The Exocrine Protein Trypsinogen Is Targeted into the Secretory Granules of an Endocrine Cell Line: Studies by Gene Transfer

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ABSTRACT The exocrine protein rat anionic trypsinogen has been expressed and is secreted from the murine anterior pituitary tumor cell line AtT-20. We examined which secretory pathway trypsinogen takes to the surface of this endocrine-derived cell line. The "constitutive" pathway externalizes proteins rapidly and in the absence of an external stimulus. In the alternate, "regulated" pathway, proteins are stored in secretory granules until the cells are stimulated to secrete with 8-Br-cAMP. On the basis of indirect immunofluorescence localization, stimulation of release, and subcellular fractionation, we find that trypsinogen is targeted into the regulated secretory pathway in AtT-20 cells. In contrast, laminin, an endogenous secretory glycoprotein, is shown to be secreted constitutively. Thus it appears that the transport apparatus for the regulated secretory pathway in endocrine cells can recognize not only endocrine prohormones, but also the exocrine protein trypsinogen, which suggests that a similar sorting mechanism is used by endocrine and exocrine cells.

The intracellular pathway of secretory proteins from the rough endoplasmic reticulum through the Golgi apparatus and to the cell surface is well established (26). Specialized secretory cells, for example pancreatic acinar cells, concentrate and store one or a few secretory products in large organelles, the secretory granules. Release of these products by exocytosis is triggered by a physiological stimulus and has been described as regulated secretion (31). In contrast to this regulated secretion, other types of cells, for example fibroblasts, which lack visible secretory granules, do not store or concentrate their secretory products but rather secrete proteins continuously in a secretagogue-independent or nonregulated fashion.

The pituitary cell line AtT-20 synthesizes proopiomelanocortin (POMC). After POMC is glycosylated and proteolytically processed, the AtT-20 cells store the peptide hormone product, ACTH, in dense core secretory granules. ACTH is secreted from these cells in response to stimulation with secretagogues (22). Thus, these endocrine-derived cells behave as regulated secretory cells in culture. In addition, AtT-20 cells externalize the endogenous type C retroviral membrane glycoprotein, gp 70, and some POMC by a pathway that is not stimulated by secretagogues and does not involve the classical dense core secretory granules (11, 12). These findings suggest that regulated and constitutive secretion, originally described by Tartakoff and Vassalli (31) for different cell types, can co-exist in the same cell. The mechanism by which the AtT-20 cell can target proteins into two secretory pathways may occur via recognition of sorting signals present in the secreted molecules (4). Candidates for such sorting signals include various posttranslational modifications, as well as the amino acid sequence, the secondary or even tertiary structure of the secretory protein.

To determine whether there is a common mechanism of recognition and targeting of endocrine prohormones to dense core secretory granules, several heterologous prohormone genes have been introduced into the murine AtT-20 cells. Human proinsulin is expressed and apparently correctly processed to mature insulin in these cells and is released by the regulated pathway (25). Furthermore, when human growth hormone is expressed in these cells, it is targeted into dense secretory granules as efficiently as the endogenous POMC/ACTH. These data suggest that these endocrine proteins use a common sorting mechanism.

Since the storage in dense core secretory granules and

1 Abbreviations used in this paper: Cys, cysteine; DME, Dulbecco's modified Eagle's medium; gp 70, glycoprotein 70; Met, methionine; MTP, human metallothionein II A promoter; N-dt, buffer of 1% Nonidet P-40, 0.4% deoxycholate, 66 mM EDTA, and 10 mM Tris-HCl, pH 7.4; POMC, proopiomelanocortin; Sac, Staphylococcus aureus cells.

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regulated secretion of proteins are properties shared by endocrine and exocrine cells, we asked if an exocrine protein can be properly targeted to the secretory granules of endocrine-derived AT-20 cells. We used the DNA coding for rat anionic trypsinogen to transfect AT-20 cells, and stable cell lines were established that heterologously expressed trypsinogen. In these cells, the exocrine protein trypsinogen is targeted into secretory granules as efficiently as the endogenous endocrine protein POMC/ACTH.

