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# Expression and purification of *Plasmodium falciparum* MSP-$1_{42}$: A malaria vaccine candidate

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**Abstract**

The C-terminal $42\cdot10^3$ Da portion of the merozoite surface protein (MSP-1) of the human malaria parasite *Plasmodium falciparum* is of interest, not only because it may constitute an essential part of a future anti-malaria vaccine, but also due to its role during the infection of erythrocytes by the parasite. We have cloned and expressed two synthetic DNA sequences encoding the two prototypic MSP-$1_{42}$ variants in *E. coli*. When over-produced, both proteins form insoluble aggregates which were isolated in high purity and yield. After solubilisation and refolding in vitro, both proteins were purified to homogeneity by a three-step procedure applying Ni-chelate, size exclusion and immuno-affinity chromatography. After purification, both proteins meet key criteria of preparations for clinical use. First, conformational studies suggest proper folding of the proteins, particularly in the region containing two EGF-like domains. Polyclonal serum raised against *E. coli* produced MSP-$1_{42}$ recognizes native MSP-1 in *Plasmodium* infected erythrocytes as shown by immunofluorescence.
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*Keywords:* Purification; *Plasmodium falciparum*; MSP-$1_{42}$

## 1. Introduction

Malaria remains one of the most important infectious diseases worldwide considering that: (i) around 40% of the world's population live in risk areas; (ii) 700–900 million febrile episodes occur annually in children under the age of 5 in Africa alone; and (iii) 1–3 million patients, primarily children, die per year. Among the various measures envisaged, the concept of a vaccine which would protect humans against malaria appears particularly attractive.

The most devastating form of malaria is caused by the parasite *P. falciparum* of which several antigens are intensely investigated with the aim of developing respective vaccines. A prime vaccine candidate is the merozoite surface protein 1 (MSP-1) which constitutes the major protein component at the surface of merozoites, the erythrocyte invading extracellular form of the parasite. MSP-1 is the product of a proteolytic cleavage process of an around $190\cdot10^3$ Da precursor protein which is fixed at the parasite membrane via a glycosyl-phosphatidyl-inositol (GPI) anchor. Despite the cleavage of this precursor into four major fragments during a first, and into five fragments in a second processing step, MSP-1 can be isolated from the parasite as an intact non-covalently associated complex (for review see [1]). The function of MSP-1 has remained elusive so far, there are, however, strong indications that it is directly involved in several steps of the invasion of the erythrocyte by the parasite. Moreover, MSP-1 is

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highly immunogenic, and various lines of evidence obtained in animal models indicate that it can elicit a protective immune response (for review see [2]). Together, these findings have made MSP-1 a most promising candidate for a vaccine against *P. falciparum* infections.

For a number of reasons, the $42 \cdot 10^3$ Da C-terminal cleavage product (MSP-$1_{42}$) is of particular interest. First, it is a target of the secondary proteolytic event cleaving this protein in a $33 \cdot 10^3$ and a $19 \cdot 10^3$ Da (historical nomenclature that does not reflect the real molecular mass) protein. This cleavage is a prerequisite for a successful invasion process during which only the GPI anchored p19 is transferred to the red blood cell, while the residual MSP-1 is stripped of the parasite's surface [3,4]. Second, antibodies directed against epitopes of MSP-$1_{19}$ and MSP-$1_{42}$ can inhibit invasion in vitro [5]. Third, in the rodent model immunization with MSP-$1_{19}$ can elicit a protective immune response [6,7]. Finally, MSP-$1_{42}$ interacts with other moieties of MSP-1 [8] and probably also with further surface molecules of the parasite. For all these reasons, it appeared important to develop an efficient and reproducible procedure for the production of MSP-$1_{42}$ which would deliver preparative amounts of properly folded protein for biochemical, structural, and immunological studies.

The overall primary structure of MSP-1 is rather well conserved throughout malaria parasites [9]. MSP-1 of *P. falciparum* consists of about 27% of highly conserved sequences, while the major portion of around 66% is dimorphic and only some 7% confined to two small regions are oligomorphic [10,11]. Based on the dimorphism, individual parasite isolates belong either to the prototypic K1 or MAD20 family. The MSP-$1_{42}$ fragment encompasses also a dimorphic and a highly conserved region. The latter covers the entire MSP-$1_{19}$, i.e., the most C-terminal portion. Accordingly, we have focussed on two proteins, MSP-$1D_{42}$ from *P. falciparum* strain 3D7 and MSP-$1F_{42}$ from strain FCB1, representing members of the two prototypic families.

As the DNA of *P. falciparum* has an unusually high AT content of >80%, the DNA sequences encoding the two prototypic proteins, MSP-1D and MSP-1F, were synthesized in our laboratory using human codon frequencies [12,13] to ensure stable cloning and expression. For the heterologous production of MSP-$1_{42}$ the structure of the C-terminal portion poses a particular challenge as it comprises two epidermal growth factor (EGF) like domains each containing three disulfide bridges. This structure has been elucidated by X-ray analysis [14] and NMR studies [15] using the homologous protein of *P. cynomolgyi*, a parasite infecting non-human primates.

Here, we describe a process which allows to produce MSP-$1D_{42}$ and MSP-$1F_{42}$ in a highly efficient way. The resulting preparations are soluble, homogeneous and pure, qualifying for clinical use when produced under GMP conditions. As the parasitic protein is not glycosylated despite potential glycosylation sites, our process is based on expression of *MSP-1* genes in *E. coli*. Its efficiency and reproducibility has opened up new perspectives for structure–function as well as for immunogenicity studies of these intriguing proteins.

