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Polymorphism of a 35–48 kDa *Plasmodium falciparum* merozoite surface antigen

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Glycoproteins of the asexual blood stages of *Plasmodium falciparum* were labelled with radioactive glucosamine and analysed by two-dimensional electrophoresis. Four major glycoproteins were detected in all eight parasite isolates studied. Two of the glycoproteins, designated GP2 and GP4, were invariant among the isolates, while the other two GP1 and GP3 were found to be polymorphic in both their biochemical and antigenic properties. By immunoblotting and immunoprecipitation with specific monoclonal antibodies, the two polymorphic glycoproteins were identified as surface antigens of merozoites.

Key words: *Plasmodium falciparum*, Two-dimensional electrophoresis, Monoclonal antibody; Glycoproteins; Antigenic diversity.

Introduction

A large number of antigens expressed by the infective stages of *Plasmodium falciparum* malaria have been identified in an attempt to produce a vaccine against this parasite [1]. Particular attention has been given to antigens associated with the surface of the merozoite. The first major antigen identified was a glycoprotein denoted PMMSA (the precursor to the major merozoite surface antigens). The antigen exists as a series of allelic variants distinguishable by monoclonal antibodies (McAbs) [2,3].

A second glycoprotein of the merozoite surface has been described which is of interest as a candidate vaccine antigen because parasite growth

is inhibited in vitro in the presence of monoclonal antibodies recognising this molecule [4,5].

In the present work, we show that, like PMMSA, different forms of this antigen exist in *P. falciparum* populations, distinguishable by differential reactivity with McAbs and mobility using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). We also describe characteristics of other major glycoproteins of blood forms detected by 2D-PAGE.

Materials and Methods

Plasmodium falciparum isolates and clones. The *P. falciparum* clones and isolates used in this study were the clones T9-94, T9-96, T9-101 (Thailand), HB3 (Honduras), 3D7 (The Netherlands), and the isolates K29 (Thailand), Palo Alto 17 (Uganda) and FCB-1 (Colombia). They differ from one another in one or more genetically determined markers such as alloenzymes, drug sensitivity and DNA markers [6–8]. The parasites were obtained from the World Health Organisation Registry of Standard Strains of Malaria Parasites held at the Genetics Department of Edinburgh University. Asexual stages of the parasite

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Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; IEP, isoelectric point; McAb, monoclonal antibody; GP, glycoprotein; PBS, phosphate-buffered saline; DMSO, dimethyl sulphoxide; PPO, 2,5-diphenyloxazole; PMMSA, precursor to the major merozoite surface antigens.

were grown in vitro [9] and synchronised by sorbitol treatment [10].

Radiolabelling of parasites. Parasites were labelled with [^3H]- or [^{14}C]glucosamine or [^{35}S]methionine for 16 h during development from trophozoite to schizont stage [11].

Experiments using the immunoblot technique described below required large amounts of unlabelled parasite material, as well as labelled parasites. To achieve an identical composition of both labelled and unlabelled material, parasites in one 25-ml culture were synchronised and grown until they consisted of 5–10% trophozoites. 5 ml of this culture was removed and radiolabelled. When schizonts were present in both cultures they were then harvested.

Parasites pellets were solubilised in buffers suitable for analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [12] or 2D-PAGE [13].

Monoclonal antibodies and indirect immunofluorescence assay. Mouse McAbs were produced as has been described [14]. Hybridomas 12.3-2-2 and 13.4-2-1, producing McAbs 12.3 and 13.4, respectively, were derived by fusion of NS1 myeloma cells with spleen cells from BALB/c mice immunised with schizonts of *P. falciparum* clones T9-96 and T9-94, respectively. The characteristics of McAbs 12.2 and 12.8 have been reported elsewhere [2,15].

Strain reactivities of the McAbs were determined by indirect immunofluorescence assay (IFA) titrations of ascitic fluids on acetone-fixed schizonts of a panel of *P. falciparum* isolates [2,14].

Immunoprecipitation. Parasite antigens were extracted in 1% (v/v) NP40 and immunoprecipitated with McAbs as described [2]. Human antibodies were purified from pooled Gambian immune sera by adsorption to nitrocellulose-bound antigen immunopurified using McAb 13.4 from T9-94 parasites [16].

