Molecular and Biochemical Parasitology, 23 (1987) 71–84
Elsevier

MBP 00774

Fragments of the polymorphic M_r 185 000 glycoprotein from the surface of isolated *Plasmodium falciparum* merozoites form an antigenic complex

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(Received 31 July 1986; accepted 5 November 1986)

Merozoites of the human malaria parasite *Plasmodium falciparum* express on their surface several antigens derived from a po lymorphic glycoprotein precursor of M_r 185 000 synthesised earlier on by trophozoites and schizonts. A panel of 18 monoclona antibodies against a range of different specificities of the precursor was used to characterise its mature products in spontaneousl released merozoites. Merozoites released by [^{35}S]methionine or [^{14}C]glucosamine-labelled schizonts, or surface ^{125}I-labelled puri fied merozoites, were extracted in detergents, and the antigens were detected by immunoprecipitation or Western blotting. W show that a nonglycosylated peptide of M_r 80 000 and two glycosylated fragments of M_r 40 000 and M_r 16 000, all derived from th precursor, are exposed on the surface of the mature merozoite. Precipitations from extracts in different detergents indicate tha the 80 and 40 kDa fragments can form a non-covalent complex with each other and two additional major surface antigens of 3 and 22 kDa. Several antibodies react strongly with the complex but not with its dissociated subunits, thus indicating presence o conformational epitopes. Other epitopes are positively mapped on different dissociated subunits by immunoprecipitation and Wester blotting. The 80 and 40 kDa antigens each carry a different polymorphic marker epitope, and both of these markers are absen on the 16 kDa fragment. The 40 and 16 kDa glycoproteins share common epitopes, and the latter may be derived from the forme fragment. Only epitopes present on the 16 kDa antigen, but not those specific for the larger fragments, are detectable by im munofluorescence in the ring-stage. This indicates that the whole or a part of the 16 kDa antigen remains on the parasite throug the invasion process.

Key words: *Plasmodium falciparum;* Malaria human; Merozoite surface; Antigen; (Glyco)protein; Polymorphism; Monoclona antibody

Introduction

The outer surface of *Plasmodium falciparum* merozoite is the site of the first contact between this extracellular invasive stage of the parasite and its host cell, the erythrocyte. Since components located on the surface are thought to participate in the complex process of invasion, and may also include target antigens of malaria immunity, the analysis of the merozoite surface is of interest to the cell biologist and immunologist alike.

Surface radiolabelling of purified merozoites has shown the protein composition of the outer surface to be relatively simple, consisting of only 6–10 constituents accessible to iodination [1–3]. In one parasite strain at least three of these components at 83, 42 and 19 kDa are derived from a single larger common precursor first synthesised in the trophozoite or schizont stages, and then cleaved into a series of smaller fragments in parallel with the merozoite differentiation [3,4]. The precursor is an antigenically polymorphic glycoprotein which varies among *P. falciparum* strains in relative molecular mass (M_r) from 185 000 to

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Abbreviations: PSA, polymorphic schizont antigen; mAb, monoclonal antibody; IFA, indirect immunofluorescence assay; IIP, indirect immunoprecipitation; NP40, Nonidet P-40; SDS, sodium dodecyl sulphate: PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulphonyl fluoride; PBS, phosphate-buffered saline; p, protein; gp, glycoprotein; M_r, relative molecular mass.

over 200000 [2–11]. The immunological importance of this molecule and/or its fragments has been indicated by the ability of human antibodies specific for the precursor to inhibit growth of *P. falciparum* in culture [12,13] and by partial protection of Saimiri monkeys immunised with the precursor [14,15].

Any functional significance of the apparently controlled fragmentation of this antigen remains to be elucidated. In view of the polymorphism of the precursor [8,33,35,36] this will require a detailed characterisation of the merozoite fragments from a number of *P. falciparum* strains. Studies that used merozoites of different strains reported, somewhat variably, the presence of the intact precursor and/or one to six of its fragments at 83 kDa or smaller [2,3,9,18,29]. Recent data indicate that some of the fragments originate from distinct portions of the precursor [16,29] and, consequently, differ one from another both chemically and antigenically [3,10,16,17], thus raising the question whether they also may have different biological and/or immunological functions.

In the present investigation we have utilised a panel of monoclonal antibodies specific for a variety of epitopes of the precursor [7,8] for a detailed characterisation of the terminal antigens present on isolated mature merozoites of a Colombian strain. We confirm that merozoites express on their surface fragments of the M_r 185000 glycoprotein rather than the intact precursor [2,3]. The fragments include a nonglycosylated peptide of M_r 80000 and two glycosylated antigens of M_r 40000 and M_r 16000. These fragments differ antigenically and are probably derived from two separate segments of the precursor. We also present evidence that the 80 and the 40 kDa fragments are physically associated with at least two other surface components (M_r 36000 and M_r 22000) in a complex held together by non-covalent interactions. We consider this study to be a prerequisite to future investigations concerned with the function(s) of these surface constituents.

Materials and Methods

Monoclonal antibodies (mAbs). Mouse monoclonal antibodies known to specifically recognise the M_r ~200000 polymorphic schizont antigen (PSA) of *P. falciparum* have been raised against four strains of the parasite and characterised as described elsewhere [7,8,11]. For use in the present study a panel of 18 reagents was chosen to include mAbs recognising a variety of different polymorphic or strain-common PSA specificities (Table I).

Additional mAbs against antigens other than the PSA were used as specificity controls. They include a mAb designated 2.1 against surface of normal human red cells, as well as mAbs against several internal *P. falciparum* antigens (mAb 5.1 against a 23 kDa protein [7,19]; mAbs 7.2 and 13.3 against a 35 kDa protein [5,11]; mAbs 2.13 and 7.12 against 155 kDa proteins [5]; mAb 2.29 against an internal 82 kDa protein (McBride, unpublished)).

Indirect immunofluorescence assays (IFA). Acetone-fixed schizonts of the FCB-1 isolate were typed with the anti-PSA mAbs as described [7,8]. The typing was confirmed in a modified test using freshly released unfixed merozoites in RPMI 1640 medium supplemented with 2% aprotinin (Trasylol, Bayer) and 5% fetal calf serum, at room temperature. 3×10^5 merozoites prepared as described below were reacted for 20 min with 50 µl of mAbs diluted 1:100. Following two washes (200 µl each), reaction with fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG for 20 min and two more washes, the parasites were resuspended in 20 µl volumes and wet samples under cover-slips were prepared for immediate observation by fluorescence microscopy.

