Plasmodium falciparum subtilisin-like protease-1 (PfSUB-1) is a protein belonging to the subtilisin-like superfamily of serine proteases (subtilases). PfSUB-1 undergoes extensive posttranslational proteolytic processing. The primary translation product is converted in the parasite endoplasmic reticulum to p54. This is further processed to p47, which accumulates in secretory organelles within the merozoite. Here, we present a detailed study of this process. In vitro translated PfSUB-1 showed no capacity to undergo autocatalytic processing. However, parasite extracts contain a protease that cleaves the in vitro translated pro-protein between Asp219 and Asn220 to form two products of 31 (p31) and 54 kDa; the latter was indistinguishable from authentic p54 and remained complexed with p31 in a noncovalent interaction characteristic of that between a subtilase prodomain and its cognate catalytic domain. Cross-linking studies showed that this complex also exists in the parasite. Expression of PfSUB-1 in recombinant baculovirus also resulted in processing to p54. Mutation of the predicted active site serine abolished processing. Recombinant p54 was secreted in a complex with p31, and could be further converted to p47 in vitro. Conversion required calcium, was an intramolecular autocatalytic process, and involved a second cleavage between Asp251 and Ala252. A decapeptide based on sequence flanking Asp219 was efficiently cleaved by recombinant PfSUB-1. We conclude that PfSUB-1 is a subtilase with an unusual substrate specificity and that it is activated by two autocatalytic processing steps.

Malaria is caused by parasitic protozoa of the genus Plasmodium. The clinical disease and associated pathology is a direct result of replication of the parasite within host red blood cells. Invasion of red blood cells by the malaria merozoite is prevented by certain serine and cysteine protease inhibitors (1, 2), and merozoite proteases appear to play a crucial role in invasion by restructuring the erythrocyte surface or cytoskeleton (3–5). In addition, a number of merozoite surface proteins undergo extensive proteolytic modification around the point of erythrocyte invasion (6, 7). The best characterized example of this is that of merozoite surface protein-1 an abundant, stably expressed surface protein that is subjected to proteolytic processing and shedding during invasion. The function of the processing step is unclear, but it is essential for invasion (7, 8). Merozoite surface protein-1 processing is mediated by a merozoite serine protease, and we recently identified a Plasmodium falciparum protein called PfSUB-1,1 belonging to the subtilisin-like serine protease superfamily, or subtilases (9), which is expressed in a subset of secretory granules within the apical domain of the merozoite (10). Discharge of these organelles is known to occur at around the point of invasion. PfSUB-1 therefore very likely plays a role in invasion and is a good candidate for the protease that mediates merozoite surface protein-1 processing. PfSUB-1 is also of interest because it represents the prototypic member of a novel class of eukaryotic subtilases with substantial homology to prokaryotic subtilisins (10–12). Proteases that mediate important roles in the life cycle of pathogenic microorganisms represent good targets for the development of protease inhibitor-based drugs. A full understanding of the mode of activation, structure, and physiological function of PfSUB-1 is essential to evaluate its potential as a therapeutic target.

Subtilases are synthesized as enzymatically inactive zymogens, activation of which invariably requires one or more proteolytic cleavages of the precursor (13). We have previously shown that the primary pfsub-1 gene product undergoes two major intracellular posttranslational processing steps. The first of these, conversion of the earliest detectable 82-kDa translation product to a 54-kDa form (called p54), takes place rapidly following translation and, by analogy with other systems, was proposed to represent an autocatalytic processing step possibly triggered by signal peptide cleavage and co-translational folding within the lumen of the parasite endoplasmic reticulum (ER). In a second, brefeldin A (BFA)-sensitive process, p54 is then quantitatively converted to a 47-kDa terminal intracellular processing product (p47). Both p54 and p47 contain the predicted catalytic domain of PfSUB-1 (10). Although p47 may represent the mature, proteolytically active form of PfSUB-1, it is a poorly abundant merozoite protein and is

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1 The abbreviations used are: PfSUB-1, P. falciparum subtilisin-like protease-1; BFA, brefeldin A; ER, endoplasmic reticulum; DSP, dithio-bis(succinimidylpropionate); PAGE, polyacrylamide gel electrophoresis; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; pHMB, p-hydroxymercuribenzoate; PMSF, phenylmethylsulfonyl fluoride; HPLC, high pressure liquid chromatography; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

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insoluble in the absence of denaturants or ionic detergent. As a result, it was not possible to demonstrate any proteolytic activity associated with the parasite-derived protein (10). Nonetheless, PISUB-1 possesses all the primary structural requirements for protease activity, and the processing profile of the protein during secretory transport resembles a process of enzyme maturation. To understand the requirements for expression of proteolytically active PISUB-1, we set out to further characterize these posttranslational modifications.

Here, we present the results of a detailed study of the fate of PISUB-1 as it traverses the secretory pathway of the malaria parasite. Using an in vitro translation-based model, analysis of posttranslational processing of PISUB-1 in the parasite, and studies of enzymatically active recombinant PISUB-1, we show that the proteolytic processing to which PISUB-1 is subjected exhibits characteristics of subtilase zymogen activation, although with some novel features, and results in the production of an enzymatically active subtilase with an unusual substrate specificity.

**EXPERIMENTAL PROCEDURES**

**Materials**—All restriction endonucleases, proteinase K, and BFA were obtained from Roche Molecular Biochemicals. RNasin, T7 polymerase, and other in vitro translation reagents were from Promega. Translation grade [35S]methionine, [3H]isoleucine, and [35S]methionine/cysteine (Pro-mixTM) was from Amersham Pharmacia Biotech. The glycosenaccharide acceptor peptide Bz-Asn-Gly-Thr-NH2 (Bz-NGT\( \text{NH}_2 \)) was from Bachem. Dithiobis(succinimidylpropionate) (DSP) and N-ethylmaleimide was from Pierce. Protease inhibitors p-hydroxymercuribenzoate (pHMB), EDTA, EGTA, phenylmethylsulfonyl fluoride (PMSF), and 4(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) were obtained from Sigma, as was tunicamycin. High FiveTM insect cells were from Invitrogen, and insect cell media were from Life Technologies, Inc. and Expression Systems (Woodland, CA). The metal affinity chromatography resin Ni-NTA agarose was from Qiagen.

