CELL STRUCTURE AND FUNCTION 27: 145–155 (2002)

Evidence of a Novel Dipeptidyl Aminopeptidase in Mammalian GH_3 Cells: New Insights into the Processing of Peptide Hormone Precursors

Kwang Ho Cheong[1], Myung Ae Lee[2*], Sang Yeol Han[1], Dennis Shields[3], Sang Dai Park[1], and Seung Hwan Hong[1]

[1]*Department of Molecular Biology and Research Center for Cell Differentiation, Seoul National University, Seoul, Korea,* [2]*Brain Disease Research Center, School of Medicine, Ajou University, Suwon, Korea and,* [3]*Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, New York, NY, USA*

ABSTRACT. **We investigated whether yeast signals could regulate hormone processing in mammalian cells. Chmeric genes coding for the prepro region of yeast α-factor and the functional hormone region of anglerfish somatostatin was expressed in rat pituitary GH_3 cells. The nascent prepro-α-factor-somatostatin peptides disappeared from cells with a half-life of 30 min, and about 20% of unprocessed precursors remained intracellular after a 2 h chase period. Disappearance of propeptide was insensitive to lysosomotropic agents, but was inhibited at 15°C or 20°C, suggesting that the hybrid propeptides were not degraded in the secretory pathway to the trans Golgi network or in lysosomes. It appeared that while most unprocessed precursors were constitutively secreted into the medium, a small portion were processed at their paired dibasic sites by prohormone-processing enzymes located in trans Golgi network/secretory vesicles, resulting in the production of mature somatostatin peptides. To test this hypothesis, we investigated the processing pattern of two different hybrid precursors: the 52-1 hybrid precursor, which has a Glu-Ala spacer between the prepro region of α-factor and somatostatin, and the 58-1 hybrid precursor, which lacks the Glu-Ala spacer. Processing of metabolically labeled hybrid propeptides to smaller somatostatin peptides was assessed by HPLC. When pulse-labeled cells were chased for up to 2 h, 68% of the initially synthesized propeptides were secreted constitutively. About 22% of somatostatin-related products were proteolytically processed to mature somatostatin, of which 38.7% were detected intracellularly after 2 h. From N-terminal peptide sequence determination of somatostatin-related products in GH_3-52 and GH_3-58 cells, we found that both hybrid precursors were accurately cleaved at their dibasic amino acid sites. Notably, we also observed that the Glu-Ala spacer sequence was removed from 52-1 hybrid precursors. The latter result strongly suggests that a novel dipeptidyl aminopeptidase activity — a yeast STE13-like enzyme — is present in the post-trans Golgi network compartment of GH_3 cells. The data from these studies indicate that mechanisms which control protein secretion are conserved between yeast and mammalian cells.**

Key words: yeast α-factor/prohormone processing/dipeptidyl aminopeptidase/heterologous expression/regulated secretion/rat GH_3 cells

Generation of a bioactive mature peptide hormone requires cleavage of a signal peptide from the prepro-hormone within the endoplasmic reticulum (ER) (Nillni *et al*., 1999), resulting in a pro-hormone precursor that undergoes several post-translational modifications such as glycosylation, proteolysis, phosphorylation, amidation and acetylation (Hatsuzawa *et al*., 2000) in the ER and the Golgi. A common feature of prohormones is that the bioactive peptide is usually flanked by pairs of basic amino acids, or less commonly by monobasic residues. Cleavage at a defined set of basic residues, mediated by the recently discovered prohormone convertase enzymes (PCs) (Taylor *et al*., 2000), results in excision of the peptide. For several prohormones, proteolytic processing is initiated in the trans Golgi network (TGN), and processing continues during targeting to, and

*To whom correspondence should be addressed: Brain Diseases Research Center, School of Medicine, Ajou University, Suwon, Korea.
Tel: +82–31–219–4529, Fax: +82–31–216–6381
E-mail: lma52347@madang.ajou.ac.kr

Abbreviations: ER, Endoplasmic reticulum; TGN, trans Golgi network; S. cerevisiae, Saccharomyces cerevisiae; DPAP, Dipeptidyl aminopeptidase; GCI, Golgi complex intermediate; SRIF, Somatostatin; PC, Prohormone convertase; TRH, Thyrotropin releasing hormone; PAS, Protein A sepharose.

within, nascent immature secretory granules (Danoff *et al.*, 1993). The mature hormones are then concentrated, resulting in the formation of dense core secretory granules which fuse with the plasma membrane and discharge their contents only in response to external stimuli (Itoh *et al.*, 2000).

In yeast, α-factor is processed from a larger precursor in the Golgi apparatus by the action of three membrane-bound enzymes. The product of the *KEX2* gene, a paired-basic-specific protease, first cleaves the precursor molecule on the carboxyl side of Lys-Arg residues. Dipeptidyl aminopeptidase A (DPAP A, the product of the *STE13* gene) (Anna-Arriola and Herskowitz, 1994; Lesage *et al.*, 2000) and carboxypeptidase B-like enzyme (product of the *KEX1* gene) (Dmochowska *et al.*, 1987) then trim, the Glu-Ala or Asp-Ala pairs and the residual Arg-Lys residues, respectively, to generate the mature α-factor peptide.

DPAPs are a group of complementary enzyme activities that remove dipeptides from the unblocked amino termini of peptides and proteins. The primary role of the DPAP enzymes in nature appears to be digestive, breaking down protein fragments into smaller peptides (McDonald and Schwabe, 1977). However, recent studies suggest that DPAP has a role in neuropeptide precursor processing (Leviev and Grimmelikhuijzen, 1995). In the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*), following cleavage at the dibasic amino acid site, mature somatostatin (SRIF) is produced by DPAP enzyme activities via a stepwise cleavage of dipeptide units (Glu-Ala), starting at the amino terminal end of the $(Glu\text{-}Ala)_3$-SRIF (Stoller and Shields, 1989; Bourbonnais *et al.*, 1988). We propose that the same events occur in mammalian cells.

The aim of this study was to determine whether yeast and mammalian cells share a common mechanism of recognition and targeting of peptide hormone precursors to the secretory pathway. Our approach was to examine the biosynthesis of heterologous yeast α-factor in rat anterior pituitary GH_3 cells (Stoller and Shields, 1989). Two different prepro-α-factor-SRIF hybrid molecules were used in these studies; the 52-1 hybrid precursor, which contains a Glu-Ala spacer (EAEAEA), and the 58-1 hybrid precursor which does not contain such a spacer.

