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Heterologous Expression of Yeast Prepro-α-factor in Rat GH_3 Cells

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Yeast pheromone α-factor is a 13-amino acid peptide hormone that is synthesized as a part of a larger precursor, prepro-α-factor, consisting of a signal peptide and a proregion of 64 amino acids. The carboxy-terminal half of the precursor contains four tandem copies of mature α-factor. To investigate the molecular basis of intracellular sorting, proteolytic processing, and storage of the peptide hormone, yeast prepro-α-factor precursors were heterologously expressed in rat pituitary GH_3 cells. When cells harboring the precursor were metabolically labeled, a species of approximately 27 kD appeared inside the cells. Digestion with peptide: N-glycosidase F (PNG-F) shifted the molecular mass to a 19 kD, suggesting that the 27 kD protein was the glycosylated form as in yeast cells. The nascent polypeptide is efficiently targeted to the ER in the GH_3 cells, where it undergoes cleavage of its signal peptide and core glycosylation to generate glycosylated pro-α-factor. To look at the post ER intracellular processing, the pulse-labelled cells were chased up to 2 hrs. The nascent propeptides disappeared from the cells at a half life of 30 min and only 10-25% of the newly synthesized, unprocessed precursors were stored intracellularly after the 2 h chase. However, about 20% of the pulse-labeled pro-α-factor precursors were secreted into the medium in the pro-hormone form. With increasing chase time, the intracellular level of propeptide decreased, but the amount of secreted propeptide could not account for the disappearance of intracellular propeptide completely. This disappearance was insensitive to lysosomotropic agents, but was inhibited at 16℃ or 20℃, suggesting that the turnover of the precursors was not occurring in the secretory pathway to *trans* Golgi network (TGN) or dependent on acidic compartments. From these results, it is concluded that a part of these heterologous precursors may be processed at its paired dibasic sites by prohormone processing enzymes located in TGN/secretory vesicles producing small peptides, and that the residual unprocessed precursors may be secreted into the medium rather than degraded intracellularly.

Most neuropeptides and small peptide hormones are synthesized as a part of larger inactive polyprotein precursors which undergo a series of post-translational modifications to generate the bioactive molecule (Douglass et al., 1984; Suzuki et al., 2000). These precursors are an excellent model system to study protein trafficking within the secretory pathway. They are cotranslationally inserted into the lumen of the RER, transported through the Golgi apparatus, and packaged into secretory vesicles. During the process, they undergo a variety of covalent modifications that serves as biochemical markers for intercompartmental transfer. Recent observations demonstrate that several sorting and processing events occur in the distal elements of the Golgi apparatus/trans-Golgi network (TGN) and in the maturing secretory granules (Orci et al., 1987; Teofoli et al., 1999). In particular, endoproteolytic cleavage at paired basic residues is initiated in acidic, clathrin-coated vesicles which bud from the TGN and the resulting mature hormone is stored in secretory granules (Orci et al., 1987).

Peptide hormone-producing cells characteristically concentrate and store secretory products in the electron-dense secretory granules (Palade, 1975). Upon stimulation by extracellular signals, these granules fuse, through a calcium-dependent process, with the plasma membrane releasing their contents into the external milieu. This type of secretion is designated as "regulated" or "stimulated" (Burgess et al., 1987). Hormone-secreting cells also undergo basal or "constitutive" secretion whereby nonhormone secretory proteins and plasma membrane proteins are neither concentrated nor stored and are transported in vesicles which continuously fuse with the plasma membrane in a

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calcium- independent manner (Burgess et al., 1987). Since the hormone-secreting cells undergo basal secretion, a mechanism must exist that discriminates between molecules destined for the regulated or constitutive pathways.

Molecular signals that target a polypeptide hormone to the regulated secretory pathway are poorly understood, although morphological evidence has implicated selective aggregation or precipitation, initiated in the TGN, in the sorting process. Current evidence suggests that in the absence of a specific topogenic signal (Stridsberg et al., 2000.), i.e., for retention in the ER (Munro et al., 1987) or Golgi apparatus (Machamer et al., 1987) or for sorting to lysosomes (Kornfeld et al., 1987), secretion through the constitutive pathway occurs by default (Rothman et al., 1987). Expression of heterologous precursors in different endocrine cells results in proteolytic cleavage to mature hormone and, in some cases, targeting to the regulated secretory pathway, e.g. preproenkephalin, preprorenin (Fritz et al., 1989), preproneuropeptide Y, and preprosomatostatin (Stoller et al., 1989). Since this diverse group of proteins which lack amino acid sequence homology can be sorted to the regulated secretory pathway, it is likely that a common structural feature rather than a primary sequence is involved in targeting. However, the identity of putative sorting signals has been elusive.