**Culture and Metabolic Radiolabeling of F. falciparum, Immunoprecipitation, and Peptide Mapping of PISUB-1**—Blood-stage cultures of the T9/96 clone of *F. falciparum* were maintained in vitro in human A+ erythrocytes and synchronized when required, as described previously (14, 15). Biosynthetic radiolabeling of parasite cultures in the presence or absence of BFA with [35S]methionine/cysteine or translation grade (14, 15). Biosynthetic radiolabeling of parasite cultures in the presence or absence of BFA with [35S]methionine/cysteine or translation grade [35S]methionine/cysteine or translation grade [35S]methionine (Promega), used as recommended. N-Glycosylation of PISUB-1 in these reactions was inhibited by the addition of the glycosaccharide acceptor peptide Bz-NGT\( \text{NH}_2 \) (16): peptide was added to a final concentration of 1 mM, 5 min prior to addition of transcription. In vitro translated proteins were visualized by fluorography or autoradiography following analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10–12.5% gels with or without the presence of 4 μg/mL in both stacking and resolving gels.

To study posttranslational stability in *in vitro* translated protein, completed translation reactions were supplemented with nonradioactive methionine to 1 mM and then incubated further at 30–37 °C for up to 16 h prior to analysis by SDS-PAGE. The susceptibility of proteins to protease digestion following translation in the presence or absence of microsomal membranes was assessed by the addition of protease K to 1 mg/mL in the absence or presence of 0.8% (v/v) Triton X-100. Following digestion for 40 min on ice, reactions were terminated by the addition of 1 mM PMSF prior to analysis by SDS-PAGE and fluorography.

**Production and Activity of P. falciparum “Processing Enzyme” Preparations**—Asynchronous cultures of T9/96 blood stage parasites were washed and saponin-lysed as described previously (14). The parasite pellet was supplemented with an equal volume of 2% (v/v) Triton X-100 in phosphate-buffered saline and gently mixed at 4 °C for 30 min. The sample was then sonicated for 30 s in a water bath (Kerry K5 100) and centrifuged at 20,000 × *g* for 40 min × *g* at 4 °C. A proportion of the Triton X-100-insoluble pellet was then further extracted in an equal volume of 5 M CHAPS in 20 mM Tris-HCl, pH 7.6, for 30 min at 4 °C. Following sonication for 30 s, the CHAPS extract was clarified by centrifugation at 40,000 × *g* for 60 min at 4 °C. The Triton X-100-soluble and -insoluble fractions and the CHAPS-soluble fraction were stored at −70 °C in aliquots.

Aliquots of parasite extract were supplemented with *in vitro* translated PISUB-1-(iv) or PSUB-1-(iv) and incubated in reaction buffer (final concentration, 20 mM Tris-HCl, 50 mM NaCl, 10 mM CaCl2, pH 7.6) for 2–120 min at 37 °C. The effect of protease inhibitors on processing activity was determined by incubation of inhibitors with the parasite extracts for 10 min on ice prior to the addition of *in vitro* translated protein. In some experiments, the *in vitro* translated protein was denatured by overnight incubation in 0.5 mM Tris-HCl, 100 mM dithiothreitol, 8 μM urea, pH 7.4, at 4 °C and then alkylated by addition of 200 mM iodoacetamide in the dark for 30 min at room temperature. The sample was dialyzed against 50 mM Tris-HCl, pH 7.4, at 4 °C for 4 h prior to incubation with parasite extracts. Reactions were stopped by boiling in SDS sample buffer and then analyzed by SDS-PAGE and fluorography using phosphatidyl pChol-1.
CAPS, 10% methanol, pH 11. Radiolabeled proteins were visualized by autoradiography and then excised and subjected to Edman degradation in an Applied Biosystems 477A protein sequencer. The elute from each cycle was collected and the radioactive content measured by scintillation counting.

Production of Antibodies Specific for the Extreme C-terminal Domain of PfSUB-1—A 25-residue-long synthetic peptide, IKSKTYIN-SNISNKWKKSRRYLC, corresponding to the extreme C-terminal end of PfSUB-1 with the addition of a C-terminal cysteine residue was coupled to maleimide-activated keyhole limpet hemocyanin (Piecer) following the recommendations of the manufacturer. The peptide conjugate, called ST-24, was used to raise antisera in mice using standard procedures (18).

Purification and in Vitro Conversion of Recombinant p54 to p47—Details of expression and purification of recombinant PfSUB-1 are described elsewhere (19). Briefly, High Five™ cells were infected with recombinant baculovirus in the presence or absence of tunicamycin at 0.5 μg ml⁻¹ and cultured in ESF 921 protein-free medium. Secreted recombinant protein (bp54-His₆) was purified by a combination of affinity and ion-exchange chromatography and stored in aliquots at −70 °C. For conversion of bp54-His₆ to bp47-His₆ in vitro, purified bp54-His₆ (1 ml) was thawed and incubated on ice with 100 μl of converting buffer (20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 12 mM CaCl₂). This mixture was then supplemented with 10% (v/v) glycerol and 10 mM CaCl₂ and then incubated at 37 °C or on ice for various periods. Following the incubation period, the beads were washed four times in the same buffer, and then the conversion products were eluted with 250 mM imidazole in 20 mM Tris-HCl, 500 mM NaCl. Eluted protein was stored in aliquots at −70 °C.

