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Falstatin, a Cysteine Protease Inhibitor of *Plasmodium falciparum*, Facilitates Erythrocyte Invasion

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Erythrocytic malaria parasites utilize proteases for a number of cellular processes, including hydrolysis of hemoglobin, rupture of erythrocytes by mature schizonts, and subsequent invasion of erythrocytes by free merozoites. However, mechanisms used by malaria parasites to control protease activity have not been established. We report here the identification of an endogenous cysteine protease inhibitor of *Plasmodium falciparum,* falstatin, based on modest homology with the *Trypanosoma cruzi* cysteine protease inhibitor chagasin. Falstatin, expressed in *Escherichia coli,* was a potent reversible inhibitor of the *P. falciparum* cysteine proteases falcipain-2 and falcipain-3, as well as other parasite- and nonparasite-derived cysteine proteases, but it was a relatively weak inhibitor of the *P. falciparum* cysteine proteases falcipain-1 and dipeptidyl aminopeptidase 1. Falstatin is present in schizonts, merozoites, and rings, but not in trophozoites, the stage at which the cysteine protease activity of *P. falciparum* is maximal. Falstatin localizes to the periphery of rings and early schizonts, is diffusely expressed in late schizonts and merozoites, and is released upon the rupture of mature schizonts. Treatment of late schizionts with antibodies that blocked the inhibitory activity of falstatin against native and recombinant falcipain-2 and falcipain-3 dose-dependently decreased the subsequent invasion of erythrocytes by merozoites. These results suggest that *P. falciparum* requires expression of falstatin to limit proteolysis by certain host or parasite cysteine proteases during erythrocyte invasion. This mechanism of regulation of proteolysis suggests new strategies for the development of antimalarial agents that specifically disrupt erythrocyte invasion.

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Introduction

The genome sequence of *Plasmodium falciparum,* the most pathogenic human malaria parasite, predicts over 30 cysteine proteases [1]. Among these predicted proteases, five have been biochemically characterized, four falcipains that closely resemble papain [2–5] and dipeptidyl aminopeptidase 1, an exopeptidase related to cathepsin C [6]. Numerous additional sequences predict enzymes related to other families of cysteine proteases, and it is likely that multiple cysteine proteases mediate different proteolytic functions during the complex life cycle of malaria parasites. The best-characterized function for plasmodial cysteine proteases is hemoglobin hydrolysis in erythrocytic trophozoites by falcipain-2 and falcipain-3 [5]. Inhibition of these proteases [7], disruption of the falcipain-2 gene [8], or removal of a falcipain-2 hemoglobin-binding domain [9] blocks hemoglobin hydrolysis. Additional processes in erythrocytic parasites that are mediated by proteases are rupture of erythrocytes by mature schizonts and subsequent invasion of erythrocytes by free merozoites. Experiments with protease inhibitors have yielded somewhat conflicting results, but in most studies inhibitors of cysteine proteases have inhibited erythrocyte rupture, and inhibitors of serine (but not cysteine) proteases have blocked erythrocyte invasion [7,10,11]. The proteases responsible for erythrocyte rupture are uncertain, although both the cysteine protease falcipain-2 [12] and the aspartic protease plasmepsin II [13] have been shown to hydrolyze erythrocyte cytoskeletal proteins in vitro. Considering erythrocyte invasion, the serine protease PfSUB2 was recently shown to cleave portions of two merozoite proteins, suggesting that this protease is required for invasion [14]. In non-erythrocytic parasites, cysteine protease inhibitors blocked the invasion of hepatocytes by *P. falciparum* sporozoites, probably by blocking the proteolytic cleavage of the circumsporozoite protein [15], and the disruption of a putative cysteine protease gene of *Plasmodium berghei* prevented sporozoite egress from oocysts [16].

Systems for the control of plasmodial protease activity have not been described. Endogenous cysteine protease inhibitors have been described in a number of eukaryotic systems. In mammalian and plant cells, lysosomal cysteine proteases are regulated by endogenous polypeptide inhibitors from the cystatin superfamily [17]. Functional significance is suggested

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Abbreviations: $FP2^{E-64}$, active site-inhibited falcipain-2; Ni-NTA, nickel-nitrilotriacetic acid; Z-Leu-Arg-AMC, benzyloxycarbonyl-Leu-Arg-7 amino-4-methylcoumarin

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Synopsis

Malaria causes hundreds of millions of illnesses and more than a million deaths each year. Illness is caused by infection of red blood cells, with repeated rounds of red cell invasion, parasite development, and red cell rupture. Among enzymes with important roles in malaria parasites are proteases, which break down other proteins. Functions of proteases include the breakdown of red cell hemoglobin, the release of parasites from red cells, and the invasion of red cells by free parasites. This work concerns the identification and characterization of a protease inhibitor of malaria parasites termed falstatin. Falstatin inhibits one class of proteases, cysteine proteases, from both malaria parasites and humans. It is produced from soon before until soon after the processes of red cell rupture and invasion. Incubation of malaria parasites with an antibody that prevents the effects of falstatin markedly inhibited red cell invasion. Thus, falstatin appears to facilitate red cell invasion, presumably by preventing the action of proteases that hinder this process. Falstatin may therefore be a potential new target for vaccines or drugs to control malaria.

by the observation that cystatin C regulates cell surface expression of MHC class II molecules in dendritic cells [18]. Additionally, in mammalian cells an endogenous inhibitor of calpain, calpastatin, regulates the activity of calpain [17]. In protozoan parasites, endogenous protease inhibitors may both regulate the activity of microbial proteases and prevent deleterious effects of host enzymes. Endogenous cysteine protease inhibitors have been described in *Trypanosoma cruzi* [19], *Leishmania mexicana* [20], and *Entamoeba histolytica* [21]. In *T. cruzi,* chagasin is an inhibitor of the cysteine protease cruzain and other cysteine proteases [19]. Overexpression of this inhibitor or addition of recombinant chagasin decreased the infectivity of *T. cruzi* in cell cultures [22]. In *L. mexicana,* disruption of an endogenous cysteine protease inhibitor led to markedly reduced virulence in mice [20].

We set out to identify an endogenous cysteine protease inhibitor in *P. falciparum*. Erythrocytic *P. falciparum* parasites express falstatin, a potent inhibitor of falcipains and many other cysteine proteases. The stage-specificity of falstatin expression and the effects of anti-falstatin antibodies on parasite development suggest that, paradoxically, this inhibitor facilitates a process that also requires proteolytic activity, the invasion of erythrocytes by *P. falciparum* merozoites.

Results

Identification, Expression, and Biochemical Characterization of Falstatin

We searched for a gene encoding a putative cysteine protease inhibitor in *P. falciparum* using the *T. cruzi* inhibitor chagasin as a probe in a BLAST search. We identified a *P. falciparum* gene *(PFI0580c)* encoding a predicted inhibitor, falstatin, with weak homology to chagasin (Figure 1). A sequence comparison algorithm (Signal IP 3.0) predicted that falstatin has a typical N-terminal signal sequence. Falstatin is much larger than chagasin, and amino acid similarities are limited to small portions of the molecules. The predicted molecular mass of falstatin is 46.97 kDa. Falstatin is much more similar in size and sequence to homologous genes from other plasmodial species (Figure 1).

The falstatin gene was amplified from 3D7 strain *P. falciparum* cDNA and inserted into an expression vector, and falstatin was expressed in *Escherichia coli* as soluble recombinant protein. Falstatin was purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography, based on a His tag introduced into the recombinant protein, followed by ion exchange chromatography (Figure 2).

