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

Teresa L. Burgess,* Charles S. Craik,** Linda Matsuuchi,* and Regis B. Kelly*
*Department of Biochemistry and Biophysics, and ‡Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143

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 adrenocorticotropic 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
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, has been 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 unrecognized 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 is essential for targeting to the regulated secretory pathway in AIT-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 polynucleotide 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 Langman (Vesnars 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 Sarotius Filters (Hayward, CA) and for colony and plaque screening were from Schleicher & Schuell, Inc. (Keene, NH). Gold conjugated to goat anti-rabbit antibody was from Janssen Pharmaceutica (Piscataway, NJ.).

Cell Culture and Transfections

AIT-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 AIT-20 cells were by a calcium phosphate precipitation protocol described previously (8, 54). Cotransfections with the selectable plasmid pSV2neo (63) and the nonselecta-
sequence of the Ksp-trypsinogen junction of pRSV::Ksp trypsinogen was confirmed using a double-stranded DNA-sequencing protocol (II).

pRSV::Δpro trypsin was constructed using a double-primer oligonucleotide mutagenesis protocol (75). The introns less trypsinogen minigene was subcloned into MDmp8 as described (15). This single-stranded antisense clone was used as the template for mutagenesis. A 24-nucleotide-long mutagenic primer, TB04, (5′-CGGCTGCTGCTAGTGAGGAGGAGA)3′, was synthesized by the Biomedical 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′-GTAACACGAGCCGACATG3′) were annealed to the single-stranded antisense trypsinogen M13 template DNA. After extension with the Klenow fragment of DNA polymerase I and ligation with T4 DNA ligase, E. coli strain JM101 was transformed with the DNA. The resulting phage plaques were screened using 35S end-labeled TB04 (44). 14% of the plaques hybridized to the TB04 oligonucleotide and 4/4 were shown by DNA sequencing to contain the desired Δpro trypsin mutation. Double-stranded phage DNA was isolated and the 32P-labeled HindIII-XhoI 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 supercoiled DNA sequencing protocol (II).

pRSV::Δpro D→N trypsin was constructed by isolating the Eco RI-Xho I restriction fragment containing the Asp→Asn 102 (D→N102) active site mutant from pTRAP D→N102 (29). This fragment was used to replace the wild-type fragment in pRSV::Δpro trypsin using standard subcloning protocols (49). The D→N102 mutation was confirmed by sequencing the double-stranded plasmid DNA of pRSV::ΔproD→N trypsin (II).

Protein Sequence Analysis
Δpro trypsin-expressing cells were labeled overnight with [35S]cysteine and Δpro trypsin was purified from both the medium and cell extract by immunoaffinity precipitation. 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 ~30,000 cpm. The sequence analysis was performed on an Applied Biosystems gas-phase sequencer, model 470A, at the Biomedical Resource Center at UCSF. Anilinothiazolone 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., Warrington, 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 I. Quantitative Comparison of Protein Targeting to the Regulated Secretion Pathway in AtT-20 Cells

<table>
<thead>
<tr>
<th>DNA construction</th>
<th>Secreted protein</th>
<th>Sorting index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Regulated and constitutive secretory proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSV2-neo::MTt trypsinogen</td>
<td>POMC/ACTH</td>
<td>0.16 ± 0.01 (2)†</td>
</tr>
<tr>
<td>pRSV::trypsinogen</td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>pRSV::Ksp trypsinogen</td>
<td></td>
<td>0.15 ± 0.01 (2)†</td>
</tr>
<tr>
<td>pRSV::TG</td>
<td>Truncated G</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.005 (1)‡</td>
</tr>
<tr>
<td>B. Trypsinogen and mutant trypsinogens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRSV::trypsinogen</td>
<td>Trypsinogen</td>
<td>0.15 ± 0.01 (2)†</td>
</tr>
<tr>
<td>pRSV::Δpro trypsin</td>
<td>Δpro trypsin</td>
<td>0.15</td>
</tr>
<tr>
<td>pRSV::Δpro Δ→N trypsin</td>
<td>Δpro Δ→N trypsin</td>
<td>0.17</td>
</tr>
</tbody>
</table>

* Calculated as in Moore and Kelly (52); see also Materials and Methods.
† Values given ± range were more than one experiment was performed. Numbers in parentheses indicate the number of independent experiments.
‡ Data from Moore and Kelly (52).
1 See Fig. 5 and text for NH2-terminal sequence analysis.
moter (Table 1A). 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-Trypt cell line was then used to perform the double-label immunoelectron microscopy experiment shown in Fig. 1. A Lowicryl-embedded section of RSV-Trypt 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 II 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

---

2. 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, BamHI; H, HindIII; and S, SstI. 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.

