Immunocytochemical Analysis of Peptide Hormone Processing: Importance of the Positively Charged N-terminal Domain of Signal Peptide in Correct ER Targeting in Yeast Cells

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ABSTRACT. We used a morphological approach to determine the topogenic role of the signal peptide in mediating the ER translocation of yeast prepro- α -factor. In prepro- α -factor-somatostatin hybrids, changes in the N-terminal amino acid sequence from wild-type NH₂-Met-Arg-Phe (MRF) to NH₂-Met-Phe-Lys (MFK) caused a subtle difference in protein trafficking in yeast cells (12). Immunofluorescence microscopy on semithin cryosections and immunoelectron microscopy on ultrathin sections showed that the transposition of the charged amino acid at N-terminus caused the precursors to be associated with either nucleus or mitochondria. This suggests that the secretory proteins are mistargeted to the irrelevant organelles as the result of inefficient ER translocation. Structural aspects of nuclear or mitochondrial targeting proteins and common principles in membrane translocation systems account for the mistargeting of overexpressed mutant hybrid precursors that are not rapidly translocated into the ER. Based on our immunocytochemical study on individual cells, we propose here that the positively charged N-terminal domain of signal peptide is important not merely in the efficiency of ER translocation, but also in appropriate targeting of peptide hormone precursors in yeast cells where post-translational ER translocation is known to occur frequently.

Although most cellular proteins are synthesized in the cytoplasm, certain proteins are transported with high specificity and efficiency to a variety of locations in the cell. Some ultimately reside in membrane-bound organelles or outside the cell altogether. To enter the secretory pathway, precursor proteins must first be translocated into the lumen of endoplasmic reticulum (ER) (7). Targeting to the ER is specified by a signal peptide sequence contained within the polypeptide chain, usually found at the N-terminus of the protein. The signal peptide is in most cases a transient extension to the N-terminus of the protein and is removed by one of a small class of enzymes known as signal peptidases, once its targeting to ER is carried out.

Comparisons of known signal peptides indicated that they 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). Beyond this overall pattern, no

precise sequence conservation could be found, and it soon became obvious that signal peptides are highly variable, rapidly evolving structures (36). The idealized signal peptides with h-regions consisting of strings of typically eight to ten Leu residues have been found to function efficiently both in *Escherichia coli* (17) and *Saccharomyces cerevisiae* (39). Genetic approaches showed that the replacement of a residue in the h-region with either a hydrophilic amino acid or a proline affected the noticeable translocation efficiency, even though the effect was apparently less severe in *S. cerevisiae* (1). More recently, it became possible to detect slight variations in the mean lengths and amino acid compositions of the three regions between different groups of organisms (35). This ‘positive-hydrophobic-polar’ structure suggested that the signal peptides might bind efficiently with the receptors such as SRP or Hsp70 and lipid bilayers, possibly in a loop structure with the basic amino terminus bound to acidic lipid headgroups on the cytoplasmic face of the membrane. However, there is still no consensus as to whether the signal peptide interacts primarily with receptor proteins or with the lipid bilayer itself.

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A positively charged n-region is thought to be required for efficient ER translocation based on the results of many biochemical studies. The presence of positively charged lysine or arginine residues in the n-region is universal among bacterial signal peptides and is much preferred in signal peptides of higher organisms (36). Possibly the nonformylated and positively charged amino groups on the initiator Met of the latter can compensate for a lack of lysine or arginine in some cases. Bacterial signal peptides that lack basic residues or even have acidic residues in their n-region have been constructed by site-specific mutagenesis. Results showed slow export of the proteins, but no absolute requirement for positive charge (4, 21, 26, 34). The n-region of eukaryotic signal peptides have not been as extensively mutagenized. Secretion of two yeast proteins was found insensitive to the effects of point mutations, small deletions and potential secondary-structure-disrupting mutations within their signal peptides (3, 5, 16). This raises the question of whether the structural requirements of eukaryotic signal peptides are different from those of prokaryotic signal sequences, or whether the eukaryotic translocation apparatus is more flexible than that of *E. coli*. One study on yeast invertase failed to find any effect on secretion when various amino acid insertions were made in the n-region (5), whereas at least one report on amino acid transpositions in the yeast prepro- α -factor n-region suggest that the precise order of amino acids may be important in efficient translocation without affecting targeting (12).

