Heterologous Expression of Human Cholecystokinin in *Saccharomyces cerevisiae*

EVIDENCE FOR A LYSINE-SPECIFIC ENDOPEPTIDASE IN THE YEAST SECRETORY PATHWAY*

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Precursors of the human regulatory peptide cholecystokinin (CCK) have been expressed in *Saccharomyces cerevisiae*, and the post-translational processing of secreted CCK-related products analyzed. Recombinant plasmids expressing native human prepro-CCK and a hybrid molecule encompassing the prepro leader of the yeast α-mating pheromone fused to pro-CCK were examined. The latter construct resulted in considerably higher levels of pro-CCK secretion and was therefore analyzed in more detail. Two of the protein modifications essential for CCK bioactivity, C-terminal α-amidation and tyrosyl sulfation, were not detected in *S. cerevisiae*. Proteolytic cleavage of pro-CCK occurred C-terminally of three basic sites; (i) Arg¹⁰⁵-Arg¹⁰⁶ which, upon exposure to carboxypeptidase activity, leads to the production of glycine-extended CCK; (ii) Arg⁹⁵ to produce CCK-8 related processing intermediates; and (iii) Lys⁸¹ resulting in CCK-22 related products. To elucidate which protease(s) are involved in these endoproteolytic cleavage events, pro-CCK was expressed in yeast mutants lacking various combinations of the Mkc7, Yap3, and Kex2 proteases. Only in *S. cerevisiae* strains deficient in Kex2 function was any of the above mentioned pro-CCK cleavages abolished, namely processing at the Arg¹⁰⁵-Arg¹⁰⁶ and Arg⁹⁵ sites. This suggests that mammalian Kex2-like serine proteases may process pro-CCK at single arginine residues. Our data suggests that an as yet uncharacterized endoprotease(s) in the *S. cerevisiae* secretory pathway is responsible for the lysine-specific cleavage of pro-CCK.

Cholecystokinin (CCK)¹ is a vertebrate neurohormonal peptide which controls a wide variety of functions, predominantly in the digestive tract and the brain (for review see; Crawley and Corwin (1994)). During precursor maturation pro-CCK is subjected to a number of post-translational modifications, including C-terminal α-amidation, tyrosyl sulfation, and endoproteolytic processing (Rehfeld et al., 1988). Bioactive CCKs possess the same amidated C terminus which results from endoproteolytic cleavage C-terminally of the dibasic Arg¹⁰⁵-Arg¹⁰⁶ residues followed by removal of these basic amino acids by carboxypeptidase digestion, exposing Gly¹⁰⁴ to peptidyl-monooxygenase activity (Fig. 1A). In contrast, endoproteolytic processing of pro-CCK is far more heterogeneous at the N terminus with examples of CCK-58, -39, -33, -22, -8, and -5 having been identified in various tissues or plasma samples (Fig. 1A) (Rehfeld and Hansen, 1986; Rehfeld et al., 1988; Eberlein et al., 1992). Many of these forms of CCK are produced via cleavage of pro-CCK C-terminally of single arginine residues (Rehfeld and Hansen, 1986; Eberlein et al., 1992). However, CCK-22, the predominant hormonal form of CCK, is produced by endoproteolytic processing at Lys⁸¹. Consequently this lysine processing event is of crucial significance for understanding the biology of CCK (Liddle et al., 1984, 1985; Cantor and Rehfeld, 1987; Rehfeld, 1994; Paloheimo and Rehfeld, 1995).

