In Vitro Mutagenesis of Trypsinogen: Role of the Amino Terminus in Intracellular Protein Targeting to Secretory Granules

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Abstract. The mouse anterior pituitary tumor cell line, AtT-20, targets secretory proteins into two distinct intracellular pathways. When the DNA that encodes trypsinogen is introduced into AtT-20 cells, the protein is sorted into the regulated secretory pathway as efficiently as the endogenous peptide hormone ACTH. In this study we have used double-label immunoelectron microscopy to demonstrate that trypsinogen colocalizes in the same secretory granules as ACTH. In vitro mutagenesis was used to test whether the information for targeting trypsinogen to the secretory granules resides at the amino (NH2) terminus of the protein. Mutations were made in the DNA that encodes trypsinogen, and the mutant proteins were expressed in AtT-20 cells to determine whether intracellular targeting could be altered. Replacing the trypsinogen signal peptide with that of the kappa-immunoglobulin light chain, a constitutively secreted protein, does not alter targeting to the regulated secretory pathway. In addition, deletion of the NH2-terminal “pro” sequence of trypsinogen has virtually no effect on protein targeting. However, this deletion does affect the signal peptidase cleavage site, and as a result the enzymatic activity of the truncated trypsin protein is abolished. We conclude that neither the signal peptide nor the 12 NH2-terminal amino acids of trypsinogen are essential for sorting to the regulated secretory pathway of AtT-20 cells.

The intracellular route taken by secretory proteins in eukaryotic cells, from the endoplasmic reticulum (ER) through the Golgi apparatus and to the cell surface, is well established (56). Classical secretory cells, such as those of exocrine or endocrine tissues, synthesize, concentrate, and then store one or a few secretory products in large, electron-dense, membrane-bound vesicles called secretory granules. Secretion from these specialized secretory cells is triggered by an external signal, and thus has been termed “regulated” secretion (67). Other cells that lack specialized secretory granules secrete proteins in an unregulated or constitutive manner. It is generally believed that constitutively secreted proteins traverse the secretory pathway from the ER to the Golgi complex and then are shuttled to the cell surface via small carrier vesicles. No extracellular signal is required to trigger exocytosis of constitutive vesicles.

The mouse anterior pituitary tumor cell line, AtT-20, behaves as a regulated secretory cell in culture. The endogenous prohormone proopiomelanocortin (POMC) is synthesized, glycosylated, and proteolytically processed to mature products including adrenocorticotrophic hormone (ACTH). ACTH is concentrated in dense-core secretory granules, and its release is stimulated by secretagogues (47, 48). In addition, some proteins reach the cell surface of AtT-20 cells via the constitutive secretory route. Both an endogenous retroviral membrane glycoprotein, gp70 (31), and the secreted extracellular matrix protein, laminin (8), are transported exclusively via the constitutive secretory pathway. The mechanism of these intracellular targeting processes is at present unknown, but it may involve recognition of specific sorting signals in the secreted proteins themselves (5).

Protein targeting to the regulated secretory pathway is apparently conserved among endocrine hormones. When human proinsulin (54) or human growth hormone (52) are stably expressed in AtT-20 cells via DNA-mediated transfection, they are sorted into the regulated secretory pathway at least as well as the endogenous hormone POMC/ACTH. Furthermore, we have recently shown that the exocrine protein, rat anionic trypsinogen, is also selectively targeted to the regulated secretory pathway in the endocrine cell line AtT-20 (8). Similar evidence has been obtained for human parathyroid hormone expressed and secreted by a rat pituitary cell line, GH4 (37), and for human proenkephalin expressed by AtT-20 cells (12). Thus proteins from different species as well as endocrine and exocrine tissues share the ability to be recognized and targeted to the regulated secretory pathway in AtT-20 cells.

A general approach to elucidating the mechanism of intracellular protein targeting is the use of recombinant DNA

1. Abbreviations used in this paper: ER, endoplasmic reticulum; Ksp, kappa signal peptide; LTR, long terminal repeat; POMC, proopiomelanocortin; RSV, Rous sarcoma virus; TG, truncated G (protein).
techniques (for review, see Garoff [25]). A cloned gene or cDNA is modified in vitro and, subsequently, the mutated protein is expressed either in vitro or in vivo to address whether a targeting signal has been disrupted. We have taken this approach to attempt to localize the signal responsible for targeting to the regulated secretory pathway. The exocrine protein, trypsinogen, was chosen for this study for several reasons. Inasmuch as the three-dimensional structure of both trypsinogen (22, 45) and trypsin (39, 65) are known, the structural information can be used to design mutations that will involve the surface of the protein (where signal recognition is likely to occur), and that are not likely to drastically alter the overall folding or enzymatic activity of the molecule. If proteins do not fold correctly, their ability to be transported beyond the ER is severely compromised (13, 26, 46, 66). In addition, because trypsinogen can be converted to trypsin, the proteolytic activity of the secreted mutant protein can be assayed as a very sensitive measure of correct protein folding. If targeting is disrupted but enzymatic activity is maintained, it is more likely that the mutation has altered a targeting signal, and not simply changed the overall structure of the protein, making the signal unrecognizable or inaccessible (58, 69).

