Université de Montréal

Role of mucinolytic activity of *Candida albicans* in the pathogenesis of mucosal and invasive candidiasis

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Thèse présentée à la Faculté des études supérieures
en vue de l'obtention du grade de
Philosophiae Doctor (Ph.D.)
en microbiologie et immunologie

Juillet, 1998

Université de Montréal
Faculté des études supérieures

Cette thèse intitulée:

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Thèse acceptée le 30 septembre 1998

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La colonisation et l'invasion des muqueuses par le champignon opportuniste *Candida albicans* implique des interactions dynamiques avec les tissus de l'hôte. L'analyse ultrastructurale de la paroi intestinale des souris inoculées par la voie orale-intragastrique avec le *C. albicans* a montré la présence d'une zone de digestion de la couche de mucine qui recouvrait les blastoconidies. Cette observation a soulevé l'hypothèse que des enzymes mucinolytiques pourraient assister le *C. albicans* dans les processus de colonisation, d'invasion et de dissémination systémique à partir du tractus gastrointestinal, en dégradant la barrière protectrice de mucus et en conséquence permettant aux levures d'adhérer et d'envahir les cellules épithéliales. Les objectifs de ce projet consistent à identifier et à caractériser l'activité mucinolytique de *C. albicans* et à explorer son rôle dans la pathogénèse de la candidose mucosale et systémique. Lorsque les blastoconidies de *C. albicans* ont été cultivées sur millieu contenant de la mucine intestinale de porc comme la seule source d'azote, une activité mucinolytique a été détectée dans les surnageants de culture, et cette activité a été inhibée par la pepstatine A. L'activité enzymatique fut partiellement purifiée par ultrafiltrations successives. L'analyse électrophorétique des filtrats de culture a révélé la présence de deux composants de 42- et 45-kDa, et pIs de 4.1 et 5.3, respectivement. Les études zymographiques faites avec la mucine biotinilée et immobilisée sur papier de nitrocellulose ont démontré que la bande de 42-kDa était responsable de l'activité mucinolytique détectée dans les surnageants de culture. De plus, cette protéine a été reconnue par la méthode de

l'immunoempreinte réalisée à l'aide d'anticorps monoclonaux dirigées contre la protéase aspartyle 2 (Sap 2). Par la suite, la séquence N-terminale des 20 premiers acides aminés de la bande de 42-kDa s'est avérée identique à celle rapportée pour la Sap2p, tandis que la séquence de la bande de 45-kDa était surtout composée de residus de glycine et n'avait pas d'homologie avec d'autres séquences connues. Le marquage métabolique à la [^{35}S]-méthionine suggèra que cette bande ne proviendrait pas de *C. albicans* mais qu'elle pourrait plutôt, être le résultat de l'activité de la Sap2p sur la mucine. Dans l'ensemble, ces résultats ont démontré que la Sap2p est impliquée dans la dégradation *in vitro* de la mucine et qu'elle pourrait assister le *C. albicans* dans le processus de pénétration *in vivo* de la muqueuse gastrointestinale. Par ailleurs, d'autres chercheurs ont montré que les souches de *C. albicans* isolées de patientes atteintes de vaginite sécrètent significativement plus de protéases *in vitro* que les souches commensales. En conséquence, nous avons produit des transformants qui permettent l'expression constitutive de la Sap2p chez le *Saccharomyces cerevisiae* et sa surexpression chez le *C. albicans*. Le cadre de lecture ouvert de la *SAP*2 et son peptide signal ont été inserés en aval du promoteur constitutif *ADH*1 de *S. cerevisiae* et de *C. albicans*. Les plasmides générés ont été utilisés pour transformer par recombinaison homologue des souches de *S. cerevisiae* et de *C. albicans ura*3. Les souches témoins négatives portaient soit le plasmide sans la *SAP*2 ou une version mutée de ce gène. La Sap2p mature a été efficacement sécrétée par les deux champignons tel que suggéré par la masse moléculaire de la protéase recombinante et la forte activité protéolytique détectée dans les surnageants de culture. La protéase a été sécrétée tant aux conditions

d'induction qu'aux conditions de répression, ce qui suggère que l'expression de la Sap2p est constitutive et non-régulée. Dans les modèles murins de candidose orale et disséminée, aucune des souches de *S. cerevisiae* ne se sont révélées virulentes. En plus, ni la souche parentale de *C. albicans* ni les transformants n'étaient virulents dans le modèle oral. Par contre, lorsque les transformants ont été inoculés par la voie intraveineuse, leur virulence a été réduite par rapport à la souche parentale. L'ensemble de ces résultats nous indique que la Sap2p n'est pas un facteur de virulence dominant chez le *C. albicans*, et que la virulence de *C. albicans* est le résultat de l'action concertée de plusieurs facteurs de virulence.

Mots clés: *Candida*, mucinase, pathogenèse, protéase aspartyle, facteurs de virulence.

SUMMARY

SUMMARY

Mucosal colonization and invasion by the pathogenic fungus *Candida albicans* requires dynamic interactions with host tissues. Ultrastructural analyses of the bowel wall of mice inoculated intragastrically with *C. albicans* showed a zone of putative extracellular digestion of the mucin layer surrounding blastoconidia. This observation prompted the hypothesis that mucin-degrading enzyme(s) may assist *C. albicans* in the process of colonization, invasion and systemic dissemination from the gastrointestinal tract, by degrading the protective mucus barrier and allowing the yeast to adhere to and invade the underlying epithelial cells. This project aimed to identify and characterize the mucinolytic activity of *C. albicans* and explore its role in the pathogenesis of mucosal and disseminated candidiasis. When *C. albicans* blastoconidia were grown in the presence of hog gastric mucin as the sole nitrogen source, mucinolytic activity was detected in culture supernatants and the activity was inhibited by pepstatin A. Partial purification of the enzyme was achieved by sequential ultrafiltration. Electrophoretic analyses of the culture filtrates revealed the presence of two components of 42- and 45- kDa, with pIs of 4.1 and 5.3, respectively. Zymographic studies, performed with biotin-labelled mucin immobilized on nitrocellulose filters, indicated that the 42-kDa band was responsible for the mucinase activity detected in culture supernatants. Additionally, this protein was reactive upon immunoblotting with anti-secretory aspartyl proteinase 2 (Sap2) monoclonal antibodies. Subsequently, the N-terminal sequencing of the first 20 amino acids of the 42-kDa band matched those reported for Sap2p, while the sequence of the 45-kDa band was particularly rich in glycine and had no

appreciable homology to any known sequence. Metabolic labelling with [^{35}S]-methionine suggested that this band did not originate from *C. albicans* cells, but may alternatively be the result of Sap2p activity on mucin. Altogether, these findings demonstrated that Sap2p was involved in the *in vitro* degradation of mucin and therefore may assist *C. albicans* in the process of *in vivo* penetration of the gastrointestinal mucosa by degrading the protective mucin barrier. Because *C. albicans* isolates from patients with vaginitis or oral candidiasis secrete significantly higher proteinase *in vitro* than commensal isolates, we developed a system to constitutively express Sap2p in *Saccharomyces cerevisiae* and overexpress it in *C. albicans*. The ORF of *SAP2* and its signal peptide sequence were inserted downstream of the *S. cerevisiae* or *C. albicans* constitutive promoter *ADH1*. The resulting plasmids were used to transform by homologous recombination *S. cerevisiae* and *C. albicans ura3* strains. Negative control transformants carried either plasmid without *SAP2* insert or with mutated *sap2*. Mature Sap2p was efficiently secreted by both fungi as deduced from the molecular mass of recombinant protein and the strong proteolytic activity detected in culture supernatants. The proteinase was secreted in both inducing and repressive conditions, suggesting its constitutive and unregulated expression. In murine models of oral and disseminated candidiasis, none of the *S. cerevisiae* strains were virulent. In addition, neither the *C. albicans* parental strain nor the transformants were virulent in the oral model. Moreover, when *C. albicans* transformants were inoculated intravenously, their virulence was significantly reduced compared to the parental strain. Collectively, our results indicate that Sap2p is not a sole dominant virulence

factor in *C. albicans* and are in agreement with the view that virulence in *C. albicans* is the result of the concerted action of several virulence factors.

Keywords: *Candida*, mucinase, pathogenesis, aspartyl proteinase, virulence factors.

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List of abbreviations

5-FdUMP	5-flurodeoxyuridine monophosphate
5-FU	5-fluorouracil
5-FUMP	5-fluorouridine monophosphate
ABC	ATP-binding cassette
ACT	Actin
ADH	Alcohol dehydrogenase
AIDS	Acquired immunodeficiency syndrome
ALA	Agglutinin-like adhesin
ARS	Autonomously replicative sequences
ASM	American Society for Microbiology
ATP	Adenosine triphosphate
BACH	D-bionityl-ε-amidocaproic acid hydrazide
BCIP	5-bromo-4-chloro-3-indolylphosphate
BECs	Buccal epithelial cells
BNHS	Biotin-ε-aminocaproic-N-hydroxysuccinimide ester
BSA	Bovine serum albumin
CD	Cluster of determinant
CDR	*Candida* drug-resistance
CFU	Colony-forming unit
CHEF	Countour-clamped homogeneous electric field
CHS	Chitin synthase
CMI	Cell-mediated immunity
CPH	*Candida* pseudohyphal regulator

CR	Complement receptors
CSH	Cell surface hydrophobicity
CTAB	Cetyltrimethylammoniumbromide
DAN	Diazoacetyl norleucine methyl ester
DIG-OSu	Digoxigenin-3-O-methyl-carbonyl-ε-aminocaproic acid-N-hydroxy-succinimide ester
DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
ECE	Extent of cell elongation
ECM	Extracellular matrix
EPNP	1,2-epoxy-3-(p-nitrophenoxy) propane
ERG	Ergosterol
FIGE	Field inversion gel electrophoresis
GalNAc	N-acetyl galactosamine
GFP	Green fluorescent protein
GI	Gastrointestinal
GlcNAc	N-acetyl glucosamine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HIS	Imidazole-glycerol-phosphate dehydrogenase
HIV	Human immunodeficiency virus
Hsp	Heat shock protein
HYR	Hyphally regulated gene
IEF	Isoelectric focusing
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
kDa	Kilodaltons
LAK	Interleukin-2-activated NK cell
LEU	3-isopropyl malate dehydrogenase
LIP	Lipase
MAIDS	Murine acquired immunodeficiency
MAP	Mitogen-activated protein
Mb	Megabase
mDNA	Mitochondrial DNA
mRNA	Messenger RNA
MUC	Mucin gene
NK	Natural killer
NNIS	National Nosocomial Infection Surveillance System
NOD	Nonobese diabetic mice
OFAGE	Orthogonal-field agarose gel electrophoresis
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-T	PBS-Tween
PCR	Polymerase chain reaction
PEP	Pepsinogen
PFGE	Pulse-field gel electrophoresis
PHR	pH regulated gene
pIs	Isoelectric points
PMNs	Polymorphonuclear neutrophils
PMSF	Phenylmethylsulphonyl floride

POD	Horseradish peroxidase
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal DNA
RGD	Arginine-glycine-glutamic acid
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SA	Sialic acid
SAP	Secretory aspartyl proteinase
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDA-Chl	Sabouraud dextrose agar-chloramphenicol
SDS	Sodium dodecyl sulfate
SPSS	Statistical Package for the Social Sciences
TBS	Tris-buffered saline
TBS-T	TBS-Tween
TBSA	Trinitrobenzene sulphonic acid
TEFE	Transverse alternating field electrophoresis
TEMED	N, N,N',N'-tetramethylenediamine
Th	T helper
TNF	Tumor necrosis factor
tRNA	Transfer RNA
URA	Orotidine-5'-phosphate decarboxylase
UV	Ultraviolet light
VECs	Vaginal epithelial cells
W-O	White-opaque switching strain
YNB	Yeast nitrogen base

YPD	Yeast peptone dextrose

To my family and friends...

Acknowledgments

I must first thank Dr. Louis de Repentigny and Dr. Pierre Belhumeur for their guidance, valuable advice and friendship. Their approach to science has been inspiring. I remain grateful.

I would like to extend my appreciation to the Consejo Nacional de Investigaciones Cientificas de Venezuela (CONICIT) for financial assistance. I am also grateful to Dr. Felipe San Blas and Dr. Gioconda San Blas for their encouragement to pursue my professional training.

My special thanks to Francine Aumont for her kind help and friendship.

Finally, I am immensely grateful to my family and friends for their constant encouragements, continuous support and understanding.

CHAPTER I

LITERATURE REVIEW

1. BIOLOGY OF *CANDIDA ALBICANS*

1.1 Classification of the genus *Candida*

The genus *Candida* comprises approximately 200 extremely diverse yeast species, whose common link is the absence of a sexual cycle (Fungi imperfecti). The current taxonomic position of the genus is as indicated (Joklik *et al.*, 1992).

Kingdom:	Fungi
Division:	Eumycota
Subdivision:	Deuteromycotina
Class:	Deuteromycetes
Family:	Cryptococcaceae
Genus:	*Candida*

Species of the genus *Candida* have been classically identified by means of physiological and morphological criteria. These include substrate fermentation and assimilation, cell wall composition, production of proteolytic enzymes as well as microscopic and morphological characteristics. However, these phenotypic attributes can vary greatly within some species, making speciation difficult. More recent technology takes advantage of several different approaches to determine species and strains differences at the DNA level. These strategies are based on comparison of G+C ratios in the DNAs, nucleic acids homologies determined by DNA-DNA or DNA-RNA molecular hybridizations, karyotyping, restriction enzyme mapping, fingerprinting of genomic DNA

using specific DNA probes and random amplified polymorphic DNA (RAPD; reviewed in Odds, 1997).

The majority of the *Candida* species reside in a wide variety of ecological niches, generally as saprophytes. Some species, such as *C. utilis* (Boze *et al.*, 1992), *C. maltosa* (Sunairi *et al.*, 1984), *C. boidinii* (Sakai *et al.*, 1995) are industrially important yeasts. Only a minority of the species are regarded as important pathogens for humans. These are *C. albicans*, *C. stellatoidea* (now considered *C. albicans*), *C. tropicalis*, *C. pseudotropicalis* (now considered a synonym of *C. kefyr*), *C. parapsilosis*, *C. guilliermondii*, *C. krusei*, *C. lusitaniae*, *C. rugosa*, *C. glabrata* (formerly classified as *Torulopsis glabrata*), and the more recently recognized *C. dubliniensis* (Sullivan *et al.*, 1995). *C. stellatoidea* was initially considered as a separate species; however, conclusive biochemical and genetic evidence now indicates that *C. stellatoidea* and *C. albicans* should be joined together as variants of the same species (Kwon-Chung *et al.*, 1989; Boucher *et al.*, 1996). *C. albicans* represents the major pathogen, as judged by the frequency of its isolation from human infections (Fridkin & Jarvis, 1996).

1.2 Morphology and morphogenesis

C. albicans is capable of growing in at least two different morphological forms, yeast and hyphae. However, unlike some other dimorphic fungal pathogens such as *Blastomyces dermatitidis*, *Histoplama capsulatum* and *Paracoccidioides brasiliensis* that express a single morphological phase in the host and a different phase outside the host, *Candida* lesions are characterized by the presence of both yeast and

hyphae. The *yeast or blastoconidia* form (4-6 x 6-10 μm), predominates as a commensal in the host during the carrier state. It reproduces by budding, which occurs preferentially at the polar region of the cell, distal to the birth scar (Figure 1A). In infected tissues, *C. albicans* also exhibits a multicellular *mycelial or hyphal* morphology. Hyphae arise from continous apical extension, leading to a compartmentalized filament. Each compartment contains a single cell, separated by a septum, with a central pore. They are not constricted and have a diameter of about one-third of a budding cell (reviewed in Shepherd, 1991, Figure 1B). The ability to form true hyphae is predominantly found in *C. albicans*.

During the yeast-to-hyphal transition, other morphologies may be observed. *Germ tubes* represent the short initial hyphae produced directly from yeast cells and *pseudohyphae* arise from blastoconidia or a hypha by a budding process in which each generation of budding remains attached to its parents (Figure 1C).

On the other hand, under special *in vitro* conditions, *C. albicans* produces thick-walled, large (8-12 μm) ellipsoidal-shaped cells, with condensed cytoplasm, referred to as chlamydospores. Such cells may represent a stationary-phase morphological phenotype or even a terminal phenotype of the budding forms (Shannon, 1981).

The germ tube test and chlamydospore production are used routinely for identification of *C. albicans*.

Morphogenesis is a consequence of polarized cell growth, which requires the integration of multiple cellular functions, such as budding site selection and assembly, cytoskeletal assembly and polarized secretion.

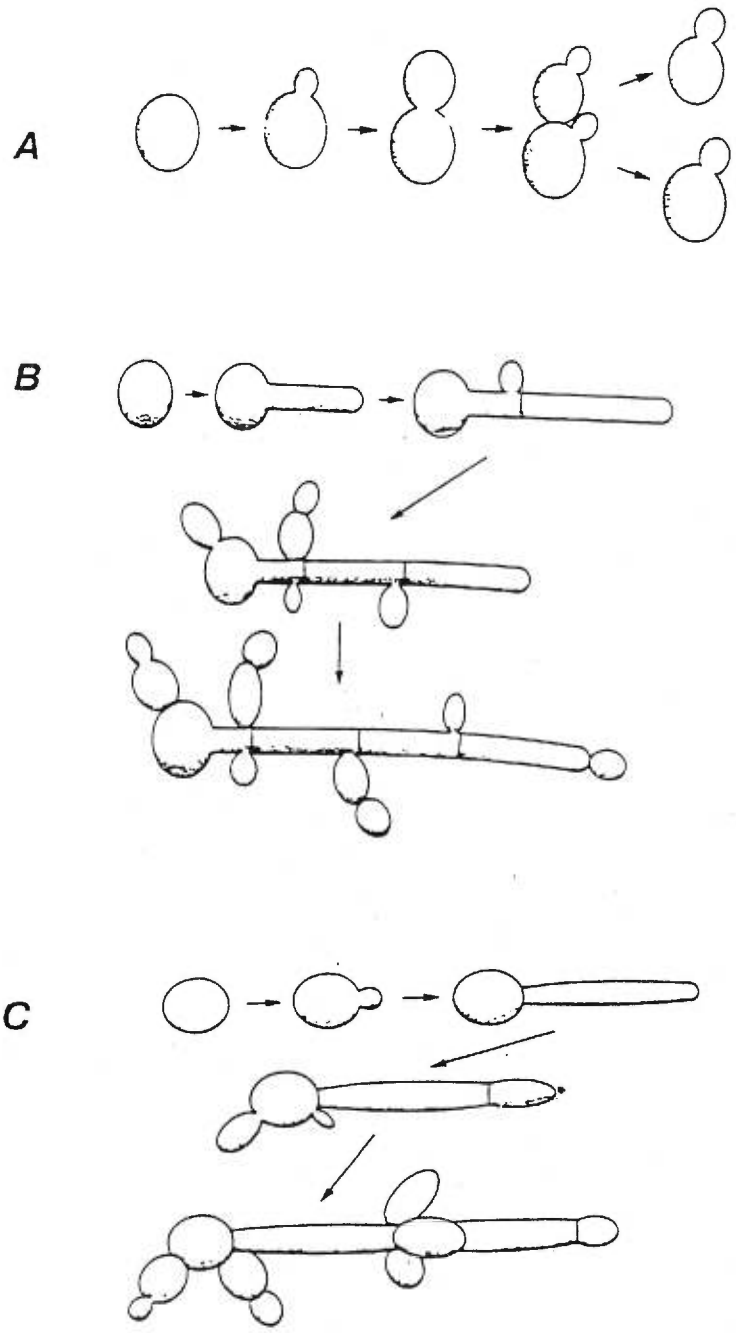

Figure 1. **Morphogenesis of *Candida albicans*. A**: Budding process. **B**: Hypha formation. **C**: Pseudohypha formation (Odds, 1988).

In general, morphogenesis involves the following events (Vanden Bossche, 1997):

1. Sensing of environmental signals.

2. Signal reception and transduction into a biochemical message. This would result in the selection of a site on the cell surface for evagination. Germ tubes arise from any part of the yeast cell surface, whereas buds tend to arise from polar regions of the cell.

3. Changes in gene expression.

4. Reorganization of the elements responsible for polarization of cell growth.

Analysis of studies performed in the late 1970's and early 1980's indicate that there is no unique environmental signal or inducer of the bud-hypha transition. Indeed, the *in vitro* transition is influenced by physical factors (temperature and pH), nutritional conditions (particularly the nitrogen status of the cell) and chemical effectors. Thus, yeast cell growth is favoured by temperatures around 24-25°C, pH 4.5 and defined media (Lee *et al.*, 1975; Manning & Mitchell, 1980; Buffo *et al.*, 1984), whereas hyphal growth is enhanced at 37°C, pH 6.5 (Buffo *et al.*, 1984), the presence of proline (Dabrowa *et al.*, 1976), N-acetyl-glucosamine (GlcNAc; Sheperd & Sullivan, 1983), zinc (Bedell & Soll, 1979) as well as serum or serum derivatives (Shepherd *et al.*, 1980) in the culture media. Currently, the view is that there must be a common control mechanism responsible for conversion from yeast to hyphal forms.

Since 1980, remarkable efforts have been done in an attempt to determine differential expression of genes during the morphological transition. Indirect approaches analyzed the distribution of pulse-labeled

polypeptides in both yeast and mycelial phases, using one and two-dimensional polyacrylamide gel electrophoresis (1-D and 2-D PAGE, respectively) (Manning & Mitchell, 1980; Finney *et al.*, 1985). Surprisingly, the results suggested that only small number of proteins were phase-specific. Later on, more direct strategies have confirmed and extended those observations, since only a few genes have been shown to be specifically induced during hyphal development, regardless of the induction method. These are *ECE*1 (Birse *et al.*, 1993), *HWP*1 (Staab *et al.*, 1996) and *HYR*1 (Bailey *et al.*, 1996). Genes such *PHR*1 (Saporito-Irwin *et al.*, 1995), *PHR*2 (Muhlschlegel & Fonzi, 1997), *SAP*4, *SAP*5, *SAP*6 (Hube *et al.*, 1994), and *ALS*1 (Hoyer *et al.*, 1995), are induced in hyphae, but only under certain specific conditions. Expression of other genes fluctuates during morphogenesis. These encode proteins involved in glycolysis (*ADH*1, *PYK*1; Swoboda *et al.*, 1994b), protein synthesis and folding (*TEF*3 and *HSP*90; Swoboda *et al.*, 1994a; Swoboda *et al.*, 1995), cell wall biosynthesis (*CHS*1, *CHS*2 and *CHS*3; Chen-Wu *et al.*, 1992; Gow *et al.*, 1995) and formation of the cytoskeleton (*ACT*1; Delbruck & Ernst, 1993).

Current efforts to understand the molecular mechanisms of the morphological transition are being focused on the study of members of the mitogen-activated protein (MAP) kinase cascade. It has been shown that *C. albicans* gene *ACPR* (also called *CPH*1), which is a homologue of *STE*12, a MAP kinase cascade member of *S. cerevisiae*, is a strong inducer of pseudohyphae formation in *S. cerevisiae* haploids and diploids (Singh *et al.*, 1994). Similarly, its mutation resulted in suppression of hyphal formation in *C. albicans* when grown on solid media (Liu *et al.*, 1994). More recently, disruption of *CaCLA*4 (Leberer *et al.*, 1997), an

homologue of *STE*20 of *S. cerevisiae*, and *CCP*1 (Csank *et al.*, 1997), a structural member of the VH1 family of dual-specificity phosphatases, resulted in defects in hyphal formation in both liquid and solid media. Futhermore, an additional *C. albicans* MAP kinase gene, *MKC*1 appears to be involved in cell wall assembly and morphogenesis, as deduced by the fact that null mutants had alterations in their cell surface and pseudohyphal formation (Navarro-Garcia *et al.*, 1998).

An additional developmental program has been described in *C. albicans*, the phenotypic switching (Slutsky *et al.*, 1985). The program allows *C. albicans* to switch spontaneously, at high-frequency and reversibly between a number of distinct colony phenotypes (Soll, 1997). The strain WO-1 has been used as a general model system. It switches between white and grey (opaque) phases and exhibits all the characteristics of switching of other strains. Structural and functional analysis of the white-specific gene *WH*11 (Soll *et al.*, 1995; Srikantha *et al.*, 1995, Srikantha *et al.*, 1996) have supported the hypothesis that switching involves a precise and highly evolved regulatory program for the expression of phase specific genes. Moreover, it seems probable that morphogenesis and phenotypic switching are interconnected. Thus, gene *WH*11 is expressed in the white-budding phase, but is inactivated in the white hyphal phase and in the opaque budding phase, suggesting that it is regulated through the same transcription activation sequences (Srikantha *et al.*, 1997).

1.3 Ultrastructure and cell organization

1.3.1 Cell wall

The cell wall of *C. albicans* has the functions of maintaining the cell shape, serving as a protective barrier and containing specific adhesive molecules for attachment and subsequent invasion of the host. It is a multi-layered structure, 100-400 nm thick, which represent 30% of the total weight of the cell, and is composed primarily of mannans, glucans, chitin, protein and lipids.

Although the number of layers and their nature vary depending on the strain and growth conditions, there is a general agreement that the outermost and highly electron dense layer is a mannoprotein complex (Reiss *et al.*, 1992; Figure 2), in which the mannose molecules are linked to the protein moiety through serine or threonine or through GlcNAc (Shepherd, 1987; Calderone & Braun, 1991). The mannoproteins, which represent 20-30% of the total dry weight of the cell wall, are the major antigenic components of the cell wall. They define the serotypes A and B by the side chain oligosaccharides attached to the backbone 1,6 mannosyl residues (Hasenclever & Mitchell, 1961). Thus, serotype A (factor 6) contains predominantly a straight chain of α-1,2-linked mannose residues with a terminal α-1,3-mannose residue. Determinants from serotype B (factor 13b) are shorter but more complex, lacking a terminal α-1,3-linkage but having a single C-1-C-2-C-3 branch point and an additional internal α-1,6 mannosyl residue (Tsuchiya *et al.*, 1965; Kobayashi *et al.*, 1992). The inner and electron transparent layer is rich in β-glucans and chitin. The β-glucans contribute 47-60% of the weight of the cell wall (Sullivan *et al.*, 1983) and represent the main structural components. They are branched

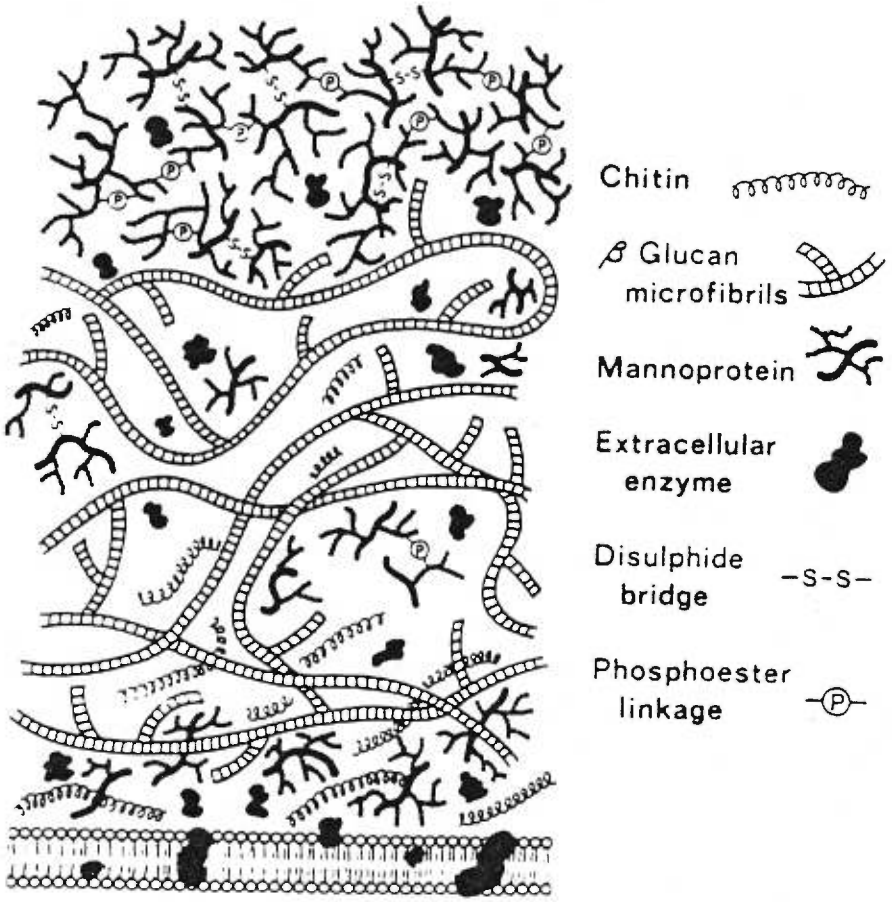

Figure 2. Architecture of *Candida albicans* cell wall (Reiss *et al.*, 1992).

polymers of glucose containing β-1,3 and β-1,6 linkages (Shepherd, 1987). Chitin, which is an unbranched homopolymer of GlcNAc, comprises 0.6-2.7% of the dry weight of the wall. It is involved in the shape, cell-cell connections in the ring between parent and daughter cells, and in the bud scars and septum formation (Braun & Calderone, 1978). One layer of chitin is distributed near the plasma membrane, but there are smaller amounts dispersed throughout the outer wall layers. Regeneration studies of cell walls of protoplasts indicated that chitin was the first polymer to be laid down, followed by β-glucans and finally mannoproteins (Rico *et al.*, 1997). In addition to the outermost mannoprotein layer, there is also a fibrillar layer, that is most evident when yeast form germ tubes (Hubbard *et al.*, 1985).

Similar proportions of most of the components are found in yeasts, germ tubes and hyphae. However, the latter contain three times more chitin than yeasts cells (Chattaway *et al.*, 1968).

Proteins represent about 10% of the dry weight of the wall, and they include enzymes involved in cell growth, morphogenesis and other metabolic functions.

The lipid content is low, about 1% of the dry weight, and they include neutral lipids and phospholipids (Ghannoum *et al.*, 1987).

1.3.2 Intracellular organelles

As with other eukayotes, the *C. albicans* cell membrane is a phospholipid bilayer with frequent invaginations. It contains lipids and proteins and a specific sterol, ergosterol, which is the target for many antifungal drugs. It has the functions of regulating cell permeability as well

as being the site of various membrane-bound enzymes important in cell wall biosynthesis such as mannan synthase, chitin synthase, glucan synthase, ATPase as well as phosphate-transport proteins. In the cytoplasm, there are few mitochondria per cell, which are associated with small and well-defined ribosomes. There is also a rough and smooth endoplamic reticulum. Vacuoles, glycogen and lipid bodies may be present. Microtubules and microfilaments exist in the cytoplasm. The nuclear apparatus consists of nuclear membranes, nuclear pores, a nucleoplast and a nucleolus. The same organelles and inclusions are found in both yeast and hyphae (reviewed in Segal & Baum, 1994).

