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The pro-region of the Kex2 endoprotease of *Saccharomyces cerevisiae* is removed by self-processing

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We have produced in the baculovirus/insect cells expression system a soluble secreted form of the *Saccharomyces cerevisiae* Kex2 endoprotease. This secreted enzyme was purified and its NH_2-terminal sequence determined. The NH_2-terminal sequence started at residue Leu^{109} of the sequence deduced from the KEX2 gene nucleotide sequence, showing that the Kex2 enzyme is produced as a proenzyme. Residue Leu^{109} is preceded by a pair of basic amino acid residues (Lys^{107}-Arg^{108}) which is a potential processing site for the Kex2 endopeptidase. Futhermore, expression of an inactive form of this truncated enzyme resulted in the production of a protein with a higher molecular weight. These observations suggest that the pro-region of Kex2 endoprotease is removed by a self-processing event.

Endoprotease; Prohormone processing; Insect cell; Protein sequence

1. INTRODUCTION

The yeast Kex2 endoprotease (Kex2p) is an integral membrane protein involved in the proteolytic processing of the α-pheromone precursor and of the K1 and K2 killer toxin precursors [1–3]. Cleavage of precursor proteins by Kex2p occurs on the COOH-terminal side of Arg residues which are found in pairs of basic amino acid residues such as Lys-Arg or Arg-Arg [1]. Doublets of basic amino acid residues are also preferred proteolytic processing sites in mammalian prohormone or proneuropeptide precursors [4,5]. Co-transfection of the KEX2 gene and of the proopiomelanocortin cDNA into mammalian cells, that cannot normally process prohormones, showed that the yeast Kex2 enzyme could process this precursor in a cellular environment and release authentic maturation products such as β-lipotropin and β-endorphin [6–8]. Thus, Kex2p can be used as a model for mammalian maturation endoproteases.

The Kex2 protein structure deduced from the 2848 nucleotide long coding sequence of the KEX2 gene [9] revealed a type I transmembrane protein with a lumenal directed NH_2-terminus and a highly charged COOH-terminal cytoplasmic region. The protein also contains sites for *N*-glycosylation and five pairs of basic amino acid residues Lys-Arg or Arg-Arg. A Ser/Thr-rich region which may be the site of *O*-glycosylation precedes the transmembrane domain. Sequence comparisons [9] and the spectrum of sensitivity to specific inhibitors [1] indicated that the Kex2 enzyme belongs to the subtilisin family of serine proteases.

In yeast, several proteases are synthesized as inactive precursors. Similarly, subtilisin contains an NH_2-terminal pro-region which is removed by an autoprocessing event [10]. Since two putative Lys-Arg dibasic cleavage sites are located in the NH_2-terminal region of Kex2p [9], we have undertaken the present work to investigate the possibility of NH_2-terminal maturation of the Kex2 endoprotease.

2. EXPERIMENTAL

2.1. *Production of truncated forms of Kex2p in insect cells*

Production of a truncated version of Kex2p (Kex2Δp) in insect cells has been described elsewhere [11]. We used the KEX2 transfer plasmid KEX2ΔpDC [11] to substitute by site-directed mutagenesis the active site Ser^{385} residue with an Ala residue in order to generate an inactive form of Kex2Δp. Both KEX2ΔpDC and the newly obtained KEX2Δ385pDC plasmids were transfered by homologous recombination into the AcMNPV viral genome (*Autographa californica* nuclear polyhedrosis virus) as described elsewhere [11,12]. The recombinant baculoviruses were respectively termed Ac[KEX2Δ] and Ac[KEX2Δ385].

For purification of Kex2Δp, Ac[KEX2Δ] baculovirus at a multiplicity of infection of 2 was used to infect a 4 l bioreactor of Sf9 cells grown to a density of 1.5×10^6 cells/ml in SF900 serum-free medium (Gibco). The supernatant was recovered 72 h after infection by centrifugation of the cells at 5,000 × *g* for 10 min at 4°C and concentrated 16-fold by ultrafiltration using an Amicon Diaflo S1Y10 spiral cartridge. Protein concentration was determined by the method of Brad-

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ford [13]. The concentrated medium was then aliquoted and kept at -20°C.

2.2. *Purification of recombinant Kex2Δp enzyme*

A 1 ml sample of the concentrated medium containing approximately 2 mg of protein was added to 10 ml of Q Sepharose previously equilibrated in 25 mM HEPES/Tris, pH 6.5, and incubated with agitation overnight at 4°C. The matrix was poured into a HR10/10 (Pharmacia) column (10 × 1 cm) and washed with 10 vols. of 25 mM HEPES/Tris, pH 6.5, at a flow rate of 1 ml/min using an FPLC pump. Proteins were eluted by a gradient of 0 to 1M NaCl at the same flow rate over a period of 30 min and fractions of 500 μl were collected. Kex2Δp activity was assayed in 50 μl aliquots from each column fraction using the Boc-GlnArgArg-MCA synthetic substrate as described previously [11].