Many intracellular targeting signals are known to be present at the NH₂ terminus of the targeted proteins. For example, mitochondrial proteins (20, 36, 38, 40); chloroplast proteins (59, 62, 70); some nuclear proteins (17, 19, 33, 55, 61, 74), and some proteins targeted to the ER have NH₂-terminal transport signals. Most recently, the yeast vacuolar targeting signal of procarboxypeptidase Y has been shown to reside in the NH₂-terminal propeptide (42, 68). In this report data are presented that demonstrate the neither the signal peptide nor the NH₂-terminal "pro" peptide of trypsinogen are essential for targeting to the regulated secretory pathway in AtT-20 cells; the intracellular targeting information is apparently not present at the amino terminus of this regulated secretory protein.

Materials and Methods

Materials

The M13 sequencing primer, synthetic DNA linkers, restriction endonucleases, and T4 ligase were from New England Biolabs (Beverly, MA). T4 polymerase kinase was from Pharmacia Fine Chemicals (Piscataway, NJ). The Klenow fragment of DNA polymerase I was generously provided by P. Walter's Laboratory at University of California, San Francisco (UCSF). [³⁵S]Cysteine was from Amersham Corp. (Arlington Heights, IL). Rabbit anti-trypsinogen antibodies and the trypsinogen standard were kindly provided by Corey Largman (Veterans Administration Medical Center, Martinez, CA). Mutagenic oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) model 380B DNA synthesizer by the Biomolecular Resource Center (UCSF). Bovine trypsin and 8-Br-cAMP were from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose for Western blots was from Schleicher & Schuell, Inc. (Keene, NH). Gold conjugated to goat anti-rabbit antibody was from Janssen Pharmaceutica (Piscataway, N.J.).

Cell Culture and Transfections

AtT-20/D-16 cells were grown in Dulbecco's modified Eagle's medium H-21, supplemented with 10% fetal calf serum and penicillin/streptomycin, under a 15% CO₂ atmosphere at 37°C. Transfections of AtT-20 cells were by a calcium phosphate precipitation protocol described previously (8, 54). Cotransfections with the selectable plasmid pSV2neo (63) and the nonselectable plasmids coding for the trypsinogen proteins described in the text were performed using 20 μg of pSV2neo and 100 μg of nonselectable plasmid per 3 × 10⁶ AtT-20 cells. Selection was with 0.25 mg/ml G418 (active drug concentration) (Gibco Laboratories, Grand Island, NY) for 2 wk after which time individual clones were isolated with cloning rings. Clones were expanded in media containing 0.125 mg/ml G418 and expressing clones were identified using a Western blot assay described previously (8, 10).

Quantitative Secretion Assay: Calculation of the Sorting Index

A complete description and derivation of this assay is presented by Moore and Kelly (52). Briefly, AtT-20 cells were metabolically labeled for >16 h with [³⁵S]Cysteine to approach a steady-state labeled condition. The cells were then incubated in normal medium to chase the labeled, constitutively secreted proteins out of the cell. After two 3-h chases, the cells were treated for 3 h with the secretagogue 8-Br-cAMP (5 mM) to stimulate the rate of secretion from the regulated pathway. Wild-type and mutant trypsinogen proteins were immunoprecipitated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8). The amount of labeled trypsinogen in various samples was quantified by scanning the autoradiograms with an LKB soft laser densitometer (LKB Instruments, Inc., Gaithersburg, MD) or by solubilizing gel slices and counting directly (72). The immunoprecipitations were quantitative and 80-100% of the initial radioactive activity was recovered in all experiments presented here. The sorting index is the ratio of the increased rate of secretion when the cells are stimulated with 8-Br-cAMP to the steady-state rate of release in the absence of stimulation. As discussed previously (52), the sorting index is proportional to the probability a nascent protein enters the regulated pathway divided by the probability that it enters the constitutive pathway.

DNA Constructions and In Vitro Mutagenesis

Standard recombinant DNA techniques were used for the following plasmid constructions (48). plRSV:.Trypsinogen. To achieve higher trypsinogen expression in AtT-20 cells, the trypsinogen-coding region from pSV2neo::MTp-trypsinogen was subcloned 3’ to the Rous sarcoma virus (RSV) long terminal repeat (LTR) (28). plRSV-truncated G (TG) (52) was digested with Bgl II; the Bgl II site was filled in using the Klenow fragment of DNA polymerase I and converted to a Hind III site with a synthetic Hind III linker. The complete pretrypsinogen DNA was isolated from the Bam HI-Sal I restriction fragment from pSV2neo::MTp-trypsinogen (8). The ends of this DNA fragment were also converted to Hind III sites by the use of synthetic Hind III linkers. (The Bam HI site was maintained and was later used in constructing the pRSV-Ksp-trypsinogen vector.) The trypsinogen fragment was ligated into the RSV LTR vector DNA to obtain the desired pRSV:trypsinogen plasmid. After transformation of Escherichia coli strain DH4 (48), colonies harboring plasmids with the desired insert in the correct orientation were identified by restriction mapping.

