Deletion of the kexin gene (KEX2) in Candida albicans has a pleiotropic effect on phenotype and virulence due partly to a defect in the expression of two major virulence factors: the secretion of active aspartyl proteinases and the formation of hyphae. kex2/kex2 mutants are highly attenuated in a mouse systemic infection model and persist within cultured macrophages for at least 24 h without causing damage. Pathology is modest, with little disruption of kidney matrix. The infecting mutant cells are largely confined to glomeruli, and are aberrant in morphology. The complex phenotype of the deletion mutants reflects a role for kexin in a wide range of cellular processes. Taking advantage of the specificity of Kex2p cleavage, an algorithm we developed to scan the 9168 open reading frames in Assembly 6 of the C. albicans genome identified 147 potential substrates of Kex2p. These include all previously identified substrates, including eight secreted aspartyl proteinases, the exoglucanase Xog1p, the immunodominant antigen Mp65, and the adhesin Hwp1p. Other putative Kex2p substrates identified include several adhesins, cell wall proteins, and hydrolases previously not implicated in pathogenesis. Kexins also process fungal mating pheromones; a modification of the algorithm identified a putative mating pheromone with structural similarities to Saccharomyces cerevisiae α-factor.

Serine proteinases of the kexin/subtilisin superfamily activate precursor forms of many exported eukaryotic proteins by specific endoproteolytic cleavage adjacent and C-terminal to dibasic residues. Members of the superfamily play a crucial role in a large and varied set of biological processes in animals, plants, and fungi. In metazoan cells, kexins participate in both the constitutive and regulated secretory pathways, with substrates including hormones, serum proteins, neuroptides, cell surface receptors, components of the extracellular matrix (ECM), and proteinases, which modify the ECM. Furin, a member of the mammalian kexin family, processes several ECM metalloproteinases requisite for the dissemination of cancer cells (2). Kexins have also been shown to mediate the maturation of viral proteins and bacterial toxins (3). In fungi, kexins recycle the plasma membrane in the Golgi and the pre-vacuolar compartment where they process proteins involved in maintenance and remodeling of the cell wall (4, 5), proteins associated with the formation of aerial hyphae (6), α-type mating prepropheromones (7), killer toxins (7), zymogens of secreted proteinases (8–10), lipases (11), poly(saccharide-degrading enzymes (12, 13), and themselves (14). Deleting the kexin of the yeast Yarrowia lipolytica abolishes the formation of hyphae (15). kex2-null mutants of the yeast Saccharomyces cerevisiae are viable but exhibit conditional morphological abnormalities (16), defectve vacuolar proton-translocating V-ATPase activity (17), cold-sensitive growth (16), and a partial defect in meiosis (genome-www.stanford.edu/Saccharomyces). Genetic data indicate that Kex2p activity somehow may influence the RNA polymerase II complex (18).

The Kex2p protein of Candida albicans, an opportunistic fungal pathogen, participates in pathways that lead to the expression of two of the organism’s best characterized virulence factors: hyphal formation and the secretion of proteinases. Under hyphal-inducing conditions, kex2/kex2 cells are larger than KEX2/KEX2 yeast, often have multiple buds and nuclei, and form short, thick, stubby protrusions instead of normal hyphae (8). C. albicans kex2/kex2 mutants aberrantly process a secreted aspartyl proteinase (SAP), leading to diminished secretion of the enzyme (8). In this study, we show that kex2/kex2 C. albicans strains are markedly attenuated in a mouse model of systemic infection. In systemic candidiasis, where the primary site of pathogenesis is the kidney, the mutants are significantly less able to invade the kidney matrix than the wild type. Furthermore, the null strains do not produce hyphae either when ingested by macrophages, when grown embedded in agar, or when disseminated in mice.

Because the pleiotropic nature of the null kex2/kex2 phenotype cannot be entirely explained on the basis of known Kex2p substrates, we took advantage of the complete sequencing of the C. albicans genome (www.sequence.stanford.edu/group/candida/) to develop a purely informatic approach to identify and characterize potential Kex2p preproprotein substrates. We identified 147 ORFs whose products are potentially cleaved by Kex2p. Among these, a number of ORFs encode hydrolases, adhesins, cell wall components, and outer membrane proteins.

