A distinct member of the aspartic proteinase gene family from the human malaria parasite *Plasmodium falciparum*

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Abstract A gene (*hap*) transcribed during the intra-erythrocytic life cycle stages of the human malaria parasite *Plasmodium falciparum* was cloned and sequenced. It was found to encode a protein belonging to the aspartic proteinase family but which carried replacements of catalytically crucial residues in the hallmark sequences contributing to the active site of this type of proteinase. Consideration is given as to whether this protein is the first known parasite equivalent of the pregnancy-associated glycoproteins that have been documented in ungulate mammals. Alternatively, it may be operative as a new type of proteinase with a distinct catalytic mechanism. In this event, since no counterpart is known to exist in humans, it affords an attractive potential target against which to develop new anti-malarial drugs.

Key words: Malaria; Aspartic proteinase; *Plasmodium*

1. Introduction

Aspartic proteinases contain two internally homologous domains, each of which contributes a catalytic Asp residue to the active site which is located between the two domains [1]. Each aspartic acid residue is found in the hallmark sequence ~hydrophobic-hydrophobic-Asp-Thr/Ser-Gly~ motif which, together with a further ~hydrophobic-hydrophobic-Gly~ motif, forms a structural feature known as a psi-loop [2]. In some enzymes, the domains are contained within a single polypeptide chain so that each of the motifs occurs twice within the molecule and in the encoding gene [3]. Aspartic proteinases are involved in a variety of protein processing functions including protein catabolism and it is in this capacity that the enzymes (plasmepsin I and plasmepsin II) from the human malaria parasite *Plasmodium falciparum* act during the intra-erythrocytic stages of parasite development. These two enzymes are critical in the pathway which breaks down host cell haemoglobin to provide nutrients for the parasite growth [4] and their inhibition results in parasite death [5,6].

In this report, we describe the sequence of a gene from *P. falciparum* which is clearly a member of the aspartic proteinase gene family but which encodes a protein (HAP) that has important variations from the conserved motifs of the aspartic proteinases. Its potential for proteolytic activity is discussed with reference to a three dimensional model.

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Abbreviations: RT-PCR, reverse transcriptase polymerase chain reaction

2. Materials and methods

2.1. Identification and cloning of the hap gene

A λ1149 library of *Eco*RI/*Hin*dIII digested DNA from *P. falciparum* strain HB3 was screened with an oligonucleotide, GGGTCACCTAGAATAAAGGTTG, designed from the sequence of the gene encoding proplasmepsin II. Several phage clones containing the same 970 bp insert were obtained. This fragment was excised from the phage by digestion with *Eco*RI and *Hin*dIII before gel purification. This DNA was made blunt-ended using the DNA polymerase I Klenow fragment prior to further digestion with *Rsa*I, *Alu*I or *Dra*I. The resulting fragments were cloned into *Sma*I cut, phosphatase-treated pUC18 and transformed into *Escherichia coli* HB101. Clones were sequenced and a contiguous sequence corresponding to the 3′ end of a gene, related to, but clearly different from those encoding proplasmepsins I and II, was identified. No further clones were detected when this insert was used as a probe to screen a variety of other *P. falciparum* genomic and cDNA libraries. In order to clone sequences 5′ to this fragment, a polymerase chain reaction (PCR)-based strategy was employed using a specific primer TTTACTTTCACAAGACTCTGATG, designed from the sequence of the initial clone, and a degenerate oligonucleotide primer ATTAAAAAATTAYATTAAAGAATC which strongly corresponds to a region of the DNA sequence that is conserved among the genes encoding *P. falciparum* proplasmepsin I and proplasmepsin II and the *Plasmodium berghei* proplasmepsin (equivalent to nucleotides 300–323 in Fig. 1). These primers were used in a PCR amplification with *P. falciparum* total genomic DNA as template. The 234 bp amplicon was cloned into pUC18 using the Sureclone PCR cloning kit (Pharmacia) and sequenced. This clone extended the sequence of the gene by a further 115 nucleotides in the 5′ direction. The gene sequence was completed using inverse PCR. Genomic DNA was digested with *Sau*3AI and the enzyme was heat-inactivated before overnight ligation of the products at 16°C. PCR was performed using the primers TTCCACATTATCAAACTCACTACC (nucleotides 390–367, Fig. 1) and GCAAATGTATTATCTTTTGGAGAGG (nucleotides 404–427), through 40 cycles with annealing at 60°C. The PCR products were gel-purified and ligated into pGEM-T (Promega) resulting in several related clones in which various rearrangements, duplications and deletions were evident. The sequence of the longest clone extended only four nucleotides upstream of the proposed initiating Met codon but no *Sau*3AI site remnant was apparent. Comparison of these clones enabled the construction of a consensus sequence for the 5′ end of the gene. The extreme instability of clones containing a 5′ non-coding sequence explains the difficulty in isolating a full length clone of this gene from conventional gene libraries.

