The EMBO Journal vol.13 no.10 pp.2280–2288, 1994

A C-terminal domain conserved in precursor processing proteases is required for intramolecular N-terminal maturation of pro-Kex2 protease

Pablo Gluschankof[1] and Robert S.Fuller[2]

Department of Biochemistry, Beckman Center for Molecular and Genetic Medicine, Stanford University School of Medicine, Stanford, CA 94305-5307, USA

[1]Present address: Centre d'Immunologie, INSERM-CNRS de Marseille Luminy Case 906, 13288 Marseille Cedex 9, France
[2]Corresponding author

Communicated by H.-D.Klenk

The Kex2 protease of the yeast *Saccharomyces cerevisiae* is the prototype of a family of eukaryotic subtilisin homologs thought to process prohormones and other precursors in the secretory pathway. Deletion analysis of Kex2 protease shows that a sequence of 154–159 residues carboxyl to the subtilisin domain is essential for the formation of active enzyme. Disruption of this region, termed the 'P-domain', blocks the normally rapid intramolecular cleavage of the N-terminal pro-segment of pro-Kex2 protease in the endoplasmic reticulum (ER). The C-terminal boundary of the P-domain coincides closely with the endpoint of similarity between Kex2 protease and its mammalian homologues. The conservation of and functional requirement for the P-domain sharpens the distinction between a 'Kex2 family' of processing enzymes and degradative 'subtilases', and implies that the Kex2-related enzymes have in common entirely novel structural features that are important in the maturation of precursor polypeptide substrates. Failure to cleave the N-terminal pro-domain, due either to truncation of the P-domain or to mutation of the active site histidine or serine, results in stable, intracellular retention of pro-enzyme, apparently in the ER. Thus pro-Kex2 protease appears to contain an ER retention signal which is removed or destroyed by cleavage of the pro-domain.

Key words: ER retention/precursor maturation/pro-enzyme/prohormone processing/subtilase

Introduction

Post-translational proteolytic modification in late compartments of the secretory pathway (*trans* cisternae of the Golgi and secretory granules and vesicles) is essential for maturation of precursors of many secretory peptides and proteins in vertebrate, invertebrate and microbial eukaryotic cells (for reviews see Douglass *et al.*, 1984; Fuller *et al.*, 1988). Enzymes responsible for cleaving precursors at the carboxyl side of pairs of basic residues and related sites have been the focus of a great deal of recent interest both because of the large number of secretory precursors that possess such sites and because of evidence for the catalysis of these specific cleavages by a family of enzymes distantly related to the subtilisin family of degradative serine proteases (reviewed in Steiner *et al.*, 1992). The Ca^{2+}-dependent transmembrane Kex2 protease of the yeast *Saccharomyces cerevisiae* ('kexin', EC 3.4.21.61) is the prototype of this family (Fuller *et al.*, 1989a,b; Mizuno *et al.*, 1989), and has been shown by a combination of biochemical and genetic evidence to be responsible for processing the precursors of the α-mating pheromone and M_1 killer toxin at -Lys-Arg-, -Arg-Arg- and possibly -Pro-Arg- sites *in vivo* (Julius *et al.*, 1984a; Zhu *et al.*, 1992). The identification and characterization of several vertebrate and invertebrate Kex2 homologues suggests that a family of Kex2-related proteases may catalyze many or more precursor processing reactions in metazoans (Fuller *et al.*, 1989b; Smeekens and Steiner, 1990; van den Ouweland *et al.*, 1990; Kiefer *et al.*, 1991; Seidah *et al.*, 1991; Smeekens *et al.*, 1991; Hallenberger *et al.*, 1992; Nakayama *et al.*, 1992; Nakagawa *et al.*, 1993). Thus, the structure of the Kex2 catalytic domain has implications for an entire family of processing enzymes.

In Kex2 protease, a single transmembrane domain (TMD) divides the protein into a larger N-terminal, lumenal domain and a smaller C-terminal, cytosolic tail (Mizuno *et al.*, 1988; Fuller *et al.*, 1989b). The TMD and cytosolic tail localize the enzyme to a late Golgi compartment in yeast (Franzusoff *et al.*, 1991; Redding *et al.*, 1991; Wilcox *et al.*, 1992), but are not required for enzymatic activity (Fuller *et al.*, 1989a) and are not found in all members of the Kex2 family. The lumenal domain is responsible for catalytic activity (Fuller *et al.*, 1989a,b). The N-terminal ~440 residues of Kex2 protein resemble prepro-subtilisin (Wells *et al.*, 1983), consisting of an N-terminal signal peptide, an intervening 'pro-domain' and a 295 residue region ~30% identical to mature subtilisin (Mizuno *et al.*, 1988; Fuller *et al.*, 1989b). The N-terminus of mature Kex2 protease is formed by rapid proteolytic cleavage at the carboxyl side of the pair of basic residues -Lys108-Arg109- in the endoplasmic reticulum (ER), followed by exoproteolytic removal of two -X-Pro- dipeptides by the *Ste*13 dipeptidyl aminopeptidase (Wilcox and Fuller, 1991; Brenner and Fuller, 1992). Although deletion of the C-terminal 200 residues of Kex2 protease, including the TMD and cytosolic tail, produced a form of the protein, Kex2Δ6, that retained enzymatic activity (Fuller *et al.*, 1989a,b), ~175 residues beyond the approximate end of the subtilisin domain remained, leaving the carboxyl boundary of the catalytic domain uncertain. Here, deletion analysis demonstrates that formation of active Kex2 protease requires a C-terminal extension of ~155 residues beyond the end of the subtilisin domain. Truncation of this domain blocks the ordinarily rapid intramolecular cleavage of the N-terminal pro-domain in the ER. Interestingly, pro-domain cleavage is required for secretion of soluble Kex2 protease, apparently at the level of exit from the ER, suggesting that a mechanism exists to retain the pro-enzyme in that compartment.

