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 mannose in the ER. The earliest detectable species, I_1 (~129 kD), undergoes rapid amino-terminal proteolytic removal of a ~9-kD pro-segment yielding species I_2 (~120 kD) before arrival at the Golgi complex. Transport to the Golgi complex is marked by extensive elaboration of Ser/Thr-linked chains and minor modification of Asn-linked oligosaccharide. During the latter phase of its lifetime, Kex2 protein undergoes a gradual increase in apparent molecular weight. This final modification serves as a marker for association of Kex2 protease with a late compartment of the yeast Golgi complex in which it is concentrated about 27-fold relative to other secretory proteins.

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^{2+}-dependent, neutral serine protease that cleaves peptide substrates at the carboxyl side of Lys-Arg and Arg-Arg sites (Julius et al., 1984*b*; Fuller et al., 1989*a*; 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., 1984*a*; 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., 1989*a*; 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., 1989*b*; Smeekens and Steiner, 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_2-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., 1989*a*) 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 (O-linked) glycosylation (Fuller et al., 1989*a*). 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 $Lys_{79}Arg_{80}$ and $Lys_{108}Arg_{109}$. After signal peptide removal, cleavage at Arg_{80} would remove a 61-residue peptide (7377 D), and at Arg_{109}, 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_2-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.

Table I. Yeast Strains Used

Strain	Genotype	Source
W303-1A	*MATa, can1-100, ade2-1, his3-11,-15, leu2-3,-112, trp1-1, ura3-1*	R. Rothstein (Columbia University, New York, NY)
CRY2	*MATα, can1-100, ade2-1, his3-11,-15, leu2-3,-112, trp1-1, ura3-1*	this lab
CWY2-1C	*MATα, can1-100, ade2-1, his3-11,-15, leu2-3,-112, trp1-1, ura3-1, sec11-7ts*	this lab
CWY1-1A	*MATα, can1-100, ade2-1, his3-11,-15, leu2-3,-112, trp1-1, ura3-1, sec18-1ts*	this lab
KRY30-1A	*MATα, can1-100, ade2-1, his3-11,-15, leu2-3,-112, trp1-1, ura3-1, sec1-1ts*	this lab
KRY33-4B	*MATα, can1-100, ade2-1, his3-11,-15, leu2-3,-112, trp1-1, ura3-1, sec14-3*	this lab
AFY89	*MATα, can1-100, his3-11,-15, leu2-3,-112, ura3-1, sec7-4ts*	Alex Franzusoff (University of Colorado, Denver, CO)

We have also identified a modification that provides a marker for the locus of mature Kex2 protease.

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. 1989*a*). 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 ARS1 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

[^{35}S]H_2SO_4 (~43 Ci/mg S) was from ICN Pharmaceuticals, Inc. (Irvine, CA). "[^{35}S]amino acids" refers to either "Tran^{35}S-label" from ICN Pharmaceuticals, Inc. (>1,000 Ci/mmol) or "express [^{35}S]protein labeling mix" (~1,150 Ci/mmol methionine) from New England Nuclear Corporation (Boston, MA). Benzamidine-HCl, PMSF, and 2-deoxy-D-glucose (2-dG)[1] were from Sigma Chemical Co. (St. Louis, MO). Other protease inhibitors were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Pansorbin 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). Antiserum against the α-1,6-mannosyl linkage was a gift of R. Schekman (University of California, Berkeley, CA).

Radiolabeling and Immunoprecipitation

Cells to be labeled were grown in low sulfate minimal medium (LSM)[1] containing 100 μm $(NH_4)_2SO_4$ and 2% (wt/vol) glucose (Julius et al., 1984*a*), 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 × 10^7 cells/ml in LSM lacking sulfate (NSM), after which labeling was initiated by adding either [^{35}S]H_2SO_4 or [^{35}S]amino acids to 300 μCi/ml. When used, a "chase" was initiated by adding unlabeled $(NH_4)_2SO_4$ 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 N^{α}-[*p*-tosyl]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. Labeling 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 in *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., 1984*a*). 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-X-100, 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 antiserum (1 to 1.5 μl), raised against a β-galactosidase fusion protein containing the 100 COOH-terminal residues of Kex2 protein (Fuller et al., 1989*b*), was added to the lysate along with 5–7 μl of Pansorbin slurry. For immunoprecipitation with antibody against the α-1,6-mannosyl linkage, 2 μl of antiserum 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., 1989*b*). 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/$CHCl_3$ (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 [^{35}S]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.

