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Scabies Mite Inactivated Serine Protease Paralogs Inhibit the Human Complement System

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Infestation of skin by the parasitic itch mite Sarcoptes scabiei afflicts 300 million people worldwide and there is a need for novel and efficient therapies. We have previously identified a multigene family of serine proteases comprising multiple catalytically inactive members (scabies mite-inactivated protease paralogs (SMIPPs)), which are secreted into the gut of S. scabiei. SMIPPs are located in the mite gut and in feces excreted into the upper epidermis. Scabies mites feed on epidermal protein, including host plasma; consequently, they are exposed to host defense mechanisms both internally and externally. We found that two recombinantly expressed SMIPPs inhibited all three pathways of the human complement system. Both SMIPPs exerted their inhibitory action due to binding of three molecules involved in the three different mechanisms which initiate complement: C1q, mannose-binding lectin, and properdin. Both SMIPPs bound to the stalk domains of C1q, possibly displacing or inhibiting C1r/C1s, which are associated with the same domain. Furthermore, we found that binding of both SMIPPs to properdin resulted in prevention of assembly of the alternative pathway convertases. However, the SMIPPs were not able to dissociate already formed convertases. Immunohistochemical staining demonstrated the presence of C1q in the gut of scabies mites in skin burrows. We propose that SMIPPs minimize complement-mediated gut damage and thus create a favorable environment for the scabies mites. The Journal of Immunology, 2009, 182: 7809–7817.

Scabies is a parasitic skin disease caused by the mite Sarcoptes scabiei. Scabies mites infest the skin, particularly the lower stratum corneum layer, and feed on epidermal protein, including host plasma. This exposure to host defense mechanisms, both internally and externally, necessitates a strategy for the mite to protect against potential threats from the host immune system. We have previously identified a multigene family of serine proteases that are secreted into the mite gut and excreted into the upper epidermis. These proteases, termed SMIPPs (scabies mite inactivated protease paralogs), are catalytically inactive and are designed to inhibit human complement pathways. Our studies indicate that SMIPPs inhibit all three pathways of the human complement system: the classical, alternative, and lectin pathways. These pathways are initiated by the binding of the C1 complex, including C1r, C1s, and C1q, to immune complexes. The alternative pathway is activated by the C3 convertases, which are essential for adaptive immunity, including the complement system found in the skin. SMIPPs bind to the stalk domains of C1q and to properdin, preventing the formation of the alternative pathway convertases and thus minimizing complement-mediated gut damage. This strategy creates a favorable environment for the scabies mites, allowing them to establish persistent infections and evade host immune responses.
C8 and C9 and thereby inhibits complement activation at the terminal stage (12). Schistosomes may also capture C2 and C3 (11). In addition, a surface FcR might bind Ig in an unfavorable orientation and thereby limit its ability to activate complement. In mice infested with larval *Strongyloides stercoralis*, C3 was not required for the development of adaptive immunity, but was necessary for the larval killing process during both protective innate and adaptive immune responses (13).

Immune evasion strategies of scabies mites are largely unknown. The scarcity of molecular data on scabies is due to the difficulty in obtaining mites in sufficient numbers, because the parasite burden is generally very low and an in vitro culture system for propagating mites is not available. Therefore, we constructed cDNA libraries from mRNA extracted from mites in skin shed into the bedding of crushed scabies patients. An expressed sequence tag library of 43,776 sequences has been established from these clones at the Australian Genome Research Facility (14). Using BLAST searches, we identified, among others, multiple scabies mite homologs of the group 3 serine protease allergens (15). Astigmatid house dust mites (HDM) are closely related to scabies and their group 3 HDM allergens have been implicated as elicitors of the allergic response in asthma. They are serine proteases that are secreted into the gut of the mites and assumed to play key roles in digestion (16). Hence, homologs of HDM group 3 allergens in scabies mites may be potential targets of immunotherapy.

The 33 sequences identified clustered into three major clades. Remarkably, all but one of the paralogs contain mutations in the catalytic triad ruling out the possibility that they can act as proteases by a known mechanism. We have accordingly named this family scabies mite inactivated protease paralogs (SMIPPs) (14). SMIPPs of each clade are present in the mite gut and excreted in fecal pellets (4). We show here that representative SMIPPs from two different subclades inhibit all three complement pathways, which may provide protection against gut damage by complement in serum imbibed by the mites.

