

structure–function analysis, as it is possible to use the homologous/endogenous system. In the TRAP tail study, mutations were not introduced in the cytoplasmic tail of MIC2 in the TRAP-MIC2 fusion (although the tails of both proteins appeared functionally interchangeable, this is not a guarantee that all interactions that these tails engage in are identical at the single residue level). Instead, mutations (Ala substitutions of the last three acidic residues, and the C-terminal Trp that are present in the tails of both TRAP and MIC2) were introduced in the cytoplasmic tail of WT TRAP. In Ref. 1, mutations in the A domain and the thrombospondin type I repeat (TSR) were introduced in PfTRAP. As PfTRAP and its variants (which originate from *P. falciparum*, which infects humans) are expressed in *P. berghei*, which infects rodents, this approach is risky.

There are at least two reasons why the defective/heterologous system should not have been used for testing the contribution of the A domain or the TSR of TRAP in TRAP-dependent phenotypes: (1) structure–function analysis of an impaired system is of no predictive value for the normal system: what further disrupts an initially crippled interaction may not have altered a normal interaction at all (and vice versa, ie. a crippled interaction is just a different interaction); and (2) with such a drop in the basal level of gliding motility and infectivity (control PfTRAP sporozoites function only slightly better than do TRAP-knockout sporozoites), additional losses of motility or infectivity are hard to interpret. For example, to evaluate the effect of TRAP mutations on sporozoite motility, Wengelnik *et al.* have to compare

numbers of gliding sporozoites of 'below 1%' (sporozoites that express the WT PfTRAP), 'gliding also seen' (sporozoites expressing a PfTRAP that contains a mutation in its A domain), and 'no gliding' (sporozoites expressing a PfTRAP that contains a deletion in its TSR).

Conclusions

We have also undertaken an analysis of the function of the A domain and the TSR of TRAP, using an insertion strategy and a control line whose phenotype is undistinguishable from the WT (K. Matuchewski *et al.*, unpublished) and obtained substantially different results than those reported in Ref. 1. For example, we found that mutations in the TSR did not affect sporozoite gliding motility, but affected cell invasion *in vitro* and infectivity *in vivo*, whereas Wengelnik *et al.* reported the opposite, ie. TSR mutant sporozoites that are impaired in gliding motility, but not in infectivity to rodents. Their result is surprising, as it is difficult to imagine how sporozoites that are impaired in motility are infective. Another major difference was noticed between mutants bearing identical mutations in the A domain of TRAP. Wengelnik *et al.* reported that the A domain mutant was affected in its capacity to infect mosquito salivary glands, but that it was normally infective to rodents. In contrast, we found that independent mutants in the A domain drastically affected infectivity to both mosquito salivary glands and the rodent liver. Finally, although Wengelnik *et al.* conclude that none of the A domain and the TSR have much importance for rodent liver infection, we found that, if the two TRAP extracellular domains were mutated,

sporozoites totally lost their capacity not only to infect rodents, but also to penetrate host cells *in vitro*. These fundamental discrepancies show that the points we have raised are not just semantic.

References

- 1 Wengelnik, K. *et al.* (1999) The A-domain and the thrombospondin-related motif of *Plasmodium falciparum* TRAP are implicated in the invasion process of mosquito salivary glands. *EMBO J.* 18, 5195–5204
- 2 Kappe, S. *et al.* (1999) Conservation of a gliding motility and cell invasion machinery in apicomplexan parasites. *J. Cell Biol.* 147, 937–943
- 3 Rogers, W. *et al.* (1992) Characterization of *Plasmodium falciparum* sporozoite surface protein 2. *Proc. Natl. Acad. Sci. U. S. A.* 89, 9176–9180
- 4 Sultan, A.A. *et al.* (1997) TRAP is necessary for gliding motility and infectivity of *Plasmodium* sporozoites. *Cell* 90, 511–522
- 5 Russel, D.G. and Sinden, R.E. (1981) The role of the cytoskeleton in the motility of coccidian sporozoites. *J. Cell Sci.* 50, 345–359
- 6 King, C. (1988) Cell motility of sporozoan Protozoa. *Parasitol. Today* 4, 315–319
- 7 Nunes, A. *et al.* (1999) Subtle mutagenesis by ends-in recombination in malaria parasites. *Mol. Cell. Biol.* 19, 2895–2902
- 8 Thathy, V. and Ménard, R. Gene targeting in *Plasmodium berghei*. *Methods Mol. Med.* (in press)
- 9 Vanderberg, J.P. (1974) Studies in the motility of *Plasmodium* sporozoites. *J. Protozool.* 21, 527–537
- 10 Carruthers, V.B. and Sibley, L.D. (1997) Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts. *Eur. J. Cell. Biol.* 73, 114–123

