MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, Sept. 2008, p. 495–544 Vol. 72, No. 3
1092-2172/08/$08.00+0 doi:10.1128/MMBR.00032-07
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Candida albicans Cell Wall Proteins

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INTRODUCTION

The cell surface has two essential roles: to maintain the integrity of the cell and to interact with the environment. A rigid cell wall provides the surface that surrounds the cell. The surface is the contact point between the microbe and host surfaces including phagocytic cells. It may also be the target of antibody response. In addition, commensal microbes found in biofilms on mucosal surfaces or microbes in biofilms formed on medical devices and prostheses have surface interactions. For *Candida albicans*, the cell wall has been a consistent focus of attention over the last several decades. Keyword searches for "*albicans*," "*albicans* cell wall," and "*albicans* adherence" in PubMed (www.pubmed.gov) showed that total publications on *C. albicans* increased by over 50% in the decade between 1998 and 2007 from the previous decade (Table 1). The number of publications related to cell wall and adherence increased over the same period but decreased slightly as the proportion of the total reports. One area that has become more prominent in the last decade and that has a cell surface component is studies on *C. albicans* biofilm. In this area, there is a more-than-sixfold increase in the proportion of reported studies on biofilm. The availability of DNA sequences from the genomic sequencing project (162) applied to both individual genes and the whole genome, e.g., microarray generation and proteomics, has also contributed increasingly in this decade to studies of the cell surface from global and individual gene perspectives. Another characteristic of these reports not evident from these numbers appears to be a greater diversity or number of the proteins and functions examined. The study of *C. albicans* cell surface proteins is moving forward on a broad front utilizing a variety of tools.

An extensive review of *C. albicans* cell wall and exported proteins (49) appeared in 1998, and this review will focus on the most recent decade. During this decade, there have been multiple reviews on various aspects of the *C. albicans* cell surface, including several very recently (100, 101, 116, 187, 215, 246, 312, 322, 355, 381). The proteins of the cell wall may play a role in maintaining structural integrity and in mediating adherence, whether to host or microbes, or they may have enzymatic functions, e.g., proteolysis. Additional factors that may influence these proteins are the morphology of yeast cells, pseudohyphae, and hyphae and the maintenance of either a planktonic or a sessile lifestyle. This review is not a comprehensive discussion of every proposed cell surface and exported protein. The number of potential and demonstrated proteins of the cell surface is too large, as will be indicated later, to give each of these proteins individual attention. However, the number of proteins with suggested functions and proteins which when deleted affect the cell are much fewer in number. The individual proteins discussed are from this latter group. Gene names are those from Candida Genome Database (CGD; August 2007 [9]). After an overview of cell wall-associated proteins, the review will focus on many of the enzymatic activities and adherence interactions mediated by cell surface proteins of the fungus in vitro.

CELL WALL ORGANIZATION

Electron microscopy of thin sections of the *C. albicans* cell wall shows layers which appear to be derived from differential abundances of cell wall constituents (177, 179, 322). The number of layers observed is variable and seems to be related to both strains and methodology (reviewed in reference 49). The inner layer, enriched for chitin and polysaccharide matrix, is more electron translucent than outer layers, which are enriched for mannoprotein. In one study, the outer layer enriched for proteins was about 150 nm in width (386). The translucent layer was of a similar size, with a thin electron-dense layer adjacent to the cell membrane. Electron microscopy images in Fig. 1 show aspects of cell wall structure with the presence of layers that differ in electron density. Structurally, the outer portion of the cell wall appears to have perpendicularly aligned fibrils that differ in length with surface hydrophobicity (136, 386). Figure 1C shows fibrils of a hydrophobic cell. The Klis laboratory made many contributions (e.g., references 73, 74, and 167 to 170) to the development of the view that the cell walls of *C. albicans* and *Saccharomyces cerevisiae* are similar in their covalent structures (see the reviews in references 177, 179, and 322). There is a flexible, three-dimensional network of branched β-1,3-glucan to which is attached β-1,6-glucan and chitin through their reducing ends (Fig. 2). Some chitin may also be attached to β-1,6-glucan. Not depicted in Fig. 2 is the association of polysaccharide chains. Chitin chains can form tight antiparallel hydrogen-bonded structures associated with high insolubility. β-1,3-Glucan can also have hydrogen bond-mediated local alignments. The cell wall proteins (CWPs) covalently attached to this meshwork of structural fibrillar polysaccharide are in two classes. The first and most abundant class of CWPs is linked to β-1,6-glucan through a glycophosphatidylinositol (GPI) remnant (GPI-CWP) (Fig. 2). The second class of proteins, termed Pir (*p*roteins with *i*nternal *r*epeats), are linked directly to the β-1,3-glucan. Pir proteins were first demonstrated in *S. cerevisiae* and subsequently simultaneously reported from the Klis and Chaffin laboratories for *C. albicans* (166, 168) (Fig. 2). In *S. cerevisiae*, the attachment has been identified as a linkage between a glutamine in the repeat sequence that is ultimately modified to

TABLE 1. Publications on *C. albicans* and cell wall

PubMed search term	1988–1997		1998–2007[a]	
	No. of hits	% of total "*albicans*"-related hits	No. of hits	% of total "*albicans*"-related hits
"*albicans*"	6,038		9,423	
"*albicans* cell wall"	363	6.0	503	5.3
"*albicans* adherence"	234	3.9	285	3.0
"*albicans* biofilm"	23	0.38	251	2.7

[a] Through December 2007.

a glutamic acid that forms a novel γ-carboxylic acid ester with a hydroxyl group in β-1,3-glucan (88). The alkali-labile linkage of the *C. albicans* Pir protein is likely through the same mechanism. The third class of proteins lacks the covalent attachment to the polysaccharide matrix (Fig. 2). Some of these proteins may be heterogeneously distributed at the surface, as described later for Pra1p. Noncovalently attached proteins may also be secreted into the external milieu and may not be cell associated. In addition, present in the wall is phospholipomannan (Fig. 2) with α-1,2-mannose linkages. The schematic emphasizes the general composition and linkage relationships and does not depict the layers and fibrils shown in Fig. 1.

The integrity of the cell wall is clearly of paramount importance to the survival of the organism. Yeasts have developed mechanisms, i.e., cell wall integrity pathways, to respond to stress that threatens the cell wall. Except for noting some individual genes that respond to certain stresses, e.g., cell wall loss, this review will not discuss these pathways. For further information on these damage control responses, see three recent reviews on *C. albicans* and *S. cerevisiae* cell walls that include information on integrity response (176, 204, 246).

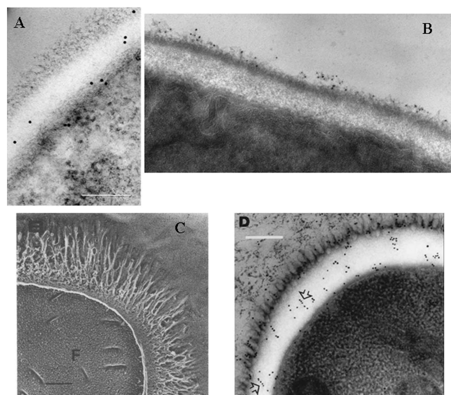

FIG. 1. Electron microscopy of *C. albicans* cell wall. (A) Immunoelectron microscopy of thin sections reacted with antibody recognizing Pir1p and then with gold-conjugated second antibody showing protein presence within the cell wall. (Reprinted from reference 168 with permission from Blackwell Publishing.) (B) Electron microscopy of cells reacted with antibody to Als1p followed by reaction with gold-conjugated second antibody before embedding and sectioning. (Courtesy of Lois Hoyer, reproduced with permission.) (C) Freeze-fractured, freeze-etched hydrophilic cell of *C. albicans* showing fibrils at the outer surface. F, fracture face. Bar, 0.19 μm. (Reprinted from reference 136 with permission.) (D) Immunoelectron microscopy of thin sections reacted with antibody recognizing Hsp70 and then with gold-conjugated secondary antibody showing protein presence within the cell wall. Arrows indicate a series of gold particles. Bar, 0.2 μm. (Reprinted from reference 213.)

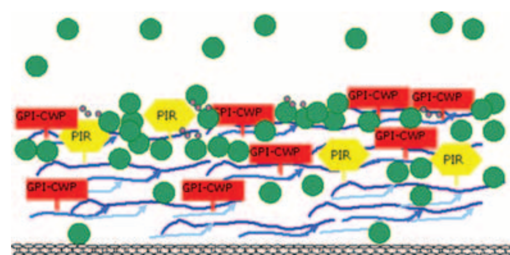

FIG. 2. Schematic representation of major cell wall components. The cell wall is external to the cell membrane (shown in black and white at the bottom). Labeled symbols: red rectangles, GPI-CWPs; yellow hexagons, Pir proteins. Unlabeled symbols: dark blue lines, β-1,3-glucan; medium blue lines, β-1,6-glucan; light blue lines, chitin; maroon circles, phosphomannolipid; green circles, unattached proteins found in the cell wall or in the medium.

COVALENTLY ATTACHED PROTEINS

GPI-CWPs

With the availability of genome sequences, several laboratories have developed algorithms to predict proteins with various characteristics. Several studies have reported analyses of the *C. albicans* genome for proteins included in the GPI-CWP class (1, 74, 92, 118, 371). These proteins have been recently reviewed by Richard and Plaine (312), who focused on the three reports that used *C. albicans* genome assembly 19 (74, 92, 118). In their analyses, they listed 115 putative GPI-CWP class proteins (see Table 1 in the work of Richard and Plaine [312]); 70 were common to all three reports, 6 were found in common between the analyses of de Groot et al. (74) and Eisenhaber et al. (92). The remaining proteins were unique to one of the studies: 24 proteins from the study of de Groot et al. (74), 9 from the study of Eisenhaber et al. (92), and 6 from the study of Garcerá et al. (118). GPI anchors may target proteins to the membrane or the cell wall. The location of GPI proteins has not been determined in most cases and, in some cases, conflicting reports have not been resolved. While individual proteins have been localized to the plasma membrane, a more global approach to protein composition of detergent-resistant membranes, i.e., lipid rafts, revealed 29 proteins (154). One of the proteins found was the CWP Ecm33p, so this protein was either in transit or distributed in both locations. Such a distribution may explain conflicts between reported locations. Indeed, Sap9p and Sap10p tagged with a marker are detected in both locations, with Sap9p being found predominately in the cell membrane (3).

The availability of the genome sequence has enabled the search not only for putative GPI-anchored proteins but also for additional sequence relationships. Among the GPI-anchored proteins, there are families of proteins, and about half of the GPI-anchored proteins belong to one of these families. The numbers of proteins in these families vary between 2 and 12 (see Table 2 in the review by Richard and Plaine [312]). The IFF (IPF family file) family has 12 genes. As a brief note, the first to be studied, *HYR1*, encoding a hyphal surface protein, was found to cause no observable defect when deleted (15). On

the other hand, *IFF11* is a gene of the family that does not encode a protein with a GPI anchor. This protein is secreted to the medium and is not found in the cell wall unless it is present below the limit of detection (17). However, this protein had an effect on the cell wall. In a null strain, there was no obvious effect on growth in rich and minimal media, hyphal morphogenesis in serum, antifungal susceptibility, carbon assimilation, or adherence to buccal epithelial cells (BECs). However, there was an effect on cell wall integrity with hypersensitivity to calcofluor white, Congo red, and sodium dodecyl sulfate (SDS). Supplementing the growth medium of the mutant with supernatant from the wild type did not alter sensitivity. This observation suggested that the function of Iff11p was prior to appearance in the culture medium and that it exerted its effect on the cell wall during the secretory process or transit of the cell wall. This is a very intriguing notion, since other proteins demonstrated to assist in cell wall biogenesis and remodeling are in the wall.

With global transcriptional analyses of *C. albicans* cells grown under different conditions or comparisons of wild-type and null strains, there is considerable information emerging about the regulation of genes, including those predicted to encode proteins with GPI anchors. However, for many of these genes a function is unidentified. Richard and Plaine (312) estimated that function is unknown for about 65% of this class of protein. Table 2. lists selected GPI-CWPs. The two criteria for selection were demonstrated or presumable presence in the cell wall and, secondly, information on function or the effect(s) of null strains. Genes of several families, namely, ALS (*a*gglutinin-*l*ike *s*equence), IFF, Sap (*s*ecreted *a*spartyl *p*roteinase), and CFEM (*c*ommon in several *f*ungal *e*xtracellular *m*embranes) genes, are among those included. Some of the GPI-CWPs share enzymatic activity of the same type as found for some noncovalently attached proteins. Among the enzymatic activities found both for GPI-CWPs and noncovalent secreted proteins are aspartyl proteinase family proteins, phospholipases, chitinases, proteins for β-1,6-glucan biosynthesis, and enzymes with glucanase activity. The noncovalent or soluble enzymes are included in a subsequent section. It is readily apparent from inspection of the column labeled "Expression" in Table 2 that the cell wall covalent structure is by no means static and that a variety of conditions can elicit a change in composition (assuming that protein abundance follows gene expression). The expression of some GPI-CWPs differs between yeast cell and hyphal morphology, and their expression responds to regulators of this process, e.g., Tup1p and Nrg1p. Other proteins respond to pH and Rim101p regulation. In addition, Ace2p, a regulator of cell separation, regulates several proteins listed in Table 2 as well as some proteins not included in the table. The implication of such changes is that there are many protein compositions of the cell wall that fulfill the need for the structural and barrier capacity of the wall.

Pir Proteins

Pir proteins were first demonstrated in *S. cerevisiae* (385). The Klis (168) and Chaffin (166) laboratories simultaneously reported the presence of this protein class in the *C. albicans* cell wall of strain CAI4 (constructed from strain SC5314) and strain NCPF 3153, respectively. The presence of Pir1p in the cell wall was confirmed (Fig. 1A) (168, 229a). The protein was detected in cell wall extracts, and abundance was increased upon heat shock (166). Under somewhat different conditions of heat shock, no difference was observed in mRNA levels (229a). Kapteyn et al. (168) found two Pir-immunoreactive bands in the cell wall extract of cells grown in synthetic complete medium but not when cells were grown in RPMI medium. Martínez et al. (229a) noted subsequently that there was deletion in one allele in the sequenced SC5314 genome but found that both alleles were similarly expressed by reverse transcriptase PCR (RT-PCR). The sequences of the two bands observed previously were not determined and the growth conditions differed from those used for the analysis of allele expression. Consequently, whether the two bands represent the same protein encoded by the two alleles or two different proteins is unknown. Heterozygous strains with a deletion in either allele grew slowly and showed abnormal shape, a tendency to form clumps, and high sensitivity to calcofluor white and Congo red compared to the wild type (229a). This observation suggested a contribution to cell wall architecture. The failure to obtain a homozygous deletion suggested that *PIR1* is essential.

PIR1 expression increased during the regeneration of spheroplasts, as determined by microarray and RT-PCR (46, 229a). Other factors also regulate *PIR1* expression. Transcriptional profiling shows a pH effect, with repression of *PIR1* by Rim101p (221) and reduced expression in hyphae and regulation by a morphogenetic regulator, Efg1p (356). In addition, expression increases by about eightfold in the presence of fluconazole (62), increases in the presence of high iron concentration (194), and modestly increases in response to reduced expression of a phospholipase, Plc1p (189). These observations show that both classes of covalently attached CWPs change in abundance in response to environmental conditions. In *S. cerevisiae*, which has four Pir family genes, the *S. cerevisiae* protein encoded by the ortholog of Ca*PIR1* mediates the translocation of Apn1p (an apurinic/apyrimidinic endonuclease) into the mitochondrion (399). In this case, a protein first identified in the cell wall was subsequently found to have a cytoplasmic function. A second *C. albicans PIR* gene, *PIR32*, is expressed abundantly in phagocytosed *C. albicans* cells recovered from macrophage compared to what is the case for uningested organisms (99). This protein has not yet been isolated from cell walls. The predicted Pir1p and Pir32p sequences have regions of identity, and another possibility for the two bands discussed above is that they represent these two proteins.

NONCOVALENT AND SECRETED PROTEINS

Not all CWPs utilize a covalent attachment for retention in the cell wall. These proteins, e.g., Bgl2p, have substrates within the cell wall and remain primarily cell associated. Some proteins may be found in culture supernatant also, while others are released to the external milieu; for example, Iff11p is predominantly recovered there. The proteins released to the environment may have hydrolytic functions that either provide nutrients for the organism or facilitate its status as a commensal or invasive pathogen. Some of these proteins recovered from the environment possess such activity, and this is used in

their identification. There are two classes of these unattached proteins: proteins exported by the classical secretory pathway and proteins that use alternative routes to the cell surface. Two studies predicted proteins secreted by the classical pathway.

Predictions

Monteoliva et al. (249) undertook an experimental approach to identify potential secreted proteins by a genetic selection for growth on sucrose by *S. cerevisiae* lacking extracellular invertase (suc$^-$). Three DNA libraries were constructed using the internal invertase sequence that lacks the N-terminal signal for classical export. The invertase sequence used for each library differed by 1 base length to allow in-frame fusions with *C. albicans* genomic DNA (0.5 to 2 kb). Each library was transformed into the suc$^-$ *S. cerevisiae* strain, and colonies that were able to grow on sucrose were selected. The sequencing of 83 plasmids rescued from colonies growing on sucrose identified *C. albicans* sequences conferring export. At the time of the experiment, by use of genome assembly 6, 11 of the sequences were found to correspond to known sequences encoding proteins with predicted N-terminal signal sequences. Currently in the CGD (9), 63 of these sequences are identified.

The second approach was a genome analysis with algorithms for characteristics of secreted proteins (202). After the removal of proteins with predicted transmembrane domain(s), GPI anchors, and mitochondrial targeting sequences from the list of proteins with N-terminal signal sequences, the final set of predicted secreted proteins contained 283 open reading frames.

This review addresses only a few of the predicted secreted proteins. A major criterion for inclusion was a suggested or demonstrated function. Although transcriptional profiling reveals the conditions or transcriptional factors that regulate the expression of a protein, the function of the protein may remain unknown. For example, Rbe1p is a predicted secreted protein that is negatively regulated by Ace1p (256), Rim101p (23), Ssn6p (120), and Efg1p (356). Secreted proteins with enzymatic functions may catalyze reactions in which cell wall components are substrates, particularly glucan and chitin or other substrates, as in the case of trehalase. Enzymes of this class are discussed in the first section below. Other enzymes target substrates beyond the cell wall. Some of these soluble secreted proteins may be recovered from the cell wall as well as the extracellular environment. Certainly, even proteins primarily recovered from the extracellular environment must transit through the cell wall. Consideration of these hydrolytic enzymes is in the section following that for the cell wall-associated enzymes. The discussion of exported proteins identified as adhesins is in a later section, and the discussion of Iff11p is in the prior section on GPI-CWPs.

Cell Wall-Localized Proteins

Several proteins that are nonattached to the polysaccharide matrix have enzymatic functions in the cell wall. Perhaps not surprisingly, most of the studied proteins with enzymatic function affect the cell wall structure. Only acid trehalase, discussed below, is not involved in cell wall structure, although it is also a glycoside hydrolase (GH). GHs (EC 3.2.1) hydrolyze the glycosidic bond between carbohydrates or between carbohydrates and a noncarbohydrate moiety. This classification scheme does not reflect other characteristics such as structure and mechanism of action, and additional family classifications have been developed (CAZy database [http://www.cazy.org/index.html] [64]). The enzymes discussed below are found in several GH families that may include both the noncovalently attached enzymes and some GPI-CWPs (Table 2).

Polarized growth occurs in both hyphae and yeast cells but is tempered in yeast cells with isotropic growth to give the ovoid structure (for more detail, see the recent review in reference 406). The cell must balance cell wall integrity with cell expansion to allow the insertion of more constituents. The loss of integrity results in sensitivity to environmental factors and, if sufficiently severe, can result in cell death. In addition, the mother and daughter bud cells must separate after cell division into two cells while maintaining cell wall integrity. Thus, synthesis, remodeling, and hydrolysis must be coordinated in time and space to provide integrity, flexibility, and cell separation. Structural rigidity, thus maintaining shape, is conferred by polysaccharides, and the synthesis of polysaccharides has recently been reviewed (204, 322). Enzymes localized in the membrane synthesize the chitin and β-1,3-glucan. β-1,6-Glucan synthesis requires not only cytoplasmic proteins but also cell wall activities, i.e., Kre6p.

Other enzymes are involved in processing and remodeling. A combination of GPI-CWPs and soluble noncovalent enzymes contributes to this vital activity. The GPI-CWPs are covered in Table 2 and the noncovalent proteins below. However, a brief overview is presented here. Enzymes with glucanosyltransferase activity split glucan and transfer part of the split chain to an acceptor chain, leading to cross-linking. Enzymes with this activity include Phr1p and Phr2p (Table 2) and Bgl2p (discussed below). Several CPI-CWPs of the CRH (Congo red hypersensitive) family (Crh11p, Crh12p, Utr2p) are putative transglucosidases that cross-link chitin and β-1,6-glucans. Other glucanases, e.g., Xog1p, release residues from β-1,3-glucan. Some enzymes, e.g., Eng1p and Sun41p, are implicated to various extents in cell separation. When deleted, the mutant strains have cell separation defects that have been tied to various extents to septum degradation. Any glucanase function of Mp65 may be more important in hyphae than in yeast cells. Although mutant strains have been produced, the roles of Scw4p and Scw11p are unclear. As noted above, chitin is synthesized by membrane-localized enzymes and cross-linked to glucan through several putative transglucosidases. Like glucan, chitin linkages must be modulated to accommodate cell growth and proliferation. Unlike the enzymes described above that have preferences for certain glucan structures and receptors, chitinases catalyze the random hydrolysis of *N*-acetylglucosamide β-1,4 linkages in chitin.

Kre9p. Kre9p, along with the covalently attached Crh11p, Crh12p, and Utr2p (Table 2), is in the GH16 family. Kre9p is involved in β-1,6-glucan biosynthesis. The synthesis of this polysaccharide requires both cytoplasmic and cell wall activities, and its synthesis is not as well understood as are those of the other two structural polysaccharides (204, 322). The null (*kre9*Δ/Δ) strain is inviable when grown on glucose (224, 279). The mutant strain grows poorly on other carbon sources (224). Growth on sucrose, mannose, maltose, or fructose is similar to or less than that on galactose. When growth was on galactose,

TABLE 2. Selected cell wall GPI-CWPs: function and expression[a]

Putative function	Gene; Orf19 no.	Enzymatic function	Homozygous null mutant characteristic			Expression[b]	References
			Cell wall integrity/function[c]	Virulence animal or in vitro tissue[d]	Morphogenesis/growth		
Adhesins	*ALS1*; 19.5741		Reduced adherence	Attenuated murine systemic, murine oral, oral RHE	Small yeast cell size; delay in filamentation; decreased biofilm	Induced biofilm; reduced expression in *tsa1*Δ/Δ strain, opaque; regulated by Rfg1p, Ssk1p, Bcr1p	1, 113, 119, 133, 144, 164, 165, 193, 221, 276, 392, 413
	ALS2; 19.1097				Homozygous deletion inviable	Compensatory expression in *als4*Δ/Δ strain; induced ketoconazole, cell wall regeneration; low iron; reduced opaque; regulated by Sfu1p	46, 193, 194, 210, 346, 416
	ALS3; 19.1816		Decreased adherence	Attenuated virulence in oral RHE	No defect in morphogenesis; decreased biofilm condition dependent	Expressed hyphae; reduced expression in *tsa1*Δ/Δ strain, opaque; regulated by Nrg1p, Rfg1p, Tup1p, Bcr1p	29, 164, 193, 259, 276, 392, 412
	ALS4; 19.4555		Decreased adherence	Wild-type virulence in RHE	Delayed filamentation medium dependent; no defect in biofilm	Compensatory expression in *als2*Δ/Δ strain; increased expression in *tsa1*Δ/Δ strain; reduced opaque	144, 193, 221, 392, 414, 416
	ALS5; 19.5736		Increased adherence	Wild-type virulence in oral and vaginal RHE	Slower growth		221, 414
	ALS6; 19.7414		Increased adherence	Wild-type virulence in oral and vaginal RHE	Slower growth	Induced macrophage	99, 414
	ALS7; 19.7400		Increased adherence	Wild-type virulence in oral and vaginal RHE	No growth defect		414
	ALS9; 19.5742		Reduced adherence; wild-type sensitivity to cell wall-perturbing agents	Wild-type virulence in RHE	No growth defect; normal biofilm	Regulated by Bcr1p	276, 414, 415
	EAP1; 19.1401		Reduced adherence to polystyrene, kidney cells		No growth defect; reduced biofilm	Expressed yeast, hyphae; regulated by Efg1p (N)	205, 206, 356
	ECM33; 19.3030.1		Sensitive to cell wall perturbation; aberrant structure; less susceptibility to caspofungin; reduced adherence and damage to host cells; defect in Als1p localization; flocculation; cell wall structure abnormal	Attenuated disseminated murine model	Yeast cell slower growth, more rounded, larger; filamentation delayed and abnormal culture; hyphae on host cells; formed biofilm	Induced cell wall regeneration, fluconazole, hypoxia; repressed caspofungin	46, 62, 210, 231, 232, 279, 346

