The Merozoite Surface Protein 1 Complex of Human Malaria Parasite Plasmodium falciparum

INTERACTIONS AND ARRANGEMENTS OF SUBUNITS*

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The major protein component at the surface of merozoites, the infectious form of blood stage malaria parasites, is the merozoite surface protein 1 (MSP-1) complex. In the human malaria parasite Plasmodium falciparum, this complex is generated by proteolytic cleavage of a 190-kDa glycosylphosphatidylinositol-anchored precursor into four major fragments, which remain non-covalently associated. Here, we describe the in vitro reconstitution of the MSP-1 complex of P. falciparum strain 3D7 from its heterologously produced subunits. We provide evidence for the arrangement of the subunits within the complex and show how they interact with each other. Our data indicate that the conformation assumed by the reassembled complex as well as by the heterologously produced 190-kDa precursor corresponds to the native one. Based on these results we propose a first structural model for the MSP-1 complex. Together with access to faithfully produced material, this information will advance further structure-function studies of MSP-1 that plays an essential role during invasion of erythrocytes by the parasite and that is considered a promising candidate for a malaria vaccine.

Merozoites, the erythrocyte invading form of malaria parasites, uniformly expose at their surface a major protein complex, the merozoite surface protein 1 (MSP-1).1 In Plasmodium falciparum, the parasite causing the most severe form of malaria in humans, MSP-1 is synthesized as an approximately 190-kDa precursor protein, which is deposited at the surface of the developing merozoite via a glycosylphosphatidylinositol anchor (1). In late schizogony, merozoites undergo maturation during which MSP-1 is proteolytically cleaved into four major fragments (2), which, however, remain non-covalently associated at the surface of the parasite. At the time of erythrocyte invasion, a second proteolytic cleavage separates the approximately 10-kDa glycosylphosphatidylinositol-anchored C terminus of MSP-1, called p19, from the rest of the complex, and only the membrane-bound portion is transferred into the newly infected erythrocyte while the remaining complex is shed from the surface of the parasite (3, 4).

Several lines of evidence demonstrate that MSP-1 plays an essential role in the life cycle of the parasite and that it is involved in the erythrocyte invasion process. Thus, attempts to knock out the msp-1 gene in P. falciparum via homologous recombination failed, whereas the same approach allows to replace the functionally conserved C-terminal end of the molecule by a sequence of a distantly related Plasmodium species (5). Moreover, several monoclonal antibodies directed to the C-terminal p19 fragment inhibit efficiently erythrocyte invasion in vitro (6). These antibodies also prevent the secondary cleavage of MSP-1 during invasion (7). At least, this latter proteolytic step appears, therefore, to be an essential prerequisite for the infection of erythrocytes. Interestingly, antibodies were identified that can block the effect of invasion inhibiting antibodies in vitro (8). However, it is not clear to which extent these blocking antibodies play a role in vivo. MSP-1 is also targeted by the human immune response and particularly the C-terminal region has been shown to elicit protective humoral immunity in various rodent systems but also in monkey models (for review see Ref. 9). MSP-1, particularly its C-terminal portion, is therefore considered a promising candidate for the development of a malaria vaccine.

Despite all this information, we know little about mechanistic aspects of MSP-1 function and about its structure. Analysis of the primary sequence of MSP-1 from different P. falciparum isolates has revealed that several regions in the molecule are highly conserved, whereas major portions are dimorphic belonging to either the K1 or MAD20 prototype. In addition, one notices two small oligomorphic blocks (10, 11). The most thoroughly investigated portion of MSP-1 is the C-terminal p19 fragment generated during the secondary proteolytic cleavage. Its sequence is highly conserved among P. falciparum isolates, but it also exhibits a remarkable structural and functional conservation across species of malaria parasites (5). It is folded into two epidermal growth factor-like domains stabilized by six disulfide bonds, and for p19 of P. falciparum the three-dimensional structure has been solved by NMR spectroscopy (12) while the respective structure of p19 from Plasmodium cynomolgi was elucidated by x-ray analysis (13). Little structural or functional information is available for the residual around 95% of MSP-1, although it is well documented that it interacts with other proteins at the surface of the parasite such as MSP-69 (14) and MSP-72 (15, 16). Moreover, it has been suggested that merozoites contact the erythrocyte surface via MSP-1 (17).

