Secretory Protein Targeting in a Pituitary Cell Line: Differential Transport of Foreign Secretory Proteins to Distinct Secretory Pathways

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ABSTRACT The mouse pituitary cell line, AtT-20, packages the adrenocorticotropic hormone (ACTH) in secretory vesicles and releases it when the cell is stimulated with secretagogues. These cells have the capacity, after transfection with the appropriate DNA, to package heterologous peptide hormones into the regulated secretory vesicles (Moore, H. P. H., M. D. Walker, F. Lee, and R. B. Kelly, 1983, Cell, 35:531–538). To test if other secreted proteins prefer a different route to the surface, we have transfected AtT-20 cells with DNAs coding for a fragment of a membrane protein, the vesicular stomatitis virus G protein from which the membrane spanning domain has been deleted (Rose, J. K., and J. E. Bergmann, 1982, Cell, 17:813–819). We found that the secreted vesicular stomatitis virus G proteins were not transported to the regulated secretory vesicles. Instead they preferentially exited the cell by the constitutive pathway previously found in these cells (Gumbiner, B., and R. B. Kelly, 1982, Cell, 28:51–59). In contrast, human growth hormone transfected into the cells by the same procedure was transported to the regulated pathway with a similar efficiency as the endogenous hormone ACTH. Transport of the secreted G protein to the regulated pathway, if it occurs at all, is at least 30-fold less efficient than peptide hormones. We conclude that the transport machinery in AtT-20 cells must selectively recognize different secreted proteins and sort them into distinct secretory pathways.

Most higher eucaryotic cells can secrete proteins into the extracellular space. It has been known for some time that secretion from a given cell type can be either regulated or constitutive (31). Regulated secretory cells store specialized secretory products at high concentrations in secretory vesicles, which accumulate in the cytoplasm until exocytosis is triggered by a stimulus. In constitutively secreting cells, newly synthesized protein is not stored but leaves the Golgi apparatus in short-lived membrane vesicles that fuse immediately with the plasma membrane in the absence of any extracellular signal. In both types of secretory cell, different secretory proteins can be found in the same secretory vesicle. It was therefore plausible to suggest that secreted proteins are not segregated from one another in the Golgi apparatus (7).

Segregation of secretory proteins needed to be reconsidered, however, when the constitutive and the regulated secretory pathways were found to co-exist in the same cell. The pituitary cell line, AtT-20, has a regulated pathway in which the adrenocorticotropic hormone (ACTH) precursor, proopiomelanocortin (POMC), is processed to mature ACTH, stored in regulated secretory vesicles, and released only when the cells are stimulated, for example, with 8-Br-cAMP. The AtT-20 cells use the constitutive pathway to externalize a viral membrane glycoprotein (gp70) made by these cells (12). Further evidence that there are two routes to the surface in endocrine cells was obtained using inhibitors (10, 17).

Although membrane proteins preferentially take the constitutive route to the surface, secreted proteins examined thus far appear to use both pathways for externalization. Some newly synthesized hormones, for example, did not enter the

1 Abbreviations used in this paper: ACTH, adrenocorticotropic hormone; CAT, chloramphenicol acetyltransferase; Endo H, endoglycosidase H; ER, endoplasmic reticulum; hGH, human growth hormone; POMC, proopiomelanocortin; RIA, radioimmunoassay; RSV-LTR, Rous sarcoma virus long terminal repeats; SV40, Simian virus 40; TG, truncated G protein; VSV, vesicular stomatitis virus.
regulated pathway but were secreted by the constitutive pathway (12, 19). Similarly, when AtT-20 cells were treated with drugs that elevated the synthesis of glycosaminoglycans, these sugar chains were detectably secreted by both pathways (2). One possibility consistent with the earlier proposal that secretory proteins are not segregated, is that secreted proteins show no preference for one pathway over another. In such a passive situation, the amount entering each pathway is proportional to the bulk-flow into each pathway. A second possibility is that sorting does exist but that the preference for one pathway over another is not absolute. Sorting in this case might be carrier-mediated and involve sorting domains of the type suggested by Blobel (1).

If protein secretion is passive in AtT-20 cells, all proteins should partition equally between the two pathways. In this report we present data that are difficult to reconcile with such a passive-flow model. A quantitative comparison of the secretion of two proteins revealed that partition into the two pathways can be different. Using an approach used to study proinsulin processing in AtT-20 cells (19), we have transfected AtT-20 cells with DNA sequences encoding a secreted form of the vesicular stomatitis virus (VSV) membrane G protein, from which the transmembrane and the cytoplasmic domains have been deleted (24). We found that the secreted G protein, unlike ACTH, is transported preferentially to the constitutive pathway, a result not readily explained if transport of secreted proteins occurs by bulk-flow. In contrast, human growth hormone (hGH) transfected into these cells is sorted into the regulated pathway at least as efficiently as is the endogenous hormone, ACTH. We conclude that transport of proteins from the Golgi apparatus cannot be passive in these cells, but most likely involves a carrier-mediated sorting mechanism that shows at least a 30-fold selectivity for different proteins.