Like many peptide hormones and neuropeptides, α-factor is synthesized as a part of a larger precursor, prepro-α-factor, which undergoes intracellular proteolytic processing to release the mature bioactive peptide. Prepro-α-factor comprises a cleavable 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 (Kurjan et al., 1982). In *Saccharomyces cerevisiae,* prepro-α-factor is targeted to the ER, where it undergoes signal peptide cleavage and core glycosylation (Waters et al., 1988). Transport from ER to Golgi is accompanied by addition of extended high mannose chains characteristic of yeast glycoproteins. In the Golgi, glycosylated pro-α-factor is proteolytically cleaved by specific proteases (KEX2, STE13, KEX1) to generate mature α-factor, which is constitutively secreted (Bourbonnais et al., 1988). In this study, we investigated how these sequences of α-factor might function in a mammalian system. For this purpose we utilized GH_3 rat pituitary cell, which is highly secretory and contains a yeast KEX2-like prohormone processing activity (Stoller et al., 1989).

Materials and Methods

Reagents

The following materials were used: peptide: N-glycosidase F (PNG-F) (Boehringer Mannheim Biochemicals); ^{35}S-methionine (New England Nuclear); protein A-sepharose CL4B (PAS), tunicamycin, chloroquine, and NH_4Cl (Sigma chemical Co); α-factor (Calbiochem); *in vitro* translation kit, canine pancreatic microsomal membrane (Promega); N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES) (Calbiochem).

Cell free protein synthesis

In vitro translation reactions were performed according to the manufacturer's instructions. A 25 µl rabbit reticulocyte lysate cell-free translation system contained 1.5 µg of *in vitro* transcribed RNA and 17.5 µl of nuclease-treated rabbit reticulocyte lysate and was adjusted to the following final concentrations: 10 mM creatine phosphate, 50 µg/ml creatine phosphokinase, 2 mM DTT, 50 µg/ml calf liver RNA, 79 mM KOAc, 0.5 mM $Mg(OAc)_2$, 0.02 mM Hemin, 20 µM each of 19 amino acids (minus methionine), 20 µCi of ^{35}S-methionine and 20 U of RNasin RNase inhibitor. Where indicated, 3.5 equivalents of canine pancreatic microsomal membrane were included. Membranes were stripped of endogenous membrane-bound ribosomes and mRNA prior to the addition. All translation reactions were performed at 30℃ for 60 min.

Production of recombinant retrovirus expressing prepro-α-factor construct

Prepro-α-factor cDNA was ligated into the BamHI site of the retroviral expression vector pLXSN (Miller et al., 1989). Infectious virus particles containing RNA transcripts of prepro-α-factor precursor were generated by transfecting packaging cell PA317 transiently with plasmid DNA according to the BES buffered $CaCl_2$ method.

GH_3 cells were seeded at 1×10^6 cells/60 mm dish and incubated for 30 min at 37℃ with medium containing 25 µg/ml DEAE-dextran. The medium from PA317 cells harboring constructs was filtered through a sterile membrane filter (Millipore, 0.45 µm pore size) and added to the dish. The cells were incubated for 2 h at 37℃. Four ml of complete Ham's F-10 was added to the cells 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. After days, G418-resistant cells were limitedly diluted into a 96-well plate, and 10 to 20 clones were selected. Immunoprecipitation after pulse labeling was employed to determine the steady state levels of α-factor from the clonal lines.

Metabolic labeling of cells

Metabolic labeling was carried out 24-48 h after 5×10^5 cells were seeded into 35 mm culture dishes as previously described (Stoller et al., 1989). The cells were washed twice with 2 ml PBS and pulse-labeled for the indicated times with 400 µl of labeling medium

supplemented with 250 μCi/ml of ^{35}S-methionine. The labeling medium was prepared from RPMI-1640 Select-Amine Kit according to the manufacturer's instructions. For the chase incubations, cells were washed twice with 2 ml PBS and 1 ml of chase medium (complete Ham's F-10) was added. A 5 μl/dish of specific anti-serum was added to the chase medium.