Merozoites, metabolic and surface radiolabelling. Merozoites of *P. falciparum* (Colombian strain FCB-1, originally isolated by C. Espinal, Instituto Nacional de Salud, Bogota, and obtained from K. Rieckman, University of New Mexico, Albuquerque) were prepared according to the procedure of Heidrich et al. [1]. Briefly, highly synchronous cultures of asexual parasites were obtained by alternate applications of the Plasmagel [20,21] and the sorbitol [22] procedures. Trophozoites (about 30 h old) were concentrated with Plasmagel to a parasitaemia of ~95% and were returned to culture as 0.25 – 0.30% cell sus-

pension without addition of any fresh erythrocytes. Where desired, the parasites were metabolically radiolabelled with either [^{35}S]methionine (New England Nuclear, spec. act. 1132 Ci mmol^{-1}; 75 μCi (15 ml culture)$^{-1}$) or [^{14}C] glucosamine (NEN, spec. act. 328.5 mCi mmol^{-1}; 7.5 μCi (15 ml culture)$^{-1}$). Medium containing these radiolabels was changed every 5 h throughout maturation of the parasites until release of merozoites begun. Merozoites were then purified from cultures by filtration as described [1] using complete RPMI-1640 medium with added 2% (v/v) aprotinin (Trasylol, Bayer). Remaining schizonts were returned into culture, and harvests of merozoites were repeated at 60–90 min intervals.

Batches of merozoites isolated by the same procedure from unlabelled cultures were washed at room temperature 3 × in 1.0 ml of Medium A (11 mM triethanolamine, 11 mM acetic acid, 0.05 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose, 0.15 M NaCl, 2% (v/v) aprotinin, pH 7.4 adjusted with 1 N KOH), and were then immediately surface iodinated by the lactoperoxidase method [23].

Iodination of 5 – 10 × 10^7 merozoites with 250 μCi of Na^{125}I (Amersham, spec. act. 100 mCi ml^{-1}) was carried out in 50 μl of Medium A at room temperature for 10 min, and was then stopped by two washes in Medium A supplemented with 1.5 mM cold NaI.

Extraction of merozoites in detergents. Iodinated merozoites were extracted immediately after labelling, while unlabelled or metabolically labelled merozoites were first washed 3 times in Medium A supplemented with 2 mM phenylmethylsulphonyl fluoride (PMSF) and protease inhibitors antipain, bestatin and pepstatin (Sigma), all at final concentration of 1 μg ml^{-1}. Approximately 10^9 washed merozoites were extracted on ice for 1 h in 0.5 ml of the same medium containing detergents, either 1% Nonidet P-40 (NP40) (Fluka) or 5 mM CHAPS (Sigma) or 5 mM Zwittergent 3–12 (Calbiochem-Behring Corp.). After extraction, insoluble material was removed by centrifugation at 13000 × *g* for 15 min at 4°C, and the supernatants were centrifuged through Sephadex G 25 gel to remove free unbound radiolabels. More than 95% of radioactivity in thus clarified extracts was precipitable in 10% trichloracetic acid. Extracts from several harvests of merozoites obtained in any one experiment were kept on ice, pooled and were routinely used within 5 h after preparation. In some experiments, one half of the NP40 extracts was frozen–thawed by 10 cycles of 5 min at −20°C and 10 min at room temperature before use.

Immunoprecipitation and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The detergent extracts were diluted four-fold with 50 mM Tris-HCl buffer, pH 8, containing 5 mM EDTA, 2% aprotinin, 1 mM PMSF and protease inhibitors as above. No detergent was present in this diluent for extracts in CHAPS and Zwittergent thus bringing their concentration to 1.25 mM final, while concentration of the detergent in NP40 extracts was kept at 1%. Aliquots of the extracts containing 3–5 × 10^5 cpm of ^{125}I , or 1–2 × 10^5 cpm of [^{35}S]methionine, or 5000 cpm of [^{14}C]glucosamine, were reacted with 25 μl of ascitic fluids containing monoclonal antibodies or with 25 μl of control sera for at least 3 h on ice. Immune complexes were then precipitated with 290 μl of sheep anti-mouse IgG (a gift from Scottish Antibody Production Unit, Carluke, Scotland) at 4°C overnight. Additional reactions were set up with IgG2 antibodies preadsorbed onto protein A–Sepharose. The immunoprecipitates were extensively washed as described [8] and were then boiled for 5 min in 60 μl of double strength Laemmli buffer (130 mM Tris-HCl, pH 6.8, 6% SDS, 20% glycerol) with or without 5% 2-mercaptoethanol as reducing agent [25]. Samples were analysed by SDS-PAGE in 5–30% gradient gels, the gels were stained with Coomassie blue and processed for autoradiography or fluorography [1,6]. Samples of total extracts or of supernatants remaining after removal of Sepharose-bound immune complexes were analysed as above.

Immunoblotting. Unlabelled or ^{125}I-iodinated antigens were separated by SDS-PAGE and were then transferred onto nitrocellulose according to Towbin et al. [24]. The transfers were at 55 V for 24 h at 2°C, and the subsequent procedures were carried out at room temperature in presence of PBS, pH 7.3, supplemented with 0.05% Tween

20. The blots were blocked with 1% (w/v) bovine serum albumin, cut into strips each of which was reacted with 5 ml of 1:200 dilution of a monoclonal ascites or control sera, washed and incubated with 5 ml of 1:100 dilution of peroxidase-conjugated rabbit anti-mouse IgG or anti-human IgG (Miles or Dakopatts) and finally developed using 4-chloro-1-naphtol to visualise antigen bands [26]. The developed strips were photographed to record the positions of the antigens, and where ^{125}I-labelled extracts were analysed, autoradiographed using Kodak X-Omat AR film.

Results

Serotyping of the FCB-1 isolate. The FCB-1 isolate used in this study was first typed by IFA on acetone-fixed films of asynchronous asexual stages with 18 mAbs known to recognise a variety of different PSA epitopes. The isolate expressed two types of conserved strain-common specificities: those present only on schizonts and merozoites but not detectable in the ring-stages, referred to as sm-specificities here (represented by mAbs 9.8 and 12.4), and specificities present in the ring-stages as well as on schizonts and merozoites, called smr-specificities (identified by mAbs 2.2, 7.5, 12.8 and 12.10). In addition, the isolate expressed on schizonts and merozoites two different polymorphic sm-specificities defined by a group of reagents (mAbs 6.1, 7.3, 7.6, 13.1 and 17.1) and the mAb 13.2 respectively. All of the common and polymorphic specificities were located on the surface of naturally released merozoites as shown by positive IFA staining in suspension without any fixation (results not shown). Several polymorphic PSA specificities were absent from the FCB-1 isolate, and the respective mAbs were used in subsequent experiments as negative specificity controls (mAbs 9.2, 9.5, 9.7, 10.3, 12.1 and 12.2).

The IFA typing was confirmed by immunoprecipitations (IIP) of biosynthetically radiolabelled PSA from NP40 extracts of schizonts (examples in Figs. 3 and 4). As expected, mAbs negative in IFA were also negative in the IIP assays, while all of the mAbs positively reactive in IFA immunoprecipitated the M_r 185000 PSA glycoprotein of the FCB-1 isolate. These results established that the isolate expressed PSA typical of serotype I [8].

