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The secreted *Candida albicans* protein Pra1 disrupts host defense by broadly targeting and blocking complement C3 and C3 activation fragments

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ABSTRACT

Candida albicans the most frequently isolated clinical fungal pathogen can cause local as well as systemic and life-threatening infections particularly in immune-compromised individuals. A better and more detailed understanding how *C. albicans* evades human immune attack is therefore needed for identifying fungal immune-evasive proteins and develop new therapies. Here, we identified Pra1, the pH-regulated *C. albicans* antigen as a hierarchical complement inhibitor that targets C3, the central human complement component. Pra1 cleaved C3 at a unique site and further inhibited effector function of the activation fragments. The newly formed C3a-like peptide lacked the C-terminal arginine residue needed for C3a-receptor binding and activation. Moreover, Pra1 also blocked C3a-like antifungal activity as shown in survival assays, and the C3b-like molecule formed by Pra1 was degraded by the host protease Factor I. Pra1 also bound to C3a and C3b generated by human convertases and blocked their effector functions, like C3a antifungal activity shown by fungal survival, blocked C3a binding to human C3a receptor-expressing HEK cells, activation of Fura2-AM loaded cells, intracellular Ca^{2+} signaling, IL-8 release, C3b deposition, as well as opsonophagocytosis and killing by human neutrophils. Thus, upon infection *C. albicans* uses Pra1 to destroy C3 and to disrupt host complement attack. In conclusion, candida Pra1 represents the first fungal C3-cleaving protease identified and functions as a fungal master regulator of innate immunity and as a central fungal immune-escape protein.

1. Introduction

The frequency of opportunistic fungal infections increases at an alarming rate and is a cause of serious health problems. *Candida albicans* the most frequently clinical isolated human fungal pathogen can cause local as, well as systemic, life-threatening infections particularly in immune-compromised individuals (Alves et al., 2017; Jong et al., 2001; Olczak-Kowalczyk et al., 2016). Despite currently available antifungal therapies, *C. albicans* induced mortality and morbidity remain high (Alonso-Valle et al., 2003; Gudlaugsson et al., 2003; Pappas et al., 2003). Over 75% of patients with systemic candidemia die. Simultaneously, treatment-resistant *C. albicans* strains are increasing, while vaccine development has remained challenging (Iannitti et al., 2012). A better and more detailed understanding how *C. albicans* evades human

Abbreviations: Pra1, pH-regulated antigen 1; TCC, terminal complement complex; FHL-1, Factor H like protein 1; C4BP, C4b binding protein; DPBS, Dulbecco's Phosphate Buffered Saline; CR3, complement receptor 3; NHS, normal human serum; C3aL, C3a like protein; C3bL, C3b like protein; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; GFP, green fluorescence protein; ABESF, 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride; Gpm1, phosphoglycerate mutase 1; GPD2, Glycerol-3-phosphate dehydrogenase 2

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immune attack is of interest and provides a basis to identify new fungal immune evasive proteins to develop new therapies.

The human host uses an efficient and sophisticated immune system to protect itself from infectious microbes. The human complement system, as a central component of innate immunity, controls immune homeostasis and recognizes and eliminates infectious agents. Complement, which forms the first defense line of innate immunity, is conserved among many species (Walport, 2001; Zipfel and Skerka, 2009). Complement is relevant for controlling fungal infections and complement deficient animals present with a higher fungal burden (Luo et al., 2009; Mullick et al., 2004; Tsoni et al., 2009). Complement is activated immediately upon infection (Zipfel, 2009; Zipfel and Skerka, 2009) via the alternative (AP), classical (CP), and lectin (LP) pathways. C3 convertases are formed which cleave the central complement protein C3 generate the anaphylatoxin C3a and the opsonin C3b. C3a is a chemoattractant with antifungal and antimicrobial activity (Sonesson et al., 2007). C3a binds to the C3a receptor (C3aR) on immune cells (Heeger and Kemper, 2012; Klos et al., 2013) and initiates inflammatory responses like granule release, generates oxygen radicals, chemotaxis, regulates histamine release, smooth muscle contraction, and increases vascular permeability (Dutow et al., 2014; Klos et al., 2013). C3b, the second product generated by the host C3 convertase, acts as an opsonin and induces opsonophagocytosis (Walport, 2001; Zipfel and Skerka, 2009). In addition, C3b attached to a microbial surface initiates the amplification loop of complement and generates additional C3 convertases. This allows C5 convertase generation and initiates the terminal complement complex (TCC) formation, which results in cell lysis and inflammation (Zipfel and Skerka, 2009).

Candida albicans activates all three complement pathways (Cheng et al., 2012; Luo et al., 2009; Zhang and Kozel, 1998). Mice deficient for the central complement components, i.e. C3 or C5, show an increased mortality and infection associated pathology up on candida infection (Mullick et al., 2004; Tsoni et al., 2009). The activated complement system directs host antifungal defense, but the fungus itself controls host complement attack (Luo et al., 2013b). Candida has established multiple evasion mechanisms to escape host complement attack. Candida acquires host complement regulators, such as Factor H, FHL-1, C4BP, CFHR1, plasminogen and vitronectin from human plasma to its surface (Behnsen et al., 2008; Meri et al., 2004; Meri et al., 2002; Zipfel et al., 2008; Zipfel et al., 1999; Zipfel et al., 2011; Zipfel et al., 2007). The human inhibitors attached to the fungal surface retain regulatory functions and assist in immune evasion (Limper and Standing, 1994; Spreghini et al., 1999; Zipfel and Skerka, 2009). Four candida Factor H and FHL1 binding proteins are identified: pH-regulated antigen 1 (Pra1), glycerol-3-phosphate dehydrogenase 2 (Gpd2), high-affinity glucose transporter 1 (Hgt1) and phosphoglycerate mutase1 (Gpm1) (Lesiak-Markowicz et al., 2011; Luo et al., 2013b; Poltermann et al., 2007; Zipfel et al., 2011). Pra1, Gpm1, and Gpd2 also bind plasminogen and allow conversion to plasmin, which degrades C3 and C3b, thereby, blocking host complement (Luo et al., 2013a). Using a proteome approach eight additional candida plasminogen-binding proteins were identified (Crowe et al., 2003). Candida Pra1 also binds the central complement protein C3 and blocks complement activation (Luo et al., 2010). In addition, secreted *C. albicans* aspartyl proteases (Sap1, Sap2 and Sap3) by degrading and inactivating C3b, C4b and C5 block complement action and effector functions (Gropp et al., 2009).