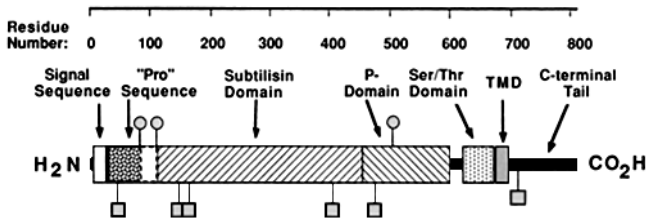

Figure 1. Schematic diagram of Kex2 protein (814 residues). Stippled circles indicate Lys-Arg sites; stippled squares indicate potential N-glycosylation sites (*Asn-X-Ser/Thr*). "P-domain" indicates a sequence of 155 residues conserved in mammalian homologues of Kex2 protein (Fuller et al., 1989*b*; Smeekens and Steiner, 1990).

amined to establish a baseline for evaluating post-translational modifications. The observed mol wt of the in vitro translation product in SDS-PAGE, 110 kD (see Fig. 2 *a*), differed from the predicted size of 90 kD (Fuller et al., 1988; Mizuno et al., 1988) by 20 kD, which may be explained by the anomalous effect of the acidic COOH-terminal tail of Kex2 protein on its gel mobility (Fuller et al., 1989*a*). Kex2 protein immunoprecipitated from cells pulse labeled for 10 min with [^{35}S]SO_4 and producing wild-type (see Fig. 2 *b*, lane *1*) or 20-fold elevated levels of Kex2 protein (see Fig. 2 *a*, lane *2*; Fig. 2 *b*, lane *5*) migrated as a somewhat heterogeneous band of 125–130 kD. After a chase of 90 min, the apparent molecular mass of Kex2 protein increased slightly (to 128–133 kD; see Fig. 2 *b*, lanes *3* and *7*), indicating continued modification. The size difference between the in vitro and in vivo forms indicated extensive posttranslational modification. N-linked oligosaccharide accounted for only a small fraction of this difference, because digestion of Kex2 protein with N-Glycanase resulted in a mobility shift of 3–5 kD at both the 0- and 90-min chase times (see Fig. 2 *b*, lanes *2*, *4*, *6*, and *8*).

The presence of O-linked oligosaccharide chains in a Ser/Thr-rich region (Fig. 1) was inferred from the apparent molecular mass of a mutant protein (Kex2Δ6) lacking this region (Fuller et al., 1989*a*) and from binding of Kex2 protein from tunicamycin-treated cells to ConA-Sepharose (Fuller et al., 1989*b*). A more direct demonstration of O-linked carbohydrate was obtained using 2-deoxy-D-glucose (2-dG) to block elaboration of O-linked chains in the Golgi complex. In yeast, O-linked chains are initiated by addition of single mannosyl residues to Ser and Thr in the ER and elaborated in the Golgi complex by addition of two or three α-1,2-linked mannose residues, followed by a terminal α-1,3-linked mannose (reviewed in Kukuruzinska et al., 1987). Because glucose and mannose are 2-epimers, initial linkage of 2-dG to Ser and Thr in place of mannose occurs in the ER, but the absence of the 2-hydroxyl blocks subsequent addition of α-1,2-linked residues in the Golgi (Lehle and Schwarz, 1976). Furthermore,