Kinetic studies showed that falstatin is a picomolar inhibitor of falcipain-2 and falcipain-3, the principal cysteine protease hemoglobinases of erythrocytic malaria parasites (Table 1). Falstatin similarly inhibited homologous cysteine proteases from other plasmodial species (Table 1). However, falstatin did not inhibit the activity of trophozoite lysates against the exopeptidase substrate Pro-Arg-AMC, which is an optimal substrate for *P. falciparum* dipeptidyl aminopeptidase 1 [6]. Considering proteases from other organisms, falstatin inhibited numerous other cysteine proteases, with picomolar activity against the human cysteine proteases cathepsin K, cathepsin L, cathepsin H, and calpain-1, and nanomolar activity against the more distantly related cysteine proteases caspase 3 and caspase 8 (Table 1). Falstatin did not inhibit two cysteine proteases with exopeptidase activity, cathepsin B and cathepsin C. In addition, it did not inhibit proteases of other catalytic classes, including the serine proteases trypsin and α chymotrypsin, the aspartic proteases pepsin and renin, and the metalloproteases collagenase and matrix metalloprotease-2 (Figure 3). In summary, falstatin is a potent inhibitor of many cysteine proteases, but not other catalytic classes of proteases.

Mechanism of Inhibition by Falstatin

Falstatin was a competitive and reversible inhibitor of falcipain-2, as demonstrated by increasing calculated K_m values but similar V_{max} values with increasing concentrations of falstatin [23]. Without inhibitor, the calculated K_m and V_{max} of falcipain-2, for the substrate benzyloxycarbonyl-Leu-Arg-7-amino-4-methylcoumarin (Z-Leu-Arg-AMC), were 7.46 μM and 2.51×10^{-7} μmol/s, respectively. In 12 nM falstatin, the calculated K_m and V_{max} were 11.3 μM and 2.20×10^{-7} μmol/s, and in 18 nM falstatin these values were 41 μM and 2.02×10^{-7} μmol/s, respectively.

Typical small molecule cysteine protease inhibitors act by binding to the protease active site to block enzyme-substrate interaction [24]. We previously showed that, in contrast, the prodomain of falcipain-2, which is a picomolar inhibitor of the enzyme, binds to the catalytic domain of the protease independent of the active site [25]. To evaluate the mechanism of inhibition of cysteine proteases by falstatin, we tested the ability of active site-inhibited falcipain-2 ($FP2^{E-64}$) to compete with active falcipain-2 for binding to falstatin. In contrast to results with the prodomain, the inhibitory effect of falstatin was not affected by the presence of $FP2^{E-64}$ (Figure 4). Thus, the binding of falstatin to falcipain-2 appears to be via interaction with the enzyme active site. Further, kinetic studies showed that $^{\Delta 10}$falcipain-2, an active enzyme lacking a C-terminal hemoglobin-binding domain [9], was nearly as well inhibited by falstatin as the wild-type enzyme, indicating that, unlike the inhibitory prodomain, falstatin does not require the C-terminal domain for interaction with falcipain-2 (Table 1).

Stage-Specific Expression and Localization of Falstatin

Polyclonal antisera were raised against falstatin, and specific antibodies were purified by affinity chromatography

Figure 1. Alignment of Falstatin with Chagasin and Putative Cysteine Protease Inhibitors of Other Plasmodial Species

Sequences were aligned using the CLUSTALW program. Amino acid identities with falstatin are in blue, and amino acids of falstatin are numbered. The predicted N-terminal signal sequence is underlined.

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with Sepharose-coupled falstatin. Extracts from highly synchronized parasites were then evaluated to characterize the stage-specific expression of the inhibitor. Immunoblots demonstrated maximal expression of falstatin in late schizonts and early rings, less expression in late rings and early schizonts, and no apparent expression in trophozoites (Figure 5). These results are consistent with microarray data, which showed maximal transcription of the falstatin gene in late schizonts and early rings [26], and proteomic data, which identified a single falstatin peptide in only one erythrocytic stage, merozoites [27].

Anti-falstatin antibodies were also used to characterize the localization of falstatin by immunofluorescence and immunoelectron microscopy. Consistent with immunoblotting results, falstatin was seen in schizonts, merozoites, and rings, with apparent maximal expression from the late-schizont to early-ring stage (Figure 6A). Immunofluorescence images with 3D7 and W2-strain parasites showed very similar results, with punctate peripheral staining in rings, no staining of trophozoites, peripheral staining of early schizonts, and strong diffuse staining of mature schizonts and free merozoites. Electron microscopy of very mature schizonts demonstrated diffuse localization of falstatin within merozoites, without clear localization to intracellular organelles or membranes (Figure 6B). Control cells incubated with the same concentrations of pre-immune sera and second antibody did not show staining by immunofluorescence or immunoelectron microscopy.

Release of Falstatin with Schizont Lysis

Falstatin contains a typical signal sequence, but the importance of this sequence for its targeting is uncertain. To determine if falstatin is released from infected erythrocytes, we cultured highly synchronized late schizonts, collected culture media over 10 h, and assessed for the

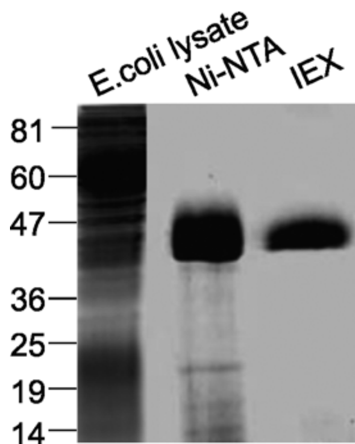

Figure 2. Expression and Purification of Falstatin

Falstatin was expressed in *E. coli* and purified by Ni-NTA affinity chromatography and ion exchange (IEX) chromatography. Protein was resolved by SDS-PAGE and stained with Coomassie blue. The positions of molecular weight markers (kDa) are indicated.

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Table 1. Inhibition of Cysteine Proteases by Falstatin

Enzyme	K_i^a (nM)
Falcipain-2	0.021 ± .008
$^{\Delta 10}$Falcipain-2	0.045 ± .008
Falcipain-2′	0.031 ± .006
Falcipain-3	0.223 ± .022
Vivapain-3	0.065 ± .011
Knowlepain-2	0.078 ± .018
Berghepain-2	0.048 ± .009
Vinckepain-2	0.150 ± .033
Papain	53.5 ± 19
Cathepsin K	0.025 ± .011
Cathepsin L	0.032 ± .008
Cathepsin H	0.052 ± .014
Cathepsin B	No inhibition
Cathepsin C	No inhibition
Calpain-1	0.196 ± .058
Caspase 3	376 ± 47
Caspase 8	80 ± 23

[a]Results utilizing substrates for each enzyme as detailed in Materials and Methods include standard deviations from two independent measurements, each performed in duplicate.

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presence of falstatin in media by immunoprecipitation with anti-falstatin antisera followed by immunoblotting. Falstatin was not detected in media from intact mature schizonts, but it was detected after schizont rupture (Figure 7). Thus, we did not identify secretion of falstatin by intact infected erythrocytes, but we did document the release of the inhibitor upon erythrocyte rupture.

Interaction of Falstatin with *P. falciparum* Cysteine Proteases

To better characterize potential targets of falstatin, we performed competition experiments between falstatin and the inhibitor DCG04, which has previously been shown to label falcipain-1, falcipain-2, and falcipain-3 (the latter two proteins co-migrate on SDS-PAGE gels), and dipeptidyl aminopeptidase 1 in *P. falciparum* lysates [6,28]. Competition experiments with different concentrations of falstatin and radio-iodinated DCG04 showed that the inhibitor interacts readily with native falcipain-2 and falcipain-3, but has much weaker binding to falcipain-1 and dipeptidyl aminopeptidase 1 (Figure 8A). These results suggest that among parasite proteases, falcipain-2 and falcipain-3 are the principal targets of falstatin inhibition.