In the past, more thorough biochemical studies of MSP-1 of P. falciparum were hampered by experimental difficulties encountered when attempting to heterologously produce this complex protein. For example, the high AT content of 76–78% of the respective parasite genes prevented their stable cloning and, thus, their heterologous expression in good yields, a prob-

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1 The abbreviations used are: MSP-1, merozoite surface protein 1; GST, glutathione S-transferase; DTT, dithiothreitol; mAb, monoclonal antibody; BSA, bovine serum albumin.

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lem solved only recently by synthetic genes encoding the proteins in different codon compositions (18, 19). Moreover, as MSP-1 is not glycosylated in the parasite despite numerous potential glycosylation sites (20), a prokaryotic expression system would be of advantage. Recovering, however, a 190-kDa protein containing numerous disulfide bonds in its proper conformation, e.g. from *Escherichia coli*, generally poses an experimental challenge.

Here, we describe experiments aimed at the elucidation of the overall structure of the MSP-1 complex of *P. falciparum* strain 3D7 (henceforth designated MSP-1D). We particularly focus on the arrangements of the subunits within the processed complex and their mutual interactions. Using synthetic polynucleotides encoding MSP-1D and processing fragments thereof, we recovered the various proteins in highly purified and soluble form from *E. coli*. These materials enabled us to reassemble *in vitro* the MSP-1 complex from its subunits and to probe its conformation in comparison to the unprocessed MSP-1 precursor isolated from *E. coli* as well as from parasites. Based on our data, we propose a structural model for MSP-1, which will now allow addressing more specifically longstanding questions concerning structure-function relationships of this intriguing protein.

**EXPERIMENTAL PROCEDURES**

**Cloning and Expression of msp-1D Sequences—**Sequences encoding the MSP-1D processing fragments p83, p30, p38, and p42 (21) were amplified by PCR using a synthetic gene coding for msp-1 from *P. falciparum* strain 3D7 (19). The DNAs encoding p83/30 and p38/42 were generated accordingly. All PCR products were confirmed by sequencing. Unique Clal cleavage sites at the 5′ ends of the PCR products allowed their cloning downstream of glutathione S-transferase (GST) or His tag encoding sequences within appropriately modified pZE13 expression vectors (22). Similarly, unique XbaI or PstI sites at the 3′ ends were used for downstream fusions of His6 or Strep (IBA, Germany) tags. Some sequences were fused to produce proteins containing tags on either end (Fig. 1). The pZE13 derivative was transferred to *E. coli* strain W3110Z1 (22) where the expression of the target gene is tightly controlled via isopropyl-1-thio-β-D-galactopyranoside.

**Purification of p190 and Fragments Therefrom from *E. coli* Extracts—**Full size p190 fused N-terminal to GST and C-terminal to His6 (pg190H) was expressed in soluble form in *E. coli* at 25 °C and purified by Ni2+-chelate chromatography following by separation on GSH-Sepharose (Amersham Biosciences). A GMP compatible production process and the thorough characterization of the resulting protein preparation will be described in detail in a forthcoming publication.

MSP-1 fragments p83, p30, p38, and p42 were prepared from inclusion bodies as described (23). Inclusion bodies of the respective proteins were solubilized in buffer 1 (50 mM Tris, pH 8.0, 4 mM guanidine hydrochloride, 3 mM β-mercaptoethanol, 10 mM imidazole) and applied to a Ni2+-chelate column equilibrated with buffer 1. After washing with buffer 2 (buffer 1 containing 15 mM imidazole), proteins were eluted with buffer 3 (buffer 1 containing 300 mM imidazole).

Fragments p83/30 and p38/42 were also recovered from *E. coli* as inclusion bodies. The p83/30 material was dissolved in buffer 1 before it was subjected to Ni2+-chelate chromatography. After washing with buffer 2, proteins were eluted with buffer 3. The eluted material was refolded by dialysis against 50 mM Tris, pH 8.0, 0.5 mM arginine, 5 mM DTT, 1 mM EDTA. After subsequent dialysis against 50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM DTT, the protein was adsorbed on streptactin-Sepharose (IBA, Germany) and eluted with the same buffer containing 5 mM n-dodecylbuthadiol.