	HWP1; 19.1321	Covalent adherence BEC; localize **a**/**a** cells in conjugation tube	Reduced biofilm formation	Wild-type disseminated murine model	No filamentation defect	Expressed hyphae (N, TP); increased α pheromone, mating, hypoxia, alkali (N, TP); regulation by Bcr1p, Cph2p, Tec1p, Tup1p (N), Nrg1p (N, TP), Efg1p (N, TP), Flo8p (N, TP)	22, 23, 28–30, 40, 68, 83, 197, 259, 276, 342, 346, 347, 356, 363, 372, 411
	YWP1; 19.3618		Increased adhesiveness	Wild-type disseminated murine model	Released cell wall stationary phase; increase in biofilm formation	Expressed yeast cells; increased fluconazole, *tsa1*Δ/Δ; decreased mating; regulated by Ssk1p, Ssn6p, Efg1p (N, TP), Efh1p; white-opaque switch; cell wall regeneration; phosphate	46, 51, 62, 83, 120, 129, 193, 264, 356, 392, 411
Cell wall biogenesis	*CHT1*; 19.7517	Chitinase			Conditional increase in filamentation	Alkaline downregulated, increased opaque	23, 85, 193, 239
	CHT2; 19.3895	Chitinase			Abnormal filamentous growth on solid medium	Repressed caspofungin; induced yeast cells (N, TP); increased farnesol-treated biofilm, high iron, alkaline pH; regulated by Efg1p, Bcr1p; cell wall regeneration (RT, TP)	23, 34, 42, 46, 85, 194, 210, 264, 273, 356
	CRH11; 19.2706	Putative transglycosidase linking chitin and glucan	Increased sensitivity to Congo red, zymolyase	Wild-type disseminated murine model	Viable	Induced caspofungin, ketoconazole, flucytosine induced; increased Ca^{2+} (N); increased amphotericin B and fluconazole-resistant strain; decreased regeneration of protoplasts; reduced in *ace2*Δ/Δ	16, 34, 210, 221, 256, 286
	CRH12; 19.3966		Increased sensitivity to Congo red, zymolyase	Wild-type disseminated murine model	Forms biofilm	Regulated Nrgp1, Tup1p, Rim101p; expressed low levels (N); repressed cell wall regeneration	23, 46, 258, 259, 279, 286
	EXG2; 19.2952	Exo-1-3-β-glucanase			Null mutant not available	Induced cell wall regeneration	46
	KRE1; 19.4377	Role in biosynthesis of β-1,6-glucan; confers killer toxin sensitivity			Mutant not available	Increased hyphae; induced caspofungin, cell wall regeneration; increased α pheromone; regulated by Nrg1p, Tup1p	22, 25, 34, 46, 120, 210, 264

Continued on following page

TABLE 2—*Continued*

Putative function	Gene; Orf19 no.	Enzymatic function	Homozygous null mutant characteristic			Expression[b]	References
			Cell wall integrity/function[c]	Virulence animal or in vitro tissue[d]	Morphogenesis/growth		
	PGA4; 19.4035	Putative transglucosidase			Null mutant not available	Increased early in RHE infection; initial germ tube induction that depends on Ras1p, Cyr1p, and Tec1p; no effect of cell wall regeneration	46, 89, 256
	PHR1; 19.3829	β-1,3-Glucanosyltransferase	Altered cross-linking of β-1,6-glucan and chitin; increased susceptibility to nikkomycin	Attenuated disseminated murine model but wild-type rat vagina	Defect in alkaline filamentation; slow growth	Expressed hyphae; transiently repressed cell wall regeneration; regulated by Rim101p	46, 72, 105, 250, 251, 306, 311, 330
	PHR2; 19.6081	β-1,3-Glucanosyl transferase	Altered cross-linking of β-1,6-glucan and chitin; increased chitin-linked mannoprotein	Wild-type disseminated murine model but attenuated rat vagina	Defect in acidic growth; abnormal yeast cell morphology; hyphae at acidic pH	Expressed acidic pH (N, TP); transiently induced cell wall regeneration; increased iron, exposure to fluconazole in amphotericin B- and fluconazole-resistant strain; greater adherence to plastic than to glass; regulated by Rim101p	10, 16, 46, 62, 72, 105, 194, 228, 250, 251
	PIR1; 19.220	Structural protein; β-1,3-glucan linked	Heterozygous mutant sensitivity to calcofluor white; essential (unable generate null)		Heterozygous mutant aberrant elongated cells with tendency to clump; slower growth	Repressed hyphae; induced Hog1p, caspofungin, cell wall regeneration; regulated by iron, Efg1p	46, 94, 166, 168, 194, 210, 356
	SPR1; 19.2237	Putative exo-1,3-β-glucanase			Viable	Not required for chlamydospore formation	274
	SSR1; 19.7030		Sensitive to calcofluor white, Congo red, zymolyase; increased β-1,3-glucans		No change in mutant	Expressed yeast, hyphae (N); induced ketoconazole, cell wall regeneration	46, 117, 118, 210
	UTR2; 19.1671	Putative transglycosidase linking chitin and glucan; accumulates site bud emergence	Increased sensitivity to Congo red, zymolyase, SDS; reduction in alkali-insoluble β-1,3 glucan; reduced adherence to host cells	Attenuated disseminated murine model	Conditional defect in filamentation	Induced cell wall regeneration; localizes to areas of chitin incorporation	1, 46, 286

	CSA1; 19.7114	Hyphal surface, growing buds; CFEM family			No obvious growth defect; biofilm reduced, fragile	Increased hyphae (N); induced ciclopirox; increased low iron; medium effects; regulated by Rim101p, Efg1p (N, TP), Cph1p (N, TP), Tup1p (N)	23, 28, 192, 194, 196, 297, 349, 356
	RBT5; 19.5636	Heme binding; conflict between cell wall and membrane; CFEM family	Deficient in hemin binding	No defect in rabbit cornea and systemic murine infection	No growth defect observed; biofilm reduced, fragile	Increased hyphae induced by serum and Spider medium (N); induced by iron, alkaline pH, ketoconazole, ciclopirox; regulated by Bcr1p, Rfg1p, Rim101p, Sfu1p, Hog1p, Tup1p	28, 227, 273, 297, 404
	PGA10; 19.5674	Heme binding; conflict between cell wall and membrane; CFEM family	Reduces heme binding under some conditions; clumping under hyphal conditions; sensitive to calcofluor white, Congo red		Biofilm reduced, fragile	Hyphal; ketoconazole, ciclopirox induced; regulated by Rim101p	23, 201, 210, 264, 297, 404
Enzymes	*CHT2*; 19.3895	Chitinase	Null small decrease in chitinase activity; slight hypersensitivity to calcofluor white		Null increased hyphal growth on solid medium; no biofilm defect	Expressed more yeast cells (N, TP); slightly induced cell wall regeneration; increased alkaline pH; regulated biofilm; downregulated caspofungin; dependent on Bcr1p (N), Tec1p (N), Cyr1p, Efg1p	23, 46, 85, 210, 264, 273, 343
	PLB5; 19.5102[c]	PLB	Defective in cell-associated PLA2	Attenuated disseminated murine model	No differences in growth, morphology	Increase in hyphae (Q); increases at early log phase and remains (N)	260, 379
	SAP9; 19.6928	Sap	Increased sensitivity to calcofluor white, Congo red, hygromycin; increased chitin; increased adherence to BECs	Reduced RHE damage	Normal growth and filamentation; abnormal budding; failed cell separation	Induced antifungal drugs; stationary phase, white-phase cells; downregulated farnesol-treated biofilm; cell wall regeneration	3, 42, 46, 62, 193, 211, 247
	SAP10; 19.3839	Sap	Increased sensitivity to calcofluor white, Congo red, hygromycin; increased chitin and protein; reduced adherence	Reduced RHE damage	Normal growth and filamentation; abnormal budding; failed cell separation	Increased low iron	3, 194

Continued on following page

TABLE 2—*Continued*

Putative function	Gene; Orf19 no.	Enzymatic function	Homozygous null mutant characteristic			Expression[b]	References
			Cell wall integrity/function[c]	Virulence animal or in vitro tissue[d]	Morphogenesis/growth		
	SOD4; 19.2062	Cu/Zn superoxide dismutase			Mutant not available	Induced ciclopirox olamine; increased opaque; repressed caspofungin,	193, 201, 210
	SOD5; 19.2060	Cu/Zn superoxide dismutase		Attenuated disseminated murine model	Sensitive to H_2O_2 in nutrient limitation; increased sensitivity to human blood granulocytes; no defect with macrophage	Induction in neutrophils; increased hyphal growth (N, TP), α pheromone; osmotic, oxidative stress (N); requires Rim101p; presence of serum requires Efg1p (N)	22, 110, 229, 264
	SOD6; 19.2108	Cu/Zn superoxide dismutase			Mutant not available	Upregulated absence of carbon source; increased initial planktonic growth	229, 260
Unknown activity/ function	*DFG5*; 19.2075		Cell wall and cell membrane		Defective alkaline filamentation; required for alkaline expression of *HWP1*	Synthetic lethal *DCW1*	360
	DCW1; 19.1989				No filamentation defect	Synthetic lethal *DFG5*	360
	HYR1; 19.4975		No change sensitivity to caffeine, calcofluor white		No effect on growth, filamentation, biofilm	Expression associated with hyphae (N); increased acquisition of azole resistance; reduced *tsa1*Δ/Δ; regulated by Bcr1p, Flo8p, Rfg1p, Efg1p, Nrg1p, Tup1p	15, 40, 164, 259, 273, 318, 356
	IHD1; 19.5760				Viable, no effect on chlamydospores	Expressed more hyphae; regulated by Nrg1p, Flo8p, Tup1p; down in *tsa1*Δ/Δ; alkali induced; regulated by Ace2p	23, 40, 256, 258, 264, 274, 392
	PGA6; 19.4765		No change sensitivity to calcofluor white, Congo red		Viable	Increased expression in *als2*Δ/Δ strain; hypoxia; high iron; regulated by Rim101p	194, 220, 346, 416
	PGA17; 19.893		No defect in biofilm formation; caspofungin sensitivity		Viable	Repressed by α pheromone, induced macrophage interaction	22, 99, 279

PGA44; 19.1714	Normal caspofungin sensitivity		No biofilm defect		279
RBR1; 19.535			Mutant filamentation defect at low pH	Acidic expression; initially repressed cell wall regeneration; regulated by Rim101p, Nrg1p	46, 221
RBR2; 19.532	No defect in caspofungin sensitivity		No biofilm defect	Increased opaque; repressed by α pheromone; induced interaction with macrophage; regulated by Rim101p	22, 99, 193, 221, 279
RBT1; 19.8907		Attenuated in rabbit cornea and systemic mouse infection; no defect in murine cornea	No apparent growth defects	Induced α pheromone, mating; alkali; regulated Rim101p; induced ciclopirox; no effect on fluconazole; regulated Tsa1p, Efg1p (N, TP), Tup1p (N)	22, 23, 28, 30, 80, 155, 272, 274, 356, 392, 411

[a] Genes encoding GPI-CWP were included in the table if they met one or both of the following criteria: (i) known or putative function and (ii) construction of a homozygous deletion strain. For mutant strains, phenotype observations for growth, virulence in animal or in vitro tissue models, and cell wall integrity or function are briefly indicated.

[b] Most of the studies that identified regulators used transcriptional profiling (TP), and that is assumed in the table. Other methods are indicated as follows: (N), Northern analysis; (RT), RT-PCR; (Q), real-time RT-PCR. "Opaque" indicates an opaque cell switch phenotype.

[c] BEC, buccal epithelial cells.

[d] RHE, reconstituted human epithelium.

the amount of β-1,6-glucan in the heterozygote fell to about 20% of that seen for the wild type and was undetectable in the null strain. Hyphae failed to form in serum. The strain showed attenuated virulence in a murine systemic disease model. The absence of β-1,6-glucan would remove the normal attachment site for GPI-CWPs (Fig. 2) (177, 179, 322). Interestingly, in *S. cerevisiae*, the loss of Stt1p, which has a role in N glycosylation, also leads to a substantial reduction in β-1,6-glucan and links N glycosylation with the cell wall (52). Transmission electron microscopy of the Stt1p-deficient cells showed a diffuse cell wall with a loss of outer mannan. *C. albicans STT1* is an ortholog of the *S. cerevisiae* gene, and there also may be a similar linkage between N glycosylation and β-1,6-glucan. *KRE9* is required for the induction of a high-affinity fibronectin receptor presumably attached to glucan, which is discussed later (293). This observation would be consistent with the loss of attachment sites, although there may be other possibilities. Growth on galactose apparently partially relieves the loss of β-1,6-glucan (189). In a study some 20 years ago, McCourtie and Douglas (238) showed that growth on galactose enhanced adherence to acrylic compared to what was seen for glucose-grown cells. Galactose-grown cells were more resistant to spheroplast formation with zymolyase. In addition, transmission electron microscopy revealed the presence of an additional surface layer. Such an outer layer may be the source of a partial remediation of the β-1,6-glucan defect.

Bgl2p. Glucan is synthesized as a linear polymer; therefore, the branching of polysaccharide found in the cell wall (shown schematically in Fig. 2) requires enzymes with the capability to form branches from the linear polymers. Bgl2p is in the same GH17 family as Mp65p and Scw4p, discussed below. Bgl2p catalyzes the splitting (removing a disaccharide) and linkage of β-1,3-glucan molecules, resulting in β-1,3-glucan chain elongation with a β-1,6 linkage at the transfer site. Bgl2p is the major β-1,3-glucosyltransferase (also sometimes called a glucan transferase); however, after deletion of both alleles, about 50% residual enzymatic activity is detected (reference 331; also reviewed previously in reference 49). The presence of residual activity suggests the presence of additional β-1,3-glucosyltransferase(s) that was associated with the formation of a product different from those formed by Bgl2p. There were no changes in β-1,3-glucan or β-1,6-glucan content in the mutant strain. However, the strain was more sensitive to the chitin synthesis inhibitor nikkomycin than was the parental strain. This suggests an increase in chitin and a compensatory role for chitin in cell walls with reduced branching. The expression of *BGL2* alters in response to several conditions. Expression is reduced in low-iron conditions, as determined by microarray analysis (194). Several other cell wall-associated genes encoding GPI-CWPs (e.g., *ALS2*, *RBT5*, *PHR2* [Table 2]) and *PIR1* covalently attached proteins, and another putative glucanase, *SCW11*, responded to iron levels (194). In expression profiling, *BGL2* expression is greater in the presence of fluconazole under conditions promoting *SAP* gene expression, as is *GSC1*, encoding a subunit of β-1,3-glucan synthase (62). Microarray analysis shows that *BGL2* is also induced, not surprisingly, during cell wall regeneration along with other cell wall-associated genes, including genes for β-1,3-glucan synthase (46). In addition to this role in glucan metabolism, Bgl2p, also shows adhesin activity, which is discussed in a later section (160).

Xog1p. The exoglucanase Xog1p is the only *C. albicans* enzyme found in the GH5 family. Xog1p has a marked specificity for β-1,3-glucoside linkages compared to β-1,6-glucoside linkages (368). The enzyme catalyzes the successive removal of glucose residues from the nonreducing end of β-1,3-glucan, although at high acceptor concentration, transglucosylation with retention of the anomeric configuration can occur. The structure has been determined at 1.9 Å (67) and the insertion of serine at the CUG codon in the mature protein had a minor effect (66). Xog1p is the major exo-β-1,3-glucanase of the cell wall, as only residual activity is detected in the null strain (127). The loss of the enzyme did not impair yeast growth or morphogenesis, although there was a slight increase in sensitivity to chitin and glucan synthesis inhibitors. In addition, there was no apparent defect in the murine systemic infection model. Phr1p and Phr2p, which are glucan-attached proteins, may also have similar activities (Table 2). Another potential exoglucanase-encoding gene, *EXG2*, is induced during cell wall regeneration (46).

XOG1 is induced about 2.5- to 6.9-fold by morphogenesis in serum, as determined by semiquantitative RT-PCR and microarray analysis, and the level of enzyme activity is also increased (200). The expression of this gene in hyphae is dependent on *SIT4*, encoding a serine/threonine protein phosphatase that modulates morphogenesis. In the *sit4*Δ/Δ strain, enzymatic activity was repressed during hyphal formation. In the absence of Nrg1p and Tup1p, repressors of morphogenesis, the gene is expressed about fourfold more than in wild-type organisms (transcriptional profiling [259]). Exposure of opaque **a** cells to α pheromone results in a sixfold increase in expression (transcriptional profiling [22]). *XOG1* is also activated by Rim101p in response to external alkaline pH (transcriptional profiling [221]). Constitutive expression of *HGT4*, encoding a glucose sensor required for growth on low glucose, elevated *XOG1* expression compared to what was seen for the null strain (transcriptional profiling [33]). The *hgt4*Δ/Δ strain showed reduced the capacity to form filaments. *XOG1* expression increased (1.6-fold) upon exposure to ketoconazole (transcriptional profiling [210]). The expression of a subunit of β-1,3-glucan synthase also increased. Ketoconazole does not directly affect the cell wall, so the mechanism by which the increased hydrolysis of β-1,3-glucan is involved in the response to fungistasis in unclear. Ketoconazole may exert its effect through changes in membrane sterols. Although *XOG1* expression increases in hyphae and in strains lacking repressors of filamentation (259), the protein does not appear to have a critical role in morphogenesis, based on the ability to undergo this transition in the absence of Xog1p.

Eng1p. The cell wall enzyme Eng1p, along with cytoplasmic Acf2p, is in the GH81 family. Eng1p is a fungus-specific endo-1,3-β-glucanase involved in cell separation. It catalyzes the release of glucose from the nonreducing end. Eng1p had endoglucanase activity, as demonstrated by the release of reducing sugars from the substrate, and activity was specific for β-1,3-glucan (96). The *eng1*Δ/Δ strain showed normal growth and morphogenesis. However, microscopic examination showed clusters of yeast cells that had completed cytokinesis but failed to separate. *ENG1* is a functional ortholog of *S. cerevisiae DSE4* (Sc*DSE4*). ScDse4p is a daughter cell protein involved in cell wall degradation from the daughter side during cell separation (61). The yeast septum is three layered, with the internal layer composed primarily of chitin and the layers on either side similar to general cell wall in composition (reviewed in references 35 and 409). Sc*DSE4* expression is cell cycle regulated and peaks in late M or at the M/G_1 boundary (359). Ace2p is a transcription factor regulating genes involved in cell separation, and *ENG1* is downregulated in the *ace2*Δ/Δ strain (256). Ace2p is localized to the daughter cell nucleus (172). Although Eng1p has not been localized to the daughter side of the septum, it seems likely to be found here, as it is a functional ortholog of a protein that is so localized and as a regulator of expression is localized in daughter nuclei. Observations with *CRL1* provided additional evidence for a role for Eng1p in cell separation (86). *CRL1* encodes a predicted GTPase of the Rho family. The *crl1*Δ/Δ strain had elongated cells and a separation defect. The overexpression of *ENG1* suppressed the separation defect but not the elongation phenotype.

Northern analysis of the *ENG1* transcript showed a decrease in abundance 2.5 h into germination (96). Another study found that *ENG1* and *XOG1* expression increased during hyphal formation and that the increase was dependent on the serine/threonine protein phosphatase Sit4p (transcriptional profiling [200]). These two studies appear to be in conflict. Both studies were performed with similar serum-containing media. Additionally, *ENG1* expression responds to other conditions that also impact the cell wall. *ENG1* is repressed by treatment with caspofungin, an inhibitor of β-glucan synthesis (transcriptional profiling [210]) and in response to pheromone in Spider medium (transcriptional profiling [22]). In both conditions, cell budding decreases and presumably there is little need for septum degradation. The evidence supports the role of Eng1p in the catabolism of the secondary septum between the daughter and mother cells, probably from the daughter side.

Mp65p. Mp65p is possibly a β-glucanase and is also discussed elsewhere as an adhesin. La Valle et al. (197a) found by Northern analysis that *MP65* expression increased upon germ tube formation in Lee medium at 37°C. On the other hand, Sohn et al. (356) reported that *MP65* expression was constitutive in both yeast cell conditions and hypha-promoting conditions. This latter study used yeast cell growth in yeast extract-peptone-dextrose (YEPD) or minimal essential medium plus glucose at 30°C and hyphal growth in YEPD plus serum or in minimal essential medium plus glucose at 37°C. The difference between the two reports may represent a medium-dependent enhancement of expression, as noted for some genes in the two conditions used by Sohn et al. (356).

The deletion of *MP65* had no effect on growth rate, cell size, or sensitivity to cell wall-perturbing agents (325). However, gene deletion severely impaired morphogenesis. Mp65p was not detected on the cell surface by an Mp65p-specific monoclonal antibody (MAb). Yeast cells had reduced ability to adhere to polystyrene. The deletion strain was attenuated in the murine model of disseminated infection. Although the protein sequence contains two conserved glutamate residues crucial for activity of the glucanase family, GH17, with which Mp65p has been associated, the enzymatic activity of the recombinant protein has not been tested. Additional evidence points to the involvement of *MP65* in cell wall metabolism or structure. *MP65* expression also increased transiently during cell wall regeneration (transcriptional profiling [46]) and increased in

response to pheromone (transcriptional profiling [22]). Expression decreased in response to treatment with caspofungin (transcriptional profiling [210]) and increased in response to ciclopirox olamine (transcriptional profiling [201]). *MP65* expression also responds to other conditions. *MP65* expression is greater in a strain with reduced *PLC1* expression than with wild-type *PLC1* expression (transcriptional profiling [189]) and at alkaline compared to acid pH (transcriptional profiling [23]). Other putative glucanase genes, e.g., *SIM1*, respond to pH. *MP65* is the ortholog of *S. cerevisiae SCW4*. A recent study showed that Scw4p is incorporated into the *S. cerevisiae* cell wall by an unknown alkali-sensitive linkage (378). A similar linkage for *C. albicans* Mp65p has not yet been examined. Although this protein has been studied for other properties unrelated to a cell wall function, the effect of deletion on morphogenesis and other observations suggest that it does have a cell wall function, perhaps as a glucanase.

Sun41p and Sim1p. There are two other possible glucanases, Sun41p and Sim1p. The *S. cerevisiae* orthologs of Ca*SUN41* and Ca*SIM1* are Sc*SUN4* and Sc*UTH1*, respectively. The *S. cerevisiae* genes are two of the four genes of the *SUN* family. ScSun4p and ScUth1p have been demonstrated to be in both the mitochondrion and the cell wall, where they are proposed to have glucanase activity (393). The Sim1p-invertase fusion was detected in export screening (249). In *C. albicans*, *SUN41* and *SIM1* both show an increase in expression during cell wall regeneration (transcriptional profiling [46]), during treatment with flucytosine (transcriptional profiling [210]), and under conditions of hypoxia (transcriptional profiling [346]). *SIM1* is downregulated by Rim101p (transcriptional profiling [221]). *SUN41* has decreased expression following infection of HEp2 cells (transcriptional profiling [326]) and treatment with caspofungin (transcriptional profiling [210]). It is also regulated by Efg1p and Cph1p (transcriptional profiling, Northern analysis [221]).

Four studies report defects associated with *SUN41* deletion (103, 138, 279, 390). Deletion of both alleles resulted in defects in hyphal formation, cell separation, and biofilm formation (103, 138, 279). *SUN41* expression in the parent strain did not change during entry into stationary phase and through 11 days of culture (390). However, the *sun41*Δ/Δ strain had a severe defect at day 8, with loss of viability affecting the maintenance of stationary phase. Mutant strains were more sensitive to caspofungin (279) and Congo red (138, 279) with slight or no change in calcofluor white sensitivity (103, 138). The mutant strain was unchanged compared to the parental strain in terms of sensitivity to other cell wall-perturbing agents or conditions such as nikkomycin Z treatment and oxidative stress (103). Mutant strains showed some reduction in adherence to a Caco-2 monolayer (138) but no difference in adherence or damage to FaDu oral epithelial cell line cells or human umbilical vein endothelial cells (HUVECs) (279). In murine models of hematogenous and oral infections, the mutant was attenuated (279). The deletion of *SIM1* resulted in only minor phenotypic alterations (103). The failure to achieve the deletion of both S*UN41* and *SIM1* supported a synthetic lethal condition. When the control of either gene was by the methionine/cysteine-repressible *MET3* promoter, there was no growth under repressing conditions, and there was growth with increased doubling time in the absence of repressors remediated the lysis phenotype. Under repressing conditions, mother and daughter cells remained attached, and after several generations, primarily mother cells lysed. Transmission electron microscopy showed defects at the septum. The conditional strains were more sensitive to several agents, such as nikkomycin, azole-class drugs, and calcofluor white. The latter reagent showed variable chitin distribution in the mutant. Under repressing conditions, sorbitol remediated the lysis condition in rich but not minimal medium. These studies provide strong evidence that these genes contribute to cell wall integrity and share an essential function.

Other putative glucanases. Less information is available for several other putative glucanases. *C. albicans* Scw4p is another unattached protein that may have glucosidase activity. The best hit for *S. cerevisiae* is Sc*SCW4*, although this gene is the ortholog for Ca*MP65*, discussed above. Ca*SCW4* expression is greater in opaque than in white cells (transcriptional profiling [193]) and is downregulated in alkaline conditions (transcriptional profiling [23]) and during cell wall regeneration (transcriptional profiling [46]). The observation of greater expression in opaque cells is interesting in view of the observation that the Sc*SCW4* Sc*SCW10* double deletion mutant has defects in mating (43). The best hit for Sc*SCW10* in *C. albicans* is *MP65*, discussed above. Yet another gene, *SCW11*, encoding a putative *C. albicans* glucanase shows altered expression in response to different conditions. Caspofungin treatment reduced expression (transcriptional profiling [210]). Expression also decreased late in protoplast regeneration (transcriptional profiling [46]). On the other hand, expression increased in response to elevated iron concentration (transcriptional profiling [194]). Expression is less in hyphae than in yeast cells and is regulated by Ace2p in both morphologies (RT-PCR [172]; transcriptional profiling [256]). The gene appears to be essential, as homozygous mutant strains were not recovered (279). As Ace2p is a regulator of cell separation and morphogenesis and expression is greater in yeast cells than in hyphae, Scw11p may be a glucanase involved in cell separation.

Chitinases. There are four annotated genes encoding chitinases in *C. albicans*. Three of these are cell wall associated and all are members of the GH18 family. Cht4p lacks a signal for classical export and is an ortholog of *S. cerevisiae* Cts2p, a cytoplasmic protein implicated in sporulation. Like glucanase activity, both covalently attached and soluble proteins are predicted cell wall chitinases. Cht2p is a GPI-CWP (Table 2) that has been found in cell wall extracts (see Table 4). Deletion does not cause a defect in cytokinesis. Cht1p is a minor chitinase (85, 343). The null strain showed no growth differences in liquid medium from what was seen for the parental strain but had increased hyphal growth on solid medium (85). In some studies, the expression of *CHT1* was not detected (172, 239). The gene was downregulated by Rim101p in alkaline medium (transcriptional profiling [23]). This observation is the likely reason for the failure of other studies to note expression, as in those studies cells were not grown at acidic pH. The mutant phenotype, if tested at acidic pH, may differ from that at alkaline pH. In addition to *CHT1*, *PHR2* and *SCW4* have a reduction in expression at alkaline pH (transcriptional profiling [23]). Changing the enzymes that contribute to cell wall

polysaccharide structure and remodeling may reflect a pH optimum that is not broad enough to support adequate cell wall integrity under different conditions.