Materials and Methods

Production of retrovirus expressing prepro-α-factor-SRIF hybrid constructs

The construction of 58-1 and 52-1 hybrid precursors has been described previously (Lee *et al.*, 1996; Cheong *et al.*, 1997). Sequences encoding the 52-1, 58-1, 82-1, and 88-1 hybrid precursors in the vector pYE7 (Lee *et al.*, 1996; Cheong *et al.*, 1997) were amplified using PCR, excised using *Bam*H1 and ligated directly into the *Bam*HI site of the retroviral expression vector pLXSN. Infectious virus particles containing RNA transcripts of prepro-α-factor-SRIF hybrid precursors were generated by transfecting the PA317 packaging cell line with appropriate plasmid DNA according to the BES buffered $CaCl_2$ method (Chen and Okayama, 1987).

Infection of target GH_3 cells

GH_3 cells were seeded at 1×10^6 cells per 60 mm dish and incubated for 30 min at 37°C with medium containing 25 µg/ml DEAE-dextran. Media from PA317 cells harboring each construct was filtered through a sterile membrane filter (Millipore, 0.45 µm pore size) and incubated with the GH_3 cells for 2 h at 37°C. Complete Ham's F-10 (4 ml) was then added and the medium was changed after 24 h. After 48 h, the medium was replaced with 5 ml of Ham's F-10 containing 1 mg/ml of G418. The medium was replaced after 5 days with fresh medium containing 1 mg/ml G418. After ten days, G418-resistant cells were trypsinized, G418-resistant cells were subcultured by limiting dilution into a 96-well plate and 10 to 20 clonal lines were subsequently selected. Immunoprecipitation after pulse labeling was employed to determine the steady state expression levels of hybrid precursors from clonal lines.

Pulse-chase protocol

Cells were pulse-labeled for the indicated periods with 400 µl of labeling medium supplemented with 250 µCi/ml of [^{35}S]-cysteine. Labeling medium was prepared from the RPMI-1640 SelectAmine Kit (Life Technol, NY, USA) according to manufacturer's instructions. For the chase incubations, 1 ml of chase medium (complete Ham's F-10) was added to the cells. To examine secreted polypeptides, medium was collected, centrifuged for 10 sec in a microcentrifuge, and adjusted to Buffer A (190 mM NaCl, 50 mM Tris-HCl, pH 8.3, 6 mM EDTA, 2.5% Triton X-100) conditions by adding one-third volume of 4X buffer A solution. After the addition of 5 µl of antiserum against SRIF, samples were incubated for 16 h at 4°C. To determine the intracellular protein level, cells were harvested and lysed in 100 µl of lysis buffer (0.5% NP-40, 0.5% NaDOC in PBS). Membranes and nuclei were pelleted by centrifugation for 5 min at 4°C in a microcentrifuge. Ten volumes of buffer A and 10 µl of anti-serum were added to post-nuclear supernatants, and incubated for 16 h at 4°C. Samples from the media and cell lysate were then incubated with 75 µl of 33% (v/v) protein A sepharose (PAS) solution for 3 h. Immunoprecipitates were isolated and analyzed by SDS-PAGE or HPLC.

High-pressure liquid chromatography

HPLC was used to analyze hybrid precursor-related products. The PAS pellets from the immunoprecipitation step 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, 100 mM DTT) and 2 µg of native SRIF. Fifty µl of 0.66 M iodoacetic acid in TEU was added to the PAS pellet and incubated in the dark at room temperature for 10 min for carboxymethylation; the supernatant was removed and saved. The PAS pellet was washed twice with 50 µl of TEU, all supernatants were combined and 20 µl of 80% CH_3CN, 1% trifluoroacetic acid

(TFA) was added. Samples were analyzed using a Waters HPLC system as previously described (Stoller and Shields, 1989; Cheong *et al*., 1997). Briefly, the samples were resolved on a Vydac C_{18} reverse phase column using either gradient 1 (0–1 min 5–20% CH_3CN, 1–6 min 20% CH_3CN, 6–15 min 20–35% CH_3CN, 15–37 min 35–46% CH_3CN, 37–47 min 46–80% CH_3CN), or gradient 2 (0–1 min 5–16.25% CH_3CN, 1–5 min 16.25% CH_3CN, 5–65 min 16.25–35% CH_3CN, 65–75 min 35–80% CH_3CN). Flow rate was 1.5 ml/min and all solutions contained 0.1% TFA. One-minute fractions were collected into mini vials, 3 ml of scintillation fluid (Atomlight, NEN) was added and the radioactivity was measured using a liquid scintillation spectrometer (Pharmacia Wallac 1410).

Peptide isolation and determination of amino acid sequence

Peptides for amino acid sequence analysis were purified by HPLC and subjected to Edman degradation cycle on a peptide sequenator (Applied Biosystems model 473A, UT, USA), as described by Bourbonnais *et al*. (Bourbonnais *et al*., 1988). PAS pellets containing [^{35}S]-cysteine-labeled products were reduced and carboxymethylated. Following centrifugation to remove the PAS beads, the supernatant was applied directly to a reverse-phase C_{18} Vydac column using gradient 2. Fractions of one min (1.5 ml) were collected and aliquots of each fraction were counted for radioactivity. Relevant fractions from HPLC were pooled and subjected directly to automated Edman degradation. The radioactivity of samples of each Edman degradation cycle was determined in a liquid scintillation counter.

Immunogold labeling of ultra-thin cryosections

Harvested cells were fixed by perfusion with PLP (10 mM periodate, 0.75 M lysine, 2% paraformaldehyde) in phosphate buffer, then trimmed to 1 mm^3, and immersed in fixative for 3 h. Fixed cells were then cryoprotected by immersion in 2.3 M sucrose, containing 20% polyvinylpyrrolidone for a minimum of 1 h, mounted on aluminum nails, and frozen in liquid N_2. Ultra-thin cryosections were cut at –100°C and placed on nickel EM grids that were coated with Formvar and carbon. All incubations and washes were performed by floating the grids on liquid droplets. Grids were washed in 10 mM glycine in PBS, pH 7.4, containing 2% FCS for 30 min, blocked for 30 min in 10% FCS and subsequently incubated with the primary antibody, affinity purified rabbit polyclonal anti-SRIF or mouse monoclonal anti-rat Golgi complex intermediate (GCI) (from 6F4C5 (Chicheportiche *et al*., 1984)), for 2 h at room temperature or overnight at 4°C. Grids were then washed 10 times, for a total of 30 min, and incubated with 5 or 10 nm colloidal gold-conjugated goat anti-rabbit or goat anti-mouse IgG (Amersham, NJ, USA) for 1–2 h at room temperature. In bridging experiments, goat anti-rabbit IgG and 6 nm colloidal gold-conjugated donkey anti-goat IgG (Jackson Laboratories, ME, USA) were used as secondary and tertiary antibodies. Grids were washed 10 times over 30 min in buffer and carried quickly through 10 droplets of double-distilled H_2O. Grids were floated on neutral uranyl acetate (2% uranyl acetate, 0.15 M oxalic acid, pH 7.4) for 10 min for staining, washed very quickly through 5 droplets of double-distilled H_2O, and adsorption stained for 5 min on a mixture containing 3.2% polyvinyl alcohol, 0.2% methyl cellulose (400 centipoises) and 0.1% uranyl acetate. The grids were picked up in loops, excess embedding material removed, and allowed to air dry. Grids were then examined using a transmission electron microscope (JEOL 1200 EX-II) at 80 kV. For double-immunolabeling experiments the primary antibodies were mixed and the secondary antibodies were mixed for each incubation.

