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## The Hyphal and Yeast Forms of *Candida albicans* Bind the Complement Regulator C4b-Binding Protein

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***Candida albicans*, an important pathogenic yeast, activates all three pathways of the complement system. To understand how this yeast evades the effects of the activated system, we have analyzed the binding of the classical pathway inhibitor C4b-binding protein (C4BP) by *C. albicans*. Purified native as well as recombinant C4BP bound dose dependently to the yeast and hyphal forms, as shown by multiple methods, such as confocal microscopy, flow cytometry, a novel enzyme-linked immunosorbent assay, absorption from human serum, and direct binding assays with purified proteins. A prominent binding site was identified at the tip of the germ tube, a structure that is considered important for tissue penetration and pathogenesis. The binding site in C4BP was localized to the two N-terminal complement control protein domains by using recombinant deletion constructs and site-specific monoclonal antibodies. As the alternative pathway inhibitors factor H and FHL-1 also bind to *C. albicans*, the binding of all three plasma proteins was compared. Simultaneous binding of the classical regulator C4BP and the alternative pathway regulator factor H was demonstrated by confocal microscopy. In addition, FHL-1 competed for binding with C4BP, suggesting that these two related complement regulators bind to the same structures on the yeast surface. The surface-attached C4BP maintains its complement regulatory activities and inactivates C4b. The surface-attached human C4BP serves multiple functions relevant for immune evasion and likely pathogenicity. It inhibits complement activation at the yeast surface and, in addition, mediates adhesion of *C. albicans* to host endothelial cells.**

*Candida albicans* is the most common human pathogenic yeast, causing cutaneous and mucocutaneous candidiasis (2, 43). In healthy individuals, the yeast is present as a commensal organism and can reside harmlessly on the skin, in the oral cavity, and in the urogenital and gastrointestinal tracts. However, *C. albicans* can cause life-threatening systemic infections, especially in immunocompromised and granulocytopenic patients (21). Invasive candidal infections are difficult to diagnose and treat (16) and are often lethal, as the mortality of a hematogenously disseminated *C. albicans* infection reaches up to 40% (27).

Several surface-expressed and secreted proteins, such as adhesion molecules, especially mannoproteins (9), and secreted proteolytic enzymes (8, 41), contribute to the pathogenicity of *C. albicans*. In addition, morphogenic changes during yeast to hypha transition are considered essential for the infection process (12). Hyphal growth can be induced by a shift of pH or change in temperature. Hyphal forms are elongated and substantially differ morphologically from the round cellular forms. Furthermore, during hyphal growth, the expression of new surface antigens causes increased adhesion of hyphae to the host cells and facilitates tissue penetration (13).

The complement system represents a central part of innate immunity. The activation of the complement system may lead to the killing of microbes and consequently protect the human

host from microbial infections. This activity is achieved either via opsonization followed by phagocytosis or by direct lysis. Newly formed products, generated during activation, display chemotactic activity and induce or enhance inflammatory reactions. The complement system can be activated via three different pathways. The classical pathway (CP) is induced by antibodies bound to target structures, and the lectin pathway (LP) is activated upon binding of mannose-binding lectin to mannan-containing structures on surfaces. The alternative pathway (AP) is activated by randomly and newly generated C3b molecules, which bind directly to unprotected surfaces. Upon invasion, microbes come in contact with the soluble components of complement. *C. albicans* activates all three pathways of complement (33). The CP is activated via mannan-specific immunoglobulin G antibodies (54), which are commonly found in human serum, and more than 70% of healthy blood donors have these antibodies (17). Mannan, one of the major components on the yeast cell surface (46), activates the LP. Furthermore, C3b molecules bind directly to the surface of *C. albicans* and cause AP activation (34, 35). Although *C. albicans* activates all three pathways, it is unclear how this yeast inactivates toxic complement activation products and how the yeast inhibits subsequent opsonization and phagocytosis.

C4b-binding protein (C4BP) is the major fluid-phase inhibitor of the CP and LP (1). This human serum protein consists of one  $\beta$ -chain and seven identical  $\alpha$ -chains, all of which are composed of complement control protein (CCP) domains. The  $\alpha$ -chain consists of eight CCPs, and the  $\beta$ -chain consists of three CCPs (10, 29, 49). Disulfide bonds between the  $\alpha$ - and  $\beta$ -chains and hydrophobic interactions keep the 570-kDa mol-

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ecule together (32). C4BP regulates complement by binding to C4b (31) via the N terminus of each  $\alpha$ -chain (6, 15), thereby making it susceptible to degradation by a plasma serine proteinase factor I and by accelerating the decay of the CP C3-convertase C4b2a (25). C4BP also inhibits the activity of the AP C3-convertase in a fluid phase (23) and acts as a cofactor in factor I-mediated cleavage of C3b (5).

The number of pathogenic microbes which are able to bind and utilize human complement inhibitors is increasing. So far, two pathogens, i.e., *Streptococcus pyogenes* (31, 39, 50) and *Neisseria gonorrhoeae* (46, 47), are known to bind the CP inhibitor C4BP and also the AP inhibitors factor H and FHL-1. The binding is mediated by the streptococcal M-proteins (31, 39, 50) and the porins of *Neisseria* (46, 47), both of which have multiple, partially overlapping binding domains for the three host regulators. We have recently shown that *C. albicans* evades the AP of complement by binding factor H and FHL-1 via two different binding moieties on the surface (38). Given the activation of all three pathways on the surface of candida, it was of interest to analyze the evasion mechanisms of this pathogenic yeast for the CP and LP.

In this study, we report a novel mechanism for *C. albicans* to control and inhibit CP activation. The cellular and hyphal forms bind C4BP from human serum. Surface attachment was confirmed with purified native and recombinant proteins. Using a novel enzyme-linked immunosorbent assay (ELISA), the binding site of C4BP was localized within the  $\alpha$ -chain to CCP1-2. As FHL-1 competes binding of C4BP to the surface of *C. albicans*, it is possible that the two host regulators share the same receptor. The surface-attached human C4BP inhibits complement activation at the yeast surface, thus regulating CP and LP activation.

#### MATERIALS AND METHODS

***C. albicans* strains and growth conditions.** The wild-type SC5314 (19) strain of *C. albicans* was used in experiments. In addition, *C. albicans* strains ATCC 18804 and EBP, *Candida glabrata*, *Candida krusei*, *Candida tropicalis*, *Candida parapsilosis*, and *Saccharomyces cerevisiae* SEY6210 were used (strain collection of the Hans Knöll Institut). Yeasts were grown at 28°C on a shaker for 16 h in 5% Sabouraud agar (Biokar; Diffchamb, Gothenburg, Sweden). After centrifugation (5 min at 3,800  $\times$  g), the cells were washed with phosphate-buffered saline (PBS; 0.15 M NaCl, 0.03 M phosphate [pH 7.2]) and counted with a cell counter (Beckman Coulter, Krefeld, Germany). Hyphal growth was induced by a further incubation of the cells for 90 min at 37°C in RPMI supplemented with 25 mM HEPES and L-glutamine (Gibco, Karlsruhe, Germany).