© Oxford University Press

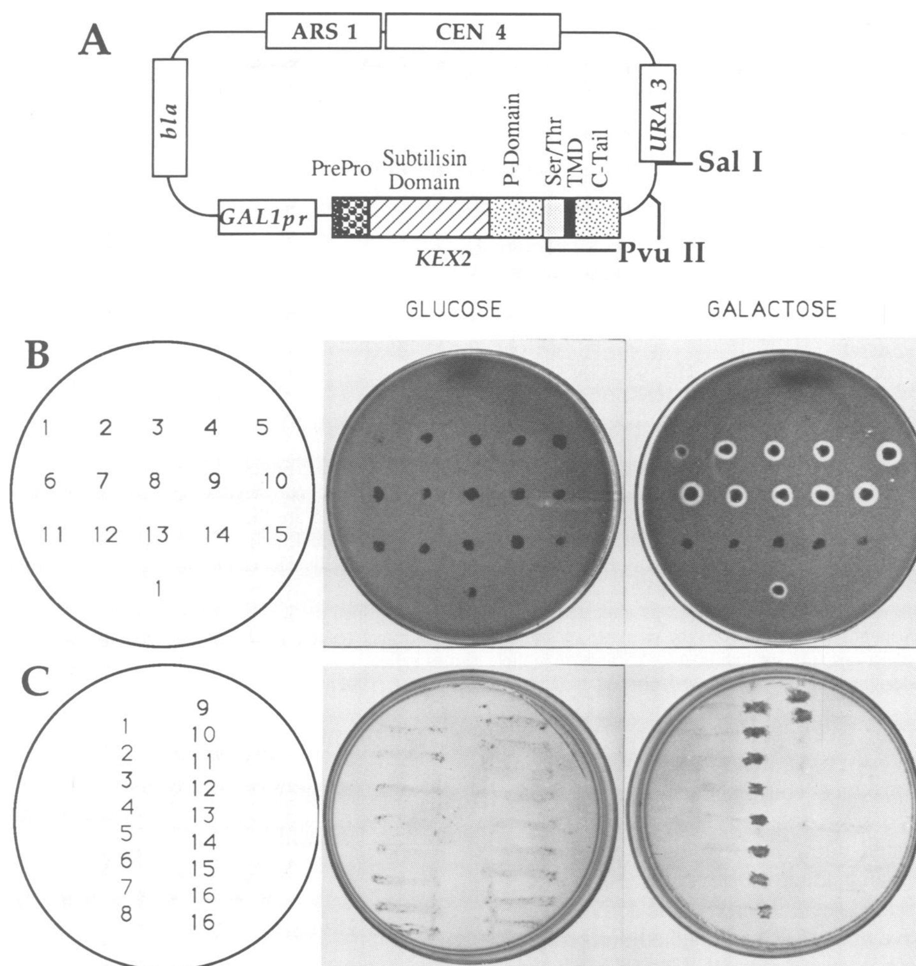

Fig 1. Creation and *in vivo* analysis of C-terminal deletion mutants. (**A**) Structure of plasmid pBM-KX22. *bla*, β-lactamase gene; *ARS1*, yeast origin of replication; *CEN4*, yeast centromere; *URA3*, yeast selectable marker; *GAL1pr*, yeast *GAL1* promoter. Schematic structure of prepro-Kex2 protease: P-domain, see text; Ser/Thr, serine/threonine rich domain; C-tail, C-terminal cytosolic tail. (**B**) *In vivo* detection of Kex2 activity by the α-factor halo assay. (**C**) *In vivo* detection of Kex2 activity by patch mating assay. In (B) and (C), the alleles of *KEX2* under *GAL1* promoter control on pBM258 were as follows: 1, *KEX2*; 2, *KEX2Δ4*; 3, *KEX2Δ5*; 4, *KEX2Δ6*; 5, *KEX2Δ613*; 6, *KEX2Δ598*; 7, *KEX2Δ595c*; 8, *KEX2Δ595b*; 9, *KEX2Δ595a*; 10, *KEX2Δ593*; 11, *kex2Δ592*; 12, *kex2Δ590*; 13, *kex2Δ579*; 14, *kex2Δ564*; 15, *kex2Δ441*; 16, none (vector pBM258 without insert). C-terminal deletions Δ4, Δ5 and Δ6, generated previously (Fuller *et al.*, 1989a,b), were included for comparison.

Results

Approximately 155 residues beyond the subtilisin domain are required for active Kex2 protease

To map the C-terminal endpoint of sequences required for proteolytic activity in Kex2, exonuclease *Bal*31 was used to create deletions extending in the 5′ direction from the *Pvu*II site previously used to create deletion *KEX2Δ6* (Figure 1A; Fuller *et al.*, 1989a). The deleted genes, under the control of the galactose-inducible/glucose-repressible *GAL1* promoter on a single copy yeast centromeric plasmid (Johnston and Davis, 1984), were introduced into a *MATα* haploid strain lacking the chromosomal *KEX2* gene. The ability of the mutant genes to support processing of pro-α-factor was assessed by measuring secretion of mature α-factor using the 'halo' (Julius *et al.*, 1984a) and mating assays (Figure 1B and C). Of the deletions that supported processing of pro-α-factor on galactose plates, none did on glucose plates, demonstrating that expression of the plasmid-encoded *KEX2* gene was required.

Cell-associated and secreted enzymatic activity were measured for controls and the newly isolated deletions. Wild-type *KEX2* and previously constructed deletions Δ4 and Δ5 (Fuller *et al.*, 1989a,b), which retain all or part of the TMD, produced exclusively (wild-type and Δ4) or mostly (Δ5) cell-associated Kex2 enzymatic activity. In the case of all of the newly constructed deletions and *KEX2Δ6*, all of which lack the TMD, active enzyme, if any, was expected to be secreted into the medium. Indeed, in every case where activity was observed, ≥80% of the activity was found in the medium, indicating that secretion was efficient and little secreted enzyme was confined to the periplasm. In >50 clones tested (a subset appears in Table I), all *Bal*31 deletions that supported processing of pro-α-factor *in vivo* also gave rise to secreted Kex2 proteolytic activity. Conversely, no *Bal*31 deletions that failed to function *in vivo* produced either secreted or cell-associated enzyme.

DNA sequence analysis of a set of the *Bal*31 deletions revealed a sharp boundary for loss of function that lay not at the end of the subtilisin domain but, surprisingly, >150 residues carboxyl to it (Figure 2). The abruptness of this boundary can be appreciated by comparing deletions Δ592 and Δ593. Cells expressing *kex2Δ592*, whose predicted product terminated with Gly592 plus two residues encoded

Gene	C-terminal Sequences	α-Factor Halo	α-Factor Mating	Kex2 Activity Medium	Kex2 Activity Cells
KEX2(wt)	S814*	+	+	-	++
KEX2Δ4	F715NR717*	+	+	-	++
KEX2Δ5	G687VIGNR692*	+	+	+	++
KEX2Δ6	P614VIGNR619*	+	+	++	+
KEX2Δ613	E613RNR616*	+	+	++	+
KEX2Δ598	S598*	+	+	++	+
KEX2Δ595c	I595VIGNR600*	+	+	++	+
KEX2Δ595b	I595IGNR599*	+	+	++	+
KEX2Δ595a	I595ANR598*	+	+	++	+
KEX2Δ593	E593VIGNR598*	+	+	++	+
kex2Δ592	G592NR594*	-	-	-	-
kex2Δ590	L590CNR593*	-	-	-	-
kex2Δ579	H579VIGNR584*	-	-	-	-
kex2Δ564	G564NR566*	-	-	-	-
kex2Δ441	KYSHR441*	-	-	-	-

(Domain markers above the sequences: Subtilisin Domain, P-Domain.)

Fig. 2. Deletion analysis reveals the C-terminal boundary of sequences required for active Kex2 protease. The C-terminal one to six amino acid residues predicted by DNA sequence are shown for each allele in the single letter amino acid code, with an asterisk marking the end of each sequence. Residues indicated in bold type represent the final wild-type Kex2 amino acid(s) and are followed by a subscript number indicating the position of the last wild-type residue. Residues indicated in hollow fonts result from read-through into the terminator oligonucleotide and are followed by a subscript number corresponding to the overall length of the open reading frame. Qualitative Kex2 *in vivo* and *in vitro* activities (from Figure 1 and Table I) are indicated. C-terminal sequences of wt Kex2 protein and deletion proteins Δ4, Δ5 and Δ6 (Fuller *et al.*, 1989a,b) were included for comparison. The end of the subtilisin domain in Kex2 as indicated in the figure was defined as Ser439, the 54th residue C-terminal to the catalytic serine, a distance that corresponds to that between the catalytic serine and the C-terminal residue in *Bacillus amyloliquefaciens* subtilisin BPN′ (Wells *et al.*, 1983). For all new deletions (i.e. excluding Δ4, Δ5 and Δ6), allele names reflect the number of wild-type Kex2 residues. For consistency, '*KEX2Δ613*' replaces the designation '*KEX2ΔC3* used previously (Brenner and Fuller, 1992).

by the terminator-adaptor (see legend to Figure 2), failed to process pro-α-factor *in vivo* as measured by the halo or mating assays or to produce cell-associated or secreted proteolytic activity. In contrast, cells expressing *KEX2Δ593*, whose product terminated with Glu593 plus five residues encoded by the terminator-adaptor, both processed pro-α-factor *in vivo* and yielded secreted enzymatic activity. All deletions more extensive than *kex2Δ592* lacked discernible activity. All deletions less extensive than *KEX2Δ593* produced functional enzyme by both *in vivo* and *in vitro* assays. Thus, the deletion endpoint for loss of activity lay between Gly592 and Glu593 in terms of wild-type Kex2 residues or between positions 594 and 598 in terms of overall length. The boundary for loss of activity, therefore, was 154–159 residues beyond the end of the subtilisin domain, defined as Ser439 (see legend to Figure 2). This additional required region beyond the subtilisin domain has been termed the P-domain (Fuller *et al.*, 1991).

