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The Kex2p Proregion Is Essential for the Biosynthesis of an Active Enzyme and Requires a C-terminal Basic Residue for Its Function

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The *Saccharomyces cerevisiae* prohormone-processing enzyme Kex2p is biosynthesized as an inactive precursor extended by its N-terminal proregion. Here we show that deletion of the proregion renders Kex2p inactive both in vivo and in vitro. Absence of the proregion impaired glycosylation and stability and resulted in the retention of the enzyme in the endoplasmic reticulum. These phenotypes were partially complemented by expression of the proregion in *trans. Trans* complementation was specific to Kex2p proregion because expression of any of the seven mammalian prohormone convertase propeptides had no effect. These data are consistent with a model whereby Kex2p proregion functions as an intramolecular chaperone and indicate that covalent linkage to the protein is not an absolute requirement for proregion function. Furthermore, extensive mutagenesis revealed that, in addition to their function as proteolytic recognition sites, C-terminal basic residues play an active role in proregion-dependent Kex2p activation.

INTRODUCTION

Folding is a crucial step in reaching a functionally competent protein structure. Although amino acid sequences possess all the information necessary to adopt final three-dimensional structures, molecular chaperones often intervene in the course of folding. They transiently interact with their substrates and guide them to achieve their final stable conformation, perhaps by preventing aggregation. Independently, some protein domains have been shown to serve as intramolecular chaperones for their cognate proteins (Baker *et al.*, 1993; Eder and Fersht, 1995; Shinde *et al.*, 1995). This is the case for subtilisin (Ikemura *et al.*, 1987), α-lytic protease (Baker *et al.*, 1992), aqualysin (Lee *et al.*, 1992), and carboxypeptidase Y (CPY) (Winther and Sorensen, 1991), whose N-terminal propeptide promotes proper folding and full enzymatic activity. In vitro studies performed with subtilisin and α-lytic protease suggest that propeptides interact with molten globular–like intermediates and help them surmount the energy barrier between the intermediate state and the final folded state (Baker *et al.*, 1992). Upon correct folding of the protease domain, autocatalytic cleavage and degradation of the proregion occur, yielding the mature enzyme (Ikemura and Inouye, 1988). It has also been shown in vitro that the prosequences of subtilisin and α-lytic protease can act transiently as an autoinhibitor of the protease activity (Shinde and Inouye, 1993; Bryan *et al.*, 1995; Sohl *et al.*, 1997).

In eukaryotes, many peptide precursors are cleaved at pairs of basic amino acid residues by proteases acting in the secretory pathway (for reviews, see Rouillé *et al.*, 1995; Seidah *et al.*, 1998). A search for proteases involved in processing at these sites led to the discovery of a family of related enzymes, which are conserved from yeast to mammals: the kexin-like proprotein convertases. The first member of this family identified was the *Saccharomyces cerevisiae* Kex2p, which is required for the processing of α-mating factor precursor and killer protoxin (Leibowitz and Wickner, 1976; Julius *et al.*, 1984; Mizuno *et al.*, 1988, 1989). Seven mammalian homologous proprotein convertases (PCs) were subsequently discovered (Steiner *et al.*, 1992; Seidah, 1995; for reviews, see Nakayama, 1997; Siezen and Leunissen, 1997). They all share structural homologies with bacterial subtilisin and are synthesized as precursors extended by an N-terminal prosequence, which is evicted from the active enzyme. Proteolytic removal generally occurs early in the secretory

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pathway in an autocatalytic manner (Seidah *et al.*, 1998) and is essential for enzyme activation. Indeed, mutations of the proregion cleavage site prevent full activation of Kex2p and of PC1 (Goodman and Gorman, 1994) and also leads to accumulation of an inactive form of furin in the endoplasmic reticulum (ER) (Leduc *et al.*, 1992; Creemers *et al.*, 1995). Moreover, deletion of the proregion inactivates furin (Rehemtulla *et al.*, 1992). These results suggest that presence of a cleavable prosequence is necessary to produce active enzyme and for its subcellular trafficking. This is consistent with a role of PC proregions in the folding of the mature protease domain. Furthermore, in vitro studies recently performed with furin, PC1/3, and PC7 indicated that, as previously reported for subtilisin, the proregion behaves as a transient autoinhibitor of activity (Anderson *et al.*, 1997; Boudreault *et al.*, 1998; Zhong *et al.*, 1999).

To gain insights into the function of the kexin prosequences, we have expressed proregion-deleted Kex2p forms in *S. cerevisiae* cells lacking Kex2p activity. Our results show that the proregion is essential for the biosynthesis of an active enzyme and for its correct cellular localization. The function of the proregion can be complemented in *trans* by expression of Kex2p proregion but not by that of other mammalian subtilisin- and kexin-like enzymes. Finally, mutations in the proregion defined critical features for its *trans* action. We provide the first demonstration that, in addition to their function in proteolytic cleavage, C-terminal basic residues play an active role in proregion-dependent Kex2p activation.

MATERIALS AND METHODS

DNA Manipulations and Plasmid Constructions

DNA manipulations were performed using standard procedures (Sambrook *et al.*, 1989; Ausubel *et al.*, 1993). Plasmid Kex2-pVT containing the complete coding sequence of the *KEX2* gene inserted into the *Bam*HI site of pVT103-U (Vernet *et al.*, 1987) was described previously (Germain *et al.*, 1993).

Plasmid Kex2HA-pVT encodes a hemagglutinin (HA)-tagged version of Kex2p. Site-directed mutagenesis was used to create an *Mlu*I restriction site immediately upstream of the Kex2p translational stop codon. This resulted in the change of the last two amino acid residues of Kex2p from Arg^{813}-Ser^{814} to His-Ala. Two copies of the HA epitope (Wilson *et al.*, 1984) were then inserted in the *Mlu*I site by ligating annealed oligonucleotides 5′-CGCG TAC CCA TAT GAT GTT CCA GAC TAC GCT GGT TCT GGT TAT CCT TAC GAC GTC CCA GAT TAT GCC AC-3′ and 5′-CGCGGT GGC ATA ATC TGG GAC GTC GTA AGG ATA ACC AGA ACC AGC GTA GTC TGG AAC ATC ATA TGG GTA-3′ to *Mlu*I-digested Kex2-pVT. The resulting *KEX2HA* construct (Figure 1) encoded an 837-amino-acid protein with the following C terminus: H^{813}–A-Y-P-Y-D-V-Q-D-Y-A-G-S-G-Y-P-Y-D-V-P-D-Y-A-T-A.