**Materials and Methods**

**Expression of SMIPP protein in Pichia pastoris and purification**

The nomenclature of the molecules investigated here is based on a phylogenetic tree illustrating the evolutionary relationships between the SMIPPs (17). Recombinant SMIPP-Ss 11 and D1 were produced in *P. pastoris* by cloning SMIPP-coding sequences retrieved from the original cDNA clones YvE025A04 (GenBank accession no. AY330801; www.ncbi.nlm.nih.gov/GenBank) and YvT004A06 (GenBank accession no. AY330805) corresponding to the mature sequences predicted (15). To direct secretion of the expressed protein into the medium, all constructs placed the predicted N terminus flush with the Kex2 signal peptide cleavage site in the transfer vector pPICZαA (Invitrogen). A stop codon at the 3’ end of the SMIPP sequence was introduced to ensure that the recombinant protein did not include any of the C-terminal tags of the vector. The plasmid was propagated in *Escherichia coli* strain TOP10F (Invitrogen), linearized with *Sac*I, and electroporated into *P. pastoris* KM71H (Invitrogen). Media for colony growth and biomass or induction cultures were made according to the manufacturer’s instructions. Cells were initially plated onto supplemented nitrocellulose filters placed on agar plates containing 100 μg/ml Zeocin (Invitrogen). After a 24-h incubation at 29°C, filters were moved onto YPDS medium containing 2 mg/ml Zeocin for selection of transformants with increased numbers of SMIPP gene copies. To ensure pure clonal isolates, single colonies were plated twice on two subsequent YPDS/Zeocin agar plates. Transformants were assessed to select for the highest expressing clone according to the manufacturer’s manual. Biomass cultures were grown for 24 h and induction cultures for 60–70 h with shaking at 29°C. SMIPP expression was detected on a Western blot using mouse antiserum generated against purified recombinant SMIPP molecules produced in *E. coli*. Correct processing of the recombinant protein at the Kex2 cleavage site was confirmed after purification by N-terminal sequencing at the Peptide Biology Laboratory (Monash University, Clayton, Victoria, Australia) (data not shown).

**FIGURE 1.** Characterization of purified SMIPP proteins. *A* and *B*. The purified proteins were separated on 10% SDS-PAGE gels under reducing conditions and visualized by staining with Coomassie blue R-250. *A*, SMIPP-S 11 expressed in *P. pastoris*: wild-type protein (lane 1), mutant protein N84Q/N191Q (lane 2), N147Q (lane 3) and N84Q/N147Q/N191Q (lane 4), and *E. coli*-expressed wild-type protein (lane 5). *B*, SMIPP-S D1 expressed in *P. pastoris*: wild-type protein (lane 1), mutant protein N145Q (lane 2), and N193Q (lane 3). SMIPP-S D1 protein with both N-glycosylation sites mutated was unstable (data not shown). Lane 4 shows *E. coli*-expressed wild-type protein. The proteins used for the complement assays reported here are indicated by arrows. *C* and *D*. The proteolytic activity of the SMIPPs was tested using degradation assays. Two concentrations of each SMIPP (100 and 200 μg/ml) were incubated with trace amounts of 125I-C4b (C) or 125I-C3b (D) for 1.5 h at 37°C. The reaction was terminated by addition of SDS-PAGE sample buffer, containing DTT, followed by heating at 95°C for 3 min. The proteins were separated on a 10–15% gradient SDS-PAGE gel and visualized using PhosphorImager. Factor I, along with its cofactors C4BP (for C4b degradation) and factor H (for C3b degradation), was used as a positive control, as was interpin A, a bacterial protease. In the negative controls, only 125I-C4b or 125I-C3b was added. (FI, factor I; FH, factor H).

Mature SMIPP protein secreted from *P. pastoris* was purified as follows: expression culture supernatant was collected following centrifugation at 12,000 × g for 45 min at 4°C and stored at −80°C until required. The supernatant was thawed and prepared for hydrophobic interaction chromatography by the gradual addition of ammonium sulfate to a final concentration of 1.5 M and adjustment of pH to 5.0. The preparation was centrifuged for 30 min at 20,000 × g. The supernatant was filtered through a 0.45-μm filter and the filtrate was loaded onto a 5-ml Hitrap phenyl-Sepharose column (GE Healthcare), pre-equilibrated with 1.5 M ammonium sulfate and 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) and 10% (v/v) glycerol (pH 5.0). Unbound material was removed by washing with equilibration buffer. Protein was eluted stepwise by addition of SDS-PAGE sample buffer, containing DTT, followed by heating at 95°C for 3 min. The proteins were separated on a 10–15% gradient SDS-PAGE gel and visualized using PhosphorImager. Factor I, along with its cofactors C4BP (for C4b degradation) and factor H (for C3b degradation), was used as a positive control, as was interpin A, a bacterial protease. In the negative controls, only 125I-C4b or 125I-C3b was added. (FI, factor I; FH, factor H).

**Mutagenesis of N-glycosylation sites**

When subjected to SDS-PAGE, each SMIPP produced a major band slightly larger than the same molecule produced in *E. coli* plus a smear of...
larger material (Fig. 1, A and B). The SMIPP-Ss I1 and D1 have three and two predicted N-glycosylation sites, respectively. Function can be hindered by artifactual hyperglycosylation; therefore, we systematically mutated the predicted glycosylation sites from N to Q at positions 84, 147, and 191 of the mature protein of SMIPP-S I1 and at position 145 and 193 of the mature protein of SMIPP-S D1 singly and in combination by oligonucleotide-based site-directed mutagenesis. Among the created Pichia mutants (Fig. 1, A and B), clones that expressed homogenous, nonglycosylated proteins were chosen for additional experiments. These clones were designated I1 and D1 for SMIPP-S I1 and SMIPP-S D1, respectively.