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Structure–Function Analysis of Malaria Proteins by Gene Targeting – A Response

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We have generated transgenic *Plasmodium berghei* parasites in which the endogenous gene encoding thrombospondin-related anonymous protein (TRAP) was replaced by either wild-type (WT) *P. falciparum* TRAP (PfTRAP) or by mutated versions of PfTRAP carrying amino acids substitutions or deletions in the A domain or in the thrombospondin-related motif¹. This strategy was employed in a structure–function analysis, which aimed to circumvent limitations due to

the availability of only one selectable marker to transform *P. berghei*. We did not attempt to modify the endogenous gene encoding TRAP by using an insertion-targeting approach because we were (and still are) convinced that genetic reversion is a limitation in structure–function studies. A reversion frequency of 1% should not be underestimated. If the revertant parasites were 50–100% more efficient than the mutated ones in infecting target cells or in gliding motility, their presence would

confound the phenotypic analysis of the mutated parasites.

The replacement strategy that we have employed offers the opportunity to verify the expression of the replaced gene in each individual parasite and provides an invaluable experimental model with which to study the immune response against *P. falciparum* antigens in rodent models. Moreover, the replacement of the endogenous genes encoding *P. berghei* TRAP or circumsporozoite

protein (CSP) with homologues from different *Plasmodium* spp will facilitate experiments aimed at investigating the role of these molecules in shaping the range of sporozoite infectivity for mosquito salivary glands and the vertebrate host. The insertion strategy has been reported by Ménard to be associated with a relatively high frequency of mutation repair events that are not well understood in molecular terms². The replacement strategy does not leave any endogenous sequence that may recombine with or function as a template with which to repair the inserted sequence.

Ménard and Nussenzweig (this issue) argue that our misconception on this last point ... 'led us to refrain from searching for the presence of the mutations in the recombinant loci, thus raising the question, whether the mutations introduced are present in the final loci'. We fail to understand a reason for this statement. The mutations that we have introduced in the gene encoding PfTRAP could easily be monitored for the presence of diagnostic restriction sites. In our paper¹, we reported that ... 'the identity of the parasites recovered from the blood of the parasitized mice was verified by PCR and restriction analysis. These analyses ruled out genetic reorganization in the TRAP locus'. We never observed repair of the introduced mutations in the gene encoding PfTRAP. It is suggested that, irrespective of the system used (insertion or replacement), the first step is to verify that the control line has a WT (*P. berghei*) phenotype. We had never thought to carry out a structure-function analysis without comparing the phenotype of the replacement control line with that of WT parasites. However, we do not think that, if differences are detected, the control line should not be used as a reference to reveal the effect of mutations and deletions. Our control line (PfTRAP) carries the gene encoding TRAP from the WT isolate ITO4 of *P. falciparum*. This *P. berghei* line develops normally into male and female gametocytes and, upon injection of infected blood by *Anopheles stephensi* mosquitoes, generates a normal number of ookinetes and oocyst sporozoites. Infection of mosquitoes with the PfTRAP line usually yields ≈4000 sporozoites per salivary gland – a number that is lower than that observed with WT *P. berghei*. We thought that these differences should not prevent us from using this line as reference for analysing the effect of mutation in the adhesive domains of PfTRAP. It is well known that *Plasmodium* parasites differ in their ability to infect different mosquito species. These differences have attributed to the selectivity of receptor-ligand interactions as well

as to the ability of the insect immune system to eliminate the parasites. We reasoned that if TRAP were involved in the process of recognition and entry into mosquito salivary glands, the comparison to WT *P. berghei* parasites did not make much sense. The transgenic parasites might display a novel phenotype due to the presence of TRAP from *P. falciparum* ITO4 in a *P. berghei* background.

