

Retrovirus-Mediated Expression of Preprosomatostatin in Rat Pituitary GH₃ Cells: Targeting of Somatostatin to the Regulated Secretory Pathway

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Somatostatin (SRIF) is a 14-amino acid peptide hormone that is synthesized as part of a larger precursor, prepro-SRIF, consisting of a signal peptide and a proregion of 80–90 amino acids; mature SRIF is located at the carboxyl-terminus of the precursor. We have used a recombinant retroviral expression vector encoding anglerfish prepro-SRIF-I to infect rat pituitary GH₃ cells. The aim of these studies was to investigate the intracellular storage and secretion of the total pool of endogenous GH compared to that of SRIF. Several clonal lines of GH₃ cells expressing high or low levels of SRIF were treated with TRH, forskolin, or depolarizing concentrations of potassium, and the levels of intracellular and secreted GH or SRIF were determined using highly sensitive RIAs. Approximately 65% of the total GH was secreted basally, whereas less than 20% of the SRIF-immunoreactive material was basally secreted. Forskolin treatment or potassium depolarization stimulated GH release, but only about 50% above basal levels. In contrast, SRIF secretion was stimulated approximately 5-fold in response to these secretagogues. Based on its lower basal rate of secretion compared to GH and its enhanced release in response to a variety of secretagogues, we conclude that the heterologously expressed SRIF is preferentially targeted to the regulated pathway in GH₃ cells. (Molecular Endocrinology 3: 1652–1658, 1989)

INTRODUCTION

A characteristic feature of polypeptide hormone-producing cells is their property of concentrating and stor-

ing, for hours or days, the secretory product in electron-dense secretory granules (1). Upon stimulation by an extracellular signal, the secretory granules fuse, in a calcium-dependent process, with the plasma membrane, thereby releasing their contents into the external milieu. This type of secretion has been designated regulated or stimulated (see Refs. 2–4 for recent reviews). Upon sequestration into the regulated pathway, polypeptide hormone precursors become available as substrates for a variety of posttranslational modifications that occur during transport from the distal Golgi apparatus to secretory granules. In contrast, fibroblasts, hepatocytes, and plasma cells, for example, manifest basal or constitutive secretion, whereby secretory proteins and plasma membrane proteins are neither concentrated nor stored and are transported in vesicles which continuously fuse with the plasma membrane in a calcium-independent manner (2, 4). Furthermore in constitutive secretion there is little or no precursor processing other than carbohydrate addition.

At present the molecular signals that target a particular precursor to the regulated secretory pathway are poorly understood, although recently very elegant studies from Chung *et al.* (5) have demonstrated that a family of hormone-binding proteins [mol wt (M_r) ~25,000], located in the Golgi apparatus, specifically bind peptide hormones destined for secretory granules. Furthermore, several observations have implicated the trans-Golgi network as the site where selective aggregation and packaging into acidic clathrin-coated vesicles occurs (6–9). Current evidence suggests that in the absence of a specific topogenic signal, *e.g.* for ER retention (10, 11) or Golgi apparatus localization (12) or for sorting to lysosomes (13), secretion through the constitutive pathway occurs by default (2, 14). However since hormone-secreting cells also undergo basal secretion, a mechanism must exist that enables them to

discriminate between molecules destined for the regulated or constitutive pathways.

We have been studying the processing of prosomatostatin (pro-SRIF) to identify putative structural domains within peptide hormone precursors that might function in mediating sorting to the regulated secretory pathway (15–18). SRIF is a 14-amino acid peptide hormone that is synthesized as part of a larger precursor, prepro-SRIF, which is one of the simplest precursors (19). The mature hormone is located at the carboxyl-terminus of the propeptide, and apart from endoproteolytic cleavage, the precursor undergoes no other posttranslational modifications.

