Candida albicans possesses at least seven differentially expressed genes that encode virulence-related secretory aspartyl proteinases (Saps). Sap DNA sequences predict post-translational processing at lysine-arginine residues in the preproteins, reminiscent of the matura-

tion of Saccharomyces cerevisiae α-factor, where a pre-

propolypeptide is converted into a biologically active

pheromone by Kex2, a subtilisin-like proprotein conver-

tase. To investigate involvement of a Candida albicans KEX2

homologue in Sap activation, a genetic selection was

performed based on KEX2 function. A kex2 strain of S.

cerevisiae was transformed with a Candida albicans genomic

DNA library and screened for the production of active

α-factor. Positive clones were assayed for killer toxin

activity, another Kex2-dependent phenotype. Plasmids

that rescued both defects contained a sequence encod-

ing a protein homologous to S. cerevisiae Kex2. Both

alleles of the Candida albicans KEX2 were inactivated by suc-

cessive mutations. Null mutants continued to secrete

active Sap2; however, the enzyme was abnormally proc-

cessed and secreted at reduced levels. Unexpectedly, null

mutants were incapable of forming hyphae, instead di-

fferentiating into aberrantly shaped cells. The ability to

normally process Sap2 and form hyphae was restored

upon transformation of null mutants with a KEX2-con-

taining plasmid.

Candida albicans is an asexual, diploid yeast that normally

colonizes the lumen of the alimentary tract and/or vagina of

humans. Although usually commensal, it is increasingly impor-

tant as an opportunistic pathogen, becoming invasive and caus-

ing significant morbidity in individuals who are immuno-

compromised (1). The secretion of aspartyl proteinases (Saps)

encoded by at least seven genes in Candida albicans, is consid-

ered to be an important virulence attribute in the opportunistic setting

(2–4), along with a high rate of phenotypic switching (5), an

enhanced ability to adhere to relevant surfaces (6, 7), and

greater tolerance to anti-fungal drugs (8).

The Candida albicans Saps have been implicated in tissue inva-

sion, adhesion, and interference with specific and nonspecific

host defense systems (9–12). They resemble pepsin in struc-

ture, have broad substrate specificity, and are reproducibly

induced by growth in media where protein is the sole nitrogen

source (13, 14). SAP gene expression is correlated with the

organism’s yeast to hyphal transition as well as phenotypic

switching. SAPs 1, 2, and 3 are expressed only in yeast cells,

whereas SAP4–6 expression is confined to hyphae (12, 15);

SAP1 and SAP3 expression is correlated with only one switch

phenotype (12, 15). In the case of SAPs 4, 5, and 6, only mRNA

synthesis, and not Sap protein synthesis has been demons-

trated in hyphae; Sap7 expression has never been detected in

the laboratory at either the mRNA or protein level.

Based on a comparison of the gene and mature protein se-

quences, it appears that all Sap enzymes are processed from a

larger prepropeptide. The predicted amino-terminal portion of

each Sap encodes a secretory hydrophobic leader sequence fol-

lowed by a proregion, which probably maintains the protein in

an inactive state. All of the Saps contain one or more consensus

propeptide processing sites ending in the dipeptide Lys-Arg

(16). These sites have been directly shown to be involved in the

cissson of Saps 1, 2, and 3 (17, 18). Cleavage of proproteins at

the carboxyl-terminal side of Lys-Arg residues is characteristic

of a family of related, subtilisin-type serine proteinases re-

ferred to as proprotein convertases (19). The prototype of these

processing enzymes is the Kex2 proteinase of Saccharomyces

cerevisiae, an enzyme involved in the activation of a secreted

pheromone (α-mating factor) and a virally encoded killer toxin

(20). A number of metazoan secretory products, including prohor-

mones, serum proteins, and neuropeptides are converted into a

biologically active form by related proprotein convertases.