2.2. Expression of recombinant HAP

In order to produce recombinant HAP protein, a truncated form of the precursor protein (semi-proHAP) was expressed in *E. coli* using the strategy described previously for plasmepsins I and II [6,7]. A fragment of the *hap* gene encoding amino acids 76–451 (Fig. 1) was amplified in a PCR reaction using the primers CCGGAATTCGGATCCAAATATTCGACAGTAGGATTT and GCGAGCATGCGGATCCTTAGTTTTTTGCAACTGCAAAACC. This amplicon was cloned into the expression vector pET3a for the induction of protein synthesis in *E. coli* BL21DE3(pLysS). The recombinant material produced was insoluble and so was dissolved in buffer (100 mM Tris-HCl, pH 8.0, 1 mM glycine, 1 mM EDTA, 50 mM β-mercaptoethanol) containing 6 M urea and refolding was attempted as de-

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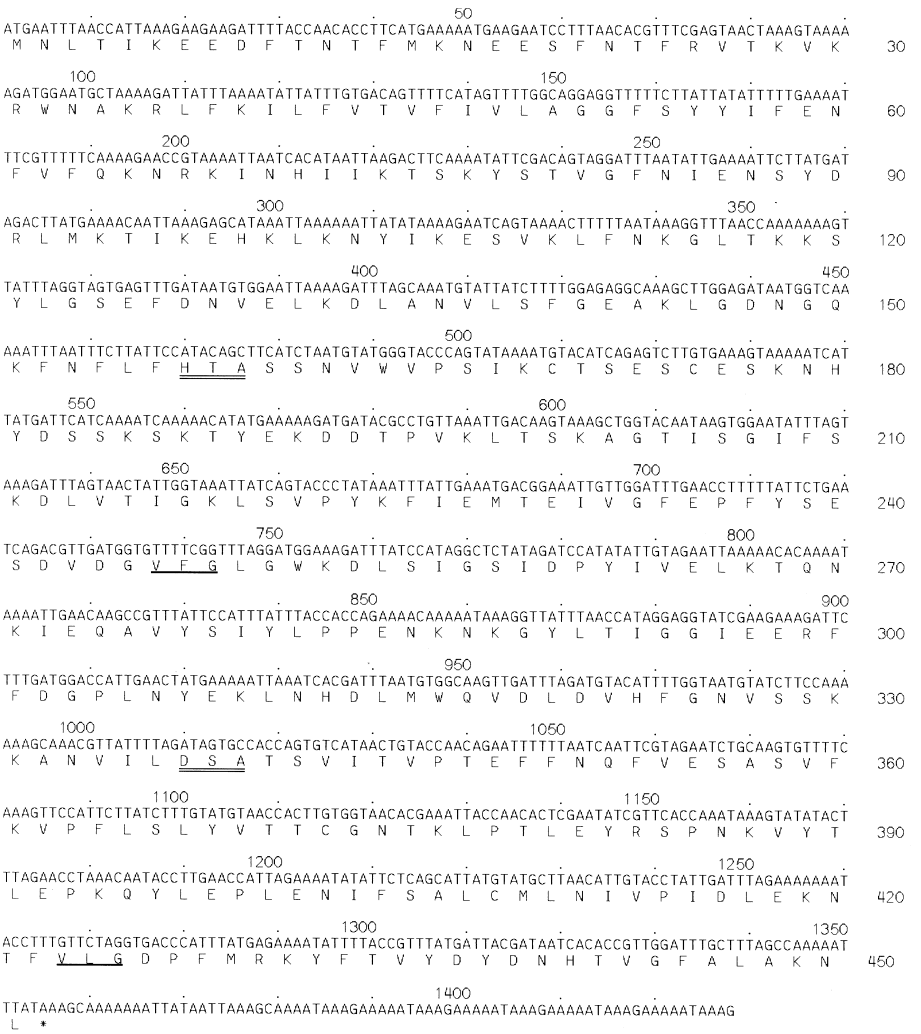