Falstatin Facilitates the Invasion of Erythrocytes by *P. falciparum* Merozoites

Adding recombinant falstatin (50 μg/ml) to cultured parasites led to no significant changes in rates of rupture or invasion of erythrocytes as compared to controls (schizont parasitemia increased from 3.5% ± 1.13% to 4.4% ± 1.06% after 12 h; ring parasitemia decreased from 6.6% ± 1.0% to 5.9% ± 0.6% after 20 h). Antibodies raised against falstatin blocked the inhibitory action of this molecule. Inhibition of falcipain-2, falcipain-3, and the cysteine protease activity of trophozoite extracts was blocked by purified antibodies in a dose-dependent fashion (Figure 9). Similarly, the antibodies blocked competition by falstatin for binding of native falcipain-2 and falcipain-3 to labeled DCG04 (Figure 8B). The blocking effects of the antibodies were overcome by increasing concentrations of falstatin (Figure 8C). The antibodies were thus a valuable tool for studying the biological role of falstatin. When incubated with erythrocytic parasites at the ring, trophozoite, or schizont stage, no effects of the antibodies were seen during the course of a single erythrocytic cycle. This result is not surprising, as antibodies would not be expected to access intracellular falstatin. It was of greater interest to assess the impact of anti-falstatin antibodies on the rupture of mature infected erythrocytes or the subsequent invasion of erythrocytes by merozoites. Purified *P. falciparum* schizonts were incubated with uninfected erythrocytes in the presence of the cell permeable cysteine protease inhibitor (2S, 3S)-trans-epoxysuccinyl-L-leucylamido-3 methybutane ethyl ester (E-64d) or antibodies against falstatin, and persisting schizonts and new rings were counted after 12 h and 20 h, respectively. Consistent with earlier reports, E-64d caused a dose-dependent inhibition of erythrocyte rupture, with accumulation of intact schizonts and decreased formation of new rings [28,29]. In contrast, cultures incubated with anti-falstatin antibodies showed no accumulation of schizonts, but ~80% inhibition in new ring formation (Figure 10). Thus, for the antibody-treated parasites, the lack of new ring formation appeared to be due to a specific inhibition of merozoite invasion. In a separate experiment, the effects of multiple concentrations of antibodies were evaluated after a 20-h incubation, beginning at the schizont stage. Anti-falstatin antibodies blocked erythrocyte invasion in a dose-dependent manner. This effect was reversed by preincubation of the antibodies with falstatin. Pre-immune serum and a control immune serum had no effect on invasion (Figure 10). Thus, falstatin activity is required for efficient invasion of erythrocytes by merozoites.

Discussion

We have identified and characterized an endogenous cysteine protease inhibitor of *P. falciparum*. Falstatin shows only modest similarity with chagasin, its closest known relative from another genus, but predicted homologs from

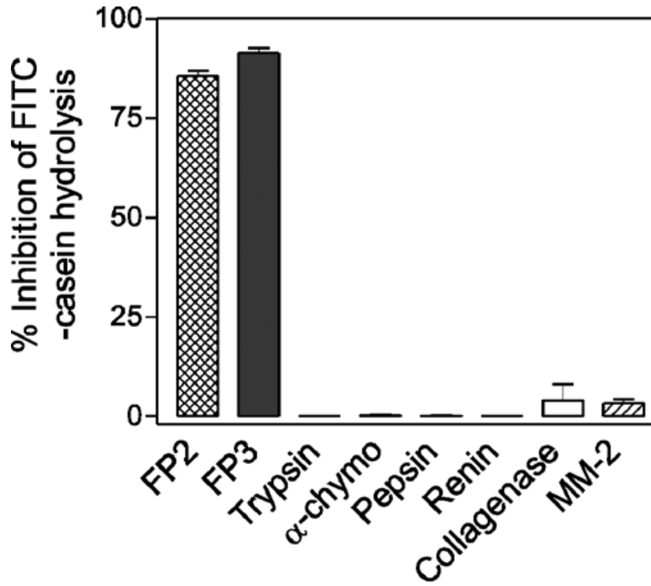

Figure 3. Activity of Falstatin against Different Classes of Proteases
Equal amounts (4 μg) of proteases (FP2, falcipain-2; FP3, falcipain-3; trypsin; α-chymo, α-chymotrypsin; pepsin; renin; collagenase; MM-2, matrix-metalloprotease-2) were mixed with 350 μl of appropriate buffers containing falstatin (1.5 μg) for 15 min, FITC-casein (20 μg) was added, and hydrolysis of the substrate with and without falstatin was compared for each protease. Error bars represent the standard deviations of results from two different assays, each performed in duplicate.
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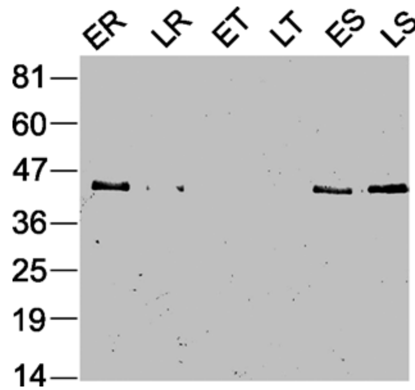

Figure 5. Stage-Specific Expression of Falstatin
Extracts from highly synchronized parasites were collected every 8 h, separated by SDS-PAGE, and evaluated by immunoblotting with anti-falstatin antibodies. Each sample of early-ring, late-ring, early-trophozoite, late-trophozite, early-schizont, or late-schizont extracts corresponded to 1.3×10^7 parasitized cells. The positions of molecular weight markers (kDa) are indicated. ER, early-ring; LR, late-ring; ET, early-trophozite; LT, late-trophozite; ES, early-schizont; LS, late-schizont.
doi:10.1371/journal.ppat.0020117.g005

other plasmodial species have higher levels of identity. As is the case with cystatins and chagasin, falstatin is a potent inhibitor of papain-family cysteine proteases, including *P. falciparum* falcipains and a number of host cathepsins. Falstatin is expressed most highly by mature schizonts, merozoites, and young rings, suggesting roles in erythrocyte rupture and/or invasion. Indeed, falstatin is released upon schizont rupture, and antibodies that inhibited falstatin action specifically blocked merozoite invasion of erythrocytes, suggesting that the inhibitor functions to prevent inappropriate activity by parasite and/or host cysteine proteases, and thereby facilitates erythrocyte invasion.