Results

Turnover of intracellular prepro-α-factor-SRIF hybrid precursors in GH_3 cells

Two different hybrid precursors containing the prepro region of yeast α-factor and anglerfish SRIF were constructed: one with a Glu-Ala spacer in front of the mature SRIF peptide (52-1), and one without the spacer (58-1) (Fig. 1) (Lee *et al*., 1996; Cheong *et al*., 1997). To examine the intracellular transit of peptide hormone precursors, GH_3 cells expressing either the 58-1 hybrid (GH_3-58) or the 52-1 hybrid (GH_3-52) were pulse-labeled with 250 μCi/ml of ^{35}S-cysteine for 15 min and chased for periods of up to 2 h. At each time point, intracellular and secreted SRIF-immunoreactive products were analyzed by SDS-PAGE. The data presented in Fig. 2 show that the level of intracellular propeptides from hybrid precursor in GH_3-58 cells decreased rapidly with the increase in chase time. The similar result was obtained from GH_3-52 cells (data not shown). These results indicate that the Glu-Ala spacer did not affect the rate of hybrid precursor turnover. A further observation from these data is that approximately 15% of SRIF-immunoreactive polypeptides labeled during the 15 min pulse in both GH_3-52 and GH_3-58 cells remained intracellularly when the cells were chased for 2 h (Fig. 2). However, the amount of secreted propeptide could not account for the disappearance of intracellular propeptide completely. Two possible explanations for these data are intracellular degradation, or further processing to the smaller, mature SRIF peptide hormone.

Intracellular degradation of hybrid peptide hormone precursors

To determine whether the prepro-α-factor-SRIF hybrid precursors were randomly degraded in the secretory pathway, the effect of temperature on levels of precursor was determined. GH_3-58 cells were incubated at 15°C or 20°C to prevent vesicular transport from the ER to *cis* Golgi and exit from TGN, respectively. The results of these experiments showed there was quantitative recovery of the precursors even after up to 4 h incubation at 15°C, which suggests that only a small amount (approximately 10%) of propeptide was degraded in the ER (Fig. 3A and Table I). In addition,

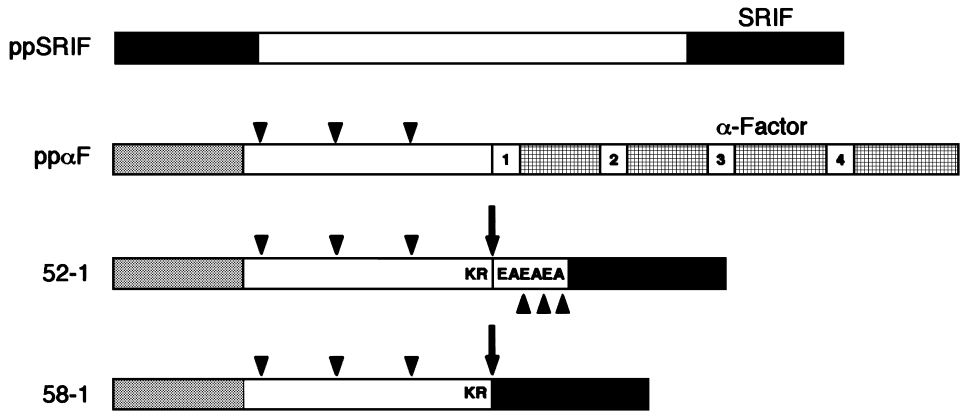

Fig. 1. Schematic representation of yeast prepro-α-factor, anglerfish prepro-SRIF and hybrid precursors. The signal peptide of α-factor is shown by the cross-striped box, while the open boxes show the pro regions. Downward arrowheads indicate the three Asn-linked glycosylation sites. The Lys-Arg (KR) dibasic cleavage site is symbolized by the downward pointing arrow, and the three Glu-Ala repeats of the spacer peptide (EAEAEA), cleaved by DPAP A, are indicated by the upward pointing arrowheads in the 52-1 construct (note that this is absent from 58-1 construct). The coding region for mature SRIF is shown as a solid box.