Protein p38/42, recovered as inclusion body, was dissolved under denaturing conditions and refolded by pulse renaturation as described previously (23). The renatured protein was applied onto a Ni2+-chelate column equilibrated with 50 mM Tris, pH 8.0, 300 mM NaCl, 10 mM imidazole. After a first (50 mM sodium phosphate, pH 6.4, 1 M NaCl, 10% glycerol, 0.1% N-lauroylsarcosine) and a second (50 mM Tris, pH 8.0, 300 mM NaCl, 50 mM imidazole) washing step, the protein was eluted with 50 mM Tris, pH 8.0, 300 mM NaCl, 250 mM imidazole. The fractions containing p38/42 were applied to an affinity column containing monoclonal antibody (mAb) 5.2 fixed to Sepharose via Protein A, equilibrated with 50 mM Tris, pH 8.0, 300 mM NaCl, 1 mM EDTA. After washing with 50 mM Tris, pH 8.0, 650 mM NaCl, 1 mM EDTA, the protein was eluted with 0.1 M glycine, pH 2.5. The eluate was immediately neutralized with 0.2 volumes of 1 M Tris, pH 8.0. Protein content of eluted fractions was determined by Bradford assay.

**Purification of Full-length MSP-1 from Merozoites—**Parasite-derived MSP-1 was isolated from the trophozoite/early schizont fraction of synchronized parasitized red blood cells. Late schizonts were removed by passing the culture through a column for magnetic cell separation (MCE-5M; Miltenyi Biotech). The parasitized red blood cells were lysed by 0.01% saponin and MSP-1 was purified by mAb 5.2 specific affinity chromatography (see above) as described previously (24).

**Assembly of MSP-1 Complexes from Subunits—**For coexpression of two msp-1 sequences in *E. coli*, pZEO3 and pZEO13 (25) were used, which because of their different antibiotic markers, could be simultaneously expressed in *E. coli*. In both vectors, the target gene was inserted in the isogroup-1-thio-β-D-galactopyranoside. For assembly of MSP-1 complexes via corenaturation of subunits, 0.5 mg of solubilized affinity purified MSP-1 fragments were mixed at a concentration of 0.1 mg/ml each in 50 mM Tris, pH 8.0, 4 mM guanidine hydrochloride, 300 mM imidazole, and dialyzed overnight at 4 °C against refolding buffer (0.5 M arginine, 50 mM Tris, pH 9.0, 1 mM GSH, 0.1 mM GSSG, 1 mM EDTA) followed by a dialysis against phosphate-buffered saline, pH 7.4, for 16 h at 4 °C.

Association of purified and renatured MSP-1 subunits was examined by incubating 0.2 mg of the respective proteins usually in the presence of a 6-fold excess of BSA in phosphate-buffered saline, pH 7.4, for 2 h at room temperature or for 8 h at 4 °C. In all three approaches, complex formation was monitored by Ni2+-chelate chromatography by pressing the various tags or the mAb 5.2 epitope as described above. Respective fractions were analyzed by SDS-PAGE followed by Coomassie or immunostaining.

**Thrombin Cleavage of MSP-1, p190, p83/30, and p38/42—**For thrombin proteolysis, purified *E. coli*-derived proteins at concentrations between 0.1 and 0.3 mg/ml or native MSP-1 were incubated with thrombin (Roche Diagnostics) at a ratio of 2 units/100 μg of protein at 25 °C in phosphate-buffered saline, pH 7.4. At different time points, 100-μl samples were removed, mixed with 5× SDS sample buffer with or without 100 mM EDTA, and immediately heated to 100 °C for 5 min. Aliquots of these samples were analyzed by SDS-PAGE. To examine the interaction of MSP-1 subunits after thrombin cleavage, the digestion products were subjected to affinity chromatography and analyzed by SDS-PAGE as described above.