Fig. 1. Sequence of the *hap* gene. The nucleotide sequence of the *hap* gene (EMBL accession number AJ009990) with translation. The His-Thr-Ala and Asp-Ser-Ala motifs at the centre of the interdomain cleft are shown double underlined whilst the ~hydrophobic-hydrophobic-Gly~ motifs, which interact with the former in the psi-loop structures, are underlined.

scribed previously using the conditions employed with recombinant proplasmepsin I [6] and proplasmepsin II [7].

2.3. Northern blot analysis

Isolation of stage-specific RNA from *P. falciparum* strain K1 and Northern blot analysis were carried out as described previously [6] using most of the *hap* gene (nucleotides 310–1428, Fig. 1) as a probe to detect *hap*-specific transcripts.

2.4. Reverse transcriptase (RT-) PCR

Messenger RNA from intra-erythrocytic stage parasites was reverse transcribed prior to PCR using the degenerate antisense strand oligonucleotide TRSATCTAAAKYWAYTTGCCA (nucleotides 966–946 Fig. 1) in combination with TGGTCAAAAATTTAATTTCTTATTC (nucleotides 444–468 Fig. 1) to amplify *hap* sequences.

To ensure that any amplification products had arisen from the presence of message-specific cDNA (rather than contaminating genomic DNA that would have generated false positive results), a control PCR reaction was performed with the *P. falciparum* DNA polymerase α-specific primers TTTATGGTTGTTTAGGA and GAAGCATCTTCACTCGT. These were used in separate amplification reactions with the cDNA and genomic DNA as templates. The gene encoding DNA polymerase α contains a 200 bp intron [8] between these primer binding sites. Therefore it was possible to distinguish between PCR products originating from cDNA (700 bp) and those derived from genomic DNA (900 bp). The data (not shown) indicated that the cDNA was uncontaminated with genomic DNA.

2.5. Modelling

A three dimensional model of 'mature' HAP (amino acid residues 125–451, Fig. 1) was constructed on a Silicon Graphics O2 workstation using Moloc [9] and plasmepsin II (PDB code 1sme, [10]) as the template structure. The quality of the model was checked with a program developed by Luethy et al. [11].

3. Results and discussion

3.1. hap isolation and sequence comparisons

As described in Section 2, an open reading frame of 1353 nucleotides was cloned which is predicted to encode a 53 kDa protein consisting of 451 amino acids. This protein is related to but clearly distinct from proplasmepsin I [12] and proplasmepsin II [13] from *P. falciparum*, having identities of 60 and 56%, respectively, with these zymogens (Fig. 2). By analogy with the proplasmepsins, the product of the *hap* gene is predicted to be a precursor protein (proHAP) with a propart approximately 123 residues long that includes a stretch of 19 hydrophobic amino acids (residues 40–58, Fig. 1). Comparable proparts have been identified in proplasmepsins I and II and, in their sequences, the corresponding hydrophobic region has been implicated in type II membrane anchoring [14]. By contrast, the proparts of mammalian/fungal aspartic protein-

