Secreted pH-Regulated Antigen 1 of Candida albicans Blocks Activation and Conversion of Complement C3

Shanshan Luo, Andrea Hartmann, Hans-Martin Dahse, Christine Skerka and Peter F. Zipfel

J Immunol 2010; 185:2164-2173; Prepublished online 19 July 2010; doi: 10.4049/jimmunol.1001011
http://www.jimmunol.org/content/185/4/2164

Supplementary Material  http://www.jimmunol.org/content/suppl/2010/07/20/jimmunol.1001011.DC1

Why The JI?

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication

*average

References  This article cites 61 articles, 25 of which you can access for free at: http://www.jimmunol.org/content/185/4/2164.full#ref-list-1

Subscription  Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2010 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Secreted pH-Regulated Antigen 1 of *Candida albicans* Blocks Activation and Conversion of Complement C3

Shanshan Luo,* Andrea Hartmann,* Hans-Martin Dahse,* Christine Skerka,* and Peter F. Zipfel*†

The complement system forms the first defense line of innate immunity and is activated within seconds upon infection by human pathogenic yeast *Candida albicans*. In this study, we identified a new complement evasion strategy used by *C. albicans*. The fungus secretes a potent complement inhibitor, pH-regulated Ag 1 (Pra1), which in the direct surrounding of the pathogen binds to fluid-phase C3 and blocks cleavage of C3 to C3a and C3b, as shown by ELISA, native gel electrophoresis, and Western blotting. Consequently, complement activation via the alternative and classical pathways is inhibited. In addition, the release of the anaphylatoxins C3a and C5a, as well as C3b/iC3b surface deposition, is reduced, as demonstrated by Western blotting, ELISA, confocal microscopy, and flow cytometry. By reducing C3b/iC3b levels at the yeast surface, Pra1 decreases complement-mediated adhesion, as well as uptake of *C. albicans* by human macrophages, as shown by flow cytometry. Thus, Pra1 is, to our knowledge, the first potent fungal complement inhibitor that favors *C. albicans* immune escape by inactivating and controlling host complement attack at the level of C3. *The Journal of Immunology*, 2010, 185: 2164–2173.

*Candida albicans* is an opportunistic human pathogenic fungus that causes life-threatening infections, as well as severe disease, particularly in immunocompromised patients (1). In recent years, the number of resistant *Candida* strains is continuously and rapidly increasing. Despite the currently existing antifungal therapies, mortality and morbidity caused by this human pathogenic fungus are still unacceptably high (2–4). Therefore, it is of general and practical relevance to characterize the immune escape mechanisms of *C. albicans* on a molecular level and to identify novel molecules that mediate fungal infection.

The human complement system is an essential and effective part of innate immunity and, in its normal setting, directly attacks any invading microbe (5–8). Thus, to survive, pathogens have to escape this central immune barrier. The complement system is activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on activated via three major pathways. The alternative pathw
In addition to acquisition of complement regulators, expression of endogenous complement inhibitors is another mechanism used by pathogenic microbes to inactive complement activation. Pathogens like *S. aureus* express extracellular fibrinogen-binding protein (Efb) and its homologous protein (Efb homologous protein) to bind C3, and further inhibits the substrate C3 conversion by the C3 convertases (38, 39) and also expresses Staphylococcal complement inhibitor to bind and stabilize the assembled C3 convertases (40, 41). In addition, *S. aureus* expresses the chemotaxis inhibitory protein to bind and neutralize the C5a receptor on the surfaces of neutrophils and monocytes, thus blocking the anaphylotoxin C5a-mediated chemotaxis (42–44).

Prl of *C. albicans* with 299 aa is a glycosylated protein. PRA1, PHR1, and PHR2 are three pH-regulated genes of *C. albicans* that are involved in morphogenesis (45). Prl is located on the fungal surface and is also sequestered into culture supernatant by both yeast and hyphal forms of *C. albicans* (46–48). Prl, which was originally identified as a fibrinogen-binding protein (46), binds human complement regulator factor H, FHL-1, and plasminogen (23), and is also a ligand for the integrin CR3 (alternative names: α5β2 or CD11b/CD18), which is expressed on the surface of human leukocytes (47).

In this study, we identify secreted *Candida* Prl as the first fungal complement inhibitor. Prl complexes C3 in solution, thereby blocking C3 activation and conversion by surface-bound C3 convertases of both the AP and CP/LP. Thus, downstream events, such as the complement amplification, generation of the anaphylatoxins C3a and C5a, as well as C3b/C3b surface deposition are inhibited. This blockade of the human complement response reduces inflammation and C3b/C3b-mediated adhesion and uptake of yeast by human macrophages.

Materials and Methods

**Cultivation of *C. albicans* strains and human cell lines**

The *C. albicans* wild-type strain SC5314 (49, 50) was cultivated in yeast–peptone–dextrose medium (2% [w/v] glucose, 2% [w/v] peptone, 1% [w/v] yeast extract) at 30°C. Yeast cells were collected by centrifugation and resuspended in a hemocytometer (Fein-Optik, Bad Blankenburg, Germany). Human monocytic cells (THP-1) were grown in RPMI 1640 medium, supplemented with 10% FCS, 1% ultraglutamine 1, and 0.055% gentamicin (Lonza) overnight at 4°C on a shaker, as described (51). Unbound anti-Factor H was removed by washing twice with DPBS. Then active NHS (500 μl) was added, and the mixture was incubated for 1 h at 4°C. Next, serum was separated by centrifugation and used directly.