**Microsequencing and Peptide Fingerprint—**For microsequencing of the MSP-1 cleavage products, the proteins were blotted onto a nitrocel- lulose membrane and stained with a solution containing 0.1% Amido Black and 2% acetic acid. The membrane was destained with methanol until the protein bands became visible. The fragments of interest were excised and analyzed on a protein sequencer (Applied Biosysms) using standard techniques. Peptide fingerprints were performed by subjecting the excised protein fragment to trypsin digestion followed by mass spectroscopic analyses of the resulting cleavage products. When- ever possible, the method was used to determine molecular weights of polyptides, coverage was around 40%. **Antibodies—**Mab 5.2 (25) was prepared from the hybridoma cell line HB9143 (ATCC); Mab 7.6, Mab 7.5, Mab 2.2, Mab 12.8, and Mab 12.10 (2) were generous gifts from Dr. Jana McBride. Sera against p83, p30, and p42 were obtained by immunizing rabbits intramuscularly with 100 μg of the respective recombinant protein purified from *E. coli* and emulsified with complete Freund's adjuvant. Animals were boosted at days 21 and 42 with the same antigen dose in incomplete Freund's adjuvant. The sera were used harvested 10 days after the second boost.

**RESULTS**

**Experimental Strategy—**The mature MSP-1 complex is generated by *in situ* proteolytic cleavage of a large surface anchored precursor protein. The interactions between some of its subunits may, therefore, be restrained by parameters that have governed the folding of the unprocessed molecule at the surface of the parasite and by interactions of the complex with other surface proteins. To study the interactions between heterologously produced MSP-1 subunits, we have, therefore, followed three experimental approaches: (i) coexpression of MSP-1 fragments in *E. coli*; (ii) corenaturation of fragments isolated from *E. coli* extracts; and (iii) incubation of stoichiometric mixtures of isolated and separately renatured fragments. The first two procedures were assumed to allow the proteins to interact in an...
Complexes formed by combining various MSP-1 fragments were isolated by affinity chromatography exploiting either tags, such as GST, specific for only one of the participating proteins, or the unique epitope within the C terminus of p42 recognized by mAb 5.2. The affinity purified material was then further analyzed by SDS-PAGE.

To delineate potential protease accessible linker regions and more compact domains within MSP-1, thrombin appeared to be a suitable enzyme because the primary sequence of MSP-1D contains around 80 sequence motifs susceptible to thrombin cleavage. A quantitative evaluation of resulting digestion patterns would, in addition, provide information on the structural homogeneity of respective protein preparations.

**Synthesis in E. coli and Isolation of MSP-1 and MSP-1 Processing Fragments**—Synthetic DNA sequences encoding the entire MSP-1 (without the N-terminal 19 amino acid signal peptide) or various processing fragments thereof were placed in expression vectors of the pZ plasmid family (22), where their expression could be controlled by isopropyl-1-thio-β-D-galactopyranoside. The coding sequences were modified at the 3' and 5' ends to produce proteins fused to His<sub>6</sub>, GST or Strep tags, respectively, as shown in Fig. 1. Full size MSP-1 fused N-terminal to GST and C-terminal to His<sub>6</sub> (pg190h) was expressed in E. coli and induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. The coding sequences were modified at the 3' and 5' ends to produce proteins fused to His<sub>6</sub>, GST or Strep tags, respectively, as shown in Fig. 1. Full size MSP-1 fused N-terminal to GST and C-terminal to His<sub>6</sub> (pg190h) was expressed in E. coli and induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside.

The eluate did not contain p83, indicating an efficient interaction between p83 and p38/42. Together, these results indicate that the p38/42 complex can be assembled from its two halves refolded in vitro and that no folding intermediate of the precursor is required for this association.

**p38/42 Interacts with p30 but Not with p83**—To delineate more closely the domains responsible for the interaction between the MSP-1 subunits, we investigated first whether p83 or p30 alone may bind to the C-terminal half. Accordingly, p30 and p38/42 were simultaneously produced in E. coli, and indeed a complex containing p30 and p38/42 can be recovered in good yields by GSH affinity chromatography (Fig. 2D). The analogous experiment with p83 and p38/42 yielded, however, no clear result because of the instability of p83 in E. coli (data not shown). Therefore, purified and separately renatured preparations of p83 and p38/42 were incubated together in the presence of BSA before the mixture was subjected to mAb 5.2-specific immunoaffinity chromatography. As shown in Fig. 2E, the eluate did not contain p83, indicating an efficient interaction between p83 and p38/42. Together, these results indicate that p30 but not p83 interacts with p38/42.

