Overexpression of Leucyl Aminopeptidase in Plasmodium falciparum Parasites

TARGET FOR THE ANTIMALARIAL ACTIVITY OF BESTATIN*

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Donald L. Gardiner†1, Katharine R. Trenholme‡1, Tina S. Skinner-Adams§2, Colin M. Stack¶3, and John P. Dalton*4

From the †Malaria Biology Laboratory, The Australian Centre for International and Tropical Health and Nutrition, Queensland Institute of Medical Research, 300 Herston Road, Herston, Brisbane QLD 4029, Australia and §Institute for the Biotechnology of Infectious Diseases, University of Technology Sydney, Westbourne Street, Gore Hill, Sydney, New South Wales 2065, Australia

Malaria aminopeptidases are important in the generation and regulation of free amino acids that are used in protein anabolism and for maintaining osmotic stability within the infected erythrocyte. The intraerythrocytic development of malaria parasites is blocked when the activity of aminopeptidases is specifically inhibited by reagents such as bestatin. One of the major aminopeptidases of malaria parasites is a leucyl aminopeptidase of the M17 family. We reasoned that, when this enzyme was the target of bestatin inhibition, its overexpression in malaria cells would lead to a reduced sensitivity to the inhibitor. To address this supposition, transgenic Plasmodium falciparum parasites overexpressing the leucyl aminopeptidase were generated by transfection with a plasmid that housed the full-length gene. Transgenic parasites expressed a 65-kDa protein close to the predicted molecule size of 67.831 kDa for the introduced leucyl aminopeptidase, and immunofluorescence studies localized the protein to the cytosol, the location of the native enzyme. The product of the transgene was shown to be functionally active with cytosolic extracts of transgenic parasites exhibiting twice the leucyl aminopeptidase activity compared with wild-type parasites. In vitro inhibitor sensitivity assays demonstrated that the transgenic parasites were more resistant to bestatin (EC50 25 μM) compared with the parent parasites (EC50 64 μM). Overexpression of genes in malaria parasites would have general application in the identification and validation of targets for antimalarial drugs.

During the development and asexual reproduction of malaria parasites within erythrocytes, as much as 65–75% of erythrocyte hemoglobin is internalized and digested (1, 2). There is still some conjecture as to the reason why the parasite needs to degrade such a large proportion of the erythrocyte hemoglobin, but several theories have been proposed (3, 4). It has been known for some time that malaria parasites require amino acids derived from host hemoglobin for protein synthesis and development (4), although recent studies suggest that the fraction of digested hemoglobin used for this purpose is surprisingly small (in the region of 16%) (5). Malaria parasites acquire additional amino acids from the extracellular medium and incorporate these into their proteins (5–7) and at the same time extrude unneeded amino acids through new permeability pathways that appear in the infected host cell membranes (8). It is now believed that the excess hemoglobin digestion is necessary to reduce the colloid-osmotic pressure within the infected erythrocyte and thus prevent premature cell lysis (9). Apart from contributing to the regulation of the internal osmotic pressure, the constant influx and efflux of amino acids may also ensure that there is a sufficient supply of each amino acid, especially those that are rare in hemoglobin (isoleucine, cysteine, glutamine, methionine, and glutamate), for protein synthesis (3, 7).

Hemoglobin is endocytosed into double membrane vesicles that bud off from the cytosome of the parasite and are transported to the acidic digestive vacuole (DV) (10, 11). Within this specialized organelle, hemoglobin is digested by endopeptidases at a pH of ~5.5 in a semidered fashion, initially at the hinge region by the aspartic peptidases plasmspens I and II and then further by the aspartyl peptides I, II, and IV, and the histoaspartic peptidase and three cysteine peptidases, falcipain-2, -2’, and -3 (1, 3, 10, 11). Peptides generated from this digestion are processed by a metalloprotease, falcicytin, into short oligopeptides of 5–10 amino acids and then by a dipeptidyl aminopeptidase I, which reduces these to dipeptides (12). However, free amino acids are not generated within the DV, as no exoaminopeptidase activity has been detected in isolated DV (13) and in vitro digestion of hemoglobin by extracts of DVs does not generate free amino acids (13, 14). Accordingly, we and others have suggested that peptides/dipeptides produced within the digestive vacuole are transported outside for conversion into individual amino acids (13–16).

