Posttranslational Processing of the Prohormone-cleaving 
Kex2 Protease in the *Saccharomyces cerevisiae* Secretory Pathway

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**Abstract.** The Kex2 protease of the yeast *Saccharomyces cerevisiae* is a prototypical eukaryotic prohormone-processing enzyme that cleaves precursors of secreted peptides at pairs of basic residues. Here we have established the pathway of posttranslational modification of Kex2 protein using immunoprecipitation of the biosynthetically pulse-labeled protein from a variety of wild-type and mutant yeast strains as the principal methodology. Kex2 protein is initially synthesized as a prepro-enzyme that undergoes cotranslational signal peptide cleavage and addition of Asn-linked core oligosaccharide and Ser/Thr-linked mannone in the ER. The earliest detectable species, I 

The *Saccharomyces cerevisiae* ("yeast") KEX2 gene product, required for maturation of pro-α-factor and pro-killer toxin (Fuller et al., 1988; Bussey, 1988), is a Ca<sup>2+</sup>-dependent, neutral serine protease that cleaves peptide substrates at the carboxyl side of Lys-Arg and Arg-Arg sites (Julius et al., 1984b; Fuller et al., 1989a; Mizuno et al., 1989). Pro-α-factor and pro-killer toxin are processed before fusion of secretory vesicles with the plasma membrane, probably in the Golgi apparatus (Julius et al., 1984a; Bussey et al., 1983; Franzusoff et al., 1991; Redding et al., 1991). Intracellular localization of Kex2 protein requires both the COOH-terminal cytosolic tail of Kex2 protein and a functional clathrin heavy chain (Fuller et al., 1989a; Payne and Schekman, 1989). Analysis of posttranslational modifications of Kex2 protein should map the route of the molecule through the secretory pathway and help characterize the compartment in which Kex2 protease functions.

Kex2 protease can process the mammalian precursors proinsulin and pro-opiomelanocortin accurately in vivo (Thim et al., 1986; Thomas et al., 1988). Newly discovered mammalian homologues of Kex2 protein suggest that it may be prototypical of eukaryotic processing enzymes specific for paired basic sites (Fuller et al., 1989b; Smeekens and Stein, 1990; Seidah et al., 1990). Thus, studies of the posttranslational modification and localization of the yeast Kex2 protease should be of general importance in understanding the compartmentalization of proteolytic processing reactions in eukaryotes.

The Kex2 protein sequence suggests several post-translational modifications (see Fig. 1). The NH<sub>2</sub>-terminus contains a probable signal peptide (residues 1-19), and a single hydrophobic transmembrane domain (residues 679–699) divides the protein into a lumenal portion containing the proteolytic domain (Fuller et al., 1989a) and a 115-residue cytosolic "tail." The lumenal domain contains potential sites for Asn-linked (N-linked) glycosylation and a region rich in serine and threonine that may be the site of extensive Ser/Thr-linked glycosylation (Fuller et al., 1989a). Residues 144–438 are ~30% identical to the mature form of subtilisin, a bacterial serine protease (Fuller et al., 1988; Mizuno et al., 1988). Subtilisin contains a 77-residue "pro"-region that is excised by an autoproteolytic, and possibly intramolecular, reaction (Power et al., 1986; Ikemura and Inouye, 1988). Analogously, a putative pro-sequence lies between the signal peptide and subtilisin domain of Kex2 protein. Potential sites for autoproteolytic processing of the Kex2 "pro"-segment occur at Lys<sub>79</sub>Arg<sub>102</sub> and Lys<sub>279</sub>Arg<sub>109</sub>. After signal peptide removal, cleavage at Arg<sub>102</sub> would remove a 61-residue peptide (7377 D), and at Arg<sub>109</sub>, a 90-residue peptide (10,821 D).

Thus, maturation of Kex2 protease might involve signal peptide cleavage, addition of N-linked and O-linked oligosaccharide, and proteolytic removal of the NH<sub>2</sub>-terminal pro-segment. In this work, we have demonstrated these modifications of Kex2 protein, and the kinetics and compartmentalization of the individual reactions have been established.
Materials and Methods

Strains and Plasmids

Yeast strains used in this study are listed in Table I. A congenic set of sec mutant strains was derived by back-crossing various sec alleles (Novick et al., 1980) into the W303 background (Redding et al., 1991). In pAB-KX22 the KEX2 structural gene is under the transcriptional control of the TDH3 promoter within the multicopy episomal vector pAB23, resulting in 100–150-fold overproduction of Kex2 protease (Fuller et al. 1989a). Plasmid YCP-KX22 consists of a 4.2-kb fragment of pAB-KX22, containing the KEX2 structural gene under control of the TDH3 promoter, inserted into the BamHI site of yeast centromere vector YCP50 (CEN4 ARSI URA3) (Rose et al., 1987), resulting in 20–40-fold overproduction of Kex2 protease. Yeast transformation was as described (Burgers and Percival, 1987).

Materials and Reagents

[35S]H2SO4 (~43 Ci/mg S) was from ICN Pharmaceuticals, Inc. (Irvine, CA). [35S]Amino acids refers to either “Tran35S-label” from ICN Pharmaceuticals, Inc. (>1,000 Ci/mmol) or “express [35S]protein labeling mix” (~1,150 Ci/mmol methionine) from New England Nuclear Corporation (Boston, MA). Benzamidine-HCl, PMSF, and 2-deoxy-d-glucose (2-DOG) were from Sigma Chemical Co. (St. Louis, MO). Other protease inhibitors were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Pancreatin was from Calbiochem-Behring Corp. (La Jolla, CA); N-Glycanase, from Genzyme Corp. (Boston, MA); Na-salicylate, from EM Science (Cherry Hill, NJ); oligo(dT)-cellulose Type 7, from Pharmacia Fine Chemicals (Piscataway, NJ); and wheat germ lysate for in vitro translation, from Promega Biotech (Madison, WI). Antisera against the α-N-mannosyl linkage was a gift of R. Schekman (University of California, Berkeley, CA).