Inactive truncated molecules not found in the medium

Conceivably, truncation within the 154–159 residue region might have left an unfolded 'tail' attached to a folded subtilisin domain, in some way targeting the entire molecule for rapid degradation. Arguing against this possibility, deletion *kex2Δ441*, which removed most of the 154–159 residue region but left the subtilisin domain intact, also failed to produce an active product. However, to determine directly whether the lack of activity observed with *kex2Δ592* and more extensive deletions was due to destabilization of potentially active gene products or to loss of essential sequences, the fates of the proteins encoded by the defective *kex2* alleles were assessed. Immunoblot analysis of culture medium was performed to ascertain whether cells expressing the defective alleles secreted a Kex2-related polypeptide. As expected, no Kex2-related polypeptide was detected in the medium of cells expressing *KEX2Δ4* or wild-type *KEX2*, both of which possess the TMD, or in the medium of cells containing the vector (Figure 3, lanes 9–11). Cells expressing *kex2Δ592* and deletions encoding smaller proteins also failed

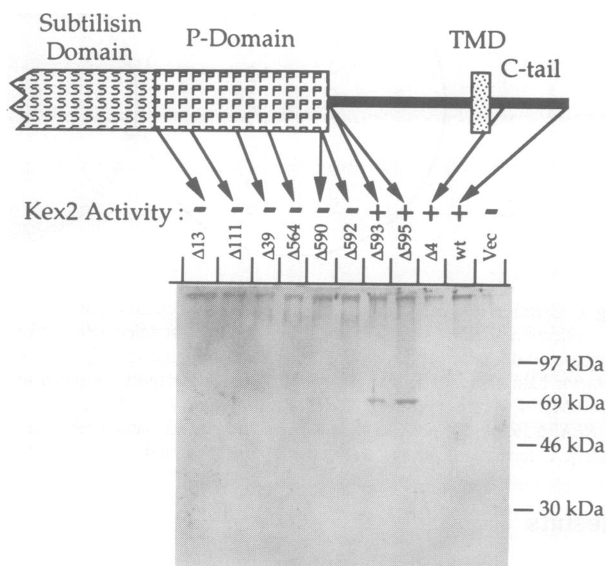

Fig. 3. Only enzymatically active, C-terminally truncated forms of Kex2 protease lacking the TMD are detectable in culture medium. Strain CB017 carrying *KEX2* alleles under *GAL1* promoter control on vector pBM258 was grown and secreted protein was immunoblotted with anti-Kex2 antiserum as described in Materials and methods. Numbers above the lanes refer to alleles as indicated in Figure 2 ('Δ595' refers to *KEX2Δ595c*). Allele numbers Δ13, Δ111 and Δ39 are arbitrary because deletion endpoints were only determined approximately from restriction analysis. Size standards (Sigma Chemical Corp.) were rabbit muscle phosphorylase b, bovine serum albumin and chicken ovalbumin, bovine erythrocyte carbonic anhydrase.

to secrete a Kex2-related polypeptide into the medium (Figure 3, lanes 1–6). In contrast, 69 kDa polypeptides were detected with anti-Kex2 antibody in the growth medium of cells expressing *KEX2Δ593* and *KEX2Δ595c*, both of which gave rise to secreted Kex2 proteolytic activity (Figure 3, lanes 7 and 8). The expected size of these secreted Kex2 molecules was 69 kDa, based on the electrophoretic behavior

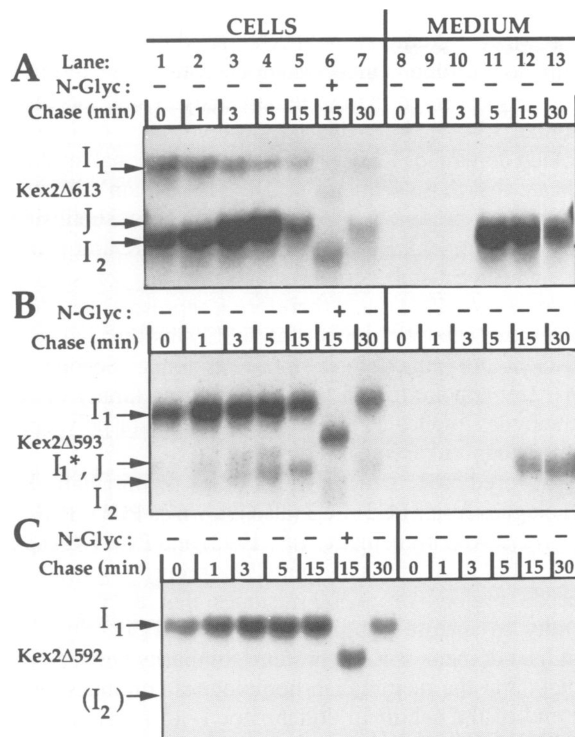

Fig. 4. Pro-domain cleavage of C-terminal deletion mutant enzymes. Strains derived by transforming CB017 with plasmids pBM-KX22Δ613, pBM-KX22Δ593, pBM-KX22Δ592 were pulse-labeled with Tran^{35}S-label, subjected to a cold chase and cell and medium samples removed at the indicated intervals were subjected to immunoprecipitation with anti-Kex2 lumenal domain antibody, SDS–PAGE and fluorography as described in Materials and methods. (**A**) Kex2Δ613; (**B**) Kex2Δ593; (**C**) Kex2Δ592. In (B), the I_1* form may represent an intermediate cleaved at Lys79Arg80 (P.Gluschankof and R.S.Fuller, unpublished data).

of the purified product of the *KEX2Δ613* gene (Brenner and Fuller, 1992).

P-domain truncation prohibits N-terminal processing of pro-Kex2 which remains in the ER

Failure to detect secreted Kex2-related proteins from cells expressing *kex2Δ592* and other defective alleles might be due to degradation of unstable polypeptides or to intracellular retention of stable but inactive molecules. To distinguish between these possibilities, the fates of newly synthesized products of the truncated structural genes were analyzed in pulse–chase labeling studies. Previous studies of wild-type Kex2 protein (Wilcox and Fuller, 1991) have shown that after co-translational glycosylation and signal peptide cleavage, nascent Kex2 protein exists transiently in the ER as a 129 kDa intermediate, 'I_1' (pro-Kex2 protease). Proteolytic removal of the N-terminal pro-domain of I_1 converts it to a 120 kDa form, 'I_2', with a half-time of ~1 min at 30°C. After transport to the Golgi, I_2 is converted to an ~126 kDa form, 'J', by modification of N-linked and O-linked saccharides (Wilcox and Fuller, 1991).

Strains expressing the *KEX2Δ613*, *KEX2Δ593* and *kex2Δ592* genes were pulse-labeled with [^{35}S]amino acids and Kex2-related polypeptides immunoprecipitated from samples of the culture removed at intervals after a cold chase were separated by SDS–PAGE (Figure 4). The *KEX2Δ613* gene encodes a secreted, soluble form of Kex2 protease (ss-Kex2) chosen for purification because of its efficient secretion and relative thermostability (Brenner and Fuller, 1992). After a 90 s pulse-labeling (Figure 4A, lane 1), most of the Kex2Δ613 protein was already in a 69 kDa 'I_2' form, indicating that conversion of I_1 to I_2 occurred as rapidly as with wild-type Kex2 protein. Continued conversion of the 78 kDa I_1 to I_2 was also observed (lanes 1–5 and 7). Modification of both I_1 and I_2 with N-linked oligosaccharide was evident by the removal of Asn-linked oligosaccharides by digestion with *N*-glycanase (lane 6). The I_2 form of Kex2Δ613 also chased into a more slowly migrating 'J' form, with an apparent increase in molecular weight of ~2 kDa, indicating glycosyl modification in the Golgi complex. Release of the Kex2Δ613 protein into the medium occurred with a half-time of <5 min (lanes 8–13), in agreement with rates measured for secretion of α-factor into growth medium (Julius *et al.*, 1984b) and invertase into the periplasm (Novick *et al.*, 1981).