Plasmid pGL9 expresses a version of *KEX2HA* deleted of its proregion (Δ*prokex2HA*). To construct this plasmid, a 2.4-kb *Bam*HI DNA fragment encoding all of the KEX2p was subcloned from Kex2HA-pVT into phagemid M13mp18. Deletion of the proregion was achieved by mutagenesis (Kunkel, 1985) using the oligonucleotide 5′-TCA ACA TCC GCT CTT GTA TCA TCA CTA CCG GTG CCT GCT CCA CCA ATG-3′. The mutated *Bam*HI fragment was cloned back into pVT103-U to give pGL9. In this plasmid, the 23 amino acid residues of the signal peptide are fused directly to Leu^{110} of the mature protein.

Plasmid pGL15 was obtained by replacing the 1.1-kb *Bgl*II fragment containing *URA3* in pVT103-U (Vernet *et al.*, 1987) by a 0.9-kb *Bam*HI–*Bgl*II fragment bearing the *TRP1* sequence from pJJ248 (Jones and Prakash, 1990).

Plasmid pGL17 carries the DNA sequences encoding the signal peptide and the prosequence of Kex2p. The forward primer 5′-GCATACAATCACTCCAAGCT-3′ complementary to the 3′ end of the ADH promoter and the reverse primer 5′-CTCGAG**TCA** TCT CTT AAA TAG GTC GTT-3′ complementary to the 3′ end of Kex2p proregion sequence allowed PCR amplification of the DNA sequence encoding Kex2p signal peptide and proregion using Kex2HA-pVT as a template. The reverse primer introduced a stop codon (shown in bold characters) and an *Xho*I site (underlined) at the 3′ terminus of the amplified fragment. This *preprokex2* fragment encoding the first 327 nucleotides of wild-type *KEX2* gene was finally subcloned into the *Bam*HI–*Xho*I sites of pGL15, resulting in pGL17.

Construction of the PC Proregion Expression Vectors

Proregions of the mammalian proprotein convertases furin, PC7, PACE4, PC4, PC1, PC2, and PC5 were expressed in *trans* using pGL15 vector (pGL15; see above). The sequence encoding the 23-amino-acid-long signal peptide of Kex2, a Thr-Arg doublet corresponding to a unique *Mlu*I site and a stop codon, was introduced between the *Bam*HI and *Xho*I sites of the polylinker (pAPR1). Subsequently, *Mlu*I–*Xho*I/*Sal*I fragments obtained by PCR amplification of the sequences encoding the Kex2 proregion (pAPR2) or the different PC proregions (pAPR3–pAPR9 according to the above order) were then introduced downstream of the *KEX2* presequence of pAPR1. The PCR-amplified proregions correspond to amino acids 24–109 of yeast Kex2 (Mizuno *et al.*, 1988), 27–107 of human furin (Van den Ouweland *et al.*, 1989), 37–140 of rat PC7 (Seidah *et al.*, 1996), 63–149 of human PACE4 (Kiefer *et al.*, 1991), 27–110 of rat PC4 (Seidah *et al.*, 1992), 28–110 of mouse PC1 (Seidah *et al.*, 1991), 25–108 of mouse PC2 (Seidah *et al.*, 1990), and 19–100 of human PC5 (Mercure *et al.*, 1996).

Mutagenesis of Kex2p Proregion

Plasmid pAPR2 was used as a template for PCR amplification of 319- to 325-bp DNA fragments. In all cases the sense primer was 5′-TGC TTT TGG TGG GCC TTT TCA ACA TCC GCT-3′. For deletion of C-terminal Lys-Arg residues, reverse primer 5′-TGCTGCAGGCTCGAGTCA AAA TAG GTC GTT ACG-3′ was used. To change the nature of the C-terminal Lys-Arg residue, antisense primer 5′-CTGCTGCAGGCTCGAGTCA*** *** AAA TAG GTC G-3′ was used (*** represents the mutagenic portion of the primer; CTT, TCT, and CCC were used to introduce Lys, Arg, and Gly, respectively). Amplified products were subcloned as *Mlu*I–*Xho*I fragments in the pAPR1 vector.

Yeast Strains and Growth Conditions

YPD, synthetic minimal, synthetic complete, and synthetic dropout media were as described (Ausubel *et al.*, 1993). Yeast strains used in this study are listed in Table 1. The GLY39 strain was constructed from M213 (*kex2::HIS3*) (Germain *et al.*, 1993). Two PCR fragments containing regions from −150 to +84 and +2018 to +2462 of the *KEX2* gene were fused to 3′- and 5′-end of the *LEU2* marker, respectively; this construct was used to disrupt the *KEX2* locus of M213 using the lithium acetate procedure (Gietz *et al.*, 1992). Transformants were selected on plates lacking leucine, and loss of *HIS3* was confirmed by the absence of growth on minimal medium without histidine (Figure 2B). Correct integration was confirmed by PCR amplifying a 1.2-kb fragment (Figure 2C) using primers 5′-CGCGGGTGCAAACAATGCAAAGT-3′ and 5′-GGAAGTGGGA-CACCTGTAGCATCG-3′ (Figure 2A). Other strains derive from GLY39 by transformation with different plasmids.

Table 1. Yeast strains used

Strain	Relevant genotype	Reference
M200-6 CK	*MAT**a**kex2::ura3 sst1 sst2 ade1 ilv3 ura3*	Whiteway *et al.*, 1988
M213	*MATαkex2::HIS3 ura3 trp1 leu2 his3*	Germain *et al.*, 1993
GLY39	*MATαkex2::LEU2 ura3 trp1 leu2 his3*	This study
GLY40	GLY39 transformed with Kex2HA-pVT	This study
GLY41	GLY39 transformed with pGL9 [Δ*prokex2HA*]	This study
GLY43	GLY41 transformed with pGL17 [*preprokex2*]	This study

Halo Assays for α-Factor Secretion

Exponentially growing cells were harvested by centrifugation (3 min, 500 × *g*) and resuspended at 1 $OD_{600\ nm}$/ml in sterile water. A 2.5-μl aliquot was then spotted on a lawn of M200-6CK cells (Whiteway *et al.*, 1988) prepared by spreading on YPD plates 5 ml of YPD containing 0.7% agar and 25 μl of saturated culture of M200-6CK cells. The appearance of halos was scored after 1–2 d of incubation at 30°C.