Radio labeling and Immunoprecipitation

Cells to be labeled were grown in low sulfate minimal medium (LSM) containing 100 μM (NH4)2SO4 and 2% (wt/vol) glucose (Fuller et al., 1989a), and growth was monitored using a Klett-Summerson colorimeter. Cultures were harvested by filtration and subjected to “sulfate depletion” for 30 min by resuspension at a density of ~2 × 10⁸ cells/ml in LSM lacking sulfate (NSM), after which labeling was initiated by adding either [35S]H2SO4 or [35S]Amino acids to 300 μCi/ml. When used, a “chase” was initiated by adding unlabelled (NH4)2SO4 to 10 mM and cysteine and methionine to 1 mM. For “rapid pulse-chase” experiments, strains containing plasmid YCP-KX22 were used because of the difficulty of labeling cells expressing Kex2 protein at the wild-type level in very short pulse times (1–2 min) required for these experiments. Labeled cell samples (1 ml) were made 10 mM in sodium azide, chilled on ice, harvested by centrifugation, and washed once in wash buffer (10 mM Na-HEPES [pH 7.0], 10 mM Azide) containing protease inhibitors (10 mM EDTA, 1 mM PMSF, 100 μM Nα-[p-tosyl]L-lys-chloromethyl ketone, 100 μM L-1-tosylamido-2-phenyl-ethyl-chloromethyl ketone, 1 mM benzamidine-HCl, 25 μM pepstatin A). Cell pellets were stored at ~80°C before lysis. Lysis of Kex2 protein was often less efficient at elevated temperatures (35–38°C) than at 25°C or 30°C both in wild-type and sec mutant strains.

Thawed cell pellets were resuspended in 50 μl lysis buffer (50 mM Tris-HCl [pH 7.5], 1% [wt/vol] SDS, plus protease inhibitors) and lysed by vortexing with 0.38 g of 0.5-mm glass beads in 13 × 100 mm glass tubes for 2 min, as described (Julius et al., 1984a). Lysates were heated to 97–98°C for 3 min, and 0.5 ml immunoprecipitation buffer (IPB) (50 mM Tris-HCl [pH 7.5], 1% [vol/vol] Triton-X100, 0.1% SDS, and 0.2% [wt/vol] deoxycholate) was added. Diluted lysates were heated again (1 min), transferred to microfuge tubes, and cell debris was removed by centrifugation.

To immunoprecipitate Kex2 protein, anti-Kex2 antiseraum (1 to 1.5 μl), raised against a β-galactosidase fusion protein containing the 100 COOH-terminal residues of Kex2 protein (Fuller et al., 1989b), was added to the lysate along with 5-7 μl of Pancreatin slurry. For immunoprecipitation with antibody against the α-N-mannosyl linkage, 2 μl of antisera were used. After incubation at 4°C for 2–24 h, immunoprecipitates were washed successively with 0.5 ml IPB, 0.5 ml IPB plus 2 M urea, and 0.5 ml IPB plus 1% (vol/vol) 2-mercaptoethanol. Washed immunoprecipitates were solubilized in SDS-PAGE sample buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 30% [vol/vol] glycerol, 5% 2-mercaptoethanol, 5 mM EDTA) at 97–98°C for 3 min, clarified by centrifugation, diluted 20-fold with IPB, and subjected to a second round of immunoprecipitation. Where indicated, N-glycanase digestion was performed after the first Kex2-specific immunoprecipitation, as described (Fuller et al., 1989b). Samples were subjected to SDS-PAGE (3% stacking gel, 7% separating gel), and gels were washed twice with 300 ml distilled water, soaked in 150 g/l Na-salicylate for 30 min (Chamberlain, 1979), dried on Whatman 3MM paper, and autoradiographed on preflashed Kodak (Eastman Kodak Co., Rochester, NY) XAR-5 x-ray film (Bonner, 1984). Autoradiograms were quantified using a Model 300A Computing Densitometer (Molecular Dynamics, Sunnyvale, CA).

In Vitro Translation

Total yeast RNA was isolated from W303-1A cells containing plasmid pAB-KX22 by glass bead lysis in the presence of 0.5% SDS, 0.1 M Tris, 1 mM EDTA, and 0.1 M LiCl (Tuite et al., 1980). RNA samples were digested with proteinase K, extracted twice with phenol/CHCl₃ (1:1) and precipitated with ethanol. Poly A⁺ RNA was selected using oligo(dT)-cellulose chromatography. In vitro translation reactions were performed according to the instructions accompanying the wheat germ lysate and contained, in 30 μl, 160 mM potassium acetate, 1 μg of polyA⁺ RNA and 1.5 μCi [35S]Amino acids. After incubation for 2 h at 25°C, reactions were made 1% in SDS, and protease inhibitors were added. The reactions were heated to 97–98°C for 3 min, diluted with IPB, and the labeled Kex2 polypeptide was recovered by one round of immunoprecipitation.

Results

In Vitro Translation Product and Mature Kex2 Protein

The in vitro translation product of the KEX2 gene was ex-

1. Abbreviations used in this paper: 2-dG, 2-deoxy-D-glycose; LSM, low sulfate minimal medium; NSM, no sulfate medium.
Figure 1. Schematic diagram of Kex2 protein (814 residues). Stippled circles indicate Lys-Arg sites; stippled squares indicate potential N-glycosylation sites (Asn-X-Ser/Thr). "P-domain" indicates a sequence of 155 residues conserved in mammalian homologues of Kex2 protein (Fuller et al., 1989a; Smeekens and Steiner, 1990).