Figure 3. pRSV::Δpro trypsin construction and Δpro trypsin secretion analysis. (A) The Δpro 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 Δpro 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.

does a signal peptide that is the same length as the Ksp, however, the last two amino acids encoded are Gln and Ala, instead of Asp and Gly (see Fig. 2A). 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.

Predicted Protein Sequences:
wt-T : MRA LLFLALVGAAV A- (Trypsinogen)
Ksp-T : MHH TSMG I K M ESQ 1 O V FVFVF LV LS GVQA - (Trypsinogen)

Predicted Protein Sequences:
wt-T : MRA LLFLALVGAAV A- (Trypsinogen)
Δ pro-T : MRA LLFLALVGAAV A- (Trypsinogen)
Deletion of the NH₂-Terminal “pro” Sequence Does Not Alter Targeting to Secretory Granules

Trypsinoigen 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 pretrypsin. 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 A7T-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-trypsinoigen immunoreactive protein from RSV-TRP cells comigrates with a rat anionic trypsinoigen standard (lanes 1 and 2). Enteropeptidase digestion (50) of RSV-TRP trypsin protein yields mature trypsin (lane 3). In lanes 4 and 5, the anti-trypsinoigen immunoreactive proteins from the Δpro trypsin and Δpro D→N trypsin cell lines are shown to comigrate with mature...
trypsin. Surprisingly, the potentially active protease, Δpro trypsin did not seem to affect the cells in any way (see below) and the efficiency of transfection was not detectably altered.

When the Δpro trypsin- and Δpro D→N trypsin-expressing cell lines were exposed to secretagogues, the rate of Δpro 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 NH₂-terminal “pro” sequence of trypsinogen.

Δpro Trypsin Is Not Enzymatically Active

Cells expressing Δpro trypsin were viable and unexpectedly indistinguishable from cells expressing trypsinogen or the active site mutant Δpro D→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. Craig, unpublished results), we were unable to demonstrate any trypsin activity from Δpro 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 Δpro trypsin. This possibility seems unlikely because this protein is efficiently synthesized, transported, and secreted from AT-20 cells. Another possible explanation is that the signal peptidase does not cleave in the expected location. If the NH₂-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 NH₂ terminus, such that incorrect cleavage would not allow the active conformation to be achieved.

To determine the NH₂-terminal amino acid of Δpro trypsin, automated NH₂-terminal radiosequencing analysis was performed on a [35S]Cys-labeled immunoprecipitated and gel-purified Δpro trypsin sample. If the NH₂ terminus is Ile (+16) as expected, [35S]Cys counts should be found in cycles 7 and 25 (see Fig. 5). If, however, we find peaks of [35S] counts at other positions, but separated by the same number of cycles, the altered NH₂-terminal sequence can be deduced.

Our sequence analysis of Δpro trypsin shows peaks of radioactivity in cycles 3 and 21 (Fig. 5). These data along with the SDS-PAGE size analysis showing that Δpro trypsin is the expected molecular weight, are consistent with the NH₂ 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 NH₂ terminus at Tyr (+20), is fully consistent with our inability to demonstrate any Δpro trypsin activity. We conclude that Δpro 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 Δpro 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-NH₂-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, AT-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 NH₂ terminus of the targeted protein. For example, in procarboxypeptidase Y, the vacuolar targeting signal resides in the NH₂-terminal propeptide (42, 69). In contrast, neither the extreme NH₂-terminal signal sequence of pretrypsinogen nor the NH₂-terminal “pro” domain of trypsinogen are required for targeting to the regulated secretory pathway in AT-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 AT-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. NH₂-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 (66), 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 AT-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 AT-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 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 (Matsuiuchi 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.

We extend very special thanks to Debbie Crumrine (UCSF) for performing the immunoelectron microscopy presented in Fig. 1. We also thank Laszlo Graf and especially Luke Evnin for help with the trypsin activity assays, and the Kelly lab for support at many levels throughout the course of this work. We are very grateful to Adam Linstead (UCSF) for writing the Macintosh computer program with which we easily calculated and compiled the scores for possible signal peptide cleavage sites, and to Leslie Specter for her assistance in preparing the manuscript. Dr. Burgess would like to thank Mary Horne, Susan Harding, and Sarah Bodary for keeping her on (the) track, and Dennis Clegg for being remarkably helpful in so many ways.

This research was supported by National Institutes of Health grant AM-33937 to Dr. Kelly; National Science Foundation grant DMB 8608086 to Dr. Craik; and New Investigator Research Award AM-35895 from National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases to Dr. Matsuiuchi. Dr. Burgess was supported by National Institutes of Health/National Cancer Institute postdoctoral training grant T32 CA-09270.

Received for publication 27 January 1987, and in revised form 13 April 1987.

References

7. Bode, W., P. Schwager, and R. Huber. 1978. The transition of bovine trypsinogen to trypsin involves strong bond binding: the refined
Burgess et al. Role of Amino Terminus in Secretory Protein Targeting