## 2. Materials and methods

### 2.1. Cloning and expression of msp-$1D_{42}$ and msp-$1F_{42}$

Synthetic DNA sequences encoding the MSP-$1_{42}$ fragments were amplified by PCR from plasmids containing msp-1F [12] or msp-1D [13], respectively, using primers which introduced at the 5′ end a *Bam*HI site, followed by a start codon, and a sequence encoding a hexahistidine tag as well as a Factor Xa cleavage site and, at the 3′ end, a *Pst*I site. After cutting with the respective restriction enzymes the PCR products were inserted into the expression vector pZE13 and thus placed under the control of the IPTG inducible promoter $P_{A1lacO-1}$ [16]. The resulting plasmids were termed pZE13/d-HX42 and pZE13/f-HX42. The amino acid exchange in MSP-$1D_{42}$ from cystein 215 to serine was introduced by PCR based site directed mutagenesis resulting in plasmid pZE13/d-$HX42_{C215S}$. For expression of msp-$1D_{42}$ and msp-$1F_{42}$ *E. coli* strain W3110Z1 [16] was transformed with plasmids pZE13/d-HX42 and pZE13/f-HX42, respectively. Overnight cultures derived from individual *E. coli* clones containing the proper expression vectors were diluted 1:50 in 1.5 l

Luria-broth (LB) medium and grown with orbital shaking (220 rpm) at 37 °C in the presence of ampicillin (100 μg$^{-1}$ ml$^{-1}$) to log phase ($OD_{600}\approx$ 1). After induction with 1 m*M* IPTG for 3 h at 37 °C the cells were harvested by centrifugation for 10 min at 5000 *g*.

### *2.2. Preparation of renatured MSP-$1_{42}$ from E. coli*

After resuspension of sedimented bacteria in PBS containing 20 u ml$^{-1}$ DNaseI, 10 μg ml$^{-1}$ Lysozyme, the cells were disrupted by French Press lysis at 1000 p.s.i.. The insoluble fraction containing more than 90% of the heterologously produced MSP-1 was seperated from the soluble material by centrifugation of the bacterial lysate at 40.000 *g* for 40 min and redissolved in 6 *M* GdmCl, 50 m*M* DTT, 50 m*M* Tris, pH 8.0 (4 ml g$^{-1}$ pellet) by using an Ultraturrax homogenizer. The suspension was further incubated under stirring at RT for at least 2 h before non-solubilized material was sedimented (40.000 *g* for 40 min) and the supernatant dialysed against at least 100 volumes of 6 *M* GdmCl, 50 m*M* Tris, 2 m*M* EDTA, pH 3.0 at 4 °C. The final protein concentration was typically in the range of 25–30 mg ml$^{-1}$. Protein renaturation was performed at 4 °C according to a procedure described by Rudolph and Fischer [17] commonly referred to as ''pulse renaturation''. In short, the solubilized protein at a concentration of 10 mg ml$^{-1}$ was repeatedly diluted 1:100 in refolding buffer (1 *M* Arginin, 50 m*M* Tris, pH 8.0, 1 m*M* EDTA, 1 m*M* oxidized glutathione (GSSG), 10 m*M* reduced glutathione (GSH)) in 1.5 h intervals until a final protein concentration of 1 mg ml$^{-1}$ was reached. After the final ''pulse'' the mixture was kept at 4 °C for 12–16 h before it was dialysed twice against 50 volumes of 50 m*M* Tris, pH8.0, 300 m*M* NaCl, 10% glycerol, 10 m*M* imidazole.

### *2.3. Purification of MSP-$1D_{42}$ and MSP-$1F_{42}$*

Both, MSP-$1D_{42}$ and MSP-$1F_{42}$ were purified by identical procedures. Chromatography was performed with an Äkta Purifier 100 system (Amersham Pharmacia Biotech, Freiburg, Germany) at 4 °C. Matrices used were Chelating-Sepharose Fast Flow with immobilized $Ni^{2+}$ ions for immobilized metal ion affinity chromatography (IMAC), Superdex 200 prep grade for size exclusion chromatography (SEC) and Protein A-Sepharose Fast Flow for immunoaffinity chromatography (Amersham Pharmacia Biotech, Freiburg, Germany). mAb 5.2 was coupled to Protein A-Sepharose by a standard procedure [18]. Chromatography was carried out as recommended by the supplier. The protein was applied to a Ni-chelate column equilibrated with 50 m*M* Tris, pH 8.0, 300 m*M* NaCl, 10% glycerol, 10 m*M* imidazole. After washing with 5 column volumes (C.V.) of 50 m*M* sodium phosphate, pH 6.4, 1 *M* NaCl, 10% glycerol, 0,1% sarcosyl and 4 C.V. of 50 m*M* Tris, pH 8.0, 300 m*M* NaCl, 10% glycerol, 30 m*M* imidazole the protein was eluted with 50 m*M* Tris, pH 8.0, 300 m*M* NaCl, 10% glycerol, 250 m*M* imidazole. The elution fraction was applied to a preparative Superdex 200 column equilibrated with 50 m*M* Tris, pH 8.0, 300 m*M* NaCl, 1 m*M* EDTA. For immunoaffinity chromatography, fractions containing the desired protein were applied onto the mAb 5.2-Protein A–Sepharose column equilibrated with the same buffer. After washing the column with 50 m*M* Tris, pH 8.0, 650 m*M* NaCl, 1 m*M* EDTA the protein was eluted with 0,1 *M* glycine, pH 2.5. The elution was immediately neutralized with 0.2 volumes of 1 *M* Tris pH 8.0, and if necessary, the protein was concentrated by ultrafiltration using a stirred cell system with a YM1 membrane (Millipore, Bedford, MA). Protein concentrations were determined according to Bradford (Biorad, Munich, Germany).

### *2.4. Immunological methods*

SDS-polyacrylamide gelelectrophoresis (PAGE) and Western blots were performed as described previously [12].

