Retrovirus-mediated Expression of Preprosomatostatin: Posttranslational Processing, Intracellular Storage, and Secretion in GH_3 Pituitary Cells

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Abstract. Somatostatin (SRIF) is a 14-amino acid peptide hormone that is synthesized as part of a larger precursor, preproSRIF, consisting of a signal peptide and a proregion of 80–90 amino acids. The mature hormone, which is located at the carboxyl terminus of the precursor, is preceded by a single pair of basic amino acids. We are studying preproSRIF to investigate intracellular sorting, proteolytic processing, and storage of peptide hormone precursors in the secretory pathway. We used a retroviral expression vector to achieve the high levels of precursor synthesis which are necessary for detailed characterization of processing intermediates and mature somatostatin. Recombinant retroviruses containing RNA transcripts encoding anglerfish preproSRIF I were used to infect rat pituitary GH_3 cells which secrete growth hormone and prolactin, neither of which are substrates for endoproteolytic cleavage. In these cells preproSRIF was accurately processed to the mature hormone with an efficiency of ~75%. Of the newly synthesized mature SRIF, 55% was sorted into the regulated secretory pathway and released in response to the secretagogue 8-Br-cAMP. The remaining 45% of mature SRIF and residual unprocessed precursor was rapidly secreted. In contrast to SRIF, only 5% of newly synthesized endogenous growth hormone was stored intracellularly, whereas 95% was sorted to the constitutive pathway and secreted rapidly with kinetics identical to proSRIF. Our results show that proSRIF processing is not necessarily dependent on a specific protease found only in SRIF-producing cells and suggest that proteolytic cleavage is not restricted to cells that process endogenous hormones. Moreover, these results demonstrate that GH_3 cells have the capacity to discriminate between endogenous and foreign hormones and target the foreign molecule significantly more efficiently to the regulated secretory pathway.

Most small peptide hormones and neuropeptides are synthesized as part of larger inactive precursors (8) which undergo one or several posttranslational modifications including glycosylation, proteolysis, phosphorylation, amidation, and acetylation to generate a bioactive molecule (8, 21). These processing events occur in different organelles during intracellular transport and therefore peptide hormone precursors are useful models to study sorting through the secretory pathway. Several sorting and processing events occur in the distal elements of the Golgi apparatus/*trans*-Golgi network and in maturing secretory granules (13, 26–28, 34). In particular, endoproteolytic cleavage of peptide hormone precursors at paired basic residues is initiated in acidic, clathrin-coated vesicles which bud from the *trans*-Golgi network and the resulting mature hormone is stored in secretory granules (28, 34). At present, the molecular signals that target a polypeptide to the so-called "regulated" secretory pathway, the products of which are released in response to secretagogues, are poorly understood. Hormone-secreting cells also undergo basal or "constitutive" secretion, in which unprocessed precursors, nonhormone secretory proteins, and plasma membrane proteins are continuously delivered to the cell surface (3, 17); the evidence suggests that entry into the constitutive pathway occurs by default (17, 30). Consequently, a mechanism must exist which enables cells to discriminate between molecules destined for the regulated or constitutive pathways (17).

Our laboratory has been investigating the expression of peptide hormone precursors in foreign cells (12, 36) to (*a*) determine which secretory cells have prohormone cleavage enzymes and (*b*) identify structural domains within precursors which might function in targeting to the regulated secretory pathway. To address these questions, we are studying the biosynthesis and processing of prosomatostatin (proSRIF) as a model for intracellular sorting and prohormone process-

1. *Abbreviations used in this paper*: GH, growth hormone; SRIF, somatostatin.

ing. Somatostatin is a 14-amino acid peptide hormone which is synthesized as part of a larger precursor, preproSRIF. PreproSRIF is one of the simplest precursors (16, 35, 36) consisting of a 20–25-amino acid signal peptide followed by a proregion of 80–90 residues. The mature hormone is located at the carboxyl terminus of the propeptide and is preceded by a single pair of basic amino acids: ArgLys. Apart from endoproteolytic cleavage, the precursor undergoes no other posttranslational modifications (25).

Several investigators using gene transfer techniques have introduced foreign precursors into pituitary AtT-20 cells or pancreatic islet RIN cells, e.g., preproenkephalin (6, 33), preproinsulin (24), pretrypsinogen (4), preproSRIF (32), preprorenin (10), and preproneuropeptide Y (7). These precursors were proteolytically cleaved to the mature hormone and in some cases targeted to the regulated pathway, albeit with variable efficiency. In contrast, viral membrane glycoproteins and laminin were constitutively released (4, 11, 17). Expression of peptide hormone precursors in fibroblasts, which manifest only the constitutive pathway, results in little (36) or no proteolytic processing (15, 32, 33) with the concomitant secretion of unprocessed precursors. These observations imply that only cells which normally process their endogenous precursors have proteases capable of cleaving precursors to the mature hormone. However, for some cells such as the rat somatotroph line GH_4C_1 (which synthesizes growth hormone and prolactin) the data are contradictory. Hellerman et al. demonstrated correct processing of proparathyroid hormone (15) to the mature hormone, whereas neither proenkephalin (33) nor proSRIF (32) were processed in these cells or in a rat pheochromocytoma line, PC-12 (32).

To determine if endocrine cells which synthesize polypeptide hormones that are not cleaved have prohormone-converting enzymes, we exploited the rat anterior pituitary line GH_3 (2). Our choice was predicated on several observations: (*a*) GH_3 cells synthesize high levels of growth hormone and prolactin, both of which contain several sets of paired basic amino acids, however these hormones are not substrates for endoproteolytic processing; (*b*) synthesis and secretion of the endogenous hormones can be modulated by other hormones and ligands such as glucocorticoids, thyrotropin releasing hormone, and forskolin; and (*c*) most importantly previous studies from this laboratory (11) demonstrated that GH_3 cells efficiently discriminate in sorting between membrane glycoproteins and endogenous growth hormone, probably in the distal elements of the Golgi apparatus.