In cells expressing the *KEX2Δ593* gene, which encodes the smallest active enzyme, only a fraction of I_1 was converted to I_2 (Figure 4B, lanes 1–5 and 7). *N*-Glycanase digestion demonstrated Asn-glycosylation of both forms (lane 6), and transport of I_2 to the Golgi was evident from its conversion to a more slowly migrating 'J' form prior to secretion (Figure 4B). However, conversion of I_1 to I_2 was inefficient and mature enzyme appeared in the medium after a considerable lag, with no secreted protein observed after 5 min of chase and only low levels after 15 min (lanes 8–13).

A strikingly different pattern was observed in cells expressing the *kex2Δ592* gene, which encodes the largest inactive form of Kex2 protein. After a 90 s pulse, the Kex2Δ592 protein was present exclusively as I_1 and never chased into I_2 (Figure 4C, lanes 1–5 and 7), demonstrating that pro-domain cleavage did not occur. Translocation of the protein into the ER was evident from the removal of Asn-linked oligosaccharides by *N*-glycanase digestion (Figure 4C, lane 6). However, the I_1 form of Kex2Δ592 was not secreted into growth medium and did not undergo an increase in molecular weight during the chase, indicating that the I_1 form of Kex2Δ592, unlike the I_2 forms of Kex2Δ593 and Kex2Δ613, was not transported to the Golgi. The Kex2Δ592 polypeptide exhibited substantial stability during the chase ($t_{1/2}$ >30 min), suggesting that the protein was not grossly misfolded.

Mutation of the catalytic triad residue His213 or Ser385 also blocks N-terminal processing and results in ER retention

In the case of degradative subtilisin family members, bacterial subtilisin BPN' (Power *et al.*, 1986) and E (Ikemura and Inouye, 1988) and the *S.cerevisiae* vacuolar proteinase B (Nebes and Jones, 1991; Hirsch *et al.*, 1992), pro-domain cleavage was shown to be autoproteolytic and most likely intramolecular. Because the mature N-terminus of Kex2 protease is created by cleavage at a -Lys-Arg- dipeptide, cleavage of the Kex2 pro-domain was also expected to be autoproteolytic (Brenner and Fuller, 1992). Consistent with this expectation, expression in insect cells of a truncated form of Kex2 protease containing a mutation of the putative catalytic Ser385 to Ala resulted in secretion of an inactive, high molecular weight polypeptide, suggesting that pro-

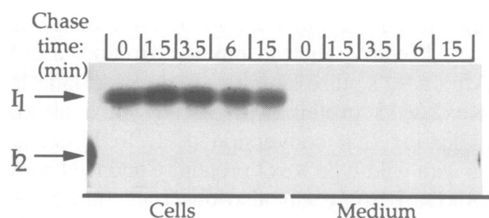

Fig. 5. Pro-domain cleavage is an intramolecular reaction. *MATα KEX2* strain CB018 containing pBM-KX22Δ613-H213A was pulse-labeled with [^{35}S]amino acids, subjected to a cold chase and cell and medium samples removed at the indicated times were immunoprecipitated with anti-Kex2 lumenal domain antibody and analyzed by SDS–PAGE and fluorography as described in Materials and methods. On longer exposures, a small amount of I_2-sized material was detected in the medium at 15 min of chase (see Discussion), but no enzyme activity was secreted and no *in vivo* activity was observed. This species was therefore judged catalytically inactive.

domain cleavage was blocked (Germain *et al.*, 1992a,b). To determine whether maturation of pro-Kex2 protease expressed in yeast was autoproteolytic and if so, intramolecular, site-directed mutagenesis was used to substitute Ala for the His residue, His213, implicated by homology to be part of the Kex2 catalytic triad. The H213A substitution was made in the *KEX2Δ613* gene under control of the *GAL1* promoter and the resulting expression plasmid introduced into *MATα* strains having either wild-type *KEX2* or a null allele (*kex2Δ2::TRP1*) in the chromosome. Expression of the Kex2Δ613-H213A protein in the *kex2Δ* strain by growth in 2% galactose failed to promote either mating with a tester *MATα* strain or production of an α-factor halo, and no secreted activity was detected in the growth medium, indicating that the product of *kex2Δ613-H213A* was inactive (data not shown). Pulse–chase immunoprecipitation analysis of the Kex2Δ613-H213A protein in the *KEX2* strain demonstrated that an I_1-sized polypeptide was synthesized, but did not undergo conversion to form I_2 (Figure 5). Much like the inactive C-terminal deletion mutant protein Kex2Δ592, the I_1 form of Kex2Δ613-H213A was stable and failed to increase in apparent molecular weight with time, again suggesting retention in the ER. Identical results were obtained with expression of Kex2Δ613-H213A in a *kex2Δ* strain (data not shown). Substitution of Ala for the Ser385 was also made in Kex2Δ613. The Ser385Ala mutant protein behaved exactly like the His213Ala mutant (data not shown). By pulse–chase analysis, the proenzyme was translocated into the ER but failed to undergo pro-domain cleavage. The proenzyme failed to exit the ER, and was completely stable during a 30 min chase. We conclude, therefore, that pro-domain cleavage is autoproteolytic, because it requires an intact catalytic triad, and intramolecular, because the defect in pro-domain cleavage was not rescued by wild-type Kex2 protease *in trans*.

Discussion

The experiments reported here demonstrate that proteolytic cleavage of the N-terminal pro-domain, and therefore formation of active Kex2 protease, requires not only the subtilisin domain but also an additional 154–159 residues C-terminal to it. Intramolecular cleavage of the N-terminal pro-segment, which occurs rapidly in the wild-type Kex2 and Kex2Δ613 proteins, is partially impaired in the smallest active molecule, Kex2Δ593. Further truncation, by removal of a single additional wild-type residue in Kex2Δ592, completely prohibits pro-domain cleavage. The abrupt loss of activity observed in the deletion analysis suggests that the essential 154–159 residue region constitutes or is part of a folded domain of Kex2 protein. Two additional arguments indicate that these sequences define a 'domain'. First, this region is not present in either prokaryotic (e.g. subtilisin and thermitase) or eukaryotic (e.g. proteinase K and protease B) degradative members of the subtilisin family, or 'subtilases' (Siezen *et al.*, 1991), and therefore it represents an addition to the core catalytic domain of subtilisins that is by itself sufficient for function in those proteins. Second, this 154–159 residue region is conserved in a family of Kex2 homologues implicated in processing precursor polypeptides in metazoan eukaryotes.

The predicted structures of the mammalian Kex2 homologues furin, PC2, PC3 (also known as PC1), PACE-4, PC4 (reviewed in Steiner *et al.*, 1992) and PC6 (Nakagawa *et al.*, 1993) all possess potential prepro-segments that precede regions of ~300 residues that display ~30% identity to mature subtilisin, but ≥45% identity to the subtilisin domain of Kex2 protein. Similarity between Kex2 protein and the mammalian homologues continues beyond the end of the subtilisin domain for ~150 residues at the level of ~28–31% identity after which the sequences of Kex2 and the mammalian homologues diverge (Fuller *et al.*, 1989b; Steiner *et al.*, 1992; see Figure 6). The end of this extended region of homology corresponds almost precisely to the C-terminal endpoint of sequences required for production of active Kex2 protease determined functionally by the deletion analysis reported here. The last residue perfectly conserved in an alignment of Kex2 with nine metazoan homologues and one fungal homologue is a Gly residue that corresponds to Gly592 in Kex2 (Figure 6). The next residue, which is Glu in *S.cerevisiae* Kex2 and its *Kluyveromyces lactis* homologue, is conserved as a Thr residue in all of the metazoan Kex2 homologues. Strikingly, the PC3 homologue from *Hydra vulgaris* terminates with only two additional residues beyond the Thr residue (Chan *et al.*, 1992). Due to its conservation solely in enzymes implicated in proteolytic processing as opposed to degradation, we have termed the 154–159 residue essential region the 'P-domain' (Fuller *et al.*, 1991).