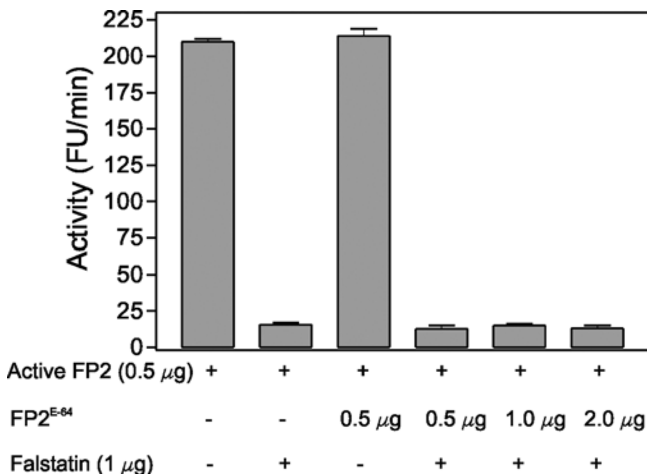

Figure 4. Mechanism of Interaction of Falstatin with Falcipain-2
Activity (arbitrary fluorescent units (FU) per minute) against Z-Leu-Arg-AMC is shown for indicated mixtures of falcipain-2 (FP2) and inactivated falcipain-2 ($FP2^{E-64}$) with falstatin. Reaction components were incubated for 15 min before the addition of substrate and measurement of fluorescence over time. Error bars represent the standard deviations of results from two different assays, each performed in duplicate.
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Falstatin represents a new family of macromolecular protease inhibitors. As is the case with cystatins, which bear no obvious homology, and chagasin, which is distantly related, falstatin inhibits a wide range of cysteine proteases. Activity against falcipains, homologs from other plasmodial species, and the human cysteine proteases cathepsin L, cathepsin H, cathepsin K, and calpain are all in the picomolar range. Less potent activity was seen against papain and human caspases. In contrast to both cystatins and chagasin, however, falstatin did not inhibit two human cysteine proteases with exopeptidase activity, cathepsin B and cathepsin C. Similarly, dipeptidyl aminopeptidase 1, a *P. falciparum* cysteine exopeptidase, was not inhibited by falstatin. In studies with falcipain-2, falstatin was seen to be a reversible, active site-binding inhibitor. Thus, falstatin is well equipped to inhibit parasite or host cysteine proteases at the times at which it is highly expressed.

It seems logical that falstatin functions to limit the activities of parasite and/or host cysteine proteases. Control of papain-family cysteine protease activity may be warranted, as this class of enzymes typically exhibits rather non-specific activity [30]. Indeed, the prototype for this family, papain, is used in many industrial applications due to its nonspecific action against protease substrates. Activity against a range of peptide bonds seems a desired feature of enzymes responsible for bulk proteolysis, as exemplified by hemoglobin hydrolysis by plasmodial trophozoites. The principal cysteine protease hemoglobinases, falcipain-2 and falcipain-3, are expressed by trophozoites, and hydrolyze hemoglobin in the acidic food vacuoles of these parasites [2,4,31,32]. Importantly, falstatin expression is not apparent in trophozoites. Presumably, control of the action of trophozoite food vacuole cysteine proteases by a parasite inhibitor is not needed. Cysteine protease activity is less pronounced in other stages of

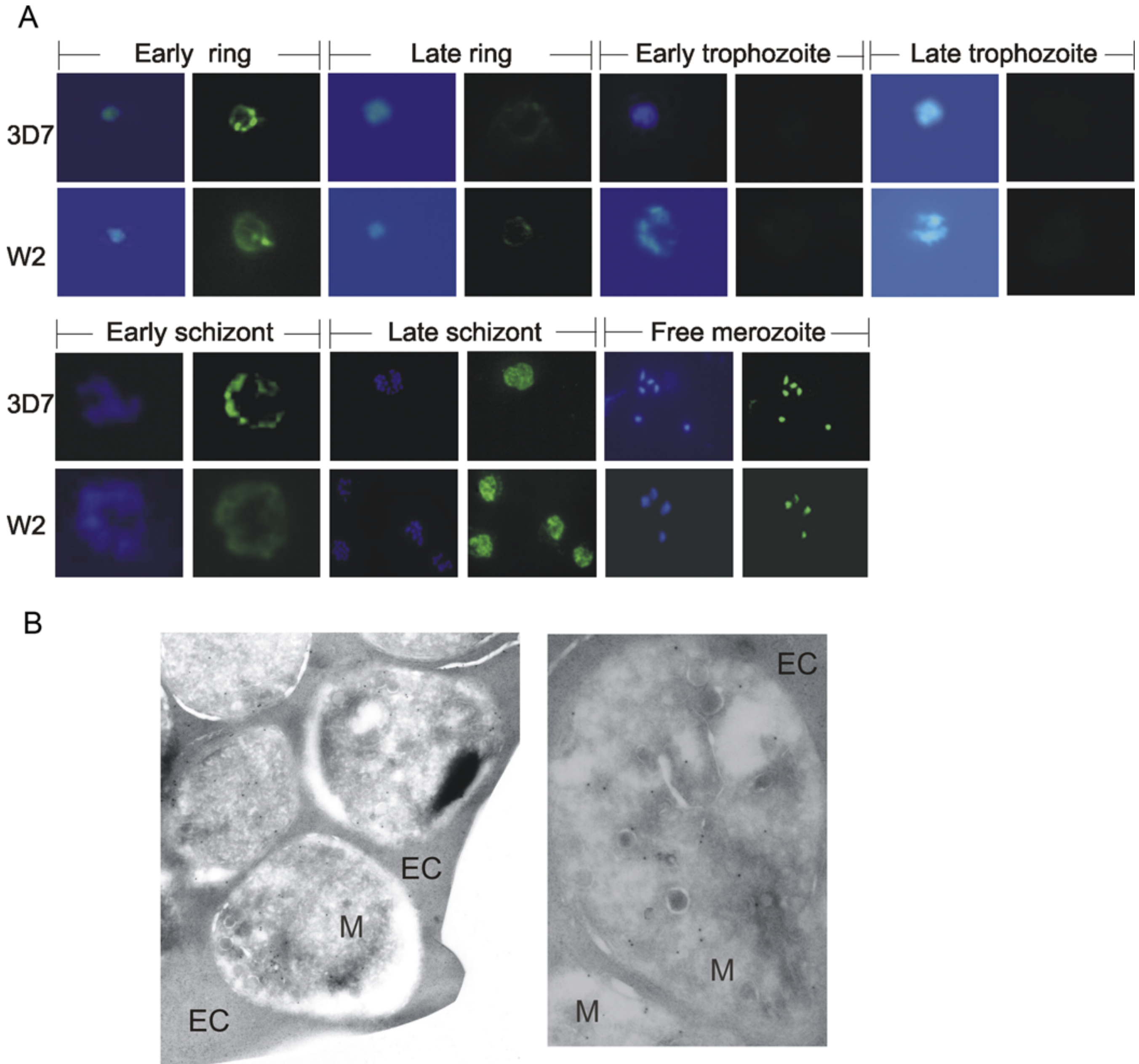

Figure 6. Immunolocalization of Falstatin

(A) Immunofluorescence microscopy. Erythrocytes infected with synchronized 3D7 or W2 parasites were collected every 8 h, stained with DAPI and anti-falstatin antibodies and FITC-second antibody, and then evaluated by immunofluorescence microscopy.

(B) Immunoelectron microscopy. Late-schizont stage parasites were incubated with anti-falstatin antibodies and gold-conjugated second antibody and then evaluated by electron microscopy. Labels show individual merozoites (M) and erythrocyte cytosol (EC).

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erythrocytic parasites, but some expression of this class of enzymes is seen throughout the erythrocytic cycle [4,8,29,31]. It is likely that cysteine proteases mediate additional parasite activities. In particular, there is strong evidence for cysteine proteases participating in erythrocyte rupture by mature schizonts [10,32,33], although the precise steps blocked by cysteine protease inhibitors are uncertain [34,35]. Some data have also suggested a role for falcipains in erythrocyte invasion by merozoites. Recently, inhibitor studies using a selective inhibitor implicated falcipain-1 in host-cell invasion [28]. However, results from other studies using cysteine protease inhibitors and the generation of viable falcipain-1 knockout parasites [29,36] leave the specific role of this protease in erythrocyte invasion uncertain.