In earlier studies (36), low levels of prohormone synthesis, resulting from transient gene expression, precluded detailed characterization and analysis of processing intermediates and the mature peptides. To circumvent this problem, we used a recombinant retrovirus expression vector pLJ (18), which contains the highly efficient murine leukemia virus promoter and a selectable marker, to generate clonal lines of GH_3 cells expressing high levels of preproSRIF. Here we show that GH_3 cells accurately and efficiently process proSRIF to the mature hormone. Approximately 75% of the intracellular precursor is processed to mature SRIF and at least half of this material is targeted to the regulated pathway. In contrast, 95% of newly synthesized endogenous growth hormone was delivered to the constitutive pathway. We conclude that GH_3 cells discriminate between and store a foreign protein more efficiently than an endogenous hormone.

Materials and Methods

Materials

Psi-2 cells (22) and the plasmid pLJ were a gift from Dr. Richard Mulligan, The Whitehead Institute, Boston, MA. Rabbit anti-SRIF serum, designated RSS-1, was prepared in this laboratory as previously described (35). Baboon anti-growth hormone serum was a generous gift from Dr. Carter Bancroft, Mount Sinai Medical Center, NY. [^{35}S]Methionine and [^{35}S]cysteine were purchased at the highest available specific activity from Amersham Corp., Arlington Heights, IL.

Methods

Cell Culture. Cells were grown at 37°C in an atmosphere of 7.5% CO_2. GH_3 cells were grown in Ham's F10 medium (3 g/ml $NaHCO_3$) supplemented with 15% equine serum, 2.5% FBS, 2 mM glutamine, 25 U/ml penicillin, and 25 μg/ml streptomycin. NIH 3T3 and Psi-2 cells were grown in DME supplemented with 10% FBS, 2 mM glutamine, 25 U/ml penicillin, and 25 μg/ml streptomycin.

Production of Recombinant Retrovirus Expressing preproSRIF. A 462-bp cDNA fragment encoding preproSRIF was ligated into the Bam HI site of the retroviral expression vector, pLJ (see Fig. 1), and the resulting plasmid DNAs amplified in *Escherichia coli* strain C600 grown in the presence of 40 μg/ml of kanamycin. Infectious virus particles containing preproSRIF RNA transcripts were generated by transfecting Psi-2 cells (22) with plasmid DNA according to the Polybrene-DMSO method (5). 48 h after the DMSO treatment, 1 mg/ml of the neomycin analogue G418 was added to the culture medium. The medium containing G418 was changed after 5 d and after a total of 10 d growth in G418, resistant cells (100–1,000 colonies/dish) were trypsinized and recultured until 80% confluent. Virus titer ranged from 10^3 to 10^5 infectious U/ml.

Infection of Target GH3 Cells. Medium from semi-confluent G418 resistant Psi-2 cells was filtered through a 0.45-μm filter. A 60-mm dish containing 10^6 GH_3 cells was incubated for 2 h with 1 ml of the filtered Psi-2 cell medium containing 10 μg of polybrene. 4 ml of complete Ham's F10 was added and the medium changed after 24 h. 48-h postinfection, the medium was replaced with 5 ml of complete F10 containing 1 mg/ml G418. This was changed after 5 d with fresh medium containing G418. 10-d postinfection, G418-resistant cells were trypsinized, reseeded, and grown to 80% confluency. Single G418-resistant cells were subcultured by limiting dilution in a 96-well plate and 10–20 clonal lines were grown to mass culture. The steady state levels of intracellular and secreted SRIF was determined by radioimmunoassay.

Biosynthetic Labeling of Cells. 60-mm dishes were seeded with 2×10^6 cells, 48–96 h later the cells were washed twice with 2 ml of PBS and pulse labeled for the indicated times with 1 ml of labeling medium supplemented with 2 mM glutamine, 250 μCi/ml of [^{35}S]cysteine or [^{35}S]methionine in the absence or presence of 2.5% heat-inactivated FBS and heat-inactivated equine serum. Labeling medium was prepared from a RPMI-1640 SelectAmine Kit (Gibco Laboratories, Grand Island, NY) according to manufacturer's instructions. Cysteine or methionine was omitted from labeling medium containing [^{35}S]cysteine or [^{35}S]methionine, respectively. For the chase incubations, cells were washed twice with 2 ml of PBS and 1 ml of chase medium containing complete Ham's F10 (containing a full complement of unlabeled amino acids) was added. In some experiments, the chase medium contained 10 μl/ml of specified antiserum.

After the labeling and chase periods, the medium was removed, centrifuged for 10 s in a microcentrifuge (model 5412; Brinkman Instruments Co., Westbury, NY), transferred to a fresh tube, and stored on ice or at −20°C until treated with antiserum. Cells were washed with PBS, and harvested by scraping with a rubber policeman into 1 ml of PBS. The cell suspension was centrifuged for 10 s in a microcentrifuge, the pellet of cells was lysed by vortexing, 10 times 1 s each, in 100 μl of lysis buffer (0.5% NP-40, 0.5% Na desoxycholate in PBS) containing a cocktail of protease inhibitors (11). Membranes and nuclei were removed by centrifugation at 4°C for 5 min in a microcentrifuge. The postnuclear supernatants were stored on ice or at −20°C until treated with antiserum.

Immunoprecipitation. To determine the intracellular levels of SRIF- and growth hormone-related products, postnuclear supernatants were adjusted to 1% SDS and incubated at room temperature for 10 min. 10 vol of buffer A (190 mM NaCl, 50 mM Tris-HCl pH 8.3, 6 mM EDTA, 2.5% Triton X-100, 100 U/ml trasylol, 5 mM cysteine or methionine) were added followed by addition of 10 μl of appropriate antiserum. Samples were incubated at 4°C for 12–24 h with constant mixing. To assay for secreted polypeptides, medium was adjusted to buffer A conditions by addition of

one-third volume of a 4× buffer A solution followed by addition of 10 μl of appropriate antiserum (if not added to the labeling or chase medium). Samples were incubated with constant mixing at 4°C for 12–24 h.