**Hemolytic assay**

NHS (10%) or factor H-depleted, complement active human serum (Δfactor H-HS; 30%) was pretreated with different amount of Prl in DPBS (20 μl) for 20 min at 37°C, then added to rabbit or sheep erythrocytes (1 × 10⁷/sample; BioTrend, Köln, Germany) suspended in 80 μl Mg-EGTA buffer specific for AP activation (20 mM HEPES, 144 mM NaCl, 7 mM MgCl₂, 10 mM EGTA [pH 7.4]) for 30 min at 37°C. Erythrocyte lysis was determined at 414 nm.

**ELISAs**

Prl and *S. aureus* Efb (0.5 μg/well dissolved in carbonate-bicarbonate buffer) were immobilized onto a microtiter plate (Maxisorb; Nunc, Wiesbaden, Germany) at 4°C overnight. After washing, nonspecific binding sites were blocked with 0.2% gelatin for 2 h at room temperature (RT). Then C3 and C3b (1 μg/well in 100 μl DPBS) were added and incubated for 1.5 h at RT. Wells were washed with DPBS buffer containing 0.05% Tween 20 and the primary polyclonal anti-C3 serum was added for 1 h at RT, followed by HRP-conjugated secondary rabbit anti-anti-serum. Bound proteins were detected using tetramethylbenzidine as a substrate (BioTrend, Köln, Germany). The reaction was stopped with 2 M H₂SO₄, and the OD was determined at 450 nm using an ELISA plate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA). C5a ELISA was performed by using a C5a ELISA kit (DRG Instruments, Marburg, Germany), according to the manufacturer’s instructions.

**Analysis of C3a and C5a generation**

For activation of AP, NHS (7.5%) or Δfactor H-HS (7.5%) was incubated with heat-treated *C. albicans* (1 × 10⁹) or zymosan (1 mg/ml) in the presence or absence of Prl in AP-specific buffer (3 mM MgCl₂ and 1 mM EGTA in DPBS) (100 μl). After incubation for 30 min at 37°C, the supernatant was separated by SDS-PAGE and transferred to a membrane, and C3a levels were analyzed by Western blotting using polyclonal rabbit anti-C3a (23), or by ELISA (TecoMedical, Bonn, Germany). For C5a generation, NHS was activated by zymosan in presence or absence of Prl in AP-specific buffer (3 mM MgCl₂ and 1 mM EGTA in DPBS), and then C5a generation was quantified by ELISA.

**Analysis of C3 convertase of the AP**

The inhibitory effect of Prl on C3 convertase formation was analyzed as described, with slight modifications (52). Briefly, NHS (30%) was activated by zymosan (3 mg/ml) for 30 min in HBS⁺ buffer (20 mM HEPES, 140 mM NaCl, 5 mM CaCl₂, 25 mM MgCl₂ [pH 7.4]). After washing, the particles were incubated in DPBS for 30 min at 37°C (to dissociate C3 convertases). Then either C3b-coated zymosan or factor B (40 μg/ml), factor D (1 μg/ml), and factor P (4 μg/ml) were preincubated with Prl for 15 min at RT and combined together. After 30-min incubation at 37°C, C3bBb levels were analyzed by flow cytometry using BiB mAb (Quidel, San Diego, CA).

The ability of Prl to dissociate a preformed C3 convertase was assayed, as described (53). Briefly, C3b (250 ng/ml) was coated on microtiter plates; factor B (500 ng), factor D (25 ng), and factor P (1 μg) were added; and the mixture was incubated for 1 h at 37°C. After washing, Prl (1, 0.1, 0.0, 5.0, 1.0 mg in 100 μl buffer) or factor H (0.1, 0.2, 0.5, 1.0 μg in 100 μl buffer) was added and incubated for 1 h at RT. Following additional washing steps, C3 convertases attached on the plate were quantified with polyclonal goat anti-factor B serum (Calbiochem, Darmstadt, Germany).

To analyze whether Prl binds and stabilizes C3 convertase formed on the zymosan surface, zymosan (1 mg/ml) or *C. albicans* was incubated with NHS (7.5%) in AP-specific buffer. The particles were pelleted and washed, and different amounts of Prl (1, 2, and 5 μg in 100 μl buffer) were added for 30 min at 37°C. Bound Prl was determined by flow cytometry using a Penta-His Ab (1:100) and an Alexa Fluor-488-labeled rabbit anti-mouse (1:200) as a secondary antiserum. In parallel, 10 μg undiluted sample from each sample was treated under the reducing condition, separated by SDS-PAGE, transferred to a membrane, and then detected by Western blotting using a Penta-His Ab.

