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Yeast prohormone processing enzyme (*KEX2* gene product) is a Ca^{2+}-dependent serine protease

(prohormone convertase/membrane-bound protease/precursor maturation/secretory pathway/posttranslational modification)

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ABSTRACT **The *KEX2*-encoded endoprotease was overproduced in yeast several hundred-fold and further purified to achieve a 10,000-fold enrichment in specific activity. The enzyme was (*i*) membrane-bound, but solubilized by detergents; (*ii*) able to cleave peptide substrates at both Lys-Arg and Arg-Arg sites; (*iii*) inhibited by EDTA and EGTA (but not *o*-phenanthroline), but fully reactivated by Ca^{2+}; (*iv*) unaffected by 5–10 mM phenylmethylsulfonyl fluoride, N^{α}-(*p*-tosyl)lysine chloromethyl ketone, or L-1-tosylamido-2-phenylethyl chloromethyl ketone, but inactivated by 1–2 μM Ala-Lys-Arg-chloromethyl ketone; (*v*) labeled specifically by ^{125}I-labeled Tyr-Ala-Lys-Arg-chloromethyl ketone; and (*vi*) resistant to *trans*-epoxysuccinate compounds (which inactivate thiol proteases), but inactivated by diisopropyl fluorophosphate (a diagnostic serine protease inhibitor). Mutant enzyme molecules lacking as many as 200 C-terminal residues still retained Ca^{2+}-dependent protease activity and were labeled by ^{125}I-labeled Tyr-Ala-Lys-Arg-chloromethyl ketone.**

Maturation of precursors to secreted bioactive peptides in eukaryotic cells requires endoproteolysis at the carboxyl side of pairs of basic residues (Lys-Arg, Arg-Arg, Arg-Lys, and Lys-Lys) (1, 2). Diverse enzymes have been implicated in the processing of mammalian prohormones and neuropeptide precursors at such sites (3–6). Genetic analysis in the yeast *Saccharomyces cerevisiae* permitted the unambiguous identification of the enzymes required for proteolytic processing of the precursor to α-factor, a secreted peptide pheromone (for review, see ref. 7). The yeast *KEX2* gene product cleaves the α-factor precursor at the carboxyl side of Lys-Arg sites (8, 9) and is a paradigm for this important class of processing endoproteases.

We used expression vectors and biochemical fractionation to produce a highly purified preparation of the Kex2 enzyme and found that it is a Ca^{2+}-dependent serine protease with other unusual properties. We also utilized an active-site affinity label for this enzyme. Preliminary reports of our work have appeared elsewhere (9–11).

MATERIALS AND METHODS

Plasmids, Strains, and Growth Media. Plasmid pAB221 contains the *KEX2* gene on a 4.9-kilobase (kb) *Bam*HI fragment inserted into pAB18 (12). Construction of overproducing plasmids is described in Fig. 1.

Yeast strains (relevant genotype) were XBH16-15A (*MATα kex2-1 ura3-52*), AB103.1 (*MATα ura3-52 pep4-3*), AB110 (*MATα ura3-52 pep4-3*), YNN217 (*MATa ura3-52*), DA2102 (*MATα ura3-52 leu2-3, -112*), and BFY100, a *MATa*/*MATα* diploid from a cross of YNN217 with DA2102. A 2.7-kb *Sal* I fragment (from plasmid pKX7Δ::*URA3*), in which an internal 2.1-kb segment (85% of the *KEX2* coding sequence) is replaced by a 1.2-kb *Hin*dIII fragment containing the *URA3* gene (13), was used to transform (14) strain BFY100 to Ura$^+$, yielding BFY101. The resulting *kex2* deletion–*URA3* insertion (15) was confirmed by hybridization analysis of restriction digests of genomic DNA. Strains BFY101-32C (*MATα kex2*Δ*1::URA3 leu2*-3, -112) and BFY101-35C (*MATa kex2*Δ*1::URA3 leu2*-3, -112) were obtained by sporulation of BFY101. Minimal media lacking uracil or leucine were used to select plasmid-containing strains, and other media were as described (14).

