Intracellular Transport and Sorting of Mutant Human Proinsulins that Fail to Form Hexamers

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Abstract. Human proinsulin and insulin oligomerize to form dimers and hexamers. It has been suggested that the ability of prohormones to self associate and form aggregates may be responsible for the sorting process at the *trans*-Golgi. To examine whether insulin oligomerization is required for proper sorting into regulated storage granules, we have constructed point mutations in human insulin B chain that have been previously shown to prevent formation of insulin hexamers (Brange, J., U. Ribel, J. F. Hansen, G. Dodson, M. T. Hansen, S. Havelund, S. G. Melberg, F. Norris, K. Norris, L. Snel, A. R. Sorensen, and H. O. Voight. 1988. *Nature [Lond.].* 333:679–682). One mutant ($B10^{His \rightarrow Asp}$) allows formation of dimers but not hexamers and the other ($B9^{Ser \rightarrow Asp}$) prevents formation of both dimers and hexamers. The mutants were transfected into the mouse pituitary AtT-20 cells, and their ability to be sorted into regulated secretory granules was compared to wild-type insulin. We found that while $B10^{His \rightarrow Asp}$ is sorted somewhat less efficiently than wild-type insulin as reported previously (Carroll, R. J., R. E. Hammer, S. J. Chan, H. H. Swift, A. H. Rubenstein, and D. F. Steiner. 1988. *Proc. Natl. Acad. Sci. USA.* 85:8943–8947; Gross, D. J., P. A. Halban, C. R. Kahn, G. C. Weir, and L. Villa-Kumaroff. 1989. *Proc. Natl. Acad. Sci. USA.* 86:4107–4111). $B9^{Ser \rightarrow Asp}$ is targeted to granules as efficiently as wild-type insulin. These results indicate that self association of proinsulin into hexamers is not required for its targeting to the regulated secretory pathway.

THE pathways of protein secretion from neuroendocrine cells have been elucidated recently by DNA transfection of cultured cells and experimental models using transgenic mice (reviewed in Kelly, 1985; Moore, 1987; also see Low et al., 1986). Neuropeptides and hormones synthesized on membrane-bound ribosomes are first targeted to the rough ER, where they enter the secretory pathway. While most secretory proteins are exported constitutively, peptide hormones are segregated away from other proteins at the *trans*-Golgi (Orci et al., 1987) where they are packaged into dense-core storage granules. Secretion from these granules is not constitutive, but requires external cues in the form of neural or hormonal stimulation.

The exact mechanisms for the sorting of peptide hormones at the *trans*-Golgi is not known. Studies using fusion proteins suggest that peptide hormones contain special sorting information and are actively sorted (Moore and Kelly, 1986). The identity of structural features encoding such information, however, remains elusive. Recent studies of patients with a genetic disorder, familial hyperproinsulinemia, have provided intriguing insights into the sorting problem. This disease is characterized by increased serum levels of proinsulin-like materials. In a kindred with this disorder (Gruppuso et al., 1984), the hyperproinsulinemia was found to be associated with a point mutation in the B-chain coding region of the insulin gene (Chan et al., 1987). Carroll et al. (1988) and Gross et al. (1989) have examined the cellular basis for the defects of this mutant in transgenic mice and in transfected AtT-20 cells, respectively. In both cases, the defects appear to result from a decrease in the efficiency of sorting of the mutant proinsulin, leading to heightened secretion of the unprocessed prohormone in an unregulated fashion. These observations suggest that the mutation may cause essential structural alterations such that the prohormone is not sorted efficiently. The mutation results in the replacement of histidine with aspartic acid at position 10 of insulin B chain (Chan et al., 1987). Wild-type human insulin exhibits subunit interactions and forms dimers; dimeric insulin binds to Zn^{2+} to form hexamers. The histidine residue at position B10 is involved in coordinating zinc ions (Blundell et al., 1972), and in the mutated form insulin dimers are formed instead of hexamers (Brange et al., 1988). Therefore, an intriguing possibility is that the aggregation state of insulin is important for its correct sorting into the regulated secretory pathway (Carroll et al., 1988; Gross et al., 1989).

In this report, we set out to examine whether the intracellular transport and sorting of insulin is affected by its quaternary structure. Brange et al. (1988) have recently characterized a series of mutant insulins with altered oligomeric states. These mutants, created by protein engineering, contain single amino acid substitutions and form insulin monomers or dimers instead of hexamers. We have chosen two of

these to study their effects on intracellular trafficking. $B9^{Ser \rightarrow Asp}$ contains a mutation at position 9 of insulin B chain, and forms monomeric insulins even at high concentrations because of charge repulsion between the subunit–subunit interface. $B10^{His \rightarrow Asp}$, as discussed earlier, forms dimeric insulins because of the lack of the Zn^{2+}-coordinating histidine residue. Thus, although the molecular basis for their structural alterations is different, neither mutant can form hexamers. These mutations were introduced into human proinsulin cDNA by site-specific mutagenesis, and their effects on intracellular targeting were assessed by DNA transfection into AtT-20 cells. This cell line has been shown to sort and process wild-type human proinsulin correctly, and therefore is a good model system to study sorting of mutant proinsulin (Moore et al., 1983). We found that the efficiency at which proinsulin is sorted does not correlate with its oligomeric state. Thus, the aggregation state of human insulin does not dictate its intracellular localization.

Materials and Methods

The M13 vector, M13 hIns pBRC2-lmp8, with human insulin cDNA inserted in the EcoRI and BamHI sites was kindly provided by Dr. C. Craik (University of California, San Francisco, CA). [^{32}P]ATP for end-labeling mutagenic primers was obtained from ICN Radiochemicals (Irvine, CA). Deoxyadenosine-5'-α-[^{35}S]thiotriphosphate for sequencing and [^{35}S]cysteine were obtained from Amersham Corp. (Arlington Heights, IL). Oligonucleotide primers for mutagenesis and sequencing were purchased from the Biomolecular Resource Center (University of California, San Francisco). Klenow fragment for primer extension and restriction enzymes were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). DNA ligase and polynucleotide kinase was from New England Biolabs (Beverly, MA) and Pharmacia Fine Chemicals (Piscataway, NJ), respectively. Sequenase was from US Biochemical (Cleveland, OH). G418 was obtained from Gibco Laboratories (Grand Island, NY). Guinea pig anti-porcine insulin antiserum was obtained from Linco Research, Inc. (Eureka, MO). [^{125}I]porcine insulin for RIA was supplied by the Metabolic Research Unit (University of California, San Francisco). PCS was obtained from Sigma Chemical Co. (St. Louis, MO). Formalin-fixed Staph A cells (Immunoprecipitin) was obtained from Bethesda Research Laboratories (Gaithersburg, MD).

