A Subtilisin-like Protein in Secretory Organelles of Plasmodium falciparum Merozoites*

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In the vertebrate host, the malaria parasite invades and replicates asexually within circulating erythrocytes. Parasite proteolytic enzymes play an essential but poorly understood role in erythrocyte invasion. We have identified a Plasmodium falciparum gene, denoted pf-sub-1, encoding a member of the subtilisin-like serine protease family (subtilases). The pfsub-1 gene is expressed in asexual blood stages of P. falciparum, and the primary gene product (PFSUB-1) undergoes post-translational processing during secretory transport in a manner consistent with its being converted to a mature, enzymatically active form, as documented for other subtilases. In the invasive merozoite, the putative mature protease (p47) is concentrated in dense granules, which are secretory organelles located toward the apical end of the merozoite. At some point following merozoite release and completion of erythrocyte invasion, p47 is secreted from the parasite in a truncated, soluble form. The subcellular location and timing of secretion of p47 suggest that it is likely to play a role in erythrocyte invasion. PSUB-1 is a new potential target for antimalarial drug development.

Plasmodium falciparum, the causative agent of the most severe form of human malaria, is an obligate intracellular apicomplexan parasite. The life cycle of the organism includes a number of specialized invasive (zoite) stages. In the vertebrate host, replication of the parasite in circulating erythrocytes is initiated when the cells are invaded by merozoites. The parasite replicates asexually within the infected erythrocyte to produce a number of progeny merozoites. Upon rupture of the host cell, these are released to invade fresh erythrocytes and perpetuate the blood stage cycle. Erythrocyte invasion by the malaria merozoite has been the subject of intensive study, since intervention strategies that prevent invasion would effectively block both replication of the parasite and the associated clinical disease.

Electron microscopic studies have shown that erythrocyte invasion by the malaria merozoite takes place in a number of discrete stages. Initial reversible attachment of the parasite to the red cell surface is rapidly followed by reorientation, the formation of an irreversible junction between the apical prominence of the merozoite and the host cell surface, and finally entry of the parasite into the cell by a mechanism resembling a form of induced endocytosis (1–4). The process is facilitated by the controlled release of the contents of three types of secretory organelles, called rhoptries, micronemes, and dense granules, situated at or toward the apical domain of the merozoite (2, 5, 6). There is extensive evidence indicating an essential role for parasite-derived proteases in invasion. Invasion by P. falciparum merozoites is blocked in the presence of the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (7), and invasion by merozoites of a number of Plasmodium species is prevented by chymostatin (8–13). The inhibitory effect of chymostatin on invasion can be reversed by pretreatment of target erythrocytes with chymotrypsin (10), suggesting that the chymostatin-sensitive step in invasion involves an essential, parasite-induced proteolytic modification of the red cell surface (10–12). A glycosylphosphatidylinositol (GPI)-anchored malarial serine protease activity has been described that may be involved in this modification (14). Treatment of isolated, invasive merozoites of the simian malaria P. knowlesi with N-tosyl phenylalanlanylchloromethyl ketone or N-tosyl lysylchloromethyl ketone prevents primary attachment of the parasites to host cells, whereas chymostatin blocks a later stage in the invasion pathway, indicating that more than one distinct protease activity may be involved (15). Consistent with this, a P. falciparum serine protease activity that mediates an essential processing and shedding of a major merozoite surface protein (merozoite surface protein-1; MSP-1) at invasion is highly sensitive to inhibition by PMSF but not by chymostatin (16, 17). Protease proteases involved in invasion are attractive potential targets for new rational approaches to antimalarial chemotherapy.

Here we report the identification of a novel, single copy P. falciparum gene (denoted pfsub-1) encoding a member of the

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subtilisin-like serine protease superfamily (subtilases). The primary gene product is expressed in the latter stages of intraacellular merozoite maturation, and is post-translationally modified during secretory transport in a manner consistent with it being processed to form a mature, enzymatically active product. The putative mature protease is concentrated in a subset of dense granules within the apical domain of free merozoites and then is released in a soluble form during erythrocyte invasion, suggesting that it may play a role in invasion. This is the first molecular characterization of a putative apicoplexoon serine protease.

**EXPERIMENTAL PROCEDURES**

**Parasites, Materials, and Nucleic Acid Manipulations—** Blood stage cultures of the T9/96 and FCU-1 lines of *P. falciparum* were maintained *in vitro* in human A+ erythrocytes and synchronized when required, as described previously (18, 19). Protease inhibitors and fluorogenic or chromogenic peptide and protein substrates were obtained from Sigma and Boehringer Mannheim. Restriction endonucleases and other DNA modifying enzymes were obtained from Boehringer, Amersham Phamacia Biotech, and New England Biolabs. The majority of the methods used for DNA manipulations were based on those of Sambrook et al. (20). DNA oligonucleotides for *P. falciparum* malarial parasite were obtained from Oligo-Med, Inc. Southern blotting was by standard procedures (20, 21). DNA probes for DNA manipulations were based on those of Sambrook et al. (20) and other workers. DNA nucleases and modifying enzymes were obtained from Boehringer, Amersham Pharmaceutical Institutes for Health (22), using the European Bioinformatics Institute database. 