Reagents. Ala-Lys-Arg-chloromethyl ketone (AKR-CK) (16) was a gift of E. Shaw (Basel); ^{125}I-labeled Tyr-Ala-Lys-Arg-CK (^{125}I-YAKR-CK) (17), *trans*-epoxysuccinate derivatives (Ep143, Ep459, and Ep475) (18, 19), and cystatin C (20) were gifts of D. F. Steiner (Chicago). Leupeptin, pepstatin A, and antipain were from Beckman; 7-amino-4-methylcoumarin (AMC), acetyl-Pro-Met-Tyr-Lys-Arg-4-methylcoumarin-7-amide (Ac-PMYKR-MCA), *t*-butoxycarbonyl-Ile-Glu-Gly-Arg-MCA (b-IEGR-MCA), and *t*-butoxycarbonyl-Gln-Arg-Arg-MCA (b-QRR-MCA) were from Peninsula Laboratories; diisopropyl fluorophosphate (DFP) was from Serva.

Enzyme Assay. Reaction mixtures (50 μl) contained 200 mM sodium Hepes (pH 7.0), 1 mM $CaCl_2$, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM L-1-tosylamido-2-phenylethyl-CK (TPCK), 1.0% (wt/vol) Triton X-100, and 100 μM b-QRR-MCA. After enzyme addition, reaction mixtures were incubated at 37°C for 30 min and terminated by addition of 0.9 ml of 0.125 M $ZnSO_4$ and 0.1 ml of saturated $Ba(OH)_2$. The precipitate was removed by centrifugation for 1 min in a microcentrifuge, and AMC in the supernatant solution was determined fluorimetrically [λ(excitation) = 385 nm, λ(emission) = 465 nm]. One unit was defined as 1 pmol of AMC released per min. Protein was determined as described (21).

Purification of Kex2 Protease. Typically, strain BFY101-35C, containing plasmid pLG-KX29, was grown at 30°C in minimal medium lacking leucine (600 ml) with 0.2% glucose and 2% galactose. At an OD_{650} of 1.0, cells were harvested, washed with 0.1 volume of buffer A (50 mM sodium Hepes, pH 7.6/10 mM EDTA/0.5 mM PMSF/0.1 mM TPCK/1 mM benzamidine hydrochloride/5 μM Ep459/25 μM pepstatin A), and frozen (−75°C). Cells were thawed on ice (remaining steps were carried out at 0–4°C), resuspended with 4 ml of buffer A in 30-ml glass tubes, and broken by multiple (12–20)

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Abbreviations: CK, chloromethyl ketone; AKR-CK, Ala-Lys-Arg-CK; ^{125}I-YAKR-CK, ^{125}I-labeled Tyr-Ala-Lys-Arg-CK; TPCK, L-1-tosylamido-2-phenylethyl-CK; TLCK, N^{α}-(*p*-tosyl)Lys-CK; DFP, diisopropyl fluorophosphate; PMSF, phenylmethylsulfonyl fluoride; AMC, 7-amino-4-methylcoumarin; MCA, 4-methylcoumarin-7-amide; Ac-PMYKR-MCA, acetyl-Pro-Met-Tyr-Lys-Arg-MCA; b-IEGR-MCA, *t*-butoxycarbonyl-Ile-Glu-Gly-Arg-MCA; b-QRR-MCA, *t*-butoxycarbonyl-Gln-Arg-Arg-MCA.

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pulses (20 s) of vigorous vortex mixing in the presence of 10 g of glass beads (0.5 mm). The lysate was removed, combined with two 2-ml washes (buffer A) of the beads, and clarified by centrifugation (10,000 × *g*, 15 min). The clarified lysate was recentrifuged (100,000 × *g*, 90 min), and the pellet (crude microsomal membranes) was resuspended in 4 ml of buffer B (buffer A with 1 mM EDTA and 20% glycerol) containing 0.5 M NaCl and centrifuged again (100,000 × *g*, 90 min). The pellet (salt-washed membranes) was solubilized in 2 ml of buffer B containing 50 mM NaCl and 2% (wt/vol) sodium deoxycholate by incubation for 60 min at 4°C and clarified by centrifugation (100,000 × *g*, 90 min). The resulting supernatant fraction was passed through a column (1 × 50 cm) of Sephacryl S-200 (40 ml, Pharmacia). Fractions (0.75 ml) were collected and assayed for activity, and the peak fractions were pooled. A portion (20%) of the Sephacryl S-200 material was diluted 2-fold with buffer B and applied to a 2-ml bed of arginine-Sepharose 4B (Pharmacia), which was then washed with 10 ml of buffer B containing 25 mM NaCl, and was eluted with a linear gradient (15 ml) of 25–600 mM NaCl in buffer B. The peak eluted between 50 and 150 mM NaCl. Specific activity of the initial clarified lysate was 7800 units/mg, whereas the final arginine-Sepharose fraction was 7×10^5 units/mg. Overall yield was 65%.