plRSV:.Ksp Trypsinogen. Three separate DNA fragments were engineered to create an expression vector coding for trypsinogen with the kappa signal peptide. First, the DNA encoding the MPCU kappa signal peptide (Ksp) was isolated as a Bam HI-Hind III restriction fragment (43). The original pre II site upstream of the initiating methionine of the Ksp was converted to a Bam HI site in a previous subcloning construction (Matsuuchi and Kelly, manuscript submitted for publication). HindIII cleaves just two amino acids before the end of the signal peptide leaving a blunt end. This blunt end was converted to a Hind III site using a synthetic linker to allow the use of a convenient Hind III site in trypsinogen. Second, the trypsinogen-coding sequence was isolated as a Hind III fragment by TRAP (29). This DNA fragment contains the entire trypsinogen sequence, but lacks the signal peptide. Ligation of the Ksp Bam HI-Hind III fragment to the trypsinogen Hind III-Sst I fragment creates an in-frame junction between the Ksp and trypsinogen sequences. The Hind III linker contributes six nucleotides that maintain the length of the Ksp, but alters the sequence from Asp and Gly to Gla and Ala. Third, the RSV vector was prepared by partially digesting pRSV:.trypsinogen with Bam HI and complete digestion with Sst I. The Bam HI-Sst I vector fragment cleaved at the Bam HI site immediately after the RSV LTR was isolated by agarose gel electrophoresis. The three DNA fragments were then ligated (Ksp, Bam HI-Hind III; trypsinogen, Hind III-Sst I; pRSV, Bam HI-Sst I). The plasmid pRSV:.Ksp trypsinogen was isolated and identified by restriction mapping. The DNA
confirmed using a double-stranded DNA-sequencing protocol (11).

The intronless trypsinogen minigene was cloned into M13mp8 as described (15). This single-stranded antisense clone was used as the template for mutagenesis. A 24-nucleotide-long mutagenic primer, TB04, (TB04 = 5'~ATCG-'IqW_JCtA~,) was synthesized by the Biomolecular Resource Center at UCSF. TB04 spans the desired 24-nucleotide deletion, which when incorporated will lead to the deletion of the 8-amino acid "pro" sequence. Both the mutagenic oligonucleotide TB04 and the M13 sequencing primer (5'GTAAAACGACGC~-) were annealed to the single-stranded antisense minigene M13 template DNA. After extension with the Klenow fragment of DNA polymerase I and ligation with T4 DNA ligase, the deletion mutation in the expression plasmid was confirmed using a double-stranded phage DNA was isolated and the 372-bp Hind III-Xho I restriction fragment containing the Apro trypsin mutation. Double-stranded phage DNA was isolated and the 372-bp Hind III-Xho I restriction fragment containing the Δpro trypsin mutation was used to replace the corresponding wild-type restriction fragment in pRSV:trypsinogen to create pRSV::Δpro trypsin. The DNA sequence of the deletion mutation in the expression plasmid was confirmed using a double-stranded DNA sequencing protocol (11).

Protein Sequence Analysis

Δpro trypsin-expressing cells were labeled overnight with [35S]sulfate and Δpro trypsin was purified from both the medium and cell extract by immunoprecipitation. The radiolabeled Δpro trypsin was further purified and concentrated by SDS-PAGE (9, 32). The final pellets were dissolved in 200 μl of 0.1% SDS, 0.1 mM EDTA, 0.05 M Tris, pH 7.9, 5 mM DTT, and 0.15 M NaCl. The media sample contained ~100,000 cpm and the cell extract sample ~3,000 cpm. The sequence analysis was performed on an Applied Biosystems gas-phase sequencer, model 470A, by the Biomolecular Resource Center at UCSF. Anilinothiazolinone amino acids were collected at each cycle and [35S] counts per minute were determined.

Immunoelectron Microscopy

RSV:Tryp cells were removed from their culture dishes using EGTA (5 mM) in PBS. The cells were pelleted in BEEM capsules (Polysciences, Inc., War- rington, PA) by centrifugation at 1,000 rpm for 5 min. The supernatant was removed and the cell pellet was immediately immersed in a fixation solution containing 4% paraformaldehyde, 0.1% glutaraldehyde, 0.1 M cacodylate buffer (pH 7.4), 4% sucrose, and 15% picric acid (18). After fixation the cells were embedded in Lowicryl K4M (Polysciences, Inc.) essentially as described (68).

Results

Trypsinogen Colocalizes With ACTH in Dense-Core Secretory Granules of AtT-20 Cells

We have previously shown that the exocrine protein rat anionic trypsinogen is targeted to dense-core secretory granules in the endocrine cell line AtT-20 (8). Cell fractionation as well as secretagogue-stimulated release showed that trypsinogen behaves similarly to the endogenous regulated secretory protein POMC/ACTH. To demonstrate directly that trypsinogen is present in the same secretory granule as ACTH, and is not exported through a parallel regulated secretion pathway, we carried out a double-label immunoelectron microscopy experiment.

