# Efficient Targeting To Storage Granules of Human Proinsulins with Altered Propeptide Domain

Sharon K. Powell,* Lelio Orci,‡ Charles S. Craik,§ and Hsiao-Ping H. Moore*

*Department of Physiology-Anatomy, University of California, Berkeley, California 94720;
‡Institute of Histology and Embryology, University of Geneva Medical School, Switzerland; and
§Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143

*Abstract.* In neuronal and endocrine cells, peptide hormones are selectively segregated into storage granules, while other proteins are exported continuously without storage. Sorting of hormones by cellular machinery involves the recognition of specific structural domains on prohormone molecules. Since the propeptide of insulin is known to play an important role in its three-dimensional structure, it is reasonable to speculate that targeting of proinsulin to storage granules would require a functional connecting peptide. To test this hypothesis, we constructed two mutations in human proinsulin with different predicted structures. In one mutation, InsΔC, the entire C peptide was deleted, resulting in an altered insulin in which the B and the A chains are joined contiguously. In the other mutation, Ins/IGF, the C peptide of proinsulin was replaced with the unrelated 12-amino acid connecting peptide of human insulin-like growth factor-I; this substitution should permit correct folding of the B and A chains to form a tertiary structure similar to that of proinsulin. By several biochemical and morphological criteria, we found that Ins/IGF is efficiently targeted to storage granules, suggesting that the C peptide of proinsulin does not contain necessary sorting information. Unexpectedly, InsΔC, which presumably cannot fold properly, is also targeted to granules at a high efficiency. These results imply that either the targeting machinery can tolerate changes in the tertiary structure of transported proteins, or that the B and A chains of insulin can form a relatively intact three-dimensional structure even in the absence of C peptide.

SPECIALIZED secretory cells, such as neural and endocrine cells, have two secretory pathways; a constitutive pathway by which proteins are exported to the cell surface directly after synthesis and a regulated pathway by which peptide hormones and neuropeptides are targeted to dense core secretory granules, where they are stored until release is stimulated by secretagogues (for reviews see 14, 22). Recent work suggests that peptide hormones are actively sorted into dense core secretory granules, whereas constitutive proteins appear to be transported to the cell surface by a bulk-flow process (for review see 22, 32). When a regulated secretory protein, human growth hormone, is fused to a constitutively secreted viral protein, TG (soluble VSV-G), the hybrid protein is routed to regulated secretory granules (20). This suggests that a positive targeting signal is encoded within the structure of regulated proteins. However, sequence comparisons of several proteins targeted to dense core secretory granules do not reveal primary sequence homology that could serve as a common signal for targeting to granules. It is also unknown to what extent the sorting signal depends on the integrity of prohormones' tertiary structure.

Signals for sorting of proteins to other organelles have been identified by analysis of mutated proteins; these results suggest that sequences which are cleaved from precursor proteins during intracellular transport often function as targeting signals. The signal sequence that directs secretory proteins to rough endoplasmic reticulum is usually cleaved soon after nascent polypeptide chains have translocated across the membrane of the rough endoplasmic reticulum and sequences required for targeting of vacuolar (12, 39) and mitochondrial (11, 34) proteins in yeast are cleaved from precursors after the proteins have reached their final intracellular destination. The biogenesis of many peptide hormones also resembles that of vacuolar or mitochondrial proteins: they are synthesized as large precursors, and the propeptides are cleaved from the hormones after they are packaged into secretory granules (7, 25, 26, 28, 38). These proregions are usually biologically inactive and, in the case of insulin, are known to play a major structural role in polypeptide folding and disulfide bond formation (35, 40). Whether these propeptides also contain targeting information or whether they are essential for folding prohormones into structures recognizable by the sorting machinery is currently unknown.

To test the role of proregions in the localization of hormones to dense core granules, we have chosen to study the C peptide of proinsulin. Insulin is structurally and biochemically well characterized and is synthesized as a precursor consisting of three distinct regions: B, C, and A chains (30,

34, and 21 amino acids, respectively). Mature insulin is produced by processing at pairs of basic amino acid residues flanking the C peptide, resulting in a mature form in which the B and A chains are joined together by two disulfide bonds (for reviews of insulin structure, see references 8, 13, 16, 35, 36). We constructed two mutated proinsulins with drastic alterations in the C peptide and compared their ability to be targeted to the regulated pathway with that of wild-type proinsulin. In the first mutation, InsΔC, the entire C peptide, including the proteolytic processing sites, was deleted, resulting in the direct fusion of the B chain to the A chain. It is reasonable to assume that this molecule may not adopt the native conformation of mature insulin since in vitro studies suggest that the C peptide of proinsulin is necessary for proper polypeptide folding and disulfide bond formation (35). This deletion might affect sorting in two ways: either the signal is a part of the deleted sequences or the signal resides in another part of the molecule but becomes obscured as a result of structural changes induced by the deletion. For comparison, we constructed another mutation in which the sequence encoding the C peptide of proinsulin was replaced by the sequence encoding the C peptide of insulin-like growth factor I (IGF-I).[1]

Human IGF-I consists of B and A chains that are 45% homologous to the B and A chains of human insulin but are connected by a 12-amino acid C peptide unrelated to the 34-amino acid C peptide of proinsulin. All six cysteines involved in insulin disulfide bond formation are conserved in the IGFs, and, despite the differences in the C peptide, the overall three dimensional structure of IGF-I is similar to that of proinsulin. Additionally, hybrid proteins made by mixing individual chains from the two proteins have been shown to be biologically active (Fig. 1; also see references 3, 6, 8, 17). IGF-I is secreted primarily by liver cells, which do not have dense core secretory granules; it is not known whether IGF-I is targeted to dense core granules in other tissues (8). Therefore, substitution of the C peptide of proinsulin with the IGF-I C peptide should allow us to assess the sorting information contained within the proinsulin C peptide with minimal perturbation of the molecule's three dimensional structure. The predicted structure of the Ins/IGF hybrid protein is shown in Fig. 1 *D*.

In this study, we examined the effect of these mutations on intracellular transport of proinsulin. We describe results obtained by the following analysis: (*a*) the ability of cAMP analogues to stimulate secretion, (*b*) detection of variant insulins in dense granules isolated on $D_2O$-Ficoll gradients, and (*c*) analysis of the intracellular location of these proteins by indirect immunofluorescence and immunoelectron microscopy.