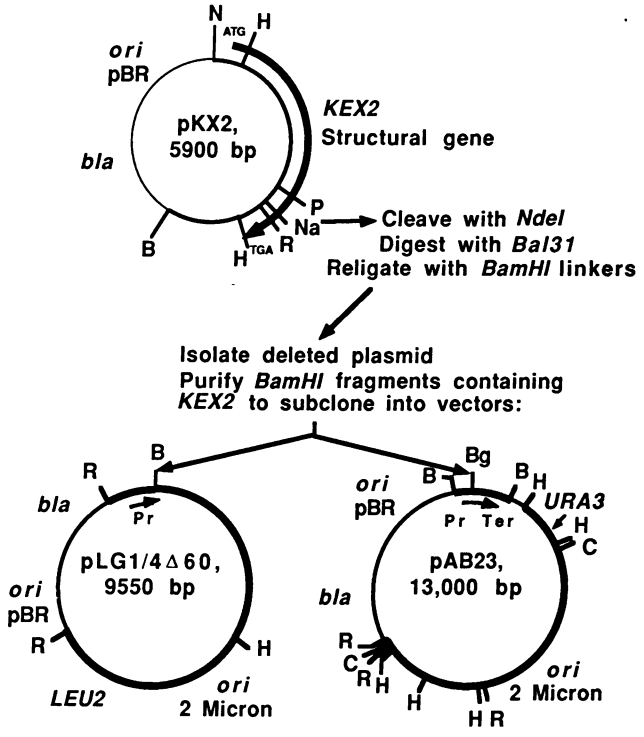

FIG. 1. Overproduction of Kex2 protein. pKX2 consisted of a 3450-base-pair (bp) *Nde* I–*Bam*HI fragment of plasmid pJ2B (8) containing the *KEX2* gene ligated to the 2097-bp *Nde* I–*Bam*HI fragment of plasmid pBR322 (22). To remove portions of the 5′ untranslated region, pKX2 was cleaved with *Nde* I (171 bp upstream from the ATG of the *KEX2* coding sequence), partially digested with exonuclease BAL-31, filled-in with *Escherichia coli* DNA polymerase I large fragment, and reclosed by blunt-end ligation to a *Bam*HI linker by using standard methods (22). Individual *Bam*HI–*Bam*HI fragments were subcloned into the *Bam*HI site of vector pLG1/4Δ60 (obtained from R. Bram and A. Buchman, Stanford) to generate pLG plasmids or into the *Bgl* II site of vector pAB23 to generate pAB plasmids (Table 1). pLG1/4Δ60 contains a 750-bp *Eco*RI–*Bam*HI fragment bearing the galactose-inducible *GAL1* promoter (23) and the *LEU2*^{d} allele, which selects for high copy number (24). pAB23 contains a unique *Bgl* II site between the promoter and terminator of the constitutively expressed *TDH3* gene (25). N, *Nde* I; H, *Hin*dIII; P, *Pvu* II; Na, *Nar* I; R, *Eco*RI; B, *Bam*HI; Bg, *Bgl* II, C, *Cla* I; Pr, promoter; Ter, terminator.

RESULTS AND DISCUSSION

Overproduction. Fragments differing only in the extent of 5′-flanking sequence preceding the initiator ATG of the *KEX2* gene were inserted downstream from both an inducible and a constitutive promoter (Fig. 1). In strains containing the *GAL1* promoter plasmids, Kex2 activity was elevated about 10-fold relative to wild-type cells even under noninducing conditions (Table 1), possibly due to the high plasmid copy number. When shifted to galactose medium, Kex2 activity increased dramatically (15- to 40-fold; Table 1). Constitutive expression of the *KEX2* gene with the *TDH3* promoter on vector pAB23 resulted in a 70- to 200-fold elevation of activity (Table 1). Specific activities in crude extracts as high as 40,000 units/mg (with pLG-KX22) and 20,000 units/mg (with pAB-KX211) have been achieved.

Partial Purification. As expected for a membrane protein, Kex2 enzyme was extracted from the particulate fraction by detergent but not by 1 M NaCl. Of the detergents tested (five nonionic, four zwitterionic, and one anionic), only sodium deoxycholate consistently solubilized 80–90% of the Kex2 activity. Fractions from arginine-Sepharose had a specific activity about 10,000-fold greater than crude extracts of wild-type cells (70–140 units/mg). Even with this enrichment, the enzyme was only 10% pure, as judged by labeling with ^{125}I-YAKR-CK (see below) and by staining with Coomassie brilliant blue. Hence, Kex2 protease is inabundant (≤0.001% of total protein in wild-type yeast cells).