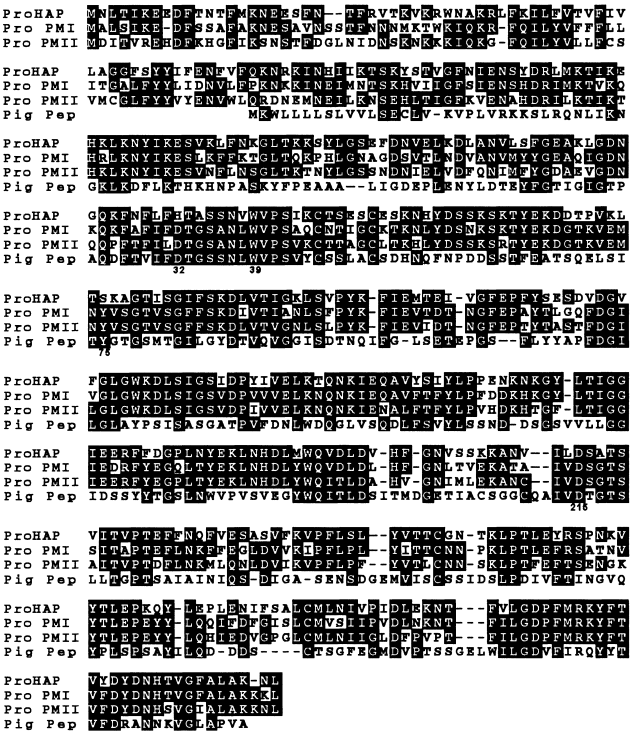

Fig. 2. Sequence alignment of proHAP with members of the aspartic proteinase family. The predicted translation product of the *hap* gene (ProHAP) is aligned with proplasmepsin I (ProPMI) and proplasmepsin II (ProPMII), also from *P. falciparum*. Residues which are identical in two or more sequences are shaded. Dashes represent spaces included to optimise the alignments. The sequence of pig pre-propepsin is included for reference and key residues in this enzyme Asp-32, Trp-39, Tyr-75 and Asp-215 are identified according to the standard numbering system for mature pepsin.

ases do not contain such a membrane spanning sequence and are only ~50 amino acids in length.

The region predicted to correspond to mature HAP (residues 124–451, Fig. 1) shares 64 and 61% identity, respectively, with the mature enzymes, plasmepsin I and plasmepsin II. A model of HAP was constructed using the crystallographically determined structure of plasmepsin II as a template. Due to the high sequence identity, the HAP model exhibits many of the major structural features of the aspartic proteinases. Since there are no insertions or deletions in the HAP sequence compared to that of plasmepsin II, it is reasonable to assume that the backbone of HAP is very similar. The so-called 'fireman's grip' interaction [15] which is located at the interface between the two domains is maintained in HAP. In contrast, the psi-loops in each domain that generate the catalytic apparatus intrinsic to an aspartic proteinase by the interaction of the essential ~Asp-Thr/Ser-Gly~ and ~hydrophobic-hydrophobic-Gly~ motifs (see Section 1), are distinctly different in HAP. Both of the ~hydrophobic-hydrophobic-Gly~ motifs are present within both domains (underlined, Fig. 1). The ~Asp-215-Thr/Ser-216-Gly-217~ motif (pepsin numbering) of the C-terminal domain is slightly modified in HAP to ~Asp-Ser-Ala~ (Fig. 2). Even more striking, however, is the alteration of the motif in the N-terminal domain from ~Asp-32-Thr-33-Gly-34~ (pepsin numbering) to ~His-Thr-Ala~. This eliminates one of the aspartic acid residues which is essential for the operation of the catalytic mechanism of an aspartic proteinase. These features within the catalytic apparatus of the *Plasmodium* protein suggest that it might be termed as a histo-aspartic protein (HAP). The model indicates that free rotation of the imidazole ring of the His-32 residue may be prevented by the positions of the side chains of Ala-34 and Ala-217 (pepsin numbering, Fig. 3). Such steric hindrance to hold His-32 in place may be important if this residue is involved in a catalytic role in HAP.