SDS-polyacrylamide gel electrophoresis. SDS-PAGE of uniform or gradient acrylamide was used to fractionate proteins according to M_r .

Standard proteins (myosin, 205 kDa; galactosidase, 116 kDa; phosphorylase *b*, 97.4 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; and carbonic anhydrase, 29 kDa (Sigma Chemical Co.) were also included.

Two-dimensional polyacrylamide gel electrophoresis and fluorography. 2D-PAGE separates proteins firstly by charge, and secondly by M_r [13]. Using a BioRad multigel cell, up to six gels were run at once using the method as described [11]. After electrophoresis, gels containing labelled proteins were fluorographed [17].

In some experiments the position of certain radiolabelled immunoprecipitated antigens was compared to co-electrophoresed unlabelled total parasite proteins on the same 2D-PAGE gel by the following method. The position of the radiolabelled antigen under study was marked on the gel by a pin-prick. The gel was then rehydrated by sequential 2-h incubations in 100%, 75%, 50% and 10% glycerol (v.v in H_2O). 2,5-Diphenyloxazole (PPO) was removed from the gel by washes in dimethyl sulphoxide (DMSO), and the gel was then stained with silver [18]. The position of the marked antigen among the other silver-stained proteins of *P. falciparum* was then identified.

Immunoblotting. Approximately 0.5 mg of unlabelled parasite proteins, together with 20 000 cpm of [^{14}C]glucosamine-labelled or 200 000 cpm of [^{35}S]methionine-labelled components added as markers, separated by either SDS-PAGE or 2D-PAGE, were transferred onto nitrocellulose [19]. Blots were incubated with ascitic fluids (1/100 dilution) and bound McAbs detected using peroxidase-conjugated anti-mouse IgG (1/300 dilution), followed by development with 4-chloro-1-naphthol [20]. Blots containing ^{14}C -labelled antigens were dried and exposed to Hyper-MP film (Amersham) in an X-ray cassette (Kodak) at -70°C .

Results

Glycoproteins of *P. falciparum*. Glycoproteins of trophozoite and schizont stages were labelled metabolically with [^3H]glucosamine in six cultures of *P. falciparum*, and compared by 2D-