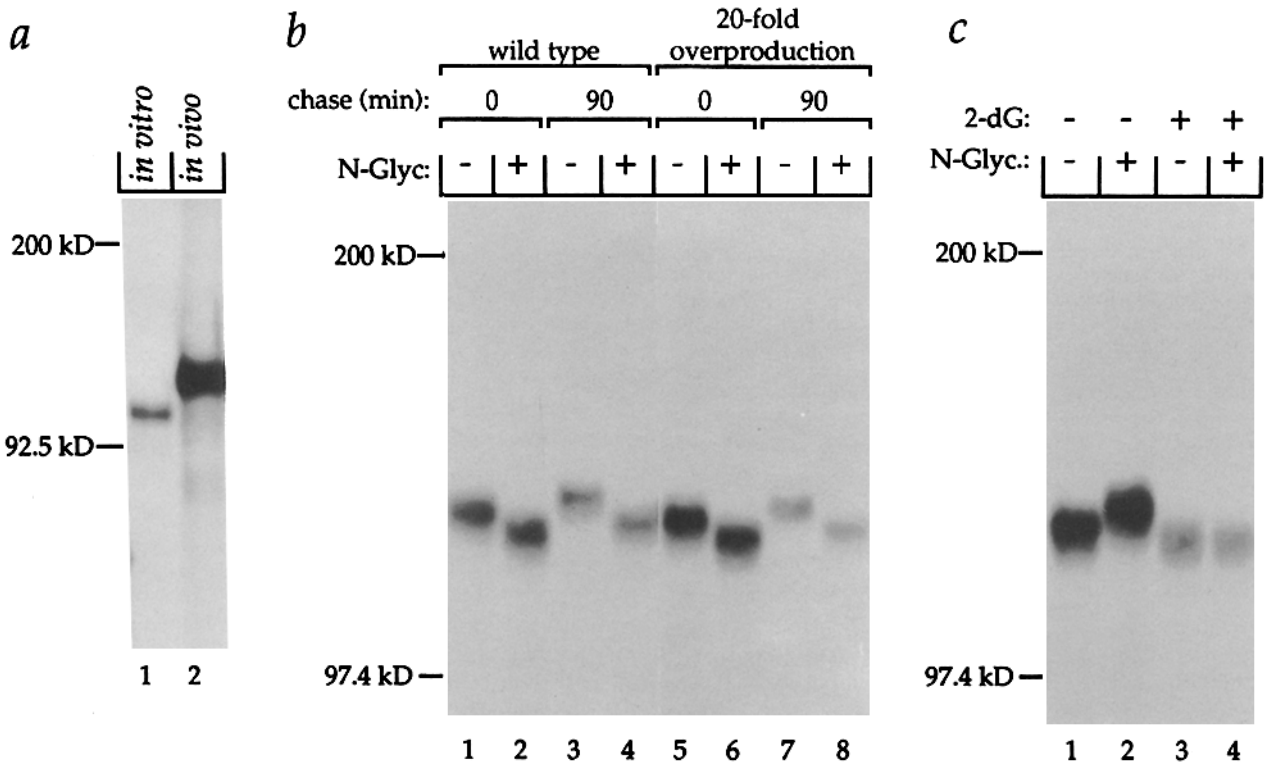

Figure 2. In vitro translation product of the *KEX2* gene and oligosaccharide modification of Kex2 protein. (*a*) (lane *1*) In vitro translation product. (lane *2*) Strain W303-1A[YCpKX22] was labeled for 10 min with [^{35}S]-H_2SO_4 at 30°C. (*b*) Strains CRY2[YCp50] (lanes *1–4*) and CRY2[YCpKX22] (lanes *5–8*) were labeled for 10 min with [^{35}S]-H_2SO_4 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 [^{35}S]-H_2SO_4. 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 *1*). 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, [^{3}H]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 ^{35}S-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_1" (127 kD in lane *3*), disappeared by 1 min of chase as the lower band, "I_2" (118 kD in lane *3*), increased in intensity (lane *4*). I_2 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 [^{35}S]SO_4, which equilibrates slowly with endogenous pools of Cys and Met, early intermediates I_1 and I_2 were not observed except in certain *sec* mutants at restrictive temperatures. Species J was the first form observed, gradually chasing 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_1, 129 ± 1 kD (eleven independent determinations); I_2, 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_1 to I_2 was due to NH_2-terminal proteolysis. First, the anti-Kex2 antibody recognizes exclusively the COOH-terminal 100 residues of Kex2 protein (Fuller et al., 1989*b*). 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, NH_2-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

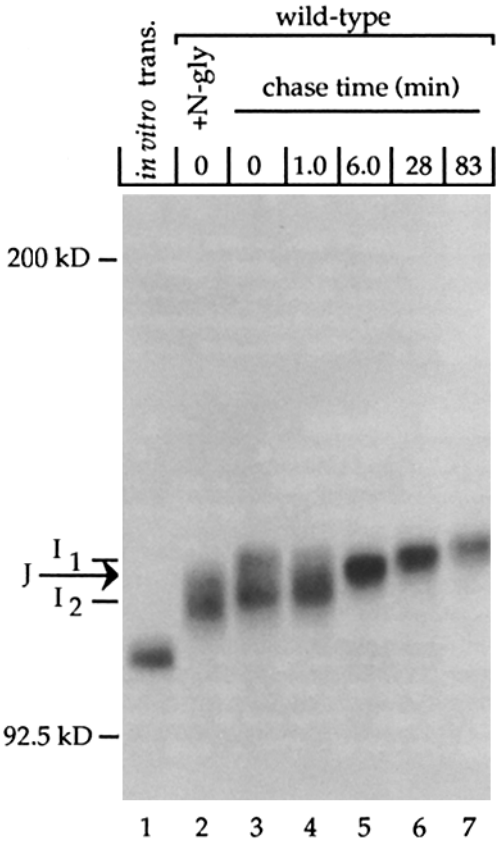

Figure 3. Kinetic analysis of maturation of Kex2 protein. Strain W303-1A[YCpKX22] was pulse labeled for 1 min, 45 s with [^{35}S]amino acids, after which a chase was initiated. 1-ml samples were harvested at the indicated chase times. The *KEX2* in vitro translation product was loaded as a reference (lane *1*). The sample in lane 2 was digested with N-Glycanase.

increased the mobility of I_1 slightly more than that of I_2 (Fig. 3, lane *2*), suggesting the presence of N-linked oligosaccharide at Asn_{44}, within the "pro" region of I_1.