A human metallothionein promoter was initially employed to control the expression of trypsinogen in AtT-20 cells (8). However, the resulting cell line (Tryp-7) expressed insufficient levels of trypsinogen to detect by immunoelectron microscopy. Therefore an expression vector was constructed with the strong promoter–enhancer element, the Rous sarcoma virus (RSV) long terminal repeat (LTR), controlling the expression of trypsinogen. The trypsinogen gene/cDNA (8, 15) was subcloned into pRSV-TG (52) (see Materials and Methods for details) to create pRSV:trypsinogen. AtT-20 cells were co-transfected with this DNA and pSV2neo as described previously (8). Clones expressing very high levels of trypsinogen were identified by a Western blot assay, and one, RSV:Tryp, was chosen for all further studies. Using the quantitative secretion assay described previously (52, also see Materials and Methods), we determined that the trypsinogen made and secreted by RSV:Tryp cells was targeted to the regulated secretion pathway with the same efficiency as the trypsinogen expressed from the metallothionein pro-

Table 1. Quantitative Comparison of Protein Targeting to the Regulated Secretion Pathway in AtT-20 Cells

| DNA construction                          | Secreted protein | Sorting index* |
|-------------------------------------------|------------------|---------------|
| A. Regulated and constitutive secretory proteins |                  |               |
| pRSV::Ksp trypsinogen                    | Trypsinogen      | 0.15 ± 0.01 (2) |
| pRSV::Δpro trypsin                       | Δpro trypsin     | 0.17 ± 0.01 (1) |
| pRSV::Δpro D−N trypsin                   | Δpro D−N trypsin | 0.15 ± 0.01 (2) |

* Calculated as in Moore and Kelly (52); see also Materials and Methods.
† Data from Moore and Kelly (52).
‡ See Fig. 5 and text for NH2-terminal sequence analysis.
moter (Table I A). This suggests that although these cells express higher levels of trypsinogen, the amount is apparently not sufficient to observe any saturation of targeting to the regulated secretory pathway. The sorting indices of trypsinogen and the endogenous hormone POMC/ACTH are very similar, although both are sorted less efficiently than heterologously expressed human growth hormone (52).

The RSV-Tryp cell line was then used to perform the double-label immunoelectron microscopy experiment shown in Fig. 1. A Lowicryl-embedded section of RSV-Tryp cells was stained with rabbit anti-ACTH antibodies and 20-nm gold coupled to goat anti-rabbit second antibody on one side of the section, and with rabbit anti-trypsinogen antibodies and 5-nm gold coupled to goat anti-rabbit second antibody on the other side of the section (see Materials and Methods for details). This section shows a large number of dense-core secretory granules that stain for both antigens. We did note that in other sections some granules stained for only one antigen or the other. This may be an artifact of the two-sided technique used in that both antigens were not always equally accessible from both sides of the section (see Discussion).

We conclude that within the limits of our observations the exocrine protein trypsinogen is targeted to the same population of dense core secretory granules as the endogenous POMC/ACTH.

The Trypsinogen Signal Peptide Does Not Contain the Secretory Granule Targeting Information

Having thus demonstrated that trypsinogen is targeted to regulated secretory granules, we have begun to dissect the trypsinogen molecule to identify domains in the protein that specify granule localization. To determine whether the signal peptide has any role in protein targeting to the regulated pathway, the trypsinogen signal peptide was replaced with the signal peptide from a constitutively secreted protein, the kappa MPC 11 immunoglobulin light chain (Matsuuchi and Kelly, manuscript submitted for publication). The DNA construct and the corresponding predicted protein sequence are shown in Fig. 2 A. The trypsinogen signal peptide is 15 amino acids in length. Signal peptidase cleaves pretrypsinogen after Ala (−1)² and before Phe (+1) to produce trypsinogen. The kappa signal peptide (Ksp) is 28 amino acids long (43) and ends with Gly (−1). The fusion between the Ksp and trypsinogen was made by taking advantage of convenient restriction endonuclease cleavage sites and using a Hind III linker to join the Ksp to the trypsinogen sequences (see Materials and Methods for details). The resultant DNA constructs are shown in Fig. 2 A. The amino acid numbering system used here is as follows: Signal peptide residues are assigned negative numbers beginning with −1, just before the cleavage site. The mature sequences are given positive numbers using the chymotrypsinogen system of Hartley (35).
Figure 2. pRSV::Ksp trypsinogen construction and Ksp trypsinogen secretion analysis. (A) The Ksp trypsinogen expression vector was constructed as described in the text. Restriction endonuclease sites: B, Bam HI; H, Hind III; and S, Sst I. The solid bar indicates the RSV LTR segment used to control Ksp trypsinogen expression. The predicted protein sequences of the wild-type trypsinogen (wt-T) and Ksp trypsinogen (Ksp-T) are given using the one letter amino acid code. A, alanine; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; Q, glutamine; S, serine; T, threonine; V, valine; and W, tryptophan. The two altered amino acids in the Ksp-T sequence, Q and A, are shown in bold lettering. (B) Secretion analysis of wt-T and Ksp-T from transformed AtT-20 cells. Cells were labeled with [35S]Cys, and then chased for three consecutive 3-h periods. During the third chase, 5 mM 8-Br-cAMP was added to one of two identical dishes. Shown are SDS-PAGE gels of anti-trypsinogen-immunoprecipitated media samples from the third chase, either in the absence (−) or presence (+) of the secretagogue, 8-Br-cAMP.

encodes a signal peptide that is the same length as the Ksp, however, the last two amino acids encoded are Gin and Ala, instead of Asp and Gly (see Fig. 2 A). We have not determined whether these changes affect the accuracy of signal peptidase cleavage. The RSV LTR was used to control the synthesis of the Ksp trypsinogen-fusion protein. AtT-20 cells were co-transfected with pRSV::Ksp trypsinogen and pSV2neo, stable cell lines were selected, and trypsinogen-expressing clones were identified. Several cell lines that synthesized and secreted an immunoreactive trypsinogen of the expected molecular weight (30,000) were obtained; one of these was further characterized.