**Serum, antibodies, and proteins.** Normal human serum (NHS) was obtained from the blood bank of the University Clinic of the Friedrich Schiller University, Jena, Germany. Upon informed consent, blood was collected from healthy donors and then pooled and stored at  $-80^{\circ}\text{C}$  until use. EDTA was added at a final concentration of 10 mM. Monoclonal antibody 70 (MAb70) and MAb104, both directed against C4BP  $\alpha$ -chain CCP1 (26), and a polyclonal rabbit anti-C4BP (Calbiochem, La Jolla, Calif.) were used. MAb104 was coupled to a Sepharose matrix and used to remove C4BP from NHS as described previously (53). The concentration of C4BP in this serum was below the detection limit of an ELISA. Depleted serum was tested in a hemolytic assay, and its complement activity was found to be intact. MAbs were a kind gift of B. Dahlbäck (Lund University, Malmö, Sweden). In inhibition assays, polyclonal rabbit antibodies against CCP1-4 and CCP19-20 of factor H as well as monoclonal anti-CD11b (Sigma Chemical Co., St. Louis, Mo.) were used. Fluorescein isothiocyanate (FITC)- and horseradish peroxidase-conjugated rabbit anti-mouse antisera were from Dako (Glostrup, Denmark). Rabbit anti-mouse and goat anti-mouse antibodies were labeled with Alexa 488 and 647 (Molecular Probes, Eugene, Oreg.) according to the manufacturer's instructions. Human C4BP was purified from plasma as described previously (13). C4b, factor H, and factor I were obtained from Calbiochem. C4b was labeled with biotin according to the instructions of the man-

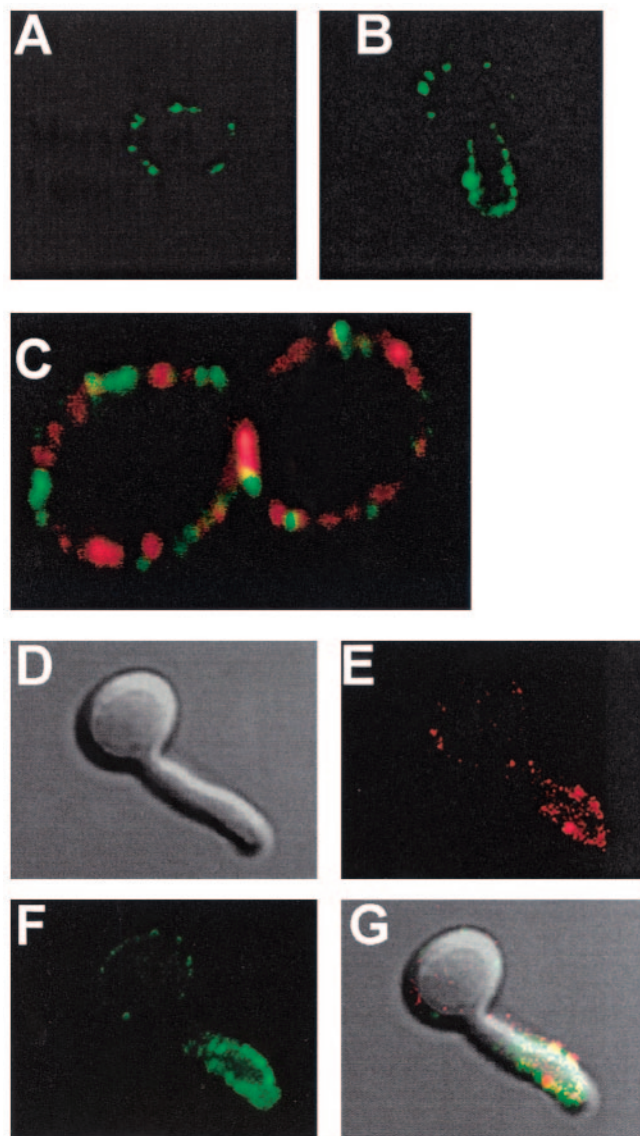


FIG. 1. Immunofluorescence analysis of C4BP and factor H bound to candida cells and hyphal forms. Cells and hyphal forms of *C. albicans* were incubated in NHS-EDTA. The samples were washed and incubated further with a mouse MAb (MAb70) detecting CCP1 of the C4BP  $\alpha$ -chain and a secondary rabbit anti-mouse antibody labeled with FITC (A and B) or Alexa 488, producing red fluorescence (C, E, and G). Factor H was detected with an anti-mouse serum and a secondary goat anti-mouse antiserum labeled with Alexa 647 for green fluorescence (C, F, and G). Parallel binding of C4BP and factor H is shown in panels C and G.

ufacturer. FHL-1 and the recombinant construct CCP8-20 of factor H used in inhibition assays were expressed in the baculovirus system as described previously (36). Polymeric recombinant deletion mutants of C4BP were constructed and expressed as described previously (7). C4BP mutants lacking individual CCP domains ( $\Delta$ CCP1,  $\Delta$ CCP2,  $\Delta$ CCP3,  $\Delta$ CCP4,  $\Delta$ CCP5,  $\Delta$ CCP6,  $\Delta$ CCP7, and  $\Delta$ CCP8) were purified by affinity chromatography as described previously (6). Heparin (5,000 IU/ml) was from Lövens, Ballerup, Denmark, and trypsin was from Sigma Chemical Co.

**Immunofluorescence and flow cytometry assays.** *C. albicans* cells and induced hyphal forms ( $10^7$ ) were incubated at room temperature (RT) for 60 min with NHS treated with EDTA (1:3 dilution), and hyphae were incubated with purified C4BP (10  $\mu\text{g}/\text{ml}$ ). After incubation, the samples were washed three times with