Many foreign proteins have been expressed in yeast (Hadfield et al., 1993). Often these proteins are directed through the yeast secretory pathway, either by the endogenous N-terminal signal sequence of the protein or via production of a hybrid protein which includes the leader region of a secreted yeast preproprotein. Passage through the secretory pathway exposes proteins to a number of post-translational capabilities, including disulfide bridge formation, glycosylation and proteolytic processing (Hadfield et al., 1993). The best characterized yeast endopeptidase is the Kex2 serine protease, which was identified for its ability to cleave precursors of the α-mating pheromone and killer toxin at dibasic sites (Julius et al., 1984). Overexpression studies in *kex2* mutants resulted in the isolation of the yeast aspartyl protease (Yap3), which is able to rescue the mating deficiency of *kex2* deficient *Saccharomyces cerevisiae* (Egel-Mitani et al., 1990). More recently a third endopeptidase, Mkc7, with considerable homology to Yap3 has been detected (Komano and Fuller, 1995). This protease, which was isolated as a multicopy suppressor of the cold sensitive growth phenotype of *kex2* mutants, has the ability to cleave an internally quenched fluorogenic substrate C-terminally of a Lys⁸¹Arg site (Komano and Fuller, 1995).

In the present study, we have examined the extent of post-translational processing of human pro-CCK expressed and secreted from *S. cerevisiae*. Evidence was found for an, as yet, uncharacterized endoprotease in yeast which is able to cleave CCK at a single lysine residue to produce CCK-22, a predominant form of circulating CCK in mammals.

MATERIALS AND METHODS

Yeast Strains, Transformations, and Growth Conditions—Yeast strains used in this study are listed in Table I. *S. cerevisiae* grown in YPD medium were transformed with plasmid DNA using the lithium acetate procedure (Ito et al., 1983). Transformants were selected and

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¹ The abbreviations used are: CCK, cholecystokinin; Yap3, yeast aspartyl protease 3; MFA1, the prepropeptide of the yeast α-mating pheromone; PCR, polymerase chain reaction; RIA, radioimmunoassay.
propagated at 30 °C in synthetic complete medium lacking uracil (Sherman, 1991).

**Plasmid Constructs**—A full-length cDNA clone encoding human prepro-CCK was generously supplied by Karin Pedersen. The *S. cerevisiae* α-mating pheromone 1 gene (MFα1) was kindly provided by Ira Herskowitz (Kurjan and Herskowitz, 1982). Two pro-CCK expression plasmids were constructed in the URA3-2α plasmid pJ399, in which gene transcription is driven by the yeast glyceraldehyde-3-phosphate dehydrogenase promoter (Bitter and Egan, 1984) and terminated by the yeast TPI1 termination signal (Alber and Kawasaki, 1982). Plasmid pJ399 was constructed from pYES2.0 (Invitrogen).

The coding region of human prepro-CCK was amplified by PCR using the *Pvu* II DNA polymerase (Boehringer Mannheim). The forward primer (5'-ACGTTAAATCTTAGATGCATGAAGCTGTTTTCGCT-3') resulted in an EcoRI site 10 nucleotides upstream of the start codon, while the reverse primer (5'-CTCACTGGTTTGAGGTGTAATTACTACGTTCA-3') introduced an XbaI site immediately downstream of the TAG stop codon. The PCR product was purified after agarose gel electrophoresis using the Sephaglas Band Prep procedure (Pharmacia), cleaved with EcoRI and XbaI (Boehringer Mannheim), and cloned into pJ399.

To ensure high secretion levels of CCK, we constructed a second expression plasmid where the sequence encoding the prepropeptide of the MFα1 yeast α-factor (Kurjan and Herskowitz, 1982) was fused to the coding region of prepro-CCK (amino acids 91–115) (Fig. 1B). This plasmid was constructed by a two-step process. First, the yeast MFα1 gene was amplified by PCR (forward primer, 5'-ACGTTAACATGGAAATGAGATTTCCTTCAATTTTTAAGCAG-3'; reverse primer, 5'-ACGTTACATGACTGGAGGTGTAATTACTACGTTCA-3') and cloned into pJ399 as described above. The resulting plasmid was cleaved with HindIII and XhoI to remove the coding region of the four tandem α-factor repeats, but leaving the sequence encoding the MFα1 prepropeptide. The region encoding pro-CCK was amplified using PCR (forward primer, 5'-ACGTTACTAGAGGGGAGTCACTATCTGCGGC-3'; reverse primer was the same as used for amplification of the coding region of prepro-CCK) resulting in a product with a 5' HindIII site and a 3' XhoI site that could be ligated into the pJ399 plasmid containing the coding region of prepro-MFα1. The resulting plasmid encodes a hybrid protein that consists of the first 89 amino acids of prepro-MFα1 and the last 85 amino acids of prepro-CCK starting at Ser113 (Fig. 1B). It was expected that pro-CCK would be liberated in the yeast secretory pathway by Fex2 cleavage C-terminally of the Lys-Arg site in the α-factor prepropeptide, followed by two dipeptidyl aminopeptidase digestion steps to remove the Glu-Ala-Glu-Ala sequence (Fig. 1B) (Hadfield et al., 1993). To ensure the coding nucleotides had not been altered during the PCR steps, plasmid inserts were exposed to double stranded DNA sequencing with the dyeoxy chain termination method using the Sequenase 2.0 DNA sequencing procedure (U. S. Biochemical Corp.).