Since all of the heterologous proteins thus far expressed in the AT-20 cells are secretory proteins, and our marker protein for the constitutive pathway, gp 70, is a membrane protein, we have examined the question of secretory protein externalization via the constitutive pathway. We find that the secretory glycoprotein laminin (32) is secreted constitutively, it is not stored intracellularly, and its release is not stimulated by the secretagogue 8-Br-cAMP. The data presented here indicate that regulated endocrine and exocrine secretory proteins can be selectively sorted by the same endocrine cell, perhaps by a mechanism involving a common sorting signal.

MATERIALS AND METHODS

Materials: Recombinant endonucleases and T-4 ligase were from New England Biolabs (Beverly, MA). DNA polymerase 1 (Klenow) was from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Na$_{251}$, [35S]cysteine ([35S]-Cys), and [35S]methionine ([35S]Met) were from Amersham Corp. (Arlington Heights, IL). IgG fraction goat anti-rabbit and fluorescein-conjugated IgG fraction goat anti-rabbit antibodies were from Cappel Laboratories (Coeyman, PA), G418 (Geneticin) was purchased from Gibco Laboratories Inc. (Grand Island, NY). Trypsinogen standards and rabbit anti-rat anionic trypsinogen were kindly provided by Corey Largman (Martinez Veterans Administration Medical Center, Martinez, CA). Rabbit anti-ACTH was prepared and affinity purified as described by Mains and Epper (21). Rabbit anti-laminin and purified laminin were kind gifts from Janet Winter and John Bixby (University of California, San Francisco). 0.45 μm pore nitrocellulose was from Sartorius Filters, Inc. (Hayward, CA), Fixed Staphylococcus aureus (Panobcin) was from Calbiochem-Behring Corp. (La Jolla, CA).

Cell Culture and Transfection: AT-20/D-16 cells were grown in Dulbecco's modified Eagle's medium (DME) H-21 (4.5 g glucose/L) supplemented with 10% fetal calf serum and penstrep, under 15% CO$_2$ atmosphere. 50 μM ZnSO$_4$ was added to the culture medium 24 h before and during sample collection for induction of the human metallothionein II,A promoter (MTP). Transfections of AT-20 cells was by a calcium phosphate precipitation protocol as described by Moore et al. (25). Since the plasmids used here for trypsinogen expression retained the selectable neo gene (28), co-transfection was not required. In brief, calcium phosphate-DNA precipitates formed using 100 μg plasmid DNA were added to 3 × 10$^6$ AT-20 cells per 10 cm dish. After 40 min at room temperature, 12 ml DME H-21 containing 10% fetal calf serum was added, and the cells were incubated 7 h at 37°C under 15% CO$_2$. Cells were then glyceroled and returned to 37°C for 48 h. Transfected cells were split 1:2 into new dishes containing 0.24 mg/ml G418 (actual drug concentration). After the labeling period, the cells were rinsed with PBS and fixed at room temperature in 10% formalin for 20 min. Cells were permeabilized by incubation in 3% BSA, 0.1% Triton X-100 in PBS. Primary antibodies were diluted 1:200 (trypsinogen) or 1:2 (ACTH) in 1% BSA 0.1% Triton X-100 in PBS buffer and used to detect the fixed cells at room temperature for 30 min. Fluorescein-conjugated goat anti-rabbit antibody was diluted 1:200 in 0.1% Triton X-100 in PBS buffer and used to detect the primary antibody/antigen complex. Cells were visualized and photographed using a Zeiss photomicroscope III. To allow direct comparison of the micrographs, exposure conditions were identical for each antigen; i.e., the exposure times for Fig. 3, C and D were identical.

