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Plasmodium sporozoite invasion of the mosquito salivary gland

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SUMMARY

About 1 to 2 million people die of malaria every year. Anopheline mosquitoes are the obligatory vectors of *Plasmodium* spp., the causative agent of malaria. For transmission to occur, the parasite has to undergo a complex developmental program in the mosquito, culminating with sporozoite invasion of the salivary glands. Strong circumstantial evidence suggests that sporozoite invasion requires specific interactions and recognition between sporozoite and salivary gland proteins. Here we review recent progress toward the elucidation of invasion mechanisms.

INTRODUCTION

Nearly half of the world population is at risk of contracting malaria and over one million people, mostly African children under the age of 5, die of the disease every year [1]. Resistance of the mosquito and the parasite to agents that kill them and the lack of an effective protective vaccine, all contribute to exacerbate disease burden [2,3,]

Unlike other major infectious diseases such as AIDS and tuberculosis, malaria parasites need a vector for transmission to occur. When a mosquito takes an infectious blood meal, the ingested gametocytes differentiate into male and female gametes that then mate to generate zygotes. Still in the midgut, zygotes differentiate into motile ookinetes that first traverse the peritrophic matrix and then cross the midgut epithelium. Upon emergence, the parasites lodge themselves between the basal epithelial surface and the basal lamina. This prompts the ookinetes to differentiate into oocysts that upon maturation about 10 days later rupture, each releasing several thousand sporozoites into the open hemolymph circulation of the mosquito [4] (Fig. 1).

OVERVIEW OF SALIVARY GLAND INVASION

Mosquito salivary glands are paired organs localized in the thorax. The gland of the female mosquito is formed by two similarly constructed lateral lobes and a shorter and wider medial lobe [5,6]. Two regions can be identified in the lateral lobes, proximal and distal, that are separated by a narrow transitional region. The distal region consists of large secretory cells whose products participate in blood feeding [7]. Detailed ultrastructural studies of sporozoite invasion of the salivary gland were conducted with *Aedes aegypti* and *P. gallinaceum* [8,•9] (Fig. 2). Initially sporozoites attach to the filamentous basal lamina via their anterior tip,

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although in some views the sporozoites can also be found attached along their entire length. As the parasite penetrates the basal lamina, it looses its thick coat and the sporozoite anterior end is found pointing towards the epithelial cell. No information is available as to how the sporozoites traverse the basal lamina. Invasion begins with invagination of the epithelial cell membrane which adopts the shape of the parasite, leading to the formation of a parasitophorous vacuole. Electron microscopy revealed that a specialized junction forms between the apical end of the sporozoite and the forming vacuole [•9]. The parasitophorous vacuole disintegrates during invasion and free parasites accumulate within the cell [10]. Next, the sporozoite invades the apical membrane of its host epithelial cell, resulting into sporozoite release into the gland's central secretory cavity (Fig. 2). During early stages of infection, sporozoites are found mostly inside the cytoplasm of the secretory cell while very few are found inside the central secretory cavity. At later stages sporozoites are mostly found inside the secretory cavity where they aggregate into large bundles [•9]. *In vitro* sporozoite invasion of salivary glands has never been reported. It is not clear why this could not be achieved.

SPECIFICITY OF SALIVARY GLAND INVASION

Of all the organs and cell types with which the sporozoites released from the oocysts come in contact, only one is invaded, the salivary glands. This specificity implies that sporozoites actively recognize the salivary glands, most likely via receptor-ligand interactions. Additional evidence for specific interactions was provided by Rosenberg's classic experiments [••11]. *P. knowlesi* fully develops in *An. dirus* mosquitoes, while in *An. freeborni* development proceeds normally up to release of sporozoites into the hemocoel but the *freeborni* salivary glands are never infected. Rosenberg demonstrated that the parasite invades *An. dirus* salivary glands implanted into infected *An. freeborni* mosquitoes while in the reverse experiment, it failed to infect *An. freeborni* glands implanted into *An. dirus* mosquitoes [••11]. These results strongly suggest that *P. knowlesi* sporozoites are unable to recognize *An. freeborni* salivary glands. The molecular basis for this specificity remains unknown.