We employed immunocytochemical analysis to determine the topogenic role of the signal peptide n-region during peptide hormone processing in yeast cells. We observed that the amino acid transpositions in the n-region of yeast prepro- α -factor affected the targeting of some secretory proteins to the irrelevant organelles such as nucleus or mitochondria. This result was confirmed by immunofluorescence on semithin cryosections and immunoelectron microscopy with ultrathin sections using various methods. We propose here that the correct structure of charged N-terminal residues in the signal peptide is important in the initial targeting of secretory proteins into the ER in yeast cells.

MATERIALS AND METHODS

Yeast Strains and Plasmids. The parent *Saccharomyces cerevisiae* yeast strain used was 20B12 (MAT α pep4-3 trp1). The construction of the two prepro- α -factor-somatostatin (SRIF) hybrid genes used has been described previously (19). These constructs were inserted into a multicopy yeast expression vector (pYE7) under the control of the PHO5 promoter and designated p52-1 (harboring wild type N-terminal sequence, MRF) and p82-1 (harboring mutant N-terminal sequence, MFK). A schematic representation of the correspond-

ing fusion proteins is shown in Fig. 1.

Cell Culture Conditions. Cells were grown to the middle or end of the logarithmic phase at 30°C on a rotary shaker in nutrient medium YPD (1% yeast extract, 2% polypeptone, and 2% glucose) or in synthetic complete medium SD (yeast nitrogen base + drop out mix). For immunofluorescence and immunoelectron microscopy, exponentially growing cells were inoculated into phosphate-depleted medium (2% glucose, 0.2% recrystallized asparagine, 1.5 g/l KH₂PH₄, 0.5 g/l MgSO₄, 0.33 g/l CaCl₂ · H₂O, 2 g/l (NH₄)₂SO₄, 0.1 mg/l KI, and drop out mix (33)) and incubated for 4 hr to induce the fusion construct.

Immunofluorescence Microscopy. Yeast cells after appropriate incubations were fixed for 1 hr at room temperature with 4% formaldehyde (methanol-free) and 0.5% glutaraldehyde and washed with 0.1 M phosphate buffer and trimmed to agar block. Cells were then cryoprotected by immersion in 2.3 M sucrose containing 20% polyvinylpyrrolidone overnight, mounted on aluminum nails and frozen in liquid nitrogen. Semithin sections (0.5 μ m) 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 with PBS, sections are treated with 10 mM glycine in PBS pH 7.4 to quench free aldehyde groups followed by incubation in 10% fetal calf serum in PBS for 30 min at room temperature to block nonspecific binding. Samples were then incubated with affinity-purified anti-SRIF antibody diluted in PBS containing 5% fetal calf serum (FCS) for 2 hr at room temperature and were washed three times in PBS/FCS. Sections are then incubated with a FITC-conjugated secondary antibody for 30 min at room temperature, washed, and mounted in 90% glycerol containing 1 mg/ml *p*-phenylenediamine and 1 μ g/ml DAPI for nuclear staining, and visualized with a Zeiss microscope through a 100 $\times$ oil lens as described by McCaffery and Farquhar (22).