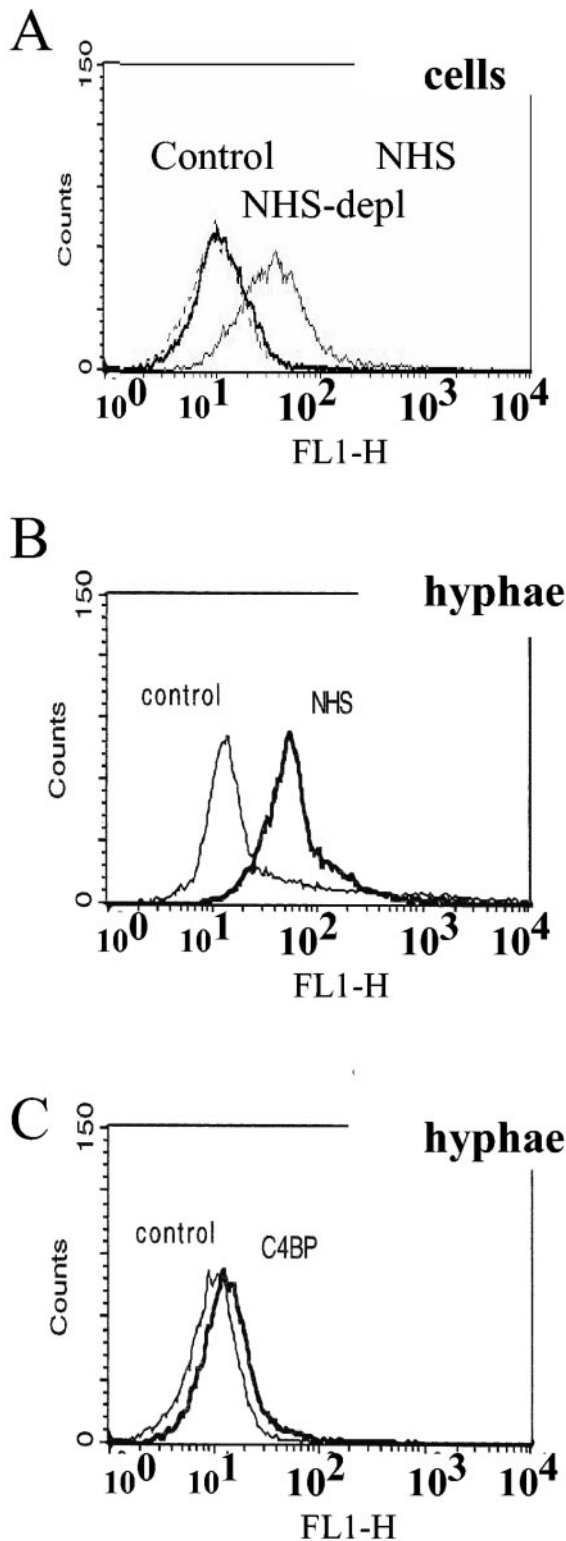


FIG. 2. Binding of C4BP to yeast and hyphal forms of *C. albicans* determined by flow cytometry. Cells incubated in NHS or NHS depleted of C4BP (A) and hyphal forms incubated in NHS (B) or purified C4BP (C) were used. The samples were washed and incubated with a C4BP-specific MAb70. After being washed, bound antibodies were detected with an FITC-conjugated rabbit anti-mouse antibody and stained cells were subjected to flow cytometry. Cells incubated in buffer were used as controls.

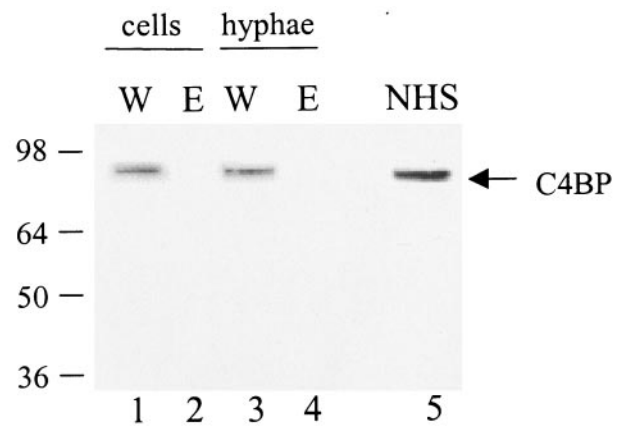


FIG. 3. Binding of C4BP to intact cells and hyphae of *C. albicans*. Cells or hyphae ( $10^9$ ) were incubated in NHS and washed extensively, and bound proteins were eluted with 3 M KSCN. The wash (W) and eluate (E) fractions were separated by SDS-PAGE under reducing conditions and analyzed by Western blotting with anti-C4BP MAb104 detecting CCP1 of the C4BP  $\alpha$ -chain. A control where C4BP from NHS is detected with the same antiserum is shown in lane 5. The mobility of the size markers is indicated on the left and indicated in kilodaltons.

ice-cold PBS supplemented with 1% bovine serum albumin (BSA; 1% BSA-PBS), and nonspecific binding sites were blocked with the same buffer for 30 min at RT. The samples were incubated with MAb70 overnight at 4°C (20  $\mu$ g/ml). After three washes with 1% BSA-PBS, rabbit anti-mouse immunoglobulin G, labeled with Alexa 647 or FITC, was added at a dilution of 1:50 in 1% BSA-PBS at RT. Factor H was visualized with a polyclonal antiserum specific for the N-terminal domains of the protein (anti-CCP1-4 and a secondary goat anti-rabbit antibody labeled with Alexa 488). The samples were washed three times with 1% BSA-PBS and examined with a laser scanning microscope (LSM 510 META; Zeiss, Jena, Germany). The stained cells were also examined by flow cytometry (FACScan; Becton-Dickinson, Heidelberg, Germany). Forward scatters were used to define the cell population, and 10,000 events were routinely counted. Cells were also treated with trypsin (50 mg/ml in PBS, 60 min at 37°C) to cleave the proteins from the outer surface. Afterwards, cells were incubated in NHS-EDTA, and binding of C4BP was measured in a flow cytometer.

**Serum absorption experiments.** Cells and hyphal forms of *C. albicans* strain SC5314 and cells of *C. albicans* strains EBP and ATCC 18804 and *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis*, and *S. cerevisiae* ( $5 \times 10^9$ ) were incubated in NHS-EDTA (1:3 dilution) for 60 min at 37°C. The cells were washed five times with a washing buffer (100 mM NaCl, 50 mM Tris-HCl, 0.05% Tween 20 [pH 7.4]). Proteins bound to the surface were eluted with 3 M KSCN, and the supernatants were collected. Aliquots of the wash and eluted fractions were subjected to a reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The membranes were blocked with 2.5% BSA-PBS-0.1% Tween-10% RotiBlock (Carl Roth, Karlsruhe, Germany) for 12 h at 4°C and incubated further with a mouse MAb directed against C4BP (MAb104) (10  $\mu$ g/ml) for 120 min at RT. After five washes with PBS, a rabbit anti-mouse antibody conjugated to horseradish peroxidase was added at a dilution of 1:2,000, and the membranes were incubated at RT for 60 min. After five washes with PBS, the proteins were detected by enhanced chemiluminescence (Amersham Biotech, Piscataway, N.J.).

