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)[1] 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 antiserum 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 Organon 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 PvuII 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 pRSV-CAT (9) and purified on a 1% agarose gel. The ligated mixture was used to transform *E. coli* strain BH101, and the plasmids from resulting ampicillin-resistant colonies were screened for the correct insert.

The plasmid pK13 carrying the hGH gene was obtained from Dr. Peter Kushner and Dr. Howard Goodman. Chloramphenicol acetyltransferase (CAT) constructs used for the comparison of promoter activities were from Dr. Don DeFranco (mouse mammary tumor virus), Dr. Thomas Edlund (herpes thymidine kinase), and Dr. Cori Gorman (Rous sarcoma virus and Simian virus 40 [SV40]).

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 uncut plasmid 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 mCi [^{35}S]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 lengths 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, and 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 antisera 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 serum 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 antiserum 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 VSV TG present. A 1.85-fold increase in radioactivity was specifically precipitated.

Calculation of Rate Constants and Sorting Index

RATE CONSTANTS FOR ENDOPLASMIC 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 (k_E) and the Golgi apparatus (k_G), 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 ($k_E[E]$), which equals the rate of Golgi export ($k_G[G]$). $[E]$ and $[G]$ are the concentrations in the ER and the Golgi apparatus, respectively. The rate constants can therefore be calculated as

$$k_E = \text{rate of secretion}/[E]$$
$$k_G = \text{rate of secretion}/[G]. \quad \text{(Eq. 1)}$$

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 $k_{r1}/(k_{r1} + k_{c1})$. This cannot be measured directly. What we have used instead is a sorting

index that is the ratio of the increment in the release rate when the cells are stimulated with 8-Br-cAMP (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 $k_{r1}[G]/(k_{r1} + k_{c1})[G]$. In the steady-state the denominator is the same as X, the measurable total flow out of the cell, and the numerator is equal to $k_{r2}[R]$, 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_{r2} to increase by k_{r2}^*, then $D = k_{r2}^*[R]$. The fractional sorting ratio is therefore $(k_{r2}/k_{r2}^*)D/X$. Since k_{r2}/k_{r2}^* 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 $k_{r2}[R] + k_{c2}[C]$ (Fig. 6), which equals at steady-state $(k_{r1} + k_{c1})[G]$. D is $k_{r2}^*[R]$. The stimulated to unstimulated ratio is therefore $\{k_{r2}^*[R]/(k_{r1} + k_{c1})[G]\} + 1$. 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 τ, then the measured release $D' = D \cdot e^{-t/\tau}$ and the fractional sorting ratio becomes

$$(k_{r2}/k_{r2}^* \cdot e^{-t/\tau})D'/X. \qquad \text{(Eq. 2)}$$

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 NH_2-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. 1 *A* 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