Assessment of Protease Activity in Recombinant PfSUB-1 and Electro spray Mass Spectrometric Analysis of PEP1 Digestion Products—The synthetic peptide N-acetyl-LVSAHIDNIS-Oh (PEP1) was dissolved in Me₂SO to 40 mM. For assays of protease activity, 5 μl of the PEP1 solution or 5 μl of in vitro translated PfSUB-1pm(iv) (Fig. 2A) was added to 85 μl of converting buffer (20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 12 mM CaCl₂). This mixture was then supplemented with 10 μl of purified recombinant bp54-His₆ (containing approximately 7.5 μg of protein) or 10 μl of buffer only. In some experiments, the assays were also supplemented with protease inhibitors. Samples were taken immediately or after incubation at 37 °C for up to 6 h for analysis by SDS-PAGE and fluorography (PfSUB-1pm(iv) digests) or reverse-phase HPLC (PEP1 digests). Samples (20 μl) of PEP1 digest were analyzed on a Vydac 4.6-mm × 25-cm C₁₈ reverse-phase column, eluted at 0.5 ml min⁻¹ with a 9–36% (v/v) gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid. Samples of the digestion products were collected manually, dried under vacuum, and then dissolved into 60% (v/v) acetonitrile, 0.1% formic acid and analyzed on a Micromass Platform single quadrupole mass spectrometer (Micromass UK Ltd., Altringham, United Kingdom). The electrospray ionization interface was heated to 70 °C, and electrospray ionization mass spectra were obtained at a sampling cone voltage of 30 V. Samples were flow-injected at a flow rate of 10 μl min⁻¹.

RESULTS

The PfSUB-1 Proprotein Does Not Undergo Autocatalytic (Intramolecular) Processing in Vitro—In a number of well documented examples, activation of subtilase zymogens is initiated following secretory signal peptide removal in the ER or periplasmic space, by an autocatalytic (i.e. intramolecular) proteolytic processing event at the junction between the pro-domain and the catalytic domain of the proprotein. During PfSUB-1 secretory transport in the malaria parasite, the earliest detectable 82-kDa translation product undergoes rapid conversion to p54 in the parasite ER (10). We used an in vitro translation-based approach to mimic and study this process. Translation in vitro of the entire pfsub-1 open reading frame (construct PM481) or a truncated sequence lacking the predicted signal peptide encoding region (construct pSub1pm) produced a major polypeptide product in each case of approximately 82 kDa; the products are referred to as PfSUB-1pm(iv) and PfSUB-1pm(iv), respectively (Figs. 1 and 2A). Products of in vitro translation are given the suffix “(iv)” to distinguish them from the authentic, parasite-derived proteins. In addition to the major product, a number of minor translation products of about 70 kDa were also seen, probably derived from translation initiation at internal ATG motifs. The mobility on SDS-PAGE of PfSUB-1pm(iv) and PfSUB-1pm(iv) were almost identical (not shown; the predicted molecular mass of the PfSUB-1 signal peptide sequence is only about 2.8 kDa). However, a small but reproducible difference in size of the two translation products was more readily detectable on gels containing 4 M urea, a system that can resolve proteins that are of similar mass but that exhibit different hydrophobicity characteristics (20) (Fig. 2B; compare lanes 4 and 6). Translation of PfSUB-1pm(iv) in the presence of canine microsomal vesicles resulted in a heterogeneous but substantial increase in molecular mass of the translation product, probably due to translocation of the product into the lumen of the microsomal vesicles and its resulting N-glycosylation (Fig. 2B, lane 2). This was not an unexpected finding because the predicted PfSUB-1 sequence contains 11 potential N-glycosylation sites (10). Consistent with this interpretation, no shift in molecular mass occurred upon translation of PfSUB-1pm(iv), which lacks the predicted signal peptide sequence, in the presence of microsomes. Similarly, the additional presence of the glycocascharide acceptor peptide Bz-NGGT-NH₂ during translation of PfSUB-1pm(iv) in the presence of microsomes completely prevented the appearance of the higher molecular weight forms of the translation product and resulted in a product that co-migrated on urea gels with PfSUB-1pm(iv) (Fig. 2B, compare lanes 5 and 6), also suggesting that translocation was concomitant with signal peptide cleavage. In protease protection experiments (not shown), PfSUB-1pm(iv) produced in the presence of microsomes was found to be completely resistant to proteinase K digestion; this resistance could be reversed by the addition of 1% (v/v) Triton X-100 (not shown). These results indicate that the authentic PfSUB-1 signal sequence successfully functions to direct translocation of the in vitro translated protein into microsomes, as has been demonstrated for other malarial signal sequences (21, 22).

P. falciparum blood-stage proteins are known to be N-glycosylated only rarely, if at all (23–25). The predicted molecular mass of the PfSUB-1 proprotein is about 75 kDa (10), but the authentic, parasite-derived protein migrates on SDS-PAGE as

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an 82-kDa polypeptide. Because both PISUB-1 pm(iv) and translated, unglycosylated PISUB-1 pm(iv) also migrated with this mobility, we consider that the migration of the proprotein on SDS-PAGE is slightly aberrant due to its amino acid content or asymmetric charge distribution (Fig. 1), and it is unlikely that PISUB-1 is extensively glycosylated in the parasite. In view of this, and in order to mimic as closely as possible the structure of PISUB-1 immediately following translocation into the parasite ER in vivo, all subsequent work investigating maturation of in vitro translated PISUB-1 made use of either PISUB-1 pm(iv) translated in the presence of both microsomes and the glycosylation inhibitor Bz-NGT-NH$_2$, or of PISUB-1 pm(iv) produced in the absence of microsomes.