THE SEARCH FOR SALIVARY GLAND RECEPTORS FOR SPOROZOITE INVASION

Several attempts to identify candidate receptors for sporozoite invasion have been reported (Table 1). It was observed that monoclonal antibodies raised against whole salivary glands inhibit sporozoites invasion [12]. A group of high molecular weight proteins (SGS family) of more than 200 kDa were identified in *Aedes aegypti*. Each SGS gene is encoded by a ~10 kb open reading frame and all SGS proteins possess predicted multipass transmembrane domains near their C-terminal ends. Antibody raised against the aaSGS1 protein inhibits sporozoite invasion [8]. Four SGS orthologues, agSGS2, agSGS3, agSGS4 and agSGS5 are also found in *An. gambiae* [13]. In a separate set of experiments, monoclonal antibodies raised against *An. gambiae* salivary glands led to the identification of a 50 kDa protein that forms a 100 kDa homodimer. Antibody against this protein binds to the surface of the salivary glands and inhibits sporozoite invasion by about 75% [•14]. This protein was termed saglin [15]. Independent experiments determined that saglin acts as a receptor for sporozoite invasion via interaction with *Plasmodium* TRAP [••16] (see below).

THE SEARCH FOR SPOROZOITE LIGANDS FOR SALIVARY GLAND INVASION

A list of candidate ligands is presented in Table 2 (see also [17])

Circumsporozoite protein (CSP)

CSP is a conserved GPI-anchored membrane protein of ~60 kDa that covers the entire surface of the sporozoite. CSP is shed when the sporozoite glides on a solid surface, creating a CSP protein trail [18]. CSP is essential for both oocyst development and sporozoite differentiation [••19]. It is required for inner membrane deposition and formation of the microtubule network associated with the oocyst's outer membrane [20].

In addition to oocyst development and sporozoite differentiation, CSP also seems to play a role in sporozoite recognition of the mosquito salivary glands. An anti-CSP monoclonal antibody completely suppressed invasion of *P. gallinaceum* sporozoites [21]. While this finding could be simply interpreted as antibodies causing steric hindrance for sporozoite access to the salivary gland surface, additional evidence suggests that CSP may play an active role. The recombinant protein binds to salivary glands in preference to other mosquito organs and this binding is inhibited by a highly conserved 15-amino acid motif that includes the 5-amino acid sequence known as region I [22,23,24,25]. Further experiments demonstrated that the 15-amino acid peptide, as well as the whole CSP protein, inhibited sporozoite invasion [26]. However, the 5-amino acid region I peptide by itself had no inhibitory activity [25]. In agreement with these findings, recombinant parasites carrying a deletion of region I showed no impairment of motility or infectivity of the mosquito [27]. A second conserved CSP motif, region II, bears a striking homology to a cell adhesion motif of thrombospondin. Sporozoites carrying a CSP gene with a deleted region II had no motility and were unable to invade the salivary glands [27]. A subsequent study determined that deletion of region II allowed for development of normal number of sporozoites, but in contrast to the previous study, these sporozoites were unable to exit the oocysts [28]. The discrepancy may be explained in part by the fact that the former studies were conducted with hybrid *P. berghei* parasites that expressed the *P. falciparum* CSP protein. These sporozoites had a10-fold lower salivary gland infectivity.

MAEBL

MAEBL is a micronemal protein of about 200 kDa that was identified in asexual stages of parasite development. MAEBL has a single transmembrane domain and is structurally related to members of the *Plasmodium* Duffy binding-like (DBL) family [29]. While MAEBL was initially characterized in asexually reproducing parasites, more recently its expression was described in sporogonic stages in the mosquito [••30,31]. Targeted disruption of MAEBL revealed that the gene is essential for salivary gland invasion. Gliding motility and infectivity to the vertebrate host were unaffected by MAEBL disruption but mutant sporozoites showed a 20-fold reduction in attachment to the salivary gland surface [••30]. The putative interacting salivary gland molecules have not been identified.

TRAP

Thrombospondin Related Anonymous Protein (TRAP) is expressed during sporozoite differentiation in the oocyst and is stored in micronemes. It is essential for sporozoites gliding and cell invasion [28,••32]. Upon contact with a target cell, TRAP is released from the micronemes onto the sporozoite's anterior tip [33]. It is also released on the substrate during gliding locomotion [34]. After invasion of the salivary gland, TRAP is found over the entire surface of *P. berghei* sporozoites (Ghosh et al., unpublished observations) and in other apicomplexans, the orthologous protein is found over the entire parasite surface. TRAP contains in its extracellular portion two adhesive modules, A-domain of the von Willebrand factor [33] and a thrombospondin type I repeat [36]. Transgenic *P. berghei* sporozoites with a mutated TRAP A-domain are impaired in salivary gland invasion but not in gliding motility [36,•37]. However, a very small number of *P. yoelii* TRAP knockout sporozoites were able to bind and invade salivary glands [38]. A detailed mutational analysis revealed that two specific

mutations in the A-domain - T126A and D157A - abrogated the sporozoite's ability to invade the salivary glands [•37].