Mutagenesis of Human Proinsulin cDNA and Construction of Plasmids

Mutations of human proinsulin cDNA were constructed essentially as described previously (Powell et al., 1988). For the B9Asp mutation, the 27mer oligonucleotide, 5'-TTCCACCAGGTGGTCGCCGCACAGGTG-3', corresponding to the coding region flanking position 9 of human insulin B chain was used as mutagenic primer. For the B10Asp mutation, the mutagenic primer was 5'-AGCTTCCACCAGGTCTGAGCCGCACAG-3', flanking position 10 of B chain. For primer extension with Klenow fragment, a second oligonucleotide corresponding to 21 bp of the insulin A-chain region was used. Phage plaques were screened using the appropriate end-labeled mutagenic primer. Positive mutations were confirmed by dideoxy sequencing (Sanger et al., 1977). The entire cDNA containing the individual mutation was excised from the M13 vector as an NcoI–BamHI fragment, and subcloned into the mammalian expression vector, pRSV-TGpoly(+) (Powell et al., 1988), via the unique NcoI–BglII sites. Identity of the final plasmids was verified by restriction mapping.

Cell Culture and Transfection

AtT-20 cells were grown in DME H21 supplemented with 10% FCS under a humidified 15% CO_2 atmosphere. CHO cells were maintained in HamsF12 supplemented with 10% FCS under 5% CO_2. DNA transfection was carried out according to methods described previously (Moore et al., 1983). For generating stable transfectants, cells were cotransfected with 120 mg of pRSV-hInsB9Asp or RSV-hInsB10Asp and 24 mg of the selectable marker pSV2-neo. Cells were selected with 0.25 mg/ml (active drug) of G418, and positive clones were screened by insulin radioimmunoassay and by immunoprecipitation. Two stable lines from each mutant, B9Asp/29 and B9Asp/38, and B10Asp/3 and B10Asp/11, were characterized. AtT-20hIns.wt/7, a cell line harboring wild-type insulin cDNA, was used to compare intracellular transport of the mutant insulins with wild-type insulin. CHO cells were transiently transfected with 30 mg of insulin DNAs, and labeled with ^{35}S-cysteine between 42 and 65 h posttransfection.

Metabolic Labeling and Determination of Sorting Efficiency

Intracellular transport and sorting was quantitated according to the method described previously (Moore and Kelly, 1985). Three 10-cm dishes of subconfluent stable transfectants were labeled for either 30 min or 16 h with 0.5 mCi of [^{35}S]cysteine in cysteine-free DME supplemented with 2% FCS. One dish of cells were extracted immediately after labeling to quantitate the amounts synthesized. For short-term labeling, cells were chased for three 1-h periods; during the third chase period 8-Br-cAMP was added to one dish at a final concentration of 5 mM to stimulate regulated release from storage granules. Cells were extracted at the end of the 3-h chase. For long-term labeling, cells were chased in unlabeled medium for three consecutive 3-h periods. During the 6–9-h chase, one dish of cells was stimulated with 5 mM 8-Br-cAMP. Medium samples were collected and the cells were extracted with NDET buffer (1% Nonidet P-40, 0.4% deoxycholate, 66 mM EDTA, 10 mM Tris, pH 7.4) containing 0.3 mg/ml each of iodoacetamide and phenylmethylsulfonyl fluoride. The medium and extract samples were subjected to double immunoprecipitation and analyzed on a 10–18% SDS-PAGE (Moore and Kelly, 1986). To ensure quantitative immunoprecipitation, the titer of antiserum was determined and used at excess. Typically, each medium sample or 1/2 of extract samples were precipitated with 3 μl of the guinea pig anti-insulin serum and the immune complexes were absorbed with 60 ml of Immunoprecipitin (fixed Staph A cells). The bands were quantitated by densitometric analysis of the autoradiogram.

Immunocytochemistry

Light microscopy. Subconfluent cell cultures were fixed with Bouin's fluid or with 4% paraformaldehyde. Cells were permeabilized by dehydration and rehydration with ethanol before incubation with anti-insulin serum (a gift from Dr. P. Wright, University of Indianapolis, Indianapolis, IN) for 2 h at room temperature. Cultures were washed with PBS (0.01 M phosphate buffer, pH 7.4, 0.15 M NaCl), and exposed to FITC-conjugated second layer antibody for 1 hr at room temperature. Cells were washed with PBS, counterstained with 0.03% Evans blue, and examined with an Axiophot fluorescence microscope (Zeiss; Oberkochen, Germany). Antibody dilutions were: guinea pig anti-insulin, 1:400; and sheep anti-guinea pig, 1:400.

EM. Cells were fixed for 20–120 min with 1% glutaraldehyde buffered in 0.1 M phosphate buffer (pH 7.4). After fixation, the cells were dehydrated with ethanol and embedded in Lowicryl K4M at low temperature (Armbruster et al., 1982). Thin sections of cells were collected on nickel grids and immunolabeled by the protein A-gold method (Roth et al., 1978). Grids were incubated on a drop of the anti-insulin serum (dilution 1:1000) for 2 h, washed extensively with PBS, and then incubated for 1 h on a drop of protein A-gold solution (size of gold particles = 15 nm). Washing was again carried out and the sections were counterstained with uranyl acetate and lead citrate before examination in a Philips LS 400 electron microscope (Philips Electronic Instruments, Inc., Eindhoven, The Netherlands).

Quantitative Evaluation. Immunogold particles were quantitated on Golgi complex and dense-core secretory granules of the transfected cells. This was done on photographic prints (calibrated magnification 48,000×) with the aid of an electronic pen connected to a microprocessor (IBM PC; IBM Instruments, Inc., Danbury, CT) programmed to record the number of gold particles per surface area (μm^2) of the compartments drawn with the pen.