A series of time course experiments were performed to investigate the ability of nascent PISUB-1 to undergo autocatalytic processing. Completed in vitro translation-translocation reactions of unglycosylated PISUB-1 pm(iv) were supplemented with an excess of nonradioactive methionine and then monitored over a period of up to 16 h at 30 or 37 °C in order to observe the fate of the microsomal protein. Under all conditions explored, the protein showed remarkable stability to further degradation or processing (not shown), and no spontaneous conversion to any product resembling p54 was evident. The addition of Ca$^{2+}$ at concentrations of up to 5 mM in the presence or absence of 1% (v/v) Triton X-100 or adjusting the pH of the samples to pH 6.8, 7.6, or 8.2 also had no effect on the stability of the protein. Identical results were obtained with translation reactions of PISUB-1 pm(iv) produced in the absence of microsomes (not shown). These results suggest that, even following translocation into the luminal environment of mammalian microsomes and signal peptide removal, in vitro translated PISUB-1 has no capacity to undergo autocatalytic processing.

**Processing in Trans of PISUB-1**—We next explored the possibility that conversion of nascent PISUB-1 to p54 may be mediated by a trans-acting processing activity in the malaria parasite. Blood-stage malaria parasites from in vitro cultures were extracted into nonionic or zwitterionic detergents. Preparations of PISUB-1 pm(iv) were then supplemented with the crude extracts, and the subsequent stability of the protein was monitored upon incubation at 37 °C. Incubation of PISUB-1 pm(iv) with the Triton X-100-soluble fraction of a parasite extract had no effect. However, incubation with either the Triton X-100-insoluble fraction or a CHAPS extract of this fraction resulted in disappearance of the primary translation product and the generation of two novel polypeptides of 31 and 54 kDa (Fig. 3, lanes 2 and 3). These are referred to as p31(iv) and p54(iv). No conversion to any product resembling p47 was seen, even after prolonged incubation under a variety of different conditions of calcium concentration and pH (not shown). Identical results were obtained following addition of the CHAPS extracts to translated, unglycosylated PISUB-1 pm(iv) (not shown). Reduction and alkylation of the PISUB-1 pm(iv) prior to incubation with the parasite extract resulted in its complete degradation (not shown), suggesting that the specificity of the conversion was dependent upon the tertiary conformation of PISUB-1 pm(iv). The p54(iv) product co-migrated precisely on SDS-PAGE with authentic p54 immunoprecipitated from metabolically labeled parasites (Fig. 3, lanes 1, 3, and 4). The relationship between the various polypeptides was investigated by two dimensional tryptic peptide mapping. As shown in Fig. 4, p54(iv) and p31(iv) are both structurally related to PISUB-1 pm(iv), but not to each other. Peptide maps of p54(iv) and parasite-derived, authentic p54 were identical. The simplest interpretation of these results is that p54(iv) and p31(iv) are derived from distinct, nonoverlapping domains of the PISUB-1 pm(iv) precursor as a result of a single endoproteolytic cleavage event. The close structural similarity between p54(iv) and authentic p54 suggests that the specificity of this cleavage was similar or identical to that responsible for conversion of PISUB-1 to p54 in vivo. These results also confirm that p54 is not subject to substantial glycosylation in the parasite.

One possible interpretation of these data is that the parasite extracts contain a factor or cofactor required for activation of an autocatalytic processing event mediated by PISUB-1 pm(iv). To address this possibility, a mutant version of construct pSub1 pm was prepared, in which the codon for the predicted active site serine residue, Ser$^{608}$, was mutated to encode an alanine residue (Fig. 1). In vivo translation of this sequence generated a major product of approximately 82 kDa, designated PISUB-1 pm-SA(iv), which was indistinguishable on SDS-PAGE from PISUB-1 pm(iv) (not shown). On incubation with the parasite extract, PISUB-1 pm-SA(iv) was converted to 31- and 54-kDa products with the same kinetics as PISUB-1 pm(iv) (not shown). It was concluded that the parasite extracts contain an endoprotease activity that can act in trans to mediate conversion of the PISUB-1 proprotein to p54.

**The 31-kDa Product of PISUB-1 Processing Remains Noncovalently Associated with p54**—Previous work has shown that p54 encompasses the catalytic domain of PISUB-1 (10). Be-
cause our results indicated that the 31-kDa product of PISUB-1 primary processing is derived from a distinct region of the sequence, the possibility arose that it might represent the PISUB-1 prodomain. Subtilase prodomains are generally thought to be required for correct folding of the protein, but also often mediate additional functions, including that of acting as transient, tight-binding inhibitors of enzyme activity that are thought to be required for correct folding of the protein, but also often mediate additional functions, including that of acting as transient, tight-binding inhibitors of enzyme activity, e.g. denaturing detergents. Nonetheless, to support the validity of our in vitro observations, it was considered necessary to attempt to demonstrate that such a complex forms in vivo. Conversion of p54 to p47 in vivo is completely blocked by BFA (10) and so presumably requires export of either p54 alone or the intact p54/p31 complex into a post-ER compartment. We reasoned that metabolic radiolabeling of malaria parasites in the presence of BFA should result in the accumulation of “trapped” complex in the ER and that it might be possible to then chemically cross-link this complex in situ, using a suitable membrane-permeable bifunctional reagent, to an extent sufficient to visualize it. Schizonts were biosynthetically radiolabeled in the presence of BFA and then washed, and the intact cells were immediately treated on ice with various concentrations of the membrane-permeable, cleavable cross-linker DSP. The parasites were then solubilized with SDS and analyzed by immunoprecipitation with the anti-PISUB-1 m antibodies. Fig. 5 shows that immunoprecipitates of DSP-treated parasites, but not of untreated parasites, contained a minor 31-kDa species that co-migrates precisely with p31(iv). Visualization of this band required prolonged fluorographic exposures, suggesting that the efficiency of cross-linking was poor. This is perhaps not surprising given the fact that access to the intracellular complex probably required the DSP to cross at least four membranes. We propose that the 31-kDa band is the in vivo homologue of p31(iv). These experiments provide persuasive evidence that the PISUB-1 processing step and complex formation observed in our in vitro system mirrors a process that occurs in vivo.