Assessment of the role of aspartyl proteinases in the viru-

lence Candida albicans is complicated by the multiplicity of genes

that encode the proteinases and the complexity and incongru-

ence of their expression patterns in vitro and in vivo. Since

these aspartyl proteinases are likely to be processed by a pro-

protein convertase-like activity, we sought to identify and ge-

netically characterize the Candida albicans KEX2-like proteinase.

Given the existence of other secreted and cell surface proteins in

Candida albicans that contain similar Lys-Arg dipeptide propro-

tein cleavage sites (21, 22), it was possible that genetic ablation of

KEX2 activity might have pleiotropic effects on Candida albicans.

EXPERIMENTAL PROCEDURES

Media, Yeast Strains, and Plasmids—Strains of Saccharomyces and

Candida used are listed in Table I. The yeasts were grown in standard

media used for genetic studies of S. cerevisiae (27, 28). Candida albicans

proteinase secretion was induced as described (29); organisms were

grown at 25 °C to prevent the formation of hyphae. Plate assays for

proteinase production incorporated 1% agarose in the standard media

and were enhanced by staining with 0.1% Amido Black in acetic acid/metha-

nol/H2O (10:25:50), followed by destaining in acetic acid/metha-

nol/water. For hyphal induction, cells were grown for 3 days in YEPD,

washed in water, then incubated at 37 °C for 6 h in either RMPI 1640

or YEPD containing 5% fetal calf serum (Life Technologies, Inc.).

The S. cerevisiae KEX2 gene cloned into the plasmid vector YCP50
Kex2 Proteinase of C. albicans

| Strain  | Genotype/Comment |
|---------|------------------|
| S. cerevisiae |
| XH16–1A  | MATaαhis3::LEU2-3,112trp1-389 ura3-52/KIL-K |
| XH16–1C  | MATaαhis3::LEU2-3,112trp1-389 ura3-52/KIL-K |
| RC634   | MATααhis3::LEU2-3,112trp1-389 ura3-52/KIL-K |
| DC17    | MATααhis3::KIL-O |
| BFY106–4C | MATααcan1–100ade2–1, his3-11,15leu2-3,112trp1-1 ura3-1 kex2 |
|         | Δ::::HIS3 |
| BFY106–4D | MATααcan1–100ade2–1, his3-11,15leu2-3,112trp1-1 ura3-1 kex2 |
|         | Δ::::HIS3 |
| C. albicans |
| WO-1    | High frequency switching strain |
| SC5214  | Isolate from blood |
| CA4     | ura3::imm434/Duraα::imm434 |
| CNA1    | ura3::imm434/Δuraα::imm434/KEX2::hisGURA3/Δuraα::imm434 |
| CNA2    | ura3::imm434/Δuraα::imm434/KEX2::hisG |
| CNA3    | ura3::imm434/Δuraα::imm434/KEX2::hisG |
| CNA4    | ura3::imm434/Δuraα::imm434/KEX2::hisG |

(30) was provided by J. Thorner (University of California, Berkeley). YCp19 (31) used to construct a C. albicans strain WO-1 genomic library was obtained from the American Type Culture Collection. The cloned C. albicans KEX22 gene (Fig. 1) was used to (a) generate deletion mutants that no longer complemented the kex2 defect in S. cerevisiae, (b) provide an episomal vehicle for introducing the intact gene back into C. albicans, and (c) prepare a construct for deleting, by homologous recombination, part of the gene in C. albicans. To construct functional inactive deletion mutants of the C. albicans KEX22, a 5-kb BamHI/SalI fragment was subcloned into pBluescript; the resulting plasmid, pCK1, was then cut with HindIII to remove an internal 350-bp fragment, and ligated to itself to create pCK2. After digestion of pCK2 with BamHI and SalI, its insert was subcloned in similarily cut YCp19. For the purposes of introducing a new copy of KEX2 into C. albicans, pCK1 was linearized with SalI and ligated to a SalI fragment of plasmid pRM10 (32) to form pCK3.