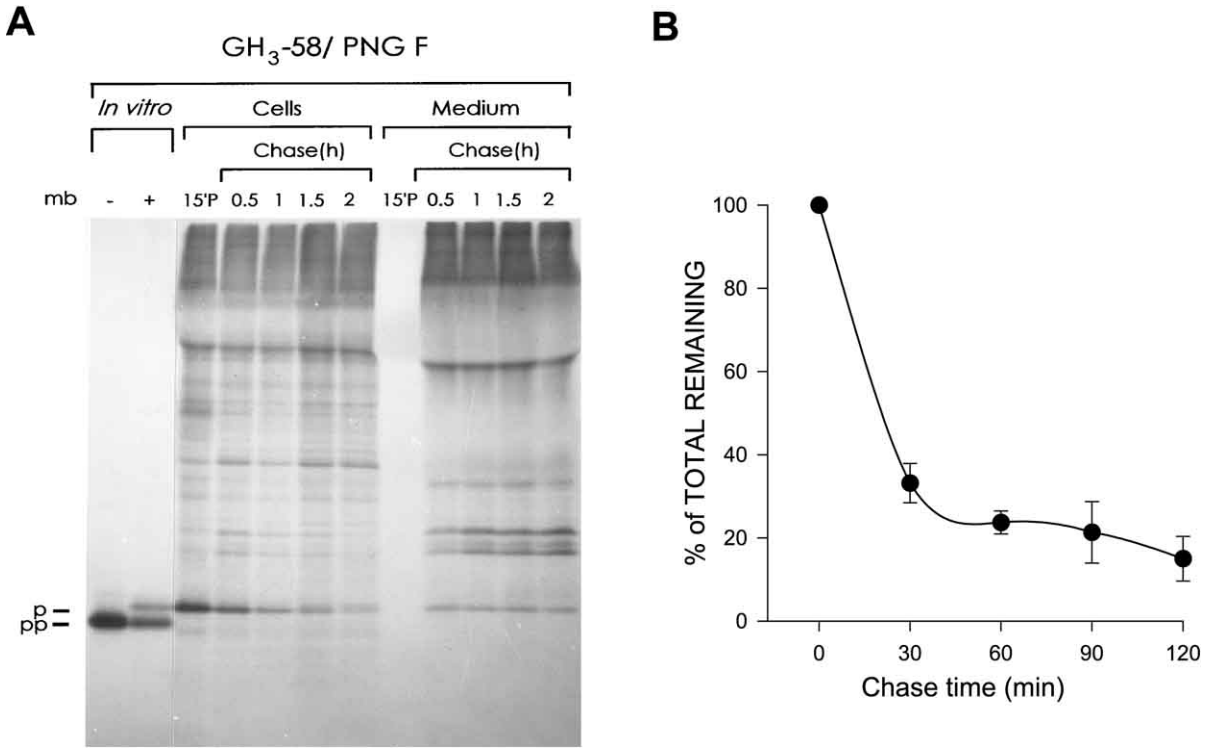

Fig. 2. Intracellular and extracellular levels of pro-α-factor-SRIF hybrid precursors: (A) GH_3-58 cells were pulse-labeled with 250 μCi/ml ^{35}S-cysteine for 15 min and chased for the indicated times. Cell lysates were treated with anti-SRIF anti-sera and immune complexes were resolved by SDS-PAGE. (B) The intensities of intracellular hybrid precursors in pulse-chase experiment were quantitated by densitometric scanning of fluorograms. The values represent the average of three experiments. The migration of prepro-α-factor-SRIF (pp) and signal peptide-cleaved naked pro-α-factor-SRIF (p) are indicated.

up to 80% of the initial propeptides were recovered at 20°C, suggesting little degradation occurred in transit from the ER to the TGN. These data suggest that the disappearance of intracellular propeptide was not due to degradation in the secretory pathway.

Next we investigated whether the disappearance of intracellular propeptide was due to degradation in acidic compartments. Cells were pulse-labeled in the presence of chloroquine, ammonium chloride or leupeptin, all of which are known to inhibit the protease activities of lysosomal

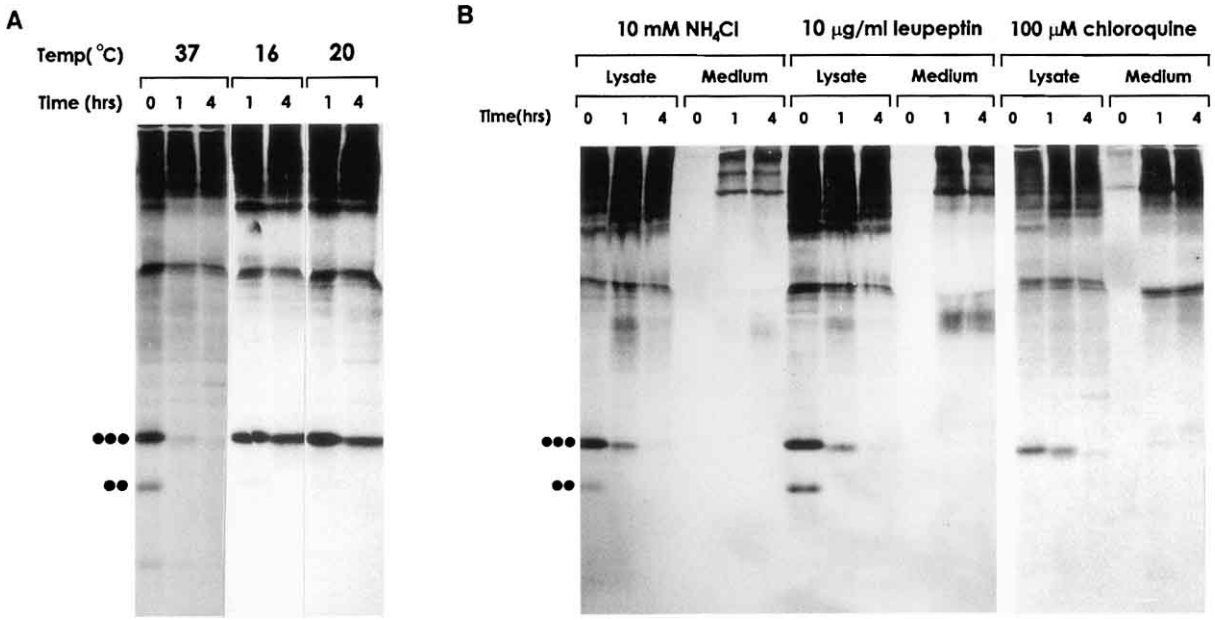

Fig. 3. Processing of pro-α-factor-SRIF hybrid precursors in GH_3-58 cells: (A) Effect of temperature on the processing of hybrid precursors: Cells were pulse-labeled with ^{35}S-cysteine for 15 min at 37°C, after which the medium was replaced with chase medium equilibrated at the indicated temperatures. Incubation was continued for up to 4 h, and cell lysates were subjected to immunoprecipitation with anti-SRIF sera. Immune complexes were resolved on 15% SDS-PAGE. (B) Effect of acidic protease inhibition on precursor processing: Cells were pre-incubated for 1 h in either 50 μM chloroquine or 100 μg/ml leupeptin or for 30 min in 10 mM NH_4Cl. Cells were then pulse-labeled for 15 min with ^{35}S-cysteine in the presence of chloroquine (lanes 1–6) or NH_4Cl (lanes 7–12) or leupeptin (lanes 13–18) and chased for the indicated times under identical conditions. At each time, cell lysates and media were treated with anti-SRIF sera and the immunoprecipitates were resolved by SDS-PAGE. The closed circles indicate the migration of core glycosylated pro-α-factor-SRIF hybrid precursors. The number of circles denote the number of attached glycosylation moieties.