DNA Constructions: For expression of trypsinogen in AT-20 cells (see Fig. 1), the Hind III-Bam HI DNA fragment containing MTP (15) was ligated to the Hind III-Sal I fragment of the trypsinogen gene/cDNA fusion (9) after the Bam HI (MTP) and Hind III (trypsinogen) ends were blunt-ended by filling in with deoxynucleotides using DNA polymerase I (Klenow fragment). The MTP-trypsinogen hybrid was then subcloned into the Hind III and Sal I sites of the pBR322 derivative, pML-2 (19), to create pML-MTP-trypsinogen. The Eco RI-Nru I fragment from pML-MTP-trypsinogen, containing the trypsinogen gene and metallothionein promoter, was subcloned into the Eco RI site of pSV2-neo (28). Plasmids were constructed and screened using standard recombinant DNA techniques as outlined in reference 23. Plasmids were purified by alkaline-SDS lysis (3) and CCl$_3$-ethidium bromide equilibrium sedimentation (23).

RESULTS

Construction of a Selectable Plasmid Coding for Rat Anionic Trypsinogen

The isolation and sequence characterization of the rat trypsinogen II cDNA and gene that encode anionic trypsinogen have been described elsewhere (8, 20). A full-length copy of the trypsinogen coding sequence, including the signal peptide and an intervening sequence at amino acid 2 (which were not present in the cDNA clone), was constructed by fusing the 5' portion of the trypsinogen II gene to the 3' portion of the trypsinogen II cDNA (9). To obtain expression of trypsinogen in AT-20 cells (see Fig. 1), the DNA fragment containing the
expression vector, pSV2-neo::MTp-trypsinogen, was constructed as described in the text. A few restriction sites are shown to orient the reader; sites in parentheses were lost during the construction. pSV2-neo is ~5,500 base pairs, the insert ~2,800 base pairs. Various regions of the insert are labeled. The signal peptide is removed before trypsinogen is secreted. The processing site refers to the zymogen activation site where proteolytic cleavage occurs extracellularly in vivo. The direction and approximate location of neo transcription are shown by the arrow.

human metallothionein IIA promoter (MTp) (15) was ligated to the trypsinogen gene/cDNA fusion. The MTp–trypsinogen hybrid was then subcloned into the pBR322 derivative, pML-2 (19), to create pML::MTp-trypsinogen.

To facilitate the isolation of AtT-20 cells that express trypsinogen, the gene for the selectable marker neo was included in the plasmid carrying the MTp-trypsinogen mini-gene. The fragment from pML::MTp-trypsinogen containing the trypsinogen gene and metallothionein promoter was subcloned into the Eco RI site of pSV2-neo (28) to make pSV2-neo::MTp-trypsinogen. Plasmids with the MTp-trypsinogen hybrid gene in both orientations, relative to pSV2-neo, have been used in this study.

**AtT-20 Cells Transformed with pSV2neo::MTp-Trypsinogen Express and Secrete Rat Anionic Trypsinogen**

To study the secretion of an exocrine protein from an endocrine cell line, we established stable AtT-20 cell lines expressing trypsinogen. AtT-20 cells were transfected with pSV2-neo::MTp-trypsinogen by a calcium phosphate precipitation protocol described previously (25). G418-resistant clones were isolated and expanded into mass cultures, and the media from these clones were screened for trypsinogen expression by Western blot analysis (7). Proteins from samples of culture media, separated by SDS PAGE, were electrophoretically transferred to a nitrocellulose filter. The filter was probed with a specific polyclonal rabbit anti-rat anionic trypsinogen serum followed by a second 125I-labeled goat anti-rabbit antibody. Approximately 50% of the clones expressed detectable amounts of trypsinogen. A Western blot of culture medium from the transformed cell line AtT-20/tryp-10 is shown in Fig. 2. A purified mixture of rat anionic trypsinogen (70%) and activated trypsin (30%) was run as a standard in an adjacent lane. Comparison of the protein secreted by the transformed AtT-20 cell lines with the rat standard revealed that expressing clones secreted trypsinogen but not trypsin. Notably, no intra- or extracellular processing to give active trypsin had occurred.