Nucleic Acid Manipulations—Standard procedures were used for cloning and subcloning of DNA fragments (28). Restriction/modification enzymes were purchased from New England Biolabs and used according to the manufacturer's recommendations. Deoxyribonucleotide stocks were purchased from Boehringer Mannheim. Recombinant plasmids were maintained in E. coli XL1 Blue (Stratagene). DNA sequence analysis of C. albicans KEX22 was determined by automated sequencing by the UCSF Biomedical Resource Center. Sequence comparisons against a non-redundant protein data base were performed by access to NCBI using the BLAST method (33).

Cloning of the C. albicans KEX2 Gene by Complementation—The secretion of biologically active a-factor was used as a positive screen for complementation of the S. cerevisiae kex2 mutation (20). Sau3A partial digests (6–20 kb) of WO strain C. albicans DNA were subcloned into the BamHI/SalI sites of YCp19 (31). The resulting library was used to transform S. cerevisiae strain BFY106–4D; after 2 days of growth in synthetic media lacking uracil, the colonies were replica-plated over a lawn of S. cerevisiae strain RC634 (a MATa strain sensitive to a-factor) grown on YEPG agar, 100 mM sodium citrate, pH 4.5, 0.03% methylene blue. Colonies producing a clear halo, due to pheromone-induced growth arrest, were re-streaked to yield individual colonies and tested again for the phenotype. Plasmids isolated from these colonies were used to transform E. coli XL1 Blue and reisolated, and individual plasmids were re-tested for their ability to restore a-factor secretion. As a secondary screen for complementation of the kex2 deficiency, the plasmids isolated above were assayed for their ability to restore killer factor secretion in kex2 KIL-K cells (20).

Production of C. albicans kex2 Strains—The two C. albicans KEX22 alleles were sequentially disrupted by the ura-blaster method (34), as adapted for use in Candida (26). A 2-kb fragment resulting from the digestion of pCK1 with XhoI and SpeI (Fig. 1) was subcloned into the same sites of this plasmid. This plasmid was then digested with HindIII to release a 350-bp fragment, treated with the Klenow fragment of E. coli DNA polymerase I in the presence of all four deoxyribonucleotides, then phosphorylated with calf intestinal alkaline phosphatase. The HindIII fragment was replaced by a similarly blunt-ended SalI/BglII fragment of pMB7, which contains the C. albicans URA3 gene flank by direct repeats of Salmonella hisG DNA (26), in the process eliminating all HindIII sites. The plasmid was then cut with SpeI and XhoI, and the insert (KEXD) was isolated by agarose gel electrophoresis and electroelution. C. albicans strain CA4 was grown in 100 ml of YEPD (adenine at 20 μg/ml) to a density of 1–3 × 10⁷ cells/ml and transformed with KEXD by electroporation (35). Digests of DNA from Ura− colonies were electrophoresed, and analyzed by Southern hybridization to assess the integrity of the KEX2 allele, an XhoI/SpeI fragment of the cloned KEX2 gene (Fig. 1) was randomly labeled with ³²P and used as the probe. Colonies having the desired disruption were then grown in the presence of 5-fluoroorotic acid, to select for cells where the hisG repeats recombined, deleting the URA3 gene in the process; these ura3 cells were then transformed again with the same KEXD casette.

SDS-Polyacrylamide Gel Electrophoresis Immunoblotting and Protein Sequencing—Culture supernatants were assayed for the presence of proteinase as described (15). For NH₂-terminal amino acid sequencing, supernatants were concentrated by ultrafiltration, electrophoresed, then blotted onto PVDF membranes. Amino-terminal sequencing was performed by the UCSF Biomedical Resource Center.