Proteins

C1, C2, C3b, C4b, C5, C6, C7, C8, C9, factor B, factor D, and properdin were purchased from Complement Technology. Human MBL was purchased from Statens Serum Institutet (Copenhagen, Denmark). C1q (18), factor H (19), C4b-binding protein (C4BP) (20), and factor I (21) were purified from human plasma as described previously. The stalk and head regions of C1q were prepared by partial proteolytic digestion of intact C1q as described previously (22, 23). Pepsin (Worthington Biochemical) was used for preparation of the stalk region and purified collagenase (Clostridium histolyticum, code CLSPA) for preparation of the head region purchased from the same company. C3b and C4b were labeled with 125I using the chloramine-T method (24). The specific activity was 0.4–0.5 MBq/μg of protein. Interpain A was expressed and purified as described previously (25).

C3b/C4b degradation assay

To confirm that the two SMIPPs tested in this study were indeed inactive proteases and not able to cleave complement proteins, such as C3b or C4b, a degradation assay was performed. Two concentrations (100 and 200 μg/ml) of SMIPPs were mixed with trace amounts of 125I-labeled C3b or C4b in TBS in a total volume of 40 μl. As positive controls, 15 μg/ml factor I, along with 200 μg/ml of a cofactor (C4BP for the degradation of C4b or factor H for the degradation of C3b), were added to the 125I-C3b or 125I-C4b. Recently, interpain A, a cysteine proteinase from Prevotella intermedia, has been shown to inhibit complement by degrading C3 and C4 (25). Therefore, interpain A was also tested here as a positive control at a concentration of 1 μM. The samples were incubated for 1.5 h at 37°C and the reaction was terminated by the addition of SDS-PAGE sample buffer with reducing agent (DTT). The samples were then incubated at 95°C for 3 min and separated on a 10–15% gradient SDS-PAGE gel. The separated proteins were visualized by using a PhosphorImager (Molecular Dynamics/GE Healthcare).

**FIGURE 2.** SMIPPs inhibit the hemolytic activity of human serum. Sheep erythrocytes sensitized with Abs (A, classical pathway) or rabbit erythrocytes (B, alternative pathway) were incubated with 0.125% or 1.3% NHS, respectively. Serum was preincubated for 15 min at RT with various concentrations of SMIPPs or BSA as a negative control. After 1 h of incubation of NHS with erythrocytes at 37°C, the degree of lysis was estimated by the measurement of released hemoglobin (absorbance at 405 nm). The lysis obtained in the absence of SMIPPs was defined as 100% hemolytic activity. C, The hemolytic activity of the fully glycosylated counterparts of D1 and I1 was tested in both the classical and alternative pathways as described above. One concentration of each protein was tested: 75 μg/ml for the classical pathway and 200 μg/ml for the alternative pathway. An average of three independent experiments performed in duplicate is presented with bars indicating SD. C, The statistical significance of the differences between BSA and all tested SMIPPs were estimated for the two assays using a one-way ANOVA with Tukey’s multiple comparison test; ***, p < 0.001.

Hemolytic assay

To assess the activity of the classical pathway of complement, sheep erythrocytes (Swedish National Veterinary Institute) were washed three times with DGVB 2+ buffer (2.5 mM Veronal buffer (pH 7.3), 70 mM NaCl, 140 mM glucose, 0.1% gelatin, 1 mM MgCl2, and 0.15 mM CaCl2), resuspended to a concentration of 109 cells/ml, and incubated, with gentle shaking for 20 min at 37°C, with an equal volume of amboceptor (Dade Behring) diluted 1/3000 in DGVB 2+ buffer. Amboceptor is an Ab against sheep erythrocytes, which activates the classical pathway of complement. After two washes with DGVB+ buffer, 8 × 107 cells/ml were incubated for 1 h at 37°C with 0.125% normal human serum (NHS), diluted in DGVB+ buffer, in a total volume of 150 μl. Before incubation with erythrocytes, NHS was preincubated with various concentrations of SMIPPs or BSA, as a negative control, for 15 min at room temperature (RT). NHS was prepared from blood of six healthy volunteers after informed consent and following review by the local ethical board at Lund University. The samples were centrifuged and the hemolytic activity (i.e., the amount of lysed erythrocytes) was determined by spectrophotometric measurement of the amount of released hemoglobin (405 nm). The lysis obtained in the absence of SMIPPs was defined as 100% hemolytic activity.