## Materials and Methods

### Antisera, Hormones, Enzymes, and Primers

Affinity-purified rabbit anti-porcine ACTH was prepared as previously described (18). Guinea pig anti-porcine insulin antiserum was obtained from Linco Research, Inc. (Eureka, MO). Hybridoma HB 125, which recognizes amino acids 8–10 of the A chain of human insulin, was obtained from American Type Culture Collection (Rockville, MD). Rhodamine and fluorescein antibodies were from Cappell Laboratories (Malvern, PA). Insulin and ACTH for RIA standards were from Sigma Chemical Co. (St. Louis, MO). [$^{125}$I]ACTH and [$^{125}$I]insulin for RIA were supplied by the Metabolic Research Unit, University of California, San Francisco. Primers for mutagenesis and for sequencing were purchased from the Biomolecular Resource Center, University of California, San Francisco. Klenow fragment and polynucleotide kinase were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). [$\alpha$-$^{32}$P]dCTP for sequencing was obtained from Amersham Corp. (Arlington Heights, IL) and [$\gamma^{32}$P]dATP for end-labeling oligonucleotide primers was obtained from New England Nuclear (Boston, MA). Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA).

### Mutagenesis

Both insulin mutations were constructed by oligonucleotide-directed mutagenesis. The mutagenic 64-mer used for construction of the insulin-IGF hybrid gene was complementary to the 14 base pairs at the 3′ end of the B chain and the 14 base pairs at the 5′ end of the A chain joined by 36 base pairs complementary to the C peptide of human IGF-I. This primer was annealed to a human proinsulin template in M13. The C peptide deletion mutagenesis was carried out in an identical manner, using a mutagenic 30-mer complementary to the 15 base pairs at the 3′ end of the B chain and the 15 base pairs at the 5′ end of the A chain. Primer extension with Klenow fragment and screening for mutations were carried out according to procedures of Zoller and Smith (41). Mutations were confirmed by dideoxy sequencing (33). The mutated proinsulin sequences were subcloned from the M13 vector into an expression vector, pRSV-poly, which contained the RSV-LTR as a promoter (described in reference 21). For subcloning, M13 RFs were digested with Nco I and Bam HI, and the fragment containing the mutated proinsulin cDNA was gel purified using an Elutip column (Schleicher & Schuell Inc., Keene, NH). 100 ng of RSV vector was digested with Nco I and Bgl II, treated with calf intestinal phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN), and ligated with 100 ng of purified fragment. Enzymes were removed from reaction mixtures by phenol-chloroform extraction and DNA was recovered by ethanol precipitation.

### Cell Culture and Transfection

AtT-20 cells were grown in 15% $CO_2$ in DME H21 supplemented with 10% FCS and 100 U/ml penicillin/streptomycin. DNA transfection was carried out by a modified calcium phosphate precipitation procedure as described previously (23). Cells were cotransfected with 120 μg of pRSV-InsΔC or pRSV-Ins/IGF and 24 μg of the plasmid pSV2-neo-encoding bacterial phosphotransferase which confers neomycin resistance. Stable clones were selected with the antibiotic, G418 (geneticin; Gibco, Grand Island, NY) at a concentration of 0.25 mg/ml active drug. Clones were screened for expression by insulin RIA or by labeling cells with [$^{35}$S]cysteine and immunoprecipitation with polyclonal antiinsulin antisera. ACTH RIA and solid-phase insulin RIA were as described previously (23).

### Immunocytochemistry

For immunocytochemistry, glass coverslips were first coated with 1 mg/ml poly-D-lysine for 1–2 h at room temperature, washed thoroughly with distilled water, and then coated with 5 μg/ml laminin (Gibco) for 12 h at 4°C. One coverslip was placed in each well of a 6-well dish and $1 \times 10^5$ cells were plated per well and grown for 48 h before immunostaining. Cells were fixed in 3.7% formaldehyde in PBS for 30 min at room temperature, rinsed in PBS plus 0.1 M glycine, and permeabilized in PBS/0.1% Triton X-100 for 5 min at room temperature. For ACTH staining, cells were reacted with a 1:20 dilution of affinity-purified rabbit anti-ACTH, followed by a 1:50 dilution of rhodamine-conjugated goat anti-rabbit antibody. For insulin staining, culture supernatant from hybridoma HB 125 was collected and concentrated fivefold by centrifugation through a Centricon 10 filter according to the manufacturer (Amicon Corp., Danvers, MA). Fixed, permeabilized cells were reacted with concentrated supernatant, followed by 1:150 dilutions of fluorescein-conjugated goat anti-mouse antibody and fluorescein-conjugated rabbit anti-goat antibody applied sequentially. All antibodies were diluted in PBS/0.1 M glycine. Incubations were carried out at room temperature for 30 min and were followed by three 10-min washes in PBS/0.1 M glycine. Coverslips were mounted onto slides with 10 μl of 1,4-diazobicyclo(2,2,2)octane in 90% glycerol/PBS.

For thin section immunocytochemistry, AtT-20 cells were fixed with 1% glutaraldehyde buffered in 0.1 M phosphate, pH 7.4, dehydrated with ethanol, and embedded in Lowicryl K4M at low temperature (1). The sections were collected on nickel grids and immunolabeled with the polyclonal antiinsulin serum (1:1,000) revealed by the protein A-gold method (31).

1. *Abbreviation used in this paper*: IGF-I, insulin-like growth factor I.

