The Glycosylphosphatidylinositol-Anchored Protease Sap9 Modulates the Interaction of *Candida albicans* with Human Neutrophils[▽]

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Received 26 June 2009/Returned for modification 29 July 2009/Accepted 25 September 2009

Human polymorphonuclear neutrophils (PMNs) play a major role in the immune defense against invasive *Candida albicans* infection. This fungal pathogen produces a set of aspartic proteases that directly contributes to virulence properties such as adhesion, tissue invasion, and immune evasion. We show here that, in contrast to other secreted proteases, the cell surface-associated isoform Sap9 has a major impact on the recognition of *C. albicans* by PMNs. *SAP9* is required for the induction of PMN chemotaxis toward *C. albicans* filaments, an essential prerequisite of effective PMN activation. Furthermore, deletion of *SAP9* leads to a mitigated release of reactive oxygen intermediates (ROI) in human PMNs and decreases *C. albicans*-induced apoptosis triggered by ROI formation. In confrontation assays, killing of a *SAP9* deletion mutant is reduced in comparison to wild-type *C. albicans*. These data clearly implicate Sap9 protease activity in the initiation of protective innate immunity and suggest novel molecular mechanisms in *C. albicans*-host interaction leading to neutrophil activation.

Systemic disease caused by the fungal pathogen *Candida albicans* is among the most important infections in immunocompromised patients. Beside other clinical conditions, neutropenia is a major risk factor for developing systemic candidiasis. Polymorphism of *C. albicans*, enabling morphological plasticity in response to ambient conditions, is known to contribute to its virulence. Filamentation of *C. albicans* is associated with tissue destruction and invasion (14, 25, 51, 58, 59). In addition to filamentation, secreted aspartic proteases of *C. albicans* (Saps) have been shown to be involved in the infection process (for reviews, see references 32 and 33). The *C. albicans* *SAP* gene family includes 10 members (*SAP1* to *SAP10*), and expression of these genes is differentially regulated during interaction with the human host. *SAP1* to *SAP3* are mainly expressed in yeast cells and during phenotypic switching (21, 54). In different studies, a role for these genes in mucosal and skin infections could be observed (11, 26, 45, 46). In contrast, *SAP4* to *SAP6* are exclusively expressed in filaments (21, 54). Their expression has been linked to macrophage destruction and evasion of *C. albicans* (4), as well as the pathogenesis of systemic disease (15, 24, 44). Very little is known about *SAP7* and *SAP8*. Although both of these genes are expressed in different infection models, the expression of *SAP7* does not correlate with virulence (34, 50).

In contrast to all other members of the Sap family, the proteases Sap9 and Sap10 are bound to the fungal cell surface by a glycosylphosphatidylinositol (GPI) anchor motif. Sap9

seems to be predominantly located in the cell membrane, and Sap10 is located in the cell wall and membrane (3). The expression of *SAP9* and *SAP10* was monitored under several conditions in vitro and in vivo and is possibly independent of pH and morphotype. Since mutants lacking *SAP9* and/or *SAP10* had altered adhesion properties and were mitigated in inducing tissue damage, a role for these proteases in infection and adherence processes has been proposed (3).

The human polymorphonuclear neutrophil (PMN) is the predominant cell type in peripheral blood and is the first line of defense in the innate immune system. Upon stimulation with inflammatory stimuli, PMNs rapidly migrate to the sites of infection and start to eliminate the invading microorganisms (47). We have shown previously that migration initiated after phosphorylation of the extracellular signal-regulated kinase mitogen-activated protein kinase is a central component in the activation of PMNs by filamentous forms of *C. albicans* (56). To counteract infection, the terminally differentiated PMNs possess a set of different mechanisms for eliminating invasive microorganisms. These include the generation of reactive oxygen intermediates (ROI), phagocytosis, and the release of granular enzymes and antimicrobial peptides, as well as the formation of neutrophil extracellular traps (5, 38, 47). The release of ROI is one of the central effector mechanisms of neutrophils. ROI are generated by the multicomponent NADPH oxidase complex, which is assembled at the cytoplasmic membrane after stimulation of the neutrophils (38). ROI formation is closely linked to the induction of apoptosis in PMNs, which modulates infection outcome (10). Once the task of killing microorganisms is completed, neutrophil apoptosis, followed by macrophage ingestion, helps to prevent release of cytotoxic compounds into host tissue and thus reduces acute inflammation. Many bacterial pathogens, e.g., *Staphylococcus*

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[▽] Published ahead of print on 5 October 2009.

