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Secreted *Aspergillus fumigatus* Protease Alp1 Degrades Human Complement Proteins C3, C4, and C5[▽]

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The opportunistic human pathogenic fungus *Aspergillus fumigatus* is a major cause of fungal infections in immunocompromised patients. Innate immunity plays an important role in the defense against infections. The complement system represents an essential part of the innate immune system. This cascade system is activated on the surface of *A. fumigatus* conidia and hyphae and enhances phagocytosis of conidia. *A. fumigatus* conidia but not hyphae bind to their surface host complement regulators factor H, FHL-1, and CFHR1, which control complement activation. Here, we show that *A. fumigatus* hyphae possess an additional endogenous activity to control complement activation. *A. fumigatus* culture supernatant efficiently cleaved complement components C3, C4, C5, and C1q as well as immunoglobulin G. Secretome analysis and protease inhibitor studies identified the secreted alkaline protease Alp1, which is present in large amounts in the culture supernatant, as the central molecule responsible for this cleavage. An *alp1* deletion strain was generated, and the culture supernatant possessed minimal complement-degrading activity. Moreover, protein extract derived from an *Escherichia coli* strain overproducing Alp1 cleaved C3b, C4b, and C5. Thus, the protease Alp1 is responsible for the observed cleavage and degrades a broad range of different substrates. In summary, we identified a novel mechanism in *A. fumigatus* that contributes to evasion from the 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 2 decades (reviewed in references 7 and 42). In healthy individuals, *A. fumigatus* conidia are inhaled but the establishment of disease is prevented by the host immune system. Inhaled *A. fumigatus* conidia are immediately confronted with the host complement system and phagocytic cells. The complement system is activated on the conidial and hyphal surface (26), and this activation results in the cleavage of C3. 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 (69). Opsonization with complement proteins is important for phagocytosis of *A. fumigatus* conidia, the key process in the defense against this pathogen (59).

Activation of the complement system occurs via three pathways: the alternative pathway (AP), the lectin pathway, and the classical pathway. The AP is activated on microbial surfaces and plays a pivotal role in the clearance of microorganisms (70). Progression of the cascade leads to generation of a C5 convertase, which produces inflammatory C5a anaphylatoxins, and also to the formation of terminal complement complexes (TCC), which can form membrane attack complexes (MAC) and hence pores on target surfaces. C3b surface deposition and MAC formation are important for clearance of bacteria but appear to play a minor role in the defense against fungi. The complement activation system is controlled by fluid-phase and cell surface-bound regulators. We and others showed before (2, 62) that *A. fumigatus* conidia bind factor H (the central human regulator of the AP), FHL-1, and CFHR1. Factor H acts as a cofactor for the plasma serine protease factor I, which mediates the cleavage of C3b (21, 35, 41). This blocks C3 convertase formation and leads to downregulation or termination of the complement cascade. In contrast to conidia, *A. fumigatus* hyphae do not bind factor H (2). Instead, they activate complement on their surface (26). Until now a single, nonprotein activity in culture supernatant that inhibits opsonization of the fungal surface by complement proteins has been described (65). The nature of this molecule has not been discovered yet. *A. fumigatus* inhibition of complement activation is important, since activation of the complement cascade causes toxic and damaging effector functions. Although hyphae are too big to become phagocytosed by macrophages, attraction and activation of neutrophils by C3a and C5a still lead to the destruction of hyphae. Therefore, here we analyzed whether *A. fumigatus* hyphae use additional strategies to interfere with the human complement system.

MATERIALS AND METHODS

Fungal and bacterial strains. The $\Delta akuB^{KU80}$ strain is an *A. fumigatus* strain deficient in nonhomologous end joining (9). The $\Delta alp1$ strain, with a deletion of the *alp1* gene was derived by transformation of the $\Delta akuB^{KU80}$ strain with a knockout DNA fragment consisting of left and right flanking regions of the *alp1* gene and the pyrithiamine resistance cassette as a dominant selection marker.

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Reconstitution of the Δ*alp1* mutant strain with the *alp1* gene was performed with a plasmid containing the *alp1* gene with its promoter and the hygromycin resistance cassette. For transformation of *Escherichia coli*, strain DH5α (Invitrogen, Karlsruhe, Germany) or BL21(DE3) (16) was used, as stated. *E. coli* strains were grown at 37°C in LB medium supplemented with 100 μg per ml of ampicillin when required.

Standard molecular biological techniques and oligonucleotides. Standard techniques for the manipulation of DNA were carried out as described previously (52). Plasmid DNA used for transformation of *A. fumigatus* was prepared using columns from Peqlab (Erlangen, Germany) according to the manufacturer's instructions. Chromosomal DNA of *A. fumigatus* was prepared using the fungal DNA mini kit (Peqlab, Erlangen, Germany). For Southern blot analysis, 10 μg of chromosomal DNA of *A. fumigatus* was cut with NcoI. DNA fragments were separated on a 1% (wt/vol) agarose gel and blotted onto a Hybond N^+ nylon membrane (GE Healthcare, Freiburg, Germany). Labeling of the DNA probe was performed using the PCR digoxigenin (DIG) labeling mix (Roche Applied Science, Mannheim, Germany). For hybridization and detection of DNA-DNA hybrids, the DIG Easy Hyb and the CDP-*Star* ready-to-use kit (Roche Applied Science, Mannheim, Germany) were used according to the manufacturer's instructions.

Generation of recombinant plasmids. Left and right flanking regions (1 kb each) of the *A. fumigatus alp1* gene were amplified from *A. fumigatus* chromosomal DNA by PCR using Phusion DNA polymerase (New England Biolabs, Frankfurt, Germany) and the oligonucleotides Alp_LB_for (ATTGCGGCCGCGTGCTCGAATCAGTGC), Alp_LB_rev (ATTGGCCTGAGTGGCCGCGCAGACTCTGACAAGAC), alp_RB_for (ATTGGCCATCTAGGCCCTGCGCAGGGCGAGTC) and alp_RB_rev (CAAACAGGCACCTACGTG), encoding NotI and SfiI sites (underlined), respectively. Both right and left flanking regions were cloned into pCR2.1 vectors, giving pCR2.1_alp1_RB and pCR2.1_alp1_LB, respectively. The orientation of the *alp1* right flanking region in the pCR2.1 vector was checked, and a plasmid carrying the SfiI restriction site next to the NotI site of the pCR2.1 vector was used. The plasmids pCR2.1_alp1_RB and pCR2.1_alp1_LB were digested with NotI and SfiI. Plasmid pALptrA, containing the pyrithiamine resistance cassette, was cut with SfiI. The resulting DNA fragments, i.e., alp1_LB and the *ptrA* cassette, as well as the opened vector pCR2.1_alp1_RB, were gel purified and ligated. The resulting plasmid was designated pCR2.1_Dalp_ptrA. For transformation of *A. fumigatus*, this plasmid was digested with XhoI to excise the deletion construct consisting of the pyrithiamine resistance cassette flanked by left and right flanking regions of *alp1*. The plasmid for reconstitution of the *alp1* gene in the Δ*alp1* strain was constructed as follows. *alp1*, including the 1-kb promoter region, was amplified by PCR using Phusion DNA polymerase with primers alp_RB_for_Bam (GGATCCGAGTCTTACCAGATGGATGGTGG) and alp_RB_rev_Bam (GGATCCCAGATACATACCATCCAAACCC). The DNA fragment was subcloned into a pJET1.2/blunt vector (Fermentas, St. Leon-Rot, Germany) and excised from the backbone by restriction with BamHI. The DNA fragment was ligated into the BamHI-restricted vector pUChph, which contains the hygromycin resistance cassette. The resulting plasmid, pUChph_alp1+P, was used for transformation.