**Interaction between Individual MSP-1 Fragments**—To investigate which of the naturally occurring primary processing products of MSP-1 are the major interaction partners within the mature complex and how they may cooperate in complex stabilization, we analyzed the association between various purified and denatured proteins when refolded together. This approach allowed us to trap associated proteins via affinity chromatography as described above. The results of these experiments, of which a representative sample is summarized in Fig. 3, show that p83 and p42 do not interact to any significant degree (Fig. 3A). By contrast, p30 shows a distinct affinity to p42 (Fig. 3D) that appears, however, not as strong as the association between p42 and p38 (Fig. 3C) as well as between p83 and p30 (Fig. 3D). Coexpression of p30 and p38 revealed an efficient interaction between these two subunits. These data
are consistent with results shown in Fig. 2 where p83/30 but not p83 would interact with p38/42. The latter result is also supported by experiments in which p42, p38, and p83 were coexpressed in E. coli, and where the resulting complex did not contain p83 (data not shown). Several controls, for simplicity not shown here, are consistent with these results. For example, incubation of separately renatured p30 with p42 followed by GSH chromatography reveals the p30/p42 complex, whereas when omitting pg30 no p42 is recovered from the column etc.

**Assembly of the Intact MSP-1 Complex from Its Major Processing Products**—The above experiments demonstrate that specific complexes can be assembled by corenaturation of various MSP-1 fragments. We, therefore, attempted to reassociate the entire MSP-1 from its major processing products p83, p30, p38, and p42. Indeed, when the proteins in their denatured form were mixed and renatured together, a substantial fraction of the material is recovered by mAb 5.2 specific immunoaffinity chromatography indicating the formation of a complex that contains all four processing fragments. The specificity of the affinity chromatography applied was examined by corenatur- ing p83, p30, and p38 with p33. As the latter protein is missing the C-terminal portion of p42 carrying the 5.2 epitope, a respective complex is not expected to be retained as shown in Fig. 4A. All experiments described here were carried out with MSP-1 fragments derived from *P. falciparum* strain 3D7, a representative of the MAD20 prototype. To examine whether some of the structural motifs responsible for the subunit interactions may be conserved between MSP-1D and MSP-1F from the FCB-1 strain, a representative of the K1 prototype, we coexpressed p83, p30, and p38 from strain 3D7 with p42 of MSP-1F. As the epitope of mAb 5.2 is located in the highly conserved C-terminal region, our standard affinity chromatography could be applied for the identification of the complex. Fig. 4B shows that the heterologous complex is indeed efficiently formed.

**Examining the Structure of MSP-1 by Proteolysis with Thrombin**—To probe the conformation of MSP-1 as well as the homogeneity of our protein preparations, we exposed in a first experiment isolated and renatured p83/30 and p38/42 as well as full size p190 to thrombin. All these protein preparations were converted into defined sets of fragments revealing all together six cleavage sites of which three are within p83/30 and p38/42, respectively (Fig. 5). Moreover, the cleavage products were generated in nearly stoichiometric amounts. These re-
sults demonstrate that (i) the vast majority of the roughly 80 thrombin cleavage motifs are inaccessible for the enzyme and (ii) that our preparations consist of homogeneously folded proteins. Analysis of the cleavage products obtained from p83/30 and p38/42 digests by Edman degradation allowed us to identify and map all the fragments via their N-terminal sequence (Fig. 6A). The analysis also showed that, as expected, all cleavages occurred C-terminal to arginine or lysine residues and with one exception in regions predicted to be unstructured (Fig. 6B). The identity of various fragments was further confirmed by Western blot analysis and mass spectroscopy (data not shown).

Considering the time course of the various thrombin digests reveals cleavage pathways as depicted in Fig. 5. Accordingly, p83/30 is rapidly digested into three major products, an N-terminal fragment A1, a middle fragment A2a, and fragment A2b covering the C-terminal part of p83 and the entire p30. Upon prolonged incubation, A1 is further processed into fragment A1a and a 6-amino acid long peptide. Digestion of p38/42, which contains 34 preferred target sites for thrombin, results in three major cleavage products (Fig. 5B); the N-terminal fragment B1, fragment B2a, and B2b containing the C terminus of p38 and the entire p42. After around 16 h, B2a processing fragments B2a1 and B2aII become visible.

