The Opportunistic Human Pathogenic Fungus *Aspergillus fumigatus* Evades the Host Complement System

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The opportunistic human pathogenic fungus *Aspergillus fumigatus* causes severe systemic infections and is a major cause of fungal infections in immunocompromised patients. *A. fumigatus* conidia activate the alternative pathway of the complement system. In order to assess the mechanisms by which *A. fumigatus* evades the activated complement system, we analyzed the binding of host complement regulators to *A. fumigatus*. The binding of factor H and factor H-like protein 1 (FHL-1) from human sera to *A. fumigatus* conidia was shown by adsorption assays and immunostaining. In addition, factor H-related protein 1 (FHR-1) bound to conidia. Adsorption assays with recombinant factor H mutants were used to localize the binding domains. One binding region was identified within N-terminal short consensus repeats (SCRs) 1 to 7 and a second one within C-terminal SCR 20. Plasminogen was identified as the fourth host regulatory molecule that binds to *A. fumigatus* conidia. In contrast to conidia, other developmental stages of *A. fumigatus*, like swollen conidia or hyphae, did not bind to factor H, FHR-1, FHL-1, and plasminogen, thus indicating the developmentally regulated expression of *A. fumigatus* surface ligands. Both factor H and plasminogen maintained regulatory activity when they were bound to the conidial surface. Bound factor H acted as a cofactor to the factor I-mediated cleavage of C3b. Plasminogen showed proteolytic activity when activated to plasmin by urokinase-type plasminogen activator. These data show that *A. fumigatus* conidia bind to complement regulators, and these bound host regulators may contribute to evasion of a host complement attack.

*Aspergillus fumigatus* is the most important airborne fungal pathogen. The frequency of invasive mycoses due to this opportunistic fungal pathogen has increased significantly during the last two decades (reviewed in references 4 and 37).

In healthy individuals, *A. fumigatus* can infect the lung, but the establishment of disease is prevented by the host immune system. The innate immune system represents the first line of defense against conidia. Inhaled conidia are immediately confronted with the host complement system and phagocytic cells. The adaptive immune system displays its protective role at the onset of an infection. The complement system is activated on the conidial surface (21) and results in the cleavage of C3. The cleavage products of this central component of the complement cascade act as opsonins on the surfaces of pathogens and enhance phagocytosis by neutrophils, macrophages, and eosinophils (46). Opsonization with complement proteins was shown to be of importance for the phagocytosis of *A. fumigatus* conidia, the key process in the defense against this pathogen (44).

Activation of the complement system occurs via three pathways: the alternative, the lectin, and the classical. The alternative pathway (AP) is activated on the surfaces of pathogens and plays a pivotal role in the clearance of microorganisms (51). Further activation of the terminal pathway leads to the formation of cytolytic membrane attack complexes on the target surfaces. These attack complexes are important for the clearance of some bacterial pathogens but appear to have a minor role in the defense against fungi, most possibly due to their thick cell walls. The complement activation system is controlled by fluid-phase and cell surface-bound regulators. The central fluid-phase regulators of the AP are factor H and factor H-like protein 1 (FHL-1). The latter is encoded by an alternatively processed nuclear RNA transcript derived from the factor H gene (10, 50). Factor H has a molecular mass of 150 kDa and consists of 20 homologous short consensus repeat domains (SCRs), and FHL-1, which has a molecular mass of 42 kDa, is composed of 7 SCRs which are identical to the N-terminal SCRs of factor H. In addition, FHL-1 has a unique C-terminal extension of four amino acids. Both factor H and FHL-1 act as cofactors for the plasma serine protease factor I. Thus, they mediate the cleavage of C3b (24, 32, 36) and accelerate the decay of the C3 convertase C3bBb (26, 49). Factor H and FHL-1 compete with factor B in binding to intact C3b (9). These regulatory functions lead to the downregulation or termination of the complement cascade. The factor H family also comprises factor H-related protein 1 (FHR-1) and five other FHRs, which display high identity to factor H, though, to date, no complement regulatory activity has been assigned. FHR-1 consists of five SCRs and has two plasma forms with either one (37-kDa) or two (43-kDa) carbohydrate side chains attached. Several pathogenic microbes bind host complement regulators to their surfaces and thus mediate immune evasion and the downregulation of complement activation (for a review, see...
Conidia were harvested in Dulbecco’s phosphate-buffered saline (PBS; without MgCl₂) and cultivated on minimal medium agar plates at 37°C for 4 days. Conidia were harvested in Dulbecco’s phosphate-buffered saline (PBS; without MgCl₂) with 0.1% (vol/vol) Tween 20. For liquid culture, 50 ml of Aspergillus fumigatus minimal medium was inoculated with conidium concentrations ranging from 2 × 10⁸ to 10⁹ to obtain young hyphae (6-h incubation) to 2 × 10⁹ to obtain swollen spores (3-h incubation).