NH_2-terminal Proteolytic Processing Occurs Before Delivery to the Golgi Complex

The rapid kinetics of conversion of I_1 to I_2 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 glycosyl modifications (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_1 to I_2 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 I_1 chased discretely into I_2 without an intervening smear and without the appearance of species J (Fig. 4 *a*, lanes *8–14*). Thus, conversion of I_1 to I_2 appears to result from a discrete endoproteolytic cleavage that occurs before delivery to the Golgi complex. Conversion of I_2 to J, therefore, must occur at a location distal to the *sec18*-dependent block, and the heterogeneous species seen in

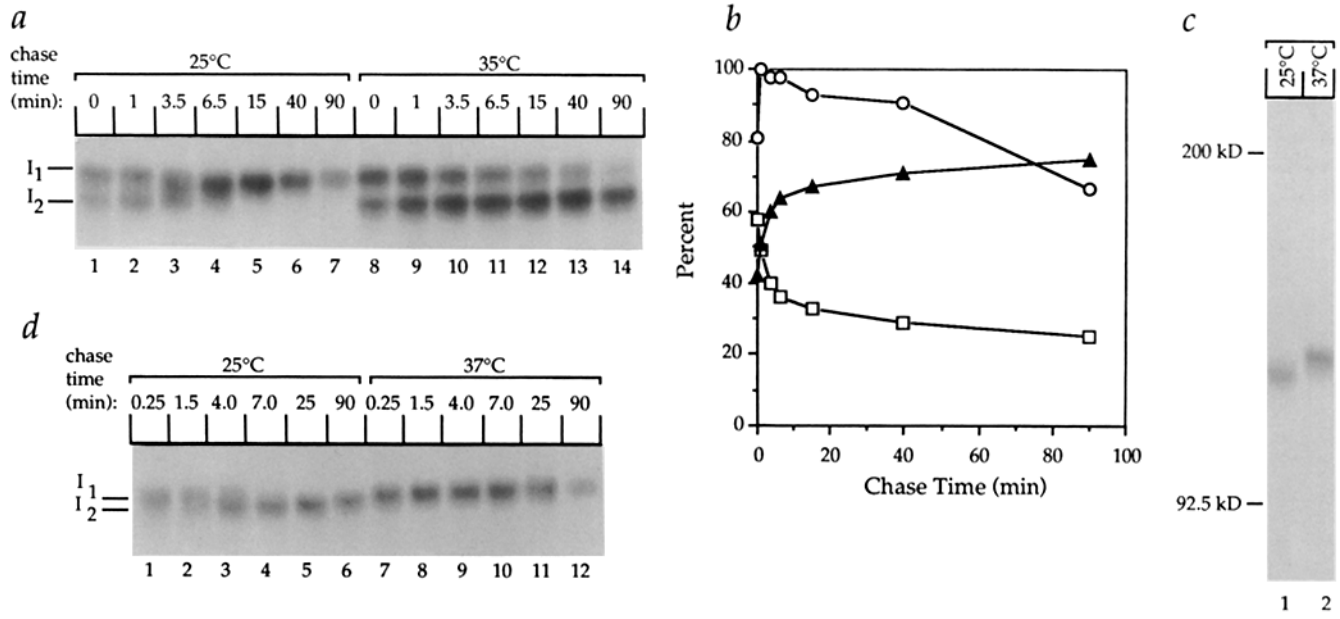

Figure 4. Proteolytic maturation of Kex2 protein. (*a*) Arrest in *sec18* cells resolves NH_2-terminal proteolytic cleavage from subsequent modifications. A culture of strain CWY1-1A[YCp-KX22] (*sec18-1*) was grown at 25°C, split, and half was maintained at 25°C, and the other half was shifted to 35°C for 30 min. Each was then labeled with [^{35}S]amino acids for 1 min, a chase was initiated, and samples were harvested at the indicated times. (*b*) Conversion of I_1 to I_2 in *sec18* at the restrictive temperature is biphasic. The amounts of species I_1 and I_2 were quantified by densitometric scanning of appropriately exposed autoradiograms of the experiment in *a*. (*circles*) Sum of I_1 plus I_2 as a percentage of the maximum value (at t = 1.0 min); (*squares*) I_1 as a percentage of I_1 plus I_2; (*triangles*) I_2 as a percentage of I_1 plus I_2. (*c*) Signal peptide cleavage is indicated by the effect of the *sec11* mutation. Strain CWY2-1C (*sec11-7*) was grown at 25°C and labeled for 15 min with [^{35}S]H_2SO_4 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] (*sec11-7*) cells were pulse labeled with [^{35}S]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 I_2 to J.