MATERIALS AND METHODS

Antisera, Peptide Hormones, and Radioimmunoassays

Rabbit serum against inactivated VSV (Indiana strain) was a generous gift of Dr. J. Rose. Goat anti-hGH antisera used for immunoprecipitation was obtained from Antibodies Inc. (Davis, CA). Affinity-purified rabbit anti-ACTH antibodies were generated and purified as described previously (18). hGH purified from E. coli was a gift of Dr. Peter Seeburg. Synthetic ACTH peptide (1–24) was from Orgonon Diagnostics (West Orange, NJ). Radioimmunoassay (RIA) of ACTH was performed as described (11). hGH RIA kits were purchased from Hybritech Inc. (La Jolla, CA).

Recombinant Vectors

The mammalian expression vector, pSV2-TG, carrying a truncated VSV G coding sequence downstream from the SV40 early promoter was a generous gift of Dr. Jack Rose. To construct a vector suitable for expression in AtT-20 cells, 5 μg of this plasmid was digested with the restriction endonucleases PvulI and HindIII (New England Biolabs, Beverly, MA), followed by treatment with calf intestinal phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN) to remove the 5' phosphates (16). The enzymes were then removed from the reaction mixture by phenol-chloroform extraction, and the DNA was recovered by ethanol precipitation. 100 ng of the digested vector was then ligated to 100 ng of a 398-bp NruI-HindIII restriction fragment, which has been excised from the plasmid pSV-CAT (9) and purified on a 1 M sucrose cushion in NDETS buffer. The cells were recovered by centrifugation, washed twice with NDETS buffer, rinsed once with distilled water, and boiled in SDS-polyacrylamide gel sample buffer before electrophoresis. Endoglycosidase H (Endo H) digestion of immunoprecipitated materials was performed as described (24).

DNA Transfection and Isolation of Stable Transformants

AtT-20 cells were transfected with plasmid DNAs by the calcium phosphate procedure as described previously (19). For transient expression, 100 μg of plasmid DNA was used per 10-cm dish of cells. For stable transformants each dish received 120 μg of nonselectable DNAs and 24 μg of the selectable DNA, pSV2-neo. The antibiotic G418 (Geneticin) used for selection was from Gibco Laboratories (Grand Island, NY). Clones were screened by RIA (for hGH) or by immunoprecipitation (for truncated G [TG]). CAT activity was assayed according to the procedure described by Walker et al. (32), except that acetyl-CoA was added at a ten times higher concentration and the reaction was carried out longer (16 h). Spots from the thin layer plates were cut out and counted in a liquid scintillation counter, or alternatively, the autoradiogram was scanned to yield the numbers shown in Table I.

Metabolic Labeling and Immunoprecipitation

Cells grown on polylysine-coated dishes were first incubated in medium lacking methionine for 30 min before labeling. 0.5 μCi [%35S]methionine in medium containing 1/20th of the normal amount of methionine and 2.5% fetal calf serum was then added to each 10-cm dish for length of time indicated in the figure legends. Chase was carried out in medium containing normal amounts of unlabeled methionine plus 2.5% fetal calf serum. Cells were harvested and prepared for immunoprecipitation according to the procedure of Rose and Bergmann (24). Medium samples were lyophilized and dissolved in NDETS buffer (1% Nonidet P-40, 0.4% deoxycholate, 66 mM EDTA, 10 mM Tris, pH 7.4, 0.3% SDS). For immunoprecipitation, samples were preadsorbed with fixed Staphylococcus aureus cells (Pansorbin, Behring Diagnostics, San Diego, CA) for 10 min. The supernatant was then recovered and reacted to an excess of antiserum specific of VSV, hGH, or ACTH (see next paragraph). After 2-3 h at room temperature, S. aureus cells were added for 5 min, and the suspension was layered on a 1 M sucrose cushion in NDETS buffer. The cells were recovered by centrifugation, washed twice with NDETS buffer, rinsed once with distilled water, and boiled in SDS-polyacrylamide gel sample buffer before electrophoresis. Endoglycosidase H (Endo H) digestion of immunoprecipitated materials was performed as described (24).

Excess antiserum were added to the antigen solutions to ensure the completion of the immunoprecipitation reactions. The amount of anti-hGH antiserum to be added was determined as follows. Each sample to be immunoprecipitated (cell lysate or medium) was first assayed by an RIA (Hybritech Inc.) specific for hGH to determine the amount of antigen present. The titer of the antiserum is then determined by adding increasing amounts of the antigen to a known concentration of the labeled antigen, followed by precipitation with S. aureus cells and PAGE gel analysis. We found 1 μl of the antigen is sufficient to precipitate 10 ng of hGH. To ensure large excess we have used 5 μl of the antiserum to precipitate 10 ng of the antigen in these experiments. Anti-VSV antiserum was titrated similarly. That the immunoprecipitation reaction was indeed complete was further verified by keeping the antiserum constant at the excess level, while doubling the amount of VSG TG present. A 1.85-fold increase in radioactivity was specifically precipitated.