Samples were then incubated with 75 μl of a 1:3 (vol/vol) slurry of protein A–Sepharose at 4°C for 3 h. Immune complexes were isolated by centrifugation, washed twice with 750 μl of buffer B (150 mM NaCl, 10 mM Tris-HCl pH 8.3, 5 mM EDTA, 0.1% Triton X-100, 100 U/ml trasylol, 5 mM cysteine or methionine, 1 mg/ml BSA), twice with 750 μl of PBS, and used immediately or stored at −20°C until analyzed by HPLC or SDS-PAGE. For consecutive immunoprecipitations, 5 μl of the second antiserum was added to the postnuclear supernatants and medium after removal of the initial immune complexes.

It should be noted that the recovery of immunoreactive proSRIF and mature SRIF from cell lysates was less efficient than from the medium. Whereas 80% of the total pulse-labeled mature SRIF and proSRIF was recovered from the medium, 60% of mature SRIF and 40% of proSRIF, respectively, were recovered from the cell lysates. This was determined by three sequential precipitations with anti-SRIF antisera; these numbers were used in generating the data for Fig. 6.

Characterization of Expressed Proteins. To analyze SRIF-immunoreactive polypeptides, HPLC methods were used. The protein A–Sepharose beads from the immunoprecipitation step (above) were incubated for 30 min at 50°C with 50 μl of TEU buffer (8 M Urea, 500 mM Tris pH 8.8, 20 mM EDTA), containing 100 mM dithiothreitol and 2 μg of native SRIF (as an internal standard to monitor retention time). 50 μl of 0.66 M iodoacetic acid in TEU was added to the beads and incubated in the dark at room temperature for 10 min, samples were centrifuged, and the supernatant saved. The pellet was washed twice with 50 μl of TEU, all supernatants were combined and 20 μl of 80% CH_3CN, 1% trifluoracetic acid (TFA) added. Samples were analyzed using an HPLC system (model 6000A solvent delivery system; Waters Instruments, Inc., Milford, MA) as previously described (35). The combined supernatants were resolved on a Vydac C_{18} reverse phase column (The Separations Group, Hesperia, CA) using one of following gradient systems. Gradient 1: 0–10 min 25% CH_3CN, 10–35 min 25–50% CH_3CN. Gradient 2: 0–5 min 5% CH_3CN, 5–6 min 5–30% CH_3CN, 6–21 min 30–45% CH_3CN, 21–25 min 45% CH_3CN, 25–26 min 45–80% CH_3CN, 26–35 min 80% CH_3CN. All solutions contained 0.1% TFA. The flow rate was 1.5 ml/min and 1-minute fractions were collected and the radioactivity determined using a liquid scintillation counter (model LS-8100; Beckman Instruments, Inc., Palo Alto, CA). 70–90% of the applied radioactivity was recovered from the column.

Automated Microsequencing. Automated Edman degradation was performed using a Beckman 890C Spinning Cup sequencer (Beckman Instruments, Inc.) as previously described (35). Repetitive yields were routinely 92–93% and the chemical yields were 20–25%.

Results

Characterization of GH3S18 Clonal Lines

To obtain a high level of preproSRIF expression in GH_3 cells, we used the retroviral expression vector pLJ. Plasmid pLJ contains the Moloney murine leukemia virus provirus. The 5′ long terminal repeat (LTR) in the provirus drives transcription of inserted sequences and the SV-40 early promoter in the ORI region drives expression of the neomycin-resistance gene for selection on G418. A 462-bp Bam HI cDNA fragment encoding anglerfish preproSRIF I was ligated into the Bam HI site in pLJ, this plasmid was designated pLJS18 (Fig. 1). Psi-2 cells were transfected with pLJS18 DNA and recombinant retrovirus was obtained (see Materials and Methods); this was used to infect the target GH_3 cells. 15 G418-resistant GH_3 clonal lines were selected and the media and cell lysates assayed for SRIF-immunoreactive material using a radioimmunoassay which showed that the level of SRIF varied over a fourfold range (data not shown).

Identity of Expressed Products

To characterize the SRIF-immunoreactive material in these cells, four GH_3S18 clonal lines and four lines of identically infected XG10 cells (clonal variants of GH_3 cells, kindly provided by Dr. R. Vandlen, Genentech Inc., San Francisco, CA) were pulse labeled for 90 min with [^{35}S]cysteine. There are two cysteine residues in proSRIF I which are retained in the mature hormone, i.e., residues 3 and 14, respectively. After labeling, the cells were lysed and treated with anti-SRIF antiserum and the SRIF-immunoprecipitated polypeptides were resolved by SDS-PAGE (Fig. 2). A specific SRIF-related polypeptide of M_r 17,000 (Fig. 2, *upper asterisk*) which comigrated with in vitro synthesized proSRIF (lane *2*, *arrow*) was present in the lysates from all infected GH_3 and XG10 cells (lanes *4–7* and *9–12*, respectively); this polypeptide was absent from uninfected cells (lanes *3* and *8*). These results indicated that all the GH_3 and XG10 clones express preproSRIF cDNA and cleave the nascent precursor to proSRIF. In addition, they synthesized variable levels of a low

