Somatostatin Discriminates between the Intracellular Pathways of Secretory and Membrane Proteins

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ABSTRACT Somatostatin is a 14-amino acid peptide hormone that inhibits the secretion of a variety of other polypeptide hormones, including growth hormone. Here we describe an experimental system used to determine whether somatostatin can discriminate in its inhibition between secretory and plasma membrane proteins. Growth hormone-secreting cells (GH_3) were infected with vesicular stomatitis virus and pulse-chased with [^{35}S]methionine to follow the simultaneous intracellular transit of growth hormone and the viral membrane glycoprotein, G protein. Secretion of growth hormone was monitored by immunoprecipitation of chase media, while appearance of G protein on the plasma membrane was detected by cell surface labeling and virus purification. In the presence of somatostatin (10 μg/ml), the secretion of growth hormone was inhibited by 80%. In contrast, G protein appeared on the plasma membrane with slightly enhanced kinetics. When cells were treated with the ionophore monensin (0.2 μM), there was a dramatic inhibition of both the secretion of growth hormone and the incorporation of G protein into plasma membranes. Our results on the differential effect of somatostatin provide evidence for sorting of secretory and membrane proteins into distinct compartments in the secretory pathway. The data further suggest that this sorting event occurs late in the Golgi complex or after proteins exit from that organelle.

In eucaryotic cells, the secretory pathway serves not only to ensure the efficient export of proteins from the cell, but also to deliver proteins to the plasma membrane and other organelles (23). Commitment of all proteins into this pathway appears to proceed by a common mechanism, which results in co-translational insertion of the nascent polypeptides into or through the membrane of the rough endoplasmic reticulum (RER)[1] (5). The subsequent directed transit of different proteins from the RER through elements of the secretory apparatus has been described in many systems (20). However, the basic mechanisms governing how movement is regulated and how the ultimate localization of different proteins is achieved remain to be determined. Secretory proteins and plasma membrane proteins, for example, traverse the entire pathway, from the RER through the Golgi apparatus, terminating in an exocytic fusion of vesicle membranes with the plasma membrane. In this process, both classes of proteins undergo many similar co- and posttranslational modifications (e.g., glycosylation, sulfation, proteolytic cleavage), indicative of their having resided in functionally similar compartments (9). However, it might be expected that different mechanisms would govern transport of soluble proteins and those embedded in lipid bilayers. In support of this hypothesis, recent studies have provided evidence that in certain differentiated cell types, secretory and plasma membrane proteins reach the cell surface at different rates (10, 14, 17, 27), and may be physically segregated from each other during transport (14). In order to investigate the molecular mechanisms underlying these phenomena, we developed a model system to study the intracellular sorting of secretory and plasma membrane proteins that exploits the physiological action of somatostatin (SRIF) to perturb protein transport.

Somatostatin (SRIF) is a 14-amino acid peptide hormone synthesized by various tissues, e.g., endocrine pancreas, hypothalamus, extrahypothalamic brain, and gastrointestinal epithelium (24, 26). Its primary physiological action is to inhibit the secretion of a corresponding array of other poly-

[1] *Abbreviations used in this paper:* DNP, dinitrophenol; Endo H, endoglycosidase H; GH_3, growth hormone-secreting cells; RER, rough endoplasmic reticulum; rGH, rat growth hormone; SRIF, somatostatin; TNBS, trinitrobenzene sulfonate; TNP, trinitrophenol; VSV, vesicular stomatitis virus.

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peptide hormones such as growth hormone, prolactin, insulin, and glucagon (24). Though it is known to interact with high-affinity surface receptors on target cells (25), the intracellular site at which it arrests secretory protein migration has not been analyzed. However, its rapid and local action (31) suggests that it perturbs late events in secretion. Furthermore, this role implies that it would be a powerful agent that could be used to selectively inhibit secretion without necessarily affecting assembly and turnover of plasma membrane components.

We therefore studied the effect of SRIF on the intracellular transit of a typical secreted protein, rat growth hormone (rGH) (2), and a model plasma membrane-like protein, the G protein of vesicular stomatitis virus (VSV) (3, 16, 21), in the growth hormone-secreting (GH_3) rat pituitary cell line. GH_3 cells synthesize and secrete large amounts of rGH (30), possess surface SRIF receptors (25), and can be infected by VSV. We report here that SRIF inhibits the exit of newly synthesized rGH but allows G protein to appear on the plasma membrane in the same cells. Our data imply that rGH and VSV G protein are sorted during intracellular transport into physically or functionally distinct compartments.

MATERIALS AND METHODS

Cells and Viral Infection: GH_3 cells (obtained from Dr. L. Reid, Albert Einstein College of Medicine) had been in continuous culture for several years and were a mixture of flattened and rounded, but adherent, cells. Cells were routinely propagated in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (Flow Laboratories, McClean, VA), in a 95% air-5% CO_2 humidified incubator. For experiments, cells were plated in 35-mm plastic dishes, and grown to near confluence. Dexamethasone (Sigma Chemical Co., St. Louis, MO) was added from a stock solution to a final concentration of 0.1 μM. VSV (Indiana serotype, a gift of Dr. E. Rodriguez-Boulan, Downstate Medical Center), was propagated in GH_3 cells using a low multiplicity of infection. Viral titers, assessed by cytopathic effect (CPE) on GH_3 cells in 96-well microtiter dishes, were routinely 10^8–10^9 CPE 50/ml; there was no significant reduction in titer after several such passages. For viral infection experiments, cells were washed in protein-free saline, and viruses (at a multiplicity of 10–15 CPE 50/cell) were adsorbed in 0.2 ml of serum-free medium for 45 min at 37°C, after which 2 ml of RPMI 1640 containing 2% fetal bovine serum was added. In preliminary experiments, cells were subjected to pulse-labeling with [^{35}S]methionine at hourly intervals after infection, which revealed peak synthesis of G protein 3.5–5 h after addition of virus.