To analyze whether Prl, by binding to C3 in the fluid phase, inhibits C3 cleavage, C3 convertase was preformed on zymosan surface, as described (52). C3, preincubated with Prl, was added to and incubated with this preformed C3 convertase for 30 min at 37°C. The supernatant was separated by SDS-PAGE, and C3a level was analyzed by Western blotting using polyclonal rabbit anti-C3a serum or by ELISA.
Analysis of Pra1 interaction with C3 in solution

To analyze complex formation of Pra1 and C3 in solution, Pra1 used at increasing concentrations (i.e., 0.25, 0.5, 1, and 2 μg) was incubated with constant amounts of C3 (1 μg) in DPBS (100 μl) for 30 min at RT. Then Pra1-C3 complexes were attached to an ELISA plate that was coated with polyclonal C3 antisera raised in goats (1:5000) for 1 h at RT. After washing, Pra1 complexed to C3 was identified with a polyclonal Pra1 antiserum raised in rabbits (1:2000), followed with swine anti-rabbit serum.

In addition, native gel electrophoresis was used to further prove the Pra1-C3 interaction, and to visualize Pra1-C3 complex formation. Pra1, used at increasing amounts (0.25, 0.5, 1, 2 μg), or HSA (negative control) was incubated with constant amounts of C3 (1 μg) in DPBS (50 μl) for 30 min. Then the samples (20 μl/sample) were separated by 8% native gel at 4°C and bands were visualized by silver staining.

C3b/C3b deposition on yeast surfaces

Heat-treated S. aureus (1 × 10⁷) was incubated with NHS (7.5%), Δfactor H-HS (7.5%), or Δfactor B-HS (7.5%) in the presence or absence of Pra1, or S. aureus inhibitor Efb (used as a positive control upon AP activation) (52, 54) for 30 min at 37°C in AP-specific buffer or dextrase–gelatin–veronal buffer (2.5 mM Veronal buffer [pH 7.4], 75 mM NaCl, 2.5% dextrase, 0.05% gelatin, 0.15 mM Ca²⁺, 0.5 mM Mg²⁺) (100 μl reaction volume). After centrifugation, the cells were washed with DPBS and kept at RT for 30 min to dissociate C3 convertases. Deposition of C3b/C3b on S. aureus surface was determined by flow cytometry (LSR II; BD Biosciences, Heidelberg, Germany) using a polyclonal goat anti-C3 serum and an Alexa Fluor-488–labeled rabbit anti-goat serum. For confocal microscopy, the C3b/iC3b-opsonized S. aureus cells prepared as above were further stained with DAPI (10 μg/ml) for 10 min at RT and fixed, and then C3b opsonization, DAPI, and differential interference contrast were examined by confocal microscopy (LSM 510; Carl Zeiss, Oberkochen, Germany).

Adhesion and phagocytosis assays

Adhesion and phagocytosis of C. albicans (1 × 10⁷) were assayed by flow cytometry. Monocytic cells (1 × 10⁶/well) were stimulated with PMA (2 μg/ml) in 24-well culturing plate (Greiner Bio-One, Solingen, Germany) for 16 h. Then the cells, which were kept in FCS-free RPMI 1640 medium, were stained with Vybrant 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO; Invitrogen, Karlsruhe, Germany) (1:100) for 40 min at 37°C. After removal of unattached yeast cells by extensive washing, human cells were detached from the surface of culture plate. Macrophages that adhered and phagocytosed C. albicans cells were quantified as double-positive (DiO+, DAPI+) cells by flow cytometry.

Results

Pra1 binds to C3 and to the activation product C3b

As C3 is the central molecule in the complement system and as several pathogen-encoded complement inhibitors bind to C3 (32, 54), it was of interest to assay whether Pra1 also binds to C3. Pra1 was immobilized, and C3 and C3b were added as ligands. Both C3 and C3b bound to Candida Pra1. C3 binding was stronger than C3b (Fig. 1, black columns). In comparison, C3 and C3b also bound to the immobilized S. aureus inhibitor Efb (Fig. 1, hatched columns).

Pra1 is a complement inhibitor

Showing that Pra1, which is secreted by both yeast and hyphal forms of C. albicans, binds to C3 and C3b suggested a complement-regulatory activity of Pra1. In a hemolytic assay with NHS and rabbit erythrocytes, Pra1 inhibited erythrocyte lysis by ~50% upon AP activation (Fig. 2A). Pra1 was recently identified as a factor H-binding surface protein (23); we therefore asked whether Pra1-mediated complement inactivation and factor H recruitment are related or two separate effects. To this end, Δfactor H-HS was preincubated with Pra1, then added to sheep erythrocytes suspended in Mg-EGTA buffer. After incubation, erythrocyte lysis was measured. Pra1, used at 20 μg/ml, efficiently inhibited erythrocyte lysis also in Δfactor H-HS by ~90% (Fig. 2B, column 4). A similar effect of Pra1 on complement inactivation was detected using rabbit erythrocytes and Δfactor H-HS (Supplemental Fig. 1). Thus, Pra1 is an AP complement inhibitor, and the inhibitory effect is independent from factor H binding.