Following the labeling and chase periods, the medium was removed, centrifuged for 10 sec in a micro-centrifuge, transferred to a fresh tube and stored on ice or at -20℃ until treated with anti-sera. Cells were washed with 1 ml PBS and harvested by scraping with a rubber policeman in 1 ml PBS. The cell suspension was centrifuged for 10 sec in a microcentrifuge and lysed by vortexing 10×1 sec in 100 μl of lysis buffer (0.5% NP-40, 0.5% NaDOC in PBS) as previously described. Nuclei were removed by centrifugation at 4℃ for 5 min in a microcentrifuge. The postnuclear supernatants were treated with anti-sera.

Immunoprecipitation

To determine the intracellular levels of α-factor-related products, 10 volumes of buffer A (190 mM NaCl, 50 mM Tris-HCl, pH 8.3, 6 mM EDTA, 2.5% Triton X-100, 78 mTIU/ml of aprotinin, 1 mg/ml BSA, 5 mM methionine) and 10 μl of anti-α-factor serum were added to the postnuclear supernatants. To look at the secreted polypeptides, the medium was adjusted to buffer A conditions by addition of one-third volume of 4X buffer A solution and 5 μl of antiserum. All these samples were incubated with constant mixing at 4℃ for 12-24 h. Then, samples from the cell lysates and media were incubated with 75 μl of 33% (v/v) PAS solution at 4℃ for 3 h. Immune complexes were isolated by centrifuging for 5 sec in a microcentrifuge, washed twice with 500 μl of buffer B (150 mM NaCl, 10 mM Tris-HCl, pH 8.3, 5 mM EDTA, 0.1% Triton X-100, 78 mTIU aprotinin, 1 mg/ml BSA, 5 mM methionine), and twice with 500 μl of PBS. The immunoprecipitates were subjected to 15% SDS-PAGE. After the electrophoresis, gels were fluorographed and exposed to phosphoimaging screen or X-ray film at -70℃.

Treatment prepro-α-factor precursors peptide: N-glycosidase F

To the washed immunoprecipitates, 40 μl of 1% β-mercaptoethanol solution was added and the samples were incubated at 50℃ for 30 min. The Immunoprecipitated samples (50 μl) to be digested with peptide: N-glycosidase F (PNG-F) were adjusted to the final concentration of 50 mM Sodium phosphate, pH 7.5, 8 U/ml PNG-F and protease inhibit or cocktail (4 mM PMSF, 4 mM benzamidine, 4 mM aminocaproic acid) and incubated at 37℃ for 2 h. The samples were then lyophilized and solubilized in 1X SDS-PAGE sample buffer for electrophoresis.

Table 1. Effects of various treatment on turnover of intracellular pro-α-factor

Treatment	Intracellular pro-α-factor (%)	
	1 hr chase	2 hr chase
37℃	18.5	9.7
15℃	100.0	91.4
20℃	77.0	75.0
37℃+100 μM chloroquine		16.9
37℃+ 10 mM NH_4Cl		15.5

Cells were pulse labeled for 15 min at 37℃ with ^{35}S-methionine and chased for 2 h with the indicated conditions. Where indicated, cells were chased in the presence of various agents. Cell lysates and media were treated with anti-α-factor antisera, and the immunoprecipitates resolved by SDS-PAGE. The resulting fluorographs were scanned densitometrically. Values (in%) corresponds to the amount of α-factor-related material recovered at 1 and 2 h, compared to that present after 15 min of pulse labeling.

Results

Many steps are involved in the processing of prepro-α-factor to generate mature α-factor in yeast. Because all these steps are very rapid, it is difficult to detect the processing intermediates in *S. cerevisiae*. In mammalian cells, the processes may be more complex and time-consuming, but it is easier to analyze the processing steps than in yeast. Therefore, we decided to express prepro-α-factor in rat pituitary GH_3 cells and to analyze each step of the secretory pathway.