Merozoites and their surface ^{125}I-labelled components. Naturally released fresh merozoites were isolated and handled in the presence of protease blockers to minimise degradation of the surface antigens. Periodic examinations by electron microscopy showed >90% of the merozoites to be in excellent condition with their surface coat intact and no evidence of morphological damage. The merozoites were free of red cells, or other stages of the parasite. Surface components of the merozoites were radiolabelled with ^{125}I and extracted in detergents. The extracts were then analysed by SDS-PAGE under reducing or non-reducing conditions, and the labelled components were visualised by autoradiography.

A set of nine bands were reproducibly labelled and effectively extracted into different detergents including NP40, Zwittergent 3- 12, CHAPS, SDS (Figs. 1A and 2; ref. 1) and deoxycholate (not shown). When reduced, the banding patterns consistently included heavily iodinated bands at 80,46,40,36 and 22 kDa, a medium-labelled band at 16 kDa, and two minor bands at 31 and 28 kDa. Somewhat variable amounts of label were found as one or two bands at ~67 kDa region. A number of very minor components were detectable less consistently only on overexposed films, and these were not investigated here. No iodinated band corresponding in size to the 185 kDa PSA was found in the extracts, indicating that this antigen itself was not a major component of merozoite surface.

A modification of the SDS-PAGE method [25] showed that some of the surface components contained disulphide bonds sensitive to 2-mercaptoethanol. Identical amounts of NP40 extract were analysed in nine adjacent wells, the only difference between the samples being whether or not they contained 5% ME (Fig. 1A). Several bands displayed altered mobilities depending on the presence (lanes 1,2,8,9) or the absence (lanes 4–6) of the reducing agent; in the two 'non-reduced' lanes (3 and 7) closest to the reduced samples it was possible to trace intermediates between the non-reduced and completely reduced states of the bands. The reduced 40 kDa band

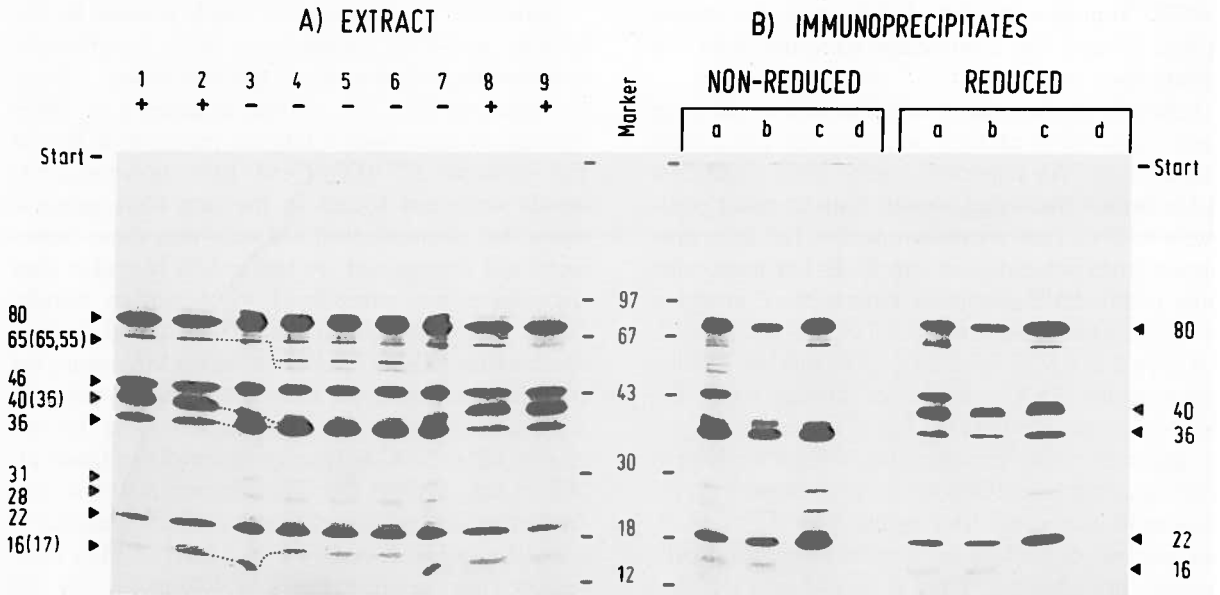

Fig. 1 (A) SDS-PAGE analysis of merozoite components accessible to surface labelling with ^{125}I. Merozoites of the FCB-1 strain were iodinated, extracted in NP40 and nine aliquots of the extract (10^4 cpm each) were separated in a 5–30% gradient gel in presence (+) or absence (−) of 5% 2-mercaptoethanol [25]. Note intermediates (lanes 3 and 7) of the transition from non-reduced (lanes 4–6) to fuly reduced states of the components (lanes 1,2 and 8,9); this was due to lateral diffusion of the reducing agent from the reduced lanes during electrophoresis. M_r in kDa of reduced components are shown on the left with estimates of non-reduced M_r in brackets for those bands of changing mobility (indicated by dotted lines drawn between lanes 2–4). Positions of standards are indicated between panels A and B: phosphorylase B (97 000); bovine serum albumin (67 000); ovalbumin (43 000); carbonic anhydrase (30 000); lactoglobulin A (18 000); cytochrome *c* (12 000). (B) Profiles of surface ^{125}I-labelled merozoite antigens immunoprecipitated by anti-PSA mAbs (lanes b–d) from a fresh NP40 extract (lanes a), and analysed under non-reducing and reducing conditions. The precipitate in lanes b (mAb 12.8) was representative of patterns obtained with all mAbs reactive with schizonts, merozoites *and* rings (smr-specificities). The pattern in lanes c (mAb 7.3) is representative of all mAbs recognising schizonts and merozoites *only* (sm-specificities, Table I). Control precipitate with mAb 9.5 (lanes d) was negative as with five other mAbs against polymorphic PSA specificities not expressed by the FCB-1 strain. Positions of the (reduced) major surface antigens investigated here are arrowed on the right of panel B.

appeared to have arisen from a species migrating as a faster 36 kDa band in the non-reduced lanes; such shift in mobility is consistent with a presence of several intramolecular disulphide-bonded domains. A non-reduced doublet at ~55 kDa on reduction shifted up to join the 67 kDa region (this was particularly clear in extracts where this component was more prominent than in the example shown). The 67 kDa (~55 kDa) component reacted poorly with immune human sera (not shown), and some of it might be serum albumin from culture medium adherent to the merozoites and thus radiolabelled. In contrast to the above bands where internal bonds seemed to predominate, a net decrease in the apparent size of a non-reduced 17 kDa to the reduced 16 kDa band indicated intermolecular bond(s) with unidentified low M_r material. However, the mobility of the partly reduced transition intermediate of this band indicated that it also contained an internal disulphide bond. The other components were not appreciably affected by ME and migrated as the major 80,46,36,22 and minor 31,28 kDa bands whether reduced or not. There was no evidence of mutual inter-chain disulphide bonding between any of the surface components.

Immunoprecipitation (IIP) of merozoite surface antigen complex. Detergent extracts of the ^{125}I-iodinated merozoites were reacted with the anti-PSA or other, control, mAbs, and the resulting immunoprecipitates were analysed by SDS-

PAGE. Typical examples of the results are shown in Fig. 1B and Fig. 2 while the complete data are summarised in Table I.