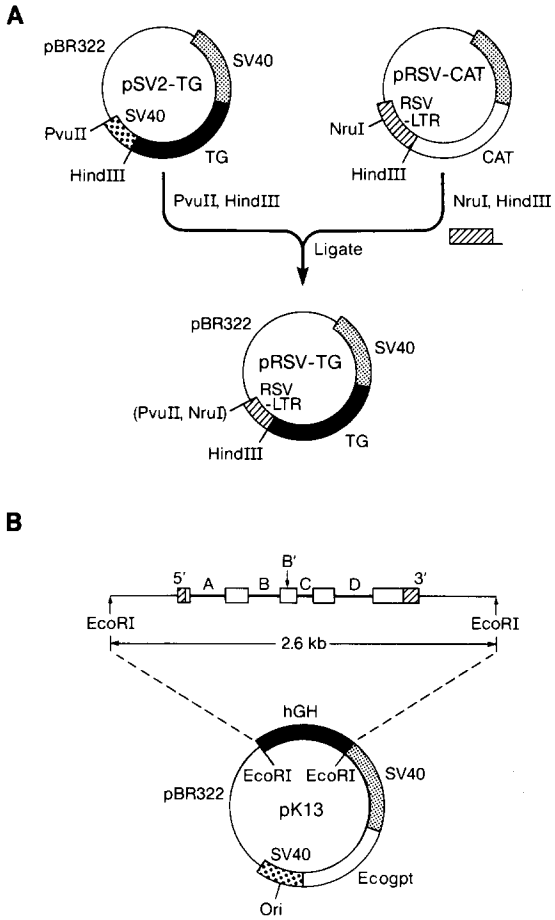

FIGURE 1 (*A*) Construction of an RSV-LTR-containing vector for the expression of truncated VSV G protein. The vector, pSV2-TG, was obtained from J. Rose and contains the truncated VSV G cDNA (▬) downstream from the SV40 early promoter (▭). The G coding sequences are followed by the SV40 t-antigen splice junction and the early region polyadenylation site (▭), which also supply a stop codon near the end of the TG sequence (25). The pBR322 sequence contains the origin of replication and the ampicillin resistance gene (——). To replace the SV40 promoter region with sequences from the RSV-LTR, pSV2-TG was digested with the restriction endonucleases PvuII and HindIII. The phosphatase-treated vector was then ligated to a gel-purified NruI-HindIII fragment (▨) containing the RSV promoter activity. The latter was excised from an RSV-LTR-containing plasmid driving the CAT gene (▭) (pRSV-CAT, 9). The resulting plasmid, pRSV-TG, carries a TG sequence joined to the RSV-LTR. All the other elements essential for expression remain the same as the parent vector, pSV2-TG. (*B*) Plasmid vector used for the expression of hGH. pK13 contains the 2.6-kb hGH gene (4) inserted at the unique EcoRI site of the vector, pSV2-gpt (20; Kushner, P., unpublished observations). (▬) hGH sequences; (▭) Ecogpt (*E. coli* gene encoding the enzyme xanthine-guanine phosphoribosyl transferase) sequences. All the other sequences are the same as denoted in *A*. Also indicated is the structure of the hGH gene as reported in reference 4 showing the position of the four introns (*A*, *B*, *C*, and *D*), exons (▭),and an alternative splice site (*B'*). (5' ▨) and (▨ 3') are the 5' and 3' untranslated regions, respectively.

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. 1 *A*). After co-transfecting AtT-20 cells with this plasmid and the selectable DNA pSV2-neo (28), positive transform-

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 promotor elements 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 gene neo driven from the SV40 early promoter (pSV2-neo, 28) and a nonselectable DNA (VSV 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 nonselectable 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 Ecogpt 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 corresonds 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