In aggregate, the list of candidate Kex2p substrates represents a range of secreted and cell surface-associated activities that...
are important at the host-fungal interface, some of which are likely to play key roles in pathogenesis. Additionally, one of the putative substrates has structural motifs characteristic of S. cerevisiae α-factor and other fungal pheromones that require processing by kexin for activation. The identification of a potential substrate of Kex2p, which is structurally similar to α-factor, is consistent with recent studies that show that α-cell derivatives of the kex2/hex2 strain are unable to mate with α-cells, while kex2/hex2 α-cells mate at wild-type efficiency with α-cells.2

EXPERIMENTAL PROCEDURES

C. albicans Strains and Growth Conditions—Strains used in this study and their relevant phenotypes are: 1) SC5314, a wild-type blood isolate, 2) CNA1, Δura3::mmr334/Δura3::mmr334 KEX2/hex2::hisG-Ura3-HisG, and 3) CNA2, Δura3::mmr334/Δura3::mmr334 kex2::hisG-URA3-hisG. Strains CNA1 and CNA3 were derived from CNA4, a ura3::his derivative of SC5314. Strains CNA3–1 and CNA3–2 are isogenic, originating from different transformations (8). Yeast forms were routinely maintained on YEPD plates (2% bactopeptone, 1% yeast extract, 2% glucose, 2% agar) at 30 °C. Hyphae were induced in serum or Lee’s media following standard procedures. To embed cells in agar, strains were grown for 48 h in YEPD at 30 °C and washed twice with distilled water; ~100 colony-forming units (CFU) were mixed with 20 ml of YPS-agar (1% yeast extract, 2% bactopeptone, 2% sucrose, 1% agar), plated in 90-mm petri dishes (19), and incubated at 25 °C. To enhance visualization of colony morphology, plates were stained with 0.1% Amido Black in acetic acid/methanol/water (10:25:65) and destained with acetic acid/methanol/water (10:20:70).

Macrophage Strains and Procedures—Murine macrophage cell line P388D1 was maintained in Ham’s nutrient mixture F12 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, GlutaMAX (Invitrogen), and 10% fetal calf serum. Macrophages were grown to confluence in 100 mm2 plastic tissue culture dishes (~1.2 × 106 cells). C. albicans were grown for 3 days in YEPD, washed twice with distilled water, suspended in Ham’s F12 without serum, counted, and combined with macrophages at a multiplicity of infection (moi) of 5.

Mouse Strains and Procedures—Host neutrophil function is the most important determinant in the outcome of human systemic infections (20), and so the virulence of C. albicans was measured in DBA/2N mice, which are deficient in complement component 5 and impaired in their ability to recruit neutrophils to foci of fungal growth (21). The DBA/2N mouse model therefore highlights damage due to direct effects of the pathogen and host cell interaction. The DBA/2N mice are highly sensitive to fungal infection; the infective dose (ID50) of C. albicans SC5314 in this strain is lower than that typically observed in other strains of mice (Table I and Ref. 21).

C. albicans was cultured at 35 °C for 24 h on Sabouraud dextrose agar (SDA) plates (22). Yeast were scraped from the surfaces of 1 or 2 plates, suspended in sterile physiological saline, and enumerated both with a hemocytometer and serial dilution and plating on SDA. The virulence of each strain was determined in female DBA/2N mice (Taconic Farms, Germantown, NY) inoculated i.v. into the lateral tail vein with 0.2 ml of a 10-fold titrated suspension of yeast at doses of 106–107 CFU per mouse (5 mice were injected at each dose). The ID50 values were estimated at 4, 7, 14, and 21 days after challenge by the method of Knudsen and Curtis (23). Data are presented as the number of yeast in the initial inoculum that are lethal to half of the animals on the indicated date. Animals were treated in accordance with the highest standards for the humane handling, care, and treatment of research animals using protocols approved by the Merck Institute Institutional Animal Care and Use Committee. Procedures for the care and use of research animals at Merck meet or exceed all applicable local, national, and international laws and regulations (22).

Histopathology—Kidneys were removed from a separate group of DBA/2N mice 2 days after infection with 106 wild-type or kex2hex2 (CNA3) organisms and placed into phosphate-buffered saline containing 10% formalin in order to investigate the histopathology of infection. A high dose of organisms was necessary to ensure that infected mice did not completely clear strain CNA3. The tissues were dehydrated, embedded in paraffin, and sectioned by standard methods. Sections were stained with hematoxylin and periodic acid-Schiff, then photographed using a Nikon FM2 mounted on a Zeiss Axiosplan fluorescent microscope. Animals used for this part of the study were maintained under conditions suggested by a veterinarian and approved by the UCSF Committee on Animal Research.

In Silico Studies—Assembly 6 of the C. albicans genome was constructed by the Stanford Genome Technology Center (SGTC) and the translated ORFs (tORFs, >100 amino acids) are available (www. sequence.stanford.edu/group/candida/). Self-Blat of Assembly 6 ORFs, to detect alleles and gene families, was performed by S. Scherer and T. Jones at SGTC. PSORT II (24), source code courtesy of Keita Nakai, was used to assign PSORT discriminant scores to the tORFs. The PSG score is calculated from both the hydrophobicity of the central part of a putative signal sequence and the net charge of its N terminus (psort.nibib.ac.jp). Those tORFs with a PSG score of >4 were considered to be proteins with a leader sequence, but not necessarily with a signal peptide cleavage site. Scripts to generate a list of Kex2p substrates based on PSG value and Kex2p-cleavage site motif, as well as to detect proteolytic cleavage patterns characteristic of pheromones, were written in Perl 5.2 and are available upon request. WU-Blast 2.0 (blast.wustl.edu) was used to compare the list to GenPept release 127.0 (www.ncbi.nlm.nih.gov/genbank/), and HMMPer 2.2 (hmmer.wustl.edu/) was used to compare the list to Pfam release 7.0 (pfam.wustl.edu/). Both Blast/GenPept and HMMPer/Pfam analyses were performed locally on a Linux 2.2.17 desktop workstation. The resulting output was parsed with simple in-house Perl scripts, uploaded into a local relational data base, and annotated manually.