Initially, the immunoprecipitations were done from fresh NP40 extracts immediately after their preparation. As expected, only those anti-PSA mAbs which had been shown both to react positively in IFA and to precipitate the 185 kDa precursor from schizonts of the FCB-1 isolate, also gave positive IIP reactions with surface antigens of merozoites (mAbs 6.1,7.3,7.6,13.1,17.1; 13.2, 9.8,12.4,2.2,7.5,12.8,12.10). All mAbs against polymorphic PSA specificities absent from the FCB-1 isolate predictably failed to recognise any of the merozoite components. Negative results were also obtained with additional control mAbs known to recognise four internal proteins of *P. falciparum*, or surface of normal human red cells (results not shown). Thus it was concluded that only surface components of merozoites were radio-iodinated, and that the positive IIP reactions of the above 12 anti-PSA mAbs were entirely specific for the surface antigens.

However, the patterns of bands present in the specific anti-PSA precipitates were surprisingly complex including most of the merozoite surface components (Fig. 1B, extract in lanes a and precipitates in lanes b–d). Only the major 46 kDa and the variable 67 kDa (~55 kDa non-reduced) bands were not found in the anti-PSA precipitates; this demonstrated not only that these bands were not recognised by the mAbs but also that they were not complexed to the other bands. Eight mAbs against at least three different sm-specificities (Table I) all precipitated the same set of bands including the major 80 kDa, 40 kDa (36 kDa non-reduced), 36 kDa, 22 kDa and the minor 31 kDa, 28 kDa species (example in lanes c). All mAbs against smr-specificities also precipitated this set and, in addition, reacted specifically with the smallest band of 16 kDa (17 kDa non-reduced) (example in lanes b). Control mAbs did not react with any of these antigens (lanes d). Specificities of these reactions were further confirmed by absorptions (results not shown but see Fig. 3 for similar results). While absorptions with

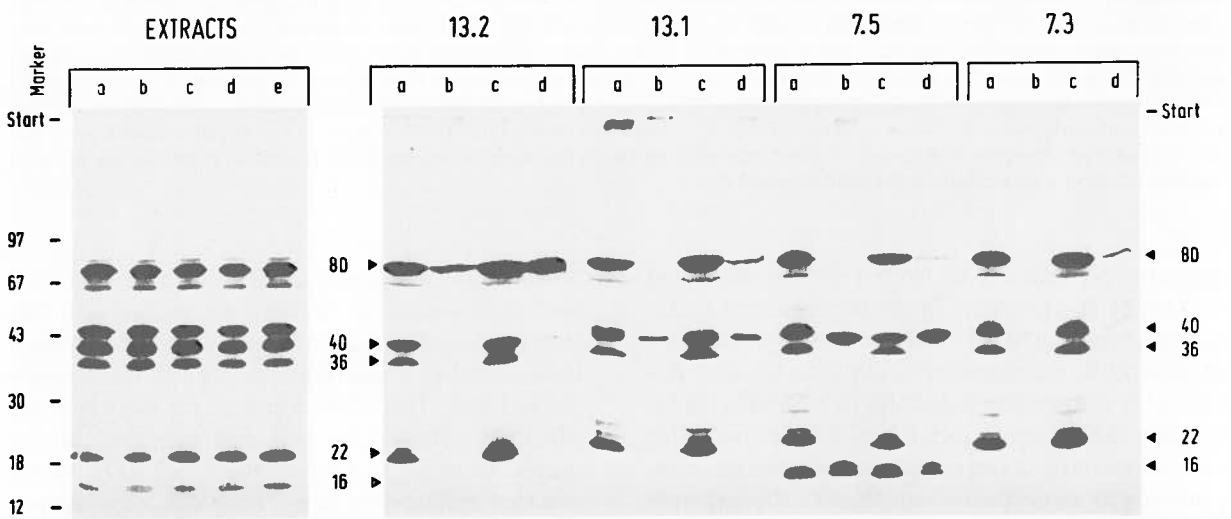

Fig. 2 Immunoprecipitation of a complex of merozoite surface antigens or of its dissociated subunits by mAbs against different PSA specificities. Five equal pellets of ^{125}I-labelled merozoites (~10^8 parasites each) were extracted in one of the detergents CHAPS (lanes a), or Zwittergent (lanes b), or NP40 (lanes c and d), or SDS (lane e). The CHAPS and Zwittergent extracts, and one of the NP40 aliquots (lanes c) were immunoprecipitated fresh, while the other aliquot of the NP40 extract was first frozen–thawed (lanes d). The total extracts, and immunoprecipitates from extracts a–d by mAbs 13.2 or 13.1 or 7.5 or 7.3, were analysed on reducing gels as indicated by the separate panels of lanes. Reduced M_r in kDa of major precipitated antigens are indicated between the panel of extracts and of precipitates by the mAb 13.2. Note that the same components were present in similar quantities in all detergent extracts; also that the smallest 16 kDa antigen reacted only with mAbs of smr-specificities (represented here by mAb 7.5) but not with the other, sm-reactive, reagents.

TABLE I

Merozoite surface components reactive with anti-PSA mAbs (FCB-1 isolate)

Antibody[a]		Immunoprecipitation of surface ^{125}I-labelled antigens from detergent extracts[b]																Immunoblots of SDS-PAGE separated antigens[c]									Summary	
Code	Specificity	CHAPS					NP40 fresh					NP40 frozen					Zwittergent					Reduced					Non-reduced				Epitope	M_r reduced fragment(s)
		80	40	36	22	16	80	40	36	22	16	80	40	36	22	16	80	40	36	22	16	80	40	36	22	16	80	36	22	17		
6.1	polymorphic 1_{sm}						++	++	++	++	−											−	±	−	−	−	−	−	−	−		
13.1	polymorphic 1_{sm}	++	++	++	++	−	++	++	++	++	−	±	+	−	−	−	−	+	−	−	−	−	±	−	−	−	−	+	−	−	sequential	40
17.1	polymorphic 1_{sm}	++	++	++	++	−	++	++	++	++	−	±	+	−	−	−	−	+	−	−	−	−	±	−	−	−	−	+	−	−	sequential	40
7.3	polymorphic 1_{sm}	++	++	++	++	−	++	++	++	++	−	±	±	±	−	−	−	±	±	−	−	−	−	−	−	−	−	−	−	−	conformational	(40 plus 36)
7.6	polymorphic 1_{sm}	++	++	++	++	−	++	++	++	++	−	−	±	±	−	−	−	±	±	−	−	−	−	−	−	−	−	−	±	−	−	conformational	(40 plus 36)
13.2	polymorphic 2_{sm}	++	++	++	++	−	++	++	++	++	−	++	−	−	±	−	++	−	−	±	−	++	−	−	−	−	++	−	−	−	sequential	80
9.8	common$_{sm}$						++	++	++	++	−											−	−	−	−	−	−	±	−	−	conformational	
12.4	common$_{sm}$						++	++	++	++	−											−	−	−	−	−	−	−	−	−	conformational	
2.2	common$_{smr}$						++	++	++	++	++											−	−	−	−	−	−	++	−	+	disulphide-dependent conformational	40 and 16
7.5	common$_{smr}$	++	++	++	++	++	++	++	++	++	++	±	++	−	−	++	−	++	−	−	++	−	−	−	−	−	−	+	−	±	"	40 and 16
12.8	common$_{smr}$	++	++	++	++	++	++	++	++	++	++	−	++	−	−	++	−	++	−	−	++	−	−	−	−	−	−	++	−	+	"	40 and 16
12.10	common$_{smr}$	++	++	++	++	++	++	++	++	++	++	−	++	−	−	++	−	++	−	−	++	−	−	−	−	−	−	++	−	+	"	40 and 16