Candida Pra1 is a 299 amino acid multifaceted fungal protein, which is expressed in alkaline pH and which binds human fibrinogen (Casanova et al., 1992). Pra1 is located in the cytoplasm, on the surface of candida yeast and hyphae, and is secreted. Pra1 expression is upregulated upon hyphae induction and Pra1 is concentrated at the tip of hyphae (Luo et al., 2009; Soloviev et al., 2007). Pra1 also binds to human integrin receptors $\alpha_{M\beta2}$ (CR3) and to $\alpha_{X\beta2}$ (CR4) and blocks CR3 or CR4 mediated recognition and signaling (Soloviev et al., 2007; Jawhara et al., 2012). Also, Pra1 binds mouse $CD4^+$ and blocks proinflammatory cytokine INF-γ and TNF-α (Bergfeld et al., 2017). Secreted Pra1 also sequesters host zinc for endothelial cell invasion (Citiulo et al., 2012).

Previously we showed that Pra1 binds and complexes human C3 and blocks C3 conversion by the host C3 convertases. Here we identify Pra1 as a C3 cleaving protease that blocks the effector functions of the Pra1 generated C3 activation fragments. Moreover, Pra1 also blocks C3a and C3b action when the effector fragments are generated by the human C3 convertase.

2. Material and methods

2.1. Proteins, serum and antibodies

Human C3, C3b, iC3b, C3c, C3d, C3a, C3a-desArg, C4a, C5a, Factor H, and Factor I were purchased from Complement Technology, Inc. Texas, USA. Polyclonal goat anti-human Factor H, polyclonal goat anti-human C3, and polyclonal rabbit anti-human C3a were purchased from Complement Technology, Inc. Texas, USA. Horseradish peroxidase (HRP)-conjugated rabbit anti-goat and HRP–conjugated goat anti-rabbit were obtained from Dako Deutschland GmbH, Hamburg. Human serum was collected from five healthy donors, pooled together, and stored at −80 °C until use. The C3a synthetic peptides were purchased from JPT Peptides, Berlin, Germany. Recombinant Pra1, Gpd2, and Gpm1 were expressed in *Pichia pastoris* strains (Luo et al., 2013a; Luo et al., 2009; Poltermann et al., 2007).

2.2. C. albicans strain, human cell lines and growth conditions

The *Candida albicans* wild type SC5314 (Gillum et al., 1984), Pra1 knockout, and Pra1 overexpression (Citiulo et al., 2012; Luo et al., 2011) strains were cultivated in YPD medium (2% (w/v) glucose, 2% (w/v) peptone, 1% (w/v) yeast extract) at 30 °C. Yeast cells were collected by centrifugation and counted with a hemocytometer (Fein-Optik, Bad Blankenburg).

2.3. Pra1 binding to C3 and C3 activation fragments

C3 and C3 activation fragments C3b, iC3b, C3c, C3d, and C3a (1 μM, 100 μl/well) were immobilized onto the 96 well microtiter plate (MaxiSorb, Nunc) overnight at 4 °C. After washing, the nonspecific binding sites were blocked with gelatin (0.2% in Dulbecco's Phosphate Buffered Saline (DPBS)) for 2 h at room temperature (RT). Following washing, 180 nM of Pra1 was added to the immobilized C3 fragments and the mixture was incubated for 1.5 h at RT. Unbound Pra1 was removed by washing with DPBS-T buffer (DPBS containing 0.05% Tween 20). Then polyclonal rabbit Pra1 anti-serum was added and following incubation for 1 h at RT, HRP-conjugated secondary goat rabbit antibody was added for 1 h at RT. In other orientation, Pra1 (180 nM, 100 μl/well) was immobilized onto a microtiter plate (MaxiSorb, Nunc) overnight at 4 °C. After washing, the nonspecific binding sites were blocked as above, and the wells were incubated with 1 μM of each of C3 and C3 activation fragments C3b, iC3b, C3c, C3d, and C3a for 1.5 h at RT. After washing, the polyclonal goat anti-human C3 serum followed by HRP-conjugated secondary rabbit anti-goat antibody was added for 1 h at RT. After addition of 3,3′,5,5′ Tetramethylbenzidine (TMB, eBioscience, Frankfurt, Germany), the reaction was stopped by addition of 2 M H_2SO_4. Absorbance was measured at 450 nm (SpektraMax 190, Molecular Devices).

2.4. Biolayer interferometry

The binding affinity of Pra1 to C3, C3a or C3b was evaluated by biolayer interferometry in a single channel BLItz system, (Forte Bio, Menlo Park, CA). Ni(II)-NTA biosensors were hydrated for at least 10 min in DPBS with gelatin (0.01%) and loaded with recombinant $(His)_6$-tagged Pra1. After washing the tip briefly (30 s) to remove nonspecifically