Pra1 inhibits C3a and C5a generation

As Pra1 binds to C3, as well as C3b, we hypothesized that Pra1 affects complement activation at the level of C3 convertase. To prove this, first NHS was activated either by C. albicans or by zymosan in presence or absence of Pra1. After incubation, the reaction mixtures were separated by SDS-PAGE, and C3a generation was followed by Western blotting. Pra1 inhibited C3a formation upon AP activation by C. albicans (Fig. 3A, upper panel, lanes 2–5) or by zymosan (Fig. 3A, lower panel, lanes 2–5). This effect was dose dependent. At 100 μg/ml, Pra1 completely inhibited C3a release (Fig. 3A, upper and lower panels, lanes 5). Thus, Pra1 inhibits AP activation at the level of C3 convertase.

FIGURE 1. Pra1 binds to C3 and to C3b. Binding of C3 and the activation product C3b to rPra1 and S. aureus Efb (as a positive control) was assayed by ELISA. Pra1, Efb, and gelatin (negative control) were immobilized and after blocking, C3 and C3b were added in the fluid phase. After incubation, binding was assayed using polyclonal goat C3 antisera. Data represent the mean values ± SD of three separate experiments.

FIGURE 2. Candida Pra1 is a complement inhibitor. A. Pra1 inhibits rabbit erythrocyte lysis in NHS. NHS (10%) was preincubated with indicated amount of Pra1 (columns 4 and 5), and then rabbit erythrocytes suspended in Mg-EGTA buffer were added. After incubation, cell lysis was measured at 414 nm. The background lysis is shown as buffer control (column 1); the maximal lysis is shown by H2O control (column 2). B. Pra1 inhibits sheep erythrocyte lysis in Δfactor H-HS. ΔFactor H-HS (30%) was preincubated with Pra1 (20 and 50 μg/ml), and then sheep erythrocytes suspended in Mg-EGTA buffer were added. After incubation, cell lysis was measured at 414 nm. Complete erythrocyte lysis was shown by H2O control (column 2). The background lysis was determined by the buffer control (column 1). Data represent the mean values ± SD of three separate experiments.
To confirm that this inhibitory effect of Pra1 on C3 conversion is independent of factor H binding, again the inhibitory effect of Pra1 on complement activation was analyzed in Δfactor H-HS. Pra1 still blocked C3a generation when the AP was activated by incubation of either C. albicans (Fig. 3B, upper panel) or zymosan particles (Fig. 3B, lower panel) in Δfactor H-HS. This effect was dose dependent, and at 50 μg/ml Pra1 almost completely inhibited C3a generation (Fig. 3B, upper and lower panels, lanes 4). Thus, the inhibitory effect of Pra1 on C3a generation is independent of factor H binding, indicating that Pra1-mediated complement inhibition and factor H recruitment are two separate effects.

To further prove the effect of Pra1 on the C3a generation, NHS or Δfactor H-HS was activated by C. albicans or zymosan in the presence or absence of Pra1, and then the C3a generation was further analyzed by ELISA. Upon incubation of C. albicans in NHS (Fig. 3C, black columns) or Δfactor H-HS (Fig. 3C, hatched columns), Pra1 inhibited C3a generation in a dose-dependent manner. At 20 μg/ml, Pra1 almost completely inhibited C3a generation (Fig. 3C). Similarly, upon incubation of zymosan in NHS (Fig. 3D, black columns) or Δfactor H-HS (Fig. 3D, hatched columns), Pra1 inhibited C3a generation in a dose-dependent manner.

**FIGURE 3.** Pra1 inhibits C3a and C5a generation. A. Pra1 inhibits C3a generation. NHS was activated by C. albicans (upper panel) or by zymosan (lower panel) in the presence of indicated amounts of Pra1 (lanes 2–5) using AP-specific buffer. Following incubation, supernatants were separated by SDS-PAGE and transferred to a membrane, and C3a was visualized by Western blotting using polyclonal rabbit anti-C3a serum. Factor H and factor I were used as a positive control (lanes 6), and gelatin as a negative control (lanes 7). Mobility of purified C3a is shown in lanes 8. B, Pra1 inhibits C3a generation also in Δfactor H-HS. Again, C. albicans (upper panel) or zymosan (lower panel) was added to Δfactor H-HS in the presence or absence of indicated amounts of Pra1 (lanes 2–5). Following reaction, C3a generation was detected by Western blotting. Factor H and factor I were used as the positive control (lanes 6), and gelatin as a negative control (lanes 8). Serum without activation was separated in lanes 7. The data show a representative result of three separate experiments. C and D, Effect of Pra1 on C3a generation shown by ELISA. NHS (black columns) or Δfactor H-HS (hatched columns) was activated by incubation with C. albicans (C) or zymosan (D) in the presence of different amounts of Pra1, and C3a generation was determined by ELISA. Pra1 dose dependently inhibited C3a generation. Factor H and factor I, which were used as a positive control, efficiently inhibited C3a generation, whereas gelatin, used as a negative control, showed no effect. E, Pra1 inhibits C5a generation. NHS was activated by zymosan (1 mg/ml) in presence or absence of indicated amounts of Pra1 (columns 2–4). C5a generation in supernatants was quantified by ELISA. Gelatin as a negative control did not inhibit C5a generation (column 6). However, factor H and factor I as a positive control inhibited C5a generation (column 7). Data represent the mean values ± SD of three separate experiments.

