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The native form of the *Plasmodium falciparum* Pf68 neutral proteinase is a 105,000-Da polypeptide

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As in many physiological processes in eukaryotic cells, proteases play an important role in plasmodia. Enzyme-dependent processes in the malaria parasite include hemoglobin digestion during schizogony and invasion of the host erythrocyte by merozoites (reviewed by Schrével et al. 1990). In recent investigations, particular emphasis has been placed on neutral proteases because if they are present in the parasite but not in the host, they can serve as possible targets for the designing of new drugs against malaria.

Fast protein liquid chromatography (FPLC) purification of a neutral *Plasmodium falciparum* proteinase (Grellier et al. 1989) with an isoelectric point of 4.4, an optimal activity at pH 7.5 and a specificity for the synthetic peptidic substrate GlcA – Val – Leu – Gly – Lys – AEC yields an enzymatically active fraction that contains several proteins. Polyacrylamide gel electrophoresis using sodium dodecyl sulfate (SDS-PAGE) revealed a major component exhibiting an apparent molecular weight of 68 kDa and other components displaying apparent molecular weights of 97 and 38 kDa. We suspected that the low-molecular-weight components might result from processing during the isolation steps. Such processing can best be recognized by using an antibody against the native, as yet unknown, form of the neutral protease.

Polyacrylamide gel electrophoresis in the absence of SDS (PAGE; Rothe 1988) was performed on trophozoite/schizont extracts from the FCB1 strain of *P. falciparum* from Colombia (extracted after saponin treatment in the presence of 10 mM Zwittergent 3-12 (Calbiochem); 2% (v/v) aprotinin (Bayer); 2 mM PMSF (phenylmethane sulfonyl fluoride); and pepstatin, bestatin and antipain (Sigma; final concentration, 1 µg/ml). The activity against the synthetic peptide substrate correlated with a smeary doublet of 105 and 97 kDa. The 105-kDa band was electro-eluted from the gel and used in rabbits to produce an anti-Pf105 antiserum, a key reagent in further experiments.

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As expected, this antibody (1:100 or 1:200 dilution) recognized a 105-kDa protein in extracts from trophozoites, schizonts and free merozoites (isolated according to Heidrich et al. 1983) in immunoblots and immunoprecipitations (using protein A-Sepharose Cl-4B; data not shown). No erythrocyte or serum component was recognized. The antibody also recognized the 97-, 68- and 38-kDa polypeptides from the chromatographic isolation (data not shown) but failed to recognize protein(s) exhibiting higher molecular mass(es). The parasitic origin of all of these proteins was demonstrated by metabolic [^{35}S]-methionine labeling. Since the antibody recognizes the 105-, 97-, 68- and 38-kDa polypeptides, it is clear that these polypeptides must be related to each other, suggesting that the low-molecular-weight products could derive from a common precursor molecule, the 105-kDa polypeptide. The enzymatic activity of this molecule was inhibited by the anti-Pf105 antibody as determined using the synthetic peptide substrate (Fig. 1). Thus, the 105-kDa polypeptide is likely to be the native neutral proteinase.

The 105-kDa protein is proteolytically processed in vitro. The dependence of the Pf105 processing on the extraction buffers and proteinase blockers used is shown in Fig. 2. A processing product of 97 kDa (first step of processing) appeared during freeze-thawing and/or further handling of the aqueous and buffered trophozoite/schizont/merozoite extracts in the absence of proteinase inhibitors at 4° C for a short period (< 2 h; Fig. 2, lane g). No such proteolysis was observed when protease inhibitors known to inhibit the neutral proteinase activity (Grellier et al. 1989) were included in the media. These inhibitors included $ZnCl_2$, iodoacetamide, leupeptin, antipain and TLCK ($N\alpha-p-$tosyl$-$L-lysine) chloromethyl ketone; Fig. 2, lanes a–e). EDTA (ethylenediamine tetraacetate), pepstatin and PMSF did not prevent formation of the 97-kDa product (Fig. 2, lanes h–j). The 68-kDa product (second step of processing) was observed only in water extracts in the absence of protease inhibitors after at least 4 h at ≥ 4° C (Fig. 2, lane f) and could be prevented by all of the protease blockers

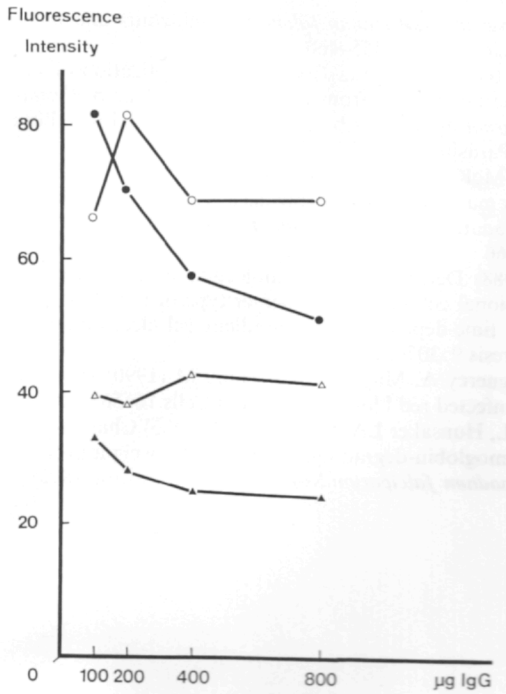

Fig. 1. Inhibition of the Pf105 proteinase activity by anti-Pf105. Freshly prepared detergent extracts from trophozoites/schizonts (protein content, 0.5 mg ml^{-1}; *upper two curves*) and merozoites (protein content, 2 mg ml^{-1}; *lower two curves*) were incubated for 2 h at 4° C with increasing amounts of purified IgGs from pre-immune (○, △) and anti-Pf105 antiserum (●, ▲) and then incubated with the synthetic peptide substrate GlcA – Val – Leu – Gly – Lys – AEC for 30 min at 37° C as described by Grellier et al. (1989)