Transformation of *A. fumigatus*. Transformation of *A. fumigatus* was carried out using protoplasts as described previously (66). When selection for pyrithiamine resistance was applied, 0.1 mg pyrithiamine (Sigma-Aldrich, Taufkirchen, Germany) per liter was added to the agar plates. When clones were selected for hygromycin resistance, the agar plates were supplemented with 240 μg/ml hygromycin (Roche Diagnostics, Mannheim, Germany).

***A. fumigatus* strains and growth conditions.** *A. fumigatus* wild-type Δ*akuB*^{KU80} strain (referred to as wild type below), the Δ*alp1* mutant strain, and the Δ*alp1*^{C} reconstituted strain were cultivated on *Aspergillus* minimal medium (AMM) agar plates with 1% (wt/vol) glucose at 37°C for 4 days. AMM consisted of 6 g/liter $NaNO_3$, 1.52 g/liter KH_2PO_4, 0.52 g/liter KCl, 0.05% (wt/vol) $MgSO_4$, and 1 μl/ml trace element solution (pH 6.5). The trace element solution consisted of 1 g $FeSO_4 \cdot 7H_2O$, 8.8 g $ZnSO_4 \cdot 7H_2O$, 0.4 g $CuSO_4 \cdot 5H_2O$, 0.15 g $MnSO_4 \cdot 4H_2O$, 0.1 g $NaB_4O_7 \cdot 10H_2O$, 0.05 g $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$, and double-distilled water (ddH_2O) to 1,000 ml. Conidia were harvested in sterile water. For protease assays, 100 ml medium was inoculated with 1×10^8 conidia. The medium was either AMM, AMM lacking a nitrogen and carbon source (AMM−N) and supplemented with 10 mM glucose and 1% (wt/vol) bovine serum albumin (BSA), AMM−N with 1% (wt/vol) BSA, or AMM−N supplemented with 10 mM glucose and 0.4% (wt/vol) elastin. The cultures were incubated for 1 to 7 days with or without rotation at 37°C. The other medium used was AMM−N supplemented with 2% (vol/vol) normal human serum (NHS) and 5 mM glucose. Fifty milliliters of AMM was inoculated with 1×10^6 conidia/ml and incubated at 37°C with rotation for 72 h for dry weight determination. Elastase secretion was visualized by growing *A. fumigatus* wild-type, Δ*alp1*, and Δ*alp1*^{C} strains on agar plates containing 0.2% (wt/vol) elastin (Elastin Products Company, Owensville, MO), 0.01% (wt/vol) yeast extract, 0.5% (vol/vol) Triton X-100, and 1.5% (wt/vol) agar.

Serum, antibodies, proteins, and protease inhibitors. NHS was obtained from healthy human donors and stored at −20°C until used. EDTA was added at a concentration of 10 mM (NHS-EDTA). Antibodies used in experiments were polyclonal goat anti-factor H antiserum, polyclonal goat anti-C1q antiserum, polyclonal goat anti-C3 antiserum, polyclonal goat anti-C4 antiserum, polyclonal goat anti-C5 antiserum, and polyclonal goat anti-C4BP antiserum (Calbiochem, Darmstadt, Germany). Horseradish peroxidase (HRP)-conjugated rabbit anti-goat antiserum was obtained from Dako (Glostrup, Denmark). HRP-coupled anti-human IgG antiserum was from Sigma-Aldrich (Taufkirchen, Germany). Factor H, factor I, and C3b were purchased from Calbiochem (Darmstadt, Germany).

Cofactor assay and complement degradation assays. Cofactor activity assay of surface-attached regulators was performed as described previously (2). Conidia (2×10^9) either were washed five times with binding buffer or were used directly without further washing steps or after heat inactivation for 10 min at 95°C. Conidia were resuspended in 150 μl binding buffer and were incubated with purified factor H (100 μg/ml) for 1 h at 37°C. Conidia were washed, and C3b (2 μg) and factor I (1 μg) or C3b alone was added. Conidia were incubated for 60 min at 37°C on a shaker. The samples were centrifuged and the supernatants analyzed by Western blotting.

Complement degradation assays were carried out as follows. Three milliliters of culture supernatant of 1- to 7-day-old *A. fumigatus* cultures was filtered through an 0.8-μm filter to separate conidia and hyphae from the sample. In order to determine the class of the protease responsible for the observed cleavage, 1.56 μl EDTA (0.5 M), 0.15 μl leupeptin (1 mg/ml), 0.15 μl pepstatin, 7 μl aprotinin (5 mg/ml), 1.5 μl phenylmethylsulfonyl fluoride (PMSF) (1 mg/ml), or 0.5 μl chymostatin (20 mg/ml) was added to the culture supernatant prior to incubation with complement proteins. Three microliters of 1:10 diluted purified C3b, purified factor H, or NHS-EDTA was added to 150 μl filtered culture supernatant. The solution was incubated at 37°C for 60 min. Samples were subjected to reducing SDS-PAGE and Western blotting.

Western blotting. Samples were separated by SDS-PAGE under reducing conditions and transferred to a polyvinylidene difluoride (PVDF) membrane, and cleavage of complement proteins C3, C4, C5, C1q, C4BP, and factor H, as well as IgG, was detected by Western blotting. The membranes were blocked with 2.5% (wt/vol) BSA–phosphate-buffered saline (PBS) with 0.1% (vol/vol) Tween 20 for 1 h at room temperature and were subsequently incubated with polyclonal goat antiserum specific for C3, C4, C5, C1q, C4BP, or factor H (dilution of 1:1,000 in 2.5% [wt/vol] BSA-PBS–0.1% [vol/vol] Tween 20) for 120 min at room temperature. After five washing steps with PBS–0.1% (vol/vol) Tween 20, HRP-conjugated rabbit anti-goat antiserum was added at a dilution of 1:5,000 and the membranes were incubated at room temperature for 60 min. For detection of IgG, membranes were incubated with HRP-coupled human IgG antiserum (dilution of 1:1,000 in 2.5% [wt/vol] BSA–PBS with 0.1% [vol/vol] Tween 20) for 120 min. After five washing steps with PBS–0.1% (vol/vol) Tween 20, the proteins were detected using enhanced chemiluminescence (Applichem, Darmstadt, Germany).