RESULTS

Complementation of a Factor and Killer Toxin Secretion in S. cerevisiae Mutants—Southern hybridizations of C. albicans genomic DNA with the cloned S. cerevisiae KEX2 gene failed to detect a C. albicans KEX2 homologue. Taking advantage of the role of Kex2 in S. cerevisiae in the production of active a-factor in haploid mating type α cells and in the production of a virally encoded killer toxin, we proceeded to isolate the C. albicans gene by complementation of function. Four of 10,000 transformants selected on the basis of ability to grow in the absence of uracil produced a slight halo over a lawn of sst1 cells (Fig. 2). Plasmids isolated from each of the four transformant clones contained a DNA insert of approximately 5 kb having the same restriction map. One of these plasmids (designated pCK4) was further characterized by restesting in S. cerevisiae BFY106–4D and confirmed to complement the a-factor processing defect. As a control for the specificity of the observed phenotype, the plasmid was introduced into mating type a cells. In this case, no halo was observed, indicating that there was no unspecific growth-arresting phenotype associated with the C. albicans DNA sequence (Fig. 2). When spotted on a lawn of S. cerevisiae sst1 cells, C. albicans did not produce a halo. When introduced into a kex2, mating type α killer strain of S. cerevisiae, pCK4 also restored its ability to lyse neighboring sensitive cells (Fig. 2). Again, C. albicans did not demonstrate the same phenotype (Fig. 2B).

Sequence Analysis—The 5-kb insert was subcloned into pBluescript, and sequence analysis from the BamHI site revealed an open reading frame, which, upon translation, yielded an amino acid sequence showing 70% identity over 37 residues with Ytp1p, a hypothetical transmembrane protein of S. cerevisiae. In S. cerevisiae, this gene is located between SIN4 (TSF3) and KEX2 (36). Sequencing from the 3' end of the XhoI site (Fig. 1), a second open reading frame was encountered that encodes a predicted amino acid sequence homologous to S.
Kex2 Proteinase of C. albicans

FIG. 1. Restriction map of the C. albicans KEX2 gene. Restriction sites used in making various constructs are indicated as: Bm (BamHI), Bg (BgIII), H (HindIII), Sa (SalI), Sp (SpcI), Xb (XbaI), and Xh (XhoI). A represents the relative positions of a portion of the coding region of C. albicans YTP1 gene and of the complete KEX2 gene (in white), with the bar representing noncoding regions. The gray bar represents part of the tetracyclin resistance gene from vector YCp19, beginning at the Sau3A cloning site (where the black are the gray bars meet). Below the map, indicated by arrowheads, are the relative positions of the active site aspartate (D), histidine (H), asparagine (N), and serine (S) residues. B represents the relative positions of the genes found in the ura-blaster cassette. The KEX2 disruption cassette used in the present study consisted of the XhoISpeI fragment of KEX2, with the internal 341-bp HindIII fragment being replaced by the ura-blaster cassette.

FIG. 2. Complementation of S. cerevisiae KEX2 deletion by C. albicans KEX2. A, secretion of biologically active α-factor. Individual colonies were spotted onto a lawn of RC634, a MATa strain that undergoes cell cycle arrest in the presence of α-factor. Top row depicts MATa kex2 S. cerevisiae strain BYF104-4D with no plasmid (a), with YCp19 (b), with YCP50 containing the S. cerevisiae KEX2 gene (c), and with YCP19 containing the C. albicans KEX2 gene (d). Middle row: BYF106-4D containing the C. albicans KEX2 gene lacking the internal 341-bp HindIII fragment encoding the region surrounding the active site serine (e), MATa kex2 S. cerevisiae strain BYF106-4C with no plasmid (f), BYF106-4C with YCp19 (g), and BYF106-4C with S. cerevisiae KEX2 cloned into YCP50 (h). Bottom row: strain BYF106-4C containing the C. albicans KEX2 gene cloned into YCP19 (i) and C. albicans strain SC5314 (j). B, killer toxin secretion. The cells were spotted over a lawn of the killer toxin-sensitive strain DC17. Top row: S. cerevisiae kex2 KIL-K strain XBH16-15A with no plasmid (a), with YCP19 (b), with S. cerevisiae KEX2 cloned into YCP50 (c), and with the C. albicans KEX2 cloned into YCP19 (d). Bottom row: XBH16-15A containing the C. albicans KEX2 gene lacking the 341-bp HindIII fragment and cloned into YCP19 (e), and C. albicans strain SC5314 (f).

