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Processing of Kex2 pro-region at two interchangeable cleavage sites

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In *Saccharomyces cerevisiae*, the Kex2 endoprotease (Kex2p) is required for the proteolytic maturation of α-pheromone and also for the removal of its own pro-region. Kex2p is specific for pairs of basic amino acid residues. Two putative processing sites are present in the pro-region of Kex2p. We have expressed processing site mutants of Kex2p and assayed the production of active Kex2p. Mutations affecting either putative cleavage site do not alter the activity However, mutations affecting both sites led to a reduction in both Kex2 activity and the amount of protein. These results suggest that removal of Kex2p pro-peptide is required for the production of a stable enzyme and can occur at either processing site.

Endoprotease; Pro-hormone processing; Pro-region

1. INTRODUCTION

The Kex2 endoprotease (Kex2p) is required for the production of active α-pheromone, and of K1 and K2 toxins in *Saccharomyces cerevisiae* [1]. The α-pheromone is synthesized from two genes as a polyprotein precursor containing several copies of the mature α-pheromone [2] which are released upon cleavage of the precursor at pairs of basic amino acid residues by Kex2p [3].

The Kex2p precursor itself has been shown to undergo several post-translational modifications [4]. The enzyme precursor has a hydrophobic signal sequence, with a high probability of cleavage by signal peptidase. The enzyme is found as a 129 kDa N-glycosylated intermediate which becomes rapidly modified into a 120 kDa form. The 129 kDa intermediate observed in the endoplasmic reticulum corresponds to the pro-enzyme precursor after signal peptide cleavage [5]. During its transport through the Golgi apparatus, the protein is then extensively O-glycosylated resulting in a 126 kDa intermediate and a 130 kDa mature glycosylated form [4]. Protein sequence analysis of Kex2p upon overexpression in yeast [5] or in baculovirus [6] has revealed the existence of an NH_2-terminal pro-sequence. Cleavage of the pro-sequence occurs on the COOH-terminal side of the pair of basic amino acid residues Lys^{108}-Arg^{109} and is the result of an automaturation event [6].

Several mamalian homolgues of Kex2p have been identified and showed to be involved in prohormone processing in the constitutive and regulated secretory pathways [7–8]. The sequence of these Kex2p homologues reveals putative pro-regions in which the presence and position of two putative processing sites are highly conserved. Therefore, pro-region processing may represent a mechanism controlling the activation of these endoproteases.

In this study, we have investigated the processing step involved in the removal of the pro-peptide of Kex2p. Our results suggest that maturation of the pro-region can occur at either processing site but that removal of the pro-region is required for production of a stable enzyme.

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2. EXPERIMENTAL

2.1. *Plasmid constructions*

A DNA fragment containing the complete coding region of the KEX2 gene was cloned into the *Bam*HI site of the yeast shuttle vector pVT103U encoding the URA3 gene as a selection marker [9]. The resulting plasmid, Kex2-pVT was used to generate single stranded DNA for site directed mutagenesis. In the first mutant, the Arg residue at position 80 was changed to a Gly residue. This mutation also created a *Bam*HI restriction site used for screening for the mutation. The resulting recombinant plasmid was termed Kex2–80pVT A second plasmid Kex2–109pVT was generated by replacing the Arg residue at position 109 with a Gln residue. This mutation created a *Hin*dIII site again used for screening. Plasmid Kex2–109pVT was then used as template for site-directed mutagenesis and led to the construction of a third recombinant plasmid Kex2–80–109pVT which contains the two mutations described above.

These plasmids were used to transform the yeast M213 strain (Matα, kex2::HIS3, ura3, trp1) by the lithium acetate method [10].

2.2. *Halo assay*

Halo assays for measuring production of biologically active α-pheromone were performed by spotting α-pheromone-producing strains on a lawn of the supersensitive indicator yeast strain M2006C (Mata, sst1, sst2, ura3, ade1) as described [2]. The assays were performed in triplicate.

2.3. *Membrane preparation and immunoblot analysis*

Yeast transformants were grown in 100 ml of selective media for 18 h Spheroplasts were generated by incubation of cell pellets from these cultures in 3 ml of buffer containing 0.9 M Sorbitol, 0.1 M EDTA and 1.5 mg of Zymolase for 30 min at 37°C. The spheroplasts were washed in Sorbitol buffer (0.9 M Sorbitol, 2 mM EDTA) and resuspended in lysis buffer (0.1 M Sorbitol, 2 mM EDTA, 1 mM DTT, 20 mM HEPES, pH 7.4, 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml aprotinin). The cells were broken using a homogenizer and centrifuged at a low speed centrifugation (1,000 $\times g$, 20 min). The supernatant was then centrifuged at 100,000 $\times g$ for 1 h at 4°C. The pellets containing membranes were then resuspended in 100 μl of lysis buffer. Aliquots of 20 μl were loaded on an 8% SDS-PAGE and run for 2 h at 150 V. Proteins were transferred to a nitrocellulose membrane and detected using a polyclonal antibody against a LacZ··Kex2 fusion protein with a second anti-rabbit antibody and the standard alkaline phosphatase protocol as described previously [11].