Sera, antibodies, and proteins. Pooled normal human sera (NHS) were obtained from healthy human donors and stored at −20°C until used. EDTA was added to the sera at a concentration of 10 mM (NHS-EDTA).

The antibodies used in the experiments were mouse monoclonal antibody (MAb) N22 for the detection of the N-terminal SCRs of factor H and FHL-1 and MAds C18 and M16 for the detection of C-terminal SCR 20 of factor H (34). Polyclonal goat anti-factor H antiserum, polyclonal goat antiplasminogen antiserum, and polyclonal goat anti-C3 antiserum were obtained from Calbiochem (Darmstadt, Germany). Alexa 488-conjugated goat anti-mouse antiserum was obtained from Dako (Glostrup, Denmark). Polyclonal goat anti-factor H antiserum, polyclonal goat antiplasminogen antiserum for the detection of factor H, MAb B22 for the detection of factor H, and FHL-1 (SCRs 1 to 7) were produced in the baculovirus system as described previously (25). Briefly, S. frugiperda (SF9) cells were grown in expression medium (BioWhittaker, Verviers, Belgium) supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml), and amphotericin B (250 μg/ml) and infected with a recombinant baculovirus at a multiplicity of infection of 10. The culture supernatant was collected 9 days after infection, and recombinant proteins were purified by Ni²⁺-chelate chromatography as described previously (25) or by Akta fast protein liquid chromatography purification (GE Healthcare, Freiburg, Germany). The proteins were concentrated using Centriplus microcentrators with a cutoff at 10 kDa (Millipore, Bedford, MA).

Effect of heparin on factor H binding to A. fumigatus. The effect of heparin on the binding of factor H to A. fumigatus was assayed by incubating 5,000 IU/ml of heparin with factor H for 30 min. This mixture was added to conidia (2 × 10⁶) and incubated for 60 min with vertical rotation at 4 rpm. The wash and elution procedures are described in “Serum absorption experiments” above. Heparin (5,000 IU/ml) was obtained from Sigma (Taufkirchen, Germany).

Plasminogen activation. Conidia (5 × 10⁶ to 1 × 10⁷ well) were incubated with plasminogen (1 μg/ml to 2 μg/ml) in 96-well MultiScreen-HTS-BV filtration plates (Millipore, Billerica, MA). Liquids were removed from the wells by vacuum. Conidia were incubated with factor H/heparin for 1 h with horizontal shaking at 400 rpm. After three wash steps with PBS, conidia were incubated with polyclonal goat anti-factor H antiserum (1:4,000 in 0.2% [wt/vol] BSA-PBS) and subsequently with HRP-conjugated anti-antiserum (1:5,000 in 0.02% [wt/vol] BSA-PBS). HRP activity was measured by the addition of a Bio Blue POD substrate (Roche, Mannheim, Germany).

RESULTS

Aspergillus fumigatus conidia bind factor H, FHL-1, and FHR-1 from human serum. To determine whether A. fumigatus binds the human complement regulators factor H, FHL-1, and FHR-1, conidia of strain ATCC 46645 were incubated in NHS-EDTA. After extensive washing, the bound proteins were detected using enhanced chemiluminescence (Amersham, Darmstadt, Germany).
were eluted. The wash and eluate fractions were separated by SDS-PAGE and analyzed by Western blotting. Factor H was detected in the eluate fractions as a 150-kDa protein by an anti-factor H antiserum (Fig. 1A). The FHR-1 protein was also detected by this antiserum as 37-kDa and 43-kDa FHR-1 isoforms (Fig. 1A). The presence of both factor H and FHR-1 in the eluate and not in the wash fractions demonstrates that *A. fumigatus* conidia were able to acquire the human complement regulators factor H and FHR-1 from NHS. In addition, the presence of FHL-1 in the eluate fraction was shown by using a specific MAb which detects 42-kDa FHL-1 but not FHR-1 (Fig. 1B).