The functional requirement for the P-domain in Kex2 protease implies that enzymes of the Kex2 family are structurally more complex than the degradative subtilases. The P-domain thus becomes an additional definitive characteristic that distinguishes a 'Kex2 family' of subtilisin-related processing enzymes from degradative subtilisins. Evidence for the importance of the P-domain in mammalian homologues of Kex2 protease also comes from the finding that deletions within the region blocked the production of active furin, although the nature of the defect was not investigated (Hatsuzawa *et al.*, 1992a). Members of the Kex2 family share additional distinctive features, including characteristic consensus sequences surrounding active site residues and a high degree of primary sequence specificity.

Conservation of the P-domain in processing proteases from fungi to mammals raises the question of its functional role. The defect in N-terminal pro-domain cleavage that results from disruption of the P-domain may provide clues. Because pro-domain cleavage is intramolecular, it depends on prior

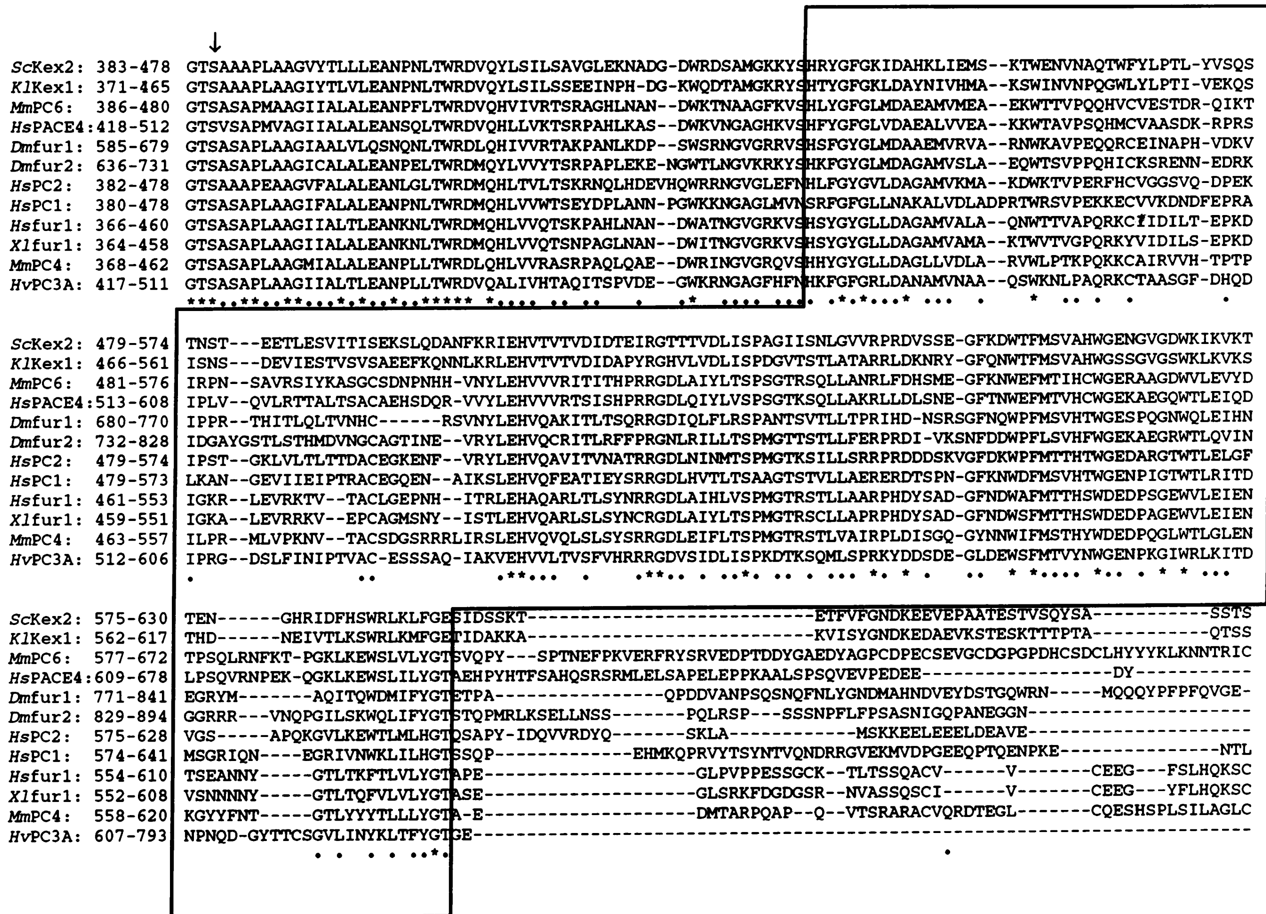

Fig. 6. The C-terminal endpoint of sequence similarity among Kex2 homologues corresponds closely to the functional endpoint of the Kex2 P-domain determined by deletion analysis. Multiple sequence alignment of indicated segments of Kex2 and 11 homologues was performed using the ClustalV program with default parameters (Higgins *et al.*, 1992). *Saccharomyces cereviase* (*Sc*) Kex2 sequence (Fuller *et al.*, 1989b); *K.lactis* (*Kl*) Kex1 (Tanguy-Rougea *et al.*, 1988); *Mus musculus* (*Mm*) PC6 (Nakagawa *et al.*, 1993); *Homo sapiens* (*Hs*) PACE4 (Kiefer *et al.*, 1991); *Drosophila melanogaster* (*Dm*) fur1 (Roebroek *et al.*, 1991); *Dm* fur2 (Roebroek *et al.*, 1992); *Hs* PC2 (Smeekens and Steiner, 1990); *Hs* PC1 (= PC3) (Smeekers *et al.*, 1991); *Hs* fur1 (van den Ouweland *et al.*, 1990); *Xenopus laevis* (*Xl*) fur1 (Korner *et al.*, 1991); *Mm* PC4O (Nakayama *et al.*, 1992); *Hydra vulgaris* (*Hv*) PC3A (Chan *et al.*, 1992). In the *H.vulgaris* PC3A sequence, it was necessary to delete residues 612–772 (represented as a dash between Asp611 and Gly773) to align residues 779–793 with the end of the P-domain. This deletion occurs at a point of multiple insertions and deletions in the alignment of the other sequences. An asterisk (*) marks positions perfectly conserved in all 12 sequences and a dot (·) marks positions in which at least six sequences conserve a specific residue. The box surrounds the region defined as the P-domain. Arrow indicates catalytic Ser385.

folding of the catalytic domain. Completion of folding of the catalytic domain of subtilisin both *in vivo* (Ikemura *et al.*, 1987) and *in vitro* (Zhu *et al.*, 1989; Ohta *et al.*, 1991; Eder *et al.*, 1993) requires interaction with the pro-domain. A similar requirement appears to exist in the Kex2 family. Deletion of the pro-domain of furin ('PACE') prevents formation of catalytically active enzyme (Rehemtulla *et al.*, 1992). Folding of the catalytic domain of Kex2 protease probably also requires interaction with the pro-domain. A deletion that precisely removes the Kex2 pro-domain results in production of a form of Kex2 that is translocated into the ER, but completely lacks enzymatic activity by both biological and enzymatic assay (P.Gluschankof and R.S. Fuller, unpublished data). Therefore, the P-domain could simply be necessary for the proper folding and integrity of either the subtilisin domain or the pro-domain or both. Alternatively, the P-domain could be involved in recognition of the pro-domain as a substrate, and if so might play a role in recognition of other substrates as well. Structural analysis of Kex2 protease along with an assessment of the phenotypes of point mutations in the P-domain should help determine the precise role of this domain in the structure and function of Kex2 protease and its homologues.

