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Partial diversion of a mutant proinsulin (B10 aspartic acid) from the regulated to the constitutive secretory pathway in transfected AtT-20 cells

(prohormone sorting/insulin biosynthesis)

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ABSTRACT **A patient with type II diabetes associated with hyperproinsulinemia has been shown to have a point mutation in one insulin gene allele, resulting in replacement of histidine with aspartic acid at position 10 of the B-chain. To investigate the basis of the proinsulin processing defect, we introduced an identical mutation in the rat insulin II gene and expressed both the normal and the mutant genes in the AtT-20 pituitary corticotroph cell line. Cells expressing the mutant gene showed increased secretion of proinsulin relative to insulin and rapid release of newly synthesized proinsulin. Moreover, the mutant cell lines did not store the prohormone nor did they release it upon stimulation with secretagogues. These data indicate that a significant fraction of the mutant prohormone is released via the constitutive secretory pathway rather than the regulated pathway, thereby bypassing granule-related processing and regulated release.**

Familial hyperproinsulinemia is a genetic disorder characterized by increased serum levels of proinsulin-like material relative to insulin in affected individuals. In two kindreds, an amino acid substitution for arginine at the dibasic cleavage site joining the C-peptide to the A-chain leads to defective processing and increased release of partially cleaved proinsulin (1–3). In contrast, a third family with hyperproinsulinemia has been described in which proinsulin is readily cleaved *in vitro* to mature insulin and C-peptide by tryptic digestion (4). Further investigation of this family revealed a single nucleotide transversion in the codon for residue 10 of the proinsulin B-chain (CAC to GAC), predicting a substitution of aspartic acid for histidine at this position, which is distant from the dibasic cleavage sites (5). To understand why this mutation leads to hyperproinsulinemia, we introduced an identical mutation into the rat insulin II gene and utilized the ability of AtT-20 cells transfected with the insulin gene to correctly process and target the native insulin gene product, leading to regulated secretion of mature insulin (6–8).

MATERIALS AND METHODS

Reagents. All common chemicals were reagent grade from commercial suppliers. Human insulin, proinsulin, ^{125}I-labeled insulin and ^{125}I-labeled proinsulin were kindly provided by Bruce Frank (Eli Lilly). Guinea pig anti-porcine insulin antiserum was obtained from ICN. Rat insulin standard was the generous gift of R. Chance (Eli Lilly). DNA polymerase (Klenow fragment), polynucleotide kinase, T4 DNA ligase, and restriction endonucleases were purchased from Boehringer Mannheim, Bethesda Research Laboratories, or Amersham. [α-^{32}P]dCTP, [α-^{32}P]dATP, and [^{3}H]leucine were purchased from Amersham. Synthetic oligonucleotides were purchased from the University of Massachusetts Medical School Molecular Biology Core Facility (Worcester, MA). The M13 sequencing primer and nonpalindromic *Hin*dIII/*Bam*HI adaptors were purchased from New England Biolabs.

Mutagenesis and Construction of Plasmids. The *Eco*RI/*Bam*HI fragments from the plasmid pRI2-SV-40-46 (9), were cloned into *Eco*RI/BamHI-digested M13mp19 replicative form and used to transform JM109 cells. Recombinant DNA with 193-nucleotide insert encoding the B-chain, the first 6 amino acids of the C-peptide, and part of the second intron was isolated and sequenced by the dideoxynucleotide chain-termination method (10). After correct sequence and orientation were verified, site-directed mutagenesis using a 14-mer (5′-ACCAACTCAGAACC-3′) was performed with the Amersham oligonucleotide-directed system according to the manufacturers' recommendations. Mutants were identified by sequencing; the entire fragment was sequenced to ensure that no other mutations were introduced during the procedure. The mutant 193-base-pair (bp) *Eco*RI/*Bam*HI fragment, the 1058-bp *Eco*RI/*Bam*HI fragment from pRI2-SV40-46, and *Bam*HI-digested, phosphatase-treated pRI2-SV40-46-Del (the parent plasmid lacking the 1251-bp *Bam*HI fragment encoding the proinsulin exons 2 and 3 and the second intron) were ligated and used to transform *Escherichia coli* HB101 cells. Expression vectors allowing expression of both insulin and a dominant selectable marker were constructed by isolating the *Hin*dIII/*Sal* I 1.4-kilobase fragments spanning the entire genomic coding sequence of wild-type and mutant rat insulin II from the pRI2-SV40-46 plasmids, converting the *Hin*dIII site to a *Bam*HI site by ligation to a *Hin*dIII/*Bam*HI adaptor, and ligating the resultant *Bam*HI/*Sal* I fragments to *Bam*HI, *Sal* I-digested DOL (11) vector.