**Polymerase Chain Reaction (PCR)—** Most PCR was carried out using AmpliTaq® DNA polymerase (Perkin-Elmer). Degenerate oligonucleotide primers for amplification by PCR and reverse transcription–PCR of malarial subtilase genes were SUB-1 (sense; 5'-CAYGGGACCACTGTTGGG-3') and SUB-2 (antisense; 5'-CCGCIACRTGIGGIGTIGC-3'), based on the amino acid sequences HGTHVAG and MATPH, respectively. Primers for amplification by inverse PCR (23) of DNA, treated total parasite RNA or yeast control RNA. The reactions were then treated with an empirically determined dilution of a mixture of RNase A and RNase T1 for 30 min at 30 °C, followed by RNase inactivation and precipitation of the remaining RNA. Samples were subjected to electrophoresis on 6% acrylamide gels in the presence of 8 M urea and analyzed by autoradiography. Markers for electrophoresis were end-radiolabeled *Hinfl*-digested dephosphorylated OX174 DNA markers (Promega). 

**Expression, Metabolic Radiolabeling, and Purification of PfSUB-1 Recombinant Fusion Proteins and Antibody Production—** DNA fragments amplified by PCR with primers SUB-EX1 and SUB-EX3, SUB-EX2 and SUB-EX3, or SUB-EX5 and SUB-EX3, were digested with BamHI and/or EcoRI and then ligated into either BamHI- and EcoRI-digested pMOSBlue (Amersham Pharmacia Biotech). E. coli strain DH5α (Stratagene) was transformed to ampicillin resistance, and recombinant clones were selected. In each case, the identity and orientation of the insert in recombinant clones was confirmed by complete sequence analysis on both strands.

Recombinant *E. coli* clone PM500–7A, containing the SUB-EX2/ SUB-EX3 PCR product inserted into the expression vector pTrcHisB, was transformed with isopropyl-1-thio-β-D-galactosidase, and the cells were extracted by sonication in 20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 10 mM imidazole (starting buffer). The clarified extract was run on a HiTrap™ chelating column (Amersham Pharmacia Biotech), and the bound fusion protein (Hisg-PfSUB1-lab) was eluted using the starting buffer supplemented with 8 M urea and 400 mM imidazole. For metabolic radiolabeling of Hisg-PfSUB1-lab, bacteria were grown to an *A* of 0.7 in M9 minimal medium and then induced for 2 h with 1 mM isopropyl-1-thio-β-D-galactosidase in the presence of 20 μCi ml−1 [35S]methionine/cysteine (Pro-mix™, Amersham Pharmacia Biotech). Recombinant *E. coli* clone PM511–3A, harboring the SUB-EX3/SUB-EX3 PCR product inserted into the expression vector pGEX-1AT, was induced with isopropyl-1-thio-β-D-galactosidase. Cells were resuspended in 25 mM Tris-HCl, pH 8.2, 1 mM EDTA, 0.2% (w/v) Triton X-100 containing 1 mM PMSF and then sonicated. The inclusion bodies were pelleted by centrifugation at 12,000 × g for 20 min and then washed twice more in the same buffer. Purification of the fusion protein (denoted GST-PfSUB1-lab) by affinity chromatography on a column of glutathione-agarose then proceeded following the sarkosyl method of Frangioni and Neel (25). Purified protein was eluted with the glutathione-agarose column with 8 M urea in 50 mM Tris-HCl, pH 8.2, and then further purified by gel filtration in the same buffer on a 2.6 × 77-cm column of Sephacryl S-200 HR (Amersham Pharmacia Biotech). The protein was finally precipitated by dialysis against PBS and used to immunize Balb/c mice using conventional procedures (26).

*P. falciparum* Radiolabeling and Merozoite Purification— Mature schizonts from highly synchronous *P. falciparum* cultures were en-
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Pulse-Chase Experiments—Mature T9/96 schizonts enriched from highly synchronous cultures were washed twice in warm methionine/cysteine-free medium, then cultured for 30 min in the same medium. The culture was then pelleted, resuspended to a hematocrit of 20% in fresh complete medium, and labeled with [35S]methionine/cysteine. Control cultures were similarly pretreated with methanol only. Following a 2-h incubation at 37 °C, labeled parasites were rapidly pelleted and resuspended in fresh complete medium at 37 °C. Immediately pelleted and resuspended in fresh cold acetone, and digested products were extracted from the gel by two successive incubations in 60% acetonitrile. Extracts were pooled, lyophilized, then taken up in 20% aqueous pyridine, and spotted onto 0.1-mm-thick cellulose TLC plates (Merck). The peptides were then separated by electrophoresis at pH 4.4 in pyridine/acetate/acetic/water (2.4: 15.79:by volume) (28), followed by ascending chromatography in the second dimension in butanol/acetate/water/pyridine (15.3:12.10:by volume). Dried plates were sprayed with ENHANCE (DuPont) and radiolabeled peptides were detected by fluorography.