All members of the Kex2 family of processing enzymes contain pairs or higher multiplets of basic residues just N-terminal to the subtilisin domain. Several, including Kex2 (Brenner and Fuller, 1992), PC1 and PC2 (Christie *et al.*, 1991; Mackin *et al.*, 1991) and furin (Hatsuzawa *et al.*, 1992b), have been shown by direct sequencing to undergo N-terminal processing at such sites. Using a rationale similar to that used in this work, intramolecular processing of pro-furin has been suggested by the effects of mutation of the putative catalytic Asp153 to Asn and the inability of co-expression of a truncated version of wild-type furin to restore cleavage of Asp153Asn pro-furin (Leduc *et al.*, 1992). Analysis of the kinetics of folding and N-terminal processing of pro-Kex2 and pro-furin *in vitro* may be necessary to prove conclusively the intramolecular nature of pro-domain cleavage. However, a consideration of the compartmentation and rate of maturation of pro-Kex2 *in vivo* provides considerable support for the intramolecular processing model. *In vivo*, cleavage of pro-Kex2 is very fast

($t_{1/2}$ ~1 min) and the cleavage product, I_2, is transported rapidly to the Golgi ($t_{1/2}$ ~2 min), indicating that Kex2 spends very little time in the ER in a potentially active form; i.e. one that lacks the pro-domain (Wilcox and Fuller, 1991). Therefore, little active Kex2 protease can be available to process, *in trans*, newly synthesized pro-Kex2 molecules. Consistent with this, Kex2 protease is not observed to process pro-α-factor or other substrates in the ER (Julius *et al.*, 1994b) unless accumulated there by fusion to an ER retention signal (Chaudhuri *et al.*, 1992). Taken together, the rapidity of pro-domain cleavage *in vivo*, the paucity of active Kex2 protease in the ER and the compelling evidence from mutagenesis of catalytic residues that pro-domain cleavage is autoproteolytic argue strongly that pro-domain cleavage is an intramolecular reaction.

Substitution of Ala for any of the catalytic triad residues (Ser221, His64 or Asp32) in subtilisin BPN′ decreased k_{cat}/K_M for peptide substrates by 10^5- to 10^6-fold (Carter and Wells, 1988), while mutagenesis of the fourth important catalytic residue, the oxyanion hole Asn155, to Asp had only an ~500-fold effect (Wells *et al.*, 1986). Mutagenesis of the predicted oxyanion hole of Kex2, Asn314, to Asp, decreased Kex2 activity to a similar degree. However, unlike the dramatic effect of mutagenesis of either Ser385 or His213, the Asn314Asp substitution had no effect on the rate of pro-domain cleavage in Kex2 *in vivo*, suggesting that some step other than peptide bond hydrolysis is ordinarily rate-limiting for pro-domain cleavage (Brenner *et al.*, 1993). Apparently, peptide bond hydrolysis becomes rate-limiting only when catalytic rate is decreased by ≫100-fold. Consistent with this interpretation, on longer exposures of the gel in Figure 5, a small amount of mature sized Kex2Δ613-His213Ala (≤5% of I_1) was observed in the medium at 15 min of chase. This was also observed in pulse–chase analysis of Kex2Δ613-Ser385Ala (data not shown). Assuming 5% conversion, the half-time for pro-domain cleavage in Kex2 His213Ala would be ~200 min, which represents a reduction in rate of ~4×10^5-fold relative to the half-time for cleavage of good peptide substrates by Kex2 (~30 ms; Brenner and Fuller, 1992).

Remarkably, failure to cleave the pro-domain, either due to truncation of the P-domain or mutation of either the catalytic His or Ser residue, resulted in intracellular retention of the pro-Kex2. In both cases, the absence of processing of the N-linked oligosaccharides, as evidenced by the lack of an increase in apparent molecular weight with time, implied that pro-Kex2 is retained in a pre-Golgi compartment, presumably the ER, until pro-domain cleavage occurs. Interestingly, when signal peptidase was inactivated in a *sec11* mutant strain by a shift to the restrictive temperature, cleavage of the Kex2 pro-domain was also blocked and prepro-Kex2 was also found to accumulate in the ER (Wilcox and Fuller, 1991; Redding *et al.*, 1991). Whether the pro-domain by itself contains a novel ER retention signal or whether some feature unique to the pro-enzyme is responsible for retention remains to be determined.

Materials and methods

Yeast strains, plasmids and reagents

Yeast strains were: CB017 (*MATα kex2Δ2::TRP1 pep4::HIS3 prb1::hisG prc1::hisG can1-100 ade2-1 his3-11,-15 leu2-3,-112 trp1-1 ura3-1*) in which the chromosomal *KEX2* gene was deleted and replaced with *TRP1* (Redding *et al.*, 1991), CB018 (isogenic with CB017 except *MAT***a** *KEX2*), RC634 (*MAT***a** *sst1-3*) and DC14 (*MAT***a** *his1*). General yeast methods and media were as described (Rose *et al.*, 1990). Growth of all yeast strains was at 30°C. Plasmid pBM-KX22 (Figure 1A) consists of the *KEX2* gene under *GAL1* promoter control in the single copy centromeric vector pBM258 (Redding *et al.*, 1991). Plasmids were introduced into yeast strains by spheroplast transformation (Burgers and Percival, 1987). Enzymes for molecular biology were from New England Biolabs. *N*-Glycanase was from Genzyme. boc-Gln-Arg-Arg-MCA [*t*-butoxycarbonyl-Gln-Arg-Arg-4-methylcoumarin-7-amide] was from Peninsula Laboratories. Oligonucleotides were from Stanford University PAN Facility. ^{35}S-Labeled amino acids (Tran^{35}S-label), >1000 Ci/mmol, were from ICN Pharmaceuticals, Inc.

Generation of C-terminal truncation mutants

Standard methods were used in DNA manipulations (Sambrook *et al.*, 1989). Plasmid pBM-KX22 (Figure 1A) was linearized with *Pvu*II and a 'deleted-gene library' was made by digesting with *Bal*31 exonuclease (2 U/ml) in 200 μl. 2 μl taken every 10 s were quenched in 50 mM EDTA in 3 min pools. Ends were filled with DNA polymerase I large fragment and phosphorylated *Sal*I 'terminator-adaptors', having tandem translational termination codons in all three reading frames, were ligated. Oligonucleotide sequences (5′ → 3′) were: GTAATAGGTAATAGGTAATAGG and TCGACCTAT-TACCTATTACCTATTAC. Attachment of terminator-adaptors ensured addition of no more than five novel residues at deletion endpoints. Ligation products were cleaved with *Sal*I and religated at low concentration to ensure that vector sequences were identical in all plasmids. Deletion sizes were determined roughly from restriction digests and nucleotide sequences across deletion endpoints were determined by sequencing double-stranded DNA using the *Sal*I counter-clockwise primer (New England Biolabs) and the Sequenase kit (US Biochemical Corp.). To define more precisely the end of the P-domain, additional deletions were made using *Sal*I-linearized pBM-KX22Δ595c and 0.1 U of *Bal*31 in a 200 μl reaction.

Creation of the His213Ala mutation

An 800 bp *Bam*HI–*Xba*I fragment from plasmid pKX22 (Fuller *et al.*, 1989a) containing the N-terminal coding portion of the *KEX2* gene was subcloned into the polylinker region of vector pUN70 (Elledge and Davis, 1988) creating plasmid pUN70BX(KX). Site-directed mutagenesis was performed by the method of Kunkel *et al.* (1987) using 10 pmol of the mutagenic oligonucleotide (5′ → 3′) ATCTCGTACCAGCGTAGTCAT-CAGA and 0.4 pmol single-stranded uracil-containing DNA produced by propagating pUN70BX(KX) in *Escherichia coli dut ung* strain CJ236 with M13KO7 helper phage (Vieira and Messing, 1987). Mutants were recovered as transformants in *E.coli* DH5α (Sambrook *et al.*, 1989), and the *Hin*dIII–*Xba*I fragment containing the mutation was subcloned into pBM-KX22Δ613, replacing the wild-type fragment and creating the *kex2Δ613-H213A* gene. Prior to subcloning, the 429 bp *Hin*dIII–*Xba*I fragment was sequenced in its entirety to confirm the mutation and eliminate the possibility of fortuitous mutations.