RESULTS

kex2 Mutants Are Highly Attenuated in Systemic Murine Infections—The inability of C. albicans kex2/hex2-null mutants either to process SAPs or to produce hyphae suggested that these strains might be severely impaired in pathogenesis and virulence. Therefore we measured the relative virulence of the KEX2/KEX2 strain SC5314, the heterozygous KEX2/hex2 strain CNA1, and the isogenic kex2/hex2 strains CNA3-1 and CNA3-2 in DBA/2N mice, which are defective in neutrophil responses to fungal infection (21). The DBA/2N mouse strain is routinely used at Merck for measuring C. albicans virulence, the ID50 is the number of yeast cells in the inoculum required to achieve 50% lethality in test animals at 4, 7, 14, and 21 days post-infection. The results of such an experiment using the wild-type and kex2 mutant strains are summarized in Table I. Inactivation of either one or both copies of KEX2 markedly attenuates C. albicans in a gene dosage-dependent manner. Inocula of ~100 times more CFU of the kex2/hex2 deletion strains, as compared with the wild type, are required to achieve 50% lethality in mice 4 and 7 days post-infection. At 14–21 days post-infection, 100 times more mutant cells are required to achieve lethality equivalent to the wild type. The heterozygous strain exhibits an intermediate level of pathogenicity, requiring 15 and 35 times more yeast in the inoculum than SC5314 did to achieve an ID50 4 and 21 days post-infection, respectively.

kex2 Mutants Invade Kidneys Less Efficiently and Cause Less Damage than Wild-type Cells—The most common cause of
C. albicans Kex2 Mutant Phenotypes

**Fig. 1.** Foci of SC5314 (wild-type) and CNA3 (kex2/kex2) C. albicans in the kidneys of infected mice 2-day post-infection. Hematoxylin and eosin-stained sections were further enhanced with periodic acid-Schiff to highlight the morphology of the fungi. A, micrograph depicting the broad lesions caused by SC5314, where host tissue is visibly disrupted and the infecting fungi proliferate as hyphae; B, contrasting view of foci of CNA3 cells, where cells are often found in the glomeruli (G) and colonies contain fewer cells; C, similar view of uncolonized kidney and glomeruli. Higher magnification of fungal lesions, highlighting the difference of morphology between SC5314 (D) and CNA3 (E). Arrows indicate fungal cells.

Death in experimental murine disseminated candidiasis is acute fungal pyelonephritis due to fungal proliferation and associated damage (21). Thus the attenuation of kex2/kex2 mutants may result either because the mutant is more susceptible to clearance from the bloodstream before reaching the kidneys, or because it has difficulty crossing the endothelium, or because it is unable to invade the kidney matrix. To explore these possibilities we infected mice with either wild-type (SC5314) or kex2/kex2 (CNA3) C. albicans and inspected the kidneys 2 days post-infection. The histopathology of kidneys removed from mice infected with SC5314 differs markedly from kidneys isolated from mice infected with CNA3 (Fig. 1). Kidneys of SC5314-infected mice contain multiple foci of proliferating hyphae associated with a general disruption of the ECM and displacement of host cells (Fig. 1, A and C). Some of the CNA3 cells concentrate in foci located in interstitial spaces; these foci are much smaller than those of wild-type cells, often containing fewer than 10 organisms per nidus (as opposed to hundreds in wild-type infections), and are associated with little or no morphological damage to the surrounding matrix (Fig. 1B). Overall, we observed far fewer organisms in the CNA3-colonized kidneys than those in SC5314 infections. CNA3 cells in the kidney fail to form hyphae (Fig. 1D), although some protuberances are thicker and longer than those of CNA3 observed under any in vitro condition except when embedded in agar (see below). Unlike SC5314, which forms a broad zone of invasion throughout the kidney, many of the CNA3 cells remain within the glomeruli and associated blood vessels, indicating that the mutant is less able to invade the renal parenchyma than the KEX2/KEX2 strain. Host inflammatory cells are absent in areas surrounding CNA3 yeast, suggesting that the attenuation is not due to cell-mediated killing of the fungi.