Earlier studies from one of our laboratories (20) demonstrated that rat anterior pituitary GH₃ cells efficiently discriminate in sorting between membrane glycoproteins and endogenous GH, probably in the distal elements of the Golgi apparatus. More recently, we demonstrated (16) that these cells also accurately and efficiently processed pro-SRIF to the mature hormone. Approximately 75% of the intracellular precursor was cleaved to mature SRIF, and at least half of this material was targeted to the regulated pathway (16, 17). In contrast, 95% of newly synthesized endogenous GH was delivered to the constitutive pathway; consequently, it was concluded that GH₃ cells discriminate between and store a foreign protein significantly more efficiently than the endogenous hormone. Since these studies used a pulse-chase experimental protocol, it was possible that we were measuring only a relatively small fraction of the endogenous GH or heterologous SRIF pools in these cells, *i.e.* the rapidly turning over newly synthesized molecules. It could be argued that the total steady state pools of these two hormones, comprising both old (long term storage) and nascent molecules, might not be differentially packaged or secreted. Thus, the preferential sorting of pro-SRIF to the regulated pathway may be a function of its biosynthetic rate compared to GH or to differences in relative pool sizes of these two hormones rather than to differences in packaging efficiency *per se*. To address these possibilities, we employed direct measurement of the total stored and secreted levels of both SRIF and GH using RIA. We have also determined the effects of various secretagogues on SRIF and GH secretion from several clonal lines of GH₃ cells infected with a retroviral vector encoding prepro-SRIF.

RESULTS

Cellular Content of Immunoreactive GH and SRIF in GH₃ Clonal Cell Lines

Numerous SRIF-containing GH₃ cell clonal lines were obtained upon infection of wild-type GH₃ cells with recombinant retrovirus produced by transfecting Psi-2 cells with the plasmid pLJS18 (16). Comparison of the intracellular SRIF- and GH-immunoreactive material in several clonal lines showed there was a 4-fold variation

in the level of expression of both hormones (Table 1). It is noteworthy that the level of GH in control uninfected GH₃ cells (wild-type) was similar to that of the retrovirally infected clonal lines, demonstrating that viral infection *per se* did not affect GH biosynthesis. The GH content of the infected cells was considerably greater than that of SRIF, ranging between approximately 13–46 ng/10⁶ cells, whereas the level total of SRIF-immunoreactive material ranged from approximately 1–3 ng/10⁶ cells. However, when expressed on a molar basis, the contents of GH and SRIF were quite similar, ranging from approximately 0.6–2 pmol/10⁶ cells. There was, however, some variation in the levels of the hormones in different clones; the molar ratio of SRIF to GH ranged from 0.5–2.15 (Table 1).

Secretion of GH and SRIF from GH₃ Cells

Recent experiments indicated that GH₃ cells stored newly synthesized pulse-labeled mature SRIF significantly more efficiently than the endogenous GH (16), and furthermore, much of this material was targeted to the regulated pathway, since it was secreted in response to cAMP. We, therefore, determined if the secretion of total intracellular SRIF was similarly regulated. Secretion (release) of GH has been reported from GH₃ cells in response to a variety of secretagogues, including TRH (24, 25), agents that promote membrane depolarization (24, 25), as well as those that elevate intracellular levels of cAMP (26). Clone GH₃S18.9 was studied because it had the highest intracellular SRIF content (Table 1), and the SRIF-immunoreactive polypeptides have been characterized in detail (16). The cells were treated with depolarizing concentrations of potassium (46 mM), TRH, or forskolin, an activator of adenylate cyclase, and the release of GH and SRIF into the medium was determined (Fig. 1 and Table 2). Under basal conditions, GH release was linear for 2 h, and TRH minimally enhanced its secretion. Forskolin or 46 mM potassium was more effective than TRH in stimulating GH release, but maximal stimulation was only about 50% above the basal level at any time (Fig. 1A). Of the total GH present after a 2-h incubation, (intracellular plus secreted material) approximately 60% was secreted basally, and the remaining 40% was stored

Table 1. Content of SRIF and GH in Clonal Lines of Rat Pituitary GH₃ Cells Infected with a Recombinant Retrovirus Encoding Anglerfish Prepro-SRIF

Clone No.	SRIF/10 ⁶ Cells		GH/10 ⁶ Cells		Molar Ratio (SRIF/GH)
	ng	pmol	ng	pmol	
9	3.26	1.99	20.4	0.92	2.16
2	1.12	0.68	20.0	0.90	0.76
14	0.90	0.55	12.9	0.58	0.94
8	1.66	1.01	45.6	2.06	0.49
WT			22.8	1.03	

The intracellular content for each clonal line of GH₃S18 cells was determined in a single experiment performed in duplicate. WT refers to control uninfected GH₃ cells.