To assess the activity of the alternative pathway, rabbit erythrocytes (obtained from a local farm) were washed three times with Mg2+ EGTA buffer (2.5 mM Veronal buffer (pH 7.3) containing 70 mM NaCl, 140 mM glucose, 0.1% gelatin, 7 mM MgCl2, and 10 mM EGTA). Erythrocytes at a concentration of 6 × 107 cells/ml were incubated for 1 h at 37°C with 1.3% NHS diluted in Mg2+ EGTA buffer in a total volume of 150 μl. Before incubation with erythrocytes, NHS was preincubated with various concentrations of SMIPPs or BSA, as a negative control, for 15 min at RT. The samples were centrifuged and the hemolytic activity was determined spectrophotometrically as described above. The lysis obtained in the absence of SMIPPs was defined as 100% hemolytic activity.

Complement activation assays

Unless stated otherwise, all incubation steps were done at RT, in 50 μl of solution, followed by extensive washing with 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% (v/v) Tween 20. Microtiter plates (Maxisorp;
Nunc) were incubated overnight at 4°C with 75 mM sodium carbonate (pH 9.6) containing the following: for the classical pathway, 2.5 mg/ml aggregated human IgG (Immuno); for the lectin pathway, 100 mg/ml mannan (Sigma-Aldrich); and for the alternative pathway, 20 mg/ml zymosan (Sigma-Aldrich). The wells were blocked with 200 l of blocking solution (1% (w/v) BSA in PBS) for 2 h. For the classical and lectin pathways, respectively, 3% NHS was diluted in DGVB2 buffer and incubated for 20 min (for detection of C4b and C3b) or 45 min (for C1q, MBL, and C9) at 37°C. For the alternative pathway, 5% NHS was diluted in Mg2+ EGTA buffer and incubated for 35 min for detection of C3b or for 1 h for detection of C9. NHS was preincubated for 15 min at RT with various concentrations of SMIPPs or BSA, as a negative control, before addition to the microtiter plate. Complement activation was assessed by detection of deposited complement proteins using rabbit polyclonal Abs against C1q (DakoCytomation), C4b (DakoCytomation), C3d (DakoCytomation), or goat polyclonal Abs against MBL (R&D Systems) and C9 (Complement Technology) diluted in blocking solution. After a 1-h incubation, HRP-conjugated secondary Abs against rabbit or goat Igs (DakoCytomation), diluted in blocking buffer, were added for 30 min. Bound enzyme was detected as described above.

Assembly and decay of the alternative C3 convertase
To measure the assembly and decay of the alternative C3 convertase in NHS and in the presence of SMIPPs, a microtiter plate-based assay was performed as described previously (26). Briefly, microtiter plates were coated with 100 µl of blocking solution and incubated at RT for 2 h. When screening for binding of various complement proteins and examining binding of C1, C1q, C1q head, and C1q stalk, 200 µg/ml SMIPP preparations were added to the wells. A titration was also performed for the binding of C1q, MBL, and properdin using concentrations ranging between 12.5 and 200 µg/ml. The SMIPPs were diluted in 50 mM HEPES (pH 7.4), 100 mM NaCl, and 2 mM CaCl2 and were allowed to bind overnight at 4°C. Specific murine polyclonal Abs against SMIPPs were then added to the wells, followed by a HRP-conjugated goat anti-mouse IgG Ab (DakoCytomation). All Abs were diluted in blocking buffer and incubated at RT for 1 h. Bound enzyme was detected as described above.

Direct binding assay
Unless stated otherwise, all incubation steps were done in 50 µl of solution, followed by extensive washing, as described for the complement activation assays. Various complement proteins were coated onto microtiter plates as described above at a concentration of 10 µg/ml. BSA (1% (w/v)) was used as a negative control. The wells were blocked with 200 µl of blocking solution and incubated at RT for 2 h. When screening for binding of various complement proteins and examining binding of C1, C1q, C1q head, and C1q stalk, 200 µg/ml SMIPP preparations were added to the wells. A titration was also performed for the binding of C1q, MBL, and properdin using concentrations ranging between 12.5 and 200 µg/ml. The SMIPPs were diluted in 50 mM HEPES (pH 7.4), 100 mM NaCl, and 2 mM CaCl2 and were allowed to bind overnight at 4°C. Specific murine polyclonal Abs against SMIPPs were then added to the wells, followed by a HRP-conjugated goat anti-mouse IgG Ab (DakoCytomation). All Abs were diluted in blocking buffer and incubated at RT for 1 h. Bound enzyme was detected as described above.
detected by addition of 100 μl of either goat anti-human factor B (Complement Technology) or goat anti-human properdin (Complement Technology) diluted 1/1000 in TBS containing 10 mg/ml BSA and 2 mM MgCl₂. After a 1-h incubation at 37°C, the plates were washed as above and 100 μl of 1/1000 diluted HRP-conjugated rabbit anti-goat IgG (Dako-Cytomation) was added and incubated for 1 h at 37°C. The plates were then washed twice with TBS containing 10 mg/ml BSA, once with TBS containing 10 mg/ml BSA and 0.1% (v/v) Tween 20, and finally once with normal TBS. The amount of remaining factor B and properdin on the plate was detected as above.