Cht3p is the major chitinase (85, 343). In a *CHT3* null strain, cell-associated chitinase activity was greater than culture supernatant activity, and both decreased about 60% in yeast cells in the mutant strain (343). In the strain lacking Cht2p, cell-associated activity decreased about 20%, while there was no decrease in culture supernatant activity. This observation is consistent with the covalent attachment of Cht2p and the greater contribution of Cht3p to chitinase activity. In hyphae, the activity was severalfold greater. In hyphae, the loss of Cht2p had no effect, while the loss of Cht3p reduced both cell-associated and culture supernatant activity by about 80%. This observation suggested that most of the increase in hyphal chitinase activity derived from Cht3p. The greater abundance of chitinase activity in hyphal organisms was unexpected, as a previous study reported the preferential transcription of both genes in yeast cells (Northern analysis [239]). Whether the difference in hypha-inducing conditions is responsible for the difference or whether there is posttranscriptional regulation is unknown. Deletion of various chitin synthase genes had either a modest effect or no effect on chitinase activity (343). Thus, there was not a concomitant decrease in chitin synthase activity when chitinase decreased. The increase in the chitin content of yeast and hyphal cells in *CHT2* and *CHT3* null mutant strains was not significant. However, there was some hypersensitivity to calcofluor white, which can reflect an increase in chitin.

Cells in which Cht1p or Cht2p was absent had no defect in cell separation, and the budding was bipolar (85). In contrast, the loss of Cht3p resulted in the failure of cells to separate. The restoration of *CHT3* expression remediated the defect, confirming the role of Cht3p in cytokinesis. A deletion of the only *S. cerevisiae* chitinase gene, *CTS1*, causes the same cell separation defect in that organism (190). *C. albicans CHT3* but not *CHT1* or *CHT2*, when expressed in *S. cerevisiae*, partially complemented the loss of Sc*CTS1* (85). This observation identifies *C. albicans CHT3* as the functional homolog of *S. cerevisiae CTS1*. In *S. cerevisiae*, Cts1p localizes to the daughter cell (61). The expression of Sc*CTS1* is cell cycle regulated and peaks at M or the M/G_1 boundary (359). The functional homology of CaCht3p and ScCts1p suggests that CaCht3p also localizes to the daughter cell and hydrolyzes the primary chitin septum from the daughter side. In a *C. albicans* strain lacking the Ace2p transcription factor, which regulates cell separation, there was no effect on *CHT2* expression. However, *CHT3* expression was almost abolished in both yeast and hyphal cells (RT-PCR and transcriptional profiling [172, 256]). The expression of *ENG1* and *MP65*, encoding glucanases, also decreased. This observation suggests that cell separation requires hydrolysis of both chitin and glucan. *CHT2* and *CHT3* expression increased in farnesol-treated biofilm (RT-PCR and transcriptional profiling [42]). Since this treatment favored yeast form growth, this observation would be consistent with the previously reported high transcript abundance in yeast cells compared to what was seen for hyphae (239) but not with the observations of enzyme abundance (343). Expression of *CHT3* increased slightly during cell wall regeneration (transcriptional profiling [46]). This delay likely indicates that the regenerating cells do not make daughter cells during the early stages of cell wall replacement.

Other proteins. *WSC1* encodes a CWP whose expression increases in the absence of cyclic AMP (134). Expression decreased during mating (transcription profiling [411]). A deletion strain was hypersensitive to caspofungin, suggesting a requirement for Wsc1p for normal cell wall structure (279). The mutant strain had no defect in biofilm formation. However, the mutant strain had a severe defect on day 4 of culture growth, affecting the entry into stationary phase on day 5 (390). Cells become smaller as growth slows and cells enter stationary phase at approximately day 5 when grown on rich medium. Electron microcopy shows that after the cell enters stationary phase, the cell wall is thicker (45). *SUN41*, discussed above, had a severe defect at day 8 of culture growth that affected the maintenance of stationary phase (390). The effect on stationary phase of two CWPs emphasizes the necessity of constant vigilance to maintain cell wall integrity and that contributions to this integrity in growing and nongrowing cells may differ.

Atc1p. Unlike the enzymes discussed above, acid trehalase (GH65 family) is not involved in cell wall remodeling. Trehalose is a nonreducing disaccharide. Atc1p, acid trehalase, which contains a possible signal sequence for classical secretion, is one of two enzymes hydrolyzing trehalose (290). This enzyme has been recovered from isolated cell walls. The *ATC1* in vitro translation product hydrolyzed trehalose. After the deletion of *ATC1*, a 170-kDa protein was missing from the extract of the mutant cell wall. The *atc1*Δ/Δ strain could not be grown on trehalose. The strain was also attenuated in the murine model of disseminated infection. Growth on glucose repressed the expression of *ATC1* detected by RT-PCR compared to the growth on trehalose, thereby indicating that *ATC1* expression is subject to glucose repression. Trehalose is a protectant against oxidative stress (7). The loss of a neutral cytosolic trehalase, Ntc1p, had no effect on the cellular response to oxidative stress (296). On the other hand, the *atc1*Δ/Δ strain, lacking acid trehalase, was more resistant to heat, osmotic, and oxidative environmental stress (289). At 1 h of exposure to 42°C, both the mutant and the wild-type strains were similar in terms of survival; however, after 3 h the mutant strain had an advantage. Osmotic stress did not affect the mutant strain until 3 h, while the parental strain was sensitive at 1 h. Although affected by oxidative stress, the mutant strain was more resistant. The mutant strain had somewhat reduced formation of germ tubes under conditions inducing morphogenesis. During longer periods of hyphal development, the mutant strain accumulated more trehalose than the parental strain. Thus, in the *ATC1* deletion strain, the organism has a reduction in hyphal formation that is a putative virulence factor and continues to accumulate trehalose as protection against oxidative stress.

Non-Cell-Wall Functions

Some secreted proteins do not remain cell associated but forage into the extracellular environment. If such proteins are hydrolytic enzymes, they have capacity to hydrolyze large or complex substrates into small units that can be transported into the cell as a source of nutrition. If the degradation of host targets facilitates colonization or invasion, then such enzymes

also function as virulence factors. In the last few years, several studies have examined clinical isolates for one or more phospholipase, proteinase, or hemolytic activities (126, 223, 283, 324, 387). These studies demonstrate the production of these activities by some but not all isolates. There are differences based on the site of isolation (283) or the presence of type 2 diabetes mellitus (387).

This section focuses on secreted hydrolyases. There are several secreted proteins that will not be further reviewed in this section: Hex1p of the GH20 family is a secreted β-*N*-acetylglucosaminidase which has received little attention since the last review, although the deletion strain was constructed a decade ago (49). Hemolytic activity also has received little attention. A mannoprotein in the 200-kDa range from culture supernatant mediated hemolytic activity (402). The activity was inactivated by periodate, thereby implicating the mannan in the activity. The protein has not been further characterized. In recent years, the two families of hydrolytic enzymes that have received the most attention are the Sap and phospholipase B (PLB) families. One of the characteristics of most of the hydrolytic activities is that there are multiple enzymes that may be expressed under different conditions. The identification of the genes encoding these proteins supported the construction of null strains. Fairly recent reviews (262, 336) address these and other hydrolytic enzymes, and the discussion of these enzymes is abbreviated. The Sap, PLB, and lipase families are discussed below, as are phosphatase and glucoamylase activities.

Saps. There are 10 *SAP* genes. Two of them, *SAP9* and *SAP10*, encode proteins attached to the cell wall matrix by covalent linkages and are summarized in Table 2. The products of *SAP1* to *SAP3* and *SAP4* to *SAP6* are approximately 67% and 89% identical, respectively, and cluster together (247, 248). There are three fairly recent reviews that cover the *SAP* gene family (261, 262, 336), and these should be consulted for more detail than will be presented here. The Saps are the only known proteinases excreted into the extracellular, non-cell-associated space. All *Candida* species secrete proteinases, but species other than *C. albicans* appear to do so at a lower level (321). The loss of *SAP2* and *SAP4* to *SAP6* severely impairs growth with protein as the sole nitrogen source (149, 327). The Saps are synthesized as prepropeptides with a signal sequence and propeptide of about 60 amino acids (151, 268, 384). The signal peptide is lost in the endoplasmic reticulum after serving to target the protein to the endoplasmic reticulum, and the propeptide is lost in the Golgi. Once secreted, the Saps may hydrolyze host proteins. Sap2p is the major Sap protein produced in vitro and has been the most studied for its properties (148, 405). Sap2p has a broad substrate specificity and among various activities can degrade host ECM (extracellular matrix), keratin, mucin, stratum corneum, proteinase inhibitors α-macroglobulin and cystatin A, and immunoglobulins (Igs) and activate proinflammatory cytokine interleukin-1β (IL-1β) and clotting factor X (18, 57, 159–161, 245, 306, 382, 383). It is a classic aspartic proteinase like the prototype pepsin. Activity is inhibited by pepstatin A and as well by several clinically useful human immunodeficiency virus (HIV) proteinase inhibitors (27). Aspartic proteinases have an acidic optimum pH. This is a potential disadvantage for *C. albicans*, since host tissues are at neutral or less acidic pH. *Candida* is a commensal in the vagina, with an acidic pH, and in the oral cavity, with neutral pH. Normal blood pH is about 7.4. There are some differences among Saps in response to pH. Activity can span a pH range from 2 to 7. Sap1-3p have their highest activity at lower pH values, pH 3 to 5, while Sap4-6p have theirs at higher pH values, pH 5 to 7 (26, 354).

There are differences in expression, and gene expression generally correlates with protein abundance (405). *SAP2* is the major *SAP* gene expressed during the early stage of growth in response to in vitro culture on protein as the nitrogen source (Northern analysis [148]). *SAP8* production is temperature regulated, it is more strongly induced during early growth at 25°C than 37°C, and it is greater in opaque than in white cells (Northern analysis [247]). However, expression has been detected in infections, suggesting that additional regulatory mechanisms may operate in *SAP8* expression (RT-PCR [263, 313]). For example, in humans *SAP8* was expressed more during infection than in oral carriage and was preferentially expressed in vaginal compared to oral disease (263). *SAP1* and *SAP3*, which have sequences related to that of *SAP2*, are regulated by phenotypic switching and are expressed in the opaque phase of strain WO-1 (Northern analysis [405]). *SAP4* to *SAP6* are almost exclusively expressed in hyphae at the neutral pH used for induction (Northern analysis [54, 148, 405]), and the corresponding proteins are found in the medium (54). Expression is not dependent on protein in the medium. The *SAP* genes are affected by exposure to α pheromone, with *SAP1* and *SAP3* expression repressed (Northern analysis, transcriptional profiling, and real-time RT-PCR [22, 211, 334]) and *SAP4* to *SAP7* expression enhanced (transcriptional profiling, real-time RT-PCR [22, 334]). There are conflicting reports as to whether *SAP2* expression is repressed (transcriptional profiling [22]) or enhanced (real-time RT-PCR [334]). Mutant strains in which single *SAP* genes are deleted are viable, as are strains with deletions of multiple *SAP*s (69, 149, 186, 334, 375, 403). The proteinase activity in biofilm culture supernatant is greater than that in planktonic organisms (241).

There are numerous studies of *SAP* gene function and expression (69, 70, 98, 149, 156, 158, 186, 313, 335, 337–339, 341, 365, 375, 376). Various studies employed in vitro-reconstituted tissue and animal models infected with mutant strains as well as the detection of gene expression at various infection sites. There are differences among studies in the number of *SAP* genes expressed and the effects of mutants on the outcome of infection. Each of the genes from *SAP1* to *SAP8* is expressed in one or more models. In some studies, expression is detected for two *SAP* genes, while five or six are detected in other models (dot and Northern blot analysis, RT-PCR [70, 335]). The profiles of expressed genes also vary during infection. For example, during the progression of infection in a reconstituted vaginal epithelial model, the expression of *SAP1*, *SAP2*, *SAP4*, and *SAP5* was detected prior to that of *SAP6* and *SAP7*, and in this model strains lacking *SAP1* or *SAP2* were attenuated, while those lacking *SAP3* or *SAP4* to *SAP6* were not (RT-PCR [335]). In a model of murine keratitis, the loss of *SAP6* resulted in mild disease, while the loss of *SAP1* to *SAP3* or *SAP4* and *SAP5* resulted in infection similar to that seen for the parent strain (156). Collectively, the studies show the expression of *SAP* genes in vivo in human tissue, reconstituted tissue, and

animal models and that the loss of one or more *SAP* genes may modify infection in model systems.

To reach the extracellular space, proteins such as Saps must pass through the cell wall and have been detected there. Immunoelectron microscopy with anti-Sap antibody shows protein in the cell wall. An anti-Sap MAb showed reactivity within the cell wall of in vitro-grown cells (367). In a rat vaginitis model, reactivity was detected in the fungal cell 1 day postinfection and, to a lesser extent, 5 days postinfection. Antibody to Sap1-3p was reactive with the walls of yeast cells above the epidermis, in contact with superficial cells of the reconstituted tissue, or within epidermal cells (338). In agreement with the predominant expression of *SAP1* to *SAP3* compared to *SAP4* to *SAP6* as detected by RT-PCR, antibody to Sap4-6p detected little reactivity. In a similar study with reconstituted vaginal epithelium, Schaller et al. (335) found more reactivity at 12 h postinfection in the cell wall with antibody to Sap1-3p than with antibody to Sap4-6. The expression of *SAP2* was detected by RT-PCR at 6 h and additionally *SAP1*, *SAP4*, and *SAP5* were detected at 12 h, while *SAP6* and *SAP7* were not detected until later. Saps may also contribute to adherence. Antibody that recognizes Sap1-3p bound to the cell surface and antibody to Sap4-6p to the surfaces of organisms several hours after phagocytosis (26). Strains lacking *SAP1*, *SAP2*, *SAP3*, or *SAP4* to *SAP6* showed altered adherence to poly-L-lysine, Matrigel (primarily laminin and other basement membrane components), or BECs, depending on the surface and organism growth in glucose or galactose (403). The strains with deletions of *SAP1*, *SAP3*, or *SAP4* to *SAP6* that were grown in glucose showed reduced adherence to poly-L-lysine; those strains lacking *SAP3* and *SAP4* to *SAP6* showed reduced adherence to BECs; and only the strain lacking *SAP3* showed reduced adherence to Matrigel. Some HIV protease inhibitors inhibit the adherence of *C. albicans* to a HeLa epithelial cell line (19). Adherence to Vero cells was inhibited by some HIV protease inhibitors, and this adherence was associated with Sap1-3p (27). On the other hand, the same drugs did not inhibit binding to endothelial cells (97), which is consistent with the observation that strains with deletions of *SAP1*, *SAP2*, or *SAP3* are unaltered in their adherence to endothelial cells (152).

In *S. cerevisiae*, both pheromone secretion and extracellular acid proteinase (barrier activity) regulate mating. A search for a candidate for a similar barrier activity in *C. albicans* identified *SAP30* (334). Sap30p was found in a large-scale fusion protein screening as one of the secreted proteins (249). *SAP30* (alias *BAR1*) expression is associated with mating type **a** cells (transcriptional profiling [388]). Upon treatment of opaque **a** cells, *SAP30* expression increased more than 200-fold (real-time RT-PCR [334]). The deletion of *SAP30* results in opaque **a** cells having a hypersensitivity to α pheromone. Mutant **a** but not mutant α cells display a mating defect, with very few mating zygotes found.

Phospholipases. Various extracellular phospholipase activities that include reactions associated with PLA, B, and C have been reported for *C. albicans* (see the reviews in references 49 and 123). However, currently only PLB-type proteins are thought to be secreted (336). There are five *PLB* genes with signals for secretion and one *SPO1* gene encoding a protein with similarity to PLB, which has a possible signal sequence. Three of the *PLB* genes, *PLB3*, *PLB4.5*, and *PLB5*, have GPI anchors, and whether they are localized to the membrane or to the cell wall has not been clarified (379) (Table 2). Secreted phospholipase activity has been reviewed recently, and the reviews cover secreted phospholipases more extensively than here (123, 336). Extracellular enzymes transit the cell wall and may be found in the wall. Phospholipase activity (combined PLA and B) is found in the cell walls of both yeast cells and hyphae in an oral reconstituted human epithelium (RHE) infection model (159). Activity localized to the periphery of the yeast cell and at the tips of hyphae. Immunoelectron microscopy also detected the protein in the cell wall (203). Environmental factors influence the expression of *PLB1*. Expression was detected in rich medium at 30°C but not at 37°C, although the protein was detected at the higher temperature (Northern analysis [252, 254]). In defined medium, expression at the higher temperature was detected only with the addition of serum. Expression was found in both yeast cells and hyphae, although in early stages of yeast growth, expression may be greater in yeast cells than in hyphae (Northern analysis [142, 252]). Tup1p (transcriptional corepressor of filamentation) regulates the expression of *PLB1*, as in its absence expression increased (Northern analysis, transcriptional profiling [142, 258]). Expression was unregulated in the *tup1*Δ/Δ strain, as the *PLB1* expression was similar in conditions that favored either yeast cell growth or hyphal formation in the wild type (Northern analysis [142, 258]). In another study, *PLB1* expression determined by RT-PCR did not correlate with adherence or hemolysin production (324). This suggests that phospholipase activity is not responsible for hemolysis, and as noted above the mannan portion of a mannoprotein may have this activity.

A strain with deletion of *PLB1* showed no obvious phenotype and was unaltered in its adherence to endothelial and epithelial cells, but its ability to penetrate the cells was diminished (203). The strain also showed attenuated virulence in a murine disseminated infection model. *PLB2* has been cloned and expression was detected in rich medium (Northern analysis [370]). The gene has not been deleted. However, Plb1p accounts for most of the activity, as only about 1% of the phospholipase activity remained after the deletion of *PLB1* (123). A more recent clinical isolate study that compared the expression levels of various lipase genes in phospholipase-positive and -negative clinical isolates supported a similar suggestion (323). The negative group expressed only *PLB2* and *PLD1*, while the positive group expressed *PLB1*, *PLC1*, and *PLD1*.

PLB1 is one of the core genes repressed more than 1.5-fold in response to osmotic, oxidative, and heavy metal stress (transcriptional profiling [94]). Many of the genes in the repressed group are involved in transcription and metabolism, and the reduction may be related to slowed growth in the presence of the stress. Hog1p also contributes to the transcriptional regulation of some genes in the absence of stress. Compared to *hog1*Δ/Δ cells, *PLB1* is expressed 3.8-fold higher in *HOG1* cells. The stress associated with drug treatment appears not to affect the expression of *PLB1*. There was no effect of treatment with ciclopirox olamine or fluconazole (RT-PCR [272]), caspofungin (RT-PCR, transcriptional profiling [210, 272]), or amphotericin B, ketoconazole, and flucytosine (transcriptional profiling [210]). However, a bisquaternary ammonium salt whose likely target is phospholipase inhibits growth (269).

PLB2 is induced sixfold in response to pheromone (transcription profiling [22]). Expression is not affected by exposure to ciclopirox olamine or fluconazole (Northern analysis [272]).

The expression of *PLB1* and *PLB2* has been detected in infection specimens. In saliva from humans with either oral carriage of *C. albicans* or infection, the expression of *PLB1* was more frequently detected in specimens from infected individuals and correlated with infection (RT-PCR [263]). There was no difference in *PLB2* expression among infected individuals and carriers. There was no difference between individuals with carriage and infection in the vagina. This suggests a site-dependent difference in expression. In an oral infection model in immunocompetent mice, *PLB1* expression was sustained for several days early in infection (RT-PCF [313]). In a murine gastric infection model, *PLB1* and *PLB2* expression was observed in both immunocompetent and immunodeficient gnotobiotic mice (RT-PCR [341]). At 3 to 4 weeks after infection, PLB2 expression was noted in tongue, palate, esophagus, and stomach, while *PLB1* was detected in stomach. This suggests a site-independent expression of *PLB2* and a site-dependent expression of *PLB1*.

Lipases. Enzymes that hydrolyze ester bonds either at the lipid aqueous interface (lipases) or in solution (esterases) have been reported for *C. albicans* but also have been rather neglected. They were included in a recent review (336), which is a source of more information than given here. The lipase family contains 10 genes, *LIP1* to *LIP10*, with *LIP7* lacking the signal for secretion. Molecular analysis of the *LIP* family began in 1997 with the cloning of *LIP1* (112) and was followed in 2000 with the cloning of the other nine genes (150). The protein sequences are similar, with up to 80% amino acid identity. Expression analysis employed RT-PCR except for Northern blotting for *LIP1*. *LIP3* to *LIP6* are expressed in vitro in all the media and conditions tested. *LIP1*, *LIP3* to *LIP6*, and *LIP8* expression occurs in medium with Tween 40 as the carbon source, while *LIP2* and *LIP9* expression occurs only in medium without lipid. The expression of some lipase genes in the absence of lipid suggests that there may be additional functions for these enzymes. During hyphal formation in defined medium, the expression of *LIP1* was not detectable by either Northern or RT-PCR analysis. *LIP2* and *LIP9* and *LIP10* expression also was not detected. However, *LIP3* to *LIP5* and, to a lesser extent, *LIP6* were expressed throughout the transition. *LIP8* expression was detected at 1, 3, and 5 h but not subsequently. When serum was used for hyphal induction, the expression pattern was similar, except *LIP1* was detected and *LIP6* expression was enhanced. There are also differences in response to other in vitro conditions. *LIP1* is repressed by α pheromone (transcription profiling [22]), *LIP2* is repressed during mating (transcription profiling [411]), *LIP4* is expressed more in opaque than in white cells (transcription profiling [193]), and *LIP6* is induced upon adherence to polystyrene (transcription profiling [228]). Upon exposure to ciclopirox olamine, the responsivenesses of the genes were different, with *LIP1*, *LIP2*, and *LIP5* showing strong, modest, and moderate repression, respectively (RT-PCR [272]). Organisms exposed to fluconazole showed a modest decrease in *LIP4* expression and an increase in *LIP8* expression. Another study found no effect of ketoconazole on *LIP* gene expression but observed a decrease in *LIP6* expression upon exposure to caspofungin (transcription profiling [210]).

LIP4 has been expressed in *S. cerevisiae* and the recombinant protein analyzed (320). The enzyme is a true lipase hydrolyzing insoluble triglycerides and is able to use methyl, ethyl, and propyl esters. The enzyme had highest selectivity with unsaturated fatty acids. There was evidence that esterification was by acyltransfer. A screen for genes whose haploinsufficiency affected filamentation identified *LIP5* through reduced filamentation on Spider medium (228). *LIP8* has been deleted (115). Heterozygous *lip8*Δ and *lip8*Δ/Δ strains showed growth similar to that seen for the wild type in complete medium, but with Tween 40 as the carbon source, both strains showed less than 80% of the wild-type growth. Surface properties of the *lip8*Δ/Δ strain but not of the *lip8*Δ strain were altered, as the null strain showed a tendency to flocculate. There was no difference between mutant and wild-type strains in morphogenesis in serum-containing medium. There was no difference between mutants and wild type when they were grown on solid medium containing Tween 20 or Tween 80. However, in a specialized medium, the *lip8*Δ/Δ strain showed a moderate but significant reduction compared to other strains. The phenotypes on solid medium differed, as the mutant strains showed a rough phenotype at pH 7 and 10 compared to what was seen for the wild type or at pH 4. The introduction of one copy of the wild-type allele into the heterozygous but not the homozygous mutant reversed this appearance. Rough colonies primarily contained mycelia and smooth colonies of yeast cells. This difference could be due to a gene dosage effect or allelic differences, as seen in the ALS family discussed later. The *lip8*Δ/Δ strain showed attenuated virulence in the murine model of disseminated infection but not in peritoneal infection (115). As described below, *LIP8* expression is frequently detected during infection.

As with *SAP* genes, there are differences in the expression of *LIP* genes in infection models. In a murine peritonitis model, the expression of lipase genes was determined in liver tissue (RT-PCR [150]). *LIP5*, *LIP6*, *LIP8*, and *LIP9* were expressed in some or all livers analyzed. Subsequently, expression in this model was examined further with an analysis of livers obtained at 4 and 72 h postinfection (RT-PCR [366]). *LIP10* was not detected. *LIP4*, *6*, and *9* were expressed in most livers at the first time point, with *LIP5* and *LIP8* expression being found in all livers. *LIP1* and *LIP3* but not *LIP2* expression was detected. At the later time, *LIP5* and *LIP8* expression was found in all livers, and *LIP2* expression was then detected in some livers. The expression of other lipase genes, particularly *LIP9*, generally decreased. At 3 days, the expression pattern in kidney was similar to that in liver. In the RHE model, both constitutive and variable expression occurred. The expression of *LIP1*, *LIP4*, *LIP5*, *LIP6*, and *LIP8* was observed at all three time points, i.e., 12, 36, and 48 h after inoculation (RT-PCR [366]). *LIP2* and *LIP9* expression was not detectable until 36 h and was still present at 48 h. *LIP3* and *LIP9* were just barely detectable at 48 h. In another murine model, expression was monitored in alimentary tract colonization in the cecum and mucosal infection in the stomach, hard palate, esophagus, and tongue (RT-PCR [340]). The expression was determined in immunodeficient gnotobiotic mice 3 to 4 weeks after inocula-

TABLE 3. Estimation of protein number in extracts of cell wall proteins prepared by various methods

Method[a]	DeGroot et al. (73) total protein species (no. of proteins identified)[d] from yeast cells	Pitarch et al. (301, 302) total protein species (no. of proteins identified)[d] from yeast cells	Urban et al. (391): Total (no. of bands identified)[d], Yeast cells	Urban et al. (391): Total (no. of bands identified)[d], Hyphae	Urban et al. (391): Changes in hyphae (gel spots)[e]	Pitarch et al. (303)[b]: Total (no. of spots identified)[d], Yeast cells	Pitarch et al. (303)[b]: Total (no. of spots identified)[d], Hyphae	Pitarch et al. (303)[b]: Changes in hyphae (gel spots)[e,f]	Ebanks et al. (87)[b]: Total (no. of spots identified)[d], Yeast cells	Ebanks et al. (87)[b]: Total (no. of spots identified)[d], Hyphae	Ebanks et al. (87)[b]: Changes in hyphae (protein)[e]
S											
IS						~700 (30)	~700 (30)	94↑ 38↓	465 (107)	427 (107)	7↑ 2↓
LIS			21 (21)	18 (18)	12U 14M						
Se		350 (24)									
Su											
ICG	12 (12)					~450 (8)	~450 (8)	↑ ↓U			
ICAl	2 (2)					~290 (10)	~210 (10)	13↑ 28↓ 5U			
ICCh						~65 (9)	~250 (9)	24↑ 2↓ 185U			
ICCn									31	31	16U 5M

[a] Proteins were extracted by the following means: S, noncovalent soluble protein extracted from intact cells by buffers containing SDS and/or reducing agent (228, 378); IS, isolated cell walls extracted by buffers containing SDS and/or reducing agent (85, 251, 301, 389); LIS, proteins labeled on intact cells and then extracted from isolated cell walls (389); Se, secreted by regenerating spheroplasts (299); Su, culture supernatant (378); ICCn, cell walls extracted with CNBr after removal of noncovalent soluble proteins (85); ICAl, cell walls extracted with mild alkali after removal of noncovalent soluble proteins (71, 301); ICG, cell walls extracted with β-1,3-glucanase after removal of noncovalent soluble proteins (71, 301); ICCh, cell walls extracted with exochitinase after extraction with β-1,3-glucanase (301).