Cell Culture, Transfection, and Sample Preparation: AtT-20 cells were grown in Ham's F-10 medium supplemented with 15% horse serum, 2.5% fetal bovine serum, glucose to a final concentration of 14 mM, and penicillin/streptomycin (100 units/ml) under a 95% air/5% CO_2 atmosphere. Dishes (100 mm) of cells were transfected with 30 μg per dish each with either DOL-insulin or DOL-[Asp^{10}]insulin plasmid DNA by Chen and Okayama's modification of the calcium phosphate procedure (12). G418-resistant clones (Geneticin; GIBCO) were screened by insulin radioimmunoassay as described (13). Clones INS, expressing rat insulin II, and [Asp^{10}]INS,

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Abbreviations: DOL, direct orientation retroviral vector with polyoma early region; BSA, bovine serum albumin; IRI, immune reactive insulin.

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expressing the mutant insulin, were used for all subsequent experiments.

To extract cellular material, semiconfluent 100-mm-diameter dishes containing transfected cells were washed twice with phosphate-buffered saline (PBS) and 5 ml of 5 M acetic acid/0.1% bovine serum albumin (BSA) was then added. The cells were scraped off the dishes and freeze-thawed three times to ensure complete extraction of cellular proteins. After centrifugation (150 × *g*; 10 min) both cell extracts and culture medium were applied to a C_{18} Sep-Pak cartridge (Waters). After a series of washing steps (14), proinsulin/insulin were eluted using 45% acetonitrile in 0.1% trifluoroacetic acid. The eluted materials were lyophilized and redissolved in 250 μl of 0.1% trifluoroacetic acid. The entire procedure resulted in the concentration and partial purification of proinsulin and insulin.

Pulse–Chase Protocol. Tissue culture dishes (100-mm diameter) carrying subconfluent cultured cells were washed twice with warm Hanks' buffered salt solution and preincubated in 6 ml of leucine-deficient F10 medium. The cells were then labeled for 30 min by addition of 1 mCi of [^{3}H]leucine to each dish (1 Ci = 37 GBq). The dishes were washed twice in complete medium and then incubated for a further 30 min postlabel (chase) period. At the end of the chase period, the medium was collected, and the cells were washed and extracted in acetic acid as described above. Both samples were then prepurified and concentrated by Sep-Pak as described for unlabeled cells, with one difference; the products lyophilized after elution from the Sep-Pak cartridges were dissolved in 0.2 M glycine/0.5% Nonidet P-40 (Sigma)/0.5% BSA, and the proinsulin plus insulin was immunoprecipitated using anti-insulin antiserum and protein A-Sepharose as described in detail previously (15). The excess of antiserum over antigen was sufficient to ensure quantitative precipitation of both proinsulin and insulin.

Analytical Methods. Unlabeled samples eluted from Sep-Pak cartridges or radiolabeled products after immunoprecipitation were further analyzed by reversed-phase HPLC. The system used an Altex Ultrasphere ODS 5-μm column and an acetonitrile/TEAP buffer system as described (16–18). For radioactive samples, 1-ml fractions were collected and added to 10 ml of liquid scintillation cocktail (Biocount from Research Products International) and the radioactivity was then measured in a liquid scintillation counter. When insulin immunoreactivity was to be determined, 100 μl of 0.5 M borate/0.1% BSA, pH 9.3, was added to each fraction tube before collection. The 1-ml fractions were lyophilized and redissolved in 1 ml of PBS/0.1% BSA, before radioimmunoassay. Rat insulin was used as the standard with guinea pig anti-porcine insulin antiserum. The cross-reactivity of this antiserum for proinsulin is ≈50%.