1.4 Growth requirements

C. albicans grows in aerobic conditions and simple media containing a source of carbon, nitrogen, phosphate and biotin. Slightly acidic pH (pH 4.5) is appropriate for ideal yeast growth. However, *C. albicans* can tolerate a wide range of pH, from 2.5 to 7.5 as well as temperatures from 20 to 38°C. Most of the *Candida* species assimilate and ferment glucose and none of them assimilate nitrates (Odds, 1988).

1.5 Genetics

Genetic studies of *C. albicans* have been hampered because it is diploid and has no known sexual cycle. However, major advances have been achieved with the use of classical (parasexual) and modern genetic techniques.

The diploid state of *C. albicans* was early suggested by several lines of evidence: i) the demonstration of natural heterozygosity by Whelan &

Magee (1981), ii) the analysis of the DNA content per cell by Riggsby *et al.*, (1982) and iii) the analysis of recombinants from protoplast fusion experiments conducted by Poulter *et al.* (1982).

Genome sizes calculated by reassociation kinetics indicated a range of 14 to 18 megabases (Mb) per haploid genome (Riggsby *et al.*, 1982).

By use of methods based on alternating field gel electrophoresis, such as pulsed-field gel electrophoresis (PFGE), orthogonal field agarose gel electrophoresis (OFAGE), field inversion gel electrophoresis (FIGE), contour-clamped homogeneous electric field (CHEF) and transverse alternating field electrophoreis (TEFE), chromosomal DNAs have been resolved into 5-12 bands ranging from 0.42 to 5.7 megabases (Mb) (reviewed in Altboum,1994). Despite the differences, most of the results indicated that *C. albicans* contains eight pairs of chromosomes, numbered R (for the rDNA-containing gene), 1, 2, 3.....and 7, in decreasing order of size (Magee *et al.*, 1988). Early examination of the karyotype demonstrated significant length polymorphism among various strains (Magee & Magee, 1987), and these results have been largely confirmed by other investigators (Iwaguchi *et al.*, 1990; Rustchenko-Bulgac, 1991). Since most of the variation is in chromosome R, it is most probably due to unequal crossing over in the rDNA repeats. Similarly, the presence of new chromosomal bands may be the result of translocations (Thrash-Bingham & Gorman, 1992; Chu *et al.*, 1993).

Initial analysis of *C. albicans* ploidy suggested that diploidy was obligatory and that haploidy or even significant aneuploidy would not be possible (Whelan & Soll, 1982). Several authors have now contradicted this view by providing evidence that *C. albicans* can tolerate large regions

of haploidy. Barton and Gull (1992) showed that a strain could survive with only one copy of the 2 Mb chromosome 3, although growth was slow until the aneuploid strain reduplicated the monosome. More recently, Magee & Magee (1997), reported that *C. albicans* strain WO-2 (spontaneous derivate of WO-1) was haploid for more than 1.5 Mb, and despite this fact, it could grow, switch from white to opaque, and carry out the dimorphic transition. Indeed, *C. glabrata* has been shown to be largely haploid and is currently exploited as a system for cloning and genetic studies (Whelan, 1987; Zhou *et al.*, 1994).

1.5.1 Genome organization

Three major strategies have been used to clone *C. albicans* genes: 1) functional complementation of auxotrophic lesions in *S. cerevisiae*, 2) sequence homology, usually to genes from *S. cerevisiae*, and 3) the ability of certain *C. albicans* sequences to confer new phenotypes on *Saccharomyces* strains (Scherer & Magee, 1990).

Most of the promoter regions of *C. abicans* genes are A-T rich, and have TATA or TAAT sequences that may serve as transcription initiation signals (Kurtz *et al.*, 1990). Nothing is known about spacing or sequence requirements for functional TATAA elements in *C. albicans*. So far, two potential TATAA-like elements at -92 and -71 were detected by Miyasaki *et al* ., (1994) when analysing *SAP*4 gene sequences. *Candida* may also use the sequences TAG...TAGT...TTT as transcription termination signals. Like *Saccharomyces*, few *C. albicans* genes contain introns. Interestingly, the presence of a group I self-splicing intron, encoding the large subunit ribosomal RNA (rRNA) was recently demonstrated and characterized

(Mercure *et al.*, 1993). Its presence has also been detected in *C. dubliniensis* and *C. stellatoidea* (Boucher *et al.*, 1997).

An unusual *C. albicans* retrotransposon element was recently described (Matthews *et al.*, 1997). It was called pCal and interestingly, the unintegrated, linear dsDNA was found at high-copy number.

1.5.2 Repetitive elements

C. albicans as well as other *Candida* species contain several dispersed, repeated families of DNA sequences (Scherer & Magee, 1990). Most of these sequences consist of the mitochondrial DNA (mDNA) and the ribosomal DNA (rDNA) tandem array and some of them have been used as probes for typing *C. albicans* strains (Scherer & Stevens, 1988). The repeat family Ca7, appears to be telomeric and produces new DNA polymorphisms at extremely high rates in some strains. The dispersed and homologous gene families Ca3 and 27A (Sadhu *et al.*, 1991) are present at approximately 10 copies per haploid genome and are specific to *C. albicans* and *C. stellatoidea*. Subsequently, two additional repetitive elements were isolated and characterized by Lasker and co-workers. The *C. albicans* repetitive element -one (*CARE*-1; Lasker *et al.*, 1991) and -two (*CARE*-2; Lasker *et al.*, 1992) are short nucleotide sequences, present at 2-10 (*CARE*-1) and 10-14 (*CARE*-2) copies per haploid genome, and dispersed on multiple chromosomes. *CARE*-1 DNA appears to be species-specific while CARE-2 has a high degree of interstrain variation.

1.5.3 Autonomously replicative sequences (ARS)

Genetic transformation systems developed for various microorganisms are usually based on the use of vectors carrying autonomously replicating sequences (ARS). These sequences enhance the stability of the transformants, facilitate cloning of genes by mutant complementation and provide the potential to study the effects of gene dosage on gene expression. Therefore, since no circular plasmids have been found in *C. albicans,* there was an interest in developing *C. albicans* host systems carrying DNA fragments capable of directing autonomous replication. Kurtz and co-workers (1987) isolated a 0.35-kb *Rsa*I DNA fragment, present in one copy per haploid genome and capable of promoting non-integrative transformation in *C. albicans*. However, plasmids based on this ARS fragment (pCARS1) were unstable, seemed to replicate as multimers, and did not replicate autonomously in *S. cerevisiae*. Later on, a 1.2-kb fragment that replicated autonomously in both *C. albicans* and *S. cerevisiae* was isolated (CaARS) (Cannon *et al.*, 1990). The plasmid (pRC2312) carrying the CaARS element (Cannon *et al.*, 1992) was used to clone and express *C. albicans ADE2* and proteinase (*APrA*) genes in *C. albicans*, but some of the transformants were still of the integrative type. Recently, all these efforts have been rewarded since the first direct cloning system in *C. albicans* that did not require the use of *S. cerevisiae* for plasmid rescue, was reported. In this case, the combined use of two *C. albicans* autonomously replicative sequences (ARS2 and ARS3) improved the stability of *C. albicans* nonintegrative transformants (Pla *et al.*, 1995).

1.5.4 Genetic code variations

According to the universal code of codon usage, CTG is encoding for the amino acid leucine (Leu). Recently, *in vitro* translation assay systems as well as codon analysis have demonstrated that this Leu codon is translated as serine (Ser) in several *Candida* species (*C. parapsilosis, C. zeylanoides*, *C. albicans*, *C. cylindracea*, *C. rugosa*, *C. melibiosica*, *C. maltosa*) (Ohama *et al.*, 1993; Santos & Tuite, 1995; Zimmer & Schunck, 1995). Serine tRNAs that recognize the CTG codon have been identified in all these species. In *C. albicans* particularly, the CTG codon in the secretory aspartyl proteinase (*SAP*1) gene is translated *in vivo* into a nonmodified Ser (White *et al.*, 1995).

These findings are very important for the development of molecular genetics in *C. albicans*. In fact, the function of foreign gene sequences may be affected by the presence of CTG codons, as evidenced by the failure of expression of wild-type green fluorescent protein (*GFP*) gene in *C. albicans*. Successful expression was achieved only after obtaining a mutated *GFP* gene that carried optimal codons for translation (Cormack *et al.*, 1997).

2. HOST-*CANDIDA* INTERACTIONS

2.1 Host barriers

2.1.1 Skin and mucous membranes

The physical integrity of the skin and mucous membranes represent a form of defense against *Candida* infections. The skin surface is relatively inhospitable to *Candida* growth because of exposure to ultraviolet light, low moisture conditions, the competition from the normal flora and the inhibitory activity of the skin lipid sphingosine (Bibel *et al.*, 1993). However, when the epithelial surface is broken as a result of trauma and/or increased moisture, a subcutaneous *Candida* infection may develop. Also, the frequent maceration of the oral mucosa by maxillary denture may result in microscopic breaches of the epithelium, allowing *Candida* infections (Watson & MacDonald, 1982). The constant desquamation of the mucosa at a rate greater than that at which *Candida* species grow *in vivo*, is an additional mechanism by which superficial mucosae protect against *Candida* infections.

2.1.2 Saliva

The constant flushing action of saliva essentially removes the unattached or loosely attached *Candida* from the oral cavity. In addition, saliva contains antifungal factors such as specific antibodies (Ponton *et al.*, 1996), lysozyme, lactoperoxidase, lactoferrin, and histidine-rich polypeptides (histatins). The latter can kill 100% of *C. albicans* cells within 24 h at concentration 5 μg/ml (Pollock *et al.*, 1984; Driscoll *et al.*, 1996).

2.1.3 Mucus gel and mucins

Many mucosal surfaces such as the gastrointestinal, reproductive and respiratory tracts are covered by a protective secretion, called slime or mucus. It is produced by specialized epithelial goblet cells and is a highly hydrated, slippery gel comprising a large number of constituents. The most prominent components in these gels are the mucin-type glycoproteins, which are responsible for the specific protective properties of the mucus gel (Van Klinken *et al.*, 1995). The capacity of the secretory mucins to protect epithelial surfaces depends largely on their high content of oligosaccharides and on their ability to form a gel.

2.1.3.1 *Structure of secretory mucins.* Two major characters distinguish secretory mucins. Firstly, high content (50% or more) of their weight consists of O-linked oligosaccharides, and secondly, the presence of tandem repeat sequences in the protein backbone. Essential for gel formation is the capacity of secretory mucins to form intermolecular disulfide bridges, resulting in oligomeric mucins (Strous, 1992).

Five monosaccharides are commonly found in the oligosaccharide structure: N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), galactose, fucose and sialic acid (SA). The latter sugar and sulfate confer a negative charge to the oligasaccharides. In the complex, the core consists of GalNAc residues directly attached to the hydroxyl group of serine or threonine of the protein backbone of mucins. The backbone region contains Galβ-1,3 and GlcNAcβ-1,4 units forming chains and the peripheral regions are represented by terminal α-glycosidic-linked galactose, GalNAc, fucose,

sialic acid, or sulfate. These peripheral sugars determine most of the characteristics of the mucins as a whole (Van Klinken *et al.*, 1995).

To date, nine human mucin genes have been identified, *MUC* 1, 2, 3, 4, 5AC, 5B, 6, 7 and 8, although only *MUC* 1, 2 and 7 have been fully cloned (Gum *et al.*, 1989; Bobek *et al.*, 1993; Shankar *et al.*, 1994). Significant homology is present at both C- and N-termini between a number of animal species and human intestinal mucins (Gendler & Spicer, 1995). Several studies have confirmed and extend the concept that the peptide core of secreted mucins consists of at least two regions: a tandem repeat-containing domain, densely O-glycosylated, rich in serine, threonine, and proline, and resistant to proteinases; and a minor portion, poorly or nonglycosylated, rich in cysteine, and susceptible to proteinases (Strous, 1992; Forstner & Forstner, 1994). Compared to the tandem repeat regions, relatively little is known about the nonrepetitive or unique sequences that flank these arrays. However, it has been suggested that these internal "naked" peptide regions would provide some flexibility to the otherwise continous rod-like stretch of glycosylated tandem repeats (Forstner & Forstner, 1994). The existence of potential link proteins in intestinal mucins was inferred from studies in which purified mucin polymers were treated with thiol reducing agents, subjected to SDS-PAGE, and observed to release a mannose-containing glycopeptide of 118 -kDa (Roberton *et al,* 1989). Recent studies have shown that these glycopeptides constitute the C-terminal 689 amino acids of a very large intestinal mucin (Xu *et al,* 1992). Moreover, it has been postulated that these peptides are an integral part of the mucin structure, serving to "link" large mucin glycoprotein "subunits" together into polymers (Forstner & Forstner, 1994).

Based upon current data, a linear model has been proposed for mucin polymer structure (Gum, 1992; Forstner & Forstner, 1994). Mucin monomers contain heavily glycosylated and therefore rigid repetitive central domains, flanked by unique sequences. At each end of the mucin monomers, the peptide is less glycosylated and thus likely to have a more folded configuration. The unique sequences contain many cysteine residues that link monomers via disulfide bonds that account for polymer formation and stabilization of the folding (Figure 3).

2.1.3.2 *Mucin-microorganism interactions.* Mucins may modulate colonization of both commensal and pathogenic microorganisms by (i) binding to microorganisms to facilitate their clearance, (ii) serving as receptor for microbial adhesion to host surfaces, and (iii) serving as a microbial nutritional substrate (Figure 4).

In the oral cavity as well as in the gastrointestinal tract, the mosaic of bacterial adhesion sites afforded by mucins may be involved in the early events of nonimmune defense. In fact, carbohydrate determinants from salivary mucins have been identified as "false" receptors for some bacteria. These determinants include blood group sugars such as terminal α-linked Gal (in the binding of *Streptococcus mutans*, Levine *et al.*, 1978) and N-acetyl-neuraminic acid for binding to *Streptococcus sanguis* (Neutra & Forstner, 1987). In the large intestine of mice, mucins did not allow *Escherichia coli* to bind to epithelial cells (Poulson *et al.*, 1994), and similarly, rabbit mucins from the jejunum inhibited the adhesion of the enteropathogenic *E. coli* strain RDEC-1 to specific receptors on ileal and proximal colon epithelial cells (Mack & Blain-Nelson, 1995).

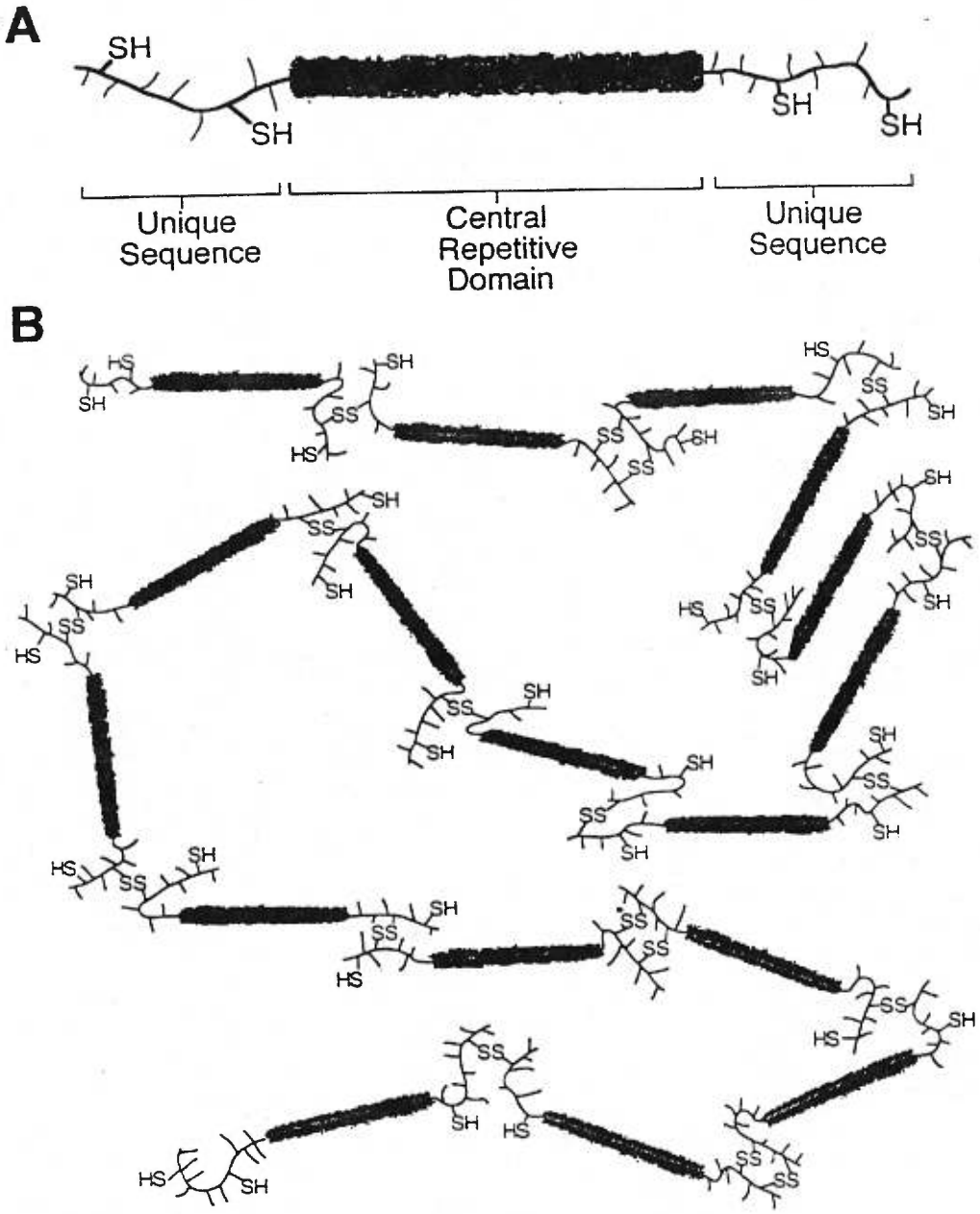

Figure 3. Structure of secretory mucins (Gum, 1992).

A: Mucin monomer

B: Mucin polymer

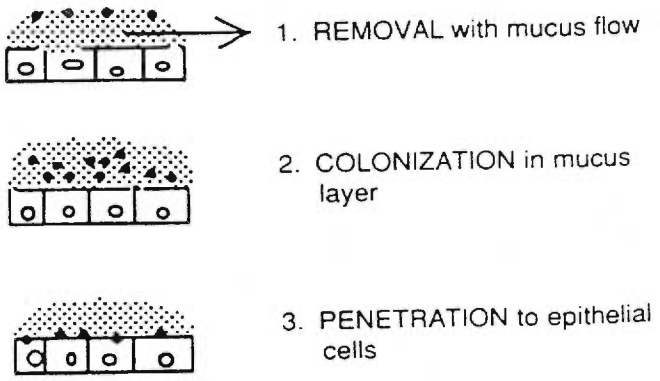

Figure 4. Fate of pathogenic microorganisms that bind to mucins (Forstner & Forstner, 1994).

Modulation of *C. albicans* populations at mucosal surfaces may occur in a similar manner. Mucin glycoproteins produced by sublingual and minor salivary gland secretions contribute the majority of blood group activity in humans and both high and low-molecular-weight mucins (*MG*1 and *MG*2, respectively) have been identified as the predominant salivary carriers of blood group determinants (Prakobphol *et al.*, 1993). Non-secretors of blood group antigens have been observed to be at greater risk for chronic oral candidosis (Thom *et al*, 1989). It has been also reported that the Galβ-1,3 (Fucα-1,4) GlcNAc-glycoside, which is found in blood group nonsecretors, binds *C. albicans* (Prakobphol *et al*, 1993). Moreover, coating dental acrylic with human submandibular-sublingual saliva, which contained predominantly *MG*1 and *MG*2 significantly enhanced adhesion of both growth forms of *C. albicans* (Edgerton *et al*, 1993). Overall, it seems that the carbohydrate units on salivary mucins could act as ligands for *Candida* cells and may promote adhesion.

Perturbation of mucin structure by mucinase production can also reduce considerably the protective role of mucins. The production of mucin-degrading enzymes has been detected in pathogens such as *Vibrio cholerae* (Crowther *et al.*, 1987), *Bacteroides fragilis* (Robertson & Stanley, 1982), *Shigella* spp. (Haider *et al.*, 1993), *Helicobacter pylori* (Slomiany & Slomiany, 1992), and *Yersinia enterocolitica* (Mantle & Rombough, 1993). Indeed, intestinal bacteria such as *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium bifidum,* are considered "protective" organisms because they are unable to degrade mucins (Ruseler-ven Embsen *et al.*, 1995).

2.1.4 Commensal microflora

The commensal microflora may regulate yeast populations by i) inhibiting the adherence of yeasts to mucosal surfaces, ii) competition for available nutrients and iii) the production of antifungal factors. Experimental evidence suggests both adherence suppression and promotion effects of oral bacteria on *Candida spp* (Nair & Samaranayake, 1996; Grimaudo & Nesbitt, 1997). Furthermore, some studies have shown that experimentally infected animals receiving broad-spectrum antibiotics had an increased incidence of disseminated candidiasis (Samonis *et al.*, 1994).

2.2 Host immunoresponse

2.2.1 Innate immunity

The cellular constituents of innate immunity, polymorphonuclear leukocytes (PMNs), monocytes and macrophages are considered the major host defense against disseminated candidiasis.

Neutropenia, leukocyte dysfunction (chronic granulomatous disease) and use of corticosteroids increase the risk of invasive candidiasis. In fact, recent data indicate that the impairment in the phagocytic activity against *Candida* in cardiovascular-surgery patients under antibiotic therapy, may increase their risk of fungal infection (Tran *et al.*, 1997). These clinical findings concur with the observation that mice with experimental granulocytopenia are more susceptible to systemic *Candida* infections than normal animals (Bistoni *et al.*, 1993).

Phagocytic cells can attach to *C. albicans* hyphae and ingest blastoconidia (Vazquez-Torres & Balish, 1997). The killing mechanism involves the activation of oxidative and oxygen-independent systems, which

result in cell wall damage and DNA fragmentation (Greenfield, 1992; Christin *et al.*, 1997).

Phagocytic cells are triggered to release cytokines by *C. albicans* components. In fact, killed *C. albicans* stimulated PMNs to release interleukin-1 (IL-1), IL-6 and tumor necrosis factor alpha (TNF-α) (Djeu *et al*, 1990; Cassone *et al.*, 1993). Similarly, *C. albicans* mannoproteins stimulated TNF-α production from murine macrophages *in vitro* (Vecchiarelli *et al.*, 1991) and activated human PMNs to generate granulocyte-macrophage colony-stimulating factor (GM-CSF) (Palma *et al.*, 1992).

Although there is no direct evidence of the role of natural killer cells (NK), the available data indicate that human NK cells are activated both *in vitro* and *in vivo* by intact *C. albicans* cells or some of their cell-wall constituents (Djeu *et al.*, 1988). The activation results in the production of IFN-γ, TNF-α, and GM-CSF, that activate other phagocytic cells to engulf and kill *C. albicans*. Moreover, it was recently shown that interleukin-2-activated NK cells (LAK) inhibited the growth of hyphal forms of *C. albicans* (Arancia *et al.*, 1995).

Overall, cells from the innate immune system accomplish their antifungal role by their phagocytic and microbicidal properties as well as by production of several cytokines that are vital in the initiation and amplification of T-cell and B-cell dependent immune responses.

2.2.2 Acquired immunity

2.2.2.1 *Humoral immunity.* Despite the fact that increased susceptibility to mucosal and/or systemic candidiasis has not been reported in patients with either congenital or acquired pure B-cell abnormalities, clinical observations

and experimental studies support the view of a protective role of anti-*Candida* antibodies. Matthews *et al.* (1984) found an association between recovery from disseminated candidiasis and the production of antibodies to a 47-kDa immunodominant protein. Patients who succumbed had low or negative titers. More recently, it was shown that presence of anti-*Candida* antibodies correlated with survival in disseminated candidiasis and were protective in a mouse model of infection (Matthews *et al.*, 1991; Han *et al.*, 1997). At mucosal sites, anti-*Candida* antibodies may be involved in preventing invasion, but nevertheless allowing low levels of colonization. Specific IgA and IgG antibodies reacting to mannan and secretory proteinase have been detected in vaginal fluids of experimentally infected rats (De Bernardis *et al.*, 1997), and their presence correlated with clearence of infection. Several mechanisms may be involved in this protective role. Firstly, antibodies may act as opsonins that enhance the phagocytosis of *Candida*. Secondly, they may promote agglutination of the fungus, avoiding dissemination and thirdly, antibodies may block binding to host cells, which may limit the extent of the infection.

2.2.2.2 *Cell-mediated immunity.* Because of the high incidence of mucocutaneous candidiasis in patients with primary or acquired T-cell immunodeficiencies, such as those with the acquired immunodeficiency sydrome (AIDS) (Klein *et al.*, 1984), those treated with immunosupressive agents or receiving transplants (Clift *et al.*, 1984), it is postulated that cell-mediated immunity (CMI) may play a predominant role in superficial candidiasis, preventing invasive infection. In fact, animals with defective T-cell function such athymic mice (Helstrom & Balish, 1979), severe combined

immunodeficiency mice (SCID mice) (Narayanan *et al.*, 1991), CD4+ -depleted mice (Cantorna & Balish, 1991) and murine acquired immunodeficiency syndrome (MAIDS) (Deslauriers *et al.*, 1997), rarely develop hematogenously disseminated candidiasis.

Selective activation of either of the CD4+ Th subsets (Th1 or Th2) correlated with defense against disseminated infection. Studies in systemically infected mice have shown that a protective pattern of immunization correlated with a predominant Th1-type response, characterized by the secretion of IL-2, IL-12 and IFN-γ. (Romani *et al.*, 1991; 1993; Bistoni *et al.*, 1993). This pattern was accompanied by specific delayed-type hypersensitivity reaction (DTH). On the other hand, a nonprotective Th2-type response was characterized by production of IL-4 and IL-10. Indeed, a Th1-type response was observed in genetically resistant mice after vaccination with an attenuated virulent *C. albicans* strain, whereas the same vaccine resulted in a Th2-type response in genetically susceptible mice (Bistoni *et al.*, 1993). Moreover, treatment with anti-IL-4 or IL-4 receptor or anti-IL-10 antibodies, transformed a nonprotective to a protective response (Puccetti *et al.*, 1994), and neutralization of IFN-γ transformed a protective pattern into its opposite (Romani *et al.*, 1992). Similar patterns have been demonstrated in murine models of vaginal candidiasis (Fidel & Sobel, 1996; Fidel *et al.*, 1997).

CD8+ T cells, through their cytotoxic activities can damage *C. albicans* hyphae (Beno *et al.*, 1995). Interestingly, recent evidence suggests that acquired resistance to oral reinfection with *C. albicans* in the murine AIDS model (MAIDS) was associated with mucosal recruitment of CD8+ T cells (Deslauriers *et al.*, 1997). It has been further suggested that

immunocompetent cellular components that protect against *Candida* at mucosal sites may be different from those involved in systemic infection. In the mouse vagina, the proportion of T cells bearing the γ/δ T-cell receptor is significantly higher than in the periphery (Fidel *et al.*, 1996b). Although the specific role of these cells has not been clarified, it is known that they recognize heat-shock proteins (Hsp), that they are cytotoxic and produce IFN-γ and TNF-α and they enhance the anti-*Candida* activity and production of nitric oxide of macrophages (Jones-Carson *et al.*, 1995). Thus, they may also be involved in host defense against candidiasis.

2.2.3 Complement activation

C. albicans activates both classical and alternate complement pathways (Kozel *et al.*, 1996), and phagocytosis and killing of *C. albicans* by PMNs and macrophages are increased in the presence of complement factors (Greenfield, 1992). Experimental models indicate that complement activation is important in resistance to both cutaneous and systemic candidiasis. Interruption of both the classical and alternate pathways in guinea pigs made them more susceptible to disseminated candidiasis than either C4 deficient (with interrupted classical but not alternate pathway) or normal animals (Gelfand *et al.*, 1978). Antimannan IgG has been identified as an initiator of the classical pathway (Zhang *et al.*, 1997). Deposition of C3 on hydrophilic and hydrophobic yeast cells as well as on germ tubes resulted in rapid activation of the classical pathway (Kozel *et al.*, 1996).

2.3 Virulence factors of *Candida albicans*

A virulence factor is usually defined as a unique molecular attribute of a pathogen used to accomplish colonization and infection of the host. Regarding *C. albicans*, despite intense searching, no unique character has been demonstrated experimentally to be essential at a particular stage of the infection pathway. In an attempt to explain this situation, Cutler (1991) proposed a hypothesis which suggests that the virulence phenotype is determined by the existence of virulence traits (factors) that belong to a set of genes. Thus, a single trait of the set is not sufficient for virulence and a critical number of genes must be expressed and act in concert to enable a strain to cause disease. Interestingly, the possibility of linkage of some virulence traits cannot be ruled out, since the disruption of a single *C. albicans* gene *INT*1, which encodes a surface protein, suppressed hyphal growth, adhesiveness and virulence in mice (Gale *et al.*, 1998).

Currently, it is generally believed that at each step of the infection process, different combinations of fungal components can play a predominant role (Figure 5). Therefore, the expression of virulence attributes is related not only to time and stage of the infection, but also to host tissues and even to host species.

The most studied *C. albicans* virulence factors are: adherence, dimorphism, phenotypic switching, and secretion hydrolytic enzymes such as aspartyl proteinases, lipases and phopholipases.

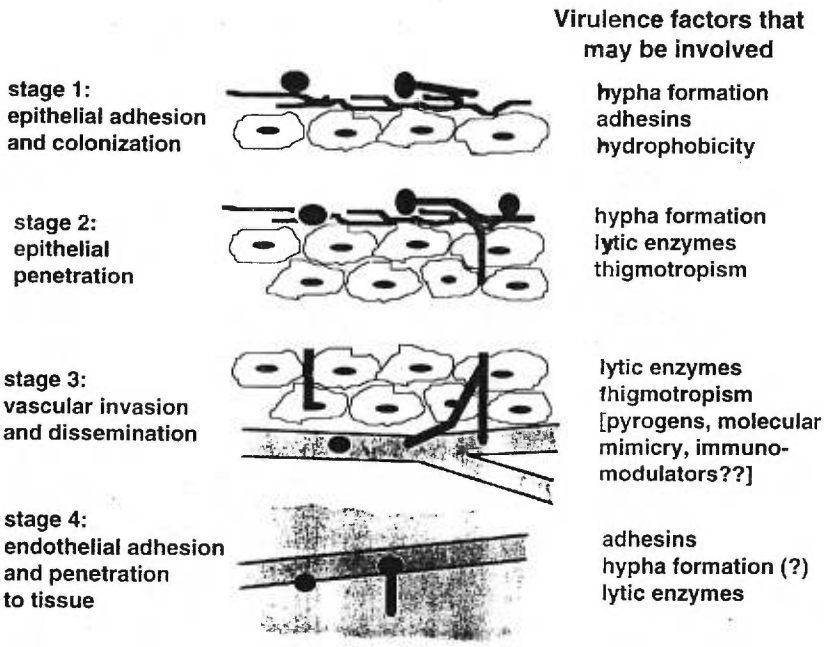

Figure 5. Stages of *Candida* infections (Odds, 1994).