TABLE 1. Strains used in this study

Designation	Clone or strain	Genotype	Reference
Wild type	SC5314		19
Wild type	CAI4[pClp10]	Δ ura3::imm434/ Δ ura3::imm434+pClp10 (integration)	31
Δ sap1-3	M119	Δ sap1::hisG/ Δ sap1::hisG; Δ sap2::hisG/ Δ sap2::hisG; Δ sap3::hisG/ Δ sap3::hisG-URA3-hisG	23
Δ sap4-6	M31	Δ sap6::hisG/ Δ sap6::hisG; Δ sap4::hisG/ Δ sap4::hisG; Δ sap5::hisG/ Δ sap5::hisG-URA3-hisG	
Δ sap9/10	M1188	Δ ura3::imm434/ Δ ura3::imm434; Δ sap10::hisG/ Δ sap10::hisG; Δ sap9::hisG/ Δ sap9::hisG-URA3-hisG	3
Δ sap9	M1018	Δ ura3::imm434/ Δ ura3::imm434; Δ sap9::hisG/ Δ sap9::hisG-URA3-hisG	3
Δ sap10	M1171	Δ ura3::imm434/ Δ ura3::imm434; Δ sap10::hisG/ Δ sap10::hisG-URA3-hisG	3
Δ sap9[SAP9]	M1182/Re5	Δ ura3::imm434/ Δ ura3::imm434; Δ sap9::hisG/ Δ sap9::hisG+pClp10-SAP9 (integration)	3
Δ sap9[pCIP10]	M1180	Δ ura3::imm434/ Δ ura3::imm434; Δ sap9::hisG/ Δ sap9::hisG+pClp10 (integration)	3

aureus or *Escherichia coli*, are known to induce the apoptosis of neutrophils (28, 53, 57). Other pathogens, such as *Leishmania major* or *Paracoccidioides brasiliensis*, can either delay or inhibit apoptosis to replicate and survive within PMNs (1, 2). For *C. albicans*, evidence exists that it may induce apoptosis when phagocytosed by PMNs (40). Although plenty of data dealing with the effects of Saps on different cell types have been published, the role of Saps in the interaction with PMNs is poorly investigated. We show here that the membrane-bound protease Sap9 modulates the response of human PMNs to *C. albicans*.

MATERIALS AND METHODS

Isolation of neutrophils. Neutrophils were isolated from the peripheral blood of healthy volunteers by a gradient centrifugation technique using Polymorph-prep (Nycomed, Oslo, Norway) (39). The remaining red blood cells were lysed with ACK buffer (Lonza) for 5 min. Cell viability was confirmed by trypan blue exclusion. In fluorescence-activated cell sorting (FACS) analyses, >95% of the cells were CD66b positive (Becton Dickinson, Heidelberg, Germany). FACS analysis was carried out with a FACSCalibur cytometer (Becton Dickinson).

Fungal growth and hyphal induction. The *C. albicans* strains used in the present study are listed in Table 1. Fungi were stored in a 35% glycerol stock, streaked onto Sabouraud agar plates before use, and incubated overnight at 28°C. A temperature shift was used to induce germination. Fungi were inoculated into 20 ml of RPMI 1640 medium with 5% fetal calf serum (FCS), followed by incubation at 37°C for at least 1 h until germ tubes were formed. The germ tube length was monitored microscopically and kept small to avoid clumping and to minimize the mass difference between yeasts and germ tubes. Yeast cells were generated in 20 ml of RPMI 1640 medium with 5% FCS, followed by incubation at 22°C for 2 h.

Alternatively, a pH shift was used to induce morphogenesis. For that purpose, *C. albicans* was inoculated into M199 medium at pH 4 (for the induction of yeast morphology) or pH 7 (for the induction of filamentation), followed by incubation at 30°C until the formation of filaments (~2 h). Fungi grown by this method were used for the chemotaxis experiment.

Oxidative burst measurements. The oxidative burst was determined by using dihydrodichlorofluorescein diacetate (DCFH-DA; Sigma). DCFH-DA is deacetylated intracellularly and can be oxidized upon ROI release to form the fluorescent substance dichlorofluorescein. To a total of 2×10^7 PMNs (4×10^6 /ml), DCFH-DA was added at a 2.5 μ M final concentration. Then, 50- μ l portions of fungal cells (2×10^6 /ml) were pipetted into the wells of a 96-well flat-bottom polypropylene plate (Greiner Bio-One). As a control for PMN activation, phorbol myristate acetate (PMA; Sigma) diluted in RPMI 1640 medium plus 5% FCS was used in a final concentration of 50 ng/ml. A negative control contained RPMI 1640 medium plus 5% FCS without activating stimulus. Then, 50 μ l of the PMN suspension (fungus/PMN ratio = 0.5, i.e., the multiplicity of infection [MOI]) was added to each well, and the fluorescence was measured at 37°C over 2.5 h in a plate reader (GENios; Tecan, Crailsheim, Germany) at 485/535 nm. For ROI inhibition experiments, PMNs were preincubated with 5 μ g of diphenylene-iodonium chloride (DPI; Sigma)/ml or 1 mg of glutathione (Sigma)/ml for 30 min at 37°C, followed by incubation in the presence of the inhibitors. The

results were expressed as x-fold values of the negative control (medium and cells) at the indicated time point.

Transwell assays. Migration of PMNs was measured by using fluorescent-labeled PMNs migrating through a Transwell membrane (3- μ m pore size, polyester, for 24-well-plates [Costar]) according to a protocol described earlier (13). Briefly, 5×10^6 PMNs per ml were loaded with 3.3 μ M 2',7'-bis-(carboxyethyl)-5-(and 6-)-carboxyfluorescein (BCECF-AM; Invitrogen) for 20 min at room temperature, washed, and resuspended in RPMI 1640 plus 5% FCS. The upper compartment of the Transwell system was filled with 100 μ l of PMNs (corresponding to 5×10^5 cells) and placed into a well containing 600 μ l of medium with or without fungal cells (MOI = 1) or fMLP (*N*-formyl-methionyl-leucyl-phenylalanine; Sigma) as a positive control for a chemotactic stimulus; the negative control contained only medium. After incubation at 37°C for 1 h, the inserts were removed, and the fluorescence of the wells was measured in a GENios plate reader (485/530 nm).