Studies using small molecule inhibitors have implicated serine proteases as playing important roles in erythrocyte invasion [32,37–39]. Proteolytic activities that immediately precede or accompany erythrocyte invasion include the ordered processing of some merozoite surface proteins, including merozoite surface protein-1, followed by the shedding of remaining merozoite surface proteins, including merozoite surface protein-1 and apical merozoite antigen-1

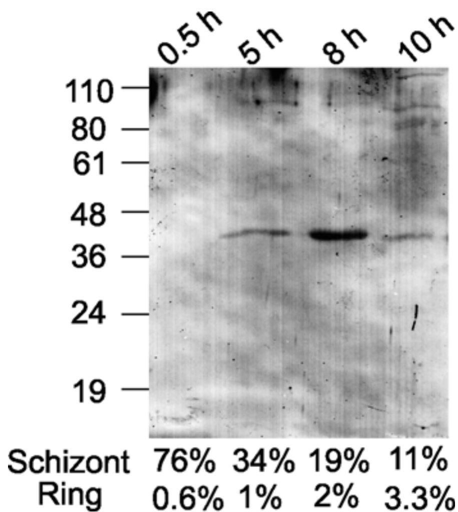

Figure 7. Release of Falstatin with Schizont Lysis

Synchronized late-schizont-infected erythrocytes were cultured in Albumax-free medium. At the indicated time points culture media were collected and concentrated, falstatin was immunoprecipitated with anti-falstatin antiserum and anti-rat IgG beads, the beads were washed, bound proteins were solubilized in sample buffer, and falstatin was resolved by SDS-PAGE and identified by immunoblotting. Controls with pre-immune serum did not immunoprecipitate any detectable proteins. The positions of molecular weight markers are indicated (kDa). Schizont and ring parasitemias at the indicated time points are shown below the gel.

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[39]. Recent work has implicated the *P. falciparum* subtilisin homolog pfSUB2 in this critical shedding process, linking serine proteases to the process of host-cell invasion [14].

Summarizing available data, cysteine proteases play clear roles in hemoglobin hydrolysis by trophozoites and probably help to mediate erythrocyte rupture by mature schizonts. Serine proteases appear to mediate essential steps in erythrocyte invasion by merozoites. Why, then, is falstatin produced by late schizonts and merozoites and released into culture medium upon schizont rupture? We propose that falstatin functions to limit cysteine protease activity at the site of parasite invasion to allow selective hydrolysis by other enzymes and thereby facilitates efficient invasion of erythrocytes. The parasite proteases that are acted upon by falstatin are most likely falcipains and other cysteine proteases that have been liberated by schizont rupture and that, unchecked, could disrupt the limited proteolysis by serine proteases that is required for erythrocyte invasion. Although abundant in late schizonts, falstatin apparently does not prevent activity of cysteine proteases that facilitate erythrocyte rupture [7,10,11], presumably due to different intracellular locations of active proteases and the inhibitor. In any event, our data strongly suggest that expression of falstatin is required for efficient invasion of erythrocytes by free merozoites.

Inhibition of host proteases by falstatin may also be biologically relevant. Lysosomal cysteine proteases, including cathepsin L and a number of related enzymes, have uncertain roles in erythrocytes, but proteases released by macrophages or other cells might act, via nonspecific proteolysis, to prevent erythrocyte invasion by free merozoites. Falstatin also inhibits human calpain-1, a related papain-family protease. Calpain-1 is localized to the inner surface of the erythrocyte cytoskeleton [40], and it is possible that inhibition of this host intracellular protease activity is required for efficient parasite development.

Our identification of falstatin and demonstration of its likely role in erythrocyte invasion by merozoites offers potential new approaches for the control of malaria. As demonstrated by our studies with specific antibodies, compounds that block falstatin function inhibit parasite development. Both drugs directed against falstatin or vaccines that elicit antibodies that block its action might offer new means, ideally in concert with other approaches, of controlling malaria. In addition, other proteases and protease inhibitors probably play key roles in the life cycle of malaria parasites, and additional research to elucidate the functions of these proteins is warranted.

Materials and Methods

Expression and purification of recombinant falstatin. The falstatin gene was identified by a BLAST search using the chagasin sequence. The falstatin gene was amplified from 3D7 strain *P. falciparum* cDNA using forward (5′-GATGGATCCAATAACAGCTACTCATTT-GAAATTGTG-3′) and reverse (5′-CGGAAGCTTATTGCACGTT-TAACTCTACAATTCT-3′) primers spanning the predicted open reading frame. The PCR product was digested with BamHI and HindIII, gel purified, and ligated into an appropriately digested pQE-30 plasmid (Qiagen, Valencia, California, United States), which encodes an amino terminal 6-His tag, to produce expression construct pQ-fal. This construct was used to transform the M15 (pREP4) strain of *E. coli,* and bacteria containing pQ-fal were grown to mid-log phase and then induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 33 °C. Cells were harvested, washed with ice-cold 10 mM Tris (pH 8.0), sonicated (five cycles of 5 s each, with cooling for 30 s between the cycles), and centrifuged at 12,000 × *g* for 30 min at 4 °C. For purification of the recombinant protein, the supernatant was incubated overnight at 4 °C with Ni-NTA resin (Qiagen) equilibrated with 10 mM Tris (pH 8.0). The column was washed with ten bed volumes of the same buffer and 50 mM imidazole, and bound protein was eluted with a continuous gradient (50 mM–1 M) of imidazole. The Ni-NTA purified protein was bound to a Q-Sepharose (Amersham Biosciences, Little Chalfont, United Kingdom) column in 10 mM Tris (pH 8.0). The column was washed with five column volumes of 100 mM NaCl in the same buffer and bound protein was eluted with a continuous salt gradient (100 mM–1 M NaCl). Purified protein was analyzed with SDS PAGE and the Bradford dye assay. The sequences of recombinant genes in expression constructs were confirmed by DNA sequencing.

Inhibitor kinetics. The cysteine proteases falcipain-2 [2], falcipain-2′ [3], $^{\Delta 10}$falcipain-2 [9], falcipain-3 [4], vivapain-3 [41], knowlepain-2 [25], berghepain-2 [42], and vinckepain-2 [42] were produced as described earlier. Cathepsins K, L, H, B, and C and papain were from Sigma (St. Louis, Missouri, United States), and calpain-1, caspase-3, and caspase-8 were from Calbiochem (San Diego, California, United States). Inhibitor kinetics were calculated as previously described [9]. Briefly, the concentrations of these enzymes (2–10 nM for all assays) were determined by active site titration with morpholine urea–leucine–homophenylalanine-fluoromethyl ketone (Mu-Leu-Hph-FMK; Peptides International; plasmodial enzymes), N-(trans-epoxy-succinyl-L-leucine 4-guanidinobutylamide (E-64; Sigma; cathepsin B, K, L, and H), acetyl–Asp-Glu-Val-Asp-chloro methyl ketone, (Ac-DEVD-CMK; Calbiochem; caspases), and chicken cystatin (Sigma; cathepsin C). Proteolytic substrates (5–20 μM; ~2-fold below K_m) were Z-Leu-Arg-AMC (Peptides International; plasmodial proteases), Z-Phe-Arg-AMC (Peptides International; cathepsins K, L, and H and papain), Z-Arg-Arg-AMC (Peptides International; cathepsin B), Pro-Arg-AMC (Bachem; dipeptidyl aminopeptidases), H-Lys(FAM)–Glu-Val-Tyr-Gly-Met-Met-Lys(Dabcyl)-OH (Calbiochem; calpain-1 [43]), Gly-Phe-4 methoxy-β-napthylamide (Bachem; cathepsin C [44]), acetyl-Ile-Glu-Thr-Asp-paranitroaniline (Ac-IETD-pNA; Calbiochem; caspase-8), and acetyl-Asp-Glu-Val-Asp-7 amino-4 methylcoumarin (Ac-DEVD-AMC; Calbiochem; caspase-3). Assay buffers were 100 mM acetate buffer, 8 mM dithiothreitol (DTT) (pH 5.5) (plasmodial proteases); 100 mM acetate buffer, 1 mM ethylene diamine tetraactetic acid (EDTA), 400 mM NaCl, 10% glycerol (pH 5.5) (cathepsins); 50 mM N-(2-hydroxyethyl)-piperazine-N′-2-ethanesul-