Membrane Preparation

Spheroplasts were prepared from 50 $OD_{600\ nm}$ cells by treatment with 200 μg/ml zymolyase 100T (Seikagaku, Tokyo, Japan) for 30 min at 37°C in TS buffer (50 mM Tris-HCl, pH 7.5, and 1.2 M sorbitol) containing 40 mM 2-mercaptoethanol. Spheroplasts were washed twice with ice-cold TS buffer and lysed by 20 min of incubation on ice in 2 ml of ice-cold 10 mM triethanolamine, pH 7.2, containing 0.3 M sorbitol plus protease inhibitors (2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 100 μg/ml PMSF, and 1 mM EDTA). Unlysed spheroplasts and cell debris were pelletted by centrifugation (1000 × *g*, 6 min, 4°C), and supernatants were first subjected to 10,000 × *g* centrifugation to remove mitochondria (10 min, 4°C) and then ultracentrifuged (100,000 × *g*, 2 h, 4°C). Final membrane pellets were resuspended in 200 μl of 50 mM Tris-acetate, pH 7.0, containing 1% Triton X-100.

Enzymatic Assay for Kex2p Activity

Kex2p activity in membrane preparations was assayed as previously described (Munzer *et al.*, 1997). Activity was expressed as the amount of fluorescence released by cleavage of the synthetic substrate pERTKR-MCA per hour. Kex2p content in the extracts was quantified by Western blot analysis. Relative specific activity was obtained by relating activity to the amount of Kex2p content in the sample and considering specific activity in the GLY40 strain as 100%.

Protein Extraction and Immunoblotting

Total protein extracts were prepared as previously described (Yaffe and Schatz, 1984). Endoglycosidase H digestions were performed according to supplier instructions (New England Biolabs, Beverly, MA). Five micrograms of protein as determined by Bradford assay (Bradford, 1976) were run on an SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane and subjected to Western blotting. The membrane was saturated for 30 min with TBSTM (100 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.2% Tween 20, and 2% nonfat milk). The primary antibody was mouse 12CA5 anti-HA monoclonal antibody produced from ascite fluid (purified immunoglobulin G diluted 1:10,000 in TBSTM), and the secondary HRP-conjugated antibody was a goat anti-mouse antibody (Dako Diagnostics Canada, Missisauga, Ontario, Canada) used at 1:2000 in TBST containing 1% BSA. Peroxidase activity was revealed by using a Western Blot Chemiluminescence Reagent Plus kit (New England Nuclear, Boston, MA).

Radiolabeling and Immunoprecipitation

For metabolic labeling of HA-tagged Kex2p and CPY, cells grown until the midlog phase were concentrated to 3 $OD_{600\ nm}$/ml and depleted of methionine and cysteine by incubation for 30 min at 30°C in minimal complete medium lacking methionine and cysteine. Cells were then pulse labeled for the indicated times at 30°C with 75 μCi/ml Tran^{35}S-label (ICN, Costa Mesa, CA). The chase was initiated by adding methionine and cysteine to 5 mM each and $(NH_4)_2SO_4$ to 10 mM. At the indicated times, sodium azide was added to a final concentration of 10 mM to cell samples (3 $OD_{600\ nm}$ cells). Cells were then lysed and prepared for immunoprecipitation as described (Wilcox and Fuller, 1991). Immunoprecipitation was performed overnight at 4°C in 0.5 ml of immunoprecipitation buffer (IPB; 50 mM Tris-HCl, pH 7.5, 1% Triton-X-100, 0.1% SDS, and 0.2% deoxycholate for anti-HA antibody; and 50 mM Tris-HCl, pH 7.5, 1% Triton-X-100, and 2 mM EDTA for anti-CPY antibody) with 1.5 μl of anti-HA antibody or 3 μl of 10 mg/ml anti-CPY antibody. Thirty microliters of 100 mg/ml protein A-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden) were then added, and samples were incubated at room temperature for 45 min. Immunoprecipitates were successively washed in 0.5 ml of IPB, 0.5 ml of IPB plus 2 M urea, and 0.5 ml of IPB plus 1% 2-mercaptoethanol, solubilized at 100°C for 3 min in 50 μl SDS-PAGE sample buffer, and finally loaded on 6 or 8% SDS-PAGE (anti-HA or anti-CPY immunoprecipitates, respectively).

Metabolic labeling and immunoprecipitation of secreted α-factor were carried out as previously described (Stepp *et al.*, 1995). The α-factor antiserum was a generous gift from S.K. Lemmon (Case Western Reserve University, Cleveland, OH). Immunoprecipitates were resolved on 8–20% discontinuous gradient SDS-PAGE.

After electrophoresis, gels were successively soaked for 30 min in 30% methanol plus 10% acetic acid (plus 5% glycerol for 20% SDS-PAGE) and 30 min in Enlightning (New England Nuclear), dried, and autoradiographed on Biomax-MS films using an intensifying screen (Eastman Kodak, Rochester, NY).

Subcellular Fractionation

Spheroplasts were prepared as described above from 50 $OD_{600\ nm}$ cells. Lysis conditions and fractionation procedure were described elsewhere (Schimmoller *et al.*, 1995; Powers and Barlowe, 1998). Briefly, lysates were loaded on top of a discontinuous 22–60% sucrose gradient and centrifuged at 35,000 rpm for 2.5 h at 4°C. Fifteen 0.77-ml fractions were collected from the top of gradient. Twenty microliters of each fraction were resolved on SDS-PAGE, transferred to nitrocellulose, and probed with anti-HA as described above or with rabbit anti-Cne1p (1:2000; Parlati *et al.*, 1995), rabbit anti-Kre2p (1:500; Lussier *et al.*, 1995), and rabbit anti-CPY (2 μg/ml; Research Diagnostics, Flanders, NJ). Anti-Cne1p, anti-Kre2p, and anti-CPY were revealed with HRP-conjugated goat anti-rabbit antibodies used at 1:30,000 (Jackson ImmunoResearch, West Grove, PA). Purified anti-Kre2p antibodies were a generous gift from Dr. H. Bussey (McGill University, Montréal, Quebec, Canada).

