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Candida albicans Als3, a Multifunctional Adhesin and Invasin▽

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Candida albicans **is part of the normal human flora, and it grows on mucosal surfaces in healthy individuals. In susceptible hosts, this organism can cause both mucosal and hematogenously disseminated disease. For *C. albicans* to persist in the host and induce disease, it must be able to adhere to biotic and abiotic surfaces, invade host cells, and obtain iron. The *C. albicans* hypha-specific surface protein Als3 is a member of the agglutinin-like sequence (Als) family of proteins and is important in all of these processes. Functioning as an adhesin, Als3 mediates attachment to epithelial cells, endothelial cells, and extracellular matrix proteins. It also plays an important role in biofilm formation on prosthetic surfaces, both alone and in mixed infection with *Streptococcus gordonii*. Als3 is one of two known *C. albicans* invasins. It binds to host cell receptors such as E-cadherin and N-cadherin and thereby induces host cells to endocytose the organism. Als3 also binds to host cell ferritin and enables *C. albicans* to utilize this protein as a source of iron. Because of its multiple functions and its high expression level *in vivo*, Als3 is a promising target for vaccines that induce protective cell-mediated and antibody responses. This review will summarize the multiple functions of this interesting and multifunctional protein.**

Candida spp. are the fourth most common cause of nosocomial bloodstream infections, and *Candida albicans* accounts for approximately 50% of cases of candidemia (20, 67). This organism also causes at least 80% of cases of oropharyngeal and vulvovaginal candidiasis (57, 66). The predominance of *C. albicans* as a cause of both hematogenously disseminated and mucosal disease suggests that this organism possesses unique virulence factors compared to other species of *Candida*. One such factor, which is present only in *C. albicans*, is Als3. This protein plays a key role in multiple processes that are necessary for the organism to colonize the host and cause disease. These processes include adherence to host cells, biofilm formation, host cell invasion, and iron acquisition. Moreover, because Als3 is highly expressed *in vivo*, it is a promising target for therapeutic antibody and vaccine development. This review will discuss Als3 structure and function, as well as its utility as a therapeutic target.

GENETIC AND BIOCHEMICAL CHARACTERISTICS OF Als3

The *ALS* gene family. Als3 is encoded by the *ALS3* gene, which is a member of the agglutinin-like sequence (*ALS*) gene family (23). This family was discovered by Hoyer et al. (24) based on its similarity to *Saccharomyces cerevisiae SAG1*, which codes for α agglutinin. The *ALS* gene family has eight members (*ALS1* to *ALS7* and *ALS9*) (Fig. 1). These genes encode cell surface proteins that are predicted to share the same overall structure (Fig. 2). At the N terminus is a signal peptide followed by a 300-amino-acid immunoglobulin-like domain and a 104-amino-acid threonine-rich domain that contains β-sheets (18, 22, 47, 55). Most Als proteins have adhesin function (18, 55, 72), and the binding domain for most substrates is located in the N terminus (31, 49, 55). The central domain of the Als proteins is composed of a variable number of 36-amino-acid tandem repeats. These repeats are rich in serine and threonine, exposed on the cell surface, and required for adherence function (31, 49). Because they are hydrophobic, the tandem repeats can directly mediate adherence to some substrates, such as polystyrene (16). There is significant strain-to-strain variation in the number of tandem repeats, and most strains possess two different-size alleles that specify different numbers of tandem repeats (24, 40, 71). In the case of Als3, a larger number of tandem repeats is associated with increased adherence when different-size alleles are expressed in *C. albicans* (40) but not when they are expressed in *S. cerevisiae* (35). The C terminus of Als proteins is serine and threonine rich and predicted to be heavily glycosylated. It contains a glycosylphosphatidylinositol anchorage sequence that is cleaved when the protein is covalently linked to the cell wall (18, 26).

Comparative genomic studies have revealed that most pathogenic *Candida* species, including *Candida tropicalis*, *Candida parapsilosis*, and *Candida dubliniensis*, contain multiple orthologs of the *ALS* genes (9, 25). However, none of these genes appear to be close orthologs of *ALS3*. *Saccharomyces* species, which rarely infect humans, do not contain *ALS* orthologs, suggesting that the products of this gene family may be uniquely important for fungal interactions with human cells.