Co-translational processing of yeast prepro-α-factor precursors in GH_3 cells

To study if the prepropeptide of yeast α-factor can mediate intracellular transport, processing and targeting to the regulated secretory pathway in mammalian cells, we expressed prepro-α-factor in rat pituitary GH_3 cells (designated GH_3-α-factor). Synthesis of the α-factor specific products (in GH_3-α-factor cells) were monitored by metabolic labeling, followed by immunoprecipitation with appropriate anti-α-factor serum. This method detected a predominant polypeptide with an approximately molecular mass of 27 kD, which migrated marginally faster than the signal peptide-cleaved, core-glycosylated propeptides synthesized in a microsome-supplemented cell-free translation system (Fig. 2, second lane). To determine the extent of co-translational processing of cell-associated propeptides, their electrophoretic mobilities were compared with those of the *in vitro* products before and after removal of N-linked carbohydrates by treatment with PNG-F. Pulse-labeled glycosylated propeptides were quantitatively digested by PNG-F (Fig. 2, fifth lane), and the resulting polypeptides migrated identically to the corresponding signal-cleaved species synthesized *in vitro* (Fig. 2, 3rd lane). For comparison, the signal-containing primary translation products are shown (Fig. 2, 1st lane). The efficiency of the signal peptide removal and core glycosylation of prepro-α-factor indicate that the prepro region of these polypeptide interacts productively with the ER targeting, translocation, and co-translational

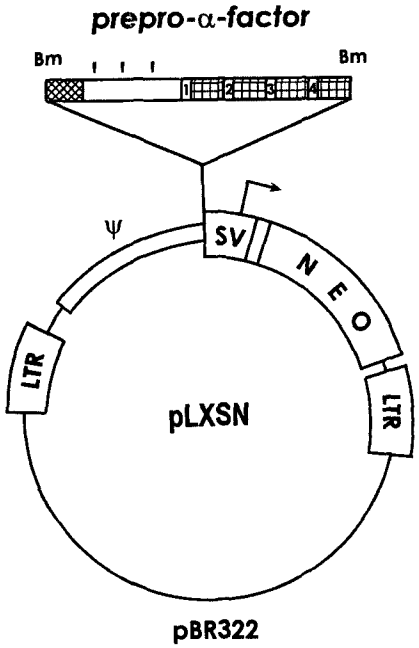

Fig. 1. Construction of α-factor: a retroviral vector with prepro-α-factor cDNA. A 586-bp BamHI fragment encoding yeast prepro-α-factor gene was ligated into mammalian expression vector pLXSN and designated pLαfSN. The expression of prepro-α-factor is under the control of the murine leukemia virus (MLV) long terminal repeat (LTR). The signal peptide of α-factor is indicated by the cross-striped boxes and the open boxes indicate the pro regions. The Glu-Ala or Asp-Ala spacer regions from prepro-α-factor are labeled with serial numbers and the coding region for mature α-factor is a cross-hatched box. Arrowheads indicate the three putative Asn-linked glycosylation sites.

processing machinery in GH_3 cells.

Fate of intracellular pro-α-factor

Fate of the newly synthesized glycosylated pro-α-factor in GH_3-α-factor cells was assessed by pulse-labeling the cells with ^{35}S-methionine, followed by chase incubations (Fig. 3, panel A). The polypeptide disappears from the cells in a linear fashion, declining to 8.4% of the initial pulse amount by 120 min of the chase. To look at the fate of the glycosylated pro-α-factor quantitatively, GH_3-α-factor cells were pulsed and chased for the indicated times and the immunoprecipitates from the cell lysates and media were compared after PNG-F treatment (Fig. 3, panel B). The naked pro-α-factor generated by PNG-F digestion can be detected in the extracellular medium after chase incubations. However, the amounts of the secreted pro-α-factor were much less than that of pro-α-factor disappeared from the cell. At this point, it was speculated that this heterologous precursor might be processed at its paired basic sites by prohormone processing enzymes located in the trans Golgi and secretory vesicles, producing α-factor itself or α-factor-related peptides. Alternatively, it might be degraded nonspecifically, either intracellularly or in the extracellular milieu.