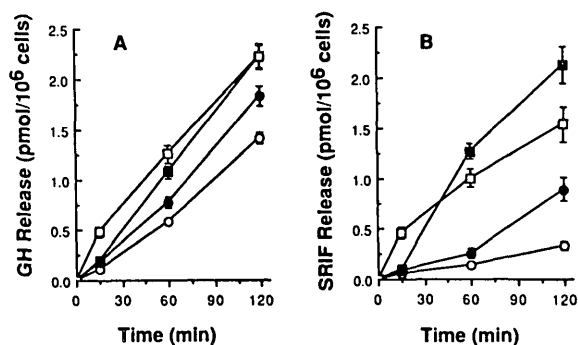


Fig. 1. Secretion of GH and SRIF from GH₃S18.9 Cells
GH₃S18.9 cells were incubated as described in *Materials and Methods*. After 3 days the medium was removed, and cells were washed with PBS and incubated in buffer I containing no additives (basal; ○—○), TRH (2.5 μM; ●—●), 46 mM K⁺ (■—■), or forskolin (50 μM; □—□). Aliquots of the incubation medium were sampled at the indicated times and assayed for both GH and SRIF (see *Materials and Methods*). Each data point represents the mean of eight samples assayed in duplicate. The brackets represent the SEM. A, GH secretion; B, SRIF secretion.

Table 2. Effect of Secretagogues on the Storage and Secretion of SRIF and GH from GH₃S18.9 Cells

Secretagogue	Total (pmol/10 ⁶ Cells)	Stored (% of Total)	Secreted (% of Total)
SRIF			
Basal	2.36 ± 0.20	84.3 ± 2.0	15.7 ± 2.0
TRH (2.5 μM)	2.46 ± 0.15	61.5 ± 6.4	38.5 ± 6.4
K ⁺ (46 mM)	2.61 ± 0.29	38.1 ± 5.4	61.9 ± 5.4
FSK (50 μM)	2.82 ± 0.18	25.3 ± 4.0	74.7 ± 4.0
GH			
Basal	2.33 ± 0.12	39.7 ± 1.6	60.3 ± 1.6
TRH (2.5 μM)	2.47 ± 0.12	27.5 ± 1.0	72.4 ± 1.0
K ⁺ (46 mM)	2.34 ± 0.29	16.0 ± 3.1	83.1 ± 2.6
FSK (50 μM)	2.72 ± 0.19	16.9 ± 2.6	84.0 ± 3.1

^a Results are the mean ± SEM of five experiments performed in duplicate. GH₃S18.9 cells were washed with buffer I and then incubated for 2 h in buffer I in the presence or absence (basal) of secretagogues. Total hormone was calculated as the sum of the secreted and intracellular hormone extracted from the cells at the conclusion of the incubation. FSK, Forskolin.

intracellularly (Table 2). In contrast to those of GH, the storage and secretion of SRIF were significantly more regulated (Fig. 1B and Table 2). Only 16% of the total SRIF was constitutively secreted in the absence of secretagogues (Table 2). Incubation of cells with TRH increased SRIF secretion 2-fold, and in the presence of depolarizing concentrations of potassium or of forskolin, SRIF release was stimulated about 4- to 5-fold (Fig. 1B). As expected with enhanced SRIF secretion there was a decrease in intracellular storage (Table 2).

There was a small increase in total GH and SRIF levels in forskolin-treated cells; however, this did not result from enhanced hormone synthesis, since stimulated secretion was also seen in the presence of protein

synthesis inhibitors (Table 3). Furthermore, it would be surprising if SRIF synthesis, which is under the control of the murine leukemia 5' long terminal repeat viral promoter, would be affected by secretagogues. Most likely, the slight apparent increase in total hormone level in response to forskolin may have been due to enhanced stability of the secreted peptides compared to that of the intracellular molecules.