Unlike the simple, rapid conversion of I_1 to I_2 in the wild-type strain at 30°C ($t_{½}$ ~1 min from Fig. 3) and in the *sec18 mutant at 25*°C ($t_{½}$ ~2 min from Fig. 4 *a*), conversion of I_1 to I_2 was biphasic in the *sec18* mutant at 35°C (Fig. 4 *b*). The majority of I_1 (≥60%) was converted rapidly to I_2 to 35°C ($t_{½}$ ~1 min), but a portion of the molecules was converted very slowly or not at all ($t_{½}$ >3 h). Biphasic kinetics were not observed at 37°C either in a Sec$^+$ strain overproducing Kex2 protein or in a *sec18* strain producing Kex2 protein at the wild-type level, suggesting that both overproduction and the *sec18* mutation were required at an elevated temperature to observe this effect (data not shown). Species I_1 and I_2 slowly increased in apparent mol wt with time at 35°C in the *sec18* 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 *sec11* mutant, in which signal peptide cleavage is blocked at 37°C (Böhni et al., 1988), was examined. When *sec11* cells were pulse labeled with [^{35}S]SO_4 (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 I_1 to I_2 occurred at 37°C in the *sec11* mutant (Fig. 4 *d*). At 25°C, conversion of I_1 to I_2 was complete by 4 min of chase. At 37°C, the rapid 9-kD decrease in apparent mol wt characteristic of conversion of I_1 to I_2 was eliminated (Fig. 4 *d*, lanes *7–12*), indicating accumulation of prepro-Kex2 protein in the *sec11* mutant at 37°C. A slow increase in mol wt was observed, similar to that found in the *sec18* mutant at 35°C, suggesting retention of the prepro-protein in the ER.

It is important to stress that conversion of I_1 to I_2 does not correspond to signal peptide cleavage. First, whereas signal peptide cleavage is ordinarily cotranslational, conversion of I_1 to I_2 is clearly posttranslational. Second, the 9-kD difference between I_1 and I_2 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 *1* and *7*). As mentioned previously, sequence