Indirect Immunofluorescence (IFA), Confocal Microscopy, and Immunoelectron Microscopy—Analysis of acetone-fixed thin smears of malaria parasite cultures or purified merozoites by IFA and confocal microscopy was essentially as described previously (29). Briefly, samples were labeled and digestion products were extracted from the gel by two successive incubations in 60% acetonitrile. Extracts were pooled, lyophilized, taken up in 20% aqueous pyridine, and spotted onto 0.1-mm-thick cellulose TLC plates (Merck). The peptides were then separated by electrophoresis at pH 4.4 in pyridine/acetate/acetic/water (2.4: 15.79:by volume) (28), followed by ascending chromatography in the second dimension in butanol/acetate/water/pyridine (15.3:12.10:by volume). Dried plates were sprayed with ENHANCE (DuPont) and radiolabeled peptides were detected by fluorography.

RESULTS

PCR Amplification and Molecular Cloning of the pfsub-1 Gene—Oligonucleotide primers were designed based on conserved motifs flanking the catalytic histidine and serine residues of the subtilisin-like class of serine proteases. PCR primers SUB-1 (sense), and SUB-2 (antisense) were based on sequences His170 to Gly176 and Met328 to Gly335 of the subtilisin E gene product (GenbankTM/EMBL accession no. K01988). Primers were completely redundant, no account being taken of the bias toward A + T-rich codon usage in the P. falciparum genome. Inosine was used in positions of more than 2-fold degeneracy in order to minimize the overall primer degeneracy and maximize sequence coverage. Reverse primer SUB-1 was designed to take advantage of the nonredundant methionine codon at the 3'-end of the oligonucleotide. In PCR reactions using as template single-stranded cDNA prepared from DNA-free total blood stage parasite RNA, a single DNA fragment of approximately 560 bp was amplified. No product was obtained from mock cDNA preparations produced in the absence of re-
verse transcriptase. A product of identical size was also obtained by PCR amplification from T9/96 or FCB-1 genomic DNA (not shown). Products from all three PCR reactions were cloned into the T-vector pMOSBlue, and 2–5 representative clones from each transformation were sequenced on both strands. All of the cloned sequences were identical. The cloned DNA was 561 bp long and contained a single open reading frame (ORF); a comparison of the deduced amino acid sequence with the protein data bases indicated strong homology with a number of bacterial subtilisins. The 561-bp fragment was then used to probe a T9/96-l-ZAP asexual blood stage cDNA library, and a total of four strongly hybridizing clones were obtained (Fig. 1, top). Complete sequence analysis of the cDNA clones showed that they represented overlapping fragments of a single contiguous sequence containing an uninterrupted ORF of 2073 bp, encoding a protein of 690 amino acids with an estimated molecular mass of 77,874 daltons (Fig. 1, bottom). Consistent with the A+T-rich P. falciparum genome, the A+T content of the ORF is 72%.

The pfsub-1 Gene Encodes a Subtilase—The deduced pfsub-1 gene product (designated PfSUB-1) clearly belongs to the subtilisin-like (subtilase, S8) protease clan or superfamily as defined by Rawlings and Barrett (32). The C-terminal 361 residues (Ser330–His690) was aligned with the catalytic domains (as defined by Siezen et al.; Refs. 33 and 34) of a number of bacterial and eukaryotic subtilases (not shown). A number of points can be made concerning this comparison. First, within the putative catalytic core domain of the

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**Fig. 1.** Molecular cloning of the pfsub-1 gene. Top, cloned pfsub-1 PCR products and cDNAs. The schematic depicts the relative positions and sizes of the 561-bp PCR product (PM443–7) amplified with degenerate oligonucleotide primers SUB-1 and SUB-2; the four partial cDNA clones (8-1, 9-1, 7-2, and 10-1) obtained by using PM443–7 to probe a P. falciparum cDNA library; the composite cDNA clone (PM481–1) constructed from partial cDNA clones 8-1 and 10-1; and the pfsub-1 ORF (shaded box) relative to flanking genomic sequence obtained by inverse PCR amplification of a 3183-bp Sau3A genomic fragment. The positions and orientations of primers SUB-SEQ11 and SUB-SEQ12 used for the inverse PCR are also shown (small arrows). Restriction endonuclease sites shown are as follows: Sau3A (S); AvrII (A); HindIII (H). Bottom, nucleotide and deduced amino acid sequence of the pfsub-1 cDNA (clone PM481–1). Noncoding and coding regions of the nucleotide sequence are shown in lowercase and uppercase letters, respectively. The TAA translational stop codon is marked with an asterisk. The putative amino-terminal signal peptide and the catalytic triad residues Asp374, His430, and Ser608 are shown in boldface type and underlined. The putative C-terminal catalytic domain (PfSUB-1m) is shown shaded.
protein (see below), the PfSUB-1 sequence exhibits a number of residues that are highly or completely conserved among all known subtilases (33, 34); these include Asp<sup>374</sup>, His<sup>430</sup>, and Ser<sup>498</sup> (the catalytic site residues); the oxyanion hole residue Asn<sup>522</sup>; and residues Gly<sup>566</sup>, Thr<sup>567</sup>, and Pro<sup>612</sup>. Furthermore, PfSUB-1 clearly does not belong to the proteinase K or lantibiotic peptidase families (subtilase families C and D; Ref. 34), both of which lack the 6-residue-long Ca<sup>1</sup>Ca<sup>2</sup>-binding loop sequence (Asn<sup>443</sup>–Val<sup>448</sup> in the deduced PfSUB-1 sequence) just C-terminal to the active site histidine residue. Additionally, PfSUB-1 clearly does not belong to the kexin family (family E; Ref. 34) of prohormone-processing proteases of yeasts and higher eukaryotes. One characteristic of this group, for example, is the DDG motif present at the active site Asp residue; PfSUB-1 possesses the more common DSG motif at this position (Asp<sup>374</sup>–Gly<sup>376</sup> of the PfSUB-1 sequence). It is thus unlikely that PfSUB-1, if expressed in the parasite as a catalytically active protease, has a dibasic cleavage specificity (35).