Figure 2. In vitro translation product of the KEX2 gene and oligosaccharide modification of Kex2 protein. (a) (lane 1) In vitro translation product. (lane 2) Strain W303-1A[YCpKX22] was labeled for 10 min with [35S]-H2SO4 at 30°C. (b) Strains CRY2[Ycp50] (lanes 1–4) and CRY2[YcpKX22] (lanes 5–8) were labeled for 10 min with [35S]-H2SO4 at 30°C, a chase was initiated, and cells were harvested at the indicated times. Samples in lanes 2, 4, 6, and 8 were digested with N-glycanase after the first round of immunoprecipitation. This figure presents a composite of two different autoradiographic exposures of one gel. (c) Effect of 2-deoxy-D-glucose. Strain CRY2[YcpKX22] was grown at 30°C, and 2-dG (500 µg/ml, lanes 3 and 4) was added 15 min before labeling for 10 min with [35S]-H2SO4. Samples in lanes 1 and 4 were digested with N-Glycanase. Media contained 0.1% rather than 2% glucose.
2-dG blocks addition of N-linked core oligosaccharide at early steps in assembly of the dolichol-linked precursor (Schwarz and Datema, 1982). Pretreatment of cells with 2-dG reduced the apparent molecular mass of Kex2 protein by 7 kD (from 127 to 120 kD, Fig. 2 c, lanes 2 and 3), and N-Glycanase digestion caused no additional decrease (Fig. 2 c, lane 4). However, N-Glycanase digestion alone caused only a 3-kD shift (to 124 kD; Fig. 2 c, lane 7). Thus, the additional 4-kD shift observed upon 2-dG treatment most likely represents inhibition of addition of α-1,2-linked residues to O-linked mannose. The difference in apparent mol wt between the in vitro translation product and Kex2 protein produced in the presence of 2-dG is due to partially offsetting effects of proteolytic processing (see below) and, most likely, the attachment of numerous single 2-dG residues in the ER (see Discussion).

Two additional experiments indicated the presence of O-linked carbohydrate. Digestion of Kex2 protein with trifluoromethanesulfonic acid under conditions that remove all oligosaccharide (Edge et al., 1981) produced a greater shift in mobility than was observed with N-Glycanase digestion alone. Also, [3H]mannose was incorporated biosynthetically into Kex2 protein in the presence of tunicamycin (data not shown).

**Intermediates in Maturation of Kex2 Protein**

To visualize early intermediates in maturation of Kex2 protein, cells overproducing the protein about 20-fold were pulse labeled briefly with [35S]labeled amino acids, then chased with excess unlabeled cysteine, methionine, and sulfate (Fig. 3). At zero chase time, Kex2 immunoreactive molecules appeared as two bands separated by a smear of intermediate-sized species (Fig. 3, lane 3). The upper band, "I," (127 kD in lane 3), disappeared by 1 min of chase as the lower band, "I2," (118 kD in lane 3), increased in intensity (lane 4). I2 disappeared by 6 min, as a new species, "J," migrating at 124 kD (in lane 5) appeared. Thereafter, the average apparent molecular mass of Kex2 protein gradually increased to 129 kD by 83 min of chase. In pulse-chase labeling with [35S]SO₄, which equilibrates slowly with endogenous pools of Cys and Met, early intermediates I, and I₂ were not observed except in certain sec mutants at restrictive temperatures. Species J was the first form observed, gradually chased into forms of higher apparent mol wt (e.g., see Figs. 2 b and 7 a). The gradual increase in apparent mol wt of Kex2 protein with time was also observed by Payne and Schekman (1989). Variations in apparent mol wt were observed from experiment to experiment. Characteristic values for these species were: I, 129 ± 1 kD (eleven independent determinations); I₂, 120 ± 2 kD (eleven independent determinations); J, 126 ± 2 kD (seven independent determinations), "mature species" (at 90 min of chase): 130 ± 1 kD (eight independent determinations).

For the following reasons, conversion of I, to I₂ was due to NH₂-terminal proteolysis. First, the anti-Kex2 antibody recognizes exclusively the COOH-terminal 100 residues of Kex2 protein (Fuller et al., 1989b). Second, the magnitude of the shift (9 ± 2 kD) was consistent with cleavage at one of the Lys-Arg sites preceding the subtilisin domain. Finally, NH₂-terminal sequence analysis of purified Kex2 protease is consistent with removal of the pro-peptide (Brenner and Fuller, 1991; see Discussion). Digestion with N-Glycanase increased the mobility of I, slightly more than that of I₂ (Fig. 3, lane 2), suggesting the presence of N-linked oligosaccharide at Asn in, within the "pro" region of I.