**Radioimmunoassays**—Radioimmunoassay (RIA) analysis was used to detect and partially characterize pro-CCK products secreted from yeast transformants. Four monospecific antisera were utilized which binds various processing intermediates of CCK were utilized; Ab 3208, Ab 7270, and Ab 89009 (Table II). Measurements were performed as described previously (Rehfeld, 1978a; Hilsted and Rehfeld, 1986; Falloheimo and Rehfeld, 1984), either directly on the liquid medium used for propagation of yeast transformants or on fractions collected from gel chromatography. The molecules used as tracers and standards in RIA experiments are presented in Table II.

The presence of CCK which had not been processed at the Arg105, Arg106 site, referred to as C-terminally extended CCK, was detected by RIA with Ab 3208 after the samples had been treated with trypsin and carboxypeptidase B (T/C). Trypsin cleaves C-terminally of the Arg105 site and carboxypeptidase B removes these residues leaving glycine-extended CCK. This procedure involves incubating the sample with an equal volume of 2 mg/ml trypsin (Boehringer Mannheim) in 0.1 M sodium phosphate buffer (pH 7.5) at room temperature for 30 min. The reaction was terminated by boiling for 10 min, after which the samples were cooled to room temperature. Carboxypeptidase B (Boehringer Mannheim) was then added to a final concentration of 100 μg/ml, the reaction was incubated at room temperature for 30 min, and terminated by a second boiling step. Processing products of pro-CCK larger than CCK-22 were detected by RIA analysis with Ab 89009 after samples were exposed to trypsin as described above.

**Gel Chromatography**—Yeast transformants grown to late exponential phase were centrifuged at 3,000 × g for 5 min to collect the cells and 100–500 ml of the culture supernatant was loaded directly onto a Sephadex G-50 superfine (Pharmacia) column (1 × 100 cm) at 4 °C. The sample was eluted in VBA buffer (20 mM barbital buffer, 0.6 mM thiomersal, and 0.11% bovine serum albumin) at a flow rate of 2.4 ml/h, with 1-ml fractions being collected every minute. Sulfated CCK-8-Gly and non-sulfated CCK-8-Gly (Cambridge Research Biochemicals) were utilized as reference standards and in standards in RIAs experiments are presented in Table II.

**Anion exchange chromatography** was conducted on a 5 × 50-mm Mono-Q HR/R anion exchange column (Pharmacia) which had been pre-equilibrated with 20 mM sodium phosphate buffer (pH 8). The column was eluted with a gradient from 0 to 20% acetic acid over 70 min, with 1-ml fractions being collected every minute. Sulfated CCK-8-Gly and non-sulfated CCK-8-Gly (Cambridge Research Biochemicals) were utilized as reference standards and in standards in RIAs experiments are presented in Table II.
were used for calibration. Samples were dried under vacuum and resuspended in VBA buffer before RIA analysis.

Purification of CCK Secreted from Yeast Transformants—A five-step procedure was utilized to purify CCK secreted from transformants of S. cerevisiae.

