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Intracellular trafficking and metabolic turnover of yeast prepro-α-factor-SRIF precursors in GH_3 cells

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Abbreviations: ER, endoplasmic reticulum; TGN, *trans* Golgi network; SRIF, somatostatin; PNG-F, peptide:N-glycosidase F; FCS, fetal calf serum; FITC, fluorescein isothiocyanate

Abstract

Chimeric genes coding for prepro region of yeast α-factor and anglerfish SRIF were expressed in rat GH_3 cells to determine whether yeast signals could regulate hormone processing in mammalian cells. We report that nascent hybrid polypeptides were efficiently targeted to ER, where cleavage of signal peptides and core glycosylation occurred, and were localized mainly in Golgi. These data indicate that prepro region of yeast α-factor functions in sorting molecules to secretory pathway in mammalian cells. A hybrid construct with a mutated signal peptide underwent similar ER translocation, whereas such a mutation resulted in defective translocation in yeast (Cheong *et al.*, 1997). This difference may be due to the differences in ER translocation between yeast and mammalian cells, i.e., posttranslational versus cotranslational translocation. Processing and secretion of metabolically labeled hybrid propeptides to mature SRIF peptides were assessed by HPLC. When pulse-labeled cells were chased for up to 2 h, intracellular propeptides disappeared with a half-life of approximately 25 min, showing that ~68% of initially synthesized propeptides were secreted constitutively. About 22% of SRIF-related products were proteolytically processed to mature SRIF, of which 38.7% were stored intracellularly with a half-life of ~2 h. In addition, immunocytochemical localization showed that a small proportion of SRIF molecules accumulated in secretory vesicles. All these results suggest that yeast prepropeptide could direct hybrid precursors to translocate into ER lumen and transit through secretory pathway to the distal elements of Golgi compartment, but could process and target it less efficiently to downstream in rat endocrine cells.

Keywords: yeast α-factor; peptide hormone processing, heterologous expression; GH_3 cell, secretory pathway

Introduction

Like many peptide hormones and neuropeptides, somatostatin (SRIF) is synthesized as part of a larger precursor, prepro-SRIF, which undergoes intracellular proteolytic processing to release the mature bioactive peptide (Patel *et al.*, 1997). Anglerfish preproSRIF is an 11 kD polypeptide consisting of a 25 amino acid signal peptide, an 82 residue proregion, and the biologically active moiety, SRIF, comprising the carboxyl-terminal 14 amino acids (Danoff *et al.*, 1991). The SRIF sequence is preceded by a pair of basic amino acids, Arg-Lys, typical of prohormone proteolytic processing sites (Fuller *et al.*, 1989). In mammalian cells, heterologously expressed prepro-SRIF is known to be accurately processed, with 55% of mature SRIF sorted to the regulated secretory pathway (Sarkar *et al.*, 1998; Stoller *et al.*, 1989).

Prepro-α-factor is comprised of a 19 amino acid signal peptide, a 64 residue proregion containing three consensus sites for Asn-linked glycosylation, and a carboxyl-terminal domain containing four copies of α-factor (13 amino acids) flanked by paired basic processing sites and spacer peptides. In *Saccharomyces cerevisiae*, prepro-α-factor is targeted to the endoplasmic reticulum (ER), where it undergoes signal peptide cleavage and core glycosylation (Waters *et al.*, 1988; Matlack *et al.*, 1999). Transport from ER to Golgi is accompanied by addition of extended high mannose chains characteristic of yeast glycoproteins (Kukuruzinska *et al.*, 1987; Brigance *et al.*, 2000). In the Golgi, glycosylated pro-α-factor is cleaved by specific proteases (KEX2, STE13, KEX1) to generate mature α-factor, which is constitutively secreted (Julius *et al.*, 1984a, 1984b).

Proteins destined for secretion by yeast *S. cerevisiae* are known to be processed through more than one constitutive pathway (Harsay and Bretscher, 1995). While protein transport in yeast may appear to be very

different from secretory or synaptic vesicle release from mammalian endocrine cells or nerve endings, recent studies suggest that the molecular machinery for secretion is conserved between these species (Lledo, 1997; Tishgarten *et al*., 1999; Forgac, 2000).

The aim of the present study was to determine whether the prepro region of yeast α-factor can regulate intracellular protein transport and processing in mammalian cells. To test this hypothesis, yeast α-factor-SRIF hybrid precursors were expressed in rat endocrine GH_3 cells and the processing of these hybrid precursors was monitored. GH_3 cells were chosen as they are widely used to study mechanisms of protein secretion and responsiveness to secretagogues. The results of these studies indicated that the prepro region of yeast α-factor was indeed capable of regulating ER translocation and Golgi targeting in mammalian cells.