Several approaches were used to distinguish between these two possibilities. To assess pro-α-factor

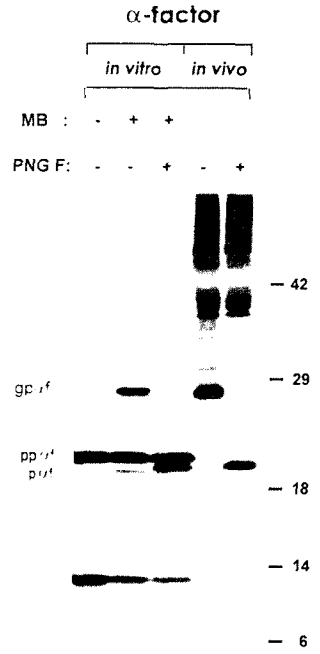

Fig. 2. Characterization of α-factor related-products in GH_3-α-factor cells. *In vitro transcribed prepro-α-factor message was translated in a rabbit* reticulocyte lysate system in the absence (lane 1) and presence (lanes 2, 3) of canine pancreatic microsomal membranes (MB). GH_3-α-factor cells (lanes 4, 5) were metabolically labelled for 30 min with 250 μCi/ml ^{35}S-methionine, after which the cell lysates were subjected to immunoprecipitation with anti-α-factor antibodies. Immunoprecipitates of *in vitro* synthesized and cell associated glycosylated pro-α-factor were digested with PNG-F. Immunoprecipitates were resolved on SDS-PAGE followed by fluorography. The migration of prepro-α-factor (ppf), signal peptide-cleaved naked pro-α-factor (pf) and core glycosylated pro-α-factor (gpf) are indicated. The positions of Mw markers are indicated in kilodaltons.

turnover in the ER or Golgi, chase incubation was performed at 15℃ or 20℃ (Fig. 4) to prevent vesicular

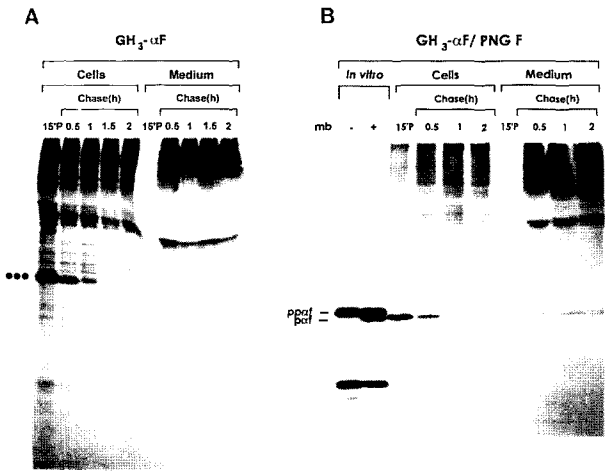

Fig. 3. The turnover of the pro-α-factor precursors in GH_3-α-factor. A, GH_3-α-factor cells were pulse-labeled for 15 min with 250 μCi/ml ^{35}S-methionine and chased for the indicated times. Cell lysate and medium were treated with anti-α-factor anti-sera and immune complexes resolved by SDS-PAGE on 15% gel. B, Duplicated samples of GH_3-α- factor cells were prepared, treated with PNG-F after immunoprecipitation and processed as above.

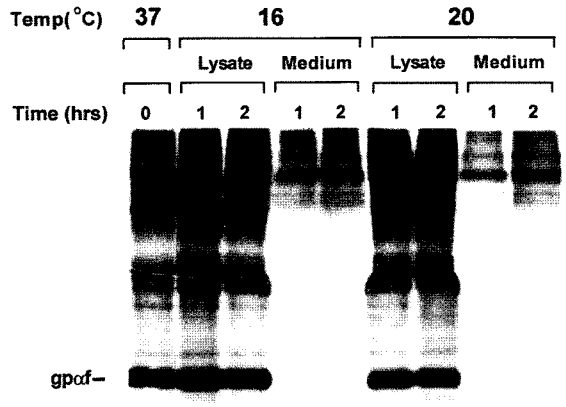

Fig. 4. Effects of temperature on turnover of pro-α-factor precursors. GH_3-α-factor cells were pulse-labeled with ^{35}S-methionine for 15 min at 37℃, after which the medium was replaced with chase medium equilibrated at the indicated temperatures (Temp). Incubation was continued for up to 2 h, at which time cell lysates were prepared, subjected to immunoprecipitation with anti-sera against α-factor, and processed as in Fig. 3.

