

**2<sup>nd</sup> EMBO WORKSHOP ON  
Molecular and population biology of mosquitoes and other disease vectors  
GR-Kolymbari, Crete, 24-31 July 2005**



**LOUKERIS lab**

**Title of the talk: Uncovering the role of proteases  
in parasitic development and invasion processes**

The main goal of our work is to assign functional roles to specific parasite and *Anopheles* proteases that may be involved in the process of mosquito midgut invasion by *Plasmodium*. This is attempted by using a combination of cell biology, transgenic technology and RNA interference approaches; methodologies that have been recently introduced into the mosquito field and together with the availability of the *Anopheles gambiae* and *P. falciparum* genomes revolutionize the study of vector and parasite biology.

**I. Studies of matrix metalloproteases in mosquito vector *Anopheles gambiae***

**Gareth Lycett/ Evi Goylielmaki**  
**collaborators: F. C. Kafatos**

Since strong evidence exists for the activation of counter-acting protease inhibitors, it is reasonable to assume that proteolytic pathways are activated in mosquitoes during midgut invasion by *Plasmodium* ookinetes. Moreover, it is sensible to speculate that metalloprotease (MMP) activation plays a critical role during parasite passage and the subsequent epithelial healing process. MMP activation is often associated with cellular invasion by parasites, viruses and metastatic cancer cells. Zinc-dependent matrix MMPs comprise a family of ECM degrading enzymes that are produced as zymogens and become activated only following cleavage of their amino-terminal pro-domains by a variety of agents. Activating agents include proteases or other MMPs or non-proteolytic agents such as reactive oxygen species, low pH and nitric oxide (NO). All these agents are probably present during mosquito midgut invasion. Therefore, the overall aim of the project is to investigate the role of specific MMPs in mosquito midgut physiology and invasion by the *Plasmodium* ookinetes.

We have started the study of matrix metalloproteases in *Anopheles* with two members of the ADAMTS family, (A Disintegrin And Metalloprotease with Thrombospondin repeats), encoded in the *Anopheles* genome (TS1 and TS2).

Nothing is known about ADAMTSs and their substrates in invertebrates, and little is known regarding their functions in vertebrates. However, one of the human ADAMTSs, ADAMTS-13, has been found to be the processing enzyme for von Willebrand factor *in vivo*. This finding is of particular relevance since the *Plasmodium* ookinete secretes and/or possesses on its surface, molecules characterized by the presence of von Willebrand factor domain(s) (WARP, CTRP, TRAP). These parasitic molecules play a crucial role in invasion.

Cloning and initial characterization of the two *Anopheles* ADAMTSs indicated that four different isoforms are generated from TS2 gene by alternative splicing. The isoforms vary in their thrombospondin (TSP) domains. This is an interesting discovery since alternative isoforms may have different substrates, as the TSP domains are thought to be essential for substrate binding and thus target specificity. Immuno-histochemical analysis of whole mount midguts suggest that at least some of the TS2 isoforms are upregulated in the epithelial cells following bloodfeeding. The effect of ADAMTS-gene expression silencing on ookinete invasion has been studied using RNA interference induced by dsRNA injection into adult mosquitoes. In three independent experiments, both dsRNAs (TS1 and TS2) led to an increase in oocyst burden in comparison with the control infections of control dsRNA injected mosquitoes.

We continue the efforts to assign specific functions to these important molecules during development and epithelial restructuring, as well as to establish their target specificities.

## **II. Serpins as tools to study proteolytic processing in apicomplexan parasites**

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*Matuszewski (Plasmodium)*

A large body of evidence points to an important role for apicomplexan proteases during invasion processes. Subtilases are an ancient group of proteolytic enzymes that are widely dispersed throughout evolution. They are functionally diverse but are generally secreted and function either within the secretory pathway or extracellularly. Members of this superfamily are also present in apicomplexan parasites. Among them, *Plasmodium falciparum* PfSUB2, *Plasmodium berghei* PbSUB2, and *Toxoplasma gondii* SUB2 are well described in the literature. Direct evidence for a functional role for SUB2s during invasion processes has been difficult to obtain, since attempts to knock out the corresponding genes have so far failed. SUB2 appears to be essential in all three apicomplexan species, in which knock-out has been tried (*P. falciparum*, *P. berghei* and *Toxoplasma*), which is in accordance with SUB2s's vital role and justification for their choice as drug target.

It has been shown that introduction of appropriate mutations in the reactive site loop (RSL) of a serpin scaffold may increase its suicidal activity against a specific protease. We thus generated serpin variants predicted to be active against apicomplexan SUB2s. In all cases the CAM isoform, a potent inhibitor of bacterial subtilases derived from *A. gambiae* SRPN10 gene locus, was used as a scaffold to introduce appropriate mutations in the RSL. In initial test experiments, we expressed one of the variants (EAP) with putative inhibitory activity against *Toxoplasma gondii* TgSUB2 and the original CAM serpin in transiently transfected *Toxoplasma* tachyzoites (col: Dr. K. Kim). Both serpins, CAM and EAP, were tagged at their amino-termini with a myc epitope tag and were fused to a signal peptide sequence (ss) derived from the *ROP1* gene encoding for the major rhoptry protein ROP1. Both

serpins, when expressed in the secretory pathway, affect proteolytic processes and/or signaling, probably by inhibiting proteases active in secretory pathway compartments.

The current main objective of the group is to identify the specific functions of *Plasmodium* SUB2 in the sporogonic development of Plasmodium. Therefore, based on these encouraging initial results derived from Toxoplasma, we concentrate presently on the generation and detailed characterization of transgenic rodent *P. berghei* parasites in which SUB2 function will be disrupted at the ookinete and/or sporozoite stage either by expression of serpins or by other means (collaboration with AP Waters/J-C Barale/K. Matuszewski labs). The above transgenic strains will be characterized through extensive proteomic studies. In addition these strains, after their initial characterization, will be used in comparative mosquito/parasite interaction studies using confocal microscopy. New *Anopheles* genes are currently cloned in our lab in order to serve as additional invasion molecular markers, (including matrix metalloproteases reported above). With these genes, together with the previously reported anti-SRPN10 antibody (the best marker of intracellular invasion by *P. berghei*), we expect to uncover putative differences in the invasion processes followed by the SUB2 depleted parasites.

### **III Functional Characterisation of *Anopheles gambiae* P450 Reductase**

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**\*see relevant abstract**