Regulation of *ALS3* expression. Als3 protein expression is regulated primarily at the transcriptional level. *ALS3* is a hypha-specific gene that is expressed by *C. albicans* hyphae and pseudohyphae but not yeast-phase organisms (3, 23). Analysis of the *ALS3* promoter using luciferase reporter constructs reveals that it contains two repression regions (R1 and R2) and two activation regions (A1 and A2) (Fig. 3). The hypha-specific repressors Tup1, Nrg1, and Rfg1 downregulate *ALS3* transcription by binding to the two repression regions. The Efg1 and Cph1 transcription factors, which induce hyphal forma-

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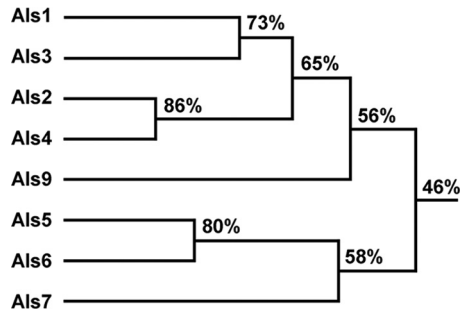

FIG. 1. Sequence homology among members of the Als family of proteins. Numbers indicate percent identity at the amino acid level.

tion, bind to the two activation regions and upregulate *ALS3* transcription. Tec1, another transcription factor that induces hyphal formation, does not activate *ALS3* expression directly but instead functions through the zinc finger transcription factor Bcr1 (3, 37). Recently, Bastidas et al. (5) found that expression of *ALS3* is inhibited under conditions of high nutrient availability. This inhibition occurs mainly through the protein kinase Tor1, which induces the expression of Nrg1 and Tup1 while downregulating expression of Efg1 and Bcr1 (5). *ALS3* is also a target of the Rim101 alkaline response transcription factor (39). However, it is not yet known whether Rim101 binds directly to the *ALS3* promoter or induces the expression of this gene indirectly.

Als3 FUNCTION

Als3 mediates adherence to diverse host substrates. Adherence to host constituents is necessary for *C. albicans* to colonize mucosal surfaces and subsequently cause disease. *C. albicans* possesses multiple adhesins that mediate binding to a variety of different host substrates (reviewed in reference 64). Many of these adhesins are encoded by the *ALS* gene family. Als3, like Als1 and Als5, has broad substrate specificity and thus mediates adherence to a variety of host constituents (55). Studies in which *C. albicans* Als3 was heterologously expressed in the normally nonadherent *S. cerevisiae* indicate that this protein mediates adherence to endothelial cells, oral epithelial cells, gelatin, fibronectin, fibrinogen, type IV collagen, laminin, and salivary pellicle (35, 55). Consistent with these results, an *als3*Δ/Δ null mutant strain of *C. albicans* has reduced adherence to endothelial cells and buccal epithelial cells (70). However, this mutant has normal adherence to fibronectin, possibly due to the compensatory effects of other Als proteins, such as Als1, that also bind to this extracellular matrix protein. As

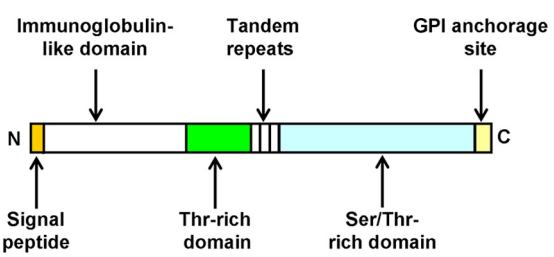

FIG. 2. Schematic diagram of the structure of Als3.

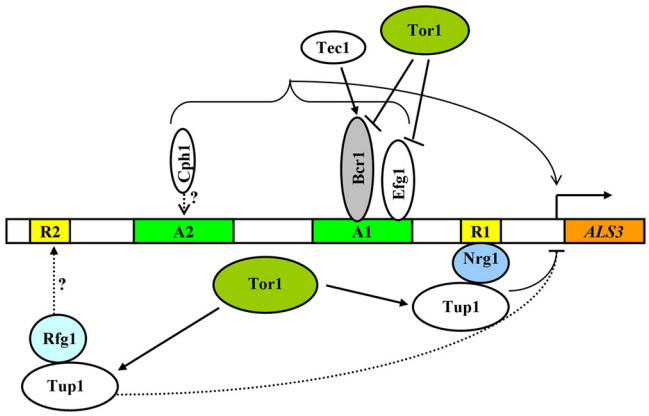

FIG. 3. Diagram of the transcriptional regulation of *ALS3* expression. A1 and A2 are activation regions in the promoter of *ALS3*, and R1 and R2 are repressor regions. Data are from references 3 and 5.

expected, both full-length monoclonal antibodies and single-chain variable fragments of human antibodies against Als3 block adherence to both endothelial and oral epithelial cells (6, 14, 28). These antibodies are directed against the N-terminal region of Als3, consistent with the model that this region contains the substrate binding domain.