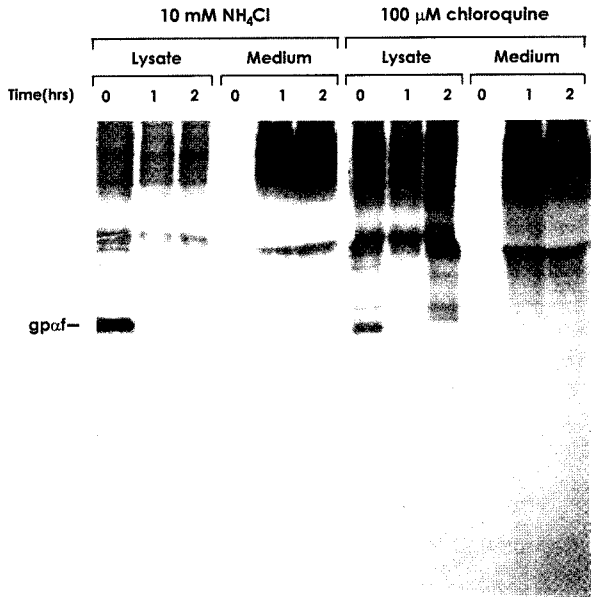

Fig. 5. Effects of lysosomotrophic agents on turnover of pro-α-factor precursors. GH_3-α-factor cells were preincubated either for 1 h in 50 μM chloroquine or for 30 min in 10 mM NH4Cl. The cells were then pulse-labeled for 15 min with ^{35}S-methionine in the presence of NH_4Cl (lanes 1-6) or chloroquine (lanes 7-12) and chased for the indicated times under identical conditions. At each time point, cell lysates and medium were treated with anti-α-factor antisera and the immune precipitates were resolved by SDS-PAGE.

transport from the ER to the cis Golgi apparatus and exit from the TGN, respectively (Saraste et al., 1984). Incubation of the cells at 15℃ or 20℃ for up to 2 h resulted in recovery of 91% and 82% of initially labeled pro-α-factor precursors, respectively. Since there was quantitative recovery of the pro-α-factor even after the prolonged incubation at 15℃ or 20℃, this suggests that very little precursor protein was degraded between exiting the ER and arriving at the distal Golgi apparatus.

To check the possibility of lysosomal degradative process, the cells were pulse-labeled for 15 min and chased for up to 2 h in the absence or presence of NH_4Cl or chloroquine, both of which are weak bases that increase the intralumenal pH of acidic compartments (Mellman et al., 1986), thereby inhibiting acid-requiring proteases (Fig. 5). Incubation of the cells at 37℃ in the presence of NH_4Cl or chloroquine resulted in recovery of ~10% of the pulsed pro-α-factor, indicating that disappearance of the pro-α-factor from the cells was not inhibited by lysosomotrophic agents at all. From these results, it was concluded that the turnover of α-factor precursors was not dependent on an acidic compartment, or lysosomal proteases. Taken togeter, it was concluded that the yeast pro-α-factor in GH_3 cells might be partly processed at its paired basic sites by prohormone processing enzymes and that the rest of it was secreted probably via the constitutive secretory pathway into the medium rather than degraded intracellularly or extracellularly.

Discussion

Since the transit from ER to plasma membrane is considerably longer in mammalian cells relative to yeast and is accompanied by a larger number of post-translational modifications, we predicted that the system employed in this study would afford an opportunity to further resolve the different stages in the intercompartmental transport and processing of this prohormone, i.e., yeast prepro-α-factor. Expression of other yeast glycoproteins in mammalian cells has revealed different degrees of conservation of the secretory function. Yeast CPY precursor cannot be translocated across mammalian membrane both *in vivo* and *in vitro*, unless its own signal sequence is replaced with a substituted signal which increases its hydrophobicity by replacing either one of its two glycine residues with a leucine (Blachly-Dyson et al., 1987). In contrast, expression of yeast invertase cDNA in Ψ-2 cells resulted in efficient intercompartmental transport and secretion of a signal peptide-cleaved, heavily glycosylated product (Bergh et al., 1987). Therefore, while the yeast translocation machinery clearly tolerates a patchy distribution of hydrophobic residues, the mammalian systems apparently require a more coherent arrangement. Based on the study of various signal peptides, von Heijne (1990) suggested that the minimal requirement for the central