Biosynthetic Labeling: For pulse-chase experiments, growth medium was replaced with RPMI lacking unlabeled methionine (Selectamine kit, Gibco Laboratories) that contained 50 μCi/ml [^{35}S]methionine (1,040 Ci/mmol); Amersham Corp., Arlington Heights, IL) and cells were incubated for 5 or 10 min as indicated. The chase medium consisted of complete RPMI 1640 containing 10% serum and 10 mM unlabeled methionine. Where indicated, somatostatin-14 (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added from a stock solution of 1 mg/ml dissolved in 0.1 N acetic acid, and monensin (Calbiochem-Behring Corp., La Jolla, CA) from a 5-mM stock solution in ethanol. Cells were exposed to SRIF or monensin only after the pulse, in order to test the effect of these reagents on intracellular transit of newly synthesized rGH. Cells were harvested by scraping, lysed in 100 μl PBS containing 0.5% (wt/vol) deoxycholate, 0.5% (vol/vol) Nonidet P-40, and a cocktail of protease inhibitors (100 U/ml trasylol, 200 μg/ml soybean trypsin inhibitor, 1 mg/ml benzamidine, 1 mg/ml ϵ-aminocaproic acid, and 0.35 mg/ml phenylmethylsufonyl fluoride, all purchased from Sigma Chemical Co.). Nuclei were removed by centrifugation for 2 min in a Brinkmann microfuge, and the postnuclear supernatants were prepared for immunoprecipitation or directly for SDS PAGE.

Immunoprecipitation of Rat Growth Hormone: Aliquots of postnuclear supernatants and chase media were subjected to immunoprecipitation using a baboon anti-rat growth hormone antiserum (a kind gift of Dr. F. Carter Bancroft, Memorial Sloan-Kettering Cancer Center). 10 μl of this serum quantitatively precipitated pulse-labeled rGH from 5×10^6 dexamethasone-treated GH_3 cells (not shown.) Immune complexes were isolated by incubation with Protein A-Sepharose beads (Sigma) and resolved by SDS PAGE using a 7–15% gradient of acrylamide. Gels were subjected to fluorography and exposed to Kodak XAR film at −70°C. The resulting fluorograms were scanned using a Beckman DU-8 spectrophotometer with an integrating function (Beckman Instruments, Inc., Palo Alto, CA).

Virus Purification: Chase media were clarified by centrifugation for 30 s in a microfuge, after which aliquots were diluted in TNE (100 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10 mM EDTA), and centrifuged for 4 h at 39,000 rpm in an SW41 rotor at 4°C. Viral pellets were dissolved directly in SDS gel loading buffer and resolved by SDS PAGE as outlined above. Viruses isolated by this procedure were essentially free of contaminating cellular proteins.

Cell Surface Labeling: At the indicated chase times, cultures were chilled, and all subsequent steps were performed at 0°C. Cells were washed three times in PBS-T (125 mM NaCl, 25 mM $NaPO_4$, pH 8.0) and incubated in 10 mM trinitrobenzene sulfonate (picrylsulfonic acid, Sigma) in PBS-T for 30 min. After three washes in PBS-T, and one in PBS-T containing 1 mg/ml BSA, intact cells were exposed to rabbit anti-dinitrophenol (DNP) antiserum diluted 1:2 in PBS-T for an additional 60 min. Cells were washed extensively and harvested as above, and 25 μl of packed protein A-Sepharose beads (Sigma) was added directly to the resultant postnuclear supernatants. Immune complexes were isolated by centrifugation and prepared for SDS PAGE.

Preparation of Anti-DNP Antiserum: In preliminary cell surface labeling experiments we tested commercially available rabbit anti-DNP antisera from several sources, as well as a murine monoclonal anti-DNP antibody. In all cases, the result was a faint or undetectable labeling of VSV G protein with high background labeling of other viral and cellular proteins. We therefore raised high titer antisera in rabbits by multisite subcutaneous injection of a DNP-hemocyanin conjugate (prepared by Dr. B. Birshtein, Albert Einstein College of Medicine). Antigen was resuspended in Freund's complete adjuvant for the initial immunization, followed at 2-wk intervals by similar injections of antigen in Freund's incomplete adjuvant. Anti-DNP titers were assessed by Ouchterlony double immunodiffusion; positive bleeds were then assayed directly in cell surface labeling experiments as described above. The use of this antiserum resulted in a 10-fold higher labeling than was seen with commercial antisera. Nonetheless, even using undiluted antiserum (1 ml per 5×10^6 cells), the proportion of pulse-labeled G protein that can be immunoprecipitated is low, as has been described previously (27). This is presumably due to an efficient coupling of trinitrophenol (TNP) moieties to an excess of nonradioactive surface molecules which compete with radiolabeled G molecules for antibody binding. However, it is assumed that an equal proportion of surface molecules are detected by this procedure in each case (27).