Results

Strategy

To determine if the oligomerization states of insulin influence its intracellular sorting and transport, we compared the pathways of secretion of insulins differing in their quaternary structures. Wild-type human insulin exists as a hexamer. We

PLASMID	B-CHAIN SEQUENCE
Wild type	5'...CAC CTG TGC GGC TCA CAC CTG GTG GAA GCT...3' His Leu Cys Gly Ser His Leu Val Glu Ala 5 6 7 8 9 10 11 12 13 14
B9ASP	5'...CAC CTG TGC GGC GAC CAC CTG GTG GAA GCT...3' His Leu Cys Gly **Asp** His Leu Val Glu Ala 5 6 7 8 9 10 11 12 13 14
B10ASP	5'...CAC CTG TGC GGC TCA GAC CTG GTG GAA GCT...3' His Leu Cys Gly Ser **Asp** Leu Val Glu Ala 5 6 7 8 9 10 11 12 13 14

Figure 1. Structure of human insulin B-chain mutants used in this study. The amino acid sequences encompassing residues 5–14 of insulin B chain and the corresponding nucleotide sequences are shown. Mutant B9Asp contains the codon GAC instead of TCA, resulting in the replacement of serine with aspartic acid at position 9. In the mutant B10Asp the CAC codon at position 10 is changed to GAC; the resulting mutation has an aspartic acid in place of the histidine residue.

have constructed two variants in the coding region of insulin B chain: **B9Asp**, contains a serine to aspartic acid substitution at position 9 of B chain, and **B10Asp** contains a histidine to aspartic acid substitution at position 10 of B chain. Brange et al. (1988) have studied the association states of these mutant insulins in highly purified preparations by osmometry, and showed that **B9Asp** is monomeric whereas **B10Asp** is dimeric. Thus, studying the behavior of these mutants in intracellular transport should shed important insights into the relationship between insulin hexamerization and sorting.

Transfection of Cell Lines with Human Proinsulin cDNAs Carrying Single Amino Acid Substitution in the B-chain Coding Region

The mutations were introduced into human proinsulin cDNA by site-directed mutagenesis (Fig. 1). For each construct, the appropriate 27-mer mutagenic primer was annealed to a copy of the human insulin cDNA that had been subcloned into M13mp8. Following primer extension and ligation, the DNA was transformed into Escherichia coli JM101 cells and transformants were screened for the mutation by plaque hybridization at increasing temperatures. Single-stranded DNA from several positive plaques was prepared and the mutation was confirmed by sequence analysis using the dideoxy method (Fig. 2). DNA fragments carrying the desired mutations were subcloned into an RSV expression vector previously described for expression in tissue culture cells (Moore and Kelly, 1985).

Expression of Mutant Proinsulins in AtT-20 and CHO Cells

To study the intracellular transport of mutated proinsulins, we transfected the plasmids into AtT-20 cells. Although derived

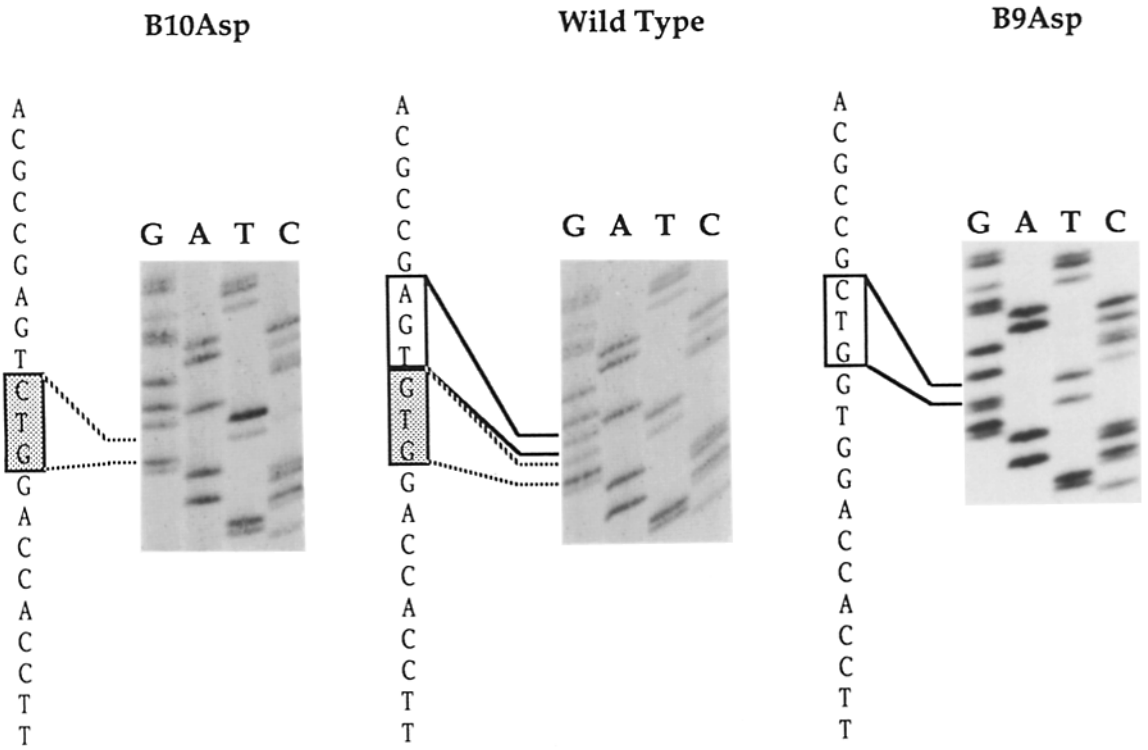

Figure 2. Confirmation of insulin B9 and B10 mutations by dideoxy sequencing. Site-specific mutagenesis was used to generate the desired mutations shown in Fig. 1. Single-stranded DNA obtained from positive M13 phage plaques was sequenced using a 21-mer oligonucleotide complementary to a region of the insulin A chain. Shown are sequences of the B-chain region targeted for mutation. Note that the actual sequences determined correspond to the noncoding strand and read from bottom to top, 5'–>3'. The triplet in open boxes corresponds to position 9 of the B chain, and the triplet in hatched boxes corresponds to position 10. The sequences showed the presence of the desired mutations.

from corticotrophs in the pituitary, these cells have been shown previously to sort and process wild-type human proinsulin correctly (Moore et al., 1983). Stable cell lines harboring mutated human proinsulins were generated by DNA transfection and selection with the antibiotic G418. Surviving clones were expanded and screened for the production of immunoreactive insulins. Two positive clones, B9Asp/38 and B10Asp/11, harboring plasmids containing the mutation at B9 and B10, respectively, were selected for study. The levels of expression as determined by insulin radioimmunoassay were 167 ng per mg of acid extractable protein for B9Asp/38 and 576 ng/mg for B10Asp/11. A control cell line, Ins.wt/7, expressing wild-type human insulin at 221 ng/mg was used for comparison. The endogenous hormone ACTH was produced at a much higher level (3–4 μg/mg). Thus, the amount of exogenous hormones produced is not likely to affect the sorting efficiency.