Azocasein assay. In order to measure the proteolytic activity of culture supernatant, 100 μl culture supernatant was incubated with an azocasein solution (5 mg azocasein in 50 mM Tris [pH 7.5], 0.2 M NaCl, 5 mM $CaCl_2$, 0.05% [wt/vol] Brij 35) for 90 min at 37°C (10). The reaction was stopped by addition of 150 μl of 20% (wt/vol) trichloroacetic acid (TCA). After 30 min of incubation at room temperature, samples were centrifuged at 13,000 rpm for 8 min and 500 μl of each supernatant was added to 500 μl 1 M NaOH. Absorbance was measured at 436 nm.

Secretome analysis. Two 500-ml Erlenmeyer flasks containing 150 ml AMM each were inoculated with 1×10^8 wild-type conidia and were incubated for 50 h at 37°C with rotation. The cultures were filtered over Miracloth. Ten milliliters of 100% (wt/vol) TCA was added to 100 ml supernatant of each culture, and the mixtures were incubated at 4°C to complete precipitation. The samples were centrifuged at 4,000 rpm for 30 min. The pellets were washed with 1 ml acetone. After evaporation of all residual acetone, 150 μl lysis buffer were added to each sample. The lysis buffer (23) contained 7 M urea, 3 M thiourea, 1% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 1% (vol/vol) Zwittergent, 0.8% (vol/vol) ampholyte, and 40 mM Tris. For protein solubilization, samples were incubated for 10 min in an ultrasonic bath. The protein concentration was measured as described previously (6). Fifty micrograms of protein sample was loaded on each strip by rehydration loading. Seven-centimeter strips with a pI range of 3 to 11 (GE Healthcare, Freiburg, Germany) were used. Focusing was carried out according to the GE Healthcare guidelines.

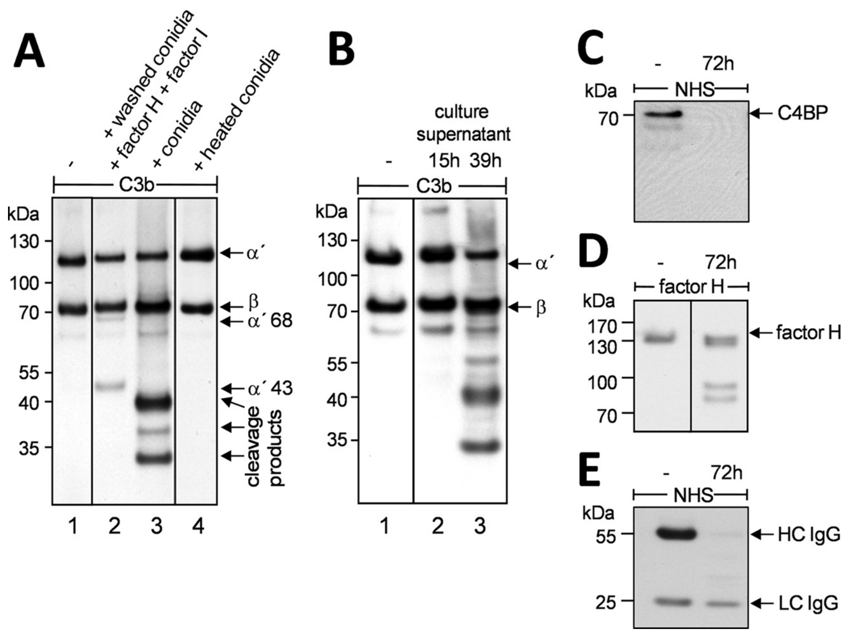

FIG. 1. Degradation of complement components by *A. fumigatus*. (A) C3b inactivation on human host tissues is mediated by the human serine protease factor I in conjunction with the cofactor factor H. The degradation products of the α chain (115 kDa) of C3b are 68 kDa and 43 kDa in size (lane 2). When C3b was incubated together with *A. fumigatus* conidia, C3b was degraded even in the absence of factor I and factor H (lane 3). Incubation of C3b with PBS (lane 1) or heat-inactivated spores (lane 4) left C3b intact. (B) Degradation of C3b by culture supernatant. Samples of culture supernatant were taken after 15 h and 39 h of cultivation of *A. fumigatus* and were incubated with C3b. Degradation of C3b was analyzed by reducing SDS-PAGE and Western blotting. Degradation products were visible only after incubation in the older culture supernatant (lane 3). (C to E) Degradation of complement regulators and IgG. Culture supernatant of a nonshaking culture incubated for 72 h was incubated with NHS or purified factor H. In controls (−), NHS or factor H was incubated with PBS instead of culture supernatant. Detection of cleavage fragments was performed with polyclonal antiserum directed against C4BP, IgG, or factor H. C4BP (C) and the heavy chains of IgG (E) were absent after incubation with culture supernatant. Factor H (D) was also cleaved but was still detectable.

Prior to second-dimension separation, strips were equilibrated for 15 min in equilibration buffer containing 1% (wt/vol) dithiothreitol (DTT) and subsequently for 15 min in buffer containing 2.5% (wt/vol) iodoacetamide. Second-dimension separation was performed on 12% acrylamide-Tris minigels. After fixation in 7% (vol/vol) acetic acid–30% (vol/vol) methanol for 1 h, gels were stained with colloidal Coomassie blue as described previously (38). Spot detection and analysis were performed with Delta2D software (Decodon, Greifswald, Germany). After excision of protein spots, the tryptic digest was carried out by in-gel digestion modified as described previously (55). Proteins were identified by matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF)/TOF analysis in reflective mode (Bruker Daltics, Germany). Mass spectra were analyzed, and searching was performed using the NCBI Mascot server (MASCOT 2.1.03; Matrix Science, United Kingdom). Hits were regarded as significant with a Mascot score of 0.05.

Overproduction of the Alp1 protease. AfAlp1 cDNA encoding a truncated Alp1 protein that lacks the 20 amino acids spanning the secretion signal (ΔN20Alp1-cDNA) was synthesized from *A. fumigatus* mRNA with the gene-specific primer pair AfAlp1tr_f/AfAlp1tr_*r* (5′ACTGGGATCCGCGCCTGTCCAGGAAACTCGTCGTGC3′/5′ACTGAAGCTTTTAAGCATTGCCATTGTAGGCTAGC3′) by using the BioScript one-step reverse transcription-PCR (RT-PCR) kit from Bioline (Luckenwalde, Germany) according to the manufacturer's protocol. The ΔN20Alp1-cDNA was cloned into the BamHI-HindIII site of plasmid pMalC2TEVHapC (16) to give the plasmid pMalC2TEVΔN20Alp1. After transformation of *E. coli* strain BL21(DE3), the recombinant ΔN20Alp1 protein was overproduced by autoinduction as described elsewhere (16).