**NH₂-terminal Proteolytic Processing Occurs Before Delivery to the Golgi Complex**

The rapid kinetics of conversion of I, to I₂ were consistent with this reaction occurring in the ER. To examine the compartmentalization of the reaction, a sec18 mutant strain was pulse labeled and chased at the permissive (25°C) and restrictive (35°C) temperatures (Fig. 4 a). At the restrictive temperature, sec18 mutants accumulate, intracellularly, secretory proteins with ER-type glycosylation (Esmon et al., 1981) and vesicles thought to be in transit between the ER and Golgi (Kaiser and Schekman, 1990). At 25°C, conversion of I, to I₂ proceeded as in the Sec+ strain, with the appearance of band J by 6.5 min of chase (Fig. 4 a, lanes 1-7). At 35°C, however, species I₂ chased discretely into I₁ without an intervening smear and without the appearance of species J (Fig. 4 a, lanes 8-14). Thus, conversion of I₁ to I₂ appears to result from a discrete endoproteolytic cleavage that occurs before delivery to the Golgi complex. Conversion of I₁ to I₂, therefore, must occur at a location distal to the sec18-dependent block, and the heterogeneous species seen in
Figure 4. Proteolytic maturation of Kex2 protein. (a) Arrest in secl8 cells resolves NH2-terminal proteolytic cleavage from subsequent modifications. A culture of strain CWY1-IA[YCp-KX22] (sec8-1) was grown at 25°C, split, and half was maintained at 25°C, and the other half was shifted to 35°C for 30 min. Each was then labeled with [35S]amino acids for 1 min, a chase was initiated, and samples were harvested at the indicated times. (b) Conversion of I1 to I2 in secl8 at the restrictive temperature is biphasic. The amounts of species I1 and I2 were quantified by densitometric scanning of appropriately exposed autoradiograms of the experiment in a. (circles) Sum of h plus 12 as a percentage of the maximum value (at t = 1.0 min); (squares) I1 as a percentage of I1 plus 12; (triangles) 12 as a percentage of I1 plus 12. (c) Signal peptide cleavage is indicated by the effect of the secl1 mutation. Strain CWY2-1C (secl1-7) was grown at 25°C and labeled for 15 min with [35S]H2SO4 at 25°C (lane 1) and at 37°C (lane 2). Cells labeled at 37°C were shifted to 37°C 45 min before adding label. The phenotype was not observed when CWY2-1C was shifted to 37°C for 30 min or less. (d) Inhibition of signal peptide cleavage blocks pro-peptide cleavage. Strain CWY2-1C[YCp-KX22] (secl1-7) cells were pulse labeled with [35S]amino acids for 1 min, a chase was initiated, and samples were harvested at the indicated times. Cells labeled at 37°C were shifted to 37°C 45 min before the addition of label. Separation between bands is not as great as in other gels, but the sizes of the Kex2 species are identical to those observed in other experiments.

Wild-type cells must represent intermediates in conversion of I1 to I2.

Unlike the simple, rapid conversion of I1 to I2 in the wild-type strain at 30°C (t, ~1 min from Fig. 3) and in the secl8 mutant at 25°C (t, ~2 min from Fig. 4 a), conversion of I1 to I2 was biphasic in the secl8 mutant at 35°C (Fig. 4 b). The majority of I1 (>60%) was converted rapidly to I2 at 35°C (t, ~1 min), but a portion of the molecules was converted very slowly or not at all (t, >3 h). Biphasic kinetics were not observed at 37°C either in a Sec+ strain overproducing Kex2 protein or in a secl8 strain producing Kex2 protein at the wild-type level, suggesting that both overproduction and the secl8 mutation were required at an elevated temperature to observe this effect (data not shown). Species I1 and I2 slowly increased in apparent mol wt with time at 35°C in the secl8 mutant (Fig. 4 a, compare lanes 8-14). N-glycanase digestion demonstrated that the increase was not due to modification of N-linked chains (data not shown), suggesting instead that more extensive addition of O-linked mannose occurred when Kex2 protein was retained in the ER.

The Signal Peptide of Kex2 Protein Is Cleaved

To determine whether the signal peptide of Kex2 protein is cleaved, a secl1 mutant, in which signal peptide cleavage is blocked at 37°C (Böhni et al., 1988), was examined. When secl1 cells were pulse labeled with [35S]SO4 (Fig. 4 c), the form of Kex2 protein labeled at 37°C (127 kD) was 3 kD larger than that labeled at 25°C (124 kD). This difference cannot be explained simply by retention of the signal peptide at 37°C, because subsequent cleavage of the pro-peptide should remove the entire prepro-segment. This reasoning suggested that retention of the signal peptide blocked pro-peptide cleavage. A rapid pulse-chase experiment was performed to determine whether conversion of I1 to I2 occurred at 37°C in the secl1 mutant (Fig. 4 d). At 25°C, conversion of I1 to I2 was complete by 4 min of chase. At 37°C, the rapid 9-kD decrease in apparent mol wt characteristic of conversion of I1 to I2 was eliminated (Fig. 4 d, lanes 7-12), indicating accumulation of prepro-Kex2 protein in the secl1 mutant at 37°C. A slow increase in mol wt was observed, similar to that found in the secl8 mutant at 35°C, suggesting retention of the prepro-protein in the ER.