To measure the decay of the alternative C3 convertase, in the presence of SMIPPs, the assay was mainly performed as above, but instead of pre-incubating serum and proteins and adding them together to the plate, 5% NHS was first added alone to the plate and incubated for 1 h at 37°C. After washing three times with TBS containing 10 mg/ml BSA and 2 mM MgCl₂, the SMIPPs and factor H were added and incubated for 1 h at 37°C. The amount of remaining factor B and properdin on the plate was detected as above.

Localization of human C1q and IgG by immunohistochemistry

Human skin biopsies infested with scabies mites were formalin-fixed overnight, paraffin embedded, and cut into 4-μm serial sections. For localization studies, slides were prepared as described previously (4, 5) and serial sections underwent an Ag heat-retrieval procedure in a solution containing 1% (w/v) Revealit-Ag in 20 mM Tris (pH 7.5; ImmunoSolution) at 85°C for 5 min. The slides were cooled and washed with distilled water for 10 min followed by 15 min washes in PBS at pH 7.2. Serial sections were blocked with 10% donkey serum in 1% (w/v) BSA in PBS. Endogenous peroxidase activity was blocked with 3% (v/v) H₂O₂ in blocking buffer. The sections were incubated overnight with rabbit anti-human C1q polyclonal Ab (Abcam) in 1/500 dilutions. As a negative control, preimmune serum in 1/500 dilutions was incubated on sections of the same series. All sections underwent an Ag heat-retrieval procedure in a solution containing 1% (w/v) Revealit-Ag in 20 mM Tris (pH 7.5; ImmunoSolution) at 85°C for 5 min. The slides were cooled and washed with distilled water for 10 min followed by 15 min washes in PBS at pH 7.2. Serial sections were blocked with 10% donkey serum in 1% (w/v) BSA in PBS. Endogenous peroxidase activity was blocked with 3% (v/v) H₂O₂ in blocking buffer. The sections were incubated overnight with rabbit anti-human C1q polyclonal Ab (Abcam) in 1/500 dilutions. As a negative control, preimmune serum in 1/500 dilutions was incubated on sections of the same series. All slides were washed in PBS and the Vector NovaRed peroxidase substrate kit (Vector Laboratories) was used for staining according to the manufacturer’s recommendations. Counterstaining with hematoxylin was done as previously described (4, 5). Ethical approval for the use of skin crusts from the beddding of a patient with recurrent crusted scabies was obtained from the Human Research Ethics Committee of the Northern Territory Department of Health and Community Services and the Menzies School of Health Research.

Statistical analysis

All experiments were done at least three times in duplicates, if not stated otherwise. Statistical significance was determined using one-way ANOVA with Tukey’s multiple comparison test (GraphPad Software). Values of p < 0.05 were considered significant (*, p < 0.05; **, p < 0.01; and ***, p < 0.001).

Results

SMIPPs show no proteolytic activity

Despite their structural similarity to proteases, SMIPPs were predicted to be inactive due to the structure of their putative active sites (17). To confirm this hypothesis, we tested whether the SMIPPs could degrade the activated complement components C3b and C4b similarly to what we observed for bacterial proteases such as interpain A (24). The two SMIPPs D1 and I1 showed no proteolytic activity, since no degradation products of either C4b (Fig. 1C) or C3b (Fig. 1D) could be detected in contrast to what was observed for positive controls.

SMIPPs inhibit the hemolytic activity of human serum

To determine whether the two SMIPPs D1 and I1 had any inhibitory effect on the human complement system, two hemolytic assays were performed measuring activity of the classical and the alternative pathway, respectively. Human serum, as a source of all complement proteins, was pretreated with various concentrations of SMIPPs before incubation with erythrocytes. Both SMIPPs were able to fully inhibit both the classical pathway (Fig. 2A) and alternative pathway (Fig. 2B). Higher concentrations of SMIPPs (200 μg/ml) were required to inhibit the alternative pathway compared with the classical pathway (75 μg/ml). This is probably due to differences in serum concentration used in the two assays: 1.3% serum in the alternative hemolytic assay vs 0.125% serum in the classical hemolytic assay. These serum concentrations were chosen on the basis of an initial titration and represent conditions in which each assay is most sensitive to changes. The alternative pathway is known to require higher concentrations of serum to function properly.