A further major deviation from an aspartic proteinase motif is evident in the β-hairpin loop (frequently termed the 'flap') that lies over the active site cleft and closes when substrate is bound, making extensive contacts with bound substrates and inhibitors [15]. In typical mammalian/fungal aspartic protein-

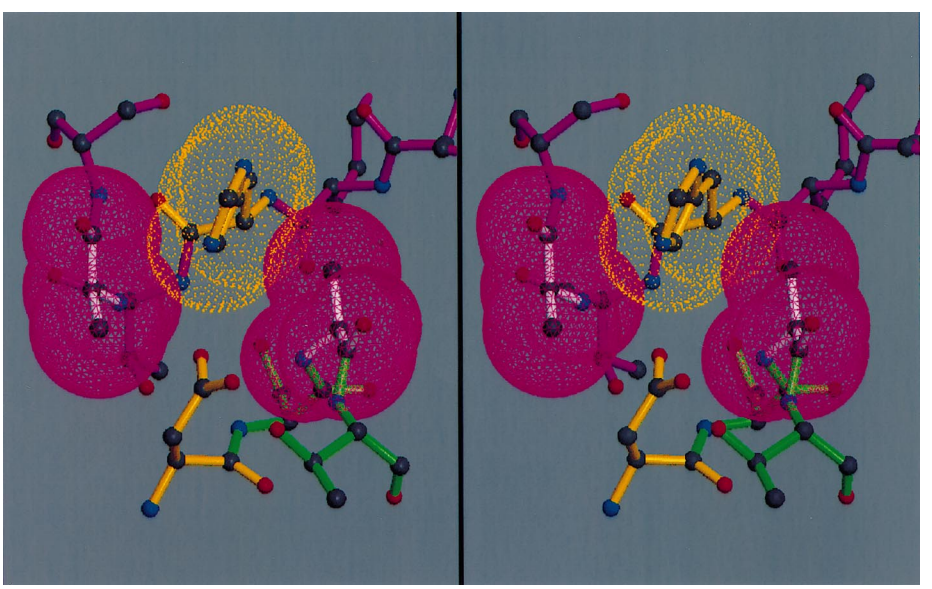

Fig. 3. Residues at the centre of the interdomain cleft of HAP. Residues 30–35 (~L-F-H-T-A~) and 215–218 (~D-S-A-T~) in HAP corresponding to the active site sequence motifs of conventional aspartic proteinases (numbering is based on that of pig pepsin). His-32 and Asp215 are shown in yellow, with Ala-33 and Ala-216 in white. Molecular surfaces are illustrated for His-32, Ala-33 and Ala-215 to indicate the constraint placed on rotation of the imidazole ring by the two Ala residues.

ases, this flap contains the sequence ~Tyr-75-Gly-X-Gly~ (where X is often Ser, Thr or Asp). In plasmepsins I and II, this sequence is slightly altered to ~Tyr-Val-Ser-Gly~. In HAP, the counterpart sequence of residues is ~Ser-Lys-Ala-Gly~. The side chain of Tyr-75 has an important role in H-bonding with the invariant Trp-39 to hold the flap closed when substrate is bound and it also divides the S_1 and S_2' subsite pockets [16,17]. The side chain of the Ser in HAP would be unable to mimic the action of Tyr-75 as it would be too short to form the necessary H-bond with Trp-39 (which is retained in the sequence of HAP, Fig. 1) and it would not be able to form a wall between the substrate residues occupying the S_1 and S_2' pockets. Attempts to replace Tyr-75 in aspartic proteinases with virtually any other amino acid results in a dramatic reduction in enzyme activity, e.g. its replacement by Ser in *Rhizomucor pusillus* pepsin led to at least a 1000-fold reduction in proteolytic activity and appeared to constrain the general aspartic proteinase inhibitor, pepstatin, to bind in a different conformation to the mutant enzyme [16]. Lys-76 in HAP is also unusual as it replaces the largely conserved Gly-76 residue of most aspartic proteinases with a positively charged residue. The side chain of residue 77 of aspartic proteinases usually has a H-bond accepting a function (Ser, Thr, Asp) which allows interactions with a backbone NH of substrates or peptidic inhibitors. The Ala in this location in HAP would be unable to contribute to such an interaction.