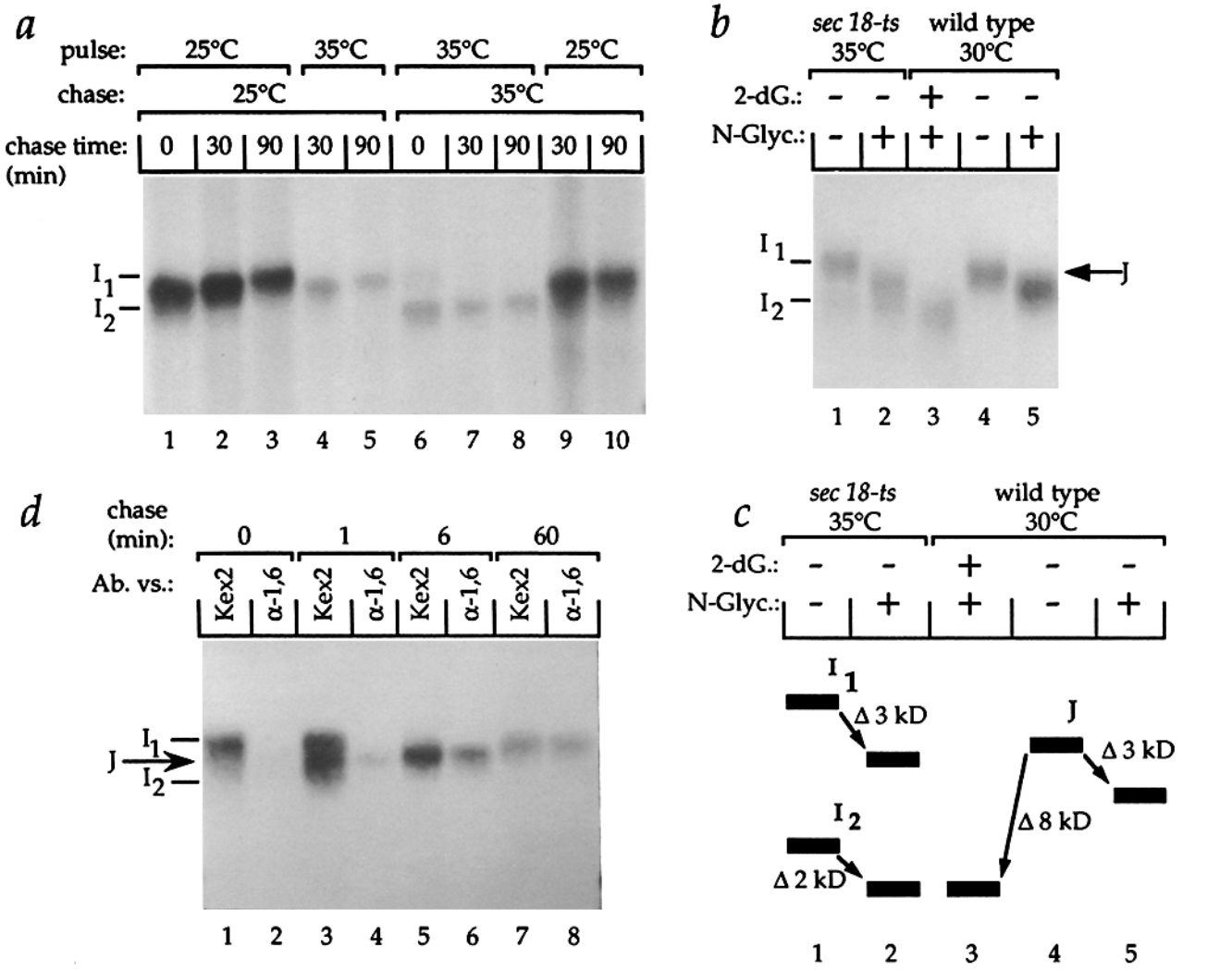

Figure 5. Transport to the Golgi. (*a*) Strain CWY1-1A (*sec18*) was grown at 25°C in LSM, harvested by filtration, and resuspended in NSM. The culture was split, and one half kept at 25°C and the other shifted to 35°C for 30 min before labeling each with [^{35}S]H_2SO_4 for 20 min. A chase was initiated at t_0 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 CWY1-1A[YCp-KX22] (*sec18-1*) (lanes *1* and *2*) was shifted to 35°C for 30 min before pulse labeling for 1 min, 15 s with [^{35}S]amino acids. Strain CRY2[YCp50] (lanes *3–5*) was labeled for 10 min with [^{35}S]H_2SO_4 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 [^{35}S]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.

analysis of purified Kex2 protease indicates proteolytic removal of the pro-peptide.

Transport of Kex2 Protein to the Golgi

To determine whether species I_2 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 [^{35}S]SO_4, 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 *1*), 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 I_2 form, with a small amount present as I_1 (Fig. 5 *a*, lanes *6*). As observed previously with the *sec18* mutant (Fig. 4 *a*), I_2 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, I_2 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-

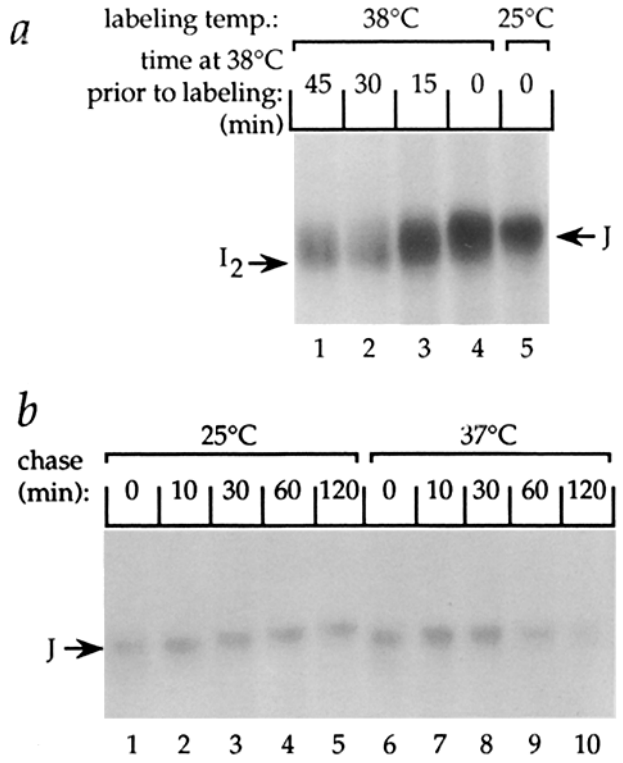

Figure 6. Effects of *sec7* and *sec14* mutations. (*A*) Strain AFY89 (*sec7-4*) was grown at 25°C and placed at 38°C for the indicated times before labeling with [^{35}S]H_2SO_4 for 20 min at 38°C, after which cells were harvested and processed. (*B*) A culture of strain KRY33-4B (*sec14-ts*), grown 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 [^{35}S]H_2SO_4. A chase was established and samples were harvested at the indicated times.

mulated at the *sec18* block was an authentic intermediate and that conversion of I_2 to J required transport to the Golgi complex.

Conversion of I_2 to J in the Golgi Complex

Digestion of I_2 and J with N-glycanase resulted in comparable shifts in apparent mol wt (2 kD for I_2 and 3 kD for J, Fig. 5, *b* and *c*). Thus, only ~17% of the 6-kD difference between I_2 and J could be explained by modification of N-linked chains. As shown in Fig. 5 *b*, after digestion with N-Glycanase, I_2 (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 mannose; Figs. 5, lane *3*), indicating that the remaining 5 kD of the shift between I_2 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 [^{35}S]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, I_1 and I_2 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 I_1 or I_2 accumulated in a *sec18* mutant at 35°C (data not shown). These results provide additional evidence that I_1 and I_2 correspond to pre-Golgi forms of Kex2 protein and that form J has reached the Golgi apparatus. During conversion of I_2 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 I_2 and J.

sec7 Interrupts 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 I_2 and forms intermediate between I_2 and J. Antibody against α-1,6–linked mannose 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 anticarbohydrate 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 mannose residues.