Enzymic Properties. In earlier studies (8), b-QRR-MCA, which contains a pair of Arg residues, was cleaved by Kex2 protease, but a similar substrate containing a pair of Lys residues was not. Ac-PMYKR-MCA, corresponding to the authentic Lys-Arg processing sites found in prepro-α-factor (26), was cleaved at about the same rate and had nearly the same affinity as b-QRR-MCA (Fig. 2). This finding and earlier studies using cruder preparations of the enzyme (8, 27–29) suggest a requirement for Arg immediately preceding the peptide bond to be cleaved in the substrate.

Table 1. Overproduction of Kex2 protease activity

Fragment	Distance,* bp	Specific activity,† units/mg: pAB23‡ + Glc	pLG1/4Δ60§ − Gal	pLG1/4Δ60§ + Gal
None	—	ND	ND	148
KX212	130	ND	ND	4,980
KX29	110	8660	ND	15,620
KX211	50	9960	1231	14,520
KX21	35	7260	918	10,700
KX22	33	ND	640	21,930
KX22′¶	—	125	ND	ND

Plasmid	Specific activity,† units/mg: AB110	DA2102
YEp24	140	55
pJ2B	ND	670

ND, not determined.

*Distance from *Bam*HI to ATG (in bp), estimated from mobility on agarose gels by using appropriate size standards.

†Activity in clarified lysates (averages of 2–4 independent determinations).

‡In strain AB110 grown in minimal medium lacking uracil with 2% glucose (+ Glc) to an OD_{600} of 2.0.

§In strain DA2102 grown in minimal medium lacking leucine with 2% sucrose to an OD_{600} of 1.0, then harvested immediately (− Gal) or grown for an additional 4 hr in medium with 2% galactose (+ Gal).

¶Fragment inserted in the anti-sense orientation.

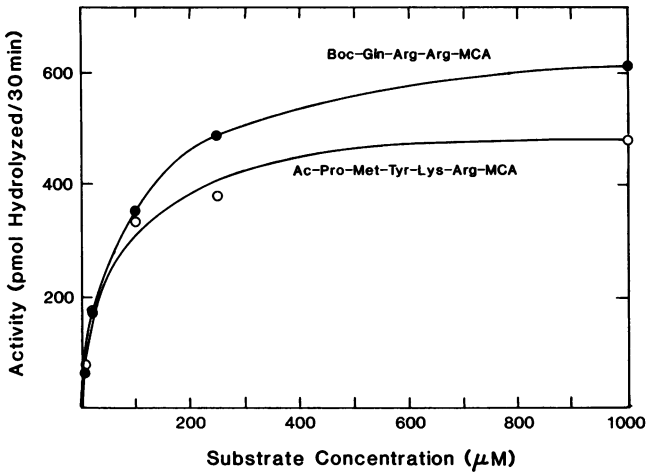

FIG. 2. Hydrolysis of synthetic peptide substrates. Reactions contained 12 units of deoxycholate extract of salt-washed membranes from strain XBH16-15a carrying multicopy plasmid pJ2B. K_m values (from least-squares analysis of double-reciprocal plots) were 114 μM (b-QRR-MCA) and 54 μM (Ac-PMYKR-MCA).

Kex2 activity was resistant to PMSF, TPCK, and N^{α}-(*p*-tosyl)Lys-CK (TLCK) (Fig. 3*A*), confirming previous results (8). Peptide inhibitors containing single arginine residues were moderately inhibitory (Fig. 3*A*). Activity was completely inhibited by preincubation for 5 min at 37°C with 2 μM AKR-CK (Fig. 3*B*). The relative efficacy of TLCK and AKR-CK demonstrates by an independent means the selectivity of the enzyme for pairs of basic residues.

Previously, the enzyme was found to be sensitive to certain heavy metal ions, iodoacetate and iodoacetamide (8), suggesting inhibitory modification of a reactive thiol. Purified Kex2 activity was inhibited by dithiothreitol (50% inhibition at 0.5 mM) and 4-(chloromercuri)-phenylsulfonate (50% inhibition at 1 mM). However, the enzyme was neither activated nor inhibited by 2-mercaptoethanol (up to 25 mM) nor could inhibition by dithiothreitol be reversed by 2-mercaptoethanol. Kex2 activity was unaffected (Fig. 3*C*) by high concentrations of several *trans*-epoxysuccinate compounds [which inhibit thiol proteases of the papain class (18, 19), including other plant proteases (e.g., ficin, Fig. 3*C*), lysosomal proteases such as cathepsin B, and the Ca^{2+}-dependent neutral thiol proteases (calpains) (30)]. Kex2 activity also was resistant to human cystatin C [a polypeptide that inhibits a broad spectrum of thiol proteases (20)].