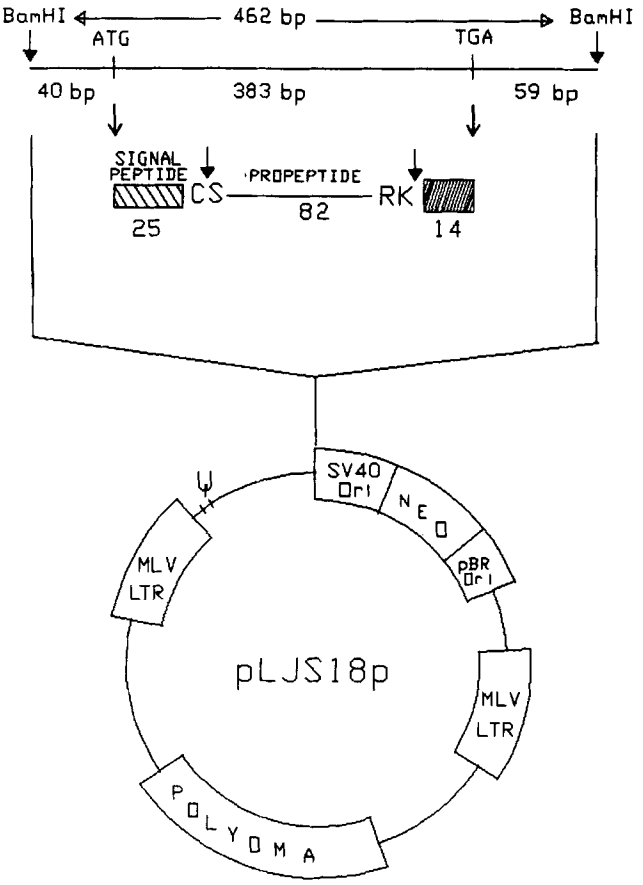

Figure 1. Construction of pLJS18: a retroviral vector encoding preproSRIF. A 462-bp Bam HI cDNA fragment encoding anglerfish preproSRIF I was excised from either plasmid pLaS$_1$, (16) or pSVppS18 (36), and ligated into the unique Bam HI site of pLJ; the resulting plasmid was designated pLJS18. The transcription initiation and termination sites for DNA ligated into this site are contained in the murine leukemia virus (MLV) long terminal repeats (*LTR*). Expression of the neomycin gene for resistance to G418 is initiated from the SV-40 early promoter in the Ori region. The organization of preproSRIF polypeptide is shown; the site of signal peptide cleavage between Cys(*C*) and Ser(*S*) (*arrow*) and the position of the only set of paired-basic residues ArgLys (*RK*) are indicated. The arrowhead indicates the start of mature SRIF-14, located at the carboxyl terminus of the molecule (*shaded area*).

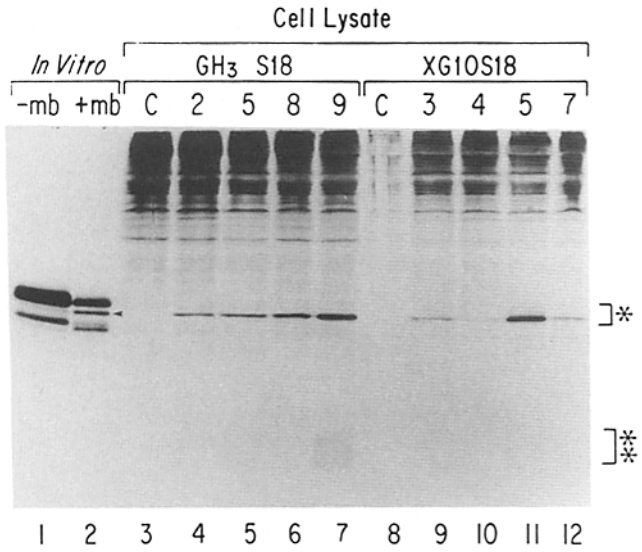

Figure 2. Characterization of clonal GH_3S18 and XG10S18 cell lines. Four GH_3S18 and XG10S18 clonal lines were pulse labeled for 90 min with [^{35}S]cysteine and the SRIF-immunoprecipitated polypeptides resolved upon 18% polyacrylamide SDS gels. Lanes *1* and *2*, in vitro translation products of anglerfish islet mRNA translated in the absence (lane *1*) or presence of microsomal membranes (lane 2). After incubation, the translation products were immunoprecipitated with anti-SRIF serum. Lane *1*, upper and lower bands correspond to preproSRIF I (M_r 18,000) and preproSRIF II (M_r 16,000) (35); Lane *2*, the arrowhead indicates the mobility of proSRIF I (M_r 17,000), the lower band corresponds to proSRIF II. Lanes *3* and *8*, SRIF-immunoprecipitable products from uninfected GH_3S18 and XG10S18 cells, respectively. Lanes *4–7*, SRIF-immunoreactive material from GH_3S18 clones 2, 5, 8, and 9, respectively. Lanes *9–12*, SRIF-immunoreactive material from XG10S18 clones 3, 4, 5, and 7, respectively. The single asterisk indicates the position of proSRIF I (M_r 17,000) present in lysates from all infected cell lines. Note that this polypeptide comigrates with proSRIF I synthesized in vitro in the presence of microsomal membranes (lane *2*). The double asterisk indicates the migration of mature SRIF.

molecular mass SRIF-immunoreactive polypeptide that was poorly resolved by SDS-PAGE (Fig. 2, *double asterisks*). We suspected that this material corresponded to mature SRIF, which was not well resolved by SDS-PAGE because of its small size (14 amino acids).

To identify this material, SRIF-immunoprecipitated products from $GH_3S18.9$ cell lysates were analyzed by HPLC using a C_{18} reverse-phase column (Fig. 3 *A*). Two peaks of SRIF-immunoreactive material, retention times 14 and 29 min, respectively, were resolved. The first peak exactly coeluted with native reduced and carboxymethylated SRIF suggesting it was the mature 14-amino acid hormone. The second peak, retention time of 29 min, was identified as proSRIF since it comigrated with in vitro synthesized proSRIF upon SDS-PAGE (data not shown). The specificity of these peaks was demonstrated by analyzing an equivalent cell lysate immunoprecipitated in the presence of nonradioactive native SRIF (Fig. 3 *A*, *open boxes*). To prove that the material eluting at 14 min was mature SRIF, it was subjected to microsequencing (Fig. 3 *B*). The amino acid sequence of native SRIF has cysteine residues in positions three and fourteen, therefore [^{35}S]cysteine radioactivity should be recovered in cycles 3 and 14. The data showed that this was the case and demonstrated that GH_3 cells accurately cleaved proSRIF to the mature peptide.

