SCABIES MITE INACTIVATED SERINE PROTEASE PARALOGUES ARE PRESENT BOTH INTERNALLY IN THE MITE GUT AND EXTERNALLY IN FECES

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Abstract. The scabies mite, Sarcoptes scabiei, is the causative agent of scabies, a disease that is common among disadvantaged populations and facilitates streptococcal infections with serious sequelae. Previously, we encountered large families of genes encoding paralogues of house dust mite protease allergens with their catalytic sites inactivated by mutation (scabies mite inactivated protease paralogues [SMIPPs]). We postulated that SMIPPs have evolved as an adaptation to the parasitic lifestyle of the scabies mite, functioning as competitive inhibitors of proteases involved in the host–parasite interaction. To propose testable hypotheses for their functions, it is essential to know their locations in the mite. Here we show by immunohistochemistry that SMIPPs exist in two compartments: 1) internal to the mite in the gut and 2) external to the mite after excretion from the gut in scybala (fecal pellets). SMIPPs may well function in both of these compartments to evade host proteases.

INTRODUCTION

The scabies mite, Sarcoptes scabiei, is the causative agent of scabies, a disease that is common among disadvantaged populations worldwide. It is known as the “itch mite” because of intense irritation and itching of the host in response to the mite and its products. The severe itching and popular rash of the primary infestation take 4–6 weeks to develop, whereas in contrast, re-infestation produces an immediate hypersensitivity reaction. Scabies mites form burrows in the skin that can be colonized by other infectious agents such as Group A streptococci and Staphylococcus aureus. The potential result of these infections include cellulitis, lymphangitis, systemic sepsis, and acute post-streptococcal glomerulonephritis.

Scabies mites have not been studied at the molecular level until recently because of the difficulty of obtaining material, a problem that has been overcome in part by expressed sequence tag approaches. Among 43,776 sequence tags, 33 distinct contigs represented by 105 clones were found to have the house dust mite (HDM) Group 3 serine protease allergens. Serine proteases of the S1 family were their next closest matches. In all but one of these genes, the catalytic triad has been mutated with the consequence that they cannot be active proteases by any known mechanism. They have been named scabies mite inactivated protease paralogues (SMIPPs). Although these genes exhibited considerable diversity, they could be grouped into four distinct clades. Commonly (but not exclusively), the active serine was mutated to alanine. Furthermore, in many of them, the histidine and aspartic acid corresponding to the active site have also been mutated. None of the SMIPPs bore any domains in addition to that corresponding to the catalytic domain of a serine protease.

Current evidence that the proteins encoded by these genes are synthesized in the mite include identification of the SMIPP genes in a cDNA library and the absence of introns and nonsense codons. However, this has not previously been confirmed in vivo. The major allergens of the HDM have been extensively studied. The Group 3 allergen has been characterized as a trypsin-like protease and is a major component of the mite scybala (fecal pellet). It has been theorized but not proven that it is expressed in the gut. These proteases are implicated in the allergic response to the house dust mite (HDM), and as such, are elicitors of conditions such as asthma. Anti-sera to scabies mites and HDM allergens cross-react. Besides being inactive, the other major difference between the HDM Group 3 allergens and the SMIPPs is that the amino acid corresponding to the key specificity site of the trypsin-like Group 3 allergen (aspartic acid) has changed in SMIPPs and now indicates a chymotrypsin-like binding specificity. This is consistent with the idea that the targets of the SMIPPs have changed from those of the Group 3 allergens. Further support comes from the loss of the disulphide bond that would normally flank the “active” serine, and this may allow flexibility within the binding pocket of SMIPP proteins. Indeed SMIPPs are not found in the closely related HDM and therefore may have evolved as an adaptation to the parasitic lifestyle of the scabies mite. Examples of such proteins acquiring novel functions have been documented.

The functions of SMIPPs cannot include proteolysis, but they may retain the ability to bind peptides and, for example, function as inhibitors of proteolysis. Localization of the SMIPPs in situ constitutes an important step toward establishing their function(s). Localization could indicate whether the SMIPPs would be able to interact with the host tissues, and importantly, with the host immune defenses. In this study, we produced antibodies in mice to five different SMIPP representing the four clades and used them to probe sections of formalin-fixed human skin tissue that was heavily infested with scabies mites.