The level of trypsinogen made by the transformed cells was examined by Western blot analysis. By comparing the amount of trypsinogen made by the transformed cells to purified trypsinogen standards on Western blots, we estimated that several clones contained as many as 2 × 10^3 molecules of trypsinogen per cell. This level of expression could be enhanced three- to fivefold by adding Zn^{++} to the culture medium. Northern blot analysis showed that this induction occurred at the transcriptional level as expected for the metallothionein promoter (10, 14) (data not shown). However, because the Zn^{++} concentration used for induction of expression caused detachment of the AtT-20 cells from the culture dish after prolonged treatment, experiments were usually carried out in the absence of heavy metals. The orientation of the MTp-trypsinogen fusion within the pSV2-neo plasmid did not affect the level of expression.

**Trypsinogen and ACTH Are Co-localized Intracellularly**

If the exocrine protein trypsinogen is recognized by the endocrine sorting apparatus of AtT-20 cells, one would expect it to be stored intracellularly in secretory granules and to show a distribution similar to endogenous ACTH. AtT-20 cells show a characteristic immunofluorescent staining pattern for ACTH with predominant staining in the juxtanuclear region and in the cell periphery (16). This staining pattern is particularly distinct if the AtT-20 cells are first grown in 8-Br-cAMP (1, 16). The cells extend long processes, cease dividing, and become “giant cells.” Under these conditions, anti-ACTH staining localizes POMC/ACTH both in the juxtanuclear region and at the tips of some but not all processes. The juxtanuclear staining is presumably due to POMC/ACTH in the Golgi region. The process tips have been shown to contain...
large numbers of dense core secretory granules (16), which probably account for the presence of ACTH. Examples of anti-ACTH immunofluorescence staining of giant AtT-20 cells are shown in Fig. 3, A and B. To determine whether ACTH and trypsinogen share the same intracellular distribution, anti-trypsinogen antibodies were used to stain both transformed and untransformed AtT-20 cells that had been grown in 8-Br-cAMP. The results are shown in Fig. 3, C and D. Only the AtT-20/tryp-7 cells (Fig. 3 C) showed the characteristic juxtanuclear and tip staining for trypsinogen. Untransformed cells showed only a uniform background staining (Fig. 3 D). When AtT-20 cells are stained for the endogenous constitutive membrane marker, gp 70, cell surface staining is seen but process tip staining is not detected (Schroer, T. A., and R. B. Kelly, unpublished observations).

Trypsinogen Secretion Is Enhanced by Secretagogues

If trypsinogen behaves as a regulated secretory protein in the AtT-20 pituitary cell line, its secretion should be enhanced by the secretagogue 8-Br-cAMP. AtT-20/tryp-7 cells were labeled with [35S]Met and [35S]Cys for 16 h to approach a steady state labeling condition. The steady state rate of secretion was determined by immunoprecipitating the protein released into the medium during the last hour of the labeling incubation. The ratio of signal to background was enhanced by taking advantage of the rapid secretion through the constitutive pathway relative to the regulated pathway (12). Two 3-h chases were carried out in the absence of label or secretagogues to chase constitutive secretory proteins out of the cell. To assay secretagogue dependent secretion, 8-Br-cAMP was added during a third 3-h chase. Trypsinogen, ACTH, and laminin, immunoprecipitated from media samples, were quantified by densitometric scanning of SDS PAGE autoradiograms. The combined results of two independent immunoprecipitations from AtT-20/tryp-7 media samples (Fig. 4) demonstrate that the secretions of trypsinogen and ACTH were indistinguishable. Trypsinogen, like ACTH, was stored...
within the transformed cells for long intervals and could be released upon stimulation with the secretagogue, 8-Br-cAMP. The control for this experiment was the secreted glycoprotein laminin. Laminin was released from AtT-20 cells with a t₁/₂ of 120 min without intracellular storage and without any stimulation of release by 8-Br-cAMP. In a similar manner we recently showed that a secreted form of vesicular stomatitis virus G protein introduced into AtT-20 cells via transfection is also released without stimulation by secretagogues. Thus, the exocrine protein trypsinogen behaves as a regulated secretory protein when expressed in the endocrine-derived AtT-20 cell line.