Animal experiments. Female specific-pathogen-free outbred CD-1 mice 5 weeks of age (18 to 22 g; Charles River, Germany) were used for all experiments. The animals were housed in groups of five and cared for in accordance with the principles outlined in the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. All animal experiments were in compliance with the German animal protection law and were approved by the responsible Federal State authority and ethics committee. Survival experiments following intranasal challenge were carried out as described previously (17). Briefly, mice were immunosuppressed by intraperitoneal injection of cortisone acetate (25 mg/mouse; Sigma-Aldrich, Taufkirchen, Germany) on days −3 and 0. On day 0 the mice were anesthetized with ketamine (100 mg/kg; Inresa Arzneimittel GmbH, Freiburg, Germany) and xylazine (2 mg/kg; Bayer Vital GmbH, Leverkusen, Germany) and infected intranasally with conidial suspensions containing 1×10^5 conidia in 20 μl PBS. Survival was monitored for 14 days. Immunosuppressed mice inoculated with PBS served as sham-infected controls in all experiments. Mice were examined clinically at least twice daily and weighed individually every day. Animals were considered moribund and sacrificed humanely when they showed rapid weight loss (>20% of body weight at the time of infection), severe dyspnoea, decreased body temperature, or other signs of serious illness.

RESULTS

Complement factor C3b is cleaved by an *A. fumigatus* endogenous activity. *A. fumigatus* conidia bind the human complement regulators factor H, CFHR1, and FHL-1. The bound regulators are active and act as cofactors in the factor I-mediated cleavage of C3b. This effect was observed on washed conidia (Fig. 1A, lane 2). However, when conidia were subjected to this test without washing steps, a different cleavage pattern was observed (Fig. 1A, lane 3). C3b cleavage fragments were smaller than 43 kDa, and cleavage occurred without addition of factor H and factor I. C3b remained intact when conidia were heat inactivated prior to incubation with C3b (Fig. 1A, lane 4). These data suggested the presence of a spore-bound protease or of a mycelial protease that cleaves human C3b.

To test whether the proteolytic activity was derived from spores or mycelia, culture supernatant of *A. fumigatus* was analyzed for C3b-degrading activity. Culture supernatants of 15- and 39-h-old culture were filtered and subsequently incubated with purified C3b for 30 min. Cleavage of C3b was visualized by Western blotting. In 15-h-old culture supernatant no degradation of C3b occurred, whereas in 39-h-old culture supernatant C3b was efficiently cleaved (Fig. 1B, lanes 2 and 3). Thus, the degrading activity was likely due to an *A. fumigatus* secreted protease. To investigate whether this activity specifically cleaves C3b or has a broader activity, the degradation of the complement regulators factor H and C4BP as well as immunoglobulin G was analyzed. Western blotting showed that C4BP was completely degraded by culture supernatant (Fig. 1C) and that factor H was cleaved but still detectable (Fig. 1D). Culture supernatant also cleaved the heavy chain of IgG. This is important for the initiation of the classical pathway and for binding of C1q as well as Fcγ-mediated phagocytosis (Fig. 1E). Thus, cleavage was not limited to C3b. Also, the complement regulators factor H, C4BP, and IgG were efficiently cleaved by culture supernatant of *A. fumigatus*.

Identification of the C3b-degrading activity of *A. fumigatus*. In order to investigate which proteases are secreted during growth in AMM, two-dimensional (2D) gel analysis of the culture supernatant was performed. Culture supernatant was filtered, precipitated, and separated by 2D gel electrophoresis (Fig. 2A). Mass spectrometry analysis of all visible protein spots revealed four secreted proteases which are present during growth in AMM (Fig. 2A, arrows 1 to 4), i.e., a secreted dipeptidyl peptidase (arrow 1), the aspartic type endoprotease AP3 (arrow 2), the aminopeptidase Y (arrow 3), and the alkaline serine protease Alp1 (arrow 4). The last protease was present in large amounts and was found more than once in a row of protein spots, possibly representing differently modified Alp1.

Since only four proteases, each belonging to a different protease class, were secreted under the incubation conditions used, protease inhibitor studies were carried out to identify the protease class responsible for C3b degradation. Culture supernatant was treated with protease inhibitors prior to incubation with C3b, and the cleavage pattern was studied by Western blot analysis (Fig. 2B). Pepstatin was added to inhibit aspartic proteases; EDTA was added to inhibit metalloproteases; and leupeptin, aprotinin, PMSF, and chymostatin were added to inhibit serine proteases. Of these protease inhibitors, PMSF and chymostatin inhibited the degradation of C3b by culture supernatant. This inhibition is characteristic of a serine protease with chymotrypsin-like activity. With Alp1, a single protease with these characteristics was present in the culture supernatant.

Absence of C3b degradation in Δ*alp1* strain culture supernatant. To elucidate whether Alp1 was the protease responsible for the cleavage of C3b, an *A. fumigatus alp1* deletion strain was generated (data not shown). Proteolytic activity present in supernatants of wild-type, Δ*alp1*, and Δ*alp1*^{C} cultures grown in AMM was quantified by cleavage of the chromogenic substrate azocasein. When casein-degrading enzymes are present, the azo dye is released and cannot be precipitated with trichloric acid. This results in an increase of absorbance at 436 nm. Proteolytic activity was detectable in wild-type and Δ*alp1*^{C} supernatants (Fig. 3A). It increased with the age of the culture and was higher after 120 h than after 72 h of incubation (Fig. 3A, bars 1 and 3). No proteolytic activity was detectable in the presence of the inhibitor chymostatin (Fig. 3A, bars 2 and 4). The Δ*alp1* supernatant showed almost no proteolytic activity (Fig. 3A, bars 5 to 8). Thus, the major proteolytic activity was absent in the Δ*alp1* strain.

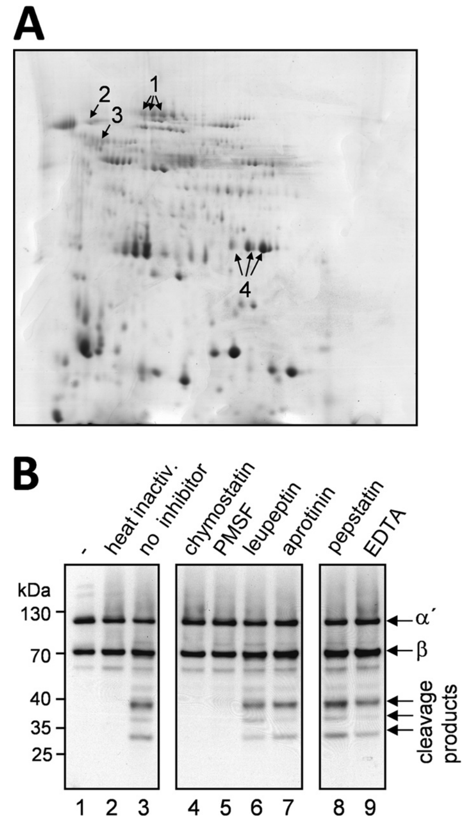

FIG. 2. Secretome analysis and protease inhibitor studies. (A) Secretome analysis. Proteins in the culture supernatant of a 50-h-old *A. fumigatus* culture grown in AMM were precipitated with TCA and separated by 2D gel electrophoresis, and all detectable protein spots were subjected to mass spectrometry (MS) analysis. All spots representing proteases are marked with arrows. Arrow 1, secreted dipeptidyl peptidase; arrow 2, aspartic type endoprotease AP3; arrow 3, aminopeptidase Y; arrow 4, alkaline serine protease Alp1. (B) Protease inhibitor studies. Supernatant of a 39-h-old culture of *A. fumigatus* was incubated with different protease inhibitors prior to incubation with purified C3b. Addition of chymostatin, PMSF, leupeptin, or aprotinin should inhibit serine proteases (lanes 4 to 7). Incubation of supernatant with pepstatin (lane 8) and EDTA (lane 9) should block aspartic acid proteases and metalloproteases, respectively. C3b incubated with PBS (lane 1), with heat-inactivated culture supernatant (lane 2), and with untreated culture supernatant (lane 3) served as controls. Samples were subjected to reducing SDS-PAGE and Western blot analysis for the detection of C3b.