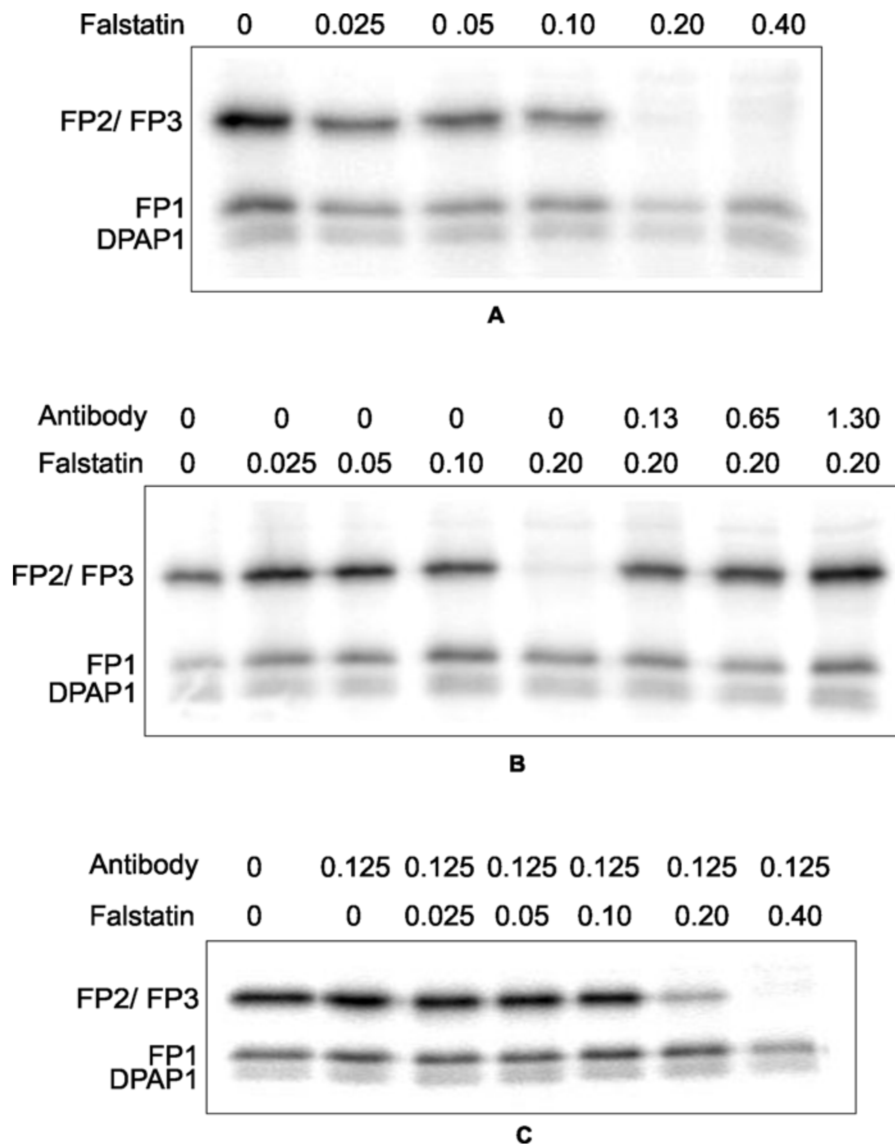

Figure 8. Inhibitor Competition

The indicated amounts of falstatin and anti-falstatin antibody were incubated with lysates from asynchronous parasite cultures before addition of [^{125}I] DCG04, electrophoresis, and analysis by autoradiography. Results with increasing concentrations of falstatin (A), increasing concentrations of antibody (B), and increasing falstatin in the presence of antibody (C) are shown. Labels above the gels represent concentrations of falstatin and antibody (μg/ml). Proteins are labeled based on known migration patterns that were previously confirmed by mass spectrometry. FP, falcipain; DPAP1, dipeptidyl aminopeptidase1[28].

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fonic acid (HEPES), 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 10% glycerol, 0.1% 3-{(3-cholamidopropyl) dimethylammonio}-1-propane sulfonate (CHAPS) (pH 7.4) (caspases), and 20 mM imidazole, 5 mM β-mercaptoethanol, 5 mM $CaCl_2$, 1 mM EDTA, 1 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N′N′-tetraacetic acid (EGTA), 30% glycerol (pH 6.8) (calpain-1). To assay the inhibitory activity of falstatin against *P. falciparum* dipeptidyl peptidases, saponin-treated trophozoites (7.8×10^5 parasites per reaction) were incubated with and without falstatin (50 μg/350 μl), and activity against the substrate Pro-Arg-AMC was assessed as previously described [6].

For kinetic assays, different concentrations of falstatin were incubated with enzymes for 10 min at room temperature. The lowest inhibitor concentration studied was at least ten times the enzyme concentration to ensure pseudo-first-order reaction conditions [45]. Substrates were then added, product formation was continuously measured for 20 min at room temperature with a spectrofluorometer, and K_i values were determined by nonlinear regression analysis using PRISM (GraphPad Software, San Diego, California, United States).

Activities of falcipains (in buffers as described above) and proteases from other catalytic classes (all from Sigma) were compared as hydrolysis of FITC-casein in the following buffers: 100 mM phosphate (pH 7.6) (trypsin), 50 mM Tris (pH 7.8) (α-chymotrypsin), 100 mM acetate (pH 4.0) (pepsin), 100 mM phosphate (pH 6.0) (renin), 50 mM Tris (pH 7.5) (collagenase), and 100 mM phosphate, 150 mM NaCl, 0.025% Brij-35, 50% glycerol (pH 7.4) (matrix-metalloprotease-2). Enzymes (4 μg) were incubated in 350-μl reactions with falstatin (1.5 μg) at room temperature for 15 min. FITC-casein (20 μg) was added, and fluorescence resulting from hydrolysis was measured (excitation 485 nm, emission 527 nm) continuously over 10 min.

To assess the mechanism of inhibition of falcipain-2, varied amounts (0.5–2.0 μg) of inactive falcipain-2 ($FP2^{E\text{-}64}$), prepared as described previously [9], and constant amounts of active falcipain-2 (0.5 μg), and falstatin (1 μg) were mixed in 350-μl assay buffer for 15 min at room temperature. Hydrolysis of the substrate Z-Leu-Arg-AMC by the protease was studied as described above.