Trypsinogen Co-purifies with Mature ACTH in Dense Core Secretory Granules

The immunofluorescence data (Fig. 3) imply that the trypsinogen stored in the transformed AtT-20 cell line has the same intracellular distribution as ACTH. To confirm biochemically that trypsinogen was present in the dense core secretory granules, they were isolated from AtT-20/tryp-7 cells by a published procedure (11), using ACTH as a marker for secretory granule content.

AtT-20/tryp-7 cells were labeled with [³⁵S]Met and [³⁵S]-Cys. The cells were harvested and dense core secretory granules were isolated. Immunoprecipitations for either ACTH or trypsinogen were performed on individual gradient fractions (Fig. 5). As expected, mature ACTH (Fig. 5, open circles) but no POMC (triangles) was found in the secretory granule peak (fractions 3-5); POMC was recovered in the major membrane peak (fractions 13-17). Trypsinogen (closed circles) co-purified with the mature ACTH. Labeled material was analyzed by immunoprecipitation at each step of the purification, and the purification table for granules containing ACTH and trypsinogen is shown in Table I. The specific activity of these two antigens increased in parallel, and a final purification of ~70-fold was achieved, in good agreement with the published purification of secretory granules (11). Thus, ACTH and trypsinogen co-segregate, indicating that trypsinogen was localized in granules, as was the endogenous peptide hormone ACTH.

DISCUSSION

We have introduced and expressed the DNA encoding rat anionic trypsinogen in the mouse pituitary cell line AtT-20 to determine whether the sorting apparatus present in an endocrine cell can recognize and target an exocrine secretory protein to the secretory granules. We conclude that AtT-20 cells target trypsinogen to ACTH-containing secretory granules based on our findings that trypsinogen co-localizes intracellularly with ACTH (Fig. 3), shows an enhanced rate of

FIGURE 4 Kinetics of laminin, ACTH, and trypsinogen secretion. Cells were labeled with [³⁵S]Met and [³⁵S]-Cys for 16 h to approach steady state labeling conditions. Three consecutive 3-h chases were carried out after the labeling period. The numbers 1, 2, and 3 refer to these 3-h collections. The amounts of ACTH, trypsinogen, and laminin secreted from AtT-20/tryp-7 during the three 3-h chase periods, normalized to the rate of release before the chase, are shown. During the final chase the secretagogue 8-Br-cAMP was added to one of two identical dishes of cells as indicated. Error bars are shown where several samples were analyzed. The number of samples is shown at the top right of each bar. Since there are multiple forms of ACTH, the sum, corrected for the number of methionines in each form of ACTH and POMC, is presented. However, the ratio of forms is not constant throughout the chases. POMC is prevalent in the early chases, whereas ACTH exclusively is stimulated by 8-Br-cAMP.
TABLE I. Secretory Granule Purification

|                | Specific activity |
|----------------|-------------------|
|                | Trypsinogen | ACTH |
| Homogenate     | 0.08        | 2.5  |
| P2-pellet      | 0.32        | 11.0 |
| Peak (pool)    | 6.1         | 175.0|

Increase in specific activity (peak/homogenate) 76 70

These results were calculated from the experiment shown in Fig. 5. ACTH and POMC are combined here to indicate the total amount of ACTH-containing molecules. The specific activity was calculated by dividing the total peak area (arbitrary units) from the densitometric scan of the immunoprecipitation gel by the total TCA-precipitated radioactivity present in the sample.

secretion in the presence of secretagogues (Fig. 4), and can be co-purified with ACTH in dense core secretory granules (Fig. 5 and Table I). The behavior of trypsinogen and ACTH can be contrasted with that of the endogenous secretory protein laminin. Laminin is secreted from AtT-20 cells without intracellular storage and in a secretagogue-independent manner (Fig. 4).