**Protein binding and inhibition ELISAs.** MaxiSorp microtiter plates (Nalge Nunc, New York, N.Y.) were coated for 4 h with *C. albicans* ( $10^9$ /ml) in RPMI 1640 medium (Gibco) treated at 37°C to induce hyphal growth. Nonspecific binding sites were blocked with 2% BSA-PBS for 60 min at 37°C. NHS (in 1:4 dilution) and recombinant C4BP (10  $\mu$ g/ml) or C4BP mutants (10  $\mu$ g/ml) in 1% BSA-PBS were added. In inhibition assays, polyclonal antisera and MAbs against factor H CCP1-4, CCP19-20, anti-C4BP (1:100 dilution), or CD11b (10  $\mu$ g/ml) were added to the reaction mixture. The proteins were allowed to bind at 37°C for 120 min. After five washes with 1% BSA-PBS, an anti-C4BP MAb (MAb104) (0.5  $\mu$ g/ml in 1% BSA-PBS; 100  $\mu$ l/well) was added, and wells were incubated for 60 min at RT. Wells were washed five times with 1% BSA-PBS, and a peroxidase-conjugated secondary rabbit anti-mouse antibody diluted

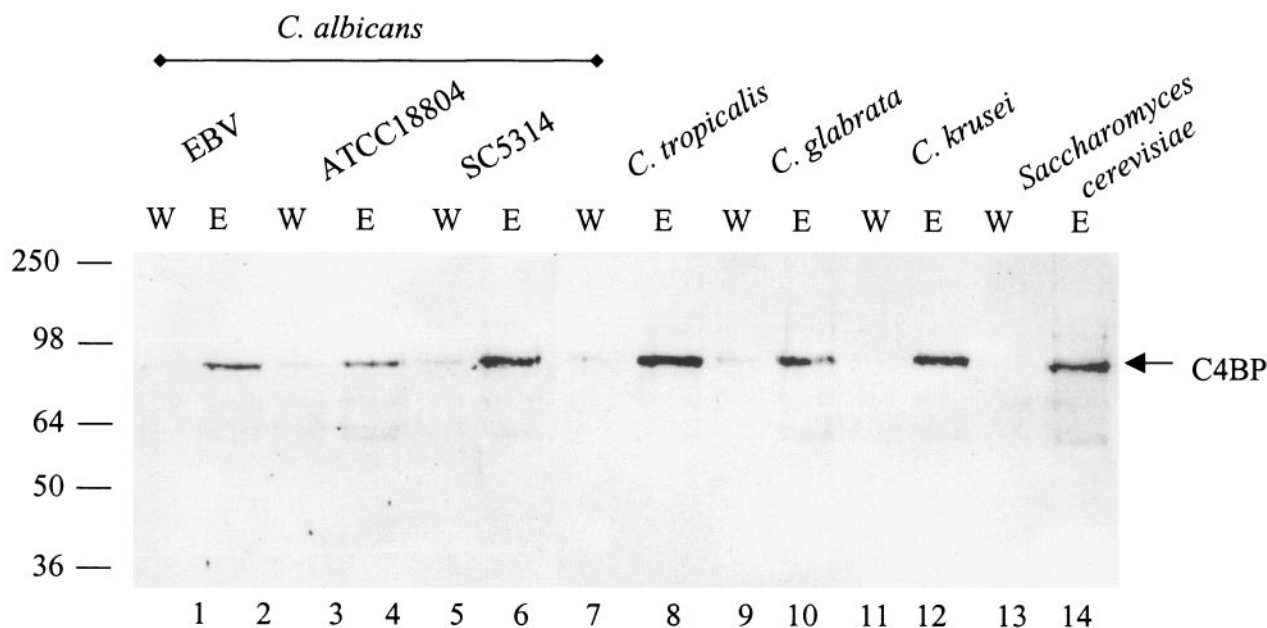


FIG. 4. Binding of C4BP to intact cells of different species and strains of *Candida*. Cells ( $10^9$ ) of the indicated species were incubated in NHS and washed extensively, and bound proteins were eluted with 3 M KSCN. The wash (W) and eluate (E) fractions were separated by SDS-PAGE under reducing conditions and analyzed by Western blotting with MAb104 detecting CCP1 of the C4BP  $\alpha$ -chain. The mobility of the size markers is indicated on the left and indicated in kilodaltons.

1:4,000 in 1% BSA-PBS (100  $\mu$ l/well) was added. After a 60-min incubation at RT, the substrate, *o*-phenyl-diamine diluted in H<sub>2</sub>O and supplemented with 0.04% H<sub>2</sub>O<sub>2</sub>, was added. After a 15-min incubation at 22°C, the reaction was stopped with 50  $\mu$ l of 2 M H<sub>2</sub>SO<sub>4</sub> per well. The absorbances were determined with an ELISA reader (Spectramax; Molecular Devices, Munich, Germany) with a 492-nm filter.

**Protein binding and inhibition assays.** Recombinant C4BP (rC4BP) was labeled with <sup>125</sup>I by using the Iodogen reagent (Pierce Chemical Corp., Rockford, Ill.) (48). Induced hyphal forms of *C. albicans* were washed with Veronal-buffered saline (50 mM NaCl, 3.3 mM diethyl barbiturate [pH 7.5]), and  $10^9$  cells were incubated with the radiolabeled C4BP (10,000 cpm/assay) in Veronal-buffered saline containing 0.1% gelatin for 20 min at 37°C, and unlabeled purified factor H, FHL-1, purified C4b, rC4BP, heparin, or BSA was added to the reaction mixture at concentrations of 0.1 to 300  $\mu$ g/ml. For factor H, FHL-1, and C4BP, the highest concentration was 100  $\mu$ g/ml. Cell-associated and free radioactive proteins were separated by centrifuging the samples through a 250- $\mu$ l column of 20% (wt/vol) sucrose in Veronal-buffered saline containing 0.1% gelatin. The radioactivities of the supernatant and the pellet fraction were measured, and the amount of bound C4BP was calculated as a percentage of the total radioactivity input. All experiments were performed in quadruplicate.

**Cofactor assay.** The cofactor activity of surface-attached C4BP was assayed as described previously (24). The induced hyphae ( $10^7$ ) were washed with a binding buffer (100 mM NaCl, 50 mM Tris-HCl [pH 7.4]) and incubated with NHS (dilution of 1:2); NHS depleted of C4BP, purified C4BP, and rC4BP (10  $\mu$ g/ml); or binding buffer for 60 min at 37°C on a shaker. Cells were washed three times with washing buffer (100 mM NaCl, 50 mM Tris-HCl, 0.05% Tween 20 [pH 7.4]), and biotin-labeled C4b (15 ng/assay) and factor I (50 ng/reaction) were added. The samples were incubated for 60 min at 37°C. The samples were centrifuged, and the supernatants and pellets were analyzed by SDS-PAGE under reducing conditions to detect the cleavage products of C4b. As a positive control, purified C4BP (50 ng) or NHS was added to the reaction mixture. As a negative control, C4b was incubated in the presence of factor I only.

**Cell adhesion assay.** Human umbilical vein endothelial cells (HUVEC) were cultivated by standard procedures (37). About  $10^5$  cells were seeded to microtiter plates and cultivated for 48 h at 37°C in a humidified atmosphere. Following extensive washing with PBS, cells were cultivated in Dulbecco's modified Eagle's medium lacking fetal calf serum for an additional 24 h. An overnight culture of *C. albicans* cells grown in yeast extract-peptone-dextrose medium was adjusted to a concentration of  $10^7$  cells/ml, and 200  $\mu$ l of this suspension was added to

recombinant or purified wild-type C4BP (10  $\mu$ g/ml). Upon cultivation for 3 h at 37°C, candida were stained with calcofluor (30 min at 37°C), and following extensive washing, adherent yeast cells were analyzed with a fluorescence reader (Fluoroscan; Ascent Labsystems, Dreieich, Germany).