Calculation of Rate Constants and Sorting Index

RATE CONSTANTS FOR ENDOCYTIC RETICULUM (ER) AND GOLGI EXPORT: To determine if the slow secretion of the TG protein is due to a slow transfer from ER to Golgi apparatus or from Golgi apparatus to cell surface, we compared its rate constants to those obtained for a constitutively secreted marker, POMC. To calculate rate constants of protein export from the ER (kE) and the Golgi apparatus (kG) we measured the steady-state pool sizes of these compartments. Since at steady-state there is a constant flux through all compartments, the rate of secretion equals the rate of ER export (kE[E]), which equals the rate of Golgi export (kG[G]). [E] and [G] are the concentrations in the ER and the Golgi apparatus, respectively. The rate constants can therefore be calculated as

\[ k_E = \frac{[E]}{[E]} \\ k_G = \frac{[G]}{[G]} \]  

Sorting Index: The peptide hormones do not all enter the regulated pathway, presumably because sorting is not perfect in these cells. It is more appropriate to think of the partitioning between the two pathways in terms of a sorting ratio. From the simple model presented in Fig. 6, the fractional sorting ratio of peptide hormones into regulated secretory vesicles would be kG/(kF + kE). This cannot be measured directly. What we have used is a sorting
index that is the ratio of the increment in the release rate when the cells are stimulated with 8-BrcAMP (D) to the total rate of release in the absence of stimulation (X).

The sorting index D/X can be shown to be proportional to the fractional sorting ratio. The fractional sorting ratio is the fraction of the flux out of the Golgi apparatus that enters regulated secretory vesicles \( k_d[G]/(k_d + k_o[G]) \). In the steady-state the denominator is the same as \( X \), the measurable total flux out of the cell, and the numerator is equal to \( k_d[R] \), since the flux out of the secretory vesicles must be equal to the flux in. \([R]\) cannot always be measured directly, but the magnitude of \([R]\) should affect the increment in the release rate on stimulation \( D \). If stimulation causes \( k_d \) to increase by \( k_d^* \), then \( D = k_d^*[R] \). The fractional sorting ratio is therefore \( (k_d/k_o^*)D/X \). Since \( k_d/k_o^* \) should be a constant, the fractional sorting ratio is linearly related to the measurable ratio \( D/X \). We have therefore used the ratio \( D/X \) in our measurements. It should be greater than the true sorting ratio.

The increased rate of release \( D \) is in turn obtained by subtracting the rate of release in the absence of stimulation \( (X) \) from the stimulated total. The amount released in the absence of stimulation \( (X) \) is \( k_d[R] + k_d[C] \) (Fig. 6), which equals at steady-state \( (k_i + k_o)[G] \). \( D \), is \( k_d^*[R] \). The stimulated to unstimulated ratio is therefore \( (k_d^*[R]/[k_i + k_o])[G] + 1 \). To enhance this ratio we can take advantage of the different time constants of the Golgi pool and the regulated secretory vesicle pool. By labeling and then chasing for a period equal to several times the Golgi pool half-life, the increment on stimulation can then be measured with much higher accuracy. If the time constant of the regulated pool is \( \tau \), then the measured release \( D' = D - e^{-\tau} \) and the fractional sorting ratio becomes

\[
\left(\frac{k_d}{k_o^* \cdot c^{n+}}\right)D'/X.
\] (Eq. 2)

Thus, the measured sorting index must be corrected for time.

RESULTS

Expression of a Secreted VSV G Protein and hGH in AtT-20 Cells by DNA Transfection

To examine if different secreted proteins can take different routes to the cell surface, we chose to transfect AtT-20 cells with two cloned DNAs, each coding for a secreted protein from a distinct origin. One, hGH, is normally secreted in a regulated manner by somatotrophs in the anterior pituitary. The other protein, in contrast, is not a product of endocrine cells but is of viral origin. It has been engineered in vitro by Rose and Bergmann (24) by deleting the membrane-anchor domain from the VSV membrane G protein. The altered protein, containing an intact luminal region of the G protein (432 amino acids from the NH2-terminus) but lacking the entire membrane-spanning and the cytoplasmic domains (79 amino acids from the COOH-terminus), has been shown to be secreted from transfected COS cells and the mouse cell C127 (8, 24).

Fig. 1A illustrates the construction of the plasmid vector used for expression of VSV TG protein in AtT-20 cells. We found that the vector pSV2-TG, originally constructed by Rose and colleagues (24), failed to yield positive stable transformants at a reasonable frequency when co-transfected with the selectable DNA pSV2-neo (28). This plasmid carries the VSV TG sequences downstream from the SV40 early promoter. To improve the number of clones expressing detectable levels of the VSV TG protein, we surveyed a number of cloned promoters for their ability to produce high levels of protein expression in AtT-20 cells. Table I compares CAT activities expressed transiently from various promoter elements. The highest level of expression was obtained with the Rous sarcoma virus long terminal repeat (RSV-LTR), which is two orders of magnitude more efficient in supporting CAT expression in these cells than is the SV40 early promoter. Mouse mammary tumor virus and the herpes thymidine kinase promoters yielded intermediate levels. We therefore replaced the SV40 promoter region of the pSV2-TG (PvuII-HindIII fragment) with a fragment of the RSV-LTR containing the promoter activity (NruI-HindIII; reference 15) (see Fig. 1A). After co-transfecting AtT-20 cells with this plasmid and the selectable DNA pSV2-neo (28), positive transformants were obtained in the same proportion as observed for pSV2-TG. The other promoter, pRSENG-HOG, was found to be inactive in this cell line (see Table I).
TABLE I. Comparison of Protein Expression in AtT-20 Cells