When purified p190 is exposed to thrombin, a digestion pattern is obtained (Fig. 5C) that can be interpreted on the basis of the above results: A1a, A2a, B2a, and B2b are generated like in digests of p83/30 and p38/42, respectively. Moreover, the novel fragment C1a is expected as it covers fragments A2b and B1 (Fig. 6), which, in the non-processed form of MSP-1, are covalently linked. Thus, the p190 cleavage pattern can be derived from the combined patterns of p83/30 and p38/42 indicating that with the exception discussed below no cleavage site is obscured by the association of the two halves within the MSP-1 complex. This conclusion has been verified by thrombin digestion of the p83/30-p38/42 complex (data not shown, see also Fig. 7C).

Fully digested p190 contains an unexpected additional product, C1 (Figs. 5C and 6), which is not further digested even upon prolonged incubation (data not shown). It is the fusion between C1a and B2a as shown by mass spectroscopy. It reveals alternative cleavage pathways for the full size p190 as shown in Fig. 5, C and D: whenever thrombin cleaves first between C1 and B2b, its target site between C1a and B2a becomes obscured. By contrast, when proteolysis occurs first between C1a and B2a, processing between B2a and B2b will take place. Together, these data indicate that the unprocessed p190 exhibits toward thrombin largely the same conformation as the two halves of the molecule probed individually or as a reassembled complex.

To probe the conformation of MSP-1 recovered from parasites with thrombin, MSP-1 from early schizonts of the 3D7 strain of P. falciparum was prepared, subjected to thrombin digestion, and analyzed by SDS-PAGE followed by Western blot. The patterns obtained (Fig. 5D) show that all fragments arising when E. coli-derived p190 is treated in an identical way are also detected with the native material. They include A1/A1a, A2a, B2a, B2b, C1, and C1a although A1/A1a may not have been identified beyond doubt, as it appears to co-migrate with material also detected by α-p42 serum (Fig. 5D). Our antibodies reveal within the preparation of native MSP-1 numerous bands, which we have not identified in detail of which most appear to be degradation products of MSP-1 and possibly aggregates thereof. The salient feature of this experiment is, however, that thrombin releases from MSP-1 material isolated from parasites all detectable cleavage products identified under respective conditions with E. coli-derived protein. It is particularly interesting that the fragment C1a is generated as well indicating that the alternative cleavage pathway outlined in Fig. 5C is also followed with the native protein. Together, these data strongly support the view that the conformation of our heterologously prepared MSP-1 closely resembles the native one.

**Interactions between Thrombin Cleavage Products**— Obviously, analyzing associations between thrombin cleavage products of MSP-1 would allow a more accurate delineation of regions responsible for the interaction between MSP-1 subunits. We, therefore, subjected p83/30, p38/42, and the complex formed by these two proteins, as well as the full size p190 to thrombin digestion followed by affinity chromatography and SDS-PAGE analysis. The results of these experiments, depicted in Fig. 8, allow us to draw several conclusions: (i) upon thrombin cleavage fragments A1 and A2a, located N-terminal within p83, are released from p83/30 as well as from the p83/30-p38/42 and p190 (Fig. 7, A, C, and D); accordingly, they are not involved in the interaction of p83 with p30; (ii) the same holds true for B1 at the N terminus of p38 that is also released from the complex (Fig. 7, B and C); (iii) by contrast, B2a and B2b interact with each other and appear to carry the determinants for the association between p83 and p42 (Fig. 7B); (iv) similarly, A2b interacts with B2a, consistent with the association between p83/30 and p38/42 (Fig. 7C); (v) fragments A2b, B2a, and B2b remain associated when the p83/30-p38/42 complex is analyzed (Fig. 7C), consistent with the interactions observed in subunit reassembly experiments described above. Finally, when p190 was exposed to thrombin and analyzed analogously, fragments C1, C1a, B2a, and B2b remain associated, as predicted (7D).

**DISCUSSION**

Several lines of evidence indicate that the MSP-1 complex found at the merozoite surface of all malaria parasites examined is an essential, possibly multifunctional protein playing a crucial role during invasion of erythrocytes by the parasite. Little is known, however, regarding mechanistic aspects of MSP-1 function and, except the most C-terminal 10-kDa portion, structural information is scarce.