Leucyl aminopeptidase-like activity has been detected in the soluble extracts of various Plasmodium species and functions in the release of hemoglobin-derived amino acids (13–21). The activity separates into the cystosolic fraction of the parasite, exhibits a pH optimum value of 7.2, and is inactive below pH 6.0, which is consistent with a function outside the DVs (13). Plasmodium falciparum aminopeptidase activity, partially purified by affinity chromatography employing the inhibitor bestatin as a ligand, cleaved synthetic peptides representative of the products of endopeptidase-degraded hemoglobin (13, 15) and exhibited a general preference for substrates containing leucine and alanine at the N terminus (amino acids that constitute 25% of the amino acid residues in human hemoglobin) (15). Aminopeptidase activity predominates in trophozoites, and the greatest in vitro growth inhibition by bestatin and nitrobestatin occurs when parasites are at the late ring and early trophozoite stage (13). This coincides with the period of increased metabolic activity within the infected erythrocyte, increased hemoglobin digestion, the induction of new permeability pathways, and rapid growth of the parasite (4, 8, 9).

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The leucyl aminopeptidase-like activity detected in soluble extracts of malaria parasites could be derived from two distinct aminopeptidases that display overlapping substrate specificities. The first is represented by a previously characterized 122-kDa zinc aminopeptidase of the M1 family, which may be membrane-bound but processed to two soluble forms of 96 and 68 kDa (21, 22). The second is the product of a gene described in the present study that encodes a 67.831-kDa leucyl aminopeptidase of the M17 family (13–15). We have overexpressed the M17 leucyl aminopeptidase gene in malaria parasites and shown that functional enzyme is expressed in the cytosol of transgenic parasites, the same compartment as the native enzyme (13, 15). More importantly, we have demonstrated that malaria parasites overexpressing M17 leucyl aminopeptidase are more resistant to killing by benznidate, an aminopeptidase inhibitor with antimalarial activity compared with wild-type parasites.

EXPERIMENTAL PROCEDURES

Parasites—P. falciparum clone D10 derived from P. falciparum isolate FC27 was obtained from The Walter and Eliza Hall Institute, Melbourne, Australia. Parasites were cultured as described previously (23).

Construction of the Transgenic Expression Plasmids and Transfection of Malaria Parasites—Pf14_0439, as annotated by PlasmoDB (plasmodb.org), is a gene located on chromosome 14 of P. falciparum. It consists of a single exon of 1818 bp that encodes a 605-amino-acid protein of the M17 leucyl aminopeptidase family. A PCR fragment corresponding to the predicted mRNA/RNA sequence was amplified from DNA isolated from P. falciparum clone D10. The forward primer for Pf14_0439 was AP14F (AGATCTATGTATTTTCTTCCTATG) and contained a BglII restriction site (in bold). The reverse primer was AP14R (CTG-CAGTTAGACCGCTAGTACGAAC) and contained a PstI site (in bold) but not the putative stop codon of the gene. The PCR products were cloned into pGEM using a TA cloning system (Promega) and sequenced to confirm that no Taq-associated errors have been introduced into the DNA. The full-length fragment was digested out of the pGEM vector and the appropriate restriction enzymes and ligated into the Gateway™-compatible entry vector pHcyanB (Gateway, Invitrogen) that had previously been digested using BglII and PstI. A c-Myc tag was ligated in-frame at the 3′ end of the introduced gene sequence. This vector is a variant of the pHG vector described by Tonkin et al. (24), in which the c-Myc 10-amino-acid epitope tag flanked by a 5′ PstI site and a 3′ Nsil site has been cloned into the original PstI site of pHGB. The entry vector containing the Pf14_0439 transgene was designated pHAP14cmycB. A cloning reaction was then performed using this entry vector and a Gateway™-compatible destination vector. The destination vector contained not only a Gateway™ destination cassette but also a second cassette comprising the human dihydrofolate reductase synthetase gene under the control of the P. falciparum calmodulin promoter. This second cassette acts as a selectable marker in P. falciparum. This final plasmid was designated pHH1-HAP14cmycB.

Ring-stage parasites were subjected to electroporation in the presence of 150 μg of plasmid as described previously (25). Parasites resistant to WR99210 were observed 15–22 days later. DNA was extracted when transgenic cultures reached 1% parasitemia and 2% hematocrit. After a 48-h incubation, the amount of [3H]hypoxanthine incorporated into the parasites was measured, and the parasites were transferred with a mouse monoclonal antibody to c-Myc (1:2500 dilution, Sigma) followed by a horseradish peroxidase-labeled anti-mouse IgG antibody (1:5000 dilution, Chemicon International Inc.). The membrane was stripped and reprobed with an anti-glyceraldehyde-3-phosphate dehydrogenase mouse monoclonal antibody (1:2000 dilution) to demonstrate equal loading and transfer of malaria proteins (26).