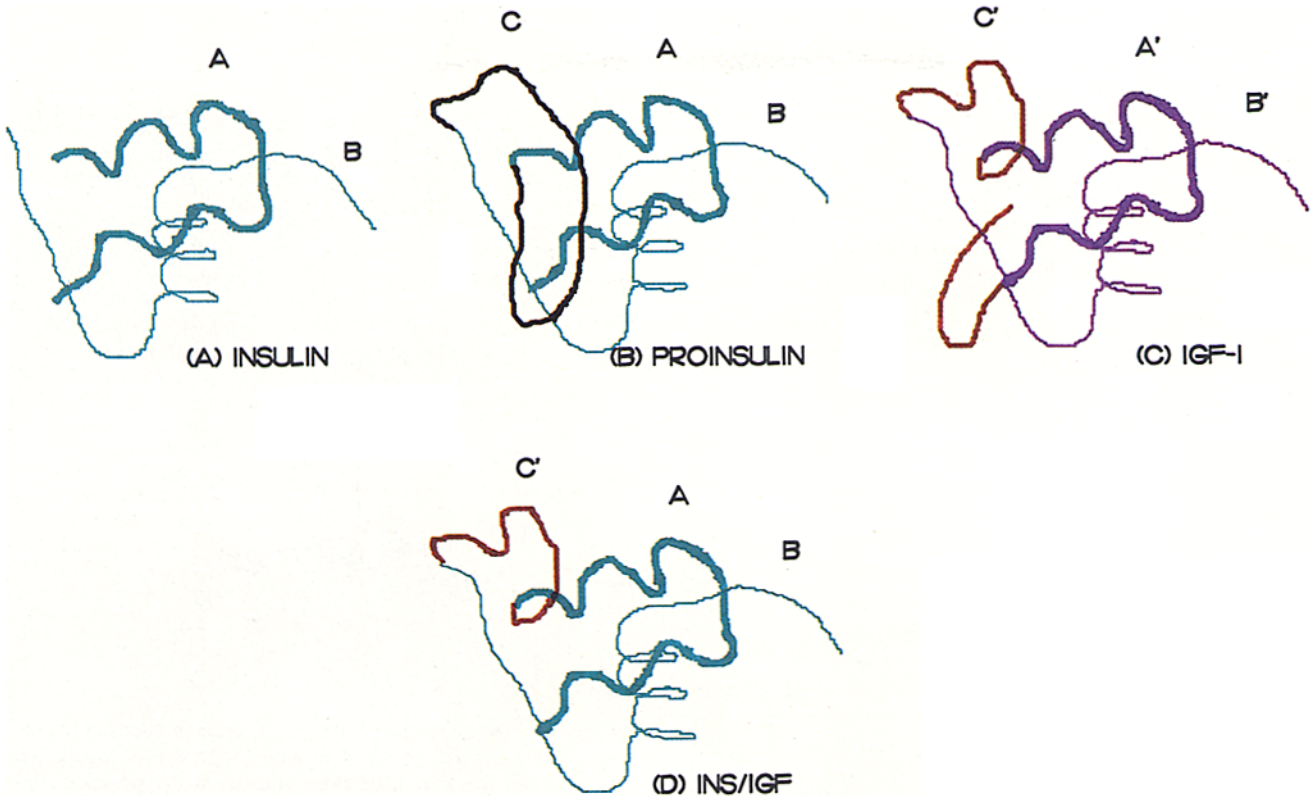


*Figure 1.* Predicted structure of Ins/IGF. *A*, *B*, and *C* are structures of insulin, proinsulin, and IGF-I, respectively, redrawn from Blundell et al. (3). *D* shows the predicted structure of the hybrid protein Ins/IGF.

### *Immunoprecipitation and Quantitation of Sorting Index*

Selected stable transfectants were labeled for 15 h with 0.5 mCi of [$^{35}$S]cysteine (Amersham Corp.) in cysteine-free DME supplemented with 2% FCS (3 ml media/10-cm dish). The sorting index was then determined as described previously (19). In brief, the 15-h labeling media was removed and the steady-state rate of secretion (N) was measured by incubating the cells with labeling medium for an additional hour. Cells were then rinsed with complete DME supplemented with 2% FCS and chased in this medium for three 3-h periods. During the last chase (6–9 h), 5 mM 8-Br-cAMP (Sigma Chemical Co.) was added to one dish to induce release from regulated secretory granules. Media and cell extracts were harvested, subjected to double immunoprecipitation, and analyzed on 10–18% SDS–polyacrylamide gels as described (20). For quantitation of sorting index, autoradiograms were scanned and the area under the peaks was determined.

### *Isolation of Dense Core Secretory Granules*

Secretory granules were isolated from AtT-20 clones expressing Ins/IGF and InsΔC on $D_2O$-Ficoll gradients as described (9). Cells were harvested from three confluent 15-cm tissue culture dishes using PBS/5 mM EDTA. The cell pellet was resuspended in 15 ml cold homogenization buffer (250 mM sucrose, 10 mM Hepes, pH 7.4, 2 mM EGTA, 1 mM EDTA) and cells were homogenized in a ball-bearing homogenizer (European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany; chamber, 8.020 mm, bearing, 8.004 mm) 1 ml at a time, 6 strokes/ml. The postnuclear supernatant was fractionated on a $D_2O$/Ficoll gradient and fractions were analyzed by ACTH RIA as previously described (9). For insulin RIA of gradient fractions, 50 μl of each fraction was diluted to 900 μl in 50 mM sodium phosphate buffer, pH 7.4, 0.5% BSA (Sigma Chemical Co.; fraction V), 0.5% NP-40 and boiled for 1 min to release proteins from membrane fractions. Insulin standards were prepared in 900 μl of the same buffer. 100 μl of a 1:15,000 dilution of guinea pig anti–porcine insulin antisera and 5 μl [$^{125}$I]insulin (40 μg/ml) in 50 mM sodium phosphate, pH 7.4/0.5% BSA was added to the samples and incubated for 12 h at 4°C. 1 μl of fixed *Staphylococcus aureus* (Pansorbin; Calbiochem-Behring Corp., San Diego, CA) cells was added to the reaction mixture and incubated for 30 min at room temperature with shaking. Cells were pelleted at 1,700 *g* for 10 min, and the amount of cell-associated [$^{125}$I]insulin was determined by counting the pellets in a gamma counter.

## *Results*

### *Strategy and Construction of Mutated Proinsulins*

To determine if the C peptide of proinsulin contains the targeting signal for the regulated pathway, we constructed two mutations in the human proinsulin cDNA by oligonucleotide-directed mutagenesis. To construct a mutation lacking the entire C peptide (InsΔC), we deleted the sequence encoding the C peptide region from the proinsulin cDNA, directly joining the sequence encoding the B peptide with the sequence encoding the A peptide (see Fig. 2). Since this mutation also removed the two prohormone processing sites flanking the C peptide, we predicted that the protein encoded by this DNA should not be proteolytically processed and should be synthesized as a single polypeptide chain containing both B and A sequences. In the second mutation, Ins/IGF, the C peptide of insulin was replaced with the 12–amino acid connecting peptide of IGF-I (see Fig. 2). The C peptide of IGF-I is not flanked by pairs of basic residues and is not proteolytically processed from the molecule; thus, Ins/IGF also should be synthesized and secreted as a single polypeptide chain. Fig. 2 shows the construction of both mutated proinsulins in an M13 vector and their subcloning into an RSV vector for expression in AtT-20 cells. The entire se-