RESULTS

To engineer the mutant proinsulin, a 193-bp DNA fragment encoding the B-chain of insulin was excised from the rat insulin II expression vector pRI2-SV40-46 (9) and subcloned into the replicative form of the phage M13mp19 (Fig. 1*A*). A G to C transversion at the first base of the B-10 histidine codon was introduced by oligonucleotide-directed mutagenesis and the mutated fragment was subcloned back into the original vector yielding the plasmid p[Asp^{10}]RI2-SV40-46 (Fig. 1*B*). In preliminary experiments, we cotransfected pRI2-SV40-46 and pSV-neo (19) into AtT-20 cells, resulting in two stable cell lines expressing low levels of insulin (data not shown). To achieve higher levels of insulin expression, we subcloned the coding sequence of both the wild-type and the mutated genes into a DOL expression vector (11) (Fig. 1*C*). With these constructs and a high-efficiency transfection technique (12), ≈37% of AtT-20 G418-resistant colonies

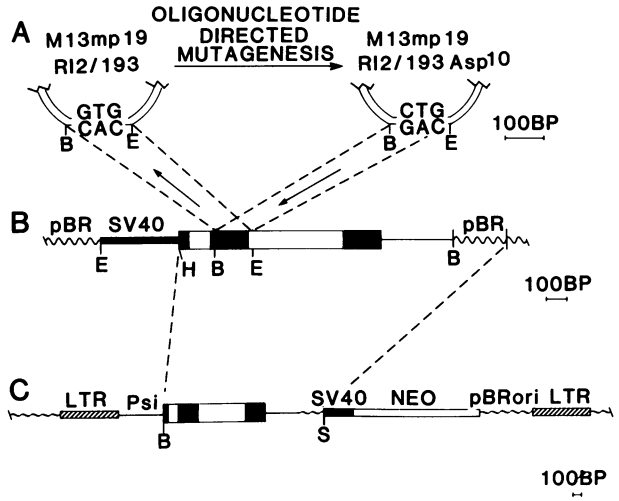

FIG. 1. Mutagenesis and construction of vectors. (*A*) Mutagenesis strategy. (*B*) Structure of the rat insulin II expression vectors pRI-SV40-46 or p[Asp^{10}]RI2-SV40-46. (*C*) Structure of the DOL-INS and DOL-[Asp^{10}]INS expression vectors. The thick box indicates the rat insulin II gene, shaded areas depict exons, and open areas depict introns. E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; S, *Sal* I. SV40, simian virus 40; LTR, long terminal repeat.

expressed immune-reactive insulin (IRI) for both the normal and mutant genes. The rate of IRI release (100-mm subconfluent dishes) was ≈10 ng per dish per hr for the wild type and ≈2 ng per dish per hr for the mutant. Nontransfected AtT-20 cells did not express any detectable IRI.

Insulin-related peptides secreted from the cells were partially purified and concentrated on C_{18} Sep-Pak cartridges and analyzed by HPLC. Culture media positive for insulin immune reactivity from three different clonal cell lines generated by transfection with either the normal or mutated gene demonstrated HPLC radioimmunoassay profiles that were qualitatively similar for each cell type. One cell line of each was used for further analysis. In the case of the INS line (native insulin gene transfectants), ≈75% of IRI released to the medium was mature rat insulin II, and 15% was proinsulin (Fig. 2*A*). The remaining material eluted earlier from HPLC than rat insulin II and has recently been shown to be an oxidized form of rat insulin II (18) (Fig. 2*A*).