cerevisiae KEX2, as well as related KEX2 sequences in Yarrowia lipolytica, Kluyveromyces lactis, and Schizosaccharomyces pombe, and to the proconvertases of metazoan species. The predicted translation product (924 amino acids) of the cloned C. albicans gene (Fig. 3) indicates that the encoded protein shares seven domains with its yeast counterparts: 1) an amino-terminal signal sequence terminating at Leu19, 2) an ensuing proregion with two Lys-Arg sites for probable autocatalytic cleavage, the latter ending at Arg129, 3) a catalytic domain characteristic of subtilisin-type serine proteases, with the active site Asp, His, Asn, and Ser residues in appropriate register, 4) a P domain that extends from Lys477 to Glu844, important in autocatalytic removal of the proregion (37), followed by 5) a region rich in serine and threonine residues containing a potential Lys-Arg cleavage site prior to Asp528, 6) a transmembrane domain extending from Tyr772 to Val792, and 7) at the carboxyl terminus, a highly charged cytoplasmic tail that contains the tetrapeptide YEFD, which in S. cerevisiae is important in ensuring retention in the Golgi (38). The predicted protein has five sites for potential N-linked glycosylation, these being located in the proregion, at the amino- and carboxyl-terminal ends of the catalytic domain, and in the Ser/Thr-rich region. ClustalW comparison (39) of the C. albicans and S. cerevisiae KEX2 products indicates that the difference in length between the two proteins (924 versus 814 amino acids, respectively) is due to an insertion immediately following the second predicted Lys-Arg cleavage site, a longer Ser/Thr-rich domain, and a longer cytoplasmic tail. The predicted extensions in the C. albicans protein are also noted in Y. lipolytica Xrp6 (976 amino acids), but not in Kex1 of K. lactis (700 amino acids) or in Krp1 of S. pombe (709 amino acids). Similar analyses indicated that differences in size between S. cerevisiae, K. lactis, and S. pombe proteins are largely due to additions/deletions in these same regions, with the serine/threonine-rich domain demonstrating the most variability. Comparison of the C. albicans KEX2 product with that of S. cerevisiae indicates that the most conserved domain is the catalytic one, where 65% identity and 74% similarity was observed.

Genetic Inactivation of C. albicans KEX2—In the deletion construct KEXD described under “Experimental Procedures,” the active site serine was deleted (Figs. 1 and 3). To assure that the deletion of the internal HindIII fragment of KEXD inactivated its function, S. cerevisiae strain BYF106-4D was transformed with pcK2, a plasmid that contains C. albicans KEX2 without the HindIII fragment. Plasmid pcK2 did not restore the ability of a cells to make α-factor, killer toxin, or, by inference, active Kex2 (Fig. 2).

Transformation of C. albicans CA14 with the KEXD cassette yielded 10 Ura+ colonies, each of which were analyzed by Southern hybridization (Fig. 4); all contained the construct inserted in the genome by homologous recombination at one of the two KEX2 alleles; 1 of the 10 colonies had multiple tandem insertions. A strain with a single copy of the desired insertion, designated CNA1, was treated with 5-fluoroorotic acid (5-FOA) to select against the Ura+ phenotype. As a result, strain CNA1 was transformed, representing the knockout of a single allele of KEX2 (Fig. 4). In the process, a single copy of hisG was left behind at the deleted locus as confirmed by Southern hybridization (Fig. 4). The CNA2 strain, containing a single disrupted allele of KEX2 was then transformed with the same KEXD cassette. Approximately 200 Ura+ colonies were obtained in the second round of transformation. Roughly 1/20 of the transformants examined by Southern analysis contained two disrupted kex2 alleles, one with a single copy of hisG and the other with the hisG-URA3-hisG cassette (Fig. 4). One of these double knockout strains was designed CNA3 and was again treated with 5-FOA to generate the final null mutation strain CNA4, which is both kex2 and ura3 (Fig. 4).