Immunoblotting Analysis of Parasites—Acetone-fixed thin blood smears of the transgenic parasite and wild-type D10 were probed with a mouse monoclonal antibody to c-Myc (1:2000) and bound antibody visualized with goat anti-mouse IgG-Cy2 (10 μg/ml) (26). The parasite nuclei were visualized with Hoechst dye (0.5 μg/ml). The slides were viewed with a fluorescence microscope (×1000 magnification).

Results

RESULTS

Transfection of Plasmid pHH1-HAP14cmycB Generates an Aminopeptidase Transgenic Parasite—P. falciparum clone D10 ring-stage parasites were transfected with the Pf14_0439 transgene and selected on WR99210. Parasites resistant to WR99210 were observed 15–22 days later. DNA was extracted when transgenic cultures reached 1% parasitemia and 2% hematocrit. The presence of the plasmid determined by PCR amplification using vector-specific primers located in the heat shock protein 86S UTR and the Plasmodium berghei dihydrofolate reductase 3′ UTR (i.e. flanking the inserted aminopeptidase gene) (24). Gel analysis of the PCR-generated DNA revealed a band of 2 kb for pHH1-HAP14cmycB (Fig. 1A). The expected size for the M17 aminopeptidase gene plus additional vector sequence. The transgenic parasite cultures were maintained on WR99210 selection thereafter to sustain the episomally carried plasmids. The growth rate and intraerythrocytic development of the transgenic parasites were not distinguishable from wild-type parasites (data not shown).

The Aminopeptidase Transgene Is Transcribed and Translated—Northern blot analysis showed that the pHH1-HAP14cmycB-transfected malaria parasite cultures transcribed a single species of mRNA with an apparent size of 2.3 kb (Fig. 1B) when hybridized using a DNA fragment corresponding to the c-Myc tag and the 3′ UTR of the transgene. When saponin-lysed parasite-infected erythrocytes of the same cultures were probed by immu-
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Figure 1. Transfection of P. falciparum parasites with gene encoding M17 leucyl aminopeptidase. A, PCR amplification of DNA extracted from the transgenic parasite Pf14_0439 clone (lanes 1–3) and wild-type parasite clone D10 (lanes 4–6). Lanes 1 and 4, PCRs using primers human dihydrofolate reductase (hDHFHR) forward and reverse, which amplify the human dihydrofolate reductase gene; lanes 2 and 5, PCR with primers, which are vector-specific primers that flank the inserted transgene; lanes 3 and 6, PCR using primers AP14F and AP14R, which amplify both the endogenous and transgene copies of Pf14_0439. B, Northern blot using 20 μg of RNA fractionated on a 1% Tris-acetate-EDTA gel and probed with a DNA fragment containing the c-Myc epitope tag and the 3′ UTR of the transgene labeled with (α-32P)dATP. AP14 refers to the transgenic parasite clone, whereas D10 is the wild-type parental clone. C, Western blot of saponin-lysed parasite extracts from the transgenic parasite clone (AP14) and the wild-type parasite line D10 probed sequentially with antibodies to c-Myc (upper panel) and glyceraldehyde-3-phosphatedehydrogenase (lower panel).

vector without the AP14cmyc cassette and maintained under a similar selection regime; the combined data of three separate tests showed that the parasites exhibited an EC50 of 64, 25, and 20 μM, respectively (Fig. 4).

DISCUSSION

Aminopeptidases are exopeptidases that catalyze the sequential removal of amino acids from the N termini of peptides and play a major role in regulating the balance between catabolism and anabolism in all living cells. In malaria parasites, leucyl aminopeptidase-like enzymes are believed to function in the terminal stages of hemoglobin digestion to generate free amino acids that are then used for parasite protein synthesis (13–15). Because malaria parasites also use amino acids taken from their external environment for protein synthesis, it appears that the aminopeptidases may also be important in regulating the internal free amino acid pool for additional purposes, including maintenance of the osmotic stability of the cell (9).

Two leucyl aminopeptidase-like enzymes are expressed by malaria parasites, a 122-kDa membrane alanine aminopeptidase of the M1 family and a 67.8-kDa leucyl aminopeptidase of the M17 family (21, 22). Aminopeptidases of the M1 family are generally considered to be membrane-bound (30), but Florent and co-workers (22) have shown by soluble extraction and immunofluorescence studies that 96- and 68-kDa forms, presumably processed from the parent molecule, are found in the cytosol of malaria cells. The M17 family of aminopeptidase, on the other hand, are found in the cytosol of all cells and do not undergo processing events (29). Both classes of enzyme exhibit optimal activity at neutral pH and have similar preferences for amino acids, although as their names imply, they show a preference for either alanine or leucine. These overlapping biochemical features have led to confusion as to the true nature of the leucine-aminopeptidase activity measured in many studies that used soluble extracts of malaria parasites as their enzyme source (13–20) and to the identity of the target of aminopeptidase inhibitors that have antimalarial activity. Recent studies (31, 32) using microarrays have provided expression profiles for the M1 and M17 aminopeptidase throughout the intraerythrocytic stages of malaria parasites and demonstrate that the both enzymes are most highly expressed in early trophozoites (13, 15). However, the expression level of the M17 leucyl aminopeptidase is ~18-fold higher than the M1 aminopeptidase. The corollary of these findings is that the M17 leucyl aminopeptidase is the predominant source of leucyl aminopeptidase-like activity in malaria parasites and thus is most likely the subject of the previous biochemical and inhibitor studies.