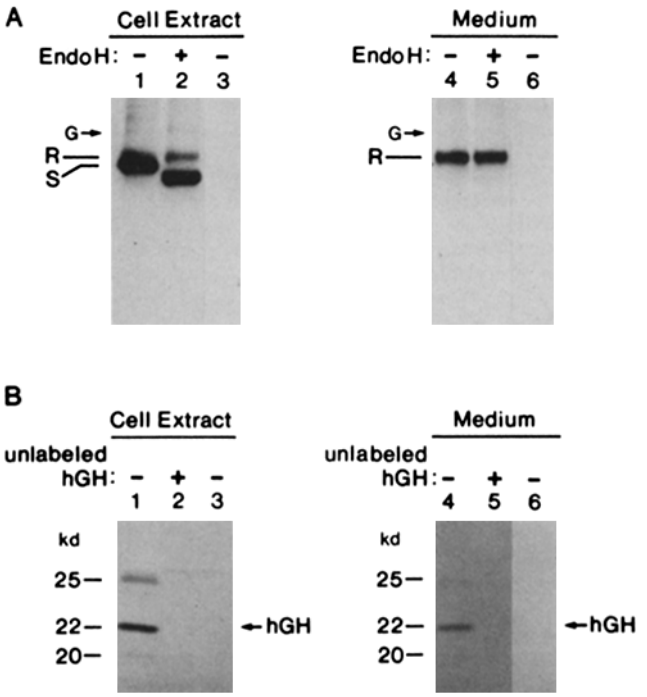

FIGURE 2 Synthesis and secretion of (*A*) TG protein and (*B*) hGH by transformed AtT-20 cells. (*A*) TG17 or untransformed AtT-20 cells were radiolabeled with [^{35}S]methionine for 6 h and chased for 3 h in unlabeled medium. Cell extract and medium samples were immunoprecipitated separately with an anti-VSV antiserum, and half of the immunoprecipitates were subjected to digestion with the Endo H. The labeled proteins were then analyzed on a 15% SDS polyacrylamide gel and visualized by autoradiography. Lanes *1*, *2*, and *3*, cell extract samples harvested at the end of the labeling period. Lanes *4*, *5*, and *6*, medium samples collected during the chase. Lanes *1*, *2*, *4*, and *5* are immunoprecipitated from the transformed TG17 cells, and lanes *3* and *6* are from control AtT-20 cells. Endo H digestion was only performed on samples shown in lanes *2* and *5*. Arrows indicate where the native VSV G protein would migrate on these gels. *R* and *S*, Endo H–resistant and sensitive forms of the TG protein, respectively. (*B*) GH13/5/4 and control AtT-20 cells were labeled and chased as in *A*. Extract (lanes *1*, *2*, and *3*) or medium (lanes *4*, *5*, and *6*) samples were immunoprecipitated with a goat anti-hGH antiserum either in the absence (lanes *1*, *3*, *4*, and *6*) or presence (lanes *2* and *5*) of excess unlabeled hGH. Immunoprecipitates were analyzed on a 10–18% gradient SDS polyacrylamide gel. Lanes 1, 2, 4, and 5, samples from GH13/5/4 cells, and lanes *3* and *6* from control cells. Arrows indicate the electrophoretic position of hGH purified from *E. coli* harboring hGH cDNA. The faint band immediately above the 25-kD band is a contaminant in the immunoprecipitates.

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 [^{35}S]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

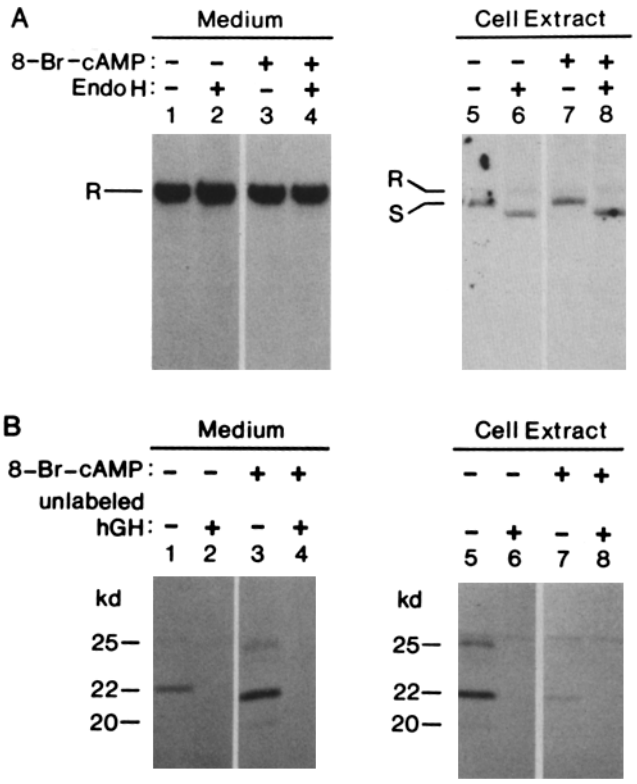

FIGURE 3 Effect of secretagogues on the secretion of (*A*) the TG protein and (*B*) hGH. (*A*) TG17 cells were labeled with [^{35}S]methionine for 6 h and then chased for 3 h either in the presence or absence of 5 mM 8-Br-cAMP. Labeled materials released into the chase medium and those remaining in the 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 ($t_{1/2}$ ~ 30–40 min, 12), whereas those secreted by the regulated pathway are stored inside secretory granules before release ($t_{1/2}$ ~ 7 h, 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}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 h) similar to that measured for mature ACTH (7 h, data not shown). In comparison, TG protein existed from TG17 cells with much faster kinetics ($t_{1/2}$ ~ 1.5 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 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 ($t_{1/2}$ ~ 6.3 h) characteristic of regulated secretion. The remaining 20%, however, was secreted at a rate approaching constitutive secretion. This apparent leakage into the constitutive pathway is reminiscent of the secretion of POMC and proinsulin. 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).