| Promotor driving non-selectable DNAs | CAT activities transiently expressed* | Frequency of stable co-transmants expressing detectable levels of nonselectable markers |
|--------------------------------------|---------------------------------------|---------------------------------------------------------------------|
| SV40 early                           | 1                                     | 0.5                                                                 |
| TK                                   | 56                                    | --                                                                  |
| MMTV                                 | 51                                    | --                                                                  |
| RSV-LTR                              | 244                                   | 66-75                                                                |

AtT-20 cells were transfected with plasmid vectors containing various promoters fused to the CAT gene. Indicated are the levels of CAT activities expressed in the cells at 65 h after transfection. Numbers are normalized to the activity from the SV40 early promoter. Right column: cells were co-transfected with the selectable marker neo driven from the SV40 early promoter (pSV2-neo, 28) and a nonselectable DNA (SV SV TG or human proinsulin) fused to the promoter indicated. The ratio of selectable DNA to nonselectable DNA was 1:5. After selection with the antibiotic G418, the survival clones were scored and compared to the number of isolates expressing detectable levels of the selectable markers. TK, thymidine kinase; MMTV, mouse mammary tumor virus.

* Arbitrary units.

...ants were detected at a frequency that is at least 10-20-fold higher than the SV40-derived vectors (Table I).

To generate stable cell lines expressing hGH, we obtained a plasmid, pK13, carrying the hGH gene from Dr. Peter Kushner and Dr. Howard Goodman. This plasmid (Fig. 1 B) contains a complete hGH gene and its 5' flanking sequences on a 2.6-kb EcoRI fragment (4), which is inserted at the EcoRI site of pSV2-gpt (20). Preliminary experiments determined that there is a secondary toxic effect of the selection medium on AtT-20 cells (Burgess, T., unpublished observations), therefore we did not use the Ecoct gene on this plasmid as a selectable marker. Instead, it was co-transfected with pSV2-neo (28), and selection was carried out with the antibiotic G418. Approximately 25% of the surviving clones expressed detectable levels of hGH as determined by RIA.

VSV TG Protein and hGH Are Secreted into the Extracellular Medium by Transformed AtT-20 Cells

Two stable transformants, AtT-20-TG17 and AtT-20-GH13/5/4, expressing the VSV TG protein and hGH, respectively, were analyzed in detail. Synthesis of TG protein in TG17 cells was demonstrated by immunoprecipitation of metabolically labeled cells with an antiserum raised against inactivated VSV. In cells infected with VSV, the G protein is normally synthesized as a single polypeptide with two N-linked oligosaccharide chains (5, 22, 25) and one or two molecules of esterified fatty acids (26). In the TG, the acylation site has been eliminated but the glycosylation sites remain intact (24). TG17 cells synthesize two differently glycosylated forms of the TG protein (Fig. 2 A, lane 1) which are absent from untransformed cells (Fig. 2 A, lane 3). The predominant band has a mobility that corresponds to TG bearing high mannose type carbohydrates. A minor, slower migrating band was also visible in the cell extract which correlates to TG with complex type sugars. The identity of these immunoprecipitated bands was further confirmed by Endo H digestion, which cleaves only N-linked oligosaccharides of the high mannose type (23). Treatment with Endo H increases the mobility of the faster migrating band, while the mobility of the slower migrating band remains the same (Fig. 2 A, lane 2). The majority of TG in the transfected cells is thus Endo H sensitive.

TG17 cells also secrete the TG into the tissue culture medium (Fig. 2 A, lane 4). The secreted form contains the complex type carbohydrates, as evidenced by its resistance to Endo H digestion (Fig. 2 A, lane 5). A similar glycosylation pattern has been shown to occur with the endogenous hormone ACTH (12; data not shown). The majority of the precursor ACTH in the cell extract exists in the high mannose form, indicative of its ER and early Golgi localization. The secreted form, on the other hand, has acquired complex type sugars. Thus the secreted TG protein appears to traverse the...
normal secretory pathway through functional Golgi compartments before reaching the cell surface. Similar observations have been made with C127 cells (24).

The clone, GH13/5/4, synthesizes three growth hormone-related polypeptides that can be immunoprecipitated from the cell extract with an antiserum against hGH (Fig. 2 B, lane 1). All three species were specifically precipitated as judged by two criteria. First, they were detected only in cells that were transformed with the hGH gene (compare Fig. 2 B, lanes 1 and 3). Second, the reaction with the antiserum is abolished by the presence of excess unlabeled growth hormone (Fig. 2 B, lane 2). The major species has a mobility on SDS polyacrylamide gels of 22 kD and co-migrates with authentic hGH. The two minor species have apparent molecular weights of 25 and 20 kD. The 20-kD protein has been reported previously to be synthesized from an alternatively spliced mRNA (4; see Fig. 1 B, legend), and has been found in normal pituitary (13). It is synthesized in a variety of cell lines including GH3 and L-cells transfected with the 2.6-kb EcoRI hGH gene (Garcia, P., unpublished observations). The origin of the 25-kD species, however, is unclear. It is only found in this cell line but not in other lines (GH3 or L-cells) similarly transfected with the hGH gene, and may be due to an aberrant splicing or rearrangement event in this isolate. Unlike the TG and POMC, growth hormone does not undergo posttranslational glycosylation or proteolytic processing. Upon secretion, the same three polypeptide species were found in the medium (Fig. 2 B, lanes 4, 5, and 6).