Assays for Kex2 activity in vivo

To determine activity *in vivo*, plasmids directing expression of wild-type Kex2 or various deletions were introduced into yeast strain CB017 (Wilcox *et al.*, 1992). α-factor halo assays were performed as described by Julius *et al.* (1984a). Transformants grown as patches on YPAD (rich medium with 2% glucose, wt/vol) or YPAGal (rich medium containing 2% galactose, wt/vol) plates were replica-plated onto a lawn of α-factor supersensitive strain RC634 (*MAT***a** *sst1-3*). Zones of growth inhibition ('halos') form only around strains secreting mature α-factor. For patch matings, the same transformants were cross-streaked onto YPAD or YPAGal plates streaked with mating type tester strain DC14. After 24 h at 30°C, matings were replica-plated onto synthetic minimal (SD) plates to detect prototrophic diploids.

Kex2 enzymatic activity

Cells expressing Kex2Δ6 protein, which lacks the TMD and cytosolic tail, accumulated enzyme activity at the cell surface but activity was not found in the growth medium (Fuller *et al.*, 1989b). We reasoned that enzyme released into the medium might be unstable. When the medium was buffered with 50 mM potassium phosphate, pH 6.6, secreted Kex2 activity was detected in the medium of strains expressing *KEX2Δ6* and certain of the new deletions (P.Gluschankof and R.S.Fuller, unpublished). Seeboth and Heim (1991) found that similar conditions stabilized secreted forms of Kex2 protease. In the experiments in Table I, yeast strain CB017 was transformed with plasmids having wild-type and C-terminally deleted *KEX2* genes under control of the *GAL1* promoter on vector pBM258. Strains were grown in synthetic complete (SC) medium lacking glucose and uracil but containing

Table I. Secreted and cell-associated activity of C-terminal deletion mutants

KEX2 allele	Kex2 enzymatic activity (units × 10^{-3})[a]	
	Medium	Cells
Wild-type	0.0	81.2
Δ*4*	0.0	21.4
Δ*5*	2.1	18.5
Δ*6*	13.4	3.4
Δ*613*	19.1	4.4
Δ*598*	6.0	0.8
Δ*595c*	6.6	1.3
Δ*595b*	8.8	1.1
Δ*595a*	9.4	0.8
Δ*593*	11.0	0.9
Δ*592*	0.0	0.0
Δ*590*	0.0	0.0
Δ*579*	0.0	0.0
Δ*564*	0.0	0.0
Δ*441*	0.0	0.0
Null	0.0	0.0

[a]Total cell-associated activity or activity in medium from 5 ml of cells, as described in Materials and methods. The data represent the average of three assays in one experiment. The entire experiment was performed twice with an average variation of ±15%.

2% galactose and buffered to pH 6.6 with 50 mM potassium phosphate. Cells harvested at OD_{600} = 1 were washed with and resuspended in an equal volume of medium. After 2.5 h with shaking at 30°C, cells (5 ml) were harvested, washed and resuspended in 100 μl of medium and kept on ice prior to assay. Kex2 activity was measured using the fluorogenic peptide substrate boc-Gln-Arg-Arg-MCA (one unit = 1 pmol 7-amino-4-methylcoumarin released per min; Fuller *et al.*, 1989a) with 2, 8 and 25 μl of media or cells. Activity was measured in the presence of 1 mM $CaCl_2$ and background measured in the presence of 1 mM ethylenediaminetetra-acetic acid (EDTA) was subtracted. Total cell-associated Kex2 activity was measured in the presence of detergent (Fuller *et al.*, 1989b). Kex2 deletions Δ4, Δ5 and Δ6 (Fuller *et al.*, 1989a,b) were included for comparison. Kex2Δ4 lacks most of the cytosolic tail but retains the TMD and Kex2Δ5 lacks the entire cytosolic tail.

Detection of secreted Kex2 protein by immunoblotting

Strain CB017 carrying various *KEX2* alleles under *GAL1* promoter control on vector pBM258 were grown at 30°C to OD_{600} = 1 in 5 ml SC medium lacking glucose and uracil but containing 2% galactose and buffered to pH 6.6 with 50 mM potassium phosphate. Cells were removed by centrifugation and protein in the medium was precipitated by adding 0.5 ml 100% trichloroacetic acid (TCA) plus 1 ml 0.2% deoxycholate, incubating on ice for 30 min and centrifuging for 15 min at 2000 *g*. Pellets were washed with ice-cold acetone, resuspended in 50 μl SDS–PAGE sample buffer, and fractionated by SDS–PAGE (separating gel was 10% acrylamide). The gel was immunoblotted (Fuller *et al.*, 1989b) using rabbit antiserum raised against a β-galactosidase fusion protein containing residues 144–814 of Kex2 protein (gift of K.Cunningham and W.Wickner, University of California, Los Angeles).

Analysis of biosynthesis and secretion of truncated forms of Kex2 protease

CB017 strains containing plasmid pBM-KX22Δ613, pBM-KX22Δ593 or pBM-KX22Δ592 were grown at 30°C in low sulfate medium (LSM) lacking glucose and containing 100 μM ammonium sulfate and 2% galactose (Wilcox and Fuller, 1991). Cultures were harvested at a density of ~10^7 cells/ml and subjected to sulfate depletion for 30 min by resuspension in LSM lacking glucose and containing 20 μM ammonium sulfate plus 2% galactose and 0.5 mg/ml BSA (to prevent loss of secreted Kex2), after which labeling was initiated by adding [^{35}S]amino acids to 330 μCi/ml. A chase was begun after 90 s by adding unlabeled $(NH_4)_2SO_4$ to 10 mM and cysteine and methionine to 1 mM. Labeled cell samples were made 10 mM in sodium azide, chilled on ice, harvested by centrifugation and washed once with HEPES buffer containing protease inhibitors (Wilcox and Fuller, 1991). Cell pellets were stored at −80°C prior to lysis. Total protein from the medium of labeled cells was precipitated with TCA and sodium deoxycholate, acetone-washed and denatured in SDS–PAGE sample buffer as for immunoblotting. Kex2 protein was immunoprecipitated twice from cell pellets and denatured medium samples as described (Wilcox and Fuller 1991) using 10 μl of rabbit antiserum that recognizes only lumenal sequences of Kex2 protease (Wilcox *et al.*, 1992), and 30 μl of a 1:1 slurry of Pansorbin (Calbiochem) in water. Where indicated, *N*-glycanase digestion was performed following the first immunoprecipitation (Wilcox and Fuller, 1991). Samples were subjected to SDS–PAGE and fluorographed (Wilcox and Fuller, 1991).

Acknowledgements

The authors thank C.Brenner, S.Elledge, M.Johnston, K.Redding and C.A.Wilcox for yeast strains and plasmids; K.Cunningham, W.Wickner and K.Redding for anti-Kex2 antibodies; and R.L.Baldwin, D.Herschlag and members of the laboratory for helpful comments on the manuscript. This work was supported by an NIH grant (GM39697) and a Lucille P.Markey Scholar Award (87-31) to R.S.F. P.G. was supported by an EMBO postdoctoral fellowship.