Enzyme linked immunosorbent assay (ELISA): 96-well-microtiter plates were coated with 0.45 μg of MSP-$1D_{42}$ or 0.43 μg of MSP-$1F_{42}$ per 0.1 ml (100 n*M*) per well and incubated overnight at 4 °C before they were washed with TBST (10 m*M* Tris–HCl, pH 8.0, 150 m*M* NaCl, 0,05% Tween-20) and blocked with TBST+1% milk powder for 1 h at RT. The test sera, serially diluted two-fold, were added

and incubated for 2 h at RT. The appropriate secondary antibodies (Sigma, Munich, Germany) diluted as recommended by the supplier were added to each well and incubated for 1 h at RT. Anti-human-IgG-AP conjugates (Promega, Mannheim, Germany) were used as secondary antibodies for monkey serum. Substrate *p*-Nitrophenyl-phosphate (1 mg $ml^{-1}$ in 0,96% v/v diethanolamine, pH 9.5, 1 m*M* $MgCl_2$) was added and incubated for 1 h at RT in the dark. The reactions were stopped with EDTA (final concentration 50 m*M*) and the absorbance was measured at 405 nm.

Indirect immunoflourescence assay (IFA): Thin films on glass slides were prepared from *P. falciparum* 3D7 infected human blood (type 0 $Rh^+$), fixed in acetone for 5 min at −20 °C, and then air dried. After blocking with 1% BSA in PBS for 1 min at 37 °C, anti-MSP-$1D_{42}$ serum, diluted 1:10.000 in 0.5% BSA in PBS, was applied to the slides and incubated in a sealed, moist box for 20 min at 37 °C. The slides were washed three times with PBS and air dried. As secondary antibody, flouresceine isothicyanate (FITC) conjugated goat anti-rabbit immunoglobulin (Sigma, Deisenhofen, Germany), was diluted appropriately in 0.5% BSA-PBS, spotted onto the slides, and incubated for 45 min at room temperature. After washing three times with PBS, parasite DNA was visualized by incubation with DAPI staining solution (Sigma, Deisenhofen, Germany) for 15 min at 37 °C. The slides were washed three times with water, air dried, and mounted with Mowiol (Calbiochem, La Jolla, CA) to retard photobleaching. Fluorescence was examined by a Leica DMRXE microscope with a 100× oil immersion objective.

### *2.5. Antibodies*

Monoclonal antibodies: Anti-MSP-$1_{42}$ mouse mAbs used in this study were: 12.10, 12.8 [19], and 5.2 [20]. mAb 5.2 was prepared from the hybridoma cell line HB9148 (ATCC).

Anti-MSP-$1D_{42}$ rabbit serum: Rabbits were immunized intramuscularly with 100 μg MSP-$1D_{42}$ emulsified with complete Freund's adjuvant (CFA) followed by two additional booster immunizations with the same antigen dose in incomplete Freund's adjuvant. Rabbits were bled on the day of the first immunization (preimmune serum) and 10 days after the secondary and tertiary immunizations (immune sera).

Anti-MSP-$1F_{42}$ monkey serum: Immunization with three 50 μg doses of MSP-1 purified from cultured *Plasmodium falciparum* FCB-1 parasites protected three of five *Aotus griseimembra* monkeys against a homologues malaria infection [21]. Blood samples were taken prior to challenge and the sera of three protected monkeys were pooled for ELISA analysis.

### *2.6. Characterization of MSP-$1_{42}$ preparations*

Endotoxin levels of the purified proteins were measured by a *Limulus* Amoebocyte Lysate (LAL) Gel Clot Assay (Charles River Laboratories, Wilmington, MA) according to the manufacturer's instructions. *E. coli* host cell protein contaminations were measured by ELISA (Cygnus Technologies) as described by the manufacturer. MSP-$1_{42}$ was desalted via a $C_{18}$ reversed phase column prior to mass spectrometry by ESI-QTOF.

## 3. Results

### *3.1. Experimental strategy*

Production of heterologous proteins in *E. coli* offers two widely used approaches, synthesis of the target protein in soluble and usually properly folded, or in aggregated form, as so-called inclusion bodies (IBs). The latter procedure is preferable, whenever the aggregated material can efficiently be renatured, as the isolation of IBs is generally a highly efficient first step of purification. For MSP-$1_{42}$, pilot experiments indicated a strong tendency of the protein to aggregate when expressed in good yields in *E. coli*. We, therefore, decided to focus on the efficient production of IBs and on renaturation procedures for the resulting proteins. Moreover, to facilitate protein purification, both MSP-$1_{42}$ variants were N-terminally fused to a hexahistidine tag which is separated from the genuine protein sequence by a Factor Xa cleavage site for the eventual removal of the N-terminal tag (Fig. 1). To probe the successful renaturation, a number of parameters had to be examined. They included, besides solubility and