Kinetics and Efficiency of Processing and Secretion

$GH_3S18.9$ cells were pulse labeled with [^{35}S]cysteine for 15 min and chased for up to 6 h. At each time point, intracellular and secreted SRIF-immunoreactive products were resolved by HPLC (Fig. 4). With increasing duration of chase, the level of intracellular proSRIF decreased with a concomitant increase in mature SRIF. After ~30 min of chase, mature SRIF appeared in the medium and reached a maximum between 90 and 120 min. Since GH is endogenously synthesized and secreted in these cells, samples from the same experiment were treated with anti-GH antiserum, after treatment with SRIF antiserum, and the immune complexes resolved by SDS-PAGE (Fig. 5). The total radioactivity present in proSRIF and mature SRIF was calculated and the radioactivity in the intracellular and secreted GH polypeptides determined by liquid scintillation counting. The relative efficiency of processing and the secretion kinetics of these molecules was then determined (Fig. 6). As previously observed (2, 11, 31), intracellular GH disappeared rapidly, $t_{1/2}$ ~20 min, from the cells and was secreted into the medium (Fig. 6, *A* and *B*). Virtually all of the initially pulse-labeled GH could be accounted for in the subsequent chase periods. After a 360-min chase, only 5% of the GH was intracellular whereas 95% was recovered in the medium. Intracellular proSRIF disappeared with a half-life of ~20 min, ~25% of the initially synthesized proSRIF was secreted (unprocessed) with kinetics almost identical to GH (Fig. 6 *D*). All but 5–10% of the residual proSRIF was proteolytically processed to mature SRIF, of which 45% was also secreted constitutively with kinetics similar to GH. Most significantly, and in contrast to GH, the remaining 55% of mature SRIF was stored intracellularly with a half-life >6 h (Fig. 6 *C*).

Characterization of the Intracellular Pool of SRIF and GH

We hypothesized that the intracellular pool of stored SRIF was targeted to the regulated secretory pathway. If this were correct, it should be released upon stimulation by secretagogues (17). Cells were pulse labeled with [^{35}S]cysteine for 15 min and chased for 120 min. After this initial chase, the medium was removed and replaced with fresh medium or medium containing 5 mM 8-Br-cAMP and the cells incubated for an additional 120 min. The cells and medium from each time point were then treated with anti-SRIF antiserum and the products analyzed by HPLC (Fig. 7). A similar experiment was performed to characterize the intracellular GH pool; $GH_3S18.9$ cells were pulse labeled with [^{35}S]cysteine for 15 min and chased for 90 min; the medium was removed and replaced with either medium alone or medium containing 5 mM 8-Br-cAMP and the cells incubated for an additional 60 min. The cells and medium from each time point were treated with anti-GH antiserum and the products analyzed by SDS-PAGE (Fig. 7). During the second 120-min chase period in the absence of 8-Br-cAMP, ~15% of the stored mature SRIF was secreted. However, in the presence of 8-Br-cAMP, 88% of SRIF-14 was

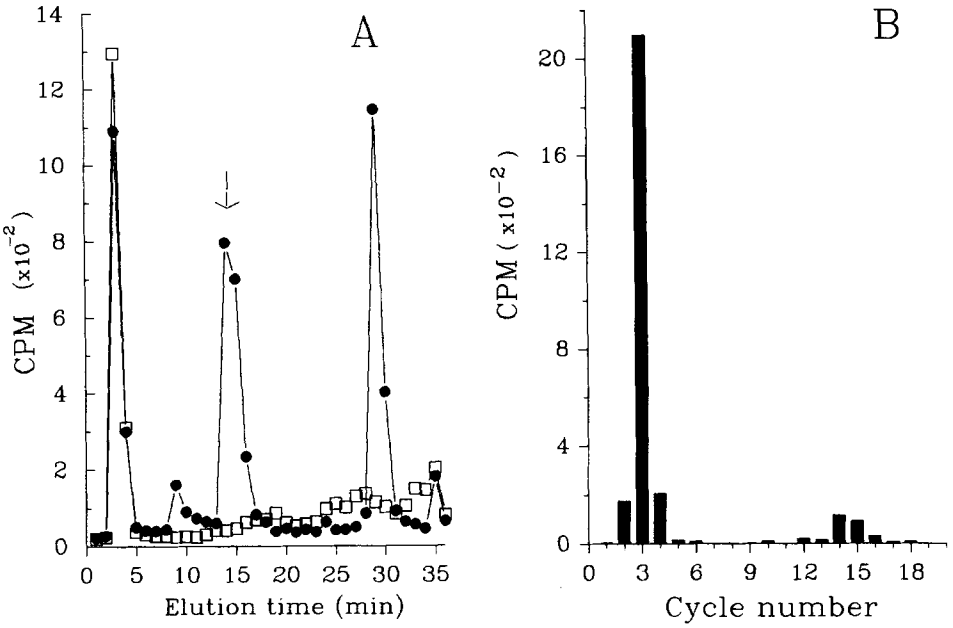

Figure 3. Characterization of intracellular SRIF-immunoreactive material from GH_3S18 cell lines. (*A*) HPLC analysis. Cells were pulse labeled with [^{35}S]cysteine for 90 min and the lysate treated with anti-SRIF antisera in the absence (*solid circles*) or presence of 10 μg nonradioactive SRIF (*open boxes*), followed by treatment with protein A-Sepharose. The immune complexes were dissociated using a urea buffer (Materials and Methods) and resolved on a Vydac C_{18} reverse phase HPLC column using gradient system I (Materials and Methods). The radioactivity in each fraction was determined by liquid scintillation counting. The arrow indicates the elution position of native reduced and carboxymethylated SRIF-14 (retention time 14 min). (*B*) Partial NH_2-terminal sequencing. [^{35}S]Cysteine-labeled material coeluting from the HPLC column with mature SRIF in *A* was applied directly to a spinning cup sequencer and subjected to 19 cycles of automated Edman degradation and the radioactivity in each cycle determined.