Secretagogues Accelerate the Release of hGH-Related Peptides but Do Not Affect the Apparent Rate of Secretion of the TG Protein

The above data demonstrate that growth hormone and TG protein can be secreted from the transformed cell lines, but do not identify the secretory pathways by which they are externalized. Previously work has shown that the two secretory pathways can be distinguished by secretagogues, which enhance secretion from only the regulated pathway (12, 18). We therefore determined the effect of 8-Br-cAMP on the secretion of hGH and the TG protein.

TG17 and GH13/5/4 cells were metabolically labeled with [35S]methionine for 6 h and chased for 3 h either in the presence or absence of 5 mM 8-Br-cAMP. Medium samples and cell extracts were collected separately, immunoprecipitated with specific antisera, and analyzed on SDS polyacrylamide gels (Fig. 3). Inclusion of 8-Br-cAMP in the chase period caused a 3-4-fold increase in the accumulation of labeled hGH in the medium of GH13/5/4 cells (Fig. 3 B, lanes 1 and 3). This is accompanied by a corresponding decrease of labeled hormone remaining in the cell extracts (Fig. 3 B, lanes 5 and 7). In marked contrast, secretion of labeled TG protein from TG17 is not detectably affected by the same secretagogue (Fig. 3 A). The same amount of Endo H–resistant form of the TG protein was recovered in the medium regardless of whether 8-Br-cAMP was present (Fig. 3 A, lanes 1–4). Furthermore, cells that have been treated with 8-Br-cAMP are not depleted of their intracellular pool of TG. Essentially the same levels of Endo H–resistant and sensitive forms of the TG protein were recovered in the stimulated or unstimulated cells (Fig. 3 A, lanes 5–8). Note that even after a chase period, the majority of the intracellular

**FIGURE 3** Effect of secretagogues on the secretion of (A) the TG protein and (B) hGH. (A) TG17 cells were labeled with [35S]methionine for 6 h and then chased for 3 h either in the presence or absence of 5 mM 8-Br-cAMP. Labeled materials released into the chase medium and those remaining in the cells were recovered and immunoprecipitated with anti-VSV antibodies. Half of the immunoprecipitates were digested with Endo H and analyzed on SDS polyacrylamide gels along with the undigested materials. Lanes 1 and 2, medium samples from unstimulated cells, and lanes 3 and 4, from 8-Br-cAMP treated cells. Lanes 5 and 6, labeled molecules remaining in cells that have not been stimulated, and lanes 7 and 8, in cells that have been stimulated. Samples in lanes 2, 4, 6, and 8 were digested with Endo H, whereas those in lanes 1, 3, 5, and 7 were not. (B) GH13/5/4 cells were labeled and chased as in A and processed with anti-hGH antibodies. Lanes 1 and 2, 3 and 4, 5 and 6, and 7 and 8 are media of unstimulated cells, media of stimulated cells, extracts of unstimulated cells, and extracts of stimulated cells, respectively. Samples in lanes 2, 4, 6, and 8 were immunoprecipitated in the presence of excess of unlabeled hGH. Note that lanes 4 and 5 of Fig. 2 B and lanes 1 and 2 of Fig. 3 B represent the same gel.

TG is Endo H sensitive.

The apparent lack of stimulation of secretion of TG17 cells is not due to their inability to respond to secretagogues. Aliquots of the medium and cell extract samples used for the above analysis were subjected to RIAs for the endogenous hormone, ACTH. TG17 cells responded to 8-Br-cAMP by secreting threefold more immunoreactive ACTH into the medium than unstimulated cells. This degree of stimulation is comparable to those from the untransformed AtT-20 population (3.5-fold) and also from the growth hormone–producing GH13/5/4 cells (3.4-fold). Interestingly, secretion of all three hGH-related peptides were stimulated to about the same extent (Fig. 3 B, lanes 1, 3, 5, and 7). We conclude that hGH and its related peptides are targeted, at least in part, to the regulated secretory pathway in this heterologous system in a way similar to the endogenous hormone ACTH. By contrast,
little or no VSV TG protein is detected exiting the cell via the regulated pathway.

hGH and TG Protein Are Externalized at Distinct Rates

A second diagnostic tool for distinguishing the two pathways is the kinetics of release. Proteins taking the constitutive route are transported directly to the cell exterior \( (t_0 \approx 30-40 \text{ min}, 12) \), whereas those secreted by the regulated pathway are stored inside secretory granules before release \( (t_0 \approx 7 \text{ h}, \text{ data not shown}) \). If hGH and TG protein indeed exit the cell by the regulated and the constitutive routes, respectively, then their release should follow these characteristic kinetics.