A major hindrance for a more thorough investigation of MSP-1 of P. falciparum has been the difficulty of obtaining this large protein and its processing products in amounts and quality required for more detailed structural and functional studies. Here, we show that full size MSP-1 of P. falciparum as well as its major processing products can be readily recovered from E. coli, and our data indicate that the resulting materials are in
A key prerequisite for the work reported here was the synthesis of the gene of MSP-1 of the 3D7 strain\(^2\) in codon frequencies optimized in various ways as reported for MSP-1 from strain FCB-1 (18). Using an E. coli expression system (22), proteins were recovered that were, like native MSP-1, not glycosylated (20) despite numerous potential glycosylation sites. Making use of various combinations of N- and C-terminal fused tags, highly purified proteins were obtained by affinity chromatography. Thus, full size MSP-1, i.e. p190 fused N-terminal to GST and C-terminal to a His\(_6\) tag, was isolated from E. coli as soluble protein, whereas fragments p83, p38, p30, and p42 as well as the fusions p83/30 and p38/42 were produced as insoluble inclusion bodies that could, however, be converted into soluble proteins likely to be correctly folded by appropriate renaturation procedures (23).

To explore interactions between various fragments of MSP-1, we followed three approaches, of which two, coexpression in E. coli and co-renaturation in vitro, should allow the detection of interactions possibly taking place between domains of the precursor molecule in statu nascendi. However, as crucial complexes were also formed via our third procedure, namely by incubation of separately renatured interaction partners, the folding of MSP-1 precursor into secondary, tertiary, and quaternary structure(s) appears to be a sequential process throughout. All three approaches applied yielded consistent results, which permit the following conclusions. There is just one subunit, p30, which interacts with all other partners, namely with p83, p38, and p42. By contrast, p83, the largest subunit, touches exclusively p30 whereas the other two, p38 and p42, interact with each other as well as with p30. The entire MSP-1 complex can be assembled from its two halves, i.e. from p83/30 and p38/42 as well as from its four major processing products p83, p30, p38, and p42. Interestingly, when in the latter reassociation experiment p42 of MSP-1D is replaced by

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\(^{2}\)C. W. Kauth, C. Epp, H. Bujard, and R. Lutz, unpublished data.
p42 of the FCB-1 strain, a representative of the alternative dimorphic prototype, the complex forms with comparable efficiency (some implications of these findings will be discussed below).

For elucidating conformational parameters of multidomain proteins such as the overall arrangement of flexible loop structures and more compact domains, limited proteolysis has proven to be a powerful tool. With around 80 potential cleavage sites, thrombin appeared to be a suitable protease for probing domain/linker structures of MSP-1D. The six cleavage sites identified giving rise to a specific pattern of fragments yielded interesting insights. Thus, when thrombin digests of p190 or of the p83/30-p38/42 complex are compared with those of the individually digested p83/30 and p38/42, no additional cleavage site is detected demonstrating that the surfaces by which the two proteins interact do not expose targets accessible for this protease. On the other hand, the cleavage pattern of p190 differs from expectation by the cleavage product C1, the fusion between C1a and B2a (Fig. 6). This fragment is not further digested even upon prolonged incubation indicating an alternative pathway of thrombin cleavage (Fig. 5, C and D) governed by kinetic and conformational parameters. Thus, whenever thrombin cleaves first between C1a and B2a, cleavage occurs also between B2a and B2b (Fig. 6). However, when proteolysis first occurs between B2a and B2b, the cleavage site within C1 appears obscured by a conformational change.

It was of obvious interest to compare the distinct and reproducible proteolytic pattern of our heterologously produced proteins with those of native MSP-1, isolated from early schizonts. In this stage of merozoite development, the majority of MSP-1 is still in its precursor state corresponding to p190. When such material was isolated and subjected to thrombin digestion, all fragments known from p190 proteolysis were generated including C1 (Fig. 5D). The emergence of the latter fragment confirms that the alternative thrombin cleavage pathway is also followed when native material is digested. The finding that the cleavage pattern of E. coli and parasite-derived material is fully compatible and that thrombin digestion of p190, p83/30, and p38/42 preparations results in stoichiometric mixtures of defined fragments (as judged from Coomassie-stained gels) strongly indicates that our heterologously produced material is homogeneous and that it has assumed a conformation that closely resembles or is identical with the native one. This reasoning is supported by a set of data not shown here. Accordingly, six monoclonal antibodies including mAb 7.6, mAb 7.5, mAb 2.2, mAb 5.2, mAb 12.8, and mAb 12.10 recognizing conformational epitopes interact efficiently with p190. Moreover, using one of these antibodies, mAb 5.2, for immunoaffinity chromatography reveals that more than 95% of a typical p190 preparation is specifically retained by the column, from which it can be eluted at low pH as a homogeneous preparation.