Inhibitor Profile of the Protease Responsible for Primary Processing of PISUB-1, and Identification of the Primary Processing Site—Our results suggested that the first step of PISUB-1 maturation in the malaria parasite can be mediated in trans by an endogenous endoprotease activity. To further characterize this activity, we examined its sensitivity to a number of protease inhibitors. The convertase activity was heat-labile and was completely inhibited by treatment with 5 mM pHMB, but it was unaffected by exposure to 10 mM EDTA, 10 mM EGTA, 1 mM PMSF, 1 mM AEBSF, or 1 mM N-ethylmaleimide (not shown).

To shed light on the specificity of the enzyme involved, it was of interest to determine the actual site at which cleavage occurs during conversion of PISUB-1 to p54. The extremely low abundance of p54 in the malaria parasite meant that it was not feasible to obtain sufficient native p54 for conventional N-terminal sequence analysis. Advantage was therefore taken of the in vitro system. PISUB-1 monomer translated in the presence of [3H]isoleucine was incubated with parasite extract and the p54(iv) processing product analyzed by N-terminal radiolabeling. In each of three completely independent experiments, a unique profile of labeled species was obtained in the first 13 cycles of Edman degradation, consistent with hydrolysis occurring between Asp213 and Asp220 within the sequence Leu-Val-Ser-Ala-Asp-Asn-Ile-Asp-Ile-Ser (Fig. 6). The predicted Mr of the PISUB-1 primary sequence extending from Asn220 to the C-terminal His690 residue is 53,123, so this result is in approximate agreement with the apparent molecular mass of p54.

Conversion of p54 to p47 involves a second processing event at the N terminus of p54—The second stage of PISUB-1 mat-
Peptide mapping of processing products. Parasite-derived p54 was purified by immunoprecipitation with the anti-PfSUB-1m antiserum from extracts of schizonts metabolically radiolabeled with translation grade \(^{35}\text{S}\)methionine. PfSUB-1m(iv) was produced by \textit{in vitro} translation in the presence of \(^{35}\text{S}\)methionine, and the p54(iv) and p31(iv) processing products obtained by digestion with a parasite extract as described under “Experimental Procedures.” All samples were subjected to SDS-PAGE and then excised from the gel and subjected to in-gel digestion with trypsin. Digesta were analyzed by two-dimensional TLC 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 fluoroxygraphy.

Recombinant Expression of PfSUB-1; Conversion to p54 in Vivo Is Autocatalytic—To further study PfSUB-1 processing, the protein was expressed in a recombinant baculovirus/insect cell system. Detectable levels of expression were only obtained following reexpression of the entire \textit{pfsub-1} gene to obtain a coding sequence distinct from the extremely A+T rich sequence of the native gene (10) and to obtain an overall codon usage more suitable for expression in insect cells. Gene synthesis, expression, and purification of the recombinant protein is described in detail elsewhere (19). The synthetic gene (called \textit{pfsub-1 synth} ) incorporated an additional five histidine codons just prior to the stop codon, thus encoding a C-terminal hexahistidine structure (the C-terminal residue of wild type PfSUB-1 is a histidine) that was designed to be used to purify the protein by immobilized nickel affinity chromatography. In all other respects, the amino acid sequence encoded by the synthetic gene—including the putative secretory signal sequence—was identical to the wild type gene. Fig. 7 shows the results of an analysis of culture supernatants from insect cells infected with recombinant baculovirus expressing either \textit{pfsub-1 synth} or a second, mutant version of the synthetic gene in which the codon for Ser\(^{608}\) was altered to encode alanine (\textit{pfsub-1 synth S\Delta A}). Under standard culture conditions, infection with either recombinant virus resulted in secretion of an approximately 96-kDa protein reactive with the anti-PfSUB-1m antibodies (Fig. 7A). In the presence of low levels of tunicamycin, an inhibitor of N-glycosylation, the results were dramatically different. Infection with the \textit{pfsub-1 synth} recombinant baculovirus resulted in secretion of a 54-kDa protein, referred to as bp54-His\(_6\) (Fig. 7A), which migrated on SDS-PAGE with only a very slightly lower mobility than parasite-derived p54 (Fig. 7B); bp54-His\(_6\) was also easily detectable in extracts of the infected cells (not shown), indicating that it was the result of an intracellular processing event. In contrast, infection with the \textit{pfsub-1 synth S\Delta A} virus in the presence of tunicamycin resulted in the secretion of an anti-PfSUB-1m-reactive protein of about 82 kDa, the same size as the parasite-derived PfSUB-1 protein (Fig. 7A).

These results were interpreted as follows. Under conditions in which N-glycosylation is inhibited, expression of PfSUB-1 in the insect cells results in its conversion during secretory transport to the equivalent of p54; this is secreted into the culture medium without further processing. Mutation of the predicted active site Ser\(^{608}\) completely abolishes this processing step, strongly suggesting that in the insect cell expression system, conversion of the PfSUB-1 precursor to bp54-His\(_6\) is autocatalytic. The slight difference in size between bp54-His\(_6\) and parasite p54 can be accounted for by the presence of the additional
Markers are indicated.