MATERIALS AND METHODS

Recombinant protein. SMIPP coding sequences from Yv4005G12, Yv5026E07, Yv5027C11, Yv6023A04, and YvT064A06 corresponding to the mature sequences predicted with an N-terminal hexa histidine tag were amplified from the scabies mite cDNA libraries using specific primers. Gene inserts were directionally ligated into the pQE9 vector and transformed into XL1 blue E. coli cells. His-tagged SMIPP proteins were expressed and purified on an Ni-NTA agarose column (Qiagen, Clifton Hill, Victoria, Australia) under denaturing conditions.
Preparation of antibodies. Ethical approval for the production of antibodies in mice was obtained from the Queensland Institute of Medical Research Animal Ethics Committee (approval A010004). Antibodies to the recombinant SMIPP proteins were raised in C57BL/6 female mice and tested for cross-reactivity using standard Western blot methodology. Commercial anti-human IgG, raised in sheep and conjugated with horseradish peroxidase (Silenus, Melbourne, Australia), was used as a positive control for the gut. To ensure that we were not preferentially localizing the gut irrespective of antibody, two further controls were used. Antibodies to S. scabiei glutathione S-transferase (GST) were raised in rabbits and antibodies to Der p 10 were a gift from Wayne Thomas (The Netherlands, Melbourne, Australia).

Tissue preparation. Ethical approval for the use of shed skin crusts from the bedding 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 (approval no. 01/15). Skin crusts were placed overnight in a petri dish on a warming plate at 27°C. To fix the mites and skin tissue, a modified version of the methodology of Bi and others was used. In brief, mites were picked from the petri dishes and placed into the cap of a 1.5-mL eppendorf tube in groups of 100. Chloroform soaked filter paper was placed into the bottom of the tube, and the cap was closed for 2 minutes. The filter paper was replaced with filter paper soaked in undiluted formaldehyde, and the cap was closed for 3 hours. The mites were removed and placed into a polymerase chain reaction (PCR) tube and secured with 5% agar. When the agar was set, it was placed into 70% ethanol.

Where skin tissue was highly infested with scabies mites, blocks ~5 mm³ were cut and placed into a small petri dish. Filter paper soaked in chloroform was added for 2 minutes and removed. Formalin solution (10%) was added to the petri dish, and the sample was left to fix for 3 hours. The skin sample was placed into 70% ethanol.

The tissue and single mites were dehydrated in increasing concentrations of ethanol before being embedded in paraffin at 60°C. Sections were cut at 6–10 μm and coated on Super-Frost plus slides. The sections were probed with the pre-bleed only stained with the counterstain (Figure 1B).

Immunohistochemistry. Slides were dewaxed in xylol and graded alcohol and placed into running water. Because of the short fixation time, no antigen retrieval method was needed. Slides were placed into phosphate buffered saline (PBS), pH 7.4, for three washes and incubated in 10% goat serum/1% bovine serum albumin (BSA)/PBS for 30 minutes to block non-specific protein binding. The goat serum was decanted, and endogenous peroxidase activity was blocked with 3% (vol/vol) hydrogen peroxide (H2O2)/1%BSA/PBS for 10 minutes. After washing, the primary antibody was diluted in 1% BSA/PBS and added to the sections for 1 hour at room temperature. After washing, the slides were incubated with Dako EnVision (DakoCytomation, Carpinteria, CA) anti-mouse serum labeled with horseradish peroxidase for 30 minutes at room temperature. After washing, the chromogenic reaction was conducted using Nova Red (Vector, Burlingame, CA) staining for 12 minutes.

RESULTS

Using each of the expressed SMIPPs and the antibodies to each of them, it was shown by Western blotting that they exhibited little if any cross-reaction (data not shown). This was expected as each represents a different clade, and these differ substantially in sequence and, when expressed in E. coli, are not glycosylated. We conclude that the antibodies were suitable reagents for immunolocalization of SMIPPs expressed by the mites.

The localization of SMIPP proteins within and outside the scabies mite was shown using sections of human skin infested with mites. The sections were probed with the polyclonal antibodies raised in mice against individual SMIPPs. Sections were also probed with serum from the same mouse before it raised antibodies as a negative control. In all cases, the sections probed with the pre-bleed only stained with the counterstain (Figure 1B).