PCRMP (Cysteine Repeat modular proteins)

The Cysteine Repeat Modular Proteins 1 and 2 are encoded by a small gene family conserved in malaria and other *Apicomplexan* parasites. *P. berghei* PCRMP1 is transcribed in developing oocysts and its abundance increases in sporulating oocysts, while PCRMP2 is transcribed in sporulating oocysts and salivary gland sporozoites. Both proteins localize on the sporozoite surface. PCRMP1 and PCRMP2 knockout sporozoites are unable to invade salivary glands, suggesting a role in salivary gland invasion [39]

USO3 and TREP / S6

P. yoelii USO3 (upregulated in oocyst sporozoites) was first identified by the Kappe laboratory [40]. The related TREP protein, first identified via subtractive hybridization experiments [41], has been alternatively named TREP [42] or S6 [43]. The two proteins have related structural features in that they are surface proteins (they possess a transmembrane domain) and have a thrombospondin repeat that presumably functions in protein-protein interactions. Structurally USO3 differs from TREP/ S6 by the lack of an adhesion domain A in the latter protein. USO3 is localized to the apical end of oocyst sporozoites while TREP/S6 is localized to the plasma membrane. Surface localization of both proteins and expression prior to salivary gland invasion are consistent with a role in invasion. Indeed, USO3 knockout of *P. yoelii* parasites leads to a complete inhibition of salivary gland invasion, while TREP/S6 knockout in *P. berghei* parasites partially inhibited salivary gland invasion. Knockout of both genes leads to partial loss of motility. It is not known whether either of the proteins directly interacts with mosquito salivary gland proteins, as was shown for TRAP (see below).

A PHAGE DISPLAY SCREEN LEADS TO THE IDENTIFICATION OF A RECEPTOR-LIGAND PAIR REQUIRED FOR SPOROZOITE INVASION OF SALIVARY GLANDS

A screen of a phage library displaying random 12-amino acid peptides led to the identification of a peptide - SM1 (for Salivary gland and Midgut peptide 1) - that binds specifically to the surfaces of the salivary gland and midgut epithelia. Importantly, binding of the peptide resulted in strong inhibition of parasite invasion of these organs [••44]. These results implied that the peptide binds to a surface receptor that the parasite needs to recognize in order for invasion to occur. Discovery of SM1 led to the engineering of the first transgenic mosquito impaired in transmission of the malaria parasite [45]. Recent work identified the molecular nature of the salivary gland receptor and of the sporozoite ligand [••16].

The receptor was identified using a double-derivatized SM1 peptide bearing a biotin residue at one end and an UV-activatable crosslinker in the middle of the peptide. The derivatized peptide was incubated with dissected salivary glands followed by UV irradiation to promote crosslinking with the protein to which the peptide was bound. The glands were then lysed and the peptide (plus the crosslinked protein) was then captured on streptavidin beads. Sequencing of the crosslinked protein identified the putative receptor as the salivary gland surface protein saglin [••16,15]. Saglin is a 50 kDa protein that has a signal peptide but no transmembrane domain. Saglin occurs on the salivary gland surface, as anti-saglin antibodies administered to mosquitoes inhibit *P. berghei* [•14] and *P. falciparum* [••16] sporozoite invasion of salivary glands. However, it is not known whether the protein is associated with the membrane or the basal lamina of the salivary gland, as the available immuno-electron micrographs do not have sufficient resolution to determine its exact location [•14]. Saglin is rich in glutamines and these

residues may be involved in protein-protein interactions. Sequencing of the proteins crosslinked to SM1 identified two additional signal peptide-containing salivary gland proteins: gSG1 [46] and gSG1b [47]. Both proteins bear some sequence identity with saglin (unpublished observations) and are specific to female salivary glands. The possible location of the gSG1 and gSG1b proteins on the salivary gland surface has not been verified and it is unclear whether they play a direct role in sporozoite invasion.