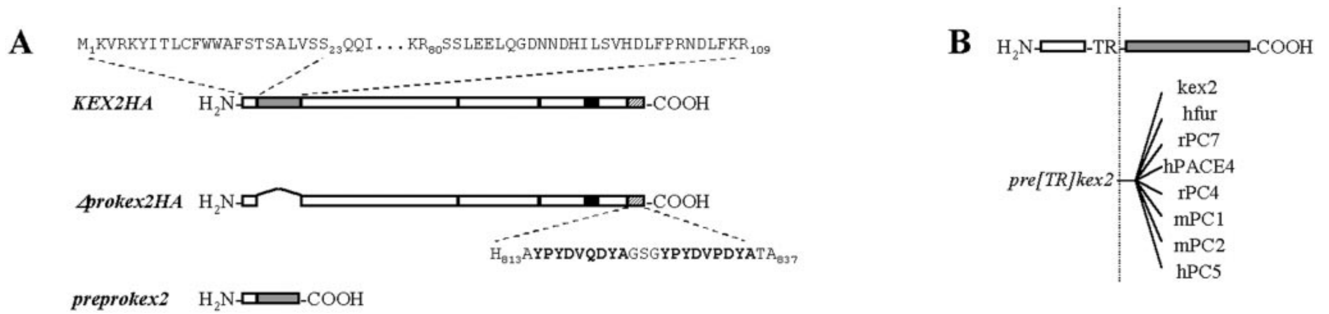


Figure 1. Schematic representation of constructs. (A) Kex2p constructs. *KEX2HA* was obtained by adding two tandem copies of the HA epitope (hatched box) to the 3′ end of the *KEX2* coding sequence. Δ*prokex2HA* encodes a protein in which Ser^{23} is adjacent to Leu^{110} and thus lacks the entire proregion. *Preprokex2* encodes the 109 N-terminal amino acids of wild-type Kex2p protein. The transmembrane domain is shown as a black box, and the proregion is shown as a dotted box. The sequence of the signal peptide and partial sequence of the proregion are presented above the first construct. The sequence of the HA epitope is presented below the second construct in bold characters. (B) Fusions of PC proregions and Kex2p signal peptide. The *pre[TR]kex2* encoding the Kex2p signal peptide bearing a C-terminal Thr-Arg doublet was fused to the proregion of either Kex2p or human furin, rat PC7, human PACE4, rat PC4, mouse PC1, mouse PC2, and human PC5.

RESULTS

Deletion of Kex2p Proregion Abolishes the Enzyme Activity In Vivo and In Vitro

To assess the importance of the Kex2p proregion for the production of a fully active enzyme, we constructed pVT103-U-derived plasmids harboring either *KEX2HA* or Δ*prokex2HA*. *KEX2HA* encodes wild-type Kex2p to which two HA epitope sequences were fused in frame at the C terminus of the cytosolic domain (Kex2HA), whereas Δ*prokex2HA* encodes a Kex2HA devoid of its proregion (Figure 1A). These plasmids were initially used to transform *S. cerevisiae* strain M213 in which *KEX2* is interrupted by *HIS3* (Germain *et al.*, 1993). However, preliminary experiments with this strain suggested a residual expression of Kex2p portions that could interfere with subsequent studies (our unpublished results). To circumvent this problem, we decided to construct a new strain by disruption (instead of an interruption) of the *KEX2* locus in M213 with the *LEU2* auxotrophy marker (Figure 2A). The resulting strain, GLY39,

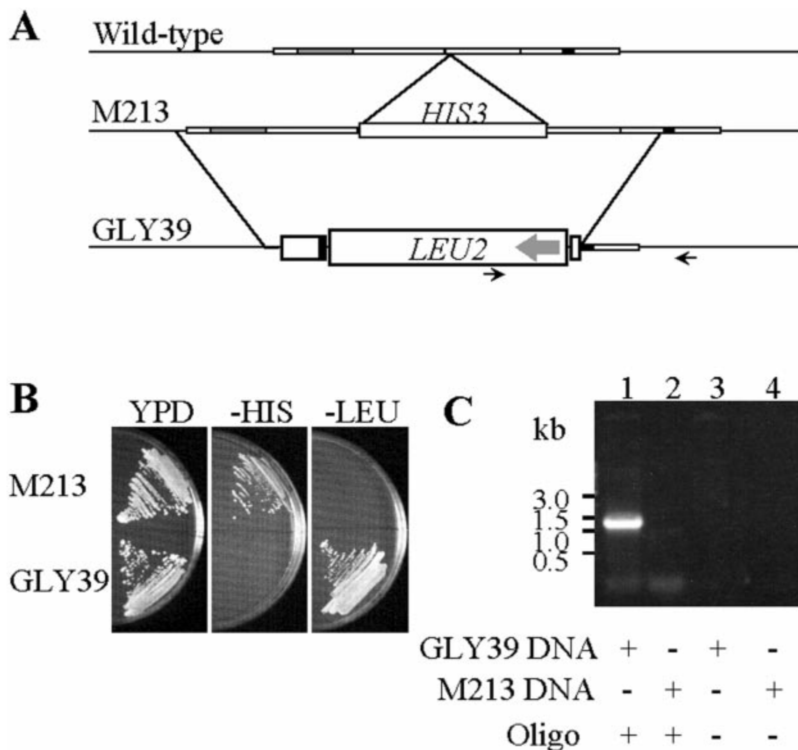


Figure 2. Construction of GLY39, a new kex2 disrupted strain. (A) In M213 the *KEX2* locus was only interrupted with the *HIS3* marker, whereas in GLY39 the *KEX2* locus was actually disrupted with the *LEU2* marker. The thick arrow represents transcription direction of the *LEU2* marker, and thin arrows indicate the hybridization site of oligonucleotides used for PCR. (B) Control of M213 and GLY39 growth on rich medium (YPD) and medium lacking histidine (−HIS) or leucine (−LEU). (C) Confirmation of GLY39 genotype. PCR was performed with genomic DNA from GLY39 (lanes 1 and 3) and the parental M213 strain (lanes 2 and 4). Negative controls without oligonucleotide are shown (lanes 3 and 4).

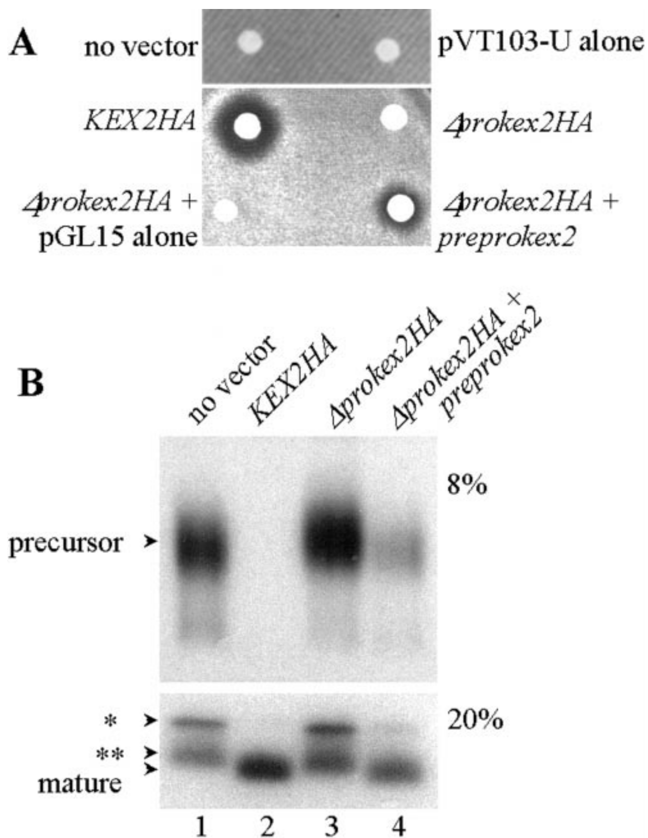


Figure 3. In vivo detection of Kex2p activity. (A) Halo assays. GLY39 or GLY39-derived strains harboring the indicated plasmids were grown at 30°C and tested for their ability to produce a halo of growth inhibition when spotted on a lawn of the supersensitive M200–6CK strain. (B) Immunoprecipitation of pro- and mature α-factor. Cells were labeled for 30 min at 30°C, and media were subjected to immunoprecipitation with α-factor antiserum. Immunoprecipitates were then run on an 8–20% discontinuous SDS-PAGE gradient gel. The precursor form was resolved in the 8% part of the gel, and mature α-factor was resolved in the 20% part. Asterisks indicate forms whose presence is associated with pro-α-factor.