Culture supernatants of wild-type, Δ*alp1*, and Δ*alp1*^{C} cultures were also tested for C3b degradation activity at 72 h and 120 h of incubation (Fig. 3B). C3b was added to filtered wild-type, Δ*alp1*, and Δ*alp1*^{C} culture supernatants. Both the α′ and β chains of C3b were completely degraded by wild-type and Δ*alp1*^{C} supernatants. Addition of the inhibitor chymostatin to the culture supernatant inhibited C3b degradation (Fig. 3B, lanes 2, 4, 10, and 12). Culture supernatant of the Δ*alp1* mutant strain did not cleave C3b. Both chains remained intact even without the addition of chymostatin (Fig. 3B, lanes 5 to

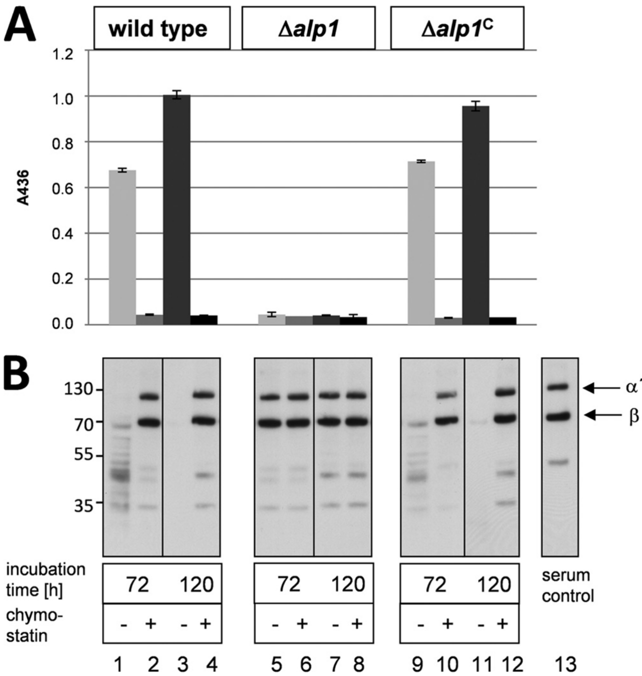

FIG. 3. Protease activity and C3b degradation after growth in AMM. The wild-type, Δ*alp1*, and Δ*alp1*^{C} strains were grown for 72 h and 120 h in AMM. Samples of the culture supernatants were either assayed directly or preincubated with the protease inhibitor chymostatin. (A) The proteolytic activities of supernatants of all cultures were measured with an azocasein assay. Error bars indicate standard deviations. (B) C3b degradation by culture supernatants was analyzed by Western blot analysis.

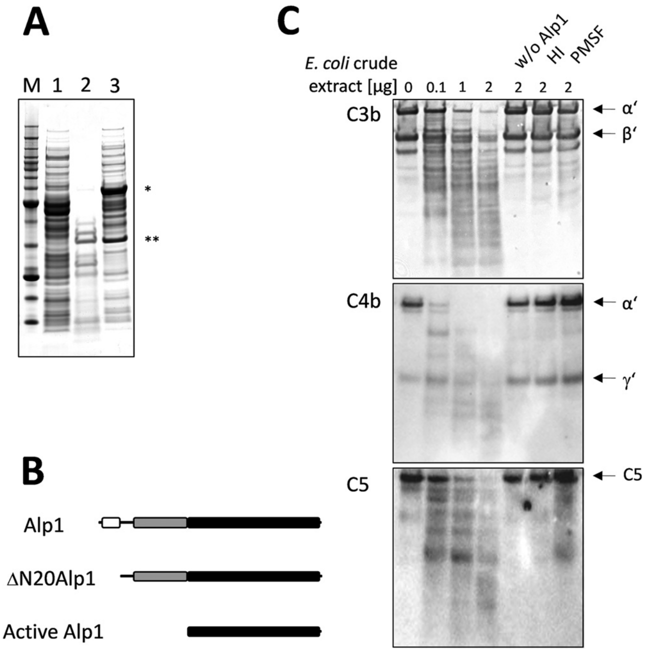

FIG. 4. Presence of active ΔN20Alp1 in *E. coli* crude extract and cleavage of C3b, C4b, and C5 *in vitro*. (A) SDS-PAGE demonstrating the autocatalytic activation of MBP-fused ΔN20Alp1 during heterologous expression in *E. coli* BL21(DE3). Mass spectrometry analysis revealed the cleavage of an N-terminal peptidase inhibitor sequence (*, MBP fused to the Alp1 prepropeptide; **, active Alp1). Lanes: M, molecular weight marker proteins; 1, soluble protein fraction of *E. coli* BL21(DE3) cells without ΔN20Alp1-encoding vector; 2, soluble protein fraction of ΔN20Alp1-overproducing *E. coli* BL21(DE3) cells without PMSF; 3, after addition of 2 mM PMSF. (B) Schematic depiction of Alp1 in accordance with SignalP and Pfam. The domains represent the signal peptide (white), the peptidase inhibitor domain (gray), and the peptidase domain (black). (C) Western blot analysis showing the degradation of C3b, C4b, and C5 by increasing concentrations of *E. coli* BL21(DE3) crude extract containing active processed protease Alp1. As negative controls, Alp1 activity was inactivated by heat (HI) or by the addition of PMSF. Incubation of complement proteins with *E. coli* BL21(DE3) protein extract (w/o Alp1) did not lead to complement degradation.

8). Two faint low-molecular-weight bands were present in wild-type and Δ*alp1*^{C} supernatants after chymostatin treatment as well as in Δ*alp1* supernatant, indicating a minor residual complement-degrading activity in addition to Alp1. These results identify the *A. fumigatus* protease Alp1 as the major protease responsible for the degradation of C3b under these conditions.