[b] Proteins from each extract were separated by two-dimensional gel electrophoresis and spot intensities compared between extracts.

[c] Differential gel electrophoresis analysis was used.

[d] Total indicates the number of protein species detected and the proteins, bands, or spots identified (in parentheses) indicate the number of protein species for which sequence information allowed a determination of identity.

[e] Changes in hyphae compared to yeast cells or in biofilms compared to planktonic organisms or in biofilms compared to yeast cells or hyphae are shown. Numbers with "U" correspond to unique proteins found in hyphae and not in yeast cell wall extract; numbers with "M" correspond to proteins that were present in multiple locations, being identified either in the yeast cell extract or more than once in the hyphal extract. Arrows indicate higher (↑) or lower (↓) abundance in quantitative analysis.

[f] For the ICG extraction, the numbers were not reported. For the ICCh extraction, 185 spots were seen only in hyphal extract.

tion. *LIP4* to *LIP8* were expressed in mucosal tissues and cecum contents, while *LIP1*, *LIP3*, and *LIP9* and occasionally *LIP10* were detected in gastric tissue but not oral tissue. *LIP2* expression was detected only in cecum contents. Analysis of lipase expression in eight saliva specimens from patients with oral infection also showed selective expression (RT-PCR [366]). The most commonly expressed genes were *LIP4*, *LIP5*, and *LIP8* in four or five specimens; *LIP6* was detected in two specimens, while *LIP1-3* were detected in a single specimen and *LIP10* was not detected. These observations are similar to those discussed above for *SAP* genes, for which there is apparent condition-dependent use of genes of a family.

Phosphatases. *C. albicans* has both internal and extracellular phosphatases. There are both constitutive and inducible acid phosphatases. The secreted acid phosphatase was one of the first mannoproteins to be characterized (reviewed in reference 49). These enzymes have received little recent attention as phosphatases. Pho100p is induced at acid pH and low phosphate (129). The protein is extracted from intact cells in buffer containing dithiothreitol (DTT). Pho100p is N glycosylated. *PHO100* expression is induced in early biofilm formation (transcription profiling [260]). Pho112p and Pho113p, constitutive phosphatases, were also extracted from cells with DTT-containing buffer (129). Both phosphatases were N glycosylated. Pho112p was detected in the large-scale protein fusion screen for secreted proteins (249). *PHO113* was negatively regulated by Rim101p (transcription profiling, RT-PCR [311]).

Glucoamylase. There are two potential glucoamylases. A 190-kDa protein was expressed more abundantly on the surfaces of galactose-grown cells (reviewed in reference 49). Peptide sequences from this protein were obtained and suggested a glucoamylase (369). Probing a genomic library with a sequence from a heterologous gene with protein homology to the peptides resulted in the isolation of the gene. *GCA1* encoded a protein with potential N-glycosylation sites. Northern analysis showed that the gene was expressed at a high level in organisms grown on galactose compared to what was seen for those grown on glucose or sucrose. RT-PCR analysis showed that expression in cells grown on starch and cellobiose was the same as expression in cells grown on galactose. Expression was less for cells grown on sucrose and even less for cells grown on glucose. *GCA1* was corepressed by Mig1p and Tup1p (transcription profiling [258]). *GCA1* expression is downregulated at alkaline pH by Rim101p (transcription profiling [23]) and induced by ketoconazole (transcriptional profiling [210]). *GCA1* is also expressed in a rat model of oral infection (RT-PCR [369]). Expression in cells grown in Lee medium was 3.5-fold greater than that in vivo. The second gene, *SGA1*, encodes a fungus-specific putative glucoamylase that has not been localized (31). Sga1p was identified as part of the large-scale genetic screen for exported proteins (249). Sga1p was one of proteins encoded by fusions of *C. albicans* DNA with the intracellular form of invertase that conferred growth when expressed in a heterologous *S. cerevisiae* suc$^-$ strain. The protein has a predicted signal sequence. *C. albicans SGA1* is the ortholog of *S. cerevisiae SGA1*, which encodes an intracellular sporulation-specific enzyme. *C. albicans SGA1* expression decreased rapidly as protoplast regeneration was initiated (transcription profiling [46]).

TABLE 3—*Continued*

Thomas et al. (380)[b]			Muhkerjee et al. (253)[c]		Martínez-Gomariz et al. (unpublished)[c]			
Total (no. of spots identified)[d]		Changes in biofilm (spots)[e]	Total planktonic cells/biofilm (spots identified)	Changes in biofilm (gel spots)[e]	Total yeast cells/ hyphae/biofilm (no. of spots identified)[d]	Changes[e]		
Planktonic cells	Biofilm					Hyphae vs yeast cells	Biofilm vs yeast cells	Biofilm vs hyphae
$\sim$300 (67)	$\sim$300 (67)	8$\uparrow$ 2$\downarrow$	71; differential	38$\uparrow$ 33$\downarrow$	580 (80)	27$\uparrow$ vs 13$\downarrow$	38$\uparrow$ vs 32$\downarrow$	14$\uparrow$ vs 37$\downarrow$
$\sim$40 (34)	$\sim$40 (34)	6$\uparrow$						

CELL WALL PROTEOME AND PROTEOMICS

What's There

One of the changes in recent years is the application of global approaches not only to gene expression but also to protein abundance and identification and metabolism. Several studies have investigated the subproteome of the cell surface (73, 87, 301, 303, 380, 391; M. Martínez-Gomariz, P. Perumal, S. Mekala, C. Nombela, C. Gil, and W. L. Chaffin, unpublished data). Fractionation of this subproteome employed various extraction procedures from either intact organisms or isolated cell walls. Additionally, proteins secreted by spheroplasts during cell wall regeneration and proteins from culture supernatants were analyzed. Table 3 shows the estimated numbers of protein species in various fractions. One study which examined four cell wall fractions estimated that overall about 1,600 proteins were detected from these fractions of yeast cell and hyphal cell walls (303). The differences in procedures for and analysis of the proteins of the fractions and multiple protein species (discussed below) and the same protein in more than one fraction make it difficult to assess the number of unique proteins from the cell wall (Table 4). The most striking difference from Table 3 is in the fraction that is released by glucanase or mild alkali from cell walls previously washed with NaCl and boiled in buffer containing SDS and reducing agent (73, 87, 303). Generally one protocol employs washes of isolated cell walls with 1 M NaCl; boiling in 2% SDS, 40 mm βME (β-mercaptoethanol), 10 mM EDTA, Tris (pH 7.8) twice for 10 min; and then washing in water before enzyme treatment (73). The other procedure uses washing with a step series (0.86 M, 0.34 M, and 0.17 M) of NaCl, boiling using 10 mM DTT and Tris (pH 8) along with the SDS and EDTA, and then washing with sodium acetate (87, 303). It seems that either the difference in washes or in the application of the isolation procedure in different laboratories has a profound difference on the proteins found in this fraction.

One hundred seventy-three proteins have been identified in the various fractions, primarily by mass spectrometry (Table 4). Extracts contained additional proteins, but sequence analysis was not sufficient to identify the encoding gene. de Groot et al. (73) detected and identified 14 proteins in extracts expected to contain only covalently attached proteins (Table 3). Urban et al. (391) identified the 39 major bands of proteins that were biotinylated on intact cell surfaces. Another study that detected about 40 proteins in culture supernatants of planktonic organisms and biofilms identified 34 of the spots (380). However, among other studies that examined fractions that contained hundreds of protein spots, the investigators did not attempt to identify all proteins (87, 253, 303, 380; Martínez-Gomariz et al., unpublished). Therefore, there are hundreds of protein spots awaiting identification.

Many of the proteins have multiple species, so not all spots on gels represent unique proteins (Table 4). Since many proteins are unidentified, other proteins may also have multiple species. Some of the proteins with multiple species have been obtained from more than one extraction protocol and in more than one study. Most extraction procedures include one or more protease inhibitors, suggesting that the multiple species may exist in the cell wall. Some of these proteins and multiple species have been observed for more than one growth condition or medium. For example, multiple species of Pdc11p have been observed from extracts obtained by six different methods and reported by several laboratories. The purpose of several studies was a comparison of protein species obtained from yeast cell and hyphal organisms, or planktonic organisms and biofilms. These studies found that some proteins differed in abundance on different cell surfaces. Interestingly, one or more species of the same protein differed in abundance in some reports. Sensitive difference gel electrophoresis technology was used in two studies allowing determination of abundance differences directly in the same gel (253; Martínez-Gomariz et al., unpublished). In the study by Martínez-Gomariz et al. (unpublished), multiple replicates of extracts from yeast cells, hyphae, and biofilm were examined, allowing a statistical analysis of differential abundance. This study also applied multivariate analysis and K-means clustering to the differentially abundant proteins to assess patterns of abundance. This latter analysis showed clusters of proteins that differed between all three conditions or two conditions and various relationships of increased or decreased abundance. There were also differences among the studies, with a protein being reported as present in one study and absent in another or differentially abundant

TABLE 4. Proteins identified in proteomic analysis of various cell wall fractions and culture supernatant

Gene or Orf19 gene assignment (ortholog)[a]	Extraction procedure[b]	No. of species of the same protein	Finding for[c]: Yeast or planktonic culture	Hyphae	Biofilm	Supernatant: Planktonic culture	Supernatant: Biofilm culture	Serum reactivity	*efg1*Δ/Δ strain[d]	Reference(s)
ACC1	ICCn		√							87
ACS2	IS		√↑	√						87
ACT1	IS		√							87
ADE1	IS		√	√						87
ADE17	IS		√↑	√						87
ADH1	S, IS, LIS, ICAl	6	√↑	√↓↑	√↑2 ↓3			√	↓2	87, 253, 303, 333, 380, 382, 391; Martínez-Gomariz et al., unpublished
ADH2	S		√		√↑					380
ADO1	S	2	√	√↑2	√					Martínez-Gomariz et al., unpublished
AHP1	S, IS	4	√	√	√↑3			√		382; Martínez-Gomariz et al., unpublished
ALD5	IS		√	√	√↑				↓	87, 253, 333
ALS1	ICG		√							73
ALS3 or *ALS6*	ICCn		√							87
ALS4	ICG		√							73
ANB1	S		√		√					380
APT1	IS		√	√						87
ARO4	IS			√						87
ASC1	IS	2	√	√↑						87
ASC2	IS		√	√↓						87
ASN1	IS	2							√	333
ATP1	LIS, ICCn		√	√						87, 391
ATP2	IS		√	√↑	√↑					87, 253
ATP3	IS		√	√						87
ATP4	IS			√						87
ATP7	IS		√	√↑						87
BAT22	S	2	√	√↑2	√↓1					Martínez-Gomariz et al., unpublished
BGL2	IS, Se	3	√	√				√		87, 301, 303
BMH1	IS, LIS		√	√						303, 391
CAR2	Su	2				√	√			380
CCT7	IS									253
CDC19	S, IS, Se, Su, ICCn, ICAl	2	√	√↓↑	√	√	√	√		87, 303, 380, 382
CDC33	S, IS		√	√	√					87, 380
CDC37	IS		√	√						87
CDC48	IS		√	√						303
CHT2	ICCn, ICG		√	√						73, 87
CHT3	Su					√	√			380
CIT1	Su, ICCn		√			√	√			87, 380
COF1	S	2	√	√	√↑					Martínez-Gomariz et al., unpublished
COR1	IS		√	√						87
CPR3	S		√	√↑	√					Martínez-Gomariz et al., unpublished
CRH11	ICG		√							73
CSH1	LIS			√						391
CSP37	IS		√	√						303
DDR48	S		√	√	√			√		380, 382
EBP1	LIS			√						391
ECM33	ICG		√							73
EFB1	IS		√	√↑						87, 303
EFT2	IS, LIS, ICCn,		√	√						87, 303, 391
EFT3	IS, LIS, ICCn		√	√						87, 303, 391
EGD1	S, IS		√		√				↓	303, 333
EGD2	IS		√	√↑					↓	87, 333
ENO1	S, IS, Se, Su, ICCn, ICAl, ICG, ICCh	5	√	√↑	√	√	√↑	√		87, 253, 301, 303, 380, 382; Martínez-Gomariz et al., unpublished
ERG13			√	√						87
FAS2	ICCn		√							87
FBA1	S, IS, Se, Su, ICAl, ICCh	5	√	√↑↓	√↑1 ↓4	√	√	√	↓	87, 253, 301, 303, 333, 380, 382; Martínez-Gomariz et al., unpublished
FMA1	IS	2	√	√						87
GND1	S		√	√	√↓					87; Martínez-Gomariz et al., unpublished
GPH1	LIS			√						391

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TABLE 4—*Continued*

Gene or Orf19 gene assignment (ortholog)[a]	Extraction procedure[b]	No. of species of the same protein	Finding for[c]: Yeast or planktonic culture	Hyphae	Biofilm	Supernatant: Planktonic culture	Supernatant: Biofilm culture	Serum reactivity	*efg1*Δ/Δ strain[d]	Reference(s)
GPM1	S, IS	4	√	√	√↑1 ↓1			√	↓	87, 253, 303, 333, 380, 382
GRE22	S, Su		√		√	√	√			380
GRE3	S		√	√↑	√					Martínez-Gomariz et al., unpublished
GRP2	S, IS	2	√	√↑1	√					87; Martínez-Gomariz et al., unpublished
GUK1	IS								↓	333
HEM13	S, LIS		√		√				↓	333, 380, 391
HOM6	S, IS		√	√↑	√↓					87, 380; Martínez-Gomariz et al., unpublished
HSP104	IS		√	√						303
HSP60	IS	2	√	√	√					87, 253
HSP70	S, IS, LIS, ICCn	2	√	√↑1↓	√↑↓	√	√	√		87, 303, 382, 391; Martínez-Gomariz et al., unpublished
HSP90	IS, LIS, ICCn		√	√↑						87, 303, 391
HXK2			√↑	√						87
IDH2	IS		√	√↑						87
ILV5	IS	2	√		√↓					253
IMH3	S, IS		√		√↓				↓	333, 380
INO1	S, IS	2	√	√↑	√↓↑			√		303, 380, 382; Martínez-Gomariz et al., unpublished
IPP1	S	2	√	√	√↓			√		380; Martínez-Gomariz et al., unpublished
KAR2	IS		√	√						87
KGD2	IS		√	√						87
LEU2	S		√	√↑	√					Martínez-Gomariz et al., unpublished
LPD1	Su, ICCn		√		√↓	√	√			87, 253, 380
LSC2	IS		√	√						87
LSP1	IS		√	√↓						87
LYS9	S		√	√↑	√					Martínez-Gomariz et al., unpublished
MDH1	Su, IS		√	√↑		√	√			87, 380
MDH2	S		√		√↑					380
MET6	S, IS, Se	5	√	√↑2	√↑2–4↓2		√	√	√	253, 301, 333, 380, 382; Martínez-Gomariz et al., unpublished
MET15	S, Su		√	√	√↑		√			380, 382
MP65	Su,ICG, ICCn	4	√	√			√	√↑		73, 87, 380
MSI3	IS		√	√↓						87
PDC11	S, IS, LIS, ICCn, ICAl, ICCh	13	√	√↓7	√↑13↓11				↓1	87, 253, 303, 333, 380, 391; Martínez-Gomariz et al., unpublished
PDI1	IS	2	√	√↓1 ↑1	√					87, 253, 303
PET9	ICCn		√	√						87
PGA4	ICG		√							73
PGA29	ICG		√							73
PGK1	S, IS, LIS, Se, Su, ICAl, ICG, ICCh	6	√	√↑	√↑	√	√	√	↓	57, 150, 198, 200, 218, 243, 245, 250
PGM2	S		√	√	√					Martínez-Gomariz et al., unpublished
PHR1	IS, LIS, ICG, ICCh		√	√						73, 303, 391
PHR2	IS, ICG, ICCh		√	√						303
PIR1	ICAl		√							73
PMA1	ICCn			√						87
PMI1	S		√		√				↓	333, 380
PMM1	S, IS, Su		√	√	√	√	√↑			87, 380
PR26	IS		√	√						87
PRA1	S, LIS, Su			√		√	√	√		380, 382, 391
PST2	S		√	√	√			√		380, 382
PST3	IS		√	√						87
PYC2	LIS		√		√					391
PYK1	LIS		√							391
QCR2	IS	2	√	√						87
RBP1	S, IS		√	√	√↑				↓	333; Martínez-Gomariz et al., unpublished
RBT5	ICG		√							73
RDI1	S, IS		√	√	√					87, 380

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TABLE 4—*Continued*

Gene or Orf19 gene assignment (ortholog)[a]	Extraction procedure[b]	No. of species of the same protein	Finding for[c]:							Reference(s)
			Yeast or planktonic culture	Hyphae	Biofilm	Supernatant		Serum reactivity	*efg1*Δ/Δ strain[d]	
						Planktonic culture	Biofilm culture			
RHR2	S		√	√	√					87; Martínez-Gomariz et al., unpublished
RIP1	IS		√	√						87
RKI1	S		√	√	√↑					Martínez-Gomariz et al., unpublished
RPL3	ICCn		√	√						87
RPL20B	ICCn			√						87
RPN8	IS			√						87
RPPO	IS	2	√	√						87
RPS4	ICCn		√							87
RPS6	LIS		√							391
SAM2	IS, LIS	2	√	√						87, 391
SDH2	LIS			√						391
SNZ1	S		√	√↑	√↑					380; Martínez-Gomariz et al., unpublished
SOD1	S, IS		√	√↑	√↑				↓	333, 380; Martínez-Gomariz et al., unpublished
SOD4	ICG		√							73
SOL3	S	2	√	√↑	√↑					Martínez-Gomariz et al., unpublished
SPE3	S	2	√	√	√					Martínez-Gomariz et al., unpublished
SRB1	S, IS, LIS	3	√	√↑↑						87, 303, 391
SSA2	S, IS, LIS, ICCn	2	√	√↓				√		87, 303, 382, 391
SSB1	S, IS, LIS, ICCn	5	√	√↓	√↓				↓	87, 303, 333, 380, 391; Martínez-Gomariz et al., unpublished
SSC1	LIS, ICCn	2	√							87, 391
SSD1	IS		√	√						303
SSR1	ICG		√							73
SSZ1	IS		√	√↓						303
STI1	S, IS	2	√		√				↓2	333, 380
TAL1	S, IS, Su		√	√		√	√			
TDH3	IS, LIS, Se, ICAl, ICG, ICCh	4	√	√↑↓				√		87, 301, 303, 391
TEF1	IS, LIS	5	√	√						303, 391
TEF2	ICCn		√	√						87
TFS1	S		√	√	√↑					Martínez-Gomariz et al., unpublished
TIF	IS, ICCn	3	√	√						87
TKL1	S, LIS, Su	3	√		√				√	333, 380, 391
TMA19	S, IS	2	√	√	√↑					87, 253, 380
TPI1	S, IS, Se, Su	2	√	√↑1	√↑2	√	√	√	↓	301, 303, 333, 380, 382; Martínez-Gomariz et al., unpublished
TPM2	S		√	√↑	√↑					Martínez-Gomariz et al., unpublished
TSA1	S, IS	3	√	√↑1	√↑2			√		87, 253, 380, 382, 391; Martínez-Gomariz et al., unpublished
TUB2	IS		√	√↓						87
TUP1	S		√	√↑	√↑					Martínez-Gomariz et al., unpublished
UBP1	LIS			√						391
UGP1	LIS		√							391
VMA2	IS		C	√						87
VMA4	IS			√						87
YNK1	S, IS		√	√↑	√				↓	87, 333, 380
YST1	IS		√	√						87
YWP1	ICG		√							73
ZWF1	Su, SB					√	√		↓	333, 380
XYL2	S		√	√	√			√		380, 382
19.1361 (*TIM23*/*MAS6*)	IS		√	√						87, 253, 380
19.1662 (*MRP1*)	IS			√						87
19.1680 (*TFP1*)	IS			√						87
19.1710	IS		√	√						87
19.251 (*HSP31*)	S, Su	2	√		√	√	√	√		380, 382; Martínez-Gomariz et al., unpublished
19.2965 (*GET3*)	IS		√	√						87
19.3037 (*PBA1*)	IS		√	√						87

Continued on following page

TABLE 4—*Continued*

Gene or Orf19 gene assignment (ortholog)[a]	Extraction procedure[b]	No. of species of the same protein	Finding for[c]: Yeast or planktonic culture	Hyphae	Biofilm	Supernatant: Planktonic culture	Supernatant: Biofilm culture	Serum reactivity	*efg1*Δ/Δ strain[d]	Reference(s)
19.3053	S		√	√	√↑					380; Martínez-Gomariz et al., unpublished
19.3235	IS								↓	333
19.3499	Su					√	√			380
19.4016 (*COR1*)	IS	2	√	√						87
19.5175 (*SSM4*)	S		√	√↑						303
19.5180 (*PRX1*)	S		√	√	√					87; Martínez-Gomariz et al., unpublished
19.5620 (*YHI9*)	S	2	√	√↑2	√↓2					Martínez-Gomariz et al., unpublished
19.6524	IS		√	√						87
19.6561 (*LAT1*)	IS		√	√						87
19.6748	IS		√	√						87
19.6809 (*PMU1*)	S	2	√	√↑1	√↑1					Martínez-Gomariz et al., unpublished
19.7234 (*RSC8*)	IS		√	√						87
19.7263 (*RPN11*)	IS		√	√						87
19.7590	IS		√	√						87
19.822	S	2	√	√	√↑2	√	√			380
19.8442	S	2	√		√					380

[a] Gene names and Orf19 assignments (and *S. cerevisiae* orthologs in parentheses) are from the CGD (9) (July 2007).
[b] Proteins were extracted by the following means: S, noncovalent soluble protein extracted from intact cells by buffers containing SDS and/or reducing agent (380; Martínez-Gomariz et al., unpublished); IS, isolated cell walls extracted by buffers containing SDS and/or reducing agent (87, 253, 303, 391); LIS, proteins labeled on intact cells and then extracted from isolated cell walls (391); Se, secreted by regenerating spheroplasts (301); Su, culture supernatant (380); ICCn, cell walls extracted with CNBr after removal of noncovalent soluble proteins (87); ICAl, cell walls extracted with mild alkali after removal of noncovalent soluble proteins (73, 303); ICG, cell walls extracted with β-1,3-glucanase after removal of noncovalent soluble proteins (73, 303); ICCh, cell walls extracted with exochitinase after extraction with β-1,3-glucanase (303).
[c] Symbols: √, protein present in extract; arrows in "Hyphae" column, abundance increase (↑) or decrease (↓) compared to what was seen for yeast cell extract; arrows in "Biofilm" column, abundance increase (↑) or decrease (↓) in biofilms compared to planktonic cells. The presence of both directional arrows indicates proteins with multiple species where some species increase in abundance and other species decrease in abundance. If the number of species that change is reported, that number follows the appropriate arrow.
[d] Arrow direction indicates change in abundance in the mutant strain compared to that in the parental strain.

when two conditions were compared in one study but not in another study. These differences may arise from several sources, such as differences in growth conditions (e.g., medium, culture age), extraction method, the sensitivity of the detection method, and the number of proteins identified.

The studies identified both proteins reported in previous smaller studies as well as proteins not previously identified. As indicated in Fig. 2 and discussed previously, there are three major classes of proteins recovered from the cell wall. Representatives of all three classes were found in these proteomic studies (Table 4). de Groot et al. (74) identified 12 proteins of the GPI-CWP class from yeast cells. These proteins included proteins of the Als family (Als1p, Als4p) and Phr1p and Phr2p. The Phr proteins were found as predicted based on culture pH. Other proteins included Chr11p, Ecm33p, Rbt6p, Ssr1p, Sod4p, and Pga4, 24, and 29p. Mild alkali treatment released the expected Pir1p and the unexpected protein Mp65p. The *S. cerevisiae* ortholog of Mp65p has also been found attached in *S. cerevisiae* (410). *C. albicans* Mp65p was initially identified as an abundant protein in hyphal culture supernatants (32) and was found recently as more abundant in culture supernatant of biofilms than in planktonic cultures (Table 4). One suggestion is that this protein may have two relationships with the cell surface, with a small portion being covalently attached through alkali-labile bonds (73).

Other proteins with an N-terminal signal sequence are likely to be cell wall localized but not covalently attached to the glucan-chitin matrix. Bgl2p, with β-1,3-glucosyltransferase activity, appeared both in soluble cell wall extracts and among proteins secreted by protoplasts (Table 4). Cht3p, the main chitinase, is a secreted protein (85) and was identified in the supernatants of planktonic and biofilm cultures. Another secreted protein, Pra1p, was also present in planktonic and biofilm culture supernatants.

The majority of proteins identified in these studies lack the signal sequence for secretion through the classical endoplasmic reticulum-Golgi pathway (Table 4). Most of these proteins have roles in the cytoplasm, as indicated by the names of the corresponding genes. Such proteins with dual locations have been termed "moonlighting" proteins. There are proteins associated with cytoplasmic glycolysis and fermentation, e.g., enolase (Eno1p), triose phosphate isomerase (Tpi1p), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Tdh3p), pyruvate kinase (Cdc19p), fructose-bisphosphate aldolase (Fba1p), and alcohol dehydrogenase (Adh1p). Other proteins with cytoplasmic metabolic roles include Leu2p, Met6p, and Cit1p. There are examples of heat shock and chaperone proteins that include Hsp70p, Ssa2p, Hsp104p, and Ssz1p. Also detected were examples of transcription factors (Eft3p, Eft2p, Teff1p, and Efb1p). Other examples include GDP-mannose pyrophosphorylase (Srb1p), a thiol-specific antioxidant-like protein (Tsa1p), and a homolog of translationally controlled tumor protein (Tma19p).