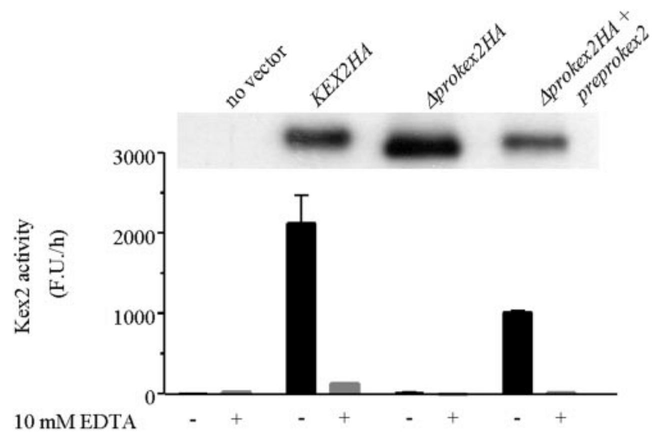


Figure 4. In vitro enzymatic assay of Kex2p activity. Equal amounts of membranes prepared from indicated strains were assayed for Kex2p activity and Western blot (inset). Activity was measured as the fluorescence released by cleavage of pERTKR-MCA at 25°C in the presence 1 mM $CaCl_2$ or 10 mM EDTA and expressed in fluorescence units per hour (F.U./h). Values are the mean of duplicates. Immunoblots were quantified by digital scanning and expressed in arbitrary units (1 for *KEX2HA*, 1.92 for Δ*prokex2HA*, and 0.67 for Δ*prokex2HA* + *preprokex2*).

was selected for its ability to grow on a medium lacking leucine (Figure 2B). Correct integration was checked by PCR (Figure 2C) using two oligonucleotide primers located in *LEU2* and at the 3′ end of *KEX2*, respectively (Figure 2A, thin arrows). Kex2p activity in GLY39-derived strains was qualitatively assessed using the halo assay, a biological test based on the efficiency of α-factor maturation and secretion (Julius *et al.*, 1984). As expected, nontransformed GLY39 cells or cells transformed with the vector pVT103-U alone did not show any Kex2p activity (Figure 3A, top), whereas a large halo was produced by the strain expressing the *KEX2HA* construct (Figure 3A, bottom). Halos of comparable sizes were obtained with *KEX2*- and *KEX2HA*-transformed strains, indicating that the presence of the HA tag did not affect enzyme activity (our unpublished data). No halo was produced by the Δ*prokex2HA* strain, suggesting that the enzyme produced without its proregion is inactive (Figure 3A, bottom). These results were confirmed by direct analysis of pro-α-factor maturation. To this end, secreted pro- and mature α-factor from ^{35}S pulse-labeled cells were immunoprecipitated (Figure 3B). Although the *KEX2HA* strain completely matured pro-α-factor, as judged by the unique fastest migrating species (Figure 3B, lane 2), control GLY39 (lane 1) and Δ*prokex2HA* (lane 3) strains were characterized by a high level of pro-α-factor and the absence of any detectable mature α-factor. Only low amounts of intermediary-migrating species were detected, indicating that pro-α-factor maturation was inefficient in those strains. Thus, the absence of halo production in the Δ*prokex2HA* strain is actually due to a lack of pro-α-factor processing.

To confirm the lack of Kex2p activity in the mutant strain, Kex2p activity in membranes prepared from each strain was determined in vitro with the fluorogenic substrate pERTKR-MCA (Figure 4, bottom). No activity was detected in membranes prepared from control GLY39 and Δ*prokex2HA* strains. In contrast, high activity was measured in extract from *KEX2HA*. As expected for a calcium-dependent enzyme, cleavage of the fluorogenic substrate was prevented by previous incubation with 10 mM EDTA. Thus, both in vivo and in vitro data show that no Kex2p activity is detected in the Δ*prokex2HA* strain.

The Function of Kex2p Proregion Can Be Complemented In Trans

We next asked whether the Kex2p proregion could act in *trans*. To test this, plasmid *preprokex2* encoding the Kex2p signal peptide and proregion (Figure 1A) was used to transform the Δ*prokex2HA* strain. In this situation a partial restoration of the Kex2p activity was found, whereas no halo was observed in control transformations with the pGL15 vector alone (Figure 3A, bottom) or when GLY39 was transformed only with the plasmid carrying the *pre-*

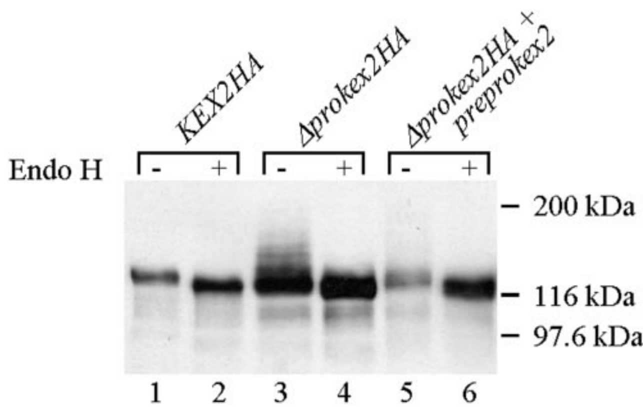


Figure 5. Immunoblotting analysis of Kex2p expression. Total protein extracts (5 μg) from the indicated strains were analyzed by immunoblotting with the monoclonal anti-HA (12CA5) antibody. Extracts were treated or not with endoglycosidase H.

prokex2 construct (our unpublished data). As expected from the results of the halo experiments, not only pro- but also mature α-factor was immunoprecipitated from the medium of the metabolically labeled GLY43 strain (Δ*prokex2HA* + *preprokex2*; Figure 3B, lane 4). Furthermore, Kex2p activity was detected in GLY43 membranes and fully inhibited by EDTA (Figure 4, bottom). Normalization of enzymatic data (Figure 4, bottom) to the amount of Kex2p in assayed samples quantified by immunoblotting (Figure 4, top) revealed that the Kex2p specific activity in GLY43 membranes was 72% of the wild-type level. Thus, the Δ*prokex2* phenotype is largely rescued by separate expression of the proregion in *trans*.