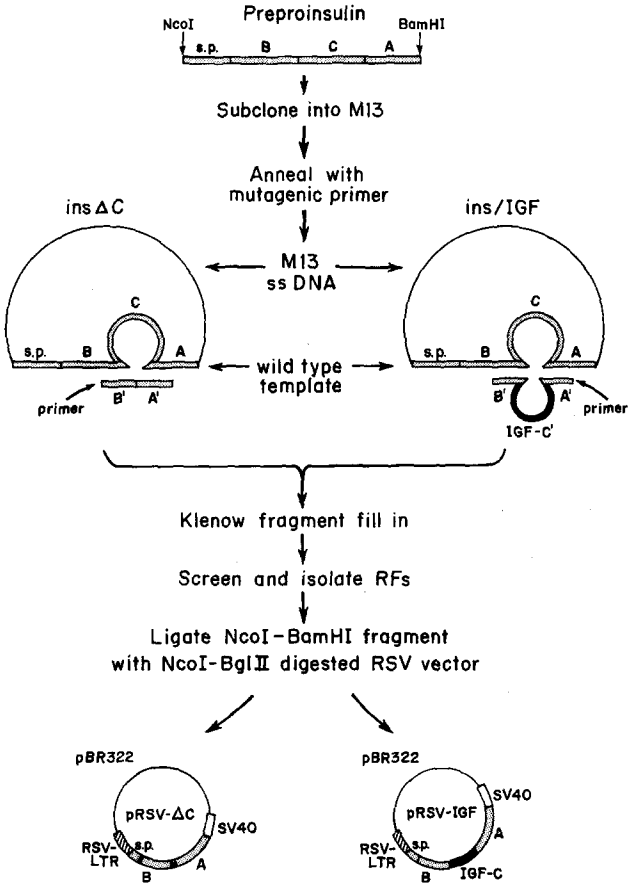


*Figure 2.* Construction of Ins/IGF and InsΔC. Both insulin mutations were constructed by oligonucleotide-directed mutagenesis (41). Primers were annealed to a wild-type insulin template cloned in M13. For Ins/IGF, a mutagenic 64-mer was used to replace the region complementary to the C peptide of human insulin with a region complementary to the C peptide of IGF-I. A mutagenic 30-mer complementary to the desired junction regions of the B and A chains was used to delete the C peptide. The entire sequence of both mutated genes was confirmed by the dideoxy method (33). Mutated fragments were subcloned from mp vectors on Nco I–Bam HI fragments and ligated into Nco I–Bgl II-digested RSV vector. The following cloning sites had been inserted in the vector at the 3′ end of the long terminal repeat: Hind III-Xba I-Nco I-Eco RI-Sma I-Bgl II.

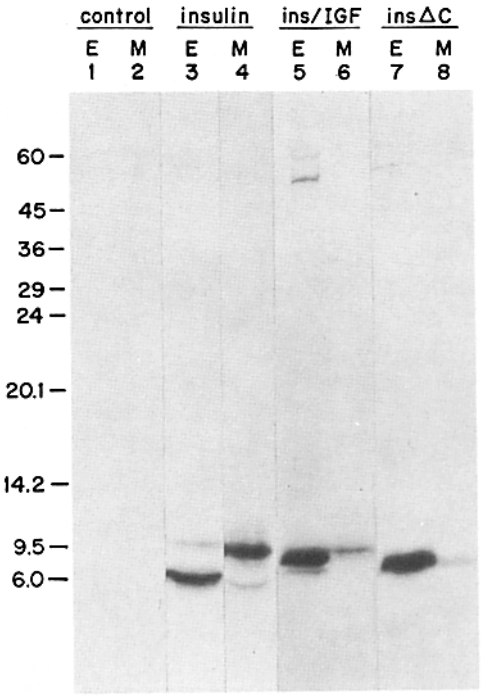


*Figure 3.* Expression and secretion of insulin and mutants in AtT-20 cells. Cells were labeled for 16 h with [$^{35}$S]cysteine. Media and cell extract samples were immunoprecipitated with a polyclonal insulin antibody as described (19). Lanes *1* and *2*, extract and medium from untransfected AtT-20 cells; lanes *3* and *4*, extract and medium from cells transfected with wild-type proinsulin; lanes *5* and *6*, extract and medium from cells transfected with Ins/IGF; lanes *7* and *8*, extract and medium from cells transfected with InsΔC. The high molecular mass bands in lanes *5* and *7* were nonspecific and were not reproducible between experiments.

quences of Ins/IGF and InsΔC were confirmed by DNA sequence analysis using the dideoxy method (data not shown).

### *Expression and Secretion of Mutated Proinsulins in AtT-20 Cells*

The effects of mutations on sorting were analyzed in mouse pituitary AtT-20 cells. Previously, we have shown that this cell line correctly sorts transfected human proinsulin into dense secretory granules and processes it to mature insulin (23). AtT-20 cells were cotransfected with plasmids carrying the mutations described above and the plasmid pSV2-neo, which confers neomycin resistance. Stable transfectants were obtained by selection in 0.25 mg/ml G418. Clones surviving the selection were screened by insulin RIA. Since the amount of nonselectable DNA was in large excess over selectable DNA, ~75% of selected G418-resistant colonies also expressed the insulin variants. The cell lines used for the following experiments produced 10–70 ng of immunoreactive insulin/3 × $10^6$ cells per 48 h, which is typical of the level of expression from an RSV-driven promoter in AtT-20 cells. These cells produce the endogenous hormone ACTH at a much higher level; ~2 μg/3 × $10^6$ cells per 48 h.

Both mutated proinsulins were synthesized and secreted by AtT-20 cells. Transfected cells were metabolically labeled with [$^{35}$S]cysteine for 16 h, and cell extract and media samples were immunoprecipitated with a guinea pig antiinsulin antiserum and analyzed on reducing SDS–polyacrylamide gels (Fig. 3). Lanes *3* and *4* show extract and media samples from cells transfected with wild-type proinsulin DNA. Two polypeptides were immunoprecipitated from both media and extracts: the upper 9.5-kD polypeptide is proinsulin, and the lower 6.0-kD band contains the B and the A chains of mature insulin (the two chains are unresolved under these gel conditions). Note that in the absence of stimulation, proinsulin is secreted constitutively and is the predominant form in the media. Mature insulin is the predominant form found in the cell extract; it is stored intracellularly until release is stimulated (Fig. 3, lanes *3* and *4*; also see reference 23). Cells transfected with the Ins/IGF plasmid synthesize and secrete a major polypeptide migrating at ~7.5 kD, the predicted molecular mass of the insulin–IGF hybrid (lanes *5* and *6*). A minor band migrating just below the 7.5-kD band is also

specifically immunoprecipitated from cells expressing the Ins/IGF gene. Pulse-chase experiments show that these two proteins do not have a precursor-product relationship (data not shown); hence the lower band could be a degradative product of the 7.5-kD protein during isolation. In cells expressing the InsΔC plasmid, extract and media samples contain a single polypeptide that migrates slightly faster than the Ins/IGF hybrid at ~7.0 kD, which is the predicted molecular mass of B plus A chain (lanes *7* and *8*).