**Fig. 2.** C. albicans interactions with cultured P388D1 cells. A, SC5314 4-h post-infection. B, CNA3 4-h post-infection. C, CNA3 24-h post-infection. Macrophage cultures co-incubated for 24 h with SC5314 were almost entirely cleared, with C. albicans growing as masses of hyphae. Arrows indicate fungal cells.

**KEX2 Is Required for Lysis of Macrophages**—Low numbers of kex2/kex2 cells in kidneys compared with wild type can be explained by slower growth or by enhanced clearance by phagocytic cells. kex2/kex2 yeast grow only slightly more slowly in various media than the parental strain, and reach a similar density by stationary phase (data not shown). We assessed whether the null mutant CNA3 is more susceptible to clearance by phagocytic cells in vitro. Wild-type C. albicans strain SC5314 is readily phagocytized by macrophage cell line 388D1, as reported for macrophage cell line IC-21 (25) and adherent mouse peritoneal macrophages (26). Ingested yeast form hyphae within 30 min, and by 4 h the hyphae traverse the length of the macrophage, often extending along the lengths of macrophage processes (Fig. 2A). The macrophages lyse within 4–6 h, releasing C. albicans hyphae, which bud new yeast that are subsequently phagocytized by other macrophages. We did not observe any phagocytosis of hyphae. Within 24 h of exposure to SC5314, the fungi almost completely destroy the macrophage lawn, as evidenced by clearing of the normally opaque macrophage monolayer after removal of medium (data not shown). CNA3 is also phagocytized, but forms aberrant cells with short stumpy projections within the macrophages that are similar to CNA3 cells incubated in hyphal-inducing liquid media (Fig. 2B); no hyphae are produced in infected macrophages. After 24 h, small patches of clearing are seen in the CNA3-inoculated monolayers; however, the lawn of macrophages remains essentially intact (Fig. 2C).

**Morphogenesis of Hyphal Forms Requires KEX2**—The inability of kex2/kex2 mutants to invade the kidney and kill macrophages can be partially explained by our previous finding that mutants are defective in both proteinase secretion and hyphal formation (8). In that study we established that kex2/kex2 mutants are unable to form hyphae in liquid or on solid media (8). However, subsequent reports have appeared indicating that some putatively hyphae-deficient mutants are in fact able to form hyphae when embedded in solid media (19, 25) and in vivo (27). We therefore asked whether CNA3 was able to form
hyphae when embedded in agar. Within 48 h, SC5314 colonies embedded in solid medium produce filaments consisting largely of hyphae, while CNA3 colonies develop only slightly corrugated surfaces. After 6 days of incubation, SC5314 grows as stellate colonies with hyphal projections extending more than twice the diameter of the main body of the colony, while CNA3 grows as colonies with irregular peripheries that barely extend beyond the colony mass (Fig. 3, A and B). Microscopically, the periphery of each CNA3 colony consists of yeast, pseudohyphae, and aberrant forms similar to those produced by CNA3 when grown in hyphal-inducing liquid media. The colonies also contain more elongated aberrantly shaped cells not found in liquid media but similar to those forms observed in kidney (Fig. 3, C). Approximately 1% of the embedded CNA3 colonies throw out sectors that are clearly more filamentous than other areas. Cells within the filamentous sectors are predominantly pseudohyphae, with no true hyphae (Fig. 3E). These sectors may be products of phenotypic switching, which, in this strain, occurs at a frequency of 1% and manifests as differences in the abundance of pseudohyphae (28). Alternatively, the sectors may be products of partial suppression of the kex2" phenotype.

Search for Cleavage Substrates of Kex2p—It is unlikely that the absence of Kex2p per se is responsible for the range of phenotypic defects described above. A more likely explanation is that Kex2p is required for normal processing and activation of a number of extracellular proteins that individually or collectively are involved in defining hyphal morphogenesis, pathogenesis, and virulence. By "extracellular" we refer to proteins that act wholly or partially outside of the plasma membrane, including but not limited to cell surface, cell wall, and secreted proteins. The complete sequencing of the C. albicans genome, coupled with the highly stereotyped cleavage recognition motif of ScKex2p in S. cerevisiae, suggested that we might be able to computationally identify substrates of Kex2p in C. albicans. To survey the entire C. albicans genome for potential Kex2p substrates, we wrote a computer script which extracts all of the ORFs from Assembly 6 that encode a protein which: 1) is >100 amino acids in length, 2) possesses a leader peptide, but not necessarily a signal peptidease cleavage site, as determined by a modified McGeoh's method (24), and 3) contains a propeptide < 150 amino acids long, as defined by the motif P4-P3-Lys-Arg, where P3 cannot be amino acid but P4 cannot be CDFGPS or W. This last criterion is a weak consensus derived from inspection of known cleavage substrates of S. cerevisiae ScKex2p (29–31) and from experimental studies (32, 33). It is possible that C. albicans Kex2p recognizes a cleavage site motif somewhat different from that of ScKex2p; however, C. albicans KEX2 was isolated by complementation of a S. cerevisiae SCKEX2-null mutant, resulting in the processing of at least two known ScKex2p substrates (8). Based on these criteria, the script extracted 147 candidate Kex2p cleavage substrates from the 9168 predicted proteins of Assembly 6 of the C. albicans genomic sequence (www-sequence.stanford.edu/group/ candida/).