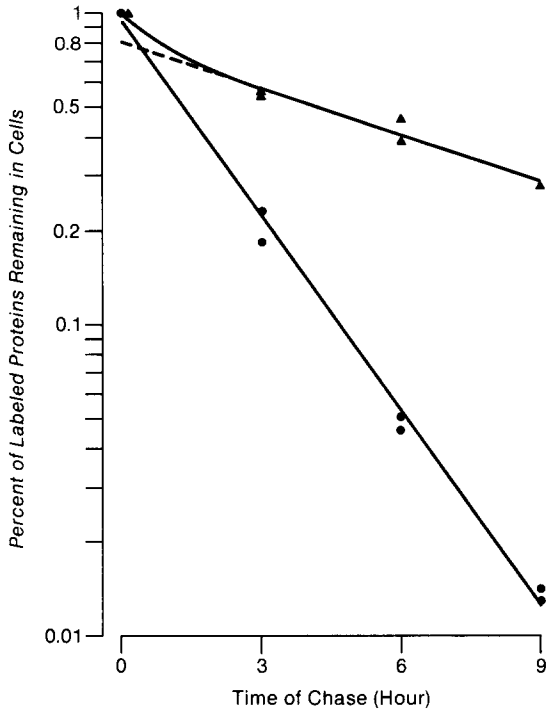

FIGURE 4 Kinetics of secretion of the TG protein and hGH. TG17 or GH13/5/4 cells were labeled with [^{35}S]methionine for 16 h and chased for increasing lengths of time. At the time indicated, medium samples were collected and the cells were extracted with detergent. TG protein and hGH were recovered by immunoprecipitation, electrophoresed on SDS polyacrylamide gels, and quantitated by scanning the autoradiograms. The percentage of total labeled proteins remaining inside the cells at each time point is calculated and plotted as a function of the length of chase. (▲) hGH; (●) TG. Secretion of the TG protein showed a single exponential decay with a rate constant of 0.46 h^{-1} (or $t_{1/2}$ = 1.5 h). The majority of hGH was secreted with a rate constant of 0.11 h^{-1} (or $t_{1/2}$ = 6.3 h).

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 ($t_{1/2}$ ~ 1.5 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 ($t_{1/2}$ ~ 30 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

TABLE II. *Rate of Export of the TG Protein from the ER and the Golgi Apparatus*

Protein	Molecules secreted per hour	ER pool size (Endo H-sensitive form)	Golgi pool size (Endo H-resistant form)	Rate constant of export from the ER	Rate constant of export from the Golgi
		[E]	[G]	k_E, h^{-1}	k_G, h^{-1}
TG	100	257	28	0.38	3.5
POMC	100	74	46	1.35	2.2

TG17 cells were labeled with [^{35}S]methionine for 15 h to reach steady-state labeling of intracellular ER and Golgi pools. To measure steady-state rate of secretion, the medium was removed from the cells and replaced with a fresh aliquot of the identical media containing [^{35}S]methionine. Labeled materials secreted into the medium during the next hour were collected, and the cells were extracted with buffers containing detergents to determine the intracellular pool sizes. Samples were immunoprecipitated with antisera against either VSV or ACTH, digested with Endo H, electrophoresed on SDS polyacrylamide gels, and quantitated by scanning the autoradiograms. Numbers were normalized to the total amount secreted into the medium per hour. The amounts of Endo H-sensitive forms of TG or POMC residing intracellularly are taken to be a measure of the ER pool, and those resistant to Endo H digestion as the Golgi pool. The rate constant of ER export is calculated by dividing the rate of secretion by the size of the ER pool. The rate constant of Golgi export is calculated by dividing the rate of secretion by the size of the Golgi pool (see Eq. 1, Materials and Methods).