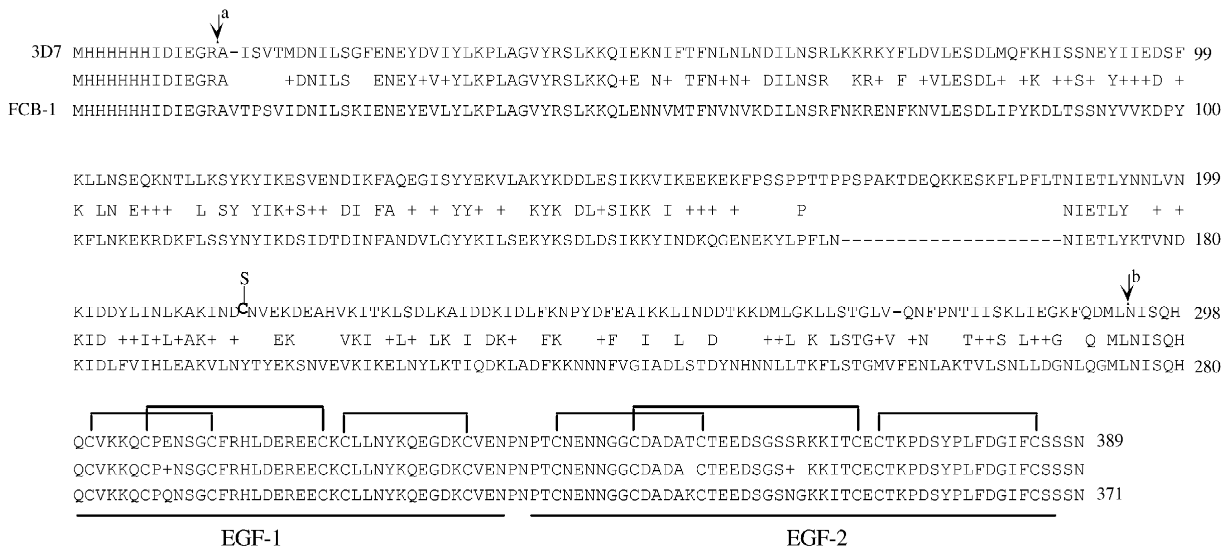


Fig. 1. Comparison of the amino acid sequences of MSP-1D$_{42}$ and MSP-1F$_{42}$. The sequence of MSP-1D$_{42}$ is shown in the upper, the sequence of MSP-1F$_{42}$ in the lower lane, alignment gaps are denoted by (−). The sequence of MSP-1D$_{42c215S}$ is identical to MSP-1D$_{42}$ with the exception of position 215 which is replaced by serine. In the middle lane, letters show identical amino acids, while (+) indicates similarity due to conservative amino acid exchanges. Arrow (a) denotes the cleavage site of Factor Xa between the N-terminal hexahistidine tag and the MSP-1$_{42}$ sequences. Arrow (b) indicates the site of secondary cleavage of MSP-1$_{42}$, resulting in MSP-1$_{33}$ and MSP-1$_{19}$. The two EGF-like domains are delineated by solid lines. Horizontal clamps between the corresponding cysteines illustrate the position of the disulfide bonds.

homogeneity of the refolded material, its interaction with several monoclonal antibodies recognizing sensitive conformational epitopes in the apparently most complex part of the molecule, as well as the comparison of the interaction of antibodies elicited by parasite derived and recombinant protein.

### *3.2. Production of insoluble MSP-1$_{42}$ in E. coli and conversion of the protein into soluble form after refolding*

It is well documented that different culture conditions such as temperature can influence the folding and thus the solubility of a protein if over-produced in a prokaryotic host. We, therefore, expressed both MSP-1$_{42}$ variants encoded in the respective plasmids (pZE13/f-HX42, pZE13/d-HX42) at 25, 30 and 37 °C, respectively. Under all three conditions, both variants of MSP-1$_{42}$ were mainly produced as IBs which, when harvested from 37 °C culture, make up around 7–10% of total *E. coli* protein (as judged by SDS–PAGE, Fig. 2). The isolated IB fraction con-

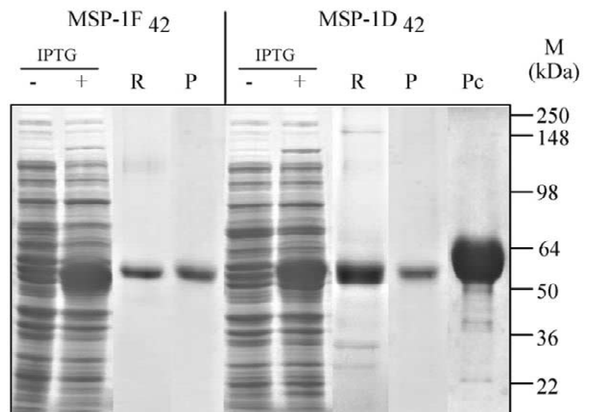


Fig. 2. Electrophoretic analysis of renatured and purified MSP-1D$_{42}$. The leftmost lanes show the electrophoretic pattern of total protein of *E. coli* W3110Z1 harbouring plasmids pZE13/f-HX42 or pZE13/d-HX42, respectively, grown either in presence (+) or absence (−) of 1 m*M* IPTG. Lanes "R": protein after refolding; lanes "P": 1 μg MSP-1$_{42}$ after purification by IMAC, SEC and immunoaffinity chromatography. Lane "Pc", 20 μg MSP-1D$_{42}$ at a concentration of 5 mg ml$^{-1}$. 10% PAA-gel, 0.1% SDS; stained with Coomassie blue; samples contained 50 m*M* DTT. M, molecular mass standard.

tains only minute amounts of degraded MSP-$1_{42}$ and *E. coli* contaminants (Fig. 2).

The successful refolding of various disulfide bond containing proteins produced as IBs in *E. coli* as for example tissue plasminogen activator (tPA) has been described (for review see [22]). Interestingly, tPA contains EGF-like domains as does MSP-$1_{42}$. Accordingly, we chose refolding conditions under which efficient renaturation as well as proper disulfide bond formation within the EGF-like domains of MSP-$1_{42}$ were likely to occur and applied a method commonly known as ‘‘pulse renaturation’’ in which the solubilized protein is added at 1.5 h intervals to the refolding buffer at low concentrations in order to minimize protein aggregation [17]. Indeed, under these conditions, less than 10% of MSP-$1_{42}$ precipitated in the subsequent dialysis against IMAC equilibration buffer. By contrast, almost 50% of MSP-$1F_{42}$ was lost during this dialysis step, indicating that the refolding conditions were not optimal for this protein in spite of the relatively high (73%) sequence similarity to MSP-$1D_{42}$ (Fig. 1). Nevertheless, both proteins were recovered in soluble form suitable for further purification.