Step 1: the wild type yeast YNG318 transformed with the plasmid encoding the hybrid prepro-\textit{MPFa}l-pro-CCK construct was grown in 200 ml of selective media to late exponential phase and cells removed by centrifugation at 3,000 \( \times g \) for 5 min. The supernatant was adjusted to pH 3 with trifluoroacetic acid and centrifuged at 10,000 \( \times g \) for 10 min. The cleared supernatant was loaded onto five Sep-Pak plus C18 columns (Waters) connected in series, which had been pre-equilibrated with 10 ml of 0.5% trifluoroacetic acid in acetonitrile followed by 10 ml of 0.5% trifluoroacetic acid in \( \text{H}_2\text{O} \). Material was passed through the columns at a flow rate of 2 ml/min. The column was eluted sequentially with 10-ml solutions of 0.5% trifluoroacetic acid in 0, 10, 30, 50, 70, 90, and 100% acetonitrile. The fractions collected were diluted in distilled water and analyzed by RIA using Ab 3208.

The same procedure was utilized with the \textit{mhc7} yap3 (HKY24) and \textit{kex2} yap3 (ME938) double mutants expressing the prepro-\textit{MPFa}l-pro-CCK construct. However, the starting quantity was 2 liters of culture medium and CCK-immunoreactive fractions were detected using RIA analysis with Ab 89009 and/or Ab 3208 after the samples had been treated with trypsin and carboxypeptidase B.

Step 2: immunoreactive fractions from Sep-Pak purification were concentrated under vacuum to a final volume of 1 ml, adjusted to pH 8 with 3 N NaOH and loaded onto a Sephadex G-50 superfine column as described previously.

Step 3: the next stage of purification involved anion exchange chromatography on a Mono-Q column and was performed as outlined above.

Step 4: the fractions containing CCK immunoreactivity were further processed by reversed phase high performance liquid chromatography using a C18 column (2.1 \( \times 150 \) mm) (Vydac) at 50 °C, eluted at 0.2 ml/min with a gradient from 0.1% trifluoroacetic acid in acetonitrile (0.5%/min). Fractions were collected every 0.5 min.

Step 5: selected immunoreactive fractions from Step 4 were subjected to a further reversed phase high performance liquid chromatography step on a Vydac C18 column using the same solvents but employing a gradient of 0.2%/min. Peak fractions were collected manually (monitored at 214 nm).

Mass Spectrometry and Peptide Sequencing—Molecular masses of purified peptides were determined by matrix-assisted laser desorption mass spectrometry performed in a Bifix instrument (Bruker-Franzen) using \( \alpha \)-cyano-4-hydroxy cinnamic acid as a matrix. Both positive and negative ions were analyzed in the linear mode using external calibration to give an accuracy of 0.1%. Individual peptides were subjected to amino acid sequence analysis using an automatic protein sequencer (Procise 494A, Applied Biosystems) equipped with an on-line high performance liquid chromatography system for detection of the amino acid phenylthiohydantoins.

Metabolic Labeling and Immunoprecipitation—Transformants of the YNG318 strain expressing the prepro-\textit{MPFa}l-pro-CCK fusion peptide were grown overnight at 30 °C in selective media. The next morning the cells were collected by centrifugation and resuspended in 500 \( \mu \)l of synthetic complete media lacking uracil and methionine, and supplemented with 500 \( \mu \)Ci of \( \text{l}^{-}[35\text{S}] \)methionine (Amersham). After the cells were incubated for a further 30 min at 30 °C they were washed twice in 50 mM Tris-HCl (pH 7.4) before being resuspended in 50 mM Tris-HCl (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride, 50 \( \mu \)g/ml apro tinin, 20 \( \mu \)g/ml leupeptin, and 2 \( \mu \)g/ml pepstatin. An approximate equal volume of pre-chilled acid washed glass beads (Sigma) was added and the sample vortexed 10 times for 30 s with cooling on ice between each vortexing. SDS was then added to a final volume of 1% and the cell lysate boiled for 3 min. An equal volume of 2 \( \times \) sample buffer (100 mM Tris-HCl (pH 8.3), 380 mM NaCl, 12 mM EDTA (pH 8), 5% Triton X-100) was included and a cleared supernatant prepared by centrifugation at 13,000 \( \times g \) for 10 min at 4 °C.