Anti-falstatin antibodies. Rats were immunized by the intraperitoneal route with 100 μg of recombinant purified falstatin emulsified in complete Freund's adjuvant. The rats were boosted on

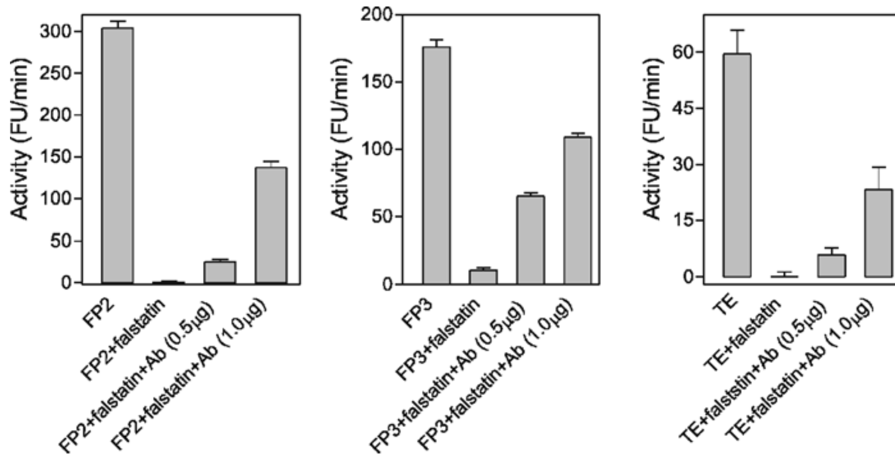

Figure 9. Inhibition of Falstatin Function by Anti-Falstatin Antibodies

Hydrolysis of the peptide substrate Z-Leu-Arg-AMC by falcipain-2 (FP2; 19.8 nM), falcipain-3 (FP3; 27.1 nM), or trophozoite extract (TE; corresponding to 5.5×10^6 parasites per reaction) was evaluated in the absence or presence of falstatin (31 nM) and the indicated quantities of anti-falstatin antibodies in 350 μl of 100 mM sodium acetate, 8 mM DTT (pH 6.0). Reaction components were incubated for 15 min before addition of substrate, and activity was measured as arbitrary fluorescence units over time (FU/min). Error bars represent the standard deviations of results from two different assays, each performed in duplicate.

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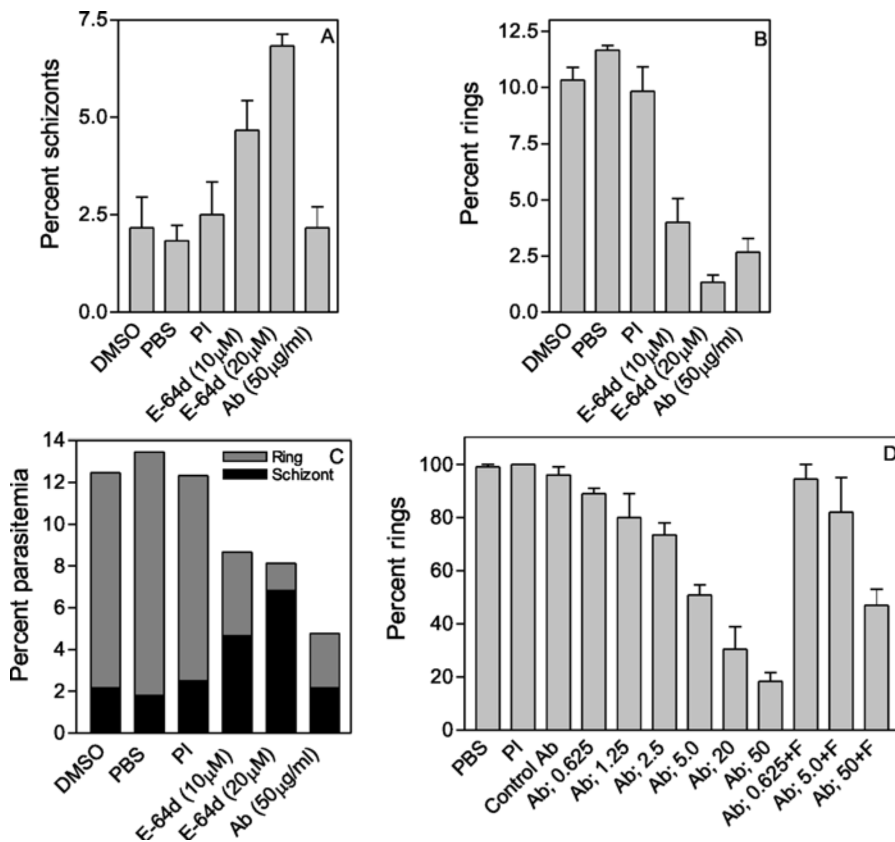

Figure 10. Effect of Anti-Falstatin Antibodies on Cultured Parasites

Schizont-infected erythrocytes were combined with fresh erythrocytes in culture medium with 0.5% DMSO or PBS, pre-immune serum, or the indicated concentration of E-64d (in DMSO) or antibody (in PBS). Smears were then made and stained with Giemsa, and percentages of schizonts after 12 h (A), rings after 20 h (B), and total parasites (C) were counted. In a separate experiment (D), purified schizonts were incubated for 20 h with PBS, control pre-immune serum (50 μg/ml), rat antiserum against *P. falciparum* farnesyl pyrophosphate synthetase (control Ab; 50 μg/ml), or the indicated concentrations of antibodies (Ab; μg/ml) in PBS with or without preincubation for 10 min with 2 μg falstatin (F). Errors bars indicate standard deviations from means of two different assays, each done in triplicate. PI, pre-immune serum.

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days 33, 66, and 95 with 60 μg of falstatin in incomplete Freund's adjuvant. Sera were collected 12–14 d after each immunization and used for immunoassays. Initial experiments were performed with immune antisera, but all reported results were with affinity purified anti-falstatin antibodies. To affinity purify these antibodies, an affinity column was prepared as described earlier [2]. Briefly, recombinant antigens (10 mg) were equilibrated with 100 mM sodium carbonate (pH 8.0), crosslinked to 5 ml of cyanogen bromide-activated Sepharose Fast Flow (Amersham Bioscience), and pre-equilibrated with the same buffer overnight at 4 °C. The column was washed with ten column volumes of 100 mM sodium carbonate (pH 8.0) and blocked with 100 mM Tris (pH 8.0), followed by 1 M ethanolamine (pH 8.0). The beads were washed again with 0.1 % BSA in PBS. Diluted antiserum (50 ml, 1:10 in PBS, 0.1% BSA) was then loaded onto the falstatin-Sepharose Fast Flow column. The column was washed with 100 ml each of PBS with 500 mM NaCl, 10 mM phosphate (pH 6.8), and 150 mM NaCl. Purified antibodies were eluted with 100 mM glycine, 150 mM NaCl (pH 2.6), collected in 2 ml fractions containing 200 μl of 1M Tris (pH 8.0), run through 0.4-μm filters, and stored at −20 °C. For immunofluorescence studies, antibodies (1 mg/ml) were further pre-absorbed with acetone-treated erythrocytes as follows: Human erythrocytes (500-μl packed cells) were treated with 10 ml of chilled acetone for 30 min at room temperature and then washed six times with PBS. Antibodies were incubated with treated cells for 40 min at 4 °C, cells were centrifuged at 15,300 × *g*, and the supernatant was used for immunofloresence assays.

To assess the functional activity of antibodies against falstatin, recombinant falcipain-2 (19.8 nM), falcipain-3 (27.1 nM), or trophozoite extracts (corresponding to 5.5×10^6 parasites per reaction) were incubated with falstatin (31 nM) and varied amounts (0.5–1.0 μg) of antibody in 350-μl assay buffer (100 mM sodium acetate [pH 6.0], 8 mM DTT) before addition of Z-Leu-Arg-AMC and measurement of hydrolysis, as described above.

