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 (GH3) were infected with vesicular stomatitis virus and pulse-chased with [35S]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) (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-
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 (GH3) rat pituitary cell line. GH3 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: GH3 cells (obtained from Dr. L. Reid, Albert Einstein College of Medicine) have 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, McLean, VA), in a 95% air-5% CO2 humidified incubator. For experiments, cells were plated in 35-mm plastic dishes, and grown to near confluence. The goal of our experiments was to monitor the simultaneous transit of rGH and G protein through the secretory pathway in GH3 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 GH3 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
an excess of viral mRNAs, for the translational machinery (18). We therefore attempted to circumvent the inhibition of rGH specifically by raising the intracellular concentration 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 [35S]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, 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. 2B), which closely followed its appearance in the plasma membrane (t1/2 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 endo H activity.
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 GH3 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. 2C). 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. 2C; 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 GH3 cells and in primary pituitary 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 pre-synthesized 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.
action, and rGH secretion was inhibited by 80% overall (Figs. 3A and 4A).

In contrast, SRIF did not inhibit the simultaneous transit of VSV G protein to the plasma membrane (Fig. 3B); 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. 3C and 4B), 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 GH3 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
therefore wished to determine the point in the secretory pathway at which \( \text{rGH} \) and G protein might diverge. To this end we tested the effect of the ionophore monensin on the simultaneous transit of \( \text{rGH} \) and G protein; this compound has been shown to block the transport of many secretory and membrane proteins through the Golgi region (29). \( \text{GH}_3 \) cells were infected with VSV, pulse-labeled, and chased in the presence or absence of 0.2 \( \mu \text{M} \) monensin (Fig. 6). Monensin effectively blocked the secretion of \( \text{rGH} \) 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. 6C), 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. 6C; \( t_{1/2} = 25 \text{ min} \) as compared with 15 min in controls). This suggests that in these cells, 0.2 \( \mu \text{M} \) monensin 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 \( \text{rGH} \) 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 \( \text{GH}_3 \) 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 \( \text{rGH} \) transcription, as well as by titrating the multiplicity of virus

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**FIGURE 5** Assessment of viral infection by immunofluorescent staining of VSV-infected \( \text{GH}_3 \) 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. \( \times 1,331 \).

**FIGURE 6** Effect of monensin on the kinetics of \( \text{rGH} \) and VSV G protein intracellular transport. Infected cells were pulse-labeled with \( ^{[35}\text{S}] \)methionine at 5 h postinfection and chased in the absence (closed symbols) or presence (open symbols) of 0.2 \( \mu \text{M} \) monensin. \( \text{rGH} \) 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. 2C. 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 \( \text{rGH} \) 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 co-workers (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, gp70env and gp93mp, 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 GH3 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 (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 Ca2+ 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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