core (or h region) is seven hydrophobic residues interrupted by no more than one serine, threonine, glycine or proline residue. The putative h region of the prepro-α-factor signal does meet these requirements. In fact, the hydrophobic region of yeast prepro-α-factor signal peptide consists of nine hydrophobic amino acids that are interrupted by one threonine residue. In addition, the intracellular precursor form of α-factor synthesized in *S. cerevisiae* is a glycosylated protein, i.e., all 3 putative glycosylation sites are actually glycosylated. When the protein is synthesized in rat pituitary GH_3 cells, the same numbers of carbohydrate chains are added as in yeast, indicating that the rat oligosaccharyltransferase recognizes the glycosylation signatures in the yeast protein very efficiently. Our results are yet another example where sequences from one organism appear to be functional in another (Bergh et al., 1987). It would be interesting to see whether signal sequence and proregion of other yeast proteins are also functional in higher eukaryoti system.

When GH_3 cells were pulse-labeled and chased to assess the fate of newly synthesized precursor proteins, the level of intracellular propeptide decreased with increased chase time, but the amount of secreted propeptide could not account for the disappearance of intracellular propeptide completely. Two possibilities exist for this difference, i.e., intracellular degradation or processing to a smaller peptide. Disappearance of the propeptide was insensitive to lysosomotrophic agents, but was inhibited at 16℃ or 20℃, suggesting that the turnover of the fusion constructs was not occurring in the secretory pathway to TGN or dependent on acidic compartments. From these results, it is concluded that a part of these heterologous precursors may be processed at its paired dibasic sites by prohormones processing enzymes located in TGN/secretory vesicles, producing small peptides and the residual unprocessed precursors were secreted into the medium rather than degraded intracellularly.

Considering the pH dependency of the two conversion endoproteases PC1/PC3 or PC2 *in vitro,* both enzymes display an acidic pH optimum, but only PC2 remains active even at neutral pH (Davidson et al., 1988). PC2 may process prepro-α-factor peptide hormone precursors. The possible other endoprotease which may process the pro-α-factor in GH_3 cells is furin, one of the mammalian homologues of the yeast Kex2 protease, which is a Ca^{++}-dependent serine protease with a subtilisin-like catalytic domain (Tsuneoka et al., 1993). Because weak bases such as NH_4Cl or chloroquine did not inhibit the cleavage, acidification of intracellular vesicles would not be necessary for the processing. This is consistent with the fact that furin reacts efficiently at neutral pH (Hatsuzawa et al., 1992). These results suggest that furin is another possible processing enzyme of pro-α-factor in various locations in the constitutive secretory pathway in GH_3 cells.

Many events specific to neuropeptide processing, such as cleavage at dibasic sequences, trimming of basic residues and carboxy-terminal amidation, take place after packaging of propeptide into dense core vesicles (DCVs) (Orci et al., 1987). Recent evidence, however, suggests that the initial endoproteolytic cleavage of some prohormones occurs earlier in the secretory pathway before or during the formation of DCVs. We show here that the newly synthesized pro-α-factor precursors are accumulated intracellularly and the processing for mature peptide is arrested at 20℃ along the secretory pathway. When the lysosomotrophic agents were present in the cells, the intracellular processing of pro-α-factor was not inhibited. Taken together, the present observations suggest that the processing step for pro-α-factor, which is critical for generating the mature peptides, does occur in any intracellular compartment after the prohormones leave TGN.

We have been investigating the expression of the yeast peptide hormone precursor prepro-α-factor in mammalian GH_3 cells to identify structural domains which might function in targeting molecules to the constitutive or regulated secretory pathway. Numerous studies (Burgess et al., 1987; Sevarino et al., 1989; Stoller et al., 1989; Thorne et al., 1990) have described the expression of heterologous precursors in different cells, in particular pituitary AtT-20 cells, GH_3 cells, and pancreatic islet RIN cells. A common observation from these experiments is that the unprocessed precursors were secreted constitutively. These results are partially consistent with our present results of prepro-α-factor precursors processing in GH_3 cells.

Acknowledgements

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