Since many incorrectly folded proteins are known to be retained within the cells, we first examined whether the mutant proinsulins can be secreted from the cells. Transfected AtT-20 cells were labeled with ^{35}S-cysteine to steady state, and both media and cell extracts were immunoprecipitated with anti-insulin antisera (Fig. 3). Cells harboring the B9Asp plasmid synthesized two molecular species that were immunoreactive with anti-insulin. The larger one had the same apparent mobility as proinsulin on SDS-PAGE, and the smaller one co-migrated with mature insulin. Both species were secreted into the medium. Cells transfected with the B10Asp plasmid showed a similar pattern (data not shown, and see below). Thus, despite the mutations affecting their oligomeric structures, a considerable portion of these proinsulin variants are proteolytically processed into mature-size insulins in a way similar to wild-type proinsulin. Furthermore, unlike many misfolded proteins, they are not retained within the ER but can be secreted from the cells. Previously

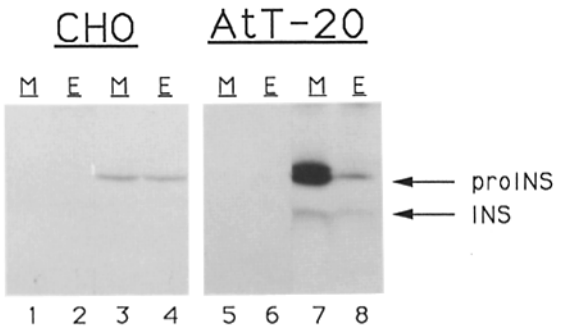

Figure 3. Expression of the B9Asp insulin mutant in transfected cells. AtT-20 cells were transfected with an expression vector containing the B9Asp insulin. Stable clones were isolated and screened for production of the transfected gene product. Cells were radiolabeled with ^{35}S-cysteine for 16 h. Labeling medium and cell extracts were immunoprecipitated with an anti-insulin serum. For comparison, CHO cells were transiently transfected with the same plasmid. The cells were radiolabeled at 42 h posttransfection for 16 h, and the medium and extract samples were processed similarly. (Lanes *1* and *2*) Samples from untransfected control CHO cells; (lanes *3* and *4*) from transfected CHO cells; (lanes *5* and *6*) from untransfected control AtT-20 cells; (lanes *7* and *8*) from a stably transfected AtT-20 cell line, B9Asp/29. Lanes *1*, *3*, *5*, and *7* are media samples, and lanes *2*, *4*, *6*, and *8* are cell extract samples.

we have shown that proinsulin is processed to mature insulin in AtT20-cells, but not in other cells such as L cells (Moore et al., 1983) or CHO cells (Quinn, D., and H. P. Moore, unpublished results). Proteolytic cleavage of B9Asp and B10Asp proinsulins is also specific to AtT-20 cells: CHO cells transiently transfected with the same plasmids synthesized and secreted only the larger species corresponding to the unprocessed precursor (Fig. 3). At the present, we do not know if processing occurred at exactly the same sites as wild-type insulin.

Immunolocalization of the Proinsulin Variants

As mentioned above, both B9Asp and B10Asp are secreted from the cells. Which pathway do they take? To determine if the mutant proinsulins are targeted to the storage granules, we performed indirect immunofluorescence studies. In both B9Asp/38 and B10Asp/11 cells, the structures stained most prominently with an anti-insulin serum are at the tips of cellular processes where dense-core granules congregate, and a perinuclear region corresponding to the Golgi complex (Fig. 4). By immunoelectron microscopy with the protein A–gold technique (Fig. 5), we confirmed a distinct labeling with the anti-insulin serum over both the Golgi complex and the dense-core secretory granule compartments. The immunogold labeling intensity in B9Asp/38 and B10Asp/11 clones was compared with that of AtT-20 cells transfected with wild-type insulin and with nontransfected cells. The respective values of labeling are shown in Table I. Both mutants are clearly present in the Golgi complex as well as dense-core granules. Note that the actual number of immunogold particles detected simply reflected the level of expression of the transfected DNA; the exact sorting efficiencies cannot be determined accurately using this method (see below). Taken together, the data showed that both mutated proinsulins entered the regulated secretory pathway where they are proteolytically processed and localized to dense-core secretory granules. The B10Asp data are consistent with those of Carroll et al. (1988) and Gross et al. (1989) who showed that a significant fraction of the B10Asp was processed into mature insulin within the cells, presumably in dense-core secretory granules.

Intracellular Sorting and Secretion of Monomeric and Dimeric Proinsulins

The above data showed that mutant proinsulins were targeted to the dense-core granules, but did not show how efficiently they were sorted into this organelle. To quantitate the targeting efficiency, we analyzed intracellular sorting and secretion of the B9Asp mutant using two labeling–chase protocols. In the short-term labeling protocol, cells were metabolically labeled with ^{35}S-cysteine for 30 min and then chased in unlabeled medium for two 1-h periods to collect proteins secreted by the constitutive pathway; the cells were then stimulated with 5 mM 8-Br-cAMP for 1 h to induce secretion from the regulated secretory pathway. Fig. 6 *A* shows a direct comparison of the B9Asp mutant and wild type (pro)insulin using this protocol. As can be seen, both wild type and B9 mutant proinsulin were proteolytically processed within the cells to peptides of similar molecular size as mature insulin. Furthermore, 8-Br-cAMP induces secretion of both wild type and B9 mutant insulin; in both