The sec14 Mutation Blocks Late Modification of Kex2 Protein

Rapid conversion of I_2 to J ($t_{½}$ ~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 *mnn1* 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

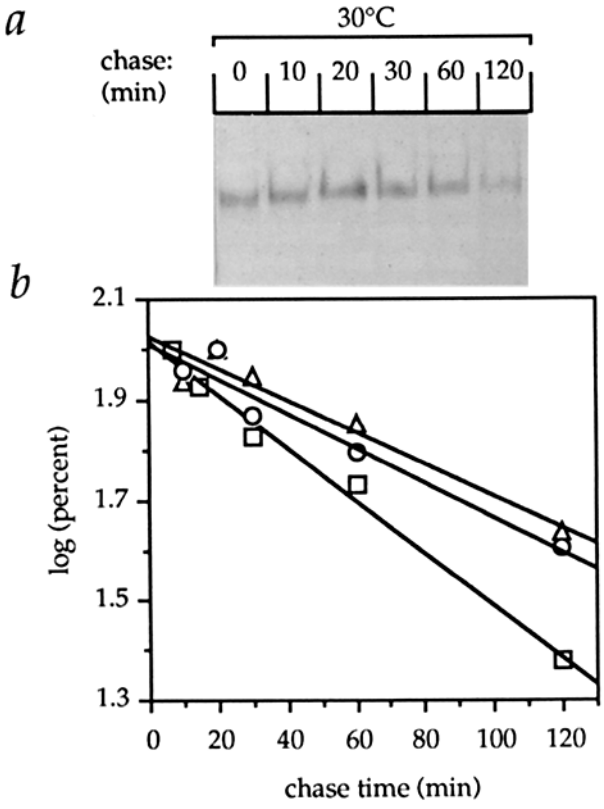

Figure 7. Kex2 protein is long-lived. (*a*) Strain W303-1A grown at 30°C was labeled for 10 min with [^{35}S]H_2SO_4, and a chase was begun. Samples were harvested at the indicated times. (*b*) The data in *A* (indicated by *triangles*) and two similar experiments were quantified using a Molecular Dynamics 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.

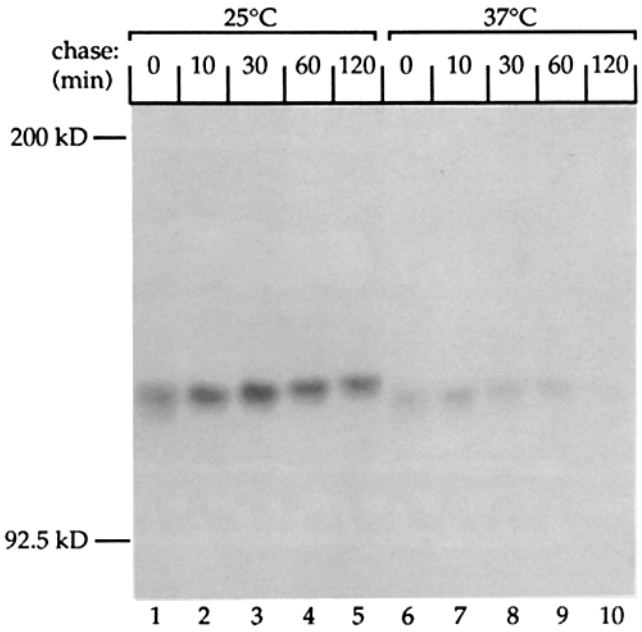

Figure 8. The effects of the *secl* mutation. Strain KRY30-1A was grown at 25°C, the culture split, and one half was kept at 25°C and the other was placed at 37°C for 30 min before labeling each with [^{35}S]H_2SO_4 for 15 min. A chase was begun and samples were harvested and processed at the indicated times.

in apparent mol wt was not observed, suggesting that the protein was sequestered in a compartment lacking the enzyme(s) responsible for these late modifications.

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., 1989*b*, 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 ($t_{½}$ ~5 min; Julius et al., 1984*a*). 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 $t_{½}$ of 80 ± 20 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 *secl* 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 *secl* 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 *secl* (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 mannose result in the first observable intermediate, I_1, which undergoes NH_2-terminal pro-peptide removal in the ER or during transport to the Golgi to produce a second intermediate, I_2. Delivery of I_2 to early Golgi compartments is marked by