To elucidate whether the nonglycosylated proteins used in this study behaved in the same manner as the fully glycosylated counterparts, we also tested fully glycosylated D1 and I1 in hemolytic assays. One concentration of each of the SMIPP proteins was tested for the classical pathway (75 μg/ml) and alternative pathway (200 μg/ml). Fully glycosylated D1 and I1 inhibited both complement pathways to a similar extent as their nonglycosylated counterparts (Fig. 2C). However, inhibition of the classical pathway was somewhat less pronounced for glycosylated I1 compared with the nonglycosylated molecule (p < 0.001). We also tested different concentrations of the fully glycosylated D1 and I1 in the complement activation assays for the three pathways, measuring C3b deposition (data not shown). We found that the nonglycosylated and the fully glycosylated D1 and I1 inhibited all three pathways in a very similar manner. The small difference seen for I1 in the classical pathway hemolytic assay could, however, also be noticed in the classical pathway complement activation assay, but not in the assays measuring activity of the lectin and alternative pathways. Based on the similar results obtained for the non- and fully glycosylated proteins, we continued testing only the nonglycosylated variants in this study.
**SMIPPs inhibit all three complement pathways**

The complement system works in a cascade-like manner with consecutive proteolytic activation of a number of proteins. To assess at what level of the cascade the SMIPPs inhibit complement, a microtiter plate-based deposition assay was performed in which complement activation was initiated by specific ligands for each pathway. After addition of human serum, pretreated with SMIPPs, the deposited complement proteins were detected using specific Abs.

In the case of the classical pathway, complement was activated by aggregated IgG deposited on the plate. When adding serum together with either D1 or I1, deposition of all four tested complement factors, C1q, C4b, C3b, and C9, was decreased (Fig. 3, left panel). The same pattern was observed for the lectin pathway; after activation with mannan, deposition of MBL, C4b, C3b, and C9 was inhibited by both D1 and I1 (Fig. 3, right panel). This was also true for the alternative pathway; after initiation with immobilized zymosan, C3b and C9 deposition was decreased (Fig. 4). Overall, D1 seemed to inhibit the pathways slightly less efficiently than I1, especially for the alternative pathway, where double the amount of D1 (400 μg/ml for D1 and 200 μg/ml for I1) was needed to completely inhibit deposition of C3b and C9.

**SMIPPs bind directly to C1q, MBL, and properdin**

Structural and functional analysis of the two SMIPPs analyzed in the current study found that they are devoid of proteolytic activity (17) and we could not detect any degradation of complement proteins such as C3 and C4 by the SMIPPs (Fig. 1, C and D). To elucidate whether the complement inhibition seen in the previous assays is due to SMIPPs binding to complement proteins, a microtiter plate-based direct binding assay was used. A first screening was performed, where the plates were coated with various complement proteins, followed by incubation with one concentration (200 μg/ml) of SMIPPs and the binding was detected with specific murine polyclonal Abs against D1 and I1. In this direct binding assay, a clear interaction of both D1 (Fig. 5A) and I1 (Fig. 5B) with C1q, MBL, and properdin was observed under conditions of physiological ionic strength and pH. The strongest binding of D1 and I1 was observed to C1q. To examine these interactions further, various concentrations (from 0 to 200 μg/ml) of the SMIPPs were allowed to bind to the immobilized complement proteins and the results showed that the binding of C1q, MBL, and properdin was dose dependent for both D1 (Fig. 5C) and I1 (Fig. 5D). Also, under these experimental conditions, the binding to C1q was shown to be the...
strongest interaction. Cross-competition assays in which D1 or I1 was allowed to bind immobilized complement proteins in the presence of another complement protein as competitor showed that C1q, MBL, and properdin bind to different sites on SMIPPs (data not shown).

SMIPPs interact mainly with the collagenous stalk of C1q
C1q is part of the C1 complex along with two molecules of each of the smaller proteolytic subunits C1r and C1s. C1q is the recognition molecule responsible for the activation of the classical pathway and it is composed of six subunits. Each subunit has a globular head at the C terminus and a collagenous stalk at the N terminus. C1q binds many different ligands and most of the ligands that activate C1q and lead to activation of the classical pathway bind to the globular head domains. On the contrary, ligands binding to the collagenous stalk of C1q may inhibit activation via the classical pathway (27).

To identify the binding site for SMIPPs on C1q, head and stalk regions of C1q were prepared, using partial proteolytic digestion of intact C1q, with collagenase (for heads) and pepsin (for stalks). By coating these isolated parts of C1q on a microtiter plate, we could demonstrate that the binding of both D1 and I1 was localized to the collagenous stalk region of C1q (Fig. 5). The binding to the globular heads was not significantly different from the binding to the negative control BSA. We also demonstrated that the binding to C1q is significantly stronger than to the intact C1 complex. This is probably due to the fact that C1r and C1s also interact with the stalks and may be competing to some extent with SMIPPs.