Inhibition of sporozoite invasion by SM1 implied that the peptide and a parasite protein compete for binding to a salivary gland receptor, presumably saglin (Fig. 3). Yet, the SM1 amino acid sequence did not match any predicted *Plasmodium* protein in the database. The hypothesis that SM1 conformation, rather than primary amino acid sequence, resembled a sporozoite protein, led to the production of an anti-SM1 antibody for testing Western blots of sporozoite proteins. These experiments led to the identification of TRAP as a mimotope of SM1 (TRAP is recognized by the anti-SM1 antibody) and raised the hypothesis that TRAP binds to saglin (Fig. 3; [••16]). Further experiments demonstrated that 1) recombinant *Plasmodium* TRAP A-domain binds to salivary glands and that this binding can be competed by SM1; 2) that recombinant TRAP A-domain binds to recombinant saglin *in vitro* and that this binding is abrogated by the same A-domain point mutations that prevent sporozoite invasion of salivary glands [•37]; that RNAi knock down of saglin expression strongly inhibits salivary gland invasion (Fig. 3; [••16]). These data strongly argue for an essential role of saglin-TRAP interactions for invasion of the salivary glands. Nevertheless, these experiments need to be interpreted with caution. Sporozoite invasion of the salivary gland is a complex process (Fig. 2) that must depend on the successful completion of a number of other steps. Thus, saglin-TRAP interaction should be considered as only one of many steps required for successful sporozoite invasion of the salivary gland.

THE FINAL STEPS

The sporozoite invades the salivary gland epithelial cell from the basal side and then exits this cell from the apical side, to reach the lumen of the acinus (Fig. 2). It is not known whether after reaching the epithelial cell cytoplasm, sporozoite migration is directional or how the sporozoite makes its way to the apical membrane. Once in the lumen of the acinus, the sporozoites associate with each other to form bundles (Fig. 2) via an unknown mechanism. A small number of sporozoites can be found in the secretory duct and again, it is not known how they detach from the bundles and how they find their way into the duct lumen. The duct wall of culicine mosquitoes (e.g., *Ae. aegypti*) contains chitin [6] and sporozoites might secrete a chitinase to make their way into the lumen. However, the duct wall of anopheline mosquitoes is not believed to contain chitin [5] and how the sporozoites find their way into the duct is not known. Most likely this occurs by sporozoite entry via the duct ending, together with the saliva.

CONCLUSIONS

Successful sporozoite invasion of the salivary gland is an essential step for the completion of the *Plasmodium* cycle in the mosquito. As is apparent from this review, our understanding of this complex, multistep invasion process is rather superficial. Since disruption of any of these many steps is likely to abrogate parasite cycle in the mosquito, further investigation of these processes should prove to be rewarding and should receive high priority. Answers to some of these questions may lead to new means to interfere with parasite transmission.