Endoglycosidase H Digestions: VSV-infected cells were pulse-labeled with [^{35}S]methionine for 5 min and chased for 10-min intervals. Cells were harvested directly into 1% SDS (100 μl per 35-mm dish), sonicated, and boiled. Two 40-μl aliquots of each lysate were adjusted to a final concentration of 0.3% SDS and 0.2 M Na citrate, pH 5.5. 2 μl of endoglycosidase H (Endo H) (9.08 U/ml, a gift of Dr. P. Atkinson, Albert Einstein College of Medicine) was added to one replicate sample, while the control received an equal volume of buffer. Incubation was at 37°C for 16 h, after which samples were prepared for SDS PAGE directly.

Immunofluorescence: Immunofluorescent staining of infected cells was performed essentially as described previously (12). Rabbit anti-VSV antiserum was kindly provided by Dr. E. Rodriguez-Boulan.

RESULTS

Synthesis and Transport of Growth Hormone and VSV G Protein in Infected Cells

The goal of our experiments was to monitor the simultaneous transit of rGH and G protein through the secretory pathway in GH_3 cells. It was therefore necessary to biosynthetically label both proteins in the RER using a short pulse, chase for increasing times, and assay for rGH secretion and the appearance of G protein on the plasma membrane. However, VSV infection rapidly shuts off host protein synthesis in many cells (34). Preliminary experiments revealed that infection of GH_3 cells with a multiplicity of infection sufficient to induce large quantities of G protein resulted in a general inhibition of synthesis of host proteins, including rGH, within 3 h after infection. Although the mechanism of VSV-induced translational inhibition is controversial, it has been suggested to result from simple competition between host mRNAs, and

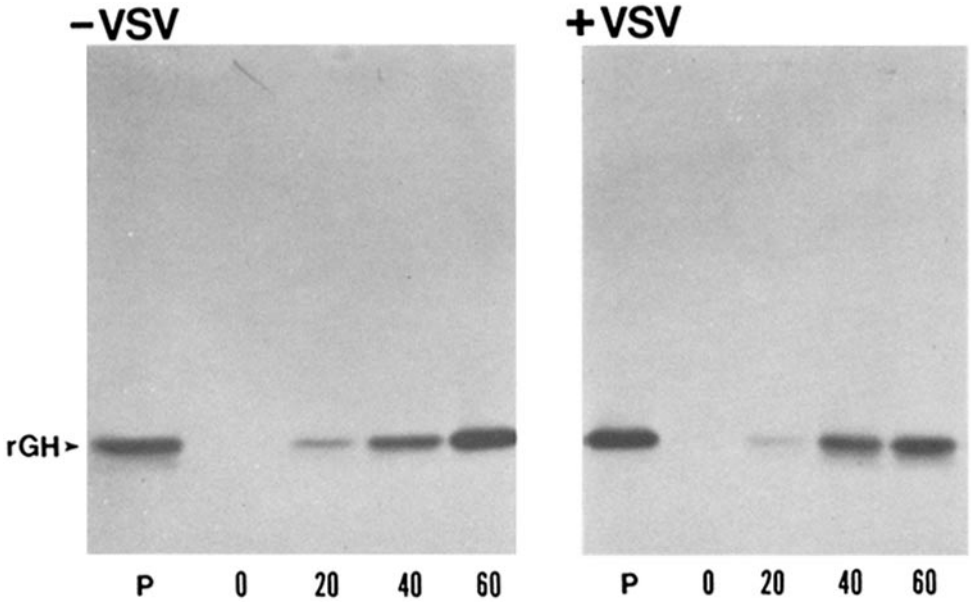

FIGURE 1 Synthesis and secretion of growth hormone in the absence and presence of viral infection. Mock-infected (*−VSV*) and VSV-infected (*+VSV*) GH_3 cells (all pretreated with dexamethasone) were pulse-labeled for 10 min at 4.5 h postinfection with 50 μCi/ml of [^{35}S]methionine, and harvested immediately (lanes *P*) or chased with unlabeled methionine for 0, 20, 40, and 60 min as indicated. Intracellular growth hormone (*rGH*) was immunoprecipitated from equivalent amounts of postnuclear supernatants (lanes *P*), and secreted rGH was measured in an equivalent amount of extracellular medium (lanes *0*, *20*, *40*, and *60*) by treatment with 10 μl of baboon anti-rGH (as described in Materials and Methods). The resulting immunoprecipitates were resolved by SDS PAGE using gradients of 7–15% acrylamide. After electrophoresis the gels were subjected to fluorography and exposed for 1 wk. A parallel analysis using rabbit anti-rat prolactin antibody revealed that this strain of GH_3 cells produces little rat prolactin which was further suppressed by dexamethasone treatment (not shown).

an excess of viral mRNAs, for the translational machinery (18). We therefore attempted to circumvent the inhibition of rGH specifically by raising the intracellular cencentration of rGH mRNA. To this end, cultures were treated with the synthetic glucocorticoid dexamethasone for several days before infection, since it is well documented that glucocorticoids stimulate rGH transcription in these cells (7). Optimally, cells were incubated with 0.1 μM dexamethasone for 48 h, then infected with VSV at a multiplicity of 10 CPE 50/cell or mock-infected, and labeled for 10 min with [^{35}S]methionine 4.5 h later. Quantitative immunoprecipitation of pulse-labeled rGH from equivalent amounts of mock-infected and VSV-infected cells demonstrated that significant levels of rGH synthesis were maintained after infection, when cells were pretreated with dexamethasone (Fig. 1). Thus, as predicted, increasing the relative concentration of rGH mRNA apparently allowed it to compete successfully with VSV mRNAs in initiation of translation.