As *Candida* Pra1 inhibits C3a generation, we asked whether this secreted fungal protein also affects C5a generation. NHS was activated by zymosan, and generation of C5a in the supernatant was quantified by ELISA. Again, upon AP activation, Pra1 blocked C5a release, and this effect was dose dependent. At 40 μg/ml, Pra1 inhibited C5a generation by 43% (Fig. 3E, column 3). Thus, Pra1 also inhibits C5a generation upon AP activation.

**Pra1 does neither affect C3 convertase formation, nor dissociate a preformed C3 convertase**

To define how Pra1 blocks AP activation at the level of C3 convertase, we asked whether Pra1 by binding to surface-deposited C3b or other components (factor B, factor D, and factor P) affects C3 convertase formation. To this end, Pra1 was preincubated with C3b-coated zymosan, and then factor B, factor D, and factor P were added. In parallel, Pra1 was incubated with factor B, factor D, and factor P in fluid phase and then added to C3b-coated zymosan. Following 30-min incubation and extensive washing, C3 convertase formation was determined on the surface of zymosan particles by flow cytometry using Bb mAb. In this set up, Pra1 did not affect C3 convertase formation (Fig. 4A, black and gray columns).
factor B; FD, factor D; FP, factor P; MFI, mean fluorescence intensity. Convertase levels were analyzed by ELISA using polyclonal FB antiserum. C3b-coated zymosan was pretreated with Pra1, and then Pral-accelerating activity to dissociate a preformed C3 convertase. C3 convertase was determined by flow cytometry using Bb mAb. This effect was dose dependent. Pra1 did not affect C3 convertase formation, nor dissociate a preformed C3 convertase. A, Pral does not affect C3 convertase formation. C3b-coated zymosan was pretreated with Pral, and then combined with FB, FD, and FP (black columns). In parallel, FB, FD, and FP were pretreated with Pral, and then added to C3b-coated zymosan (gray columns). After incubation, C3 convertase formation on zymosan surface was determined by flow cytometry using Bb mAb. B, Pral lacks decay-accelerating activity to dissociate a preformed C3 convertase. C3 convertase was assembled on the surface of microtiter plates, and then Pral (black squares) or factor H (white diamonds) was added. After incubation, C3 convertase levels were analyzed by ELISA using polyclonal FB antiserum. Data represent the mean values ± SD of three separate experiments. FB, factor B; FD, factor D; FP, factor P; MFI, mean fluorescence intensity.

We next investigated whether Pral exerts decay-accelerating activity and dissociates a preformed C3 convertase. To this end, a C3 convertase was assembled on the surface of a microtiter plate using purified complement components. Pral was added, and after incubation the remaining C3 convertases were identified using polyclonal goat anti-factor B serum. Pral did not dissociate the preformed C3 convertase (Fig. 4B, black squares). In contrast, factor H, as a decay-accelerating factor, did dissociate the preformed convertase (Fig. 4B, white diamonds). Thus, Pral does neither inhibit C3 convertase formation, nor does Pral dissociate a preformed C3 convertase.

Pral inhibits complement activation in fluid phase and binds C3 in solution

As Pral does not inhibit C3 convertase formation, and also lacks decay activity to dissociate a preformed C3 convertase, we asked whether Pral, as a C3b-binding protein, binds to and stabilizes a surface-attached C3 convertase. To this end, a C3 convertase was assembled on zymosan surfaces with NHS. After washing, Pral was added to surface-assembled C3 convertases and incubated for 30 min. Bound Pral were determined by flow cytometry. In this case, Pral did not bind to C3 convertase (Fig. 5A), but remained in fluid phase (Fig. 5B). Similarly, Pral did not bind to a C3 convertase assembled on the surface of C. albicans (data not shown). This suggests that Pral inhibits complement activation in fluid phase. To further prove this hypothesis, Pral was first incubated with either NHS or A-factor H-HS, and then rabbit or sheep erythrocytes were added. After incubation, both rabbit and sheep erythrocytes that were protected from lysis by Pral were washed and incubated with polyclonal Pral antiserum to detect whether Pral bound to C3b assembled on the surface of erythrocytes. Again, Pral was not detectable on the surface (data not shown). Based on these results, we conclude that Pral inhibits complement activation in fluid phase, which is different from the factor H recruitment by surface-expressed Pral.

Showing that Pral inhibits complement activation in fluid phase, we hypothesized that Pral binds to C3 substrate in fluid phase and by blocking C3 cleavage inhibits complement activation. To prove this, Pral and C3 complex formation in solution was analyzed by ELISA and by native gel electrophoresis. Pral and C3 were incubated in solution for 30 min at room temperature, and then the complexes were captured by a polyclonal goat C3 antiserum that was coated to a microtiter plate. After washing, Pral, complexed to the C3, was identified with a polyclonal Pral antiserum. When C3 concentrations were kept constant (1 μg/100 μl) and the concentration of Pral was increased, Pral showed dose-dependent binding to C3 (Fig. 5C).