2.3.1 Adherence

The first step in an infectious disease, whether colonization only or entry into deeper host tissues, requires a special ability to bind to the host cells. In this regard, clinical and experimental evidence point towards the adherence properties of *C. albicans* as an important virulence factor. The most pathogenic *Candida* species exhibit higher *in vitro* adhesion to epithelial cells and plastic surfaces than less pathogenic species (Calderone, 1993). Similarly, strains isolated from AIDS patients had significantly increased adherence *in vitro*, compared to those from normal individuals (Pereiro *et al.*, 1997). Moreover, strains with reduced *in vitro* adherence properties, because of growth conditions or mutations were less pathogenic in experimental infections (Calderone & Braun, 1991).

C. albicans binds to a variety of biological surfaces such as epithelial and endothelial cells, fibronectin, fibrin-platelet matrix, neutrophils, bacteria, and non-biological surfaces such as polystyrene, denture acrylic and Teflon. Although a combination of mechanisms such as nonspecific adhesion, coadhesion with bacteria and adhesin-receptor interactions is likely to operate *in vivo*, most of the available evidence indicates the primary importance of the latter. In this regard, experimental data indicate that *Candida* adhesins are mannoproteins which recognize either fucosyl or glucosamine glycosides on epithelial cells, or arginine-glycine-aspartic acid (RGD) containing peptides of endothelial cells or extracellular matrix proteins (reviewed in Fukazawa & Kaguya, 1997).

2.3.1.1 *Binding to host epithelial cells. In vitro* adhesion of many strains of *C. albicans* to buccal epithelial cells (BECs) or vaginal epithelial cells (VECs)

involves lectin-like interactions between the protein portion of a mannoprotein adhesin and a glycoside receptor on the host cell surface (Tosh & Douglas, 1992). Preparations of crude and purified adhesins bound to fucosyl or N-acetyl glucosamine-containing receptors, extracted from BECs. The adhesin contains a 15.7-kDa component, which represents the receptor-binding domain of a mannoprotein (Cameron & Douglas, 1996). Recently, a *C. albicans* fimbrial lectin-adhesin, which mediates adherence to BECs via asialo-GM_1 and asialo-GM_2 gangliosides, was purified and partially characterized (Yu *et al.,* 1994a,b). The fimbriae bound to BECs and to synthetic βGalNAc(1-4)-βGal-protein conjugates in a saturable and concentration-dependent manner. In addition, *C albicans* fimbriae were shown to share antigenic epitopes with *Pseudomonas aeruginosa* pili (Yu *et al.*, 1996), and antibodies raised against it were able to block adhesion of *C. albicans* to BECs (Lee *et al.*, 1996). The existence of additional fibrillar/fimbrial adhesins with different lectin-like specificities was suggested by Douglas *et al.* (1995).

The oligosaccharide portion of the mannoprotein may also participate in attachment to host cells. Mutants lacking antigenic factor 6 showed decreased ability to adhere to both exfoliated buccal epithelial cells and a human cell line (mouth squamous carcinoma cells, Miyakawa *et al*, 1992).

2.3.1.2 *Binding to extracellular matrix proteins (ECM) and complement factors.* Susceptibility of a host to *C. albicans* partly depends on the epithelial damage that leads to exposure of the subendothelial matrices. There are two major types of extracellular matrices (ECM): interstitium, which contains fibronectin and several types of collagen, and basement membrane

containing mainly laminin and type IV collagen. *C. albicans* recognizes ECM proteins and use them to adhere to capillary endothelium or to subendothelial basement membrane, which may be important in the initiation of hematogenous candidiasis.

In vitro studies have demonstrated that *C. albicans* blastoconidia adhere avidly to fibronectin (Skerl *et al.*, 1984), and type IV collagen, laminin and fibronectin coated on plastic (Klotz, 1990). Adherence was inhibited with antibodies to fibronectin and laminin and the expression of laminin and fibronectin-binding protein in blastoconidia has been showed in immunochemical analysis (Klotz, 1990; López-Ribot *et al.*, 1996). Binding of ^{125}I-fibronectin to blastoconidia was inhibited by unlabelled human fibronectin and by Arg-Gly-Asp (RGD), Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) and Gly-Arg-Gly-Asp-Thr-Pro (GRGDTP) peptides (Klotz & Smith, 1991). Klotz *et al.* (1992) have treated rabbits with RGD peptides immediately before inoculating them with *Candida*. Rabbits that received the RGD peptides had significant lower colony counts in their kidneys compared with control rabbits.

Recognition of RGD sequences is mediated by the mannoprotein adhesins, CR2 and CR3, which are integrin analogues that mimic complement receptors of mammalian cells (Hostetter, 1994). The complement receptors (CR) of mammalian cells recognize the complement C3 conversion products C3d (CR2) or iC3b (CR3). The key role of *C. albicans* iC3b (CR3) in disseminated infection is deduced by the fact that *C. albicans* strains with reduced ability to recognize the CR2 ligand, C3d, were avirulent in murine model of disseminated candidiasis (Franzke *et al.*, 1993). Similarly, significant increased survival time and rates of mice infected with

C. albicans were obtained when they were treated with anti-iC3b monoclonal antibodies (Lee *et al.*, 1997). Along with ECM proteins, *C. albicans* CR2 and CR3 also bind to fibrinogen and fibrin. This ability could promote *C. albicans* attachment to prosthesis material or to catheters, which are coated by fibrin after their introduction into the bloodstream (Kennedy *et al*, 1992). Additionally, the noncovalent binding of iC3b may allow *C. albicans* to masquerade as a neutrophill and in consequence to evade phagocytosis.

Adherence to ECM may be also mediated by the product of the recently cloned agglutinin-like adhesin gene (*ALA*1) (Gaur & Klotz, 1997). Further characterization of the protein and the gene is currently in progress.

2.3.2 Dimorphism

Several dimorphic and pathogenic fungi such *Histoplasma capsulatum*, *Blastomyces dermatitidis,* or *Paracoccidioides brasiliensis* exhibit yeast development *in vivo* and a hyphal phase under saprophytic conditions. However, *C. albicans,* which is also a dimorphic fungus, show both yeast and hyphal morphologies in infected tissues (Odds, 1988).

Early observations indicated that yeast cells inoculated to animals rapidly transform to hyphae and that phagocytic cells were unable to ingest hyphae (Young, 1958), leading to the dogma that the hyphal forms were the infective elements of the fungus. Subsequently, evidence was presented suggesting increased virulence of the yeast form over hyphae (Simonetti & Strippoli, 1973; Evans, 1980). Shepherd (1985) compared virulence of two yeast and hyphal morphological mutants. In a disseminated murine model of candidiasis, both mutants retained their original phenotypes *in vivo* and could adhere to, invade and proliferate in soft tissues, causing systemic

infection. It thus became evident that both the yeast and hyphal forms of *C. albicans* are able to cause disease, although both forms are usually found together and either form may be associated with health or disease. Indeed, in some infections, such as HIV-associated erythematous candidiasis, yeast forms may predominate. More direct evidence implicating hyphal formation in virulence came from experiments in which strains carrying deletions of genes involved in signaling cascades, *CaCLA*4 and *CCP*1, showed defects in hyphal growth that resulted in reduced virulence in systemic infection (Leberer *et al.*, 1997; Csank *et al.*, 1997).

The kinetics of destruction of skeletal muscle fibers by the hyphal form of *C. albicans* has been obtained by using light and electron microscopy. Following subcutaneous inoculation of *C. albicans* into the footpad of mice, blastospores germinated within 2 h. Subsequently, hyphae were seen penetrating into the adjacent striated muscle fibers and destroying the collagen bundles of the interstitium (Ashman *et al.*, 1995). Thus, in the initial stage of infection the formation of hyphae may promote the adherence and penetration of host tissues, and subsequently, the expression of cell surface antigens that appears to be coordinately regulated, may enable hyphae to attach firmly while penetration of host tissues is facilitated by the secretion of hydrolytic enzymes.

2.3.3 Phenotypic switching

In order to escape the immune system, several pathogenic microorganisms can switch at extremely high frequencies between two or more alternative phenotypes. For an opportunistic pathogen such as

Candida species, switching may be a general strategy for adaptation of the fungus to host microniches.

Clinical findings suggest that switching may be involved in the selection of phenotypically different strains. Switching has been demonstrated at sites of commensalism and infection, and between recurrent episodes of vaginal candidiasis (Soll *et al.*, 1987; Soll *et al.*, 1989; Hellstein *et al.*, 1993). Similarly, the frequency of switching also correlates with the apparent virulence of the infective strains (Jones *et al.*, 1994). In AIDS patients in particular, it has been shown that the same genetic strain persisted in the majority of HIV-infected individuals, but the phenotypic expression of this strain changed with time (McCullough *et al.*, 1994).

In the well-studied white-opaque (W-O) transition system, switching not only involved changes in colony and cellular morphology but also altered cell surface antigenicity and adherence properties (Kennedy *et al.*, 1988; Anderson *et al.*, 1990), consistent with a role for variable surface molecules in tissue invasion and localization. Several other characteristics, such as proteinase secretion, bud-hypha transition, lipid and sterol content, sensitivity to neutrophils and oxidants, susceptibility to antifungal agents, which are believed to play a role in survival and pathogenicity, are also affected during switching (reviewed in Soll, 1997).

Overall, the recent data indicate that switching mechanisms may potentiate the pathogenic abilities of *C. albicans* by i) enhancing invasion and proliferation in extremely different body environments, ii) evasion of the immune system by changing surface antigenicity, which may lead to eventual exhaustion of host defenses, iii) enhancing adhesion to mucosa,

tissue penetration and secretion enzymes such as proteinases and iv) escaping antifungal treatment.

2.3.4 Secreted aspartyl proteinases (Saps)

Aspartyl proteinases are a distinct group of endoproteolytic enzymes that are found widely in eukaryotes from fungi to higher plants and mammals. Mammalian aspartyl proteinases have been isolated from such organs as stomach (chymosin, gastricin, pepsin), kidney (renin) and from cellular compartments such as lysozomes (cathepsin D) (Tang & Wong, 1988). In the vacuoles of the yeast *S. cerevisiae*, an aspartic proteinase is produced that is involved in the processing of vacuolar proteins to their mature forms (Ammerer *et al.*, 1986). Several retroviruses, including HIV isolates, have been found to code for an aspartyl proteinase that is essential for proteolytic processing of viral polypeptides and viral infectivity (Hansen *et al.*, 1988). Fungi from such diverse genera as *Mucor*, *Aspergillus*, *Endothia*, *Rhizopus*, *Penicillium* (Ward & Kodama, 1991) and *Candida* (Rüchel, 1981) produce aspartyl proteinases.

2.3.4.1 *Biochemical properties of Saps*. Characteristically, aspartyl proteinases function at low pH, hence the initial name of *acid protease* was derived. The activity of an individual aspartyl proteinase is defined by its interaction with specific protease inhibitors. Pepstatin A acts as a substrate analogue by binding tightly to aspartyl proteinases in a 1:1 molar ratio. It is an N-acylpentapeptide (isovaleryl-Val-Val-Sta-Ala-Sta, where *Sta* represents satatine, 4-amino-3-hydroxy-6-methyl-heptanoic acid) isolated from *Streptomyces lignacolum*. Two active-site affinity labels, diazoacetyl

norleucine methyl ester (DAN) and 1,2-epoxy-3-(p-nitrophenoxy) propane (EPNP), are also used to characterize the catalytic residues of aspartyl proteinases (reviewed in Kay & Dunn, 1992). These affinity labels react with the side-chain carboxyl group of aspartyl acid residues 215 and 32 (porcine pepsin numbering system), respectively, rendering the enzyme inactive. These data indicate that aspartic acid residues 215 and 32 function in the catalytic mechanism, hence, the concise name of *aspartyl proteinase* was derived.

Fungal aspartyl proteinases are highly homologous, regardless of the source. Analysis of the primary structure reveals areas of strong amino acid sequence homology, especially in and around the active-site residues. Comparison of the tertiary structures also shows strong similarities between species. The three-dimensional structures of endothiapepsin (Blundell *et al.*, 1990), rhizopuspepsin (Suguna *et al.*, 1987), porcine pepsin (Sielecki *et al.*, 1990) and *Candida* Sap (Cutfield *et al.*, 1993; 1995) have been determined by X-ray crystallography. Generally, each molecule consists of approximately 320-360 amino acids. These are arranged into a secondary structure consisting in primarily of a 18- to 20-strand β-pleated sheet structure that twists in an anticlockwise direction, forming a globular-shaped protein with symmetrical lobes. Residing between these two lobes is an extended binding cleft, capable of accomodating the substrate. Near the center of this pocket lie the two active aspartic residues 215 and 32. Each lobe contains one of the aspartic acid residues, which protrudes into the substrate binding cleft. The substrate-binding site is capable of interacting with up to seven residues of the substrate.

2.3.4.2 *Saps from Candida spp.* The proteinases have been detected in isolates of *C. albicans*, *C. tropicalis*, *C. parapsilosis* (Staib, 1965; Rüchel, 1981; Rüchel *et al*, 1983), *C. rugosa*, *C. lusitaniae*, *C. lipolytica* (Banerjee, 1991) and *C. pulcherrima* (Gotoh *et al.*, 1995).

Studies of *C. albicans* secreted proteolytic activity have now revealed that there are at least nine Sap isoezymes (EC 3.4.23.6) (Hube, 1996). The *SAP* genes are referred to as *SAP1* (Hube *et al.*, 1991), *SAP2* (Wright *et al.*, 1992), *SAP3* (White *et al*, 1993), *SAP4* (Miyasaki *et al.*, 1994), *SAP5*, *SAP6*, *SAP7* (Monod *et al*, 1994) and the newly identified *SAP8* and *SAP*9 (Genbank AF043330 and AF043331, respectively, 1997). Some members of this multigene family were identified by screening a genomic library with a *SAP1* probe under low-stringency hybridization conditions (Monod *et al.*, 1994). Comparison of the cloned sequences of *SAP2-SAP7* shows homology of 50-77% to *SAP1* (Monod *et al.*, 1994). The genes are located on different chromosomes: *SAP1*, *SAP4-SAP6* on chromosome 6, *SAP2* on chromosome R, *SAP3*, *SAP*8 and *SAP*9 on chromosome 3 and *SAP7* on chromosome 1. Northern and Western blot analysis indicate that the expression of a particular gene and its protein product is regulated differentially, depending not only on the strain, phase of growth and morphology but also on the composition of the medium (Hube *et al.*, 1994). Expression of *SAP1* (originally called *PEP1*) and *SAP3* have been detected predominantly during the growth of the opaque, but not white phenotype of the strain WO-1 (Morrow *et al.*, 1992; Hube *et al.*, 1994), while *SAP2* encodes the major *SAP* mRNA in the yeast form of most clinical and laboratory strains when they grow in media containing protein as sole nitrogen source. The levels are not affected by temperature or carbon

sources and are maximal during mid-log growth. Finally, *SAP4-6* are detected during hyphal formation at neutral pH (Hube *et al.*, 1994; White & Agabian, 1995). PCR amplification of common regions of *SAP*s has been recently used to develop a rapid diagnostic test of candidemia (Flahaut *et al.*, 1998).

SAP genes from other *Candida* species have also been cloned. These include one gene from *C. tropicalis* (*SAPT1*, Togni *et al.*, 1991) and two tandemly linked genes from *C. parapsilosis* (*SAPP1*, *SAPP2*, DeViragh *et al.*, 1993).

Candida Saps contain amino-terminal extentions of approximately 60 amino acids that are not found in the mature forms of the enzymes. This extention is composed of two regions: (a) a signal peptide of 14-21 amino acids, which aids in the secretion of the enzyme and (b) a 40-50 amino acids propeptide, which is thought to stabilize the enzyme in an inactive conformation (zymogen or proenzyme) until it is "activated" by proteolytic processing (Togni *et al.*, 1996). They are separated from mature protein by Lys-Arg dipeptides, which represent the cleavage site for protein processing.

Although four Sap isoenzymes have been isolated to date from *C. albicans*, only one gene product, Sap2p, has been characterized in detail. It has a Mr of 42-kDa (Wright *et al*, 1992), an optimal pH range of 3.0-5.5 (Ray *et al.*, 1991) using bovine serum albumin as substrate and is denatured at alkaline pH (Wagner *et al.*, 1995). Intracellular forms of 54-kDa (Banerjee *et al.*, 1991) or 45-kDa (Homma *et al.*, 1992) have been detected and they may be the precursor of the mature proteinase. Epitope mapping of Sap2p derived six antigenic sequences some of which showed close homology to other *Candida* Saps. Only the sequences GVSIKN and PVDKCQ were

shown to be unique to Sap2p (Ghadjari *et al.*, 1997). However, the antibodies raised against them were ineffective to prevent *Candida* infections in murine models of candididasis. The main properties of *Candida* Sap isoenzymes are summarized in Table 1.

2.3.4.3 *Role of Saps in pathogenesis.* Although no formal proof has been presented regarding the role of Saps in pathogenesis, the following findings point out their involvement in colonization and invasion. i) Aspartyl proteinases are highly secreted by the most pathogenic *Candida* species *C. albicans*, *C. tropicalis* and *C. parapsilosis* (Rüchel *et al.*, 1983). ii) Sap-secreting strains possess enhanced adherence to buccal epithelial cells and human keratinocytes, and this phenomenon is partially inhibited by pepstatin A in a dose-dependent manner (Borg & Rüchel, 1988; Ollert *et al.*, 1993). Similarly, pretreatment of neutropenic mice with pepstatin A resulted in a dose-dependent protection against intranasal challenge with *C. albicans* (Fallon *et al.*, 1997). iii) Sap antigens have been detected in mucosal specimens from cases of oral thrush and vaginitis (Borg & Rüchel, 1988; De Bernardis *et al.*, 1990), and demonstrated on fungal elements in tissue specimens from cases of deep-seated candidiasis (Rüchel *et al.*, 1991). iv) Evidence of *in vivo SAP*1 and *SAP*2 expression and active proteinase secretion was demonstrated during early stages of experimental vaginitis (De Bernardis *et al.*, 1995; Stringaro *et al.*, 1997). v) *In vitro* proteolytic degradation of host proteins such as epithelial keratin (Negi *et al.*, 1984), dermal collagen (Kaminishi *et al.*, 1986), immunoglobulins (Rüchel, 1986), and subendothelial extracellular matrix (Morschhauser *et al.*, 1997) by Saps, suggest their potential role in facilitating cell damage and tissue invasion.

Table 1. Characteristics of Sap isoenzymes

Isoenzyme gene	Protein (kDa)	pIs	Cell type	Culture conditions
*SAP*1	40	4.0	O, yeast[a]	BSA/37° C
*SAP*2	42	4.3	W, O, yeast[a]	BSA/37° C
*SAP*3	41	5.7	W, O, yeast[a]	BSA/37° C
*SAP*4	n.d	n.d	Hyphae	Serum, pH 7
*SAP*5	n.d	n.d	Hyphae	Serum, pH 7
*SAP*6	n.d	n.d	Hyphae	Serum, pH 7
*SAP*7	n.d	n.d	n.d	n.d
*SAP*8	41	n.d	yeast	BSA/25° C
*SAP*9	n.d	n.d	n.d	n.d

[a]: yeast form of most clinical isolates

W, O: white and opaque forms, respectively

n.d: not determined

This table is adapted from White *et al.*, 1995.

vi) Several independent investigators have shown that proteinase-deficient mutants obtained by natural selection or chemical or UV mutagenesis (Macdonald and Odds, 1983; Crandall and Edwards, 1987; Kwon-Chung *et al.*, 1985; Ross *et al.*, 1990), were less lethal to mice than parent strains. These studies, although supporting the hypothesis that proteinase activity is needed for pathogenicity, were not conclusive because the mutant strains may be deficient in more than just proteinase, either because multiple mutations were present or because the yeast had a general rather than a specific defect. More recently, *C. albicans* strains harbouring knockout mutations in *SAP* genes were obtained (Hube *et al.*, 1997; Sanglard *et al.*, 1997). When tested in murine and guinea pig models of systemic candidiasis, the virulence of *C. albicans sap*1, *sap*2, *sap*3 mutants and *sap*4,5,6 triple homozygous null mutants was modestly but significantly attenuated (Hube *et al.*, 1997; Sanglard *et al.*, 1997). Indeed, when their adhesiveness was examined on poly-L-lysine-coated glass and Matrigel, *sap*4,5,6 mutants showed reduced binding (Watts *et al.*, 1998a). These results suggested that none of the *SAP* genes investigated are single dominant virulence factors in systemic infections, but yet virulence was the result of the concerted action of a multiplicity of genes encoding virulence and growth factors.

2.3.5 Lipases and phospholipases

Extracellular lipolytic activity (lipases and phospholipases) of *C. albicans* were detected as early as 1967 (Costa, 1967). Later on, Ogawa and co-workers (1992) measured lipase activity from 85 clinical isolates grown in the presence of Tween 80. They found that the most pathogenic

Candida species, *C. albicans*, *C. tropicalis* and *C. parapsilosis* had the highest levels of lipase activity. Recent genetic analysis allowed the cloning and characterization of the first *C. albicans* lipase gene (*LIP*1, Fu *et al.*, 1997). It is part of a gene family, also present in *C. tropicalis*, *C. parapsilosis* and *C. krusei*.

Phospholipase activity is detected by growing the yeast in the presence of egg yolk and lecithin. Data indicate that *C. albicans* secretes phospholipase A (Barrett-Bee *et al.*, 1985), phospholipase B (Banno *et al.*, 1985) and phospholipase C (Pugh & Cawson, 1977), whose gene was recently characterized (Bennett *et al.*, 1998), as well as lysophopholipase and lysophospholipase-transacylases (Takahashi *et al.*, 1991; Mirbod *et al.*, 1995). Support of their role in pathogenesis is based primarily on findings that indicated that i) the enzymes are expressed in the tips of fungal hyphae of *C. albicans* cells invading chick embryonic membranes (Pugh & Cawson, 1977), ii) higher phospholipase activity correlates with higher adherence to buccal epithelial cells (Barrett-Bee *et al.*, 1985), iii) *C. albicans* blood and invasive isolates produced significantly higher phospholipase activity than commensal (oral) and non-invasive strains, and enzyme levels had predictive value regarding mortality in a mouse model of disseminated candidiasis (Ibrahim *et al.*, 1995), and iv) higher mortality rates were found in mice inoculated with high-phospholipase secretors. Phospholipase activity also correlated with the involvement of kidneys in experimental invasive candidiasis (Kothavade & Panthaki, 1998).

Extracellular phospholipases may contribute to pathogenicity by lysing host cells or changing their surface in order to facilitate adherence and penetration.

2.3.6 Other putative virulence factors

2.3.6.1 *Thigmotropism.* The ability of *C. albicans* to sense surface topography is called contact guidance or thigmotropism. Hyphae of *C. albicans* followed grooves and ridges on various artificial membranes and penetrated pores of Nucleopore filters (Sherwood *et al.*, 1992). Therefore, it has been suggested that this attribute may allow the hyphae to penetrate mucosal surfaces through intercellular junctions and membrane invaginations. The thigmotrophic response is attenuated by gadolinium ions ($GdCl_3$) and other inhibitors of calcium channels such as verapamil, suggesting that calcium uptake may be involved in thigmotropic regulation (Watts *et al.*, 1998b). Similarly, Perera *et al.*, 1997 have shown thigmotropic behaviour of hypha of the dermatophytes *Epidermophyton floccosum*, *Microsporum canis* and *Trichophyton mentagrophytes*.

2.3.6.2 *Surface hydrophobicity.* Cell surface hydrophobicity (CSH) of *C. albicans* may play a role in *Candida* virulence and pathogenicity, since hydrophobic cells were more virulent in an animal model (Antley & Hazen, 1988). Similarly, a polyclonal antiserum against yeast hydrophobic proteins recognized them exposed on fungal cells present in host tissues (Glee *et al.*, 1995). Furthermore, hydrophobic cells have increased binding to several ECM proteins compared to hydrophilic cells, and this ability may be a contributor for their enhanced virulence (Silva *et al.*, 1995).

3. *CANDIDA* INFECTIONS

3.1 Epidemiology of *Candida* infections

3.1.1 Incidence

Several surveys have documented increased rates of fungal, and particularly *Candida* infections over the past decade and the highest rates were recorded as hospital-acquired infections. According to the Centers for Disease Control and Prevention, *Candida* emerged between 1980 and 1990 as the sixth most common nosocomial pathogen and was the fourth most common pathogen isolated from bloodstream infections (Beck-Saguè & Jarvis, 1993). Furthermore, this tendency has been maintained from 1990 through 1992, as suggested by analysis of reports from National Nosocomial Infection Surveillance System (NNIS) (Emori & Gaynes, 1993). The reasons for this increase may be found in the advances of medical and surgical therapy as well as the AIDS pandemia, that have resulted in the proliferation of a severely ill, immunocompromised, hospitalized patient population (Fridkin & Jarvis, 1996).

3.1.2 Sources

Candida species have been recovered from soil, inanimate objects, food, the hospital environment and humans. The most common natural habitat of pathogenic *Candida* species appears to be the surface of the human body exposed to the environment. Thus, they are part of the normal flora of the buccal and vaginal mucosa and the gastrointestinal tract.

In the oral cavity, the recovery of *C. albicans* increases from 5.7% immediately after birth to 14.2% after 7 days and by 4 weeks of age, the oral yeast carriage rate is 82% (Russell & Lay, 1973). The frequency of yeast recovery from clinically normal mouths in healthly adults ranges from 10 to 50% (Ghannoum & Radwan, 1990).

In the vagina, *Candida* species and mainly *C. albicans* are found in 4 to 27% of normal, non-pregnant women; however, these values increased to 7.8-67.8% both during pregnancy and in the presence of local predisposing factors (Odds, 1988).

The gastrointestinal tract is the major habitat for commensal *Candida* spp. Their concentration varies considerably in different parts of the tract. The frequency of isolation of *C. albicans* increased progressively from 30% the oropharynx, 54% in the jejunum, 55% in the ileum and to 65% in the fecal specimens (Ghannoum & Radwan, 1990).

3.1.3 Transmission

Although most *Candida* infections are thought to be endogenously acquired, exogenous transmission may exist. Evidence supporting person-to-person transmission came from the observation that the main source of yeasts which colonize the mouths of neonates is the mother's birth canal. It is also well known that *Candida* can be sexually transmitted. In addition, in some hospital areas such as burn and intensive care units, molecular epidemiological studies have documented cases of cross-infection (Richet *et al* ., 1991; Schimid *et al.,* 1995; Vasquez *et al* ., 1993).

Transmission may also occur by inanimate objects (Ruiz-Diez *et al.*, 1997). Since some hospital apparatus such as cardiac and renal dialysis

machines are difficult to sterilize and are in direct contact with the bloodstream of patients, they represent a potential source of transmission. Similarly, parenteral nutrition, intravascular devices and intravenous drugs are also significant risk factors for candidiasis.

After *C. albicans*, *C. tropicalis* is the next most common species involved in both oral and systemic infections. *C. parapsilosis* infections has been associated with total parenteral nutrition, endocarditis in cardiac surgery patients and intravenous drug use (Weems, 1992). In severely immunocompromised patients, *C. krusei* has been implicated in fungemia and endophthalmitis; the mechanism of infection appears to be endogenous (Wingard *et al.*, 1991). In addition, there are reports of invasive *C. lusitaniae* infections, and the source was the colonizing flora of the gastrointestinal tract (Sanchez *et al.*, 1992).

3.1.4 Predisposing factors

The conditions that predispose to the development of candidiasis are summarized in Table 2.

3.2 Oropharyngeal candidiasis

Oral candidiasis presents clinically in many forms. It results from yeast growth and penetration of the oral tissues when the host physical and immunological defenses have been undermined. A recent classification of oral *Candida* infections is presented in Table 3.

The condition is common among patients undergoing cytotoxic chemotherapy and in AIDS patients. Two forms are frequently seen in HIV-infected individuals: acute pseudomembranous and chronic erythematous

Table 2. Factors predisposing to candidiasis

Cutaneous and mucosal candidiasis	Systemic candidiasis
Physiologic Pregnancy Old age Infancy	Iatrogenic Immunosuppression Organ transplantation Surgery Steroid treatment Cytotoxic drugs Broad-spectrum antibiotics Catheters Hyperalimentation Artificial heart valves
Traumatic Maceration Burns Other infection	
Hematologic Cellular immunodeficiency Acquired immunodeficiency syndrome (AIDS)	Hematologic Chronic granulomatous disease Aplastic anemia Agranulocytosis Leukemia, lymphoma, Hodgkin's disease
Endocrine Diabetes mellitus	
Iatrogenic Broad-spectrum antibiotics Birth control pill	Other Malnutrition Trauma Intravenous drug abuse Peritoneal dialysis

This table is adapted from Joklik *et al.*, 1992

Table 3. Clinical classification of oral candidiasis

Acute types:
Pseudomembranous
Erythematous
Chronic types:
Pseudomembranous
Erythematous
Plaque-like
Nodular
Candida-associated lesions:
Angular cheilitis
Denture stomatitis
Median rhomboid glossitis

From Holmstrup & Axéll, 1990.

candidiasis. Oral candidiasis is more frequent in late HIV-infection and is predictive for progression to AIDS (Katz, 1991; Katz *et al*, 1993). *C. albicans* is the most common cause of oral infections, although other species such as *C. krusei*, *C. tropicalis*, *C. parapsilosis* and *C. glabrata* may be also involved (Dronda *et al.*, 1996).

3.2.1 Acute pseudomembranous candidiasis (oral thrush)

Thrush occurs more frequently in newborn children and debilitated and elderly individuals. It is also associated with diseases such as diabetes mellitus and impairments of cell-mediated immunity. It is characterized by the presence on mucosae of semi-adherent, whitish, soft and creamy pseudomembranes, either localized or as confluent patches (Holmstrup & Axéll, 1990). The plaques when removed leave an erythematous and bleeding surface. They are composed of blastoconidia, hyphae, bacteria, desquamated epithelial cells, leukocytes, keratin and food debris. Sites of involvement are the buccal mucosa, gingiva and tongue with occasional extention to the pharynx or esophagus (Sofaer, 1990).

3.2.2 Acute erythematous candidiasis (median rhomboid glossitis)

This condition is usually associated with broad-spectrum antibiotic or corticosteroid treatment. It may arise as a consequence of acute pseudomembranous infection or it may develop *de novo*. The lesions are characterized by small or large erythematous areas, and although any part of the oral mucosae may be affected, the most common location is the dorsum of the tongue where it presents as a depapillated lesion. This is the only form of oral candidiasis which is consistently painful.