The Alp1 protease cleaves C3b, C4b, and C5 *in vitro*. To further provide evidence that Alp1 is able to cleave the immune regulators, we overproduced the truncated protein ΔN20Alp1 in *E. coli* BL21(DE3) cells. The Alp1 protein fused to the maltose binding protein (MPB) was autocatalytically processed during heterologous overproduction by cleaving off the MBP together with a probable peptidase inhibitor sequence (Fig. 4A and B). The processed and active Alp1 version was identified by mass spectrometry and showed a molecular mass of 32 kDa in SDS-PAGE. These results are in accordance with an *in silico* analysis which was carried out with the search tool at the Pfam database (http://pfam.sanger.ac.uk/search). The analysis revealed that Alp1 consists of two functional domains. The first domain shows similarity to the peptidase inhibitor I9 protein family (24, 31). This domain is predicted to act as both a temporary inhibitor and a molecular chaperone. Thus, it might facilitate the folding of the nascent protein. The second domain is predicted to encode a peptidase S8 of the subtilase family (48).

We observed that the soluble protein extract of MBP-ΔN20Alp1-producing *E. coli* BL21(DE3) cells was efficiently degraded by the processed Alp1 protein (Fig. 4A). Since the autocatalytic processing of Alp1 resulted in the cleavage of the MBP fusion tag, no purification procedure based on affinity chromatography could be used for Alp1. Therefore, Alp1-containing crude extract of *E. coli* BL21(DE3) cells was used for coincubation with C3b, C4b, and C5. As shown in Fig. 4C, increasing concentrations led to complete degradation of all three human complement proteins, i.e., C3b, C4b, and C5. As a negative control, protein extract derived from an *E. coli* BL21(DE3) strain not producing ΔN20Alp1 was used, which did not lead to any degradation of the complement factors. Similarly, no cleavage occurred when the protease inhibitor PMSF was added to the ΔN20Alp1-containing protein extract or when the protein extract was heat inactivated. These findings confirm that Alp1 degrades the human complement factors C3b, C4b, and C5.

Growth and C3b degradation by the Δ*alp1* strain on different carbon and nitrogen sources. The majority of proteases that were secreted when *A. fumigatus* was grown in AMM were serine proteases like Alp1, whereas on other carbon and ni-

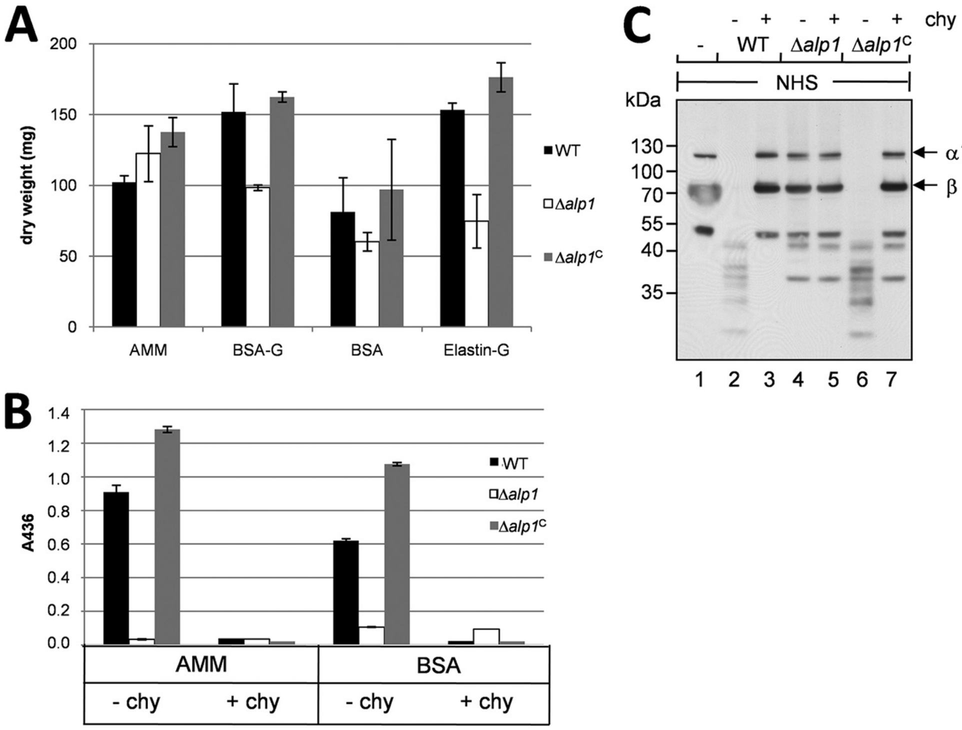

FIG. 5. Growth and C3b degradation of the Δ*alp1* strain on different carbon and nitrogen sources. (A) Dry weight measurement after growth in different media. The wild-type (WT), Δ*alp1*, and Δ*alp1*^{C} strains were grown in AMM, AMM−N supplemented with 10 mM glucose and 1% (wt/vol) BSA (BSA-G), AMM−N with 1% (wt/vol) BSA (BSA), or AMM−N with 10 mM glucose and 0.4% (wt/vol) elastin (Elastin-G). Dry weight was measured after 72 h of incubation. Error bars indicate standard deviations. (B) Protease activity after growth in BSA medium compared to AMM. Protease activity in the culture supernatants after 72 h of incubation was measured with an azocasein assay. Alp1 protease activity was inhibited by addition of chymostatin to the culture supernatants (+chy). (C) Degradation of C3b after growth in BSA medium. The wild-type, Δ*alp1*, and Δ*alp1*^{C} strains were grown in AMM−N with 1% (wt/vol) BSA as the sole carbon and nitrogen source for 67 h. Culture supernatants were incubated with NHS, and C3b cleavage was analyzed by Western blotting.

trogen sources also other proteases were secreted (10). To test whether these proteases also cleave C3b, the wild-type, Δ*alp1*, and Δ*alp1*^{C} strains were grown in four different media. Medium 1 was AMM. Medium 2 contained AMM without a nitrogen and carbon source (AMM−N) and supplemented with 10 mM glucose to support initial growth and 1% (wt/vol) BSA. Medium 3 was AMM−N with 1% (wt/vol) BSA as the sole nitrogen and carbon source. Medium 4 consisted of AMM−N supplemented with 10 mM glucose and 0.4% (wt/vol) elastin.

The dry weight after growth in these media was measured. In AMM, dry weight was the same for all three strains. In medium 3, with BSA and glucose, and in medium 4, with BSA alone, the biomass of the Δ*alp1* strain was reduced by one-third compared to those of the wild-type strain and the reconstituted strain. In medium 4, with elastin, the Δ*alp1* strain did not grow well, most probably because of the absence of the major elastase Alp1, and reached only half of the biomass of the wild-type and Δ*alp1*^{C} strains (Fig. 5A).