Materials and Methods

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

GH_3 cells were grown at 37°C in Ham's F10 medium supplemented with 15% equine serum and 2.5% fetal bovine serum (Stoller and Shields, 1988). *Bam*HI fragments encoding 52-1, 58-1, 82-1 and 88-1 hybrid precursors were generated following PCR amplification of pYE7 constructs (Lee *et al*., 1996). These fragments were ligated directly into the *Bam*HI site of the retroviral expression vector pLXSN (Miller and Rosman, 1989). 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 (Calbiochem) buffered $CaCl_2$ method (Chen and Okayama, 1987).

Infection of GH_3 cells

GH_3 cells were seeded at 1×10^6 cells/60 mm dish and incubated for 30 min at 37°C with medium containing 25 μg/ml DEAE-dextran. The media from PA317 cells containing virus harboring each construct was filtered through a sterile membrane filter (Millipore, 0.22 μm pore size) and added to the cells. Cells were incubated for 2 h at 37°C, after which 4 ml of complete Ham's F-10 was added. The medium was changed after 24 h, and after a further 24 h the medium was replaced with 5 ml Ham's F-10 containing 1 mg/ml G418. The medium was changed after five days with fresh medium containing 1 mg/ml G418. After ten days, G418-resistant cells were trypsinized, single G418 resistant clones were subcultured by limiting dilution into 96-well plates and 10-20 clonal lines were selected. Immunoprecipitation after pulse labeling was employed to determine the steady state expression levels of hybrid precursors from clonal lines.

Metabolic labeling of cells and immunoprecipitation

5×10^5 cells were seeded into 35 mm PDL-coated culture dishes as previously described (Stoller and Shields, 1989), and 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 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 seconds in a micro-centrifuge, 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 de-termine the intracellular protein level, cells were harvested and lysed in 100 μl of lysis buffer (0.5% NP-40, 0.5% NaDOC in PBS) as previously described. 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.

Immunofluorescence microscopy

Cells were grown on glass coverslips and fixed for 1 h with 4% formaldehyde in PBS at 4°C. Fixed cells were permeabilized by incubation with 0.1% Triton X-100 in PBS containing 1% bovine serum albumin (PBS/BSA) for 10 min. All subsequent washes and antibody incubations were performed in PBS/BSA. Samples were then incubated for 2 h at 4°C with affinity-purified rabbit anti-SRIF antibody or mouse monoclonal anti-rat Golgi complex intermediate antibody (GCI) (from 6F4C5 cells) (Chicheportiche *et al*., 1984) diluted in PBS/BSA. After three PBS/BSA washes (over 30 min), samples were incubated with fluorescein isothiocyanate (FITC)- or rhodamine-conjugated IgG for 30 min at 4°C and washed. The coverslip was mounted on a glass slide in 75% glycerol in PBS, pH 7.4, containing 1 mg/ml *p*-phenylenediamine and examined using phase-contrast/epifluorescence microscopy.

Immunofluorescence of semi-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% polyvinyl-pyrorolidone for 1 h, mounted on aluminum nails, and

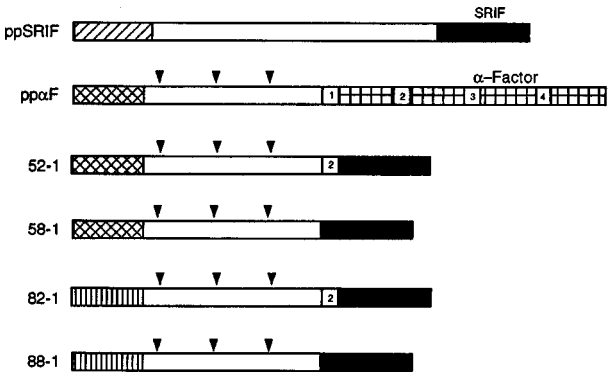

Figure 1. Structure of chimeric genes coding for prepro region of yeast α-factor and anglerfish SRIF: The structures of yeast prepro-α-factor, prepro-SRIF and prepro-α-factor-SRIF hybrid precursors are shown schematically. The signal peptide of α-factor is indicated by cross-striped boxes and open boxes indicate the pro regions. The Glu-Ala or Asp-Ala spacer regions of prepro-α-factor are labeled with serial numbers and the coding region for mature α-factor is a cross-hatched box. The coding region for mature SRIF is a solid box. Arrowheads indicate the three putative Asn-linked glycosylation sites.