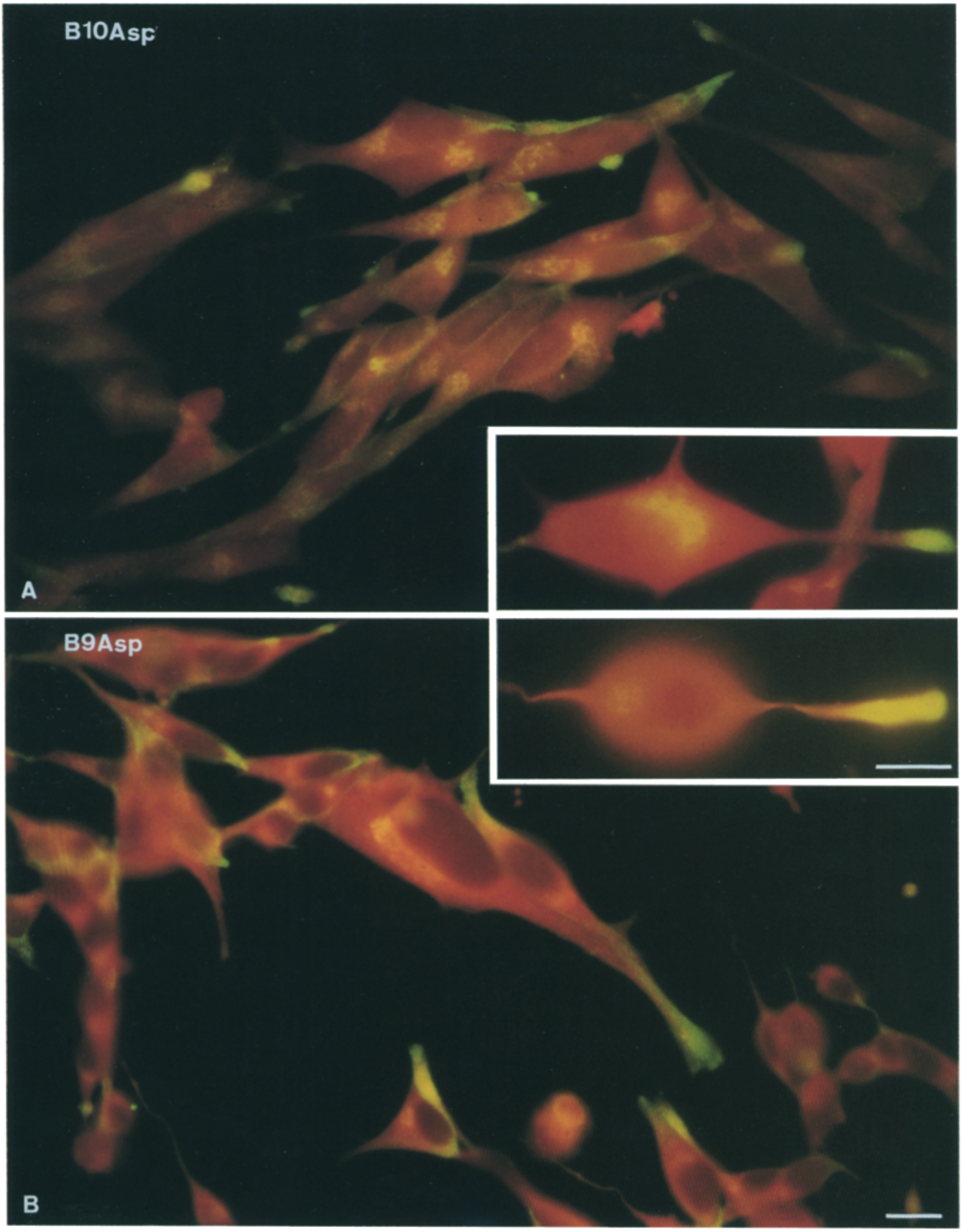

Figure 4. Subconfluent cultures of AtT-20 cells transfected with mutant insulin cDNA (*A*, B10Asp/11 clone; *B*, B9Asp/38 clone) were analyzed by immunofluorescence using an anti-insulin serum. In both clones, the immunofluorescent staining is in the perinuclear (Golgi) region and cell periphery, most intense at the tip of cell processes (*insets*). Bars, 20 μm.

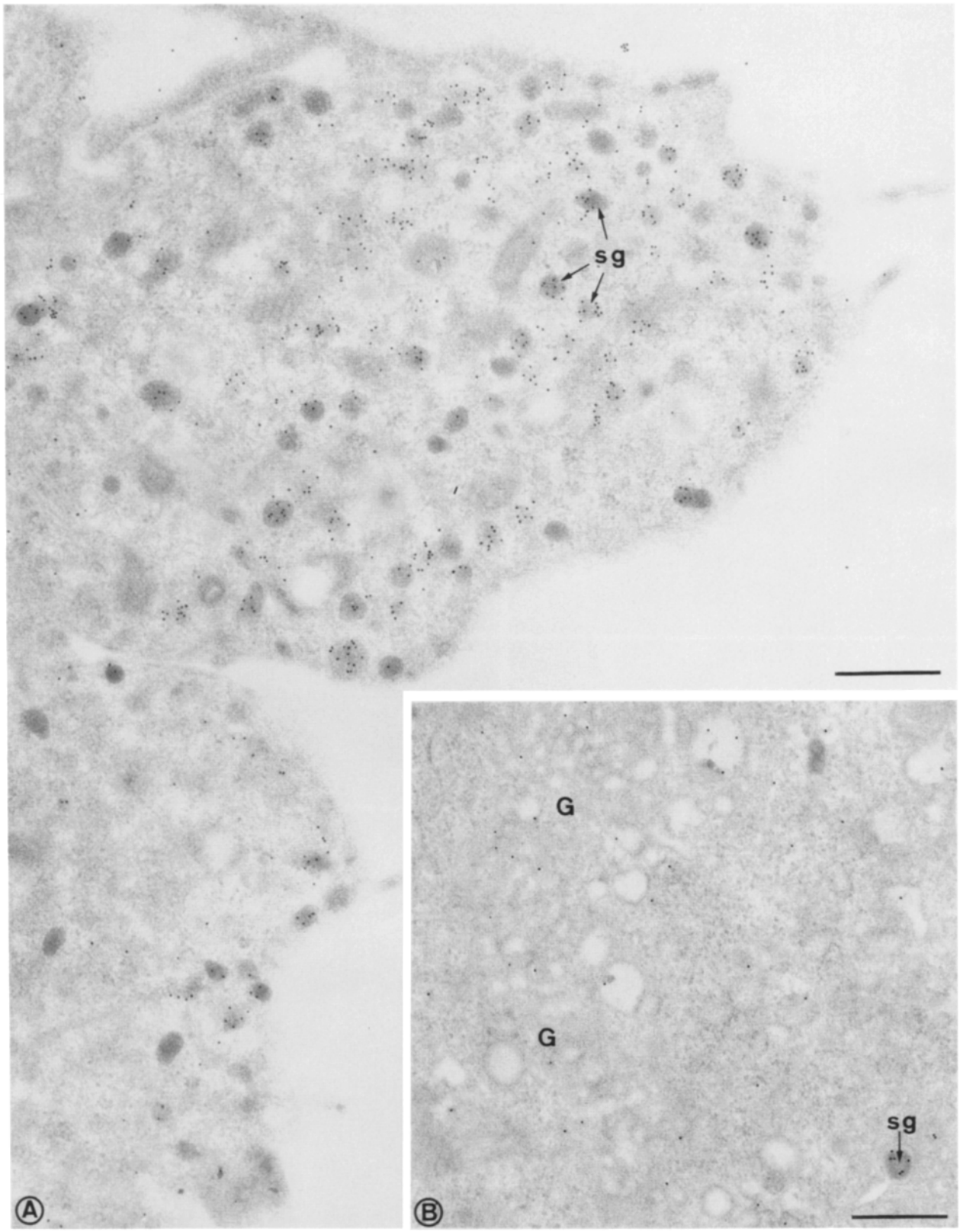

Figure 5. Fields of AtT-20 cells of the B10Asp/ll clone immunostained with anti-insulin revealed by the protein A-gold technique. (*A*) Abundant dense-core secretory granules (*sg*) in the peripheral cytoplasm (the tip of a cell process is shown in the upper part of the picture) are distinctly labeled by immunogold particles. (*B*) Golgi region (*G*) comprising cisternal-vesicular elements associated with a relatively low level of insulin immunoreactivity. A more heavily labeled dense-core secretory granule (*sg*) still connected to a cisternal element is visible in the lower right corner. The quantitative evaluation of the immunogold labeling over Golgi and secretory granule compartments in B10Asp and B9Asp clones is shown in Table I. Bars, 0.5 μm.