To measure the kinetics of secretion, TG17 and GH13/5/4 cells were labeled with \(^{35}\text{S}\)methionine for 15 h and then chased for various lengths of time. At a given time point, labeled materials in the medium and in the cells were recovered and analyzed (Fig. 4). We found that the majority of hGH was secreted by GH13/5/4 cells with a half-time \( (6.3 \text{ h}) \) similar to that measured for mature ACTH \( (7 \text{ h}, \text{ data not shown}) \). In comparison, TG protein existed from TG17 cells with much faster kinetics \( (t_0 \approx 1.5 \text{ h}) \), indicative of its constitutive mode of secretion.

Although the majority of the hGH was stored intracellularly and secreted slowly, a portion of the hGH synthesized by

\[
\begin{align*}
\text{TABLE II. Rate of Export of the TG Protein from the ER and the} \\
\text{Coli} \\
\text{Apparatus} \\
\text{Protein} & \quad \text{ER pool size} \quad \text{Golgi pool size} \quad \text{Rate constant of export from the} \\
& \quad \text{Molecules secreted per hour} \quad \text{(Endo H-resistant form)} \quad \text{ER pool size} \quad \text{(Endo H-resistant form)} \quad \text{ER}\ \text{Golgi} \\
\text{Pro-} & \quad [E] \quad [G] \quad k_E \text{ h}^{-1} \quad k_C \text{ h}^{-1} \\
\text{crete sensitive} & \quad \text{from} \quad \text{from} \quad \text{from} \quad \text{from} \\
\text{form} & \quad \text{ER} \quad \text{ER} \quad \text{ER} \quad \text{Golgi} \\
\text{TG} & \quad 100 \quad 257 \quad 28 \quad 0.38 \quad 3.5 \\
POMC & \quad 100 \quad 74 \quad 46 \quad 1.35 \quad 2.2 \\
\end{align*}
\]

GH13/5/4 cells was secreted more rapidly into the medium. The kinetic measurements in Fig. 4 revealed two components of secretion. About 80% of the labeled intracellular pool exited the cells at a slow rate \( (t_0 \approx 6.3 \text{ h}) \) characteristic of regulated secretion. The remaining 20%, however, was secreted at a rate approaching constitutive secretion. Previously we have also observed rapid secretion of a portion of the newly synthesized hormones \( (12, 19) \), except in these cases they are in the form of the unprocessed precursors (see Discussion).

The TG Protein Is Transported out of the ER Slowly

In the above studies we noted that although the kinetics of release for TG protein \( (t_0 \approx 1.5 \text{ h}) \) was much faster than what would be expected from the regulated pathway, it was considerably slower than the externalization of other constitutive proteins such as gp70 and POMC \( (t_0 \approx 30 \text{ min}) \). The difference was not due to storage in secretory vesicles but to a relatively slow rate of export of TG protein from the rough ER. To show this the rates of export from the ER and the Golgi apparatus were calculated from the relative size of the ER and the Golgi pools. To measure the ER and Golgi pool sizes, the intracellular amounts of Endo H-sensitive and resistant forms of the TG proteins were determined by immunoprecipitation of steady-state labeled cells and compared to the steady-state rate of secretion (Table II). The rates of export from the ER and the Golgi apparatus were calculated according to Eq. 1 (see Materials and Methods) and compared with those obtained for another constitutive marker, POMC.

TG17 cells exhibited a large steady-state ER pool size (Endo H-sensitive form) for the TG protein relative to POMC, and thus a correspondingly 3-4-fold slower exit rate from the ER. Transfer of TG protein out of the Golgi apparatus, however, appeared to be as efficient as POMC, since TG17 cells exhibited a normal relative pool size for the Golgi form (Endo H-resistant) of the TG protein (Table II). Thus, except
for the slow rate of exiting the ER, TG behaved exactly as
POMC, which is secreted constitutively (12). Similar results
have been previously obtained with transfected C127 or COS
cells (8, 24).

Quantification of Sorting

The slow ER exit rate of the TG protein made us wonder
if the lack of detectable stimulation of its secretion by 8-Br-
cAMP (Fig. 3) could be due to the background of a high
constitutive secretion that is chased out slowly. To compare
sorting independent of the ER pool size, we devised a labeling-
chase protocol which is explained in the Materials and Meth-
ods section. In essence, the rate of secretion from the regulated
pool in comparison with the total rate of secretion at steady-
state provides a measure of the efficiency for entering the
regulated pathway that is independent of the kinetics of
constitutive release.