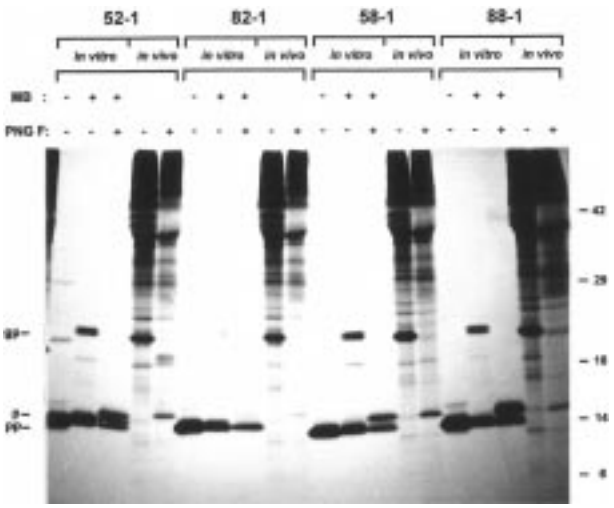

Figure 2. Expression of prepro-α-factor-SRIF hybrid proteins in GH$_3$ cells. Prepro-α-factor-SRIF hybrid constructs (52-1, 58-1, 82-1 and 88-1) were *in vitro* transcribed and translated in rabbit reticulocyte lysates in the absence (1st lane in each construct) and presence (2nd and 3rd lanes in each construct) of canine pancreatic microsomal membranes (MB). GH$_3$ cells harboring each construct were metabolically labeled for 30 min with 250 μCi/ml [^{35}S]cysteine and the cell lysates were subjected to immunoprecipitation with anti-SRIF serum. The 3rd and 5th lanes of each construct show immunoprecipitates after digestion with PNG-F. Immune complexes were resolved on 15% SDS-PAGE and visualized using fluorography. The migration of prepropeptides (pp), signal peptide-cleaved naked propeptides (p) and core glycosylated propeptides (gp) are indicated. The positions of molecular weight markers are indicated in kilodaltons.

frozen in liquid N_2. Semi-thin (0.5 μm) sections were cut on a Reichert Ultracut E microtome, equipped with a FC-4 cryoattachment at -80°C, and placed on gelatin-coated glass microscope slides. After washing, sections were immersed in 50 mM NH_4Cl in PBS, pH 7.4, for 10 min to quench free aldehyde groups, followed by incubation in 10% fetal calf serum (FCS) in PBS for 20 min at room temperature to block non-specific binding. Samples were then incubated with primary antibody diluted in PBS containing 5% FCS (PBS/FCS) for 2 h at room temperature, or overnight at 4, and washed three times (over 30 min) in PBS/FCS. Sections were then incubated with FITC- or rhodamine- conjugated IgG for 30 min to one hour at room temperature, washed, mounted and examined using a Zeiss Axiophot microscope.

Results

Synthesis and cotranslational processing of prepro-α-factor-SRIF hybrid precursors in GH$_3$ cells

Four different hybrid precursors containing the prepro region of yeat α-factor and anglerfish SRIF were used to examine if prepro region of yeast α-factor direct nascent peptide hormones to secretory pathways in rat GH$_3$ cells (Figure 1). Constructs 52-1 and 82-1 have a Glu-Ala spacer between the pro region and SRIF, while 58-1 and 88-1 do not. Constructs 52-1 and 58-1 have a wild-type signal sequence, while 82-1 and 88-1 are Met-Phe-Lys (MFK) signal peptide mutants due to the transposition of a basic amino acid Lys from residue two to residue three (Lee *et al*., 1996; Cheong *et al*., 1997). The hybrids were expressed in rat pituitary GH$_3$ cells, which are widely used to study protein secretion and are known to process heterologous SRIF precursors efficiently (Stoller and Shields, 1988; 1989). The cell lines were designated GH$_3$-52, GH$_3$-58, GH$_3$-82 and GH$_3$-88, corresponding to the hybrid expressed. Infected GH$_3$ cell lines were metabolically labeled, lysed and postnuclear cell supernatants were incubated with anti-SRIF serum. Electrophoretic analysis of the im-0munoprecipitates indicated that the predominant SRIF-containing polypeptide had a molecular mass of ~25kD (Figure 2). When compared to the migration of peptides synthesized in a microsome-supplemented cell-free translation system, the cell-synthesized products migrated marginally faster than *in vitro*-synthesized signal peptide-cleaved, core-glycosylated propeptides (Figure 2).