Immunoprecipitations were performed at 4 °C for 2 h following the addition of 5 \( \mu \)l of Ab 7270 which binds to glycine extended CCK (Table II). Immune complexes were bound to Protein A-Sepharose (Pharmacia) during a second 2-h incubation at 4 °C with gentle agitation. The sample was then centrifuged at 13,000 \( \times g \) for 10 s and the pellet washed 8 times in 1 \( \times \) sample buffer, boiled for 5 min, and loaded directly onto a Sephadex G-50 superfine column. Radiolabeled CCK was detected in the column fractions by a combination of liquid scintillation counting and RIA analysis utilizing Ab 3208.

RESULTS

Secretion of CCK from Yeast Transformants—Transformants of the YNG318 strain expressing prepro-CCK and the prepro-\textit{MPFa}l-pro-CCK fusion protein were grown to late exponential phase and the culture media was subjected to RIA analysis for the presence of glycine-extended, C-terminally extended, and \( \alpha \)-amidated CCK. In each case two independent transformants were analyzed. No major differences in CCK immunoreactivity were detected with transformants harboring the same plasmid constructs (Table III).

Secretion of glycine-extended CCK was determined by immunoreactivity to Ab 3208 (Table III). Both constructs resulted in the secretion of glycine-extended CCK from yeast transformants. However, approximately hundredfold higher levels of secretion were seen when CCK was expressed as a fusion to the \( \alpha \)-factor prepropeptide compared with the native prepro-CCK.

Treatment of the samples with trypsin and carboxypeptidase B increased the quantity of glycine-extended immunoreactivity at least 2-fold indicating that considerable quantities of secreted CCK was C-terminally extended (Table III).

In contrast to glycine and C-terminally extended CCK, no \( \alpha \)-amidated CCK was found in media supernatants of yeast expressing prepro-CCK, as indicated by the absence of immunoreactivity to Ab 2609 in media in which yeast expressing the prepro-CCK or the prepro-\textit{MPFa}l-pro-CCK constructs had been
Analysis of the Proteolytic Processing of CCK Expressed in
Yeast—To further characterize the extent of endoproteolytic
processing of CCK produced in S. cerevisiae, secreted material
of strain YNG318 expressing the prepro-MFa1-pro-CCK fusion
protein were subjected to gel chromatography. Analysis of the
fractions collected for glycine-extended CCK uncovered two
major peaks of CCK immunoreactivity, one at an elution con-
stant ($K_D$) of 0.75, the other 1.15 (Fig. 2). These $K_D$ values are
similar to the known elution constants of CCK-22-Gly and
CCK-8-Gly, respectively, determined by the same procedures
(Cantor and Rehfeld, 1987). To confirm the identity of the
CCK-immunoreactive products they were purified to homoge-
neity and subjected to mass spectrometry and protein sequence
analysis. As predicted, the later immunoreactive peak was
identified as CCK-8-Gly and the earlier as CCK-22-Gly (Fig. 3,
A and B). It should be noted that the peptides isolated during
this study were oxidized to varying degrees (Fig. 3). All human
CCK peptides contain methionine and tryptophan residues. It
is a common observation that methionine residues are prone to
oxidation in the presence of oxygen. In addition, we often find
that tryptophan containing peptides undergo oxidation in the
mass spectrometer. Both processes appear to be more pro-
nounced in short peptides and at low peptide concentrations.

Since the initial RIA data indicated that a considerable
quantity of CCK secreted from yeast was not processed at the
C terminus (Table III), the column fractions were treated with
trypsin and carboxypeptidase B before RIA analysis with Ab
3208. This resulted in the detection of two additional CCK-
immunoreactive peaks not present in untreated fractions, one
at a $K_D$ of 0.6 and the other at a $K_D$ of 1 (Fig. 2B). Thus, it would
appear the new peaks represented C-terminally extended

2 A. H. Johnsen, unpublished observations.
CCK-22 and CCK-8, respectively. Fractions collected from Sephadex G-50 gel chromatography were also analyzed with Ab 89009 which binds the N terminus of CCK-22. Two immunoreactive peaks were observed, one corresponding to CCK-22-Gly and the other at $K_D$ 0.6 (Fig. 2C), strongly suggesting this elution constant represents C-terminally extended CCK-22. A similar experiment was performed using an antibody which binds the N terminus of non-sulfated CCK-8 (Ab 94179). The results showed two immunoreactive peaks at $K_D$ values of 1 and 1.15, the latter being CCK-8-Gly, and the former being C-terminally extended CCK-8 (data not shown).