We have demonstrated that a fraction of salivary gland sporozoites (2–10%) developing from the PfTRAP line were able to glide on glass slides¹. The low frequency of motile sporozoites was shown to be due to the experimental conditions rather than to a deficit of TRAP function. A dose of 100 PfTRAP sporozoites per mouse is sufficient to start an infection in 66% and 63% of mice injected with WT and PfTRAP parasites, respectively¹. The group of mice injected with PfTRAP parasites showed a pre-patent period 6–12 h longer than that of mice injected with WT parasites. Although the pre-patent period is a function of the number of sporozoites invading the hepatocytes, this index is also influenced by the growth rate of blood-stage parasites. PfTRAP and WT parasites also differ for the expression of the selectable marker DHFR-TS of *Toxoplasma gondii*, which, in the absence of selection pressure, could represent a growth disadvantage. As we have previously replied to Ménard and Nussenzweig⁴, it would be arbitrary to utilize a short delay in the pre-patent period to infer quantitative differences in the infectivity of PfTRAP and WT sporozoites.

When comparing PfTRAP parasites with sporozoites carrying a substitution in a conserved residue of the A-domain or a deletion in the region II⁺ of PfTRAP, we observed that these mutations impaired the ability of sporozoites to invade mosquito salivary glands¹. However, oocyst sporozoites from these transgenic parasites were still able to infect mice as efficiently as were PfTRAP parasites. Sporozoites carrying a mutation in the A-domain of PfTRAP are motile, while no gliding motility could be detected in sporozoites with a region II⁺ deletion¹. On the basis of these results, we concluded that 'TRAP is implicated in recognition and invasion of salivary glands by the sporozoite and that this process is functionally distinct from its involvement

in parasite motility'. The phenotype of the oocyst sporozoites carrying a deletion of thrombospondin surprised Ménard and Nussenzweig, because sporozoites that are impaired in motility are infective for the vertebrate host. These sporozoites were injected intravenously into the mice, therefore it would be reasonable to assume that they do not need to glide in order to reach the liver sinusoids. Furthermore, there is no experimental evidence indicating that sporozoite invasion of hepatocytes, *in vivo*, requires gliding motility. On the contrary, TRAP-knock-out parasites were reported to infect hepatocytes *in vivo*, although much less efficiently than did wild-type sporozoites³.

We are also told that unpublished results from Ménard and Nussenzweig suggest different conclusions, but we cannot comment on results that are not yet in the public domain. However, in an abstract reporting their results, Ménard and Nussenzweig have stated that infection into salivary glands was reduced by 50% in the region II⁺ mutant clone and by 80% in the A domain mutant clone*. They also reported that each mutant was still infective for the mammalian host, although with a longer pre-patent period[†]. These observations led to the conclusion that 'the invasive and gliding properties may not be mechanistically identical'.

Our region II⁺ mutant differs from that developed by Nussenzweig in that we did not observe gliding motility. These parasites carry a deletion of the entire region II⁺ sequences, while that developed by Nussenzweig carries a single amino acid substitution. Therefore, the two region II⁺ mutant parasites cannot be compared. Notably, in the case of the A domain mutant, the preliminary results of Ménard and Nussenzweig independently confirm the observations of our paper, thus validating the overall approach and conclusions.

References

- 1 Wengelnik, K. et al. (1999) The A-domain and the thrombospondin-related motif of *Plasmodium falciparum* TRAP are implicated in the invasion process of mosquito salivary glands. *EMBO J.* 18, 5195–5204
- 2 Nunes, A. et al. (1999) Subtle mutagenesis by ends-in recombination in malaria parasites. *Mol. Cell. Biol.* 19, 2895–2902
- 3 Sultan, A.A. et al. (1997) TRAP is necessary for gliding motility and infectivity of *Plasmodium* sporozoites. *Cell* 90, 511–522
- 4 Wengelnik, K. et al. (2000) Analysis of a malaria sporozoite protein family required for gliding motility and cell invasion. *Trends Microbiol.* 8, 96–97

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*K. Matuschewski et al. Abstract No. 105, 10th Molecular Parasitology Meeting, Woods Hole, USA, 1999

†K. Matuschewski et al. Abstract No. 49, III Immunoparasitology Meeting, Woods Hole, USA, 1999