Cell Wall Localization of Moonlighting Proteins

The general protein secretory pathway has been conserved among organisms (41). The secretion of proteins through the eucaryotic classical endoplasmic reticulum-Golgi pathway has been studied in detail, and signal sequences targeting proteins into this pathway have been identified. Analysis in *S. cerevisiae* has played a key role in dissecting the pathway, and *C. albicans* has orthologs of these genes, e.g., *SEC* genes and SNARE complex genes, e.g., Orf19.1182. Eukaryotic peptides secreted through this pathway have an N-terminal sequence that targets them to the endoplasmic reticulum to exit on the trans-Golgi face being loaded into vesicles destined for the vacuole and plasma membrane. Along the way, the signal is lost and other processing may occur, e.g., glycosylation. Thus, proteins with covalent attachment to the polysaccharide matrix and proteins such as Cht3p and Saps noted above have the signal that shunts them to this pathway of cell exit. There are two alternate pathways in *S. cerevisiae* and *C. albicans*. Sc*STE6* encodes an ATP-binding cassette transporter that exports only the peptide pheromone **a**-factor, and the *C. albicans* ortholog *HST6* is required for mating (225). Another pathway, the nonclassical export pathway, involves two genes, *NCE101* and *NCE102* (60), and has orthologs in several yeasts, including *C. albicans*. This pathway was responsible for the heterologous secretion of mammalian nonclassical export substrate galectin-1, suggesting a common mechanism in the eukaryotes. Among probable endogenous substrates, only Nce103p, a carbonic anhydrase-like protein, has been identified. It is possible that some of the proteins lacking the signal for classical secretion use this pathway. Endosome recycling is a mammalian alternative mechanism (270) that is proposed for the recycling of *S. cerevisiae* Ste13p, a classically exported pheromone receptor (53).

How the proteins that lack the signal for classical secretion reach the cell surface is a major question in cell wall formation for *C. albicans* as well as for other yeasts on whose surfaces such proteins have also been detected. Some of the proteins in this group, such as those involved in glycolysis, are abundant cytoplasmic proteins and would be released by lysed cells. These proteins could then stick nonspecifically to the *C. albicans* surface and thus be present when the cell walls are analyzed. Some studies were performed with isolated cell walls that are bathed in cytoplasmic contents before the particulate cell walls are obtained, and this possibility therefore could not be excluded. To remove proteins that adhere during cell wall isolation, some procedures, such as those indicated as "IS" in Table 4, employ salt washes prior to the extraction of isolated cell walls. However, the proteins that remain after this procedure would suggest a very tight interaction or incorporation more securely within the cell wall matrix. Two studies that have examined the adherence of cytoplasmic proteins to cell surfaces made differing observations. In one study, Hsp70p, enolase, and cytoplasmic proteins adhered to cell walls previously treated by boiling in SDS (95). In another study, Tsa1p-green fluorescent protein (Tsa1p-GFP) present in cytoplasm did not adhere to the surfaces of intact hyphae nor did exposure to cytoplasmic proteins change the surface proteins detected in the absence of exposure (391). One possible explanation for these differences is that in one case noncovalent proteins had been substantially removed, leaving unoccupied sites to which proteins might bind through either glucan or protein interactions, and that in the other case the sites might be occupied and little exchange occurred with added protein.

Methods that do not rupture cells have also been used. These methods, such as those indicated as "S" in Table 4, usually employ a reducing agent, DTT or βME, SDS, NaCl, or a combination. Many of the proteins identified from isolated cell walls have also been identified in extracts obtained by these methods. Recently, it has been suggested that reducing agents and detergent induce membrane leakage, which accounts for the presence of proteins extracted by these methods (178). Another method to avoid cell breakage and membrane permeability is the use of membrane-impermeable reagents such as sulfo-*N*-hydroxysuccinimide–biotin that tag proteins external to the membrane for subsequent identification. A number of proteins, including Hsp70p, Pdc11p, and Tdh3p, have been identified by this method as located outside the cell membrane ("LIS" in Table 4), as reviewed previously (49).

Although applied to a more limited number of proteins, other methods have been used to identify surface proteins on organisms that have been washed with buffer such as phosphate-buffered saline but not otherwise treated. Antibodies to specific proteins have been used to confirm the cell surface localizations of Hsp70p, Pgk1p, Tdh3p, and Eno1p (reviewed in reference 49) and of Adh1p (182). Antibodies have also been used to localize several of these proteins by immunoelectron microscopy by reaction with thin sections. These reports show reactivity within the cell wall and not just on the surface, as might otherwise be predicted by adsorption from medium (Fig. 1D) (5, 128, 213). Other reports of antibodies and reagents reacted with the cell surface prior to sectioning have shown these reagents were restricted to the cell surface (Fig. 1B) (128, 245, 394). Genetic studies using the internal form of invertase as a reporter have shown that that *S. cerevisiae* enolase and Fba1p and *C. albicans* Tdh3p allow the enzyme to support the growth of a suc$^-$ (invertase-negative) strain (219, 287). A construct with only the internal invertase form did not support growth. The N-terminal region of Tdh3p and the first 169 amino acids of enolase are sufficient for this activity.

Reaching the Cell Surface

C. albicans is not the only organism on whose surface has been found many of the same or similar proteins. Other such organisms include fungi *S. cerevisiae*, *Histoplasma capsulatum*, *Kluyveromyces lactis*, *K. marxianus*, and *K. bulgaricus*; parasites *Schistosoma mansoni* and *Entamoeba histolytica*; and bacteria *Streptococcus oralis*, *S. pneumonia*, *S. pyogenes*, *S. mutans*, *Listeria monocytogenes*, *Bacillus anthracis*, and the gram-negative *Bacteroides fragilis* and *Aeromonas hydrophila* (briefly reviewed in reference 278). In these organisms, the signal for export through established pathways is also missing.

Multiple export pathways in various bacteria have been established, including a type III (114, 285, 310), a type IV (82), and the twin-arginine translocation (Tat) (81) pathways. However, there are still proteins, including those mentioned above, that are secreted by an unidentified route(s) and lack anchorage structures in the bacterial cell wall (21, 57). Bendtsen et al. (21) examined the patterns represented in these proteins and developed a predictive algorithm, SecretomeP, for both gram-

positive and -negative bacteria. Feature-based analysis has the potential to uncover additional protein export pathways (383).

In addition to galectin, which is heterologously exported from *S. cerevisiae*, several other proteins have been demonstrated to exit the mammalian cell by nonclassical pathways (reviewed in reference 270), including cytokines (IL-1β, thioredoxin, macrophage migration inhibitory factor), proangiogenic growth factors (fibroblast growth factor 1 [FGF-1] and FGF-2), galectins (e.g., galectin-1 and -3), some viral proteins (e.g., HIV Tat), visfatin and epimorphin (139, 373), and Hsp70 and Hsp90 (389). Some of the processes by which some of these proteins are exported have been reported (reviewed in references 270, 271, 278, and 353). These include membrane blebbing (galectins), membrane flip-flop (HASPB, a *Leishmania* protein produced in the infected cell), and endosomal recycling (IL-1β, En2, and HMGB1). Other routes include substrate-specific interactions of helper proteins with the secreted protein (FGF-1, IL-1α, translationally controlled tumor protein) (308, 309), nanosized exosomes (Hsp70, Hsp90) (59, 195), and a pathway involving a short basic amino acid sequence (HIV Tat) (104). SecretomeP, noted above, was originally developed as a predictive algorithm for mammalian proteins secreted by nonclassical pathway(s) (20).

The examples of nonclassical secretion in bacteria and mammalian cells suggest that additional alternative export pathways may exist in yeasts. The sensitivity of current proteomic techniques has not been applied to determining other possible substrates for the nonclassical export pathway. There is a suggestion that proteins lacking the signal sequence perhaps could hitch a ride on post-Golgi vesicles carrying classical pathway proteins destined for the membrane and thus reach the cell wall (75). Endosome recycling may be another possibility (53). Another suggestion is that chaperones such as the Hsp70 family could assist translocation across the cell membrane (216). Some of these proteins, e.g., enolase, Gpm1p, and Cdc19p, can bind to phospholipids (418), and subsequent translocation across the cell membrane would result in secretion. Recently, extracellular vesicles with capsular polysaccharide and proteins have been isolated from *Cryptococcus neoformans*, and vesicles were suggested as the solution to the issue of the trans-cell wall transport (315, 316). There may be multiple routes with various degrees of specificity. Whatever mechanism underlies the presence of proteins on the cell surface, they have a role in the interaction between the fungus and the host.

ADHERENCE AND LIGANDS

The cell wall is the organelle of the yeast cell that maintains structural integrity and by virtue of its location has a role in the physical interaction of the cell and its host. *C. albicans* can adhere to itself by flocculation, to other microbes by coaggregation, and to host proteins and cells, e.g., fibronectin. These interactive properties are associated with surface adhesins. The interactions may be between proteins and proteins or between proteins and sugars. The interacting partners may both be cell associated, or the interaction may be between *C. albicans* and a soluble or immobilized host ligand. Biofilm formation also involves surface interactions. As a commensal, *C. albicans* can be found on the skin and on mucosal surfaces. To remain in these locales and not be removed, there must be interactions to retain host association. During infection, the fungus may encounter any host tissue and potentially new ligands, such as hemoglobin in blood, in order to infect tissues. Also in this relationship with the host are the host innate defenders in phagocytic cells. These cells are stimulated by the recognition of microbial surface components. At times, these adherence interactions may promote the interest of *C. albicans* in maintaining commensal status or, if conditions are favorable, overgrowing in the sites of colonization or establishing metastatic disease. The host also has an interest in permitting commensal organisms to persist to compete with infectious microbes for binding sites. On the other hand, the host must protect itself from the invasion of sterile sites. There is still much to learn about the interplay between microbe and host in health and disease. In the following section, there is a brief look at the fungal ligands recognized by innate immune system receptors. The major part of this section focuses on adherence. The first subsection of this part focuses on adhesins and the ligands with which they bind, and the second section focuses on the ligand and the adhesin(s) that recognize the ligand.

Cell Wall Polysaccharides as Ligands for Innate Immune System Receptors

Although this review focuses on proteins, on the unopsonized *C. albicans* cell fungal polysaccharide is a ligand for host proteins on host immune system cells (see the reviews in references 79, 100, 124, 266, and 317). For example, β-1,3-glucan exposed at the yeast surface but not accessible at the hyphal surface is recognized by Dectin-1 (137). The recognition of glucan by neutrophils (polymorphonuclear leukocytes [PMNs]) is reduced by antibody to PMN surface complement receptor 3 as well as by antibody to glucan (198). There are several mannose binding proteins: Dectin-2, mannose receptor, and SIGNR1. Mannose receptors recognize oligosaccharides with terminal mannose, glucose, or *N*-acetylglucosamine (364) and preferentially recognize α-linked branching oligosaccharide (173). Dectin-2 preferentially binds to hyphae compared to yeast cells (332) and recognizes high-mannose structures (240). In addition to the mannose receptor, dendritic cells can interact with *C. albicans* through DC-SIGN (36). SIGNR1 is another mannan binding receptor on resident peritoneal macrophages (377). Interactions between Toll-like receptors (TLRs) and non-TLRs such as Dectin-1 may also be important in the response, and Dectin-1 is suggested to be major receptor for myeloid cells (79). Overall, it is clear that the recognition of cell wall polysaccharide interacts with pattern recognition receptors such as Dectin-1 and TLRs, but there are differences between studies to be resolved in understanding the conditions and ligands that prompt the innate immune response toward protective stimulation.

Adhesins and Ligands

Interest in the adherence of *C. albicans* to ligands that retain the organism in the host and on inanimate surfaces is of long standing. These interactions prevent fluids, such as saliva, from removing the organism from the host. A major method used to identify these interactions and participants was to test the ability of the organism to bind to a substrate (cell, protein, or

other substrate) selected by the investigator and then use various methods to characterize the interaction.

During the 1990s, many *C. albicans* adhesins and host ligands were identified (reviewed in reference 49). These ligands included components of host ECM, laminin, fibronectin, collagen, entactin, and vitronectin. Tenascin was the last ligand from ECM identified (214). The second major group was serum proteins that included fibrinogen and complement fragments C3d and iC3b (reviewed in reference 49). The adhesins were identified by a variety of techniques including far-Western blotting, affinity chromatography, and immunoprecipitation. Multiple *C. albicans* protein species were detected as adhesins for the various ligands, and several of the putative adhesins interacted with more than one ligand. Surprisingly, during the last 10 years, even with access to more-rapid and small-scale techniques for the determination of protein sequences, knowledge of the identities of these adhesins and of their relationships to each other advanced only modestly. One area that received considerable attention and clarification is the Als family of GPI-CWPs. For convenience, in the first section, the adhesive interactions will be discussed by reference to adhesin and, in the second section, the discussion will be organized by host ligand.

Although this review focuses on proteins, glycosylation too has an effect on adherence and will be considered briefly. The proteins secreted via the classical secretory pathway may become both N and O glycosylated. Some of the proteins may be extensively glycosylated, with resultant molecular sizes much larger than predicted from the protein sequence. For a more detailed description of mannan, see the reviews in references 49, 122, and 307. N-linked mannan attached through asparagine residues of the protein consists of a core sequence and highly branched outer chains. The outer chains have acid-stable side chains and acid-labile chains containing β-1,2-mannose chains linked through phosphate esters to the stable mannan. β-1,2-Mannose chains have also been found in phospholipomannan (Fig. 2). O-linked mannan is a short linear α-1,2-mannose-linked chain attached to protein serine or threonine residues. Mannan has been implicated in adherence. For example, strains with deletions of *MNT1* and *MNT2*, encoding α-1,2-mannosyl transferases, are impaired in adherence to BECs and an ECM preparation from Engelbreth-Holm-Swarm cells (257).

Adhesins

Als protein family. The *ALS* gene family encoding GPI-CWPs (Table 2) includes eight genes and the family has recently been reviewed by Hoyer et al. (144) and should be consulted for a more extensive discussion. As expected, the Als proteins are found at the cell surface (Fig. 1B). Briefly, the encoded proteins have three domains (143). The central domain, consisting of tandem repeats, can vary in number between genes and alleles and can be used to classify three subgroups: based on cross-hybridization of the tandem repeats, *ALS1*, *ALS2*, *ALS3*, and *ALS4* are in one group and *ALS5*, *ALS6*, and *ALS7* are in a second group, while *ALS9* is in neither of these two groups (143, 145, 146). The last two domains have multiple glycosylation sites that are predicted to lead to a stretched conformation that can extend the N-terminal probable binding domain from the attachment site. The presence of tandem repeats is a potential site for allelic variation. Allelic variation can occur within the same cell with differences in the two alleles borne by the diploid cell or between strains (144). For example, in the widely used strain SC5314, the larger alleles of *ALS1*, *ALS5*, and *ALS9* are on one chromosome, while the smaller alleles are on the other (417). In one study with many isolates, the tandem repeat number within *ALS1* was 16 for the most common allele, but alleles with 4 to 37 copies were also found (220). As noted below, alleles may differ in their contributions to the phenotype of the organism. Recombinant soluble Als5p fragments can aggregate in solution as amyloid-like fibers, and there are conserved sequences associated with this aggregation in other Als proteins (284). Such interactions may contribute to the adherence function of Als proteins, and this possibility undoubtedly will be explored.

The transcription of all family genes in vitro and during infection has been detected, but some genes (*ALS6* and *ALS7*) have been observed with only low levels of expression (144). *ALS* gene expression may be affected by the stage of growth and by morphology (133). *ALS1*, *ALS2*, and *ALS3* have a dynamic range of expression, with large increases and decreases. The expression of Als proteins was monitored by constructing strains that expressed a GFP fusion protein. Als1p-GFP expression (monitored through fluorescence flow cytometry) increased more than 20-fold upon transfer into fresh medium before slowly decreasing, and Als7p-GFP had a transient increase shortly after transfer, although at a low level. Als5p-GFP and Als6p-GFP were expressed at low levels, with a small, late increase in Als6p-GFP expression. Both Als3p-GFP and Als9p-GFP were at background levels. During germ tube formation, Als1p-GFP expression was the first to increase and attained the highest level; Als3p-GFP expression increased more slowly and rose abruptly coincident with microscopic germ tube emergence. The expression levels of Als5p-GFP, Als6p-GFP, Als7p-GFP, and Als9p-GFP were each comparable to the control level. The early expression of Als1p-GFP followed by a decrease in germ tube formation suggests that Als1p should be found in the initial growth of the germ tube. This was observed with a MAb to the N terminus of Als1p, where bright fluorescence was localized to the base of the germ tube (232). Transcript numbers determined by real-time RT-PCR generally agree with the relative abundance as assessed by GFP monitoring (132). Real-time RT-PCR and RT-PCR showed that when expression occurred *ALS1*, *ALS2*, and *ALS3* were the most abundantly expressed group, while *ALS4* was the most abundantly expressed of the low or weakly expressed group (130, 133). *ALS3* expression was much greater in germ tubes than in a 16-h yeast culture. In biofilms, *ALS1* was also a major differentially expressed gene compared to what was seen for planktonic gene expression by microarray analysis (119) and real-time RT-PCR (280). If protein abundance parallels gene expression, then some of the Als proteins may be present at very low levels and perhaps not detected, as in the studies by de Groot et al. (73), who detected only Als1p and Als4p, and Ebanks et al. (87), who detected either Als3p or Als6p (Table 4). The *ALS* genes as well as *EAP1* are genes of the broader class of flocculation genes found in yeasts (see the review in reference 395). *SFL1* (Orf19.454) is an ortholog

TABLE 5. Effect of *ALS* gene deletion on adherence to various ligands and cells[a]

Gene	Adherence to indicated host cell or ligand						Aggregation	Growth	Biofilm	Reference(s)
	HUVEC	FaDu	BEC	RHE	Fibronectin	Laminin				
ALS1	NC, ↓	NC, ↓	NC	↓	NC	NC	NC	Small cell size, delayed filamentation	↓	1, 113, 144, 273, 300, 413, 414
ALS2	↓	NT	NC	↓	NC	NC	NC	Reduced hyphal formation	↓	414, 416
ALS3	↓	↓	NC, ↓	⇓	NC	NC	NC	NC	↓ in vitro; NC in vivo	144, 273, 282, 300, 412–414
ALS4	↓	NT	NC	NC	NT	NT	NC	Reduced hyphal formation on RPMI	NC	414, 416
ALS5	↑	NT	↑	NC	↑	NT	↑	Decreased growth rate	NT	414
ALS6	↑	NT	↑	NC	NT	NT	↑	Decreased growth rate	NT	414
ALS7	↑	NT	↑	NC	NT	NT	NC	NC	NT	414
ALS9	↓	NT	NC	NC	NT	NC	NT	NC	NC	414, 415

[a] All comparisons were made between strains with deletion of the indicated gene and the wild-type strain. Symbols and abbreviations: ↓, decreased adherence; ⇓, almost eliminated; ↑, increased adherence; NC, no change in adherence; NT, not tested. If different observations for the same strain were made in different reports, then two symbols are given.

of *S. cerevisiae SFL1*, a suppressor of flocculation (18). In the absence of Sfl1p, the expression of *ALS1* and *ALS3* increased. Recently, the regulation of *ALS3* expression focused on the promoter region (8). Two regions were identified, one required for activation in hyphae and proximal to the coding region and a distal region that amplified the response (8).

In disease models and clinical samples, *ALS* genes show variation in expression. In a RHE model, the expression of *ALS1*, *ALS2*, *ALS3*, *ALS4*, *ALS5*, and *ALS9* was present over time (12, 24, 36, and 48 h) for each of the three inoculum concentrations tested (RT-PCR [130]). For both *ALS3* and *ALS5*, one replicate among all the analyses fell below the detection limit. However, for *ALS6* and *ALS7*, this occurred more often and in the case of *ALS6* both replicates were below the detection limit for two analyses. This suggests that *ALS7* and particularly *ALS6* are expressed at low levels. Two studies compared expression from human clinical specimens with expression in an animal model. Analysis of mRNA was more sensitive than total RNA for human vaginal fluid samples (RT-PCR [55]). *ALS1-3* and *ALS9* were detected in 70 to 87% of specimens; *ALS4*, *ALS5*, and *ALS7* were detected in about 30% of samples; and *ALS6* was detected in only 13% of samples. In a murine vaginitis model, *ALS* expression was determined at day 4 and day 7. The percentage of samples in which expression was detected was greater at day 7 than at day 4. At day 7, *ALS*, *ALS2*, and *ALS3* expression was detected in all mice. *ALS9* expression was detected in about one half of the animals on both days. *ALS6* and *ALS7* expression was detected on both days in one or two mice. *ALS4* and *ALS5* were detected on day 7 only in one of five mice. Expression in a vaginal RHE model was assessed at 12, 24, and 36 h. *ALS*, *ALS2*, *ALS3*, and *ALS9* were consistently strongly expressed and *ALS5* was consistently but somewhat more weakly expressed. *ALS4*, *ALS6*, and *ALS7* expression showed negative or weak signals. Since *ALS4* showed strong expression in inoculum cells, the reduction of its expression in samples recovered from the vaginal RHE suggested that expression may be downregulated.

In a hyposalivatory rat model of oral infection, *ALS* expression was greater at day 5 than at day 3 after inoculation (RT-PCR [131]). *ALS1*, *ALS2*, *ALS3*, and *ALS4* expression was detected at both time points, while *ALS5* and *ALS9* expression was not detected until day 5. *ALS6* was detected only once, and *ALS7* expression was never detected. There were differences in the numbers of expressed *ALS* genes depending on the tissue. Generally, more genes were expressed in organisms from the tongue and mandible than in those from mucosae. Oral scrapings from HIV-positive individuals were analyzed for *ALS* expression. The expression of all *ALS* genes was detected in some specimens. *ALS1*, *ALS2*, *ALS3*, and *ALS9* expression was found in all specimens. Expression of the other genes was more variable, with *ALS4*, *ALS5*, and *ALS7* found in 66 to 83% of samples and *ALS6* being detected least often, at 50%. In a murine model of disseminated infection, only GFP fusion constructs with *ALS1* and *ALS3* were detectable by protein expression, primarily in the kidney (132). The kidney was the most frequently infected organ, followed by the heart, with the liver and spleen occasionally infected. The more sensitive RT-PCR method was also used for detection. The other *ALS* genes were detected by more-sensitive RT-PCR. *ALS1-3*, *ALS9*, and, less frequently, *ALS4* were expressed, while *ALS5*, *ALS6*, and *ALS7* expression was not detected. Generally, both clinical samples and model samples show two classes of expressed genes. *ALS1*, *ALS2*, *ALS3*, and *ALS9* are the most abundantly expressed genes, while the others are less frequently expressed, particularly *ALS6*. Generally, the genes most abundantly expressed in culture are also the most abundantly or frequently expressed in infection models, except for *ALS9*, which was often expressed at low levels in culture but was consistently found to be expressed in infection models.

The sequence- and homology-based prediction that Als proteins would have adherence properties was borne out by several studies (144). Some of these properties are summarized in Table 5. Since the Als proteins expressed on yeast cells can vary with the growth state, there should also be variation in the contribution of Als proteins to adhesive interactions. Adhesive properties of these proteins have also been examined by heterologous expression in *S. cerevisiae*. This approach offers an opportunity to study their adhesive properties in greater isolation than possible on the *C. albicans* cell but also has the risk that context modifies the properties. These studies are discussed in the review by Hoyer et al. (144) and will not be included here. Adherence to host cells or other ligands shows that strains with deletions of *ALS* genes have altered binding.

The loss of Als1p reduced binding by 20% (strain 1) and 35% (strain 2) to HUVECs (113, 413), and overexpression increased binding (113). Antibody to the N terminus of Als1p reduced binding to endothelial cells (113) and again implicated the N terminus in binding. Using a third *als1*Δ/Δ strain and an assay for cell association, no reduction in cell association with HUVECs was noted (300). There are also conflicting reports on the effect of the deletion on binding to epithelial cells. Yet another strain showed a reduced binding to FaDu cells (pharyngeal epithelial cell line established from a squamous cell carcinoma) (1), while strain 3 showed no reduction in association with FaDu cells (300). A fourth *als1*Δ/Δ strain showed attenuation in an oral model of infection and reduced binding to murine tongue in an ex vivo assay (165). Strain 1 showed no loss of binding to FaDu cells, BECs, or RHE, although there was a reduction in the destruction of RHE compared to what was seen for the control strain (413). There was no effect on binding to fibronectin or laminin (144, 413). The apparent differences among strains may arise from one or more sources, such as unknown alteration(s) introduced into the mutant during construction, differences in the mammalian cells, differences in the growth levels, and differences in assays of the interaction between fungus and host cells. A side-by-side comparison of these strains should answer at least some of these questions.

The deletion of *ALS2* could not be achieved, and the gene was placed under the *MAL2* promoter (416). The deletion of *ALS4* resulted in an increased expression of *ALS2* and vice versa with the uninduced conditional *ALS2* strain, as determined by real-time RT-PCR. This suggests that there may be some compensatory effects and perhaps some functional overlap. The loss of *ALS4* reduced adherence to HUVECs but not to BECs or RHE and did not cause the destruction of RHE. The uninduced conditional *ALS2* strain showed reduced adherence to HUVECs and RHE and reduced RHE destruction, compared to what was seen for the control, but loss of adherence to BECs.

Compared to the loss of Als1p, the absence of Als3p had a great effect on reducing adherence to HUVECs (413). While the absence of Als1p did not alter binding to BECs, the absence of Als3p reduced binding about 60%. The expression of the N-terminal region of Als3p in the null strain partially restored binding to BECs (412). Antibody recognizing the N-terminal region reduced binding. In the case of RHE, the strain lacking Als3p bound and there was little subsequent destruction of RHE (413). There was no effect on binding to fibronectin or laminin (144, 413). When a second strain was tested for cell association, there was a reduction in the interaction with HUVECs but no change with FaDu cells (300). Like the *als1*Δ/Δ strains, there may be several sources for differences between strains and assays. Indeed, as is noted below, the second strain showed a large reduction in endocytosis with both HUVECs and FaDu cells (300). In strain SC5314, the two *ALS3* alleles have different numbers of tandem repeats (282). A heterozygous strain expressing the allele with more tandem repeats showed adherence to HUVECs and FaDu pharyngeal epithelial cells similar to that seen for the parent strain. The heterozygous strain expressing the allele with fewer repeats had diminished adherence compared to the parent, thus implicating this region in adherence. However, the role of this highly glycosylated repeat is likely that of extending the N-terminal binding domain further from the attachment site (144, 282).