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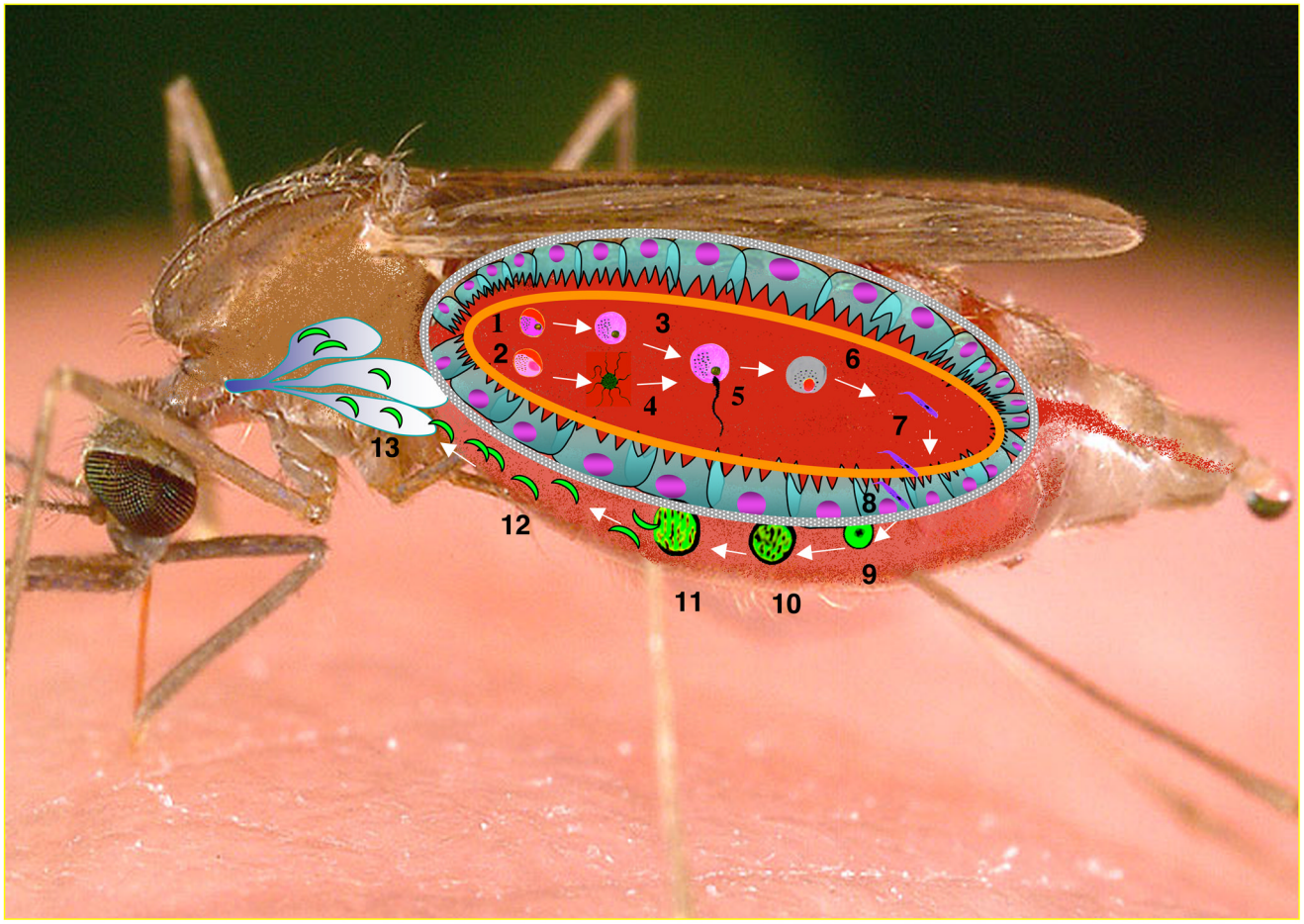

Figure 1. Life cycle of the *Plasmodium* parasite in its mosquito vector

Female **(1)** and male **(2)** gametocytes differentiate into gametes **(3,4)**. After completion of meiosis, the male gametocyte generates 8 gametes **(4)** in a process known as “exflagellation”. A male gamete fertilizes a female gamete **(5)** to generate a zygote **(6)**, which in turn differentiates into a motile ookinete **(7)**. About 24 h later, the mature ookinete first traverses the peritrophic matrix (orange line) and then the midgut epithelium **(8)**, after which it differentiates into a oocyst **(9)**. During the next ~10 days the oocyst grows **(10, 11)** and when mature, it releases sporozoites into the open hemolymph circulation **(12)**. The circulating sporozoites recognize and invade the salivary glands **(13)** where they are stored until release at the time when the mosquito bites the next individual.