Parasite culture and immunoblotting. *P. falciparum* strains 3D7 and W2 were cultured using standard methods in human erythrocytes at 2% hematocrit in RPMI 1640 medium supplemented with 25 mM Hepes, 30 mg/liter hypoxanthine, 0.225% $NaHCO_3$, and 0.5 % Albumax (Life Technologies, Carlsbad, California, United States). Synchronization was maintained by serial treatment with 5% D-sorbitol [46]. Erythrocytes infected with highly synchronous late schizonts were enriched by flotation on 63% Percoll (Sigma). The interphase containing schizont-infected erythrocytes was collected and washed twice with RPMI media. For immunoblots, infected erythrocytes were washed with ice-cold PBS, lysed with 0.1% saponin in ice-cold PBS for 10 min, centrifuged (2,060 × *g* for 10 min at 4 °C), washed three times with ice-cold PBS, and stored at −70 °C. Samples corresponding to 1.3×10^7 parasitized cells per lane were then incubated with SDS PAGE buffer, boiled for 10 min, and centrifuged at 15,300 × *g* at 4 °C for 20 min; soluble proteins were separated by 10% SDS PAGE and transferred to a nitrocellose membrane. The membranes were blocked with 1.5% BSA and 1% fat-free milk in PBS overnight at 4 °C and incubated with affinity purified anti-falstatin antibody (10 μg/ml) in PBS with 0.5% BSA and 0.5% fat-free milk for 1 h at room temperature. The membranes were washed and incubated with alkaline phosphatase-conjugated goat anti-rat IgG (1:8,000; Jackson Immuno Research, West Grove, Pennsylvania, United States) in the same solution at room temperature for 1 h. After incubation, membranes were extensively washed with PBS containing 0.2% Tween-20, and antigen–antibody complexes were developed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium tablets (Sigma) following manufacturer's instructions.

Immunofluorescence microscopy. Erythrocytes infected with synchronized parasites were washed twice in PBS and allowed to attach to poly-L-lysine coated slides (Electron Microscopy Science) for 1 h at room temperature. Cells were then fixed with 4% EM grade paraformaldehyde (Sigma) in PBS for 30 min, treated with 0.1% Triton-X 100 in PBS for 30 min, rinsed with PBS, and blocked with blocking buffer (1% BSA in PBS) for 1 h at room temperature in a humidified chamber. Anti-falstatin antibodies (affinity-purified and pre-absorbed with red blood cells, as described above; 30 μg/ml) and pre-immune rat sera (1:100) were diluted in blocking buffer and incubated with the fixed and blocked cells overnight in a humidified chamber. Cells were washed four times with PBS, stained with 4′-6-diamidino-2-phenylindole (DAPI) nuclear stain (2 μg/ml) and FITC-labeled goat anti-rat antibody (1: 500, Sigma), and incubated for 30 min in a humidified chamber. Stained slides were rinsed several times in PBS, dried, and overlaid with mounting solution (10% mowiol 4–88, 25% glycerol, 2.5% 1,4 –diazobicyclo-[(2.2.2]–octane, 0.1M Tris [pH 8.5])[47] and a cover slip before examination by fluorescence microscopy.

Immunoelectron microscopy. Erythrocytes infected with mature schizonts were washed three times with PBS and fixed at room temperature in 1% paraformaldehyde and 0.2% glutaraldehyde in 50 mM phosphate buffer (pH 7.4) for 2 h. Fixed cells were washed, resuspended in PBS, and kept at 4 °C until analysis by electron microscopy at the University of California, San Francisco Cell Imaging Core Facility using standard protocols [48]. Briefly, fixed samples were cryoprotected with 20% polyvinylpyrrolidone in 2.3 M sucrose overnight, and frozen in liquid nitrogen. Frozen thin sections were cut with a Leica Ultracut UCT microtome with EM FCS (Leica Microsystems, Wetzlar, Germany). Sections were treated with 0.2% glycine, blocked with 2% fish gelatin, 2% BSA in PBS (pH 7.4), and then incubated with purified anti-falstatin antibody (50 μg/ml in PBS) followed by 10 nm colloidal gold (Ted Pella, Incorporated, Redding, California, United States) conjugated with goat anti-rat IgG (1:50 in PBS) for 30 min. Sections were stained with oxalate uranyl acetate and embedded in 1.5% methyl cellulose, 0.3% aqueous uranyl acetate (Ted Pella), and examined with a Philips Tecnai 10 electron microscope.

Identification of falstatin in culture supernatants. Late-schizont-infected erythrocytes (650 μl; 7.6×10^6 parasites/μl) were prepared as described above, washed with culture medium lacking Albumax, gently resuspended in 150 ml (10 ml for the first time point) of Albumax-free medium, divided into separate flasks, and maintained at 37 °C until medium was collected. At indicated time points parasites were examined by evaluation of Giemsa-stained smears, and the medium was concentrated to 2 ml with an Amicon filter (10-kDa cut-off; Millipore, Billerica, Massachusetts, United States). Falstatin was then immunoprecipitated as follows: Concentrated culture supernatants were incubated with 10 μl anti-falstatin antibody (2 mg/ml) and 1× Protease Inhibitor Cocktail Set III (Calbiochem) for 30 min at 4 °C, anti-rat IgG-agarose beads (60 μl of a 50:50 slurry in Albumax-free medium) were added, reaction mixtures were incubated for 1 h at 4 °C with gentle shaking, and samples were centrifuged for 10 min at 15,300 × *g*, 4 °C and washed twice with 50 mM Tris (pH 7.4), 300 mM NaCl, 0.1 % Triton–X 100. Washed beads were resuspended in 25-μl reducing SDS-PAGE sample buffer, boiled for 10 min, centrifuged at 15,300 × *g*, and samples were analyzed by immunoblotting with anti-falstatin antibodies as described above.

Inhibitor competition experiments. DCG04 was radioiodinated as described earlier [28]. Asynchronous 3D7 parasites were lysed in 1% NP-40, and soluble and insoluble fractions were mixed. Approximately 100 μg of total protein, estimated with Bradford reagent, was used in each reaction. Indicated amounts of falstatin with or without antibody were incubated with the lysates for 30 min. [I^{125}]DCG04 was then added and the reactions were continued for 1 hour at room temperature before adding SDS loading buffer and boiling. Approximately 20 μg of labeled lysate was loaded in each gel lane, samples were resolved by SDS-PAGE, and gels were analyzed by autoradiography.

Rupture and invasion assays. Infected erythrocytes (2% parasitemia) at different parasite stages were incubated with anti-falstatin antibodies or PBS, and morphologies were evaluated over the course of the life cycle. For studies of effects of antibodies on erythrocyte rupture and invasion, schizont-infected erythrocytes were purified as described above and diluted with uninfected erythrocytes to a final parasitemia of 5%. Infected erythrocytes were then incubated with or without E-64d (in 0.5% DMSO) or antibody against falstatin (in PBS) for the indicated intervals, and parasite counts were performed by microscopic analysis of Giemsa-stained smears (parasites per 500 erythrocytes). Controls were parasites incubated with PBS, 0.5% DMSO, rat pre-immune sera, and rat antiserum against *P. falciparum* farnesyl pyrophosphate synthetase (a gift from A. Lau).

Supporting Information

Accession Numbers

The nucleotide and amino acid sequences reported in this paper have been submitted to the Gene Bank/National Center for Biotechnology Information (NCBI) Data Bank (http://www.ncbi.nlm.nih.gov) with accession number DQ288863.

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Author contributions. All authors conceived and designed the experiments. KCP, NS, and SAK performed the experiments. All authors analyzed the data. KCP and PJR wrote the paper with assistance from all other authors.

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Competing interests. The authors have declared that no competing interests exist.

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