Effects of kex2 Mutation on Proteinase Secretion—Although
the regulation of the SAP gene family has been widely studied in WO and 3153 cells, little is known about its regulation in other strains including the parent strain SC5314 or its Ura1 derivative, CAI4, commonly used for mutational analysis in C. albicans (26). When grown in proteinase-inducing liquid media under conditions that do not simultaneously stimulate hyphal development, C. albicans strains SC5314 and CAI4 both produced a single Sap isoenzyme, which is equivalent to Sap2 based on electrophoretic mobility (Fig. 5) and Northern analyses (data not shown). In other C. albicans strains, the expression of Saps 4–6 is inferred from the appearance of their respective mRNAs in hyphae grown under inducing conditions (12, 15); however, Sap 4, 5, and 6 polypeptides have never been specifically detected in any C. albicans strain. The antibody

Fig. 3. Sequence analysis of the C. albicans KEX2 gene. The predicted translational product of the cloned gene is shown with features discussed in the text being highlighted. The active site residues (DHNS) are denoted by asterisks (*), possible dibasic processing sites are indicated by an underline, potential sites for N-linked glucosylation are denoted by a delta (Δ), the P region is indicated by .., and the transmembrane domain is indicated by carets. The serine threonine stretch lies between P region and the transmembrane domain, and the cytosolic tail, containing a probable TGN retention sequence ("'"), follows the transmembrane domain. The sequence depicted extends from the XbaI to the Sau3A sites indicated in Fig. 1.

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used in the present study cross-reacts with Saps 1–3, but it is not known whether it will react with other Saps; however, Coomassie-stained gels yielded a single band. We thus confined our analyses of C. albicans Sap secretion to Sap production in yeast forms of the organism. When seeded in proteinase-inducing medium at a similar density, the parent strains SC5314 and CAI4 and the knockout strains CNA1 and CNA3 grew at different rates, with CNA3 (kex2/kex2) growing the slowest.

Analysis of secreted Saps in the culture medium by SDS-PAGE and Western blotting revealed that at 24 h the wild type and heterozygous CNA1 strains produce a single Sap, Sap2, whereas homozygous kex2 cells produce two antibody-reactive species, one with a faster electrophoretic mobility and another with a slower one than Sap2 (Fig. 5). When the kex2/kex2 strain CNA4 was transformed with a plasmid containing C. albicans KEX2 (pCK3), the antibody-reactive doublet was replaced by a single protein having the same electrophoretic mobility as Sap2; this effect was a result of the failure of the homozygous kex2 strain to give rise to hyphae, although they retained the ability to form pseudohyphae and invade the agar substratum (Fig. 6). However, after 1 week of growth on solid medium, the zones of clearing surrounding kex2/kex2 cells approximated that of wild type cells. This pattern is consistent with the interpretation that the wild type cells reach stationary phase more rapidly than the double knockout strain.

Morphogenesis of C. albicans kex2 Cells—When left on albumin-containing media for over 10 days, the edge of the colonies of kex2/kex2 cells was smoother in comparison to that of wild type cells. Microscopic examination revealed that the difference was a result of the failure of the homoygous kex2 strain to give rise to hyphae, although they retained the ability to form pseudohyphae and invade the agar substratum (Fig. 6). However, after 1 week of growth on solid medium, the zones of clearing surrounding kex2/kex2 cells approximated that of wild type cells. This pattern is consistent with the interpretation that the wild type cells reach stationary phase more rapidly than the double knockout strain.