To further prove Pral-C3 complex formation, again Pral and C3 were incubated in solution, and then the complexes were separated by native gel electrophoresis and visualized by silver staining. The Pral-C3 complex was identified as a band with a mobility of ~250 kDa. This mobility fits with the predicted molecular masses of Pral (58 kDa) and C3 (194 kDa) in nonreduced form. This band was only detectable when both Pral and C3 were present (Fig. 5D, lanes 3–6), but it was not detected when single protein (either C3 or Pral) or HSA were used (Fig. 5D, lanes 2, 7, and 8). The intensity of this 250-kDa band correlated with the amount of Pral (Fig. 5D). In this assay, additional bands of slower mobility were detected both for C3 and for Pral. These bands are considered multimers of either C3 or Pral. Thus, Pral binds C3 and forms a complex in solution.

Pral, by complexing C3 in fluid phase, inhibits further C3 cleavage by a surface-attached C3 convertase

Knowing that Pral complexes C3 in solution, we further tested whether Pral, by binding to C3 in solution, prevents C3 cleavage by a preformed, surface-attached C3 convertase. C3 was preincubated with soluble Pral in fluid phase, and then added to a C3 convertase that was assembled on a zymosan surface. After incubation, C3a generation was determined by Western blotting and by ELISA. In this setup, Pral inhibited C3a generation in a dose-dependent manner (Fig. 6A, lanes 2–4, 6B), and used at 20 μg/ml (0.34 μM), Pral inhibited C3a generation completely (Fig. 6A, lane 3, 6B, column 3). Thus, Pral, by complexing C3 in solution, blocks C3 cleavage and thus inhibits further complement progression and amplification.

Pral inhibits C3b/iC3b deposition on C. albicans surface

C3b/fC3b is an important complement effector that is generated upon complement activation. C3b/fC3b deposited onto a surface of a particle or microbe mediates phagocytosis and efficient removal of a tagged particle or microbe (15). As a complement inhibitor, Pral blocks complement activation at the level of C3. To define whether and how this complement inhibitory effect of Pral favors and correlates with C. albicans immune escape, we first asked whether Pral limits C3b/fC3b deposition on the surface of yeast cells. To this end, the role of Pral on C3b/fC3b surface deposition was assayed following incubation of yeast cells with NHS. Upon AP activation, Pral inhibited C3b/fC3b deposition, as demonstrated by confocal microscopy (Fig. 7A) and by flow cytometry (Fig. 7B). This effect was dose dependent. Pral blocked C3b/fC3b surface deposition by ~62% in NHS (Fig. 7B) and by 67% in A-factor H-HS (Fig. 7C) at a concentration of 100 μg/ml. S. aureus Efb (52, 54), as a positive control at 40 μg/ml (equal molar concentration with 100 μg/ml Pral), inhibited C3b/fC3b surface deposition by ~61% in NHS (Fig. 7B, hatched column) and by 72% in A-factor H-HS (Fig. 7C, hatched column), respectively.

Pral inhibits AP activation by blocking C3 conversion. As C3 convertases of both AP and CP/LP are functionally and structurally related, we hypothesized that Pral also inhibits CP/LP activation. Yeast cells were incubated in Δfactor B-HS to allow CP/LP activation, and

**FIGURE 4.** Pral does neither affect C3 convertase formation, nor dissociate a preformed C3 convertase. A, Pral does not affect C3 convertase formation. C3b-coated zymosan was pretreated with Pral, and then combined with FB, FD, and FP (black columns). In parallel, FB, FD, and FP were pretreated with Pral, and then added to C3b-coated zymosan (gray columns). After incubation, C3 convertase formation on zymosan surface was determined by flow cytometry using Bb mAb. B, Pral lacks decay-accelerating activity to dissociate a preformed C3 convertase. C3 convertase was assembled on the surface of a microtiter plate using Bb mAb. In contrast, factor H, as a decay-accelerating factor, did dissociate the preformed convertase (Fig. 4B, white diamonds). Thus, Pral does neither inhibit C3 convertase formation, nor does Pral dissociate a preformed C3 convertase.
FIGURE 5. Pra1 inhibits complement activation in fluid phase and binds C3 in solution. A and B, Pra1 does not bind and stabilize a preformed C3 convertase. Zymosan was incubated with NHS; a C3 convertase was formed on zymosan surface. Following addition and incubation of Pra1 with surface-attached C3 convertase, bound Pra1 was assayed by flow cytometry. Pra1 did not bind to C3 convertase on the surface (A), but was present in the supernatant (B). C, Pra1 complexes C3 in solution. Increasing amount of Pra1 was incubated with constant amount of C3 in DPBS for 30 min at RT. Then the sample was added to ELISA plates, which were coated with polyclonal C3 antiserum raised in goats (1:5000) for 1 h at RT. After washing, Pra1, complexed with C3, was identified with a polyclonal Pra1 antiserum by electrophoresis. Pra1, used at increasing amounts, was incubated with constant amounts of C3 in solution, and the complex formation is dose-dependent. w/o., represents no ligand incubation, only for the Ab control. HSA was used a negative control. D, Pra1-C3 complex formation is further confirmed by native gel electrophoresis. Pra1, used at increasing amounts, was incubated with constant amounts of C3 in DPBS for 30 min. Then the sample was separated by 4% native gel at 4°C, and bands were visualized by silver staining. When both Pra1 and C3 are present, a complex band with a mobility of ~250-kDa was visualized. With increasing amount of Pra1, this band becomes stronger (lanes 3–6). In the presence of HSA (lane 8), or when either Pra1 or C3 was absent (lanes 2 and 7), no band of this mobility was identified. A, B, and D show a representative result of three separate experiments; C represents the mean values ± SD of three separate experiments.