Both acute pseudomembranous and erythematous candidiasis are of short duration in contrast to chronic, long-standing conditions. However, if not treated they may last for months or even years, as seen in patients treated with corticosteroid aerosols, or HIV-infected individuals. In these cases the accurate term would be chronic pseudomembranous/erythematous candidiasis.

3.2.3 Chronic hyperplastic candidiasis (*Candida* leukoplakia)

This form of candidiasis has been associated with iron or folate deficiencies and defective T-cell immunity. It is characterized by firm, persistent, irregular whitish plaques with a surrounding margin of erythema on the buccal mucosa or tongue. Unlike oral thrush, the hyperplastic plaques cannot be removed easily. Although the classical presentation site is the retrocommissural area, in HIV-infected patients it is most often seen bilaterally on the mucosa (Odds, 1988).

3.2.4 Angular cheilitis (perlèche)

Prior to the HIV pandemia, these lesions were more frequently encountered in elderly individuals as a complication of denture-induced stomatitis. Perlèche is comprised of lesions which affect the angles of the mouth. It is characterized by erythema, fissuring, and maceration of the oral commisures from which *C. albicans* can be isolated (Ohman *et al.*, 1985).

3.2.5 Chronic atrophic candidiasis (*Candida*-associated denture stomatitis)

Commonly encountered in denture wearers, it is characterized by chronic erythema and edema of the mucosa of the palate that is in contact

with the dentures (Budtz-Jørgensen, 1990). There is no invasion of the epithelium.

3.3 Gastrointestinal candidiasis

The gastrointestinal tract (GI) is the main source of *Candida* spp. Yeast populations are maintained in the GI mucosa by i) adhesion to epithelium, ii) adhesion to mucus, iii) co-adhesion with adherent fungi or bacteria, and iv) entrapment in the mucus gel.

3.3.1 Esophageal candidiasis

Although esophageal candidiasis may arise as an extention of oral candidiasis, in most cases the esophagus is the only site involved. It is the most frequent gastrointestinal infection in HIV-infected patients and is also associated with hematological malignancies, corticosteroids, diabetes mellitus and preexisiting gastrointestinal pathology. The common symptoms include painful swallowing, a feeling of obstruction and substernal chest pain. Gastrointestinal bleeding, nausea, and vomiting are also seen (Rippon, 1988).

3.3.2 Gastric candidiasis

The stomach is the second most common site for *Candida* invasion after the esophagus. *Candida* gastritis has been reported following surgery of gastric ulcer and the use of cimetidine. It is believed that this drug encourages colonization by *Candida* spp by increasing the gastric pH. The infection resembles *Candida* esophagitis except that there is nearly always a

definable preexisting gastric lesion which is superinvaded by the fungus (Odds, 1988).

3.4 Systemic candidiasis

This pathology results from hematogenous spread of *Candida* and may involve any organ or tissue of the body. The main portal of entry is the GI tract. In fact, passage of yeast across the GI wall have been demonstrated in immunocompromised animals (de Repentigny *et al.*, 1992; Berg *et al.*, 1993). Candidemia is frequently associated with immunosuppression such as that associated with organ or bone marrow transplantation, and also with contaminated intravenous catheters.

Once in the bloodstream, *Candida* produces a clinical picture which is indistinguishable from bacteremia, with fever, leukocytosis and hypotension. One common clinical sign of deep tissue invasion is the appearence of white retinal *Candida* lesions (chorioretinitis). Other common sites of involvement include the kidneys, for which *Candida* has a predilection, the gastrointestinal tract, and the brain. The heart may also be a focus of infection following hematogenous spread, but the majority of cases of *Candida* endocarditis follow surgery for valve replacement (Swerdloff *et al.*, 1993).

3.5 Vaginal candidiasis

It is estimated that 75% of premenopausal women will experience at least one episode of vulvovaginal candidiasis in their lifetime (Sobel, 1988). Occasional vaginal candidiasis does not require a special predispositon of the host. However, about 5% of all women of childbearing age suffer from

recurrent infections. Local and specific immune deficiency may favour enhanced susceptibility to vaginal candidiasis. Prepubertal girls do not suffer *Candida* vaginitis unless chronic mucocutaneous candidiasis is present. Diabetes mellitus, antibiotic therapy, oral contraceptives and pregnancy predispose to vaginal candidiasis. *C. albicans* is the causal agent of 80 to 92% of cases of vulvovaginitis (Odds, 1988). However, because of the widespread use of antifungals, the frequency of other *Candida* species, particularly *C. glabrata* is increasing significantly (Horowitz *et al.,* 1992).

Candida vaginitis is characterized by inflammation of the vagina and the formation of a thick, yellow-white, milky discharge, and patches of gray-white pseudomembranes on the mucosa. The whole area is inflamed and pruritus and burning are also present (Rippon, 1988).

4. ANIMAL MODELS OF HUMAN CANDIDIASIS

4.1 Experimental oral candidiasis

Experimental oral candidiasis has been mainly induced in rats and mice. In most cases the infection is inapparent with no clinical symptoms or lesions.

Rat models developed in the 70's required pretreatment of the animals with antibacterial antibiotics prior to their inoculation (Jones & Russel, 1974). Subsequently, a model of candidal tongue infection was developed in untreated Sprague-Dawley rats (Allen *et al.*, 1989). The infection was characterized by clinically evident tongue lesions that were apparent 2 weeks post fungal inoculation. More recently, a model was devised in which the major salivary glands of the rats are removed in order

to favour *C. albicans* colonization (Totti *et al.*, 1996). Regarding murine models, a model of oral candidiasis has been described in which normal adult mice are inoculated by topical application of *C. albicans* blastoconidia and oral colonization is monitored by culturing saliva samples and digested oral mucosae (Lacasse *et al.*, 1990; 1993). In contrast to rats, mice do not carry *C. albicans* as part of their normal flora, and therefore this model is appropriate to distinguish between innate and acquired immune resistance mechanisms. In this model, the carrier state is associated with low persistent levels of *Candida* and with the development of delayed-type hypersensitivity, which mimics the situation in humans (Lacasse *et al.*, 1993; Chakir *et al.*, 1994). This model has proved useful to study oral colonization of *C. albicans* in MAIDS (Deslauriers *et al.*, 1997).

4.2 Experimental gastrointestinal and systemic candidiasis

The mouse is the most frequently used animal for developing models to study the evolution, course, and nature of systemic candidiasis obtained through oral inoculation. Several types of murine models have been developed. 1) Infection in healthy adult mice, which results in colonization of the GI tract and is not followed by dissemination (Samonis *et al* ., 1990). 2) Infection in newborn animals in which the microflora is not yet established and the immune system is not fully mature (Cole *et al* ., 1989; 1996). In this case, the inoculum is delivered by the oral intragastric route and 2-4 days later the keratinized epithelium of the neonatal gastric cardial-atrium fold became colonized. The appeareance of the gastric mucosa in untreated and treated mice is shown in Figure 6.

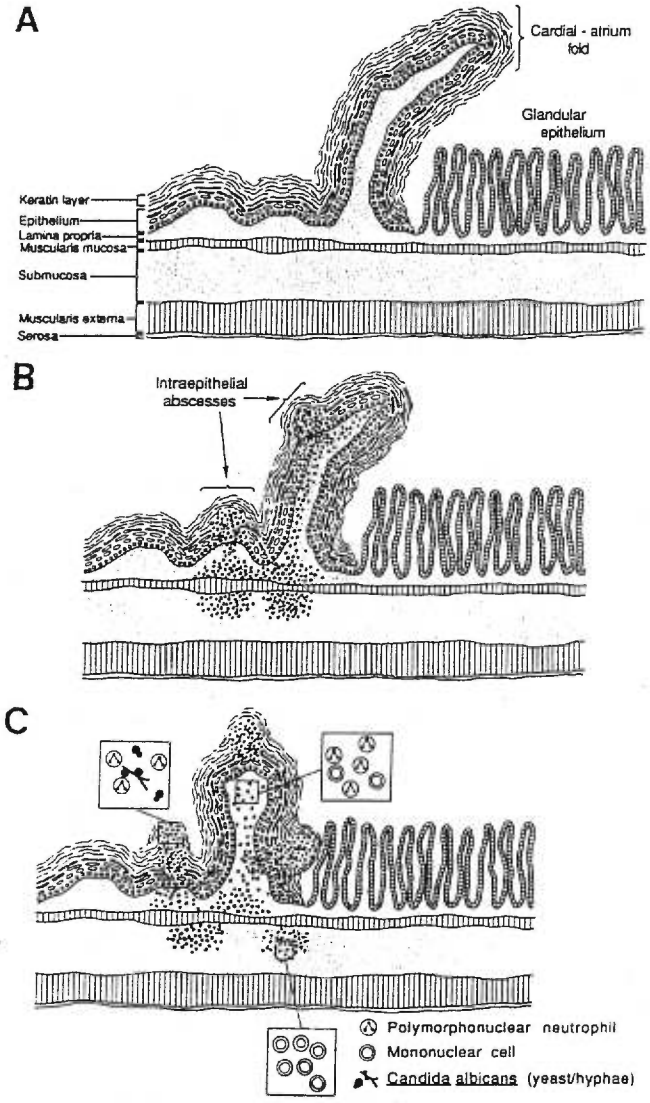

Figure 6. Histologic appearance of the gastric mucosa in the region of the cardial-atrium fold. A: Untreated mouse. **B**: 3 weeks after challenge with *C. albicans*. **C**: 5 weeks after inoculation (Cole *et al.*, 1996).

3) Infection in animals immunocompromised by irradiation, by treatment with cytotoxic and/or immunosuppressive agents (Cole *et al.*, 1989; de Repentigny *et al.*, 1992). This model attempts to simulate conditions of *Candidia* infections in neutropenic patients with acute leukemia. After inoculation by the oral-intragastric route, persistently colonized mice are immunocompromised by administration of cyclophosphamide and cortisone acetate. Systemic spread is demonstrated by culture of *C. albicans* in kidneys, liver, lungs and spleen. In addition to the murine models, nonimmunocompromised rabbits have been used to study esophageal *Candida* infections. The inoculum is administrated in drinking water and at given times, the esophagus is examined (Hoshika *et al.*, 1996).

Experimental systemic candidiasis has been induced in a variety of animals such as mice, rats, guinea pigs, rabbits and even monkeys. The models differ in the mode of induction of infection, the route of inoculum administration and the strains used. Nevertheless, the murine model is the most frequently used. The infection is induced by intraperitoneal (i.p.) or intravenous (i.v.) injections that reproduce hematogenous candidiasis. Endpoints include mortality, fungal burden in tissues (kidneys are the target organs), and the severity and extent of tissue damage. The mice may be normal, or with congenital or acquired immunosuppression such as severe combined immunodeficiency syndrome (SCID) (Bosma & Carroll, 1991), retrovirus-induced immunodeficiency syndrome (Cole *et al.*, 1992), or congenital complement factors deficiency (Cantorna & Balish, 1990). Frequently used inbred mouse strains have varying susceptibilities to candicidal infection. Thus, C57BL/6 mice are relatively resistant to intravenous challange, BALB/c mice have intermediate resistance, and

C3H/HeJ mice are highly susceptible to infection. Mouse strains A/J and DBA/2 are also frequently used host in experimental infections since they are deficient in the fifth component of complement (C5) (Ashman *et al.*, 1996).

4.3 Experimental vaginal candidiasis

Because constant estrus is required to establish vaginal *C. albicans* infections, most of the current available animal models use mice or rats ovariectomized or treated with estrogens. Following intravaginal inoculation of *C. albicans* to oophorectomized, estrogen-treated rats (De Bernardis *et al.*, 1990) or to estrogen-treated mice (Fidel *et al.*, 1993), animals develop a persistent long-lasting infection over several weeks, characterized by abundant hyphal elements and leukocytes in the vaginal fluid as well as blastospores adhering to the vaginal mucosa. These models have been successfully used to study the local immunoresponse to *Candida* infections. In addition, a murine model of *C. glabrata* vaginitis has been developed using nonobese diabetic (NOD) mice, which after intravaginal inoculation with clinical isolates of *C. glabrata* showed superficial invasion of the vaginal epithelium (Fidel *et al.*, 1996a).

5. ANTIFUNGAL AGENTS

5.1 Currently available drugs

The therapeutic options for treating fungal infections are limited to a relatively low number and structural variety of antifungal drugs (Figure 7).

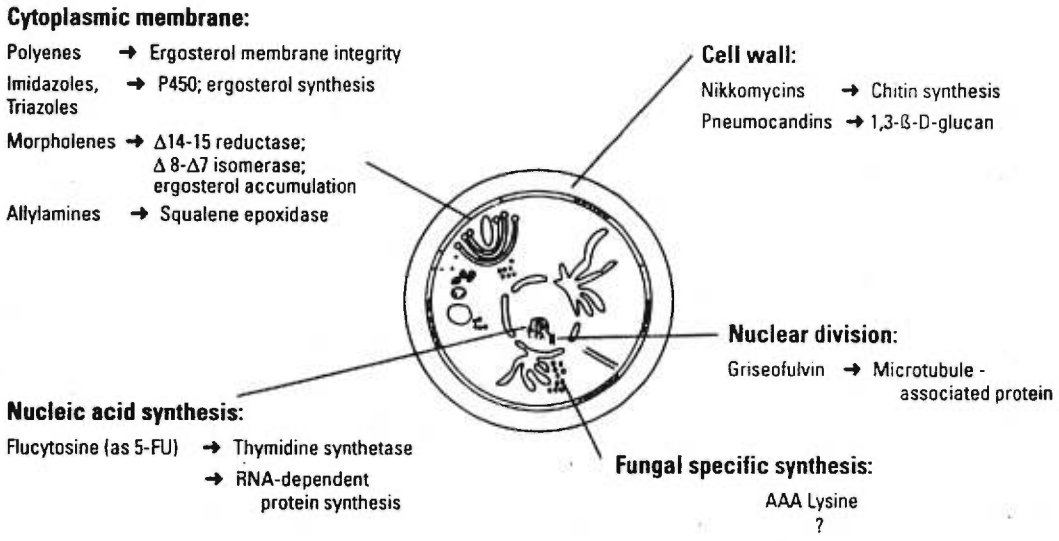

Figure 7. Antifungal agents and postulated mode of action

(Rippon & Fromtling, 1993).

In this regard, the most widely used antifungal agents against *Candida* infections comprise the polyene amphotericin B, the triazoles fluconazole and itraconazole, and the base analog 5-fluorocytosine (Como & Dismukes, 1994; Tuite, 1996).

The target of most of these drugs is the cell membrane. The polyene antifungals, amphotericin B (systemic use) and nystatin (topical use) bind in a fairly unselective way to ergosterol in the plasma membrane. This binding leads to an impairment of barrier function rendering the membrane permeable to protons (Kerridge, 1986). In addition, these drugs can also cause oxidative damage to the fungal cell membrane. The selectivity of the polyenes is based on their higher affinities to ergosterol than to cholesterol in the plasma membrane of mammalian cells. However, this differential effect is not absolute and results in toxicity which is the major limitation on their clinical use. The administration of amphotericin B within liposomes is a promising approach to overcome toxicity problems (Brajtburg & Bolard, 1996).

The azoles, imidazole and triazoles, also target the fungal cell membrane by an indirect mechanism. They specifically inhibit the cytochrome P-450-dependent fungal enzyme lanosterol 14α-demethylase, which results in blockage of the biosynthesis of membrane ergosterol and accumulation of abnormal lanosterol-like structures that cause leakage of cell constituents and finally cell death (Vanden Bossche, 1985). The first generation of imidazoles (clotrimazole, miconazole, and econazole) were not useful systemically. However, ketoconazole, a drug with higher hydrosolubility and decreased metabolism by liver enzymes was developed and proved to be useful in the treatment of systemic *Candida* infections

(Vanden Bossche *et al.*, 1987). More recently, the triazoles fluconazole and itraconazole have shown excellent clinical efficacy and are the most widely used systemic antifungal drugs. Decreased interaction with mammalian P-450 enzyme systems may be involved in their reduced toxicity. However, a high number of resistant *Candida* strains are emerging.

Among other molecular targets in antifungal therapy that have included direct interference with protein synthesis, only flucytosine (5-flucytosine) has been developed for clinical use. It is transported into fungal cells by cytosine permease and deaminated to 5-flourouracil (5-FU) by a cytosine deaminase. Subsequently, 5-FU is converted into 5-fluorouridine monophosphate (5-FUMP), which is a precursor of aberrant RNA that interferes with protein/DNA synthesis, and 5-fluorodeoxyuridine monophosphate (5-FdUMP) which is a potent inhibitor of thymidylate synthase and hence DNA synthesis (Vanden Bossche *et al.*, 1987). Acquisition of resistance to this antifungal is decreased by combination therapy with polyenes.

5.2 Resistance to antifungal drugs

The efficacy of the existing antifungals is threatened by selection of intrinsically resistant species and development of resistance in species usually regarded as susceptible. The extensive use of azoles for prophylaxis and treatment, as well as their administration in low doses, or lack of patient compliance have contributed for the selection of resistant strains.

Among the most important mechanisms of resistance to azoles are overproduction or mutations of the target enzyme, and reduction of

intracellular concentration of the drug through multidrug resistance efflux pumps.

Increased levels of mRNA from *ERG*16 (14α-demethylase) were found in *C. albicans* isolates from HIV patients (White, 1997). Furthermore, azole-resistant strains carrying a mutated cytochrome P-450 lanosterol 14α–demethylase have been recently isolated (Sanglard *et al.*, 1998). Some amino acid substitutions in the enzyme resulted in decrease in the affinity for azoles.

In addition, evidence suggests that efflux pumps in the cytoplasm or plasma membrane can lower azole drug concentrations in the cell (Parkinson *et al.*, 1995; Sanglard *et al.*, 1995). These pumps belong to two families, the ABC transporters and the Major Facilitators. The ABC transporters are pumps containing two nucleotide binding domains, also referred to as ATP Binding Cassettes and two membrane spanning domains that include a total of 12 transmembrane segments (Michaelis & Berkower, 1995). The Major Facilitator family also contains two membrane spanning domains which are not associated with nucleotide binding domains (Marger & Saier, 1993). Multidrug transporters of the ABC-type such *CDR*1 (Prasard *et al.*, 1995),*CDR*2 (Sanglard *et al.*, 1997) and *CDR*3 (Balan *et al.*, 1997) and Major Facilitator CaNDR1/Benr (Ben-Yaacov *et al.*, 1994), have been identified in *C. albicans*. Deletions in *CaMDR*1/*Benr*, *CDR*1 and *CDR*2 lead to hypersensitivity to many drugs, including the most important antifungals in clinical use and their overexpression has been observed in resistant clinical isolates (Sanglard *et al.*, 1995; 1996).

Resistance to flucytosine can result from several mechanisms, such as i) mutations in cytosine permease and/or deaminase, ii) mutations in

uracil:phosphoribosyl transferase, an enzyme involved in the synthesis of both 5-FdUMp and 5-FUTP, and iii) increases in de novo synthesis of pyrimidines (reviewed in Vanden Bossche *et al.*, 1994).

6. WORKING HYPOTHESIS

The observations that have been discussed in the previous sections clearly indicate that mucosal colonization and invasion by *C. albicans* require dynamic interactions with host tissues to enable the yeast to escape host clearance and cause infection.

Regarding the ability of *C. albicans* to spread hematogenously from the GI tract, we hypothesize that mucin-degrading enzymes may assist the yeast in this process, by degrading the protective mucus barrier, and allowing the yeast to gain access to, adhere to and, invade epithelial cells. This hypothesis is based on observations obtained from the neonatal mouse model of gastrointestinal candidiasis. One hour after oral-intragastric inoculation of mice with *C. albicans*, blastoconidia were seen within the mucus layer in the jejunum (Pope *et al.*, 1979), surrounded by apparent lysis of mucus (Cole *et al.*, 1988; Cole *et al.*, 1996).

6.1 Experimental objectives

The objectives of the project were to:

a) Isolate extracellular mucinolytic activity from *C. albicans*.

b) Characterize the extracellular mucinase enzyme.

c) Determine the role of the mucinase enzyme in the pathogenesis of mucosal and systemic candidiasis.

6.2 Experimental approach

A flowchart of the experimental approach of this project is shown in Figure 8. Briefly, as an initial step the ability of *C. albicans* blastoconidia to utilize mucin as the sole nitrogen and/or carbon source(s) was evaluated in media containing high molecular-weight hog gastric mucin. The culture supernatants were then subjected to sequential ultrafiltrations in order to remove the undigested mucin and to concentrate the secreted proteins. Subsequently, these extracellular proteins were biochemically characterized. Finally, in order to assess the role of the mucinase activity in the pathogenesis of candidiasis, the gene encoding for the mucinase enzyme was constitutively expressed in *C. albicans* and in the non-pathogenic yeast *S. cerevisiae.* The genetically modified strains were then tested for their virulence in murine models of mucosal and systemic candidiasis.

EXPERIMENTAL APPROACH

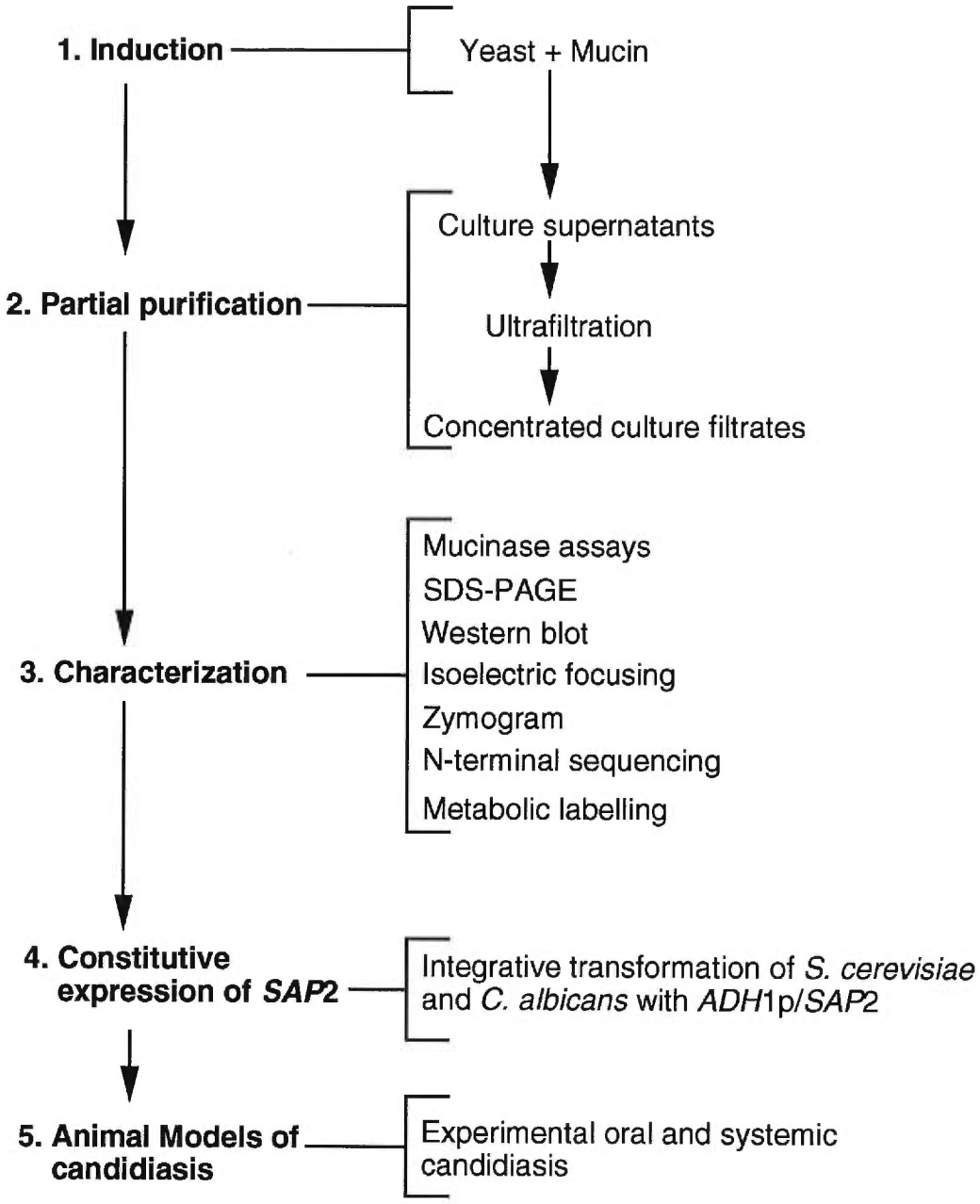

Figure 8. Flowchart of experimental approach for characterization and virulence assays of mucinolytic activity of *C. albicans*.

CHAPTER II

Development of a method to detect secretory mucinolytic activity from *Candida albicans*

Journal of Medical and Veterinary Mycology
34:401-406, 1996

Development of a method to detect secretory mucinolytic activity from *Candida albicans*

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Journal of Medical & Veterinary Mycology 1996, 34, 401–406 Accepted 20 May 1996

Development of a method to detect secretory mucinolytic activity from *Candida albicans*

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Ultrastructural examinations of sites where *Candida albicans* invaded the bowel wall after oral intragastric inoculation of infant mice suggested that blastoconidia are capable of progressive extracellular digestion of the intestinal mucus barrier. Microplate assay methods, based on biotin or digoxigenin-labelling systems, were therefore devised for quantitation of protease and glycosidase activities against the glycoprotein mucin. Labelled mucin was adsorbed on microplate wells, incubated with sample to be assayed for enzyme activity, and the remaining labelled mucin was quantitated by spectrophotometry. Proteolytic activity against mucin was demonstrated using concentrated culture filtrate of *C. albicans* strain LAM-1, grown in yeast nitrogen base medium containing mucin as sole nitrogen source. The activity was inhibited by boiling for 10 min or by incubation with the aspartyl proteinase inhibitor pepstatin A.

Introduction

Mucin is a high molecular weight glycoprotein which is the major component of the mucus overlaying the gastrointestinal epithelium. It plays an important role in protection against invasion by potential pathogens because of its rich and heterogeneous oligosaccharide composition and its ability to form a gel [1]. Many pathogenic microorganisms are known to exhibit either glycosidic or proteolytic activities against mucin, suggesting that these enzymes are involved in the breakdown of mucus and penetration of the mucin barrier. Mucinolytic activities have been described in *Shigella* spp. [2], *Pseudomonas maltophilia* [3], *Helicobacter pylori* [4,5], *Streptococcus intermedius* [6], *Bacteroides* spp. [7], *Yersinia enterocolitica* [8] and *Vibrio cholerae* [9–12].

It has been shown by transmission electron microscopy that *Candida albicans* can digest intestinal mucus after oral intragastric inoculation of infant mice [13]. *Candida* spp. are known to secrete aspartyl proteases (SAP) which can degrade a wide variety of substrates: epithelial keratin, dermal collagen, albumin, haemoglobin, immunoglobin heavy but not light chains, and extracellular matrix proteins [14]. Strong evidence of the role of hydrolytic enzymes in the virulence of *Candida* spp. has been provided for the SAP. However, no enzyme with mucinolytic activity has yet been described in *Candida*. Indeed, according to Borg & Rüchel [15], the action of SAP on mucin was restricted to nicking and became apparent only after treatment with detergent and reducing agent.

Methods used to evaluate mucinolytic activity have several limitations. Non-specific substrates such as casein or ovomucin [9,11,16] have the disadvantage of being chemically quite distinct from typical mammalian gastrointestinal mucins [12]. Cetyltrimethylammoniumbromide (CTAB) or protamine sulphate precipitation [2,9,10,16, 17] depends on monitoring of substrate breakdown by qualitative assessment of the precipitability of the macromolecule, and consequently provides no precise information about the nature or extent of degradation [12]. Other methods are relatively laborious and time consuming or show a lack of sensitivity, such as radiolabelled mucin monitoring [12,18], trinitrobenzene sulphonic acid (TBS) determination of amino residues [9,19], and mucin degradation profile monitoring by chromatography [4,5,7, 12,18,20] or by revelation of hydrolysis zones on Petri dishes [3].

The purpose of this study was to devise a quantitative, simple and rapid assay to evaluate mucinolytic activity in *C. albicans*. We used biotin- or digoxigenin-labelled mucin immobilized on a solid phase as substrate. The method

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described here allows the quantitation of protease as well as glycosidase activities against mucin and was able to detect mucinolytic activity in a *C. albicans* culture supernatant.

Materials and methods

Enzymes

Protease type XIV (Pronase E, from *Streptomyces griseus*) was obtained from Sigma (Sigma Chemical, St Louis, MO), neuraminidase (sialidase, from *Vibrio cholerae* (E.C. 3.2.1.18)) from Boehringer Mannheim (Mannheim, Germany) and endoglycosidase H (Endo H, from *Streptomyces plicatus*) from ICN Immunologicals (Costa Mesa, CA).

Reagents

Mucin type III, partially purified from porcine stomach, was obtained from Sigma. Biotin-ε-aminocaproic-*N*-hydroxysuccinimide ester (BNHS), D-biotinyl-ε-amidocaproic acid hydrazide (BACH), digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxy-succinimide ester (DIG-OSu), streptavidin-horseradish peroxidase (POD) conjugate, anti-DIG-POD Fab fragment and ABTS were obtained from Boehringer Mannheim. Intestinal mucin was isolated from 25 Balb/c mice (Charles River Canada, St Constant, Quebec, Canada) and purified by the method of Carlstedt *et al.* [21] as modified by J. F. Forstner (personal communication). The mice were fasted for 16–24 h and sacrificed by cervical dislocation. The small intestine was excised, flushed with a small volume of cold saline to remove luminal debris, and laid on a glass plate. The intestinal lumen was then exposed by making a longitudinal incision. After gently scraping the mucosa with a glass slide, the scrapings were weighed and transferred to 100 ml of a cold protease inhibitor mixture (pH 6·5) containing 6 M guanidine hydrochloride, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 5 mM *N*-ethylmaleimide. The scrapings were then homogenized twice for 25 s with a Sorvall Omni-Mixer (Sorvall, Newtown, CT) set at low speed, and stirred for 18 h at 4 °C. Any insoluble material was removed by centrifugation at 30 000 *g* for 30 min at 4 °C. The mucin contained in the supernatant was then purified by three CsCl density gradient ultracentrifugations as described by Carlstedt *et al.* [21].