The rates of GH and SRIF biosynthesis were not measured directly in these studies; however, under basal conditions the cellular content was unaltered during 2 h of incubation (data not shown). Furthermore, the content measured under basal conditions was equal to the total hormone level in the presence of cycloheximide (Table 3). Hence, net production of GH (synthesis minus degradation) was reflected by the amount secreted. Over a 2-h incubation the secretion *i.e.* production, of GH was 3–4 times that of SRIF in clone 9 cells (Table 3). The cellular content of SRIF from cells incubated in the absence or presence of cycloheximide was similar (1.9 pmol/10⁶ cells compared to 1.5 pmol/10⁶ cells, respectively; Table 3). In contrast, the GH content was reduced from 0.93 to 0.24 pmol/10⁶ cells in the presence of cycloheximide, indicating that GH synthesis was approximately 3-fold greater than that of SRIF. To examine whether the increased rate of GH synthesis played a role in the distribution of GH and SRIF, basal and forskolin-stimulated release were examined in the presence and absence of cycloheximide (Table 3). Even though total GH was decreased 3-fold in the presence of cycloheximide, the percentages of total GH secreted basally and in response to forskolin were not significantly different. SRIF secretion in cycloheximide-treated cells was low in the absence of secretagogue, and consistent with the data in Fig. 1, its secretion responded to forskolin stimulation much more dramatically than did GH (Table 3). These data demonstrate that maintenance of the intracellular pool of GH was dependent on ongoing protein synthesis; in contrast, the intracellular SRIF pool was relatively independent of synthesis, *i.e.* it was stored. The results also show that even though GH synthesis is significantly more inhibited by cycloheximide than SRIF, the basic secretory physiology does not change, and therefore, our results cannot be explained by differences in the biosynthetic rates of the two hormones.

It is well documented that regulated secretion is dependent on calcium (see Ref. 2 for review). We, therefore, determined the effect of omission of extracellular calcium on the basal release of SRIF and GH from GH₃S18.9 cells. As shown in Table 4, omission of calcium inhibited the release of SRIF approximately 80%, while GH release was inhibited approximately 35%. These data indicate that basal SRIF release is substantially more dependent on extracellular calcium than is GH release.

It was possible that SRIF storage and secretion in GH₃S18.9 cells were atypical either because of the high level of its synthesis or as a result of a defect in the secretory pathway. To address these possibilities, we

Table 3. Effect of Cycloheximide on Secretion and Storage of SRIF and GH

	(pmol/10 ⁶ Cells)			
	Secreted	Content	Total	% Total secreted
SRIF				
Basal				
- CY	0.38 ± 0.07	1.9 ± 0.07	2.4 ± 0.21	17.5 ± 4.5
+ CY	0.13 ± 0.02	1.5 ± 0.19	1.8 ± 0.22	7.2 ± 0.9
FSK				
- CY	2.3 ± 0.29	0.87 ± 0.08	3.0 ± 0.46	70.4 ± 7.3
+ CY	1.7 ± 0.7	0.95 ± 0.29	2.6 ± 0.65	64.0 ± 2.4
GH				
Basal				
- CY	1.5 ± 0.08	0.93 ± 0.06	2.4 ± 0.27	61.6 ± 1.8
+ CY	0.55 ± 0.01	0.24 ± 0.04	0.8 ± 0.05	70.4 ± 3.5
FSK				
- CY	2.2 ± 0.24	0.42 ± 0.03	2.6 ± 0.54	83.6 ± 4.5
+ CY	1.2 ± 0.09	0.10 ± 0.001	1.3 ± 0.10	91.8 ± 0.7

GH₃S18.9 cells were pretreated for 15 min with or without 5 μg/ml cycloheximide and then incubated for 2 h in the absence or presence of cycloheximide. SRIF and GH levels were determined on the secreted and intracellular (content) materials. Total protein synthesis was inhibited more than 95% at this concentration of cycloheximide. FSK, Forskolin; CY, cycloheximide.

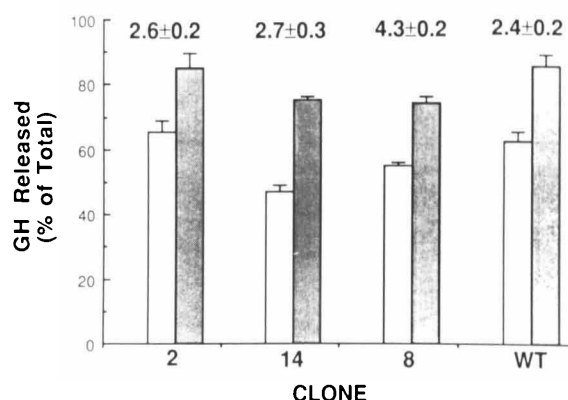
Table 4. Effect of Extracellular Calcium on the Release of SRIF and GH from GH₃S18.9 Cells