Fig. 2*A* also shows HPLC radioimmunoassay analysis of culture media from AtT-20 cell lines stably transfected with the mutant insulin gene. In sharp contrast to the results obtained with the wild-type gene, close to 50% of the IRI in the medium of [Asp^{10}]INS cells was found to be proinsulin. Because the rate of release of total IRI was approximately the same for both cell lines, it did not appear that the predominance of proinsulin in the [Asp^{10}]INS cell culture medium was due to saturation of the cell hormone-processing machinery. Since neither insulin nor proinsulin in the medium is significantly degraded by AtT-20 cells, it was also unlikely that the observed differences resulted from enhanced degradation of native proinsulin compared to [Asp^{10}]proinsulin. We also found that human [Asp^{10}]insulin was similarly resistant to degradation by AtT-20 cells, thus discounting the possibility that once released from cells the mutant insulin, but not mutant proinsulin, was rapidly destroyed (data not shown). We then examined the cellular content of insulin-related peptides in INS and [Asp^{10}]INS cells (Fig. 2*B*): Strikingly, the cellular proinsulin content of both [Asp^{10}]INS and INS cells was <10% of the total immune reactivity.

There is compelling evidence that proteolytic cleavage of prohormones in both AtT-20 cells (21) and pancreatic B cells (22) is a unique function of secretory granules. Our results suggested that the accumulation of proinsulin in the medium,

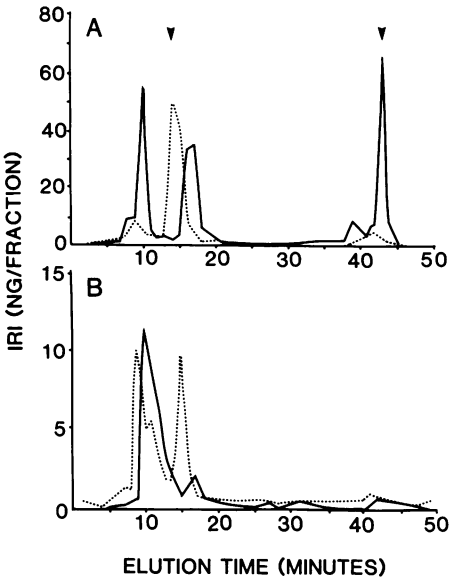

FIG. 2. HPLC analysis of insulin-related peptides in culture medium (*A*) and cellular extracts (*B*) of AtT-20 cell lines stably transfected with plasmids DOL-INS (dashed line) or DOL-[Asp^{10}]RI2 (solid line). Secreted proteins in 4-day-old culture medium of cells and cell extracts were analyzed by reverse-phase HPLC followed by insulin radioimmunoassay. The known elution times for rat insulin II (14 min) and rat proinsulin II (43–44 min) are indicated by arrowheads. The elution position of rat [Asp^{10}]insulin relative to the wild-type molecule is as predicted from HPLC characterization of normal human insulin and chemically synthesized human [Asp^{10}]insulin (data not shown; ref. 20). The early peak preceding the insulins at 9–10 min is oxidized rat insulin II (18). The actual concentration of the proinsulins is approximately twice the values depicted since the cross-reactivity of proinsulin relative to insulin was found to be 50% in our assay.

in conjunction with low cellular proinsulin content in [Asp^{10}]INS cells, was a result of release of the prohormone via the constitutive pathway (23), thereby bypassing secretory granule-related proteolytic cleavage. Studies on the release of newly synthesized proinsulin/insulin lends credence to this concept (Fig. 3). After a 30-min labeling of [Asp^{10}]INS cells with [^{3}H]leucine, 24% of prelabeled mutant proinsulin was released as proinsulin, while only 4% was released as insulin during a 30-min chase period. This finding of extensive and rapid release of newly synthesized prohormone is a hallmark of release via the constitutive pathway (23). By contrast, similar treatment of INS cells resulted in only a modest release of labeled proinsulin

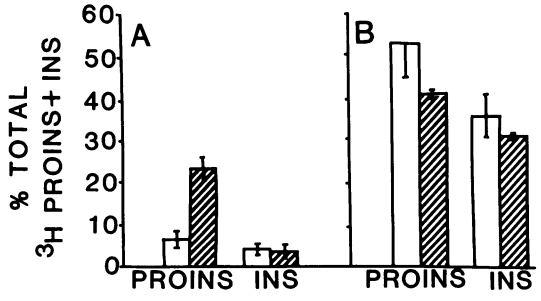

FIG. 3. Fate of newly synthesized proinsulin. INS cells (open bars) or [Asp^{10}]INS cells (hatched bars) were labeled with [^{3}H]leucine for 30 min and then subjected to a 30-min chase. Labeled proinsulin (PROINS) and insulin (INS) were immunoprecipitated and then quantified by HPLC analysis of chase media (*A*) and cell extracts (*B*). The results are expressed as the mean ± SEM (n = 3 for [Asp^{10}]INS cells; n = 4 for INS cells) of the percentage of total detectable labeled proinsulin or insulin in cells and medium. The results have been corrected to take into account the presence of 11 leucine residues in proinsulin but of only 6 in insulin.