Micro-organism and culture conditions

C. albicans strain LAM-1, originally isolated from the blood of a patient with systemic candidiasis [22] was graciously provided by Dr Noëlla Deslauriers, Université Laval, Ste-Foy, Québec and maintained on Sabouraud glucose agar. Yeast cells were first grown overnight in Sabouraud glucose broth at 37 °C, and used to inoculate a liquid culture containing yeast nitrogen base medium without amino acids and ammonium sulphate (Difco Laboratories, Detroit, MI), supplemented with 1% glucose and 0·2% mucin, at a density of 1×10^3 cells/ml. After incubation at 37 °C for 24 h with rotary agitation (240 rev min^{-1}), the cells (1×10^8/ml) were harvested at 1500 *g* for 10 min. Supernatants were filtered through a crossflow Sartocon-Micro unit (Sartorius AG, Goettingen, Germany) of 100 kDa exclusion limit, and the filtrate was concentrated 100-fold in a stirred cell device containing a 10 kDa exclusion limit ultrafiltration membrane (Amicon, Beverly, MA).

Enzyme assays

Petri dish assay

Agarose 1% was melted with mucin 0·3% in sodium phosphate buffer 0·1 M, pH 7·0 and poured in a Petri dish. Wells (diameter 6 mm) were cut out with the wide edge of a Pasteur pipet. Twenty microlitres of Pronase E solution in the same buffer was added to the wells and the dish incubated at 37 °C overnight. The dish was flooded with a solution of 0·1% Coomassie Blue R250 in 30% methanol and 7% acetic acid and left at room temperature for 3 h. To visualize the digestion of mucin, the dish was repeatedly flooded with a solution of 30% methanol and 7% acetic acid until a clear zone was observed.

Mucin-labelling procedure

Preparation of BNHS mucin was performed as follows. Mucin (0·6 g) was dissolved in 2 ml of PBS and mixed with a solution containing 1 mg of BNHS (freshly prepared in 50 μl of dimethylphormamide). The solution was incubated at room temperature for 3 h and then dialyzed overnight at 4 °C against PBS. Conjugates were stored at −20 °C.

BACH-labelled mucin was prepared by dissolving 0·4 g of mucin in 3 ml of 0·1 M sodium acetate, pH 5·5, which was oxidized in an ice bath for 30 min with 1 mM sodium periodate. The reaction was stopped by addition of 45 μl of 1 M glycerol (final concentration, 15 mM) and cooling for 5 min in an ice bath. Solutions were dialyzed for 3 h at room temperature against 0·1 M sodium acetate, pH 5·5. Oxidized mucin preparations were mixed with BACH (10 mM stock solution freshly prepared in dimethylsulphoxide) to give a final concentration of 1 mM and reacted with shaking for 2 h at room temperature. BACH-mucin was dialyzed against PBS overnight at 4 °C and stored at −20,°C.

In the DIG-OSu assay, a stock solution was prepared by dissolving 0·5 mg ml^{-1} of DIG-OSu in dimethylphormamide. A DIG-OSu solution was further obtained by diluting 5 μl of the stock solution in 20 ml of potassium phosphate buffer 0·05 M, pH 8·5 containing 0·01% Nonidet P-40. Mucin was labelled *in situ* immediately after adsorption on microplate wells, with 100 μl of DIG-OSu solution, 60 min at room temperature.

Microplate assay

Mucin was adsorbed onto Immulon 4 multiwell plates (Dynatech Laboratories, Alexandria, VA), in 100 μl of sodium carbonate buffer 0·1 M, pH 9·6, for 30 min at room temperature or overnight at 4 °C. The wells were washed with enzyme buffer solution (sodium phosphate buffer 0·1 M, pH 7·0 for Pronase E, sodium acetate buffer 0·1 M, pH 5·5 for neuraminidase and Endo H). When *C. albicans* supernatants were assayed, the wells were washed with sodium acetate buffer 0·1 M, pH 4·5. Then, 100 μl of enzyme solution was added and incubated at 37 °C at different times. The wells were then washed with PBS. Blocking was achieved with 200 μl of blocking reagent for ELISA (Boehringer Mannheim), 30 min at room temperature. The wells were then washed twice with PBST. Streptavidin-POD conjugate solution was freshly prepared by diluting 1:1500 in potassium phosphate buffer 0·1 M, pH 7·4 (PBS), containing 0·1% Tween-20 (PBST). Anti-DIG-POD Fab fragment solution was freshly prepared by diluting 1:1000 in PBST. Coupling was achieved with the addition of 150 μl of Streptavidin-POD (for BNHS or BACH assays) or of anti-DIG-POD (for DIG-OSu assay), left 60 min at room temperature. Revelation of POD was carried out with 150 μl of ABTS solution, prepared by dissolving one tablet of ABTS in 50 ml of ABTS buffer. Absorbance was read at 410 nm at different times with a MR600 microplate reader (Dynatech).

Enzymatic activity was determined by reporting absorbance readings on a plot obtained with a standard curve of labelled mucin. Activity was expressed as percentage decrease in absorbance compared with control wells devoid of enzyme or containing uninoculated culture medium.

Statistical analysis

The analysis of variance method of the Statistical Package for the Social Sciences (SPSS, Chicago, IL) was used to analyse interactions between concentrations of mucin, amount of enzyme, day of assay and the results of mucinolytic activity for each microplate assay format. Significant interactions ($P < 0·05$) were further analysed, and significant individual differences ($P < 0·05$) within each of these parameters were determined by using the Scheffé general linear models procedure.

The coefficient of variation (relative SD) was used to estimate the within- and between-run precision of each microplate assay.

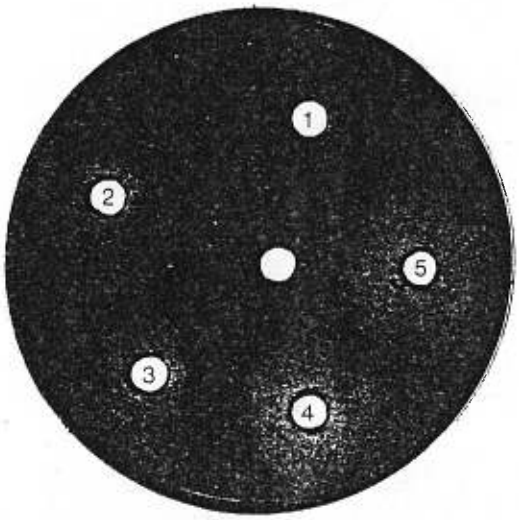

Fig. 1 Petri dish assay for mucinolytic activity. Outer wells (1–5) contained 0·05, 0·5, 1·0, 5·0 and 20 μg of Pronase E, respectively. The centre well contained 20 μg of Pronase E, boiled for 10 min.

Results

A Petri dish assay was used to assess the ability of Pronase E to digest mucin. Mucin was used as substrate for hydrolysis by different quantities of the enzyme and a boiled sample as negative control (Fig. 1). Zones of clearing revealed by Coomassie Blue staining were roughly proportional to the amount of enzyme present. The detection limit of this method was 0·5 μg of enzyme. Although not precisely quantitative, these results indicated that Pronase E was a suitable control enzyme to devise an assay for the quantitation of mucinolytic activity.

Figure 2 shows representative standard curves obtained with the two labelling procedures, and the sensitivities with different concentrations of Pronase E. All three assays detected as little as 0·25 μg of Pronase E activity after incubation of 60 min. For the DIG-OSu method (Fig. 2A), standard curves obtained with 0·25–5 μg of Pronase differed significantly ($P < 0·05$ by the Scheffé general linear models procedure), but curves obtained with 5 and 20 μg of Pronase were not significantly

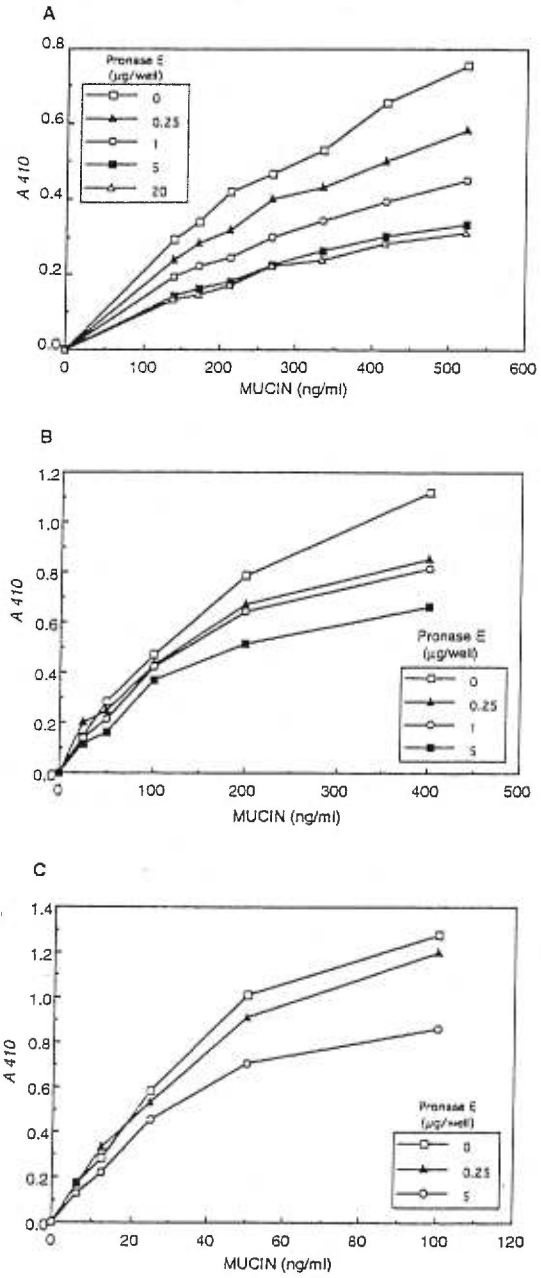

Fig. 2 Representative standard curves obtained by labelling porcine stomach (A, B) or purified mouse intestinal mucin (C), and incubating for 60 min with different concentrations of Pronase E. Mucin was labelled *in situ* with DIG-OSu (A), or as a stock solution with BNHS (B, C). Proteolytic cleavage was determined as a decrease in A_{410}.

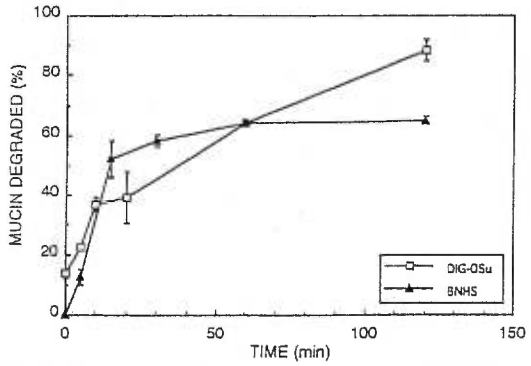

Fig. 3 Time-dependence of proteolytic degradation of DIG-OSu- or BNHS-labelled porcine stomach mucin, incubated with 5 μg of Pronase E. Each point represents the mean ± SD of triplicate (BNHS) or quadruplicate (DIG-OSu) determinations.

different ($P > 0{\cdot}05$). Average within- and between-run coefficients of variation were 13·4% and 15·0%, respectively, determined on quadruplicate samples tested daily for 3 days at a mucin concentration of 500 ng ml^{-1} and 0·25–20 μg of Pronase. The Scheffé method did not demonstrate significant differences between the standard curves obtained on three different days ($P > 0{\cdot}05$). Using BNHS labelling (Fig. 2B), standard curves generated with 0·25–5 μg of Pronase were significantly different ($P < 0{\cdot}05$). Average within- and between-run coefficients of variation were 7·0% and 13·5%, respectively, determined at mucin concentrations of 50–400 ng ml^{-1} and 0·25–5 μg of Pronase. No significant differences were observed between standard curves obtained on three different days ($P > 0{\cdot}05$). Because commercial porcine stomach mucin is only partially purified, we used purified intestinal mouse mucin to exclude the possibility that the decrease in absorbance might result from enzymatic degradation of contaminating impurities (Fig. 2C). Standard curves obtained after BNHS labelling of porcine stomach (Fig. 2B) or mouse intestinal mucins (Fig. 2C) were similar, confirming specific proteolytic activity against mucin.

Time-dependence of degradation of labelled mucin is shown in Fig. 3. Incubation was continued for 120 min. The highest rate of hydrolysis occurred in the first 30 min, after which a plateau was reached. The two systems gave slightly different results, the *in situ* labelling with DIG-OSu giving a maximum activity of 85% and the stock-labelling with BNHS giving a maximum activity of 63%.

This microplate assay allows the versatile labelling of mucin either on the proteic or glycosidic portion, using

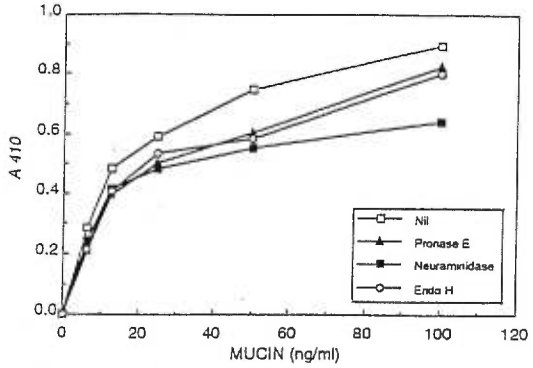

Fig. 4 Representative standard curves obtained by labelling porcine stomach mucin on the glycosidic portion with BACH, and incubating for 60 min with Pronase E (5 μg), neuraminidase (10 mU) or Endo H (2·3 mU).

BACH for the latter. Figure 4 shows the patterns of hydrolysis of mucin by Pronase E and two glycosidases, neuraminidase and Endo H. Standard curves obtained with the three enzymes differed significantly from control curves ($P < 0{\cdot}05$ by analysis of variance). Average within- and between-run coefficients of variation for neuraminidase, Endo H and Pronase were 13·9 and 16·8, 10·7 and 12·9, and 7·3 and 8·1%, respectively, determined on duplicate samples tested daily for 3 days at mucin concentrations of 6·3–100 μg ml^{-1}. No significant differences were found between standard curves obtained on three different days ($P > 0{\cdot}05$, analysis of variance). Because protease activity frees peptides from the substrate, it should result in a decrease in the number of glycosidic labelled molecules, as occurred after incubation with Pronase E.

A *C. albicans* culture filtrate was assayed for mucinolytic activity using BNHS-labelled porcine stomach mucin as substrate. The yeast cells were grown in a liquid medium containing mucin as sole nitrogen source. The concentrated culture filtrate gave a protease activity of 20·6 ± 7·6% (mean ± SD of three independent experiments). The same culture filtrate, boiled for 10 min, gave a residual activity of 9·5%. Pepstatin A, a specific aspartyl proteinase inhibitor, was tested for its ability to inhibit proteolytic activity of the *Candida* culture filtrate. At a concentration of 2 μg ml^{-1}, residual proteolytic activity was 7·5 ± 1·9%, and proteolysis was thus inhibited by 64%. Assay of the concentrated culture filtrate using BACH-labelled porcine mucin as substrate gave an activity of 12·1 ± 1·4% (mean ± SD of three independent experiments), which was similarly decreased to 7·5 ± 1·8% by treatment with pepstatin A ($P < 0{\cdot}05$).

Discussion

The microplate assay described has a number of advantages over other assays for mucinolytic activity. It is simple, rapid, quantitative, reproducible and particularly useful in laboratories where a large number of samples are processed and where a microplate reader is already in use. The assay detected as little as 0·25 μg of Pronase E activity, and provides greater assurance of specificity for substrate by employing mucin rather than a heterologous substrate such as casein. In addition, labelling of peptidic or glycosidic residues can differentiate protease and glycosidase activities against the glycoprotein mucin. Glycosidic labelling detects degradation of both the protein backbone and glycosidic side chains of mucin, while labelling peptidic residues allows quantitation of proteolytic activity.

Both biotin and digoxigenin can be used to label mucin either as a stock solution or *in situ* after adsorption on microtitre plates. We chose to use the stock solution because it eliminates a 1 h incubation, and provides enhanced reproducibility as the same stock solution is used for a large number of assays. Although labelling with biotin or digoxigenin are based on the same principles, we selected the biotin system because streptavidin conjugate is less expensive than anti-DIG antibodies. Although digoxigenin has been previously utilized to label and quantitate porcine stomach mucin [23], this is the first report of its use in a quantitative microplate assay for mucinolytic activity.

The assay detected proteolytic activity against BNHS-labelled mucin in culture supernatants of *C. albicans*, and inhibition by pepstatin A suggested the involvement of a *Candida* secreted aspartyl proteinase (SAP). The weaker activity observed against BACH-labelled mucin was most likely also caused by a SAP rather than a glycosidase, as it was inhibited by pepstatin A. This interpretation is also supported by the observation that pronase gave significant but lower activity against mucin labelled on the glycosidic portion with BACH (Fig. 4) compared with labelling of the peptidic portion using BNHS (Fig. 2). Further characterization of the secretory mucinolytic activity from *C. albicans* is ongoing in our laboratory.

Acknowledgements

This study was supported by grants from the Medical Research Council of Canada and the Fonds de la recherche en santé du Québec. A.-R.C. was the recipient of a studentship from the Consejo Nacional de Investigaciones Cientificas y Tecnologicas (CONICIT), Caracas, Venezuela.

We thank Guy Lemay for helpful discussions, Christian Léger and Michel Lamoureux for the statistical analysis, and Raffaela Ballarano for preparation of the manuscript.

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CHAPTER III

Evidence for degradation of gastrointestinal mucin by *Candida albicans* secretory aspartyl proteinase

Infection and Immunity 64: 4514-4519, 1996

Evidence for degradation of gastrointestinal mucin by *Candida albicans* secretory aspartyl proteinase

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INFECTION AND IMMUNITY, Nov. 1996, p. 4514–4519
0019-9567/96/$04.00+0

Vol. 64, No. 11

Evidence for Degradation of Gastrointestinal Mucin by *Candida albicans* Secretory Aspartyl Proteinase

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Received 13 May 1996/Returned for modification 3 July 1996/Accepted 7 August 1996

A zone of extracellular digestion of the mucin layer around *Candida albicans* blastoconidia was observed by transmission electron microscopy in the jejunum of mice inoculated intragastrically (G. T. Cole, K. R. Seshan, L. M. Pope, and R. J. Yancey, J. Med. Vet. Mycol. 26:173–185, 1988). This observation prompted the hypothesis that a putative mucinolytic enzyme(s) may contribute to the virulence of *C. albicans* by facilitating penetration of the mucus barrier and subsequent adherence to and invasion of epithelial cells. Mucinolytic activity was observed as zones of clearing around colonies of *C. albicans* LAM-1 grown on agarose containing yeast nitrogen base, glucose, and hog gastric mucin. In addition, concentrated culture filtrate obtained after growth for 24 h in yeast nitrogen base, supplemented with glucose and mucin as the sole nitrogen source, contained proteolytic activity against biotin-labelled mucin which was inhibited by pepstatin A. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the culture filtrate revealed two components of 42 and 45 kDa, with pIs of 4.1 and 5.3, respectively. A zymogram showed that mucin was degraded only by the 42-kDa component, which was also recognized by immunoblotting with an anti-secretory aspartyl proteinase (anti-Sap) 2p monoclonal antibody. The N-terminal sequence of the first 20 amino acids matched that reported for Sap2p. These results demonstrate that Sap2p is responsible for proteolysis of mucin by *C. albicans* in vitro and may be involved as a virulence factor in the breakdown of mucus and penetration of the mucin barrier by *C. albicans*.

Invasive candidiasis is the most frequent opportunistic fungal infection in immunocompromised patients and is identified at autopsy in 15 to 30% of those with acute leukemia (1, 37). Penetration of the gastrointestinal mucosa by *Candida* species is thought to be the most frequent portal of entry, leading to systemic dissemination (51, 54). The mechanisms involved in transcytosis, however, are not well understood (7, 8).

Candida albicans possesses a multiplicity of factors that could be involved in the invasive process. Putative virulence attributes include adhesins, dimorphism, phenotypic switching, and secretion of hydrolytic enzymes such as proteinases and phospholipases (11, 24, 47). Extracellular proteolytic activity of *C. albicans* has been extensively studied and is due to secreted aspartyl proteinases (Saps) (EC 3.4.23.6), which can break down a number of host substrates, including epithelial keratin (38), dermal collagen (26), albumin (48), hemoglobin (41), and immunoglobulin A (45).

Sap isoenzymes are now known to be the products of at least seven distinct genes, which are expressed and regulated differentially. Northern (RNA) analysis has shown that expression of SAP1 (23) and SAP3 (57) is regulated during phenotypic switching between the white and opaque forms of strain WO-1 (36), while SAP2 (60) is expressed by yeast cells in media containing protein as the sole nitrogen source (22, 55). The expression of SAP4 to SAP6 is detected at neutral pH during serum-induced yeast-to-hyphal-phase transition, but expression of the SAP7 gene has not been detected to date under any in vitro conditions (22, 56). Although genes SAP1 to SAP7 have been cloned and sequenced (22, 33, 34, 56, 57), only the products of genes SAP1, SAP2, and SAP3 have been isolated and characterized (56). An additional Sap isoenzyme, tentatively referred to as Sap8p, has been identified by N-terminal sequencing, but the corresponding gene has not yet been cloned (35, 56). Several lines of evidence suggest that Saps may be directly involved in fungal colonization and invasion of host tissues. First, the expression of Saps was detected on the surface of blastoconidia adhering to human nonkeratinized buccal epithelium and on invading germ tubes in vitro (2), in vaginal secretions of patients suffering from *Candida* vaginitis (12), and in experimental vaginitis (13). Second, *C. albicans* isolates from human immunodeficiency virus-infected patients expressed higher levels of Sap activity than isolates from control patients (14, 40). Third, a specific inhibitor of aspartyl proteinases, pepstatin A, blocked adherence of *C. albicans* to cultured human epithelial keratinocytes (39) and to human oral mucosa (2). Fourth, Sap-deficient mutants obtained by chemical mutagenesis were less virulent in mice than their parental strains (28, 44). Among *Candida* species other than *C. albicans*, *Candida tropicalis* secretes one acid proteinase (Sapt1) (52), whereas *Candida parapsilosis* secretes two isoenzymes (Sapp1 and Sapp2) (16, 46). However, virtually nothing is known of the impact of *Candida* virulence factors on the ability of the fungus to invade epithelial cells in the gastrointestinal tract and disseminate to deep organs.

The infant mouse model has been used for examination of various aspects of gastrointestinal and systemic candidiasis (5–8, 15). In 5-day-old mice, passage of *C. albicans* across the gastrointestinal wall occurs after oral-intragastric inoculation and results either in mortality or persistent colonization. While the absence of systemic spread of the fungus in persistently colonized mice indicates that normal host defense mechanisms effectively prevent invasion by *C. albicans*, subsequent immu-

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nosuppression with cortisone and cyclophosphamide results in invasion by hyphae in the cardial-atrium fold of the stomach as well as in systemic dissemination. Histological examination using transmission electron microscopy showed progressive extracellular digestion of the mucin layer around *C. albicans* CA30 yeast cells located in the jejunum of mice inoculated intragastrically (8). This observation prompted the hypothesis that a putative mucinolytic enzyme(s) contributes to the virulence of *C. albicans* by facilitating penetration of the mucus barrier and subsequent adherence to and invasion of epithelial cells.

We examined the extracellular mucinolytic activity of *C. albicans* by (i) studying the ability of yeast cells to use mucin as a nitrogen or carbon source and (ii) characterizing the enzyme(s) involved in mucin degradation. Our results showed that at least one member of the Sap family, Sap2p, is involved in degradation of highly glycosylated mucin in vitro.

(This work was presented in part at the ASM Conference on Candida and Candidiasis: Biology, Pathogenesis and Management, San Diego, Calif., 24–27 March 1996).

MATERIALS AND METHODS

Microorganisms. *C. albicans* LAM-1 (serotype A) was originally isolated from the blood of a patient with systemic candidiasis (29). Sap-producing *C. albicans* C9 was kindly provided by B. B. Magee, University of Minnesota, St. Paul, and *C. albicans* CA30, isolated from the kidneys of a leukemic patient, was obtained from G. T. Cole, Medical College of Ohio, Toledo. Cultures were maintained at 4°C on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.).

Culture conditions. Blastoconidia were grown in Sabouraud liquid broth (BBL, Cockeysville, Md.) for 18 h at 37°C with rotary agitation (240 rpm). The cells were collected by centrifugation (1,500 × *g*, 10 min) and suspended at 1.0×10^3 cells per ml in 1.7% (wt/vol) yeast nitrogen base (YNB) medium without amino acids and ammonium sulfate (Difco) supplemented with 0.35% (wt/vol) porcine stomach mucin (type III; Sigma Chemical Co., St. Louis, Mo.), and, in selected experiments, 1% (wt/vol) glucose and/or 0.5% (wt/vol) ammonium sulfate. For induction of aspartyl proteinase under known conditions (22), blastoconidia were grown in YNB supplemented with 1% (wt/vol) glucose and 0.2% (wt/vol) bovine serum albumin (BSA) (fraction V; Sigma).

Preparation of culture supernatant. After incubation at 37°C with rotary agitation, cells were removed by centrifugation (1,500 × *g*, 10 min). The pH of the supernatants was raised from 3.5 to 6.0 with 1 M KH_2PO_4 to limit autodegradation of enzymes (44), and the supernatants were kept on ice throughout the concentration process. High-molecular-weight mucin was removed by filtration through a cross-flow Sartocon-Micro unit (Sartorius AG, Göttingen, Germany) with a 100-kDa exclusion limit. The filtrate was first concentrated through a Sartocon-Micro unit with a 10-kDa exclusion limit and/or in a stirred-cell device containing an ultrafiltration membrane with a 10-kDa exclusion limit (Amicon Inc., Beverly, Mass.) and finally through a Centriplus-10 tube (Amicon). This gave a minimum of 100X concentrate, which was stored at −80°C. Protein content was measured by the method of Bradford using the Bio-Rad (Hercules, Calif.) protein assay and BSA as a standard (3).

Detection and quantitation of mucinolytic activity. The presence of mucinolytic activity was determined by two methods. In the first, cultures were incubated for 3 days at 37°C on a medium containing 1.5% (wt/vol) agarose, 1.7% (wt/vol) YNB, 0.5% (wt/vol) mucin, and 1% (wt/vol) glucose. Plates were subsequently stained with 0.1% (wt/vol) amido black in 3.5 M acetic acid for 30 min and destained with 1.2 M acetic acid. Zones of mucin lysis were observed as discolored halos around colonies.

In the second method, mucinolytic activity was quantitated in a microplate assay using immobilized biotin-labelled mucin as substrate, as described previously (9). Briefly, microplate wells were coated with biotin-labelled mucin, and after washing to remove nonadsorbed mucin, concentrated culture supernatants were added and incubated for 60 min at 37°C in 100 mM sodium acetate buffer (pH 3.5). After incubation, the undigested labelled mucin was detected with streptavidin-peroxidase. Enzymatic activity was determined by reporting absorbance readings on a plot obtained with a standard curve of labelled mucin. Activity was expressed as the percent decrease in absorbance compared with that of control wells devoid of enzyme or containing uninoculated culture medium (9).

SDS-PAGE and Western blot (immunoblot). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (30) with 10% (wt/vol) polyacrylamide separating gels (80 by 70 by 1.5 mm) in a Mini-Protean II Cell (Bio-Rad). Samples (2 to 5 μg) and broad-molecular-weight size standards (Gibco BRL, Canadian Life Technologies Inc., Burlington, Ontario, Canada) were boiled at 100°C for 5 min in an SDS-sample buffer containing 60 mM Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 5% (vol/vol) β-mercaptoethanol, and 0.025% (wt/vol) bromophenol blue. Electrophoresis was performed at room temperature at a constant current of 150 V until the bromophenol blue tracking dye reached the bottom of the gel. Protein bands were visualized by silver staining. For immunoblotting, protein bands on SDS-PAGE gels were transferred electrophoretically by the method of Towbin et al. (53) in a buffer containing 250 mM Tris-HCl (pH 8.8), 192 mM glycine, and 20% (vol/vol) methanol to a nitrocellulose membrane (pore size, 0.2 μm). After electrophoretic transfer (100 V for 2 h at 4°C) in a Mini Trans-Blot cell (Bio-Rad), the nitrocellulose membrane was blocked overnight (4°C) by incubation in 2% (wt/vol) skim milk in Tris-buffered saline (TBS; 10 mM Tris-HCl [pH 7.5], 60 mM NaCl). The next day, the blocked membrane was incubated for 4 h with mouse anti-Sap2p monoclonal antibody (IFG_3, kindly provided by T. L. Ray, University of Iowa, diluted 1:100 in TBS–2% (wt/vol) skim milk. After three 10-min washes with TBS-T (TBS containing 0.1% [vol/vol] Tween 20), the blot was incubated with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Gibco BRL), diluted 1:5,000 in TBS–2% (wt/vol) skim milk. After 1 h of incubation at room temperature, the membrane was washed as described above and developed with alkaline phosphatase color development reagent (10 ml of a 100 mM Tris-HCl [pH 9.6]–100 mM NaCl–5 mM $MgCl_2$ buffer containing 33 μl of nitroblue tetrazolium solution and 44 μl of BCIP solution [5-bromo-4-chloro-3-indolylphosphate]) (Gibco BRL).

IEF and two-dimensional electrophoresis. Native isoelectric focusing (IEF) was carried out in a vertical minigel system by the procedure of Robertson et al. (43), with some modifications. Gels (80 by 70 by 1.5 mm) were cast from a mixture of the following ingredients: 7 ml of water, 2 ml of acrylamide mixture (30% [wt/vol] acrylamide, 1% [vol/vol] bisacrylamide), 2.4 ml of 50% (vol/vol) glycerol, and 0.6 ml of Pharmalyte (pH range, 4 to 6.5; Pharmacia Biotech Inc., Baie d'Urfé, Québec, Canada). After deaeration, 50 μl of freshly prepared 10% [wt/vol] ammonium persulfate and 20 μl of TEMED (*N, N, N', N'*-tetramethylenediamine) were added. Sample wells were formed at the top of the gel with a five-well comb. After polymerization was completed, the comb was removed and the wells were rinsed with distilled water. The wells and upper chamber were filled with the anode solution (20 mM acetic acid), and the lower chamber was filled with the cathode solution (25 mM sodium hydroxide). pI markers (Pharmacia Biotech) and samples (2 to 5 μg) were mixed with an equal volume of IEF sample buffer containing 60% (vol/vol) glycerol and 4% (vol/vol) Pharmalyte (pH range, 4 to 6.5). The electrodes were connected so that the polarity was reversed, consistent with the electrode solutions. Electrophoresis was performed at 4°C for 1.5 h at 200-V constant voltage and then at 400-V constant voltage for an additional 1.5 h. After focusing was completed, the gel was removed and either silver stained, subjected to a second dimension in SDS-PAGE, immunoblotted, or prepared for a zymogram evaluation. For the two-dimensional analysis, the IEF gel lane was equilibrated for 30 min in a solution containing 62.5 mM Tris-HCl (pH 6.8), 2.3% (wt/vol) SDS, 10% (vol/vol) glycerol, and 5% (vol/vol) β-mercaptoethanol. Subsequently, the gel was placed in direct contact with the stacking gel of the SDS-PAGE system and run in a 10% (wt/vol) polyacrylamide separating gel under the conditions described above. Molecular weight standards were run simultaneously, and protein bands were silver stained.