The effect of deleting *ALS5*, *ALS6*, or *ALS7* was surprising in that the strains with a deletion showed increased adherence to HUVECs and BECs (414). The loss of Als7p had no effect on cellular aggregation, while the loss of Als5p and Als6p increased aggregation slightly but not enough to account for the increased adherence of strains lacking Als5p, Als6p, or Als7p. The reintegration of one allele did not restore adherence comparable to that of the control strain, except for the strain with a reintegration of one *ALS6* allele in terms of its adherence to HUVECs. Deletion had no effect on the destruction of RHE. Since expression of these genes is at very low levels, proteins may also be at very low levels. This potential low level of protein suggests that these proteins may have a role in cell surface conformation by exposing adhesins or by an alteration that is compensated by additional adhesins. The absence of Als9p altered adherence to endothelial cells but not that to epithelial cells or laminin (415). There was also no effect with the RHE model (414). In a strain with reintegration of the functional gene, one allele but not the other could restore function (415). This is another example of allelic difference.

In biofilms, organisms are in contact with other organisms and Als adhesins. *BCR1* encodes a transcription factor required for biofilm formation that regulates expression of several adhesin genes, including *ALS1* and *ALS3* (273). Nobile et al. (273) found that an *ALS1* null strain formed an in vitro biofilm that was not strongly attached to the surface. In addition, the strain with a loss of *ALS3* had severely inhibited biofilm formation. Only a rudimentary biofilm that had mainly yeast cells formed in vitro on a silicone surface. *A bcr1*Δ/Δ strain did not form biofilm but was rescued by the overexpression of *ALS3* and partially rescued by the overexpression of *ALS1*. Since *ALS3* is not normally expressed in yeast cells, and cells of an *als3*Δ/Δ strain can adhere, the defect in the *als3*Δ/Δ strain is likely to be later in biofilm development. In an in vivo rat venous catheter model, the *bcr1*Δ/Δ strain also failed to form biofilm, but the *als3*Δ/Δ strain produced apparently normal biofilm. However, as in vitro, the overexpression of Als3p rescued the in vivo biofilm defect of the *bcr1*Δ/Δ strain. Hoyer and colleagues (412) found that throughout the biofilm Als3p-GFP was distributed diffusely on the germ tube surface. In this study, biofilms formed by *als3*Δ/Δ were disorganized and unstable, with hyphae that were parallel rather than exhibiting the usual entwined structure. Efg1p is required for the expression of *ALS3*, and a strain lacking *EFG1* formed only a sparse structure. The overexpression of Als3p in an *efg1*Δ/Δ strain restored biofilm formation. Although both studies with *ALS3* null strains reached similar conclusions that Als3p is important for biofilm formation, there were differences in the reduced biofilm formed. While this may reflect strain differences, it may also reflect the influence of environmental conditions, e.g., medium, on biofilm formation, as suggested by the differences in in vitro and in vivo biofilm formation described above. The loss of Als2p but not of Als4p reduced biofilm mass about 15% (416). This suggests that although there is some compensatory

expression of *ALS4* when *ALS2* is deleted and vice versa (416), the compensation of Als4p is insufficient or does not extend to this function.

A recent report identified cadherin as another ligand for Als3p (300). *C. albicans* invades endothelial and oral epithelial cells by the hyphal induction of host cell endocytosis (102, 288, 298). Host cell N-cadherin is the surface receptor engaging hyphae (299). The deletion strain lacking Als3p but not Als1p failed to induce endocytosis by both HUVECs and epithelial cells of the FaDu cell line (300). The restoration of functional Als3p also restored endocytosis. An affinity purification scheme found that N-cadherin and E-cadherin from endothelial and epithelial cells, respectively, were missing from host proteins bound to the Als3p-deficient strain compared to what was seen for parental and Als1p-deficient strains. In the absence of endocytosis, little damage occurred to the mammalian cells. The binding site resides in the N-terminal portion of Als3p. Latex beads were coated with recombinant N-terminal protein fragment (~400 amino acids) of Als1p or Als3p or control bovine serum albumin (BSA). The rAls3p-coated beads were endocytosed by CHO (Chinese hamster ovary) cells expressing either N-cadherin or E-cadherin, while control beads coated with BSA were not. Although Als1p and Als3p share considerable similarities, molecular modeling revealed differences that predicted that the Als3p N-terminal domain would interact with either cadherin, similar to what is seen for cadherin-cadherin interactions.

Als proteins may have an additional role in cell growth. Despite the expression of the corresponding proteins at very low levels in wild-type cells, the deletion of *ALS5* or *ALS6* resulted in strains with slowed growth (414). The growth rate did not change with the addition of uridine, suggesting that the defect was not associated with the *URA3* marker. Only in the case of the *ALS5* strain did the restoration of one allele restore a normal growth rate. Yeast cells of the *als1*Δ/Δ strain were smaller in size than the control strain when grown in the same conditions (144). Germ tube formation was also delayed in deletion strains (113, 413). The deletion strain also had delayed pathogenesis in several models (1, 113, 165). An earlier report by Chaffin and Sogin (50) associated decreasing cell size with delayed germ tube emergence. The delay in pathogenesis may be associated with the delay in hyphal formation. These observations with Als1p, Als5p, and Als6p suggest, at least with these proteins, that proteins may have more than a single role.

Eap1p. Eap1p was originally identified in a screen of a genomic library expressed for sequences that conferred adhesive properties on *S. cerevisiae* (205). In *C. albicans*, *EAP1* is regulated by Efg1p, a regulator of cell morphology. Eap1p, like Als proteins, is a GPI-CWP (Table 2) and corresponds to one of the yeast flocculation genes reviewed by Verstrepen and Klis (395) and to yeast adhesins reviewed by Dranginis et al. (84). An indirect immunofluorescence assay demonstrated that Eap1p tagged with a hemagglutinin epitope is found at the cell surface (206). Glucanase digestion released a tagged protein construct from the cell wall, and the loss of the GPI anchor site also resulted in finding the protein in the medium. To assess the adherence of wild-type and *eap1*Δ/Δ strains to polystyrene surfaces, the shear force required for detachment was determined. Fewer mutant cells than wild-type cells adhered. The heterozygous *EAP1*/*eap1*Δ cells adhered with intermediate frequency. This observation suggested that Eap1p contributes to adherence to polystyrene but is not the only adhesive surface component. However, once adhered, both wild-type and mutant cells were fairly resistant to removal. Comparison of the adherence of wild-type cells and that of mutant cells to a human embryonic kidney cell line showed that mutant cells were reduced 37% compared to what was seen for the wild type (205). Adherence was dependent on Efg1p, as the *efg1*Δ/Δ strain had reduced adherence compared to the wild-type strain and adherence was restored by the expression of *EAP1* under the actin promoter in the *efg1*Δ/Δ strain (205). Since the deletion of *EAP1* did not abrogate adherence, this observation suggests that other factors also contribute to adherence. Northern analysis showed that *EAP1* was expressed in both yeast cells and hyphae and that expression was dependent on Efg1p (205). An earlier transcriptional profiling study of the yeast-to-hypha transition reported a twofold increase in *EAP1* at 6 hours (264). However, in this study no morphological difference in expression was noted at 6 h with Northern analysis (205). *EFG1* is a regulator of morphogenesis and cell wall remodeling. Although *EAP1* expression is not induced in germ tube formation, its expression is still controlled by Efg1p.

Biofilms for both the wild-type and null strains were formed under constant medium flow with defined shear force (206). The wild type formed biofilm as described in other biofilm studies. Biofilm developed from adhered yeast cells to a multilayered structure with yeast cells and hyphae and ECM. Yeast cells of the mutant strain adhered and formed pseudohyphae and hyphae at 8 h in a biofilm several layers thick. However, by 20 h most of the cells washed out. Thus, the *eap1*Δ/Δ strain was able neither to form a thick biofilm nor to maintain a reduced thickness biofilm in vitro. The strains were also tested in vivo in the rat venous catheter model and, as described elsewhere for *ALS3* and *HWP1* strains, the wild type formed a biofilm in the catheter lumen. The null strain did not. *EAP1* gene expression in sessile cells from both the in vitro and in vivo models was about twice that of planktonic organisms (real-time RT-PCR). The planktonic control contained a mixture of morphological forms, as did the biofilm. This difference is the same for morphological forms found by transcriptional profiling (264) but differs from the lack of difference determined by Northern analysis (205). This difference may be associated with biofilm formation or may be influenced by the different growth media used in the determinations.

Hwp1p. Hw1p is a unique adhesin expressed on the hyphal surface that becomes covalently attached to host cells (see the reviews in references 49 and 371). An initial description of *HWP1* expression found the transcript only in hyphal organisms by Northern analysis (363), and a subsequent analysis found very faint levels in yeast cells by Northern analysis (68). Antibody to the protein showed expression only on hyphal surfaces both from in vitro-produced hyphae (68, 363) and hyphae in the superficial layer of the stomach of a highly colonized beige mouse (363). *HWP1* encodes a GPI-CWP whose expression is regulated by a number of transcription factors that control morphogenesis and mating (Table 2). Exposure of opaque **a**/**a** cells but not α/α cells to α pheromone increased *HWP1* expression in Lee medium (Northern analysis [68]) and also on Spider medium (transcription profiling [22]).

While opaque cells do not form germ tubes in response to α pheromone, they do form conjugation tubes in preparation for mating, and these conjugation tubes are elongated structures. Treatment with anti-Hwp1p antibody specifically stained the emerging and elongating conjugation tubes of the **a**/**a** cells (68). When the growth reverted to yeast growth at the apex, expression terminated and the yeast cell was unstained. When α/α cells along with **a**/**a** cells were exposed to α pheromone in a mating culture, only the conjugation tubes of **a**/**a** cells expressed Hwp1p. The differential Hwp1p expression on parent cells facilitated the determination that the first daughter cell of a mating emerges from the conjugation tube contributed by the **a**/**a** parent. Adherence to bring the mating cells together is involved in mating, but whether Hwp1p functions as an adhesin in this process is unknown.

Recently, an analysis of a segment of the promoter region was found to bind several proteins that differed in yeast cells and during hyphal induction (175). Like the promoter analysis of *ALS3* above (8), two regions were found. The model proposed for yeast cells was that the promoter was occupied by DNA-histone octamers and Gfc1p and Hbp3p that inhibited the binding of activators (175). During hyphal formation, the removal of this blockage allowed activation that was amplified by the interaction of the distal region with transcription factors previously described, e.g., Efg1 and Nrg1. *HWP1* is also regulated by the recently described Sfl1p, with increased expression in the absence of Sfl1p (RT-PCR [18]).

This GPI-CWP protein is a substrate for mammalian transglutaminases that cross-link the *C. albicans* protein to the surface of oral mucosa (362). The cross-linking occurs in the N-terminal domain of Hwp1p. There is similarity in this domain to small proline-rich proteins (PRPs) expressed on the surface of stratified squamous epithelium. Like the adhesive interactions between Als3p and cadherins described above, the *C. albicans* protein may mimic a host protein. Not all BECs are capable of cross-linking with *C. albicans* via Hwp1p (362). Recombinant Hwp1p attached to only the more terminally differentiated BECs displaying SPR3 (a small PRP) and keratin 13 and not to the less terminally differentiated cells displaying involucrin (305).

Recent reports have suggested the possibility of a new adhesive role for the protein in biofilms (273, 277). The expression of the transcription factor Bcr1p was required for biofilm formation (276). Targets for control by this factor include several genes encoding surface proteins: *ALS3*, *ALS1*, and *HWP1*. A strain lacking Hwp1p formed a biofilm which was prone to detaching from the abiotic substrate (273). The *hwp1*Δ/Δ strain produced a biofilm with reduced biomass compared to that seen for the wild type (277). The biofilm had few hyphae, while both planktonic yeast cells and hyphae were in the surrounding medium. Several years ago, a study showed that the deletion of *HWP1* does not cause a filamentation defect (347). Biofilms formed when *bcr1*Δ/Δ mutant cells were mixed in various ratios with cells of strains with wild-type *HWP1* or with heterozygous or homozygous null mutations (277). The surrounding medium of a biofilm formed with the *bcr1*Δ/Δ strain had many planktonic organisms. The mixture with *hwp1*Δ/Δ mutant cells also had many planktonic organisms but fewer than the *bcr1*Δ/Δ mutant alone. When mixed with a strain expressing Hwp1p, the planktonic population was substantially lower, even when 87.5% of cells were from the *BCR1* null strain. The authors suggested that Hwp1p acts as an adhesin to retain organisms in the biofilm. In the in vivo model for biofilm formation in a rat venous catheter, the *HWP1* null strain was also defective in in vivo biofilm production. This is unlike what is the case for the *als3*Δ/Δ mutant discussed previously, which formed biofilm in vivo but not in vitro. Also, unlike what was seen for Als3p, the overexpression of Hwp1p in the *BCR1* null strain did not restore the biofilm formation defect. However, in view of the differences with *als3*Δ/Δ mutants in vitro and in vivo, environmental conditions may be a factor.

Ywp1p. Ywp1p is a GPI-CWP (Table 2) whose expression is greatest on yeast cell surfaces just after the end of the most rapid exponential growth and thereafter declines (129). In low-phosphate medium, GFP-tagged protein persisted longer on growing cells and cells from several-day-old cultures. Expression was not observed on hyphae and pseudohyphae. The protein in the medium increased as the culture approached stationary phase through either shedding of attached protein or secretion without attachment or both. The *ywp1*Δ/Δ strain adhered more to polystyrene and other surfaces than did the wild type and formed yeast cell biofilm with several layers, in contrast to wild-type cells. Wild-type cells showed less adherence and monolayer formation under biofilm conditions. At conditions conducive to hyphal formation, there was less effect, as Ywp1p is not expressed on hyphal surfaces. Thus, this protein appears to block adherence of wild-type cells, a property that it shares with Als5p, Als6p, and Als7p (Table 5). The suggestion put forth was that the protein promoted dispersal through limited adherence or perhaps inhibition by soluble protein, which would allow the organism to seek new sites for colonization. This suggestion was supported by the observation that the addition of culture medium containing the protein was more inhibitory of adherence and biofilm formation than that of culture medium without the protein. To colonize new sites, cells from biofilm must disperse and, as most wild-type biofilms have abundant hyphae, this protein on yeast cells might promote this. *YWP1* expression is upregulated in white cells of the WO-1 strain, where there are distinct microscopic morphological differences between the white and opaque yeast cells (transcriptional profiling [193]). This suggests that the role of Ywp1p is more important in white cells. The expression of this gene is also regulated under other conditions (Table 2). One transcription regulator displays a complex regulation. *YWP1* expression is dependent on *EFG1* as well as downregulated in both an *EFG1* null strain and an *EFG1*-overexpressing strain, perhaps through both the activation and the repression of *YWP1* (83, 356).

Ecm33p. Ecm33p is a GPI-CWP (Table 2). *ECM33* is a member of a two-gene family that includes *ECM331*. *ECM33* contains an intron (231). Genes involved in splicing, translation, and mitochondrial respiration are overrepresented among genes with introns (244). *ECM33* is not part of this bias representation, as it encodes a CWP. The deletion of this gene affects both yeast cell and hyphal morphology. On solid medium, *ecm33*Δ/Δ cells seemed to be rounder and larger than in liquid media, with this appearance being even more pronounced in stationary phase. However, the period of growth appeared to be 24 h, which is less time than in a more rigorous

description of stationary phase (390). As normal cells progress into stationary phase, they become smaller. This *ecm33*Δ/Δ strain may also have abnormalities after an additional growth period to reach and maintain stationary-phase status. The doubling time of the mutant strain was greater than that of the parental strain (232). In the *ecm33*Δ/Δ cells, filamentation was delayed in YEPD with serum, and fewer hyphal organisms were produced, with the width of those filaments being greater than that seen for the parental strain (231). In wild-type strains, the more rapidly growing cells are larger than the slower-growing cells (48). In addition, germ tubes are initiated more rapidly from large cells than from small cells (50). Comparison of the *ecm33*Δ/Δ cells with wild-type cells suggests that there is a disregulation in cell size. Cells of the *als1*Δ/Δ strain also have delayed filamentation, but the yeast cells are also smaller (144, 413), and the size relationships with wild-type cells appear to be retained in this mutant. Calcofluor white staining of *ecm33*Δ/Δ cells showed an alteration, with much of the reactive material appearing as an aggregate (231). Both *ECM33*/*ecm33*Δ and *ecm33*Δ/Δ cells had a tendency to flocculate in a medium-dependent manner, as this flocculation was observed in YEPD and not in yeast nitrogen base. This may represent a protein interaction or a loss of interaction with another medium-dependent CWP. For example, *PIR1* is not expressed in YEPD but is in yeast nitrogen base (166, 229a). The gene dosage effect was also seen for the heterozygous and homozygous deletion strains in sensitivities to Calcofluor white, Congo red, and hygromycin B. These observations implicate Ecm33p in cell wall integrity and shape.

Electron microscopic imaging of the *ecm33*Δ/Δ and *ECM33*/*ecm33*Δ strains and comparison to wild-type and complemented strains showed differences (232). Both the heterozygous, complemented strain with one allele and the null strain showed differences. Strains with one wild-type allele showed an abnormally wide and electron-dense outer layer in comparison to the wild type (Fig. 1 shows examples of the wild type). In the *ecm33*Δ/Δ strain, not only was the outer layer unusually electron dense but the inner electron translucent layer was wider and less electron dense in comparison to the wild type. Additional evidence of aberrant wall structure was obtained with the reaction of a MAb to the N terminus of Als1p. In the wild-type hyphae, Als1p was localized to the region adjacent to the mother cell. In a heterozygous strain, the immunofluorescence associated with the binding of the MAb extended further up the developing hyphae, and in the null strain the fluorescence was diffusely observed everywhere on the hyphae except the tip.

The slow growth of the *ecm33*Δ/Δ yeast cells makes it difficult to interpret the reduced virulence of these cells in the murine systemic infection model (231). In vitro models where growth is not expected to be as critical were examined. When added to HUVECs and FaDu cells, the null strain rapidly germinated and produced hyphae of normal length. The conditions appear to affect the germination of the null strain, since in liquid and solid media, as discussed above, filamentation was delayed. The number of organisms adhered to the two cell surfaces decreased as a wild-type allele was deleted such that deletion strains had the least adherence and heterozygous strains had intermediate adherence. The same gene dosage effect was noticed when endocytosis and HUVEC and FaDu cell damage were examined. These observations support a contribution of Ecm33p to adherence and host cell interactions. However, the aberrant architecture of the cell wall raises the questions of whether the effect is a direct one.

Pra1p. The binding of fibrinogen was particularly well characterized when studies began on host proteins that could serve as ligands for *C. albicans* surface receptors (see the review in reference 49). Briefly, as a fibrinogen binding protein, Pra1p was found on both yeast cell and hyphal surfaces (44) and in vivo and in vitro (218). The protein was ubiquitinated (345). The binding of fibrinogen and antibody to Pra1p was heterogeneous, with a patchy distribution on the surface (229b). This suggests that at least Pra1p and perhaps others are asymmetrically exposed at the cell surface. Whether the pattern of surface exposure reflects the distribution of any nonexposed protein is unknown. The gene encoding Pra1p was identified both through its fibrinogen binding property (alias *FBP1* [4]) and by its being a pH-regulated antigen (344). *PRA1* expression was regulated in response not only to pH but also to medium (Northern analysis [4, 344]). Expression was found in yeast cells and germ tubes in Lee medium and tissue culture medium 199 at 25°C and 37°C. However, expression was not detected at either temperature when organisms were grown in YEPD medium. Also, transcript was not abundant immediately following inoculation into fresh medium, despite cell growth (Northern analysis [344]). Transcriptional profiling subsequently showed that the pH regulation is mediated through Rim101p (transcription profiling, RT-PCR [311]). Recently, Pra1p was found among proteins biotinylated at the hyphal surface but not at the yeast cell surface (391). However, yeast cells were grown in YEPD, where previous studies would predict that *PRA1* would not be expressed. Pra1p contains a signal sequence for classical secretion.

The strain with both alleles deleted is viable (344). The general growth characteristics of the strain were normal. The *pra1*Δ/Δ strain formed germ tubes at 37°C but not at 42°C. At the higher temperature, the chitin distribution was altered. These latter two characteristics suggest a contribution to the cell wall but are clearly milder than the effects of other genes when deleted. *PRA1* has not been reported among the genes regulated by morphogenesis in transcription profiling studies, e.g., general morphogenesis (264) and regulation by Nrg1p (259). However, in addition to the regulation of expression by pH and medium noted above, *PRA1* expression rapidly decreases when cells are transferred into blood (transcription profiling, RT-PCR [111]). Response to estrogen compounds supports additional regulation (transcriptional profiling [56]). The abundance of *PRA1* transcript decreased in response to exposure to 17-β-estradiol by over twofold in a strain lacking *CDR1* and *CDR2*, encoding drug efflux pumps. Only a small number of genes responded to estrogen compound exposure in the three strains tested. The changes were confirmed by RT-PCR.

In a proteomic study, three of six spots that were more abundant in a silver-stained gel of hyphal whole-cell lysates were identified as Pra1p (58). Two of the spots were identified as Phr1p. A similar comparison between the wild type and the *pra1*Δ/Δ strain detected the absence of Pra1p only in the mutant strain. Surprisingly, when similar profiling was performed on the *phr1*Δ/Δ strain, not only was Phr1p missing but Pra1p

was decreased in abundance relative to what was seen for the wild type. Northern analysis of *PRA1* showed no difference in expression in yeast and hyphal cells and the absence of the transcript in the *pra1*Δ/Δ strain but not the *phr1*Δ/Δ strain. These observations support a possible posttranscriptional regulation in hyphae and *phr1*Δ/Δ cells. The extent to which extracts and separations are replicated may affect the detection of differential protein abundance, as may the presence of multiple species, not all of which may be identified and therefore considered. The more recent studies of Pra1p have focused not on adherence properties but on antigenic properties and potential as a vaccine candidate rather than as an adhesin (see references 397 and 398, for example).

Recently, Pra1p has been identified as a ligand for a host integrin receptor, $\alpha_M\beta_2$ (CD11b/CD18, Mac-1) (357). $\alpha_M\beta_2$ is the major receptor on PMNs. This receptor is the PMN partner for binding with *C. albicans* germ tubes, and it mediates the migration of leukocytes to infection sites and the subsequent PMN interaction with the fungal cell (106–109). Supernatants of 2- to 3-day cultures were the starting material to identify the *C. albicans* ligand. Supernatant immobilized in wells of a tissue culture plate supported the adherence of THP-1 cells and their migration through filters in a transwell migration assay. The supernatant activity increased over several days of fungal growth. The addition of antibodies to either the integrin subunit or a high-affinity ligand of the integrin blocked activity. The activity of the supernatant was sensitive to proteases but not to other hydrolytic enzymes. Hybridomas prepared from mice immunized with culture supernatant were tested for the ability of secreted MAb to block the activity of the supernatant. The antigen recognized by the most potent MAb was obtained by affinity chromatography with immobilized MAb. The isolated material was a large complex that dissociated after heating at or above 65°C to give several bands detected by staining a SDS-polyacrylamide gel electrophoresis gel. Western blotting with the MAb identified a single band of 52 kDa in the heated sample and with the large complex (~250 kDa) in the unheated sample. The protein in the reactive bands was digested with trypsin and the peptides were identified by mass spectrometry. The smaller, 52-kDa band yielded peptides only from the Pra1p sequence. The 250-kDa band yielded peptides from the Pra1p sequence as well as Mp65p and Hyr1p. A second method to identify the fungal ligand utilized affinity chromatography with recombinant purified $\alpha_M\beta_2$. Conventional N-terminal sequencing as well as mass spectrometry of the bound protein again identified Pra1p. In the affinity purifications, the eluted material supported the adherence of THP-1, while the column pass-through material was depleted compared to the starting material. The supernatant obtained from the *PRA1* null strain failed to support the adherence or migration of THP-1 cells. The role of Pra1p in PMN killing and phagocytosis was further investigated by testing the ability of human PMNs to kill and phagocytose cells of either the parental strain or the mutant strain. After 5 h of coincubation, 52% of the wild-type *C. albicans* cells were viable, compared to 90% viability for the mutant. The addition of antibodies to the $\alpha_M\beta_2$ receptor blocked the killing of wild-type cells. Purified Pra1p also protected wild-type cells from killing. The release of Pra1p from fungal cells provides an evasion from PMN action by blocking adherence to the hyphae, which is required for killing.

In addition to the above-described study, an earlier study also found Pra1p to be part of a larger complex, but the other components were not identified (217). The identities of the complex components are interesting with respect to the nature of their association when in the cell wall. As noted above, Pra1p has a signal sequence for secretion and Hyr1p is a GPI-CWP (Table 2). The other component, Mp65p, may be held in the cell wall by as-yet-uncharacterized alkali-labile linkage, as described earlier for this protein. Along with the fibrinogen binding activity, Pra1p contributes in several ways to the interaction between fungus and host.

Csh1p. Cell surface hydrophobicity has been implicated as a contributor to adherence to host ECM and cells and resistance to macrophage killing (reviewed in references 49 and 135). This could be a factor in the high virulence of hydrophobic cells compared to that of hydrophilic cells in mice. MAbs to cell surface hydrophobic protein inhibited the adherence of cells to fibronectin and laminin (237). Pretreatment of hydrophobic cells with MAb also reduced adherence to endothelial cell monolayers (125). These studies implicated the protein recognized by MAb and perhaps the hydrophobic properties in the adherence of ligands and cells. The acid-released fraction of mannan from hydrophobic cells was longer and more abundant than that from hydrophilic cells (236), and hydrophobicity was independent of serotype A or B (235).

A scheme to identify the protein and encoding gene employed antibody that recognized the hydrophobic protein and peptide sequences of the isolated protein (352). The identification of *CSH1*, which encodes a predicted protein of 38 kDa, was the result. The predicted protein sequence lacked an N-terminal secretion signal suggesting cytoplasmic localization and was a protein of the aldo-keto reductase family. Several other sequences in the genome had similarity to *CSH1*. The deletion of *CSH1* decreased cell surface hydrophobicity about 75% and decreased adherence to fibronectin. By Western blotting, a 38-kDa protein was missing from the whole-cell lysate of the mutant strain separated in one dimension. The protein is apparently not glycosylated but must influence the acid-labile mannan composition, as there are differences between hydrophobic and hydrophilic cells, as noted above. Western blot analysis of a two-dimensional separation showed that the major activity observed in the parental strain was lost in the mutant strain (351). There was a minor cross-reactivity unaffected by the deletion of *CSH1*. In a strain in which one copy of *CSH1* was reintegrated, the expression of Csh1p increased with increasing temperature between 23°C and 37°C. Interestingly, by Northern analysis *CSH1* expression was greater at the lower temperature. Thus, there appeared to be an inverse relationship between transcript and protein abundance. Maximum *CSH1* expression occurred at 11 h in actively growing cells at 37°C but later (26 h) in a culture growing at 23°C. Western blotting of extracts of proteins biotinylated on the intact cell, proteins released by limited glucan digestion of intact cells, and cytoplasmic lysate showed that most of the 38-kDa protein was in the cytoplasm. However, it was present in the cell wall extracts and the amount of protein in the cell surface extracts

was increased in extracts of hydrophobic cells compared to what was seen for hydrophilic cells.

