Retrovirus-mediated Expression of Preprosomatostatin: Posttranslational Processing, Intracellular Storage, and Secretion in GH3 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 GH3 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 GH3 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.
PreproSRIF is one of the simplest precursors (16, 35, 36) pancreatic islet RIN cells, e.g., preproenkephalin (6, 33), precursors to the mature hormone. However, for some cells preproinsulin (24), pretrypsinogen (4), preproSRIF (32), cated at the carboxyl terminus of the propeptide and is pre-
cursors were proteolytically cleaved to the mature hormone
Detection of unprocessed precursors. These obser-
which manifest only the constitutive pathway, results in little
Expression of peptide hormone precursors in fibroblasts,
endogenous precursors have proteases capable of cleaving
However, for some cells such as the rat somatotroph line GH
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products for endoproteolytic processing; (b) synthesis and
which contains the highly efficient murine leukemia virus
secretory granules.](21) Somatostatin is a 14-amino acid peptide hormone which
mon (15) to the mature hormone, whereas
neither proenkephalin (33) nor proSRIF (32) were processed
parathyroid hormone (15) to the mature hormone, whereas
in the presence of 40 g/ml of kanamycin. Infectious virus particles containing prepro-
the regulated pathway, albeit with variable efficiency. In contrast, viral membrane glyco-
optimized previous studies from this laboratory (11) demon-
Material and Methods
Production of Recombinant Retrovirus Expressing preproSRIF
Materials

**Materials**

Psi-2 cells (22) and the plasmid pLJ were a gift from Dr. Richard Mulligan, The Whithead Institute, Boston, MA. Rabbit anti-SRIF serum, designated RSI-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. [35S]Methionine and [35S]Systeine were purchased at the highest available specific activity from Amer sham Corp., Arlington Heights, IL.

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Cell Culture. Cells were grown at 37°C in an atmosphere of 7.5% CO2. GH3 cells were grown in Ham's F10 medium (3 g/ml NaHCO3) supple-
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A 462-bp cDNA fragment encoding preproSRIF was ligated into the Bam H1 site of the retroviral expression vector, pLJ (see Fig. 1), and the resulting plasmid DNAs amplified in *Escherichia coli* strain C600 grown in the pre-

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of appropriate antiserum (if not added to the labeling or chase medium). 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 trasylo, 5 mM cysteine or methionine, 1 mg/ml BSA), twice with 75 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 90% 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 lysate. 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% CH3CN, 1% trifluoroacetic acid (TFA) added. Samples were analyzed using an HPLC system (model 6000A solvent delivery system; Waters Instruments, Inc., Milford, MA) 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 GH3 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. The orga-
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 GH3S18.9 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 four, therefore [³⁵S]cysteine radioactivity should be recovered in cycles 3 and 14. The data showed that this was the case and demonstrated that GH3 cells accurately cleaved proSRIF to the mature peptide.

Kinetics and Efficiency of Processing and Secretion

GH3S18.9 cells were pulse labeled with [³⁵S]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, 

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 [³⁵S]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; GH3S18.9 cells were pulse labeled with [³⁵S]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 GH3S18 cell lines. (A) HPLC analysis. Cells were pulse labeled with [35S]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 C18 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 NH2-terminal sequencing. [35S]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 GH3S18.9 cells. Cells were pulse-labeled (P) with [35S]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 CH3CN gradient is shown in the upper right panel. Arrowheads indicate the elution position of mature SRIF and proSRIF, respectively.
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 GH3 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 GH3 cells at levels approaching those of the endogenous growth hormone as determined by radioimmunoassay (Fleischer, N.,...
T. Stoller, H. Liker, and D. Shields, manuscript in preparation) and established clonal lines synthesizing different levels of the precursor. Our data show that GH3 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 NH2-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 polypeptide 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 GH3 (pituitary) or PC-12 (adrenal medulla) cells (32); this was unexpected for several reasons. Firstly, Hellerman et al. (15) showed that proPTH (parathyroid hormone) is accurately cleaved to PTH (at LysLysArg) in GH3 cells. Secondly, GH3 cells are clonal derivatives of the rat GH line, which we demonstrate here cleaves proSRIF accurately and efficiently. Thirdly, Low et al. (19) showed in transgenic mice containing the rat proSRIF 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 non-specific proteolysis and it is possible that some cells secrete high levels of proteases, leading to low recoveries of the mature hormone. Alternatively, GH3 cells might lack or not express the gene encoding the processing enzyme; we are currently investigating this possibility.

The efficient processing of proSRIF in GH3 cells was particularly intriguing since this precursor has an atypical cleavage site, ArgLys. This combination of paired basic residues (on the NH2-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 secretagoga 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 GH3 cells it is rapidly secreted (1, 11, 31). A possible explanation is that GH produced by GH3 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 GH3C1 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 proactinin. 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 GH3 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 GH3 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 GH3 cells and their clonal variants should provide important insights into targeting proteins to secretory granules.

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