Table I. EFFECTS OF VARIOUS TREATMENTS ON THE TURNOVER OF INTRACELLULAR HYBRID PRECURSORS

Treatment	Intracellular hybrid precursors (%)	
	1 h chase	4 h chase
37°C	18.8	11.3
15°C	95.0	92.6
20°C	86.2	69.4
37°C + 50 μM Chloroquine	55.1	14.4
37°C + 100 μM Chloroquine	76.3	18.2
37°C + 10 mM NH_4Cl	38.1	5.2
37°C + 10μg/ml Leupeptin	30.5	9.1

Cells were pulse-labeled for 15 min at 37°C with ^{35}S-cysteine and chased for up to 4 h. Where indicated, cells were pre-incubated, labeled and chased in the presence of various agents. Cells and media were treated with anti-SRIF antisera and the immunoprecipitates were resolved by SDS-PAGE. The resulting fluorographs were scanned densitometrically. Values (in %) correspond to the amount of SRIF-immunoreactive material recovered at 1 and 4 h, compared to that present after 15 min of pulse labeling. Each experiment was repeated three times and data denote one representative result.

enzymes. The data presented in Figure 3B show that intracellular disappearance of propeptides was not greatly affected by the presence of 10 mM NH_4Cl. Similar results were obtained upon treating the cells with leupeptin. However, in chloroquine-treated cells, a significant amount of propeptide remained intact. In addition, treatment with chloroquine decreased the synthesis of nascent propeptides during pulse-labeling period, suggesting that in addition to inhibition of lysosomal proteases, chloroquine may affect other cellular functions. It is reported that endoproteolytic cleavage of proSRIF and formation of nascent secretory vesicles in the TGN are inhibited by chloroquine, a weak base, and CCCP, a protonophore (Xu and Shields, 1993; 1994). This suggests that prohormone cleavage is initiated in the TGN, and that

this reaction requires an acidic pH which is facilitated by a Golgi-associated vacuolar-type ATPase. These results strongly suggest that the processing of pro-α-factor-SRIF hybrid precursors occurs in TGN and requires acidic conditions.

These results were quantitated and summarized in Table I. While 20% of pulse-labeled propeptide remained intracellularly in mock-treated cells, approximately 30-39% of the pulse-labeled propeptides could be recovered after 1 h incubation in the presence of lysosomotrophic agents. These data suggest that a negligible amount of propeptides (<10%) are degraded in acidic compartments, and most hybrid precursor proteins are processed to yield mature, small peptides or are directly secreted to the medium.

Processing of hybrid precursors in GH_3 cells

To test if hybrid precursors are processed to mature, small peptides, GH_3-58 or GH_3-52 cells were metabolically labeled for 90 min with [^{35}S]-cysteine, lysed, and anti-SRIF-immunoprecipitable products were analyzed by HPLC using a C_{18} reverse phase column. Using gradient 1, analysis of GH_3-58 cell products showed two peaks of SRIF-immunoreactive material with retention times of 20 and 37 min, respectively (Fig. 4, upper panel). In products from GH_3-52 cells (Fig. 4, lower panel), the first eluting peak had a slightly different retention time from that of GH_3-58 cell products, suggesting that the identity of the processed peptides may be different. SDS-PAGE analysis of products from two cell lines showed the second peak, with a retention time of 37 min, was due to pro-α-factor-SRIF hybrid precursors (Fig. 4, inset). The specificity of these peaks was also demonstrated by comparison with the peaks of a pLXSN retroviral vector-infected cell lysate (data not shown). The first eluting peak was further analyzed using gradient 2 (Fig. 5). For the GH_3-58 cell product, the first peak with a retention time of 42 min exactly co-eluted with native reduced and carboxymethylated SRIF, suggesting that it was the mature 14-amino acid hormone (Fig. 5, upper panel). In contrast,

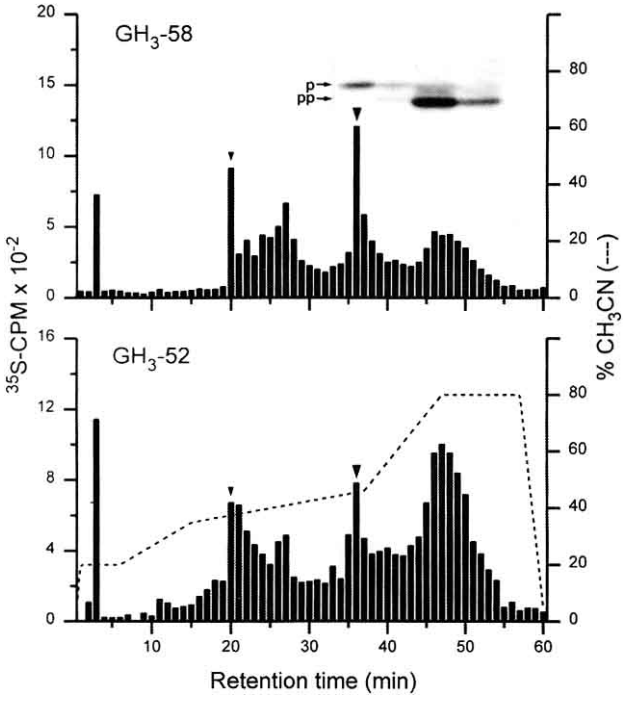

Fig. 4. Characterization of intracellular SRIF-immunoreactive material by HPLC. GH_3-58 (upper panel) and GH_3-52 (lower panel) cells were labeled with 250 µCi/ml [^{35}S]-cysteine for 90 min and cell lysates were subjected to immunoprecipitation and digested with peptide: N-glycosidase F (PNG-F). The immune complexes were carboxymethylated and separated on a Vydac C_{18} reverse phase HPLC column. One-minute fractions were collected and the radioactivity in each fraction was measured by liquid scintillation counting. The CH_3CN gradient profiles are shown in the lower panel. The small and large arrowheads indicate the elution positions of the reduced and carboxymethylated cold SRIF-14 (20 min) and pro-α-factor-SRIF hybrid precursors (36 min), respectively. Inset, serial five fractions of HPLC eluates were pooled, dried, analyzed by SDS-PAGE and fluorographed.