Another murine monoclonal antibody, C7, has been found to bind to Als3 and inhibit adherence to host cells (8). Interestingly, this monoclonal antibody also reduces *C. albicans* germination, is fungicidal, and protects mice from disseminated candidiasis (33, 54). However, C7 binds to other antigens in addition to Als3, such as enolase and nucleoporin Nup88 (41). Also, other antibodies that bind only to Als3 do not inhibit germination or reduce *C. albicans* viability. Therefore, it is probable that many of the beneficial properties of C7 are mediated by its recognition of antigens other than Als3.

Als3 plays a key role in biofilm formation. A specialized form of adherence is biofilm formation. Biofilms are structured microbial communities that are attached to solid surfaces. *C. albicans* biofilm formation on dentures is associated with denture stomatitis. More importantly, biofilm formation on intravascular catheters plays a key role in the development of hematogenously disseminated candidiasis. Indeed, almost 80% of patients with candidemia have a central venous catheter in place at the time of diagnosis (20).

When *C. albicans* forms a biofilm, the initial basal layer consists of yeast-phase cells that are adherent to the substrate. On top of these cells is a mixture of pseudohyphae and hyphae (11, 37). Als3 plays a key role in biofilm formation. *C. albicans* mutants that lack Als3 produce scant, disorganized biofilms on catheter material *in vitro* (36, 69). Also, a *bcr1*Δ/Δ mutant, which has reduced expression of Als3 and other adhesins, has defective biofilm formation both *in vitro* and in the rat venous catheter model. Although an *als3*Δ/Δ mutant forms a normal biofilm *in vivo*, overexpression of *ALS3* in the *bcr1*Δ/Δ mutant rescues its biofilm defects (36). These results indicate that, while multiple adhesins participate in biofilm formation *in vivo*, Als3 has a central role in this process. Other *C. albicans* adhesins that contribute to biofilm formation are Hwp1 and Als1 (36). Interestingly, a mixture of biofilm-defective *hwp1*Δ/Δ and *als1*Δ/Δ *als3*Δ/Δ mutants can form a hybrid biofilm both *in vitro*

and in the rat catheter infection model (38). These data suggest that the adherence of *C. albicans* cells to one another in a biofilm is mediated by the complementary binding of Hwp1 to Als1 and Als3.

Als3 is bound by *Streptococcus gordonii*. When *C. albicans* grows on mucosal surfaces, it adheres to the normal bacterial flora in addition to host cells. It can also form a mixed-species biofilm with oral bacteria on prosthetic materials such as dentures. One bacterium with which *C. albicans* can form a biofilm is *Streptococcus gordonii*. This bacterium enhances *C. albicans* biofilm formation by abrogating the *C. albicans* farnesol-based quorum-sensing mechanism and thereby stimulating hyphal growth (4). *S. gordonii* cells bind to *C. albicans* hyphae in an Als3-dependent manner. Furthermore, *S. gordonii* adheres avidly to *S. cerevisiae* expressing *C. albicans* Als3 but not to a control strain of *S. cerevisiae* that does not express this protein. The attachment of *S. gordonii* to Als3 is mediated by the multifunctional polypeptide adhesins SspA and SspB (56). *S. gordonii* also binds to *C. albicans* Als5 (27). Other Gram-positive cocci, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus pyogenes*, also adhere to *C. albicans* (44). Whether Als3 mediates these adhesive interactions is not yet known. On the other hand, the Gram-negative bacterium *Pseudomonas aeruginosa* binds to *C. albicans* hyphae and subsequently kills the fungus (21). Hyphae of a *C. albicans* *als3*Δ/Δ mutant have wild-type susceptibility to killing by *P. aeruginosa*. Thus, Als3 is not essential for the attachment of this bacterium to *C. albicans*, and other fungal ligands for *P. aeruginosa* must exist (7).