Kex2p Glycosylation Is Affected by Deletion of Its Proregion

When total protein extracts from strains *KEX2HA* and Δ*prokex2HA* were analyzed by Western blotting with the anti-HA antibody (Figure 5, lanes 1 and 3, respectively) the Δprokex2HA protein showed a more heterogenous electrophoretic pattern, with most of the protein migrating with a slightly lower apparent molecular mass (MM) than that of the Kex2HA protein (127 and 134 kDa, respectively). To explore the possibility that this difference in MM is due to different *N*-glycosylation states of the proteins, we next performed endoglycosidase H digestions. A 7-kDa decrease was observed for the wild-type Kex2HA (Figure 5, lanes 1 and 2), whereas treatment of the mutant protein only resulted in a 4- to 5-kDa loss (Figure 5, lanes 3 and 4). The remaining discrepancy (127 and 122 kDa for the wild-type and mutant protein, respectively) could result from another step in post-translational modifications such as the extent of *O*-glycosylation. Interestingly, extracts from the GLY43 strain (double transformant) revealed mostly the presence of the slow-migrating Kex2p species (Figure 5, lanes 5 and 6). These results show that expression in *trans* of the Kex2p proregion largely corrects the glycosylation defect attributable to proregion deletion.

Deletion of the Proregion Results in Kex2p Localization to the ER

Because the Δ*pro* mutation caused the production of an inactive and abnormally glycosylated protein, but still residing in membrane preparations, we addressed the question of whether the mutant protein is mislocalized. We investigated the subcellular distribution of Kex2p by fractionation of membranes isolated from our different strains. As expected, wild-type Kex2p cosedimented with the Golgi resident mannosyltransferase Kre2p (Figure 6, left panels). In contrast, proregion-deleted Kex2p was exclusively found to cosediment with the ER resident chaperone calnexin (Cne1p) and was absent from fractions containing either Kre2p or vacuolar marker CPY (Figure 6, middle panels). In *trans* expression of the proregion in the Δ*prokex2HA* strain lead to a partial relocalization of Kex2p in Golgi fractions (Figure 6, right panels). Therefore, transport out of the ER is prevented

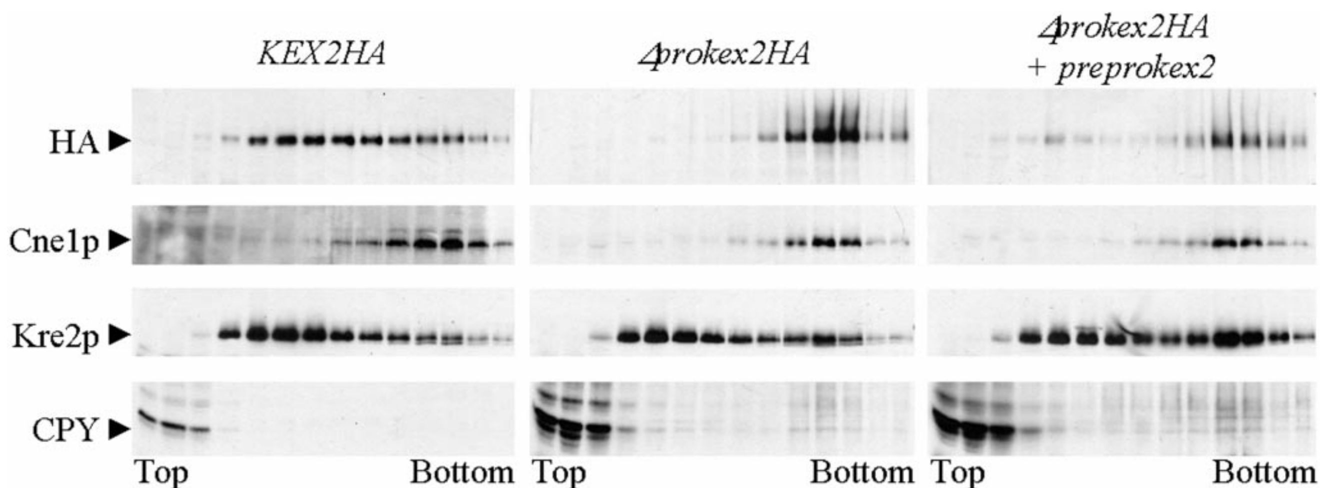


Figure 6. Sucrose gradient of HA-tagged Kex2p. Spheroplasts lysates from the indicated strains were separated on sucrose density gradients (22–60%), and fractions were collected from the top. Kex2HA, calnexin (Cne1p, ER marker), Kre2p (Golgi marker), and CPY (vacuole marker) in each fraction were detected by Western blotting.

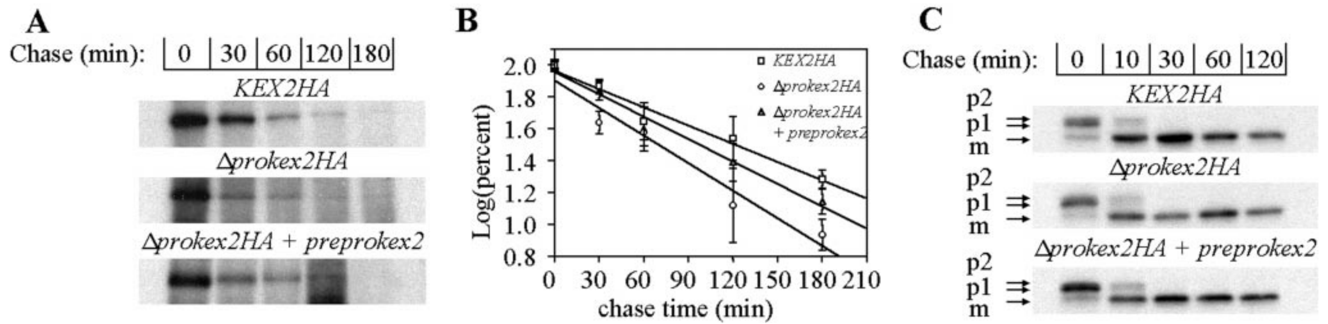


Figure 7. Pulse–chase analysis of HA-tagged Kex2p and CPY. Cells transformed with the indicated plasmids were labeled for 10 min at 30°C with Tran^{35}S label, and a chase was begun. Samples were harvested at the indicated times and subjected to immunoprecipitation. (A) HA-tagged proteins were immunoprecipitated with the monoclonal 12CA5 anti-HA antibody. (B) Data from three independent experiments were digitally scanned. Values are expressed as percentage of the maximum intensity in each experiment, and the log of the value was used for linear regression analysis to determine the half-life of Kex2p protein in a strain transformed with the *KEX2HA* construct (squares), Δ*prokex2HA* construct (circles), or Δ*prokex2HA* + *preprokex2* constructs (triangles). (C) Anti-CPY antibodies immunoprecipitated ER and Golgi forms of pro-CPY (p1 and p2, respectively) and mature CPY (m).

by deletion of Kex2p proregion but is partially recovered by *trans* expressing the prodomain.