To determine the kinetics of rGH secretion in control and VSV-infected cells, we isolated pulse-labeled rGH by immunoprecipitation of chase media and analyzed it on SDS PAGE (Fig. 1). Low levels of rGH could be detected at the first chase point (20 min) and were maximal by 60 min of chase. It appeared that the majority of pulse-labeled rGH was extracellular at this time in control (uninfected) cells, and similar results were obtained for VSV-infected cells. Quantitation of rGH secretion kinetics by densitometric scanning of the fluorograms revealed that rGH exits with a half-time of 35–40 min (see Fig. 4*A*). Most significantly, the rate of secretion of rGH, as well as the absolute amounts secreted, were not altered by viral infection, indicating that there was little or no disruption of normal secretory processes under our experimental conditions.

Two complementary methods were employed to measure the appearance of newly synthesized G protein in the plasma membrane of infected cells (Fig. 2). First, virus particles were isolated from chase media and analyzed for their content of labeled G protein. This serves as an assay for the functional integration of G protein into regions of the plasma membrane from which virus budding occurs. However, since it was conceivable that SRIF might allow G protein to reach the plasma membrane but interfere with a later step of virus assembly, we also measured cell surface G protein directly. Intact cells were exposed to trinitrobenzene sulfonate (TNBS), which results in the covalent linkage of TNP moieties to externally exposed proteins (6, 15). This was followed by immunoprecipitation of TNP-tagged molecules with anti-DNP antibodies and gel electrophoresis of the immune complexes. Treatment of intact cells with TNBS (Fig. 2*A*) resulted in the isolation of increasing amounts of labeled G molecules with longer durations of chase. Though there is background labeling of other viral proteins, the only species that exhibits pulse-chase behavior migrates slightly slower than pulse-labeled G (Fig. 2*A*, compare lanes *P* and *60*). This increase in apparent molecular weight is consistent with processing of oligosaccharide moieties that accompanies G transit from the RER through the Golgi region (see below.) The time required for half of the labeled G molecules to reach the plasma membrane was 45 min (see Fig. 4*B*). Chase incubations >60 min, followed by TNBS labeling, revealed a depletion in the amount of plasma membrane–associated G (not shown). This presumably resulted from G protein incorporation into virions (Fig. 2*B*), which closely followed its appearance in the plasma membrane ($t_{1/2}$ of incorporation = 45–50 min), indicating a short residence time of G in the plasma membrane. The viral L, N, and NS proteins appeared in virions in parallel with G, while newly synthesized M protein molecules exited much more rapidly, as has been reported for VSV replication in other cell types (1).

A more detailed analysis of G protein migration was facilitated by the presence of N-linked carbohydrate moieties on this protein. Core high mannose oligosaccharides added to nascent G molecules co-translationally in the RER are sensitive to Endo H, while galactose- and sialic acid-containing carbohydrates on mature G are resistant to the action of this enzyme (28). Endo H resistance is acquired during migration of the protein through the Golgi cisternae (most probably *trans* elements) which contain the carbohydrate-modifying