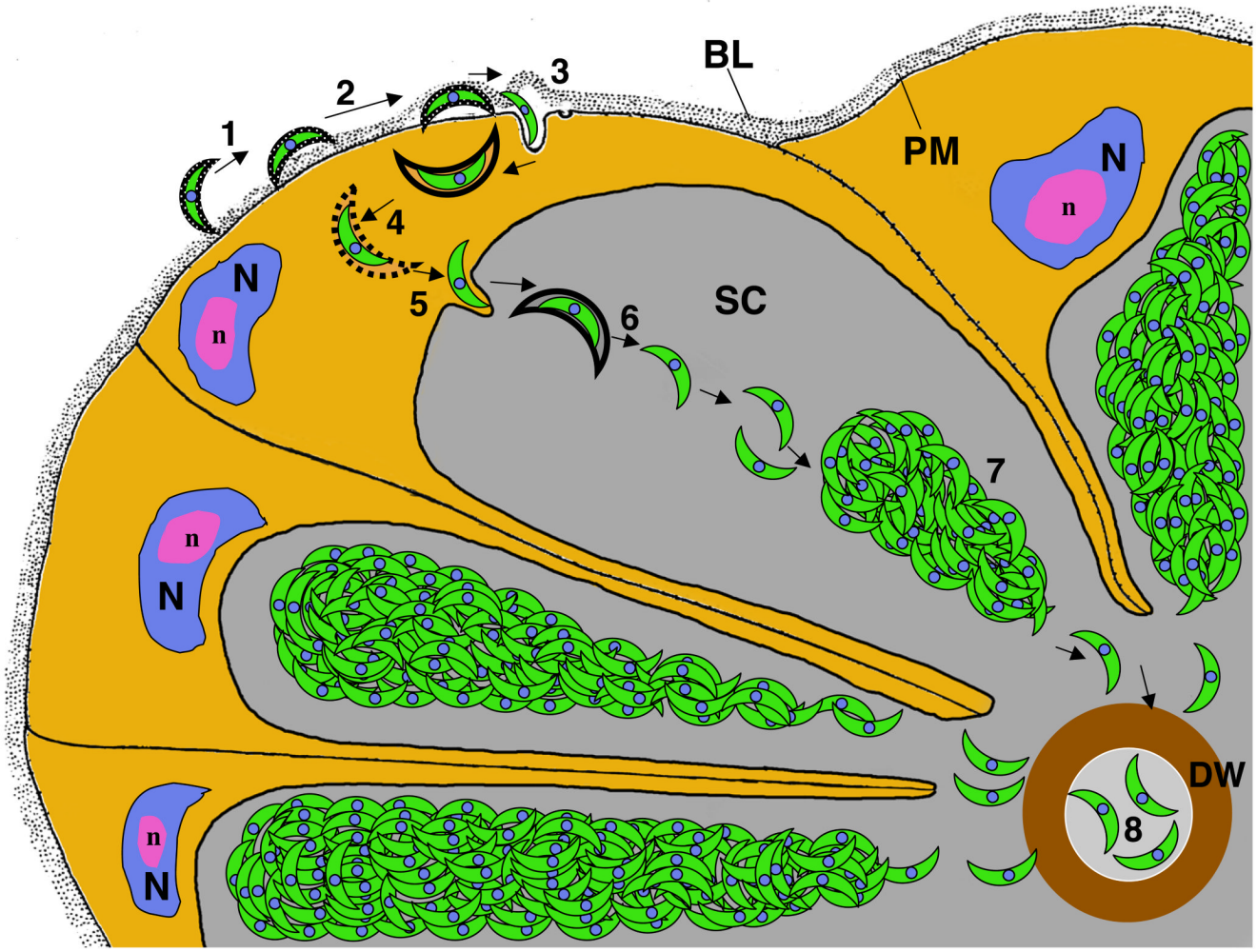

Figure 2. Progression of sporozoite invasion of the salivary gland

1) The sporozoite attaches to the basal lamina; **2)** Sporozoite passage to the space between the basal lamina and the basal epithelial cell plasma membrane, a process associated with the loss of the sporozoite's thick coat; **3)** Penetration of the basal plasma membrane; the sporozoite resides within a vesicle; **4)** Release of the sporozoite from the surrounding membrane by an unknown mechanism; **5)** Invasion of the apical membrane and entry into the secretory cavity; **6)** Sporozoites are released from the surrounding membrane by an unknown mechanism; **7)** Sporozoites assemble into bundles within the secretory cavity; and **8)** A small number of sporozoites enter the secretory duct by an unknown mechanism. BL: Basal Lamina; DW: Duct wall; N: nucleus; n: nucleolus; PM: Plasma membrane; SC: Secretory cavity. Modified from reference [•5].