FIGURE 6. Pra1, by complexing soluble C3, blocks further C3 cleavage. A and B, Pra1 complexes C3 in fluid phase and inhibits C3 cleavage. A C3 convertase was preformed on a zymosan surface, purified C3, preincubated with Pra1 in fluid phase, was added to a preformed C3 convertase for 30 min. The reaction mixture was separated by SDS-PAGE, and C3a level was analyzed by Western blotting using polyclonal rabbit anti-C3a serum (A) or by C3a ELISA (B). Pra1 inhibited C3a generation in a dose-dependent manner (A, lanes 2–4, and B, columns 2–4). Gelatin used as a negative control had no significant effect (A, lane 5, and B, column 5). Mobility of purified C3a is shown in A, lane 6. A, A representative result of three separate experiments. B, The mean values ± SD of three separate experiments.
deposition, reduces complement-mediated adhesion and uptake of opsonized *Candida* cells by human macrophages.

**Discussion**

In this study, we identified Pra1, which is secreted by human pathogenic yeast *C. albicans*, as a potent fungal complement inhibitor. Pra1 binds C3 in solution and blocks the further cleavage of C3 to C3b and C3a by surface-attached C3 convertases of both AP and CP/LP. Thus, Pra1 inhibits the complement amplification loop, further progression, inflammation, as well as adhesion and phagocytosis of the yeast cells. Pra1 is, to our knowledge, the first fungal complement inhibitor identified from human pathogenic yeast *C. albicans*.

Pra1 binds predominantly to C3 (Fig. 1) and does not interact with the C3 convertase (Fig. 5). This interaction is of functional relevance: as C3, complexed with *Candida* Pra1, is neither cleaved by a C3 convertase of the AP nor by that of the CP/LP. This effect results in inhibition of the generation of anaphylotoxins C3a and C5a (Fig. 3, Supplemental Fig. 2), as well as C3b/C3b surface deposition (Fig. 7). Consequently, *Candida* Pra1, by inhibiting C3a and C5a release, has an anti-inflammatory effect, and by blocking C3b/C3b surface deposition reduces attachment and uptake of yeast cells by human macrophages (Fig. 8). Thus, the fungal inhibitor Pra1, by controlling important complement reactions, favors survival of the yeast in an immunocompetent host.

*Candida* Pra1 was initially identified as a fibrinogen-binding protein (46) and was recently characterized as a fungal factor H, FHL-1, and plasminogen-binding surface protein (23). In this work, we identified a new function of Pra1. Pra1 is a potent complement inhibitor that blocks complement activation at the level of C3 (Figs. 3, 6). Factor H, as an AP complement regulator, prevents C3 convertase formation and also dissociates a preformed C3 convertase of the AP (56). It was therefore relevant to demonstrate that Pra1-mediated blockage of complement activation is distinct from factor H recruitment. Pra1 showed the same inhibitory activity on sheep and rabbit erythrocyte lysis (Fig. 2B, Supplemental Fig. 1), as well as C3a release and C3b/C3b surface deposition (Figs. 3, 7) in Δfactor H-HS, compared with NHS. The inhibitory effect of Pra1 on CP/LP activation further proves the specific Pra1 functions as factor H controls the AP, but not the CP/LP. Therefore, Pra1 and factor H-mediated complement inactivation are two separate and independent functions.

Pra1 displayed the complement inhibitory effect in the fluid phase, which correlates with the role of the secreted Pra1 and is separated from surface Pra1-mediated complement evasion by recruitment of factor H onto yeast surface. Pra1, secreted by *C. albicans*, complexes C3 in solution (Fig. 5), inhibits C3 cleavage by a preformed C3 convertase (Fig. 6), and consequently blocks further formation of the complement amplification loop and downstream effector functions. This soluble Pra1-mediated complement...
FIGURE 8. Pra1 inhibits C3b/iC3b-mediated adhesion and phagocytosis of yeast cells by human macrophages. C3b/iC3b-opsonized, DAPI-stained yeast cells were incubated with DiO-labeled THP-1 for 15 min. After washing, macrophages alone were detected as single-positive cells (DiO+, DAPI−) (dotted graphs, A for the AP; B for CP/LP, panels I, Q1). THP-1 with adhered and phagocytosed C. albicans cells were identified as double-positive cells (DiO+, DAPI+) (A, B, panels II, Q2). Pra1 reduced the number of double-positive human macrophages in a dose-dependent manner (A, B, panels III–V, Q2). Efb (used at 4 μg, equal molar with 10 μg Pra1) was used as a positive control (A, panel VI). Data show a representative result of three separate experiments. C and D, The mean percentage of Pra1 inhibition on adhesion and phagocytosis from three separate experiments is shown. Pra1, used at 10 μg, reduced both attachment and uptake of opsonized yeast cells by human macrophages by 54% upon AP activation (C, column 5). S. aureus Efb, used at a comparable molar with 10 μg Pra1, inhibited both adhesion and internalization by ~40% (C, column 6). Similarly, upon CP/LP activation, Pra1 inhibited both adhesion and phagocytosis by ~34.8% at 10 μg (D, column 5).