Plate-based infection assays. For plate-based infection assays, 8×10^4 PMNs (prestimulated with 25 ng of PMA/ml for 30 min at 37°C) and 8×10^4 fungal cells (MOI = 1; also with PMA addition) were incubated in 40 μ l of RPMI 1640 medium plus 5% FCS for 0 to 3 h at 37°C. PMNs were lysed by adding 2 ml of ice-cold sterile water to the samples. Serial dilutions of the samples were plated on Sabouraud agar, followed by incubation for 24 h at 37°C. Counting of colonies was done by using the ProtoCol colony counter. Calculation of the killing rate was performed by normalizing against the control (samples containing only fungi without PMN = 100%).

XTT assays. XTT {2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium hydroxide} assays were prepared as described by Meshulam et al. and Gaviria et al., with modifications (17, 30). XTT (Sigma) was prepared freshly in 1 $\times$ phosphate-buffered saline (PBS) at 0.5 mg/ml with heating at 55°C for 30 min. Coenzyme Q (2,3-dimethoxy-5-methyl-1,4-benzoquinone; Sigma) was added to a final concentration of 50 μ g/ml. A total of 10^6 fungal cells and a total of 10^6 prestimulated PMNs (25 ng of PMA/ml, 30 min at 37°C; MOI = 1) were suspended in 200 μ l of RPMI 1640 medium plus 5% FCS, followed by incubation at 37°C for 0 to 3 h. Next, 800 μ l of ice-cold sterile water was added to lyse the PMNs, followed by centrifugation at 14,000 rpm and 4°C for 4 min. The supernatant was carefully aspirated, and the procedure was repeated once more with 1 ml of water. Fungal viability was analyzed by adding 400 μ l of the XTT solution to the pellet for 1 h at 37°C, followed by a centrifugation at 14,000 $\times$ g and 4°C for 1 min. Then, 100 μ l of the supernatant was transferred into a 96-well plate (Nunc MaxiSorb). The absorbance of each sample was determined at 450 nm by using a GENios plate reader. The percentage of fungal damage was calculated as follows: $\{1 - [A_{450}(\text{fungi} + \text{PMNs}) - A_{450}(\text{PMNs})]/A_{450}(\text{fungi})\} \times 100$.

Growth inhibition experiments. For this assay, a total of 10^7 germ tubes in 1 ml of RPMI 1640 medium plus 5% FCS was stained with 0.1 mg of fluorescein isothiocyanate (FITC; Sigma) for 15 min at 37°C, followed by two washing steps in RPMI 1640 medium plus 5% FCS. After coincubation of 10^5 fungi with the same amount of PMNs (MOI = 1) for 1 h, the samples were centrifuged on glass slides, fixed with 3.7% paraformaldehyde for 10 min, and washed with PBS. Slides were then mounted with Fluoprep (bioMérieux) and analyzed by fluorescence microscopy. The growth of the fungi during the experiment (represented by the nonfluorescent part of the fungi) was quantified in relation to fungal size before coincubation (fluorescent fungal parts) by using Zeiss AxioVision software. Eighty randomly chosen fungi were measured for every experimental

setting. Fungal growth in the absence of PMNs was used as the control and set as 100% for the calculation.

Apoptosis quantification. PMNs (5×10^5) were coincubated with 5×10^5 fungi (MOI = 1) in RPMI plus 5% FCS. As a negative control (spontaneous apoptosis only), uninfected PMNs were used. For ROI inhibition experiments, PMNs were preincubated with 5 μ g of DPI/ml or 1 mg of glutathione/ml for 30 min at 37°C, followed by coincubation in the presence of the inhibitors. After the indicated time points, samples were centrifuged at 2,500 rpm for 2 min, taken up in 500 μ l of PBS, and centrifuged again. After the wash with PBS, samples were resuspended in 500 μ l of annexin V binding buffer (stock solution [10-fold concentrated]: 0.1 M HEPES [pH 7.4], 1.4 M NaCl, 25 mM CaCl_2), followed by another centrifugation step and resuspension in annexin V binding buffer. For cell staining, 5 μ l of annexin V-FITC (BD) and 7 μ l of a 50- μ g/ml propidium iodide (PI) stock solution (Sigma) were added, followed by incubation at room temperature in the dark for 15 min. Next, samples were washed, resuspended in annexin V binding buffer, and analyzed with a FACSCalibur flow cytometer. Evaluation of data was performed by using CellQuest software (Becton Dickinson).