The next experiments sought to determine whether the propeptides produced by infected GH$_3$ cells had undergone any cotranslational processing. Pulse-labeled propeptides were quantitatively digested by peptide: N-glycosidase F (PNG-F) in order to remove any N-linked carbohydrates, and the products of this digestion were analyzed by electrophoresis. The migration of these peptides was again compared to that of species synthesized in a cell-free system. We found that the digested, cell-synthesized polypeptides (Figure 2, fifth lane in each group) migrated identically to the corresponding signal peptide-cleaved species synthesized *in vitro* (Figure 2, third lane in each group). The signal peptide-containing primary translation products are also shown in Figure 2,

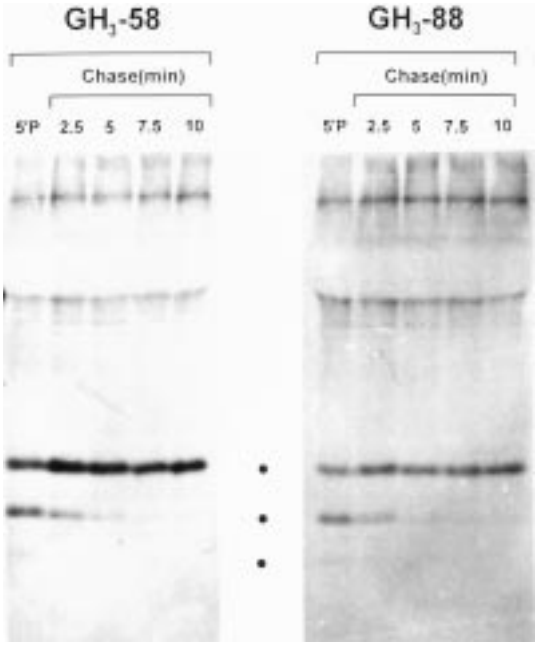

Figure 3. Kinetics of ER translocation of prepro-α-factor-SRIF hybrid precursors in GH_3-58 and GH_3-88 cells. GH_3-58 (left panel) and GH_3-88 (right panel) cells were pulse-labeled for 5 min in RPMI1640 medium containing 250 μCi/ml of [^{35}S]cysteine, and chased for the indicated intervals. The chase was initiated by the addition of 9 vol. of Ham's F-10 medium containing 1 mM cysteine. Cell lysates were treated with anti-SRIF anti-sera and immune complexes were resolved by 15% SDS-PAGE. The closed circles indicate the migration of core glycosylated pro-α-factor-SRIF hybrid precursors.

first lanes. Treatment of infected GH_3 cells with tunicamycin resulted in the synthesis of an identical signal peptide-cleaved unglycosylated species, molecular mass of ~14 kD (data not shown). These data show that the cell-synthesized hybrid proteins underwent efficient signal peptide removal and core glycosylation, suggesting that the prepro region of yeast α-factor interacts effectively with the machinery of ER targeting, translocation and cotranslational processing in rat GH_3 cells.

ER translocation of hybrid precursors with native and mutated N-terminus

In our previous studies, transformation of cDNA encoding prepro-α-factor-SRIF hybrid protein into yeast resulted in the secretion of mature SRIF (Lee *et al.*, 1996). Further studies showed that yeast cells transformed with the 82-1 construct, an MFK signal peptide mutant, accumulated a significant amount of unglycosylated, signal-containing precursor on the cytoplasmic side of the ER membrane, some of which was mistargeted to the nucleus and mitochondria (Cheong *et al.*, 1997). The mutation involved the transposition of a basic amino acid from residue two to residue three. To determine whether this N-terminal mutation similarly affected ER translocation in GH_3 cells, hybrid precursors