[a] Specificities of mAbs had been determined by IFA against a panel of *P. falciparum* isolates (for details see refs. 7,8). Specificities present in all isolates tested to date (>300) are termed 'common', while those expressed in some but not in all isolates are termed 'polymorphic'. Polymorphic specificities which segregate from each other among isolates were numbered arbitrarily. Where several mAbs are listed against a specificity this indicates that their target epitopes do not segregate between PSA variants but it does not prove structural identity of such epitopes. Polymorphic specificities defined by mAbs 9.2, 9.5, 9.7, 10.3, 12.1 and 12.2 are not expressed by the serotype I FCB-1 strain [8]; these mAbs were used as controls and reacted negatively in all tests (results not listed). Subscript letters indicate positively reactive stages of the asexual blood cycle: s, schizont; m, merozoite; r, ring.

[b] Summary of experiments: immunoprecipitations from fresh NP40 extracts were repeated 6 times; from frozen–thawed NP40 1–4 times with different mAbs; Zwittergent 1–3 times; CHAPS twice. All detergent extracts contained the same antigens (Fig. 2), and precipitations were started with 3×10^5 protein-bound cpm of each extract. Only major components precipitated by anti-PSA mAbs are listed by their M_r in kDa determined under reducing conditions. The results are expressed as follows: −, consistently negative findings; ++, presence in precipitates from fresh NP40 extracts where the different bands appeared in approximately the same proportions as in the extract, or for precipitations of comparable strength from the other detergents; + and ±, positive reactions which were, however, weaker than control precipitation from fresh NP40 included in all experiments. See Fig. 2 for examples. No immuno-precipitation of the surface antigens was ever achieved with 7 control mAbs against human red cells or internal parasite antigens (p35; p82; p155; p23 [5,11,19]).

[c] Western blots of surface ^{125}I-labelled merozoites extracted in NP40 and separated under reducing or non-reducing conditions. Antigens reactive with mAbs after transfer onto nitrocellulose were identified as the surface bands by superimposition of the blots and their autoradiographs. Note that depending on the absence or presence of 2-mercaptoethanol during separation some antigens changed mobility or lost reactivity respectively with some mAbs. See text and Fig. 1 for details.

the sm-specific mAb 7.3 quantitatively depleted that extract of any components reactive with self, and left only the 16 kDa antigen to react with the smr-specific mAb 12.8, the reciprocal absorption with the mAb 12.8 depleted all antigens reactive with mAbs of either specificities. This established that the 16 kDa antigen was not physically linked with the other six bands. However, it also implied that all of the other bands shared at least one epitope with the 16 kDa, the epitope being additional to three sm-specificities seemingly also present on all of the larger bands. This we considered to be most unlikely, and therefore investigated the alternative possibility that the surface components were coprecipitated as a non-covalently linked complex by mAbs reactive with any of its subunits.

To dissociate the putative complex prior to immunoprecipitations, aliquots of ^{125}I-labelled merozoites were extracted in different detergents including the zwitterions CHAPS and Zwittergent 3-12, the anions deoxycholate and SDS, as well as the non-ionic NP40. The NP40 extracts were used either fresh as before, or after 10 cycles of freeze–thawing.

Results of these experiments are illustrated by Fig. 2 and summarised in Table I. While CHAPS and fresh NP40 extracts contained the intact complex, simple freeze–thawing of the NP40 extract or extraction in Zwittergent (or deoxycholate or SDS, results not shown) appeared to dissociate the complex, and reactivities of individual mAbs were now more selective for different subunits. Thus only the 80 kDa fragment (and very