Expression of the pro-CCK fusion in strain MT960 (Table I), which is the wild type for the PEP4 protease, but defective in the BAR1 encoded “barrier” protease, an exported pepsin-like protease which cleaves α-factor (Mackay et al., 1988), resulted in the formation of CCK-22-Gly and CCK-8-Gly in a manner similar to that of strain YNG318 (data not shown). Thus, neither protease A nor the barrier protease seems to be responsible for the observed proteolytic cleavages of pro-CCK.

Proteolytic Processing of Human Pro-CCK Expressed in Yeast Endoprotease Mutants—The prepro-MFa1-pro-CCK plasmid was transformed into S. cerevisiae strains deficient in the function of known yeast secretory pathway endopeptidases in an attempt to determine which enzyme(s) were responsible for the endoproteolytic processing of pro-CCK. Analysis of the extracellular products from transformants of the _mkc7_ yeast mutant HKY21 by gel chromatography and RIA analysis with Ab 3208 showed that both CCK-22-Gly and CCK-8-Gly were produced (Fig. 4A). Pro-CCK processing was then analyzed in yeast deficient in both the Mkc7 and Yap3 aspartyl proteases (HKY24). However, the three proteolytic cleavage events occurring in wild type yeast persisted in the _mkc7 yap3_ double mutant (Fig. 4B). Treatment of the column fractions with trypsin and carboxypeptidase B uncovered two additional immunoreactive peaks (Fig. 4B) corresponding to C-terminally extended versions of CCK-22 and CCK-8. Processing at Lys$^8$ persisted (data not shown). Thus, neither protease A nor the barrier protease seems to be responsible for the observed proteolytic cleavages of pro-CCK.

When the prepro-MFa1-pro-CCK construct was expressed in a _kex2_ mutant, ME598, the pattern of pro-CCK endoproteolytic processing was altered (Fig. 4C). No glycine extended products were detected, and a single major CCK-immunoreactive peak was found eluting at a $K_D$ of 0.6 after the Sephadex G-50 column fractions had been exposed to trypsin and carboxypeptidase B treatment (Fig. 4C). A similar situation was observed when the fusion protein was expressed in the _kex2 yap3_ double mutant ME938 (Fig. 4D). Immunoreactive material in the medium of this double mutant was purified, exposed to protein sequencing and mass spectrometry (Fig. 3C).

The results showed two immunoreactive peaks at $K_D$ values of 1 and 1.15, the latter being CCK-8-Gly, and the former being C-terminally extended CCK-8 (data not shown).

Intracellular Processing of Pro-CCK in Yeast Transformants—Metabolic labeling and immunoprecipitation experiments were performed to determine which, if any, of the proteolytic cleavage events of pro-CCK occurred within the yeast cell. Both radiolabeled and CCK-Gly immunoreactive material was found to elute at $K_D$ values of 0.75 and 1.15 (Fig. 5), presumably shown to correspond to CCK-22-Gly and CCK-8-Gly, respectively (Figs. 2 and 3, A and B). Since no larger forms of CCK were detected it appears that only the smaller forms of CCK are glycine extended in the yeast cell. In addition, when CCK-33-NH$_2$ was incubated for 5 h at 30 °C with _S. cerevisiae_, as well as with media in which the yeast had been grown, only CCK-33-NH$_2$ was recovered (data not shown), indicating that the cleavage at Lys$^8$ is occurring entirely within the cell.

Analysis for Tyrosyl Sulfation of CCK Secreted from Yeast Transformants—Anion exchange chromatography was em-

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\* J. F. Rehfeld, unpublished data.
linked to the prepro region of pro-CCK was expressed as a fusion protein, N-terminally.