By homology with other subtilases, PfSUB-1 is likely to be synthesized as a pre-pro-protease; a putative signal peptide 25 residues long (the predicted prepeptide) is present at the amino terminus of the deduced protein sequence (Fig. 1, bottom), and the alignment referred to above would suggest that the mature protease sequence (denoted PfSUB-1<sub>m</sub>) probably extends from Ser<sup>330</sup> to the C-terminal His<sup>580</sup> residue, having a predicted M<sub>r</sub> of 40,432. If this is so, the pro-domain extends from Lys<sup>296</sup> to Arg<sup>329</sup> and has an estimated M<sub>r</sub> of 34,652. Consistent with this proposal, a hydropathy profile of the complete deduced sequence exhibits noticeable asymmetry, with a clear bias toward hydrophilic residues in the predicted pro-peptide sequence (not shown). This is a commonly observed characteristic of subtilase pro-domains (36). Other than the N-terminal signal peptide, no hydrophobic domain typical of a transmembrane or GPI signal sequence is present in the deduced PfSUB-1 sequence.

The pfsub-1 Gene Is Single Copy, Contains No Introns, and Is Transcribed in Blood Stages of the Parasite—Southern blot analysis of T9/96 genomic DNA was performed using as a probe the cloned 561-bp PCR product. Fig. 2A shows that following digestion of genomic DNA with any of six different restriction enzymes (none of which cleaves within the fragment used as a probe), the probe hybridized strongly to only one DNA fragment in each case. However, even under highly stringent washing conditions, weak hybridization was also reproducibly evident with at least one other DNA fragment in some digests. This result was interpreted as indicating that pfsub-1 is a single copy gene, but that the genome also contains at least one other locus with significant homology to the probe. The 561-bp pfsub-1 fragment was also used to probe blots of P. falciparum chromosomes separated by pulse-field gel electrophoresis (a kind gift of D. Williamson and P. Moore, Division of Parasitology, National Institute for Medical Research); the probe hybridized strongly to a single chromosome, further supporting the existence of a single genomic copy of the pfsub-1 gene (not shown).

A composite cDNA clone containing the complete pfsub-1 ORF was constructed by utilizing the single HindIII restriction site within the gene sequence and the plasmid-derived EcoRI site at the 3′-end of each cloned partial cDNA insert shown in Fig. 1 (top). Partial cDNA clones 8-1 and 10-1 were restricted with HindIII and EcoRI. The 350-bp fragment thus released from clone 8-1 was replaced with the 700-bp HindIII/EcoRI fragment released from clone 10-1. This resulted in plasmid PM481-1, which contains the complete pfsub-1 ORF plus 121 and 81 bp of noncoding sequence, respectively, up-
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stream of the start ATG and downstream of the TAA stop codon (Fig. 1). PCR amplification of the complete pfsub-1 ORF from PM481–1 or total cDNA with primers SUB-EX1 and SUB-EX3 produced a single DNA fragment of 2027 bp. Amplification from T9/96 genomic DNA under the same conditions produced a fragment of the same size, suggesting that the pfsub-1 gene contains no introns. Complete sequencing of the genome derived product confirmed this.

The pfsub-1 cDNA contains no Sau3A site, and the Southern blot data therefore indicated that the whole locus could be isolated on a single Sau3A genomic fragment of about 3.2 kilobase pairs (Fig. 2A, lane 4). This information was utilized in an inverse PCR approach to confirm the beginning and end of the pfsub-1 ORF and to ensure that no rearrangements had occurred in the regions immediately flanking the ORF during cDNA cloning and phagemid excision. Genomic T9/96 DNA was digested with Sau3A and religated under conditions designed to preferentially obtain intramolecular ligation. Two further PCR primers were then used to amplify a DNA fragment of ~1.4 kilobase pairs from the religated DNA. This was cloned and sequenced on both strands. The fragment was found to correspond to the expected inverse PCR DNA product, consisting of sequence including and flanking the ends of the pfsub-1 ORF (Fig. 1, top). The first Sau3A site upstream of the pfsub-1 gene lies precisely 389 bp 5' to the start ATG of the pfsub-1 ORF, and the distance from the TAA stop codon of the ORF to the first downstream Sau3A site is precisely 721 bp. The total length of the Sau3A fragment containing the pfsub-1 gene is therefore 3183 bp, corresponding well to the Southern blot data. No open reading frame other than the ORF was identified within this stretch of DNA.