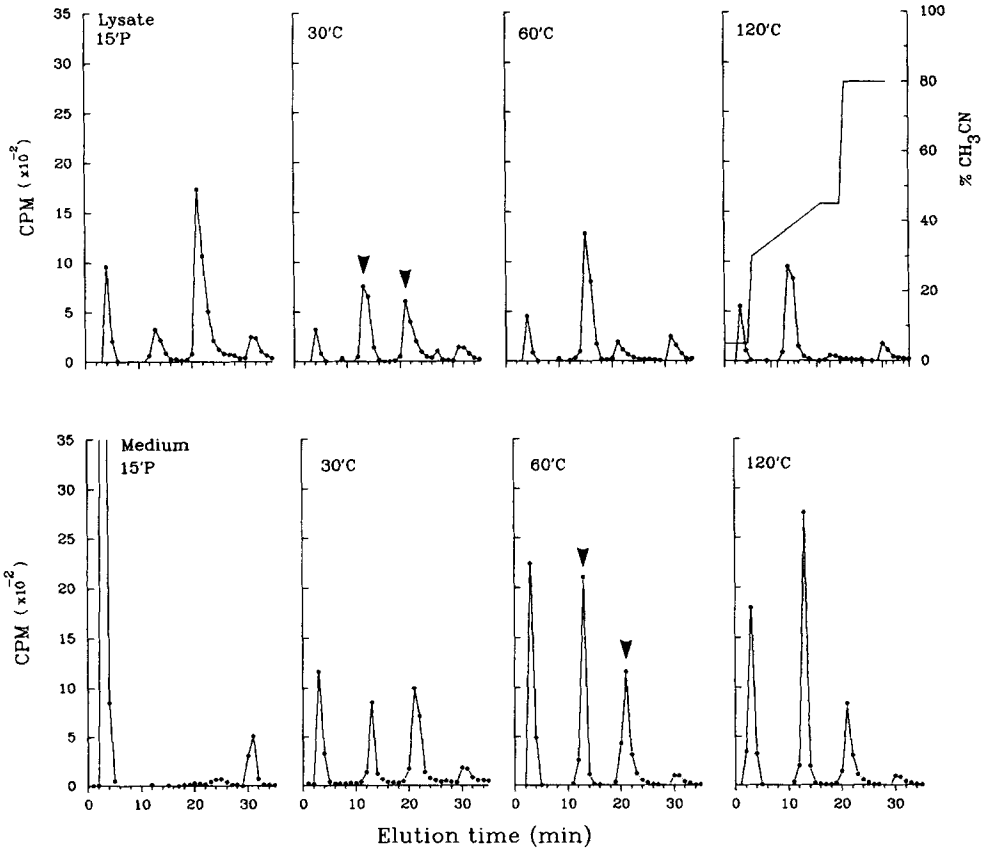

Figure 4. HPLC analysis of intracellular and secreted SRIF-immunoreactive material from $GH_3S18.9$ cells. Cells were pulse-labeled (*P*) with [^{35}S]-cysteine for 15 min (*P*) and chased (*C*) for the indicated times. At each time point the cells were lysed and the medium collected; the cell lysate and medium were treated with anti-SRIF antiserum and the SRIF-immunoreactive material was resolved by HPLC using gradient system 2 (Materials and Methods). The radioactivity in each fraction was determined by liquid scintillation counting; (*Top*) Cell lysate; (*bottom*) secreted material. The CH_3CN gradient is shown in the upper right panel. Arrowheads indicate the elution position of mature SRIF and proSRIF, respectively.

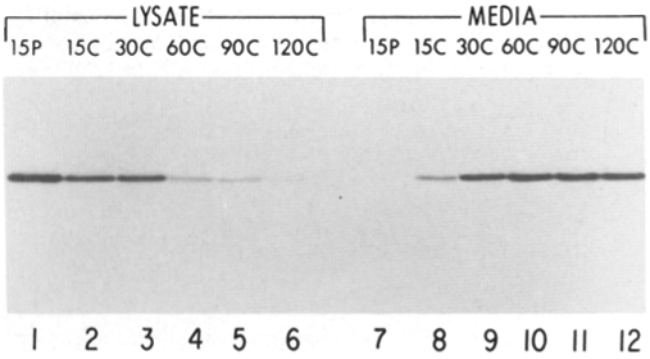

Figure 5. Synthesis and secretion of GH in $GH_3S18.9$ cells. Samples were obtained from the pulse-chase experiment shown in Fig. 4. After treatment with SRIF antiserum and protein A–Sepharose, the supernatants were incubated with 5 μl anti–GH antiserum and the immunoprecipitable material resolved by SDS-PAGE. An autoradiogram of the gel is shown. The kinetics of GH secretion were identical in uninfected GH_3 cells (not shown). Lanes *1–6*, intracellular GH; lanes *7–12*, secreted GH. *P* indicates the 15-min pulse and *C* corresponds to the indicated chase times.

secreted into the medium. The near complete release of stored SRIF by the secretagogue, 8-Br-cAMP, demonstrated that these molecules were targeted to the regulated pathway. Although secretion of the residual GH was similarly stimulated by 8-Br-cAMP (Fig. 7), it should be noted that this represents only 5–10% of the initially synthesized GH pool. In contrast, the corresponding SRIF pool was 55% of total mature hormone. These results demonstrated not only that GH_3 cells have a regulated secretory pathway but that they also possess an efficient mechanism for selectively sorting SRIF from GH.