Higher expression levels, yeast expressing the prepro-MFa1-pro-CCK construct, Radioactivity (left y axis) and CCK-Gly immunoreactivity utilizing Ab 3208 (right y axis) were determined for each column fraction.

In an attempt to identify which yeast endopeptidase(s) were responsible for the above mentioned cleavage events, the processing patterns were analyzed in yeast strains deficient in known secretory pathway endopeptidases. When pro-CCK processing was examined in strains lacking the structurally known secretory pathway endopeptidases uncovered four pro-CCK products, glycine-extended CCK-8 and CCK-22, as well as C-terminally extended CCK-8 and CCK-22. Therefore, S. cerevisiae is able to cleave pro-CCK at three sites: (i) C-terminally of the dibasic Arg105-Arg106 residues, an cleavage which is an essential step in production of bioactive CCK in vertebrates, (ii) between the Arg95-Asp96 amino acids producing CCK-8 related products, the most common form of CCK in the mammalian brain (Rehfeld, 1978b; Rehfeld and Hansen, 1986); and (iii) between the Lys81-Asn82 residues producing CCK-22 related products, which is a predominant form of CCK in human plasma and plasma from most other mammals (Liddle et al., 1984, 1985; Cantor and Rehfeld, 1987; Rehfeld, 1994; Paloheimo and Rehfeld, 1995). Processing at the Lys81 residue is extremely efficient, abolishing detection of endopeptidase cleavage events which may have occurred N-terminally of Lys81, with the antisera utilized in this study. However, a small quantity of C-terminally extended CCK-93, lacking one amino acid at the N terminus, was purified from the hck2 yap3 double mutant ME938 expressing the pre-proMFa1-pro-CCK construct.

In an attempt to construct.
Kex2, but proficient in Mkc7 and Yap3 function, do not produce any CCK-Gly this result suggests that pro-CCK expressed in yeast is not cleaved at Arg\(^{105}\)Arg\(^{106}\) by either the Mkc7 or Yap3 proteases within the yeast cell. Incubating CCK-33 with purified Yap3 in vitro resulted in the liberation of both CCK-22 and CCK-8 (Cawley et al., 1996). Since processing to produce these forms of CCK persists in a mck7 yap3 double mutant it is presently difficult to determine if either of the proteases are able to cleave pro-CCK at Lys\(^{91}\) or Arg\(^{95}\) in vivo.

Two pro-CCK endoproteolytic processing events were abolished in a yeast strain lacking the Kex2 serine protease, namely the dibasic Arg\(^{105}\)-Arg\(^{106}\) directed cleavage and processing between Arg\(^{95}\)-Asp\(^{96}\), suggesting that Kex2 is responsible for both processing events. However, the possibility that the intracellular trafficking of pro-CCK may be altered in the kex2 mutant, thus directing the pro-CCK molecule to a distinct area of the secretory pathway where it cannot be cleaved at Arg\(^{105}\), Arg\(^{106}\) or Arg\(^{95}\) cannot be ruled out. Recently the first unequivocal evidence for at least two distinct vesicles in the yeast secretory pathway has been reported, where it appears that one of these vesicles may represent proteins bound for the plasma membrane, with the other vesicle type bound for secretion from the cell (Harsay and Bretscher, 1995).

The Kex2 enzyme is widely known to be responsible for processing of both yeast and foreign proteins at dibasic sites (Fuller et al., 1988; Hadfield et al., 1993). Thus, it was not surprising that pro-CCK processing was abolished at the Arg\(^{105}\)-Arg\(^{106}\) residues in a kex2 mutant. As seen in other instances where Kex2 has been responsible for post-translational processing, not all of the proprotein was processed at this site (Miyajima et al., 1986; Thim et al., 1986; Zeebo et al., 1986; Moody et al., 1987; Driedonks et al., 1995). Notably, even when low amounts of pro-CCK were passed through the yeast secretory pathway, as was the case when prepro-CCK was expressed in yeast, there were still relatively high quantities of C-terminally extended CCK secreted. Since large amounts of glycine-extended CCK were secreted from S. cerevisiae transformants expressing the prepro-MFo1-pro-CCK construct, it seems improbable that yeast expressing the prepro-CCK construct lack the capacity to cleave all pro-CCK present in the secretory pathway at the Arg\(^{105}\)-Arg\(^{106}\) site.