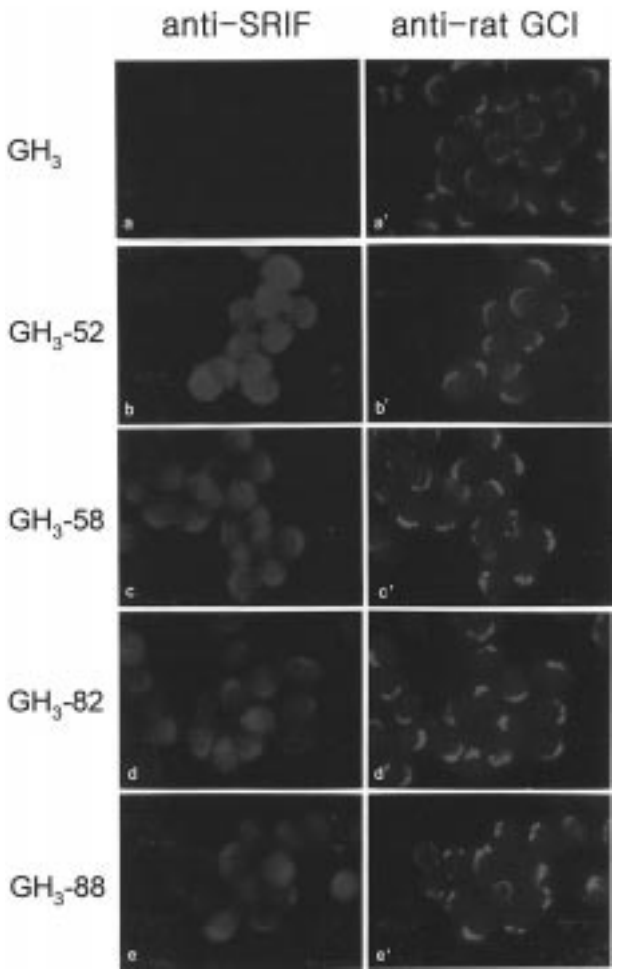

Figure 4. Localization of prepro-α-factor-SRIF precursors in GH_3-52, GH_3-58, GH_3-82 and GH_3-88 cells. Preconfluent cells were grown on coverslips, fixed and processed for immunofluorescence staining for SRIF (a, b, c, d, e) and Golgi complex intermediates (GCI) (a', b', c', d', e'). All cells show the same labeling patterns, in which both the cytoplasm and the Golgi region is stained, which is a typical pattern for secretory proteins.

with either native (58-1) or MFK (88-1) signal sequence were expressed in GH_3 cells and the kinetics of their ER translocation were compared. GH_3-58 and GH_3-88 cells were pulsed for 5 min with ^{35}S-cysteine and chased for the indicated intervals. The data presented in Figure 3 show that the rate of ER translocation was the same for both constructs, indicating that the MFK mutation did not cause accumulation of unglycosylated precursor on the cytosolic face in GH_3 cells. Therefore, in GH_3 cells, but not yeast cells, the transposition of a basic amino acid from residue two to residue three had no effect on ER translocation of nascent hybrid precursors, and the presence of an amino-terminal basic residue is sufficient for ER translocation. To confirm these observations, localization of hybrid precursors by immunocytochemical means was undertaken. Staining of clonal lines with anti-SRIF antibody resulted in a pattern typical of a secretory protein (Figure 4). No differences in immunofluorescent

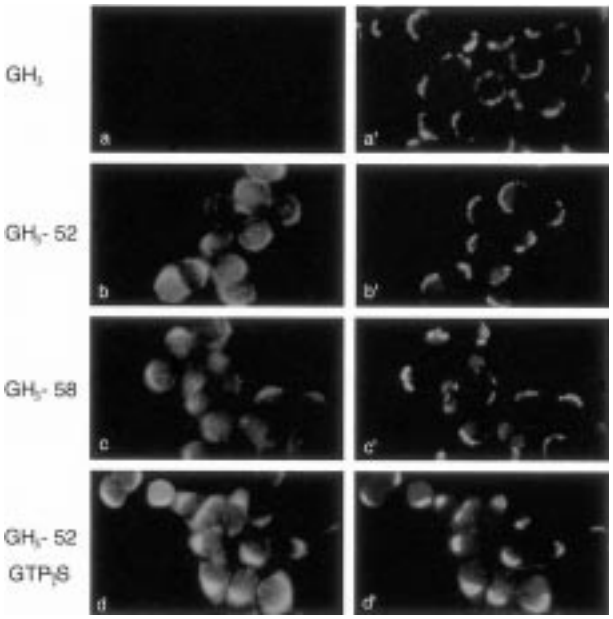

Figure 5. Localization of prepro-α-factor-SRIF precursors by fluorescence microscopy in GH$_3$-52 and GH$_3$-58 cells. Cells grown on coverslips were immunofluorescence-labeled with affinity-purified rabbit anti-SRIF IgG (a, b, c, d) and mouse anti-rat GCI monoclonal IgG (a', b', c', d'), followed by FITC-conjugated goat anti-rabbit IgG or rhodamine-conjugated goat anti-mouse IgG, and viewed as whole cells using epifluorescence microscopy.

staining patterns were observed when GH$_3$-52 and GH$_3$-82 or GH$_3$-58 and GH$_3$-88 cells were compared. These results may be due to the differences in ER translocation aspects in yeast and mammalian cells, i.e., posttranslational versus cotranslational translocation, respectively (Rapoport *et al*., 1996).