Figure 3. pRSV::Apro trypsin construction and Apro trypsin secretion analysis. (A) The Apro trypsin expression vector was constructed as described in the text. Restriction endonuclease sites and RSV LTR as in Fig. 2. (X) indicates the location of the eight-amino acid "pro" deletion. The approximate location of Asp 102 is also indicated. The predicted protein sequences of wild-type trypsinogen (wt-T) and Apro trypsin (Δpro-T) are shown in the one-letter amino acid code as in Fig. 2; in addition, D, aspartic acid; N, asparagine; and R, arginine. The "pro" deletion is indicated on the wt-T sequence by dashed lines, and the arrow shows the normal signal peptidase cleavage site. (B) Secretion analysis of wt-T, Δpro-T, and D→N Δpro trypsin (D→N Δpro-T). Cells were labeled and chased as in Fig. 2. Shown are SDS-PAGE gels of anti-trypsinogen-immunoprecipitated media samples from the third chase, in the absence (−) or presence (+) of the secretagogue 8-Br-cAMP.
The Ksp was cleaved from pretrypsinogen, as judged by comparing wild-type trypsinogen and Ksp trypsinogen by immunoprecipitation and SDS-PAGE (Fig. 2 B). The secretion properties of Ksp trypsinogen were determined using the quantitative secretion protocol described previously (52, see also Materials and Methods). The secretagogue 8-Br-cAMP stimulated the release of Ksp trypsinogen (Fig. 2 B) and the sorting index calculated from these secretion data did not differ from wild-type trypsinogen (Table I A). For comparison, the data for a transfected, constitutively secreted protein, the TG protein of vesicular stomatitis virus, is included in Table I A (52). There is at least a 30-fold difference in the sorting of TG and the regulated secretory proteins. We conclude from this experiment that the sorting domain required for granule localization in AtT-20 cells is not present in the signal peptide of trypsinogen.

**Deletion of the NH₂-Terminal "pro" Sequence Does Not Alter Targeting to Secretory Granules**

Trypsinogen is secreted from the pancreas as an inactive zymogen. The protease is converted to trypsin when enteropeptidase (50) cleaves off the NH₂-terminal eight-amino acid "pro" sequence. This cleavage triggers a local conformational change, whereby the NH₂-terminal Ile (+16) of trypsin forms a critical salt bridge with Asp (+194), a residue near the active site (22, 23, 39, 45, 65). To determine whether the NH₂-terminal "pro" sequence is also involved in secretory granule targeting, oligonucleotide-directed mutagenesis was used to precisely delete the "pro" sequence of pretrypsinogen. The DNA construct, (pRSV: Δpro trypsin), and predicted protein sequence are shown in Fig. 3 A. (Details of the mutagenesis are in Materials and Methods.) This construction will direct the synthesis of a protein, which upon correct signal peptidase cleavage and proper protein folding is an active protease. Because this active protease may be detrimental to the cell, a second construct that introduces an active site mutation as well as the "pro" deletion was also made (pRSV: Δpro D→N trypsin). The chosen mutation, Asp→Asn 102 (D→N 102) has recently been shown to reduce the enzymatic activity at least four orders of magnitude at neutral pH (16).

Both pRSV: Δpro trypsin and pRSV: Δpro D→N trypsin were used to transfect AtT-20 cells, and clones expressing an immunoreactive Δpro trypsin were identified by a western blot assay. Fig. 4 shows a Western blot of proteins from the various expressing cell lines. The anti-trypsinogen immunoreactive protein from RSV-Tryp cells comigrates with a rat anionic trypsinogen standard (lanes 1 and 2). Enteropeptidase digestion (50) of RSV-Tryp protein yields mature trypsin (lane 3). In lanes 4 and 5, the anti-trypsinogen immunoreactive proteins from the Δpro trypsin and Δpro D→N trypsin cell lines are shown to comigrate with mature

**Figure 4.** Western blot analysis of trypsinogen and Δpro trypsin expressing AtT-20 cell lines. Lane 1, purified rat anionic trypsinogen (20 ng) (T' gen Std). Lane 2, trypsinogen (T' gen). Lane 3, enteropeptidase digested trypsinogen (Trypsin). Lane 4, Δpro trypsin (Δpro-T). Lane 5, D→N Δpro trypsin (D→N). Lane 6, uninfected AtT-20 cells (Control). Samples in lanes 2–6 are from the appropriate AtT-20 cells. The blot was probed with a specific rabbit anti-rat anionic trypsinogen serum, followed by an L25I-labeled goat anti-rabbit antibody. Trypsinogen and trypsin migrate with apparent molecular weights of ≈30,000 and ≈29,000, respectively.