Kex2p Half-Life Is Affected by Deletion of Its Proregion

The kinetics of Kex2p transport were analyzed by pulse–chase experiments. The wild-type Kex2HA protein chased progressively into a more slowly migrating form (Figure 7A, top), reportedly a consequence of additional glycosylation caused by protein recycling into the late Golgi compartment (Wilcox and Fuller, 1991; Wilcox *et al.*, 1992). However, the apparent MM of the Δprokex2HA protein remained constant (Figure 7A, middle). This latter observation is consistent with a lower amount of glycosylation of the mutant protein. The calculated half-life of the wild-type protein was 71 ± 21 min (Figure 7B), in agreement with previous observations (Wilcox *et al.*, 1992), considering that in our system Kex2p is ~10-fold overexpressed (our unpublished data). In contrast, the Δprokex2HA protein half-life was only 37 ± 7 min (Figure 7B), suggesting that mutant protein was more rapidly degraded than wild-type Kex2p. Complementation was also successful in these experiments, because both Kex2p gel mobility (Figure 7A, bottom) and degradation (Figure 7B; half-life = 56 ± 14 min) were slackened when Δ*prokex2HA* and *preprokex2* were coexpressed. The biosynthesis of CPY was also analyzed by a pulse–chase experiment (Figure 7C). This vacuolar protein is synthesized as a preproenzyme, which is rapidly converted to a proenzyme (p1-CPY) upon arrival into the ER. During its transit through the Golgi apparatus p1-CPY acquires oligosaccharide chains (p2-CPY). Final p2-CPY processing into mature CPY (m-CPY) occurs in the vacuolar compartment by cleavage of its proregion. The kinetics of CPY processing were the same for all strains (Figure 7C), revealing the integrity of their secretory pathway. Thus, the accelerated turnover of Δprokex2HA protein does not result from any general transport deficiency but is a consequence of proregion deletion.

Complementation of Δpro Mutation Is Sequence Specific

Kex2p is the prototype of the eukaryotic family of subtilisin-like enzymes, which were shown to be involved in proprotein processing by cleaving at pairs of basic amino acid residues (Seidah *et al.*, 1998). Although Kex2p shares higher sequence identity with its mammalian counterparts in the catalytic domain (up to 50%) than in the proregion (between 23 and 29%) (Seidah *et al.*, 1998), we undertook a systematic analysis of the *trans* complementation by each of the PC proregions. Mammalian proregions were inserted downstream of Kex2p signal sequence (Figure 1B). These genetic manipulations created proregions comprising two additional residues, Thr and Arg, at their N termini, and we showed that the [TR] proregion of Kex2p (encoded by pAPR2) complemented the Δ*pro* mutation as well as the wild-type proregion (Figure 8, halos 6 and 4, respectively). In addition, when used as a negative control, the plasmid pAPR1 bearing the signal peptide nucleotide sequence alone (*pre[TR]kex2*) had no effect on Δ*pro* phenotype. Plasmids encoding human furin-, rat PC7-, human PC5-, human PACE4-, rat PC4-, mouse PC1-, and mouse PC2 proregions were then used to transform the Δ*prokex2HA* strain, and Kex2p activity was finally assayed by the halo test (Figure 8). No halo could be observed, indicating that no complementation took place with any mammalian PC proregion added in *trans*. This supports the hypothesis of a specific interaction of the Kex2p proregion with the remaining part of the enzyme.

A C-terminal Basic Residue Is Critical for the Kex2p Proregion Function In Trans

The proregion of Kex2p, as well as those of PCs, has a C-terminal Lys-Arg doublet. To assess the importance of these amino acid residues for the Kex2p proregion function, mutant proregions were tested for their ability to complement in *trans* the Δ*pro* mutation by the halo test. Deletion of the C-terminal Lys-Arg doublet (ΔKΔR), as well as its substitution by a Gly-Gly doublet (Figure 9, halos 4 and 3,

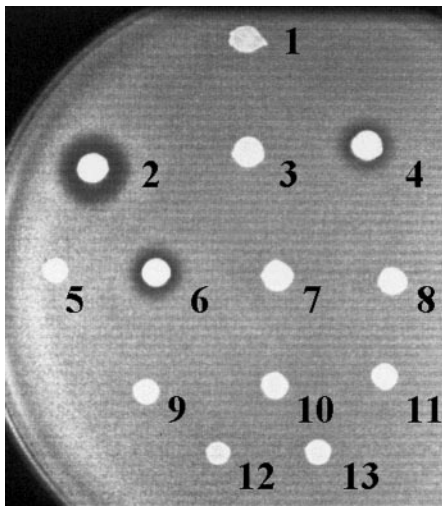
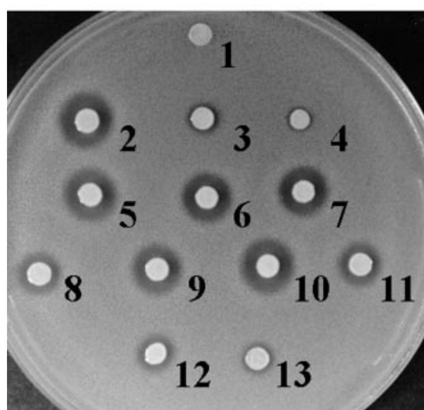


Figure 8. Complementation of Δpro phenotype is sequence specific. Halo tests were performed with control GLY39, *KEX2HA*, Δ*prokex2HA*, and Δ*prokex2HA* + *preprokex2* strains (1–4, respectively) or the Δ*prokex2HA* strain transformed with plasmids expressing *pre[TR]kex2* (5), *pre[TR]prokex2* (6), or proregions of human furin, rat PC7, human PC5, human PACE4, rat PC4, mouse PC1, and mouse PC2 (7–13, respectively) fused to *pre[TR]kex2*.

respectively), led to a very low complementation of the Δ*pro* phenotype. The requirement for the C-terminal basic doublet was further studied with a series of mutants. We introduced mutations that either conserved the dibasic stretch (KK, RR, and RK) or substituted one basic residue to a Gly (KG, GK, GR, and RG). We also generated two shorter mutant proregions lacking the last amino acid but still bearing a C-terminal basic residue (KΔR and RΔR). No difference was observed between the halos produced by the wild-type proregion and the KK, RR, and RK mutants. This indicates that the nature of the basic residue (Lys or Arg) at either position of the doublet does not affect the proregion *trans* activity. On the other hand, *trans* complementation was much more efficient when a basic residue was at the C-terminal extremity (GK and GR) rather than at the penultimate position (RG and KG; Figure 9, compare 9 with 8 and 10 with 11). Surprisingly, KΔR and RΔR mutations drastically reduced the complementation (Figure 9, halos 12 and 13). This suggests that shorter proregions do not correctly interact with the enzyme.