Unlike its resistance to thiol protease inhibitors, Kex2 enzyme was inactivated by DFP (Fig. 3*D*), although a relatively high concentration was required. The selectivity of

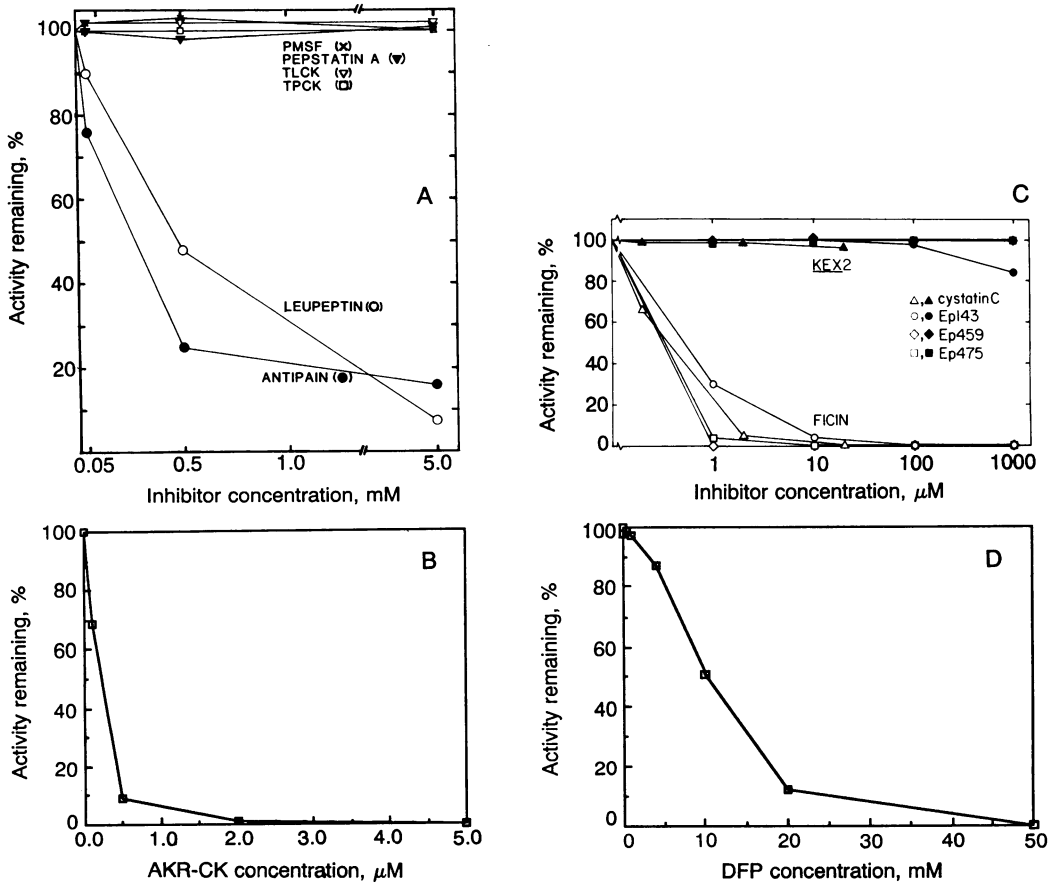

FIG. 3. Effect of protease inhibitors. (*A*) Inhibitors in dimethyl sulfoxide were added to reaction mixtures (lacking TPCK and PMSF) containing 8.4 units of a deoxycholate extract (1.2×10^4 units/mg) of salt-washed membranes from strain AB103.1 carrying multicopy plasmid pAB221. Dimethyl sulfoxide alone had no effect. (*B*) AKR-CK (in 1 μl of 1 mM HCl) was added to reaction mixtures containing 7 units of another deoxycholate extract (6×10^4 units/mg) lacking TPCK, PMSF, and substrate and was incubated for 5 min at 37°C. Substrate (b-QRR-MCA) then was added to a final concentration of 100 μM, and incubation was continued for 30 min. (*C*) Solutions (50 mM) of *trans*-epoxysuccinate derivatives [Ep143 in dimethyl sulfoxide, Ep459 in water, and Ep475 in 50% aqueous dimethyl sulfoxide] and an aqueous solution (1 mM) of cystatin C were added to reaction mixtures containing 22 units of the pooled peak fractions from Sephacryl S-200 chromatography (9×10^4 units/mg). Reaction mixtures (50 μl) containing ficin were 200 mM Tris·HCl, pH 6.8/1 mM EDTA/200 μm b-IEGR-MCA. (*D*) DFP was added in 1 μl of isopropanol to reaction mixtures containing 45 units of the same Sephacryl S-200 fraction, but lacking substrate, and incubated for 30 min at 37°C. Substrate (b-QRR-MCA) then was added to 100 μM, and incubation was continued for 30 min. Isopropanol alone caused a slight (9%) stimulation.