It is important to stress that conversion of I1 to I2 does not correspond to signal peptide cleavage. First, whereas signal peptide cleavage is ordinarily cotranslational, conversion of I1 to I2 is clearly posttranslational. Second, the 9-kD difference between I1 and I2 is much greater than the molecular mass of the putative signal peptide of Kex2 protein (2.2 kD). In contrast, the difference in molecular mass of Kex2 protein briefly pulse labeled at 25°C and 37°C (~3 kD) is in agreement with the size expected of the signal peptide (Fig. 4 d, lanes I and 7). As mentioned previously, sequence
Figure 5. Transport to the Golgi. (a) Strain CWY1-lA (sec18) was grown at 25°C in LSM, harvested by filtration, and resuspended in NSM. The culture was split, and one half kept at 25°C and the other shifted to 35°C for 30 min before labeling each with [35S]H2SO4 for 20 min. A chase was initiated at t0 and labeled cultures were split again, with half of each kept at the labeling temperature (lanes 1-3 and 6-8) and the other half shifted to the other temperature (lanes 4, 5, 9, and 10). At 30 and 90 min after the chase, 1-ml samples were harvested. (b) Strain CWY1-lA[YCp-KX22] (sec181) (lanes 1 and 2) was shifted to 35°C for 30 min before pulse labeling for 1 min, 15 s with [35S]amino acids. Strain CRY2[YCp50] (lanes 3-5) was labeled for 10 min with [35S]H2SO4 at 30°C. 2-dG (500 µg/ml; lane 3) was added 15 min before the addition of label. Cultures were harvested immediately after labeling. Samples in lanes 2, 3, and 5 were digested with N-glycanase after the first round of immunoprecipitation. Cultures were grown in media containing 0.1% glucose. (c) A schematic rendition of the gel presented in b. (d) Strain CRY2[YCpKX22] was pulse labeled for 1.5 min with [35S]amino acids, a chase was initiated, and 2-ml samples were harvested at the indicated times. After two rounds of immunoprecipitation with the Kex2-specific antiserum, each sample was split. One half of each was subjected to a third round of immunoprecipitation with anti-Kex2 antiserum, while the other half was immunoprecipitated with α-1, 6-Man Ab.

Transport of Kex2 Protein to the Golgi

To determine whether species I2 accumulated at 35°C in the sec18 mutant was an authentic intermediate in transit between the ER and Golgi, the reversibility of the block was examined. A sec18 mutant strain expressing Kex2 protein at the wild-type level was pulse labeled at 25°C and 35°C with [35S]SO4, and portions of the labeled cultures were chased at 25°C and 35°C (Fig. 5 a). Pulse labeling at 25°C produced species J (Fig. 5 a, lane J), which underwent a gradual increase in mol wt at both 25°C (Fig. 5 a, lanes 2 and 3) and 35°C (Fig. 5 a, lanes 9 and 10), indicating that the pulse-labeled Kex2 protein had passed the sec18 arrest point before establishment of the secretory block at 35°C. Upon pulse labeling at 35°C, most of the labeled Kex2 protein was in the I2 form, with a small amount present as I1 (Fig. 5 a, lanes 6). As observed previously with the sec18 mutant (Fig. 4 a), I2 failed to undergo conversion to species J upon prolonged chase at 35°C, and instead slowly increased in apparent mol wt (Fig. 5 a, lanes 7 and 8). When the chase was performed at 25°C, however, I2 was converted entirely to species J and more slowly migrating mature forms (Fig. 5 a, lanes 4 and 5). This result indicated that the form of Kex2 protein accu-
sec7 and sec14 mutations. (A) Strain AFY89 (sec7-4) was grown at 25°C and placed at 38°C for the indicated times before labeling with [35S]H2SO4 for 20 min at 38°C, after which cells were harvested and processed. (B) A culture of strain KRY33-4B (sec14-4a), grown at 25°C, was divided and one half was kept at 25°C and the other half was shifted to 37°C for 30 min before labeling each for 15 min with [35S]H2SO4. A chase was established and samples were harvested at the indicated times.

Conversion of I2 to J in the Golgi Complex

Digestion of I2 and J with N-glycanase resulted in comparable shifts in apparent mol wt (2 kD for I2 and 3 kD for J, Fig. 5, b and c). Thus, only ~17% of the 6-kD difference between I2 and J could be explained by modification of N-linked chains. As shown in Fig. 5 b, after digestion with N-Glycanase, I2 (Fig. 5, lane 2) comigrated with Kex2 protein produced in the presence of 2-dG (i.e., species J lacking both N-linked chains and α-1,2 extensions of O-linked mannos; Figs. 5, lane 3), indicating that the remaining 5 kD of the shift between I2 and J corresponds to elongation of the O-linked carbohydrate.

Modification of the N-linked chains on Kex2 protein in the Golgi complex was probed using antibodies against the α-1,6-mannosyl linkage (α-1,6-Man Ab). Acquisition of immunoreactivity to α-1,6-Man Ab in the Golgi complex has been demonstrated for numerous yeast glycoproteins (e.g., Franzusoff and Schekman, 1989). Kex2 protein from wild-type cells pulse labeled with [35S]amino acids and chased for various times was immunoprecipitated twice with Kex2-specific antibody, and precipitated a third time with either α-1,6-Man Ab or anti-Kex2 serum (Fig. 5 d). After 1 min of chase, a faint but discrete band the size of species J was precipitated by the α-1,6-Man Ab (Fig. 5, lane 4). At later times (Fig. 5, lanes 6 and 8), greater amounts of Kex2 protein were precipitated by α-1,6-Man Ab. As expected for pre-Golgi species, I2 and J were not precipitated by α-1,6-Man Ab in this experiment (Fig. 5, lane 4), and α-1,6-Man Ab failed to precipitate either I2 or J accumulated in a sec18 mutant at 35°C (data not shown). These results provide additional evidence that I2 and J correspond to pre-Golgi forms of Kex2 protein and that form J has reached the Golgi apparatus. During conversion of I2 to J, elongation of the O-linked carbohydrate appeared to precede the α-1,6 modification of the N-linked chains, because α-1,6-Man Ab precipitated a discrete band the size of species J and failed to precipitate species intermediate in size between I2 and J.

sec7 Intermits Transport of Kex2 Protein

At the restrictive temperature, temperature-sensitive alleles of the SEC7 gene block formation of secretory vesicles and cause accumulation of heterogeneous species of invertase lacking α-1,3-linked modification of N-linked chains (Franzusoff and Schekman, 1989). Preincubation of a sec7-4 strain at the restrictive temperature (38°C) for increasing times before labeling resulted in accumulation of progressively smaller species of Kex2 protein (Fig. 6 a), similar in size to I2 and forms intermediate between I2 and J. Antibody against α-1,6-linked mannos precipitated only the most slowly migrating forms labeled at 38°C, whereas the majority of Kex2 protein labeled at the permissive temperature was precipitated by the antiaminoacylase antibody (data not shown). Therefore, full maturation of Kex2 protein requires its transport beyond the block defined by the sec7 mutation, and suggests that the late phase of modification may include addition of α-1,3-linked mannos residues.