Cytokine analysis. The secretion of interleukin-8 (IL-8) from PMNs was analyzed with the IL-8 CytoSet (BioSource). Briefly, 10^6 PMNs and 10^6 fungi per sample (MOI = 1) were coincubated at 37°C, and supernatants from uninfected PMNs were used as a negative control. Supernatants were collected at the indicated time points and stored at -80°C until further use. Samples were diluted 1:5 in assay buffer, and 100- μ l portions of these dilutions were used according to the manufacturer's instructions.

Statistics. All statistical evaluations were carried out with Microsoft Excel. A two-tailed Student *t* test was used to calculate significance ($P < 0.05$). All experiments were performed at least three times with cells from different blood donors.

RESULTS

SAP9 is involved in triggering PMN chemotaxis toward germ tubes. Previously, we have shown that targeted motility of human PMNs induced by *C. albicans* filaments is a prerequisite for efficient antifungal activity of these phagocytic cells (56). To investigate the effect of Saps on PMN chemotaxis, transwell assays were used to quantify PMN migration toward yeasts and filaments of *C. albicans* strain SC5314 and the $\Delta sap1-3$, $\Delta sap4-6$, and $\Delta sap9/10$ deletion mutants, respectively. As expected from previously published experiments, yeast cells did not trigger relevant migratory activity at an MOI of 1 after 1 h of coincubation (56). In addition, no differences between wild-type *C. albicans* and the protease-deficient mutants could be detected (data not shown). In contrast and as shown previously, wild-type filaments at an MOI of 1 were a potent inducer of PMN motility. For comparative analyses, the length of filaments was adjusted to be equal for all strains by incubating the slower germinating strain $\Delta sap9/10$ for a longer time period. Deletions of *SAP1* to *SAP3* ($65\% \pm 35\%$) and *SAP4* to *SAP6* ($68\% \pm 22\%$) did not change the PMN migratory activity in response to filaments compared to the wild-type (Fig. 1). In contrast, the $\Delta sap9/10$ deletion mutant ($42 \pm 10\%$) triggered a significantly reduced rate of migration compared to wild-type filaments ($65\% \pm 26\%$, $P < 0.05$; Fig. 1). To test whether this effect was due to the combined deletion of both *SAP9* and *SAP10* or due to either *SAP9* or *SAP10*, the $\Delta sap9$ and $\Delta sap10$ single mutants were used in the experiments. Although filaments of the $\Delta sap10$ mutant ($58\% \pm 34\%$) induced a migratory activity similar to that of the wild type, filaments of the $\Delta sap9$ mutant ($29\% \pm 4\%$) had significantly reduced abilities to cause attraction (Fig. 1). To confirm a role for *SAP9* in the induction of targeted motility toward *C. albicans* filaments, the complemented strain $\Delta sap9[SAP9]$ was tested for its capacity to induce targeted motility of PMNs. As expected, filaments of this

FIG. 1. Deletion of *SAP9* leads to a decreased migratory activity of human PMNs in response to *C. albicans* filaments. The chemotaxis of PMNs was determined in a transwell system. Formylated peptide (fMLP) was used as a positive control; medium without stimulus served as a negative control. *C. albicans* $\Delta sap9/10$ and $\Delta sap9$ strains induced significantly reduced migration rates compared to the wild type and other deletion mutants. The wild-type phenotype was restored by reintegration of *SAP9* ($\Delta sap9[SAP9]$). Bars show the arithmetic means of six experiments $\pm$ the standard deviation. WT, wild type.

strain induced wild-type levels of migratory activity ($53\% \pm 24\%$, Fig. 1). Our own previous studies have shown that IL-8 secretion by PMNs is involved in the overall recruitment of neutrophils initiated by *C. albicans* filaments (56). Therefore, we tested the secretion of IL-8 by PMN using *C. albicans* wild-type and the $\Delta sap9$ mutant cells. However, no significant differences could be observed, indicating that the effect of *SAP9* deletion on migratory activity is not primarily due to the differential induction of IL-8.

The deletion of *SAP9* leads to an altered release of ROI in human PMNs. One of the most important effector mechanisms of human PMNs is the release of ROI after contact with microorganisms or microbial components. To analyze the role of Saps in the induction of ROI, primary human PMNs were infected with *C. albicans* strain SC5314 and $\Delta sap1-3$, $\Delta sap4-6$, and $\Delta sap9/10$ deletion mutants, respectively. The release of ROI was quantified in real-time assays based on the conversion of dichlorodihydrofluorescein to the fluorescent marker dichlorofluorescein upon oxidation. Small filaments and yeast cells of the respective strain were used as stimuli. The same cell numbers of yeast and hyphal cells were used. In general, differences in dry weight between yeasts and small filaments were $\leq 20\%$. Over time, the oxidative burst induced by yeast cells of the $\Delta sap1-3$ and $\Delta sap4-6$ strains was similar to that of the wild-type yeasts. In contrast, the $\Delta sap9/10$ strain was not able to induce an oxidative response similar to wild-type levels but leads to a significantly lower induction of ROI (Fig. 2A). The same pattern was observed for germ tubes from wild-type and mutant strains (Fig. 2B). Again, the $\Delta sap1-3$ and $\Delta sap4-6$ mutants did not differ from the wild type, and the $\Delta sap9/10$ strain led to a significantly decreased release of ROI. Time point analyses showed a reduction of ROI after 100 min of coincubation for the $\Delta sap9/10$ yeasts (3-fold of control versus the wild type [7 ± 3 -fold of control]; $P < 0.05$, Fig. 2A). This effect was even more pronounced with germ tubes from the $\Delta sap9/10$