Fig. 5. In situ cross-linking shows that p54 and p31 form a complex in vivo, and Anti-ST-24 antibodies recognize both p54 and p47. Schizonts metabolically radiolabeled in the presence of BFA were washed and treated with 10% Me2SO only (lane 2) or 1 or 2.5 mM DSP in 10% Me2SO (lanes 3 and 4, respectively). The cells were then solubilized into SDS and processed for immunoprecipitation with the anti-PSUB-1m serum. The position of the co-precipitating 31-kDa polypeptide that appears in lanes 3 and 4 as a result of treatment with the cross-linker is indicated by an arrow. Lanes 1 and 5 contain identical samples of total PSUB-1m(iv) following in vitro digestion with a parasite detergent extract. The fluorograph is deliberately overexposed. Lane 6, parasite cultures were metabolically radiolabeled in the absence of BFA and then extracted into SDS and processed for immunoprecipitation using the mouse antiserum raised against a peptide corresponding to the extreme C-terminal 24 residues of the PfSUB-1 open reading frame (anti-ST-24). The antibodies clearly recognized both p54 and p47, as well as the PSUB-1 precursor. Positions of molecular mass markers are indicated.

FIG. 6. N-terminal radiosequencing of p54(iv). [3H]Isoleucine-labeled PSUB-1m(iv) was incubated with parasite detergent extract to obtain the p54(iv) band and p31(iv) digestion products. Following SDS-PAGE and transfer to ProBlot membranes, the region of the blot containing the p54(iv) band was excised and subjected to Edman degradation. Phenylhydantoin products were collected and analyzed by scintillation counting. Shown is the result of a single experiment; the yield of radioactivity (in cpm) obtained with the eluate from each cycle is shown below each residue of the unique amino acid sequence motif within PSUB-1 consistent with the labeling profile.

Fig. 7. Autocatalytic processing of PSUB-1 in recombinant baculovirus-infected insect cells. A, High Five insect cells were mock-infected or infected with recombinant baculovirus expressing pfsub-1synth or the mutant pfsub-1synthΔA gene, in the presence or absence of tunicamycin (Tun). Culture supernatants were harvested 72 h postinfection and analyzed by Western blot using the anti-PSUB-1m antibodies to probe the blot. The position of the correctly processed recombinant bp54-His6 product is indicated by an arrow. A, analysis by Western blot of a sample of culture medium containing secreted bp54-His6, and an SDS-extract of purified T9/96 merozoites. The blot was again probed with anti-PSUB-1m antibodies. The positions of migration of parasite-derived p54 and p47 are indicated by arrows. Positions and sizes (in kDa) of molecular mass markers are indicated.

...glycosylation upon translocation into the insect cell ER (the deduced PSUB-1 sequence possesses a total of 11 potential N-glycosylation sites (10)), resulting in a substantial increase in molecular mass. Presumably as a result of this glycosylation, processing of the pfsub-1synth product is completely blocked, although secretion of neither this nor the product of the pfsub-1synthΔA gene is prevented. It is not clear why glycosylation should have such a marked inhibitory effect on processing of the proprotein, but because PSUB-1 is not subject to N-glycosylation in the parasite, inappropriate glycosylation in this heterologous system may cause aberrant folding of the molecule or may result in deleterious modifications to catalytically important residues. In this regard, it is worth noting that Asn522, the predicted oxyanion hole residue of Pf-...
analysis of the minor 82-kDa product also yielded the sequence Lys-Glu-Val-Arg-Ser-Glu. This corresponds to the N terminus of the predicted PISUB-1 proprotein following signal peptide removal at the predicted Gly\(^{29}\) cleavage site (10), and therefore this 82-kDa species probably represents a minor population of proprotein molecules that have been secreted without undergoing processing to the 54-kDa form. The fact that the 31-kDa co-purifying species also possessed this N-terminal sequence allowed its identification as the recombinant version of p31(iv)—that is, the proposed prodomain moiety derived from the N-terminal domain of PISUB-1. The N terminus of the 30-kDa species represents Glu\(^{40}\) of the PISUB-1 sequence; it is therefore probably a further processing product, or more likely a degradation product of the 31-kDa protein. Both species are hereafter referred to collectively as bp31.

These data indicate that under conditions in which N-glycosylation of the malarial protein is prevented, expression of PISUB-1 in the insect cell system results in the secretion of recombinant p54 in an apparently stable complex with its cognate prodomain. The stoichiometry of this association was not explored further here, although we would predict that the complex is in the form of a heterodimer. Although the staining of the bp31 bands in the purified preparations appears substantially less than that of the bp54-His\(_{6}\), this may in part be due to the unusually hydrophilic nature of the prodomain (Fig. 1 and Ref. 10). Indeed, this may have other consequences; our results indicate that the primary sequence of the prodomain extends from Lys\(^{26}\) to Asp\(^{219}\), a predicted molecular mass of only 21,960 Da. The mobility of p31 on SDS-PAGE is therefore clearly highly anomalous, an often observed characteristic of hydrophilic malarial proteins (27, 28).

Conversion in Vitro of Recombinant p54 to p47—Prolonged storage of the purified bp54-His\(_{6}\) preparations at 4 °C resulted in noticeable degradation (not shown), indicating the presence of a protease activity. To explore this possibility further, and to investigate whether the activity might reside in the recombinant protein, the following experiment was performed. Purified protein was rebound in batch form to fresh Ni-NTA agarose beads on ice. The immobilized protein was extensively washed, and then the beads were incubated at 37 °C in a buffer containing 10 mM CaCl\(_2\). Fig. 8A, lane 2, shows that under these conditions, there was clear conversion of the bp54-His\(_{6}\) to a major smaller product of about 47 kDa. Conversion was associated with a decrease in the intensity of the bp31 bands. This is seen more clearly in Fig. 8B, which shows a time course of the conversion process. Conversion was temperature- and time-dependent, being about 50% complete after an 8-h incubation period, and could be completely inhibited in the presence of 2 mM pHMB (Fig. 8B). In further experiments (not shown), the conversion was found also to be prevented by the presence of 10 mM EGTA but was not inhibited by the serine protease inhibitors AEBSF or PMSF; the presence of up to 10 mM \(\beta\)-mercaptoethanol also had no effect on the efficiency of conversion. The 47-kDa species migrated on SDS-PAGE only slightly more slowly than parasite-derived p47 (not shown), and in Western blot analysis, it was also reactive with the anti-ST-24 antibodies (not shown); it was therefore referred to as bp47-His\(_{6}\). N-terminal sequence analysis of bp47-His\(_{6}\) yielded the sequence Ala-Glu-Asp-Tyr-Asp, showing that the conversion event involved cleavage of bp54-His\(_{6}\) between Asp\(^{219}\) and Asp\(^{251}\). In addition to conservation of the aspartate residue, the cleavage product appears to be more similar to a serine, a valine, and an aspartate at the P8, P4, and P2 positions, respectively.