The sec14 Mutation Blocks Late Modification of Kex2 Protein

Rapid conversion of I2 to J (t½ ~2 min) was followed by a slow phase in which the apparent molecular mass of Kex2 protein increased from 126 to 130 kD in 90 min at 30°C. A similar progressive modification of the Kex1 carboxypeptidase was attributed to alteration of N-linked chains (Cooper and Bussey, 1989). N-Glycanase-digestion of Kex2 protein immunoprecipitated after pulse labeling and after a 90-min chase produced species that differed by 2–3 kD (compare lanes 2 and 4 and lanes 6 and 8 in Fig. 2 a). Therefore, the gradual modification of Kex2 protein could not be due exclusively to alterations of N-linked chains. The extent of this modification was decreased in an mnnl mutant strain (data not shown), indicating that it corresponded in part to addition of α-1,3-linked mannose to O-linked and N-linked oligosaccharide chains (Raschke et al., 1973). In addition, mature species of Kex2 protein were precipitated by anti-α-1,3-mannose antiserum (data not shown).

Temperature-sensitive mutations in the SEC14 gene appear to block transport of secretory proteins out of a late Golgi compartment (Bankaitis et al., 1989). At the restrictive temperature, the sec14 mutant strain accumulated Kex2 protein in a form recognized by α-1,6-Man Ab (data not shown) and similar in apparent mol wt to species J (Fig. 6 b, lanes 6–10), consistent with accumulation of the protein in a late Golgi compartment. However, the gradual increase
Figure 7. Kex2 protein is long-lived. (a) Strain W303-1A grown at 30°C was labeled for 10 min with \[^{35}S\]H_2SO_4, and a chase was begun. Samples were harvested at the indicated times. (b) The data in A (indicated by triangles) and two similar experiments were quantified using a Molecular Dynamics Model 300A Computing Densitometer. Values are expressed as a percentage of the maximum in each experiment, and the log of that value was used for linear regression analysis to determine the half-life of Kex2 protein.

Lifetime of Kex2 Protein

Immunocytochemical data has suggested that prohormone maturation in mammals occurs in nascent secretory granules (Orci et al., 1987). If secretory vesicles were the site of processing by Kex2 protease, then in the simplest model the enzyme should be delivered to the cell surface upon fusion of the vesicles with the plasma membrane. However, the steady-state level of Kex2 protease at the cell surface is quite low (Fuller et al., 1989b; Payne and Schekman, 1989). This might be due to rapid degradation of Kex2 protein en route to or at the cell surface. If so, the rate of turnover of the protein should be comparable to the overall rate of secretion of mature α-factor \((t_\text{1/2} \approx 5\ \text{min}; \text{Julius et al., 1984a})\). The half-life of Kex2 protein in wild-type cells at 30°C was measured by pulse-chase analysis (Fig. 7 a). Disappearance of Kex2 protein was exponential, with a \(t_\text{1/2} \approx 80 \pm 20\ \text{min}\) (Fig. 7 b). The longevity of Kex2 protein relative to the rate of secretion of α-factor rules out degradation as an explanation for absence of the protein from the cell surface. Instead, if delivered to the cell surface, Kex2 protein must be rapidly recycled to an intracellular compartment by an endocytotic mechanism. Alternatively, the protein may be retained within one or more intracellular compartments.

Kex2 Protein Is Not Incorporated into Secretory Vesicles

Secretion of α-factor is blocked at 37°C in secretory mutants such as sec1 and sec6 that accumulate secretory vesicles (Novick et al., 1980). A model in which Kex2 protein is transported to the cell surface predicts that the protein would accumulate in secretory vesicles at the restrictive temperature in sec1 and sec6 mutants, and thus become inaccessible to Golgi glycosyl transferases such as the α-1,3-mannosyl transferase. However, the late, gradual modification of Kex2 protein continued in both sec1 (Fig. 8) and sec6 (data not shown) mutant strains at 37°C. These results support the conclusion, based on both morphological and fractionation data (Redding et al., 1991), that net intracellular retention of mature Kex2 protein does not involve transport to and return from the plasma membrane.

Discussion

Life History of Kex2 Protease

Fig. 9 presents a model for the posttranslational modifications of Kex2 protease, which provide molecular signposts for transport and targeting of the protein in the secretory pathway. Cotranslational signal peptide cleavage and addition of N-linked oligosaccharide and O-linked mannosese result in the first observable intermediate, I₁, which undergoes NH₂-terminal pro-peptide removal in the ER or during transport to the Golgi to produce a second intermediate, I₂. Delivery of I₂ to early Golgi compartments is marked by
Figure 9. Posttranslational modifications of Kex2 protease in the yeast secretory pathway.

elongation of O-linked chains and α-1,6-modification of N-linked oligosaccharides. Incremental modification, probably consisting largely of addition of α-1,3-linked mannose to O-linked and N-linked chains, marks the enzyme in its likely compartment of action. Kex2 protein is relatively stable. Eventual degradation of the protein requires transport to the vacuole (C. A. Wilcox, K. Redding and R. S. Fuller, manuscript in preparation).