Kex2 protease for paired basic residues may explain its low affinity for DFP because bovine trypsin, which cleaves at single basic residues, is at least 100 times less sensitive to DFP than chymotrypsin, which cleaves at bulky hydrophobic residues (31). DFP is considered a reagent diagnostic for serine proteases (32). The predicted amino acid sequence of Kex2 protein (R.S.F., A.B., and J.T., unpublished data) contains a domain homologous to the subtilisin class of serine proteases. Also, the presence of a cysteine four residues C-terminal to its putative active-site histidine may account for the observed inhibition of the Kex2 enzyme by some nonspecific thiol-directed reagents (8–11, 27–29).

Ca^{2+} Dependence. The enzyme was inhibited by EDTA but not by *o*-phenanthroline (up to 10 mM, Fig. 4*A*). The latter binds heavy metals but not Ca^{2+}. Unlike the intracellular subtilisin of *Bacillus subtilis* (33), inhibition of Kex2 protease by chelators was freely reversible. After inhibition with either EDTA or EGTA, the enzyme was reactivated fully only with Ca^{2+} (Fig. 4*B*). Of the divalent cations tested (Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, and Fe^{3+}), only with Ca^{2+} was substantial activation observed in the presence of excess chelator; the free concentration of Ca^{2+} required for 50% activation of the enzyme was less than 1 μM. Strict Ca^{2+} dependence of *KEX2* activity was observed regardless of the degree of overproduction or the state of purification.

Affinity Labeling. ^{125}I-YAKR-CK covalently labels trypsin and cathepsin B (17). In detergent-solubilized membranes from cells overproducing Kex2 protein, the major species tagged by ^{125}I-YAKR-CK was ≈100 kDa (Fig. 5). A second species of 125–135 kDa was also consistently detected, along with variable amounts of lower molecular mass forms (Fig. 5, lane 3 and Fig. 6*A*, lane 1).

These species were all Kex2-related, as judged by several criteria. First, labeling of these species was greatly reduced by EDTA (Fig. 5). Second, labeling was prevented by AKR-CK (50 μM) or by substrates, b-QRR-MCA and Ac-PMYKR-MCA (400 μM), but not by TLCK (2.5 mM) (data not shown). Third, labeling of the 100-kDa and 125- to 135-kDa species was proportional to the units of Kex2 activity present in the reaction (Fig. 5). Fourth, neither species was detectable in membranes from a strain lacking a functional *KEX2* gene (BFY101-32C) (data not shown).

Several findings demonstrate that the 125- to 135-kDa species is the native form of the Kex2 enzyme and that the 100-kDa species is an enzymatically active fragment generated by autoproteolytic removal of ≈100 residues of C-terminal sequences. First, only the 100-kDa species was detected by ^{125}I-YAKR-CK labeling when a *KEX2* gene deleted of a segment encoding the C-terminal ≈100 amino

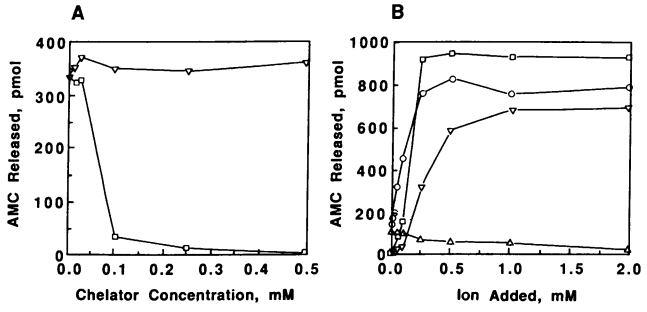

FIG. 4. (*A*) Chelators were added to reaction mixtures (minus $CaCl_2$) containing 11 units of a deoxycholate extract (Fig. 3*A*). ▽, *o*-phenanthroline; □, EDTA. (*B*) EDTA or EGTA were added at 0.25 mM to reaction mixtures (minus $CaCl_2$) containing 30 units or 25 units, respectively, of a deoxycholate extract (Fig. 3*B*). After 15 min on ice, $CaCl_2$ or $MgCl_2$ was added, and incubation was continued at 37°C for 30 min. □, + EDTA/+ Ca^{2+}; ▽, + EDTA/+ Mg^{2+}; ○, + EGTA/+ Ca^{2+}; △, + EGTA/+ Mg^{2+}.