No RNA species hybridizing to the 561-bp probe was detectable in Northern blot analysis of poly(A+) T9/96 asexual blood stage mRNA (not shown). RNase protection assays, however, allowed detection of the presence of pfsub-1 transcripts in total RNA of asexual blood stage parasites. Plasmid PM494-B, containing the inverse PCR product, was ligared with AavII and used as a template for production of a 422-nucleotide antisense RNA probe using the T7 promoter region of the plasmid. The probe therefore contained 56 nucleotides of plasmid-derived sequence, and a total of 366 nucleotides of sequence derived from the inverse PCR product insert, extending from the position of the SUB-SEQ12 oligonucleotide to a position 121 bp upstream of the first ATG of the pfsub-1 ORF (the AavII site; see Fig. 1, top). Following hybridization of this probe with total RNA isolated from asynchronous asexual blood stage parasites and digestion with RNase, a major protected fragment of about 366 bases was reproducibly detectable by gel electrophoresis and autoradiography (Fig. 2B). This result confirmed the presence of the 5'-noncoding region of the pfsub-1 cDNA in mRNA.

Expression of PfSUB-1 in E. coli and Production of Monospecific Antibodies—pfsub-1 sequences encoding Lys396 to the final His660 residue (the putative propeptide sequence, denoted PfSUB-1pm), or Ser330 to His660 (the predicted mature propeptide domain, PsSUB-1m), were amplified by PCR and cloned into the expression vector pTrcHisB to produce constructs encoding fusion proteins with an N-terminal hexahistidine domain, separated from the insert sequence by an enterokinase cleavage site (Fig. 3). The region encompassing the cleavage site also contains an 8-amino acid residue epitope recognized by a commercially available monoclonal antibody (Anti-Xpress™). When clones PM500-6E and PM500-7A, containing the propeptide and mature propeptide sequences, respectively, of PsSUB-1, were assessed for fusion protein expression following induction by Western blot, using the Anti-Xpress™ monoclonal antibody, fusion proteins of the expected size were detected (not shown, but see Fig. 4); the calculated size of the fusion partner sequence encoded by these constructs is 3659 daltons, so the predicted M₉ of the proprotease PsSUB-1 fusion protein (denoted His₆-PsSUB-1pm) is 78,707, and that of the mature PsSUB-1 fusion protein (denoted His₆-PsSUB-1m; Fig. 3) is 44,073. The latter fusion protein was partially purified by metal chelate chromatography for use in experiments described below. DNA encoding Ser330 to His660 of pfsub-1 was similarly amplified and cloned into the EcoRI site of pGEX-1AT to produce clone PM511–3A. The resulting fusion protein, denoted GST-PsSUB-1m (Fig. 3), was purified to homogeneity and then used to raise anti-PsSUB-1m antiserum in mice; the sera were used to identify and characterize the parasite pfsub-1 gene product.

A number of approaches were taken to look for protease activity in both crude and purified preparations of the fusion proteins, including analysis on gelatin and azocasein SDS-PAGE (37) and examination for hydrolytic activity in solution or in agarose gels against denatured casein, azocasein, gelatin, and hemoglobin (38, 39). Activity against the fluorogenic substrate benzoyl glycine (Bzl-Gly) was also investigated, as was activity against FITC-casein and FITC-bovine serum albumin (39). No protease activity was detected (data not shown).

Identification of pfsub-1 Gene Products in the Malaria Parasite—Fig. 4 shows that in both Western blot and immunoprecipitation analysis of F. falciparum merozoite or schizont extracts, the anti-PsSUB-1m antibodies recognized only two polypeptide species; these migrated at 47 kDa (the major species) and 54 kDa (minor species) on SDS-PAGE under reducing conditions, and the mobility of both proteins was reduced-sensitive. The proteins were denoted p47 and p54, respectively.
Efficient immunoprecipitation of these proteins was obtained only after SDS extraction of parasite preparations; solubilization with TX-100 or sodium deoxycholate alone resulted in poor yields (not shown). To investigate the relationship between these two proteins and to seek further evidence that one or both proteins were products of the pfsub-1 gene, p47 and p54 were immunoprecipitated from extracts of biosynthetically radiolabeled T9/96 schizonts, digested with trypsin or chymotrypsin, and then analyzed by two-dimensional peptide mapping in parallel with similar digests of biosynthetically labeled recombinant His<sub>6</sub>-PfSUB-1<sub>m</sub> gene products. Peptide maps derived from the parasite-derived proteins and the recombinant His<sub>6</sub>-PfSUB-1<sub>m</sub> (Fig. 5) showed high relatedness but were not identical. Given the known differences between these polypeptides (i.e., the presence of approximately 3 kDa of nonmalarial sequence at the N terminus of His<sub>6</sub>-PfSUB-1<sub>m</sub> and the difference in size between the malarial and recombinant proteins), as well as the possibility of differential post-translational modification of the malarial and E. coli-derived proteins, identity was not expected. The maps are, however, sufficiently similar to strongly suggest that p47 and p54 are products of the pfsub-1 gene. Further peptide mapping of radiolabeled in vitro translated pfsub-1 gene products has confirmed this (not shown). Peptide maps of p47 and p54 were virtually indistinguishable (Fig. 5). It was concluded that both proteins are pfsub-1 gene products, probably derived from differential post-translational processing of a full-length precursor protein.