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 Methods 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 [^{35}S]methionine for 16 h to approach steady-state labeling of all intracellular pools. Materials secreted into this medium during the last hour of labeling were collected and analyzed. Since [^{35}S]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 ($t_{1/2} \sim$ 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/h in absence of stimulation*	Increment of the amount secreted/h in response to stimulation‡	Amount depleted from intracellular pool/h upon stimulation§	Sorting index‖
	X	*D'*	*D'*	*D'/X*
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. 5*A*, lanes *13* and *14* minus lanes *11* and *12*, or Fig. 5*B*, 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.

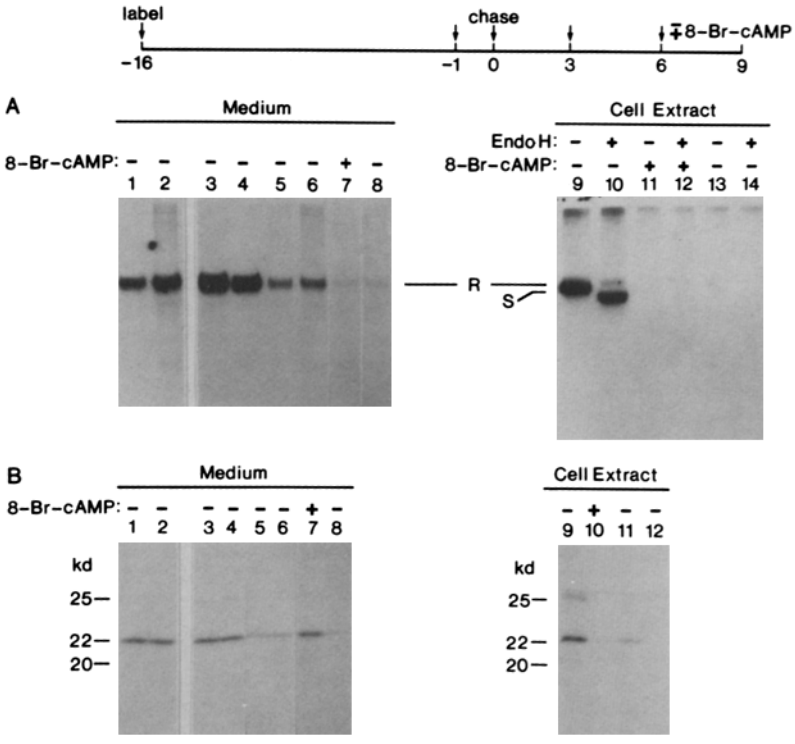

FIGURE 5 Quantification of transport by the two pathways. TG17 or GH13/5/4 cells were labeled with [^{35}S]methionine for 15 h. Medium was removed, and a fresh aliquot of the labeling medium was added for an additional hour. At time zero indicated in the top axis, cells were chased for two consecutive 3-h periods followed by stimulation with 5 mM 8-Br-cAMP during the third. (*A*) Secretion of the TG protein. Lanes *1* and *2*, medium samples collected during the 1-h ($t = -1$ to 0 h) labeling period. Lanes *3* and *4*, from the first chase period ($t = 0$ to 3 h). Lanes *5* and *6*, from the second chase period ($t = 3$ to 6 h). Lanes *7* and *8*, from third chase period ($t = 6$ to 9 h) in the presence and absence of 5 mM 8-Br-cAMP, respectively. Lanes *9* and *10*, intracellular proteins extracted at the end of the labeling ($t = 0$). Lanes *11* and *12*, intracellular proteins remaining in cells that have been stimulated at the end of the chase ($t = 9$ h) and lanes *13* and *14*, in cells that have not been stimulated. Samples in lanes *10*, *12*, and *14* were digested with Endo H. (*B*) Secretion of hGH. Lanes *1–8* are the corresponding medium samples as described in *A*. Lanes *9–11*, cell extracts prepared at the beginning of chase, at the end of chase from stimulated cells, and at the end of chase from unstimulated cells, respectively. Lane *12*, control AtT-20 cells extracted at the end of the labeling period.