Conventional Electron Microscopy. Yeast cells grown in various culture conditions were fixed with 3% glutaraldehyde in a buffer containing 0.1 M cacodylate, pH 6.8 for 2 hr. Prior to fixation, cells were treated with 0.1 M β -mercaptoethanol in 0.02 M EDTA (pH 8.0) to facilitate later removal of cell walls. After glutaraldehyde fixation, cell wall was removed by treating with Zymolase in 1 M sorbitol, pH 5.8. Cells were subsequently postfixed in 2% OsO₄ in 0.1 M cacodylate buffer for 30 min at 0°C. The cells were dehydrated with ethanol and propylene oxide, and embedded in Epon resin. Ultrathin sections (70 nm) of the cells were picked up on collodion coated grids, double-stained with uranyl acetate and lead citrate and observed under a JEOL 1200EXII electron microscope after carbon coating.

Immunoelectron Microscopy. Immuno-EM observation using affinity-purified anti-SRIF antibody was carried out in two different resins. For Epon embedding, cells were prefixed in 1% formaldehyde and 1% glutaraldehyde before cell wall removal and postfixed in OsFeCN (0.5% OsO₄, 0.8% potassium ferricyanide) (38). After dehydration in ethanol, cells were

embedded in Epon resin. For acrylic resin embedding, cells were prefixed in 4% formaldehyde. After cell wall removal, cells were dehydrated by ethanol and embedded in the Unicryl (27) or Lowicryl K4M. Serial sections were prepared with Ultracut J (Reichert-Jung) and picked up on formvar coated nickel grids. Immunocytochemical staining of Epon or acrylic resin embedded yeast cells was carried out according to Wright and Rine (38): The grids were floated on drops of 10% goat serum, 1% BSA, 0.5% Triton X-100 and 0.3% NP-40 in PBS and then transferred to droplets of affinity-purified anti-SRIF antibodies in the same solution. After washing, the grids were incubated with goat anti-rabbit IgG gold complex (15 nm in diameter, Amersham) and observed under a JEOL 1200EXII electron microscope after staining with uranyl acetate and lead citrate.

RESULTS

General morphological observation in various culture conditions. The parent *Saccharomyces cerevisiae* yeast strain 20B12 (MAT α pep4-3 trp1) and two yeast cell lines that express wild type 52-1 prepro- α -factor-somatostatin (SRIF) hybrid construct and mutant 82-1 (MFK) hybrid construct (Fig. 1) were used throughout this study. The initial aim of these observation was to look at the intracellular structures related to the processing of peptide hormones. Yeast is known to have quite different ultrastructural features depending on the growth condition. For the high expression of the prepro- α -factor-SRIF precursors (Fig. 1), yeast cells had to be grown in phosphate depleted medium for several hours. To recognize the proper subcellular organelles involved in the secretory pathway in this nutrient-deficient culture condition, we first observed cells grown in normal synthetic media without tryptophan to maintain the plasmid that has the *trp* gene as an auxotrophic marker. Ultrastructures such as nucleus, mitochondria, vacuole and other organelles known to be involved in the protein transport pathway were observed (Fig. 2). In yeast cells containing 52-1 (MRF) hybrid precursors (Fig. 2, a1 and a2), various vesicular structures as well as autophagic bodies are well visualized in the vacuole. Yeast cells harboring 82-1 (MFK) hybrid precursors (b1 and b2) have almost the same ultrastructures except that they are slightly larger than wild type or 52-1 containing cells. Interestingly, several different kinds of vesicular structures are accumulated in the vacuole in large amounts, even in wild type 20B12 cells. Cells with p52-1 hybrid precursors that have normal signal peptide (MRF) or p82-1 hybrid precursors with mutated signal peptide (MFK) showed no difference in ultrastructures. These membranous structures are ascribed to the proteinase A-deficient (pep4-3) phenotype of the host 20B12 cells (32).

To differentiate the morphological changes affected

by the nutritional condition, we next observed the same cells grown in rich (YPD) medium (Fig. 3). As expected, autophagic bodies completely disappeared in the vacuoles of all three cell lines observed. The vacuoles were transparent and contained few detectable particles in wild type 20B12 (a and b) and 52-1 cells (c and d). But in 82-1 cells (e and f), several spherical bodies and vesicular structures have remained in distinguishable amounts. This could indicate that mutated hybrid precursors affected the accumulation of membranous particles in the vacuole and hence these vacuolar vesicles may be related with the processing pathway of mutated peptide hormone precursors.