### *3.3. Purification of refolded MSP-$1_{42}$*

In a first step, refolded and soluble MSP-$1_{42}$ preparations were subjected to IMAC. The resulting material was essentially free of *E. coli* contaminants, i.e. bacterial proteins and endotoxins (Table 1). At this stage, the N-terminal hexahistidine tag could be cleaved off quantitatively by Factor Xa (data not shown) though for the present study this step was considered not to be necessary. The preparations of both MSP-$1_{42}$ variants contained small amounts of multimeric forms of the protein which, however, could be effectively removed by SEC on Superdex 200. The resulting preparations exhibited a high degree of purity and homogeneity although minor amounts of C-terminally truncated material was still detectable which, however, could be readily removed by immunoaffinity chromatography as described below. When the two purified proteins (Fig. 2) were concentrated by ultrafiltration, they showed a differential behaviour with respect to their solubility. Whereas MSP-$1_{42}$ could be readily concentrated to 5 mg $ml^{-1}$, there was a limit of 1 mg $ml^{-1}$ for MSP-$1F_{42}$ beyond which the protein precipitated.

### *3.4. Immunoaffinity chromatography of MSP-$1_{42}$*

Monoclonal antibody 5.2 recognizes a conformational, reduction sensitive epitope within the EGF-like domains of MSP-$1_{42}$ though its precise binding site is not known. Affinity chromatography using immobilized mAb 5.2 would, therefore, specifically separate MSP-$1_{42}$ molecules containing the reconstituted epitope from material that is not properly folded. The finding that more than 95% of both MSP-$1_{42}$ preparations were retained by the affinity column from which they could be eluted quantitatively and in high purity indicates that they were folded correctly at least with respect to this particular conformational epitope. When analyzed by SDS–PAGE under reducing conditions (Fig. 2) these

Table 1
Characteristics of MSP-$1_{42}$ preparations

| | Yield of inclusion bodies (mg $g^{-1}$ *E. coli* paste) | Yield of purified MSP-142 (mg $g^{-1}$ *E. coli* paste) | MWa (Da units)[a] | Endotoxins[b] (EU/mg of purified MSP-142) | *E. coli* protein[c] (ng $mg^{-1}$ of purified MSP-142) |
|---|---|---|---|---|---|
| MSP-$1D_{42}$ | 35 | 18 | 44.813+20 (calc. 44.820) | 3240 | 267 |
| MSP-$1F_{42}$ | 28 | 9 | 42.740+20 (calc. 42.731) | 2000 | 62 |

[a] Molecular weights were determined by ESI-QTOF mass spectrometry (Applied Biosystems).

[b] Endotoxin levels of the purifed proteins were measured by a Limulus Amoebocyte Lysate (LAL) Gel Clot Assay (Charles River Laboratories).

[c] Residual *E. coli* protein was measured by an anti-*E. coli* ELISA (Cygnus Technologies).

proteins exhibit an apparent molecular mass of around $48 \cdot 10^3$ Da (3D7) and $45 \cdot 10^3$ Da units (FCB-1), respectively. Their molecular mass determined by mass spectrometry (ESI-QTOF) was in accordance with the calculated values and did not indicate any protein truncation or modification of amino acid side chains (Table 1).

### *3.5. Probing the conformation of purified MSP-$1_{42}$*

To gain insight into the conformation of the heterologously produced MSP-$1_{42}$, we subjected the proteins to denaturing conditions and monitored a possible effect on their migration behaviour during PAGE. Moreover, we studied the interaction of three mAbs which recognize conformational epitopes. As shown in Fig. 3A, both proteins are, as expected, highly sensitive towards reduction as shown by shifts in electrophoretic mobility, whereas 8 *M* urea under non-reducing conditions merely induces some heterogeneity. Interestingly, under non-reducing conditions and in absence of urea, monomeric MSP-$1D_{42}$ migrates as a double band, indicating an alternative conformation apparently locked in by disulfide bonds. This alternative structure is not seen in MSP-$1F_{42}$ and could, therefore, be caused by the MSP-$1D_{42}$ specific cysteine in position 215. Indeed, when *cys*215 was exchanged for a serine the resulting MSP-$1D_{42/C215S}$ does not form the alternative structure (Fig. 3).

Several faint bands between 80 and $120 \cdot 10^3$ Da units can occasionally be detected by Coomassie staining and are more prominently seen in Western blots. They most likely represent disulfide bonded dimeric complexes of MSP-$1_{42}$. To analyze the conformation of MSP-$1_{42}$ in more detail, the proteins were probed with mAbs 12.10, 12.8 and 5.2, respectively, via Western blots (Fig. 3). All three antibodies recognize reduction sensitive conformational epitopes within the C-terminal part of the molecule. The epitope of mAb 12.8 is part of the first EGF-like domain while EGF-like domains 1 and 2 participate both in the formation of the epitope of mAb 12.10 [23].

Both, mAb12.8 and mAb 12.10 are particularly interesting since they inhibit processing of MSP-$1_{42}$ and thereby block erythrocyte invasion of the parasite in vitro [24]. Under non-reducing conditions, MSP-$1D_{42}$ and MSP-$1F_{42}$ react with all three antibodies demonstrating correct folding in this part of the molecule, though interesting differences can be noticed. Thus, mAb 5.2 and 12.8 interact with both species of MSP-$1D_{42}$ seen at position $48 \cdot 10^3$ Da units whereas mAb 12.10 recognizes only one, suggesting that the second EGF-like domain has not formed in the faster migrating species (Fig. 3). Moreover, the latter antibody also fails to recognize the putative trimeric complex of MSP-$1D_{42}$. Furthermore, although identical amounts of both MSP-$1_{42}$ variants were loaded onto the gel, the signal obtained in the Western blot with mAb 12.10 is considerably weaker for MSP-$1D_{42}$ than for MSP-$1F_{42}$.