**Figure 5.** NH₂-terminal protein sequence analysis of Δpro trypsin. Both secreted (●) and cell-associated (▲) [³⁵S]Cys labeled Δpro trypsin samples were subjected to automated sequence analysis. Anilinothiazolinone amino acids were collected at each cycle and ³⁵S counts per minute were determined. The protein sequence of mature trypsin is shown on top in the one letter amino acid code as in Figs. 2 and 3; in addition, C, cysteine, and Y, tyrosine. The deduced sequence of Δpro trypsin is shown on the bottom line. The C's are shown in bold lettering to emphasize that these are the only amino acids directly identified using this method.
trypsin. Surprisingly, the potentially active protease, \( \Delta \text{pro trypsin} \) did not seem to affect the cells in any way (see below) and the efficiency of transfection was not detectably altered.

When the \( \Delta \text{pro trypsin} \) and \( \Delta \text{pro D} \rightarrow \text{N} \) trypsin-expressing cell lines were exposed to secretagogues, the rate of \( \Delta \text{pro trypsin} \) secretion was stimulated (Fig. 3 B). The sorting index calculated for both of these mutant trypsin proteins did not differ from wild-type trypsinogen (Table 1 B). We conclude that the granule targeting information is not present in the \( \text{NH}_2 \)-terminal "pro" sequence of trypsinogen.

**\( \Delta \text{pro Trypsin Is Not Enzymatically Active} \)**

Cells expressing \( \Delta \text{pro trypsin} \) were viable and unexpectedly indistinguishable from cells expressing trypsinogen or the active site mutant \( \Delta \text{pro D} \rightarrow \text{N} \) trypsin. Using a variety of assays including binding to bovine pancreatic trypsin inhibitor, hydrolysis of highly sensitive fluorescent peptide substrates (15), and a gel overlay activity assay (Evnin, L., and C. S. Craik, unpublished results), we were unable to demonstrate any trypsin activity from \( \Delta \text{pro trypsin} \)-expressing cells. In contrast, the media from trypsinogen expressing cells when digested with enteropeptidase always showed trypsin activity (data not shown). The inactivity could be due to incorrect folding of \( \Delta \text{pro trypsin} \). This possibility seems unlikely because this protein is efficiently synthesized, transported, and secreted from AtT-20 cells. Another possible explanation is that the signal peptidase does not cleave in the expected location. If the \( \text{NH}_2 \)-terminal Ile (+16) of trypsin is modified (57, 60), all proteolytic activity is lost. The structural (22, 39, 45, 65) and binding (6, 7) data further suggest that there is a specific binding pocket for the \( \text{NH}_2 \)-terminus, such that incorrect cleavage would not allow the active conformation to be achieved.

To determine the \( \text{NH}_2 \)-terminal amino acid of \( \Delta \text{pro trypsin} \), automated \( \text{NH}_2 \)-terminal radiosequence analysis was performed on a \( ^{35} \text{S} \)Cys-labeled immunoprecipitated and gel-purified \( \Delta \text{pro trypsin} \) sample. If the \( \text{NH}_2 \)-terminus is Ile (+16) as expected, \( ^{35} \text{S} \)Cys counts should be found in cycles 7 and 25 (see Fig. 5). If, however, we find peaks of \( ^{35} \text{S} \) counts at other positions, but separated by the same number of cycles, the altered \( \text{NH}_2 \)-terminal sequence can be deduced.

Our sequence analysis of \( \Delta \text{pro trypsin} \) shows peaks of radioactivity in cycles 3 and 21 (Fig. 5). These data along with the SDS-PAGE size analysis showing that \( \Delta \text{pro trypsin} \) is the expected molecular weight, are consistent with the \( \text{NH}_2 \)-terminus being at Tyr (+20), four amino acids shorter than expected. Analyses performed on both media and cell extract samples gave similar results, ruling out the possibility of extracellular proteolysis. This result, which places the \( \text{NH}_2 \)-terminus at Tyr (+20), is fully consistent with our inability to demonstrate any \( \Delta \text{pro trypsin} \) activity. We conclude that \( \Delta \text{pro trypsin} \) is not active because it is proteolytically cleaved (probably by signal peptidase) between Gly (+19) and Tyr (+20) instead of between Ala (−1) and Ile (+16) as expected. Furthermore, because the \( \Delta \text{pro trypsin} \) protein is actually missing the first 12 amino acids of trypsinogen (not just the 8-residue "pro" sequence), we can also conclude that the first 12-\( \text{NH}_2 \)-terminal amino acids of trypsinogen are not essential for targeting to the regulated secretory pathway.

**Discussion**

In this article, we show that the exocrine protein trypsinogen is targeted into the same dense-core secretory granule as the endogenous endocrine protein POMC/ACTH in the anterior pituitary tumor cell line, AtT-20. To identify the protein domain responsible for this intracellular sorting process, we have begun to express mutant trypsinogen proteins to try to alter intracellular targeting. Many other intracellular targeting signals are located at the \( \text{NH}_2 \)-terminus of the targeted protein. For example, in procarboxypeptidase Y, the vacuolar targeting signal resides in the \( \text{NH}_2 \)-terminal propeptide (42, 69). In contrast, neither the extreme \( \text{NH}_2 \)-terminal signal sequence of pretrypsinogen nor the \( \text{NH}_2 \)-terminal "pro" domain of trypsinogen are required for targeting to the regulated secretory pathway in AtT-20 cells.