In the present study, we have shown that the M17 leucyl aminopeptidase can be overexpressed in malaria cells by transfecting ring-stage
parasites with the transgene housed in the pH1-HAP14cmycB vector prepared in our laboratory. The transgene was transcribed and expressed as a single 65-kDa protein. Immunofluorescence studies showed that it was transported correctly to the cytosol, the site of the native enzyme (13). Most importantly, enzymatic assays with specific peptide substrates showed that the transfected malaria cells expressed twice as much aminopeptidase activity as wild-type cells, which we can presume is because of the proper folding and activation of the enzyme synthesized from the transfected gene. The introduction of the transgene did not alter the in vitro growth rate or viability of the malaria cells. Accordingly, although we suggested that leucyl aminopeptidases are important in the regulation of the free amino acid pool in malaria cells, the increased enzyme activity within the cells did not upset this function sufficiently to affect malaria growth. It is probable that cells that overexpressed leucyl aminopeptidase to a lethal level were lost during the selection of transgene-carrying parasites using WR99210.

Bestatin is a dipeptide originally isolated from filtrates of Streptomyces olivoreticuli and has immunopotentiating and antitumor activity (33, 34). We have reported that bestatin, which inhibits aminopeptidase members of both the M1 and M17 families (27, 30), was a potent inhibitor of the in vitro growth of malaria parasites (13, 16). Exposure of synchronized cultures of P. falciparum cultures to bestatin and its derivative nitrobestatin indicated that late rings/trophozoites are the most susceptible intraerythrocytic stage. This correlates with the time at which hemoglobin is being degraded and used as a source of nutrient
and also with the increased expression of both the M1 and M17 aminopeptidases (13, 21, 31, 32). Here we show that P. falciparum parasites overexpressing the M17 leucyl aminopeptidase are >2-fold less susceptible to killing by bestatin (EC₅₀ 64 μM) compared with wild-type parasites (EC₅₀ 25 μM). The natural interpretation of these results is that higher amounts of bestatin, which binds aminopeptidases in an equimolar ratio, are required to reduce the intracellular activity of leucyl aminopeptidase to lethal levels in malaria cells that express more of this enzyme. By extension, our studies show that the M17 leucyl aminopeptidase is a target of bestatin in malaria cells.

During this study, malaria parasites were also transfected with the gene encoding the M1 membrane alanine aminopeptidase, but these transgenic parasites did not exhibit an increase in aminopeptidase activity or a difference in susceptibility to bestatin compared wild-type malaria cells (data not shown). Although immunoblotting demonstrated that a protein of the expected molecular size (~120 kDa) was expressed by the transgenic parasites, this was not processed to the soluble smaller molecular size forms of 96 and 68 kDa that were detected in the native parasites by Florent and co-workers (22). The translated M1 aminopeptidase may not have been correctly folded and/or post-translationally modified to produce a functionally active enzyme, and therefore we cannot make a conclusion on its potential as a target for bestatin-mediated killing of malaria. In addition, we cannot exclude the possibility that overexpression of the M17 leucyl aminopeptidase prevents killing of malaria parasites by providing more enzyme to compete for bestatin binding and preventing it from inhibiting the M1 aminopeptidase.

Cysteinyl (falcipain 1–3) and aspartyl (plasmaspins I–IV) endopeptidases are involved in the degradation of hemoglobin within the digestive vacuole (1–4). Specific or broad range inhibitors of these endopeptidases block hemoglobin degradation and have potent effects on malaria growth, and therefore both enzyme classes are intensely studied as targets for new antimalarial drugs (3, 10, 35). Because cysteinyl and aspartyl inhibitors exhibit synergistic activity when used together, combination therapies may offer a more reasonable proposition for combination therapies; the targets would not only include the M17 leucyl aminopeptidase and M1 membrane alanine aminopeptidases but also two uncharacterized enzymes expressed by the intraerythrocytic stages of malaria, namely prolyl aminopeptidase and aspartyl aminopeptidase (plasmodb.org).

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