	+ Ca ²⁺	- Ca ²⁺	- Ca ²⁺ /+ Ca ²⁺ (%)
SRIF (pmol/10 ⁶ Cell)	0.416 ± 0.016	0.088 ± 0.003	21.1
GH (pmol/10 ⁶ Cell)	1.32 ± 0.098	0.852 ± 0.021	64.5

Growth medium was removed from multiple replicate plates of GH₃S18.9 cells and replaced with incubation medium (+ Ca²⁺) or incubation medium in which calcium was omitted and EGTA (1 mM) was added (- Ca²⁺). After 2 h of incubation at 37 C, the incubation medium was removed and assayed for SRIF and GH contents. Each data point represents the mean ± SEM of six replicates.

examined the relative secretion of SRIF and GH from several other GH₃ clones as well as untransformed wild-type cells (Figs. 2 and 3). Even though the three clonal lines exhibited a 4-fold difference in SRIF synthesis (Table 1), their secretory physiology was virtually identical. The basal level of GH secretion was high (50–65% of the total) and was stimulated approximately 20% by forskolin (Fig. 2). However, as observed for clone 9 cells, the basal release of SRIF was low (ranging from 4% in clone 8 to ~10% of the total for clone 18) and was stimulated about 6-fold by forskolin. Thus, although these clones expressed lower levels of SRIF than GH₃S18.9 cells, the response to secretagogues was identical, further demonstrating that the differential sorting of SRIF and GH was not a function of the level of hormone synthesis.

SRIF has a multitude of biological effects, one of which is to inhibit GH secretion from the pituitary. Earlier work had demonstrated that treatment of GH₃ cells with high concentrations of SRIF resulted in decreased GH secretion (20). Therefore, we might have expected that SRIF production by GH₃S18 cells would alter the release of GH from these cells; however, this did not seem to be the case (Fig. 2). A major effect of SRIF is to stimulate the G-protein, N_i, which inhibits adenylate cyclase. Although SRIF action is not totally explicable on the basis of adenylate cyclase inhibition (27, 28), its

**Fig. 2.** Secretion of GH from various GH₃S18 Clones

GH₃S18 clones 2, 14, and 8 and control wild-type GH₃ cells were incubated for 2 h in the presence (■) or absence (□) of forskolin (50 μM). The bars represent the percentage of total hormone released at 2 h. Total hormone was calculated as the sum of that released into the medium plus the residual hormone extracted from the cells at the conclusion of the incubation. The numbers at the top of each pair of bars represent the total hormone content of that particular clone. All values are the mean ± SEM for four replicates.

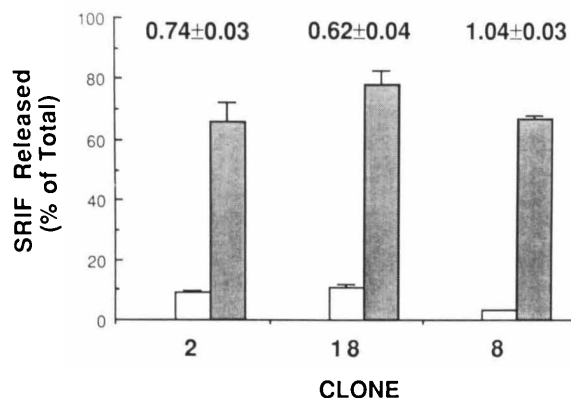


Fig. 3. Release of SRIF from Various GH₃S18 Clones

GH₃S18 clonal lines 2, 14, and 8 and control wild-type GH₃ cells were incubated for 2 h in the presence (■) or absence (□) of forskolin (50 μM). The bars represent the percentage of total hormone released at 2 h. Total hormone was calculated as described in Fig. 3. The numbers at the top of each pair of bars represent the total hormone value for that particular clone. All values are the mean ± SEM for four replicates.

action on adenylate cyclase has been noted in multiple target cells. SRIF added to control (wild-type) GH₃ cells or to GH₃ clone 9 cells, even at concentrations greater than those produced by the latter, did not affect the ability of forskolin to activate adenylate cyclase (data not shown). We suggest that these cells which have been maintained in tissue culture for long periods have decreased levels of SRIF receptor and, hence, are refractory to the action of SRIF.