During frozen storage, the null strain reacquired cell surface hydrophobicity levels similar to those of the parent strain (350). However, the 38-kDa protein was absent from the cell extract, and thus the increase in hydrophobicity is associated with an unknown mechanism. The strain also showed an increase in binding to fibronectin that supports multiple adhesins for this ligand. The strain lacking *CSH1* and strain in which one copy of *CSH1* was reintegrated showed similar hydrophobicities. In a murine model of disseminated infection, both strains showed similar levels of virulence, although the deleted strain had a small increase in mean survival. In a murine vaginitis model, the strains were similar.

A study undertaken to study the variation in biofilm formation with multilocus genotypes isolated from different sources found little correlation (207). However, there was a positive correlation between the cell surface hydrophobicity of the inoculum and biofilm formation. *CSH1* expression decreased in biofilm treated with farnesol (transcription profiling, RT-PCR [42]). Cell surface hydrophobicity decreased with increasing farnesol concentration added for biofilm formation. These two studies suggest that cell surface hydrophobicity contributes to biofilm formation and further links *CSH1* expression with that phenotype. Under oxidative stress conditions, expression increased compared to what was seen for unstressed organisms (transcription profiling [93]). Three studies find increased expression in response to inhibitors or drug resistance. Expression was greater in ketoconazole-treated cells than in untreated cells (transcription profiling [210]). In benomyl-treated cells in which *MDR1* (encoding a multidrug efflux pump) expression increases, *CSH1* expression increased. Expression was similarly increased more than 100-fold in a strain with upregulated *MDR1* (transcript profiling [171]). In a series of clinical isolates with stepwise acquisition of fluconazole resistance, *CSH1* expression was increased in expression coordinately with *MDR1* (transcription profiling, RT-PCR [318]). These changes may be related to the putative cytoplasmic function as an aldo-keto reductase. Whether these challenges also result in changes to surface hydrophobicity is untested.

Sap protein family. In addition to their role as proteinases discussed previously, an adherence role has been proposed for Saps (see the review in reference 262). Adherence to three substrates was examined (403). Poly-L-lysine and ECM obtained from Engelbreth-Holm-Swarm cells were immobilized on glass slides. Adherence to BECs was also examined. The adherence of strains defective in one or more *SAP* genes to these substrates was tested. The observations supported a generally modest contribution to adherence. Galactose-grown cells adhered more than glucose-grown cells. Preincubation of cells with pepstatin A inhibited adherence to poly-L-lysine more than that to ECM. The differences with what was seen for mutant strains were generally small: strains lacking *SAP1*, *SAP3*, or *SAP4* to *SAP6* grown on glucose but not galactose adhered less to poly-L-lysine than did parental cells. The loss of *SAP2* had no effect for growth on either sugar. On ECM, only the *sap3*Δ/Δ strain showed a small reduction in adherence. Adherence to BECs was less for the glucose-grown strains lacking *SAP3* or *SAP4* to *SAP6* and for galactose-grown cells for all mutants.

Mp65p. A 65-kDa mannoprotein present in the cell wall and in culture supernatant has been studied extensively as an important target for host defense. It is a putative β-glucanase that was described in an earlier section ("Cell Wall-Localized Proteins"). The sequence contains an RGD motif that has been implicated in a variety of adhesins (197a). Recently, the adherence properties of this protein have been examined (325). Although yeast cells of a null and wild-type strain did not differ in generation time or size, cells of the null mutant did not form germ tubes. Cells of the mutant strain adhered less well to polystyrene than did the wild-type cells. Cells of heterozygous strains (including a null strain with one allele replaced) had an intermediate adherence level. Antibody to recombinant Mp65p inhibited the binding of wild-type yeast cells to plastic. This observation suggested that the loss of Mp65p and not failure to make germ tubes contributed to reduced plastic adherence. Human variable heavy- and light-chain domains lacking the Fc region that recognized Mp65p were generated from a phage library (71). This antibody construct inhibited the binding of *C. albicans* wild-type cells to epithelial cells of rat vagina. These recent studies support that Mp65p not only is a major target for cell-mediated immune response but also has adhesive properties. Whether the protein has the putative β-glucanase enzymatic function is unknown.

Hsp70 protein family. Histatins are small histidine-rich salivary peptides with potent antifungal activity. Over the last decade, a clear picture has been emerging of how these peptides interact with and kill *C. albicans*. Histatin 5 binding to *C. albicans* is saturable (91). A binding protein of about 70 kDa was identified from surface extracts of *C. albicans* as well as sensitive *S. cerevisiae*. The binding protein was isolated and identified as Hsp70 (208). Hsp70 proteins have been demonstrated on the cell surface (Table 4) (reviewed in reference 49). However, unlike the adhesins discussed above with the exception of Csh1p, Hsp70 proteins lack a signal for classical secretion. The *S. cerevisiae* parental strain and the *ssa1 ssa2* mutant (missing two genes of stress-70 subfamily A) strains provided further confirmation, as the mutant strain was much less sensitive to histatin 5. Recently, both pulldown assays and yeast two-hybrid assays showed greater interaction between histatin 5 and *C. albicans* Ssa2p than between histatin 5 and Hsp70p (alias Ssa1p) (209). *C. albicans* mutants with deletion of either *SSA2* or *HSP70* showed normal growth and filamentation. However, the *SSA2* null strain was highly resistant to killing, while the *HSP70* null strain was not, suggesting that Ssa2p has the major receptor role. P-113 (amino acids 4 to 16), a peptide fragment that was as active as histatin 5, was inhibited when the salt concentration was increased (319). P-113 lost activity when two lysine residues were replaced by glutamine. This observation suggested that binding involved ionic interaction. The glutamine-substituted peptide binds to the surfaces of both wild-type and *SSA2* null cells at levels higher than those seen for the unsubstituted peptide but is not translocated into the cytoplasm (157). A peptide array of overlapping peptides of Ssa2p showed that P-113 had strong binding to two peptides, while the glutamine-substituted peptide did not bind to the array peptides. Wild-type and mutant cells failed to import the substituted peptide into the cytoplasm, while the wild-type cells

imported the unsubstituted peptide more rapidly and more extensively than mutant cells. These observations support an ionic interaction between surface protein and histatin 5.

Histatin 5 interaction with *C. albicans* leads to ATP release in a nonlytic manner as a prelude to fungal cell death (184). The ATP itself has a cytotoxic role because the removal of the released ATP can inhibit the killing. When histatin 5 was directly introduced into the fungal cell, bypassing the entry step, it was still capable of inducing the release of ATP and killing *C. albicans* (11). This observation suggested that the signal for killing did not come externally. The loss in cell viability was concomitant with a decrease in cell volume and in the number of G_1-arrested cells (12). As the cell volume decreased, the proportion of unbudded cells increased. RT-PCR analysis of histatin 5-treated cells showed a drop in G_1 cyclin, *CLN1*, and *CLN3* expression levels. Anion channels and ATP release have been implicated in cell volume regulation, and DIDS (4,4′-dithiocyanatostilbene-2,2′-disulfonic acid), a chloride anion-channel-blocking drug, inhibited the histatin 5-associated loss in viability. Potassium and magnesium ions are lost along with ATP, and one route for nonlytic loss may be through potassium channel Tok1p. The deletion of *TOK1* reduced but did not abolish ATP efflux (13). Histatin 5 treatment of *tok1*Δ/Δ cells still produced substantial killing. These observations suggested that Tok1p contributes to but is not the main site for action. *TRK1* encodes the potassium transporter. Attempts to delete the second allele were unsuccessful, suggesting that *TRK1* is an essential gene (14). However, the deletion of one allele resulted in reduced histatin 5 killing, histatin 5 induced ATP release, and the influx of K^+ (measured as $^{86}R^+$) was reduced five- to sevenfold. These observations suggest that Trk1p is the mediator of ATP loss.

Very recently, the Edgerton laboratory reported that transcriptional profiling of histatin 5-treated *C. albicans* showed that the major response was in genes involved in adaptation to osmotic stress (400). Activation of the HOG pathway was shown. Prestressed (sorbitol-treated) cells were hypersensitive to histatin 5, while concurrent stress did not change sensitivity. Oxidative prestress did not alter histatin 5 sensitivity. This suggests a common response to osmotic stress and to histatin 5.

Other small peptides of the oral cavity may use the same pathway as histatin 5 in their antimicrobial action. Human neutrophil defensin 1 is active at a concentration similar to the active concentration of histatin 5 and also induces nonlytic efflux of ATP (90). The binding protein for histatin 5 was identified by a blot overlay method. *C. albicans* proteins were electrophoretically separated and transferred to a membrane for blotting with labeled histatin 5. Preincubation of the blot with neutrophil defensin 1 or unlabeled histatin 5 abrogated the binding of labeled histatin 5. This observation suggested that the two peptides shared a binding protein on the fungal surface. When neutrophil defensin 1 was incubated with wild-type, *hsp70*Δ/Δ, and *ssa2*Δ/Δ strains, the mutant strains remained susceptible to killing (401). This observation is at odds with the first observation of competition for the binding site. The binding protein for neutrophil defensin 1 remains unresolved. When human β-defensin 2 was incubated with the three strains, at low peptide concentrations (2 to 10 μM) there was reduced susceptibility of both mutant strains, but at 12 μM the killing was about 90% of that observed with the wild-type strain. When β-defensin-3 was incubated with the strains, there was again reduced susceptibility with both mutant strains, which was particularly marked in the case of the *hsp70*Δ/Δ strain. When these three peptides were tested with the parental and *TRK1*/*trk1*Δ strains, there was no difference. These results suggest that β-defensin-2 and β-defensin-3 may share binding proteins with histatin 5 but that the subsequent mechanism of killing follows a different path(s).

ECM Proteins as Ligands

The implications of and interest in host ECM proteins as adherence targets of *C. albicans* were largely due to the work of Klotz and colleagues published about 20 years ago (reviewed in reference 49). Epithelium and endothelium are in contact with a thin (40- to 120-nm) basal lamina (basement membrane) that separates monolayers from underlying connective tissue, which also contains ECM. Basal lamina also surrounds some cells, e.g., individual muscle cells, and can vary from tissue to tissue (see the review and summary information in references 2 and 199). Damage to epithelium or endothelium can expose the underlying basal lamina, and this can be a site for arresting *C. albicans* though binding interactions. For example, damage to endothelium may provide a site at which the fungus can escape the vascular system and disseminate. The ubiquitous presence of ECM makes its components attractive targets for adherence. Several studies that focused on the group of ECM proteins rather than on a single constituent are discussed first. In the decade following the last review, no subsequent studies have addressed interaction with entactin (49). Binding to individual components collagen, vitronectin, fibronectin, laminin, and tenascin-C are considered separately.

If the binding of *C. albicans* to host cells is via specific ligands, the reduction or modification of those host cell surface ligands would be predicted to reduce *C. albicans* adherence. Blocking laminin, fibronectin, collagen type 1, and collagen type IV on the surfaces of Hep-2 cells with MAbs reduced the binding of the fungus (63). The incorporation of galactosamine into the adherence assay also reduced binding. The combination of galactosamine and anti-collagen type IV MAb reduced adherence about 70%. This suggests multiple binding interactions potentially involving both protein-protein and protein-carbohydrate interactions. Another study examined the adherences of four *C. albicans* strains to uncoated polystyrene wells, ECM, and fibronectin in the presence of IgG-class antibody (314). IgG was purified from rabbit serum prior to and after immunization with *C. albicans* cytoplasmic extract (preimmune/nonspecific and specific, respectively). Yeast cells were incubated with either IgG preparation before adherence. Both preparations significantly reduced adherence to all three surfaces, but there was rarely any difference between specific and nonspecific IgG. Numerous other studies that have tested antibodies showed that they did not appear to bind to the surface, thus suggesting that conditions in other studies did not promote nonspecific antibody binding (see the examples in reference 49).

A more recent study examined the ability of *C. albicans* to invade or cross an ECM barrier (333). A layer of ECM was prepared on top of a porous filter. Yeast cells were inoculated on the top of the ECM barrier and incubated overnight. Gentle

washing and swabbing removed unadhered yeast cells. The top and bottom of the filter were then examined for organisms by scanning electron microscopy. The parental strain showed hyphae on both surfaces. However, the *efg1*Δ/Δ strain, which does not form filaments, showed only a few cells on the bottom surface. The defect may be in adherence with only cells that adhere having a potential to transverse the gel and filter. Organisms of the mutant strain showed reduced adherence to immobilized fibrinogen and ECM components, fibronectin, tenascin, and laminin compared to what was seen for the parental strain. Proteins extracted from the surface with βME were analyzed by electrophoresis. Thirty proteins that were differentially abundant were identified (Table 4). None of these proteins was implicated in the apparent difference in adherence.

Collagen. *C. albicans* adheres to type I, denatured type I (gelatin), and type IV collagen to different extents (reviewed in reference 49). Competition with fibronectin, various peptides, heparin, and dextran sulfate reduced cell adherence. However, dextran sulfate may bind to collagen rather than to the fungal cell. Collagen affinity chromatography yielded two proteins as potential adhesins. Collagen IV monomers have three domains; an N-terminal 7S domain (7S), a central collagenous triple alpha helix domain (CC), and a noncollagenous C-terminal domain (NC1). Binding to the three domains of collagen IV was examined further (6). Binding to immobilized collagen IV was greater than that to laminin or fibronectin, and the presence of divalent cations enhanced binding. Binding to the 7S domain, NC1, or CC was cation dependent, partially dependent, or independent, respectively. Levels of adherence to the 7S and CC domains were similar and somewhat greater than that to NC1. Enzymatic removal of N-linked oligosaccharides from the 7S domain reduced adherence, and this along with cation dependence suggested a lectin-like *C. albicans* adhesin. Addition of glucose, galactose, lactose, or heparin sulfate resulted in no reduction of binding. However, *N*-acetyllactosamine followed by *N*-acetylglucosamine, methylmannoside, and L-fucose, sugars known to be in the N-linked oligosaccharide, inhibited binding up to 50%. Trypsin treatment of yeast cells reduced binding. The putative protein(s) mediating adherence was not identified.

A second study examined adherence to collagen type I and gelatin immobilized on nitrocellulose filters and placed in polystyrene wells for assay (226). *C. albicans* adhered to both ligands. Preincubation with either of two peptides containing RGD sequences but not the peptide in which E was substituted for D inhibited binding to gelatin. There was no effect on binding to collagen I. Thus, it appeared that *C. albicans* recognized RGD in the denatured but not in the native collagen. Further, either one or more adhesins recognize more than one ligand sequence, or else there are multiple collagen adhesins.

Vitronectin. Adherence to vitronectin was reviewed previously (49). Briefly, both carbohydrate β-glucan and protein were potential adhesins for this constituent of vascular wall and serum. Far-Western (ligand affinity) blotting identified a 30-kDa protein. Since integrins (αβ heterodimers) recognize host ECM ligands, frequently involving an RGD binding motif, there was considerable speculation that *C. albicans* adhesins would share some integrin characteristics (see the review of integrins in reference 222). Since the last review (49), that notion guided subsequent studies of adherence to vitronectin. MAbs and polyclonal antibodies recognizing human α_v, β_3, β_5, $\alpha_v\beta_3$, or $\alpha_v\beta_5$ were used (361). MAbs to both subunits and complexes bound to *C. albicans*, as determined by flow cytometry. Immunoprecipitation of yeast cell lysate with antisera to either α or β_3 subunits yielded the same 130-, 110-, and 100-kDa proteins. This is the same size range as for human integrin subunits. The antibody to β_3 precipitated a dominant 84-kDa protein along with 130- and 100-kDa proteins. Antibodies, particularly the combination of antibodies to $\alpha_v\beta_3$ or $\alpha_v\beta_5$, blocked fungal adherence to immobilized vitronectin, as did RGD-containing peptides. Antibodies also blocked adherence to an endothelial cell line expressing surface vitronectin. Similar experiments were performed on germ tubes (329). MAbs to the α subunit or complexes with either β subunit stained organisms. Organisms reactive with antibody to $\alpha_v\beta_3$ increased, while organisms reactive with antibody to $\alpha_v\beta_5$ decreased. About 65% of the germ tubes adhered to vitronectin, with the extent of adherence affected by the presence of divalent cations. Antibody to $\alpha_v\beta_3$ and its subunits but not antibody to $\alpha_v\beta_5$ inhibited binding. Antibodies, heparin, or RGD-containing peptide reduced adherence to an endothelial cell line, and their combination abolished binding. The adherence of germ tubes differed from what was seen for yeast cells by the apparent absence of a contribution to binding of moiety(ies) reactive with antibody to $\alpha_v\beta_5$. Such observations continue to support the notion of shared function or structure between mammalian cells and fungus. However, the binding proteins have not been identified to permit more than a superficial understanding of the relationship.

When mammalian integrins bind ligands, outside-in signaling can be initiated by receptor clustering and with involvement of kinases, prominently including FAK (focal adhesion kinase) (reviewed in references 222 and 348). Tyrosine kinase inhibitors, e.g., genistein, inhibited yeast cell binding to vitronectin and an endothelial cell line (328). Immunoprecipitation and Western blotting with antibody to mammalian FAK revealed a 105-kDa cytoplasmic protein band that is similar in size to mammalian FAK. Confocal microscopy of permeabilized yeast cells adhered to vitronectin showed that protein reacting with anti-FAK antibody was in the membrane and colocalized with protein reactive with anti-α_v, -β_3, or -β_5 antiserum. The protein immunoprecipitated with anti-FAK antibody from cells bound to vitronectin or endothelial cells, compared to unbound cells, showed a high level of reactivity with anti-phosphotyrosine, suggesting phosphorylation and putative activation upon binding to vitronectin. Binding to fibrinogen, BSA, or poly-L-lysine did not result in a similar change. None of the reactive proteins was identified. In this area, the studies have not progressed to the identification of putative proteins participating in the binding.

In another study, antibody to the human fibronectin integrin receptor, $\alpha_5\beta_1$, was used to screen yeast cell and germ tube cDNA libraries (182). The reactive clones had greater activity with the anti-vitronectin receptor ($\alpha_v\beta_3$) antibody but did not react with antibodies to the human iC3b receptor ($\alpha_M\beta_2$) and did not hybridize in Southern analysis with the fibronectin receptor probe. In vitro protein expression from some clones yielded a 37-kDa protein that could be precipitated with appropriate antisera. The nucleotide sequence was that of *ADH1*

(Orf19.3997). Western blot analysis of a detergent-based cell wall extract with anti-fibronectin receptor and anti-vitronectin receptor antibody showed a reactive 37-kDa protein similar to *S. cerevisiae* Adh1p, which was used as a control. However, there was reactivity with multiple bands, with prominent bands being larger or smaller depending on the antibody. In particular, anti-fibronectin receptor antibody detected larger species near the top of the gel. A 37-kDa reactive band was also detected in culture supernatant. There are differences in the sources of extracts and proteins for Western blotting between this and a previous study of the vitronectin receptor (361). These and previous studies reviewed elsewhere (49) consistently show antigenic relatedness between *C. albicans* proteins and human integrin proteins.

Fibronectin. Fibronectin is a large dimeric glycoprotein that circulates in plasma as well as being part of the host ECM. Fibronectin as a ligand for *C. albicans* adhesins was the focus of many studies in the 1990s (reviewed in reference 49), in contrast to what has been the case more recently. Multiple protein species were identified by various means of monitoring interactions with fibronectin. Some studies reported that RGD peptides inhibited fibronectin binding to *C. albicans*, while other studies did not. As discussed for vitronectin, the notion that *C. albicans* adhesins for ECM may be related to human integrins underlay a strategy to clone a gene encoding a protein that reacted with anti-fibronectin integrin receptor antibody (182). As noted above, the cloned sequence was homologous with *ADH1*. The encoded protein as well as authentic alcohol dehydrogenase reacted with antibodies to human fibronectin and vitronectin receptors.

Among the receptors for fibronectin previously reviewed was a low-affinity receptor induced by the addition of hemoglobin to a defined medium (49). The induction of this activity greatly increased the binding of soluble fibronectin. The binding was saturable, with a dissociation constant (K_d) of 2.7×10^{-8} M. The yeast cells grown in hemoglobin also had increased binding to immobilized fibronectin and bovine corneal endothelial cells. The researchers went on to demonstrate that the receptor induced by growth in the presence of hemoglobin was promiscuous (407). It was also a receptor for soluble laminin, fibrinogen, and collagen type IV but not collagen type I. A 55-kDa protein present in DTT and lyticase extracts of the cell surface was identified as the binding protein. The binding to fibronectin was through the fibronectin cell binding domain (408). A C-terminal portion of the cell binding domain containing an RGD sequence was an effective inhibitor with or without the RGD sequence. The binding of the cells grown in hemoglobin was enhanced to immobilized fibronectin and fragments containing the cell binding domain. The 55-kDa protein has not been identified.

As reviewed previously (49), Negre et al. (265) reported a high-affinity fibronectin receptor ($K_d = 1.3 \times 10^{-9}$ M) detected on the surfaces of organisms grown in complex media as well as a low-affinity receptor. The ligand for the high-affinity receptor was in the collagen binding domain of fibronectin. Although other peptides inhibited the binding of soluble fibronectin, they were much less effective. Maximal binding to immobilized fragments was observed with the gelatin/collagen binding domain. Pendrak et al. (293) examined the effect of caspofungin on the induction of the high-affinity fibronectin receptor. Yeast cells were grown in defined medium with or without subinhibitory levels of caspofungin. Cells grown in caspofungin alone showed high binding of soluble fibronectin compared to control cells, although binding was less than that of cells grown with hemoglobin. The maximum caspofungin effect was at doses at which there was also development of misshapen cells. A lower dose of 10 ng/ml, at which there were no obvious morphological or growth defects, was used for subsequent experiments. Nikkomycin, a chitin biosynthesis inhibitor, also induced enhanced fibronectin binding but to a lesser extent than caspofungin. Fluconazole had no effect. The binding constant for soluble fibronectin to caspofungin-treated cells was about 1 nM, the same as previously reported for the high-affinity receptor. The binding constant was similar for the nikkomycin-treated cells, but the receptor surface density was less. Competition with the soluble fibronectin for binding occurred with the collagen binding domain fragment but not with the cell binding domain fragment. Cells from which proteins had been sheared in a blender had reduced fibronectin binding capacity. Proteins sheared from the cell were bound to polystyrene wells, and the binding of fibronectin to these immobilized proteins was greater for the fraction recovered from caspofungin-treated cells than for what was seen for untreated controls. The binding activity was concentrated in a fraction of a molecular weight greater than 500,000. Treatment with trypsin and DTT and to a lesser extent with β-1,3-glucanase reduced binding to the complex. In a *KRE9* null strain lacking β-1,6-glucan, there was no effect of caspofungin on the induction of fibronectin binding. Also, in a heterozygous strain in which one allele of the essential *HBR1* (*h*emoglo*b*in *r*esponse) gene was deleted, the induction of fibronectin binding by caspofungin was absent. However, the heterozygous deletion had no effect on the induction of the low-affinity receptor by hemoglobin. This study and that of Soustre et al. (358), also discussed elsewhere, report opposite effects of caspofungin treatment on binding to fibronectin. In both studies, organisms were grown in yeast nitrogen base with glucose (2% [111 mM] in one case and 50 mM in the other). There is a large difference in the caspofungin MICs of 625 ng/ml (293) and 8 ng/ml (358) but less difference in the amounts of caspofungin used, as the former used 10 ng/ml and the latter used MIC/2. One study used soluble fibronectin (293) and the other immobilized fibronectin (358); however, with the high-affinity receptor, maximal activity was found with the gelatin/collagen binding domain fragment in both soluble and immobilized assays.

Laminin. Adherence to laminin received considerable attention in the previous decade (see the review in reference 49) but has received less attention more recently. Laminin is a large (900-kDa) protein composed of A, B1, and B2 chains. Previously, GAPDH encoded by *TDH3* was identified as an adhesin for laminin, and the surface enzymatic activity was observed. This was confirmed by use of *S. cerevisiae*, where strains lacking either Tdh1p, Tdh2p, or Tdh3p or double mutants lacking Tdh1p and Tdh2p or Tdh2p and Tdh3p had cell wall-associated enzymatic activity (78). The recombinant *Candida* Tdh3p protein was purified and was enzymatically active (396). The purified protein also bound to laminin and fibronectin. The expression of the *C. albicans* Tdh3p on the surface was examined further using a Tdh3p fusion with the internal form of *S. cerevisiae* invertase (76). When the plasmid containing this

construct was transformed into a suc$^-$ strain of *S. cerevisiae*, the transformant was able to grow on sucrose. Further constructs localized the region of Tdh3p required to support the growth of transformants on sucrose to the N-terminal half of the protein. The cell wall-associated ScGAPDH activity increased in response to starvation and temperature increase (77). When a transformant with a *C. albicans* Tdh3-invertase fusion was examined, there was a similar response in terms of an increase in both GAPDH and invertase activity. This increase was not dependent upon either protein synthesis or the ubiquitin stress response. These observations provide additional support for Tdh3p as an adhesin and for its presence on the cell surface.

Tenascin-C. Tenascin is a hexameric protein with subunits generally of around 200 kDa (see the review in reference 147). The protein has repeated structural motifs, typically including 14.5 epidermal growth factor-like repeats, fibronectin type III repeats, and a globular fibrinogen-like domain. Tenascins have tightly regulated expression during the development and lifetime of vertebrates. Tenascins modulate cell-matrix interactions. With a sharing of the various structural motifs present in other host ligands to which *C. albicans* binds, tenascin-C would be predicted to bind to the fungus. Soluble tenascin-C bound to germ tubes but not to yeast cells, as determined by indirect immunofluorescence (214). Cell surface extracts from both yeast cells and germ tubes bound to immobilized tenascin-C. Antibody to either *C. albicans* or tenascin but not to laminin inhibited binding. This observation supported a specific interaction. Inhibition of the binding of fungal proteins to tenascin-C by fibronectin but not fibrinogen implicated the fibronectin type III repeats in binding. The binding of fungal protein was RGD peptide and divalent cation independent. The failure of soluble tenascin-C to bind to yeast cells coupled with the binding of a component(s) of a yeast cell surface extract to immobilized tenascin-C suggests that either the adhesin or the adhesive motif is blocked on the yeast cell surface but exposed at the germ tube surface.