**Fig. 4. Identification of PfSUB-1 in blood stage parasites.** A, T9/96 schizonts were biosynthetically radiolabeled with [35S]methionine/cysteine and then detergent-solubilized and analyzed by immunoprecipitation using a mouse anti-PfSUB-1<sub>m</sub> serum (lane 2), a mouse anti-GST serum (lane 3), or normal mouse serum (lane 4). Immunoprecipitates were subjected to SDS-PAGE under reducing conditions on a 10% gel. Lane 1 contains a sample of the total parasite extract, and the positions of molecular weight markers are shown. The positions of the two proteins (denoted p54 and p47) specifically precipitated by the anti-PfSUB-1<sub>m</sub> antibodies are also indicated. B, PfSUB-1 is expressed in merozoites, and its migration on SDS-PAGE is reduction-sensitive. Lanes 1–5, proteins immunoprecipitated from extracts of metabolically radiolabeled T9/96 schizonts were subjected to SDS-PAGE on a 10% gel in the presence (+) or absence (−) of dithiothreitol as a reducing agent. Lanes 6–11, purified, naturally released T9/96 merozoites (lanes 6–10) or a sample of fusion protein His<sub>6</sub>-PfSUB-1<sub>m</sub> (lane 11) was SDS-solubilized and subjected directly to SDS-PAGE on a 10% gel in the presence (+) or absence (−) of dithiothreitol. The proteins were transferred electrophoretically to nitrocellulose, and the blot was probed with the anti-PfSUB-1<sub>m</sub> antibodies. The migration of both p54 and p47 is reduction-sensitive, indicating the presence of intramolecular disulfide bonds. In addition, the His<sub>6</sub>-PfSUB-1<sub>m</sub> fusion protein was strongly recognized by the anti-PfSUB-1<sub>m</sub> antibodies. The positions of migration of molecular mass markers bovine serum albumin (68 kDa) and ovalbumin (44 kDa) are also shown.

**Fig. 5. Two-dimensional peptide mapping indicates structural similarity between p54/p47 and a recombinant pfsub-1 gene product.** Biosynthetically radiolabeled E. coli-derived recombinant His<sub>6</sub>-PfSUB-1<sub>m</sub> or parasite-derived p54 and p47 proteins were purified by immunoprecipitation and then subjected to digestion with trypsin (T) or chymotrypsin (C). Digests were analyzed by two-dimensional thin layer chromatography using electrophoresis at pH 4.4 in the first dimension (A), followed by ascending chromatography in the second dimension (B). Labeled peptides were detected by fluorography.
then chased in the presence of excess nonradioactive methionine and cysteine. Samples of the culture taken at 0, 5, 15, 30, and 60 min following commencement of the chase were again analyzed by immunoprecipitation using the anti-PfSUB-1\textsubscript{m} antibodies (lanes 1 and 2) or an anti-GST serum as a control (lanes 3 and 4). In BFA-treated parasites, the appearance of labeled p47 is completely blocked, with a concomitant increase in levels of p54. The fluorograph is deliberately overexposed, and the arrows indicate the positions of minor labeled species immunoreactive with the anti-PfSUB-1\textsubscript{m} but not the anti-GST serum. Positions of molecular weight markers are shown.

FIG. 6. \textit{p47 is derived from p54 via a brefeldin A-sensitive step.} Cultures containing mature T9/96 schizonts were resuspended in medium and then divided into two identical 2-ml cultures. To one culture was added 10 \mu l of a 1 mg ml\textsuperscript{-1} solution of BFA in methanol. To the other culture was added 10 \mu l of methanol only. The cultures were incubated at 37 °C for 15 min, and then both were metabolically radio-labeled with \textsuperscript{35}S\textsubscript{S}methionine/cysteine for a period of 2 h. The labeled parasites were then analyzed by immunoprecipitation using the anti-PfSUB-1\textsubscript{m} antibodies (lanes 1 and 2) or an anti-GST serum as a control (lanes 3 and 4). In BFA-treated parasites, the appearance of labeled p47 is completely blocked, with a concomitant increase in levels of p54. The fluorograph is deliberately overexposed, and the arrows indicate the positions of minor labeled species immunoreactive with the anti-PfSUB-1\textsubscript{m} but not the anti-GST serum. Positions of molecular weight markers are shown.

FIG. 7. \textit{Pulse-chase analysis shows that p54 and p47 are derived from higher molecular weight precursors.} Highly synchronous cultures of mature T9/96 schizonts were metabolically pulse-radio-labeled for 9 min with \textsuperscript{35}S\textsubscript{S}methionine/cysteine. A chase was then initiated, and cells were harvested at the indicated times and analyzed by immunoprecipitation. The positions of p54 and p47 are indicated, and positions of larger precursor proteins labeled with arrows. The bands at about 45 and 42 kDa present in all lanes are likely to be non-PfSUB-1-derived proteins co-precipitated or cross-reactive with the anti-PfSUB-1\textsubscript{m} serum used.
characteristics of merozoite dense granules (Fig. 8, bottom). Interestingly, although these organelles are dispersed throughout the merozoite cytoplasm (2), immunoreactivity was observed only with a subset of granules situated toward the apical end of the parasite. This result was entirely consistent with the IFA data showing a predominantly apical reactivity. Since p47 is the major *pfsub-1* gene product detectable in merozoite extracts by Western blot and appears to be the terminal processing product in the parasite, it is likely that this is the species that concentrates in merozoite dense granules.