Discussion

We have previously described (36) the expression of prepro-SRIF in monkey kidney cells (COS 7) and demonstrated correct, although inefficient, cleavage of proSRIF and correspondingly low levels of mature hormone secretion. Similarly, Sevarino et al. (32) recently showed that rat proSRIF was also inefficiently processed in 3T3 cells. In agreement with these reports, we observed no proSRIF cleavage in 3T3 cells (data not shown). Analysis of processing efficiency, storage, and secretion requires gene expression at levels sufficiently high to facilitate detailed biochemical characterization of the unprocessed and mature molecules. To this end, we have used a recombinant retroviral expression vector, pLJ (18), which allows cDNA integration into the host cell genome; transcription of the cDNA is driven by the highly active promoter within the Moloney murine leukemia virus LTR. Using this system, we expressed preproSRIF in GH_3 cells at levels approaching those of the endogenous growth hormone as determined by radioimmunoassay (Fleischer, N.,

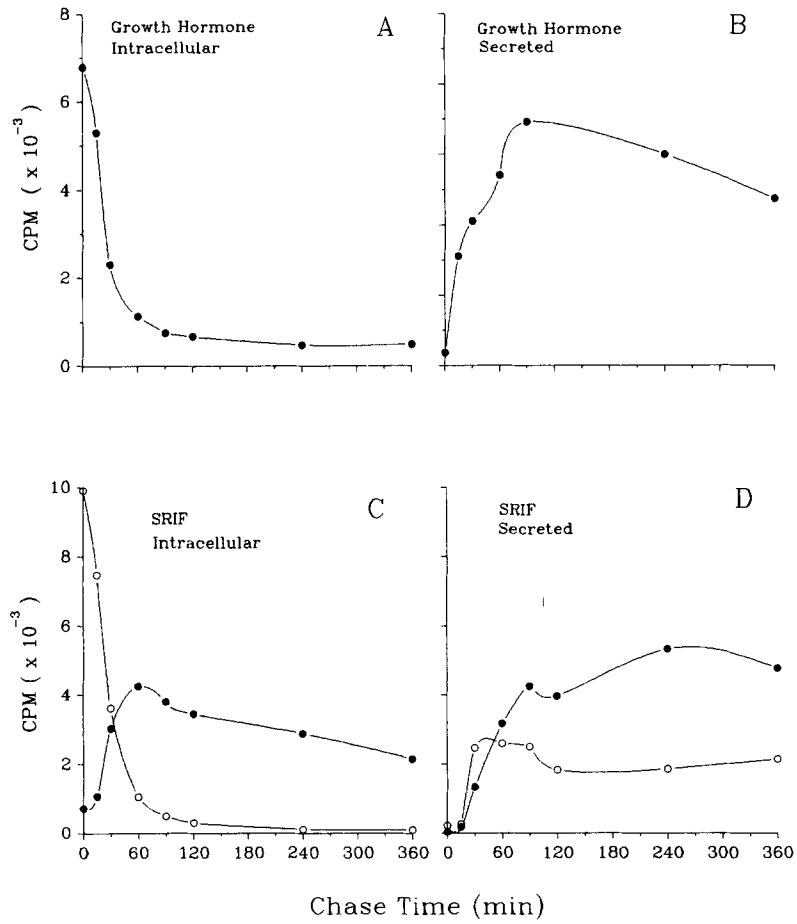

Figure 6. Comparison of the processing and secretion kinetics of proSRIF, SRIF, and GH in $GH_3S18.9$ cells. The bands, corresponding to GH-specific polypeptides from a similar experiment to that shown in Fig. 5, were excised and the radioactivity in the solubilized gel pieces determined. (*A*) Radioactivity in intracellular GH; (*B*) radioactivity in secreted GH. Cells were pulse labeled with [^{35}S]cysteine for 15 min and chased for up to 360 min; at each time point the cell lysate and medium were treated with anti-SRIF antiserum, the immunoreactive material resolved by HPLC, and the radioactivity in the appropriate peaks determined. (*C*) Radioactivity in intracellular mature SRIF (*solid circles*) and proSRIF (*open circles*). (*D*) Radioactivity in secreted mature SRIF (*solid circles*) and proSRIF (*open circles*). Note that the data were corrected for differential recovery of pro-SRIF and mature SRIF (Materials and Methods).

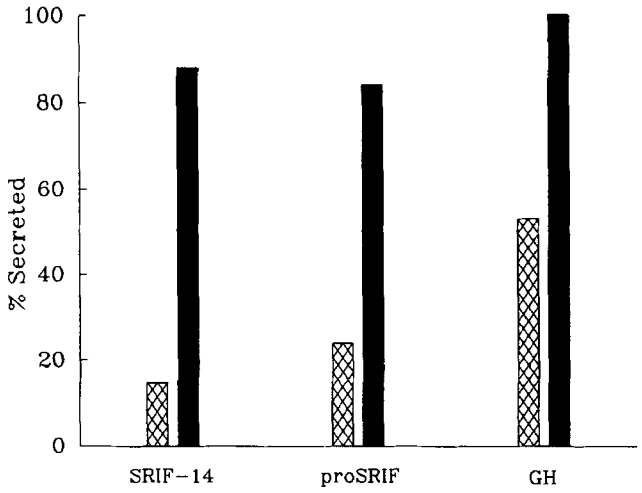

Figure 7. Secretagogue-mediated secretion of intracellular SRIF and GH. $GH_3S18.9$ cells were pulse labeled for 15 min with [^{35}S]cysteine and chased for 120 (SRIF) or 90 min (GH). The medium was removed and the cells incubated for an additional 120 (SRIF) or 60 min (GH) in the absence or presence of 5 mM 8-Br-cAMP. The cells and medium from each time point were treated with anti-SRIF antisera or with anti-GH antiserum. The immunoprecipitates were analyzed by HPLC (SRIF-immunoreactive polypeptides) or SDS-PAGE (GH-related products). The total radioactivity in the SRIF-related peptides was determined by integration of the appropriate HPLC peaks (Fig. 4) and radioactivity in GH polypeptides determined by liquid scintillation counting (Fig. 5). Bars represent the percent of intracellular material secreted into the medium in the absence (*crosshatched bars*) or presence of 8-Br-cAMP (*solid bars*); the results are averaged from five experiments. 100%, which corresponds to the level of intracellular material before 8-Br-cAMP treatment was: 3,000 cpm (SRIF-14), 300 cpm (proSRIF), and 450 cpm (GH).