**Immunofluorescence of the binding of factor H to conidia of *A. fumigatus***. The binding of factor H to *A. fumigatus* conidia and the surface distribution of the bound immune regulator were analyzed by immunofluorescence. Conidia were incubated with purified factor H, and surface binding was visualized by immunofluorescence microscopy. The specific fluorescence signal confirmed the binding of factor H to the surfaces of the conidia (Fig. 2A to C). A patchy distribution of factor H on the surfaces of the conidia was observed, suggesting clustering of the fungal ligand molecules. No fluorescence was detected on conidia with the secondary antibody alone or without the addition of factor H in the presence of both antibodies (Fig. 2D and E). Specific binding of factor H to conidia was confirmed by fluorescence-activated cell sorter analysis (data not shown).

**Localization of the binding sites within factor H and FHL-1**. To localize the binding domain(s) of factor H and FHL-1, conidia were incubated with recombinant mutants of the proteins encompassing SCRs 1 to 4, 1 to 5, 1 to 6, 1 to 7 (FHL-1), 8 to 11, 11 to 15, 15 to 19, 15 to 20, 19 and 20, and 8 to 20. After incubation, wash fractions (data not shown) and eluate fractions were separated by SDS-PAGE and analyzed by Western blotting. This approach identified two distinct binding regions. One region is located within the N terminus of factor H and FHL-1 in SCRs 1 to 7, as demonstrated by the binding of SCRs 1 to 4, 1 to 5, and 1 to 6 (Fig. 3A). A second binding site, unique to factor H, was localized to the C terminus. Recombinant mutant proteins representing SCRs 15 to 20, 19 and 20, and 8 to 20 (Fig. 3B) bound to *A. fumigatus* conidia, but mutant proteins representing SCRs 8 to 11, 11 to 15, and 15 to 19 did not (Fig. 3B). SCRs 19 and 20 bound with less intensity. The fainter band might be due to the instability of this small protein. Mutant proteins encompassing SCRs 1 to 4 and 1 to 5 were identified as doublet bands. Their identities were validated by mass spectrometry analysis (data not shown).

In summary, factor H uses two binding regions for its attachment to *A. fumigatus* conidia, whereas FHL-1 uses one region. One binding region is located within SCRs 1 to 7 and is common to both factor H and FHL-1. The second region is located in the C-terminal region, in SCR 20, of factor H.

**Heparin inhibits factor H binding to *A. fumigatus* conidia**. Factor H has four heparin interaction sites, which have been localized to SCR 7, SCR 9, SCR 13, and SCR 20 (35, 50). As...
some heparin binding domains of factor H mediate its attachment to microbial surfaces, we asked whether heparin affects the binding of factor H to \textit{A. fumigatus}. At a concentration of 600 IU/experiment (4,000 IU/ml), heparin completely inhibited the attachment of factor H to conidia, as shown by Western blot analysis (Fig. 4A). Quantitative studies showed that the effect was dose dependent. At a concentration of 80 IU/ml, heparin inhibited the binding of factor H to conidia by ca. 50\% (Fig. 4B). These results showed that the binding sites of factor H to \textit{A. fumigatus} overlap or are even identical to the heparin binding sites. This effect is in agreement with the observation that one of the binding sites is located in SCR 20.

\textbf{Binding of plasminogen to conidia of \textit{A. fumigatus}.} Plasminogen is a member of the soluble proteins of the blood. It was previously shown (41) that plasmin, if activated to plasmin, can exhibit proteolytic activity when bound to the surfaces of pathogenic bacteria, such as \textit{B. hermsii} (12) or \textit{P. aeruginosa} (27). This proteolytic activity seems important for the degradation of the proteinaceous matrix, thereby allowing the bacteria to spread into the tissue. Therefore, we analyzed whether plasminogen can also bind to \textit{A. fumigatus}. As shown here, plasminogen bound to \textit{A. fumigatus} conidia. After the incubation of conidia with NHS, plasminogen was detected in the eluate fractions as an \(90\)-kDa protein by Western blot analysis using a polyclonal antiplasminogen goat antiserum (Fig. 5).