Each of the 147 candidate substrates was analyzed by Blast against Assembly 6, followed by manual inspection of each alignment. This assessment revealed that 60 candidate ORFs are alleles or fragments of one another. This reflects the diploid nature of C. albicans as well as the fragmented nature of Assembly 6, which consists of as many as 2519 unlinked contigs. Two gene families, with at least three members each, were identified at this step. One family encodes 9 SAPs, one of which has not been described in the literature (ORF6.1902), and the other encodes 3 inositol monophosphatases involved in methionine biosynthesis and halotolerance.

Correction for multiple alleles and allelic fragments left 130 haploid ORFs potentially encoding Kex2p substrates. Of these, 14 are cited genes whose protein products either are cleaved by Kex2p or contain leader sequences with a putative Kex2p cleavage site: SAP1–6, SAP8–9, XOG1, MP65, HWP1, PHR1-2, and KEX2. Each of the proteins encoded by these ORFs is homologous to a S. cerevisiae SAP. Notably, two previously described SAPs were not recovered by our script: Sap7p, which does not have a KR site, and Sap10p, which does not have a leader sequence.

The 130 haploid ORFs were ascribed functions by a procedure involving Blast of the candidate list against Genpept and HMmer of the list against Pfam, followed by manual examination of the output (see "Experimental Procedures"). 70 of the haploid ORFs have likely S. cerevisiae homologues, and 60 do not. Of the 70 with S. cerevisiae homologues, 58 (including the 14 cloned genes named above) have homologues with an as-

![Flowchart describing the extraction of potential Kex2p cleavage substrates from the C. albicans genome, and their classification](image-url)

FIG. 4. Flowchart describing the extraction of potential Kex2p cleavage substrates from the C. albicans genome, and their classification. See text for details of computational and informatic procedures.
and ScExg2p (Orf6.6664). The remaining 12 candidates are as ScCrh1p (Orf6.1231 of Assembly 6), ScSun4p (Orf6.2071), ing well characterized extracellular Database (genome-www.stanford.edu/Saccharomyces/), includ-
Saccharomyces fungi. The two acid sphingomyelinases identified as potential homologues of eukaryotic acid sphingomyelin phosphodiester-
ity (NUP; gi 3764057). The remaining two proteins are
play a role in virulence and pathogenesis. With the exception of
sphingomyelinases may similarly
sphingomyelinases are related to mammalian neutral sphingo-
myelinases (34); these bear little sequence similarity to the C. albicans enzymes.
One of the Candidate Cleavage Substrates of Kex2p Is Struc-
turally Related to Fungal Pheromones—C. albicans is classified as an imperfect yeast, although it possesses MATα- and MATa-
like mating-type loci (35). The genome also encodes many gene products consistent with a complete sexual cycle (36). C. albicans strains engineered to be homozygous for the a mating-type locus are able to mate with strains similarly engineered at the a mating-type locus (35, 37). Deletion of KEX2 abrogates the ability of a cells to mate with a cells, but not the ability of a cells to mate with a cells,2 suggesting that C. albicans a cells may secrete an a-specific mating pheromone processed by Kex2p. Although well-characterized peptide mating pheromones such as the o-factor of S. cerevisiae and the P-factor of S. pombe are cleaved by kexins (7, 38), our semiautomated annotation of the list of candidate Kex2p cleavage substrates did not describe a protein having a significant degree of sequence similarity to known fungal pheromones. This is not surprising, as fungal pheromones such as α-factor and P-factor do not bear much sequence similarity to one another (see Fig. 6). To ask whether C. albicans synthesizes peptide-mating pheromones, we elaborated our script to query the C. albicans genome for proteins that possess structural commonalities with α-factor and P-factor in addition to a leader sequence and a Kex2p cleavage motif. The algorithm computationally cleaved the can-

**TABLE II**

Selected C. albicans proteins computationally identified as potential cleavage substrates of Kex2p