DISCUSSION

A number of studies (16, 29–37) have demonstrated that heterologous secretory proteins can be expressed and processed in certain neuroendocrine cells, and in some cases secretion can be regulated. Our results indicate that the heterologously expressed hormone SRIF is preferentially targeted to the regulated secretory pathway in GH₃ cells compared to endogenous GH. This conclusion is based on the lower basal rate of SRIF secretion compared to GH, the greater dependence of SRIF release on extracellular calcium, and its enhanced stimulation upon treatment of GH₃ cells with a variety of secretagogues known to affect the regulated secretory pathway. In agreement with studies from other laboratories (37), we found no correlation between the biosynthetic rate of SRIF and its level of intracellular storage. However, this study and earlier data (16) revealed significant differences in both pro-SRIF processing and storage efficiency between GH₃ and the closely related GH₄C₁ cells (37). In contrast to GH₄ cells, which processed pro-SRIF rather inefficiently and stored less than 10% of SRIF-immunoreactive material (37), GH₃ cells cleaved 70–75% of pro-SRIF and stored nearly 80% of the total SRIF material. The

reasons for the differences in prohormone processing and sorting between these clonal cell lines are unclear at present. Our data are consistent with targeting of SRIF and GH to different secretory vesicles. However, it is quite possible that the smaller fraction of GH that is stimulated by various secretagogues is in the same granules as the fraction of SRIF whose secretion is regulated. Morphological, biochemical, and cell fractionation experiments are currently in progress to determine the relative distribution of the two hormones in separate populations of secretory vesicles.

Our initial hypothesis was that the endogenous hormone would have been either equally or more efficiently targeted to the regulated pathway than pro-SRIF. While previous studies (24, 38) indicate that GH secretion in GH₃ cells is much less regulated than in the normal pituitary, we speculated that this resulted from defects in the storage capacity of these cells. In fact, our results indicate the opposite and demonstrate that GH₃ cells have a high capacity for regulated secretion; hence, the difference from normal somatotrophs may relate to the molecular requirements for entering this pool. In this context, the preferential storage of SRIF in the regulated pathway was rather surprising given the observation that GH₃ cells lack detectable levels of the 25K hormone-binding proteins recently shown to be involved in mediating transport to secretory granules (5).

The current study expands previous work from one of our laboratories (16) which concluded that heterologously expressed SRIF in GH₃ cells is preferentially targeted to the regulated secretory pathway. In those studies, approximately 50% of the pulse-labeled mature SRIF was stored intracellularly in a cAMP-responsive compartment. In contrast, only 5–10% of the pulse-labeled endogenous GH was stored intracellularly, and most of it was secreted constitutively. It was possible that the differential storage of mature SRIF and GH could have been related to the relative pool sizes of these two molecules rather than to differences in the efficiency of intracellular storage. Thus, if, for example, the GH pool was significantly larger than SRIF or it was turning over rapidly (relative to SRIF), then it might be argued that the previous pulse-chase data were related to the differential kinetics of the pools rather than to preferential storage of mature SRIF. Furthermore, in the earlier studies (16) the levels of pulse-labeled GH and SRIF were measured using different techniques, which, although permitting comparison of their processing and secretion kinetics, precluded determination of the synthetic rates or intracellular content. To address these issues the current studies employed RIAs to measure the level of total SRIF-related and GH-immunoreactive molecules. Most significantly, the RIA data and those obtained from pulse-labeling experiments gave quite similar results and demonstrated that substantially more GH than SRIF was secreted constitutively (65% vs. 20%). The pulse-labeling experiments showed that of the newly synthesized hormones, approximately 90% of GH and 45% SRIF were secreted constitutively. The slight differences between these two