### *3.6. Cross-reaction of MSP-$1_{42}$ specific antibodies with the two protein variants*

The two MSP-$1_{42}$ variants studied here consist of a well conserved and a dimorphic region. The conserved region covers the entire MSP-$1_{19}$ which differs in only four positions (Fig. 1). However a high degree of similarity (59%) is also observed in the dimorphic part delineating MSP-$1_{33}$. To estimate the relative contribution of the conserved and dimorphic regions to the immune response against MSP-$1_{42}$ we examined the reactivity of monkey serum raised against parasite derived MSP-1F (anti-MSP-1F) and rabbit serum raised against MSP-$1D_{42}$ (anti-MSP-$1D_{42}$) by ELISA. As mAb 5.2 interacts with a conserved epitope it was used as a control and, as expected, yielded identical results with both MSP-$1_{42}$ variants (Fig. 4). By contrast, anti-MSP-$1D_{42}$ shows 84% cross-reactivity with MSP-$1F_{42}$ which roughly corresponds to the degree of similarity between the two recombinant MSP-$1_{42}$ proteins. Similarly, the monkey serum cross-reacted to an extent of 87% suggesting that the two recombinant proteins share the majority of immunogenic regions and that the conformation of the proteins folded in vitro must be very similar to the one of the parasite derived MSP-1. This reasoning is supported by the finding that the antibodies raised against heterologously produced MSP-$1D_{42}$ efficiently interact with *P. falciparum* parasites as shown by indirect immunofluorescence (Fig. 5).

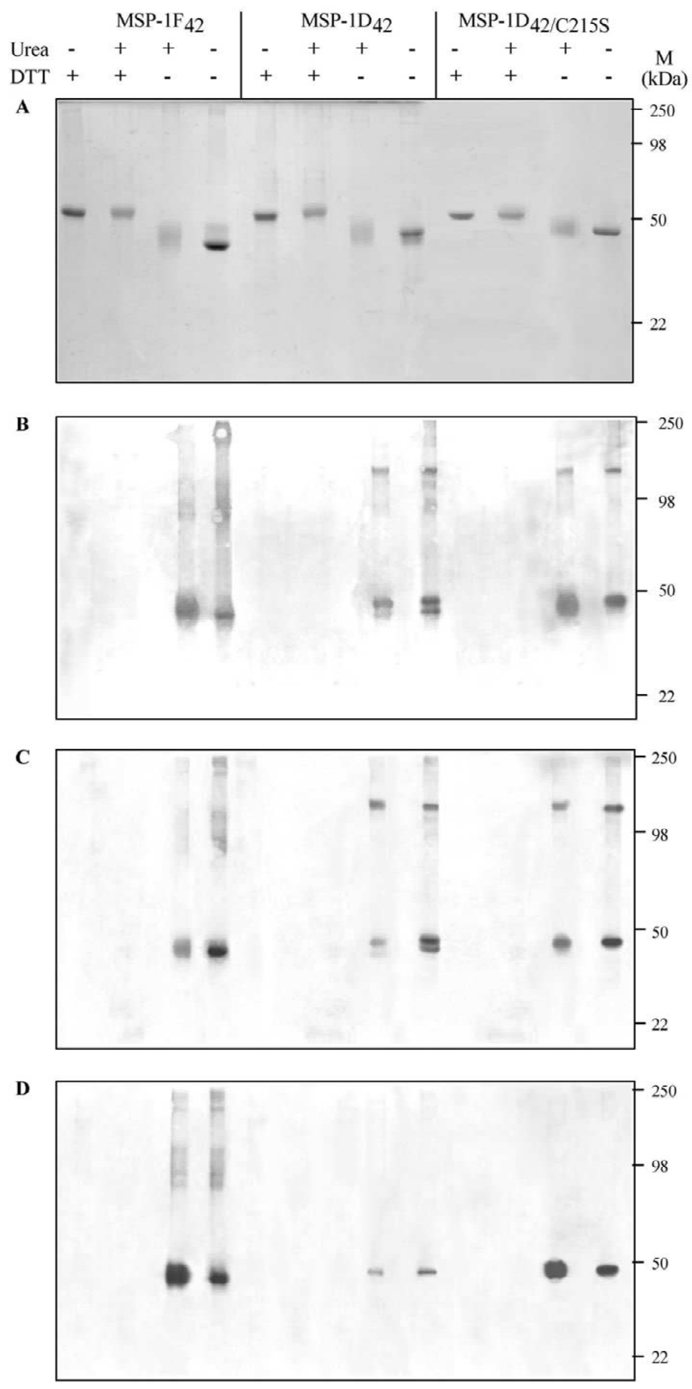


Fig. 3. Probing MSP-$1_{42}$ structure by SDS–PAGE and Western blot. A, Purified MSP-$1F_{42}$ (0.1 mg $ml^{-1}$) and MSP-$1D_{42}$ (0.1 mg $ml^{-1}$) were incubated for 2 h at RT either in the presence (+) or absence (−) of 8 *M* urea or 50 m*M* DTT or both. A sample of 0.8 μg of each protein was applied per lane (10% PAA-gel, 0.1% SDS, Comassie staining). B–D, Western blots obtained from gels as shown in (A) probed with the conformational antibodies mAb 5.2 (B), mAb 12.8 (C) and mAb 12.10 (D), respectively.