As discussed earlier, Ins/IGF is expected to fold similarly to proinsulin and form proper disulfide linkages; in vitro studies using short synthetic cross-linkers to join the B and A chains of insulin also enable the formation of the correct structure (40). In support of this, we found that reduction of Ins/IGF with mercaptoethanol results in a decrease in the mobility on SDS-polyacrylamide gels that is similar to the decrease observed for proinsulin (data not shown). In contrast, reduction of InsΔC did not produce a comparable shift in mobility (data not shown).

Although both Ins/IGF and InsΔC can be detected in the culture supernatant from unstimulated cells, the majority of the labeled polypeptide was found in the cell extracts (Fig. 3). The slow release of these proteins in the absence of stimulation indicates that they might be stored in dense core granules. However, it is also possible that these genetically altered proteins do not fold correctly and are retained in the endoplasmic reticulum. These different possibilities are examined below.

### *Intracellular Localization by Immunocytochemistry*

To determine where the variant insulins are located within the cell, we first examined the cells by indirect immunofluorescence. AtT-20 cells grown on laminin extend long processes, and dense core granules are concentrated in the tips of processes and also are located at the periphery of these cells (Rivas, R., and H. P. Moore, unpublished data; also see reference 15). Cells stained with anti-ACTH antibodies revealed punctate staining in the cytoplasm and intense fluorescence at the tips of processes (Fig. 4 *A*). Proinsulin transfected into AtT-20 cells is packaged in the same granules as ACTH and shows a similar staining pattern (27). In contrast, a soluble form of the vesicular stomatitis virus G protein that is known to be secreted by the constitutive pathway (19) shows diffuse cytoplasmic staining; no visible staining could be detected in the processes (Fig. 4 *B*; see *arrows*). This staining most likely represents the rough endoplasmic reticulum, since the majority of this protein inside the cell has endoglycosidase H-sensitive oligosaccharides (19). When cells expressing either Ins/IGF (Fig. 4 *C*) or InsΔC (Fig. 4 *D*) were stained with an mAb that recognizes both proinsulin and mature insulin, the distribution of immunoreactive sites resembled that of ACTH and wild-type insulin; the most intense staining was found at the tips of processes. The mutated insulins also showed some staining of the juxtanuclear regions which was not observed for ACTH staining. This is due to the poor reactivity of the ACTH serum against the precursor POMC and consequent lack of Golgi staining (Orci, L., and H. P. Moore, unpublished data).

To determine the subcellular localization of the mutated insulin InsΔC, we stained thin sections for electron microscopy with a polyclonal antiinsulin serum revealed by the protein A-gold technique. Insulin immunoreactivity was distinctly and specifically located over dense core secretory granules (Fig. 5).

### *Response to Stimulation by cAMP*

As described above, immunocytochemistry revealed that the mutated insulins are located within dense core secretory granules. To quantitate the efficiency at which they are sorted, we measured the "sorting index" according to a label-chase protocol described previously, which measures the relative increase in the rate of secretion when cells are stimulated by secretagogues (19). Since only proteins targeted to granules are responsive to 8-Br-cAMP, the extent of secretagogue-induced secretion relative to basal secretion is a measure of the efficiency at which a protein is targeted to the regulated pathway. Fig. 6 shows a comparison of media samples collected from control cells and from cells stimulated with 8-Br-cAMP. Secretion of both Ins/IGF and InsΔC is strongly stimulated by 8-Br-cAMP (compare lanes *1* and *3*, stimulated, to lanes *2* and *4*, unstimulated). Calculation of sorting indices shows that Ins/IGF and InsΔC are efficiently sorted into the regulated pathway: wild-type human proinsulin has a sorting index of 0.66, and Ins/IGF and InsΔC have higher sorting indices of 2.2 and 4.4, respectively (Table I). All three proteins are sorted with efficiencies at least two orders of magnitude higher than constitutive secreted proteins (e.g., sorting index of soluble VSV-G, 0.002–0.006, see reference 19). The higher sorting indices of the mutated proteins are not a result of higher sorting capability of the isolated clones, since analysis of the sorting indices of ACTH in these cells shows that they are similar to that of untransfected cells (data not shown). The mutant proteins are stable within the cell and in the medium; the radioactivity incorporated during the labeling period can be quantitatively recovered at the end of the 9-h chase period (data not shown). Thus, the high sorting indices of the mutated proteins are not a result of differential stabilities in certain cellular compartments.

### *Biochemical Fractionation of Dense Core Secretory Granules*

The results of both immunofluorescence and labeling experiments described above indicate that Ins/IGF and InsΔC are targeted to the regulated pathway. To further demonstrate that the mutated proteins are stored in dense-core secretory granules, unstimulated cells were homogenized and fractionated on $D_2O$–Ficoll gradients (Fig. 7). As shown previously, dense core secretory granules have a high protein content and can be separated from other cellular organelles on this gradient (9). Since AtT-20 cells store the endogenous hormone ACTH in dense core granules, we assayed the gradients by an ACTH RIA to determine where dense core granules migrate. The peak of immunoreactivity migrating close to the bottom of the gradient (fractions 2–6) represents dense core granules containing mature ACTH, whereas those in the middle (see Fig. 7 *B*, fractions 9–12) and the top of the gradient are other membranes containing the ACTH precursor, POMC (see references 9 and 10; Figure 7). To determine whether the mutated proinsulins could be detected in dense core granule fractions, aliquots from each fraction were also assayed by an insulin RIA using a polyclonal insulin antiserum that recognizes both mutated proteins. Fig. 7 *A* shows