The second pro-CCK proteolytic processing event which occurred in wild type yeast, but not the kex2 mutant, was the single arginine directed cleavage resulting in CCK-8 related products. There is an increasing number of instances where Kex2 and related serine proteases of the mammalian secretory pathway have been shown to preferentially process proproteins at single arginine residues (Zhu et al., 1992; Bourbounais et al., 1994; Dupuy et al., 1994; Galanopoulou et al., 1995; Vollweider et al., 1995). For instance, prohormone convertase 1, not prohormone convertase 2, was shown to cleave rat prodynorphin at a single arginine residue when co-expressed in mouse AtT-20 cells (Dupuy et al., 1994). Our data raises the possibility that some members of the mammalian Kex2-like proteases may also play a role in the processing of pro-CCK at the Arg\(^{95}\)-Asp\(^{96}\) site to release CCK-8. A non-serine endopeptidase which displayed a high selectivity for cleavage of CCK-33, resulting in CCK-8, has been partially purified from rat brain synaptosomes (Viercek and Beinfeld, 1992), although there is currently no direct evidence to suggest that this protease cleaves pro-CCK in vivo. Experiments similar to those performed on prodynorphin by Dupuy and co-workers (1994) would be interesting to determine if any of the known mammalian Kex2-like proteases are able to process pro-CCK at the Arg\(^{95}\)-Asp\(^{96}\) site. It is possible that the tissue specificity observed in the N-terminal processing of pro-CCK in mammalian tissues is a result of differential expression of a single arginine-specific endopeptidase and Kex2-like serine proteases, some of which may have the ability to process pro-CCK to CCK-8.

The ability to cleave pro-CCK at Lys\(^{81}\) to produce CCK-22 related products was maintained in a variety of recombinant yeast strains deficient in three known putative endopeptidases of the S. cerevisiae secretory pathway. Hence our data suggests that another endopeptidase is present in the secretory pathway of yeast. Previously, two groups have suggested that an uncharacterized protease was cleaving the human \(\beta\)-amyloid precursor after the lysine residue in the Hia-Gln-Lys-Leu-Val sequence, when expressed and secreted in yeast (Hines et al., 1994; Zhang et al., 1994). However, one of these studies did not examine kex2 or yap3 mutant yeast strains (Hines et al., 1994), whereas the other did not express \(\beta\)-amyloid precursor in yap3 mutants (Zhang et al., 1994). The lysine-directed processing of \(\beta\)-amyloid precursor in yeast is identical to that performed by \(\alpha\)-secretase in the mammalian brain, to produce the \(\beta\)4 peptide found in senile deposits of Alzheimer’s disease patients (Hines et al., 1994; Selkoe, 1994; Zhang et al., 1994). At present it is debatable if the same protease is responsible for the lysine-directed processing of pro-CCK and \(\beta\)-amyloid precursor in yeast. This can only be determined when the gene encoding the endopeptidase responsible for the lysine-directed cleavage in pro-CCK is identified.

In conclusion, the expression of pro-CCK in S. cerevisiae has uncovered important features of the yeast secretory pathways ability to post-translationally process secreted peptides/proteins. First, it appears that yeast lack the tyrosyl sulfation and \(\alpha\)-amidation protein modifying capabilities. Second, our evidence suggests that the Kex2 protease has the ability to cleave Arg-Asp bonds. Finally, we provide evidence that a novel lysine-specific endopeptidase is present in the yeast secretory pathway. Attempts to isolate and characterize the gene encoding this putative endopeptidase are currently underway.

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Heterologous Expression of Human Cholecystokinin in *Saccharomyces cerevisiae*: EVIDENCE FOR A LYSINE-SPECIFIC ENDOPEPTIDASE IN THE YEAST SECRETORY PATHWAY

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