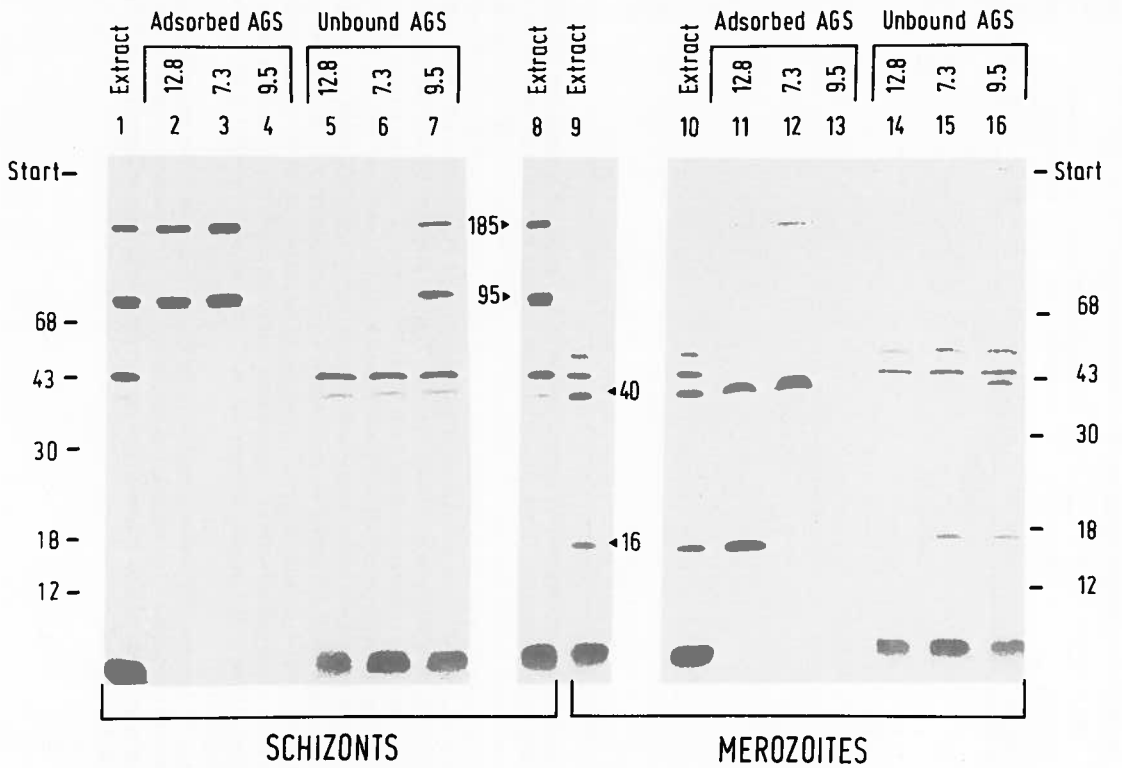

Fig. 3 Immunoprecipitation of [^{14}C]glucosamine-labelled antigens of schizonts and merozoites. Fresh NP40 extracts of schizonts (lanes 1,8) or merozoites (lanes 9,10) were reacted with anti-PSA mAbs, and the immunoprecipitates (lanes 2–4 and 11–13) as well as thus depleted extracts (lanes 5–7 and 14–16) were analysed under reducing conditions. Antibodies 12.8 and 7.3 both absorbed the 185 kDa precursor from schizont extract (lanes 2,3 and 5,6 showing precipitates and depleted supernatants respectively); both mAbs precipitated a 40 kDa merozoite antigen but only mAb 12.8 (a smr-specificty) recognised another merozoite band of 16 kDa (precipitates lanes 11,12 and depleted supernatants lanes 14,15). None of the antigens reacted with negative control mAb 9.5 (precipitate lanes 4,13: supernatant lanes 7,16). Molecular weight standards are indicated on the left and right of experimental lanes.

weakly the 22 kDa band) but none of the other species reacted with mAb 13.2. The 40 kDa antigen was precipitated almost free of other bands by mAbs 13.1 and 17.1. Both the dissociated 40 kDa and the 16 kDa fragments were quantitatively precipitated by ring-reactive mAbs (7.5, 12.8 and 12.10). Interestingly, two mAbs (7.3 and 7.6) both strongly reactive with the intact complex, precipitated only traces of the 40 and 36 kDa bands from the dissociated extracts; this indicated that these mAbs recognised conformational epitopes of the complex probably formed between the 40 and 36 kDa species.Note that all the bands were equally represented in all detergent extracts, Fig. 2. Also, as indicated by the presence of light and heavy Ig chains of the mAbs on Coomassie blue-stained gels of separated immunoprecipitates, none of the detergents inhibited effective isolation of the immune complexes containing the mAbs; results not shown.

Mapping of PSA epitopes onto merozoite surface antigens by Western immunoblotting. Fresh or frozen-thawed NP40 extracts of ^{125}I-iodinated or unlabelled merozoites were used with identical results in experiments where the antigens were first separated by SDS-PAGE and then transblotted and probed with the mAbs. The results confirmed the mAbs' specificities as seen in IIP from the dissociated complex, and allowed direct assignment of marker epitopes to different subunits. In addition, by probing the antigens separated under either non-reducing or reducing conditions, it was possible to make conclusions about the role of disulphide bonds in maintaining integrity of certain epitopes. The results are summarised in Table I.

Blotting showed that the 80 kDa fragment alone carried the polymorphic site recognised by the mAb 13.2. Since the epitope was preserved on SDS-denatured and reduced fragment, it might have little conformation aside that consisting of the primary structure. In contrast, the strain-common epitopes defined by the smr-reactive mAbs (2.2,7.5,12.8 and 12.10) were detectable only on the non-reduced 36 and 17 kDa forms but were destroyed when these antigens were reduced (to 40 and 16 kDa respectively), indicating that the epitopes were stabilised by the disulphide bonds associated with these fragments (se Fig. 1A). The 40 kDa (36 kDa non-reduced fragment also contained polymorphic epitope recognised by the mAbs 13.1 and 17.1. No PSA markers could be assigned by immunoblotting t the major 36 and 22 kDa (nor to the minor 31 an 28 kDa) components of the surface complex. Not that all of the surface components were effec tively transblotted from both non-reduced an reduced gels, and were detectable by autoradiog raphy; not shown.

Merozoite surface (glyco)proteins and their schi zont precursor. To determine which of the sur face-complex components were glycosylated pro teins, the progenitor schizont stages, metabolicall

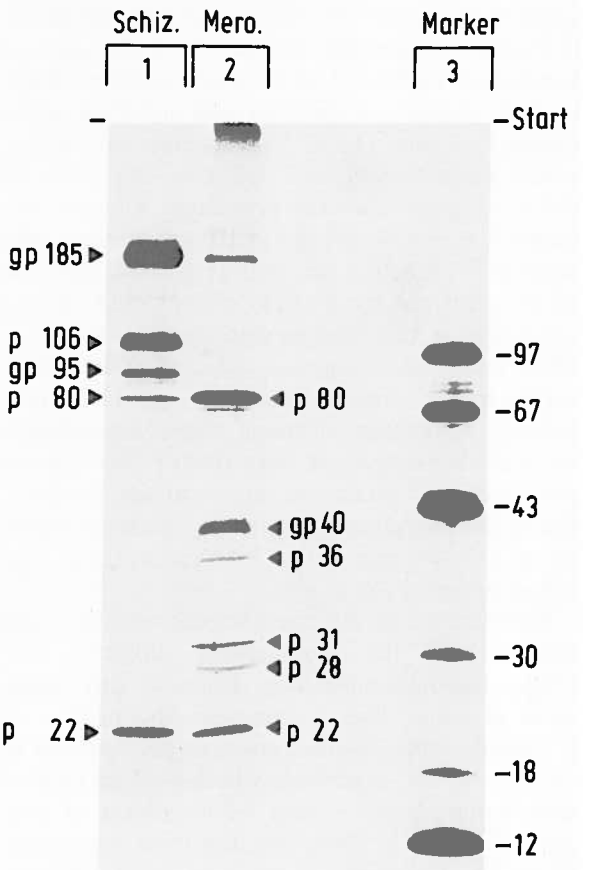

Fig. 4 [^{35}S]methionine-containing proteins precipitated by all anti-PSA mAbs from fresh NP40 extracts of FCB-1 schizonts (lane 1) or merozoites (lane 2) (example mAb 7.3). M_r in kDa of reduced antigens are indicated by arrowheads; molecular weight standards are shown on the right.

labelled with [^{14}C]glucosamine or with [^{35}S]methionine, and merozoites released by thus labelled schizonts, were used as the source of the precursor glycoprotein and of its terminal fragments respectively.

Fig. 3 compares glycosylated components of schizonts to those of merozoites, and shows antigens precipitated from these stages by reagents against sm- or smr-specificities, and negative controls. The comparison of total extracts (schizonts lanes 1, 8 versus merozoite lanes 9, 10) shows an apparent replacement of the 185 kDa (and also a 95 kDa) glycosylated schizont bands by smaller 53, 40 and 16 kDa components in mature merozoites. This result is consistent with published pulse-chase work indicating that some of these merozoite bands could be derived from the large schizont precursor [6]. Here, antigenic similarity is shown between the 185 and 95 kDa schizont bands and the 40 and 16 kDa merozoite products by both specific precipitation with anti-PSA mAbs (lanes 2, 3 and 11, 12 for schizonts and merozoites respectively) and selective depletion of these antigens from the remaining supernatants (lanes 5, 6 and 14, 15). As in IIP experiments with surface ^{125}I-labelled antigens (Fig. 1B), only the 40 kDa but not the 16 kDa glycosylated antigen was found in precipitates with sm-specific mAbs, while both these fragments reacted with smr-reactive mAbs (Fig. 3, lanes 12 and 11 respectively). Note that although these experiments were deliberately done with fresh NP40 extracts presumed to contain the intact surface complex, the remaining glycosylated components of merozoites (53, 46, and <10 kDa) were not coprecipitated by anti-PSA mAbs.