Fractions containing the Kex2Δp activity were pooled, concentrated to 500 μl on a PM10 Diaflo ultrafiltration membrane and applied to a Superose 12 gel filtration HR 10/10 column (Pharmacia) previously equilibrated in 25 mM HEPES/Tris, pH 6.5. Proteins were eluted in the same buffer at a flow rate of 0.5 ml/min and 500 μl fractions collected which were tested for Kex2Δp activity as described above.

2.3. *Protein sequencing*

Fractions from the Superose 12 column containing Kex2Δp activity were pooled and precipitated in 20% trichloroacetic acid for 1 h at -20°C and then overnight at 4°C. Precipitated proteins were centrifuged at 4°C for 15 min at 10,000 rpm and the resulting pellets were washed twice with 200 μl of acetone at -20°C. The precipitated proteins were analyzed by SDS-PAGE on an 8% gel run for 1 h at 150 mA and transferred to a PVDF-Problot (ABI) membrane. The Kex2Δp protein band on the PVDF-Protoblot membrane was identified by immuno-blotting of a duplicate membrane. Automated Edman degradations were performed in a Gas-Phase Sequencer (Model 470A) equipped with an on-line phenylthiohydantoin Analyser (Model 120A) from Applied Biosystems Inc. The immunoreactive band was cut and loaded with a TFA-treated cartidge filter coated with 1.5 mg of polybrene and 0.1 mg NaCl (Biobrene Plus, ABI). The standard program (03RPTH, ABI) was employed for sequencing. The phenylthiohydantoin amino acids (PTH-aa) derivatives were determined by comparision with standards (PTH Analyser Standards, ABI) analysed on-line at the start of a sequence analysis run.

3. RESULTS AND DISCUSSION

We have recently reported the expression of an active truncated form of the yeast Kex2 enzyme in Sf9 insect cells using the recombinant baculovirus Ac[KEX2Δ] [11]. This truncated form of Kex2p, termed Kex2Δp, was found to be secreted into the culture medium as a 68 kDa protein. This extracellular form of Kex2p was first applied to an anion exchange matrix Q Sepharose (Fig. 1A) and subsequently applied to a Superose 12 molecular sieve column (Fig. 1B). Analysis of the proteins present in the fractions containing the Kex2Δp activity at both purification steps, showed the enrichment of a 68 kDa protein which corresponded to the increase in enzyme specific activity (Fig. 1C).

The Kex2p primary structure deduced from the DNA sequence of the gene reveals an hydrophobic region at the NH_2-terminus of the protein which may function as a signal peptide [9]. Computer analysis (PC Gene) of the Kex2p sequence predicts cleavage of the NH_2-terminal signal peptide on the COOH-side of residue Ala^{19}. Thus, the expected NH_2-terminal sequence of Kex2p and also

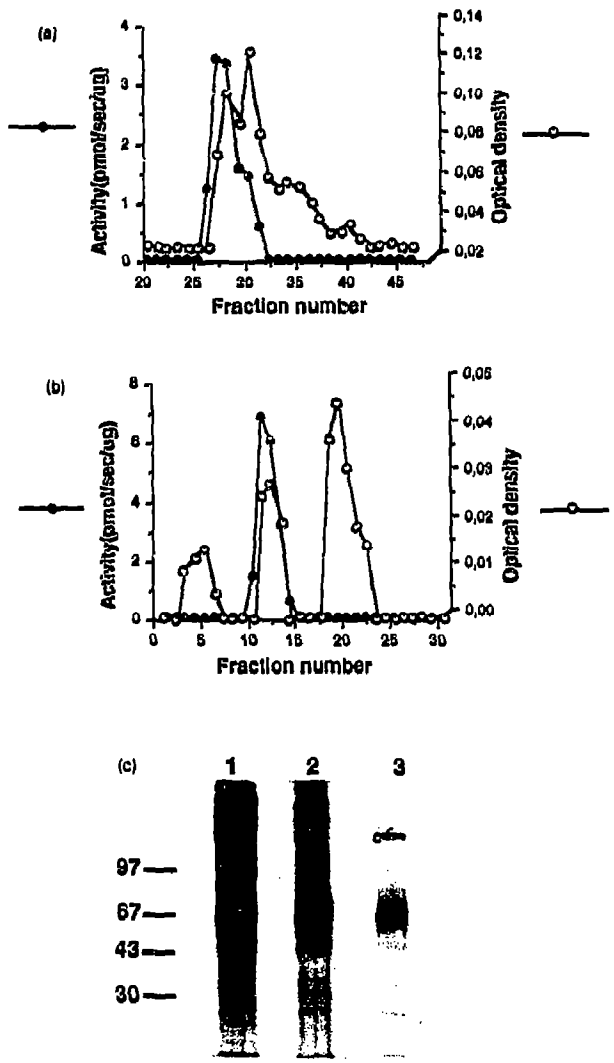

Fig. 1. (A) Elution profile of Kex2Δp from Q Sepharose column. Fractions were assayed for their absorbance (○) at 280 nm and their activity (●) against the synthetic substrate Boc-GlnArgArgMCA as described in Experimental. (B) Elution profile of Superose 12 gel filtration column. Fractions were assayed as above. (C) Silver-stained polyacrylamide gel. Samples of 5 μl were run on a Phast gel system; (lane 1) concentrated supernatant; (lane 2) fraction 27 after Sepharose Q column; (lane 3) fraction 12 after Superose 12 column.