To measure the total rate of secretion, TG17 and GH13/ 5/4 cells were labeled with [35S]methionine for 16 h to ap-
proach steady-state labeling of all intracellular pools. Ma-
terials secreted into this medium during the last hour of labeling
were collected and analyzed. Since [35S]methionine was present
throughout this time, the amount of labeled materials secreted into the medium is a measure of the steady-state rate of secretion rather than the ER pool size. Secretion from the regulated secretory granule pool was then measured as follows. The labeled cells were first chased with unlabeled medium for
two 3-h periods to lower the background secretion contributed
by the constitutive pathway (t1/2 = 30 min–1.5 h). They were
then stimulated during a third 3-h interval with 5 mM 8-Br-
cAMP. The difference between secretion from stimulated cells
and from unstimulated cells during this time was measured
and compared with the steady-state rate of secretion. Fig. 5
shows the comparison of secretion of hGH and the TG protein
using this protocol. Even with this long chase protocol, which
maximizes the detectability of regulated secretion, we could
not detect any effect of 8-Br-cAMP on the secretion of the
TG protein (Fig. 5 A). In contrast, stimulation of growth
hormone under identical conditions was evident (Fig. 5 B).
Quantitation of these data is shown in Table III, where sorting
indices are calculated by taking the ratio of the stimulated
response to the steady-state rate of secretion (see Eq. 2,
Materials and Methods). All peptide hormones examined thus
far, POMC (endogenous), hGH (exogenous) (Table III), and
proinsulin (19), show a similar partition coefficient into the
regulated secretory granules. In contrast, transport of TG

| Table III. Quantitative Comparison of Transport of Secreted Proteins to the Regulated Pathway in AtT-20 Cells |
|---------------------------------|
| Protein | Total amount secreted/ pool/h | Increment of the amount secreted/ pool/h | Amount depleted from intracellular pool/h | Sorting index |
|---------|-----------------------------|-----------------------------|-----------------------------|----------------|
| Endogenous mouse ACTH | 600 ± 50 | 83 ± 7 | 84 ± 8 | 0.14 ± 0.02 |
| Truncated VSV G | 31 ± 6 | 0.16 | 0.13 | 0.005 |
| hGH | 690 ± 60 | 270 ± 30 | 220 ± 30 | 0.35 ± 0.06 |

The data in Fig. 5 were quantitated by scanning the autoradiogram.
* Amount secreted during the 1-h labeling period (Fig. 5, lanes 1 and 2).
* Differences in the amounts secreted from stimulated and unstimulated cells
during the third chase period (Fig. 5, lane 7 minus lane 8).
* Differences in the amounts inside stimulated and unstimulated cells at the
end of the chase (Fig. 5A, lanes 13 and 14 minus lanes 11 and 12, or Fig. 5B,
lane 11 minus lane 10).
* The ratio of column 2 to column 1, or column 3 to column 1, according to
Eq. 2 in Materials and Methods.
protein to the regulated pathway, if it occurs at all, was at least 30-fold less efficient (Table III). We conclude that peptide hormones are transported to the regulatory secretory pathway in preference to other secreted proteins, such as the TG protein.

DISCUSSION

At least three classes of proteins, i.e., plasma membrane, lysosomal, and secretory proteins, use a common site, the rough ER, for their membrane translocation. Presumably, mechanisms exist that sort these proteins from one another and target them to their final destinations. This is known to be true at least for lysosomal enzymes, which can be selectively segregated by mannose-6-phosphate receptors and transported to the lysosomes in fibroblasts (for review, see reference 27). Evidence for active sorting of the other two classes of proteins, however, is scant. In hepatocytes, it has been documented that the secreted albumin, transferrin, and the membrane VSV G protein are localized to the same secretory vesicle, suggesting that proteins may not be segregated from each other before reaching the cell surface (30). Similarly, temperature-sensitive yeast mutants that are defective in invertase secretion also cannot internalize some of their plasma membrane proteins (21). A simple model to explain these observations is that secretory proteins and plasma membrane proteins are the only proteins that are neither actively segregated from the Golgi lumen (like lysosomal enzymes) nor selectively retained (like Golgi enzymes). As a result they are automatically transported to the cell exterior without active sorting.

In cells such as endocrine cells that have more than one type of secretory vesicle, transport to each pathway could also be governed by passive flow. Previous work with AtT-20 cells has established that plasma membrane proteins such as the viral glycoprotein gp70 preferentially use the constitutive type vesicles for export (12). The percentage of gp70 taking the regulated route is at most one-tenth of that for ACTH. This difference in the routes of transport, however, is not an incontrovertible argument for active sorting. The sizes of the two types of vesicles can differ, thus carrying different amounts of membrane proteins relative to secretory proteins to the cell surface. For instance, if the constitutive vesicles have a tenth of the diameter of the regulated vesicles, their surface to volume ratio would be ten times higher than for the regulated vesicles. This could result in the apparent 10-fold segregation of membrane and secretory proteins. Shape differences in the transport vehicles have also been proposed to explain the segregation of ligand and receptors during endocytosis (Helenius, A., and I. Mellman, personal communication).

The need to examine a passive-flow model was reinforced by our DNA transfection experiments. Despite their species difference and the lack of apparent sequence homology, human proinsulin (19), hGH (this report), and rat trypsinogen (3) are all transported into the regulated secretory vesicles by AtT-20 cells. This lack of specificity for species or sequence could be readily explained if transport were by a nonselective bulk-flow process.