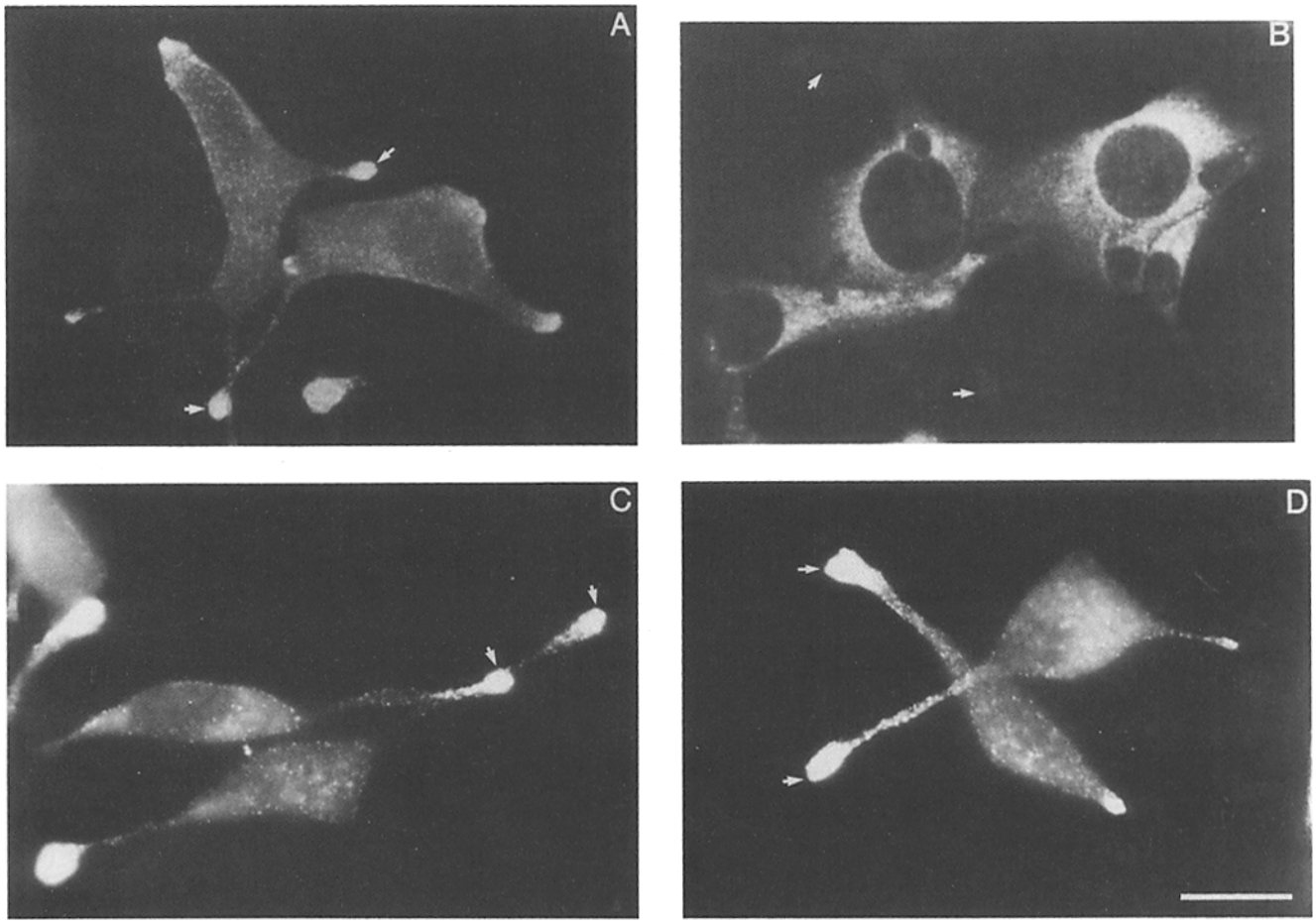


*Figure 4.* Intracellular localization of mutant insulins by indirect immunofluorescence. AtT-20 cells were grown on laminin-coated coverslips, fixed in 3.7% formaldehyde, and permeabilized in 0.1% Triton in PBS. Cells were stained with affinity-purified rabbit anti-ACTH or culture supernatant from hybridoma HB 125 which recognizes A8-10 of insulin, followed by fluorescein- or rhodamine-conjugated secondary antibodies. (*A*) Cells expressing InsΔC stained with an anti-ACTH antiserum and fluorescein goat anti-rabbit antisera. Most immunoreactivity is concentrated in the tips of cell processes. (*B*) Cells expressing a constitutively secreted protein, soluble vesicular stomatitis virus G (19), stained with a rabbit anti-VSV-G antiserum followed by fluorescein-conjugated goat anti-rabbit antibody. Note the abundant cytoplasmic fluorescence but the lack of staining in the tips of cell processes (*arrows*). (*C* and *D*) Cells expressing Ins/IGF and InsΔC, respectively, stained with a mouse mAb (HB 125) followed by fluorescein-conjugated goat anti-mouse antibodies. Note staining of the tips of processes as well as perinuclear Golgi regions. Bar, 40 μm.

that Ins/IGF colocalized to the same fractions at the bottom of the gradient as ACTH: a peak of insulin immunoreactivity can be clearly identified in fractions containing dense core granules. Cells expressing InsΔC also exhibited a prominent peak of insulin immunoreactivity in this region (Fig. 7 *B*). A similar profile was also obtained from cells expressing wild-type proinsulin (data not shown).

Recovery of each hormone in the granule peak was quantitated as a percentage of total amount of that hormone present in the cell homogenate. Since the actual yield of granules varies from experiment to experiment, for a given gradient the recovery of the mutated proteins in granule fractions was normalized to the recovery of the endogenous hormone ACTH. These numbers represent the amount of transfected hormone stored in granules relative to the endogenous stored hormone ACTH. The ratio of immunoreactive insulin to ACTH recovered in the granules is 1.14 for cells expressing wild-type proinsulin, and 3.5 and 3.0 for cells expressing Ins/IGF and InsΔC, respectively. Thus, both mutants are targeted to the granules even more efficiently than wild-type insulin.

## Discussion

Many peptide hormones are synthesized as larger precursors which are proteolytically processed during transport. Biologically inactive propeptide(s) (such as the C peptide of insulin) are often copackaged into granules and released with active hormones. Copackaging of propeptides into granules suggests that cleavage occurs after precursors have already been imported into granules. Alternatively, each cleaved product derived from a precursor may contain a sorting signal and may be sorted individually after processing. Recent studies indicate that sorting of hormones precedes proteolytic processing. Proinsulin is converted to mature insulin in newly formed acidifying granules that do not contain consti-