Hybrid precursors from 52-1 (MRF) follows correct secretory pathway. To look at the intracellular localization of somatostatin precursors, we next employed immunofluorescence microscopy (Figs. 4, 5) and immunoelectron microscopy (Fig. 6). Immunofluorescence or immuno-gold staining was not detectable in wild type 20B12 cells except few nonspecific bindings (invisible immunofluorescence or about one gold particle per fifty observed cells) and, hence were used as a criteria for the nonspecific or artifactual results in every experiment. In cells harboring the 52-1 construct, hybrid precursors are mainly observed in the ER, vacuole and periplasm (Fig. 4a, b, c and Fig. 6a, b, c), indicating that the precursors with wild type N-terminus of α -factor (MRF) are located in the subcellular organelles involved in the secretory pathway. This result implies that when 52-1 prepro- α -factor-SRIF precursors with the wild type n-terminal amino acid sequence of yeast prepro- α -factor was synthesized in the cytoplasm, they were very effi-

Fig. 1. Structure of prepro- α -factor-somatostatin hybrid precursors: The structures of anglerfish preprosomatostatin (ppSRIF), yeast prepro- α -factor (prepro α FAC) and the prepro- α -factor/somatostatin hybrid precursors are shown. The boxes at left indicate the signal peptides. The straight line and hatched lines indicate the pro regions of ppSRIF and pp- α -factor, respectively. The diamond symbols denote three N-glycosylation sites in the pro region of α -factor precursor. Amino acid sequence of the N-termini of the hybrid precursors are shown with single letter code above the signal peptide. The numbers in boxes indicate the spacer sequence of pp- α -factor. Construction of the DNA encoding the 82-1 and insertion into multi-copy yeast expression plasmid pYE7 and construction of 52-1 by site-directed mutagenesis from p82-1 has been detailed previously (19).

Fig. 2. Electron micrographs of yeast cells grown in a nutrient deficient condition. Yeast cells were incubated in a synthetic complete medium without tryptophan, and then processed for conventional EM observation as described in Materials and Methods. Cells with p52-1 (a1 and a2) and p82-1 (b1 and b2) constructs showed same ultrastructures as wild type 20B12. N, nucleus; M, mitochondria; V, vacuole; AB, autophagic body. Bars 0.5 μ m.

ciently transported to the correct secretory pathway and secreted in a short time as mature somatostatin molecules. This is in accordance with the biochemical results of rapid processing (19).

Hybrid precursors are associated with irrelevant organelles in 82-1 (MFK) harboring cells. As shown in Fig. 5 and Fig. 6, subcellular localizations of mutant hybrid precursors in 82-1 (MFK) cells are quite different from those of 52-1 (MRF) cells. In most cases, hybrid precursors are located in subcellular organelles in the secretory pathway as in 52-1 cells (Fig. 5a and Fig. 6d). But in some cells, more immunofluorescence and gold particles are observed to be associated with the nucleus

and mitochondria than with other subcellular organelles involved in the secretory pathway (Fig. 5b, c and Fig. 6e, f, g, h).

These results imply that mutant hybrid precursors with altered position at the N-terminus of the positively charged amino acid can be mistargeted to the nucleus or mitochondria. It may be that some mutant hybrid precursors normally follow the secretory pathway, whereas others with inefficient translocation ability remain in the cytoplasm or cytoplasmic face of ER membranes, which are subsequently mistargeted to the irrelevant organelles such as the nucleus and mitochondria.