In SDS-PAGE, I₂ minus N-linked oligosaccharide (∼117 kD) is ∼7 kD larger than the in vitro translation product, even though I₂ lacks ∼10–12 kD of prepro-sequences. This "bookkeeping" implies that ∼17–19 kD of the apparent molecular mass of I₂ must be due to a modification other than proteolytic cleavage of N-glycosylation. The most likely explanation for this phenomenon is the presence of numerous O-linked monomannosyl residues on I₁. O-linked monomannosyl residues have a disproportionate effect on SDS-PAGE mobility, as documented in the case of the a-agglutinin, a yeast glycoprotein that contains exclusively O-linked carbohydrate (Watzele et al., 1988). Deglycosylated a-agglutinin migrates as a 13-kD peptide, but the form isolated from a sec18 mutant at the restrictive temperature (Julius et al., 1984a), though Kex2 protease accumulates in a form, I₁, that lacks the pro-peptide. The activity of I₁ might be limited by persistent binding of the pro-peptide or by conditions in the ER or transport vesicles (e.g., low concentrations of enzyme and substrate, low Ca²⁺ ion) that are unfavorable for processing.

The lack of pro-peptide cleavage in the sec11 mutant is intriguing. In a sec11 strain at the restrictive temperature, Kex2 protein was localized by indirect immunofluorescence to the nuclear envelope and cytoplasmic reticular structures, suggesting retention in the ER (Redding et al., 1991). The signal sequence might interfere with folding, and thus pro-peptide cleavage, by interacting with the rest of the protein or by binding the polypeptide to the ER membrane. Alternatively, membrane attachment might prevent transport of the protein to regions of the ER in which pro-peptide cleavage is favored.

Localization of Kex2 Protease to a Late Golgi Compartment

At steady state, the bulk of Kex2 protein (>97%) is intracellular (Fuller et al., 1989b). Indirect immunofluorescence indicates steady-state localization of Kex2 protease to multiple "punctate" intracellular structures (Redding et al., 1991). The progressive modification of mature Kex2 protein, presumably by a Golgi glycosyl transferase, and colocalization of Kex2 protein with Sec7 protein (Franzusoff et al., 1991), indicates that the compartment in which Kex2 protein resides represents an aspect of the yeast Golgi complex. The lack of substantial inhibition of the progressive modification of Kex2 protein by the sec11 mutation suggests that Kex2 protein does not cycle between Golgi apparatus and the cell surface. Indeed, both in morphological studies and by subcellular fractionation, localization of Kex2 protein was unaffected by the sec11 mutation (Redding et al., 1991).

The slow modification of Kex2 protein was blocked by the sec14 mutation, implying that SEC14 function is required either for initial transport of Kex2 protein to the compartment containing the α-1,3-mannosyl transferase or for continued accessibility of Kex2 protein to the transferase. Although,
**References**

Bankaitis, V. A., D. E. Malehorn, S. D. Emr, and R. Greene. 1989. The Saccharomyces cerevisiae SEC14 gene encodes a cytosolic factor that is required for transport of secretory proteins from the yeast Golgi complex. J. Cell Biol. 108:1271–1281.

Böhn, P. C., R. J. Deshaies, and R. W. Schekman. 1988. SEC11 is required for signal peptide processing and yeast cell growth. J. Cell Biol. 106:1035–1042.

Bonner, W. M. 1984. Fluorography for the detection of radioactivity in gels. Methods Enzymol. 104:460–465.

Brenner, C., and R. S. Fuller. 1991. Structural and enzymatic characterization of a purified prohormone processing enzyme: secreted, soluble Kex2 protease. Proc. Natl. Acad. Sci. USA. In press.

Burgers, P. A. M., and K. J. Percival. 1987. Transformation of yeast spheroplasts without cell fusion. Anal. Biochem. 163:391–397.

Bussey, H. 1988. Proteases and the processing of precursors to secreted proteins in yeast. Yeast. 4:17–26.

Bussey, H., D. Saville, D. Greene, D. J. Tipper, and K. A. Bostian. 1983. Secretion of Saccharomyces cerevisiae Killer Toxin: processing of the glycosylated precursor. Mol. Cell. Biol. 3:1362–1370.

Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water soluble fluor, sodium salicylate. Anal. Biochem. 98:132–135.

Cooper, A., and H. Bussey. 1989. Characterization of the yeast KEXI gene product: a carboxypeptidase involved in processing secreted precursor proteins. Mol. Cell. Biol. 9:2706–2714.

Cunningham, K. W., and W. T. Wickner. 1989. Yeast KEX2 protease and mannose-6-phosphate are localized to distinct compartments of the secretory pathway. Yeast. 5:25–33.

Edge, A. S. B., C. R. Flatynak, L. Hof, L. E. Reichert, Jr., and P. Weber. 1987. Deglycosylation of cytosolic proteins by trifuoromethanesulfonic acid. Anal. Biochem. 118:131–137.

Esmon, B. P., N. Novick, and R. Schekman. 1981. Compartimentalized assembly of oligosaccharides on exported glycoproteins in yeast. Cell. 25:451–460.

Franzusoff, A., and R. Schekman. 1989. Functional components of the yeast Golgi apparatus are defined by the sec7 mutation EMBO (Eur. Mol. Biol. Organ.) J. 8:2695–2702.

Franzusoff, A., K. Redding, J. Crosby, R. S. Fuller, and R. Schekman. 1991. Localization of components involved in protein transport and processing through the yeast Golgi apparatus. J. Cell Biol. 112:27–37.

Fuller, R. S., R. E. Sterne, and J. Thorner. 1988. Enzymes required for yeast prohormone processing. Annu. Rev. Physiol. 50:345–362.