Western blot from IEF gel. Following the isoelectric focusing, the gel was washed four times (10 min) in a buffer containing 5 mM Tris-HCl (pH 8.8)–1% (wt/vol) SDS to remove the carrier ampholytes. The proteins were electrotransferred to nitrocellulose paper and probed with the mouse anti-Sap2p monoclonal antibody.

Zymogram from IEF gel. The mucinolytic activity of the proteins separated by native IEF was evaluated by overlaying the gel on mucin-coated paper. One hundred microliters of biotin-labelled mucin (400 μg/ml) was copolymerized in 12% (wt/vol) polyacrylamide gel and electrotransferred to nitrocellulose paper as described earlier. When blotting was complete, the mucin-coated paper was washed three times (10 min) in 100 mM sodium acetate buffer (pH 3.5) and placed on several filter papers (Whatman 3MM) underlaid with soft absorbent tissue paper. The IEF gel, which was immersed in 100 mM sodium acetate buffer (pH 3.5) for 30 min, was then carefully overlaid on the nitrocellulose paper and incubated for 14 to 16 h at 37°C in a moist chamber. After removal of the gel from the paper surface, the paper was washed three times (10 min) in TBS and blocked in TBS–2% (wt/vol) skimmed milk (1 h, 22°C). After three washes with TBS-T, the paper was incubated for 60 min with streptavidin-POD diluted 1:1,500 with TBS-T. Three additional washes in TBS-T and two in TBS were employed before the paper was exposed to a chromogenic peroxidase substrate solution containing 10 ml of methanol, 1 ml of 4-chloro-1-naphthol solution (30 mg/ml in methanol), 39 ml of TBS, and 30 μl of 30% (vol/vol) hydrogen peroxide. The reaction was developed in darkness and stopped by washing the membrane with distilled water.

Metabolic labelling. Blastoconidia of *C. albicans* (3 ml, 1.0×10^4 cells per ml) were cultured for 24 h in YNB containing 0.35% (wt/vol) mucin, glucose, and 40 μCi of Trans^{35}S-label (specific activity, > 1,000 Ci/mmol; ICN Pharmaceuticals Inc., Costa Mesa, Calif.) per ml. The culture supernatant was ultrafiltered through a Centriplus-100 tube (Amicon), and the filtrate was concentrated by precipitation with acetone. The proteins were resuspended in 20 μl of IEF sample buffer and subjected to IEF. After the electrophoresis, the IEF gel was fixed (30 min) in methanol-acetic acid-water (5:1:4) and soaked (30 min) in En^{3}Hance solution (New England Nuclear Corp., Boston, Mass). Finally, the gel was dried and exposed to a Kodak X-Omat film at −80°C.

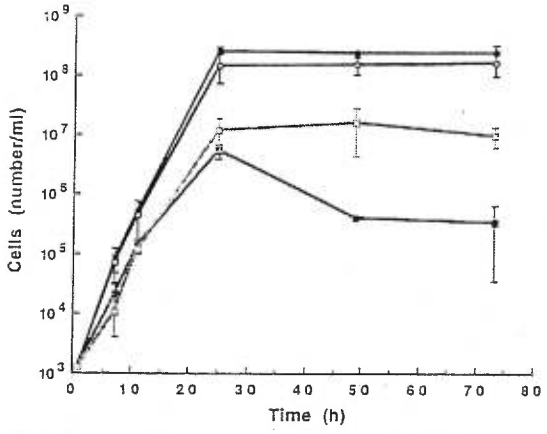

FIG. 1. Growth of blastoconidia of *C. albicans* LAM-1 in YNB cultures all containing mucin and supplemented with glucose and ammonium sulfate (●), only glucose (○), only ammonium sulfate (■), or neither glucose nor ammonium sulfate (□). The cultures were incubated at 37°C, and the cells were counted with a hemacytometer. Values represent the means ± standard deviations of samples from three independent experiments.

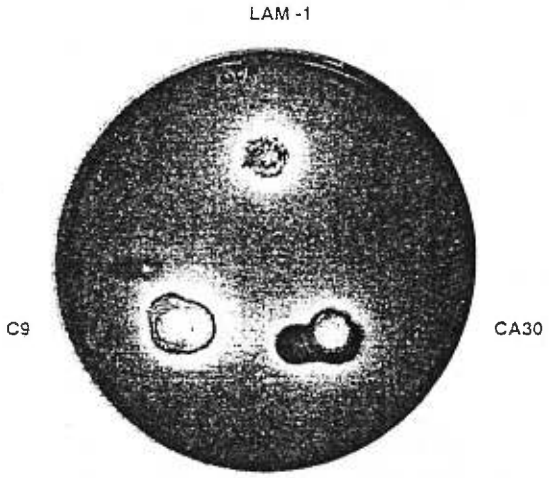

FIG. 2. Zones of mucin lysis around colonies of *C. albicans* LAM-1, C9, and CA30 grown on YNB-glucose-mucin. The plates were incubated at 37°C for 3 days and stained with amido black.

N-terminal sequencing. After IEF, the regions of the gel corresponding to pIs 4.1 and 5.3 were excised and immersed in two-dimensional electrophoresis equilibrium buffer for 15 min. The gel slices were then inserted on top of an SDS–5% (wt/vol) polyacrylamide stacking gel, and migration was performed as mentioned above for SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membranes (pore size, 0.2 μm; Bio-Rad) in 10 mM CAPS buffer (pH 11)–10% (vol/vol) methanol, by using a Trans-Blot cell (Bio-Rad) at 90 V and 250 mA for 60 min. After rinsing with water, the membrane was stained with 0.1% (wt/vol) Coomassie blue R-250 in 50% (vol/vol) methanol and destained in 50% (vol/vol) methanol–10% (vol/vol) acetic acid. The membrane was allowed to dry, and the bands were excised and sequenced by automated Edman degradation with an Applied Biosystems sequencer model 473A (Service de Séquence de Peptides de l'Est du Québec, CHUL, Ste-Foy, Québec, Canada).

RESULTS

Mucin as a nitrogen or carbon source. The ability of *C. albicans* yeast cells to use mucin as the sole nitrogen or carbon source was evaluated by growing the blastoconidia in media containing mucin instead of glucose and/or ammonium sulfate. Figure 1 shows that mucin and ammonium sulfate were equivalent as nitrogen sources in supporting cell growth in the presence of glucose, and the culture reached the stationary phase after 24 h of incubation. Thus, the cells were able to use mucin as the sole nitrogen source. The initial pH of the culture containing glucose and mucin was 4.5 and fell to 3.5 after 24 h of incubation. Preculture of blastoconidia in conditions of ammonium starvation (1.7% [wt/vol] YNB, 1% [wt/vol] glucose, 0.02% [wt/vol] ammonium sulfate), rather than Sabouraud liquid broth, had no effect on subsequent cell growth in media containing glucose and mucin or mucin alone. Limited growth occurred when the yeast cells were inoculated in media in which no carbohydrate source other than mucin was included, suggesting that mucin is less efficiently utilized as a carbon source by *C. albicans*. Finally, growth in YNB without amino acids, ammonium sulfate, glucose, or mucin was further limited and only reached 5.8×10^4 and 8.4×10^4 cells per ml after 24 and 72 h of incubation, respectively.

Clear zones of mucin lysis were apparent around colonies of strains C9, LAM-1, and CA30 grown on solid medium containing mucin and glucose (Fig. 2). The diffusion of mucinolytic activity into the medium surrounding the colonies suggested the release of a soluble mucinolytic enzyme(s). However, no zones of mucin lysis were observed on medium containing mucin, glucose, and ammonium sulfate (data not shown). On the basis of these observations, we selected YNB containing mucin and glucose as the inducer medium and the virulent strain LAM-1 used in the model of oral candidiasis (29) for the subsequent experiments.

Characterization of mucinolytic activity. Culture supernatants from LAM-1 cells were purified and concentrated by ultrafiltration, and the mucinolytic activity was quantified in a microplate assay, with biotin-labelled mucin as the substrate. Raising the pH to 6.0 and concentrating the supernatant before freezing were all essential to maintaining mucinolytic activity. Maximal protease activity was detected at pH 3.5 in 24-h culture supernatants (20% ± 7.6% [mean ± standard deviation of three independent experiments]). After incubation with pepstatin A (2 μg/ml), the activity decreased to 7.5% ± 1.9% and proteolysis was thus inhibited by 64%. Finally, when the culture supernatant was boiled, the residual activity was 9.5% (9). The inhibition of mucin degradation by pepstatin A suggested the involvement of an aspartyl proteinase (EC 3.4.23.6) in mucinolytic activity. An incubation period of 24 h was selected for the subsequent experiments.

Upon SDS-PAGE, two protein bands with apparent molecular weights of 42 and 45 kDa appeared in the mid-log phase (15 h) (Fig. 3, lane 6). Both bands persisted throughout a 7-day extended incubation period. Failure to initially raise the pH of the culture supernatant from 3.5 to 6.0 resulted in a marked weakening of the 42-kDa band. The 42-kDa band was more prominent relative to the 45-kDa band and was the only one to react with the anti-Sap2p monoclonal antibody along with a lower-molecular-weight product most likely resulting from autodegradation (44) (Fig. 3, lane 8). Induction of Sap2p expression under standard conditions was done by growing the known aspartyl proteinase-producing strain C9 (28) in YNB medium containing glucose and BSA. Culture supernatant from strain C9 also showed a 42-kDa band which was recognized by the anti-Sap2p monoclonal antibody (Fig. 3, lanes 2 and 4). These

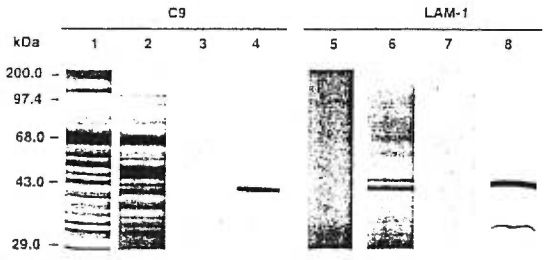

FIG. 3. SDS-PAGE and Western blot analysis of culture supernatants from *C. albicans* C9 and LAM-1 grown in YNB-glucose-BSA and YNB-glucose-mucin, respectively. Lanes: 2 and 6, SDS-PAGE of supernatants from C9 and LAM-1, respectively; 4 and 8, Western blot of supernatants from C9 and LAM-1, respectively, probed with anti-Sap2p monoclonal antibody; 1, 3, 5, and 7, samples from uninoculated media included as controls (1 and 5, SDS-PAGE; 3 and 7, Western blot). The gels were silver stained.

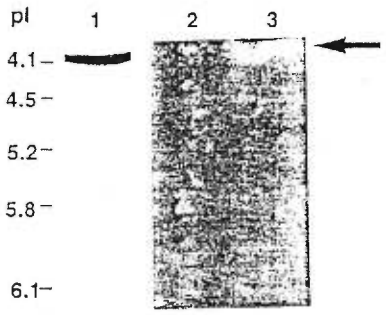

FIG. 5. Western blot and zymogram from a native IEF gel of culture supernatant from *C. albicans* LAM-1. Lanes: 1, Western blot, probed with anti-Sap2p monoclonal antibody; 2, zymogram of heat-inactivated culture supernatant; 3, zymogram of culture supernatant, showing degradation of mucin (arrow).

results provide evidence that the 42-kDa band was antigenically related to *C. albicans* Sap2p.

IEF and two-dimensional electrophoresis. To further characterize the 42- and 45-kDa bands, they were subjected to native IEF and two-dimensional electrophoresis. Figure 4 shows that the pIs of the 42- and 45-kDa components were 4.1 and 5.3, respectively. No other bands were detected in the second-dimension gel, indicating the absence of Sap isoforms in the culture supernatant. The IEF procedure resulted in complete separation of the 42- and 45-kDa bands, allowing us to perform the N-terminal protein sequencing without risk of contamination.

Western blot and zymogram from IEF gel. The identity of the pI 4.1 protein was confirmed by Western blot from the IEF gel, since it was the only one recognized by the anti-Sap2p monoclonal antibody (Fig. 5, lane 1).

To determine whether the mucinolytic activity was a consequence of cooperative action of both 42- and 45-kDa bands, we performed a zymogram from the native IEF gel, with biotin-labelled mucin as the substrate. The proteolytic activity was uniquely identified at pI 4.1 as a clear band against a dark blue-violet background (Fig. 5, lane 3), demonstrating that the 42-kDa protein was the only component responsible for mucin degradation.

Metabolic labelling. Autoradiography revealed that during incubation in mucin-containing medium, the 42-kDa band was the only component which incorporated [^{35}S]methionine or -cysteine. The 45-kDa component failed to incorporate the radiolabelled precursor, suggesting that it may be a breakdown product of mucin.

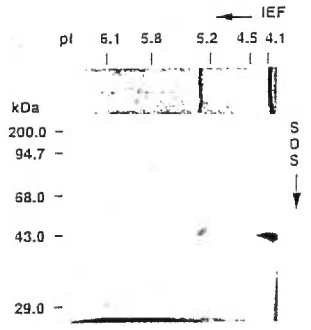

FIG. 4. Native IEF gel (top) and two-dimensional electrophoresis gel (bottom) of culture supernatant from *C. albicans* LAM-1. The pIs and positions of molecular weight markers are indicated. The gels were silver stained.

N-terminal sequencing. The N-terminal sequence of the first 20 amino acids from the 42-kDa protein was QAVPVTLH NEQVTYAADITV, matching that reported by others (22) for *C. albicans* Sap2p. The most-probable glycine-rich sequence of the 45-kDa protein was GEGGSGGEGGQGGQGGPXGA, and a BLAST search (1 May 1996) revealed homology with type II keratin, a mesenchyme-specific cell surface glycoprotein precursor from the sea urchin (*Strongylocentrotus purpuratus*), and a glycine-rich cell wall structural protein from the tomato (*Lycopersicon esculentum*).

DISCUSSION

Invasion of the bowel wall by *Candida* species and systemic spread to deep organs are triggered by complex interactions resulting from modifications of normal bacterial flora and host defenses. However, virtually nothing is known of the impact of *Candida* virulence factors on the ability of the fungus to engage in this process. Several clues suggest that *Candida* virulence factors may play a role. (i) *C. tropicalis* disseminates more easily than *C. albicans* from the gastrointestinal tract of humans (59) and mice (15, 58), suggesting the presence of specific virulence determinants. (ii) An advancing border of necrosis is seen at the leading edge of gastrointestinal invasion by *C. tropicalis* hyphae (54). (iii) Progressive extracellular digestion of the mucin layer is observed in 5-day-old mice inoculated intragastrically with *C. albicans* CA30 (8). The latter observation prompted the hypothesis that a mucinolytic enzyme(s) contributes to the virulence of *Candida* species by facilitating penetration of the mucus barrier and subsequent adherence to and invasion of epithelial cells.

In this study, we provide evidence that Sap2p contained in concentrated culture filtrate of *C. albicans* blastoconidia has mucinolytic activity. Mucins are the major constituents of mucus and play a role in protection against invasion by potential pathogens because of their rich and heterogeneous oligosaccharide composition and ability to form a gel (19). For a microorganism such as *C. albicans*, which colonizes mucosal surfaces, mucin degradation by Sap2p may allow closer approximation to epithelial cells and/or modification of cellular surfaces to create receptors, promoting invasion and spread of the fungus within the host. Because porcine stomach mucin is

structurally similar to the major gastric glycoproteins of humans and resembles human intestinal mucin in general composition (25), these findings suggest a potential role for Sap2p in the pathogenesis of gastrointestinal candidiasis. We have previously shown that concentrated culture filtrate from *C. albicans* LAM-1 degrades porcine stomach mucin (9). The glycoprotein structure of mucins renders them potentially susceptible to attack by both glycosidases and proteinases. The proteolytic nature of the mucinase activity of Sap2p is suggested by loss of portions of mucin molecules which were labelled with biotin specifically and exclusively on their protein moieties. The activity was probably directed against the minor or naked regions of the mucin molecule which are poorly glycosylated as well as the link peptide of 118 kDa (18), which are known to be susceptible to proteolytic digestion.

The present in vitro experimental evidence suggests that Sap2p may be involved in the previously described in vivo progressive extracellular digestion of the intestinal mucus barrier observed after oral-intragastric inoculation of *C. albicans* in the infant mouse model (8). Most indirect evidence which associates proteinase production and virulence during mucosal candidiasis is derived from the detection of the expression of SAP1 and SAP2 in experimental vaginitis (13).

The production of mucin-degrading enzymes has been implicated as a virulence determinant for a number of enteropathogens, including *Vibrio cholerae* (10), *Bacteroides fragilis* (42), *Shigella* spp. (20), *Helicobacter pylori* (49), and *Yersinia enterocolitica* (32). Extensive mucin degradation would require the secretion of neuraminidase, endo-β-*N*-acetylhexosaminidase, and proteases (50). While we cannot exclude the possibility of expression of secreted or wall-associated glycosidase activity by *C. albicans* grown in the presence of mucin, it would appear more likely that the major mucin-degrading enzyme may be Sap2p.

Our results are in agreement with reports indicating that Sap2p is the major isoenzyme of the Sap family produced by the yeast form of a majority of clinical isolates of *C. albicans* (22, 55). The apparent molecular mass of 42 kDa and pI of 4.1 were in close agreement with those reported for Sap2p (57). It is well known that Sap2p expression is induced in media containing protein as a single nitrogen source, stimulated by peptides of eight or more amino acid residues and repressed by low-molecular-weight nitrogen components (31). Induction of Sap2p by mucin is consistent with the observation that several protein substrates, including casein, bovine serum, and hemoglobin, all have the ability to induce Sap (55). In addition, inhibition of mucinolytic activity around the colonies of *C. albicans* C9, LAM-1, and CA30 grown on solid medium containing mucin, glucose, and ammonium sulfate concurred with the known repression of Sap2p levels by ammonium salts (55). Despite its glycosylated structure, mucin seems to be a highly utilizable nitrogen source, inducing Sap2p expression and supporting cell growth in the presence of glucose. We have also examined the role of mucin as a sole carbon source. Cultures supplemented with mucin but lacking an added carbon source grew poorly. The simplest explanation for these results is that glucose acts as a preferred growth substrate for *C. albicans*, while mucin is a poor carbon source, probably because no glycosidases capable of mucin degradation were secreted under these conditions. In addition, these results are consistent with the observation that *C. albicans* requires a carbon and energy source for growth and expression of Sap2p (22). Detection of Sap2p in the mid-log phase and persistence in the medium for 7 days are in agreement with the results of White et al. (57) and occurred concurrently with a decrease in the pH of the culture medium to 3.5, which is optimal for expression of Sap2p mRNA (22, 55).

A less-prominent band of 45 kDa was also present in the concentrated culture supernatants from cells grown in mucinase-inducing medium. Its N-terminal amino acid sequence did not show any homology with the N-terminal segments of mature Sap proteins reported to date, making it thus unlikely that it is a Sap isoenzyme. The absence of metabolic labelling suggested that it may be a breakdown product of mucin resulting from Sap2p digestion. However, the possibility that it may be an extracellular product of *C. albicans* could not be formally excluded because of its low methionine-cysteine content and/or possible weak de novo synthesis. The N-terminal amino acid sequence did not show conclusive homology to sequences of known proteins.

Candida-mucin interactions may involve not only mucinolysis but also adhesion to host surfaces. In the gastrointestinal tract, mucin glycoproteins were associated with adhesion of *C. albicans* to intestinal epithelium (27), and two different studies have demonstrated adhesion of *C. albicans* to salivary mucin, which may either act to inhibit adhesion with some surfaces in the oral cavity or promote adhesion to other surfaces (17, 21). *C. albicans* contains a mannoprotein adhesin with a lectin-like affinity for fucose, a component of mucins (4). It is thus conceivable that *C. albicans* may both adhere to and degrade mucins in the oral cavity and small intestine, and both properties may act to modulate *C. albicans* populations in the gastrointestinal tract.

The results of this study demonstrate that Sap2p is responsible for proteolysis of mucin by *C. albicans* in vitro and may be involved as a virulence factor in the breakdown of mucus and penetration of the mucin barrier by *C. albicans*.

ACKNOWLEDGMENTS

This study was supported by grants from the Medical Research Council of Canada and the Fonds de la Recherche en Santé du Québec. P.B. is a scholar of the Fonds de la Recherche en Santé du Québec. A.-R.C. was the recipient of a studentship from the Consejo Nacional de Investigaciones Cientificas y Tecnologicas, Caracas, Venezuela.

We thank Guy Lemay for helpful discussions and Annie Aubry, Patricia Hurtubise, Julie Lippens, and Johanne Ratelle for technical assistance. We are also grateful to Thomas L. Ray for supplying the anti-Sap2p monoclonal antibody and Sylvie Bourassa for performing the N-terminal sequencing.

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Editor: T. R. Kozel

CHAPTER IV

Overexpression of *Candida albicans* secretory aspartyl proteinase 2 and its expression in *Saccharomyces cerevisiae* do not augment virulence in mice

Microbiology 144: 2299-2310, 1998

Overexpression of *Candida albicans* secretory aspartyl proteinase 2 and its expression in *Saccharomyces cerevisiae* do not augment virulence in mice

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Footnote:

Contributions of Ana Rosa Colina to this article:

- Preparation of *C. albicans* genetically transformed strains.
- Evaluation of the virulence of *C. albicans* transformants in murine models of candidiasis.

SUMMARY

In order to elucidate the implications of secreted aspartyl proteinase (Sap)2p in the pathogenesis of *Candida* infections, we have expressed the *SAP2* gene in *S. cerevisiae* and overexpressed it in *C. albicans*. The coding region of *SAP2* including its signal sequence and propeptide was amplified by PCR and cloned downstream of the *S. cerevisiae* or *C. albicans ADH1* promoter. Plasmidic expression of *SAP2* in *S. cerevisiae* showed that the signal peptide was functional. Integrative transformation of *S. cerevisiae* and *C. albicans* was accomplished by homologous recombination within the *URA3* locus for *S. cerevisiae* and *SAP2* locus for *C. albicans*. Negative control transformants carried either plasmids without *SAP2* insert or with mutated *sap2*. *S. cerevisiae* and *C. albicans* transformants showed similar growth rates to their parental strains or negative controls, when grown in media containing amino acids. However, in medium with BSA as sole nitrogen source, constitutive expression of *SAP2* enabled *S. cerevisiae* to grow and increased the growth rate of *C. albicans*. In both media, only *S. cerevisiae* transformants harbouring *SAP2* secreted the enzyme, as confirmed by proteinase activity assays and immunoblotting. When *C. albicans* was grown in amino acids medium, the enzyme was detected exclusively in transformants constitutively expressing *SAP2*. However, in BSA medium, these strains secreted earlier and higher amounts of enzyme and total proteinase activity. In pathogenicity studies in intact mice, expression of Sap2p as a sole putative virulence factor did not cause *S. cerevisiae* to become virulent, and constitutive overexpression of

SAP2 did not augment virulence of *C. albicans* in experimental oral or systemic infection.

INTRODUCTION

Candida albicans is an important opportunistic pathogen causing local or systemic infection mainly in immunocompromised patients (Odds, 1988). Several virulence factors have been proposed in pathogenicity: adhesion, dimorphism, phenotypic switching, molecular mimicry of mammalian integrins, and secretion of phospholipases and aspartyl proteinases (Cutler, 1991; White *et al.*, 1995). The secreted aspartyl proteinases (Saps) have been studied extensively, and are encoded by at least eight genes which are expressed and regulated differentially (Hube, 1996). Northern (RNA) analysis has shown that *SAP*1 and *SAP*3 are regulated during phenotypic switching between the white and opaque forms of strain WO-1, while *SAP*2 was found to be the dominant transcript in budding cells grown in media containing protein as the sole nitrogen source. The expression of *SAP*4 to 6 is detected at neutral pH during serum-induced yeast to hyphal transition, but the expression of the *SAP*7 gene has not been detected (Hube *et al.*, 1994; White & Agabian, 1995).

In clinical and laboratory strains grown as the yeast form *in vitro*, Sap2p is the predominant expressed proteinase. Evidence has also been provided that Saps are secreted by *C. albicans in vivo* during the course of mucosal and deep tissue infections (Borg & Rüchel, 1988; De Bernardis *et al.*, 1995). The most commonly used approach to define the role of Saps as virulence attributes has been to compare lethalities of mice infected intravenously with parental strains or proteinase-deficient mutants. The mutants, isolated by chemical mutagenesis techniques or UV-irradiation, have been shown to be less virulent than their parental strains. However,

definitive proof of the role of Saps in virulence of *C. albicans* was lacking, either because (i) the mutants had high levels of reversion, (ii) they still produced detectable proteinase activity *in vitro*, (iii) the nature of the mutation was undefined, or (iv) they may harbour multiple, independent and unknown lesions (Macdonald & Odds, 1983; Kwon-Chung *et al.*, 1985; Crandall & Edwards, 1987; Ross *et al.*, 1990).

Recently, *C. albicans* strains harbouring targeted disruptions of *SAP* genes have been constructed using the ura-blaster protocol (Hube *et al.*, 1997; Sanglard *et al.*, 1997). When tested in a murine model of systemic candidiasis, the virulence of *C. albicans sap*1, *sap*2 and *sap*3 mutants was modestly attenuated (Hube *et al.*, 1997). However, the extent of attenuation of virulence did not correlate directly with the reduction of proteolytic activity *in vitro*. Similarly, mice infected with a *C. albicans sap*4,5,6 triple homozygous null mutant had survival times significantly longer than that of control animals (Sanglard *et al.*, 1997). In *C. tropicalis*, disruption of the *SAP*T gene was also achieved by co-transformation with a linear DNA fragment carrying a deletion in *SAP*T (Sanglard *et al.*, 1992). Surprisingly, the virulence of the *SAP*T-null mutant and the proteinase-positive strain did not differ significantly after intravenous infection in mice (Togni *et al.*, 1994). These results suggested that the Sap of *C. tropicalis* does not contribute significantly to fungal virulence in systemic infections. Alternately, potentially decreased virulence may have been masked by the redundancy of members of a putative multigene Sap family in *C. tropicalis* (Monod *et al.*, 1994) and/or the redundancy of other virulence attributes of *Candida*.

Selective expression/overexpression of genes coding for putative virulence attributes is an attractive strategy to overcome redundancy

problems and clarify the contribution of each isoenzyme to virulence. This approach would allow the study of virulence-enhancing genes in already virulent strains (homologous overexpression) and in nonpathogenic but closely related strains (heterologous expression). The rationale for this approach is also based on clinical studies which have shown that the Sap activity of *C. albicans* isolates from patients with vaginitis (Cassone *et al.*, 1987) or HIV-infection (Ollert *et al.*, 1995) was significantly higher than that of isolates from asymptomatic carriers. In the present study, we describe the construction of stable *S. cerevisiae* and *C. albicans* strains that constitutively secrete Sap2p. Sap2p expression by *S. cerevisiae* allowed it to hydrolyse and use BSA as nitrogen source, while Sap2p overexpression in *C. albicans* resulted in early and high proteinase secretion. The virulence of these genetically engineered strains was evaluated in murine models of oral or systemic infections.

(This work was presented in part at the 97th General Meeting of the American Society for Microbiology, Miami Beach, FL., 4-8 May, 1997).

METHODS

Strains and culture conditions

Yeast strains used in this work are listed in Table 1. *Escherichia coli* DH10B (Gibco BRL) was routinely used as a plasmid host for cloning procedures. Bacterial and yeast media were prepared as described (Rose *et al.*, 1990; Sambrook *et al.*, 1989). Yeast parental strains were grown in YPD medium containing 1% (w/v) yeast extract (Difco), 2% (w/v) bacto peptone (Difco) and 2% (w/v) dextrose. Uracil/uridine independence selection was performed by using CAT medium [0.67% (w/v) Yeast Nitrogen Base (YNB) without amino acids (Difco), 0.5% or 1% (w/v) casaminoacids, 0.008% (w/v) adenine, 0.002% (w/v) tryptophan] supplemented with 2% (w/v) dextrose. The medium to regenerate *C. albicans* spheroplasts was supplemented with 1 M sorbitol. Induction of Sap2p was done in YNB-BSA medium containing 0.17% (w/v) YNB without amino acids and ammonium sulfate (Difco), supplemented with 2% (w/v) dextrose and 0.2% (w/v) BSA (fraction V, Sigma Chemical). Media were supplemented with 100 mg ml^{-1} uracil/uridine as required. All yeast cultures were incubated at 30 °C, except for experiments on the kinetics of Sap2p secretion by *S. cerevisiae* and *C. albicans* which were conducted at 37 °C. Growth rates were monitored by measuring the absorbance at 600nm (OD_{600}). Media were solidified with 2% (w/v) agar. Germ tube formation was evaluated by incubating in 5% (v/v) newborn calf serum (Gibco BRL), for 3 h at 37 °C.

PCR amplification of *SAP2* gene

Genomic DNA from *C. albicans* strain LAM-1, obtained as described by Magee *et al.* (1987), was used as template for PCR amplification of the *SAP2* gene. The two primers used were: 5'-CGGGATATCAACAACCCACTAGACATCACC-3' and 5'-CTGGAGCTCCACCCCTTCATCTTAGGTCAA-3'. These primers flank the *SAP2* coding sequence with its own signal peptide. The underlined sequences indicate *EcoR*V and *Sac*I restriction sites, respectively. The 100 ml-PCR reaction contained 1 μg of genomic DNA template, 1 μg each of the two primers, 400 mM each of dATP, dGTP, dCTP, dTTP, 10 ml DMSO, 2 U Vent DNA polymerase (New England Biolabs) and 1X Vent buffer. The PCR program was 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min, for 30 cycles. A 1244 bp PCR product was gel purified and cloned into pBluescript KS II+ (Stratagene), after digestion with *EcoR*V and *Sac*I. The PCR product sequence was verified by double strand sequencing with universal and internal primers (Sanger *et al.*, 1977).

Plasmid constructions

Recombinant DNA manipulations were done by standard methods (Sambrook *et al.*, 1989). Plasmid pVTU-*SAP2* was constructed by inserting the *SAP2* PCR product, digested by *EcoR*V and *Ecl*136II, into the *Ecl*136II site of *S. cerevisiae* shuttle vector pVT102-U (Vernet *et al.*, 1987). A negative control, pVTU-a*SAP2*, carried *SAP2* in antisense orientation. pVT102-U is a 2 μ-based plasmid bearing the *ADH1* promoter (*ADH1*p), its terminator region and the *URA3* marker gene. From pVTU-*SAP2*, a 2 kb *Sph*I fragment containing *SAP2* downstream of *ADH1*p, was blunt-ended

with T4 DNA polymerase and inserted in the *Sma*I site of pRS306, a *S. cerevisiae* integrative plasmid containing the *URA*3 marker gene (Sikorski & Hieter, 1989). This plasmid was named pRS-*SAP*2. Finally, the *C. albicans* vector containing *C. albicans ADH*1p and *URA*3 marker, YPB-*ADH*pL (kindly provided by A. J. P. Brown, University of Aberdeen, U.K.), was used to insert the 1244 bp *EcoRV*-*Ecl*136II *SAP*2 fragment into *EcoRV* site, under the control of the *C. albicans ADH*1p. This construct was named pYPB-*SAP*2. A negative control plasmid, pYPB-m*SAP*2, was generated by inserting a mutated *sap*2. The mutation was introduced by cleavage of the *SAP*2 gene with *BamH*I, filled-in with klenow enzyme and religated. This introduced a frameshift (verified by sequencing) after the 77th amino acid and a premature stop codon 3 amino acids further.