| Assembly 6 ORF no. | S. cerevisiae homologue | C. albicans name | Description |
|-------------------|-------------------------|------------------|-------------|
| 708               | SCW10/YMR305c           | Mp56             | Mannoprotein; glucosidase; RGD binding site; dominant immunogen |
| 1982              | EXG1/YLR300w            | XOG1             | Exo-1,3-β-glucosidase |
| 2204              | YPS1/YLR120c            | SAP6             | Secreted aspartyl proteinase |
| 2688              | YPS1/YLR120c            | SAP8             | Secreted aspartyl proteinase |
| 3803              | YPS3/YLR121c            | SAP4             | Secreted aspartyl proteinase |
| 4427              | YPS1/YLR120c            | SAP5             | Secreted aspartyl proteinase |
| 4600              | KEX2/YNL238w            | KEX2             | Kexin/convertase |
| 4644              | YPS3/YLR121c            | SAP1             | Secreted aspartyl proteinase |
| 4853              | MUC1/YLR191c            | HWP1             | Hyphal wall protein; transglutaminase substrate |
| 5306              | YPS3/YLR121c            | SAP2             | Secreted aspartyl proteinase |
| 6260              | GAS1/YMR307w            | PHR2             | pH-regulated; GPI-anchored glycosidase |
| 7314              | YPS1/YLR120c            | SAP9             | Secreted aspartyl proteinase |
| 7524              | GAS1/YMR307w            | PHR1             | pH-regulated; GPI-anchored glycosidase |
| 7558              |                           | NUP              | Purine nucleoside permease |
| 9036              | YPS3/YLR121c            | SAP3             | Secreted aspartyl proteinase |

**Selected ORFs with S. cerevisiae homologues of known function**

| 311 | WSC2/YNL283c | Cell wall integrity; stress response |
| 362 | CIS3/YJL158c | Cell wall; protein with internal repeats |
| 1064 | ZRT3/YKL175w | Zinc ion transporter |
| 1231 | CRH1/YGR189c | Congo red-sensitive; glycosidase |
| 1688 | KRE2/YIL027c | Killer toxin-resistant |
| 2071 | SUN4/YNL066w | Cell septation |
| 2502 | MET2/YOL064c | Methionine metabolism; halotolerance; inositol monophosphatase |
| 3098 | ROT1/YMR208w | Activation of Rho1p and cell wall synthesis, and actin polarization |
| 3659 | HEM1/YER014w | Protoporphyrogen oxidase |
| 3863 | KTI2/YKL110c | Killer toxin resistance; associated with RNA PolII elongator |
| 4538 | FMN1/YDR236c | Riboflavin kinase |
| 6148 | LHS1/YKL073w | Hsp70 |
| 6664 | EXG2/YDR261c | Exo-1,3-glucosidase |
| 6972 | ECM39/YNR030w | Calcoflour-sensitive |

**Novel ORFs with ascribed function**

| 962 | YPL207w | Flavodoxin |
| 1752 | YMR313c | Patatin-family phospholipase |
| 1902 | YPS3/YLR121c | Secreted aspartyl proteinase |
| 3514 | | Sphingomyelin phosphodiesterase |
| 4306 | | Mating pheromone |
| 7581 | | Sphingomyelin phosphodiesterase |
| 8388 | YML125c | Oxidoreductase |
didate proteins at the Kex2p cleavage sites and compared the lengths of the resulting peptide fragments. Eighteen candidates were cleaved into at least one pair of equally sized fragments. Of these, a single protein (Orf6.4306) also possesses the X(A/P)X(A/P) motif characteristic of α-factor and P-factor (Fig. 6). This dipeptide motif is recognized and processed by the S. cerevisiae ScSte13p aminopeptidase, and a clear homologue of ScSte13p is present in the C. albicans genome (Orf6.4953). Also found in Assembly 6 are C. albicans homologues to other S. cerevisiae proteins related to α-factor synthesis and signaling, including ScKex1p (peptidase involved in α-factor maturation; Orf6.7946) and ScSte2p (receptor for α-factor; Orf6.4012) (36). The transcript of Orf6.4306 is specifically expressed in the opaque switch phenotype of the WO-1 strain of C. albicans (44).

Mating competent cells in strain SC5314 have an opaque-like phenotype, and demonstrate morphological behavior reminiscent of the pheromone-dependent "schmoos" of S. cerevisiae (39).

DISCUSSION

C. albicans proteinase secretion, hyphal development, egress from macrophages, and tissue invasion require kexin activity, allowing us to surmise that processing of proproteins by Kex2p is essential for expression of the full range of virulence traits described for this pathogen. Inactivation of the kexin results in a marked attenuation in C. albicans virulence; 1000 times more mutant yeast cells than wild-type cells are required to achieve an equivalent ID₅₀ at day 21 post-infection. Wild-type cells form hyphae, which cause large pockets of tissue damage throughout the kidney, while kex2/kex2 mutants are defective in hyphal morphogenesis and tend to be restricted to the glo- meruli. Kidneys of mutant-infected mice suffer less tissue destruction; foci are smaller and more discrete than those produced by wild type, and are populated by fewer fungal cells. These results indicate that kex2/kex2 cells are unable to effectively negotiate the pathway from the bloodstream into and through renal connective tissue. This defect might be attributable to more rapid clearance from the circulation by phagocytic cells, a reduced capacity to extravasate from the circulatory system, a decreased ability to migrate through tissue spaces, or some combination thereof. These possibilities are discussed below within the context of known and potential C. albicans kexin substrates identified in the present study.