SMIPPs inhibit assembly of alternative pathway C3 convertases due to their ability to bind properdin, but they do not accelerate decay of preassembled convertases
We observed that the SMIPPs inhibited the alternative pathway of complement, both in hemolytic (Fig. 2B) and complement deposition assays (Fig. 4). This could be due to binding of the positive regulator of this pathway, properdin, which occurs as shown in Fig. 5. To investigate whether the binding of the SMIPPs to properdin would inhibit the assembly and/or accelerate the decay of the alternative C3 convertase, a microtiter plate-based assay was performed. Agarose, a polysaccharide that will activate the alternative pathway of complement (28), was coated onto microtiter plates, followed by addition of NHS, as a source of complement proteins and the formed C3 convertases were detected by measuring the amount of deposited factor B and properdin using specific

FIGURE 6. SMIPPs inhibit assembly of alternative pathway C3 convertases due to their ability to bind properdin, but they do not accelerate decay of preassembled convertases. Microtiter plates were coated with agarose, followed by the addition of NHS, and the C3 convertases formed were detected by measuring the amount of deposited factor B and properdin with specific polyclonal Abs. The SMIPPs (or BSA as a negative control) were either added together with NHS to study the assembly of the convertase (A) or after NHS incubation to study the decay (B). Factor H was added as a positive control but only at one concentration; 100 μg/ml. The absorbance obtained in the absence of SMIPPs was defined as 100% and an average of three independent experiments performed in duplicate is presented with bars indicating SD. The statistical significance of differences between BSA and the rest of the groups was estimated using a one-way ANOVA with Tukey's multiple comparison test: ***, p < 0.001. FH, factor H.

FIGURE 7. Immunohistological colocalization of C1q and host IgG indicates that human complement is ingested into the mite gut. A schematic diagram (A) outlines the features visible in three serial histological sections of mite-infested epidermis. The epidermis is shown in blue, the burrows in white, the bodies of two mites in gray, and the mite gut in red. B, C, and D. The matching serial histological sections. Red staining indicates binding of Ab to protein. Section (B) was probed with anti-human IgG, a marker for the mite gut (4). Section C shows staining of the mite gut when probing with anti-C1q, while section D, probed with preimmune mouse serum as a negative control, remained unstained. b, Burrow wall; c, cuticle; es, esophagus; g, gut; m, mouth parts. Scale bar, 100 μm.)
polyclonal Abs. To assess the assembly of the alternative C3 convertase, in the presence of SMIPPs, D1 and H were preincubated with NHS before being added to the agarose-coated plates. Factor H was used as a positive control, since it is known to bind C3b and hinder the formation of the alternative C3 convertase. Both D1 and H significantly hindered the formation of the convertase (Fig. 6A) at both tested concentrations, as indicated by the reduced amount of deposited factor B and properdin. To assess the ability of the SMIPPs to accelerate the decay of the alternative C3 convertase, the proteins were added after NHS incubation, i.e., after the assembly of the convertases. Factor H was also in this setup used as a positive control, due to its known ability to accelerate the decay of the alternative convertase, by directly binding C3b and displacing Bb from the convertase. This can be seen in Fig. 6B, where the addition of factor H resulted in a decrease of deposited factor B. However, the SMIPPs were not able, at any tested concentration, to accelerate the decay of the alternative C3 convertase (Fig. 6B). Together, these results demonstrate that the SMIPPs are able to inhibit the assembly of the alternative C3 convertase, but once the convertase is preformed, SMIPPs are not able to accelerate its decay.

**SMIPPs colocalize with human complement factor C1q in the mite digestive system**

The presence of complement factors in the scabies mite gut was clearly demonstrated by probing serial sections of human mite-infested skin with Abs against human C1q in comparison to staining with human IgG, which for the purpose of this experiment served as a positive control for gut localization (Fig. 7). Human IgG was localized to the gut (Fig. 7B) as previously documented (4, 5). This confirmed that, in the adjacent serial section (Fig. 7C), labeling by anti-C1q Abs was indeed in the gut. Staining of the gut was the most easily seen; however, sections that were in the right plane also showed staining in the esophagus (Fig. 7, B and C). The localization of SMIPPs proteins to the digestive system of the mite has previously been shown in sections of scabies-infested human skin (4, 5) probed with polyclonal Abs against individual SMIPPs. In all cases, the sections probed with the preimmune serum only stained with the counterstain, as demonstrated in Fig. 7D, clearly excluding any unwanted background staining.

**Discussion**

The complement system has several sensory molecules able to recognize molecular patterns foreign to its host. Furthermore, it can be strongly activated by both natural, low-affinity IgM Abs as well as specific, high-affinity IgG Abs. Not surprisingly, many pathogens utilize multiple sets of evasion strategies directed at inhibition of complement, as redundancy and multiplicity are important for immune and complement evasion (9). In the current study, we found that SMIPPs of scabies mites are able to interact with several complement proteins, which leads to inhibition of all three pathways of complement. This finding is consistent with hemopathogenic parasites having complement evasion mechanisms (10). Both Necator americanus and Hemonchus contortus express a calreticulin-like protein that binds C1q (29, 30). Ticks such as Ixodes scapularis and Ixodes ricinus express proteins preventing deposition of C3b, such as Isac (26) and IRACs/IxACs (31, 32). IRACs and IxACs, along with Salp20, also have the ability to bind properdin (33). Interestingly, the SMIPPs analyzed in the current study combine the properties of many of the molecules described above, as they have the ability to bind C1q, MBL, and properdin.