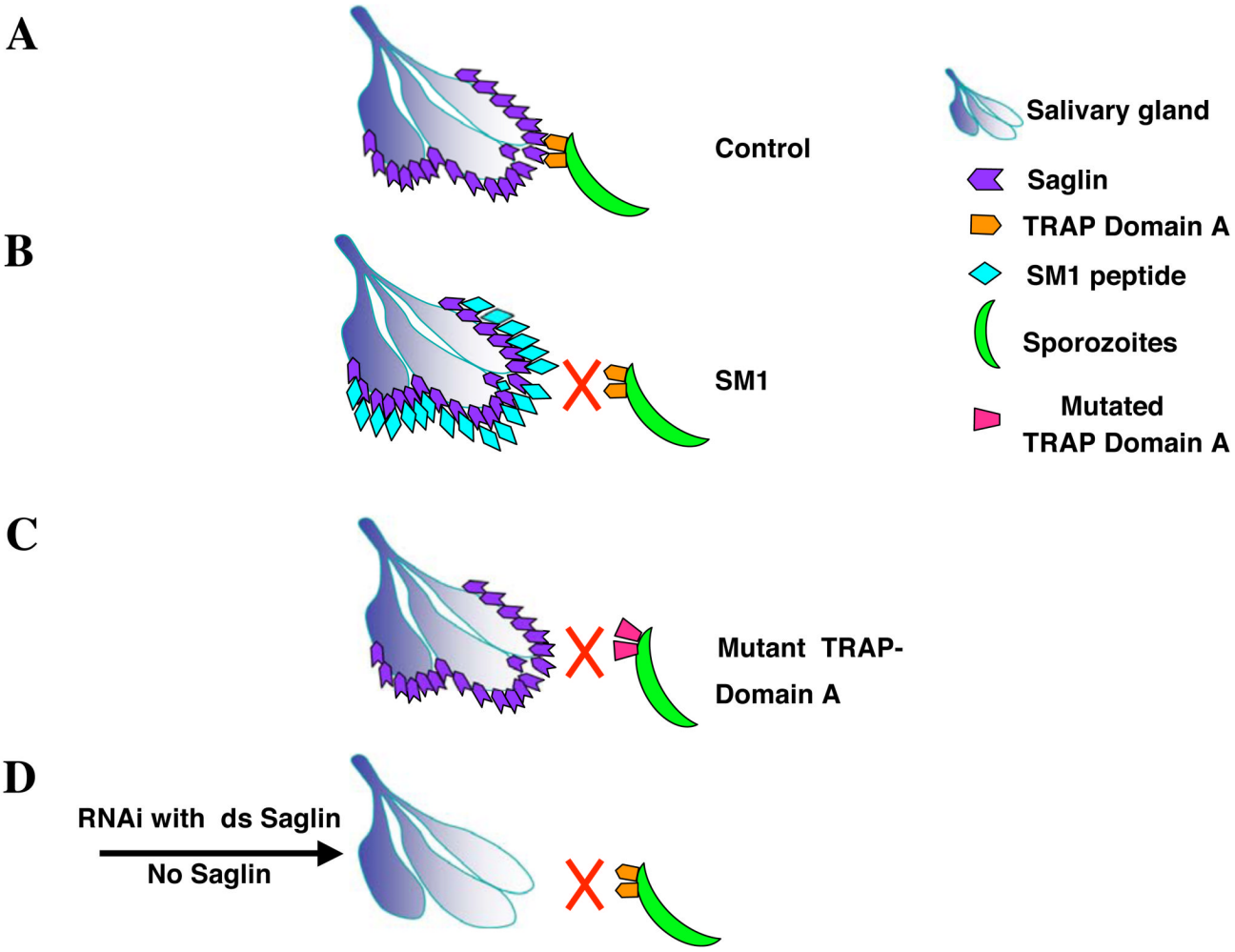

Figure 3. Schematic representation of the interaction of the salivary gland protein saglin with the sporozoite protein TRAP

A) Binding of *Plasmodium* TRAP to the salivary gland protein saglin results in productive invasion. **B)** SM1 binds to saglin occupying this receptor and in this way precludes interaction with the sporozoite TRAP. **C)** Mutation of the critical TRAP A-domain T126 to A alters its conformation preventing recognition of saglin and sporozoite invasion. **D)** Down-regulation of saglin expression by RNAi reduces the abundance of the saglin receptor on the salivary gland surface preventing sporozoite invasion. Based on data of reference [••16].

TABLE 1

Candidate salivary gland proteins with a role in sporozoite invasion

Protein	Localization	MW (kDa)	References
Saglin	surface	50	[15,••16]
gSG1	?	46	[46]
gSG1b	?	43	[47]
SGS family	surface	40	[13]

TABLE 2

Sporozoite proteins with possible role in salivary gland invasion

Protein	Localization	MW (kDa)	Reference
CS	surface	40	[25]
TRAP	surface	94	[••32]
MAEBEL	surface	175	[29]
PCRMP1	surface	>150	[39]
PCRMP2	surface	>250	[39]
USO3	surface	?	[40]
S6	surface	~260	[43]
TREP	surface	?	[42]