The inability of C. albicans kex2/kex2 mutants to exit the phagolysosomes of macrophages likely accounts for their diminished virulence, as phagocytic clearance of the organism from the circulation is the dominant host defense mechanism preventing systemic candidiasis. Within minutes of intravenous injection, nonspecific phagocytosis in the reticuloendothelial system removes the majority of C. albicans yeast from the bloodstream (40). A fraction of ingested fungi may subsequently exit macrophages, probably through a combination of enzymatic and mechanical means, placing them in a position to invade deeper organs (25, 41). Two of the principal virulence factors of C. albicans affected by inactivation of Kex2p, hyphal formation and proteinase secretion (8), govern the efficiency of exit from phagocytic cells (25, 26). Hyphal morphogenesis in C. albicans is regulated by different environmental cues through at least four separate signal transduction pathways that converge to induce the hyphal form (42). kex2/kex2 cells are unable to form hyphae under all conditions tested; therefore the morphogenic defect of the mutant likely lies after the point where the pathways converge, perhaps because the yeast fails to properly assemble one or more components critical for the architecture of the hyphal wall. Consistent with this hypothesis, we have observed irregularities in chitin deposition in kex2/kex2 C. albicans (8). Kexin mutants of another fungal pathogen, Candida glabrata, are hypersensitive to Calcoflour, a dye that binds to chitin and is used to identify cell wall mutants (43). S. cerevisiae kex2 deletion mutants grown at low temperatures display defects in cell polarity coincident with delocalization of actin and chitin (16); these phenomena may reflect misprocessing of several cell wall proteins, as judged by altered gel electrophoretic and isoelectric focusing mobilities (44). A C. albicans exoglucanase likely to be involved in cell

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3 Lan, C.-Y., Newport, G., Murillo, L. A., Jones, T., Scherer, S., Davis, R. W., and Agabian, N. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 14907–14912.
wall remodeling, Xog1p, is probably processed by Kex2p (45); however, xog1/xog1 mutants do not have the grossly aberrant morphology of kex2/kex2 mutants (46). hwp1/hwp1 mutants, on the other hand, do form stunted hyphae in renal tissue (47). Other cell wall proteins identified in the present study as being candidate Kex2p substrates, and thus possibly essential for hyphal formation, include homologues of ScCw10p (Mp65), ScCrh1p, and ScSun4p; the last two are potential glycosidases.

Unreported factors that may also contribute to escape from the phagolysosome are the two putatively secreted spongomyelinases identified in the present study. Spongomyelinases of pathogenic bacteria, enzymatically similar but structurally unrelated to those of C. albicans, lyse phagosomal membranes (48) or act as hemolysins that facilitate the acquisition of iron from the host (49, 50). The C. albicans enzymes are structurally related to mammalian secretory acid sphingomyelinases, which have been implicated in inflammatory processes and possibly atherogenesis (51). In addition, one of the products of spongomyelinase catalysis, ceramide, is a eukaryotic signaling molecule that controls key aspects of the inflammatory reactions of macrophages, including apoptosis, differentiation, and cytokine secretion (52, 53). Ceramide alters a macrophage mitogen-activated protein kinase (MAPK) pathway, producing an environment that is favorable for intracellular growth of Leishmania donovani (54). Since phagocytosis by macrophages of C. albicans, but not S. cerevisiae, down-regulates macrophage MAPK activity (55), we hypothesize that C. albicans alters host cell ceramide signaling pathways. C. albicans in fact induces apoptosis in cells of monocyte lineage (41, 56), an expected outcome of ceramide overproduction and sphingomyelinase activity; however not all studies have confirmed this (57).

The normal route of extravasation of C. albicans that escape clearance by phagocytic cells is transcellular, involving receptor-mediated attachment to endothelial cell surfaces coincident with germ tube formation, entry into host cells via phagocytosis, and exit facilitated by puncture of the host cell membrane by the hyphal tip (58, 59). The informatic methods used in this study identified several potential substrates of Kex2p that may participate in C. albicans adhesion to host cells. Several of these proteins are rich in serine and threonine and, based on PSORT analyses, are predicted to localize to the cell wall, properties common to yeast agglutinins (60). Another group of kexin-processed substrates, the SAPs, play a role in adhesion to epithelial cells (61). Cell wall mannoprotein Mp65, which is experimentally processed at a consensus kexin cleavage site, probably contributes to adhesion as it has a complement-binding RGD site not found in its S. cerevisiae homologue ScCw10p (62). The hyphal wall protein Hwp1p, on the other hand, has been shown to be dispensable for attachment of the organism to endothelial cells (47).

