running similarly to the isomer 1 band was secreted with ~50% efficiency. This is consistent with a portion of the C(A11)S single mutant to have a simple unpaired A6-cysteine yet form the critical B7-A7 and B19-A20 disulfide bonds, resulting in a band of normal mobility and secretion behavior. By contrast, most C(A11)S mutant co-migrating with the isomer 1 band is likely to have disturbed formation of either the B7-A7 or B19-A20 disulfide bond, probably because of intramolecular attack by the unpaired A6-cysteine residue. Interestingly, expression of the C(A20)A mutant yielded only a single band: a form of the slowest mobility, nearly co-migrating with the isomer 2 band of proinsulin, unable to be secreted.

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with the discovery of dominant-negative diabetes in the migrating proinsulin isomers were significantly increased in pancreatic beta cell toxicity (18). Increased abundance of misfolded proinsulin may predispose to pancreatic beta cell toxicity.

In islets from wild-type and of pancreatic beta cells (22). We therefore chose to examine proinsulin biosynthesis from the islets of wild-type and PERK$^{-/-}$ mice by nonreducing tris-tricine-urea-SDS-PAGE, in relation to total protein synthesis followed by conventional Laemmli SDS-PAGE (23). As shown in Fig. 4 in islets from wild-type mice, proinsulin synthesis was greater at a prevailing glucose concentration of 17 mM than at 5 mM, and this was accompanied by an increase in total protein synthesis. When comparing islets of PERK$^{-/-}$ mice to wild-type mice, proinsulin biosynthesis was increased relative to total protein synthesis, especially at high glucose. In conjunction with increased proinsulin biosynthesis was an increase in slower migrating proinsulin isomers (marked with downward arrows). These bands were distinct from mouse proinsulin conversion intermediates (standards from beta-TC3 cells shown at right). The slower migrating proinsulin isomers were significantly increased in absolute amounts but not relative amounts. In conjunction with the discovery of dominant-negative diabetes in the Akita mouse, the current results appear consistent with a view that increased abundance of misfolded proinsulin may predispose to pancreatic beta cell toxicity (18).

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FIG. 3. Additional single cysteine point mutants of proinsulin analyzed by nonreducing Tris-Tricine-urea-SDS-PAGE. 293T cells were mock-transfected (Control) or transfected with a cDNA encoding wild-type human proinsulin or that contained the Akita mutation C(A7)Y(A) or C(B19)A (B). In A, the cells were radiolabeled for 1 h, in duplicate, and lysed without chase. The hAkita mutant was recovered at decreased levels and the band co-migrated largely with the proinsulin isomer 1 band. In B, the cells were radiolabeled for 90 min and chased for the times indicated, in the absence or presence of 20 μM MG115, a proteasome inhibitor. The C(B19)A mutant also exhibited a mobility consistent with nonnative proinsulin, was not secreted, and was degraded over 5 h in a manner partially protected by the proteasome inhibitor.

FIG. 4. Newly synthesized proinsulin in the isolated pancreatic islets from wild-type and PERK−/− mice. Fifty islets were analyzed in each sample. All islets were preincubated for 40 min at the respective glucose concentrations of either 5.5 or 17 mM. The islets were then radiolabeled for 20 min with 35S-labeled amino acid mixture in the same medium. The islets were then lysed and immunoprecipitated with anti-insulin, and analyzed by nonreducing Tris-Tricine-urea-SDS-PAGE (shown in upper panel). The supernatant of the insulin immunoprecipitations was then analyzed by conventional Laemmli SDS-PAGE (shown in lower panel). (As a standard in the Tris-Tricine-urea-SDS-PAGE above, beta-TC3 cells were pulse-labeled for 30 min with 35S-labeled amino acid mixture and chased for 30 min before cell lysis and insulin immunoprecipitation.) Note that the islets of PERK−/− mice hypersynthesize native proinsulin as well as the slower migrating species of proinsulin (downward arrows) that do not co-migrate with proinsulin conversion intermediates (Conv. Int.) (bracket).