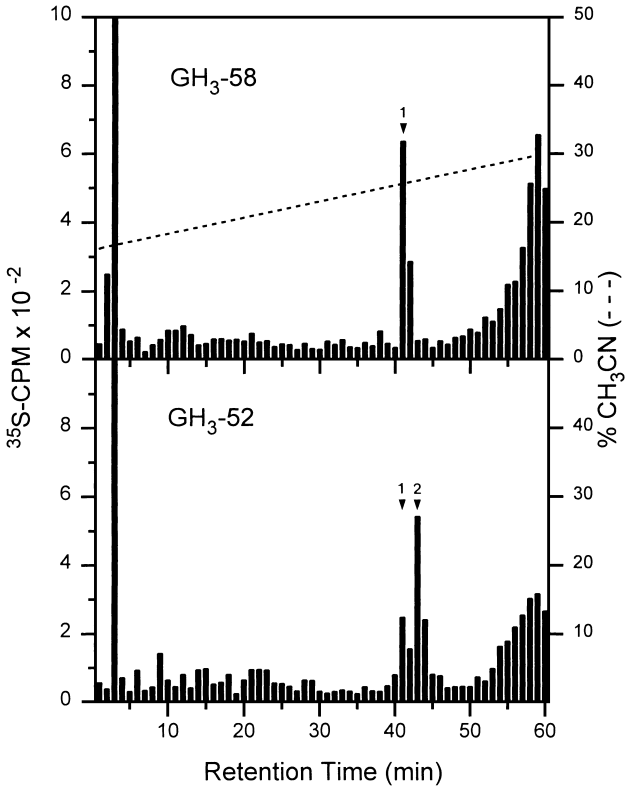

Fig. 5. HPLC analysis of processed SRIF-related peptides from GH_3-58 and GH_3-52 cells. GH_3-58 (upper panel) and GH_3-52 (lower panel) cells were metabolically labeled for 90 min with [^{35}S]-cysteine as in Fig. 2. The media and cell lysates were treated with anti-SRIF antiserum and the immune complexes were treated with PNG-F for 2 h and applied to a Vydac C_{18} reverse-phase HPLC column. Elution was performed as described in the legend to Fig. 2. The CH_3CN gradient is shown in the upper panel. Arrows indicate the elution positions of SRIF (1) and $(Glu\text{-}Ala)_3$-SRIF (2).

the SRIF-immunoreactive material from GH_3-52 cells was divided into two peaks eluting at 42 and 44 min (Fig. 5, lower panel). While the first peak appeared to be mature SRIF, the slower, second peak was reasoned to be due to the peptide having 6 additional residues at the amino terminus of SRIF (Glu-Ala spacer), which is the result of cleavage at the dibasic amino acid specific cleavage site of the 52-1 hybrid precursor.

A likely role for dipeptidyl aminopeptidase in yeast α-factor precursor processing in GH_3 cells

To confirm the identity of the materials eluting at 42 and 44 min, each peak was collected and subjected to Edman degradation cycles (Fig. 6). There are two cysteine residues in SRIF, at positions 3 and 14. Therefore, [^{35}S]-cysteine radioactivity should be recovered in cycles 3 and 14 if the material is mature SRIF. From the immunoprecipitable peptides eluting at 42 min from both GH_3-58 and GH_3-52 cells (Fig. 6, upper and middle panels), SRIF-related, immunoreactive cysteine radioactivity was increased above background level at cycle 3. However, from the second peak in GH_3-52 cells, the [^{35}S]-cysteine radioactivity peak was first detected at the 9th Edman degradation cycle (Fig. 6, lower panel). These data demonstrate that both hybrid precursors were accurately cleaved at dibasic amino acid sites in mammalian GH_3 cells, as occurs in yeast. GH_3-52 cells contained the two processed peptides, mature SRIF and $(Glu\text{-}Ala)_3$-SRIF, at a ratio of 1:4.5. In yeast, following cleavage at dibasic sites by the KEX2 enzyme, maturation of prepro-α-factor involves trimming by a DPAP (product of the *STE13* gene), which removes the Glu-Ala, Asp-Ala spacer region (Dmochowska *et al*., 1987). Surprisingly, our results using GH_3-52 cells suggest that yeast DPAP A-like enzyme activity is present in mammalian cells and could recognize and cleave the Glu-Ala spacers in the hybrid precursors accurately.

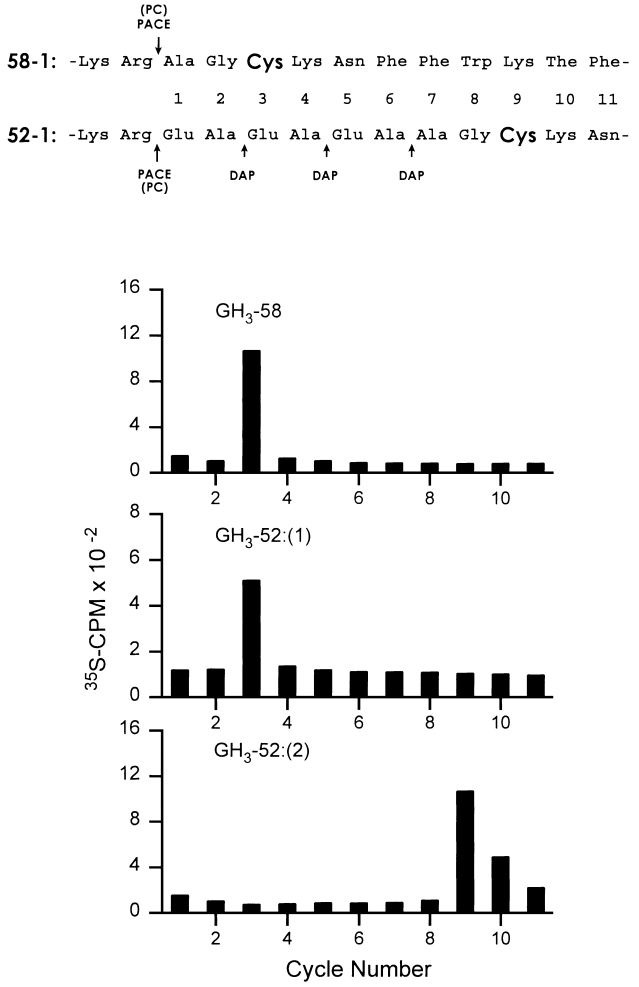

Fig. 6. N-terminal amino acid sequence determination of SRIF-immunoreactive peptides from GH_3-52 and GH_3-58 cells. GH_3-58 (upper panel) and GH_3-52 (middle and lower panel) cells were labeled with [^{35}S]-cysteine for 90 min and SRIF-immunoreactive material was purified by HPLC. Labeled peptides in fractions indicated by the arrows in Fig. 5 were subjected to modified Edman degradation cycles. The total ^{35}S radioactivity in each sequencing cycle was determined by liquid scintillation counting.