Protease activity in BSA medium was measured for all strains. Protease-free BSA should promote secretion of metalloproteases (10). However, for the wild-type strain and the reconstituted strain, protease activity in the culture supernatant after growth with BSA was slightly lower than that after growth in AMM (Fig. 5B). This activity was almost completely inhibited by the addition of the inhibitor chymostatin to the culture supernatants of both the wild-type and reconstituted strains. For the Δ*alp1* strain, secretion of proteases other than Alp1 was expected to promote growth of the fungus in medium containing BSA as the sole nitrogen and carbon source. However, the proteolytic activity of culture supernatant of the Δ*alp1* strain was six times lower than that of wild-type supernatant (Fig. 5B). The proteases present in the culture supernatant were not inhibited by addition of chymostatin. Apparently, this low protease activity was sufficient to promote growth of the strain up to a level of 65% of the biomass of the wild type. Degradation of BSA, which supported growth, was indirectly seen (Fig. 5C, lanes 1, 3, 4, 5, and 7). The β chain of C3b and the BSA in the medium have the same molecular mass. In a Western blot directed against C3b, the β chain of C3b is therefore visible as a smear (Fig. 5C, lane 1). When BSA in the medium is degraded by proteases, the β chain of C3b is visible by Western blot analysis as a sharp band (Fig. 5C, lanes 4 and 5). The proteases secreted by the Δ*alp1* strain degraded BSA but did not cleave complement component C3b (Fig. 5C). Therefore, also under these conditions, Alp1 is the major protease of *A. fumigatus* that cleaves C3b.

Degradation of complement proteins after growth in AMM. C3b is an important opsonin, but other complement proteins also have essential functions in the defense against microbes. C5b, a cleavage product of C5, initiates formation of the membrane attack complex at a later stage of complement activation,

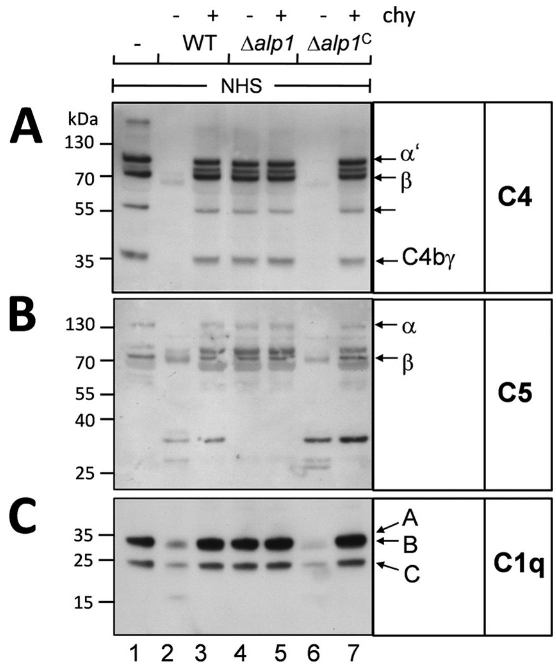

FIG. 6. Degradation of other complement proteins after growth in AMM. The wild-type, Δ*alp1*, and Δ*alp1*^{C} strains were grown in AMM for 72 h without shaking. Culture supernatant of each strain was either pretreated with chymostatin (chy) or incubated directly with NHS for 60 min. Detection of cleavage fragments was performed by Western blotting with polyclonal antibodies directed against C4 (A), C5 (B), or C1q (C).

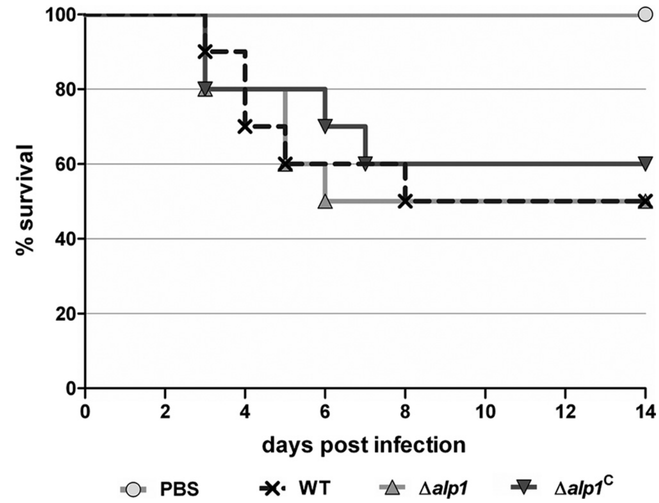

FIG. 7. Animal experiments. Virulence of the Δ*alp1* strain as well as the corresponding wild-type and reconstituted strains was tested in a cortisone acetate mouse infection model. Mice (n = 10) were immunosuppressed with cortisone acetate on days −3 and 0 and were infected intranasally with 1×10^5 spores of each strain in a 20-μl total volume. Immunosuppressed mice inoculated with PBS served as controls. Survival was monitored for 14 days.

and C5a represents a potent anaphylatoxin. C1q initiates the classical pathway of complement activation, and C4 is part of the C3 convertase of the classical pathway (12, 63, 64). When culture supernatant of the wild-type or Δ*alp1*^{C} strain was incubated with human serum that contains C4, C5, and C1q, subsequent Western blot analysis showed that C4 (Fig. 6A, lanes 2 and 6) was completely degraded. Degradation of C5 and C1q was also observed but was less prominent (Fig. 6B and C, lanes 2 and 6). Supernatant of the Δ*alp1* strain did not cleave C4, C5, or C1q (Fig. 6A to C, lanes 4 and 5). In the case of C4, a faint band of 80 kDa was visible after incubation with Δ*alp1* culture supernatant, but C4 remained mainly intact. These data confirmed the *in vitro* data obtained with the Alp1 protein produced by *E. coli* (Fig. 4), showing that degradation of complement proteins by Alp1 is not limited to C3b but also occurs with complement proteins C4, C5, and C1q.

Virulence of the Δ*alp1* and Δ*alp1*^{C} strains in a cortisone acetate mouse infection model. A cortisone acetate mouse model was applied to test the virulence of the Δ*alp1* mutant and to analyze the strain for a possible higher recruitment of neutrophils to the site of infection. Absence of Alp1 should lead to increased activation of complement on the hyphal surface and to elevated levels of anaphylatoxins. Due to the low numbers of neutrophils in a neutropenic mouse model, the impact of the homing of neutrophils on virulence could not be determined in previous studies (37, 58). However, in this cortisone acetate mouse model, the Δ*alp1* strain also showed no attenuation in virulence (Fig. 7), and no increased infiltration of neutrophils to the site of infection was visible (data not shown).

DISCUSSION

In this study we report a novel mechanism of immune evasion for *Aspergillus fumigatus*. *A. fumigatus* secreted Alp1, a protease that efficiently cleaved components of the complement system, i.e., C3, C4, C5, and C1q, as well as IgG, and thus modulates complement activation on the hyphal surface.