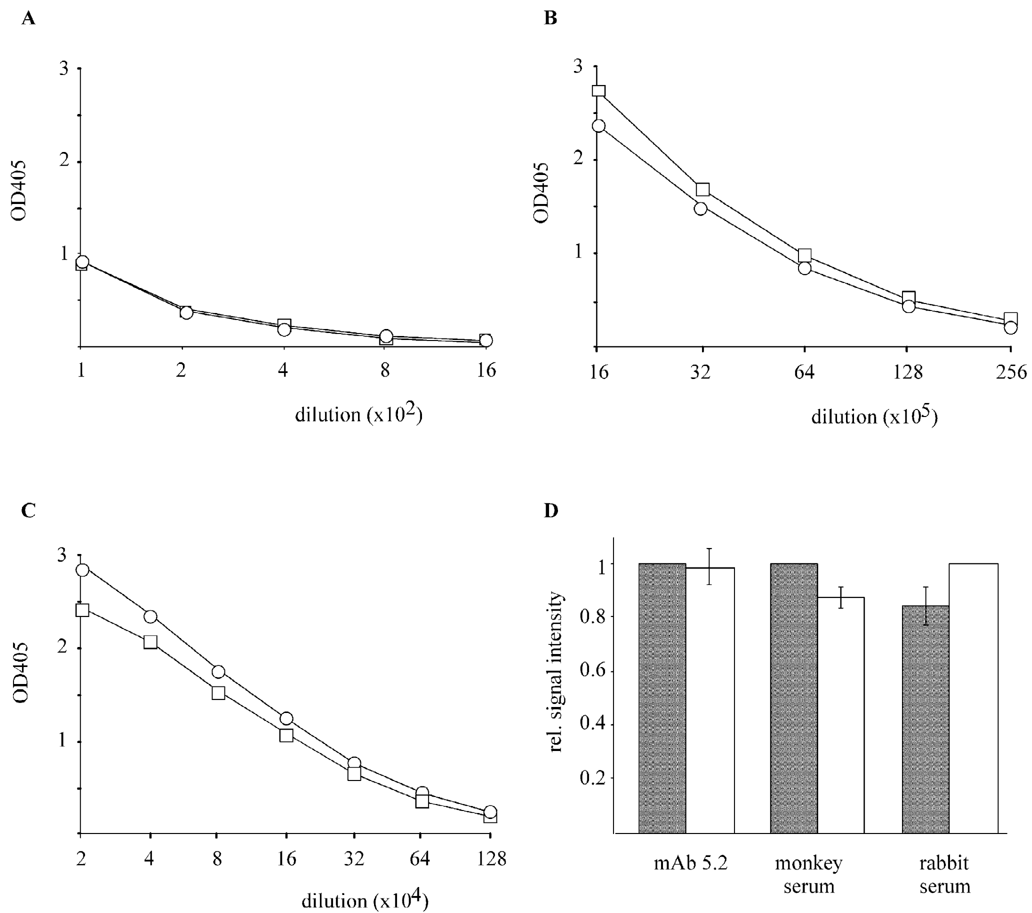


Fig. 4. Cross-reactivity of antibodies with MSP-1$D_{42}$ and MSP-1$F_{42}$. The titers of rabbit (anti-MSP-1$D_{42}$) and monkeys (anti-MSP-1$F_{42}$) serum as well as of mAb 5.2 were determined by ELISA using MSP-1$D_{42}$ (open squares) and MSP-1$F_{42}$ (open circles). A, mAb 5.2. B, monkey serum, C, rabbit serum. $OD_{405}$ values are the means of two independent measurements after subtraction of background obtained with the corresponding pre-immune sera. D, Cross-reactivity of mAb 5.2 and the two sera with the two MSP-$1_{42}$ variants. The values were derived by dividing the signal intensities obtained with MSP-1$D_{42}$ and MSP-1$F_{42}$ at identical dilutions ranging from 0.4 to 2.0 at $OD_{405}$. The arithmetical means are indicated as bars (±triple standard deviations are indicated), white: MSP-1$D_{42}$, grey: MSP-1$F_{42}$.

## 4. Discussion

The C-terminal $42 \cdot 10^3$ Da portion of the merozoite surface protein 1 of *P. falciparum* is of considerable interest not only as a potential component in a future anti-malaria vaccine, but also as a possible target for drug development as it is believed to play an essential role during the invasion of erythrocytes by the merozoite form of the parasite. A prerequisite for its detailed study at the biochemical and immunological level is the availability of well defined material in sufficient amounts. In the past, the heterologous production of this protein in native form and at large scale has been hampered by a number of technical difficulties. Thus, the high AT content of *P. falciparum* DNA (>80%) destabilizes large DNA sequences in *E. coli* [12]. Moreover, as the parasite protein is not glycosylated despite its potential glycosylation sites, expression in *E. coli* appeared indicated. This expression system yields,

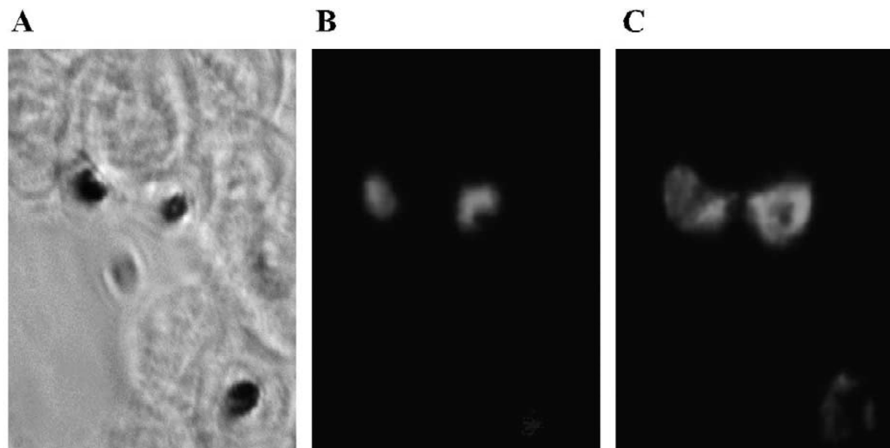


Fig. 5. Interaction of anti-MSP-1D$_{42}$ antibodies with merozoites of *P. falciparum*. Air dried films of human blood infected with *P. falciparum* 3D7 were fixed with acetone and probed with antibodies raised against purified MSP-1D$_{42}$. Early schizonts are shown by phase contrast (A), after DNA staining with DAPI (B) and by immunofluorescence with an anti-rabbit secondary antibody (C).

however, primarily insoluble aggregates and, therefore, seemed not to be suitable considering the complex structure of the protein.