\textbf{Different developmental forms of \textit{A. fumigatus} bind to complement regulators.} Conidia of \textit{A. fumigatus} bound to factor H, FHR-1, FHL-1, and plasminogen. Therefore, we analyzed whether other developmental forms of \textit{A. fumigatus}, such as swollen conidia and young hyphae, also bind these host immune regulators. The binding intensity of complement regulators was highest for conidia and lower for swollen conidia. Young hyphae from an overnight culture bound to only a small amount of factor H, FHR-1 (Fig. 6A), FHL-1 (Fig. 6B), and plasminogen (Fig. 6C). Thus, specifically conidia, the developmental form of \textit{A. fumigatus} which is first in contact with the
host immune system, acquire immune regulators factor H, FHR-1, FHL-1, and plasminogen from human sera. Surface-bound factor H displays cofactor activity. We analyzed whether factor H maintains complement-regulating activity when bound to *A. fumigatus* conidia and thereby inhibits complement activation. Conidia were incubated with factor H, and after extensive washing, purified factor I and C3b were added. As a control, conidia were incubated with factor H and factor I alone. After incubation, the products were separated by SDS-PAGE. The proteolytic cleavage of the α′-chain of C3b was assayed by Western blotting. In the presence of factor I and C3b, factor H-coated conidia mediated the cleavage of C3b. This was concluded from the appearance of the α′-chain fragments with molecular masses of 68 kDa and 43 kDa cleavage products. This cleavage pattern is characteristic of factor H-mediated cofactor activity in the factor I-mediated cleavage of C3b (27, 31). Thus, surface-bound factor H maintains cofactor activity. Activation of surface-bound plasminogen. Plasminogen needs to be activated to plasmin to display proteolytic activity. uPA mediates the activation of plasminogen to plasmin. The activation and proteolytic activity of conidia-bound plasminogen by uPA were demonstrated using a colorimetric assay. Activated plasminogen cleaves the chromogenic plasmin substrate S-2251, which results in an increase in absorbance. The addition of uPA and S-2251 to plasminogen-coated conidia resulted in the cleavage of the chromogenic substrate. Thus, plasminogen in its surface-bound form can be activated to plasmin and gains proteolytic activity. In probes lacking plasminogen or uPA, no cleavage of the substrate occurred (Fig. 7B).
The amount of plasminogen applied was sufficient to saturate all available binding sites on the conidia, as no increase in absorbance was observed when the plasminogen concentration was increased (Fig. 7B). However, when the amount of conidia was doubled, the intensity of the signal also increased.

**DISCUSSION**

In this study, we report a novel mechanism of immune evasion for *Aspergillus fumigatus*. *A. fumigatus* conidia bind to the host immune regulators factor H, FHL-1, and FHR-1 as well as plasminogen.

The human innate immune system is essential for the defense against an *A. fumigatus* infection. Phagocytosis and the killing of conidia by phagocytes are key processes to prevent an infection (4). Therefore, evasion from phagocytosis is likely to allow *A. fumigatus* a prolonged survival in the human host. The role of the complement system in the defense against *A. fumigatus*, however, is poorly understood. For *A. fumigatus*, the lung, as the major site of infection, represents an environment which is different from serum. However, several components of both the alternative and the classical pathways are present in human bronchoalveolar fluid except for C4bp (3). Factor H mRNA (3) was identified in RNA prepared from lung tissue, and type II pneumocytes (cell line A549) secrete factor H (42). Thus, the lung is a site for complement activation, and inactivation can occur in vivo. Moreover, plasminogen and plasminogen activator are also readily available in the human lung (2).

Previously, it was shown that the deposition of C3 correlates with pathogenicity. Conidia of the highly pathogenic species *A. fumigatus* and *Aspergillus flavus* bound fewer C3 molecules per unit of conidial surface than conidia of less-pathogenic *Aspergillus* species (15). Opsonization, however, is important for enhanced phagocytosis (44). Conidia of a white (pkpP) mutant of *A. fumigatus* (28), which bound more C3 molecules per unit of conidial surface than the wild type, also showed a higher phagocytosis rate (45). Thus, complement inactivation appears to contribute to the survival of *A. fumigatus* in the human host.