C1q is a promiscuous molecule able to interact with many ligands. In general, it appears that ligands binding to the globular heads of C1q, such as lgs, C-reactive protein, amyloid, and some extracellular matrix proteins, such as fibromodulin and osteoadherin, are all strong activators of the classical pathway of complement (34). On the other hand, ligands that interact with the stalks of C1q, such as the extracellular matrix proteins decorin and biglycan, are able to inhibit the classical pathway (27). This is probably due to competition for binding with the C1r/C1s enzymes that adhere to the stalk region and perhaps also due to effects on conformational changes in the C1 complex that are characteristic and required for complement activation upon binding to activating ligands. We found that SMIPPs bind to the stalk region of C1q, similar to what has been previously observed for decorin, which serves as an explanation as to why SMIPPs are able to inhibit the classical pathway. A similar mechanism is likely to be applicable for MBL because it has a similar overall architecture to C1q, particularly in its collagenous stalk and globular head domains, but these domains of MBL were not available for us to study. However, the inhibitory effect of SMIPPs on the lectin pathway is clearly due to their ability to bind MBL.

Properdin is a positive regulator of the alternative pathway that exerts its effect by binding C3b and thereby stabilizing the alternative C3 convertase, resulting in a prolonging of the half-life of the convertase of up to 10-fold (35). It also inhibits the binding of factor I to C3b, thereby protecting it from proteolytic cleavage (36). We observed in the present study that SMIPPs inhibit the alternative pathway of complement by binding to properdin and thereby inhibiting the assembly of the alternative pathway C3 convertase. However, unlike Salp20 from ticks, the SMIPPs were not able to displace properdin from the previously formed C3 convertase and were thereby not able to accelerate the decay of the convertase complex. SMIPPs thereby act in the same manner as the IxAC proteins, which also bind properdin and inhibit the assembly of the alternative pathway C3 convertase, but are not able to displace properdin from previously formed convertase complexes.

The x-ray crystal structures of SMIPP-SI1 and SMIPP-SD1 have recently been determined (17) and therefore may provide clues to the mechanisms of complement-binding activities reported here. Both SMIPP structures show significant structural changes within the active site region, providing a structural rationalization for their lack of protease activity. However, analysis of the hydrophobic and electrostatic properties of their molecular surfaces reveals no apparent common mode of interaction with other proteins (typically protein-protein interaction sites comprise significant regions of hydrophobic or charged nature). Inspection of sequence variability within the SMIPP family, in the context of the structures, suggests that the family is conformationally diverse and thus able to present multiple protein interaction binding sites that could bind a range of complement proteins. However, the absence of structural information for MBL, properdin, and C1q precludes modeling of such potential interactions. At least for the two SMIPPs studied here, no common structural mode of complement inhibition is apparent; it is possible that the structural diversity within the SMIPP family allows multiple modes of binding that would be required to inhibit several structurally unrelated members of the complement system. Further insights may be obtained by structural characterization of other members of the SMIPP family, as well as complement-binding studies of mutants, which can now be rationally guided by the available structural data.

In some of the assays presented in this study, relatively high concentrations of SMIPPs were needed to obtain a significant complement inhibitory effect, a phenomenon that has been observed previously in studies of other known complement inhibitors (27, 37). The concentrations of SMIPPs in the mite gut are not known and the local concentrations of complement proteins at the sites of mite infection are also not determined. However, 33 SMIPPs have now been identified and, since both of the SMIPPs tested here
show complement inhibitory functions, it is possible that many of these molecules inhibit complement. This makes it quite plausible that high concentrations of SMIPPs will be found in vivo. Obviously, further studies will be needed to assess the in vivo relevance of SMIPPs in defense against complement.

We have demonstrated that SMIPPs are present in the gut of the mite and excreted in feces (4, 5), hence their site(s) of action may be both internal and external to the mite. Internally, as the gut contains host plasma (4, 5), complement cascades must be inhibited in a milieu in which digestion of protein food can occur. This seems to be the obvious role of the SMIPPs. Externally, the inhibition of complement may have further consequences for the host. By interfering with host complement, the SMIPPs may effectively enhance the survival of pathogenic bacteria that colonize the mite gut. We have previously shown that cysteine proteinases from *Porphyromonas gingivalis* degrade complement factors and may provide an advantage to other periodontal pathogens residing in the same location (38). Ultimately, the inhibition of the host complement by scabies mite products such as SMIPPs may account for much of the associated secondary bacterial skin infections and downstream chronic disease. There is direct evidence that communities that have received treatment for scabies experience a dramatic reduction in bacterial skin sores (39, 40). Hence, elucidating the mechanism of the interaction between SMIPPs and the individual complement components is a vital step in preventing attenuation of the pathways which are pivotal to host defense against both the scabies mite and secondary bacterial pathogens. This in turn may lead to development of new treatments against this important but neglected parasite.

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**Disclosures**

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