In addition to the catalytically active aspartic proteinases, a family of closely related proteins is expressed in ungulate (hoofed) mammals during pregnancy [18]. For example, one such a pregnancy-associated glycoprotein of ovine origin (ovPAG-1) shares approximately 50% identity with pepsin but lacks one of the two catalytic Asp residues (Asp-215 pepsin numbering) whilst a bovine homologue (boPAG-1) has an alanine in place of the conserved glycine at position 34 in the first ~Asp-Thr-Gly~ motif. Models of these proteins (constructed on the basis of the known crystal structures of porcine pepsin and bovine chymosin) indicate that both are likely to be catalytically inactive [19] but, as both can bind pepstatin, it has been suggested that their binding sites are still viable and they may perform some function by binding specifically to other proteins [18].

The pregnancy associated glycoproteins (PAGs) have several features in common with HAP, for instance, the highly conserved ~hydrophobic-hydrophobic-Gly motifs which form a part of the psi-loops. In addition, some of the residues that make HAP unusual are also changed in the PAGs as illustrated in Table 1. It can be seen that Gly-34 of the aspartic proteinases is Ala in HAP and in boPAG1, poPAG1 and ovPAG3 also [19,20]. Guruprasad et al. [19] speculated that this alteration may render these PAGs proteolytically inactive due to displacement of the catalytic water molecule immobilised between Asp-32 and Asp-215. However, in rabbit cathepsin E, the same Gly-34 is replaced by the much larger Val and this enzyme is still able to cleave substrates with the same efficiency as cathepsin E from other species which retain Gly-34 [21]. The replacement of Asp-32 by His has not been reported in the PAGs to date but Asp-215 is changed to His in boPAG9 [22]. Unusual residues have also been detected at positions 75–77 on the flap of several PAGs (Table 1) as described above for HAP. Thus, a role for HAP as a binding protein similar to the mammalian pregnancy-associated glycoproteins may be postulated. The binding cleft in HAP, like those of the closely related true aspartic proteinases plasmepsin I and plasmepsin II, is rather hydrophobic which may indicate a preference in HAP for a hydrophobic ligand(s) in this protein-binding scenario.

3.2. Expression of the hap gene

A RT-PCR was performed, using the primers described in Section 2, on parasitic mRNA extracted from intra-erythrocytic stages and a 522 bp product was cloned and sequenced. This was established unequivocally to have been derived from mRNA and not contaminating genomic DNA by carrying put the control PCR on DNA polymerase α, as described in Section 2. It was thus demonstrated that the *hap* gene is actively transcribed. This is consistent with the fact that *P. falciparum* appears to have minimised the content of non-functional DNA in its genome, with an early survey showing only three out of 20 genes to contain any intronic sequences [23].

Table 1
Substitution of conserved aspartic proteinase features in HAP-selected pregnancy-associated glycoproteins (Xie et al., 1997; Szafranska et al., 1995)

Residues (pepsin number)	Protein	Amino acid(s)
13	Typical AP	Glu/Gln
	HAP	Leu
	Plasmepsin I and II	Met
	ovPAGs 1, 8, 9	Leu
32–35	Typical AP	Asp-Thr-Gly
	HAP	His-Thr-Ala
	boPAG1, poPAG1, ovPAG3	Asp-Thr-Ala
	Rabbit CatE	Asp-Thr-Val
75–77	Typical AP	Tyr-Gly-Ser/Thr/Asp
	HAP	Ser-Lys-Ala
	poPAG1	Pro-His-Ser
	boPAG10	Tyr-Thr-Ala
215–217	Typical AP	Asp-Thr/Ser-Gly
	HAP	Asp-Ser-Ala
	boPAG9	His-Thr-Gly

Numbering of amino acids is based on that of pig pepsin.