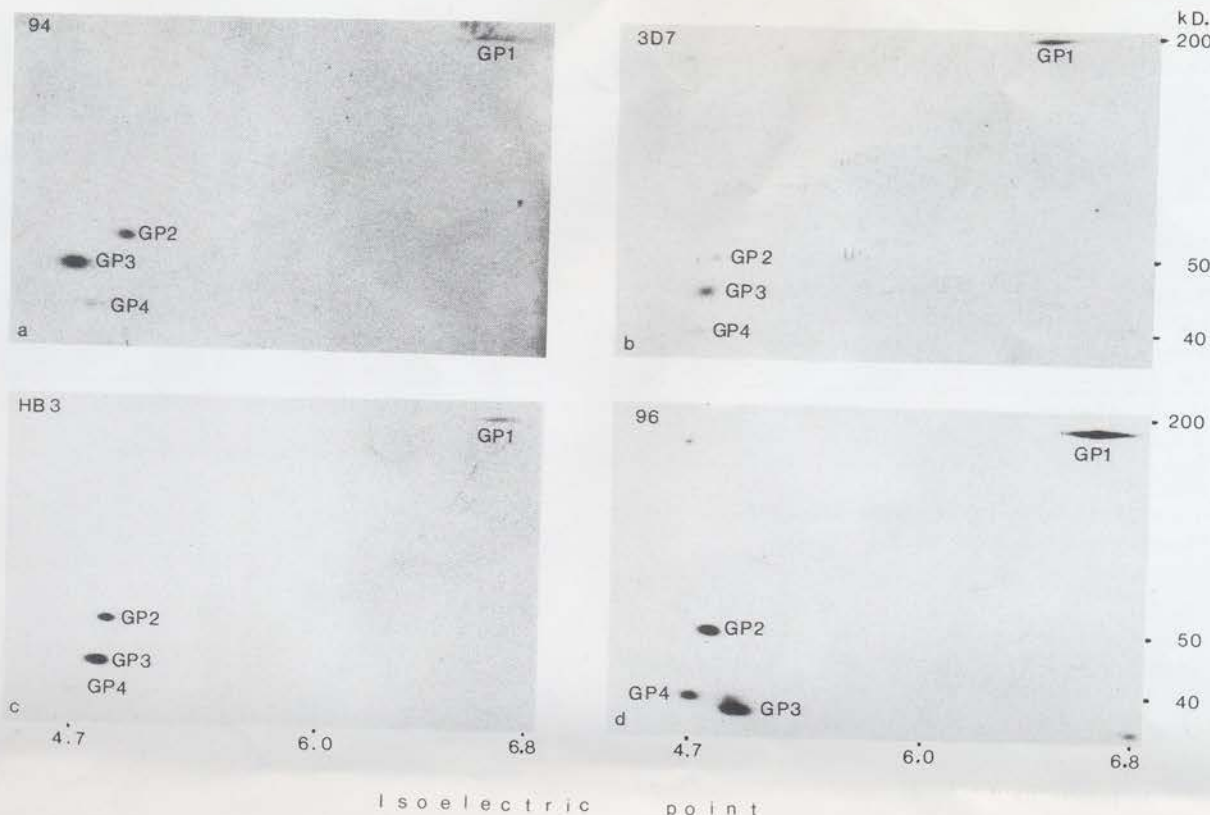


Fig. 1. Two-dimensional PAGE of glycoproteins. The four parasite clones T9-94, 3D7, HB3 and T9-96 were labelled with glucosamine. 2D-PAGE was carried out, resolving four predominant glycoproteins GP1, GP2, GP3 and GP4, as indicated.

PAGE. In all parasites, four major glycosylated components were identified which are denoted here as GP1, GP2, GP3 and GP4. Fig. 1 illustrates these glycoproteins in four cloned parasites, T9-94, T9-96, 3D7 and HB3.

Characterisation of GP3. GP3 was the glycoprotein which exhibited most variation in different parasites. Five different variants could be identified by 2D-PAGE, ranging in size from 35.2 kDa to 48.4 kDa and in isoelectric point (IEP) from 4.6 to 5.2 (Table I). The characteristics of GP3 in clones and isolates of *P. falciparum* were studied further by immunoblotting, immunoprecipitation and immunofluorescence experiments. The results are summarised in Table I.

GP3 from three clones was reacted with McAbs 12.3 and/or 13.4 on immunoblots of components separated by 2D-PAGE (Fig. 2). In clone 3D7, the antigen reacted positively with both antibodies

(Fig. 2b and c) and had an M_r of 46 100 and IEP of 4.7.

In clone T9-96, GP3 was recognised only by McAb 12.3, and on 2D-PAGE gels possessed two lobes, with an IEP of 5.0 and 5.2 and an M_r of 36 300 and 35 200, respectively (compare Fig. 1d and Fig. 2d). Clone T9-94 reacted only with McAb 13.4 and possessed an IEP of 4.6 and an M_r of 48 400 (Figs. 1a and 2a). McAb 13.4 also specifically immunoprecipitated the antigen from detergent extracts of this parasite, metabolically radiolabelled with a mixture of amino acids, histidine or glucosamine (results not shown).

Fig. 3 shows further examples of differential recognition of GP3 variants in all the parasite lines studied either by monoclonal antibodies in immunoprecipitation (panel a) or immunoblotting tests (panel b). Thus, McAb 13.4 immunoprecipitated a single [3 H]glucosamine-labelled antigen from parasites 3D7, T9-94 and FCB-1 (Fig. 3a,

TABLE I

Characteristics of GP3 in different isolates of *P. falciparum*

Parasite	Electrophoresis		McAb 12.3 reactivity			McAb 13.4 reactivity			Variant form
	M_r	IEP	Blot ^a	IPTN ^a	IFA ^b	Blot ^a	IPTN ^a	IFA ^b	
HB3	45.0	4.8	—	—	<10	nt	—	<10	V
3D7	46.1	4.7	++	nt	10 ⁴	++	+	10 ³	VI
T9-94	48.4	4.6	—	—	10 ⁴	++	++	10 ⁵	VII
FCB-1	48.4	4.6	nt	nt	10 ⁴	++ ^c	++ ^c	10 ⁵	VII
T9-96	35.2/36.3	5.2/5.0	++	++	10 ⁴	—	—	<10	I
K29	45.0	4.7	nt	nt	<10	nt	nt	<10	III
T9-101	36.8	nt ^d	++	nt	10 ⁴	nt	nt	<10	II
Palo Alto 17	37.3	nt ^d	++	nt	10 ⁴	nt	nt	<10	IV

^aResults of immunoblots and of immunoprecipitations were scored as + and ++ where positive and as — where negative; nt, not tested.

