

## *Plasmodium falciparum* Proteinases: Cloning of the Putative Gene Coding for the Merozoite Proteinase for Erythrocyte Invasion (MPEI) and Determination of Hydrolysis Sites of Spectrin by Pf37 Proteinase

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*Numerous proteinase activities have been shown to be essential for the survival of Plasmodium falciparum. One approach to antimalarial chemotherapy, would be to block specifically one or several of these activities, by using compounds structurally analogous to the substrates of these proteinases. Such a strategy requires a detailed knowledge of the active site of the proteinase, in order to identify the best substrate for the proteinase. Aiming at developing such a strategy, two proteinases previously identified in our laboratory, were chosen for further characterization of their molecular structure and properties: the merozoite proteinase for erythrocytic invasion (MPEI), involved in the erythrocyte invasion by the merozoites, and the Pf37 proteinase, which hydrolyses human spectrin in vitro.*

Key words: *Plasmodium falciparum* - proteinases - spectrin - erythrocyte invasion.

### MPEI

MPEI was initially identified in parasite extracts, by its cysteine proteinase activity, able to cleave a fluorogenic substrate, Gluconoyl-Valine-Leucine-Glycine-Lysine (or Arginine)-3-amido-9-ethylcarbazole, with an optimum activity at pH=7.4 (Schrével et al. 1988). This activity was mainly found in *P. falciparum* extracts from the schizont/merozoite stage (Grellier et al. 1989). Evidence that this activity was playing a role in erythrocyte invasion by the merozoite came from a series of *in vitro* synchronized culture assays of *P. falciparum*, done in presence of a non cleavable VLGK derivative: the release of merozoites was unaffected while the number of new rings appearing

in the culture was markedly reduced (Mayer et al. 1991). The active fraction, biochemically purified, consisted of a set of four related proteins (105 kDa, 97 kDa and 68/38 kDa), of which the three smaller ones appeared as maturation products of the 105 kDa one (Lawton et al. 1991).

A polyclonal antibody was raised in rabbit against the active fraction. It recognized this set of four peptides in immunoblot experiments, and labelled the anterior end of merozoites in immunofluorescence assays of schizont and merozoite stages. It was used to screen a *P. falciparum* expression library containing DNAase I genomic fragments of the Palo Alto FUP/CB strain (Fandeur et al. 1991), inserted into the EcoRI site of  $\lambda$ gt11. A single positive clone was isolated. Its 750-bp insert was sequenced. Its A+T content is high (80%) but still compatible to the A + T ratio of *P. falciparum* coding sequences (Weber 1987). An open reading frame was found throughout the insert. Its A/T ratio (1.48) as well as the biased codon usage are in good

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agreement with typical *P. falciparum* genes (Saul & Battistutta 1988). The predictive peptide encoded by this putative MPEI gene has a very interesting structure. First it is very asparagin-rich (43%) the other abundant amino acids being aspartic acid (12%) and methionine (11%). Second, the N-terminal part consists of 22 repeated hexapeptides. A first series of 11 hexapeptides similar to each other, is followed by a second series of 11 hexapeptides, also similar to each other. It is noteworthy that asparagin-rich proteins and proteins containing repeated peptides, often hexapeptides, are rather commonly found in *P. falciparum* (Wahlgren et al. 1996, Kun et al. 1991, Kemp et al. 1990). The C-terminal end of the predicted peptide is non repetitive. Amino acid motifs, characteristic of a cysteine protease active site, could not be identified in the 250 amino acids encoded by the 750 bp insert, which is too small to contain the complete gene for the 105 kDa MPEI. Database homology searches did not indicate any significant homologies between our predicted peptide and other genes. Based on the antigenic properties of the various regions of the predicted peptide, revealed by computer program analysis, two peptidic sequences were selected in the non repetitive region. In parallel, a recombinant putative MPEI peptide fused to a protein derived from the Glutathione S Transferase of *Schistosoma japonicum* was produced in the cytosol of *E. coli*. Both synthetic peptides and the recombinant protein are now being injected into rabbits, in order to confirm that the gene product corresponds to MPEI.