Table I. Comparison of the Density of (Pro)Insulin Immunolabeling over Golgi Complex and Secretory Granules in AtT-20 Cells Transfected with Mutant Human Insulins

	Number of immunogold particles/μm^2 of cell compartment			
	B9Asp	B10Asp	Wild type	Nontransfected
Golgi complex (15)	6 ± 1	8 ± 1	10 ± 2	0.7 ± 0.2
Secretory granules (300)	108 ± 5	308 ± 17	277 ± 13	2 ± 1

The number of organelles evaluated in each clone is indicated in parenthesis. Shown is the mean value followed by the standard error of the mean. An example of immunolabeling (B10Asp) is shown in Fig. 5.

cases, the secretagogue stimulated secretion three- to four-fold. Thus, sorting and processing is indistinguishable for the mutant and wild type proinsulin at this level of resolution. To further confirm this result, we adopted a long-term labeling protocol that we have devised previously to quantitate sorting efficiencies (Moore and Kelly, 1985). This method is preferable over the short-term labeling protocol in that the amount of proteins entering the regulated pathway can be more accurately quantified. This is necessary for analyzing protein sorting in transfected cells in which the level of expression is relatively low, and in which the cell lines under study (such at AtT-20 cells) exhibit poor sorting

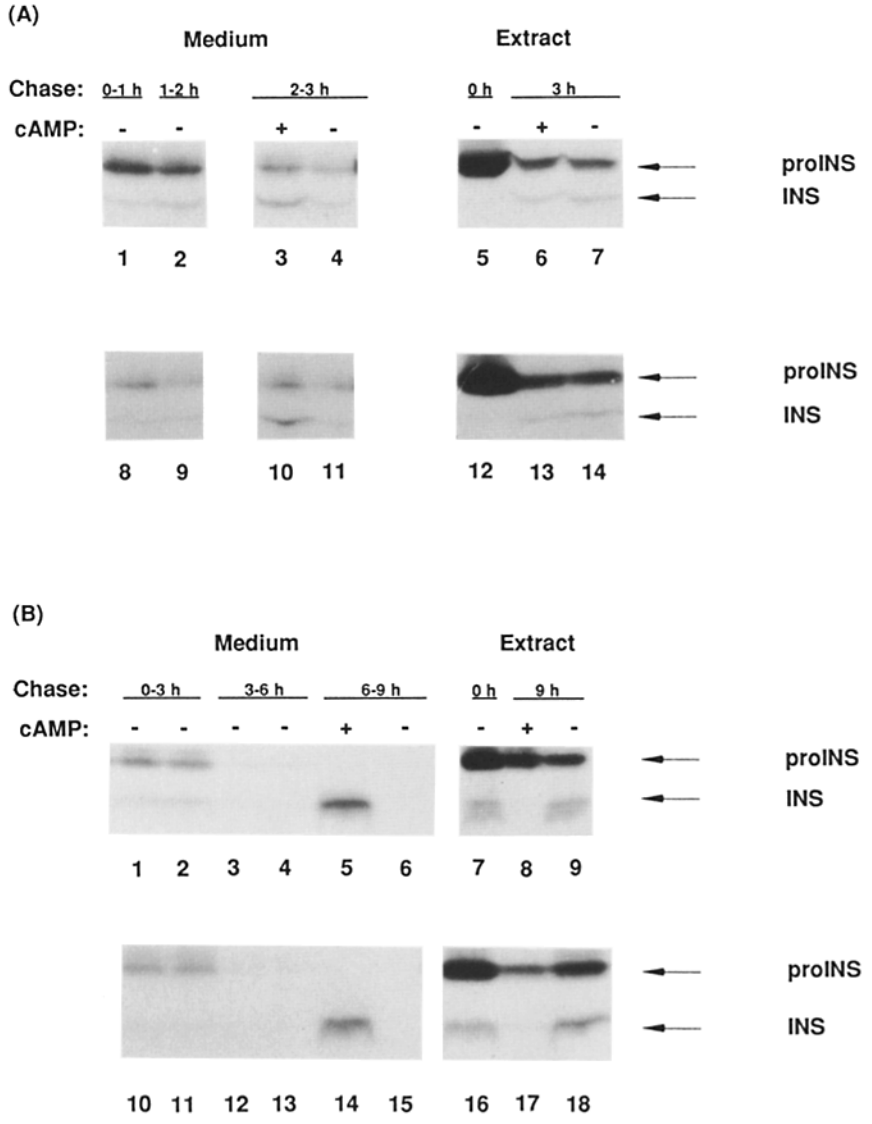

Figure 6. Intracellular transport and sorting of B9Asp mutant and wild-type insulin in transfected AtT-20 cells. (*A*) Short-term labeling protocol. (*B*) Long-term labeling protocol. For both protocols, we used three identical dishes of B9Asp/38 and Ins.wt/7 cells harboring the B9 mutant and wild-type insulin, respectively. For short-term label, cells were pulse labeled for 30 min with ^{35}S-cysteine, and one dish of cells was extracted immediately after labeling. The other two dishes were chased for three 1-h periods. During the third chase, one dish was stimulated with 5 mM 8-Br-cAMP. Labeled materials remaining in cells were extracted after the chase. Insulin immunoreactive materials were precipitated with an anti-insulin serum, and analyzed on 10-18% SDS-PAGE. For long-term label, cells were radiolabeled with ^{35}S-cysteine for 16 h. Cells from one dish were extracted to determine the total amount synthesized, and the other two dishes were chased with unradiolabeled medium for three consecutive 3-h periods. During the 6–9 h chase period, one dish of cells were incubated with 5 mM 8-Br-cAMP to stimulate secretion from storage granules. The other dish of cells was left unstimulated as control. Cells were extracted at the end of the chase periods. (*A*) Lanes 1–7 are the B9 mutant, and lanes 8–14 are wild-type insulin. Lanes *1* and *8* are secretion during 0–1 h chase, and lanes *2* and *9* are secretion during 1–2 h chase; lanes *3* and *10* are secretion during 2–3-h chase from stimulated cells, and lanes *4* and *11* are from control cells during the same period. Lanes *5* and *12* are cell extracts immediately after labeling; lanes *6* and *13* are extracts at the end of the 3-h chase from cells that had been stimulated, and lanes *7* and *14* are from control unstimulated cells. (*B*) Lanes *1–9* are the B9 mutant, and lanes *10–18* are wild-type insulin. Lanes *1*, *2*, *10*, and *11* are medium samples collected during 0–3-h chase; lanes *3*, *4*, *12*, and *13* are medium samples collected during 3–6-h chase; lanes *5* and *14* are medium samples from cells stimulated with 8-Br-cAMP during 6–9-h chase, and lanes *6* and *15* are from control unstimulated cells during the same period. Lanes *7* and *16* are cell extracts immediately after labeling; lanes *8* and *17* are extracts at the end of the 9-h chase from cells that had been stimulated, and lanes *9* and *18* are from control cells.