## RESULTS

***C. albicans* cells and hyphal forms bind C4BP.** The binding and surface distribution of C4BP on intact *C. albicans* were analyzed by laser scanning microscopy of both cells and hyphal yeast forms (Fig. 1). The cell bodies (Fig. 1A) and tips of the hyphae stained positive (Fig. 1B). Bound proteins showed a patchy distribution, suggesting a clustering of the C4BP-binding structures on the surface. Simultaneous binding of C4BP and factor H to cells (Fig. 1C) and the tip of the hyphae were seen by digital interference contrast microscopy images (Fig. 1D to G). In addition, specific binding of C4BP and a very similar surface distribution were detected with NHS and purified plasma protein (data not shown). Incubation with the secondary conjugated antibodies alone following NHS treatment, as well as in buffer instead of NHS, showed no staining (data not shown).

Flow cytometry was used to further quantify C4BP binding to the cells and hyphal forms of *C. albicans*. After incubation in NHS, C4BP bound to cells (Fig. 2A), and when human serum depleted of C4BP was used, no binding was detected, thus confirming the specificity of the interaction. Again, the binding from NHS was prominent with the hyphal forms (Fig. 2B). The incubation of hyphae with purified C4BP showed a clear positive, but the staining was weaker (Fig. 2C). When cells were treated with trypsin to cleave proteins from the outer membrane, no binding of C4BP was seen (data not shown).

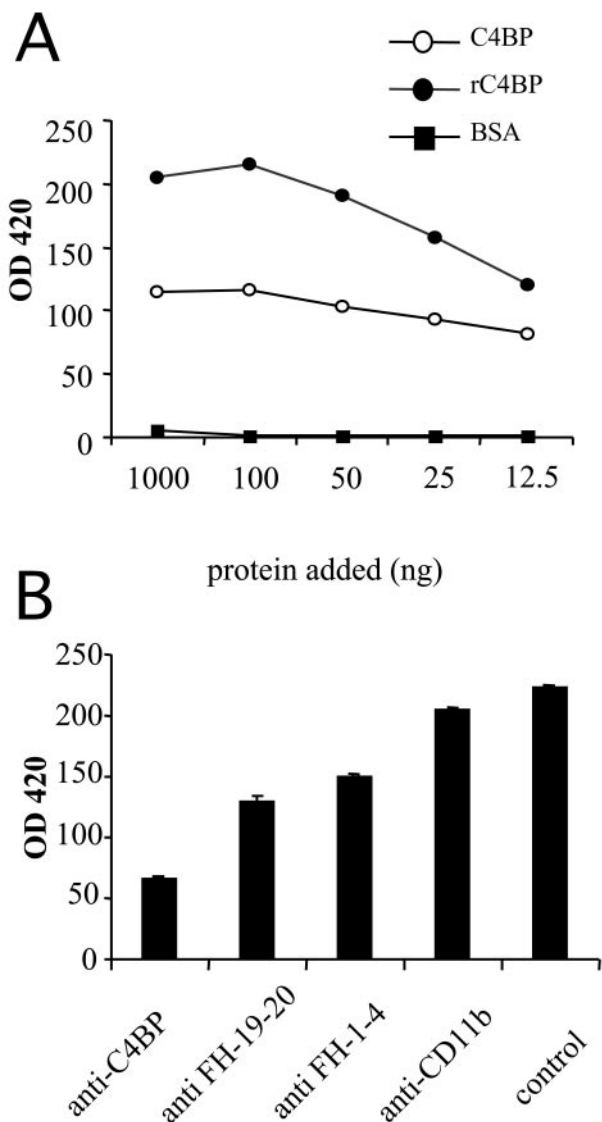


FIG. 5. Dose-dependent binding of C4BP to *C. albicans* and inhibition of binding with different antibodies. ELISA plates were coated with *C. albicans* hyphal forms. Purified native or recombinant C4BP was added to the cells in increasing concentrations. BSA was used as a control (A). For inhibition of binding of C4BP to the *C. albicans* hyphae, the indicated antibodies were added to the reaction mixture (B). After being washed, bound proteins were detected with a polyclonal anti-C4BP antibody. A positive control, where hyphae were incubated with C4BP alone, was considered to have 100% binding. The results show the mean values with indicated standard deviations of the results from five independent experiments. OD 420, optical density at 420 nm.

To further assay the binding of the complement regulator C4BP to *C. albicans*, yeast cells were incubated in NHS, and after extensive washing, absorbed proteins were eluted. Aliquots of the various wash and elution fractions were reduced, separated by SDS-PAGE, and analyzed by Western blotting. Specific binding of C4BP was observed, as demonstrated by the presence of the 75-kDa  $\alpha$ -chain of C4BP in the eluted fraction (Fig. 3, lane 2). In this assay, the hyphal forms showed an intensity of binding similar to that of the cells (Fig. 3, lanes 4

and 2). The specificity of binding was further assayed with human serum depleted of C4BP. After incubation with depleted serum, no binding of C4BP was detected (data not shown).

**Binding of C4BP to other candida strains.** It was of interest to test and compare the C4BP binding of pathogenic strain SC5314, used in all assays, to other *C. albicans* strains (EBP and ATCC 18804) as well as to different species of the family *Candidae* (*C. tropicalis*, *C. glabrata*, and *C. krusei*) and *S. cerevisiae*. Yeast cells were incubated in NHS, and bound proteins were eluted, separated by SDS-PAGE, and detected by Western blotting. All analyzed *C. albicans* strains, as well as the additional species, bound similar amounts of C4BP with amounts almost similar to that of the pathogenic SC5314 strain (Fig. 4).

**Dose dependency of binding.** To characterize the nature of the interaction between C4BP and *C. albicans* in more detail, an ELISA was established. Hyphal forms of *C. albicans* were immobilized in wells of a microtiter plate, purified C4BP was added in different concentrations, and the binding of C4BP was detected by specific MAb. The experiment revealed a dose-dependent binding of native C4BP purified from human serum and of recombinant wild-type C4BP to the hyphal forms (Fig. 5A). A similar dose-dependent binding was observed with NHS as a source of C4BP or recombinant C4BP (data not shown). The specificity of this interaction is also concluded from the inhibitory effect of an anti-C4BP antiserum (Fig. 5B), whereas two antisera raised against factor H or an antiserum against the  $\beta$ -chain of the integrin receptor (anti-CD11b) had no effect or only minor effects on binding (Fig. 5B).