Previously it was shown that *A. fumigatus* conidia activate only the alternative pathway of complement activity, whereas hyphae activate both the alternative and the classical pathway (21). Conidia are the first cells of *A. fumigatus* which invade the host and which are confronted by the host immune system. Evasion from recognition by the innate immune system is crucial for the survival of the pathogen and the onset of the infection. The binding of host complement regulators by conidia might represent a mechanism to inhibit complement activation. Once supplied with nutrients and water, conidia swell and germinate. Unlike conidia, germcells and hyphae bind only a small amount of factor H. Apparently, interaction partners for factor H are specifically expressed on the conidial surface. During germination, the proteinaceous layer of the conidia is shed, and the properties of the surface change. Thus, it is conceivable that receptors present on conidia are no longer found on hyphae. Nevertheless, hyphae are also protected against the activation of the complement system, since it has been shown that they secrete a complement inhibitory factor (47). This still unidentified low-molecular-weight compound was shown to decrease complement activation.

The use of recombinant mutant proteins of factor H identified two binding sites on factor H and FHL-1 which mediate surface attachment to *A. fumigatus*. The first domain, shared by factor H and FHL-1, was located within SCRs 1 to 7, and the second site, which is specific to factor H, within SCR 20. Since it is known that heparin also binds to SCR 20 and, as shown here, inhibits the binding of factor H to conidia, the importance of SCR 20 for binding was further underlined. Factor H binds to other pathogenic fungi with the same two binding sites. For *C. albicans*, it was shown that factor H binds to the surface via SCRs 6 and 7 and via SCRs 19 and 20 (31). With these binding sites, the regulators are oriented with their C-terminal ends to the surfaces of the conidia and the N-terminal complement regulatory regions pointing to the outside (51). This type of attachment allows C3 inactivation in the direct vicinity of the cell surface. As shown here, bound factor H remained functionally active and acted as a cofactor to the factor I-mediated cleavage of C3b.

The acquisition of host proteins, particularly of human complement regulators, seems to represent a central mechanism in immune evasion of human pathogens (for a review, see reference 51). An increasing number of human pathogens which acquire human plasma complement regulators, such as the AP regulators factor H, FHL-1, and FHR-1 as well as the classical pathway regulator C4bp, has been identified. Such organisms include *Streptococcus pyogenes* (20), *S. pneumoniae* (33), *Borrelia burgdorferi* (14, 23), *Yersinia enterocolitica* (5), *Neisseria gonorrhoeae* (39, 40), *N. meningitidis* (38), *Echinococcus granulosus* (8), *Pseudomonas aeruginosa* (27), and the human immunodeficiency virus (43). *C. albicans* (30, 31) binds the three complement regulators factor H, FHL-1, and C4bp. C4bp did not bind to the surfaces of *A. fumigatus* conidia but did bind to those of *A. niger* conidia (data not shown). For some pathogens, the microbial binding proteins responsible for the attachment of the regulators have been identified. These include the M protein of *S. pyogenes* (20); CRASP-1, OspE; and CRASP-2 to -4 of *B. burgdorferi* (14, 22, 23); the sialylated lipooligosaccharide major outer membrane porin of *N. gonorrhoeae* (39); the Hic protein of *S. pneumoniae* (18); Tuf of *P. aeruginosa* (27); and gp120 and gp41 of the human immunodeficiency virus (43). FHR-1 binding proteins were identified as BbCRASP-3 to -5 for *B. burgdorferi* (12) and as Tuf for *P. aeruginosa* (27).

Besides the known regulators of complement activation, another protein was reported to be of importance in immune evasion of microorganisms. Plasminogen can be activated to plasmin by host or microbial plasminogen activators and has proteolytic activity. This activity regulates several physiological processes, e.g., fibrinolysis, the degradation of the extracellular matrix, cell migration, the processing of growth factors, and the formation of metastases of tumors. Plasminogen is bound by various human pathogens, including *C. albicans* (7), *B. hermsii* (41), and *P. aeruginosa* (27). In this study, we showed the binding of plasminogen to *A. fumigatus* conidia. These surface-bound plasminogen molecules could be activated to plasmin and exhibited proteolytic activity.

In conclusion, *A. fumigatus* conidia bind human complement regulators factor H, FHR-1, FHL-1, and plasminogen. The bound host regulators may contribute to the immune evasion of *A. fumigatus* and to the inactivation of an activated human
complement system. The identification and cloning of A. fumigatus molecules involved in the interaction with and binding to human immune regulators are central and essential aspects of future work, which is likely to result in the identification of new virulence determinants.

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