Protease Activity in Trans of PISUB-1—The sequences flanking internal autocatalytic processing sites within subtilases often mimic those that act as physiological substrates of the activated enzymes in trans (9, 13, 31). It was decided to determine whether bp54-His\(_{6}\) or its processed product possesses protease activity in trans, using substrates containing one or both of the sequences flanking the identified internal processing sites. Fig. 10 shows the results of incubating purified bp54-His\(_{6}\) with the radiolabeled in vitro translation product PISUB-1\(_{3pm}\). The labeled protein was rapidly and quantitatively cleaved to form two polypeptides with the characteristics of p54 and p47.
and p31. Interestingly, as in the case of conversion in the presence of schizont extracts, no further processing to a product resembling p47 was seen. As in the case of the activity detected in parasite extracts, processing was inhibited by 2 mM HMB but was unaffected by 10 mM EGTA or EDTA, or the serine protease inhibitors PMSF and AEBSF. In further experiments comparing the activity of purified bp54-His_{6} with similar amounts of protein that had been partially converted to bp47-His_{6}, while immobilized on Ni-NTA agarose, no quantitative difference in the amount or rate of trans processing activity could be discerned (not shown). However, the interpretation of such experiments was complicated by the fact that the bp54-His_{6} preparations always contained low levels of bp47-His_{6}. Fig. 10B shows the results of incubation of purified bp54-His_{6} with a synthetic peptide, N-acetyl-LVSADNIDIS (PEP1) based on the sequence flanking Asp^{219} (Fig. 9). PEP1 was specifically cleaved into two products that could be readily resolved by reverse-phase HPLC. Cleavage could be completely prevented by the addition of pHMB to 2 mM, and in the absence of added bp54-His_{6}, the peptide was completely stable over the period of incubation. The digestion products were collected, pooled, and analyzed by electrospray mass spectrometry. Charged ions of only two products were detectable (not shown), with masses of 544.8 ± 0.5 and 559.9 ± 0.5 Da. These correspond closely to the species that would be obtained by cleavage of PEP1 at the Asp-Ala bond, generating the products Ac-LVSAD (calculated mass, 545.3 Da) and NIDIS (calculated mass, 560.3 Da). In further preliminary experiments using a number of peptide substrates unrelated to PEP1 and ranging in size from 4 to 11 residues long but with no internal aspartate residues, no endopeptidase activity was detectable (not shown). Further work is clearly required to determine the fine specificity of PISUB-1, but these data show conclusively that PISUB-1 is an endoprotease with an apparent preference for cleavage on the C-terminal side of certain aspartate residues.

**DISCUSSION**

We have studied in detail the posttranslational processing of a *P. falciparum* merozoite subtilisin-like protein and have obtained direct biochemical evidence that the product of this processing is an enzymatically active protease. Our results suggest that PISUB-1 processing may represent a process of activation or maturation, analogous to that seen with many subtilases, although with some unusual features that merit further discussion.

The primary step of PISUB-1 maturation in the malaria parasite, conversion to p54, is BFA-insensitive, indicating that it takes place in the ER. To test the hypothesis that this might be an autocatalytic process, we used an *in vitro* translation model to examine the fate of newly synthesized PISUB-1. Observations in both prokaryotic and eukaryotic systems suggest that signal peptide removal may be a prerequisite for subtilase activation (32, 33). We therefore endeavored to mimic this process in two ways: by obtaining translocation into microsomal vesicles in the presence of an inhibitor of N-glycosylation and by expressing a truncated protein lacking the leader peptide domain. Under no conditions was spontaneous processing of the *in vitro* translated protein detected. In contrast, conversion to p54 could be efficiently reproduced when PISUB-1 was expressed in insect cells in the presence of tunicamycin. We have not determined here the intracellular compartment in which processing takes place, but the fact that it was completely prevented by mutation of the predicted catalytic serine residue is convincing evidence that in this recombinant system at least, conversion to p54 is autocatalytic. It is likely that the same holds true in the parasite. What then is the explanation for the apparent lack of catalytic activity associated with *in vitro* translated PISUB-1? One possibility is that fundamental differences exist between the environment of the mammalian microsome *in vitro* and that of the malarial or insect cell ER *in vivo*. Critically important chaperones or “foldases” (34–37) may not be represented in the canine microsome, and even subtle differences in, for example, ionic composition or pH might be important determinants in obtaining correct folding of the proprotein such that the catalytically important residues adopt...
their proper spatial conformation or such that the active site groove is correctly positioned to mediate intramolecular cleavage (26). Our findings are not unprecedented; prodomain cleavage of the proprotein convertases furin and PC2—a process rigorously demonstrated to be autocatalytic in vivo (13, 38, 39)—cannot readily be reproduced in the reticulocyte lysate/canine microsome in vitro system (40, 41), although a Xenopus egg-derived cell-free system supports full maturation of PC2 (41–43). On the other hand, the in vitro translated PfSUB-1 products studied here clearly possessed tertiary structure, as evidenced by the discrete and authentic nature of the processing profile observed following exposure to parasite extracts. This cleavage was mediated in a highly specific manner by a Triton X-100-insoluble parasite protease activity; the specificity of this activity, its sensitivity to pHMB, and its insensitivity to chelating agents, PMSF, and AEBSF, are characteristics shared by the activity associated with the baculovirus-derived bp54-His6 recombinant. The parasite extract activity therefore probably represents endogenous PfSUB-1. Taken together, our results indicate that the primary step of PfSUB-1 maturation in the malaria parasite is an autocatalytic processing event, which may be mediated in trans.