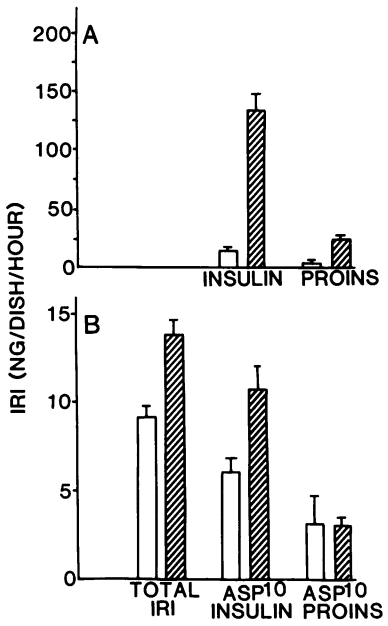

FIG. 4. Rate of release of insulin-related peptides into the culture medium over a 2.5-hr period in the basal condition (open bars) and in the presence of 5 mM 8-Br-cAMP (hatched bars) by INS cells (*A*) or by [Asp^{10}]INS cells (*B*). The media were analyzed by reverse-phase HPLC followed by radioimmunoassay. Proinsulin (PROINS) values have been corrected for cross-reactivity with antiserum used in the radioimmunoassay relative to insulin.

and insulin during the 30-min chase period, as would be expected for cells secreting most proinsulin and insulin via the regulated pathway.

Secretagogues such as cAMP stimulate the release of peptide hormones in secretory granules but not the release of proteins exiting via the constitutive pathway (6). When INS cells were exposed to 8-bromo-cAMP (8-Br-cAMP) for 25 hr, there was a 9-fold increase in both insulin and proinsulin concentrations in the medium, indicating that both insulin and proinsulin were being released via the regulated pathway and that ≈16% of the granule-related material was proinsulin (Fig. 4*A*). When [Asp^{10}]INS cells were exposed to 8-Br-cAMP over a 2.5-hr period, there was an ≈1.5-fold increase in the secretion of total IRI peptides. However, when the effect on individual insulin-related peptides was analyzed, the secretion of insulin was markedly increased, whereas there was no increased secretion of proinsulin, indicating that insulin was being released primarily via the regulated pathway while proinsulin was not (Fig. 4*B*). These data do not exclude the possibility that a small fraction of both mutant and normal insulin is released via the constitutive pathway in AtT-20 cells.

DISCUSSION

The results for INS cells, transfected with the native insulin gene, confirm and extend previous studies in which others (6, 7) have shown that AtT-20 cells transfected with the human insulin gene can correctly process proinsulin via the regulated secretory pathway. By the use of reverse-phase HPLC (16–18) as an analytical tool rather than polyacrylamide gel electrophoresis (6, 7), we additionally show that proinsulin in AtT-20 cells is correctly converted to native insulin with no detectable accumulation of any conversion intermediates. Although AtT-20 cells display an unusually active constitutive release pathway when compared with native pancreatic B cells (24), insulin release from INS cells was nonetheless stimulated some 9-fold by cAMP. This value is higher than that typically observed for release of corticotropin, the endogenous product of AtT-20 cells. We attributed this to the

known functional heterogeneity of many transformed secretory cell types, including AtT-20 cells, rather than the result of any specific manipulation used in the present study.