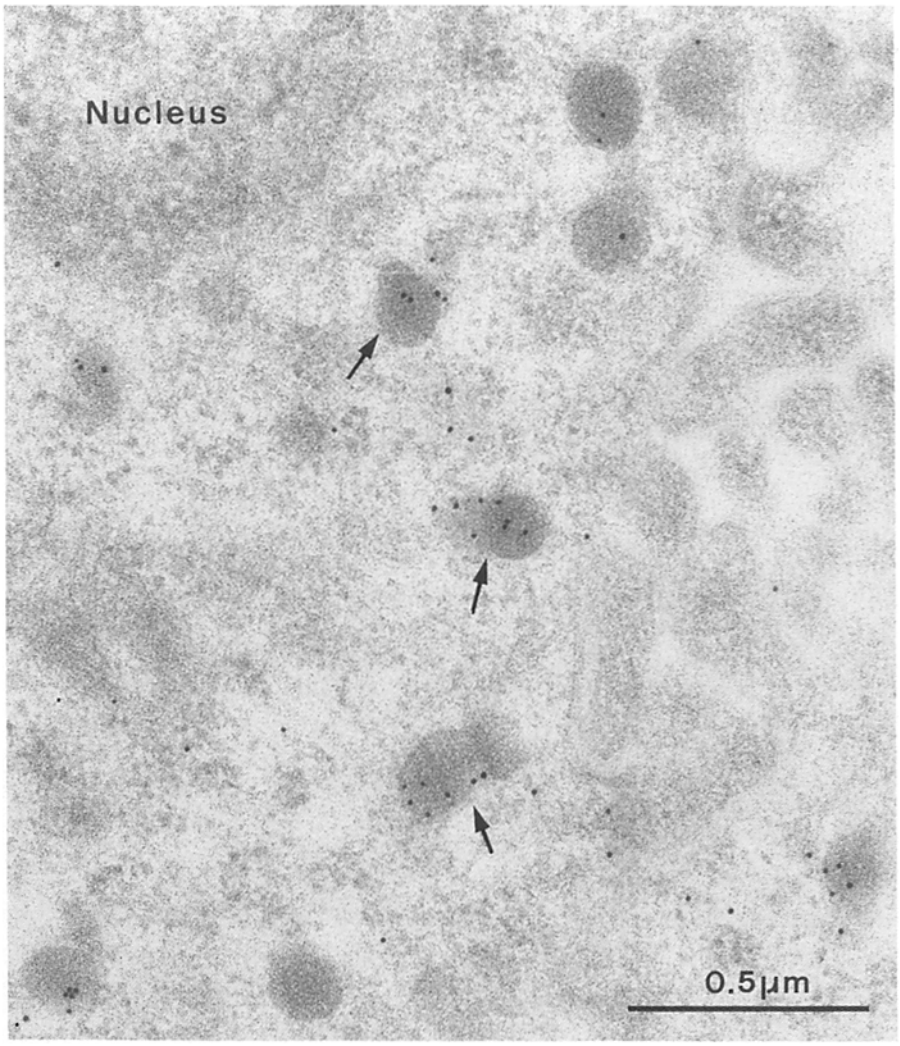


*Figure 5.* Thin section of an AtT-20 InsΔC cell labeled with antiinsulin serum revealed by the protein A–gold technique. The field shows several dense core secretory granules (*arrows*) with a morphology typical of this pituitary cell line. Most of the insulin immunogold particles are concentrated over granules. Minimal cellular background is present over the remainder of the cytoplasm. Quantitation of the labeling gives the following numbers: secretory granules, 110 ± 8 gold particles/μm² (146 granules evaluated); mitochondria (cellular background), 3 ± 1 gold particles/μm²; secretory granules in nontransfected AtT-20 cells immunolabeled in the same condition (control), 4 ± 1 gold particles/μm² (159 granules evaluated). Number of pictures evaluated, 19.

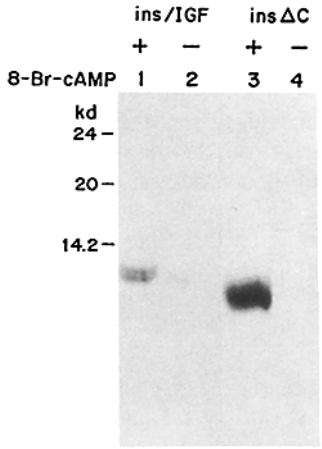


*Figure 6.* Enhanced secretion of mutant proinsulins in response to stimulation by cAMP. Stable transfectants were labeled for 16 h and chased for 6 h. Shown here are media samples collected between 6 and 9 h after labeling either in the presence or absence of 5 mM 8-Br cAMP. The samples were double immunoprecipitated with an antiinsulin antiserum, run on 10–18% SDS-polyacrylamide gels, and prepared for autoradiography as described (20). Note that the amounts of proteins analyzed for the two clones shown in this figure were not normalized with respect to each other.

tutively transported proteins (25, 27, 28), and some unprocessed precursor can be detected in dense core granules and is released upon stimulation (29, 38). These results imply that the sorting machinery recognizes precursor hormones rather than individual mature peptides. Since deletion or substitution of the C peptide of proinsulin does not impair its ability to be sorted, the sorting signal must be within regions of the B and/or A chains that are exposed in the proinsulin molecule. This domain is therefore different from the domain that is recognized by the physiological insulin receptor; the latter recognizes regions of mature insulin that are obscured by the C peptide of proinsulin (13).

A surprising finding is that both Ins/IGF and InsΔC appear to be targeted severalfold more efficiently than proinsulin in transfected AtT-20 cells. This could be due to an increase in the affinity for the sorting machinery as a result of removal of C peptide. Recently, it has been demonstrated that some proteins contain multiple functional signals for targeting to an organelle, and that the efficiency of targeting increases with an increasing number of signals (2, 30). It is possible that the C peptide contains a redundant signal that is sufficient but not required for targeting. However, this pos-

*Table I. Comparison of Sorting Indices of Mutant Proinsulins*

| Protein | Sorting index |
|---|---|
| Human proinsulin | 0.66 |
| Ins/IGF | 2.20 |
| InsΔC | 4.40 |

Selected stable transfectants were labeled for 15 h with 0.5 mCi of [$^{35}$S]cysteine in cysteine-free DME supplemented with 2% FCS (3 ml media/10-cm dish). The labeling media was removed and the steady-state rate of secretion was measured by incubating the cells with labeling medium for an additional hour. Cells were then rinsed with complete DME supplemented with 2% FCS and chased in this medium for three 3-h periods. During the last chase (6–9 h), 5 mM 8-Br-cAMP was added to one dish to induce release from regulated secretory granules. Media and cell extracts were harvested, subjected to double immunoprecipitation, and analyzed on 10–18% SDS–polyacrylamide gels as described (20). Sorting indices were determined as follows:

$$\text{sorting index} = \frac{\text{(secretion/h with cAMP)} - \text{(secretion/h without cAMP)}}{\text{steady-state secretion/h}}$$