POMC/ACTH and trypsinogen were colocalized in dense-core secretory granules using a double immunocytochemical-staining procedure with antibodies specific for POMC/ACTH and trypsinogen. This technique involves reacting each primary antibody with only one side of a section, and using different-sized gold particles, coupled to secondary antibodies, to detect each antigen separately. Although we found granules that stained for only a single antigen when using this two-sided technique, we do not believe this is significant. Control experiments in which the same primary antibody was used on both sides of the grid, indicated that all granules were not equally accessible from both sides of the section (see discussion in Bendayan [3]). This seems reasonable since the sections are of nearly the same thickness (800 Å) as the diameter of individual secretory granules (diam = 1,000 Å), and therefore many granules should be accessible from only one side of the section.

Recently, Fumagalli and Zanini (24) showed that mixed somatomammotrophic cells of cow pituitary (which express both growth hormone and prolactin), can package their hormones into separate secretory granules as well as mixed within the same granule. Interestingly, they also found some granules in which the hormones were segregated into distinct domains within the secretory granules. They suggested that mechanisms exist within somatomammotrophic cells for sorting growth hormone and prolactin into separate granules. Bassetti et al. (1) have presented morphologic evidence for mixed packaging of growth hormone and prolactin into the secretory granules of human pituitary tumors. They also noted that in one tumor they saw separate growth hormone containing granules as well as mixed granules. In contrast, we have no compelling evidence that AtT-20 cells have the ability to distinguish between different regulated secretory proteins; POMC/ACTH and trypsinogen appear to be targeted to secretory granules indistinguishable by both the biochemical (8) and morphologic criteria.

Our finding that replacing the trypsinogen signal peptide with the signal peptide from a constitutive protein does not alter targeting to secretory granules was not unexpected. \( \text{NH}_2 \)-terminal signal peptides are known to be responsible for signal recognition particle dependent ER targeting (for review, see Walter et al. [73]). Once the nascent polypeptide is inserted into the ER membrane, signal peptidase makes a specific cleavage that removes the signal peptide (21, 41, 51), probably co-translationally (4, 27). The signal peptide could only be involved in granule targeting if the initial
interaction between the regulated secretory protein and the sorting apparatus takes place in the ER. Protein sorting into the regulated secretory pathway is thought to be a post-Golgi apparatus event (44), and the data presented here are consistent with this view.

In designing our trypsinogen mutation strategy, we have relied on the structural data available for both trypsinogen (22, 45) and trypsin (39, 65). Inasmuch as the conversion of trypsinogen to trypsin involves predominantly local structural changes, we reasoned that deleting the "pro" sequence would perturb the protein structure to such a small extent that the mutant protein would still be transported beyond the ER to the cell surface. Similarly, although the active site mutation reduces the proteolytic activity at least four orders of magnitude at neutral pH (16), the three-dimensional structure of the D→N102 mutant protein is virtually identical to the wild-type protein (64). The kinetics of intracellular transport of both the Δpro and Δpro D→N variants were slowed about twofold compared with trypsinogen (data not shown); however, the efficiency of sorting into the regulated secretory pathway was not altered (Table I B). Although the NH₂-terminal "pro" domain of trypsinogen does not appear to be required for protein targeting to the regulated secretion pathway in ΔAT-20 cells, the possibility remains that the targeting information is redundant. Either the "pro" sequence or another sequence may be sufficient for protein targeting to the regulated secretion pathway.

Our ability to recover Δpro trypsin expressing ΔAT-20 clones was surprising as we expected pRSV::Δpro trypsin to direct the synthesis of the potent protease, trypsin. Direct protease activity measurements indicated that Δpro trypsin was not enzymatically active. NH₂-terminal sequence analysis of [35S]Cys-labeled, immunopurified Δpro trypsin (Fig. 5) indicated that the first four amino acids of wild-type trypsin were missing from Δpro trypsin purified from either radiolabeled cell extracts or media samples. The NH₂-terminal Ile (+16) is required for enzymatic activity (57, 60), and thus this result explains why Δpro trypsin is not active. However, it is unclear why this unexpected NH₂-terminus is created.

Using a recently published method for predicting signal sequence cleavage sites (71), we calculated weighted scores along the wild-type pretrypsinogen and preΔpro trypsin sequences. Whereas this calculation method correctly predicts the wild-type pretrypsinogen signal peptide cleavage site between Ala (−1) and Phe (+1), it does not predict the observed cleavage site in preΔpro trypsin between Gly (+19) and Tyr (+20). Although the score at this position is only the sixth highest along the preΔpro trypsin sequence, it does occur as a local peak. The weighted calculation method used here relies largely on the sequence of the signal peptide itself; in that this sequence is unchanged in Δpro trypsin, the specificity of the proteolytic cleavage must be affected by the sequences COOH-terminal to the signal sequence. The prediction method, however, does not rely on the overall length of the signal peptide. The observed cleavage site of preΔpro trypsin increases the signal peptide length from 15 to 19 residues which may play a role in cleavage site selection.