efficiencies (compared to cells in vivo). Cells were labeled to steady state for 16 h with ^{35}S-cysteine. The cells were then chased in unlabeled medium for two 3-h periods to allow constitutive secretion to occur. The cells were then incubated for 3 h in the presence of 8-Br-cAMP, which stimulates the secretion of hormone stored in regulated secretory granules. Fig. 6 *B* shows an example of the autoradiographic analysis of secretion from wild type Ins.wt/7 cells and from the mutant B9Asp/38 cells using this protocol. This experiment was repeated several times using two different clones transfected with B9Asp, and a summary of the quantitation of the results is shown in Table II. In both cases, ~12–14% of the total immunoreactive molecules after the labeling period were mature-sized insulin. If one assumes that the mature form is a measure of the amount stored in regulated secretory granules (Moore et al., 1983; also see below), this indicates that in both cells approximately the same fraction of immunoreactive insulin in the biosynthetic pathway is in storage granules. This point is further demonstrated by the fact that 8-Br-cAMP caused a similar level of stimulation of wild-type and mutant insulins during 6–9-h chase (13.6 and 10%, respectively). The amount of intracellular storage at the end of chase is also similar for both wild-type and mutant insulins (54% for wild type and 58% for B9Asp). We noticed that a significant fraction of the transfected products are still in the form of unprocessed proinsulin even after 9 h of chase; this is true both for wild type and B9Asp mutant insulin. It is possible that a fraction of human proinsulin is not folded properly in AtT20-cells and are retained within the ER. Despite this, the data show that B9Asp mutant protein is handled very similarly to the wild type protein in AtT-20 cells. The major difference that we detected is that during a 9-h chase period, 25% of labeled mutant proinsulin were released in a constitutive fashion compared to only 12% of wild-type proinsulin. This difference is mostly because of differences in the amounts recovered: at the end of the 9-h chase, we could not recover 31% of wild type immunoreactive (pro)insulin from either the cell extracts or the media; this amount is less (16%) for mutant (pro)insulin. At present, we cannot distinguish whether they were degraded intracellularly or lost from the media. A similar result was also obtained using the short labeling protocol (see Fig. 6 *A*). Thus, the B9 mutant is transported and processed by the cells in a fashion very similar to wild-type insulin. The only significant difference is that the mutant proinsulin appears to be more stable and/or more quantitatively recovered in the constitutively secreted media.

We have also compared the sorting efficiency of B10Asp mutant with that of wild-type insulin and B9Asp mutant. Using a protocol that we describe previously to measure sorting index (Moore and Kelly, 1985), we quantitated the amount of proteins secreted by the regulated pathway relative to the amount released constitutively. Determination from two independent clones of B10Asp mutant showed sorting indices of 0.40 ± 0.005. Wild-type human insulin shows a sorting index of 0.66 (Powell et al., 1988). Thus, the dimeric insulin B10Asp shows a slightly reduced efficiency of sorting, consistent with earlier published results (Carroll et al., 1988; Gross et al., 1989). Monomeric insulin B9Asp shows a sorting index of 0.67 ± 0.02 and therefore is sorted as efficiently as wild-type insulin. We concluded that hexamerization of insulin is not necessary for its correct sorting into the regulated secretory pathway.

Discussion

Recent studies on intracellular membrane traffic revealed a close relationship between the oligomerization state of a pro-

Table II. Quantitation of Intracellular Transport of Insulin Monomers and Hexamers in Transfected AtT-20 Cells

Protein	Chase 1	Chase 2	Chase 3 (−sti)	Chase 3 (+sti)	Extract (−sti)	Not recovered
Wild-type insulin hexamer						
No chase						
Pro	0	0	0	0	3,183 (88%)	
Mature	0	0	0	0	450 (12%)	
Total	0	0	0	0	3,633 (100%)	
Chase						
Pro	363 ± 9 (10%)	76 ± 4 (2%)	10 (0.2%)	<10	1,377 (38%)	
Mature	52 ± 3 (1%)	31 ± 1 (1%)	16 (0.4%)	520 (14%)	567 (16%)	
Total	415 ± 12 (11%)	107 ± 5 (3%)	26 (1%)	520 (14%)	1,944 (54%)	1,141 (31%)
B9Asp insulin monomer						
No chase						
Pro	0	0	0	0	3,501 (86%)	
Mature	0	0	0	0	564 (14%)	
Total	0	0	0	0	4,065 (100%)	
Chase						
Pro	742 ± 68 (18%)	190 ± 5 (5%)	70 (2%)	<20	1,806 (44%)	
Mature	27 ± 25 (1%)	21 ± 18 (1%)	<20	420 (10%)	552 (14%)	
Total	769 ± 93 (19%)	211 ± 23 (6%)	70 (2%)	420 (10%)	2,358 (58%)	657 (16%)

Three identical dishes of Ins.wt/7 or B9Asp/38 or B9Asp/29 cells were labeled with ^{35}S-cysteine for 16 h. Cells from one dish were extracted for normalization. The remaining two dishes were chased for three 3-h periods. During the last (6–9 h) chase, one dish of cells was stimulated with 5 mM 8-Br-cAMP. Media samples from each of the chases were collected, and the cells were extracted at the end of the 9-h chase. Media and extract samples were subjected to immunoprecipitation and SDS-PAGE as in Fig. 6 B, and the autoradiograms were quantitated by scanning and integrating the peak areas. The numbers under no chase represent immunoreactive (pro)insulin in cells extracted immediately after labeling; the sum of proinsulin and insulin at this time point was taken as 100% and used for normalization of subsequent numbers. Chase 1′, Chase 2′, and Chase 3′ represent media samples collected during 0–3, 3–6, and 6–9-h chases, respectively. The final cell extract numbers are those recovered at the end of 9-h chase from cells that had not been stimulated. Since proinsulin and mature insulin have the same number of cysteine, the peak areas were used without further correction as a measure of the relative molar quantities of pro/insulin.