**Localization of binding domain within C4BP.** It was of interest to identify whether the  $\alpha$ - or  $\beta$ -chain of C4BP mediates binding to candida and to localize the binding domain within the intact C4BP protein. We found that the  $\alpha$ -chain mediates binding, as a recombinant C4BP that lacks the  $\beta$ -chain bound to *C. albicans* equally well as native C4BP with the  $\beta$ -chain when tested by ELISA (data not shown).

To localize the domain within the  $\alpha$ -chain of C4BP relevant for this interaction, we used recombinant deletion mutants which lack single CCP domains ( $\Delta$ CCP1,  $\Delta$ CCP2,  $\Delta$ CCP3,  $\Delta$ CCP4,  $\Delta$ CCP5,  $\Delta$ CCP6,  $\Delta$ CCP7, and  $\Delta$ CCP8). Hyphal forms were immobilized on ELISA wells, and binding of the recombinant deletion mutants was detected with an anti-C4BP antiserum. C4BP variants lacking CCP1 or CCP2 did not bind to *C. albicans* (Fig. 6), and the construct which lacks CCP6 also showed weaker binding. All of the other deletion mutants bound to the hyphae with apparent affinities similar to that of the recombinant intact C4BP. This experiment localized the binding domain in C4BP for *C. albicans* to CCP1-2 and most likely identified a second binding domain within CCP6. The same pattern of binding and interaction was observed with  $^{125}$ I-labeled recombinant proteins, and similarly, the binding of C4BP was inhibited by MAb102, which binds to CCP1 of C4BP (data not shown).

**Effect of salt on binding.** To analyze the nature of the interaction, the binding of native or recombinant C4BP to the hyphal forms of *C. albicans* was assayed in different salt concentrations. The binding of purified and recombinantly expressed C4BP to *C. albicans* is sensitive to salt. At low salt concentrations, binding was increased compared to the physi-

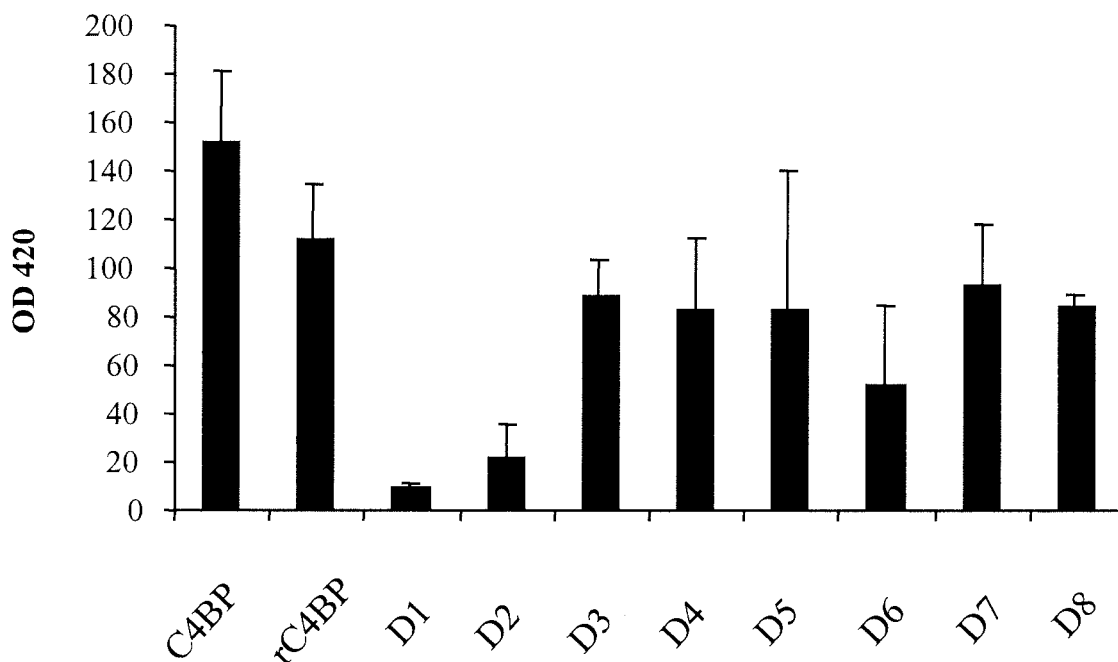


FIG. 6. Localization of the binding region in C4BP. *C. albicans* hyphae were coated on ELISA plates, and purified C4BP, intact rC4BP, and recombinant deletion mutants of C4BP were added. After being washed, bound proteins were detected with a polyclonal anti-C4BP antibody. The individual deletion mutants are represented as follows:  $\Delta$ CCP1, D1;  $\Delta$ CCP2, D2;  $\Delta$ CCP3, D3;  $\Delta$ CCP4, D4;  $\Delta$ CCP5, D5;  $\Delta$ CCP6, D6;  $\Delta$ CCP7, D7;  $\Delta$ CCP8, D8. The results show the mean values with indicated standard deviations of the results from four independent experiments.

ological salt concentration (150 mM), and in higher concentrations of salt, binding was almost totally abolished (Fig. 7). This implies that the binding is mostly based on ionic interactions between amino acids.

**Competition for C4BP binding.** Two separate receptors for complement AP regulators factor H and FHL-1 on the surface of *C. albicans* have been previously identified (38). Given the structural and functional similarity, it was of interest to test whether the three human complement regulators (C4BP, factor H, and FHL-1) bind to the same receptor or to related receptors on the surface of *C. albicans*. Competition assays

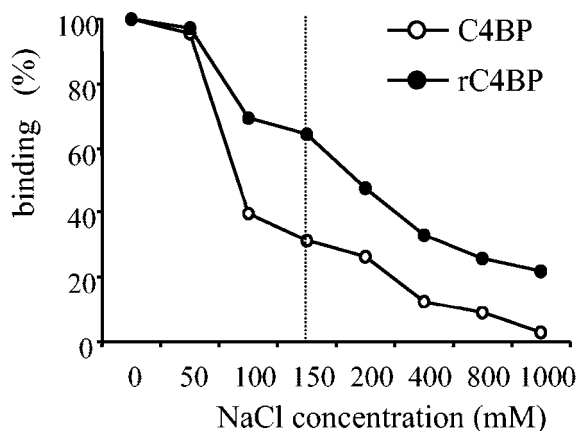


FIG. 7. Effect of salt on the binding of C4BP to hyphae of *C. albicans*. ELISA plates were coated with *C. albicans* hyphal forms and native C4BP or rC4BP was added to the buffer containing the indicated concentrations of NaCl. After being washed, bound proteins were detected with a polyclonal anti-C4BP antibody.

showed that the binding of C4BP was inhibited by C4BP and by FHL-1, but only at rather high concentrations. Factor H showed a weaker effect (Fig. 8A). The strong inhibitory effect of FHL-1, but not of factor H, suggests that FHL-1 and C4BP utilize the same receptor. In addition to FHL-1, C4b and heparin, known ligands for C4BP, affected binding. BSA used as a control showed no effect (Fig. 8B). Heparin, which binds to the N terminus of the C4BP  $\alpha$ -chain (7, 28, 52), competed for binding in a dose-dependent manner (Fig. 8B), thus confirming that the N-terminal domains of the  $\alpha$ -chain are relevant for this interaction. The lower inhibitory effect of C4b is explained by the multiple binding sites of the native C4BP protein to this ligand.