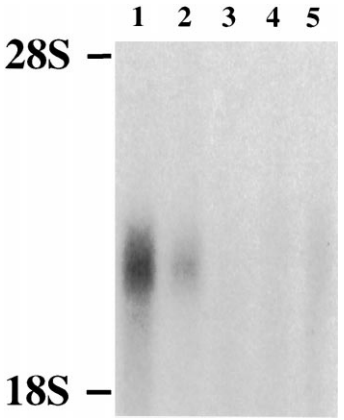

Fig. 4. Temporal expression of the *hap* gene. The Northern blot was prepared as described in Moon et al. [6] using the following stage-specific mRNA samples. Lane 1, mid and late trophozoite; lane 2, late trophozoites and young schizonts; lane 3, schizonts and mature schizonts; lane 4, mature schizonts and early rings; lane 5, late rings. The positions of the 18S and 28S *P. falciparum* rRNAs are shown.

Changes in the expression of the *hap* gene during the intra-erythrocytic cycle were detected by Northern blot analysis of synchronised blood stage cultures (Fig. 4). mRNA levels for *hap* peaked during the mid to late trophozoite stage of the growth cycle with minima during the ring and schizont stages. This is a similar expression pattern to that observed previously for the proplasmepsin II gene [6].

It is thus clear that transcription of the *hap* gene produces a full length mRNA and there is no a priori reason why this should not be translated in frame to generate a proHAP protein comparable in length to aspartic proteinase precursors such as proplasmepsin I and proplasmepsin II. The identification of HAP protein in parasites requires an antiserum specific for this protein and which does not cross react with plasmepsins I or II. Identities greater than 60% between HAP and these two plasmepsins have prevented the production of such a selective antiserum to date. With this restriction, identification of HAP protein in naturally occurring extracts prepared from parasites at appropriate stages is not a viable option. This, together with the paucity of material likely to be extracted, indicated that a recombinant approach would be more likely to generate the amounts of HAP required for studies of peptide/protein-binding and catalysis. Plasmepsin II is readily generated by auto-activation of recombinant proplasmepsin II produced in *E. coli* [7]. However, considerably greater difficulties were encountered, with very low yields of active enzyme, when this approach was applied to recombinant proplasmepsin I [6]. Reflecting the latter situation, expression of the *hap* gene in *E. coli* generated large amounts (10 mg/l) of recombinant semi-proHAP in an insoluble form, but although refolding was attempted, no activity was seen (data not shown). Thus, the production of properly folded HAP may require the use of a heterologous system such as *P. pastoris*.

The replacement of one of the catalytic Asp residues of an aspartic proteinase by a His residue means that, of necessity, HAP cannot operate through the type of catalytic mechanism employed by archetypal and retroviral aspartic proteinases [24–26]. If HAP is active as a proteinase, then it would be required to operate through a completely novel catalytic mechanism and would thus represent a new class of (histo-aspartic) proteinase. The combination of all of the residue replacements described above for HAP relative to the aspartic proteinases, may act in a compensatory fashion such that hydrolysis of a scissile peptide bond would still be facilitated by the protein but the distinct residues in the catalytic constellation of HAP would afford a distinctly novel opportunity for drug design targeted against this unique parasite protein. If, like the PAGs on the other hand, HAP is not active proteolytically, then it represents the first such protein to be identified from a species other than ungulate mammals and may have a role, as yet unidentified, in binding proteins. Resolution between these intriguing possibilities is the subject of ongoing investigations.