All types of hybrid precursor are mainly targeted to the Golgi

The intracellular localization of SRIF-related proteins in cells expressing hybrid constructs was studied by whole cell immunofluorescent detection. The data presented in Figure 4 show the results of experiments in which preconfluent GH$_3$-52, GH$_3$-58, GH$_3$-82 and GH$_3$-88 cells were grown on coverslips, fixed and probed for SRIF (a, b, c, d, e) and rat Golgi complex intermediate (GCI) (a', b', c', d', e'). These data indicate that regardless of the type of hybrid precursor being expressed, cells showed labeling in both the cytoplasm and the Golgi region, which is a typical staining pattern for secretory proteins. The data presented in Figure 5 show that SRIF was detected in the perinulcear area and in a diffuse pattern throughout the cytoplasm of GH$_3$-52 (b) and GH$_3$-58 (c) cells, whereas no signal was observed for SRIF in GH$_3$ cells, as expected (a). Interestingly, the intracellular localization of 52-1 hybrid molecules, which have a Glu-Ala spacer between the pro region and SRIF, is identical to that of GH$_3$-58 that express hybrid molecules with no

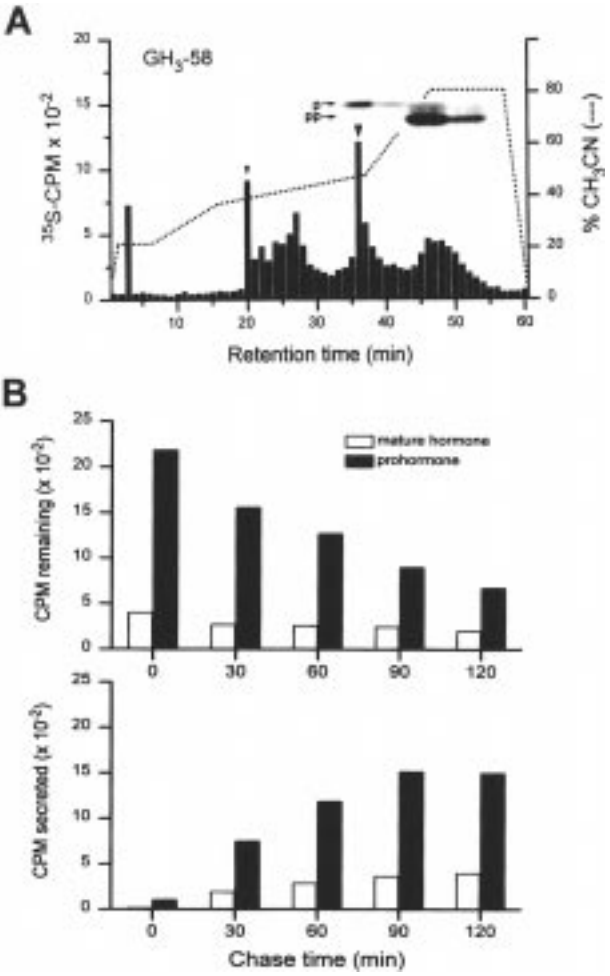

Figure 6. (A) Characterization of intracellular SRIF-immunoreactive material. Cells were labeled with 250 μCi/ml [^{35}S]cysteine and subjected to immunoprecipitation, digested with PNG-F, and separated on a C$_{18}$ RP column. The radioactivity of one-minute fractions was measured by liquid scintillation counting. The small and large arrowheads indicate the elution positions of the reduced and carboxymethylated cold SRIF-14 and hybrid precursors, respectively. Inset, serial five fractions of HPLC eluates were pooled and analyzed by SDS-PAGE and fluorography. The CH$_3$CN gradient profiles are shown in the dotted line. (B) The processing and secretion kinetics of hybrid precursors. Cells were pulse-labeled as in (A) and chased for up to two hours. At each time point, the cell lysate and media were treated as in (A) and the radioactivity in appropriate peaks was determined. Intracellular (upper panel) and secreted (lower panel) propeptide and processed peptide.

spacer sequence (Figure 5), indicating that the Glu-Ala spacer sequence does not affect protein targeting. To verify that prepro-α-factor-SRIF precursors were transported through the Golgi, in which membrane trafficking is regulated by GTPase activities, cells were treated with 20 μg/ml GTPγS for 2 h. Following this treatment, significantly more precursors were colocalized with rat GCI (Figure 5), confirming that hybrid precursors are efficiently targeted to Golgi. Further evidence of the Golgi targeting of these precursors was that staining of hybrid precursors in the Golgi region