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forms within 6 h (Fig. 7). Homozygous kex2/kex2 cells failed to produce hyphae under the same conditions, even after 24 h of induction; instead, the cells were enlarged, aberrantly shaped, often had multiple buds, tended to aggregate, and often had multiple nuclei. When grown in minimal media or in RPMI 1640 at 25 °C, all of the strains grew as yeast cells. Staining kex2/kex2 cells grown under hyphae-inducing conditions with calcofluor white indicated that chitin deposition, normally most intense at the septae, whereas in strain CNA3 the staining is more uneven, focal deposits being sometimes found at regions between cells, at bud scars, and in cell extensions. Cells were prepared for microscopy by fixing with 4% formaldehyde in PBS (pH 7.4) for 4 h, followed by two washes with PBS; they were stored at 4 °C and photographed with a Nikon FM2 mounted on a Zeiss Axioplan fluorescent microscope.

**DISCUSSION**

Sequence analysis of the *C. albicans* gene described in the present study indicates that it encodes a type 1 transmembrane protein homologous to Kex2 of *S. cerevisiae* (41), Xpr6 of *Y. lipolytica* (42), Krp1 of *S. pombe* (43), and Kex1 of *K. lactis* (44), and to a subset of subtilisin-related proprotein convertases of higher eukaryotes (19). Based on its amino acid sequence, Kex2 would thus be in a position to interact with the *Candida* Saps, which based on biochemical, ultrastructural, and genetic criteria appear to transit through the yeast classical secretory pathway (45, 46).

Results of the present study indicate that Sap2 secretion by *C. albicans* strain SC5314 involves a Kex2-dependent processing event, but that the aspartyl proteinase can also be activated by an alternative pathway not appreciably expressed by wild type cells. Sap2 is normally processed after the dipeptide KR, but in the absence of Kex2 processing occurs after the R, yielding an amino terminus that is 1 amino acid longer; whether additional processing events occur at the carboxyl terminus remains possible, but atypical electrophoretic characteristics of fungal aspartyl proteinases has been noted in previous studies (18). The alternative processing of Sap2 in *kex2 C. albicans* may involve either (a) activation by another proteinase or (b) autocatalysis. With respect to the first possibility, it is conceivable that as Sap2 is secreted, it is cleaved by as yet to be established homologues of *S. cerevisiae* Mkc7 or Yap3, membrane-associated aspartyl proteinases that when overexpressed suppress some *kex2* phenotypes (47). However, these enzymes appear to have a similar specificity as Kex2 and would not be expected to yield the lower molecular weight Sap2 product noted in *kex2*/*kex2* cells. We are unaware of the existence of any other active proteinase that Sap2 would encounter during its transit from the endoplasmic reticulum to the outside of the cell. As for the second possibility, the observation that precursors present in the medium after 1 day in culture are no longer evident on day 4 is consistent with autocatalysis. Several secretory aspartyl proteinases of fungal origin have...
been noted to mature when placed under acidic conditions, self-activation apparently involving intra- and intermolecular interactions dependent on the presence of a lysine residue near the cleavage site (48). More closely related to the present study, a recombinant form of the proenzyme of a C. tropicalis Sap1, a homologue of C. albicans Saps, has been demonstrated to process itself when placed under acidic conditions (49). The C. tropicalis enzyme can also be autocatalytically activated when produced in S. cerevisiae under conditions where one of its Kex2 processing sites are mutated; however, in this instance the product is 4 amino acids longer than the expected mature form (46). Removal of the Lys-Arg sequence immediately preceding the mature form of Sap1 results in reduced secretion and possibly reduced activity of the enzyme, leading to an inability of C. tropicalis to grow in the presence of bovine serum albumin as a sole nitrogen source (46). Similarly, Y. lipolytica grown under hyphae-inducing conditions is unable to secrete an unprocessed and essentially inactive Kex2-dependent alkaline serine proteinase (42).