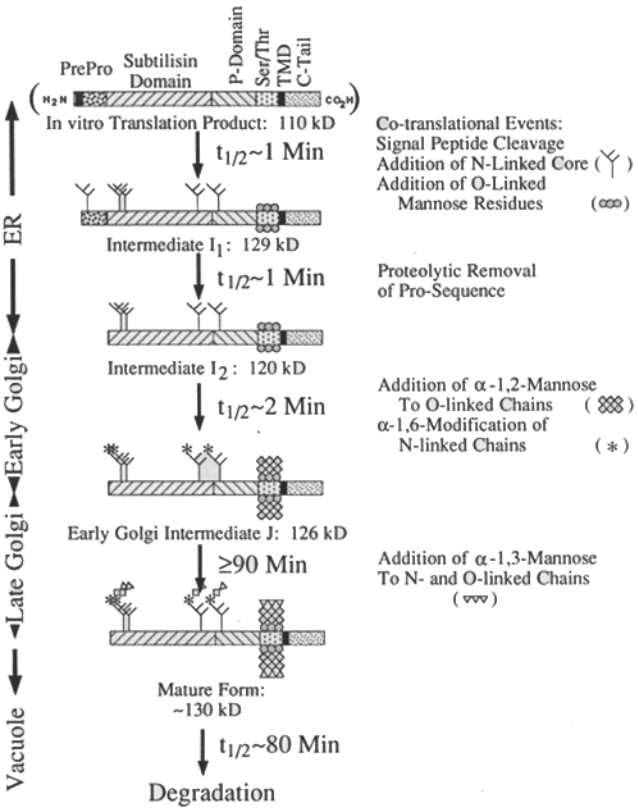

Figure 9. Posttranslational modifications of Kex2 protease in the yeast secretory pathway.

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, manuscription in preparation).

In SDS-PAGE, I_2 minus N-linked oligosaccharide (~117 kD) is ~7 kD larger than the in vitro translation product, even though I_2 lacks ~10–12 kD of prepro-sequences. This "bookkeeping" implies that ~17–19 kD of the apparent molecular mass of I_2 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 I_2. 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, containing an estimated 20 mono-mannosyl residues (3.2 kD), migrates at 21 kD. In contrast, addition of α-1,2-linked and α-1,3-linked mannose in the Golgi results in an additional shift of only 1.3 kD in the mobility of a-agglutinin.

Pro-peptide Cleavage

Consistent with autoproteolytic removal of the pro-peptide, sequence analysis of purified Kex2 protease demonstrates that the mature NH_2-terminus is generated by cleavage at the second of the two Lys-Arg sites upstream from the subtilisin domain (Brenner and Fuller, 1991). Like pro-subtilisin (Power et al., 1986) and the precursor of yeast vacuolar protease B (Moehle et al., 1989), the pro-peptide of pro-Kex2 protein is cleaved rapidly upon membrane translocation. Cleavage is not cotranslational, but occurs after a lag of 1–2 min at 30°C, which may reflect the time required for the folding of the pro-enzyme. Like the pro-peptides of subtilisin (Zhu et al., 1989) and α-lytic protease (Silen and Agard, 1989), the Kex2 pro-peptide may facilitate folding of the catalytic domain.

Unlike trypsinogen, which is activated by enterokinase only after transport to the lumen of the duodenum (Light and Janska, 1989), cleavage of pro-Kex2 protein occurs long before the enzyme reaches its presumed site of action, the late Golgi. In any event, there is no direct evidence that pro-Kex2 protein itself is inactive, although prepro-Kex2 protein accumulated in the *sec11* mutant was catalytically inactive (L. Kean and R. S. Fuller, unpublished results). Interestingly, pro-α-factor is not processed in a *sec18* mutant at the restrictive temperature (Julius et al., 1984*a*), though Kex2 protease accumulates in a form, I_2, that lacks the pro-peptide. The activity of I_2 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 Ca^{2+} 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 peptide 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., 1989*b*). 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 *sec1* 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 *sec1* 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,

sec14 mutants at the restrictive temperature accumulate invertase containing outer chain α-1,3-linked mannose (Franzusoff and Schekman, 1989), recent experiments suggest that the bulk of Kex2 protease lies in a compartment beyond that containing α-1,3-transferase activity (Graham and Emr, 1991). Also, fractionation of yeast microsomal membranes on Percoll density gradients partially resolved Kex2 proteolytic activity, in two peaks, from the bulk of α-1,3-mannosyl transferase activity (Cunningham and Wickner, 1989). An interesting possibility is that Kex2 protein cycles between two compartments, and that *SEC14* is required for retrograde transport of Kex2 protein to the compartment containing α-1,3-transferase.

Presumably, it is during the late phase of modification, which represents >95% of the lifetime of the enzyme, that Kex2 protease becomes actively engaged in processing substrates. The time required for transport of Kex2 protein to the late Golgi can be estimated from the time required for completion of the rapid phase of modification ($t_{½}$ ~3 min at 30°C). Because the half-life of Kex2 protease is ~80 min, the enzyme can be estimated, roughly, to accumulate 27-fold at its site of action relative to other proteins in transit in the secretory pathway. This observation may help explain the remarkable efficiency of processing by Kex2 protease, despite its low abundance (Fuller et al., 1989*a*).

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