#### Pf37 PROTEINASE

Based on the observations that various species of plasmodia cause alterations of the erythrocyte membrane and notably that certain components such as spectrin are degraded in late schizogony (Weidekamm et al. 1973), a proteinase activity that would be capable of degrading spectrin *in vitro* was looked for and found in soluble extracts of *P. falciparum* (Deguercy et al. 1990). Fractionation of the soluble extract from schizont stages allowed the purification of a 37 kDa protein displaying this activity, which was inhibited by some but not all inhibitors of both serine and cysteine proteinases. This Pf37 proteinase was active *in vitro* on spectrin at an optimal pH of 5. Experiments were designed to determine whether the purified Pf37 proteinase hydrolysed purified spectrin at specific sites *in vitro*. To do so, a new protocol for the Pf37 proteinase

purification procedure was established to obtain sufficient amounts of pure active proteinase. The purification was performed by a 2-step procedure. In the first step, a 100,000 xg *P. falciparum* extract was separated by gel filtration. In the second step, the active fractions were pooled, concentrated and subjected to a non-denaturing electrophoresis on a micropreparative electrophoresis system. By such a procedure, the analysis of final active fractions by SDS-PAGE and silver staining showed only the Pf37 proteinase.

Kinetics of spectrin hydrolysis by pure Pf37 proteinase followed by SDS-PAGE and Western blotting using antisera directed against the  $\alpha 1$  to  $\alpha 5$  tryptic domains of human spectrin will allow the location of cleavage sites on the different tryptic domains of  $\alpha$ -spectrin. Then, the microsequencing of N-terminal-end of hydrolysis products will allow to determine the peptidic sequence at the hydrolysis sites by comparison with the human spectrin amino-acid sequences (Sahr et al. 1990). Based on these results, new peptidic substrates specific for the Pf37 proteinase will eventually be synthesized and tested.

Such experiments are presently done in the laboratory and first results indicated that stable fragments are yielded by the action of Pf37 on  $\alpha$ -spectrin.

#### REFERENCES

- Deguercy A, Hommel M Schrével J 1990. Purification and characterization of *Plasmodium falciparum* and *P. berghei* 37,000 acid proteases which cleave erythrocyte cytoskeletal components. *Mol Biochem Parasitol* 38: 233-244.
- Fandeur T, Bonnefoy S Mercereau-Puijalon O 1991. *In vivo* and *in vitro* derived Palo Alto lines of *Plasmodium falciparum* are genetically unrelated. *Mol Biochem Parasitol* 47: 167-178.
- Grellier P, Picard I, Bernard F, Mayer R, Heidrich H-G, Monsigny M Schrével J 1989. Purification and identification of a neutral endopeptidase in *Plasmodium falciparum* schizonts and merozoites. *Parasitol Res* 75: 455-460.
- Kemp D, Cowman AF, Walliker D 1990. Genetic diversity in *Plasmodium falciparum*. *Adv Parasitol* 29: 75-149.
- Kun J, Hesselbach J, Schreiber M, Scherf A, Gysin J, Mattei D, Pereira Da Silva L, Müller-Hill B. 1991. Cloning and expression of genomic DNA sequences coding for putative erythrocyte membrane-associated antigens of *Plasmodium falciparum*. *Res Immunol* 142: 199-210.

- Lawton P, Barrault C, Grellier P, Schrével J, Heidrich H-G 1991. The native form of the *Plasmodium falciparum* pf68 neutral proteinase is a 105,000 a polypeptide. *Parasitol Res* 77: 720-722.
- Mayer R, Picard I, Lawton P, Grellier P, Barrault C, Monsigny M, Schrével J 1991. Peptide derivatives specific for a *Plasmodium falciparum* proteinase inhibit the human erythrocyte invasion by merozoites. *J Med Chem* 34: 3029-3035.
- Sahr KE, Laurila P, Kotula L, Scarpa AI, Coupal E, Leto TL, Linnenbach AJ, Winkelmann JC, Speicher DW, Marchesi VI, Curtis PJ, Forget BG 1990. The complete cDNA and polypeptide sequences of human erythroid  $\alpha$ -spectrin. *J Biol Chem* 265: 4434-4443.
- Saul A, Battistutta D 1988. Codon usage in *P. falciparum*. *Mol Biochem Parasitol* 27: 35-42.
- Schrével J, Grellier P, Mayer R, Monsigny M 1988. Neutral proteases involved in the reinvasion of erythrocytes by *Plasmodium* merozoites. *Biol Cell* 64: 233-244.
- Wahlgren M, Aslund L, Franzen L, Sundvall M, Wahlin B, Berzins K, Mcnicol LA, Björkman A, Wigzell H, Perlmann P, Pettersson U 1986. A *Plasmodium falciparum* antigen containing clusters of asparagine residues. *Proc Natl Acad Sci USA* 83: 2677-2681.
- Weber JL 1987. Analysis of sequences from the extremely A + T rich genome of *Plasmodium falciparum*. *Gene* 52: 103-109.
- Weidekamm E, Wallach DFH, Lin PS, Hendricks J 1973. Erythrocyte membrane alterations due to infection with *Plasmodium berghei*. *Biochem Biophys Acta* 323: 539-546.