**Cell-bound C4BP mediates cofactor activity.** The cofactor activity of a surface-attached C4BP was tested for factor I-mediated cleavage of human C4b. Hyphae of *C. albicans* were incubated either in NHS, NHS depleted of C4BP, purified native C4BP, and wild-type rC4BP. After intensive washing of the treated cells, purified factor I and biotin-labeled C4b were added, and following further incubation, the cleavage of C4b was assayed by SDS-PAGE in combination with chemiluminescence detection. The cofactor activity of the bound regulator is indicated by the disappearance of the  $\alpha$ -chain and the appearance of the cleavage products of 45-kDa C4d and the  $\alpha$ 3-fragment (Fig. 9, lanes 2, 3, 5, and 6). The bound regulators clearly showed cofactor activity (Fig. 9, lanes 2 and 3). C4BP was responsible for this activity, as no cleavage was observed when NHS depleted of C4BP was used (Fig. 9, lane 4). Purified C4BP and recombinant C4BP showed cleavage of the  $\alpha$ -chain (Fig. 9, lanes 5 and 6); however, in this assay, the cleavage products appeared at a lower intensity, as less C4BP was used for cleavage than in serum.



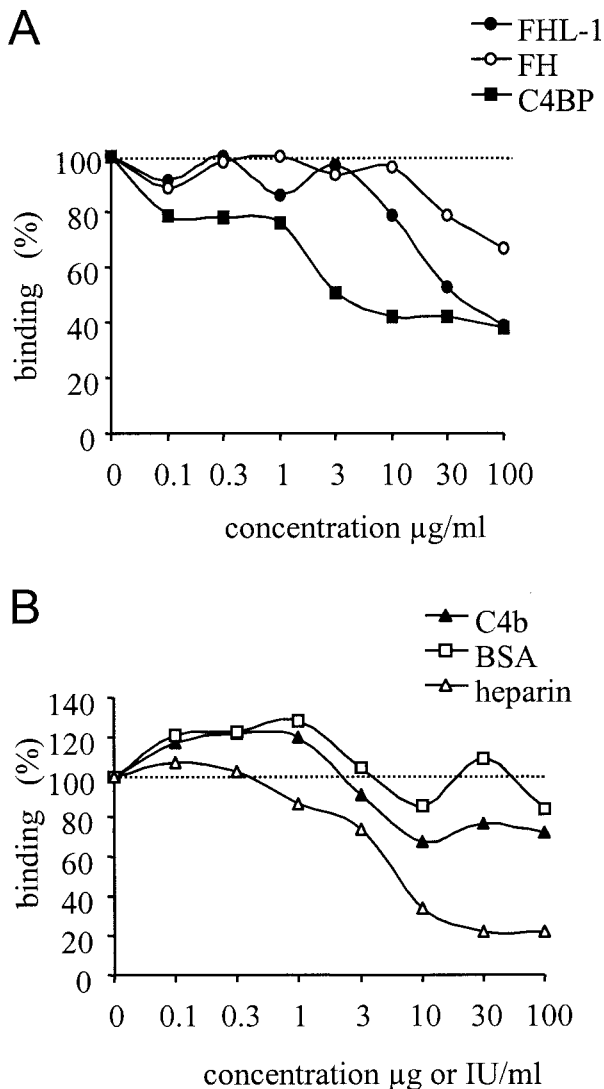


FIG. 8. Inhibition of binding of C4BP to *C. albicans* cells with FHL-1, factor H, and C4BP (A) and with C4b, heparin, and BSA (B). *C. albicans* hyphal forms ( $10^9$ /assay) were used in direct binding assays. Hyphae were incubated with  $^{125}$ I-C4BP at the indicated concentrations of purified proteins in the presence or absence of the indicated inhibitors. After 20 min of incubation, the bound protein was separated from the unbound protein by centrifugation through a sucrose column. Binding is expressed as a percentage of bound protein compared to the total protein input. The 100% value is indicated by the dotted line.

**Cell-bound C4BP mediates adhesion to human endothelial cells.** To identify additional consequences of C4BP binding to candida, we tested the effect of surface-attached C4BP on the adhesion of *C. albicans* to human endothelial cells. Upon incubation in either native C4BP purified from plasma or recombinant C4BP, candida cells show a preferred adhesion to human endothelial cells compared to PBS-treated cells (Fig. 10).

**DISCUSSION**

Upon infection of its human host, *C. albicans* is in direct contact with the human complement system. Although candida activate all three pathways of the human complement system,

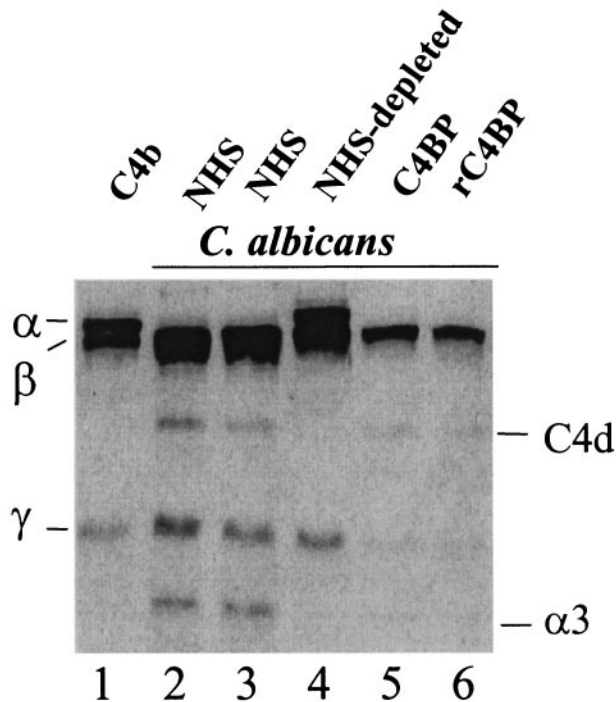


FIG. 9. Cofactor activity of surface-bound C4BP. *C. albicans* cells were preincubated in NHS (lanes 2 and 3), serum depleted of C4BP (lane 4), C4BP purified from human serum (lane 5), or rC4BP (lane 6). The substrate of C4BP-purified C4b is separated in lane 1, and the three chains of the protein ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) are indicated. After extensive washing, biotin-labeled C4b and factor I were added, the mixture was incubated for 30 min, the products were separated by SDS-PAGE, and C4b and its cleavage products were visualized by luminescence. Inactivation of C4b is observed by the disappearance of the  $\alpha'$  band and the appearance of the 45-kDa C4d and the 20-kDa  $\alpha_3$  fragment, as seen in the positive control in lane 2. Cofactor activity of C4BP bound from human serum was detected (lanes 2 and 3), but human serum depleted of C4BP showed no cofactor activity (lane 4). Purified C4BP and rC4BP used at lower concentrations also showed cofactor activity (lanes 5 and 6).