^bIFA results are given as titres of ascitic fluids, i.e., the greatest dilutions positively reactive with approx. 10⁴ acetone-fixed schizonts.

^cFor results on isolate FCB-1 see also refs. 4 and 21.

^dResults were obtained only by SDS-PAGE.

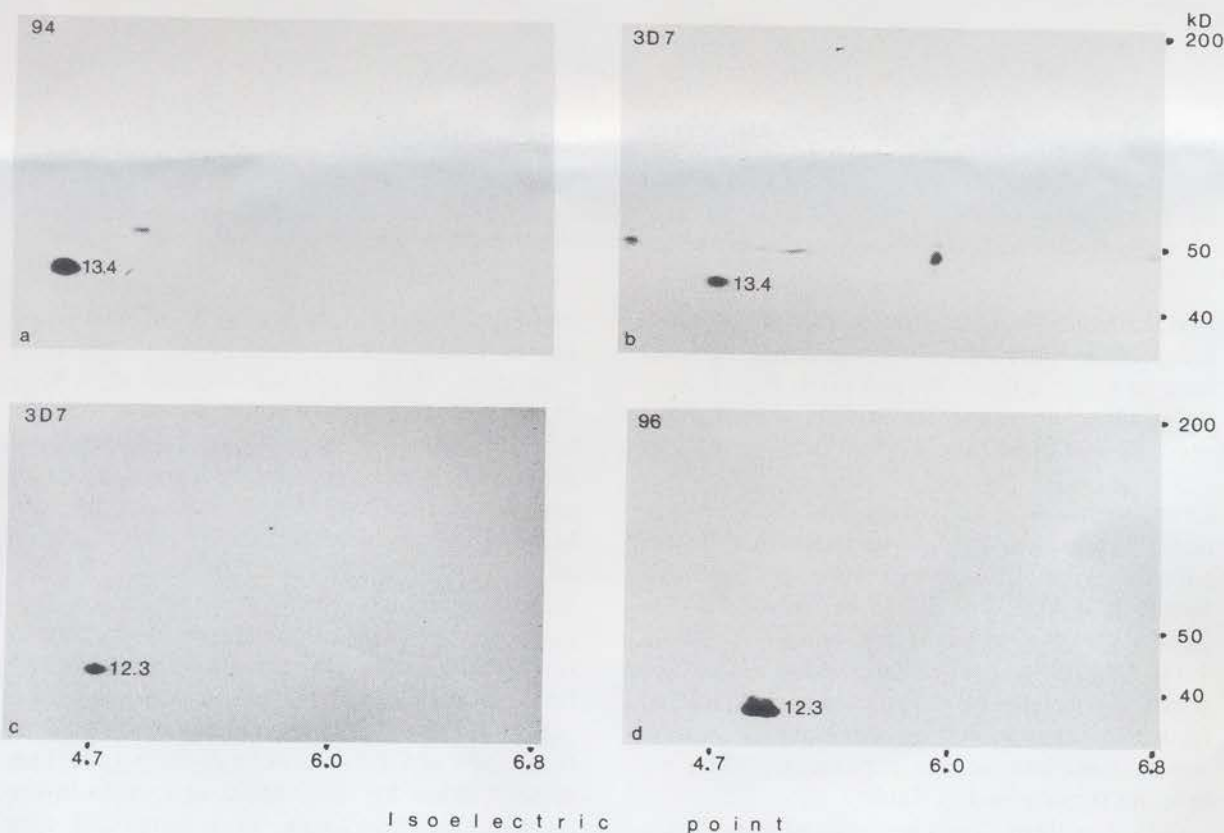


Fig. 2. Two-dimensional PAGE and immunoblotting. Proteins from the three parasite clones T9-94, 3D7 and 96 were separated on 2D-PAGE gels and blotted on to nitrocellulose. These blots were then reacted with either monoclonal antibody 12.3 or 13.4, identifying antigens in the 2D-PAGE gels.