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References

[1] Khan, A.R. and James, M.N.G. (1998) Protein Sci. 7, 815–836.
[2] Blundell, T.L., Guruprasad, K., Albert, A., Williams, M., Sibanda, B.L. and Dhanaraj, V. (1998) in: Aspartic Proteinases (James, M.N.G., Ed.), pp. 1–14, Plenum Press, New York.
[3] Tang, J.N., James, M.N.G., Hsu, I.-N., Jenkins, J.A. and Blundell, T.L. (1978) Nature 271, 618–621.
[4] Gluzman, I.Y., Francis, S.E., Oksman, A., Smith, C.E., Duffin, K.L. and Goldberg, D.E. (1994) J. Clin. Invest. 93, 1602–1608.
[5] Rosenthal, P.J., McKerrow, J.H., Aikawa, M., Nagasawa, H. and Leech, J.H. (1988) J. Clin. Invest. 82, 1560–1566.
[6] Moon, R.P., Tyas, L., Certa, U., Rupp, K., Bur, D., Jaquet, C., Matile, H., Loetscher, H.-R., Grueninger-Leitch, F., Kay, J., Dunn, B.M., Berry, C. and Ridley, R.G. (1997) Eur. J. Biochem. 244, 552–560.
[7] Hill, J., Tyas, L., Phylip, L.H., Kay, J., Dunn, B.M. and Berry, C. (1994) FEBS Lett. 352, 155–158.
[8] White, J.H., Kilbey, B.J., deVries, E., Goman, M., Alano, P., Cheeseman, S., McAleese, S. and Ridley, R.G. (1993) Nucleic Acids Res. 21, 3643–3646.
[9] Gerber, P.R. and Mueller, K. (1995) J. Comput. Aided Mol. Des. 9, 251–268.
[10] Silva, A.M., Lee, A.Y., Gulnik, S., Majer, P., Collins, J., Bhat, T.N., Collins, P.J., Cachau, R.E., Luker, K.E., Gluzman, I.Y., Francis, S.E., Oksman, A., Goldberg, D.E. and Erickson, J.W. (1996) Proc. Natl. Acad. Sci. USA 93, 10034–10039.
[11] Luethy, R., Bowie, J.U. and Eisenberg, D. (1992) Nature 356, 83–85.
[12] Francis, S.E., Gluzman, I.Y., Oksman, A., Knickerbocker, A., Mueller, R., Bryant, M.L., Sherman, D.R., Russell, D.G. and Goldberg, D.E. (1994) EMBO J. 13, 306–317.
[13] Dame, J.B., Reddy, G.R., Yowell, C.A., Dunn, B.M., Kay, J. and Berry, C. (1994) Mol. Biochem. Parasitol. 64, 177–190.
[14] Francis, S.E., Banerjee, R. and Goldberg, D.E. (1997) J. Biol. Chem. 272, 14961–14968.
[15] Pearl, L.H. and Blundell, T.L. (1984) FEBS Lett. 174, 96–101.
[16] Park, Y.N., Aikawa, J., Nishiyama, M., Horinouchi, S. and Beppu, T. (1996) Protein Eng. 9, 869–875.
[17] Park, Y.-N., Aikawa, J.-I., Nishiyama, M., Horinouchi, S. and Beppu, T. (1997) J. Biochem. 121, 118–121.
[18] Roberts, R.M., Xie, S., Nagel, R.J., Low, B., Green, J. and Beckers, J.-F. (1995) in: Aspartic Proteinases: Structure, Function, Biology and Biomedical Implications (Takahashi, K., Ed.), pp. 231–240, Plenum Press, New York.
[19] Guruprasad, K., Blundell, T.L., Xie, S., Green, J., Szafranska, B., Nagel, R.J., McDowell, K., Baker, C.B. and Roberts, R.M. (1996) Protein Eng. 9, 849–856.
[20] Szafranska, B., Xie, S., Green, J. and Roberts, R.M. (1995) Biol. Reprod. 53, 21–28.
[21] Kageyama, T. (1993) Eur. J. Biochem. 216, 717–728.

[22] Xie, S., Green, J., Bixby, J.B., Szafranska, B., DeMartini, J.C., Hecht, S. and Roberts, R.M. (1997) Proc. Natl. Acad. Sci. USA 94, 12809–12816.
[23] Hyde, J.E. and Sims, P.F.G. (1987) Gene 61, 177–187.
[24] James, M.N.G. (1993) in: Proteolysis and Protein Turnover (Bond J.S. and Barrett. A.J., Eds.), pp.1–8, Portland Press, London.
[25] Hyland, L.J., Tomaszek, T.A., Roberts, G.D., Carr, S.A., Magaard, V.W., Bryan, H.L., Fakhoury, S.A., Moore, M.L., Minnich, M.D., Culp, J.S., DesJarlais, R.L. and Meek, T.D. (1991) Biochemistry 30, 8441–8453.
[26] Hyland, L.J., Tomaszek, T.A. and Meek, T.D. (1991) Biochemistry 30, 8454–8463.