FIG. 2. Time point analyses of ROI formation in human PMNs induced by *C. albicans* and *SAP* deletion mutants. ROI release was quantified by real-time detection of dichlorodihydrofluorescein to fluorescein after coincubation of fungi with PMNs. Medium was used as a negative control. (A) After 100 min, yeast forms of the *C. albicans* $\Delta sap9/10$ and $\Delta sap9$ mutants induced significantly reduced levels of ROI compared to the wild type and the other deletion mutants. A wild-type phenotype was restored by ectopic expression of *SAP9* ($\Delta sap9[SAP9]$). (B) ROI formation induced by filaments of *C. albicans* after 100 min. A similar pattern as for yeast forms was seen with generally higher levels of ROI induction. Again, the deletion of *SAP9* results in decreased ROI levels. Values represent the x-fold of the negative control (medium and PMN without stimulus). WT, wild type.

mutant (13 ± 3 -fold of control versus the wild type [21 ± 6 -fold of control]; $P < 0.05$) (Fig. 2B). The respective single mutants were tested to determine which of the two Saps was responsible for the mitigated release of ROI. Similar to the results observed for the induction of targeted motility, the $\Delta sap10$ mutant did not lead to any reduction in ROI levels and thus behaved identically to the wild type. In contrast, a $\Delta sap9$ mutant induced significantly decreased levels of ROI compared to wild-type *C. albicans*. This defect could be reversed by using a complemented strain carrying the plasmid pCip10 with a functional copy of *SAP9* ($\Delta sap9[SAP9]$, Fig. 2). To verify that the reconstitution of wild-type-like ROI release by the complemented strain was an effect of the plasmid *SAP9* and not the plasmid itself, a *sap9* deletion mutant carrying the empty plasmid was tested. This mutant induced ROI amounts similar to those induced by the *sap9* single mutant without plasmid (data not shown). To further investigate the impact of *URA3* manip-

ulation in the mutants, the isogenic strain CAI4[pCip10] was used in this assay. No significant difference could be found compared to the wild-type strain SC5314 (data not shown). Therefore, the observed effects are due to deletion and reintegration of the *SAP9* gene. In accordance with previously published data, no growth deficit could be observed for the $\Delta sap9$, $\Delta sap10$, and $\Delta sap9/10$ mutants (3). However, to exclude an effect of divergent biomass, experiments were performed in which all strains had been equilibrated for dry weight prior to interaction with PMNs and yielded similar results (data not shown). In addition, the budding dysfunction that can be observed for all three mutants in liquid synthetic dropout (SD) medium (3) was not evident in RPMI 1640 under the conditions used in the present study. To exclude that some functions of Sap9 might depend on the growth medium, experiments were repeated with yeasts grown in liquid SD medium for 2 h. After 2 h of incubation in SD medium, clumping is not yet pronounced and counting of single cells is therefore feasible. As expected, the wild-type, $\Delta sap9$, and $\Delta sap10$ strains grown for 2 h in SD medium induced patterns of ROI release identical to those of fungi grown in RPMI for 2 h (data not shown). Therefore, the observed effects do not depend on the growth medium.

***C. albicans*-induced neutrophil apoptosis is decreased by deletion of *SAP9*.** Apoptosis of human neutrophils is a crucial event in the infection process (12). Heat-killed *C. albicans* cells have been shown to induce PMN apoptosis upon phagocytosis (40). To investigate the effects of live *C. albicans* on PMN apoptosis and a putative role of Saps in the induction of programmed cell death, PMNs were coincubated with wild-type and mutant yeast cells and germ tubes for different time periods, and the apoptosis of PMNs was quantified by using flow cytometry. Annexin V, a marker for apoptosis, which binds to exposed phosphatidylserine from cells undergoing apoptosis, and propidium iodide, which stains disrupted cells, were used to discriminate between apoptosis and necrosis. Samples from the time points of 1 to 3 h of coincubation were analyzed, and the percentage of annexin V-FITC-labeled cells was calculated. The apoptosis rate significantly increased when PMNs were coincubated with wild-type germ tubes compared to wild-type yeast cells. To test whether *SAP9* has an influence on the induction of apoptosis, the $\Delta sap9/10$ double mutants and $\Delta sap9$ single mutants were used in this assay. We observed no differences between the strains after 1 h of coincubation, and the apoptosis rate of PMNs remained low in all samples. After 2 h of incubation, the apoptosis induced by all yeast cells remained low and without differences, whereas the apoptosis induced by the germ tubes of the $\Delta sap9/10$ ($15\% \pm 5\%$) and the $\Delta sap9$ ($10\% \pm 6\%$, $P < 0.05$) deletion mutants was significantly reduced compared to that induced by the wild-type germ tubes ($31\% \pm 18\%$, Fig. 3A and B). In dot plot analyses, a clear population shift from annexin V-FITC-negative to -positive PMNs could be seen when the wild type was used as a stimulus instead of the $\Delta sap9$ mutant (Fig. 3C and D). The complemented strain $\Delta sap9[SAP9]$ was able to restore this effect (Fig. 3B). Interestingly, the maximal percentage of apoptotic PMNs was already observed after 2 h (wild-type yeasts, $11\% \pm 6\%$; wild-type filaments, $31\% \pm 18\%$ annexin V-FITC-positive cells; Fig. 3A and B). After 3 h, the number of apoptotic PMNs was stagnating, with no changes in the apoptosis