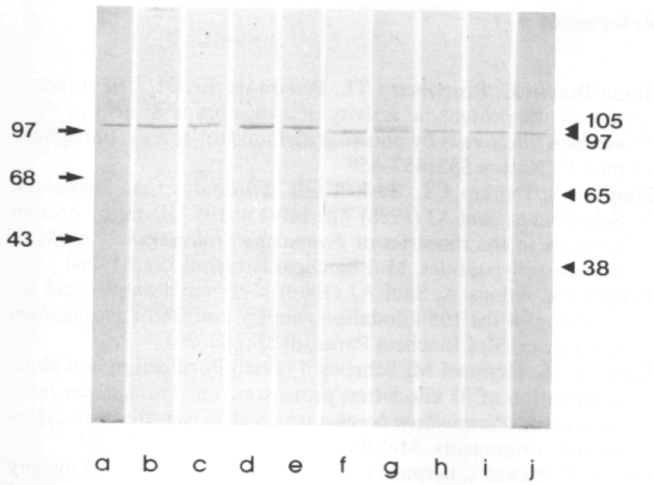

Fig. 2. Inhibition of processing of Pf105, and processing of Pf105 to the low-molecular-weight products of 97, 68 and 38 kDa. The blots were made from water extracts. *Lanes a–e*, Inhibition of processing in the presence of protease blockers. *Lane 1*, 1 mM antipain; *lane b*, 3 mM iodoacetamide; *lane c*, 0.1 mM leupeptin; *lane d*, 1 mM TLCK; *lane e*, 1 mM $ZnCl_2$. *Lanes f, g*, Processing in the absence of proteinase inhibitors. *Lane f*, Trophozoite/schizont extract processed for 6 h at 4° C, frozen and thawed; *lane g*, original merozoite extract without inhibitors, after about 2 h. *Lanes h–j*, Processing in the presence of inactive proteinase inhibitors. *Lane h*, 1 mM EDTA; *lane i*, 1 mM pepstatin; *lane j*, 2 mM PMSF

described except PMSF (data not shown). The formation of the 38-kDa product (Fig. 2, lane f) was promoted in media with an acidic pH, as was the formation of the 68-kDa product, and could be prevented by all of the protease blockers used.

The reproducibility of the processing suggests that the protein is cleaved by specific, parasite-derived enzyme(s). However, the involvement of an autocatalytic mechanism cannot be ruled out, since the protease inhibitors that prevented processing from 105 to 97 kDa also inhibited the Pf105 activity against the peptidic substrate. As described, processing appeared to take place in two steps (from 105 to 97 kDa and from 97 to 68/38 kDa) under different conditions, suggesting that two (or more) enzymatic systems may be involved. These enzyme(s) are as yet unknown.

The first processing step, i.e. from 105 to 97 kDa, was clearly seen in extracts from trophozoites and schizonts and could also be observed in extracts from merozoites, from which erythrocytic enzymes were excluded. It was blocked by most of the proteinase inhibitors used but not by pepstatin, PMSF or EDTA. In contrast, the second step (to 68/38) was blocked by all of the inhibitors used except PMSF. This latter processing to the smaller products was seen mainly in trophozoite/schizont extracts. The enzyme(s) responsible for the second processing step is (are) likely to be active at an acidic pH. Malarial acid proteinases such as the food-vacuole 28-kDa cysteine proteinase (Rosenthal et al. 1988) or protease S (Vander Jagt et al. 1986), both of which are responsible for hemoglobin degradation, or more recently described enzymes (Pf37, Duguercy et al. 1990; p76, Braun-Breton et al. 1988) might participate in this second step after being released during in vitro lysis of trophozoites and schizonts in aqueous acidic extracts. The enzymatic activity of the mixture of the processed products against the substrate GlcA – Val – Leu – Gly – Lys – AEC was rather stable at 4° C (over at least 24 h), indicating that the processing does not affect the catalytic site of the enzyme.

The question arises as to whether or not the neutral proteinase Pf105 described herein is identical with the 105-kDa rhoptry antigen described by Cooper et al. (1988). These authors have not assigned an enzymatic activity to the latter protein. Both proteins, however, undergo processing that results in products of 97 and 38 kDa (Cooper et al. 1989). Neither cryo-immuno-electron microscopy (data not shown) nor immunofluorescence showed any clear compartmentation of the Pf105 enzyme in parasite organelles such as rhoptries, suggesting that our Pf105 is not identical to the 105-kDa rhoptry protein. Preliminary N-terminal sequence analysis of Pf105 has supported this finding (data not shown).

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