Fuller, R. S., A. Brake, and J. SEC14. 1989b. Yeast prohormone processing enzyme (KEX2 gene product) is a Ca2+-dependent serine protease. Proc. Natl. Acad. Sci. USA. 86:1434–1438.

Fuller, R. S., A. Brake, and J. Thorner. 1989b. Intracellular targeting and structural conservation of a prohormone-processing endoprotease. Science (Wash. DC). 246:482–486.

Graham, T. R., and S. D. Emr. 1991. Compartmental organization of Golgi-specific protein modification and vacuolar protein sorting events defined in yeast sec18 (NSF) mutant. J. Cell Biol. 115:207–218.

Ikemura, H., and M. Inouye. 1988. In vitro processing of pro-subtilisin produced in Escherichia coli. J. Biol. Chem. 263:12959–12963.

Julius, D., R. Schekman, and J. Thorner. 1984a. Glycosylation and processing of pre-pro-o-factor in the yeast secretory pathway. Cell. 36:309–318.

Julius, D., A. Brake, L. Blair, R. Kurosawa, and J. Thorner. 1984b. Isolation of the putative structural gene for the lysine-aromatic-cleaving endoprotease required for processing of yeast prepro-o-factor. Cell. 37:1075–1089.

Kaiser, C. A., and R. Schekman. 1990. Distinct sets of SEC genes govern transport vesicle formation and fusion early in the secretory pathway. Cell. 61:723–733.

Kukuruzinska, M. A., M. L. E. Bergh, and B. J. Jackson. 1987. Protein glycosylation in yeast. Annu. Rev. Biochem. 56:915–944.

Lehle, L., and R. T. Schwartz. 1976. Formation of dolichol monophosphate 2-deoxy-D-glucose and its interference with the glycosylation of mannoproteins in yeast. Eur. J. Biochem. 67:239–245.

Light, A., and H. Januca. 1984b. Enterokinase (enteropeptidase): comparative aspects. Trends Biochem. Sci. 14:110–112.

Mizuno, K., T. Nakamura, T. Oshima, S. Tanaka, and H. Matsu. 1988. Yeast KEX2 gene encodes an endopeptidase homologous to subtilisin-like serine proteases. Biochem. Biophys. Res. Commun. 156:246–254.

Mizuno, K., T. Nakamura, T. Oshima, S. Tanaka, and H. Matsu. 1989. Characterization of KEX2-encoded endopeptidase from yeast Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. 59:303–311.

Moehle, C. M., C. K. Dixon, and E. W. Jones. 1989. Processing pathway for protease B of Saccharomyces cerevisiae. J. Cell Biol. 108:309–325.

Novick, P., C. Field, and R. Schekman. 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell. 21:205–215.

Orci, L., M. Ravazzola, M. J. Storch, R. G. W. Anderson, J. D. Vassalli, and A. Perrelet. 1987. Pro teaseolytic maturation of insulin is a post-Golgi event which occurs in acidifying clathrin-coated secretory vesicles. Cell. 49:865–868.

Payne, G. S., and R. Schekman. 1989. Clathrin: A role in the intracellular retention of a Golgi membrane protein. Science (Wash. DC). 245:1358–1365.

Power, S. D., R. M. Adams, and J. A. Wells. 1986. Secretion and autoproteolytic maturation of subtilisin. Proc. Natl. Acad. Sci. USA. 83:3096–3100.

Raschke, W. C., K. A. Kern, C. Antalis, and C. E. Ballou. 1973. Genetic control of yeast mannan structure. J. Biol. Chem. 248:4660–4666.

Redding, K. C., Holcomb, and R. W. Jones. 1991. Immunolocalization of Kex2 protease identifies a putative late Golgi compartment in the yeast Saccharomyces cerevisiae. J. Cell Biol. 113:527–538.

Rose, M. D., P. Novick, J. H. Thomas, D. Botstein, and G. R. Fink. 1987. A Saccharomyces cerevisiae genomic plasmid bank based on a centromere-containing shuttle vector. Gene. 60:1–12.

Rosbash, M., and J. W. Holtzer. 1984. Dephosphorylation and processing of precursor proteins in the yeast secretory pathway. Nature (Lond.) 309:462–464.

Selden, D. P., and D. F. Steiner. 1990. Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease Kex2. J. Biol. Chem. 265:2997–3000.

Thim, L., M. T. Hansen, K. Norris, I. Hede, J. Forstrom, G. M. Smeekens, and N. P. Fil. 1986. Secretion and processing of insulin precursors in yeast. Proc. Natl. Acad. Sci. USA. 83:6766–6770.

Thomas, G., B. A. Thorne, L. Thomas, R. G. Allen, D. E. Hruby, R. Fuller, and P. Weber. 1987. Deglycosylation of cytosolic proteins by trifuoromethanesulfonic acid. Anal. Biochem. 163:391–397.
and J. Thorner. 1988. Yeast KEX2 endopeptidase correctly cleaves a neuroendocrine prohormone in mammalian cells. *Science (Wash. DC)*. 241: 226–230.

Fuite, M. F., J. Plesset, K. Moldave, and C. S. McLaughlin. 1980. Faithful and efficient translation of homologous and heterologous mRNAs in an mRNA-dependent cell-free system from *Saccharomyces cerevisiae*. *J. Biol. Chem.* 255:8761–8766.

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Watzele, M., F. Klis, and W. Tanner. 1988. Purification and characterization of the inducible a-agglutinin of *Saccharomyces cerevisiae*. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1483–1488.

Zhu, X., Y. Ohta, F. Jordan, and M. Inouye. 1989. Pro-sequence of subtilisin can guide the refolding of denatured subtilisin in an intermolecular process. *Nature (Lond.)* 339:483–484.