FIG. 3. Induction of apoptosis in PMNs by *C. albicans*. (A) Only low levels of apoptosis were induced by *C. albicans* yeast cells after 2 h of contact with PMNs. (B) In contrast, filamentous forms induced relevant levels of apoptosis after 2 h. Filaments of the $\Delta sap9/10$ (not shown) and $\Delta sap9$ mutants induced significantly reduced apoptosis, whereas apoptosis rates for $\Delta sap9[SAP9]$ were restored to wild-type levels. (C and D) The effect of *SAP9* deletion, depicted in exemplary dot plots. The wild type induces a pronounced shift in the PMN population (C), whereas the fraction of annexin V-FITC-positive PMNs remains lower upon stimulation with the $\Delta sap9$ mutant (D). Bars represent arithmetic means of the percentage of annexin V-positive/PI-negative cells from 13 experiments $\pm$ the standard deviation. WT, wild type.

rates. Pathogen-induced apoptosis of PMNs has been linked to the generation of ROI by NADPH oxidase. Since the $\Delta sap9$ and $\Delta sap9/10$ mutants induced lower levels of ROI than did the wild type, we hypothesized that the reduced induction of apoptosis might be related to the capacity of the strains to induce ROI formation. Therefore, PMN apoptosis was quantified in the presence of DPI, a known inhibitor of neutrophil NADPH oxidase. A 5- μ g portion of DPI/ml was sufficient to completely prevent *C. albicans*-induced ROI formation in human PMNs (Fig. 4A). Indeed, significantly reduced rates of apoptosis were found in the presence of DPI (Fig. 4B). Furthermore, no differences in apoptosis induction were found between wild-type *C. albicans* cells and $\Delta sap9/10$ or $\Delta sap9$ mutant cells in the presence of DPI. In control experiments, neither DPI nor the solvent dimethyl sulfoxide affected fungal or neutrophil viability. To confirm a role of ROI in *C. albicans*-induced apoptosis of PMNs, the experiments were repeated without DPI but in the presence of 1 mg of glutathione/ml. This substance effectively prevents ROI accumulation (Fig. 4A) but not ROI formation. As expected, glutathione was able to significantly reduce the apoptosis of PMNs, and was only slightly less effective than DPI (Fig. 4B). Thus, *C. albicans*-induced ROI can trigger PMN apoptosis, and the reduced ability of the $\Delta sap9/10$ and $\Delta sap9$ mutants to trigger ROI formation is correlated with the reduced induction of PMN apoptosis.

PMN-mediated inhibition of *C. albicans* filamentation is independent of *SAP9*. One of the most significant effects of

human PMNs on *C. albicans* is the inhibition of filamentation (16, 42). Indeed, PMNs are the only host cells known thus far that are capable of inhibiting germ tube elongation in *C. albicans*. To test whether Saps are involved in PMN-mediated growth inhibition of *C. albicans* filamentous forms, small germ tubes of *C. albicans* wild type and the $\Delta sap9/10$ mutant were labeled with FITC, followed by incubation for 1 h in RPMI (37°C) in the presence or absence of human neutrophils. Filamentation during the incubation period was quantified by determining the length of the nonfluorescent apical part of filaments in relation to the fluorescent basal part of the filaments (Fig. 5A). In this setting, neutrophils reduced the filamentation of wild-type *C. albicans* by 75% (Fig. 5B). Similar results were obtained with the $\Delta sap9/10$ mutant (78%), indicating that the inhibition of filamentation is not dependent on the presence of cell wall-associated proteases.

***SAP9* is involved in PMN recognition and killing of *C. albicans*.** Since we observed that the oxidative and chemotactic response of PMNs to *C. albicans* $\Delta sap9/10$ and $\Delta sap9$ mutants is reduced, we questioned whether the ability of PMNs to kill these mutants might also be altered. PMNs and yeast cells were coincubated in a plate-based infection assay at an MOI of 1 for different time periods. Next, neutrophils were lysed, and fungi were plated in serial dilutions to monitor the CFU. As a control, samples containing only fungi incubated under the same conditions were plated at every time point and set to 100%. After 1 h, no differences between the mutants and the wild type

FIG. 4. *C. albicans*-induced PMN apoptosis is dependent on ROI generation. (A) ROI production of PMN after stimulation with fungi in the presence of the NADPH oxidase inhibitor DPI (5 μg/ml) and the ROI scavenger glutathione (1 mg/ml). The oxidative burst is significantly reduced by both substances. Values represent the x-fold of the negative control. (B) Apoptosis of PMNs was quantified after 2 h of coincubation with *C. albicans* wild-type, $\Delta sap9$, and $\Delta sap9[SAP9]$ mutant strains in the presence of DPI (5 μg/ml) and glutathione (1 mg/ml). No relevant apoptosis was detected for any of the strains. Bars show arithmetic means of five experiments $\pm$ the standard deviation. WT, wild type.