References

Brenner,C. and Fuller,R.S. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 922–926.
Brenner,C., Bevan,A. and Fuller,R.S. (1993) *Curr. Biol.*, **3**, 498–506.
Burgers,P.M.J. and Percival,K.J. (1987) *Anal. Biochem.*, **163**, 391–397.
Carter,P. and Wells,J.A. (1988) *Nature*, **332**, 564–568.
Chaudhuri,B., Latham,S.E., Helliwell,S.B. and Seeboth,P. (1992) *Biochem. Biophys. Res. Commun.*, **183**, 212–219.
Chan,S.J., Oliva,A.A., Jr, Lamendola,J., Grens,A., Bode,H. and Steiner,D.F. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 6678–6682.
Christie,D.L., Batchelor,D.C. and Palmer,D.J. (1991) *J. Biol. Chem.*, **266**, 15679–15683.
Douglass,J., Civelli,O. and Herbert,E. (1984) *Annu. Rev. Biochem.*, **53**, 655–715.
Eder,J., Rheinnecker,M. and Fersht,A.R. (1993) *Biochemistry*, **32**, 18–26.
Elledge,S.J. and Davis,R.W. (1988) *Gene*, **70**, 303–312.
Franzusoff,A., Redding,K., Crosby,J., Fuller,R.S. and Schekman,R. (1991) *J. Cell Biol.*, **112**, 27–37.
Fuller,R.S., Sterne,R.E. and Thorner,J. (1988) *Annu. Rev. Physiol*, **50**, 345–362.
Fuller,R.S., Brake,A. and Thorner,J. (1989a) *Proc. Natl Acad. Sci. USA*, **86**, 1434–1438.
Fuller,R.S., Brake,A. and Thorner,J. (1989b) *Science*, **246**, 482–486.
Fuller,R.S., Brenner,C., Gluschankof,P. and Wilcox,R.S. (1991) In Jörnvall,H., Höög,J.-O. and Gustavsson,A.-M. (eds), *Advances in Life Sciences: Methods in Protein Sequence Analysis.* Birkhaüser, Berlin, pp. 205–214.
Germain,D., Vernet,T., Boileau,G. and Thomas,D.Y. (1992a) *Eur. J. Biochem.*, **204**, 121–126.
Germain,D., Dumas,F., Vernet,T., Bourbonnais,Y., Thomas,D.Y. and Boileau,G. (1992b) *FEBS Lett.*, **299**, 283–286.
Hallenberger,S., Bosch,V., Angliker,H., Shaw,E., Klenk,H.D. and Garten,W. (1992) *Nature*, **360**, 358–361.
Hatsuzawa,K., Murakami,K. and Nakayama,K. (1992a) *J. Biochem.*, **111**, 296–301.
Hatsuzawa,K., Nagahama,M., Takahashi,S., Takada,K., Murakami,K. and Nakayama,K. (1992b) *J. Biol. Chem.*, **267**, 16094–16099.
Higgins,D.G., Bleasby,A.J. and Fuchs,R. (1992) *CABIOS*, **8**, 189–191.
Hirsch,H.H., Schiffer,H.H., Muller,H. and Wolf,D.H. (1992) *Eur. J. Biochem.*, **203**, 641–653.
Ikemura,H. and Inouye,M. (1988) *J. Biol. Chem.*, **263**, 12959–12963.
Ikemura,H., Takagi,H. and Inouye,M. (1987) *J. Biol. Chem.*, **262**, 7859–7864.
Johnston,M. and Davis,R.W. (1984) *Mol. Cell. Biol.*, **4**, 1440–1448.
Julius,D., Brake,A., Blair,L., Kunisawa,R. and Thorner,J. (1984a) *Cell*, **37**, 1075–1089.
Julius,D., Schekman,R. and Thorner,J. (1984b) *Cell*, **36**, 309–318.
Kiefer,M.C., Tucker,J.E., Joh,R., Landsberg,K.E., Saltman,D. and Barr,P.J. (1991) *DNA Cell Biol.*, **10**, 757–769.
Korner,J., Chun,J., O'Bryan,L. and Axel,R. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 11393–11397.
Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) *Methods Enzymol.*, **154**, 367–382.
Leduc,R., Molloy,S.S., Thorne,B.A. and Thomas,G. (1992) *J. Biol. Chem.*, **267**, 14304–14308.

Mackin,R.B., Noe,B.D. and Spiess,J. (1991) *Endocrinology*, **129**, 2263–2265.
Mizuno,K., Nakamura,T., Ohshima,T., Tanaka,S. and Matsuo,H. (1988) *Biochem. Biophys. Res. Commun.*, **156**, 246–254.
Mizuno,K., Nakamura,T., Oshima,T., Tanaka,S. and Matsuo,H. (1989) *Biochem. Biophys. Res. Commun.*, **159**, 305–311.
Nakagawa,T., Hosaka,M., Torii,S., Watanabe,T., Murakami,K. and Nakayama,K. (1993) *J. Biochem.*, **113**, 132–135.
Nakayama,K., Kim,W.S., Torii,W.S., Hosaka,M., Nakagawa,T., Ikemizu,J., Baba,T. and Murakami,K. (1992) *J. Biol. Chem.*, **267**, 5897–5900.
Nebes,V.L. and Jones,E.W. (1991) *J. Biol. Chem.*, **266**, 22851–22857.
Novick,P., Ferro,S. and Schekman,R. (1981) *Cell*, **25**, 461–469.
Ohta,Y., Hojo,H., Aimoto,S., Kobayashi,T., Zhu,X., Jordan,F. and Inouye,M. (1991) *Mol. Microbiol.*, **5**, 1507–1510.
Power,S.D., Adams,R.M. and Wells,J.A. (1986) *Proc. Natl Acad. Sci. USA*, **83**, 3096–3100.
Redding,K., Holcomb,C. and Fuller,R.S. (1991) *J. Cell Biol.*, **113**, 527–538.
Rehemtulla,A., Dorner,A.J. and Kaufman,R.J. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 8235–8239.
Roebroek,A.J.M., Pauli,I.G.L., Zhang,Y. and van de Ven,W.J.M. (1991) *FEBS Lett.*, **289**, 133–137.
Roebroek,A.J., Creemers,J.W., Pauli,I.G., Kurzik-Dumke,U., Rentrop,M., Gateff,E.A., Leunissen,J.A. and van de Ven,W.J. (1992) *J. Biol. Chem.*, **267**, 17208–17215.
Rose,M.D., Winston,F. and Hieter,P. (1990) *Methods in Yeast Genetics. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sambrook,J., Fritsch,E.F. and Maniatis,J. (1989) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Seeboth,P. and Heim,J. (1991) *Appl. Microbiol. Biotechnol.*, **35**, 771–776.
Seidah,N.G., Marcinkiewicz,M., Benjannet,S., Gaspar,L., Beaubien,G., Mattei,M.G., Lazure,C., Mbikay,M. and Chretien,M. (1991) *Mol. Endocrinol.*, **5**, 111–122.
Siezen,R.J., de Vos,W.M., Leunissen,J.A. and Dijkstra,B.W. (1991) *Protein Engng*, **4**, 719–737.
Smeekens,S.P. and Steiner,D.F. (1990) *J. Biol. Chem.*, **265**, 2997–3000.
Smeekens,S.P., Avruch,A.S., LaMendola,J., Chan,S.J. and Steiner,D.F. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 340–344.
Steiner,D.F., Smeekens,S.P., Ohagi,S. and Chan,S.J. (1992) *J. Biol. Chem.*, **267**, 23435–23438.
Tanguy-Rougeau,C., Wesolowski-Louvel,M. and Fukuhara,H. (1988) *FEBS Lett.*, **234**, 464–470.
van den Ouweland,A.M.W., van Duijnhoven,H.P.L., Keizer,G.D., Dorssers,L.C.J. and van de Ven,W.J.M. (1990) *Nucleic Acids Res.*, **18**, 664.
Vieira,J. and Messing,J. (1987) *Methods Enzymol.*, **153**, 3–11.
Wells,J.A., Ferrari,E., Henner,D.J., Estell,D.A. and Chen,E.Y. (1983) *Nucleic Acids Res.*, **11**, 7911–7925.
Wilcox,C.A. and Fuller,R.S. (1991) *J. Cell Biol.*, **115**, 297–307.
Wilcox,C.A., Redding,K., Wright,R. and Fuller,R.S. (1992) *Mol. Biol. Cell*, **3**, 1353–1371.
Zhu,X., Ohta,Y., Jordan,F. and Inouye,M. (1989) *Nature*, **339**, 483–484.
Zhu,Y.S., Zhang,X.Y., Cartwright,C.P. and Tipper,D.J. (1992) *Mol. Microbiol.*, **6**, 511–520.

Received on July 12, 1993; accepted on March 4, 1994