inactivation enhances complement control in the direct vicinity of the yeast and generates an additional protective layer for the yeast against host complement attack. The C3 blocking effect of Pra1 is similar to Efb-C (the C3-inhibitory domain of Efb) from the Gram-positive bacterial S. aureus, which also binds C3 and C3b and blocks C3 cleavage by the C3 convertase, thereby blocking downstream activation of the complement response (54). However, it is also reported that S. aureus Efb binds to C3b containing convertases and blocks complement activation (52). Further investigations will show whether Pra1 and Efb-C, although generated by two different organisms, have overlapping functions. Candida Pra1 definitely acts differently from S. aureus inhibitor Staphyloccocal complement inhibitor, which blocks complement activation by stabilizing C3 convertases (40). As C3 convertases of the CP/LP and the AP are functionally and structurally related, a similar mechanism is also suggested for the inhibition of Pra1 on the CP/LP activation.

Pra1 also inhibits C5a generation (Fig. 3E, Supplemental Fig. 2). However, as Pra1 already blocks complement activation at the level of C3, it is currently unclear whether the blockade of C5a release is a direct effect by inhibiting the assembly of a C5 convertase, or a site effect due to the inhibition of C3 conversion and a blockade of the complement amplification loop. Independent of the exact mechanism, Candida Pra1-mediated inhibition on C3a and C5a generations presumably prevents inflammatory effector functions, like recruitment of immune effector cells to the sites of infection. Thus, secreted Pra1 forms an anti-inflammatory environment that is beneficial for the pathogen and favors C. albicans survival (57, 58).

C3b or iC3b, when deposited onto the microbial surfaces, is recognized by CRs, that is, CR1 and CR3, which are expressed on the surface of human phagocytic cells. This favors both adhesion and phagocytosis (Supplemental Fig. 3). Both effects can be dangerous for pathogens (15). As a potent complement inhibitor, Pra1 inhibits C3b/iC3b surface deposition upon complement activation. The inhibitory effects are of different intensities (by 62% for the AP, by 44% for the CP/LP) (Fig. 7). By decreasing C3b/iC3b surface deposition, Pra1 interferes with C3b/iC3b-mediated adhesion and phagocytosis (Fig. 8) and aids in C. albicans immune escape. As multiple other receptors also play a role for recognition of C. albicans by macrophages, such as TLRs or dectin-1 (59–61), the inhibition on C3b/iC3b-mediated adhesion and phagocytosis by Pra1 is only partial.

Pra1 is secreted by both Candida yeast cells and hyphae, and is identified in culture medium. The semiquantitative approach revealed that within 24 h, 10 million yeast cells generate ~5 μg Pra1 (~50 μg/ml; Supplemental Fig. 4). This concentration correlates with the biological effect observed in this study for rPra1 (10–100 μg/ml). During infection and tissue invasion, local Pra1 levels may even be higher as Pra1 expression is upregulated upon hyphal induction (62). This provides further evidence for the biological relevance of Pra1 in vivo. Thus, locally produced Pra1 represents a potent fungal complement inhibitor that efficiently controls and modulates host complement attack.

Pra1 is both secreted and localized at the surface of both C. albicans yeast and hyphae, and does also bind to human cells (23, 46, 47). Such different localizations allow Candida Pra1 to act at different sites, as follows: 1) as a secreted protein, Pra1 complexes C3 in solution, inhibits C3 conversion by C3 convertases of both AP and CP/LP, and blocks further complement amplification and progression and downstream immune responses; 2) as a surface protein, Pra1 binds human complement regulators factor H, FH1-L, and plasminogen for complement evasion and tissue invasion (23); and 3) Pra1 also binds to the surface of human cells via CR3 receptors (47). This binding may result in a modulation of the intracellular signaling and affect additional immune effector functions.

Thus, as a multifunctional fungal complement inhibitor, Pra1 apparently forms multiple protective layers that shield the human pathogenic yeast C. albicans from the different host complement and immune attacks. Pra1 provides an example for the multiplicity and complexity of the immune escape that are contributed by one single fungal virulence factor. A detailed understanding of these multiple roles of Pra1 allows to define new strategies to interfere with and fight against Candida infection.

Acknowledgments

We thank Michael Reuter and Sascha Böhm from the Department of Infection Biology of the Leibniz Institute for Natural Product Research and Infection Biology, Hans Knöll Institute, for providing the rEfb protein of S. aureus.
Disclosures

The authors have no financial conflicts of interest.

References