The data presented in this report, however, are difficult to reconcile with the passive flow segregation model. Using a secreted form rather than the membrane form of the VSV G protein, such that the surface to volume ratio is not a concern, we found that selective transport can still occur. A similar conclusion can also be drawn from analyzing the VSV TG protein transfected into HIT (an insulin-producing pancreatic cell line) cells (unpublished observations). Now for the first time we can say unequivocally that two known soluble proteins introduced by DNA transfection partition differently between two pathways. Recently, using a labeling protocol identical to that described here, Burgess et al. (3) have shown that laminin, a naturally occurring secretory protein of the AtT-20 cells, is secreted constitutively. Thus, in this cell type proteins are actively sorted from one another and targeted to specific secretory pathways.

One hypothesis to explain our data is that both regulated and constitutively secreted proteins contain active sorting signals and are recognized by individual receptors (1). Alternatively, segregation can be mediated by only one type of receptor that specifically recognizes proteins entering either the regulated pathway such as ACTH, hGH, and human insulin, or the constitutive route such as VSV TG. The other class of proteins would then be transported to the cell surface by simple noncarrier-mediated bulk-flow. Several indirect lines of evidence led us to suggest that only entry into the regulated pathway may be carrier mediated. First, in cells treated with β-D-xyloside to inhibit proteoglycan synthesis, the majority of the glycosaminoglycan chains are efficiently exported via the constitutive pathway (2). Since the TG protein also preferentially takes this route but does not share common chemical structures, either they both enter this pathway by bulk-flow, or they have to be segregated by different receptors, a possible but less likely situation. Second, when targeting to regulated pathway is perturbed by chloroquine, those ACTH molecules that would normally enter the regulated secretory granules are diverted to the constitutive pathway. Either entry to the latter pathway does not require signal(s) or much less likely, the constitutive signal is added to the diverted ACTH molecules in the presence of chloroquine. Finally, the VSV TG protein might not be expected to contain a natural sorting signal as a secretory protein. Nonetheless, it is secreted constitutively. Direct proof, however, that sorting signals are contained within the regulated and not the constitutive proteins awaits further experimentation.

Although all peptide hormones that we have examined thus far were found to be transported and packaged in the regulated secretory granules by AtT-20 cells, some newly synthesized hormones always escape this packaging and are secreted constitutively. Since sorting is not all-or-none, it must be analyzed quantitatively. Since proteins are known to exit ER at different rates (14, 24, 29, and this report), quantitation of sorting had to be independent of export rates from the ER. By measuring the ratio of the release rate from the regulated pathway to total release rate, we can measure sorting independent of the ER exit rate (in Eq. 2, Materials and Methods, \( D'/X \) is independent of \( k_e \)). Secondly, since only a vestige of the regulated pathway remains in this tumor cell line, the protocols had to be optimized to be very sensitive to small amounts of regulated release. The accuracy of the measurements for regulated release was maximized by chasing out background constitutive release that had to be subtracted from the total. Using this procedure, we are able to detect at least a 30-fold discrimination between TG and hGH by the sorting apparatus (Table III). It should be emphasized that this is a limit measurement and the true partition ratio could be even
higher. The quantitative measurements also indicate that the sorting apparatus does not prefer the endogenous ACTH to the foreign hGH. The preference of the sorting apparatus for growth hormone may be significant (Table III).

While the sorting index provides a convenient measure to compare sorting between different proteins, it is not the true partition of Golgi molecules between the two pathways. If the simple model outlined in Fig. 6 is correct, the true sorting ratio is related to the sorting index by a proportionality constant that is a function of the various rate constants (see Eq. 2). If these are estimated the true sorting ratio is between 0.15 and 0.30, or only 1 in 3–6 newly synthesized hormone molecules enters the regulated pathway. Either AtT-20 cells sort proteins very inefficiently into the regulated pathway, or a considerable amount of what is sorted is lost before it arrives in mature secretory vesicles. Loss could be induced by crinophagy (6) or by rapid release of the contents of immature vesicles. An alternative way of interpreting the inefficient sorting is that AtT-20 cells are rapidly growing, unlike conventional cells, and the doubling of membrane area every 25 h requires considerable addition of membrane proteins and so unusually high constitutive release. Despite the inefficiency in sorting, the quantitative procedure described here provides sufficient sensitivity to allow future mapping of sorting sequences by in vitro mutagenesis.

If targeting to the regulated secretory granules indeed is mediated by specific receptors and if only one type of receptor exists, then our observations that several endocrine peptides enter this pathway at similar efficiencies suggest that these proteins share a common structural feature. Direct examination of their primary amino acid sequences revealed no conserved homology between these proteins. However, the signal peptides of known proteins, though recognized by the same signal recognition particle, also lack direct amino acid sequence homology. The protein moieties of those lysosomal enzymes that are selectively recognized by the same enzyme, N-acetylglucosamine phosphotransferase, also fail to exhibit consensus amino acid sequence (Kornfeld, S., unpublished observations). Presumably, targeting is mediated by recognition of a common higher order structure.

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Figure 6 A simple scheme of partitioning between the two secretory pathways, k1, k2, k3, and k4 are rate constants, and [C], [R], and [C] are the concentrations in the Golgi apparatus, regulated secretory vesicles, and constitutive secretory vesicles, respectively. When the cell is stimulated to secrete, the rate constant k2 increases to k2 + k2*.