were observed, and the survival rate of all fungi was calculated to be ca. 90%. One hour later, the survival rate was decreased to 72% $\pm$ 21% for the wild type compared to 93% $\pm$ 25% for the $\Delta sap9$ mutant strain. After 3 h, 53% $\pm$ 14% of the wild-type yeast cells were killed, and the $\Delta sap9$ mutant yeasts survived significantly better (88% $\pm$ 15%; $P < 0.05$). By the use of the $\Delta sap9[SAP9]$ complemented strain, which was killed to a similar extent as the wild-type yeast cells, it was verified that the influence on overall killing was *SAP9* dependent (Fig. 6A). This was additionally underscored by the findings that the $\Delta sap10$ mutant was not able to withstand PMN killing signifi-

FIG. 5. *SAP9* is not required for PMN-induced growth arrest of *C. albicans* filaments. Filament growth was quantified in the absence or presence of PMNs by using fluorescently stained filaments. PMNs induce a significant growth arrest in both wild-type (A) and $\Delta sap9/10$ (B) *C. albicans* strains. (C) Bars show the arithmetic means of four experiments $\pm$ the standard deviation; 80 fungi were randomly evaluated for each experiment and strain. WT, wild type.

cantly better than the wild-type yeasts (data not shown). Furthermore, an XTT-based assay was used to assess the efficiency of PMNs to damage filamentous forms of *C. albicans* (Fig. 6B). As observed in the experiments with yeast cells, germ tubes of the $\Delta sap9/10$ and $\Delta sap9$ mutants were less damaged than wild-type germ tubes (36% $\pm$ 18% and 47% $\pm$ 9%, respectively, compared to 64% $\pm$ 18% damaged wild-type germ tubes) after 3 h of coincubation. Again, germ tubes of the complemented strain $\Delta sap9[SAP9]$ showed a restored susceptibility to neutrophil-mediated damage. Therefore, the deletion of *SAP9* modulates the killing of *C. albicans* by human PMNs.

DISCUSSION

Ten *SAP* genes encoding different Sap isoenzymes have been identified in *C. albicans* (32, 33). The expression of some of these genes was shown to be associated with different fungal morphologies: while *SAP1* to *SAP3* are mainly expressed in

FIG. 6. (A) Plate-based infection assays show that the ability of human PMNs to kill *C. albicans* yeasts is significantly reduced for a $\Delta sap9$ mutant. This effect is reversed by the ectopic expression of *SAP9* ($\Delta sap9[SAP9]$). Bars show arithmetic means of five experiments $\pm$ the standard deviation. (B) An XTT-based assay for quantification of fungal cell damage shows that the ability of human PMNs to inflict damage to *C. albicans* $\Delta sap9$ filaments was attenuated. This effect was reversed by ectopic expression of *SAP9* ($\Delta sap9[SAP9]$). Bars show arithmetic means of six experiments $\pm$ the standard deviation. WT, wild type.

yeast cells and the opaque morphology during phenotypic switching, the expression of *SAP4* to *SAP6* was shown to be hypha associated (21, 54). Certain members of the Sap family, in particular, Sap2, are essential for fungal growth when protein is the sole nitrogen source (49). However, in addition to digesting proteins for nutrient acquisition, Saps also contribute to interactions of *C. albicans* with the human host. Sap protease activity has been linked to the adherence to host cells, tissue invasion and immune evasion. Furthermore, Sap substrates include immunoglobulin A, lactoferrin, cathepsin D, complement components, and antimicrobial peptides (8, 18, 20, 22, 29, 43).

SAP4 to *SAP6* have been shown to be expressed after ingestion by murine macrophages, and a mutant lacking these genes had a reduced potential to survive phagocytosis by these cells (4). Furthermore, it has been shown that the expression of *SAP* genes in vivo is tissue specific and regulated in a stage-specific manner (15, 48).

Sap9 and Sap10 differ from other Saps since they are attached to the cell wall (Sap10) and the cell membrane (Sap9 and Sap10) via GPI anchors. Both proteases cleave at dibasic or basic processing sites and have functions in cell surface integrity and cell separation during budding. In addition, deletion of *SAP9* and *SAP10* modified the adhesion properties of