Yeast transformation

S. cerevisiae strains grown overnight in YPD medium at 30 °C, were transformed using the LiAc/SS-DNA/PEG procedure described by Schiestl *et al.* (1993). Strains clABYS86 (S86), PUB754 (754) and SEY6210 (6210) were transformed with pVTU-*SAP*2. PUB754, a his+ leu+ derivative of S86, was obtained by two separate "one step gene replacement" transformations with *HIS*3 and *LEU*2 DNA fragments (Rothstein, 1983). Integration of *SAP*2 in *S. cerevisiae URA*3 locus was done by transforming the 754 strain with pRS-*SAP*2, linearized by NcoI in *URA*3 gene. Negative controls were also constructed by transforming the 754 strain with *Nco*I-linearized pRS306 plasmid.

Transformation of *C. albicans* CAI4 was done by the spheroplast method as described by Rose *et al.* (1990), with minor modifications.

Briefly, CAI4 yeast cells grown overnight in YPD were diluted in YNB-BSA medium (supplemented with uridine), to an OD_{600} of 0.2. Then, exponentially growing cells (OD_{600} of 0.5) were used to prepare the spheroplasts. pYPB-*SAP2* and pYPB-m*SAP2* plasmids were linearized by cutting at the *Kpn*I site within the *SAP2* gene and used to transform the spheroplasts by integration at the *SAP2* locus. The *S. cerevisiae* and *C. albicans* transformants, selected by complementation of the *ura3* mutation, were grown on CAT plates.

Proper integration was evaluated by Southern blot analysis (Belhumeur *et al.*, 1993). Genomic DNA from *S. cerevisiae* transformants, prepared as described by Hoffman & Winston (1987), was digested with *Avr*II and probed with a 693 bp *EcoR*V-*Sma*I *URA3* (Fig. 1, panel *S. cerevisiae*) or a 597 bp *BamH*I-*Kpn*I *SAP2* restriction fragment. *C. albicans* genomic DNA prepared according to Magee *et al.* (1987), was digested with *Kpn*I and probed with a 265 bp *Pst*I-*Dra*I *SAP2* DNA fragment (Fig. 1, panel *C. albicans*).

SDS-PAGE and Western blots

Intra- and extracellular protein extracts from *S. cerevisiae* plasmidic transformants grown 18 h in CAT medium at 30 °C, were obtained by lysing with glass beads (Harlow & Lane, 1988) and acetone precipitation, respectively. One-tenth of the original extracts volume was loaded in a 12% polyacrylamide separating gel to perform SDS-PAGE (Laemmli, 1970). For time course evaluation of Sap2p expression, 60 ml of culture supernatants were analysed by SDS-PAGE. Following electrophoresis, proteins were transferred on nitrocellulose membranes (Towbin *et al.*, 1979). Membranes

were blocked with 2% (w/v) skim milk in Tris-buffered saline (TBS: 10 mM Tris-HCl [pH 7.5], 150 mM NaCl), and washed between steps with TBS containing 0.1% (v/v) Tween 20 (TBST). Membranes were incubated for 1 h with mouse anti-Sap2p monoclonal antibody IFG3, kindly provided by T. L. Ray (University of Iowa), diluted 1:500 in TBS-2% (w/v) skim milk. The bound antibody was detected using alkaline-phosphatase conjugated goat anti-mouse IgG (Gibco BRL) diluted 1:5000 in TBS-2% (w/v) skim milk.

Enzyme assays

Proteolytic activity was quantitated by the method of Milewski *et al.* (1994), with minor modifications. Briefly, culture supernatants (150 ml) were mixed with 1% (w/v) BSA in 50 mM sodium citrate, pH 3.5 (600 ml). After 1 h incubation at 37 °C, reactions were stopped by adding 400 ml of 10% (w/v) trichloroacetic acid (TCA). Samples were cooled in an ice-bath for 10 min, and precipitated proteins were removed by centrifugation at 5000 g, 10 min. The supernatants absorbance was read at 280 nm and corrected for background using a control (for each point) in which TCA solution was added prior to the culture supernatant. One unit of enzyme activity was defined as the amount of enzyme causing ΔA_{280} 0.1 in 1 h.

Pathogenicity *in vivo*

The virulence of *S. cerevisiae* and *C. albicans* transformants as well as their parental strains was evaluated in murine models of oral and systemic candidiasis. Crl:CD-1 (ICR) BR female mice, weighing 22-24 g, were obtained from Charles River Breeding Farms (St-Constant, Québec, Canada) and were kept at the University of Montreal Animal Care Unit. For

experimental infections, the yeast inoculum was prepared from cells grown overnight in CAT medium at 30 °C.

The oral infection model described by Chakir *et al.* (1994) was used with minor modifications. Briefly, both *S. cerevisiae* and *C. albicans* yeast cells were washed twice in sterile PBS (0.01 M, pH 7.4), and counted in a hemacytometer. Cells (1×10^8) were distributed in sterile plastic microfuge tubes and pelleted by centrifugation at 13000 g. Six mice per strain were anesthetized by the intraperitoneal route with 0.35 ml of ketamine (10 mg ml^{-1}). They were then inoculated by topical application into the oral cavity, using a sterile calcium alginate swab (Fisher Scientific). A longitudinal quantification of the microorganisms in the oral cavities of individual mice was done by daily oral swabbing until day 8 post-inoculation. Swabs used for sampling were dissolved in 2 ml volumes of Ringer's citrate buffer (Rodrigue *et al.*, 1989), and serial dilutions in PBS were plated on CAT medium supplemented with 50 mg l^{-1} chloramphenicol (CAT-Chl). Plates were incubated at 30 °C and colony-forming units (CFU) were counted.

Experimental murine systemic infection (Clemons *et al.*, 1994) was used to study *S. cerevisiae* virulence. Mice were inoculated intravenously with 2×10^7 or 6×10^7 cells, previously washed twice in sterile saline. Six mice per yeast strain were killed at one, two or four weeks after infection. To determine organ burden, brains and kidneys were removed aseptically, homogenized in 5 ml of sterile saline, and plated on CAT-Chl and Sabouraud supplemented with chloramphenicol (SDA-CHL) for determination of CFU.

The virulence of *C. albicans* strains was also evaluated in a model of systemic candidiasis. Mice were inoculated intravenously with 5×10^5

cells, previously washed twice in PBS. Mice were observed daily for signs of morbidity over 15 days. Moribund animals were scored as nonsurvivors and euthanized by CO_2 inhalation. To evaluate tissue invasion, additional groups of infected mice were sacrificed at one, three or six days post-inoculation. One kidney from each animal was removed and prepared for histological analysis. They were fixed in 10% formalin, embedded in paraffin, sectioned and stained by the Grocott procedure (Luna, 1992). The remaining kidney and the liver were also removed aseptically, weighed and homogenized in 5 ml PBS. Homogenates were plated on CAT-Chl and SDA-Chl, and incubated at 30 °C for colony counting. Data were expressed as CFU/g kidney or liver. Several colonies of *C. albicans* were isolated from both media to verify the *in vivo* stability of *SAP2* integration by Southern blot as described above.

Zymolyase and NaCl resistance

A zymolyase test was used to compare the sensitivity of the cell wall of transformants and parental strains (Lussier *et al.*, 1997). *S. cerevisiae* and *C. albicans* strains were grown in CAT medium to exponential phase (0.6 OD_{600}), washed twice in sterile saline and resuspended in sterile zymolyase buffer (50 mM Tris-HCl, pH 7.4) at 0.635 OD_{600}. Aliquots of 400 ml were mixed with 100 ml zymolyase 100T (1 mg ml^{-1}) or 100 ml of zymolyase buffer (controls). After 1 h incubation at 37 °C with gentle shaking, 5 ml of serial dilutions were spotted on CAT agar medium. *C. albicans* and *S. cerevisiae* strains were also tested for NaCl resistance. Briefly, cultures grown overnight in CAT medium were patched onto CAT plates supplemented or without 0.2, 0.6, 1.0, 1.4, 1.8, 2.2 or 2.6% NaCl.

Sensitivity to zymolyase and NaCl was determined by comparison of the level of growth after incubation at 30 °C.

Statistical analysis

The data were fed to the SAS system (SAS Institute Inc., Cary, NC, USA). The Kaplan and Meier (1958) product limit estimate was used to analyze survival data and plot the survival function. The Wilcoxon method was applied to compare the survival functions of experimentally infected mouse populations, and differences were considered significant at the 0.01 level.

Differences in weights and CFU g^{-1} of kidneys were compared among mouse populations by using the general linear models procedure two-way analysis of variance, conducted with two factors, one between (strain) and one within (time). Significant interactions ($p<0.01$) were further analyzed using the Bonferroni (Dunn) *t* test, and the resulting comparisons were considered significant at the 0.01 level.

RESULTS

Expression and secretion of Sap2p by *S. cerevisiae*

As an initial approach toward heterologous expression of *C. albicans SAP2* in *S. cerevisiae*, we performed a PCR amplification of this gene from *C. albicans* strain LAM-1. The 1244 bp PCR product containing the native prepro and mature coding regions was cloned into the multicopy expression vector pVT102-U downstream of the constitutive *ADH1* promoter, to create the plasmid pVTU-*SAP2* (sense). A negative control pVTU-a*SAP2* (antisense) bearing the PCR product in opposite orientation was also constructed. These recombinant plasmids were transformed in *S. cerevisiae* strains 6210 and S86, the latter defective in intracellular proteinases (Table 1).

The expression of Sap2p was evaluated by Western blot, in intra- and extracellular extracts. As shown in Fig. 1 (lanes 3, 4 and 6), a single positive signal of approximatively 42 kDa was detected in the extracts of strains transformed with pVTU-*SAP2* (sense orientation). A decrease in loading revealed additional forms of 39 and 45 kDa (data not shown). The largest amount of Sap2p was detected in the extracellular fraction (secreted) indicating that the secretion signal was fully functional in *S. cerevisiae*. In addition, there was more abundant production of Sap2p by strain S86, the intracellular proteinases mutant, compared to 6210 (Fig. 1, lanes 4 and 6). We could not determine whether this difference was due to the lack of these proteinases, since these strains are not isogenic. In addition, degradation of casein was demonstrated in a Petri dish assay (Colina *et al.*, 1996a) using unconcentrated culture supernatant of S86

(sense orientation), but was absent in the antisense orientation (data not shown). Based on these results, we decided to select strain S86 harbouring pVTU-*SAP*2 for further experiments. No anti-Sap2p reacting material was detected in the strain transformed with pVTU-a*SAP*2 (antisense orientation; Fig. 1, lanes 1 and 2).

Overall, these findings indicated that the recombinant enzyme was efficiently expressed and secreted into the culture medium, and that the signal sequence of *C. albicans SAP*2 was functional in *S. cerevisiae*.

Integrative transformation of *S. cerevisiae* and *C. albicans*

To produce stable *S. cerevisiae* and *C. albicans* transformants that could be used in animal models and for further genetic analysis, plasmids carrying *SAP*2 were integrated into the genome by homologous recombination.

(i) *S. cerevisiae*: The integrative plasmid, pRS-*SAP*2 (see Methods), was linearized at the NcoI site in *URA*3 and transformed into the 754 strain. A negative control was obtained by integrating pRS306 without insert. Proper integration was evaluated by Southern blot, after digestion of parental and transformant genomic DNAs with *Avr*II. As shown in Fig. 2 (panel *S. cerevisiae*), a single 2.7 kb fragment hybridized to the *URA*3 probe in the parental strain (754), as predicted. In positive transformants (884, 885), we expected a 9.1 kb fragment, resulting from the integration of pRS-*SAP*2. Unexpectedly, both strains gave a positive signal larger than 15 kb which can be interpreted as a double integration. Because of satisfactory expression of Sap2p, these strains were selected for further

studies (see below). A 7.1 kb band was detected in negative control 894, corresponding to the integrated vector without *SAP*2. These findings were confirmed by probing the same blot with a radiolabelled *SAP*2 DNA fragment (data not shown).

(ii) *C. albicans*: Plasmids pYPB-*SAP*2 and pYPB-m*SAP*2 carrying the wild-type and mutated *SAP*2 under the control of *ADH*1p, respectively, were linearized at the *Kpn*I site to target the integration into the *SAP*2 locus. Genomic DNAs were digested with *Kpn*I and probed with the *SAP*2 fragment (see Methods). In the parental strain (CAI4), the *SAP*2 probe hybridization resulted in a single band larger than 13 kb derived from the wild-type alleles of *SAP*2. The transformants S1, S2, N1 and N2 had an additional 13 kb fragment, which corresponds in size to the integrated construct (Fig. 2, panel *C. albicans*).

Kinetics of Sap2p secretion by *S. cerevisiae*

The time course for proteinase secretion was investigated in media containing free amino acids from acid hydrolysis of casein (CAT), or BSA as nitrogen source (YNB-BSA). In CAT medium, positive transformants 884 and 885 and negative control 894 showed growth rates similar to parental strain 754 (Fig. 3a, upper left). As shown in Fig. 3a (upper right), growth in YNB-BSA medium was restricted to strains harbouring the *SAP*2 gene, suggesting their ability to use BSA as sole nitrogen source. However, it is noteworthy to mention that the growth rates in this medium were lower than in CAT.

In both media, secreted proteolytic activity was detected exclusively in those transformants carrying *SAP*2 (Fig. 3b, middle left and right). However, the level of activity was 1.7-fold higher in CAT than in YNB-BSA, for the same cell density. This could reflect the greater physiological activity of the cells grown in the former medium. In CAT medium, the maximum level of proteinase activity was attained at the beginning of the stationary phase (Fig. 3b, middle left).

Additional information on Sap2p secretion was obtained from Western blot analysis of 885 transformant supernatants. Sap2p was detectable at the beginning of the exponential phase, after 6 and 24 h of incubation in CAT and YNB-BSA media, respectively (Fig. 3c, lower left and right). In both media, the protein was processed to three forms with approximate molecular masses of 45, 42 and 39 kDa. The middle 42 kDa form co-migrated with Sap2p purified from concentrated culture filtrate of *C. albicans* strain LAM-1, consistent with our previous observations (Colina *et al.*, 1996b). The higher 45 kDa form may result from altered post-translational modification or incomplete processing of the protein by *S. cerevisiae*, and the lower 39 kDa form may represent a degradation product. Using a zymogram procedure described previously (Colina *et al.*, 1996b), it was shown that each of the three bands had enzymatic activity using mucin as substrate (data not shown). No Sap2p was detected in negative control 894 (data not shown).

Kinetics of Sap2p secretion by *C. albicans*

Similarly to *S. cerevisiae*, *C. albicans* was grown in CAT or YNB-BSA medium, respectively non-inducing or inducing *SAP*2 expression. In

CAT medium, the growth rates of the secretor-transformant S2 as well as the negative controls N1 and N2 (with mutated sap2), were indistinguishable from that of the parental strain, CAI4. However, the secretor-transformant S1 grew far more slowly than CAI4 (Fig. 4a, upper left). In YNB-BSA medium, transformants S1 and S2 displayed similar growth rates, which were greater than those of CAI4 and negative controls (Fig. 4a, upper right). Overall, cultures of S2, N1, N2 and CAI4 reached a higher cell density when media were supplemented with amino acids rather than BSA as nitrogen source.

Under non-inducing conditions (CAT medium), secreted proteolytic activity was detectable only in the supernatants of transformants S1 and S2 (Fig. 4b, middle left). These results indicated that *SAP2*, placed under *ADH*1p, was constitutively expressed in transformants S1 and S2. In YNB-BSA medium, conditions in which wild-type *SAP2* is induced, proteolytic activity was detected in CAI4 and negative controls as well as in secretor-transformants. However, in the latter strains, activity was 3.3-fold (S1) and 1.75-fold (S2) higher than in CAI4 or negative controls (Fig. 4b, middle right). Although higher proteolytic activity in S1 could have possibly resulted from the integration of multiple copies of the *ADH*1 fusion, this was unlikely because the intensities of the bands for wild-type *SAP2* and integrated vector were individually comparable in the Southern analysis of S1 and S2 (Fig. 2, right panel). Enhanced proteolytic activity in YNB-BSA compared to CAT medium (Fig. 4) may have resulted from the presence of other Saps produced in YNB-BSA (Hube *et al.*, 1994).

Finally, the supernatants of S2 and N1 strains were selected to evaluate the presence of Sap2p. In CAT medium, Sap2p of S2 (apparent

molecular mass, 42 kDa) was detectable after 9 h of incubation, corresponding to the exponential phase (Fig. 4c, lower left). No protein was detected in N1 supernatants (data not shown). Interestingly, when BSA was included in the culture medium (Fig. 4c, lower right), S2 produced detectable Sap2p 3 h earlier than N1. The slower growth rate of N1 compared to S2 correlated with the apparent lower secretion of Sap2p.

Germination rates of the transformants and parental strain were indistinguishable. After 3 h of incubation at 37 °C, more than 70% of blastoconidia had germinated.

Pathogenicity *in vivo*

The virulence of *S. cerevisiae* negative (894) and positive (884, 885) transformants was evaluated in experimental oral or systemic infection in intact mice. In the former, all three strains were completely cleared from the oral cavity 5h after inoculation (data not shown). A similar result was obtained after systemic infection. No CFUs were recovered from kidneys or brains of mice over a period of 28 days after infection with each of the *S. cerevisiae* strains. The inoculum of 2×10^7 cells was selected in accordance with a previous study (Clemons *et al.*, 1994) which evaluated the pathogenic potential of various clinical and laboratory isolates of *S. cerevisiae*. In addition, when the experiment was repeated using 6×10^7 cells to exclude the possibility of an insufficient inoculum, cultures of homogenized organs remained completely negative for all strains. A control experiment was conducted using a 2×10^7 cells inoculum of *S. cerevisiae* Y55 (avirulent laboratory strain) and virulent clinical isolates YJM128 and YJM273, as described by Clemons *et al.* (1994).

Quantification of CFUs in kidneys and brains of mice produced results very close to those previously reported (Clemons *et al.*, 1994) (data not shown). This confirmed that lack of virulence obtained with strains 884, 885 and 894 was not due to a technical problem.

C. albicans parental strain SC5314, and positive (S1, S2) and negative (N1, N2) transformants were compared for their virulence in two models of candidiasis. In the murine oral model, all four transformants were completely cleared 24 h after inoculation. Positive control *C. albicans* strain LAM-1 produced an initial decrease in CFUs at 24 h followed by a mean peak of 5.6×10^3 CFU 48 h after infection, as reported previously (Lacasse *et al.*, 1990). *C. albicans* parental strain SC5314 was less virulent than LAM-1 in the oral model, producing a peak of 3.0×10^3 CFU. Virulence of *C. albicans* transformants was also determined in a murine model of systemic candidiasis. In a first experiment, in which survival of mice was used as endpoint (Fig. 5), no mice died after intravenous infection with strain S1 which was thus completely avirulent. However, strain SC5314 was significantly ($p<0.007$) more virulent than S2, N1 and N2, which were not significantly different among themselves ($p>0.15$). A second experiment evaluated kidney and liver burdens of the same strains after intravenous infection. Comparison of organ weights revealed no reproducibly significant differences on days 1, 3 and 6 after infection with strains SC5314, S1, S2, N1 and N2 (data not shown). However, comparison of mean log CFUs recovered from kidneys on these same days showed strikingly decreased CFU g^{-1} for strain S1 compared to the other four strains, which among themselves were not significantly different (Table 2). CFUs in liver were two logs (day 1) and four logs (days 3 and 6) lower than

those obtained from the kidneys. However, the relationship among strains remained the same. All strains produced equivalent liver CFUs on the three observation days, except for strain S1 which yielded negative cultures (data not shown). Finally, randomly selected colonies of S1, S2, N1 and N2 isolated from the kidneys of mice showed no modification at the *SAP2* locus by Southern blot.

In the previous experiment, one kidney was removed for CFU quantification and the other for histological analysis. Macroscopic examination of the kidneys showed several surface microabcesses on days 3 and 6 after infection for all strains except S1. Interestingly, larger microabcesses were noted for strain S2 on day 3, compared to the other strains. Histopathological examination demonstrated a mixture of blastoconidia, hyphae and pseudohyphae in the cortex on days 1 and 3 with strains SC5314, N1, N2 and S2. However, no fungi were observed on these same days in kidneys of mice infected with strain S1. Moreover, strain SC5314 produced several cortical abcesses on day 1, while strains N1, N2 and S2 produced very rare blastoconidia and hyphae. On day 3, more widespread fungi were seen with strain SC5314 compared to day 1, with extension into the renal medulla; N1, N2 and S2 were very sparse or absent.

Zymolyase and NaCl resistance

We observed decreasing viability of *S. cerevisiae* and *C. albicans* transformants expressing or overexpressing Sap2p on CAT medium held for 5 days at 4 °C, after initial growth at 30 °C for 48 h. This could have potentially resulted from cell wall damage produced by massive expression

and/or secretion of the protease. The same hypothesis could also account for the absence of increased virulence of positive transformant S2 and the decreased virulence of S1 *in vivo*, compared to the negative controls. To assess this possibility, two different cell wall resistance assays were done. In both the zymolyase and NaCl resistance assays, all *S. cerevisiae* negative control and positive transformants demonstrated the same level of sensitivity. However, treatment with zymolyase resulted in a 2-log greater decrease in viability of *C. albicans* strain S1, compared to SC5314, S2, N1 and N2. In addition, growth of strain S1 was inhibited by 1.4% NaCl, compared to 2.2% for the other four *C. albicans* strains.

DISCUSSION

This is the first report describing the construction of stable *S. cerevisiae* and *C. albicans* integrative transformants, constitutively secreting Sap2p. We used the *S. cerevisiae* or *C. albicans ADH*1 promoter (Bertram *et al.*, 1996) on expression plasmids to overexpress *C. albicans SAP*2 in both yeast species. The *ADH*1 promoter has been previously used in studies of gene expression in *C. albicans* (Cormack *et al.*, 1997).

On the basis of *SAP*2 DNA and Sap2p N-terminal sequences, the mRNA is translated as a preproform, 56 amino acids larger than the mature protein. The prepropeptide has a signal peptide sequence of 14-21 amino acids with 1 to 4 putative signal peptidase cleavage sites and two Lys-Arg sequences, one of which is immediately before the N-terminus of the mature form (Wright *et al.*, 1992; Hube, 1996). Thus, Sap2p was expected to be processed in *S. cerevisiae* because it has been shown that the cleavage of *C. tropicalis* Sap heterologously expressed in *S. cerevisiae* is mediated by *KEX*2-dependent proteolysis (Togni *et al.*, 1996).

The Sap2p signal peptide was functional in *S. cerevisiae*, allowing the secretion of three active forms of Sap2p. While we cannot rule out the possibility of posttranslational modifications, the higher molecular mass form (45 kDa) may result from incomplete processing of the proteinase by *S. cerevisiae*. The secretion of a premature form may be due to saturation of the processing system as a consequence of overexpression of the protein. Previous reports have shown that heterologous expression of C. tropicalis *SAP*T1 (Togni *et al.*, 1996) or *C. albicans SAP*1 (Smolenski *et al.*, 1997) in *S. cerevisiae* resulted in secretion of extracellular active enzyme

identical to the native form, indicating that no oligosaccharide chains were added. In both cases, the expression was under the control of inducible promoter *GAL*10 and the hosts were not deficient in intracellular proteinases. Although no extensive studies have been done on the intracellular processing of *C. albicans* Sap2p, Banerjee *et al.* (1991) and Homma *et al.* (1992) reported the detection of a 45 kDa intracellular precursor of Sap2p. More recently, it was shown that heterologous expression of *SAP*T1 of *C. tropicalis* by *S. cerevisiae*, resulted in intracellular forms 4 to 6 kDa larger than the mature protein (Togni *et al.*, 1996). Further experiments will be required to clarify these issues.

Considering that *S. cerevisiae* is nonpathogenic, the study of the virulence of strains secreting Sap2p is an attractive system to address its role in pathogenicity in the absence of other putative factors. Although episomal expression of Sap1p has been previously achieved in *S. cerevisiae* (Smolenski *et al.*, 1997), we have now obtained expression of the major transcription product Sap2p from an integrated construct, suitable for studies of virulence in animal models. The constitutive expression of Sap2p by *S. cerevisiae* clearly allowed it to hydrolyse and use BSA. This may be important for survival in animal models, because it would enable it to use host proteins as nitrogen source.

A model of the regulation of *SAP*2 expression suggests that *C. albicans* has a basal level of proteinase expression, which yields peptides that act as inducers of *SAP*2 expression, via a positive feedback mechanism (Hube *et al.*, 1994). Our data indicate that *SAP*2 expression was deregulated in those *C. albicans* strains constitutively producing Sap2p. In the presence of high molecular weight protein, the enzyme was

secreted precociously, which resulted in earlier induction of the wild-type *SAP*2 gene and higher amounts of total proteinase activity. These strains constitute a novel approach to explore the role of early and high expression of Sap2p during experimental infections. In fact, De Bernardis *et al.* (1995) have shown that highly vaginopathic strains expressed both *SAP*1 and *SAP*2 earlier in experimental vaginitis than moderately or nonvaginopathic strains. Furthermore, it has been hypothesized that Saps may play a role early in systemic dissemination of *C. albicans* to deep organs from mucosal sites (Kimberly *et al.*, 1997). Finally, *C. albicans* isolates from oral cavities of symptomatic HIV-infected patients secreted significantly higher levels of proteinase than those from asymptomatic or noninfected patient (Ollert *et al.*, 1995; De Bernardis *et al.*, 1996). When tested in experimentally infected animals, the high-producers had enhanced virulence (De Bernardis *et al.*, 1996). However, whether the increased proteinase activity was due to a particular Sap(s) was not determined.

In pathogenicity studies *in vivo*, expression of *SAP*2 as a sole putative virulence factor did not cause an avirulent strain of *S. cerevisiae* to become virulent, and constitutive expression of *SAP*2 did not augment virulence of *C. albicans* in experimental oral or systemic infection in intact mice. The absence of enhanced virulence of *S. cerevisiae SAP*2 transformants is consistent with the long held view that *C. albicans* possesses a multiplicity of properties, each with a low propensity for enhancing fungal infectivity and none necessarily dominant, and all, even in combination unable to overcome fully intact host defenses (Odds, 1988).

Unknown mutations occurring concurrently in all strains would be unlikely to explain the significantly enhanced survival of mice infected with

C. albicans negative control (N1, N2) or positive *SAP*2 transformant (S2), compared to parental strain SC5314. In addition, although N1, N2 and S2 only contain one copy of *URA*3, while the wild-type SC5314 has two copies, a gene dosage effect is also unlikely to be responsible for the attenuated virulence of N1, N2 and S2 since heterozygous *URA*3 mutants were as virulent as parental strains in experimentally infected mice (Leberer *et al.*, 1997). Because the negative controls (N1, N2) would have been expected to be as virulent as the wild-type SC5314, mutated *SAP*2 may have been translated into a truncated protein, resulting in saturation of the secretory pathway.

Although positive *C. albicans* transformants S1 and S2 were expected to be isogenic, they had different phenotypes. S1 grew much slower in CAT medium compared to all other strains including the wild-type SC5314. In addition, this strain had higher proteolytic activity in YNB-BSA medium and was avirulent after systemic infection in intact mice. The strikingly decreased virulence of strain S1 and its enhanced cell wall sensitivity compared to the other *C. albicans* transformants suggest that *SAP*2 overexpression may have damaged the cell wall, resulting in enhanced killing by polymorphonuclear leukocytes and other intact host defenses. Alternately, an unknown mutation in S1 causing slower growth in CAT medium may also have attenuated virulence. With regard to strain S2, a mutation may have caused a reduced secretion of Sap2p, which in turn caused less damage to the cell wall of *C. albicans*. Lack of enhancement of virulence of positive *C. albicans* transformant S2 compared to negative controls N1 and N2 may have resulted from unlikely spurious mutations during transformation, the route of infection, the

absence of immunosuppression, or defects in other virulence factors due to saturation of secretory pathways. In this regard, all *C. albicans* transformants maintained the ability to form pseudohyphae and hyphae *in vivo*. Although Sap2p is the most abundant transcript of *C. albicans* yeast cells, increased virulence *in vivo* may require not only its enhanced expression, but also the early participation of other putative virulence factors including members of the Sap isoenzyme family, such as Saps 4, 5 and 6 which are produced by the hyphal form of *C. albicans*. Finally, an absence of *SAP2* constitutive expression in the animal models was unlikely because *ADH1* is a strong constitutive promoter. Furthermore, *C. albicans* transformants isolated from the kidneys of mice showed no modification at the *SAP2* locus. Further studies will be required to determine whether differences in virulence would be observed in a rat vaginal model between the wild-type and strains overexpressing *SAP2*. Although *SAP2* constitutive expression did not augment virulence, this new approach may be a useful paradigm in the study of virulence attributes in *Candida* and other fungal pathogens.

ACKNOWLEDGEMENTS

This study was supported by grants from the Medical Research Council of Canada and the Fonds de la Recherche en Santé du Québec. A.R.C. was the recipient of a studentship from the Consejo Nacional de Investigaciones Cientificas y Tecnologicas, Caracas, Venezuela.

We are gratefull to W. Fonzi for providing *C. albicans* strain CAI4, to A. H. Bussey for *S. cerevisiae* strain cIABYS86, and to K. V. Clemons for *S. cerevisiae* YJM128, YJM273 and Y55 strains. We also wish to thank A. J. P. Brown for vector YPB-*ADH*1pL, and T. L. Ray for anti-Sap2 monoclonal antibody.

We thank Annie-Claude Miailhe for excellent technical assistance and Marc Fredette, Michel Lamoureux and Yves Lepage for statistical analysis.