kex2/kex2 mutants accumulate in kidney glomeruli and the immediately surrounding tissue, indicating that cells that do exit endothelial cells are compromised in their ability to invade solid tissue. The broad zones of tissue destruction and cellular displacement in the kidneys normally associated with wild-type C. albicans infections are absent in mice infected by the mutant, and are replaced by compact lesions. This likely reflects the inability of the mutants to form hyphae, and is consistent with the traditional view that the hypha is a principal effector of pathogenesis (63). The migration pathway of wild-type C. albicans into deeper tissue presents a leading edge of hyphae that mechanically dislodge desmosomes and enzymatically degrade ECM (64); kex2/kex2 mutants engage in none of these behaviors. The invasiveness of the mutant, while greatly reduced, is not entirely abolished, as a few yeast do enter the kidney parenchyma. These exceptions are consistent with the observation that translocation of the yeast form can be a minor component of C. albicans spread (65), and that invasion is to some degree effected by mechanisms independent of hyphae, such as the release of several hydrolases (66, 67).

In sum, the present study demonstrates that, while not essential for viability in vitro, kexin activity is required for full virulence of C. albicans. At minimum, the enzyme is involved in a variety of functions that manifest at the host-pathogen interface: proteinase secretion, hyphal development, escape from macrophages, and invasion of solid tissue. The invasion defect of kex2/kex2 mutants reminds us of tumor cells whose metastasis is attenuated by a specific inhibitor of furin, a homologue of Kex2p found in metazans (2), and reflects the role of this enzyme in mediating the maturation of proteins that interact with the environment. Our informatic method identified several secreted hydrolases, cell wall constituents, adhesins, and other proteins that are known to make important contributions to C. albicans pathogenesis, and discovered a larger set whose contribution remains to be assessed. In aggregate this list identifies a subset of potential kexin substrates, which ultimately function at the host-pathogen interface. Since homologues of Kex2p are likely to play significant roles in the biology of eukaryotic pathogens in general, and in light of the increasing number of genomes being sequenced, it should prove useful to apply this computational approach to search for similar proteins in other pathogens. Demonstrations that the polypeptide allergens of parasitic nematodes and the major adhesin of the pathogenic fungus Coccidioides immitis are processed by kexins (68, 69) support this conjecture. Some of the substrates of unknown function, which we identified by this procedure may mediate previously unsuspected interactions between parasitic organisms and their hosts, between individuals of a colonizing population, and between different species or populations of microbes.

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REFERENCES
1. Seidah, N. G., and Chretien, M. (1999) Brain Res. 848, 45–62
2. Bassi, D. E., Lopez De Cicco, R., Mahliogi, H., Zuccher, S., Thomas, G., and 1657
3. Molloy, S. S., Anderson, E. D., Jean, F., and Thomas, G. (1999) Trends Cell Biol. 9, 28–35
4. Bascu, D. R., Cueva, R., Andaluz, E., and Larriba, G. (1996) Biochim. Biophys. Acta 1310, 110–118
5. Brickner, J. H., and Fuller, R. S. (1997) J Cell Biol. 139, 23–36
6. Westen, H. A., Bohllmann, R., Eckerskorn, C., Lentoppech, F., Bolker, M., and Kahnmann, R. (1996) EMBO J. 15, 4274–4281
7. Julius, D., Brake, A., Blair, L., Kunisawa, R., and Thorner, J. (1984 Cell 37, 1073–1089
8. Newport, G., and Agabian, N. (1997) J Biol. Chem. 272, 28954–28961
9. Enderlin, C. S., and Ogrydziak, D. M. (1994) Yeast 10, 67–79
10. Togni, G., Sanglard, D., Quadroni, M., Fondling, S. I., and Monod, M. (1996) Microbiology 142, 493–503
11. Pignede, G., Wang, H., Fudalej, F., Gaillardin, C., Seman, M., and Nicaud, J. M. (2000) J Bacteriol. 182, 2802–2810
12.oller, S. P., Schusswohl, D., Baron, M., Parriche, M., and Kubicek, C. P. (1998) Appl. Environ. Microbiol. 64, 3202–3208
13. Iguchi, K., Hirano, H., Kishida, M., Kawasaki, H., and Sakai, T. (1997) Microbiology 143, 1657–64
14. Germain, D., Dumas, F., Vernet, T., Bourbonnais, Y., Thomas, D. Y., and Boileau, G. (1992) FEBS Lett. 299, 283–286
15. Richard, M., Quijano, R. B., Bazzate, S., Borden-Pallier, F., and Gaillardin, C. (2001) J Bacteriol. 183, 3088–3107
16. Komanu, H., and Fuller, R. S. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 10765–10768
17. Grant, S. H., Sackin, P. M. (1999) Appl. Environ. Microbiol. 65, 4274–4281
18. Lehrer, R. I., and Cline, M. J. (1971) Cancer 27, 1211–1217
19. Ashman, R. B. (1997) FEMS Microbiol. Lett. 165, 181–189
20. Abruzzo, G. K., Flattery, A. M., Gill, C. J., Kong, L., Smith, J. G., Krupa, D.,