sets of data may be related to inherent discrepancies in the techniques for measuring these hormones (*i.e.* incomplete recovery of material, differential susceptibility of the hormones to proteolysis, and differences in the antibodies). Alternatively, they might be related to different kinetic pools of the hormones, *i.e.* it is possible that there is both a rapidly secreted pool and a long term storage pool, as suggested for other secretory proteins, such as in proinsulin and insulin (39). Similar to results on the release of newly synthesized insulin from pancreatic B cells (39), the majority of both the newly synthesized pulse-labeled SRIF and total SRIF-immunoreactive material was secreted via the regulated pathway. However, even if the constitutive secretion of pulse-labeled GH was explicable on the basis of a rapidly turning over pool, the data presented here demonstrate that the total endogenous GH (including a putative long term pool) is not stored as efficiently as the heterologously expressed SRIF. These data revealed that there was about a 4-fold variation in the level of SRIF expression in 15 clonal lines investigated. Most significantly, the intracellular storage of SRIF-immunoreactive material relative to that of GH was not correlated with the level of hormone expression. Thus, even in cells expressing one fourth the level of SRIF compared to GH, SRIF secretion was significantly enhanced by forskolin treatment, whereas the stimulation of GH was minimal. It will be important to determine the mechanism by which there is preferential targeting of SRIF to the regulated secretory pathway. Such a determination may provide new insight into the mechanism involved in prohormone processing and secretion.

MATERIALS AND METHODS

Materials

Rabbit anti-SRIF, suitable for use in RIAs, was a gift of Dr. Michael Berelowitz, State University of New York (Stony Brook, NY). TRH was obtained from Hoechst-Roussel Pharmaceuticals (Somerville, NJ). Forskolin, rhesus monkey serum, γ -globulin, and goat antiserum were purchased from Calbiochem (La Jolla, CA). [125 I]SRIF was purchased from New England Nuclear (Boston, MA); reagents for the GH assay were provided by the National Hormone and Pituitary Program (University of Maryland School of Medicine and sponsored by NIDDK). Rat GH (RP-2) was used as the reference standard.

Methods

Development of GH₃S18 Clonal Lines Clonal lines derived from rat pituitary GH₃ cells expressing prepro-SRIF were generated as previously described (16). Briefly, a 462-basepair *Bam*HI cDNA fragment encoding angler fish prepro-SRIF-I (M, 18,000) was inserted into the retroviral expression vector pLJ (16). The resulting plasmid DNAs were transfected into Psi-2 cells (21) to produce recombinant retrovirus stocks which were used to infect GH₃ cells. Virally infected cells were selected by their resistance to the neomycin analog G418 (1 mg/ml). Multiple clonal lines, designated GH₃S18 followed by the clone number, were obtained, and the results from several are reported here.

Culture of GH₃S18 Clonal Lines Wild-type GH₃ cells and the various GH₃S18 clonal lines were grown in Ham's F-10 medium supplemented with 15% defined equine serum-2.5% fetal bovine serum, as previously described (16). Cells were grown in T-25 flasks, fed twice weekly, and split 1:10. For experiments, multiple replicates of cells were seeded at a concentration of 1×10^6 on 60 \times 15-mm tissue culture dishes; after 3 days the medium was removed, and the cells washed with 4 ml PBS. Each plate was then incubated in 4 ml buffer I (118 mM NaCl, 4.6 mM KCl, 0.5 mM CaCl₂, 1 mM MgCl₂, 10 mM, dextrose, 5 mM HEPES, 0.1% NaHCO₃, and 0.1% BSA, pH 7.2) at 37 C in air and 5% CO₂ for the indicated times. To measure basal and regulated secretion, respectively, cells were incubated with either buffer I alone (basal) or with TRH (2.5 mM), forskolin (50 mM), or 46 mM K⁺. When cells were depolarized in 46 mM K⁺, the NaCl concentration was reduced to 76 mM.

During incubation the medium was serially sampled, at 15, 60, and 120 min, to measure the secretion of SRIF and GH. At the indicated times, the medium was removed, and either SRIF or GH was extracted from the cells. To extract SRIF, 1 ml ice-cold 0.1 N HCl was added to each plate; for GH, 1 ml 0.01 N NaOH was added. The cells were harvested with a rubber policeman, sonicated for 20 sec at 50 watts, and centrifuged at 1000 \times g for 2 min 4 C. The supernatant was lyophilized and reconstituted in RIA buffer. These extraction procedures resulted in quantitative recovery of the respective hormones. The concentrations of SRIF and GH in the incubation medium and cell lysates were determined using double antibody RIAs. For SRIF, the method of Berelowitz *et al.* (22) was employed. cAMP levels were measured by RIA (23) using antiserum generously provided by Dr. J. Vaitukaitis.

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