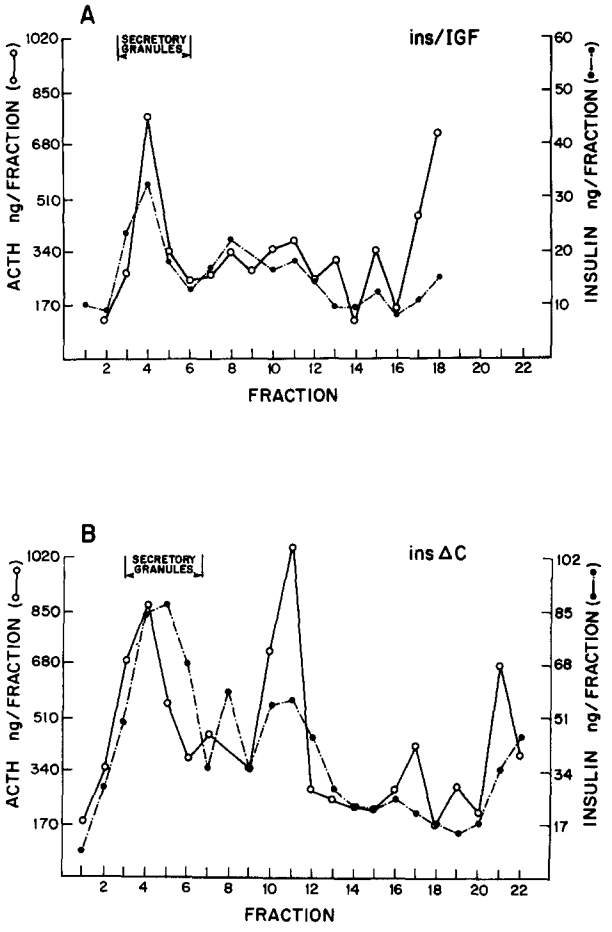


*Figure 7.* Presence of mutated proinsulins in isolated secretory granules. Secretory granules were isolated from AtT-20 clones expressing Ins/IGF and InsΔC on $D_2O$-Ficoll gradients as described (9). The amount of ACTH or insulin-immunoreactive materials present in each fraction was determined by RIA and plotted against the fraction number. Dense core granule-specific fractions are indicated (fractions 3–6 for Ins/IGF and 3–7 for InsΔC). Note that ACTH immunoreactive materials were also found in other lighter fractions but the exact amounts varied between experiments. These fractions have not been characterized, but most likely contain Golgi membranes and incompletely homogenized materials.

sibility seems unlikely since the efficiency of targeting appears to increase rather than decrease when the C peptide is removed. It should be noted that these comparisons are made in a pituitary tumor cell that normally does not express insulin. At the present time, we cannot rule out the possibility that the high sorting efficiencies of the mutated proteins are due to specialized components of the secretory machinery of AtT-20 cells. It will be of interest to determine if the mutated insulins are also sorted more efficiently when expressed in pancreatic cells.

For vacuolar and mitochondrial enzymes, proteolytic activation is concomitant with the removal of targeting signals, which reside within the propeptides (11, 12, 39). Presumably, removal of targeting signals renders the sorting process irreversible so that the transported proteins will remain in the target organelles. In contrast, the targeting signal of proinsulin is not removed upon arrival at the secretory granules; both mutated insulins described here are targeted efficiently but do not undergo proteolytic processing. Similarly, Burgess et al. (5) recently found that the $NH_2$-terminal peptide cleaved from the exocrine protein, trypsinogen, does not contain a targeting signal for granules. These results indicate that targeting to secretory granules may involve some other mechanism to ensure unidirectional transport. One possibility, supported by an early observation in AtT-20 cells treated with chloroquine (21), is that the sorting event is made irreversible by the low pH of the secretory granules. Instead of removing targeting signals by proteolytic processing at the target organelle, the low pH of this compartment could cause the dissociation of delivered proteins from their transporting carriers.

It has been postulated that the C peptide provides the minimal length necessary for translocation across the endoplasmic reticulum membrane (35). This possibility is ruled out since InsΔC is efficiently transported through the secretory pathway. Since the C peptide is not essential for either translocation or targeting, its major role is probably in directing polypeptide folding. This is consistent with the recent finding that a protein in which the B and A chains of insulin are connected by two basic amino acids is not recognized by a yeast-processing enzyme (37). In light of this, it is surprising that InsΔC is efficiently targeted to granules. Our result implies that the sorting machinery is not sensitive to overall structural alterations of insulin. Another intriguing hypothesis is that sorting does require proper folding of the B and A sequences and that InsΔC, despite the lack of C peptide, is able to form this structure. InsΔC is transported efficiently through the secretory pathway and, unlike many misfolded proteins, is not associated with the endoplasmic reticulum protein, BiP (reference 4; see Fig. 3). This suggests that InsΔC adopts a native conformation and escapes trapping in the rough endoplasmic reticulum. Analysis of the exact disulfide bond linkages and the biological activity of InsΔC will be important in determining whether it is folded properly.

A puzzle that still remains to be solved is what comprises the common sorting signals of various regulated proteins that lack apparent sequence homology. Ogata et al. (24) showed recently that two intensely sweet proteins, monellin and

thaumatin, which apparently bind to the same receptor and are recognized by the same antibodies, have no statistically significant sequence homology and do not share similarities in three-dimensional crystal structure. They postulate that the common epitope is comprised of many short stretches of sequences from different parts of the molecules rather than a contiguous stretch of amino acids. It is possible that the sorting domain of proinsulin is similarly formed by short discontinuous segments of the B and A chains. Identification of the exact amino acids that contribute to the targeting signal will require further mutagenesis of the B and A chains of insulin.

The authors thank Mr. Rodolfo Rivas for his support and assistance in immunofluorescence experiments, Mr. David Quinn for secretory granule preparation, Dr. Koong-Nah Chung for preparation of affinity-purified anti-ACTH antibodies, Dr. G. Grodsky for the generous gift of [$^{125}$I]insulin, and M. Ravazzola, A.-M. Lucini, G. Perrelet, G. Negro, and P. A. Ruttiman for assistance with the immunoelectron microscopy experiments. We also thank Drs. Peter Walter, Gary Firestone, and Jeremy Thorner for helpful discussions, and members of the Moore lab for critical reading of the manuscript.

This work was supported by National Institutes of Health grant GM 35239 and National Science Foundation Presidential Young Investigator Award DCB 8451636 awarded to H. P. Moore and by a Swiss National Science Foundation grant (3.404.86) awarded to L. Orci. H. P. Moore is a recipient of Juvenile Diabetes Career Development Award (284087) and Alfred P. Sloan Research Fellowship (BR2497). A contribution of Novo Research Institute (to L. Orci) is also acknowledged.

Received for publication 12 November 1987, and in revised form 16 February 1988.

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