The transfected gene product in the [Asp[10]]INS cells was handled quite differently from that in the INS cells. Although the proinsulin/insulin ratio within the cells was similar for both types of transfectants, there was markedly more proinsulin released, relative to insulin, from the [Asp[10]]INS cells. There was rapid and extensive release of newly synthesized mutant proinsulin from [Asp[10]]INS cells, whereas the release of newly synthesized proinsulin/insulin from INS cells was only modest. Finally, although [Asp[10]]INS cells responded to cAMP with an increase in the release of IRI, upon HPLC analysis it was found that only the release of insulin, but not of proinsulin, had been stimulated.

The salient features of the constitutive release pathways (23) are that newly synthesized products are released rapidly, that such release is not controlled by secretagogues, and that for prohormones there is only limited conversion to the native hormone. Taken together, all the data are consistent with the partial diversion of proinsulin in [Asp[10]]INS cells from the regulated to the constitutive pathway. Carrol *et al.* (25) have recently reached a similar conclusion based on their studies on transgenic mice expressing human [Asp[10]]-proinsulin. They further found that the mutant prohormone and/or hormone were subject to more extensive degradation within pancreatic beta cells. In our study, long-term intracellular degradation has not been measured. However, during the course of an extended chase period (180 min) in a limited number of additional experiments, it was found that up to 60 min of chase there was no detectable degradation of either mutant or native forms of proinsulin/insulin but that between 60 and 180 min there was more extensive degradation of the mutant forms (data not shown). Although an interesting observation, it remains unclear what causes this increased degradation. In native pancreatic beta cells, insulin is degraded primarily by crinophagy (fusion of granules with lysosomes) (26). As discussed below, [Asp[10]]insulin cannot form Zn hexameric crystals and there are theoretical grounds for expecting increased degradation of noncrystalline insulin species after introduction into lysosomes (26, 27). There is, however, no direct evidence to suggest that the increased degradation of the mutant proinsulin/insulin is occurring by the normal route. It is thus possible that under conditions of inefficient targeting to secretory granules, there is inappropriate degradation of proinsulin either within the Golgi complex itself or as a result of erroneous delivery to other organelles (including, possibly, lysosomes). Finally, degradation within the transport vesicles of the constitutive pathway itself remains a possibility. Whatever the mechanisms, increased degradation of mutant proinsulin or insulin cannot account for the increased proinsulin in the medium of [Asp[10]]INS cells, but it does serve to underline the importance of tertiary and quaternary structure in the intracellular handling of proteins.

While it is impossible to determine from our data the precise point of divergence of the mutant proinsulin from the regulated pathway, it is reasonable to assume that this occurs in the trans-Golgi apparatus, which recently has been shown to be the site for sorting products destined for the regulated or the constitutive secretory pathway in both AtT-20 and pancreatic B cells (7, 22). The mechanism for the partial diversion of [Asp[10]]proinsulin to the nonregulated pathway is unclear. Although it could be due to increased interaction with a putative constitutive pathway receptor, this is unlikely, since recent experimental evidence suggests that protein transport via this pathway is a passive process with positive selection required for targeting to secretory granules (7, 28). Alternatively, it is possible that the B10 histidine residue is part of a domain directly involved in proinsulin targeting to secretory granules. Since the B10 histidine residue enables the proinsulin molecule to form zinc ion coordinated hexamers (29), it is tempting to speculate that the abrogation of this ability by the mutation leads to aberrant targeting. It has recently been shown by immunocytochemistry in both AtT-20 cells (30) and pancreatic B cells (31) that secretory peptides condense in the trans-Golgi prior to secretory granule budding. Thus, it appears very likely that the self-association of proinsulin hexamers (32), in proximity to a presumed secretory granule targeting apparatus, results in the morphologically apparent local concentration of prohormone destined for secretory granules. Our findings are consistent with such a mechanism—namely, that monomeric (or dimeric) proinsulin can be targeted to granules, but at a much lower efficiency than the aggregated hexameric form. Mutant prohormone not targeted to granules apparently either leaves the cell, via the "default" constitutive pathway, or is degraded intracellularly. To validate the hypothesis that proinsulin aggregation enhances targeting to the regulated pathway, it will be of interest to look at proinsulin processing in transfected secretory cells expressing proinsulins with modifications of other amino acids known to be necessary for hexamer formation.

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