The presence of p47 within intact secretory organelles of naturally released free merozoites suggested that PISUB-1 is unlikely to be involved in merozoite release from schizonts but that it might play a functional role in either erythrocyte invasion or postinvasion events. A number of reports have shown that dense granule release in apicomplexan parasites occurs predominantly subsequent to, rather than during, invasion (e.g. Refs. 2, 6, and 41–43), and a number of defined dense granule components have been localized to the parasitophorous vacuole of the invaded host cell (e.g. Refs. 44 and 45). Information on the fate of p47 following invasion might provide clues as to the function of the protein. To explore this, metabolically radiolabeled, mature T9/96 schizonts were washed extensively and then either immediately snap-frozen (zero time schizonts) or recultured for 4–6 h, with or without the addition of a 10-fold excess of fresh red blood cells, to allow merozoite release and invasion to take place. Following this, culture supernatants were harvested. The cell pellets from cultures to which additional erythrocytes had been added were then processed by centrifugation on Percoll to remove residual schizonts. The final preparations contained only uninfected erythrocytes and young ring-stage parasites. These samples were then analyzed by immunoprecipitation with the anti-PfSUB-1m antibodies, in parallel with the culture supernatants and zero time schizont samples. Fig. 9 shows that whereas the zero time schizont extracts contained p54 and p47 as expected, only one major immunoreactive species of approximately 43 kDa was detectable in culture supernatants following merozoite release. No immunoreactive proteins were precipitated from ring-stage parasites (not shown). This is consistent with the absence of reactivity with the antibodies in IFA analysis of rings. It is tentatively concluded that p47 can be further processed and shed from merozoites in a truncated, soluble form. It is unclear whether this occurs quantitatively at invasion, but our inability to detect any PISUB-1-derived proteins in the newly invaded erythrocyte may suggest that the protease is not carried into the invaded cell or else that it is rapidly relocalized and/or degraded following invasion. If p47 plays a functional role in erythrocyte invasion, anti-PISUB-1m antibodies might interfere with this and inhibit merozoite entry into erythrocytes *in vitro*. Accordingly, the mouse anti-PISUB-1m sera were tested for their ability to inhibit erythrocyte invasion in cultures
**DISCUSSION**

This report describes the first gene to be identified of any apicomplexan parasite that encodes a serine protease-like protein. We have not directly demonstrated any proteolytic activity associated with any pfsub-1 gene product, but a number of indications lead us to propose that p47 or its truncated, secreted product is likely to be an enzymatically active protease and that it may play a role in erythrocyte invasion.

First, at the primary sequence level PISUB-1 shows significant homology to known subtilases, and possesses all of the features known to be required of an active subtilase. Only four amino acid residues are completely conserved among known subtilases (34); these are the catalytic triad residues (PISUB-1 residues Asp274, His430, and Ser608) and a glycine residue (Gly606 in the PISUB-1 sequence) two positions N-terminal to the active site serine. PISUB-1 also possesses an asparagine (Asn626) at the position of the oxyanion hole residue; the only substitution acceptable at this position is that of an aspartate, found in the PC2 subfamily of kexin-like convertases (34). While many bacterial subtilisins are devoid of cysteine residues, an increasingly large number of subtilases are known to possess up to two intramolecular disulfide bonds within the catalytic domain (34). There are seven cysteine residues within the putative catalytic domain of PISUB-1, and the reduction-sensitive mobility on SDS-PAGE of p47 is consistent with the presence of intramolecular disulfide bonds. Detailed homology-based molecular modeling of the putative PISUB-1 catalytic domain supports this postulate. Thus, at the structural level, PISUB-1 resembles a subtilase.

Second, the proteolytic processing to which PISUB-1 is subjected during secretory transport probably represents a process of enzyme activation common among subtilases. In a number of well studied examples, co-translational signal peptide cleavage allows folding of the subtilase catalytic domain, mediated by the intramolecular chaperone activity of the pro-domain (36, 48). This is often (but not always) rapidly followed by autocatalytic cleavage of the pro-domain from the catalytic domain; this cleavage can take place in cis (intramolecular) or in trans (intermolecular) and in some cases simple pro-domain cleavage is sufficient to allowzymogen activation (49, 50). However, there is a growing body of evidence that subtilase activation is often a substantially more complex process, which in eu-
karyotes may be intimately linked to correct routing of the proenzyme through the secretory pathway and final compartmentalization of the active enzyme (51–54). Here we have shown that the primary pfsub-1 gene product is subjected to at least two major post-translational processing steps. The first of these, conversion of the 82-kDa form, possibly via a 60/61-kDa intermediate, to the p54 form, takes place very rapidly following synthesis and therefore could represent an autocatalytic processing step triggered by signal peptide cleavage and cotranslational folding within the lumen of the ER. The p54 form is then quantitatively converted to p47 in a slower, BFA-sensitive process. Further work is required to establish whether either or both of these processing events represent enzyme activation, but certainly they are wholly consistent with a putative activation pathway. If so, the process is clearly more complex than a simple one-step pro-domain removal. BFA sensitivity is a common feature of transport to secretory organelles in apicomplexan parasites (55–59), but little is known about the structural requirements for correct sorting to these organelles (59); it is possible that part of the PISUB-1 sequence could provide a targeting signal, as has been determined for other subtilases and yeast carboxypeptidase Y (52, 60).