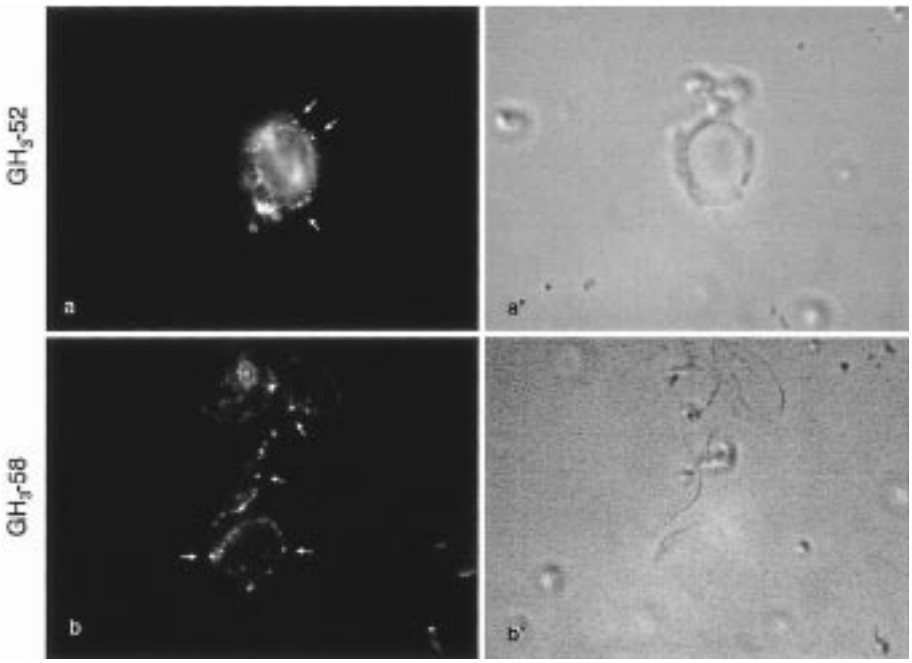

Figure 7. Localization of prepro-α-factor-SRIF precursors by immunofluorescence microscopy on semi-thin cryosections. Cells were fixed by perfusion with PLP fixative and immersed in the same fixative for three hours. Semi-thin cryosections (0.5 μm) of GH_3-52 (a, a') and GH_3-58 (b, b') cells were transferred to gelatin-coated slides. The samples were incubated with an affinity purified anti-SRIF antibody for two hours, followed by FITC-conjugated goat anti-rabbit IgG for 30 min. Distinct granular staining pattern of SRIF molecules (arrows) indicates accumulation in secretory granules near the plasma membrane.

disappeared after disrupting the Golgi compartment with 0.4 μg/ml Brefeldin A (data not shown).

Kinetics of processing and secretion

To examine the intracellular transit of peptide hormone precursors, GH_3-58 and GH_3-52 cells were pulse-labeled with [^{35}S]-cysteine for 30 min and chased for up to 2 h. At each time point, intracellular and secreted SRIF-immunoreactive products were resolved by SDS-PAGE and HPLC using a C_{18} reverse phase column (Figure 6). The level of intracellular propeptides from either hybrid precursor decreased rapidly and similarly with the increase in chase time in SDS-PAGE analysis (data not shown), indicating that the Glu-Ala spacer did not affect the rate of hybrid precursor turnover. HPLC analysis of GH_3-58 cell products showed two peaks of SRIF-immunoreactive material with retention times of 20 and 37 min, respectively (Figure 6A). The first peak with a retention time of 20 min exactly co-eluted with native reduced and carboxymethylated SRIF, suggesting that it was the mature 14-amino acid hormone (Figure 6A). SDS-PAGE analysis of products from GH_3-58 cells showed the second peak, with a retention time of 37 min, was due to pro-α-factor-SRIF hybrid precursors (Figure 6A, inset). With increasing chase time, the level of intracellular propeptides decreased (Figure 6B). After 30 min of chase, SRIF-related products appeared in the medium. The radioactivity in the intracellular and secreted SRIF-related products was determined by liquid scintillation counting and the total radioactivity in propeptide and processed peptide was calculated. The relative efficiency of processing and the secretion kinetics of the SRIF-related molecules were determined (Figure 6B). Intracellular propeptide forms disappeared with a half-life of approximately 25 min. After 2 h of chase, 68% of the initially synthesized pro-peptides were secreted constitutively and 32% re-mained inside the cell. About 22% of the 15 min pulse-labeled SRIF-related products were proteolytically processed to mature SRIF, of which 61.3% was also secreted constitutively. However, the remaining 38.7% of mature SRIF was stored intracellularly with a half-life of ~2 h. These results indicate that propeptide of yeast α-factor could mediate intracellular transport to the TGN, but might not function as efficiently in sorting to the regulated secretory pathway in rat pituitary GH_3 cells as the propeptide of anglerfish SRIF did (Stoller and Shields, 1989). During intracellular transit, there was minor degration of hybrid precursors in the secretory pathway because there was quantitative recovery of the precursors even after up to 4 h incubation in the lower temperature or lysosomotrophic agents (data not shown).

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 on semi-thin (0.5 μm) cryosections (Figure 7). The localization to the Golgi and secretory vesicles was visualized by using an affinity-purified anti-SRIF antibody with FITC-conjugated secondary antibody. A distinct punctuated staining pattern was observed near the plasma membrane by fluorescence microscopy on semi-thin cryosections (Figure 7), suggesting that SRIF molecules accumulate in secretory granules near the plasma membrane (arrows). The relatively small number of densely stained secretory granules shows that the targeting efficiency to the regulated secretory pathway is quite low, as predicted from the biochemical experiments above. The localization pattern of 52-1 hybrid precursors (Figure 7a, a') in secretory vesicles is the same as that of 58-1 precursors (Figure 7b, b') 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 again Similar results are obtained from electron microscopy (data not shown).