Accumulation of somatostatin in the nucleolus and

Fig. 3. Electron micrographs of yeast cells cultured in a rich medium. Wild type 20B12 cells (a and b), normal p52-1 (c and d) and mutant p82-1 containing cells (e and f) were grown in YPD medium and processed for EM observation as in Fig. 2. Autophagic bodies do not appear in the vacuole. Membranous structures are more abundantly observed in the vacuole of p82-1 (MFK) containing cells. Bars 0.5 μ m.

Table I. STATISTICAL ANALYSIS ON THE LOCALIZATION OF THE IMMUNOGOLD PARTICLES IN EACH CELL LINES.

Subcellular Localization	20B-12	52-1	82-1
Nucleus	2	3	102
Mitochondria	1	2	47
Secretory Pathway	4	478	455

Gold particles were counted on lowicryl and epon sections. Fifty cells in each cell lines were chosen for counting.

mitochondrial inner membrane was confirmed by serial section analysis and various immunoelectron microscopic methods. To confirm the mistargeting of 82-1 hybrid precursors, serial ultrathin sections were prepared, treated with affinity-purified anti-SRIF antibodies and then processed for electron microscopy (Fig. 7). The serial-section method showed that 82-1 (MFK) hybrid precursors are indeed associated with the nucleolus and mitochondrial intermembrane space. These results confirm that hybrid precursors with mutated N-terminus are transported to the nucleus (a, b, c) or mitochondria (d, e, f) in addition to normal ER pathway.

As the mechanism of nuclear (20, 23) and mitochondrial targeting (11) depends on the positively charged basic amino acids at the N-terminal region of the target molecule and both share the common principles of protein translocation across membranes with ER translocation system (28), nuclear and mitochondrial accumulation of mutated 82-1 hybrid precursors can be explained as a result of mistargeting caused by the transposition of the basic amino acid, lysine, from second to third at the N-terminus.

We also tried to test if the association of gold particles in our observation resulted from the artifact during the sample preparation. For that, various sample preparation methods with different resins were compared in maintaining the antigenicity against preservation of structural integrity (Fig. 8). Yeast cells were fixed in mild condition and embedded in hydrophilic Unicryl (27) or Lowicryl K4M resin suitable for better preservation of antigenicity. Slightly more gold particles are found to associate with the subcellular organelles of the secretory pathway in both 52-1 (a), and 82-1 cell (b, c, d, e) than in Epon embedded samples shown in Fig. 6. However, the overall immuno-gold staining pattern was not much different from that of Epon-embedded samples (f). Many gold particles are also associated with the nucleolus and mitochondria only in 82-1 cells (Table I). Since other hydrophilic resins are known to have only minor differences in preserving antigenicity (29), we concluded that the different sample preparation methods or resins used did not affect the immunoelectron microscopic localization of prepro- α -factor-SRIF precursors in the experiment.

DISCUSSION

We analyzed the effect of the altered N-terminal charged amino acids of signal peptide in yeast cells by immunocytochemical approach. To elucidate accurate morphological aspects in yeast cells, we first observed the general morphology of the wild type 20B12 (MAT α pep4-3 trp1) cells. In limited nutritional conditions (Fig. 2), cells showed many membranous structures as well as the autophagic bodies, characteristic structures accumulating in the vacuole due to proteinase A deficiency (34). In rich medium (Fig. 3), autophagic bodies disappeared completely in all the cells with various constructs. Vesicular structures inside the vacuole are also markedly reduced in the wild type and 52-1 (MRF) cells, but not in 82-1 (MFK) cells that have altered amino acid sequence in the signal peptide region. This difference may be related with the processing of hybrid precursor from 82-1 construct with the altered N-terminus.