Als3 is an invasin. After *C. albicans* adheres to either oral epithelial cells or vascular endothelial cells, it can invade these cells. *C. albicans* invasion into oral epithelial cells is a key feature of oropharyngeal candidiasis (10, 17, 32, 48, 53). Also, during hematogenously disseminated candidiasis, blood-borne organisms must invade the endothelial cell lining of the vasculature to reach the deep tissues (19). *C. albicans* must invade oral epithelial cells and endothelial cells in order to damage these cells *in vitro* (15, 43). Moreover, *C. albicans* mutants with impaired capacity to invade and damage host cells *in vitro* frequently have attenuated virulence in murine models of oropharyngeal and hematogenously disseminated candidiasis (12, 39, 43, 51, 61). These data suggest that the capacity of *C. albicans* to invade and damage host cells is an important virulence attribute.

One mechanism by which *C. albicans* can invade both oral epithelial cells and endothelial cells is by inducing its own endocytosis. This process occurs by a zipper-like mechanism whereby the host cell is induced to produce pseudopods that progressively surround the organism and pull it into the cell (43, 50, 68). *C. albicans* hyphae are endocytosed much more readily than are yeast-phase organisms, suggesting that hyphae express specific invasin-like molecules on their surface that bind to one or more host cell receptors and induce endocytosis. Als3 is one of these invasins. A *C. albicans* *als3*Δ/Δ null mutant is endocytosed poorly by both oral epithelial cell lines and vascular endothelial cells *in vitro*. Moreover, latex beads coated with the recombinant N terminus of Als3 (rAls3-N) are efficiently endocytosed by these host cells, whereas control beads coated with bovine serum albumin (BSA) are not (47). Similarly, a strain of *S. cerevisiae* that expresses *C. albicans* Als3 is avidly endocytosed by endothelial cells, in contrast to control strains of *S. cerevisiae* that do not express Als3 (55). Due to their defect in inducing host cell endocytosis, strains of *C. albicans* with either reduced or absent expression of *ALS3* have significantly reduced capacity to damage epithelial cells and endothelial cells *in vitro* (39, 47, 70). Although *C. albicans* must invade host cells to some extent to damage these cells, invasion by itself is not sufficient to induce damage. For instance, killed *C. albicans* cells are endocytosed by both endothelial and epithelial cells, but they do not cause detectable damage to these cells (15, 43). Thus, a factor(s) produced by viable organisms is required to cause host cell damage after invasion occurs.

Some of the host cell receptors for Als3 have been identified. Two of these receptors are E-cadherin on epithelial cells and N-cadherin on endothelial cells (47). Binding of Als3 to these receptors is sufficient to induce endocytosis because latex beads coated with rAls3-N are endocytosed by Chinese hamster ovary cells that heterologously express either E- or N-cadherin. Computer-assisted molecular modeling of the interactions of Als3 with either E- or N-cadherin suggests that the immunoglobulin domains in the N-terminal region of Als3 bind to the extracellular immunoglobulin domains of the cadherins (Fig. 4). Intriguingly, the binding parameters of the Als3–E-cadherin interaction are similar to those of one E-cadherin molecule binding to another E-cadherin molecule. Thus, *C. albicans* Als3 functions as a molecular mimic of mammalian E-cadherin (47).

Als3-induced endocytosis is mediated by the clathrin-dependent endocytic machinery, which requires dynamin and cortactin as well as clathrin (34). The cadherins can activate this pathway. Other proteins expressed on the host cell surface can also activate this pathway, and it is probable that some of them function as additional host cell receptors for Als3. As evidence that cadherins are not the only host cell receptor for *C. albicans*, small interfering RNA (siRNA) knockdown of endothelial cell N-cadherin by 90% reduces the endocytosis of this organism only by 34% (46). Recently, it has been reported that the epidermal growth factor receptor and HER2 are additional host cell receptors that are bound by Als3 and induce endocytosis (73). The relationship among these receptors and the cadherins during the endocytosis of *C. albicans* is currently under investigation.

Another *C. albicans* invasin is Ssa1, which is a member of the HSP70 family of heat shock proteins and is expressed on the surface of hyphae. An *ssa1*Δ/Δ mutant is defective in binding to E-cadherin and N-cadherin and is endocytosed poorly by oral epithelial cells and vascular endothelial cells *in vitro* (61). Latex beads coated with recombinant Ssa1 are avidly endocytosed by epithelial and endothelial cells, demonstrating that this protein functions as an invasin. Importantly, the *ssa1*Δ/Δ mutant has significantly attenuated virulence in mouse models of oropharyngeal and hematogenously disseminated candidiasis. Interestingly, the endocytosis defect of an *ssa1*Δ/Δ *als3*Δ/Δ double mutant is similar to that of an *als3*Δ/Δ single mutant (61). A likely explanation for this result is that Ssa1 and Als3 bind to the same host cell receptors. It is even possible that Ssa1 and Als3 may form a multiprotein receptor complex, analogous to the integrins in mammalian cells.