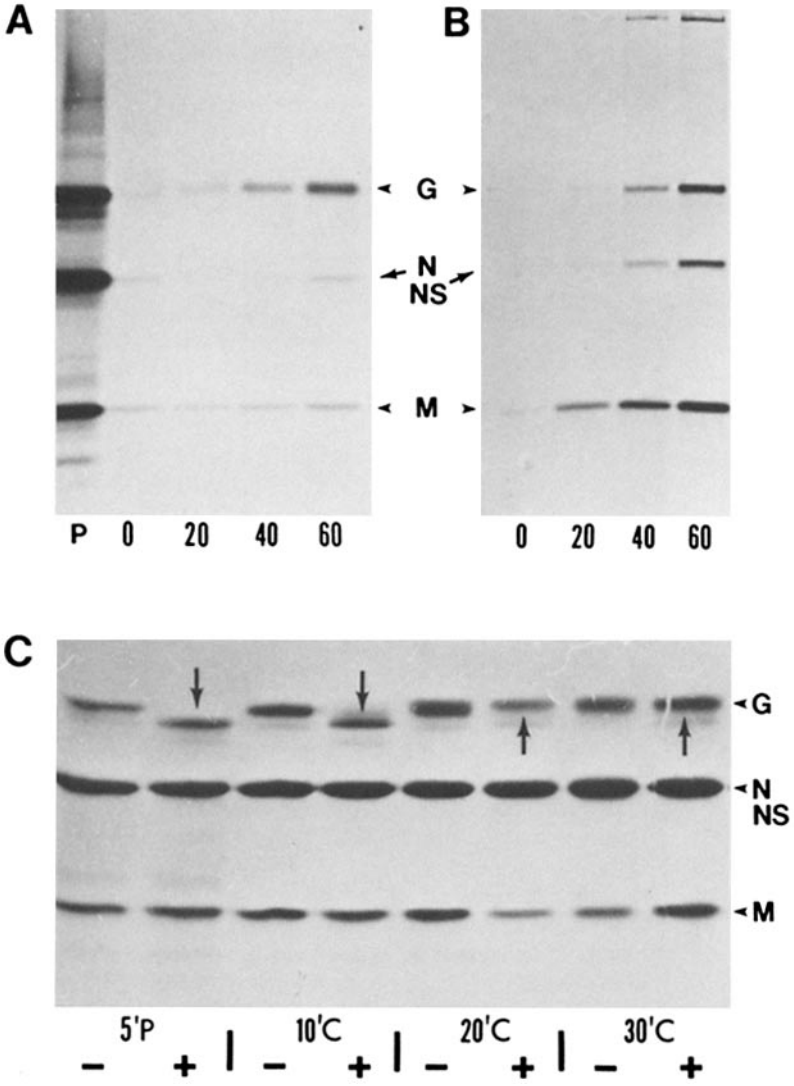

FIGURE 2 Intracellular transport of VSV G protein in GH_3 cells. (*A*) Cell surface labeling. Cells were pulse-labeled (at 4.5–5 h after infection) with 50 μCi/ml of [^{35}S]-methionine for 10 min, chased for 0, 20, 40, and 60 min, and quickly chilled. The cells were then subjected to TNBS labeling, and the [^{35}S]methionine TNP-conjugated plasma membrane proteins were detected by immunoprecipitation with anti-DNP antiserum, followed by resolution of the immune complexes on SDS PAGE. Lane *P* shows the postnuclear supernatant of cells harvested immediately after the pulse, and contains one-fifth the cellular material represented in lanes *0–60*. (*B*) Assembly of mature virus particles. Viruses radiolabeled with [^{35}S]methionine were pelleted from the chase media at 0, 20, 40, and 60 min chase points, and analyzed directly on SDS PAGE. (*C*) Kinetics of VSV G protein glycosylation. Infected cells were pulsed for 5 min with 50 μCi/ml [^{35}S]methionine and chased for 0, 10, 20, and 30 min (*5'P, 10'C, 20'C,* and *30'C*), after which total cell lysates were prepared and divided into two equal aliquots. One aliquot was subjected to digestion with Endo H for 16 h at 37°C (+), while the other was incubated with an equal volume of buffer (−). The resulting material was analyzed by SDS PAGE as outlined above. Downward-pointing arrows indicate Endo H-sensitive species of G protein; upward-pointing arrows indicate Endo H-resistant forms. The migration of the virion marker proteins *G, N, NS,* and *M* are indicated in each panel.

enzymes (22). Thus, the time required for acquisition of Endo H resistance can be used to approximate the rate of transfer of glycoproteins from the RER to the *trans* Golgi complex. Infected GH_3 cells were pulse-labeled for 5 min and chased for 10-min intervals, and total cell lysates were subjected to digestion with Endo H (Fig. 2*C*). G protein labeled during this shorter pulse was completely sensitive to this enzyme, as expected for localization in the RER. Endo H-resistant forms appeared rapidly; by 10 min of chase at least one intermediate in carbohydrate processing could be observed, though full-sized Endo H-resistant forms were not apparent until 20 min of chase. By 30 min of chase, 80% of labeled G was rendered resistant to Endo H.

It therefore appears that newly synthesized G protein travels from the RER to *trans* elements of the Golgi complex with a half-time of 15–20 min (Fig. 2*C*; see also Fig. 6), reaches the plasma membrane 20–30 min later, and is rapidly incorporated into budding virions. Taken together, these data indicate that under the conditions of infection used for these experiments, both rGH and VSV G protein are synthesized in large amounts and traverse the secretory pathway at approximately equivalent rates.

Effect of SRIF on Intracellular Transport of rGH and VSV G Protein

Previous studies on SRIF action in GH_3 cells and in primary pituicyte cultures have measured the inhibition of release of total cellular rGH by radioimmunoassay (4, 25, 32). Thus, it was necessary to establish (*a*) that SRIF would be effective in inhibiting the externalization of newly synthesized rGH using a pulse/chase protocol, and (*b*) that viral infection would not disrupt the surface receptor–mediated response to SRIF. It should be emphasized that our experimental protocol was designed to assess the effect of SRIF on the intracellular transit of presynthesized rGH. Consequently, cells were treated with SRIF after the pulse-labeling period, thus avoiding any possible effect of SRIF on rGH synthesis per se. Preliminary experiments in uninfected cells revealed that continuous exposure of cells to SRIF for the entire chase period resulted in 75–80% inhibition of secretion of pulse-labeled rGH-immunoreactive material. These experiments also demonstrated that prior treatment with dexamethasone did not alter the magnitude of this response. When cells were infected with VSV as described above, they were equally responsive to SRIF