Kex2Δp should start with Leu^{20}. However, sequencing of the enzyme purified from the culture medium of infected Sf9 cells reveals a unique sequence starting at residue Leu^{109} (Fig. 2). No signal of a sequence starting at residue Leu^{20} or any other putative signal peptide cleavage site could be detected. This result indicates proteolytic processing of the NH_2-terminal end of Kex2Δp resulting in the removal of a 89 amino acid pro-peptide. Based on the observation of a short-lived processing intermediate of Kex2p in the endoplasmic reticulum, the existence of such a precursor form of Kex2p has also been suggested to occur in yeast [14]. Interestingly, the sequence started on the COOH- terminus of Arg^{108} which forms with Lys^{107} a pair of basic

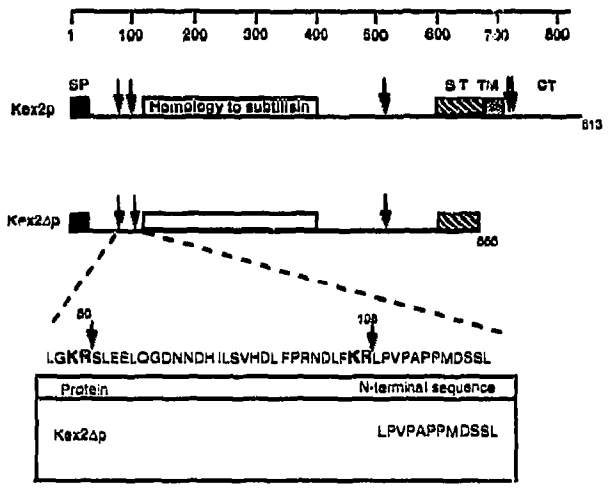

Fig. 2. Schematic representation of the of Kex2p precursor and of the truncated form Kex2Δp. SP, signal peptide; ST, serine/threonine-rich region; TM, transmembrane domain; CT, cytosolic tail. The dibasic sites are represented by arrows. The amino acid sequence from residue 77 to residue 120 deduced from the KEX2 gene sequence and the NH_2-terminal sequence of Kex2Δp endoprotease purified from insect cell supernatant are shown.

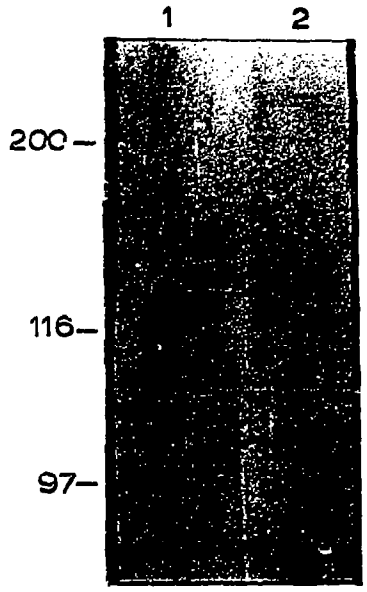

Fig. 3. Immuno-blot of the intracellular form of (1) Kex2Δp and (2) Kex2–385Δp. Samples of 50 μg total protein were run on an 8% SDS polyacrylamide gel and transferred to a membrane for antibody detection as described elsewhere [11].

amino acids. Such dibasic sites are preferred cleavage sites for the Kex2 endoprotease [1]. This observation suggests that processing of the Kex2 endoprotease precursor occurs by an enzyme with the specificity of the Kex2 enzyme.

To test the hypothesis of the processing of the NH_2-terminal of Kex2Δp by KexΔ2p itself, we infected Sf9 cells with the Ac[KEX2Δ385] baculovirus which carries a mutant of the enzyme where residue Ser^{385} was replaced by an Ala residue (Kex2Δ385p). We have shown recently that residue Ser^{385}, which is a member of the active site triad, is essential for the activity of Kex2Δp and COOH-terminal processing of the enzyme [11]. Here, intracellular forms of Kex2p were investigated to avoid COOH-terminal maturation which occurs upon secretion of the enzyme [11]. The immunoblot of the intracellular forms of both the active and inactive enzyme show that the molecular weight of the Kex2Δ385p protein is approximately 9 kDa greater than that of its active counterpart Kex2Δp (Fig. 3). This result can be explained by the presence of the pro-region (89 amino acids) in the inactive molecule. We thus propose that the NH_2-terminal maturation of Kex2Δp is catalyzed by Kex2p.

The pro-region of the bacterial serine protease subtilisin has been shown to be required for the maintainence of the enzyme in an inactive state as well as for the folding of the enzyme [10,15]. Kex2p is homologous to subtilisin and it will be interesting to determine if these functions of the pro-region have been conserved in the yeast enzyme. Furthermore, the cDNA sequences of the mammalian homologues of Kex2p (PC1, PC2 and furin [16–18]) also reveal putative pro-regions. Elucidation of the function of Kex2p pro-region and eventually of the mammalian homologs may shed more light on the function of pro-peptides from bacteria to mammals.

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