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 externalize 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 away 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

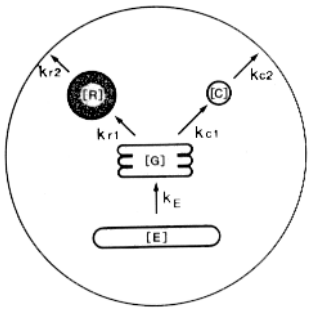

FIGURE 6 A simple scheme of partitioning between the two secretory pathways. k_{r1}, k_{c1}, and k_{r2}, and K_{c2} are rate constants, and [G], [R], and [C] are the concentrations in the Golgi apparatus, regulated secretory vesicles, and constitutive secretory vesicles, respectively. When the cell is stimulated to secrete, the rate constant k_{r2} increases to k_{r2} + k_{r2}*.

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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REFERENCES

1. Blobel, G. 1980. Intracellular protein topogenesis. *Proc. Natl. Acad. Sci. USA* 77:1496–1500.
2. Burgess, T. L., and R. B. Kelley. 1984. Sorting and secretion of ACTH in a pituitary tumor cell line after perturbation of the level of a secretory granule-specific proteoglycan. *J. Cell Biol.* 99:2223–2230.
3. Burgess, T. L., C. S. Craik, and R. B. Kelly. 1985. The exocrine protein trypsinogen is targeted into the secretory granules of an endocrine cell line: studies by gene transfer. *J. Cell Biol.* 101:639–645.
4. DeNoto, F. M., D. D. Moore, and H. M. Goodman. 1981. Human growth hormone DNA sequence and mRNA structure: possible alternative splicing. *Nucleic Acids Res.* 9:3719–3730.
5. Etchison, J. R., J. S. Robertson, and D. F. Summers. 1977. Partial structural analysis of the oligosaccharide moieties of the vesicular stomatitis virus glycoprotein by sequential chemical and enzymatic degradation. *Virology.* 78:375–392.
6. Farquhar, M. G. 1971. Processing of secretory products by cells of the anterior pituitary gland. *Mem. Soc. Endocrinol.* 19:79–124.
7. Farquhar, M. G., and G. E. Palade. 1981. The Golgi apparatus (complex)—(1954–1981)—from artifact to center stage. *J. Cell Biol.* (3, Pt. 2) 91:77s–103s.
8. Florkiewicz, R. Z., A. Smith, J. E. Bergmann, and J. K. Rose. 1983. Isolation of stable mouse cell lines that express cell surface and secreted forms of the vesicular stomatitis virus glycoprotein. *J. Cell Biol.* 97:1381–1388.
9. Gorman, C. M., G. T. Merlino, M. C. Willingham, I. Pastan, and B. H. Howard. 1982. The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. *Proc. Natl. Acad. Sci. USA.* 79:6777–6781.
10. Green, R., and D. Shields. 1984. Somatostatin discriminates between the intracellular pathways of secretory and membrane proteins. *J. Cell Biol.* 99:97–104.
11. Gumbiner, B., and R. B. Kelly. 1981. Secretory granules of an anterior pituitary cell line, AtT-20, contain only mature forms of adrenocorticotropic hormone and beta-lipotropin. *Proc. Natl. Acad. Sci. USA.* 78:318–322.
12. Gumbiner, B., and R. B. Kelly. 1982. Two distinct intracellular pathways transport secretory and membrane glycoproteins to the surface of pituitary tumor cells. *Cell.* 28:51–59.
13. Lewis, U. J., L. F. Bonewald, and L. J. Lewis. 1980. The 20,000-dalton variant of human growth hormone: location of the amino acid deletions. *Biochem. Biophys. Res. Commun.* 92:511–516.
14. Lodish, H. F., N. Kong, M. Snider, and G. J. A. M. Strous. 1983. Hepatoma secretory proteins migrate from rough endoplasmic reticulum to Golgi at characteristic rates. *Nature (Lond.).* 304:80–83.