Combining proteolytic cleavage with affinity chromatography allowed us to determine whether some of the digestion products would be released from the complex and, thus, to delineate more precisely the regions responsible for the subunit interactions. Indeed, several sets of data show (Fig. 7) that A1, A2a, and B1 are released by thrombin cleavage and, thus, most likely do not participate in subunit interactions. On the other
hand, these findings identify A2b, B2a, and B2b as critical for the interactions within the MSP-1 complex. Together our data allow us to derive the model for the processed MSP-1 complex presented in Fig. 8. The salient features of this model can be summarized as follows: subunit p30 may be seen as a core to which all other processing fragments bind. The structural determinants responsible for all the interactions are contained in the regions delineated by A2b, B2a, and B2b (Fig. 6). The subunits p38 and p38 exhibit regions of higher flexibility as they are susceptible not only to thrombin digestion but also to protease attack in E. coli (data not shown); both proteins appear to consist of at least three more compact folds connected by protease-sensitive linkers.

There is independent support for some of our results. Thus, studies of Lyon and co-workers (26) have indicated that the epitope of mAb 7H10 is shared between subunits p38 and p42. Moreover, based on data obtained with sequences of MSP-1 of Plasmodium yoelii in a yeast two-hybrid system, Daly et al. (27) have demonstrated an interaction between sequences that, in analogy, would be most likely contained within p38 and p33 of MSP-1 of P. falciparum. This latter finding is particularly interesting as, in the absence of extended homologies in the primary structures, it indicates a functional conservation of MSP-1 among different malaria parasite species.

A number of further implications of our data appear worthwhile to be discussed. Thus, the finding that p42 of strain 3D7 can be replaced by p42 of strain FCB-1 suggests that the motifs responsible for the interaction of p42 with the residual complex are conserved between the two dimorphic MSP-1 prototypes. For p42 and p38, they may be located within the highly conserved part of the molecule (Fig. 1), whereas in p30 they must be part of the dimorphic region. As this subunit interacts also with p38 and p38, determinants for these interactions have indeed to be contained within the dimorphic region. This holds true also for p38, which interacts with p30 via its dimorphic C-terminal portion, as all its highly conserved sequences are liberated from the complex by thrombin digestion. Whether determinants for subunit interaction located within the dimorphic region are conserved across MSP-1 variants may be revealed by further mixed reconstitution experiments. One might also speculate about the role of the most extended conserved regions within MSP-1, which reside in the N-terminal part of p38. Because they do not participate in the formation and stabilization of the complex and at the same time are embedded in what appears a flexible domain/linker structure, they might be involved in interactions with other proteins of the parasite (28) or with structures at the surface of the erythrocyte. One, furthermore, may ask why thrombin does not detect the putative linker regions within MSP-1 targeted by enzyme(s) processing the 190-kDa precursor at the merozoite surface. Inspecting the MSP-1 sequence for preferred motifs for thrombin reveals, however, that none are located proximal to the natural processing sites and, thus, a respective cleavage pattern cannot be expected.

Obviously, the access to “biochemical” amounts of faithfully produced MSP-1, its subunits, and their complexes will greatly facilitate to tackle many longstanding questions. They include the interaction of MSP-1 with other parasite proteins, its possible affinity to the erythrocyte surface, and mechanisms of maturation of the MSP-1 precursor. Thorough structural analyses of MSP-1 and its ligands will, however, be of particular interest. Together such studies should yield new insights into the function(s) of MSP-1. They may also reveal novel targets for interfering with the life cycle of the parasite. Last but not least, some of the processes we have developed for the production of MSP-1 and MSP-1 subunits are GMP compatible opening up the way for a detailed examination of the protective potential of MSP-1 when used as a vaccine.

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