The final set of IIP experiments correlated the surface and the glycosylated antigens with [^{35}S]methionine-containing schizont and merozoite proteins. Fig. 4 compares the profiles of [^{35}S]methionine-labelled antigens precipitated by all PSA mAbs, regardless whether of sm or smr-specificities, from a fresh NP40 extract of schizonts (lane 1) to those isolated from merozoites (lane 2). The precipitates from schizonts contained the 185 kDa antigen, and smaller bands at 106, 95, 80 and 22 kDa. In contrast, the merozoites contained much less of the 185 kDa precursor and no detectable 106 or 95 kDa proteins. Instead, the 80 kDa antigen became more prominent and polypeptides of 40, 36, 31 and 28 kDa appeared, with the 22 kDa band present in quantity comparable to its content in the schizonts. These results are compatible with published works demonstrating the derivation of the smaller polypeptides from the 185 kDa precursor [2, 3, 10, 29]; they also indicate that the 22 kDa band appears before the 40, 36, 31 and 28 kDa series and thus is not likely to be derived from these fragments. No [^{35}S]methionine-labelled merozoite band equivalent to the 16 kDa surface fragment was detected indicating that, at least in the FCB-1 isolate, it contained little or no methionine.

Identity of the surface-iodinated, and of the similarly sized biosynthetically labelled antigens of merozoites was further confirmed by a parallel SDS-PAGE analysis under both reducing and non-reducing conditions of total labelled extracts (not shown) and of immunoprecipitates (Fig. 5). The appropriately labelled antigens present in these samples always comigrated, inclusive of the characteristic shifts in mobility from the non-re-

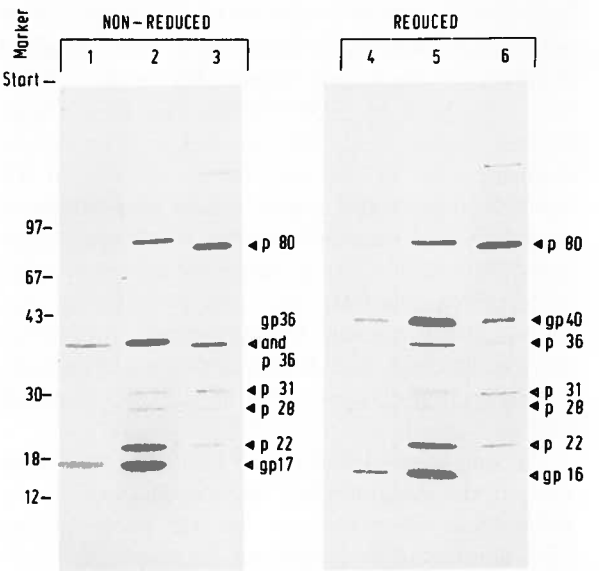

Fig. 5 Comparative radiolabelling of merozoite antigens reactive with anti-PSA mAbs. Immunoprecipitates with mAb 12.8 from fresh NP40 extracts of surface ^{125}I-iodinated merozoites (lanes 2,5), or merozoites biosynthetically labelled with [^{14}C]glucosamine (lanes 1,4) or [^{35}S]methionine (lanes 3,6) were analysed under non-reducing or reducing conditions. Non-reduced and reduced M_r of the glycoproteins are indicated to the right of the respective precipitate panels. Molecular weight standards were as in Fig. 1.

TABLE II

Characteristics of surface (glyco)proteins of FCB-1 strain merozoites[a]

Antigen (kDa)		Labelled with[b]			PSA marker epitopes	
Reduced	Non-reduced	Surface ^{125}I	[^{35}S]Methionine	[^{14}C]Glucosamine	Specificity	mAb(s)
p80	80	+++	+++	−	polymorphic 3_{sm}	13.2
p67	67	+	±	−		
gp40	36	+++	++	++	polymorphic 1_{sm}	13.1, 17.1
p36	36	++	+	−	and common$_{smr}$	12.10, 12.8, 7.5, 2.2
p31	31	+	+	−		
p28	28	+	+	−		
p22	22	+++	++	−		
gp16	17	++	−	++	common$_{smr}$	12.10, 12.8, 7.5, 2.2

[a] All major and minor bands detectable in anti-PSA immunoprecipitates from non-dissociated extracts are included.
[b] Relative intensity of radiolabelling (see Fig. 5): +++, strong; ++, medium; +, weak; −, not detectable.

duced to the reduced states of some of them. It was concluded that the FCB-1 strain merozoite surface antigens precipitated by anti-PSA antibodies include the 80, 36, 31, 28 and 22 kDa proteins, the 40 kDa (36 kDa non-reduced) glycoprotein and the 16 kDa (17 kDa non-reduced) glycosylated component. At least some of these antigens share epitopes with the 185 kDa precursor glycoprotein, and are probably derived from it via the intermediate 106 kDa protein and the 95 kDa glycoprotein early fragments present in schizonts (compare schizont lanes 2, 3 in Fig. 3 to lane 1 in Fig. 4). The biochemical and antigenic characteristics of the merozoite antigen complex are summarised in Table II.

Discussion

Here we demonstrate that a majority of surface components of *P. falciparum* merozoites are immunoprecipitated by anti-PSA antibodies as a non-covalently linked complex (Fig. 2). Other workers have alluded to the existence of such complex in *P. knowlesi* [28] and *P. falciparum* [16]. The latter report indicated that only a 42 and a 19 kDa fragment of PSA were complexed, while we find a much larger assembly consisting of the p80 and gp40 fragments of PSA and four proteins which may or may not be derived from the same precursor (p36, p31, p28, p22). This discrepancy is likely to be due to use of different detergents and/or to handling of the detergent extracts prior to immunoprecipitation (Fig. 2). We find the complex in fresh NP40 or CHAPS extracts but nc in Zwittergent or in deoxycholate, the detergei apparently used by others [2,3,16]. Freeze– thaw ing also seems to dissociate the complex, and th could explain why it was not detected in som earlier studies [10,29]. As yet we do not exclud the possibility that the complex might be an ai tefact formed only in vitro, although we think thi rather unlikely for the following reasons. First, th complex is present in freshly prepared cell ex tracts but, once dissociated by freeze–thawing, i does not spontaneously reform (at 4°C overnight as could reasonably be expected in the case of detergent-induced artefact. Second, the comple includes very reproducibly the six surface com ponents listed above, while, equally selectively two other surface antigens are excluded from i (gp16 fragment of PSA and a 46 kDa band un related to PSA) as are also several glycosylatec components (Fig. 3) and all of the internal para site antigens we tested for (see legend to Table for these controls). This implies a degree of spe cific association such as is common for functiona non-covalent molecular assemblies including en zymes and other proteins [30] or cell surface re ceptors [31,32]. Third and most significant, the intact complex but none of its individual compo nents alone, displays conformational epitopes of the precursor (recognised by mAbs 7.3 and 7.6) which are detectable by IFA on whole mero zoites, indicating that the complex exists in situ on the parasite surface. Our minimal hypothesis compatible with these findings is that foundations

of the complex are laid in vivo on tertiary folding of the PSA precursor. This would bring into contact sequentially distant domains of the precursor, and the conformation would be stabilised by non-covalent bonds formed between the domains. After cleavages of the joining peptide backbone, the domains would become subunits of the complex now linked only by the non-covalent bonds. The irreversible cleavages of the peptide links could result in loss of information needed for spontaneous reassembly of the complex if the interactions between subunits are experimentally disrupted.