15. Luciw, P. A., J. M. Bishop, H. E. Varmus, and M. R. Capecchi. 1983. Location and function of retroviral and SV40 sequences that enhance biochemical transformation after microinjection of DNA. *Cell.* 33:705–716.
16. Maniatis, T., E. F. Fritsch, and J. Sambrook, editors. 1982. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 133–134.
17. Moore, H.-P., B. Gumbiner, and R. B. Kelly. 1983. Chloroquine diverts ACTH from the regulated secretory pathway to a constitutive one in AtT-20 cells. *Nature (Lond.).* 302:434–436.
18. Moore, H.-P., B. Gumbiner, and R. B. Kelly. 1983. A subclass of proteins and sulfated macromolecules secreted by AtT-20 cells is sorted with ACTH into dense secretory granules. *J. Cell Biol.* 97:810–818.
19. Moore, H. P. H., M. D. Walker, F. Lee, and R. B. Kelly. 1983. Expressing a human proinsulin cDNA in a mouse ACTH secreting cell. Intracellular storage, proteolytic processing and secretion on stimulation. *Cell.* 35:531–538.
20. Mulligan, R. C., and P. Berg. 1980. Expression of a bacterial gene in mammalian cells. *Science (Wash. DC).* 209:1422–1427.
21. Novick, P., C. Field, and R. Schekman. 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell.* 21:205–215.
22. Reading, C. L., E. E. Penhoet, and C. E. Ballou. 1978. Carbohydrate structure of vesicular stomatitis virus glycoprotein. *J. Biol. Chem.* 253:5600–5612.
23. Robbins, P. W., S. C. Hubbard, S. J. Turco, and D. F. Wirth. 1977. Proposal for a common oligosaccharide intermediate in the synthesis of membrane glycoproteins. *Cell.* 12:893–900.
24. Rose, J. K., and J. E. Bergmann. 1982. Expression from cloned cDNA of cell-surface secreted forms of the glycoprotein of vesicular stomatitis virus in eucaryotic cells. *Cell.* 30:753–762.
25. Rose, J. K., and C. J. Gallione. 1981. Nucleotide sequences of the mRNAs encoding the vesicular stomatitis virus G and M proteins determined from cDNA clones containing the complete coding regions. *J. Virol.* 39:519–528.
26. Schmidt, M. F. G., and M. J. Schlesinger. 1979. Fatty acid binding to vesicular stomatitis virus glycoprotein: a new type of post-translational modification of the viral glycoprotein. *Cell.* 17:813–819.
27. Sly, W. S., and H. D. Fischer. 1982. The phosphomannosyl recognition system for intracellular and intercellular transport of lysosomal enzymes. *J. Cell. Biochem.* 18:67–85.
28. Southern, P. J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* 1:327–341.
29. Strous, G. J. A. M., and H. F. Lodish. 1980. Intracellular transport of secretory and membrane proteins in hepatoma cells infected by vesicular stomatitis virus. *Cell.* 22:709–717.
30. Strous, G. J. A. M., R. Willemsen, P. van Kerkhof, J. W. Slot, H. J. Geuze, and H. F. Lodish. 1983. Vesicular stomatitis virus glycoprotein, albumin, and transferrin are transported to the cell surface via the same Golgi vesicles. *J. Cell Biol.* 97:1815–1822.
31. Tartakoff, A., and P. Vassalli. 1978. Comparative studies of intracellular transport of secretory proteins. *J. Cell Biol.* 79: 694–707.
32. Walker, M. D., T. Edlund, A. M. Boulet, and W. J. Rutter. 1983. Cell-specific expression controlled by the 5′-flanking region of insulin and chymotrypsin genes. *Nature (Lond.).* 306:557–561.