As is the case with C. tropicalis, we found that efficient secretion of Sap2 is Kex2-dependent in C. albicans. Unlike the homologue in C. tropicalis, however, the abnormally processed enzyme has activity sufficient to sustain viability of C. albicans grown under conditions where bovine serum albumin is the sole source of amino acids.

In addition to influencing Sap production, the double KEX2 mutation affected morphogenesis. When grown under conditions that induce hyphal development, strain CNA3 became clearly distinct in shape from both yeast and hyphal forms. The cells tended to be larger than yeast forms, often possessed multiple buds, and formed short extensions that were occasionally bent and appeared as thicker than normal germ tubes that remained attached after budding. In the second, the phenotype is temperature-dependent such that cells grown at 16 °C are considerably larger than the wild type and often contain multiple buds (47). Deformations in S. cerevisiae kex2 cells appear to be associated with abnormal patterns of chitin deposition, as demonstrated by calcofluor staining patterns. Our results with C. albicans are consistent with this finding.

The molecular basis of the atypical morphology of null kex2 C. albicans grown under hyphae-inducing conditions is unclear; however, possibilities include: 1) a defect in cell wall formation, 2) interference with a direct role of Saps in cell wall remodeling, or 3) an effect on cell polarity. To date, two C. albicans cell wall components have been identified that, based on DNA sequence analysis, would appear to require Kex2 processing: an exo-β-(1, 3)-glucanase (21) and Hwp1, a hyphal cell-wall-associated protein consisting of tandemly repeated proline- and glutamine-rich amino acid motifs (22). In S. cerevisiae, single or double disruptions of EXG1 and EXG9, which encode exoglucanases, are phenotypically neutral (50); however, this organism does not form true hyphae. It should be noted that the kex2 mutants developed in the present study were able to form pseudohyphae and to display invasive behavior on agar plates. Of possible relevance, deletion of a family of Kex2-processed hydrophobic cell wall proteins of inactivation of Ustilago maydis results in an inability to form aerial hyphae (51); C. albicans homologues have not been described, although several hydrophobic cell wall proteins have been identified at the biochemical level (52). In S. cerevisiae, cell wall components that appear to require Kex2-dependent processing have been described (53), but their function is currently unknown; the inability of kex2 S. cerevisiae to sporulate due to a defect at the late stages of meiosis has been tentatively ascribed to abnormal cell wall metabolism (54). As for the second possibility, secretory asparyl proteinases of aspergilli have been suggested to play a direct role in cell wall formation, perhaps by reorganizing the architecture of the growing hyphal tip (55). As for the last possibility, defects in kex2 S. cerevisiae grown at 16 °C are consistent with a defect in polarized growth, as inferred from de-localization of cortical actin patches in affected cells (47).

Results of the present study indicate that disruption of Kex2 function in C. albicans has pleiotropic effects that may impinge on the ability of the organism to colonize and invade tissues. Whether the lowered capacity of kex2 C. albicans to secrete asparyl proteinases and form true hyphae affects the ability of the organism to cause disease under opportunistic conditions remains to be demonstrated. If so, it may be possible to test whether peptide-based kexin inhibitors being developed for anti-viral purposes (56) have anti-fungal properties. This line of investigation may be facilitated by the observation that, unlike S. cerevisiae, C. albicans has a specific oligopeptide transport system that can accommodate pentamers and hexamers (57).

Acknowledgments—We thank Fang Feng for technical assistance and to Kuo for timely and constructive suggestions. We also extend our appreciation to D. Soll for provision of the WO-1 strain of C. albicans, W. Fonzi for strains C5314 and CAI4 and plasmid pMB7, C. Nombela for plasmid pRM10, and C. Inouye and J. Thorner for the S. cerevisiae strains and KEX2 plasmid.

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Kex2 Proteinase of C. albicans

28961