Despite the fact that the in vitro translated PfSUB-1 products were catalytically inactive, cleavage of PfSUB-1 pm(iv) at Asp219 by the parasite-derived activity resulted in a p54 product that remained in a remarkably stable complex with its prodomain. A similar complex was detectable in the parasite, and expression of PfSUB-1 in insect cells resulted in secretion of a recombinant form of the same complex. The recombinant complex was capable of spontaneous conversion to p47; in the parasite, conversion involved a further cleavage near the N terminus of p45, at Asp251. We propose that conversion of bp54-His6 to bp47-His6 is an in vitro representation of p54 processing as it transits the secretory pathway of the malaria parasite. There are clearly some significant differences between the recombinant model and authentic PfSUB-1 maturation, perhaps the most striking of which is the relatively slow rate of bp54-His6 to bp47-His6 conversion in vitro; processing of p54 to p47 in trans parasite is quantitative and rapid, going virtually to completion within approximately 20 min (10). It is also not clear why conversion of bp54-His6 to bp47-His6 apparently does not take place at all within the insect cell secretory system. However, it is widely recognized that the secretory system of the blood-stage malaria parasite is unusual in a number of respects; these include the apparent absence of N-glycosylation for most of the parasite life cycle and the absence of morphologically recognizable stacked cisternae typical of the Golgi apparatus (44, 45). Processing of p54 to p47 in trans may be triggered by an environmental change (such as an increase in free calcium concentration or a pH shift) associated with further transport along the malarial secretory pathway, as seen in the case of some mammalian subtilisin-like convertases (13, 39, 42). Conversion of bp54-His6 to bp47-His6—but apparently not enzymatic activity in trans—was sensitive to EGTA, indicating that it is calcium-dependent. The pH dependence of the process was not investigated here. In vivo, conversion of p54 to p47 takes place via an intramolecular mechanism. An alternative, trans processing mechanism cannot be ruled out, but the lack of any detectable conversion of the in vitro translation product PfSUB-1 pm(iv) to a 47-kDa form in the presence of bp54-His6 argues against this. It will be of interest to determine whether a peptide corresponding to the sequence flanking Asp251 acts as a substrate for the recombinant enzyme.

Is conversion of p54 to p47 an activation event? An unambiguous evaluation of the relative activities in trans of bp54-His6 and bp47-His6 was rendered impossible by the fact that preparations of purified bp54-His6 were always contaminated by small amounts of bp47-His6, presumably as a result of gradual conversion during or prior to purification. However, parallels with other systems would suggest that p54 complexed to its p31 prodomain is likely to be proteolytically inactive in trans. In the only previous case in which it has been possible to isolate a stable, correctly processed prodomain-catalytic domain complex—that of the association between the catalytic domain of a S221C active-site mutant form of Bacillus subtilis subtilisin E and its propeptide (48)—structural analysis of the complex showed that that after prodomain cleavage the C-terminal Tyr177 residue of the prodomain moiety remains in the P1 active site binding pocket in a product-like manner, thus blocking access to exogenous substrates. Structural analysis of the bp31/bp54-His6 complex may provide a unique opportunity to gain useful insights into the interaction between a eukaryotic subtilase and its cognate prodomain, as well as shedding light on the mechanism by which conversion to p47 is mediated. The fact that the complex can be very effectively stabilized by treatment with pHMB has encouraged us to make this aim an immediate priority. Unambiguous evaluation of whether the bp31/bp54-His6 complex possesses enzymatic ac-
tivity in trans might be enabled by appropriate mutagenesis of the Asp$^{251}$ cleavage site so as to specifically abolish conversion of bp45-His$_5$ to bp47-His$_5$. Our results showed that conversion of bp45-His$_5$ to bp47-His$_5$ was associated with loss of the bp31 prodomain. Prodomain removal is a prerequisite for activation of other subtilases (13, 26), and cleavage at Asp$^{251}$ may require prior dissociation of the prodomain; alternatively, dissociation may occur as a result of the second processing step (Fig. 11). A third possibility is that conversion to p47 may involve complete prodomain degradation, as in the case of subtilisin. If p54 to p47 conversion is an activation event, it represents yet another variation on the increasingly diverse set of possible mechanisms by which subtilase activation in eukaryotes can be accomplished.

PISUB-1 is a prototypic member of a new subclass of subtilases that includes a second merozite protein, called PISUB-2 (11, 12). The most closely related mammalian subtilase appears to be a membrane-bound ER-resident protease called Site-1 protease (31) or SKI-1 (49), which is notable because of its unique specificity among mammalian subtilases for cleavage after a threonine, lysine, or leucine residue (31, 49). The apparent specificity of active PISUB-1 for cleavage after aspartate is equally unusual. At present, it sheds no light on the biological function of the protease, although it does suggest that PISUB-1 is unlikely to mediate secondary processing of merozite surface protein-1, because this is known to involve cleavage of a Leu-Asn bond (50). Interestingly, PISUB-2, which is expressed as a large putative type I integral membrane protein, also undergoes a two-step processing during secretion (12); the second of these, but not the first, is BFA-sensitive, suggesting that subtilase processing in the malaria parasite may be controlled and compartmentalized in a common manner. PISUB-1 maturation may thus act as a model for subtilase activation in this medically important group of protozoa.

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