In all cases, the SMIPPs were localized to the digestive system of the mite (Figure 1C–E). Staining of the gut was the most easily seen; however, sections that were in the right plane also showed staining in the esophagus. For Yv6023A04, there was also possible localization in the eggs of the mite, both before and after the egg has been laid, although this was poorly reproducible (data not shown). Human IgG, which for the purpose of this experiment was used as a positive control, was localized to the gut (Figure 1A) as previously documented. This confirmed that, in the adjacent serial section, labeling by anti-SMIPP sera was indeed in the gut. The anti-S. scabiei GST positive control sera localized to the integument of the epidermis and body cavities, with minor staining in the digestive system (data not shown) as previously described. SMIPPs were also localized to external acellular masses, which we conclude are scybala (fecal pellets; Figure 1F). This was to be expected because all components within the gut are presumably expelled in the scybala. The scybala were negative to pre-bleed and anti-Der p 10 sera and reacted comparatively faintly to anti-GST (data not shown), consistent with this conclusion.

DISCUSSION

This study confirms that at least the five SMIPPs studied here are translated as proteins within the mite body. There is no reason to assume that the other SMIPPs would not also be expressed, in particular because none had introns or nonsense codons. That they are translated further strengthens our hypothesis that the SMIPPs retain function(s) even though they have lost their catalytic ability.

It is clear from the above that SMIPPs exist in two compartments: 1) internal to the mite in the gut and 2) external to the mite after excretion from the gut in scybala. Different proteolytic “threats” face the mite in each of these locations.
Internally the imbibed plasma in the gut presumably contains the serine protease cascades of both the blood clotting and complement fixation pathways. Complement has been shown to be an important component in host defense against ticks.

Both these pathways must be inhibited while simultaneously digestion of epidermal protein as food must take place.

In the HDM, it is the allergens in the scybala that cause the most problems to those susceptible to allergic reaction. That the SMIPPs are also localized to scybala shows that the SMIPPs are available to interact with and presumably disrupt the host immune system. The endoparasitic wasp, Cotesia rubecula, presents an example of a proteolytically inactive serine protease facilitating avoidance of the host immune system. It uses a clip domain serine protease paralog to disrupt the proteolytic cascade that leads to the formation of melanin in the host. By disrupting this process, the wasp avoids immune system activation directed against the deposition of her eggs.

The scabies mite’s life cycle is contained almost entirely within the skin of the host. It therefore must contend with skin shedding; interrupting this process would be beneficial to the mite. In crusted scabies, one classic sign is hyperkeratosis. Inhibition of the serine protease stratum corneum chymotryptic enzyme (SCCE) can affect the cleavage of desmosome adhesion molecules leading to hyperkeratosis.

**Figure 1.** An example of localization of SMIPPs. **A, B,** and **C** represent serial sections of human skin infested with scabies mites. Red shows binding of antibody to protein *in vivo.* **A,** Probed with anti-human IgG. **B,** Probed with pre-bleed. **C,** Probed with anti-Yv4005G12. Further examples, probed with **(D)** anti-Yv5026E07 and **(E)** YvT004A06. **F,** An example of positively stained feces, probed with anti-Yv5026E07 (m, mouthparts; l, leg; e, egg; c, cuticle; g, gut; b, burrow wall; f, feces).
zymes often modulate the activity of their active counterpart. A number of processes relevant to the life cycle of the mite involve serine proteases.

Other functions of inactive protease paralogues include anti-microbial activities. Burrows of the scabies mite often contain microbes such as Group A streptococci, and these may be targets of SMIPPs. There is increasing evidence that skin streptococcal infections may directly cause rheumatic fever, which is common in many scabies endemic regions.

While there are cases of inactive proteases throughout the literature, most are in considerably smaller families of two or three members. With the large number of SMIPPs found, there would be a huge amount of redundancy if all had the same function. While the functions of SMIPPs are yet to be determined and the binding partners to be elucidated, the evidence here that SMIPPs are found both inside and outside of the mite suggests that they could potentially be involved in multiple processes.

Using sections of tissue infected with mites, it is hard to differentiate which stages of the life cycle of the mite have positive localization except for adult females bearing eggs. Therefore, although we know that the proteins are located in the gut, we cannot address at which other stages of the life cycle the proteins are expressed. Further work using RT-PCR could address this issue.

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