Accumulation of SRIF molecules in the secretory granules

To determine the final destination of SRIF molecules, the intracellular localization of SRIF-related peptides was determined in a more definitive fashion by immunofluorescence microscopy (data not shown), and immunoelectron microscopy (Fig. 7). The localization to the Golgi and secretory vesicles was visualized by using an affinity-purified anti-SRIF, anti-rat GCI monoclonal antibody with gold-conjugated antibody (10 nm gold particle for the Golgi, 5 or 6 nm gold particle for SRIF). A distinct punctuated staining pattern was observed near the plasma membrane by fluorescence microscopy on semi-thin cryosections (data not shown), suggesting that SRIF molecules accumulate in secretory granules near the plasma membrane. The localization pattern of 52-1 hybrid precursors in secretory vesicles is the same as that of 58-1 precursors that do not have Glu-Ala spacers at the N-terminus of mature SRIF. These results imply that spacer sequence has no effect on the targeting of hybrid precursor to the secretory pathway. Electron microscopy was employed for more accurate subcellular localization, especially to see whether SRIF molecules accumulate in secretory vesicles (Fig. 7). In double-labeling experiments with anti-Golgi and anti-SRIF antibodies, SRIF-immunoreactive vesicles were observed to bud out from the Golgi in GH_3-52 (Figs. 7a, c, e) and GH_3-58 (Figs. 7b, d, f) cells. Many SRIF-specific immunogold particles were found on electron dense secretory vesicles in both cells. This shows that the pro region of yeast α-factor could target and accumulate SRIF molecules in secretory vesicles that are involved in the regulated secretory pathway in rat

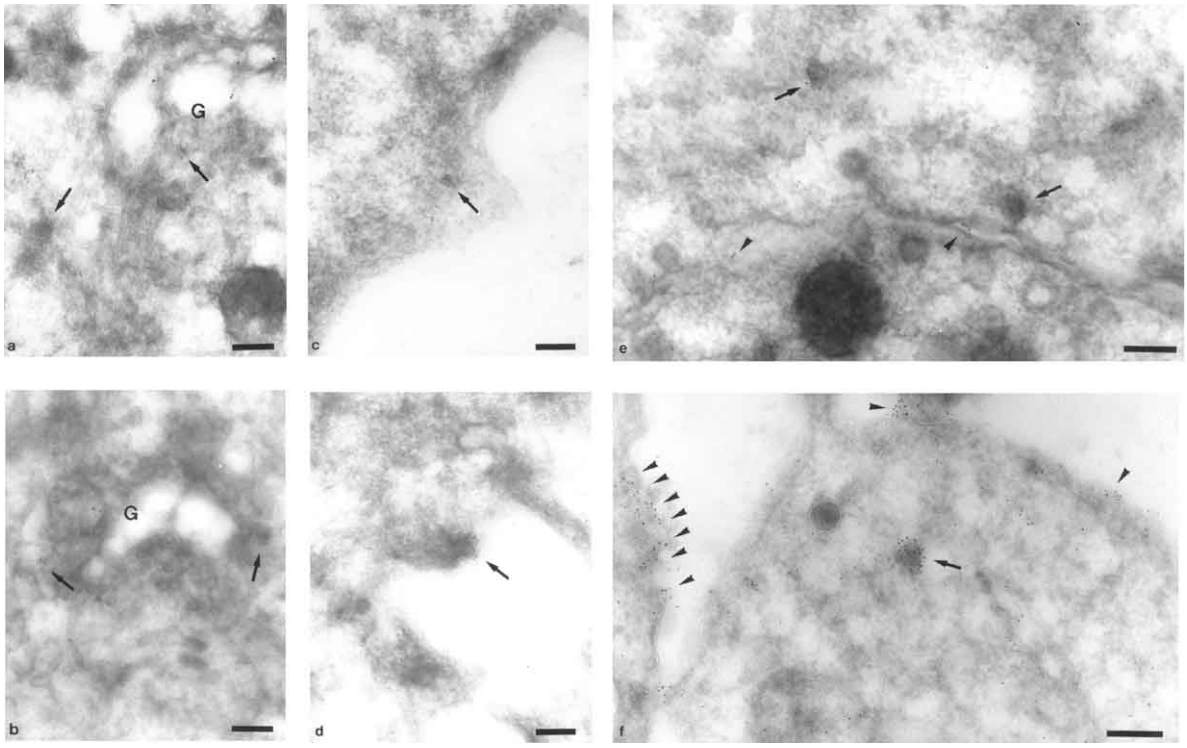

Fig. 7. Localization of SRIF-related peptides by immunoelectron microscopy. Cells were fixed with PLP fixative for 3 h. Ultra-thin cryosections (80 nm) of GH_3-52 (a, c, e) and GH_3-58 (b, d, f) cells were transferred to Formvar-coated Ni grids, and were incubated with affinity-purified anti-SRIF antiserum for 2 h at RT, followed by immunogold labeling. SRIF-rich vesicles (arrows) are budding out from the Golgi (G). 10 nm gold-conjugated antibody for the Golgi (G), and 5 or 6 nm gold-conjugated antibody for SRIF were used. Five and six nm SRIF-specific immunogold particles were observed in electron-dense secretory vesicles (arrows) as well as at the cell surface (arrowheads). Bars = 200 nm.

Table II. INCREASE OF SRIF SECRETION BY THE TREATMENT OF SECRETAGOGUES

Treatment	Secreted (cpm) Intracelluar (cpm)	Secreted/Intracelluar Ratio	Increase (%)
2 h chase	403.2±20.2 279.3±12.7	1.44	-
2 h chase + TRH	416.0±21.2 247.7±12.1	1.68	16.7
2 h chase + 8-Br-cAMP	414.3±19.5 238.7±13.1	1.74	20.8
2 h chase + Forskolin	390.7±18.8 247.0±12.5	1.58	9.7

Cells were pulse-labeled for 15 min at 37°C with [^{35}S]-cysteine, then chased for 2 h in the absence or presence of 5 mM 8-Br-cAMP, 100 nM TRH or 10 µM forskolin. Cells and media were treated with anti-SRIF antiserum and the immunoprecipitates analyzed by HPLC. The radioactivity in the SRIF peptides was determined from the HPLC peak and is shown in cpm. Data denote the mean ±SEM of representative determination made in three separate cultures.

pituitary GH_3 cells, albeit with low efficiency. Patches of gold particles (arrowheads) were also observed on the surface, suggesting that most of the hybrid precursors are constitutively secreted, as predicted by the biochemical data (Fig. 2). No such particles were observed on the surface or secretory vesicles of parental GH_3 cells (data not shown).