Third, the terminal intracellular PISUB-1 processing product, p47, localizes to a subpopulation of dense granule-like organelles within the extreme apical domain of the merozoite and appears to be shed in a truncated, soluble form following merozoite release but before completion of erythrocyte invasion; no PISUB-1-derived proteins were detectable in the newly invaded erythrocyte. These observations are in apparent conflict with the majority of the available data suggesting that dense granule secretion in apicomplexan parasites is a postinvasion event (2, 6, 41–44). However, dense granules in Plasmodium are currently defined essentially on morphological criteria; there is a paucity of specific markers for these organelles, and indeed only two other malarial proteins, RESA/PI155 and a 14-kDa protein denoted RIMA, have been previously localized to merozoite dense granules (41, 61, 63). It is therefore possible that distinct subpopulations of dense granules may exist in Plasmodium; there is evidence for this in the apicomplexan parasite Cryptosporidium parvum (64), and if so, these subpopulations may be functionally distinct and may undergo exocytosis at different stages of the invasion process. Our evidence that PISUB-1 is released at invasion suggests that it could play a role in the process of erythrocyte entry. Anti-PISUB-1p antibodies had no inhibitory effect on erythrocyte invasion by released merozoites in vitro, but it is unclear whether these antibodies have access to PISUB-1 in the intact merozoite or whether they can interfere with its function. The demonstration of enzymatic activity in native, merozoite-derived p47 or its truncated soluble product would be a step toward addressing this issue. Consistent with observations of other workers (65), we have not been able to detect activity corresponding to p47 on gelatin substrate SDS-PAGE. However, the enzyme may be irreversibly denatured by exposure to SDS, and the insolubility of p47 in nonionic detergents precludes ready isolation and analysis of the merozoite-derived protein in a native state. These limitations could be overcome by recombinant expression of enzymatically active PISUB-1, and this is a major priority of current work.

If PISUB-1 is involved in erythrocyte invasion, what precise role might the enzyme perform? The best characterized proteolytic event associated with erythrocyte invasion is the processing and shedding of a merozoite surface protein complex derived from the precursor protein MSP-1. A single proteolytic cleavage at a Leu-Asn motif within the membrane-bound component of this complex, known as secondary processing, releases the complex quantitatively from the surface of the merozoite as it enters the host erythrocyte (16–18, 29). MSP-1 secondary processing is thought to be essential for successful erythrocyte invasion, and it is mediated by a parasite-derived, calcium-dependent serine protease (16, 17). Interestingly, this activity is exquisitely sensitive to inhibition by PMSF, but it is only poorly inhibited by even very high concentrations (up to 10 mM) of DFP (19). Given its apparent nonreactivity with DFP, its location in the merozoite, and the timing of its secretion, PISUB-1 is a good candidate for this activity. This intriguing possibility clearly merits further investigation and is supported by molecular modeling of the proposed catalytic domain of PISUB-1, which has indicated that a heptapeptide corresponding to the sequence flanking the MSP-1 secondary processing site (i.e., Gln-Gly-Met-Leu-Asn-Ile-Ser) fits well into the active site groove of the model, with the Leu-Asn scissile bond in the appropriate position for cleavage. The only other serine protease activity previously localized to P. falciparum merozoites is a DFP-reactive enzyme thought to be activated following cleavage of a GPI anchor (14); it is unlikely that PISUB-1 corresponds to this activity, since it shows no DFP reactivity and the sequence exhibits C-terminal hydrophobic domain typical of GPI signal sequences (66).

Of the two major families of active site serine endoproteases, the chymotrypsin-like and the subtilisin-like families (32), chymotrypsin-like proteases are common in higher eukaryotes but appear to be rare in lower eukaryotes and prokaryotic organisms. Sakranari et al. (67) have reported evidence for the existence of a chymotrypsin-like gene in Trypanosoma cruzi, but there is no other previous genetic data on protozoal serine proteases. Extensive attempts in this laboratory to isolate chymotrypsin-like P. falciparum genes using a number of different PCR-based approaches have been unsuccessful. It is conceivable that, like Saccharomyces cerevisiae (40), the malaria parasite (and perhaps other apicomplexan parasites) does not possess genes of this class. Proteases play a crucial role in the life cycle of the blood stage malaria parasite and are undoubtedly potential targets for the design of protease inhibitor-based drugs. Expression of PISUB-1 in an enzymatically active form should allow the development and evaluation of such inhibitors.

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