To investigate the effect of the transposition of charged amino acid at the N-terminus of the signal peptide, we next employed immunofluorescence microscopy on the semithin cryosections and immunoelectron microscopy. Considering that yeast cell is very compact and has a thick cell wall, we employed semithin cryosectioning and ultrathin serial sectioning. This approach also excluded the substantial problems in relating the general immunofluorescence image of the subcellular structures carrying the antigen against the detailed organization of the membrane compartments involved in the biosynthetic or endocytic routes (13). SRIF molecules are found in the subcellular organelles of the secretory pathway in both the p52-1 (MRF) and p82-1 containing cells (Fig. 4, Fig. 5a, and Fig. 6a, b, c, d). The result indicates that once the α -factor-somatostatin hybrid precursors having the wild type or mutated signal peptide were synthesized in the cytoplasm, they are transported to the correct secretory pathway, processed and secreted as mature somatostatin molecules (Fig. 4 and Fig. 6a, b, c). These results are in good agreement with the biochemical analysis of the same hybrid precursor processing in yeast cells (19).

However, immunofluorescence and immuno-gold staining pattern was quite different in some of the 82-1 (MFK) cells when compared with 52-1 cells (Fig. 5b, c, Fig. 6e, f, Fig. 7, and Fig. 8). Our previous study (19) implied that unglycosylated mutant hybrid precursors are accumulated intracellularly. In the present report, we have provided various evidence that some 82-1 (MFK) hybrid precursors are accumulated either in the nucleus or mitochondria.

What could lead to this mistargeting? Considering that post-translational ER translocation is common in yeast, and that our previous biochemical study (19) showing ER translocation and glycosylation of our hy-

Fig. 4. Localization of 52-1 prepro- α -factor-SRIF hybrid precursors by immunofluorescence microscopy on semithin cryosections. Semithin cryosection of yeast cells shows that 52-1 hybrid precursors are found in the secretory pathway such as the ER, Golgi and periplasm (a, b, c). In some cells, large amounts of precursors are also found in the vacuole (a, arrow head). Semithin cryosection ($0.5\ \mu\text{m}$) were cut after the yeast cells harboring p52-1 construct were incubated in a phosphate depleted medium to induce the prepro- α -factor-SRIF hybrid construct under the control of *PHO5* promoter, and then prepared for immunofluorescence microscopy as described in Materials and Methods. DAPI staining (a', b', c') indicates the location of nucleus (arrows). Bar $10\ \mu\text{m}$.

Fig. 5. Localization of mutant 82-1 (MFK) prepro- α -factor-SRIF precursors by immunofluorescence microscopy on semithin cryosections. (a) Most 82-1 (MFK) hybrid precursors are localized to the secretory pathway (b and c). Semithin cryosection of yeast cells harboring 82-1 (MFK) demonstrating mistargeted hybrid precursors to the nucleus. Digitally captured image obtained with anti-SRIF (a and b) was superimposed with DAPI staining (a' and b') using NIH scion image to confirm the complete colocalization on the nucleus (arrows). Yeast cells were incubated and prepared as described in Fig. 4. Bar 10 μ m.

Fig. 6. Localization of prepro- α -factor-SRIF hybrid precursors by immunoelectron microscopy. Yeast cells were incubated in a phosphate depleted medium for the overexpression of prepro- α -factor-SRIF hybrid construct under the control of *PHO5* promoter and then prepared as described in Materials and Methods for immunoelectron microscopy. In cells harboring p52-1 construct, gold particles are mainly found in the nuclear laminae, ER, vacuole, and periplasm (a, b, c). More gold particles are associated with the nucleolus (d, e) and mitochondrial inner and outer membrane (e, f, g, h) in some of 82-1 (MFK) containing cells. N, nucleus; M, mitochondria; V, vacuole. Bars 0.5 μ m.

Fig. 7. Visual confirmation of mistargeting of 82-1 (MFK) hybrid precursors by serial-section analysis. With the same sample containing p82-1 construct as in Fig. 5, serial sections were obtained, treated with anti-somatostatin antibodies and processed for electron microscopy. Arrow indicates sequential sectioning in the same yeast cell. Many gold particles are associated with the nucleolus (a, b, c) and mitochondrial innermembrane space (d, e, f) in every section. Bars 0.5 μ m.