Als3 is a receptor for ferritin and mediates iron acquisition from the host. In order for a microorganism to survive in the

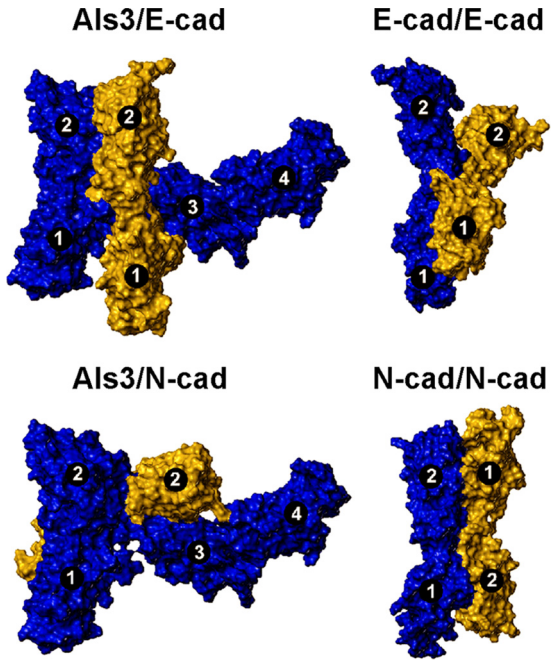

FIG. 4. Three-dimensional models of the N-terminal region of Als3 binding to the extracellular domains of E-cadherin and N-cadherin. The solvent-accessible surface areas of the proteins are shown, and the β-barrel domains are numbered sequentially from the N terminus. In models of the docking of Als3 to a cadherin (left column), the Als protein is blue and the cadherin is gold. In models of cadherin self-association (right column), one cadherin molecule is blue and the other cadherin is gold. (Reprinted from Phan et al. [47].)

host, it must be able to obtain iron. In mammals, virtually all iron is complexed with host cell proteins, and 30% of total iron is bound to ferritin (2). *C. albicans*, unlike *S. cerevisiae*, can utilize ferritin as the sole source of iron. Almeida et al. (1) discovered that *C. albicans* hyphae, but not yeast-phase cells, can bind to both purified ferritin and ferritin contained within epithelial cells. Most intriguingly, hyphae of an *als3*Δ/Δ mutant are unable to bind ferritin and thus grow poorly on media containing ferritin as the sole source of iron. Heterologous expression of *C. albicans ALS3* in *S. cerevisiae* enables this organism to bind ferritin. These observations demonstrate that Als3 functions as a ferritin receptor and facilitates the capacity of *C. albicans* to obtain iron from the host.

Role of Als3 in virulence. As expected from the multifunctional nature of Als3, mutant strains of *C. albicans* that lack this protein have prominent defects in assays of host cell interactions, biofilm formation, and iron acquisition *in vitro*. In addition, Als3 is strongly expressed by hyphae in the kidneys of mice with disseminated candidiasis (14), and high levels of *ALS3* mRNA are present in oral scrapings of patients with oropharyngeal candidiasis (68). Thus, one would expect that an *als3*Δ/Δ mutant would have significantly attenuated virulence in experimental animal models of candidiasis. We have found that an *als3*Δ/Δ mutant has modestly reduced virulence in the corticosteroid-treated mouse model of oropharyngeal candidiasis (Q. T. Phan, N. V. Solis, and S. G. Filler, unpublished data). Cleary et al. (13) recently reported that an *als3*Δ/Δ mutant has wild-type virulence when inoculated into immunocompetent adult mice via the tail vein. In contrast, Tsai et al. (65) found that an *als3*Δ/Δ mutant has significantly attenuated virulence when inoculated intraperitoneally into 2-day-old mouse pups. Thus, Als3 appears to play a variable role in virulence, depending on the route of inoculation and the age and immune status of the host.

There are at least two possible explanations for why *als3*Δ/Δ mutants have little or no virulence defects in some mouse models of candidiasis. One is that the functional redundancy of other Als proteins and additional adhesins and invasins masks the effects of the absence of Als3. Another possibility is that there is compensatory upregulation of other adhesins and invasins when Als3 is absent. This compensatory upregulation may occur to a variable extent, depending on the microenvironment of the host to which the organism is exposed. These issues of functional redundancy and compensatory upregulation illustrate the complexity of studying proteins such as Als3 that are members of a larger protein family.