tein and its competency for transport (reviewed in Hurtley and Helenius, 1989). The clearest examples are found in protein export from the ER, where assembled oligomers are rapidly transported out of the ER but unassembled subunits and misfolded proteins are retained. In this case, protein folding and quaternary structures are important for sorting between transport and retention. Whether oligomerization states also determine protein sorting at other steps of transport remains to be determined. In vitro studies showed that proinsulins from many species interact with zinc ions to form hexamers, and it has been postulated that the hexameric organization may play a role in the biosynthesis and intracellular transport of insulin (Emdin et al., 1980). An interesting exception is that proinsulin in guinea pig islets does not self associate but is processed and secreted (Wood et al., 1975); it was unclear whether this reflected a difference in the targeting mechanism in guinea pig compared to other mammalian cells. The studies reported in this paper, however, indicate that the intracellular sorting of human proinsulin between the constitutive and the regulated pathway is also not affected by its oligomerization state.

Previous results showed that B10Asp is partially diverted from the regulated secretory pathway to an unregulated pathway. Our analysis of this mutant also showed a modest decrease in the efficiency of sorting: B10Asp is sorted at approximately two-thirds the efficiency of wild-type insulin (sorting index of 0.40 compared to 0.66). This level of diversion is comparable to that found in transgenic mice and in AtT-20 cells: Carroll et al. (1988) showed that only 85% of the mutant prohormones were sorted into storage granules in the pancreas of transgenic mice compared to 97% for the wild-type protein; Gross et al. (1989) showed that in AtT-20 cells, 90% of labeled wild-type insulin was retained within the cells after a 30-min chase period whereas only 70% of B10Asp was retained. In all the studies, it is clear that a significant portion of the mutant insulin is still targeted to the storage granules and released by the regulated pathway. If the lowered sorting efficiency of B10Asp is because of dimer rather than hexamer formation, then B9Asp should show equal or more severe reduction in sorting efficiency since only monomeric insulin is formed. This is clearly not the case. The sorting efficiency of B9Asp is indistinguishable from that of wild-type insulin. Thus, the lower sorting efficiency of B10Asp is most likely a result of other changes in the protein rather than its inability to form the proper oligomeric structure. One possibility, as suggested by Carroll et al. (1988), is that the elevated affinity for insulin receptor of B10Asp may cause it to bind to the insulin receptor en route to the cell surface, thereby partially diverted to the constitutive route. The exact mechanism for the diversion of this mutant remains to be determined.

The prediction that B9Asp and B10Asp form monomeric and dimeric proinsulin, respectively, is based on in vitro studies using purified mutant insulins (Brange et al., 1988). Although it would be desirable to confirm the oligomeric states of the mutants in our transfected AtT-20 cells, this is technically unfeasible. Hexamer formation is reversible, and can be detected only at relatively high monomer concentrations (0.1–1 mg/ml) under equilibrium conditions. The amounts of proteins expressed in the transfected cell lines are too low (0.1–1 mg/10^6 cells) to permit purification and analysis by equilibrium centrifugation or osmometry. Other methods for analyzing quaternary structure, such as velocity sedimentation of crude cell lysate, are only appropriate for detecting stable polymers which do not dissociate during the sedimentation procedure. We feel that the results obtained by Brange et al. (1988) should accurately predict the states of proinsulins in our transfected cells for reasons which follow. Our constructs contained the identical mutations to theirs. Although expressed in different systems (theirs in yeast cells and ours in AtT-20 cells), these mutant proinsulins behaved similarly and were processed to mature insulins in both systems. Thus, the mutant insulins produced in AtT-20 cells should have the same structures as those purified from yeast cells. Purified B9Asp and B10Asp insulins are present as monomer and dimer, respectively, even at concentrations as high as 1 mM (Brange et al., 1988). The level of transfected insulins is ~10-fold less than the endogenous ACTH, which has been estimated to be in the mM range in storage granules (Gumbiner and Kelly, 1981). Therefore, the concentration of insulins expressed in these cells is not likely to exceed the concentration used to determine their structures in vitro. Although the structural determinations were performed on mature mutant insulins, the same oligomeric structures most likely also apply to the corresponding proinsulins. The insulin moiety of the proinsulin molecule has been shown to exist in essentially the same conformation as the mature insulin molecule, and both proinsulin and insulin show similar type of interactions with zinc to form hexamers. Thus, the oligomeric states of the mutant proinsulins should show similar behavior as the corresponding insulins (Frank and Veros, 1970).

Electron microscopic studies have shown that many prohormones become highly concentrated at regions of the *trans*-Golgi where dense-core granules are forming (Salpeter and Farquhar, 1981; Orci, 1974, 1982; Orci et al., 1987). This has led to the hypothesis that sorting of this class of molecules into storage granules may be achieved by their ability to self aggregate in the milieu of the *trans*-Golgi (Burgess and Kelly, 1987). The physical properties of insulin to form hexamer and larger aggregates make this a very attractive idea. In addition, analysis of secretion of von Willebrand factor from human endothelial cells has shown that different oligomeric forms of this protein are secreted via the constitutive and the regulated pathways: molecules secreted constitutively are dimeric, whereas those secreted by the regulated pathway consist of only very large multimers (Sporn et al., 1986). One possible explanation of such differences is that sorting is driven by aggregate formation and that only the aggregated form is sorted into the regulated pathway. However, this is not the only possibility. Another equally likely explanation is that aggregate formation is not causal but rather a result of sorting—the high concentration of the sorted proteins may facilitate aggregate formation within the storage granules. Our finding that the monomeric proinsulin is sorted at the same efficiency as hexameric proinsulin argues against the idea that sorting is achieved by self aggregation of the transported proteins. It should be noted, however, that although our data do not support homophilic protein–protein interactions as a primary mechanism for sorting, they do not exclude the possibility that heterophilic aggregate formation may be important for sorting. In the transfected AtT-20 cell system, the exogenous proinsulin is sorted along with endogenous ACTH and other storage granule proteins (Moore et

al., 1983). It is possible that proinsulin binds to endogenous secretory proteins to form heteropolymers which are then segregated. Heteropolymer formation has recently been documented for pancreatic secretory products (Tooze et al., 1989), and hormones can be cross-linked by heterophilic protein–protein interactions (Chung et al., 1989). Future experiments will be necessary to determine the exact role of such heteropolymer formation in the sorting of regulated secretory proteins.

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