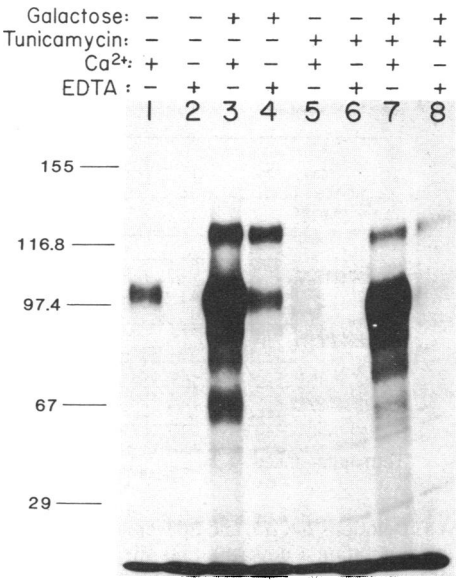

FIG. 5. Affinity-labeling of Kex2 protein. Strain DA2102 containing plasmid pLG-KX211 was grown at 30°C in minimal medium lacking leucine (280 ml) with 0.1% glucose and 2% sucrose to an OD_{600} of 2. The culture was divided into four equal portions and fresh medium (100 ml) was added to each. Galactose (2%) was added to culture B (lanes 3 and 4) and culture D (lanes 7 and 8). Tunicamycin (0.5 μg/ml) was added to culture C (lanes 5 and 6) and culture D (lanes 7 and 8). No addition was made to culture A (lanes 1 and 2). After incubation (140 min), salt-washed membranes were prepared. Specific activities (units/mg) were 810 (culture A), 4×10^4 (culture B), 330 (culture C), 1.3×10^4 (culture D). Reaction with ^{125}I-YAKR-CK (10^5 cpm) was for 30 min at 37°C in assay mixes containing either 1 mM $CaCl_2$ (lanes 1, 3, 5, and 7) or 1 mM EDTA (lanes 2, 4, 6, and 8). Proteins were precipitated with 1 ml of cold 10% trichloroacetic acid after addition of soybean trypsin inhibitor (25 μg), washed with 100 μl of cold acetone, and subjected to electrophoresis on an 8% polyacrylamide gel containing SDS. The autoradiogram (Kodak XAR-5 film) was overexposed to permit visualization of the higher molecular mass bands. Markers were *E. coli* RNA polymerase and β-galactosidase, rabbit phosphorylase B, bovine serum albumin, and carbonic anhydrase.

acids was expressed (Fig. 6*B*, lanes 1 and 2). Second, in immunoprecipitation and immunoblot experiments, antibody directed against the C-terminal 100 residues of the Kex2 polypeptide recognized the 125- to 135-kDa species but not the 100-kDa species (R.S.F., A.B., and J.T., unpublished data). Third, by using membranes from a *pep4-3* strain (vacuolar protease-deficient mutant) (34), loss of the 125- to 135-kDa species (detected by both ^{125}I-YAKR-CK labeling and immunoblotting) occurred only in the presence of both detergent and Ca^{2+} and was blocked by AKR-CK.

Domain Structure. Kex2 protein contains a transmembrane segment near the C terminus (Fig. 6*A*), which separates the N-terminal domain that contains homology to the subtilisin family of serine proteases from a highly acidic C-terminal "tail." Three 3′ deletions of the *KEX2* gene were constructed (Fig. 6*A*). Deletions Δ4, Δ5, and Δ6 all produced active, Ca^{2+}-dependent proteolytic species, as judged by enzyme assays (data not shown) and by labeling with ^{125}I-YAKR-CK (Fig. 6*B*). Thus, the first 614 amino acids of the polypeptide contain a functional active site and the Ca^{2+}-activation sequences.