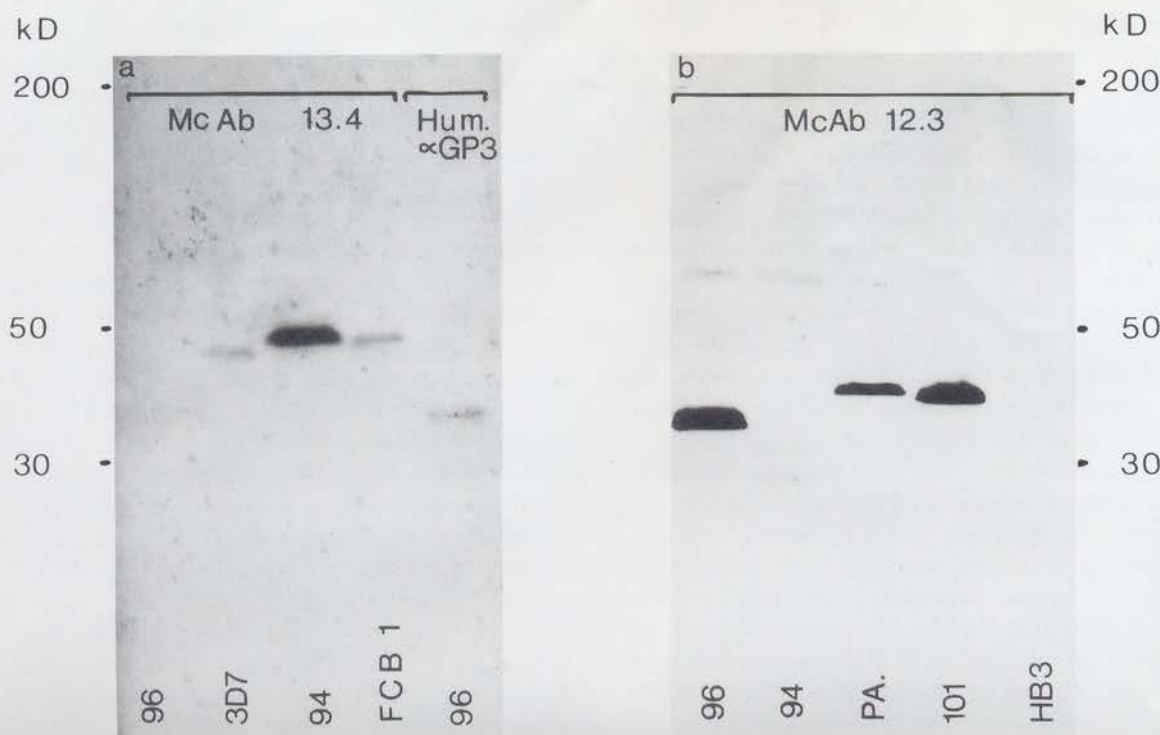


Fig. 3. Antigenic and electrophoretic polymorphism of GP3 in different isolates of *P. falciparum*. Parasite proteins were reacted with antibodies either by immunoprecipitation (a) or by immunoblotting (b). The parasites used were T9-96, 3D7, T9-94, FCB-1, T9-96, Palo Alto 17, T9-101 and HB3.

lanes 2-4) but failed to react with antigen of parasite T9-96 (lane 1). However, GP3 from T9-96 was recognised both by human polyclonal antibodies against GP3 of T9-94 (Fig. 3a, lane 4), and by McAb 12.3 (Fig. 3b, lane 1). McAb 12.3 also reacted positively with GP3 variants of parasite Palo Alto 17 and T9-101 (37.3 and 36.8 kDa, respectively; Fig. 2b, lanes 1, 2 and 4), but failed to recognise the antigen of T9-94 and HB3 (Fig. 2b, lanes 2 and 5).

The results were confirmed by IFA tests with the McAbs on schizonts from the eight parasite lines (Table I). In addition, in repeated IFA testing of parasites maintained in long-term cultures for up to 5 years, no change in phenotype was detected in any of the strains. Thus, the serotypic variants of GP3 detected by McAbs in IFA are phenotypically stable characteristics of *P. falciparum* grown in vitro.

Characterisation of GP1. GP1 is the precursor of the major merozoite surface antigens denoted PMMSA, PSA or P195 [2,22,23]. The identity of GP1 has been confirmed by 2D-PAGE of immunoprecipitates from labelled proteins of parasite T9-94 using antibodies to this molecule. On 2D-PAGE, four forms of this glycoprotein can be detected, ranging in M_r from 185 000 to 205 000 and in IEP from 6.47 to 6.6 [11].