The role of the complement system in the defense against *A. fumigatus* has not been extensively studied, but existing data indicate an important effect of this central host innate immune system. Upon incubation with serum, conidia were opsonized with C3b, which enhanced phagocytosis (61). A white mutant (*pksP*) of *A. fumigatus* (20), which was highly attenuated in virulence in a murine infection model (30), bound more C3b molecules per unit of conidial surface area and was subsequently better phagocytosed by macrophages (46, 61). In addition, the pathogenic aspergilli *A. fumigatus* and *A. flavus* bound fewer C3 molecules per unit of conidial surface area than did the less pathogenic species *A. glaucus*, *A. nidulans*, *A. niger*, *A. ochraceus*, *A. terreus*, *A. versicolor*, and *A. wentii* (15). This difference further supports the importance of complement modulation and inhibition for the virulence of pathogenic fungi. The major site of infection for *A. fumigatus* is the lungs, which represent an environment different from serum. However, all components of both the alternative and the classical pathways are present in human bronchoalveolar liquid (5, 40). Thus, the lungs are a site for complement activation, and inactivation also can occur *in vivo* since complement regulators are present. Downregulation of the complement cascade by binding of the human host complement regulators factor H, FHL-1, CFHR1, and/or C4BP has been shown for bacterial and fungal pathogens, e.g., *A. fumigatus* (2, 62), *Candida albi-*

cans (33, 34), *Streptococcus pneumoniae* (13), *Streptococcus pyogenes* (25), *Borrelia burgdorferi* (1, 14, 27), *Borrelia spielmanii* (56), *Neisseria gonorrhoeae* (39, 44, 45), and *Pseudomonas aeruginosa* (28). However, endogenous activities downregulating complement activation have been described for only a few pathogens (for a review, see reference 4). For *P. aeruginosa*, C3 degradation by the major secreted metalloproteinase elastase was reported (28, 53), and for *C. albicans*, C3 degradation (22) by a secreted protease was shown. This protease also cleaved the human proteinase inhibitors α_2-macroglobulin and α_1-proteinase inhibitor and degraded the Fc portion of IgG. Recently, aspartic proteases of *C. albicans* were shown to cleave C3b, C4b, and C5 (11). Cleavage of C3 and C5 was described for *Porphyromonas gingivalis* (67; for a review, see reference 57), a bacterium implicated in periodontal disease. Proteases of *P. gingivalis* also cleaved the C5a receptor on neutrophils (19). The *Treponema denticola* surface protease dentilisin hydrolyzed the α chain of C3b, thereby producing iC3b (68). The streptococcal pyrogenic exotoxin B (SpeB) degrades C3 (29, 60). Preincubation of serum with SpeB enhanced resistance of group A streptococci to complement-mediated lysis. A strain lacking SpeB was opsonized with more C3 fragments (29). Infection with the SpeB mutant resulted in migration of neutrophils to the site of infection, which phagocytosed SpeB mutant bacteria, whereas only few neutrophils were recruited in case of infection with the wild type (60). The cysteine protease EhCP1 of the protozoan parasite *Entamoeba histolytica* cleaved C3, immunoglobulin G, and pro-interleukin-18 (32). *Salmonella enterica* produces the surface protease PgtE, which cleaves C3b, C4b, and C5 (47). Recently, interpain A, a cysteine proteinase, was described as an important virulence factor of *Prevotella intermedia* (43). The protease cleaved C3 and thereby inhibited complement activation via all three pathways and increased serum resistance of *P. intermedia*.

The *A. fumigatus* protease Alp1 that we describe here showed a broad proteolytic activity. The degrading activity includes several human complement proteins, such as C3, C4b, C5, C1q, and IgG, as shown using supernatants and also protein extract of an Alp1-overproducing *E. coli* strain. Thus, proteolytic cleavage was not limited to complement component C3, as described for most proteases (with the exception of PgtE of *S. enterica*). Cleavage of these proteins was nearly complete so that after incubation hardly any degradation products were visible. The complement regulators factor H and C4BP were also degraded by culture supernatant. Since *A. fumigatus* hyphae did not bind and employ these molecules for complement inactivation on the hyphal surface, the cleavage of these regulators does not result in a counterproductive effect. The *alp1* deletion strain lacked most of the complement-degrading activity. In Δ*alp1* cultures during growth on human serum, C3 remained intact longer than in wild-type cultures. Other proteases of *A. fumigatus* seem to act in concert with Alp1 in the degradation of complement proteins. However, Alp1 is the major protease involved in this degradation process.

The *A. fumigatus* genome contains sequences coding for 56 known or putative proteases (8). Since *A. fumigatus* is a saprophyte, proteases were thought to be essential for survival in the natural habitat and to be a possible virulence determinant that might enable the fungus to grow in the lungs, a habitat rich in elastin. The secreted (36, 49, 51) and cell wall-attached (50) protease deletion strains of *A. fumigatus* generated until now grew as invasively as the wild type. Alp1 is a major secreted protease of *A. fumigatus* and is also secreted during infection of the lungs (49, 58). It exhibits elastase activity and cleaves elastin. However, an *alp1*-disrupted *A. fumigatus* strain was as virulent in a cyclophosphamide-based murine infection model as the wild type and showed the same invasive growth (37, 58). Recently, a global protease regulator was identified in *A. fumigatus*. It governs expression of multiple secreted proteases (Alp1, MEP, Dpp4, CpdS, AFUA_2G17330, and AFUA_7G06220). Culture filtrate from a deletion strain exhibited reduced killing of lung epithelial cells and lysis of erythrocytes. However, as in protease single-deletion strains, the downregulation of multiple proteases had no effect on virulence of the fungus in mouse infection models with either cortisone acetate alone or cyclophosphamide and cortisone acetate (3, 54).

Culture filtrate of *A. fumigatus* suppresses chemotaxis and O_2^- release by neutrophils. With an *alp1*-disrupted strain, this suppression was less prominent and the cells showed more motility and O_2^- release (18). Here we applied a cortisone acetate mouse model to test the impact of these effects on the virulence of the Δ*alp1* and Δ*alp1*^{C} strains. In these mice, neutrophils are not depleted as they are in cyclophosphamide-treated mice. Degradation of complement by *A. fumigatus* should lead to less production of anaphylatoxins and consequently in impaired homing of neutrophils. Such an effect was seen for *S. pyogenes* protease SpeB (60). However, no increase in neutrophil infiltration was detected for the Δ*alp1* strain. Despite these data based on animal experiments, the influence of Alp1 might be more subtle than could be measured in these experiments. Secretion of proteases might play a role in a more secluded area such as the central nervous system (CNS). Vogl et al. (62) showed that complement proteins have low abundance in *A. fumigatus* brain abscesses and that hyphae isolated from liquor showed decreased C3 deposition. This finding could be due to the proposed *A. fumigatus* complement inhibitor (65). A more recent study by Rambach *et al.* (45a) suggests that a secreted protease, most likely Alp1, causes complement degradation in cerebral aspergillosis. Our findings corroborate the central role of Alp1 in this cleavage.

In conclusion, we describe a new function for a major protease of *A. fumigatus*. Alp1 efficiently cleaves important human components of the complement cascade, which leads to downregulation of complement activation at the hyphal surface. Thus, *A. fumigatus* possesses at least two distinct mechanisms to evade the human complement system: the binding of complement regulators to conidia and the secretion of the protease Alp1 by hyphae.

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