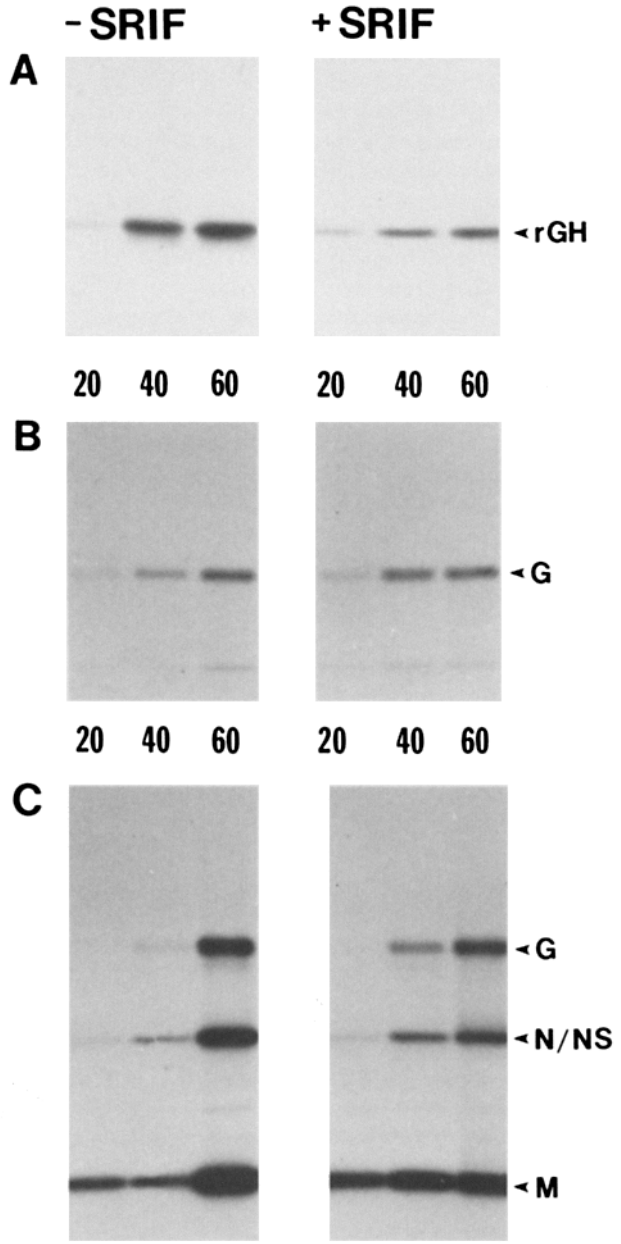

FIGURE 3 Effect of somatostatin on the intracellular transport of rGH and VSV G protein. VSV-infected GH_3 cells were pulsed with [^{35}S]methionine for 10 min at 4.5 h postinfection (in the absence of SRIF), and chased for 20, 40, and 60 min in the absence (−*SRIF*) or presence (+*SRIF*) of 10 μg/ml SRIF. (*A*) Secreted rGH. Pulse-labeled rGH was isolated from chase media at the times indicated by immunoprecipitation with baboon anti-rGH antiserum. (*B*) Cell surface VSV G protein. At the indicated times, cells were chilled, and the surface proteins were labeled using the TNBS procedure, followed by immunoprecipitation with anti-DNP antiserum. (*C*) Assembly of mature virions. Viruses were isolated from the chase media by centrifugation as described in Materials and Methods. In all three panels, samples were analyzed by SDS PAGE on 7–15% gradient gels, followed by fluorography.

action, and rGH secretion was inhibited by 80% overall (Figs. 3*A* and 4*A*).

In contrast, SRIF did not inhibit the simultaneous transit of VSV G protein to the plasma membrane (Fig. 3*B*); accessibility of pulse-labeled G protein to TNBS conjugation was consistently increased by the hormone. In the presence of SRIF, G protein was incorporated into plasma membranes with a half-time of 30–40 min, as compared with 45 min in untreated controls. This result was confirmed by the isolation of mature virions (Figs. 3*C* and 4*B*), in which G protein appeared more rapidly than in control cells ($t_{1/2}$ = 35–40 min as compared with 45–50 min). The other viral structural proteins were also externalized more rapidly in the presence of SRIF, implying that virus assembly as a whole was enhanced by the hormone. This might be due to a more rapid mobilization of G protein into plasma membranes, providing more nucleation sites for virus budding. We conclude from these experiments that SRIF allowed G protein to reach the plasma membrane, and to be incorporated into virions, while simultaneously inhibiting the secretion of rGH in the same cultures.

It could be argued that the differential effect of SRIF resulted from a non-uniform infection of the cells with VSV. If this were the case, uninfected cells could synthesize rGH and respond to SRIF. Infected cells in the same culture, rendered refractory to the action of SRIF by the cytopathic effect of VSV, could synthesize and transport G protein irrespective of the presence of the hormone. Though the multiplicity of infection used and the maintenance of rGH synthesis and secretion observed (Fig. 1) rendered this possibility unlikely, we nevertheless tested it directly by subjecting cells to indirect immunofluorescent staining using anti-VSV antibody and rhodamine-conjugated second antibody (Fig. 5). Under the conditions of infection used, >90% of the cells exhibited high levels of viral-specific immunofluorescence. Most of the cells were highly infected and rounded; in some, it is possible to see perinuclear and peripheral staining characteristic of Golgi complex and plasma membrane, respectively. This result suggested that the differential effect of SRIF could not be explained on the basis of two subpopulations of GH_3 cells.

Effect of Monensin on Intracellular Transit

The data presented above demonstrate that the intracellular pathways of rGH and VSV G protein can be distinguished by their sensitivity to the inhibitory effect of SRIF, which probably acts at a relatively late step in the secretory pathway. We

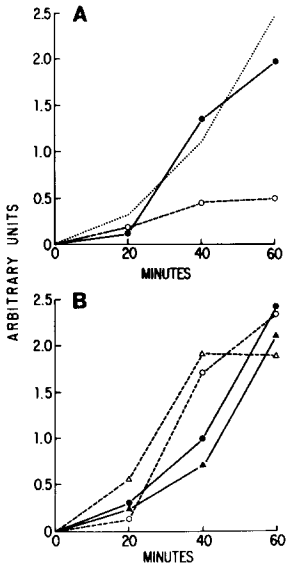

FIGURE 4 Kinetics of rGH and VSV G externalization. The fluorograms shown in Fig. 3 were analyzed by densitometric scanning (see Materials and Methods), and the integrated absorbances were plotted in arbitrary units. (*A*) rGH secretion in the absence (●) and presence (○) of SRIF. The dotted line represents rGH secreted by parallel cultures of mock-infected, dexamethasone-treated GH_3 cells (see Fig. 1). (*B*) VSV G protein release. Detection on the cell surface, minus SRIF (▲) and plus SRIF (△); assembly of virions, minus SRIF (●) and plus SRIF (○).