Fig. 8. Immunoelectron microscopy of the lowicryl sections shows same mistargeting pattern in 82-1 (MFK) containing yeast cells. The p52-1 (a) and p82-1 containing (b, c, d, e, f) yeast cells were incubated as in Fig. 5 and fixed in mild condition using 4% formaldehyde without postfixation and embedded in hydrophilic Unicryl and Lowicryl K4M (a, b, c, d, e), or Epon resin (f). Sections were immunolabeled as in Fig. 5. Slightly more gold particles are found than in Fig. 5 without changing the immuno-gold staining pattern. N, nucleus; M, mitochondria; V, vacuole. Bars 0.5 μ m.

brid molecules may be posttranslational events, possible explanation for this mistargeting is at hand. In addition, analogous to the fact that overexpressed fibroblast growth factor (FGF3) was shown to be directed to either the nucleus or the secretory pathway (18), our result could be another example showing the influence of the competing signals within the same polypeptide. One may consider the following two aspects. [1] Structural similarity of targeting molecule: Deduced chemical structure analysis of the wild type (52-1) and mutant (82-1) signal peptide suggests that positively charged residue can be extended more outward in mutant molecules by virtue of the proline at the fourth amino acid position. From this feature, we may be able to explain the nature of mistargeting of the hybrid precursors to nucleus and mitochondria. [2] Similarity in protein translocation systems across membranes (28): Resemblance in protein translocation systems of each compartment strongly suggests that the factors involved in ER translocation and other organelles such as nucleus and mitochondria may compete for the mutated signal peptide.

Firstly, nuclear protein import is mediated by short amino acid sequences called nuclear localization signal. Most nuclear localization signals (NLS) contain a high proportion of basic amino acid residues without obvious consensus (8, 10). Functional shuttling carriers (25) and the GTP-binding proteins (24) are also required for nuclear translocation similar to the ER translocation. In ER translocation, with the signal recognition particle (SRP) (14) acting in the process, many evolutionarily conserved components and the GTP-binding proteins of protein translocation complex are essential.

In mitochondrial targeting, positively charged amino acids at the N-terminus also play an essential role in binding to the molecular chaperon Hsp70 (11). Cytosolic Hsp70 families provide important functions in mitochondrial protein targeting along with the various molecular chaperons located in mitochondrial matrix (31). The role of the Hsp70 class of chaperons in maintaining the translocation competence of a subset of proteins is intriguing since they are not compartment specific. For example, the cytosolic chaperon SSA and its cooperating factor YDJ1 of the Hsp70 family are implicated not only in the post-translational translocation of prepro- α -factor into the ER, but also in the import of several precursors into the mitochondrion in yeast cells (6, 9, 37). Molecular chaperons such as cytosolic Hsp70 family (15) and ER membrane-bound calnexin (2) may provide SRP-independent targeting mechanisms into the ER lumen. These facts imply that mutant hybrid molecules which have altered position of positively charged amino acid can be targeted to the nucleus and mitochondria by the common principles of protein translocation across membranes (28).

When a defect in protein translocation was introduced by a mutation in *Sec61*, which encodes a key component of a multisubunit protein translocation apparatus of the ER, the target protein was shown to be conjugated with ubiquitin at the endoplasmic reticulum in *Saccharomyces cerevisiae* (30). Since the secretory proteins that are not transported into the right compartment are readily degraded by the ubiquitin pathway, they cannot be mistargeted to another subcellular locations without special care of appropriate guide molecules. This also confirms that our results support the crucial role of positively charged structure in the n-region of signal peptide.

Taken together, we suggest that the important role of signal peptide in correct ER targeting is not affected merely by the existence of basic amino acids at N-terminus (36), but by the correct structure or position of the positively charged amino acid as in our experiments. We propose here that the positively charged N-terminal domain (n-region) of signal peptide is important not only in the efficient ER translocation, but also in proper targeting of peptide hormone precursors in yeast cells where post-translational ER translocation commonly occurs.

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