Trypsinogen expression, under control of the metallothionein promoter in stably transformed AtT-20 cells, was found to be induced three- to fivefold by heavy metals. The level of trypsinogen expression was 5 to 10 times higher than the insulin expression level obtained using an SV40-proinsulin construction in these mouse cells (25). The difference in expression level does not appear to be due to differences in the stability of the two proteins (Moore, H.-P. H., and T. L. Burgess, unpublished observation). It may reflect the copy number of the insulin and trypsinogen genes in the transfected lines studied or may be the result of differences in the strength of the SV40 and MTp's in these murine cells. This latter possibility seems likely, as we find the SV40 promoter to be extremely weak in quantitative comparisons of promoter strength in transient expression assays in AtT-20 cells. The orientation of the MTp-trypsinogen mini-gene fusion within pSV2-neo does not seem to influence the level of expression.

Intracellular proteolytic processing is common among regulated endocrine secretory proteins. In the AtT-20 cell line POMC is proteolytically processed at several dibasic amino acid sequences into smaller peptides, including ACTH. When the DNA encoding proinsulin is expressed in these cells, proinsulin is processed to peptides that co-migrate with mature insulin on SDS gels (25). The endocrine protein human growth hormone, which has a potential Arg-Lys cleavage site (24), is not processed in normal pituitary nor when expressed in AtT-20 cells. Furthermore, the trypsinogen processing site that is cleaved extracellularly in vivo by enteropeptidase (2) is not recognized in AtT-20 cells. Presumably the AtT-20 protease(s) cannot recognize the Asp-Asp-Asp cleavage site in trypsinogen, a site that is recognized by trypsin. These observations suggest that the AtT-20 protease(s) is not indiscriminate; it will not cleave at all dibasic sequences (e.g., growth hormone), and it does not cleave all proteolytic processing sites (e.g., trypsinogen). It can however process certain heterologously expressed prohormones (e.g., proinsulin).

What signal(s) might be responsible for targeting of regulated secretory proteins? Since several proteins that are not proteolytically processed can be correctly localized into AtT-20 secretory granules, e.g., trypsinogen (this report) and human growth hormone, it is unlikely that proteolysis per se is required for targeting proteins to the regulated pathway. It is also unlikely that N-linked glycosylation serves as the only signal for regulated secretory proteins as many regulated secretory proteins are not glycosylated, e.g., growth hormone, trypsinogen, and proinsulin. Furthermore, the N-linked glycosylation (16) and sulfation of N-linked oligosaccharides
POMC/ACTH sorting or in the constitutive secretion of gp 70. Sulfated proteoglycans and glycoproteins have frequently been implicated in the process of concentration and sorting of secretory proteins (26, 30). However, when chondroitin sulfate glycosaminoglycan side chains are inhibited from attaching to their protein core by the use of a β-d-xylolside, POMC/ACTH sorting is again unaltered (6). These findings and our observations of the common secretory granule localization of ACTH, insulin, growth hormone, and trypsinogen in AT-20 cells (proteins that do not share any known common posttranslational modification), led us to suspect that the proposed common sorting domain lies in the protein structure of regulated secretory proteins. We consider it unlikely that the N-terminal signal peptide serves this signaling role since it is removed very early in the intracellular secretory pathway (e.g., 18). The possibility remains, however, that the proteins acquire a posttranslational modification of unknown nature, which is recognized by the sorting apparatus.

An experimental approach combining gene transfer and in vitro mutagenesis should reveal the putative common sorting signal. Because the three-dimensional structures of both insulin (5) and trypsin (13, 29) have been determined, we will use this information to help design deletion and hybrid protein mutants with minimal perturbation of structure. Analysis of the secretion properties of such mutant and hybrid proteins should provide insight into the sorting domain required for intracellular targeting of proteins.

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