The data presented here suggest that the secretory granule targeting information of trypsinogen is not present at the NH₂-terminus of the protein, thus the information must be contained within the mature portion of the protein. We have previously compared the primary structures of many different regulated secretory proteins in search of some common targeting sequence; none was found. We suggest that the secretory granule targeting signal might be similar to signal peptides, showing no primary sequence homology, yet displaying functional identity with one another. The common targeting information may interact with a presumptive sorting receptor via a primary sequence-independent association similar to that recently described for peptide antigens with the class II major histocompatibility antigen (30).

The conventional approach to identify a protein domain containing targeting information is to generate fusion proteins where the presumptive sorting domain is fused to a reporter element. To study sorting in the Golgi complex, however, such fusion proteins must first escape the ER. Recent experiments from this laboratory (Matsuuchi and Kelly, manuscript submitted for publication; 53) have demonstrated that this may be a major limitation. The mutation approach described here offers an alternative strategy. If a mutation causes loss of targeting, it could be due to removal of a targeting signal, or aberrant protein folding that could obscure a targeting signal. If, however, targeting can be disrupted without loss of enzymatic activity, one can make the reasonable argument that the protein structure is intact, and the targeting signal itself has been altered (58, 69). The knowledge of the three-dimensional structure of trypsinogen may facilitate the design of such catalytically active but incorrectly targeted proteins.

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References

1. Bassetti, M., A. Spada, M. Arosio, L. Vallar, M. Brina, and G. Giannattasio. 1986. Morphological studies on mixed growth hormone (GH)- and prolactin (PRL)-secreting human pituitary adenomas: coexistence of GH and PRL in the same secretory granule. J. Clin. Endocrinol. Metab. 62: 1093-1100.

2. Bendayan, M. 1982. Double immunocytochemical labeling applying the protein A-gold technique. J. Histochem. Cytochem. 30:81-83.

3. Bendayan, M. (1984). Protein A-gold electron microscopic immunocytochemistry: methods, applications and limitations. J. Electron. Microsc. Tech. 1:243-270.

4. Bergman, L. W., and W. M. Kuehl. 1979. Formation of an intrachain disulfide bond on nascent immunoglobulin light chains. J. Biol. Chem. 254: 8869-8876.

5. Blobel, G. 1980. Intracellular protein topogenesis. Proc. Natl. Acad. Sci. USA. 77:1496-1500.

6. Bode, W., and R. Huber. 1976. Induction of the bovine trypsinogen-trypsin transition by peptides sequentially similar to the N-terminus of trypsin. FEBS (Fed. Eur. Biochem. Soc.) Lett. 68:231-236.

7. Bode, W., P. Schwager, and R. Huber. 1978. The transition of bovine trypsinogen to a trypsin-like state upon strong ligand binding: the refined
63. Southern, P. J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. 1:327–341.

64. Sprang, S., T. Standing, C. S. Craik, J. Finer-Moore, R. Stroud, N.-H. Xuong, R. Hamlin, W. J. Rutter, and R. Fletterick. 1987. The three-dimensional structure of Asn102 trypsinogen: role of Asp102 in serine protease catalysis. Science (Wash. DC). In press.

65. Stroud, R. M., L. M. Kay, and R. E. Dickerson. 1974. The structure of bovine trypsin: Electron density maps of the inhibited enzyme at 5 Å and at 2.7 Å resolution. J. Mol. Biol. 83:185–208.

66. Tartakoff, A. M. 1983. Mutations that influence the secretory path in animal cells. Biochem. J. 216:1–9.

67. Tartakoff, A., and P. Vassalli. 1978. Comparative studies of intracellular transport of secretory proteins. J. Cell Biol. 79:694–707.

68. Valentino, K. L., D. A. Crumrine, and L. F. Reichardt. 1985. Lowicryl K4M embedding of brain tissue for immunogold electron microscopy. J. Histochem. Cytochem. 33:969–975.

69. Valls, L. A., C. P. Hunter, J. H. Rothman, and T. H. Stevens. 1987. Protein sorting in yeast: the localization determinant of yeast vacuolar carboxypeptidase Y resides in the propeptide. Cell. 48:887–897.

70. Van den Broeck, G., M. P. Timko, A. P. Kausch, A. R. Cashmore, M. Montagu, and L. Herrera-Estrella. 1985. Targeting of a foreign protein to chloroplasts by fusion to the transit peptide from the small subunit of ribulose 1,5-bisphosphate carboxylase. Nature (Lond.). 313:358–363.

71. von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. 14:4683–4691.

72. Walter, P., R. C. Jackson, M. M. Marcus, V. R. Lingappa, and G. Blobel. 1979. Tryptic dissection and reconstitution of translocation activity for nascent presecretory proteins across microsomal membranes. Proc. Natl. Acad. Sci. USA. 76:1795–1799.

73. Walter, P., R. Gilmore, and G. Blobel. 1984. Protein translocation across the ER. Cell. 38:5–8.

74. Wychowski, C., D. Besichou, and M. Girard. 1986. A domain of SV40 capsid polypeptide VP1 that specifies migration into the cell nucleus. EMBO (Eur. Mol. Biol. Organ.) J. 5:2569–2576.

75. Zoller, M. J., and M. Smith. 1984. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. DNA (NY). 3:479–488.