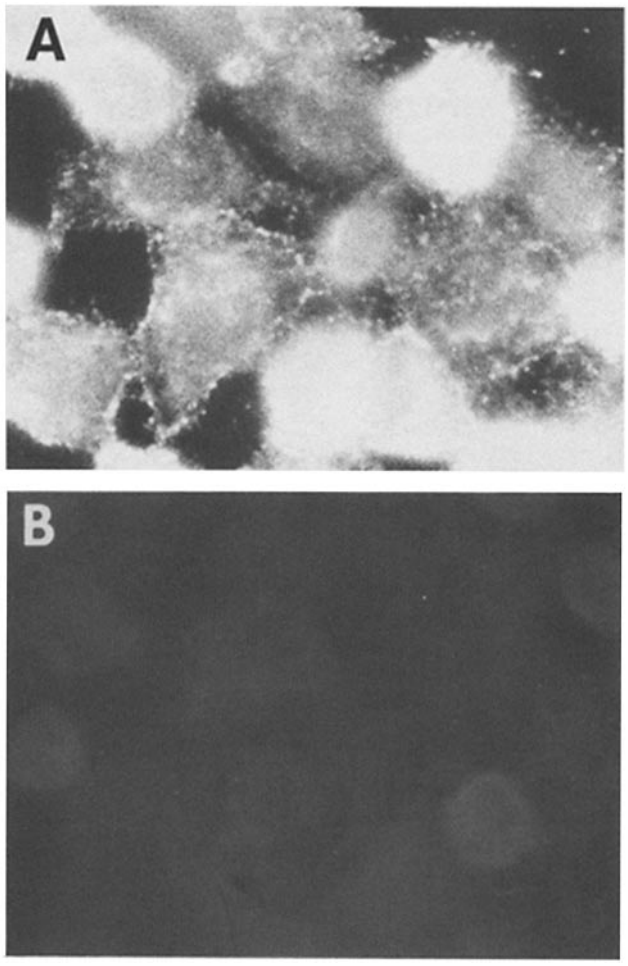

FIGURE 5 Assessment of viral infection by immunofluorescent staining of VSV-infected GH_3 cells. Cells grown on coverslips were infected at a multiplicity of 10–15 CPE 50/cell, fixed 5 h later, and subjected to indirect immunofluorescence using rabbit anti-VSV antiserum followed by rhodamine-conjugated goat anti-rabbit IgG. (*A*) Rabbit anti-VSV antiserum; (*B*) nonimmune rabbit serum. Mock-infected cultures stained with anti-VSV antiserum appeared as in *B*. × 1,331.

therefore wished to determine the point in the secretory pathway at which rGH and G protein might diverge. To this end we tested the effect of the ionophore monensin on the simultaneous transit of rGH and G protein; this compound has been shown to block the transport of many secretory and membrane proteins through the Golgi region (29). GH_3 cells were infected with VSV, pulse-labeled, and chased in the presence or absence of 0.2 μM monensin (Fig. 6). Monensin effectively blocked the secretion of rGH and the externalization of G protein, to the same extent, at all time points measured (Fig. 6, *a* and *b*). In addition, this agent also inhibited virus assembly, as evidenced by a concomitant decrease in the incorporation of the other labeled viral structural proteins into mature virions (not shown).

To determine the site of monensin-mediated arrest within the Golgi apparatus, control and monensin-treated infected cell lysates were digested with Endo H and analyzed by SDS PAGE. VSV G protein acquired resistance to Endo H in the presence of monensin (Fig. 6*C*), indicating that the drug allowed G protein access to the *trans* element of the Golgi. However, the time required for acquisition of complex carbohydrates and hence Endo H resistance was significantly longer in the presence of monensin (Fig. 6*C*; $t_{1/2}$ = 25 min as compared with 15 min in controls). This suggests that in these cells, 0.2 μM nomensin delays transport through proximal elements of the Golgi complex in addition to inhibiting exit from this organelle. In this regard, G protein that accumulated intracellularly in the presence of monensin had a slightly smaller apparent molecular weight than mature G protein (not shown); this was presumably due to the absence of sialic acid residues on these molecules (27). On the basis of these results, we tentatively conclude that rGH and VSV G protein traverse the Golgi apparatus in parallel, and are subsequently diverted into functionally different pathways.

DISCUSSION

The experiments described here demonstrate that VSV-infected GH_3 rat pituitary cells represent a useful model system for studying intracellular transport and sorting of secretory and plasma membrane proteins, in this case, rat growth hormone and VSV G protein. By manipulating the dose and duration of dexamethasone pretreatment to stimulate rGH transcription, as well as by titrating the multiplicity of virus

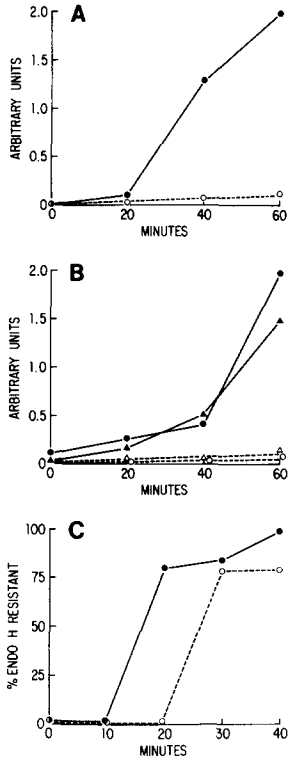

FIGURE 6 Effect of monensin on the kinetics of rGH and VSV G protein intracellular transport. Infected cells were pulse-labeled with [^{35}S]methionine at 5 h postinfection and chased in the absence (closed symbols) or presence (open symbols) of 0.2 μM monensin. rGH was detected by immunoprecipitation from chase media, and cell surface and virion-associated G protein were measured by TNBS labeling and virus isolation as described in the legend to Fig. 2. Endo H digestion of control and monensin-treated cultures was as described in Materials and Methods and the legend to Fig. 2*C*. All samples were resolved on 7–15% gradient SDS gels and fluorographed, and the fluorograms were quantitated by densitometry. Values are plotted in arbitrary units. (*A*) Secretion of rGH in the absence (●) and presence (○) of monensin. (*B*) Cell surface G protein, minus monensin (▲), plus monensin (△); G protein in virions, minus monensin (●), plus monensin (○). (*C*) Acquisition of Endo H resistance minus monensin (●) and plus monensin (○).