3. RESULTS AND DISCUSSION

We have previously shown that the pro-region of Kex2p is removed by self-processing on the COOH-terminal side of the dibasic site Lys^{108}-Arg^{109} [6]. The DNA sequence of the KEX2 gene reveals the presence of another pair of basic amino acid residues at position Lys^{79}-Arg^{80}. In order to study the role of these dibasic sites in the biosynthesis of Kex2p, we have constructed Kex2 enzymes harboring either the mutation R80G (Kex2–80p; Fig. 1) or R109Q (Kex2–109p; Fig. 1). These mutated enzymes were expressed in yeast by transformation of a Mat$\underline{\alpha}$ yeast strain deficient in endogenous Kex2p activity with the respective plasmids. The transformants were tested for their ability to cleave α-pheromone precursor by the halo assay. The production of active α-pheromone from a Mat$\underline{\alpha}$ strain induces a G1 cell cycle arrest of cells of the opposite mating type (Mat$\underline{a}$). The size of the halo correlates well with the amount of a active pheromone, and therefore to Kex2p-dependent maturation of α-pheromone precursor. Fig. 2 shows that the halo of growth arrest generated by a transformant expressing either Kex2–80p or Kex2–109p is similar to that generated by a transformant expressing wild-type Kex2p. Immunoblotting of the membrane proteins extracted from M213 yeast cells expressing either Kex2–80p (Fig. 3, lane 3) or Kex2–109p was performed (Fig. 3, lane 4). Both Kex2–80p and Kex2-109p migrated on SDS-PAGE with an apparent

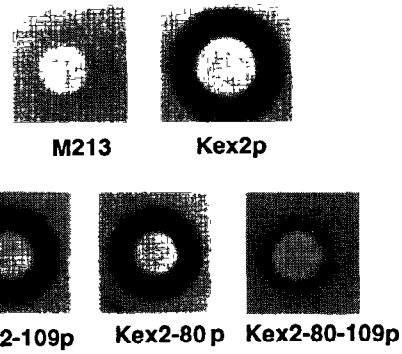

Fig. 2. Halo assay. M213 cells alone or M213 cells transformed with a multicopy plasmid encoding either the wild type Kex2p protein. Kex2–80p, Kex2–109p or Kex2–80–109p were spotted onto a lawn of the Mat$\underline{a}$ supersensitive strain M2006C as described in section 2.

M_r similar to that of Kex2p (Fig. 3, lane 2). The predicted difference of approximately 2 kDa between the cleavage product of Kex2–80p and Kex-109p was not detectable. This is presumably due to extensive glycosylation leading to a broadening of the bands. Our results indicate that cleavage of the pro-region and production of an active enzyme can be achieved by proteolysis at either processing site.

These results are in contrast to those found for the mamalian homologues of Kex2p, PC2 and Furin, in which mutations affecting either processing site abolish activity [12–13]. This discrepancy might reflect a tighter contol over the pro-region cleavage in the mammalian system. The presence of the Furin consensus cleavage sequence (Lys/Arg)-X-(Lys/Arg)-Arg [8] in the pro-region of PC1 and PC2 raises the possibility of a Furin catalysed step in addition to a putative autocatalysed step in the maturation of these pro-regions.

The double mutant Kex2–80–109p (Fig. 1) shows reduced but significant α-pheromone production in the halo assay compared with wild type Kex2p or the single mutants (Fig. 2). This suprising result could be explained by a cleavage of the pro-region at the putative cleavage site Pro^{103}-Arg^{104}. Such Pro-Arg sites have been shown to be cleaved by Kex2p in yeast in the K1 toxin precursor [14]. However, a triple mutant bearing mutations R80G, R104G and R109Q (Kex2–80–104–

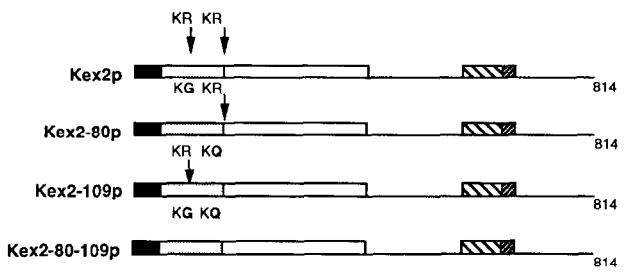

Fig. 1. Kex2 processing sites mutants. Representation of the Kex2 protein encoded by the plasmids described in section 2. Arrows indicate the positions of the potential processing sites.

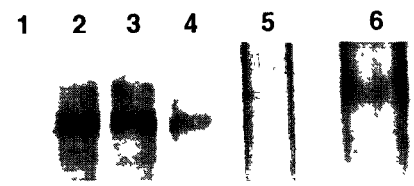

Fig. 3. Immunoblot of the membrane proteins from M213 cells (lane 1) or transformed with the plasmids Kex2-pVT (lane 2), Kex2–80pVT (lane 3), Kex2–109pVT (lane 4), Kex2–80 109pVT (lane 5) or Kex2–386pVT (lane 6) Preparation of the membrane proteins was performed as described in section 2

109p) has the same activity as Kex2–80–109p (data not shown). Therefore, the abolition of a putative cleavage at position Pro^{103}-Arg^{104} has no effect on Kex2p activity.

Very little Kex2–80–109p protein could be detected by immunoblotting (Fig. 3, lane 5) suggesting that the presence of the pro-region destabilizes Kex2p and the enzyme is rapidly degraded. However, since a mutation in the active site of Kex2p [6] led to the accumulation of an inactive pro-enzyme, Kex2–386p (Fig. 3, lane 6), the presence of the pro-region is not sufficient to induce degradation. Therefore, the observed degradation of Kex2–80–109p is either dependent on Kex2p activity or induced by the cooperative effect of the two mutations. Nevertheless, these results suggest that the pro-enzyme is active but removal of the pro-region is required for the production of a proteolysis resistant conformation. Consistent with this hypothesis we have obtained results suggesting that the pro-region of Kex2p acts as a molecular chaperone (Germain et al., in preparation). Whether this is also the case for the pro-region of the mamalian Kex2 homologues remains to be determined.

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