Effect of antifungal drugs on adherence. Several studies in recent years examined the effect of pretreatment with subinhibitory drug concentrations on adherence to various host ligands. Imbert and colleagues (47, 153, 358) examined adherence to uncoated polystyrene, fibrinogen, fibronectin, laminin, gelatin, collagen type IV, and ECM (a mixture of laminin, collagen type IV, entactin, and heparan sulfate proteoglycan) for yeast cells treated with one-fourth the MIC. Treatment with flucytosine had no effect. Amphotericin B, itraconazole, fluconazole, and terbinafine decreased adherence to polystyrene, fibrinogen, and fibronectin. All except amphotericin B also decreased adherence to laminin, gelatin, collagen type IV, and ECM (153). Caspofungin at one-half the MIC decreased adherence to ECM for all six fluconazole-sensitive strains tested (358). However, when strains were fluconazole resistant in vitro, three of five strains showed reduced adherence when treated with the low concentration of caspofungin. Another echinocandin, aminocandin, was studied at one-half the MIC with fibronectin as well as the ECM complex (47). Five of six fluconazole-sensitive strains showed reduced adherence to ECM, and three were less adherent to fibronectin. Five fluconazole-resistant strains all showed reduced adherence to ECM and four strains reduced adherence to fibronectin. The outcome differed when the same fluconazole-resistant strains were tested with caspofungin, as two strains were unaffected. Although the number of strains is small, this suggests a difference between two drugs of the same class on fluconazole-resistant strains. As the mechanism of fluconazole resistance for these strains is unknown, there is a possibility that the fluconazole resistance mechanism may underlie differences in the echinocandin drug effect. The effect of caspofungin treatment of reducing the adherence to fibronectin differed from what was found in the previously discussed study of Pendrak et al. (293), who observed increased binding to fibronectin.

Peptides. Klotz and colleagues have examined the adherence of *C. albicans* and *S. cerevisiae* expressing Als1p and Als5p to beads coated with 7-mer peptides from a peptide library (180). The sequence was determined for five beads that bound *C. albicans* as well as the Als1p- and Als5p-expressing *S. cerevisiae*. All peptides shared common characteristics in which the first residue had a high turn property, e.g., A, D, or P; the second was a bulky hydrophobic or aromatic amino acid, e.g., F, T, or W; and the third was an R or a K. This motif was termed "$\tau\varphi^+$." Scrambling sequences had little effect on *C. albicans* cells but reduced binding of the *S. cerevisiae* strains. Perhaps this difference was due to the redundancy of adhesins on the *C. albicans* surface. When BSA was denatured, exposing several of these motifs, and mixed with *C. albicans* yeast cells, the cells aggregated, while they did not with native BSA. The addition of a 23-mer peptide derived from the cell binding domain of fibronectin inhibited binding to a bead coated with a 7-mer peptide (181). Smaller peptides derived from the sequence were ineffective. This peptide also killed *C. albicans* in a manner independent of the adhesive interaction. The fungicidal activity was inhibited by physiological salt concentrations, as are host microbicidal peptides.

Beads coated with different ECM proteins, fibronectin, type IV collagen, laminin, BSA, or casein supported the binding of *C. albicans* yeast cells, germ tubes, and pseudohyphae (121). Homopolymers composed of 10 serine, threonine, or alanine residues attached to beads supported the binding of the various *C. albicans* forms, while the single amino acids did not. After a few hours, the cells separated from the polyalanine-coated beads but not from other two homopolymers. Urea, formamide, and a pH of 12 inhibited binding to fibronectin- or polythreonine-coated beads. The addition of glucose, galactose, mannose, fucose, 1 M NaCl, EDTA, or Tween 20 had no effect. This suggested that the adhesive interactions with the two ligands were chemically similar. The addition of accessible threonine residues to the C terminus of a nonadhesive peptide stimulated binding.

Serum Proteins as Ligands

The entry of *C. albicans* into the bloodstream directly through catheters or by translocation exposes the organism to proteins and other constituents of blood. It is has been appreciated for many years that serum can induce morphogenesis and this can be incorporated into an identification scheme. Microarray analysis shows that after introduction into blood, *C. albicans* responds with a change in transcription profile (111). Serum proteins can bind to the *C. albicans* surface, and the binding of several of these proteins has been the focus of

many studies (reviewed in reference 49). In particular, the binding of complement fragments C3d and iC3b contributed to the notion that functional similarity might extend to structural similarity of adhesins. In mammals, integrins are adhesins for these proteins, and antibodies to mammalian integrins were utilized as probes for *C. albicans* adhesins. The considerable study of C3d and iC3b binding to *C. albicans* in the 1990s has not continued. The identity of proteins implicated in binding of these two complement fragments remains unknown. Fibrinogen is another serum ligand that bound to cells, and continued studies of this ligand resulted in the identification of one of the adhesins, Pra1p, discussed above. More recently, studies have described the binding capacity for fluid-phase complement system regulators C4b binding protein (C4BP) and factors H and FHL-1. Additionally, other studies report some of the characteristics of a receptor for hemoglobin and adhesins for plasminogen.

C4BP and factors H and FHL-1. In addition to C3d and iC3b, three other complement pathway proteins, C4BP (from the classical and lectin pathways) and factors H and FHL-1 (regulatory components from the alternative pathway), bind to the cell surface (242, 243). The binding of these regulatory factors is a possible mechanism of complement escape by microbes (185, 242, 243, 304). These factors affect the decay of the C3 convertase and factor I-mediated degradation of C3b, thus diminishing complement activation. Factors H and FHL-1 bind to the yeast surface in a patchy pattern (243). A heterogeneous distribution of binding is reported for other ligands such as fibrinogen and laminin (49). Binding of the two factors from EDTA-treated normal human serum was dose dependent and specific, as purified recombinant proteins competed with radiolabeled protein (243). Factors H and FHL-1 contain multiple short complement regulator domains (SCRs), and binding of various recombinant proteins with deleted domains identified a common *C. albicans* binding region within SCRs 6 and 7. A second binding site unique for factor H mapped to SCRs 19 and 20. Factor H has heparin interaction sites localized to SCRs 7, 13, and 20. Heparin inhibited the binding of factor H. The attached factors bound to the yeast cells retained their activity and mediated the factor I-dependent cleavage of C3b.

C4b, a part of the C3 convertase, forms during the activation of the classical and lectin complement pathways. The multimeric C4BP can bind to C4b and accelerate the decay of the enzyme and the factor I-dependent degradation of C4b. C4BP bound to yeast cells and the tips of hyphae in a patchy pattern, as did factors H and FHL-1 (242). The binding was dose dependent and observed with recombinant protein as well as EDTA-treated normal human serum. The observation that unlabeled C4BP, factor FHL-1 at a high concentration, and to a lesser extent factor H inhibited binding of radiolabeled C4BP suggested a common binding site for C4BP and factor FHL-1. C4BP has seven α chains and a single β chain. Binding was mediated by α chains, and the binding was due primarily to ionic interaction. The chains are composed of eight complement control protein (CCP) domains. Binding of recombinant proteins lacking one of the domains to hyphae showed that binding was localized to CCP1 and CCP2 and possibly to CCP6. Like factors H and FHL-1 of the alternative pathway, C4BP retained cofactor activity. Bound C4BP mediated the factor I-dependent cleavage of C4b. Compared to yeast cells treated with buffer alone, yeast cells with bound C4BP were adherent to HUVECs. Thus, the bound C4BP may contribute to host interaction by the inhibition of complement activation and enhanced adherence.

To identify the fungal adhesin for these ligands, the binding of factors H and FHL-1 to a *S. cerevisiae* protein array was determined (304). Three proteins bound both factors and one protein bound only factor H. Phosphoglycerate mutase bound both factors and was selected for further characterization with the *C. albicans* ortholog, Gpm1p. Recombinant Gpm1p (rGmp1p) bound both factors H and FHL-1 but not C4BP. The binding of recombinant factors H and FHL-1, described above, to rGpm1p was determined, and this confirmed that SCRs 6 and 7 are a common region for the two factors and a second and lower-intensity binding region for factor H in SCRs 19 and 20. This confirmed the observations of binding to yeast cells. The factors bound to rGmp1p also had factor I-dependent cleavage of C3b, as they did when bound to intact cells. The presence of Gpm1p on the surfaces of intact yeast cells and hyphae was confirmed by antibody prepared to rGmp1p. Antibody reactivity showed the same pattern of surface distribution as noted for bound factors, with a heterogeneous distribution on yeast cells and at the hyphal tip. A deletion strain of *GPM1* lacked protein reactive with the antibody in both cytoplasmic and cell wall fractions. Compared to cells of the wild-type strain, cells of the deletion strain bound somewhat less factor H or FHL-1. As suggested by the array studies, the presence of multiple adhesins on the surface is the likely explanation for the partial reduction.

Hemoglobin. Initial studies of hemoglobin on the induction of surface changes affecting binding to fibronectin were reviewed previously (49), and a more recent review focused on hemoglobin sensing and fungal response in greater detail than presented here (295). There are two reports describing receptors for hemoglobin (291, 404). Both reports provide evidence for a low-affinity receptor. However, there are some differences between conditions and parameters tested that complicate determining whether the same receptor is described. Pendrak et al. (291) examined receptor characteristics for the induction of fibronectin binding, while Weissman and Kornitzer (404) focused on Fe utilization from hemoglobin and hemin and specific receptors. Pendrak and colleagues went on to follow up on the signaling function (292–295).

As discussed above, cells grown in defined medium with hemoglobin have enhanced binding to fibronectin, laminin, collagen type IV, and fibrinogen mediated by a promiscuous receptor. Fibronectin receptor expression was induced by ferric, ferrous, and cobalt-protoporphyrin derivatives of hemoglobin (291). Since the cobalt-containing molecule was active, the requirement was not for iron. In addition, the internalization of iron from hemoglobin occurred after the detection of enhanced binding capacity. The hemoglobin binding protein haptoglobin inhibited binding, and globin was inactive, thus suggesting that some structural aspect of the globin-porphyrin complex is required. Yeast cells were able to bind to immobilized hemoglobin. Since the induction of fibronectin binding was saturable and dose dependent, the presence of a receptor was suspected. In solution, the binding of hemoglobin, at a K_d of 1.2×10^{-6} M, suggested the presence of a low-affinity

receptor. This receptor has not been further identified. Prior to the demonstration of a low-affinity receptor on yeast cells, an earlier report found that hemoglobin bound to hyphae at levels higher than those to yeast cells (374). Hyphae can use hemoglobin as a source of iron. Whether the binding to hyphal organisms is through the same receptor that is more abundantly expressed on hyphae or via a different receptor is not known. The hemoglobin receptor also appears to have a signaling function. Pendrak et al. (295) reported that hemoglobin induces the expression of other genes, e.g., *HBR1* and *HMX1*. Hbr1p is a suppressor of white-opaque switching (294). As opaque cells are more susceptible to host defenses than are white cells (183), the suppression of switching while in the vascular system may be an advantage for the microbe. More recently, hemoglobin has been identified as an inducer of morphogenesis under conditions of neutral pH and low glucose and ammonium ion (292).

C. albicans has the components for high-affinity iron import but can also use hemoglobin and hemin (oxidized heme). *PGA10* (alias *RBT51*), with homology to *RBT5*, was identified by complementation in *S. cerevisiae* of a strain missing the high-affinity uptake receptor and in the presence of an iron chelator, ferroxine (404) (Table 2). *PGA10* belongs to a family with *RBT5* and *CSA1*, which encode predicted GPI-CWPs with the conserved CFEM domain (312). Rbt5p has been recovered from the cell wall (73), which conflicts with the suggestion of membrane localization (404). Each gene of the family was deleted in a strain missing the *C. albicans* high-affinity iron transporter (Ccc2p) (404). In the mutants, the *RBT5* null strain was the one reduced in growth on hemoglobin in the presence of ferroxine. As monitored by Western blotting, Rbt5p increased in iron-starved cells. It is glycosylated, as indicated by its size, which was much larger than predicted, and a polydisperse band. Glycosylation was confirmed by other methods. Using antibody recognizing Rbt5p and zymolyase-treated cells, reactivity was associated with the cells. The authors suggested a membrane location. ^{55}Fe-hemin bound to *C. albicans* in a saturable manner that was susceptible to competition by unlabeled hemin and hemoglobin. The competition suggested a single receptor class. The receptor has high affinity for hemoglobin and requires about 0.2 μM to compete at a labeled ligand concentration of 1 μM. In iron-starved cells, there are an estimated 3×10^6 receptors, compared to 1.2×10^5 receptors in unstarved cells. Although strains with single deletions of *PGA10* or *CSA1* were not impaired in binding, there was some contribution, as the strain with deletions of all three genes (*RBT5*, *RBT51*, and *CSA1*) bound less hemin than the single-deletion strains.

Although there is little free hemoglobin in serum, *C. albicans* possesses a hemolytic factor (reviewed in reference 49) that has been partially characterized (402). There are also some pathological conditions that can increase availability (reviewed in reference 295). As an iron source, once hemin has entered the cell, the release of Fe from heme is catalyzed by Hmx1p, a cytoplasmic protein (174) whose expression increases upon hemoglobin binding. In addition to the question of whether the CFEM family proteins Rbt5p, Rbt51p, and Csa1p are the receptors for signaling, there are many unanswered questions. Among these questions are how the signal transmits from the adhesin-ligand binding and how heme is released from hemoglobin and imported into the cell.

Plasminogen. *C. albicans* produces extracellular Saps that can hydrolyze host proteins, as discussed briefly above. Some microbes can hijack host proteolytic proteins by binding plasminogen and use the host fibrinolytic system to enhance invasion. Two contemporaneous studies showed that *C. albicans* can bind human plasminogen (65, 163). Yeast cells in rich medium in exponential growth (163) or from a 24-h culture (65) bound soluble plasminogen. Plasminogen bound to immobilized cells in a dose-dependent manner with a K_d of 70 nM, which was in the same range as for other microbes (65). Cells can also bind the activated plasmin from solution (163).

To identify proteins binding plasminogen, Crowe et al. (65) extracted CWPs by treatment of cells with βME and Quantazyme (β1-3 glucanase). Plasminogen bound to the extracted immobilized proteins with a K_d of 112 nM. Proteins extracted from the *S. cerevisiae* and *C. albicans* glycosylation-deficient *och1*Δ/Δ, *mnn4*Δ/Δ, *mnt4*Δ/Δ, *mnt2*Δ/Δ, *mnt3*Δ/Δ, *mnt4*Δ/Δ, and *mnt5*Δ/Δ strains gave similar levels of binding. A lysine analog, ε-aminocaproic acid (εACA), almost completely inhibited binding to proteins. The observation suggested that lysine residues were critical for binding. Far-Western blotting of SDS-polyacrylamide gel electrophoresis-separated proteins indicated five major and several less intensely reacting spots. The addition of εACA during the plasminogen binding step again blocked binding. To identify the reactive moieties, spots identified by far-Western blotting after two-dimensional separation were cut from the gel for sequence analysis. The major plasminogen binding spots were encoded by the genes *GPM1*, *ADH1*, *TSA1*, and *CTA1*. Each of the protein products contain C-terminal lysine residues. Tef1p was a minor binding protein although it has terminal lysine residues, suggesting that the lysine may not be readily exposed. Three other minor binding proteins were Pgk1p, Tdh3p, and Fba1p. These proteins lack signals for classical secretion, and such proteins are the topic of discussion elsewhere in this review (Table 4). Some of the proteins, in addition to having cytoplasmic functions, are associated with other surface functions, such as laminin binding by Tdh3p.

In addition to binding factors H and FHL-1, rGPm1p also bound plasminogen (304), as predicted by the study discussed above. The inhibition of plasminogen binding to rGmp1p by εACA again implicated lysine residues as the binding motif of Gmp1p. Consistent with the observations of Crowe et al. (65) of multiple plasminogen adhesins, the strain with a deletion of *GPM1* showed a reduction but not a complete loss of plasminogen binding capacity (304).

In other microbes, enolase is a surface protein that binds plasminogen. Jong et al. (163) studied a recombinant *C. albicans* enolase expressed in *Escherichia coli*. The recombinant protein was immobilized by a six-histidine tag-bound plasmin. The plasmin was enzymatically active and inhibited by known plasmin inhibitors. The binding was lysine dependent. Since the predicted sequence for Eno1p lacks C-terminal lysine but does have two residues within the last nine positions, it may be a relatively weak binder and not detected among the minor proteins by Crowe et al. (65). Plasminogen bound to a receptor undergoes a conformational change that alters the affinity for activators. The K_m for streptokinase activation of plasminogen

bound to immobilized enolase was about 10% of that of unbound plasminogen (163). This suggests that plasminogen bound to enolase undergoes a conformational change.

The three studies examined the effect of bound plasmin on *C. albicans* interaction with various substrates. In one study, *C. albicans* cells with bound plasminogen showed hydrolysis of a fibrin clot when an activator of plasminogen was present (65). There was no activity from fungal cells in the absence of bound plasminogen. The requirement for an exogenous activator suggested that *C. albicans* does not have an appropriate proteolytic activity at the cell surface. Plasminogen bound to rGpm1p was converted to proteolytically active plasmin in the presence of an activator (304). The hydrolytic activity increased with increasing rGpm1p available to bind plasminogen. A third study used a fibrin matrix gel. *C. albicans* cells with bound plasminogen or plasmin showed hydrolysis, and the activity was abolished by inhibitors (163). There was no activity in the absence of bound plasminogen or plasmin. Thrombin in the gel may have been responsible for activating the bound plasminogen. The effect of bound plasminogen in the presence of an activator to facilitate the transversal of an ECM barrier or to damage an endothelial monolayer was examined (65). No advantage was observed with the plasminogen-coated cells. In contrast, there was an advantage when an in vitro blood-brain barrier system of human brain microvascular endothelial cells was tested (163). *C. albicans* cells with or without bound plasmin were added to the upper chamber containing the endothelial cell monolayer, in which tight junctions had been formed. Of two strains tested, the plasmin-coated cells of a rapidly growing strain had a small advantage (about 20%) in transversal into the bottom chamber over what was seen for uncoated cells. The advantage was larger for plasmin-coated cells of a slower-growing strain that still formed germ tubes, although the overall number of cells that traversed the monolayer was less than half of those for the faster growing strain.

Salivary Proteins as Ligands

The oral cavity is a site of commensal colonization. The organism must be acquired and a stable population established. Organisms are constantly being cleared by the host, and thus to be retained, the organism must have some method of adherence within the mouth. All oral surfaces acquire a pellicle coating containing salivary components. As a commensal resident in the oral cavity, *C. albicans* must adhere to oral surfaces, which are constantly coated with saliva, and oral colonization has been the subject of two reviews (37, 38). Several identified and partially characterized adhesin-ligand interactions have previously been identified between *C. albicans* and BECs (see Table 1 in reference 38). In this review, several adhesins, Hwp1p and Saps (Table 2 and elsewhere), and Als family proteins (Table 5) which can mediate binding to BECs have been noted. Other than for histatin 5, which was discussed in a previous section, the progress in identifying adhesins has been modest since last reviewed (37, 38, 49). In this section, a study that confirms the adherence-promoting properties of saliva is considered and followed by separate sections for specific ligands.

Radiolabeled *C. albicans* yeast cells bound to three human epithelial cell lines: A549, derived from lung pneumocyte type II cells; HEp-2, derived from a laryngeal carcinoma; and HET-1A, originating from simian virus 40 T-antigen-immortalized esophageal cells (140). Adding pooled saliva to the binding assay increased yeast cell adherence, ultimately appearing to saturate binding at 40% saliva, and about 30 to 60% of added cells bound. Several *C. albicans* isolates showed similar behavior. Pretreatment of yeast cells with saliva also promoted adherence compared to what was seen for cells not pretreated, while pretreatment of the epithelial cell monolayers had a minimal effect on increasing adherence. When individual saliva samples were tested, saliva with more *C. albicans*-specific IgA was less effective at promoting adherence, and the removal of IgA increased the ability of the IgA-depleted saliva to promote adherence. These observations suggest that it is the adherence of salivary components to yeast cells and not to epithelial surfaces that promotes adherence.

Salivary bPRPs. Along with histatin and statherin, PRPs are the major salivary polypeptides. bPRPs (basic PRPs) can be either glycosylated or not. *C. albicans* can adhere to saliva-coated hydroxyapatite and to immobilized salivary proteins (39, 283a). bPRPs are major ligands for *C. albicans* adherence (283a). A recent study implicates Bgl2p, discussed previously for its enzymatic activity in the cell wall, as an adhesin for salivary proteins (160). An extract obtained from yeast cells by mild glucanase treatment inhibited binding to salivary proteins in two assays: binding to saliva-coated hydroxyapatite beads and binding to a blot overlay of separated saliva. In the latter assay, bPRPs were the major saliva binding components. Mannoprotein purified by chromatography on concanavalin A-Sepharose was enriched for inhibitory activity and contained two major components of 35 kDa and 97.4 kDa. The inhibitory activity of the proteins eluted from a gel was about 30- to 100-fold more than that of the crude extract. The 97.4-kDa material yielded no identifiable sequence. However, the 35-kDa sequence corresponded with Bgl2p. Bgl2p has the signal sequence for secretion by the classical pathway. In the cell wall, it appears to have two functions, one enzymatic within the *C. albicans* cell wall and one as an adhesin for an abundant protein in the oral environment.

Polymicrobial biofilms form on silicone surfaces of voice prostheses. The components of saliva from oral rinses of patients with voice prostheses that promoted *C. albicans* adherence were examined (141). Salivary components that bound to silicone were eluted, separated by electrophoresis, stained for protein, and transferred onto nitrocellulose filters. Two major salivary proteins of about 40 and 53 kDa were removed from the silicone. A blot overlay assay with radiolabeled yeast cells was employed to identify ligands. In both the initial saliva and eluted saliva, PRPs were ligands. However, in the eluted material, only one PRP bound yeast cells. The major binding was to the 40- and 53-kDa bands. This suggests that bPRPs are not major silicone-bound components and that the two unidentified proteins promote adherence to silicone voice prostheses.

Statherin. The major saliva component statherin is a 43-amino-acid phosphorylated polypeptide. Protein peaks from a chromatographic separation of saliva were bound to hydroxyapatite beads, and the ability of the bound protein to promote adherence was determined. The binding occurred with several fractions identified as glycosylated PRPs and some uncharacterized peaks and primarily to statherin but not to acidic PRPs.

Fuc-α-1,2-Gal-β-1,4-Glc partially inhibited binding to material other than statherin. Purified statherin mediated the dose-dependent binding of *C. albicans* to hydroxyapatite beads (161). Antistatherin antibody inhibited binding to saliva-coated beads and to BECs by 93% and 43%, respectively. The addition of Fuc-α-1,2-Gal-β-1,4-Glc reduced adherence to BECs by 79%. There is some difference between this study and prior studies, including one discussed above, which found that the major ligands of saliva after electrophoretic separation and blot overlay assay of fractionated or unfractionated saliva was to bPRPs (160, 283a). Collectively, however, these studies suggest that multiple ligands and adhesins mediate adherence in the oral cavity. Interestingly, in saliva, PRPs and statherin share the property of inhibiting the spontaneous precipitation of calcium phosphate salts.

BIOFILMS

Biofilm formation is an area that has received much more attention in this decade than in the previous (Table 1). Biofilms have been reviewed frequently, with recent reviews available (24, 188, 212, 255, 267, 275, 395). With this wealth of current sources, biofilms will be discussed only superficially. With the increasing awareness of biofilm contribution to *C. albicans* biology and pathogenesis, biofilm formation is now one of the phenotypes that is frequently examined for mutant strains. This practice has added and will continue to add genes that impact biofilms to those genes identified in direct studies of biofilms. The description of biofilm clearly has a role for proteins in the interactions between cells in the biofilm and the extracellular production of matrix material. A glimpse of the impact of biofilm formation on extracellular proteins emerged from the proteomic analysis of noncovalently attached protein from biofilm cell wall and in culture supernatant (Table 4). In this review, a criterion for the discussion of individual genes required more than the identification of regulatory proteins and conditions. Fourteen proteins that in some way impacted biofilm formation, including proteins whose loss abolished biofilms in vitro and in vivo, were discussed. This confirms that extracellular proteins are important in biofilm formation and maintenance. The corresponding 14 genes are the following: *ALS1*, *ALS3*, *CHT2*, *CHT3*, *CSA1*, *CSH1*, *EAP1*, *MP65*, *PGA10*, *PHO100*, *RBT4*, *SAP9*, *SUN41*, and *YWP1*.

CONCLUDING REMARKS

There is no doubt that the cell wall, with its polysaccharides and proteins, is a dynamic organelle. Under the seemingly placid surface seen through the microscope is a constantly readjusting structure. Indeed, even placing those cells under the coverslip creates changes in local environment, pH, nutrients, and oxygen that initiate changes in the wall. Global transcription and proteomic analyses are beginning to identify the proteins expressed under different conditions. However, since functional analysis through mutant construction is more labor-intensive, the pace at the individual gene level has been slower. Further, when genes are studied, the assumption about their location is not always confirmed. Adhesins for various ligands identified by size in the last decade have still not been associated with a particular protein and gene. The new technologies will not answer all questions. For instance, do all proteins that are implicated in cell wall structure, e.g., by their calcofluor white sensitivity, affect integrity in the same way? Are the secreted enzymes of cell wall metabolism functioning at localized sites when they change in response to environment or do they act around the whole periphery? Several of the transcription factors alter the expression of genes encoding proteins of the cell wall. Do these coordinately regulated proteins work together, or do they have independent functions? The challenge with analysis of adherence is particularly intriguing. How can the deletion of multiple proteins each reduce or ablate adherence? As the unraveling of the mysteries of the cell wall continues, a new challenge to weave the findings into a network rather than isolated proteins and regulatory factors will be developing. There is doubtlessly another decade of adventure ahead.

ACKNOWLEDGMENT

This article is dedicated to the memory of Helen R. Buckley, who first piqued my interest in *C. albicans* and then remained a friend and a source of inspiration, encouragement, and knowledge.

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