Characterisation of GP2 and GP4. GP2 and GP4, were invariant in all the parasites studied. GP2, (M_r 51 200, IEP 4.9) is also labelled with methionine. GP4 could not be identified with any previously studied methionine-labelled proteins by 2D-PAGE analysis; in all the isolates studied, GP4 was found in the same position, with an M_r of 40 300 and an IEP of 4.7. It is not known if either of these glycoproteins is a breakdown product of PMMSA.

Discussion

The principal novel finding in this study is the structural diversity of a glycoprotein, denoted GP3, among different *P. falciparum* isolates. We have also identified variant forms of a second glycoprotein, denoted GP1, and two other glycoproteins GP2 and GP4 which appear to exhibit no variation.

The antigen GP3 is a surface component of merozoites. It is recognised by the McAb 13.4 which can inhibit parasite growth in vitro [4,21]. However, McAb 13.4 reacts with only a minority of parasite isolates [4].

In the present study a molecule was found to react with both McAb 13.4 and McAb 12.3 in the parasite clone 3D7, suggesting that the epitopes recognised by these monoclonals are on the same molecule. It was also found that human polyclonal antibodies to the antigen recognised by McAb 13.4, reacted with the antigen in T9-96 parasites, a clone which did not react with McAb 13.4. The molecular weight of the antigen recognised by these human antibodies was the same as that of the antigen recognised by 12.3. These observations, and the information obtained by 2D-PAGE, suggested that McAb 12.3 and 13.4 react with different epitopes of the same antigen.

A survey of parasite strains tested with both McAbs 12.3 and 13.4 found that a large proportion bound to one or both of these monoclonal antibodies [4 and McBride unpublished]. However, a small number of *P. falciparum* parasites react with neither antibody (e.g., HB3).

Using genetic analysis, it was demonstrated that different forms of GP1 (PMMSA) were inherited as alternatives and behaved as allelic forms of the same gene [3]. A second antigen, GP3 ((recognised by McAb 12.3), underwent recombination with GP1. GP3 and GP1 were therefore products of different genes. However, in the study, only GP3 of the parental clone 3D7 was detected by McAb 12.3; the other parental clone, HB3, was negative. The lack of reactivity could have been due to the deletion of this gene, as has been noted for other parasite genes [24-27], or to lack of reactivity with the antigen. We have now demonstrated that the glycoprotein GP3 exists in HB3, and that it does not react with the McAb. It seems

likely that the different forms of the GP3 antigen were inherited as alternatives and were therefore by allelic forms of the same gene. Conclusive proof may be obtained by using a McAb which reacts positively with the GP3 of HB3, but not with 3D7.

Glycoproteins in the range of 45-56 kDa have previously been identified in a number of *P. falciparum* isolates, and some or all of these may correspond to GP3 described in this work. Howard and Reese [28] used 2D-PAGE to study the glycoprotein composition of the FVO isolate. In their study, an acidic glycoprotein of 46 kDa was identified in two-dimensional gels of glucosamine-labelled parasites. Stanley et al. [29] described a surface glycoprotein of 50 or 56 kDa in different strains. Ramasamy [30] described a myristilated glycoprotein of 45 kDa in the FCQ27 strain. Epping et al. [5] identified a molecule of 51 kDa on the merozoite surface. The two different M_r s calculated [5,30] appear to be artefactual, as the same molecule has now been studied using molecular biological techniques and the complete DNA sequence of one variant, denoted the 45000 Da MSA, obtained [31].

Studies on this antigen have been complicated by the fact that different laboratories have obtained different M_r estimations for the same variant of the antigen. Because of this, it has been difficult to determine the extent of real variation in this antigen. In this study we have used McAbs, radiolabelling and electrophoresis to demonstrate that the same merozoite surface antigen varies extensively in antigenic structure, apparent M_r and IEP in genetically distinct isolates and clones. The different forms of the antigen appear to behave as alternatives, at the population level and following genetic exchange, suggesting that the different forms are encoded by allelic variants of the same gene. Given the surface location of the antigen, the polymorphism of this molecule may be of significance in enabling the parasite to avoid host immunity.

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