C. albicans to epithelial cells and caused attenuated epithelial cell damage during experimental oral infection, suggesting a unique role for these proteases in both cellular processes and host-pathogen interactions (3). In epithelial models, murine parenchymal organs, and a human immunodeficiency mouse model, *SAP9* gene expression is altered in biofilms (16a) and increases upon treatment of *C. albicans* with fluconazole and caspofungin (9). We show in the present study that the expression of *SAP9* is necessary for efficient recognition and killing of *C. albicans* by human PMNs. These terminally differentiated immune effector cells play a central role in antifungal immunity (47). Consequently, neutropenia has repeatedly been shown to be an independent risk factor for the development of invasive candidiasis (6, 37). This is corroborated by the observation that human PMNs are able to suppress *C. albicans* filamentation and rapidly kill both yeast forms and filaments of *C. albicans* in vitro (7, 16, 56). In the present study, we show that Sap9 significantly influences interaction with PMNs. Noticeably, these effects were not directly related to the budding dysfunction described for $\Delta sap9$ (and $\Delta sap10$) mutants, since this defect, which is clearly visible in liquid SD medium, was not evident in RPMI 1640 medium, which was used as a pre-culture and during interactions with neutrophils in the present study. Previously, we have shown that targeted motility toward filamentous forms of *C. albicans* is an essential component of PMN anti-*C. albicans* filament activity (56). In the present study we show that, in contrast to *C. albicans* wild-type filaments, the filaments of a $\Delta sap9/10$ deletion mutant strain induced a significantly reduced targeted motility. By using single deletion mutants as well as a complemented strain expressing *SAP9*, we verified that this effect was due to the deletion of *SAP9*. Since the decreased targeted motility toward PMNs was due to the deletion of a gene encoding a GPI-anchored protease, we conclude that a substrate of Sap9, which is released by *C. albicans*, rather than Sap9 itself, may lead to PMN activation. Deletion of *SAP9* also resulted in decreased formation of ROI in human PMNs. The role of ROI in the killing of fungal pathogens and especially *Candida* species is currently unclear. Patients suffering from chronic granulomatous disease show a high susceptibility to microbial infections, indicating a general importance of ROI generation for the immune defense against infectious diseases (55). However, although fungal infections and especially aspergillosis are common in patients with chronic granulomatous disease and are responsible for a high mortality, the pathogenic yeast *C. albicans* plays only a minor role in this risk group (55). Even in the case of *Aspergillus fumigatus*, recently published data shed some doubt on the direct role of ROI in killing (27). Furthermore, data from our own lab, as well as other labs, suggest different mechanisms of killing for *C. albicans* (and *C. glabrata*), which might, however, indirectly depend upon ROI formation (O. Kurzai et al., unpublished data). In fact, ROI generation by human PMNs not only constitutes a mechanism for microbial killing but also seems to have additional function (35). For example, the generation of neutrophil extracellular traps has been shown to depend on ROI induction, and these traps have been suggested to contribute to PMN-mediated killing of *C. albicans* (52). Similarly, ROI induction plays an important role in triggering PMN apoptosis. Apoptosis of PMNs is an essential mechanism in regulation neutrophil homeostasis and balancing infection

(12). In elucidating the mechanisms resulting in constitutive and pathogen-induced PMN apoptosis, it soon became evident that the generation of ROI plays a central role as a trigger for the induction of programmed cell death (53). Heat-killed *C. albicans* yeast forms have been shown to induce PMN apoptosis in a dose-dependent manner (40). Here we could show that viable *C. albicans* filaments induce significant rates of apoptosis in human PMNs, whereas the effect of viable yeast forms was negligible. The induction of apoptosis was clearly dependent on ROI release, since *C. albicans*-induced apoptosis could be reversed by the addition of DPI or glutathione, resulting in NADPH oxidase inhibition or ROI scavenging and the loss of ROI formation. In addition, *C. albicans* Δ sap9, which induced lower levels of ROI than wild-type *C. albicans*, also induced lower levels of apoptosis. Taken together, our data suggest a key role for Sap9 in the interaction of *C. albicans* with human neutrophils. A Δ sap9 *C. albicans* mutant—especially in its filamentous form—induced lower levels of PMN migratory activity, lower levels of ROI, and reduced apoptosis, which indicates a decrease in overall PMN activation. Consequently, PMN-mediated killing of the Δ sap9 mutant was attenuated. It has to be noted that deletion of SAP9 did not lead to a complete abolition of ROI induction, killing, or induction of apoptosis. This clearly indicates that Sap9 activity is one of manifold factors which eventually contribute to PMN activation and fungal eradication. Mainly carbohydrate components of the fungal cell wall are known to directly contribute to immune activation: mannoproteins and β -glucans, especially β -1,6-glucan, can induce phagocytosis, oxidative response, and lactoferrin release and thereby promote the killing of *C. albicans* (36, 41). Pra1, a protein of the *C. albicans* cell wall, has also been described to directly activate human PMNs (47a). Therefore, multiple components of this pathogen are likely to cooperate in the activation of innate human immunity. Recently, the cleavage of a host protein, histatine-5, by Sap9 has been shown to mediate immune evasion of *C. albicans* (29). Despite this, substrates of Sap9 among fungal and host proteins remain to be fully defined. Our data suggest that Sap9, possibly by cleavage of fungal or host proteins and/or by regulating the content and shape of the fungal cell surface, is also involved in the activation of human PMNs and may therefore mediate protective immune activation during systemic infection, as well as immune evasion in a localized setting of mucosal colonization and/or infection.

ACKNOWLEDGMENTS

We are grateful to Nina Trzeciak for expert technical assistance.

This study was supported by a research grant from the Interdisciplinary Center for Clinical Research (IZKF) at the University of Wuerzburg (IZKF-A5 to O.K. and J.L.). Work in the lab of B.H. was supported by the Deutsche Forschungsgemeinschaft (DFG Hu528/14 to B.H.).

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