Increase of SRIF secretion by secretagogues

To further examine whether intracellular SRIF was targeted to the regulated secretory pathway, we investigated the release of stored molecules following stimulation with secretagogues. Cells were pulse-labeled with [^{35}S]-cysteine for 15

min and chased for 120 min in the absence or presence of secretagogues, 8-Br-cAMP, forskolin or thyrotropin releasing hormone (TRH). After this chase, the cells and the media were treated with anti-SRIF antibody and the related products were analyzed by HPLC (Table II). During the 120 min chase period in the absence of secretagogues, 67% of 15 min pulse-labeled CPM was secreted. However, in the presence of secretagogues, 69–70% of both mature and propeptides were secreted into the medium. Considering that only 10% of the initially synthesized propeptides remain intracellularly after the 120 min chase, the slight stimulation in SRIF secretion by secretagogues indicates the release of SRIF molecules from the cell was regulated by the external signals. These results also demonstrate the targeting of pro-α-factor-SRIF to the regulated pathway through the prepro region of yeast α-factor in highly specialized mammalian endocrine cells. Taken together, these observations constitute compelling evidence that a minor portion of the prepro-α-factor-SRIF hybrid precursor was specifically transported to dense-core secretory granules, i.e. processed via the regulated secretory pathway.

Discussion

Our study demonstrated that two different hybrid precursors (Fig. 1) were accurately cleaved at dibasic amino acid sites in mammalian GH_3 cells, as occurs in yeast. Although pro-α-factor-SRIF hybrid precursors contain a Lys-Arg sequence, which is the preferred cleavage site in mammals, only 22% of hybrid propeptides were processed to mature SRIF in GH_3 cells. In surveying 35 different peptide hormone and neuropeptide precursors containing approximately 60 bioactive peptides, we found that there is a hierarchy for use of certain pairs of basic amino acids on the NH_2-terminal side of the hormone: Lys-Arg (70%), Arg-Arg (15%), Lys-Lys (10%) and Arg-Lys (5%). Susceptibility to cleavage may potentially be modulated by the primary sequence of flanking residues as well as the paired basic amino acid sequence itself. Conversely, a comparison of the predicted secondary structures of a large number of prohormones has suggested that prohormone conformation may influence processing. For example, there is less cleavage of paired basic amino acids thought to be in α-helices compared to those in β-turns (Brakch *et al*., 2000). Therefore, the low efficiency of processing of pro-α-factor-SRIF hybrid precursors at dibasic sites may be due not to the amino acid sequence of the cleavage site itself, but to the flanking sequences.

There are currently four recognized classes of DPAP enzymes: DPAP-I, DPAP-II, DPAP-III and DPAP-IV (McDonald and Schwabe, 1977). So far, DPAP has not been recognized as a processing enzyme for peptide hormone precursors in mammalian cells, although it is known to be involved in the final processing of honey bee melittin, cecropin from moth and xenopsin from frog skin (Kreil, 1990; Boman *et al*., 1989). N-terminal amino acid sequence determination of processed peptides from GH_3-52 cells (Fig. 4) suggested that a DPAP-like enzyme activity might be responsible for the final processing of the immature precursors in GH_3 cells. This is the first report to demonstrate the involvement of DPAP in processing of peptide hormone precursors in mammalian cells, providing us with new insights into peptide precursor processing in higher eukaryotic cells.

In our study, lysosomotrophic agents did not inhibit the disappearance of hybrid precursors, indicating that the acidification of TGN was not essential for processing and/or secretion. Significantly, this suggests that the proteases involved in processing pro-α-factor-SRIF hybrid precursors in GH_3 cells are those capable of operating at neutral pH. In the yeast *S. cerevisiae* the pro-α-factor processing enzyme is Kex-2p (Steiner *et al*., 1992), and two mammalian prohormone-cleaving enzyme families have been identified with similar active sites to Kex-2p, namely the prohormone convertases (PCs), and the related furins. PCs are specific to the regulated secretory pathway and cleave prohormones in secretory granules. The PC family member PC2 may be involved in hybrid precursor processing in GH_3 cells since these cells express this enzyme, which, although displaying optimum activity at acidic pH, is also active in neutral environments (Davidson *et al*., 1988). The other candidate endoprotease, furin, reacts efficiently at neutral pH (Hatsuzawa *et al*., 1992) and is specific for the constitutive secretory pathway in virtually all cell types. Notably, the unique cleavage sequence for furin is $-Arg^{-4}-X^{-3}-(Lys/Arg^{-2})-Arg^{-1}$ (RX(K/R)R) (Hatsuzawa *et al*., 1992), which is found in the prepro-α-factor-SRIF precursors.

Protein secretion from mammalian cells can be either constitutive or regulated. It has been generally assumed that while the constitutive secretory pathway is present in all eukaryotic cells, the regulated secretory pathway is found only in specialized cells such as neuronal, endocrine or exocrine cells (Kuliawat *et al*., 2000). Several characteristics serve to distinguish these two pathways. For the constitutive secretory pathway, release of secretory products occurs at a consistent rate, reflective of an ongoing membrane flux to the cell surface. Regulated secretion, on the other hand, involves two distinct steps. Newly synthesized regulated secretory products are first stored within the cell, accumulating in electron-opaque mature secretory granules, which provides a distinction between the two different secretory pathways (Salamero *et al*., 1990). Upon stimulation, the contents of these regulated storage granules can undergo rapid release from the cell. The secretory pathway of proteins in the yeast *S. cerevisiae* is known to be continuous (Harsay and Bretscher, 1995). Superficially, protein transport in yeast and secretory or synaptic vesicle release from mammalian endocrine cells or nerve endings appear to be very different. However, recent studies suggest that the molecular machinery for secretion is conserved between constitutive fusion

machinery in yeast and specialized elements that may mediate regulated events specific to processing of peptide hormone and neurotransmitter release in mammalian cells (Matlack *et al*., 1999; Brigance *et al*., 2000). It has become apparent that many of the components in the secretory pathway that mediate recognition of intracellular transport vesicles and their fusion with target membranes are functionally conserved between mammalian and yeast cells (Ferro-Novick and Jahn, 1994). Ling and Shields (1996) reported that a salt extract of membranes from the yeast *S. cerevisiae*, a cell lacking a regulated secretory pathway, stimulated secretory vesicle budding in the absence of mammalian cytosolic factors, which promote secretory-vesicle release. The present study showed that a yeast prepro-α-factor-SRIF precursor was processed in a mammalian cell, and that this processing appeared to involve both constitutive and regulatory pathways. These observations support the contention that the molecular mechanisms which control peptide hormone processing and secretion are conserved between yeast and mammalian cells.

Acknowledgments. This work was supported by a grant from the Korea Science and Engineering Foundation through the Scientific Research Center for Cell Differentiation (95K3-0401-03-03-4 and 95K3-0401-03-01-4). We thank Dr. M. G. Farquhar for helpful suggestions and for the generous use of her immunocytochemical facilities.

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(*Received for publication, March 11, 2002*
and in revised form, May 23, 2002)