T. Stoller, H. Liker, and D. Shields, manuscript in preparation) and established clonal lines synthesizing different levels of the precursor. Our data show that GH_3 cells efficiently (75%) and accurately proteolytically process proSRIF exclusively to mature SRIF-14. Using a variety of different HPLC elution conditions (e.g., in which SRIF-14 elutes 3 min later than SRIF-28) there was no evidence of processing to SRIF-28, an NH_2-terminally extended form of the hormone. Thus, efficient proteolytic cleavage is not restricted to cells that process their endogenous hormones nor dependent on a specific protease found only in SRIF-producing cells. It is noteworthy that proSRIF processing was not related to the efficiency of intracellular storage, since ~45% of mature SRIF was secreted constitutively. This observation supports the hypothesis that processing per se does not mediate targeting to the regulated pathway (17).

It has been reported that only certain endocrine cells cleave polyprotein precursors efficiently (6, 9, 24, 32). Surprisingly, rat proSRIF, which was processed in RIN 5F cells (islet) and AtT-20 cells (pituitary), was not cleaved in GH_4C_1 (pituitary) or PC-12 (adrenal medulla) cells (32); this was unexpected for several reasons. Firstly, Hellerman et al. (15) showed that proPTH (proparathyroid hormone) is accurately cleaved to PTH (at LysLysArg) in GH_4C_1 cells. Secondly, GH_4C_1 cells are clonal derivatives of the rat GH_3 line, which we demonstrate here cleaves proSRIF accurately and efficiently. Thirdly, Low et al. (19) showed in transgenic mice containing the rat preproSRIF gene, that proSRIF was expressed and processed to mature SRIF in pituitary gonadotrophs, thus demonstrating that cells which do not normally synthesize proSRIF can process at paired basic residues. Furthermore, since AtT-20 cells cleave several different heterologous prohormones (17), it seems quite unlikely that cell-specific enzymes are required for processing. Our results thus contrast with those of Sevarino et al. (32) who suggested that proSRIF processing requires specific pathways present in only some neuroendocrine cells. The reasons for this discrepancy are unclear, but could be related to poor recovery of mature SRIF. Somatostatin is susceptible to nonspecific proteolysis and it is possible that some cells secrete high levels of proteases, leading to low recoveries of the mature hormone. Alternatively, GH_4C_1 cells might lack or not express the gene encoding the processing enzyme; we are currently investigating this possibility.

The efficient processing of proSRIF in GH_3 cells was particularly intriguing since this precursor has an atypical cleavage site, ArgLys. This combination of paired basic residues (on the NH_2-terminal side of the hormone) is found in <10% of prohormones. Nevertheless, cleavage at the lysine residue was specific and no evidence for miscleavage or for cleavage at the preceding arginine was observed. Our data shows that cleavage specificity is not determined exclusively by the nature of the basic amino acids present at the processing site and implies that the conformation of this region may be important for defining specificity.

Endocrine cells respond to secretagogues by rapidly releasing stored hormone; storage requires efficient sorting to the regulated secretory pathway. Our results show that 55% of newly synthesized mature SRIF was sorted into the regulated pathway and virtually all this material was secretagogue sensitive. The efficient storage of SRIF is somewhat unusual, since for example, pulse labeling of AtT-20 cells expressing GH results in only ~10% of the newly synthesized GH entering the regulated pathway. However since the half-life of material in the regulated pathway is ~10 times greater than in the constitutive pathway, the fraction of unlabeled hormone in storage granules is ~80% (Kelly, R., personal communication). In contrast to SRIF, only 5% of newly synthesized GH was stored in regulated vesicles. It might be argued that since ~25% of pulse-labeled mature SRIF remained in the cells after 6 h of chase that 25% of radiolabeled GH was also present but was not extracted. However, this was not the case, since comparison of several other extraction conditions, including the use of alkaline and acidic buffers, did not enhance total GH recovery (Fleischer, N., T. J. Stoller, H. Liker, and D. Shields, manuscript in preparation). Our results thus suggest that SRIF is stored significantly more efficiently than GH. The constitutive secretion of GH, yet storage of the foreign peptide SRIF, was unexpected since although GH secretion is highly regulated in situ, in GH_3 cells it is rapidly secreted (1, 11, 31). A possible explanation is that GH produced by GH_3 cells has mutated and lacks a functional "sorting signal." Although we cannot exclude this possibility, it is unlikely since GH synthesized by these cells was recognized by anti-GH antiserum, has the same molecular weight, isoelectric point, amino-terminal sequence, and biological activity as authentic rat GH (2).

An alternative hypothesis is that our tissue culture medium lacks or is rate limiting for a component which is required for GH packaging and storage, but not for SRIF. In this context, Scammell et al. (31) showed that treating GH_4C_1 cells with a combination of estradiol, insulin, and epidermal growth factor increased the number and size of mature granules, resulting in a fivefold enhancement of stored endogenously synthesized prolactin. A characteristic feature of mature secretory granules is an electron opaque content or "dense core" composed of highly concentrated semi-crystalline secretory product (29). Thus, it is possible that the medium used for growing GH_3 cells may be deficient in one or more factors necessary for the concentration of growth hormone.

A third explanation for the differential storage of SRIF and GH is that acidification of the *trans*-Golgi network or secretory vesicles could be defective in GH_3 cells. SRIF packaging may be less dependent upon a low pH sorting step for transport into mature granules than other hormones such as growth hormone (14). The observation (1) that in pancreatic islets, SRIF-secretory granules were less acidic than those containing insulin or glucagon is consistent with this hypothesis. However, a direct role for acidification in the processing, sorting, and packaging of peptide hormones into secretory granules is somewhat controversial. Moore et al. (23) showed that treatment of AtT-20 cells with high concentrations of chloroquine, which neutralizes acidic intracellular compartments, inhibited proACTH/endorphin proteolytic processing and diverted the precursors to the constitutive pathway. In contrast, Mains and May (20) recently demonstrated that low doses of chloroquine, which abolished intracellular pH gradients, had no effect on proACTH/endorphin processing, or on secretagogue-stimulated secretion of the mature peptides. Whatever the mechanism for the differential sorting of SRIF and GH, the data presented here demonstrate that GH_3 cells and their clonal variants should provide important insights into targeting proteins to secretory granules.

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