#	C-terminus
1	No proregion
2	-KR
3	-GG
4	-ΔKΔR
5	-KK
6	-RR
7	-RK
8	-KG
9	-GK
10	-GR
11	-RG
12	-KΔR
13	-RΔR

Figure 9. Importance of the C-terminal basic doublet for proregion function. Halo tests were performed with the Δ*prokex2HA* strain nontransformed (1) or transformed with plasmids expressing either the wild-type (2) or mutant Kex2p proregions (3–13). C-terminal sequences of the proregions are listed.

DISCUSSION

The Kex2p endoprotease of the yeast *S. cerevisiae* is a subtilisin-like enzyme involved in the maturation of pro-α-mating factor and pro-killer toxin by limited proteolysis at pairs of basic amino acid residues (Leibowitz and Wickner, 1976; Julius *et al.*, 1984; Mizuno *et al.*, 1988, 1989). Like subtilisin and its mammalian homologues, Kex2p is first synthesized with an N-terminal proregion that is rapidly removed by an autocatalytic reaction (Wilcox and Fuller, 1991; Germain *et al.*, 1992). To determine the role of this proregion, we have expressed a mutant Kex2p deleted of its proregion (Δ*prokex2HA* mutant) in yeast cells disrupted for the *KEX2* gene (GLY39 strain). Kex2p activity in transformed cells was monitored in vivo by a halo assay based on the efficiency of α-mating factor maturation and by immunoprecipitation of ^{35}S pulse-labeled α-factor. Kex2p activity in membrane preparations was determined in vitro by cleavage of a synthetic peptide. We show here that Δ*prokex2HA* encodes an inactive enzyme.

It has been previously proposed that prodomains may act as intramolecular chaperones (Shinde *et al.*, 1995). Studies with bacterial subtilisin and α-lytic protease indicated that when produced without their propeptide these enzymes remain inactive in a partially folded state, suggesting that the function of the proregion is to help the protease domain fold into an active conformation (Ikemura *et al.*, 1987; Baker *et al.*, 1992). The phenotypes observed in the Δ*pro* mutant are consistent with a model whereby deletion of Kex2p proregion would lead to a misfolded inactive protein and support the conclusion that the Kex2p proregion acts as an intramolecular chaperone. Indeed, the Δprokex2 protein is retained in the ER and presents an accelerated turnover. The lower glycosylation observed for the Δprokex2 protein likely results from the absence of oligosaccharide chain elongation in post-ER compartments.

Results from in vitro refolding of bacterial subtilisin (Zhu *et al.*, 1989) and in vivo studies with several degradative proteases synthesized with an N-terminal proregion such as bacterial α-lytic protease (Silen and Agard, 1989), subtilisin (Chang *et al.*, 1996), thermolysin (Marie-Claire *et al.*, 1999), *S. cerevisiae* proteinase A (Van den Hazel *et al.*, 1993), or secreted alkaline extracellular protease from *Yarrowia lipolytica* (Fabre *et al.*, 1992) have all indicated that prodomains can act in *trans* to activate the protease domain. Accordingly, we show here for the first time that such an in *trans* activation can take place in vivo for a member of the dibasic-specific kexin family. Thus, a covalent linkage of the prodomain is not absolutely required for its productive interaction with the protease domain. However, the addition of the propeptide does not totally rescue the Δ*pro* phenotype. Neverthe-

less, increased amounts of *trans*-supplied proregion augment complementation efficacy (our unpublished data). This dose-dependent action of the proregion might reflect independent entry into the secretory pathway, which certainly renders less efficient its association with the protease domain compared with that of a *cis*-supplied prosegment.

Alhough Kex2p shares a strong identity with its mammalian homologues, none of the PC proregions could complement the Δ*pro* mutation. This supports the view that the chaperone-like function of the Kex2p prosegment is specific for its cognate enzyme. Such specific interaction between PCs and their proregion has been reported. Indeed, it has been observed that proregion or proregion-related peptides of furin, PC7, or PC1/3 can inhibit in a specific manner these enzymes in vitro with nanomolar Ki (Anderson *et al.*, 1997; Boudreault *et al.*, 1998; Zhong *et al.*, 1999). Prodomains of kexin-like family members seem thus to interact specifically with their own protease domain.

Interestingly, we did observe that the Kex2p proregion could not reduce halo size when overexpressed in the wild-type *KEX2* strain (our unpublished data). Alhough these observations need to be confirmed by in vitro studies, they suggest that the PC proregions have an additional function, which the Kex2p proregion lacks. Activation and inhibition may be two different mechanisms involving distinct parts of the region. It is thus conceivable that portions that are conserved among the Kex2p and PC proregions would be important for the activation function.

Proregions of Kex2p and mammalian PCs have a conserved Lys-Arg doublet at their C terminus. One role of this pair of residues is to provide a site for the autocatalytic cleavage of the proregion. Our results show for the first time that they are critical for the proregion *trans* activity. Three important observations were made during our mutational analysis of the penultimate and C-terminal residues. First, the C terminus of the proregion cannot be shortened even by one amino acid residue. Mutant proregions with deletion of one residue or both residues have lost most of their complementation activity. Second, a basic residue at the C-terminal position is sufficient to ensure full activity of the proregion. Finally, a penultimate basic residue does not fully compensate the absence of such a residue in the C-terminal position. Structural analysis of subtilisin and α-lytic protease suggests that final folding of the mature protein is promoted by interaction with the proregion. During this last step, the proregion C terminus is located in the catalytic pocket (Sauter *et al.*, 1998). Such a model is consistent with our results. The absence of correctly positioned basic residues in the C terminus of the Kex2p proregion would hinder its insertion in the catalytic pocket, preventing final folding. Whether the C-terminal basic residue acts to model the active site or to stabilize the interaction between the immature protease and the proregion is not clear at the present time. More experiments are needed to clarify the mechanism by which the proregion functions and to identify other essential domains for the intramolecular chaperone activity.

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