A key difference between many *in vitro* models of *C. albicans*-host cell interaction and *in vivo* virulence studies is the effects of hyphal formation. Mutants of *C. albicans* with defects in hyphal formation have severe host cell interaction defects *in vitro*. For example, they are unable to damage and escape from macrophages (30), and they have markedly reduced capacity to invade and damage both epithelial cells and endothelial cells (43, 45). Although *C. albicans* mutants that are locked in the yeast phase have significantly reduced lethality when inoculated into adult mice via the tail vein, they are able to escape from the bloodstream and invade and persist in the target organs (30, 52). Thus, in experimental animal models, these yeast-phase organisms are somehow able to adhere to and invade host cells and obtain iron from the host in the absence of Als3, which is expressed only by hyphae. How yeast-phase organisms are able to carry out these processes is currently unknown, but it is likely that they express one or more functional equivalents of Als3.

Als3 AS A VACCINE TARGET

Vaccination with rAls3-N protects mice against candidiasis. The risk factors for developing both hematogenously disseminated and mucosal candidiasis are well defined (reviewed in reference 42). Thus, a vaccine that prevented these diseases could be targeted to patients who either have the appropriate risk factors or are likely to develop them. The fact that Als3 is highly expressed on the *C. albicans* surface *in vivo* makes it a good target for a vaccine. Spellberg et al. (60) found that vaccination with rAls3-N protects immunocompetent mice from both vaginal candidiasis and lethal disseminated candidiasis. It also significantly reduces oral fungal burden in the corticosteroid-treated mouse model of oropharyngeal candidiasis. Interestingly, the same rAls3-N vaccine is also protective in the mouse model of methicillin-resistant *Staphylococcus aureus* bacteremia, probably due to antigenic cross-reactivity (59). The vaccine both improves survival and reduces organ bacterial burden in this model.

Adoptive transfer experiments and studies with different strains of knockout mice, including gamma interferon (IFN-γ)$^{-/-}$, interleukin 17 (IL-17)$^{-/-}$, and gp91$^{phox-/-}$ strains, indicate that the mechanism of rAls3-N vaccine-induced protec-

tion against both *C. albicans* and *S. aureus* bloodstream infection is the induction of a Th1 and Th17 immune response (29, 58–60). Antibodies are neither necessary nor sufficient for the efficacy of this vaccine. Vaccination with rAls3-N primes Th1, Th17, and Th1/Th17 lymphocytes to produce high levels of IFN-γ and IL-17A, as well as the chemokines KC and MIP-1α. These proinflammatory cytokines enhance the capacity of phagocytes to kill both pathogens and thereby prevent disease (29). Human trials of the rAls3-N vaccine are in final preparation.

Vaccination with β-1,3-glucan induces antibodies against Als3. It has also been found that vaccination of mice with laminarin (purified β-1,3 glucan) conjugated with genetically inactivated diphtheria toxin protects mice from infections caused by both *C. albicans* and *Aspergillus fumigatus* (62). The mechanism of this protection is the induction of anti-β-1,3-glucan antibodies. The protective effects of this vaccine against *C. albicans* infection can be mimicked by a monoclonal antibody directed against β-1,3-glucan (63). Although this monoclonal antibody has several targets, one of them is Als3, which is a glucan-linked glycosylphosphatidylinositol-anchored protein. Therefore, Als3 is a promising target for two different types of vaccines.

FUTURE DIRECTIONS

Although the role of Als3 in many key aspects of *C. albicans* biology has been investigated, a number of questions about this protein remain unanswered. For example, none of the Als proteins has been successfully analyzed by X-ray crystallography. Thus, the exact three-dimenstional structure of Als3 has not been definitively determined. Furthermore, detailed structure-function analyses of this protein have not yet been done. Also, it is highly probable that there are additional host cell targets of Als3 that have not been discovered to date. Finally, the list of *C. albicans* proteins that function similarly to Als3 and can compensate for its absence is currently incomplete. Answering these questions will provide important new information about how *C. albicans* adheres to host and bacterial substrates, forms biofilms, and invades host cells. This information also holds promise to lead to the development of new approaches to prevent and treat candidal infections.

ACKNOWLEDGMENTS

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