Discusson

We investigated whether the prepro-region of yeast α-factor could regulate protein processing in mammalian cells. In particular, we examined the roles of the N-terminal charged amino acids in the signal peptide, and of the pro region, in targeting and processing of prepro-α-factor-SRIF hybrid molecules during intracellular transit in GH_3 cells. Following biochemical and immunocytochemical analysis of 58-1 and 88-1 hybrid constructs, we conclude that the prepro region of yeast α-factor functions efficiently in targeting SRIF to ER and Golgi, and that altering the position of N-terminal charged amino acids in signal peptide does not affect the ER translocation step in rat pituitary GH_3 cells. The 82-1 construct codes for an MFK signal peptide mutant due to the transposition of a basic amino acid from residue two to residue three. In previous studies using yeast cells transformed with this construct, we found a significant amount of unglycosylated, signal-containing precursor accumulated on the cytoplasmic side of the ER membrane (Lee *et al*., 1996; Cheong *et al*., 1997). However, here we report that in mammalian GH_3 cells, the transposition of a basic amino acid had no effect on ER translocation of nascent hybrid precursors, and that the presence of an amino-terminal basic residue was sufficient for ER translocation. These results may reflect the differences between posttranslational and cotranslational translocation in yeast and mammalian cells, respectively (Rapoport *et al*., 1996).

Signal peptides typically have three distinct domains: an amino-terminal positively charged region (n-region, 1-5 residues long), a central hydrophobic domain (h-region, 7-15 residues), and a more polar carboxy-terminal domain (c-region, 3-7 residues). However, beyond this overall pattern, no precise sequence conservation can be found, indicating that signal peptides are highly variable, rapidly evolving structures (von Heijne, 1990). The n-region of eukaryotic signal peptides is generally insensitive to point mutations, small deletions and potential secondary-structure-disrupting mutations (Brown *et al*., 1984; Kaiser and Botstein, 1986; Blachly-Dyson and Stevens, 1987). In *S. cerevisiae* the ER translocation apparatus is more flexible and the effect of mutations appears less severe (Allison and Young, 1988). The one exception to this is the α-factor signal peptide, in which the higher sensitivity is related to the ability of this protein to cross yeast membranes post-translationally (Lee *et al*., 1996). In most mammalian cells the cotranslational pathway predominates, while post-translational pathways are not known (Noe *et al*., 1986; Rapoport *et al*., 1996). All these findings are consistent with our observation that a mutation in the signal peptide of yeast α-factor did not affect its translocation to the ER, or its targeting to the Golgi, in mammalian cells.

In our study, lysosomotrophic agents such as NH_4Cl, leupeptin or chloroquine did not inhibit the disappearance of hybrid precursors, indicating that the acidification of TGN was not essential for processing. 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 (Seidah *et al*., 1992; 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, and the related furins. Prohormone convertases are specific to the regulated secretory pathway and cleave prohormones in secretory granules. The prohormone convertase family member PC2 may be involved in hybrid precursor processing in GH_3 cells since these cells express this enzyme (Seidah *et al*., 1990), which, although displaying optimum activity at acidic pH, is also active in neutral environments (Davidson *et al*., 1988). The other major candidate endoprotease likely to be involved in processing pro-α-factor in GH_3 cells is furin. Furins, which are Ca^{2+}-de-pendent serine proteases with subtilisin-like catalytic domains (Seidah and Chretien, 1992; Tsuneoka *et al*., 1993; Kim *et al*., 2000; Sun and Wolfe, 2000), process constitutively secreted and membrane bound gly-coproteins in the TGN in virtually all cell types. Furin reacts efficiently at neutral pH (Hatsuzawa *et al*., 1992) and is specific for the constitutive secretory pathway. Notably, the unique cleavage sequence for furin is $-Arg^{-4}-X^{-3}-(Lys/Arg^{-2})-Arg^{-1}$

(RX(K/R)R) (Hosaka *et al.*, 1991; Molloy *et al.*, 1992), which is found in the prepro-α-factor-SRIF precursor.

The present study showed that the prepro region of yeast α-factor was capable of regulating ER translocation and Golgi targeting of hybrid precursors in mammalian cells. However, this region didn't work efficiently later in the secretory pathway. Greater understanding of the factors regulating this latter process will require investigations into the definitive proteolytic processing events, and the identification of the final intracellular destination of SRIF molecules.

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