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Table 1. Yeast strains

Strain	Genotype	Source or reference
S. cerevisiae		
clABYS86	*MATα leu2 ura3-52his3 pra1 prb2 prc1 cps*	A. H. Bussey
PUB754	*MATα ura3-52 pra1 prb2 prc1 cps*	This study
SEY6210	*MATα leu2-3,112 ura3-52 his3Δ200 lys2-801*	
	trp1Δ901 suc2Δ9	Robinson *et al.*, 1988
Y55	(Laboratory isolate), HO	Clemons *et al.*, 1994
YJM128	(clinical isolate)	Clemons *et al.*, 1994
YJM273	(clinical isolate)	Clemons *et al.*, 1994
C. albicans		
CAI4	*Δura3::imm434/Δura3::imm434*	Fonzi *et al.*, 1993
LAM-1	(clinical isolate)	Lacasse *et al.*, 1990
SC5314	(clinical isolate)	Gillum *et al.*, 1984

Table 2. Comparative CFU of *C. albicans* parental strain and transformants recovered from kidneys at various times during disseminated infection of mice

Day	Strain	Nº of animals	Mean log CFU in kidney per g of tissue ± SD
1	SC5314	7	4.45 ± 0.75
	S1	7	0.88 ± 1.39
	S2	7	4.57 ± 0.27
	N1	4	5.93 ± 0.07
	N2	5	5.53 ± 0.52
3	SC5314	7	5.04 ± 0.51
	S1	6	0.88 ± 0.88
	S2	6	5.99 ± 0.74
	N1	6	5.15 ± 0.60
	N2	6	5.10 ± 0.73
6	SC5314	7	4.98 ± 0.59
	S1	6	0
	S2	4	5.58 ± 0.15
	N1	5	5.78 ± 0.28
	N2	6	5.55 ± 0.31

Figure 1. Western blot analysis of intra- (I) and extracellular (E) proteins of *S. cerevisiae* S86 and 6210 plasmidic transformants (with sense or antisense *SAP*2 constructs) grown in CAT medium for 18 h at 30°C, and probed with an anti-Sap2p monoclonal antibody. Loaded sample volumes were adjusted to equivalent mass cultures.

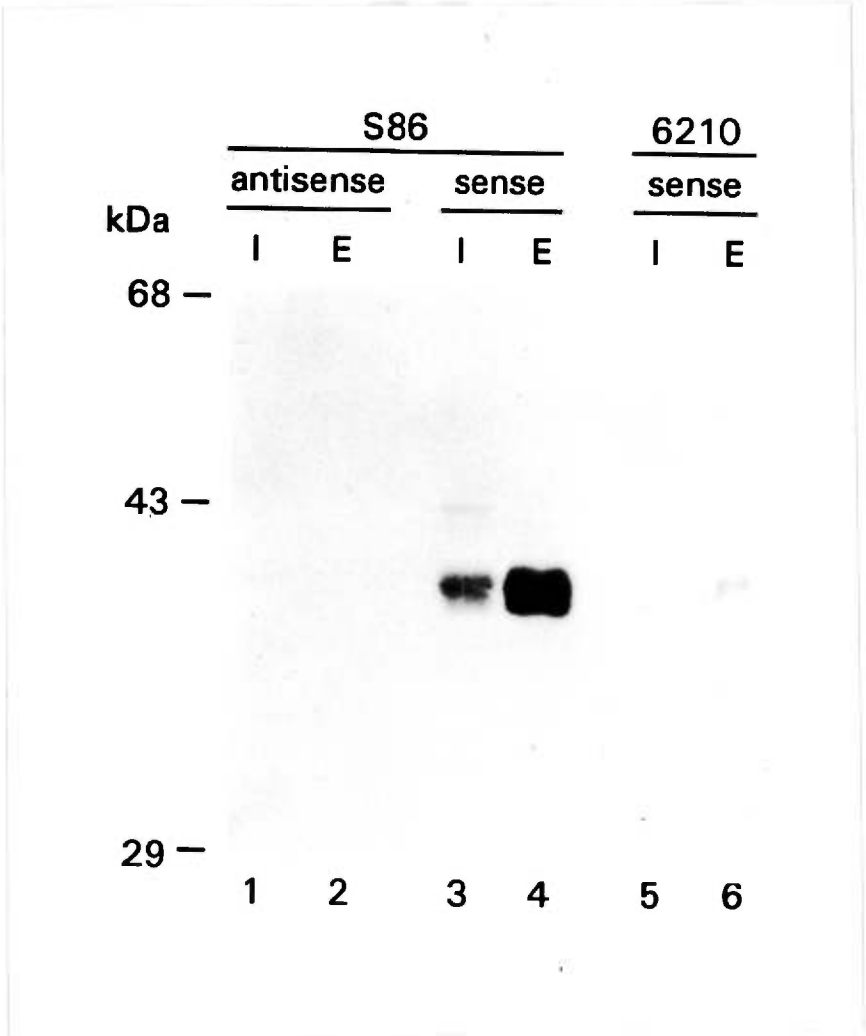

S86
6210
antisense
sense
sense
kDa
I
E
I
E
I
E
68 —
43 —
29 —
1
2
3
4
5
6

Figure 2. Southern blot analysis of wild-type and selected transformants of *S. cerevisiae* and *C. albicans*. Top, integration sites with probes indicated by thick lines. Bottom, *S. cerevisiae* and *C. albicans* genomic DNAs digested with *Avr*II and *Kpn*I, respectively and probed with [^{32}P] *EcoR*V-*Sma*I (*S. cerevisiae*) or [^{32}P] *Pst*I-*Dra*I *SAP*2 (*C. albicans*) DNA. Grey boxes represent the chromosomal copy of *URA*3 and *SAP*2 genes.

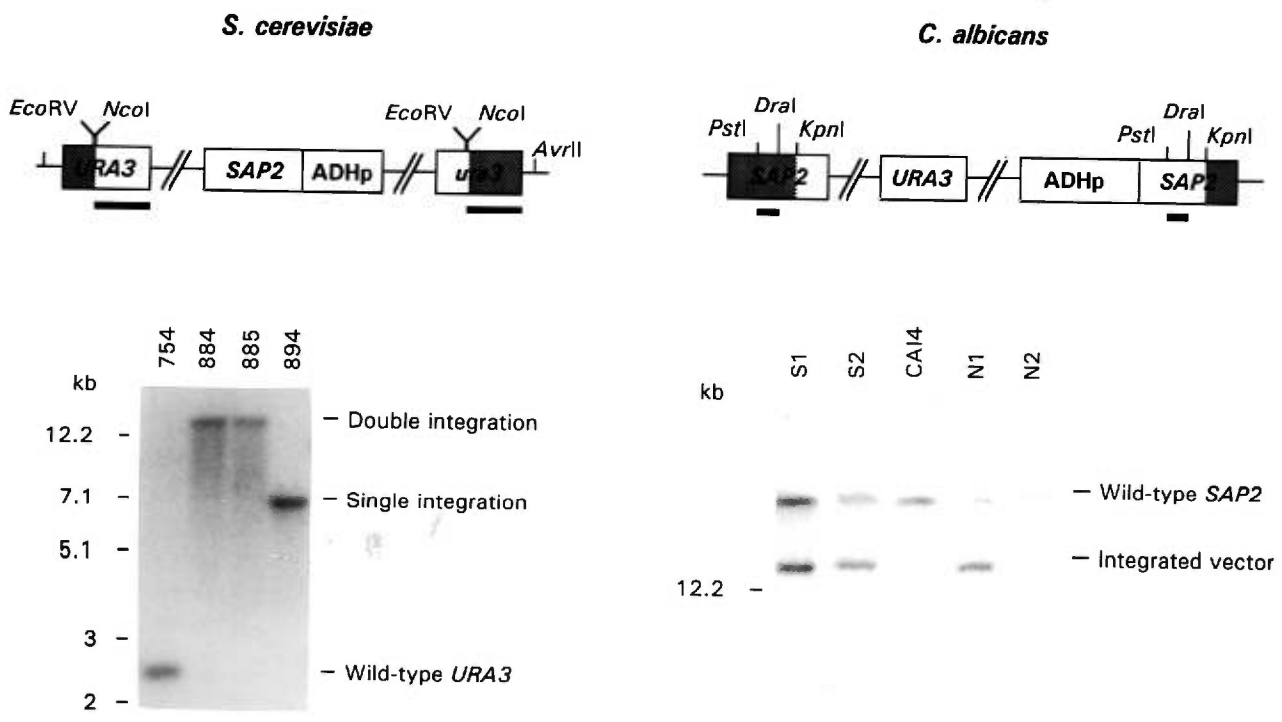

S. cerevisiae
EcoRV
NcoI
EcoRV
NcoI
AvrII
URA3
SAP2
ADHp
754
884
885
894
kb
12.2
7.1
5.1
3
2
Double integration
Single integration
Wild-type URA3
C. albicans
DraI
PstI
KpnI
DraI
PstI
KpnI
SAP2
URA3
ADHp
S1
S2
CAI4
N1
N2
kb
12.2
Wild-type SAP2
Integrated vector

Figure 3. Kinetics of Sap2p secretion by *S. cerevisiae* positive transformants 884 (●) and 885 (○), negative control 894 (■), and parental strain 754 (□), grown in 0.5% CAT (left) and YNB-BSA (right) media at 37°C. At the indicated times, culture samples were evaluated for growth (a), extracellular proteolytic activity (b) and Sap2p expression in 885 (Western blot) (c). Data are mean values of duplicate experiments.

Figure 4. Kinetics of Sap2p secretion by *C. albicans* positive transformants S1 (●) and S2 (○), negative controls N1 (■) and N2 (□) and parental strain CAI4 (◆), grown in 1% CAT (left) and YNB-BSA (right) media at 37°C. At the indicated times, culture samples were evaluated for growth (a), extracellular proteolytic activity (b) and Sap2p expression in S2 and N1 (Western blot) (c). Data are mean values of duplicate experiments.

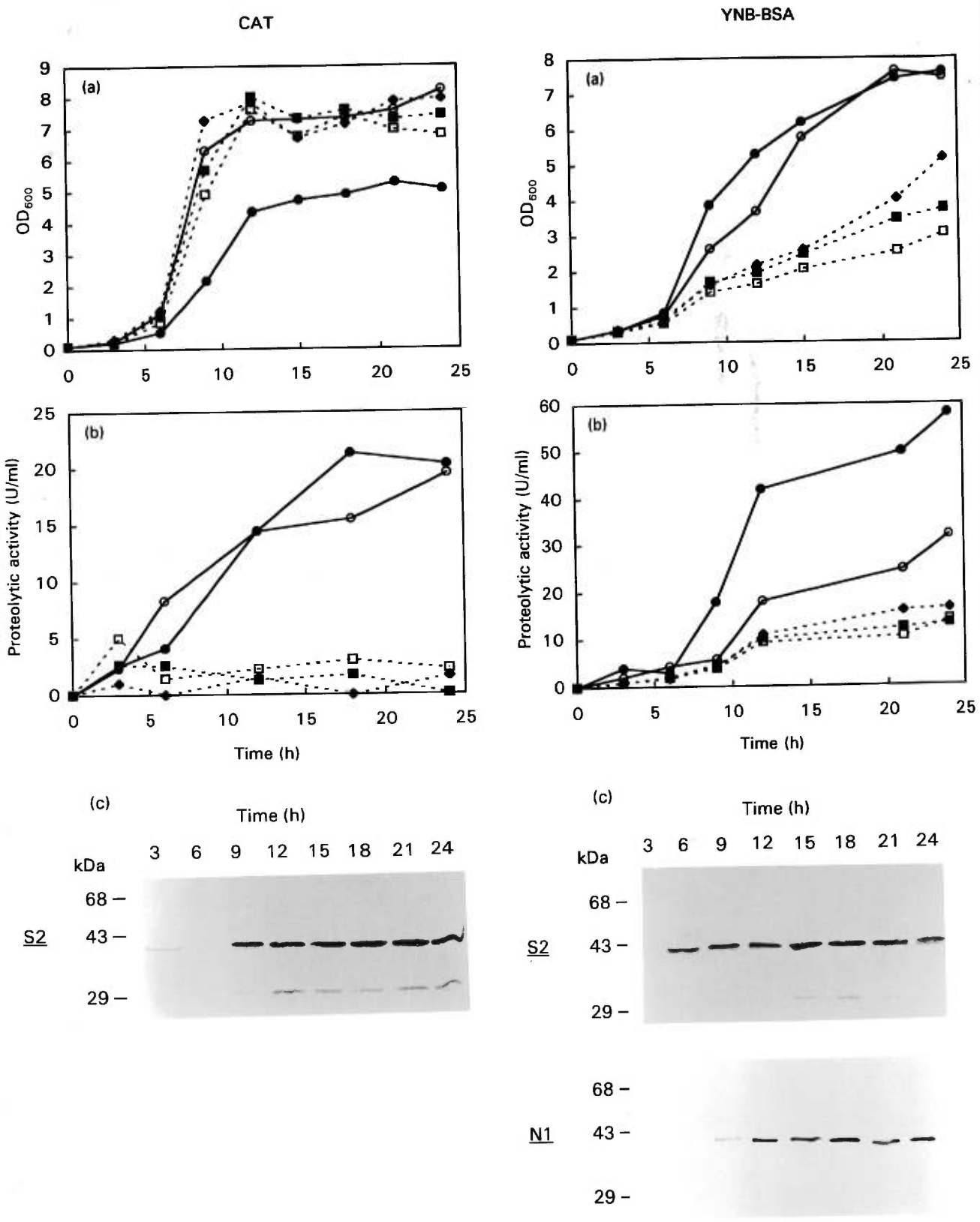
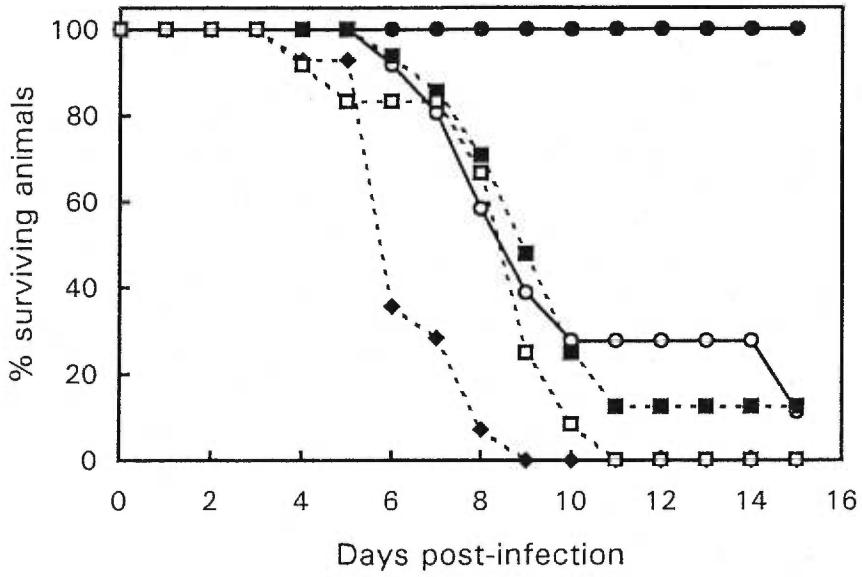

CAT
YNB-BSA
(a)
(b)
(c)
OD600
Proteolytic activity (U/ml)
Time (h)
kDa
S2
N1
68
43
29
3 6 9 12 15 18 21 24

Figure 5. Survival of CD-1 mice infected intravenously with 5 x 10^5 cells of *C. albicans* transformants S1 (●) (n=12), S2 (o) (n=15), N1 (□) (n=14), N2 (■) (n=12) and parental strain SC5314 (◆) (n=14).


100
80
60
40
20
0
% surviving animals
0
2
4
6
8
10
12
14
16
Days post-infection

CHAPTER V

General discussion and conclusions

1. GENERAL DISCUSSION

The opportunistic pathogen *Candida albicans* has evolved sophisticated ways to interact with the human host. In an attempt to understand these mechanisms, the studies described in this project globally aimed to identify and characterize the mucinolytic activity of *C. albicans* and explore its role in the pathogenesis of mucosal and systemic candidiasis.

As a first step in the project, a microplate assay was developed in order to monitor mucinase activity from *C. albicans* blastoconidia, grown in the presence of hog gastric mucin as the sole nitrogen source (Chapter II). This method, although requiring partially purified enzyme preparations, proved to be appropiate in quantifyng mucinase activity from the culture filtrate. In addition, biochemical, immunochemical and enzymatic analyses of concentrated culture filtrates provided evidence indicating that the secreted aspartyl proteinase 2 (Sap2p) was involved in the *in vitro* degradation of mucin (Chapter III). It was rather surprising that the low protein-content mucin induced the secretion of Sap2p, which suggest that their "naked" peptide regions should contain at least 8 amino acids. This is supported by findings indicating that polypeptides with 7 or less amino acid residues did not act as inducing signals for proteinase production (Lerner & Goldman, 1993). Considering that human intestinal and gastric mucins share structural properties with hog gastric mucin (Pearson *et al.*, 1980), these findings support the hypothesis that Sap2p may assist *C. albicans* in the process of *in vivo* penetration of the gastrointestinal mucosa by degrading the protective mucin barrier and hence, facilitating adhesion to the underlying epithelial cells.

The fact that Sap2p was the only proteinase secreted in mucin-containing media suggested that it may contribute to the overall proteolytic damage of mucosal barriers. However, it cannot be ruled out that other members of the Sap family may function synegistically during *in vivo* infection. This assumption is reasonable because it has been shown that sera from HIV-positive patients suffering from candidiasis, as well as sera from experimentally infected mice, reacted predominantly with Saps 1 and 3, rather than Sap2 (Agabian *et al.*, 1994). Also, crystallographic analysis of the binding sites of the isoenzymes Sap1, Sap2 and Sap3 suggests that structural differences among them may affect their substrate specificities (Abad-Zapatero *et al.*, 1996; Cutfield *et al.*, 1995).

This apparent redundancy of function of the Saps may be an effective strategy for *C. albicans* to adapt to rapidly responding immune system defenses and a broad range of different host tissues. In fact, among both pathogenic bacteria and fungi, the multiplicity of genes encoding for similar virulence factors seems to be the rule, rather than the exception. *Listeria monocytogenes* has two phospholipase genes that can mediate its escape from the intracellular vacuole. Deletion of either gene did not significantly affect virulence, but when both genes were deleted, virulence was lost (Smith *et al.*, 1995). In *Cryptococcus neoformans,* at least seven genes may be involved in the production of melanin, which has been associated with virulence (Torres-Guerrero & Edman, 1994). Similarly, it has also been proposed that at least four extracellular proteases from *Aspergillus fumigatus* have virulence attributes (Reichard *et al.*, 1990).

The data above link for the first time, the production of Sap2p by *C. albicans* with the degradation of a host protective protein, mucin. Given that

Sap2p has a broad substrate specificity, the identification of physiologically relevant substrates, such as mucins, is important for the understanding of the mechanisms by which Sap2p facilitates infection.

Our findings are in general agreement with those from the existing literature that strongly implicate extracellular proteinases, particularly Sap2, as potential virulence factors (Chapter I, Section 2.3.4.3). However, definitive proof supporting this hypothesis has not been achieved. In this context, the expression of *SAP*2 in a non-pathogenic but closely related fungus such as *Saccharomyces cerevisiae* and its overexpression in *C. albicans* seemed to be a rational approach to investigate its role as a virulence-enhancing gene.

The constitutive expression of *SAP*2 in *S. cerevisiae* and *C. albicans* was undertaken as described in Chapter IV. The ORF of *SAP*2 was placed downstream of the constitutive promoter *ADH*1 and integrated into the genome. Both *S. cerevisiae* and *C. albicans* efficiently secreted a mature Sap2p, as deduced from the molecular mass measurements of the recombinant proteinase and the strong proteolytic activity detected in culture supernatants. Kinetic studies indicated that the proteinase was secreted in both inducing and repressive conditions, thus strongly suggesting that Sap2p was constitutively expressed. Previous reports have described the heterologous expression of other *Candida SAP*s in *S. cerevisiae* (Togni *et al.*, 1996; Smolenski *et al.*, 1997). However, these studies involved the use of episomal plasmids harbouring the gene under the control of an inductible promoter. In contrast, our approach was based upon integrative plasmids and the use of a constitutive promoter, which resulted in stable expression and thus made these genetically modified strains more suitable for biological studies in animal models.

Virulence studies were performed in mouse models of oral and disseminated candidiasis. In both models, none of the *S. cerevisiae* strains were infectious. In addition, neither *C. albicans* parental strain nor transformants showed virulence in the oral model candidiasis. Nevertheless, when *C. albicans* transformants were intravenously inoculated, their virulence was significantly reduced compared to the parental strain. To our knowledge, this is the first report of *in vivo* pathogenicity studies of *S. cerevisiae* and *C. albicans* strains overexpressing a *C. albicans* putative virulence factor. These results indicate that the constitutive expression of Sap2p was not sufficient to promote virulence in *S. cerevisiae* and to enhance virulence in *C. albicans.* Curiously, the parental strain of *C. albicans* transformants (strain SC5314) was infectious only when inoculated intravenously but not orally. These observations suggest that *C. albicans* virulence depends on the strain and route of inoculation and that the virulence attributes required during a mucosal infection may not be the same as those involved in disseminated disease.

Collectively, these findings support the view that virulence in *C. albicans* is not the result of the expression/overexpression of a single virulence determinant but rather that a choreographic expression of several virulence factors may be required. This hypothesis, initially postulated by Cutler (1991), gained support with the recent findings regarding the virulence of *SAP*1, *SAP*2 and *SAP*3 homozygous null mutans (Hube *et al.* 1997). Although no extracellular proteinase activity was demonstrated *in vitro* for *sap*2 null mutants, pathogenicity studies showed that this strain was still virulent. Moreover, only slight differences in survival were detected between mice infected with the wild-type strain and those with each one of the null

mutants. In addition, the observation that a triple deletion of *C. albicans* *SAP*4, *SAP*5 and *SAP*6 attenuated virulence in mice also support the hypothesis of concerted action of several genes encoding for virulence factors (Sanglard *et al.*, 1997).

Our results may appear to contradict those reported by De Bernardis *et al.* (1996), that suggested that there is a link between high-proteinase secretion *in vitro* and pathogenicity of *C. albicans* in vaginal candidiasis. However, it is worth noting that in their study they used non-isogenic and wild-type strains, while we and others (Hube *et al.*, 1997) worked with isogenic and genetically modified *C. albicans*. In addition, although it has been determined that Sap2p is the major proteinase secreted in protein-containing media, it is possible that *in vivo* conditions may favor the expression of other Saps which are not usually expressed *in vitro* or under commensal conditions.

In contrast to the *in vivo* results, *in vitro* data presented in Chapter IV (Figures 3 and 4) showed quite clearly that strains that constitutively express Sap2p have a competitive advantage for growing in protein-containing media. However, it seems likely that the infection process may include several steps of gene activation/deactivation in response to environmental conditions, and that therefore strains with unregulated expression of Sap2p may not succeed as pathogens. It is possible that the yeast may (i) waste energy in the continuous synthesis of unneeded proteins or (ii) become unable to produce required proteins because of saturation on secretory pathways. In fact, some recent studies indicated that Sap2p may be particularly important only at the early stage of mucosal infection (De Bernardis *et al.*, 1995; Fallon *et al.*, 1996). Indeed, coordinated regulation of

virulence traits is a common strategy used by most bacterial pathogens. For instance, pathogenic *Escherichia coli* and *Proteus mirabilis* express several adhesins on mucosal surfaces. However, after invading deeper tissues, they either shed some of these adhesins or produce masking capsules (Beachey, 1981).

Increased susceptibility to phagocytosis and killing by PMNs may also be involved in the lack of enhanced virulence of *C. albicans* transformants. Yeast cells that continuously and abundantly express Sap2p on their surfaces may be more easily opsonized by antibodies and subsequently engulfed by phagocytes. In addition, the apparent damage of the yeast cell wall resulting from the overexpression of the proteinase, may render the fungus more prone to the killing mechanisms of PMNs.

Finally, the immune status of the host must also be taken into account in the analysis of our results. We and others (Hube *et al.*, 1997, Sanglard *et al.*, 1997) have used immunologically intact animals to study pathogenicity of an opportunistic fungus. While there is not doubt that this model tests the ability of *C. albicans* to overcome the defenses of an immunocompetent host, the potentially enhanced virulence of our transformants may be underestimated under these circumstances. Given that the mechanisms operative in the intact mouse may differ from those in the compromised animal, further analysis of our genetically transformed strains should be done in immunodeficient mouse models.

2. Questions derived from this study

Data from this project are consistent with the view that *C. albicans*, as other pathogens, may have developed precise programs for expressing combinations of genes involved in pathogenesis. However, several questions regarding the *in vivo* expression of *SAP*s and regulation of their expression remain unanswered.

- Which of the member(s) of the *SAP* family are expressed during a mucosal/invasive infection, and when are they expressed? The timing and magnitude of Sap(s) expression may vary depending on the site and stage of infection.

- Is their expression relevant to the development of infection? The mere *in vitro* expression of a gene will not always guarantee that it is expressed *in vivo* to such an extent that it will be required for infection.

- Which host factors are responsible for triggering the expression of *SAP* products? The identification of host factors that induce Sap(s) secretion, may suggest the mechanisms by which Sap(s) facilitate infection.

- Is *in vivo* expression of Sap(s) linked to the expression of other virulence factors? The current view of *Candida* virulence indicates that Sap(s) expression may be temporally coordinated with the expression of other potential virulence factors such as adhesins, dimorphism and phenotypic switching.

- How is *SAP* expression regulated and what aspect(s) of the regulatory system are necessary for virulence? Characterization of such regulatory systems may contribute significantly to our understanding of the molecular basis of *Candida* pathogenicity and provide new targets for antifungal drugs.

Several molecular and biochemical tools are now available that may allow us to answer these questions in the near future.

A worthwhile approach would be to construct vectors containing a hybrid gene which includes *SAP* promoters upstream of a promoterless reporter gene such as the yeast-enhanced green fluorescent protein gene (*yEGFP*, Cormack *et al.*, 1997) or the β-galactosidase of *Kluyveromyces lactis* (*LAC*4, Leuker *et al.*, 1992). The vectors are selectively integrated into the genome and the transformed *C. albicans* strains are inoculated into an appropriate animal and/or cell culture system. The expression of the gene fusions can be monitored in animal/culture tissues by FACS, in situ analysis of the fluorescence or by immuno/enzymatic detection of the β-galactosidase (Figure 1). The expression of the reporter genes will indicate that at this stage of the infection, host factors have induced the activation of a particular *SAP* promoter.

A concomitant approach to functionally characterize those relevant promoters is to fuse *SAP* promoters to a highly sensitive reporter gene, the *Renilla reniformis* luciferase gene (*R-lux*) (Srikantha *et al.*, 1996).

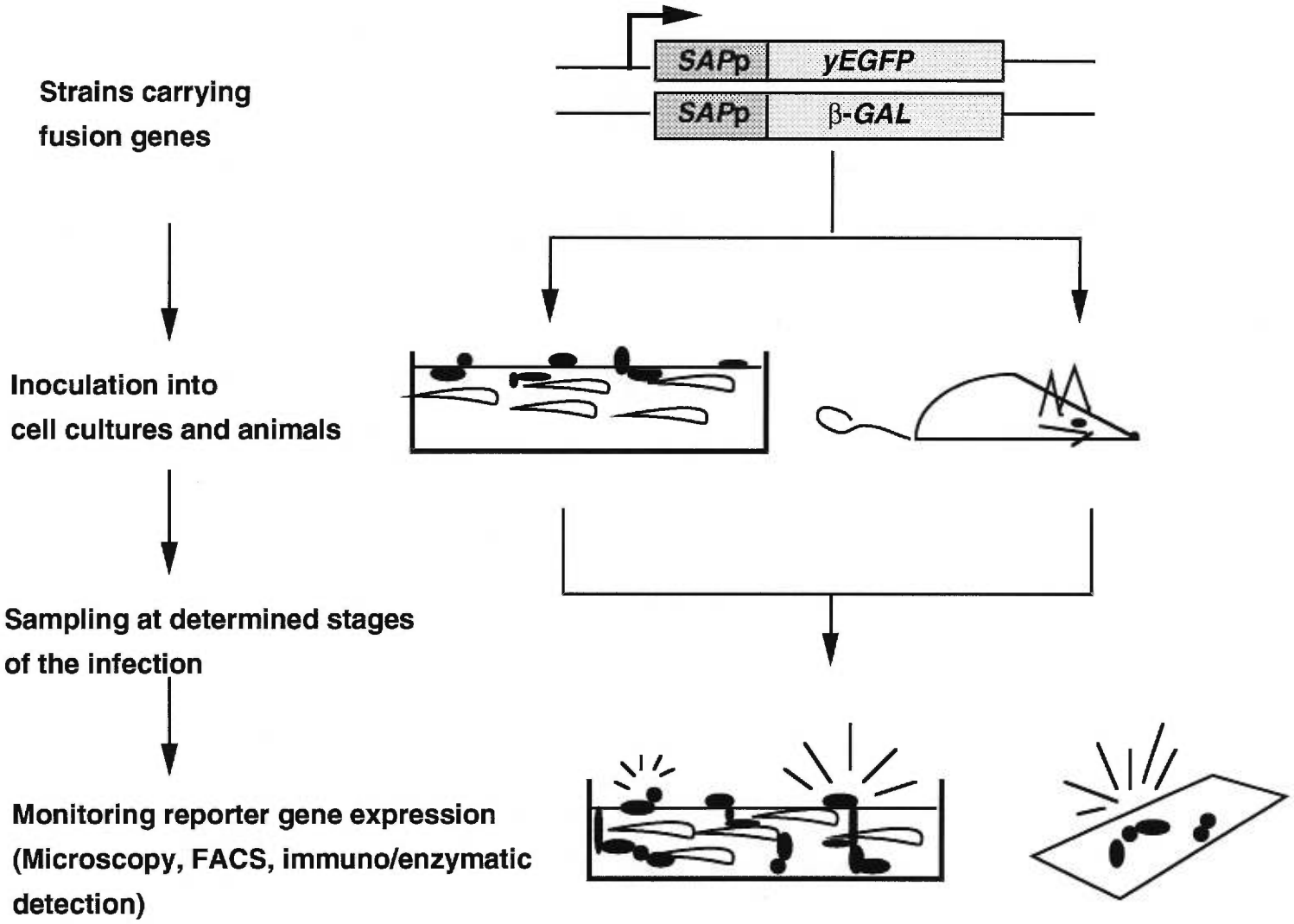


Figure 1. Experimental approach to study the expression of *SAP*s during infection.

3. Concluding remarks

1. Highly glycosylated mucins were shown to be a utilizable nitrogen source for *C. albicans*, inducing the production of secreted aspartyl proteinase 2 (Sap2p) and supporting cell growth.

2. Biochemical evidence indicated that Sap2p from blastoconidia of *C. albicans* has mucinolytic activity.

3. Genetically modified *S. cerevisiae* and *C. albicans* strains were generated which constitutively secreted abundant and enzymatically active Sap2p. These strains were suitable for use in virulence studies in animal models.

4. The constitutive expression of Sap2p by *S. cerevisiae* and *C. albicans* was not sufficient to confer a virulent phenotype to *S. cerevisiae* or to enhance virulence in *C. albicans*.

5. Taken together, our results support the view that virulence in *C. albicans* depends on the coordinated expression of more than one virulence determinant.

CHAPTER VI

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