used for infection, we were able to establish conditions in which uniformly infected cultures synthesize and externalize large amounts of both proteins. This protocol enabled us to test the effect of SRIF, a ubiquitous physiological modulator of peptide hormone secretion, on the simultaneous transit of rGH and G protein. The results indicate that SRIF selectively inhibits the secretion of rGH while allowing the externalization of VSV G protein. In contrast, monensin, an ionophore that disrupts the Golgi apparatus, blocks transit of both proteins. These data provide evidence that the two proteins are effectively sorted at some point during their passage through the secretory pathway, an event that probably occurs late in the Golgi apparatus or after exit from that organelle.

On the basis of kinetic analyses, evidence has accumulated from other systems suggesting that traffic of secretory and membrane proteins through the secretory pathway is highly regulated and is not accomplished by bulk flow of RER membrane and content. For example, Strous, Lodish, and coworkers (17, 27) demonstrated that several serum proteins (as well as VSV G protein) have distinct rates of secretion from HepG2 hepatoma cells. Using acquisition of Endo H resistance to determine transfer though the Golgi apparatus, they ascribed these differences to different rates of transfer between RER and Golgi, and concluded that after exiting from the Golgi the proteins travel at equivalent rates. Similarly, Fitting and Kabat (10) have shown that two retroviral glycoproteins, $gp70^{env}$ and $gp93^{gag}$, reach the plasma membrane of transformed cells with distinctly different kinetics; they postulate a selection mechanism (perhaps receptor-mediated) that may operate at the level of the RER. Regulation of transit also occurs in AtT-20 pituitary cells, but at a later stage in the secretory pathway. This cell line, studied extensively by Gumbiner and Kelly (14), secretes both the precursor polypeptide proopiomelanocortin and adrenocorticotropin which is derived from it by proteolysis; and also expresses an endogenous retroviral glycoprotein on the cell surface. Proopiomelanocortin and viral glycoprotein molecules are secreted rapidly, via a "constitutive" pathway. In contrast, mature adrenocorticotropin molecules, and a set of sulfated proteoglycans, are packaged into secretory granules and released more slowly, in a secretagogue-sensitive manner, which may represent an alternative, "regulated," pathway (13, 14, 19).

Our experimental system differs from those described above in that we have not observed major differences in transit times of rGH and VSV G protein. There is no apparent intracellular accumulation of newly synthesized rGH analogous to that seen with mature adrenocorticotropin in AtT-20 cells. Although such accumulation has been reported in GH_3 cells, the strain of cells used in this study had been in continuous culture for several years and had presumably lost this differentiated function. In fact, in our experiments, rGH (which is transported via a SRIF-sensitive pathway) reaches the plasma membrane slightly faster than G protein ($t_{1/2}$ of 35–40 min as compared with 45 min). This result implies that intracellular sorting, even into differentially regulated pathways, may not necessarily result in measurable differences in transport kinetics. It could be argued, however, that these two proteins traverse most of the secretory pathway in tandem, possibly in the same vesicles, and are only segregated from each other at a very late stage. Consequently, differences in transfer rates after this point might not contribute significantly to the overall rates of secretion. The possibility of late sorting of VSV G protein and rGH is suggested by the observation that SRIF causes G protein to reach the surface slightly faster than in untreated controls (Fig. 3), without altering the rate at which it acquires Endo H resistance (not shown). Thus, this phenomenon may represent a specific effect on post-Golgi complex vesicles into which G protein has been segregated.

An alternative explanation of our data is that rGH and G protein travel the length of the secretory pathway in the same vesicles, and that sorting of the two occurs as a *result* of SRIF action. That is, SRIF may cause the content of a secretory vesicle to be retained intracellularly, while allowing the vesicle membrane and its resident proteins to fuse with the plasma membrane. Such a process would be analogous to the dissociation of asialoglycoprotein from its receptor that occurs in an endosomal compartment following receptor-mediated endocytosis (11). Subsequent to this, receptors recycle to the plasma membrane, while ligand remains in the cell. This implies that the membrane and content of such vesicles should be considered as distinct compartments. In our case, the enhanced externalization of G protein caused by SRIF may be a direct result of such an uncoupling of secretory vesicle membrane and content. Localization of G protein and rGH-containing vesicles by immunoelectron microscopy, as well as physical isolation of such vesicles by subcellular fractionation of control and SRIF-treated cells, should indicate whether the two proteins are physically, as well as functionally, segregated.

Our experiments also raise several questions concerning the mode of SRIF action. Numerous studies have suggested that changes in cortical cAMP and/or Ca^{+2} ion concentrations mediate the inhibitory effect of SRIF on secretion (4, 8, 33). Our data imply that whatever the nature of the second messenger(s) involved, SRIF causes a selective, and perhaps local, modulation of exocytic processes. Thus, further detailed analysis of SRIF action per se, using this system, may provide important insights into the molecular mechanisms that regulate protein sorting and secretion.

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