The apparent molecular mass of the native Kex2 protein (125–135 kDa) is significantly greater than that predicted for the 814-residue polypeptide (90,020). The decrease in molecular mass caused by the glycosylation inhibitor tunicamycin (Fig. 5) and by digestion with endoglucosaminidase H (data not shown) indicated that Asn-linked oligosaccharide contributes only 5–6 kDa of this difference. By the same criteria, the Δ4, Δ5, and Δ6 proteins contain the same amount of

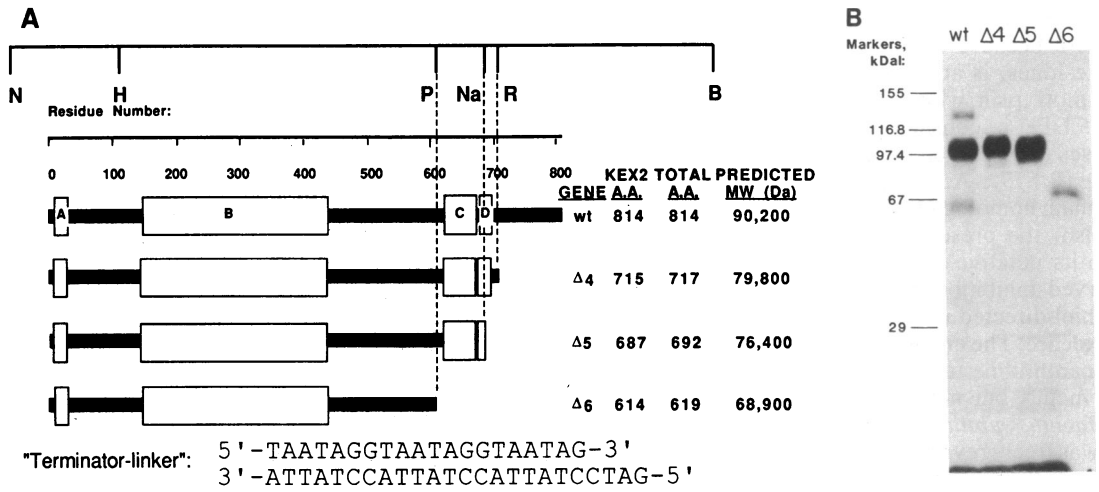

FIG. 6. (*A*) Deletions Δ4, Δ5, and Δ6 were constructed by inserting the "termination-linker" into the *KEX2* coding sequence at the indicated restriction sites, creating a novel *Bam*HI site at each deletion end point. These deletions were inserted into the *Bam*HI site of pLG1/4Δ60, generating pLG-KX212Δ4, pLG-KX212Δ5, and pLG-KX212Δ6. (*Upper*) Partial restriction map of the *KEX2* gene; abbreviations are as in Fig. 1. A.A., amino acid. (*Lower*) Predicted structure of the wild-type (wt) and truncated proteins. Features of the deduced amino acid sequence: A, signal sequence; B, domain of homology to subtilisin; C, Ser/Thr-rich domain; D, transmembrane domain. (*B*) ^{125}I-YAKR-CK-labeling of wild-type and C-terminal deletion proteins. Strain BFY101-32C containing pLG-KX22, pLG-KX212Δ4, pLG-KX212Δ5, or pLG-KX212Δ6 was grown at 30°C to an OD_{600} of 1 in minimal medium lacking leucine with 2% sucrose, and expression was induced by addition of galactose (2%). Cells were grown for 8 hr, and salt-washed membranes were prepared. Specific activities (in units/mg) were wild-type (wt), 5.9×10^4; Δ4, 1.6×10^4; Δ5, 1.5×10^4; and Δ6, 2.2×10^3. Samples were labeled with ^{125}I-YAKR-CK and analyzed as before (Fig. 5). Lanes: 1, wild-type (580 units); 2, Δ4 (160 units); 3, Δ5 (150 units); 4, Δ6 (22 units). Molecular mass markers (in kDa) are at left.

N-linked carbohydrate as the wild-type protein. Unlike Δ4 and Δ5, however, the observed molecular weight of the Δ6 protein (Fig. 6*B*) agrees closely with its predicted size (73,000). In the Δ6 deletion, a Ser/Thr-rich domain is absent. In other membrane proteins, such Ser/Thr-rich sequences are sites of extensive O-linked glycosylation (35). Thus, O-linked oligosaccharide in this region of the KEX2 polypeptide may contribute substantially to the apparent molecular mass of the mature protein. The high net negative charge (−15) of the tail contributes to the discrepancy between the observed and actual molecular mass of the native Kex2 protein because the shift in apparent molecular mass caused by deletion of the C-terminal tail (Δ4) is nearly 3 times greater than that expected from the number of residues removed.

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