Alternative expression systems like recombinant Baculo viruses and yeast [25,26] or mammalian cells [27] have up to date not been developed to a state where large amounts of native, non-glycosylated MSP-1$_{42}$ can be produced in an efficient and inexpensive way.

Herein, we describe a process for the large scale production of MSP-1$_{42}$ of *P. falciparum* in *E. coli* yielding soluble and highly purified protein which, according to a variety of criteria, is properly folded and stable. As MSP-1 of *P. falciparum* is dimorphic and since MSP-1$_{42}$ also consists of a dimorphic and a conserved region, our process was developed to produce the two prototypic variants, MSP-1D$_{42}$ and MSP-1F$_{42}$, originating from the two *P. falciparum* strains 3D7 and FCB-1, respectively. Upon induction, both proteins are synthesized in *E. coli* in good yields as insoluble inclusion bodies.

Conceiving an efficient renaturation procedure was, therefore, an essential prerequisite. After successfully adapting the principle of pulse renaturation, the initial production of inclusion bodies turned into an advantage, as the simple isolation of MSP-1$_{42}$ aggregates established a most efficient first step of purification. Followed by two chromatographic separation steps, both versions of MSP-1$_{42}$ were obtained as hexahistidine tagged proteins in a quality that meets all criteria for clinical use, when produced under GMP conditions (Table 1). Whenever required, the histidine tag may be cleaved off by a Factor Xa digestion to yield authentic MSP-1$_{42}$.

Probing the conformation of the two heterologously produced MSP-1$_{42}$ variants with three monoclonal antibodies which recognize different conformational epitopes has yielded results which strongly suggest that at least the complex C-terminal portion of the two proteins is properly folded. One of these antibodies, mAb 5.2, was used to estimate within a preparation the fraction of protein that has formed the mAb 5.2 specific epitope. Chromatography on immobilized mAb 5.2 showed that more than 95% of either MSP-1$_{42}$ variant is retained by these columns from which can be eluted under defined conditions. Since the epitope recognized by mAb 5.2 is located within the region of the two EGF-like domains, this result indicates that the two MSP-1$_{42}$ variants in our protein preparations are correctly folded in the putatively most complex part of the molecules.

The two other antibodies, mAb 12.8 and 12.10, which both can inhibit invasion of erythrocytes by parasites in vitro, also recognize epitopes within the

region of the two EGF-like domains. While mAb 12.8 requires intact EGF-like domain 1 for binding, the epitope of mAb 12.10 is only established when both EGF-like domains have formed. The fact that both mAbs interact with our MSP-$1_{42}$ preparations further supports that the C-terminal portions of both MSP-$1_{42}$ variants have assumed the native conformation. Obviously, this conformation is stabilized by the six disulfide bonds within the EGF-like domains, as even upon treatment with 8 *M* urea the correct structure is maintained or recovered.

Monoclonal antibody 12.10 has allowed us to elucidate an intriguing difference between MSP-$1D_{42}$ and MSP-$1F_{42}$ preparations. As shown in Fig. 3, Coomassie staining as well as Western blot analysis with mAb 5.2 and 12.8 reveal for MSP-$1D_{42}$ but not for MSP-$1F_{42}$ two molecular species of around $48\cdot10^3$ Da units. Interestingly, mAb 12.10 does not interact with the lower band indicating a conformational variant of MSP-$1D_{42}$ where the EGF-like domain 2 has not properly formed. As MSP-$1D_{42}$ in contrast to MSP-$1F_{42}$ contains one additional cystein at position 215, an alternative disulfide bonding may occur competing with the formation of EGF-like domain 2. Indeed, when *cys*215 was replaced by ser, only one $48\cdot10^3$ Da species is present in our MSP-$1D_{42/C215S}$ preparations. In respective Western blots, the signal obtained with mAb 12.10 is now comparable in strength with the one obtained for MSP-$1F_{42}$. Analyzing the Western blots of Fig. 3 reveals another difference: in contrast to MSP-$1F_{42}$, both MSP-$1D_{42}$ versions show a distinct molecular species around position $100\cdot10^3$ Da. This species is not recognized by mAb 12.10 and is only occasionally detectable as faint band by Coomassie staining. It is also never detected by size exclusion chromatography. The most likely interpretation is, therefore, that this molecular species forms during sample preparation whereby disulfide bonds of EGF-like domain 2 appear involved in forming some reduction sensitive dimeric or trimeric structures. Why this reshuffling of disulfide bonds takes place preferentially in MSP-1D preparations cannot be explained at present.

Further support for proper conformation of our heterologously produced MSP-$1_{42}$ comes from rabbit antibodies elicited against MSP-$1D_{42}$: They recognize MSP-1 on the surface of merozoites. Moreover, when the cross-reactivity between the two versions of MSP-$1_{42}$ of these rabbit antibodies are compared with the cross-reactivity of sera from monkeys protected against *P. falciparum* infection after immunization with native MSP-1, comparable values are found (Fig. 4).

## 5. Conclusions

Together, our data show that both MSP-$1_{42}$ variants of *P. falciparum* can be produced in *E. coli* and recovered as soluble, highly purified and homogeneous preparations of protein which most likely are in native conformation. We are, thus, confident that the availability of in principle unlimited amounts of MSP-$1_{42}$ will spark further biological and biochemical studies which hopefully will contribute to our understanding of erythrocyte invasion by the malaria parasite and thereby eventually have an impact also on medical aspects of one of the most serious infectious diseases of mankind.

## Nomenclature

| | |
|---|---|
| MSP-1 | merozoite surface protein 1 |
| IMAC | immobilized metal affinity chromatography |
| SEC | size exclusion chromatography |
| IB | inclusion bodies |
| IPTG | Isopropyl-β-D-thiogalactopyranoside |
| mAb | monoclonal antibody |
| GMP | Good Manufacturing Practice |
| RT | room temperature |
| GdmCl | Guanidinium hydrochloride |

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