this pathogenic yeast survives the destructive effects of the activated complement system. Binding of soluble human complement regulators is one mechanism for immune evasion, and we show here that *C. albicans* binds the CP inhibitor C4BP to

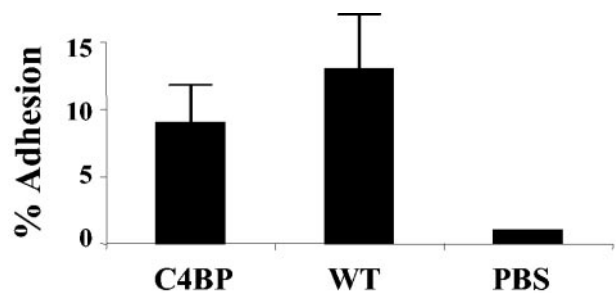


FIG. 10. C4BP attached to the *C. albicans* surface mediates adhesion to human endothelial cells. HUVEC cells grown on microtiter plates were incubated with *C. albicans* cells preincubated in native C4BP purified from human plasma or rC4BP. The results show the mean values of the results from three independent experiments. The standard deviations are indicated. WT, wild type.



its surface. Binding of C4BP from human serum as well as purified native C4BP was shown by immunofluorescence, flow cytometry, and serum absorption experiments. The specificity of binding was confirmed by using human serum depleted of C4BP. Results for flow cytometry are shown (Fig. 2A), and similar results were observed in immunofluorescence and immune absorption assays (data not shown). Immunofluorescence analysis also indicated binding to the tip region of the hyphae. The bound C4BP maintains its regulatory activity and contributes to the degradation of human C4b. Again, this specific role of the attached C4BP was confirmed by using C4BP-depleted human serum. The absence of C4BP in serum resulted in the lack of cofactor activity at the surface of *C. albicans* (Fig. 9).

The domain of C4BP relevant for binding to *C. albicans* was localized to the two most N-terminal CCPs of the  $\alpha$ -chain. Deletion mutants lacking CCP1 and CCP2 showed reduced binding in an ELISA, and heparin, which binds to the CCP1-2 of C4BP (28), strongly inhibited binding of C4BP to *C. albicans* (Fig. 6). The multimeric intact C4BP protein has seven  $\alpha$ -chains and can utilize the same N-terminal domains for binding to multiple ligands. The three most N-terminal CCP domains bind the major ligand C4b (22, 26, 40), and C4b inhibited the binding of C4BP to *C. albicans* (Fig. 8B). The N-terminal CCPs 1 and 2 of C4BP are utilized for interaction with filamentous hemagglutinin of *Bordetella pertussis* (3), and CCP1 is utilized for interaction with *S. pyogenes* M-proteins (4). Due to its multimeric nature, binding via these CCP domains does not arrest the functional activity of the bound C4BP, as was also observed for *C. albicans*. C4BP bound from NHS as well as purified native and recombinant wild-type C4BP maintained the cofactor activity in cleaving C4b in the presence of factor I.

Competition assays indicate that FHL-1, but not factor H, competes with C4BP for binding to the surface of *C. albicans* (Fig. 8B), and it is therefore speculated that at least two receptors on the surface mediate binding of the host regulators. One of these receptors binds C4BP and FHL-1, and the other is specific for factor H. As binding of C4BP and/or factor H and FHL-1 is a common phenomenon among species of the *Candidae*, at least one of these binding moieties may represent a conserved molecule (Fig. 4). Mannoproteins and  $\beta$ -glucans are abundant and common cell wall components of yeast (46), and these components may bind the host regulators through ionic interactions. The binding of C4BP to *C. albicans* was affected by salt (Fig. 7), demonstrating the ionic nature of this interaction.

Binding of all three regulators is observed with both hyphal and cellular forms, and a prominent attachment site is located within the tip of the hyphae. At the moment, the binding moiety(ies) of candida cells and hyphae are unknown and are the subject of further studies. A number of microbes is known to date to bind human C4BP. The filamentous hemagglutinin surface protein of *B. pertussis* mediates binding of C4BP, but the nature of another binding component on the cell surface is yet unknown (3, 4). C4BP binds to other yeast species, including the nonpathogenic *S. cerevisiae* (Fig. 4). A similar mechanism has recently been reported when the binding of plasminogen to *C. albicans* was analyzed. This human plasma protein also binds to the nonpathogenic *S. cerevisiae* (11). Thus, the

binding of host regulatory plasma proteins may be a general property of yeast cells and may enable opportunistic pathogenicity.

Outer membrane protein A (OmpA) of *Escherichia coli* binds CCP3 of the  $\alpha$ -chain of C4BP, and this binding contributes to the serum resistance of *E. coli* (44). C4BP is also captured via M-proteins by *S. pyogenes* and by porins and pili of *N. gonorrhoeae*. In both cases, the binding of C4BP mediates the serum resistance of the bacteria (4).

In addition to *C. albicans*, binding of the three soluble host complement regulators, C4BP, factor H, and FHL-1, has been previously shown for two other pathogenic microbes, e.g., *S. pyogenes* and *N. gonorrhoeae*. *S. pyogenes* binds C4BP, FHL-1, and factor H via the M-proteins, C4BP and FHL-1 bind to overlapping domains and to the hypervariable N-terminal region (30, 31, 50), and factor H binds to the conserved C region of the M-proteins (19). Similarly, *N. gonorrhoeae* uses the surface-expressed porins 1A and 1B to bind both C4BP and factor H (46). The binding of C4BP is important in complement evasion for *Neisseria*, as it mediates the serum resistance of the analyzed strains (45).

*C. albicans* utilizes the three human complement regulators, the CP regulator C4BP (this work) and the two AP regulators factor H and FHL-1 (38), of the host for protection and immune evasion. Binding of the three host complement inhibitors, also observed for *S. pyogenes* and *N. gonorrhoeae*, arrests complement activation at the initial level, to the amplification loop. This prevents the attachment of opsonins to the microbial surface and, as a consequence, improves survival during the initial phase of infection.

Bound to the surface of *C. albicans*, the immune regulator C4BP serves multiple functions. In addition to downregulation of complement (Fig. 9), the attached host protein enhances the adhesion of candida to host endothelial cells (Fig. 10). A similar phenomenon was recently observed for the AP regulator FHL-1. Attached to the Fba protein of *S. pyogenes*, FHL-1 mediates and enhances ingestion into epithelial cells (42). The relevance of this adhesion for the pathogenesis of candida and the general role of surface-attached C4BP needs further evaluation.

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