What significance can be proposed for the complex? Its surface location and association with the merozoite coat [27] suggest that the antigenic complex of *P. falciparum* could be akin to, or a building block of, the highly ordered clusters of proteinaceous filaments which make up the surface coat of *P. knowlesi* merozoites [34]. There are parallels between the timing of appearance and compartmentalisation of the clusters [34] and of PSA and/or of its fragments [8] (McBride, unpublished) shown here to be included in the complex. Both appear first as patches on the surface of intracellular schizonts and, later, as a layer enveloping budding merozoites and the residual body, and both persist on released merozoites. During invasion the coat is shed as is also the p80 fragment [16] and, probably, most of the complex with the exception of a portion of the gp40 antigen, the gp16 fragment (see below).

Notwithstanding strain-related variations in the apparent molecular mass of both the PSA precursor and of the antigens derived from it [8,11,35], most of the surface components investigated here can be related to products of other PSA variants. Our data concerning processing of the FCB-1 strain gp185 (PSA serotype I [8]) can largely be accommodated by models proposed for the serologically similar Wellcome strain [16,35] or for a serotype VI [8] (McBride, unpublished) Camp strain [29], and here we shall refer to these schemes in particular.

The evidence for including the 36, 30, 28 and 22 kDa proteins among the PSA-derived antigens is circumstantial and consists of their presence in the merozoite surface complex reactive with anti-PSA antibodies. If derived from PSA, the p36 may correspond to a non-glycosylated 45 or a surface 38 kDa fragment present in other strains [29] (A.A. Holder, personal communication). The minor 31 and 28 kDa surface proteins could be equivalent to 30 and 28 kDa fragments detectable by Western blots in the Wellcome strain (A.A. Holder, personal communication). Whether the 22 kDa antigen is derived from PSA or is an unrelated protein remains to be clarified.

In the present study the p80 and the glycosylated gp40 and gp16 fragments were shown unequivocally by immunoblotting to share epitopes with the gp185 precursor. Since the p80 is not glycosylated and also lacks marker epitopes of the smaller antigens (Table II), it cannot be their predecessor and is likely to represent a structurally and antigenically distinct domain of the precursor. We consider that the p80 of FCB-1 is homologous to 76–83 kDa proteins derived from other PSA variants [2–4,8,10,18,29]. These proteins represent the N-terminus of PSA [16,29,35] (W. Strych, A. Miettinen-Baumann, L. Lottspeich and H.G. Heidrich, manuscript in preparation) and, in keeping with polymorphism of DNA within this region [18,33,35,36], they contain polymorphic epitopes detectable by mAbs (Table II) [10,29]. Serological evidence obtained with monoclonal [3,10,16,29] or, more significantly, with polyclonal antibodies [16,18,29] indicates that immunologically these proteins may bear little, if any, resemblance to antigens representing other portions of the precursor.

The gp40 (36 kDa non-reduced) and the gp16 (17 kDa non-reduced) antigens share a number of similarities indicating that they arise from the same domain of the precursor. They are both glycosylated, both seem to contain internal disulphide bonds and also share the same epitopes sensitive to reduction of these disulphides (conserved smr-specificities, Table I). Thus we propose that the gp16 species is a terminal fragment remaining after a cleavage of the gp40. It is of further interest that while epitopes specific for gp40 (or p80 or the whole complex) are no longer detectable by IFA in newly invaded ring-stages, the rings retain reactivity with mAbs which recognise the gp16. This indicates that it is the gp16 portion of the gp40 antigen that remains on merozoites through invasion and raises the possibility

that it includes the region which anchors the precursor and, later, its derivates to the parasite membrane. According to the deduced amino acid sequence of the precursor [16, 33] the putative membrane anchor peptide is located at the C-terminus of the precursor, in a close vicinity of a cysteine-rich sequence which also contains a potential *N*-glycosylation site (Asn 1572 or 1563 in variants reported respectively in refs. 16 and 33). Our evidence for glycosylation, content of cysteines and paucity of methionine combined in gp16 therefore tentatively locates it close to or within the last ~100 residues at the C-terminus of the precursor (and also of gp40). The gp16 fragment is not unique to the FCB-1 strain since using the same mAbs against strain-conserved epitopes we detect a surface antigen of exactly the same and, depending on reduction of the disulphides, changing mobility on merozoites of strains expressing other PSA variants (strains PB1/4 and T9/100, serotypes II and VII respectively; McBride and Heidrich, unpublished). In these strains we also find cross-reactivity of the 16 kDa with another surface antigen at ~40 kDa, presumed to be homologous with gp40. Homologues in other strains probably include 40–46 kDa glycoproteins [10,29] and a 42 kDa surface antigen [3,16] which too is glycosylated (A.A. Holder, personal communication). On a rationale different from ours these glycoproteins were also tentatively assigned to the C-terminus [16,29].

Although our results show that the serological polymorphism of the precursor extends to both the p80 and the gp40 fragments, they also ind cate that the gp16 portion carrying strain-com mon epitopes may be relatively conserved. Thi is in accord with a recent demonstration tha within the DNA region coding for homologues c gp40 in different PSA serotypes [8], polymorph ism is detectable in DNA coding for the N-ter minus of the fragment, while the DNA segmen coding for the C-terminal end, probably contain ing gp16, seems highly conserved [35]. If in th future the gp16 fragment can be shown to have function in the merozoite's biology, the appar ently conserved structure of this antigen indicate that this part of the polymorphic precursor coul hold a promise for the development of a malari vaccine.

Acknowledgements

We thank Mrs. M. Neubauer and Mrs. A Bas kaya for technical assistance, Mrs. Janice Bar bour and Mrs. Ann Muir for typing the manu script, and the Scottish Antibody Production Uni for a generous gift of antisera. This work re ceived support from The Wellcome Trust, fron the UNDP/World Bank/WHO Special Pro gramme for Research and Training in Tropica Diseases, and from the Deutschen Bundesminis terium für Forschung and Technologie. One of u (J.S.McB.) received travel funds from the Britisl Society for Immunology and from the Britisl Council.

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