The EMBO Journal vol.12 no.4 pp.1607–1614, 1993

A conserved region of the MSP-1 surface protein of *Plasmodium falciparum* contains a recognition sequence for erythrocyte spectrin

Socrates Herrera, Werner Rudin[1], Myriam Herrera, Pedro Clavijo, Lida Mancilla, Cecilia de Plata, Hugues Matile[2] and Ulrich Certa[2,3]

Department of Microbiology, School of Health, Universidad del Valle, Cali, Colombia, [1]Swiss Tropical Institute, CH-4002 Basel, Switzerland and [2]Department of Pharmaceutical Research, F.Hoffmann-La Roche Ltd, CH-4002 Basel, Switzerland
[3]Corresponding author

Communicated by R.M.Franklin

The major surface protein MSP-1 of *Plasmodium falciparum* blood-stage malaria parasites contains notably conserved sequence blocks with unknown function. The recombinant protein 190L, which represents such a block, exhibits a high affinity for red blood cell membranes. We demonstrate that both 190L and native MSP-1 protein bind to the inner red blood cell membrane skeleton protein spectrin. By using overlapping peptides covering the 190L molecule, we show that the spectrin contact site of 190L is included in a linear sequence of 30 amino acid residues. Association of 190L with naturally occurring spectrin deficient red blood cells is drastically reduced. In the same cells parasite invasion is normal, but the intracellular parasite development arrests late in the trophozoite stage. A similar situation arises when synthetic peptides covering the spectrin recognition sequence of 190L are added to *P.falciparum* cultures. These data and the cellular localization of MSP-1 suggest the possibility that MSP-1 associates with spectrin under natural conditions.

Key words: intracellular parasite development/malaria/MSP-1/spectrin binding

Introduction

Invasion of merozoites of the human malaria parasite *Plasmodium falciparum* is restricted to red blood cells. It is therefore likely that erythrocytes have surface structures that are specifically recognized by parasite ligands, probably proteins. Because these surface proteins are also expected to be targets for inhibitory antibodies, a number of them have been cloned and sequenced with the goal to exploit these antigens for recombinant subunit malaria vaccine development.

Several of these antigens have been found to interact with erythrocyte membrane proteins. The knob protein (KP), which is essential for the formation of the knob-like structures on the surface of infected red blood cells, interacts selectively with actin and spectrin (Kilejian *et al.*, 1991). Other examples include the *P.falciparum* glycophorin-binding proteins (Perkins, 1984), RESA (ring-infected erythrocyte surface antigen; Favarolo *et al.*, 1986), which associates with spectrin shortly after merozoite invasion (Foley *et al.*, 1991; Ruangjirachuporn *et al.*, 1991), and a histidine-rich protein (HRP-1), which binds to knob structures present on the surface of the infected red blood cell (Leech *et al.*, 1984). In addition, human erythrocytes with genetically inherited deficiencies in membrane proteins such as band III (ovalocytosis) or spectrin (spherocytosis) are poor host cells for the asexual development of *P.falciparum* (Schulman *et al.*, 1990; Jarolim *et al.*, 1991). These findings indicate that outer as well as inner membrane proteins of the erythrocyte are a requirement for *P.falciparum* invasion and the subsequent intracellular development.

MSP-1, p190 or PMSA (polymorphic schizont antigen) is a well characterized surface protein of blood-stage parasites (Freeman and Holder, 1983; Hall *et al.*, 1983, 1984a; Tanabe *et al.*, 1987). MSP-1 is a glycoprotein of ~190 kDa and the molecular weight varies slightly between parasite isolates due to antigen polymorphism (McBride *et al.*, 1985). MSP-1 is synthesized as a large precursor polypeptide and is then processed into smaller fragments during late schizont development (Lyon *et al.*, 1986). The exact role of the processing and the function of the individual MSP-1 precursor-derived polypeptide fragments is unclear. The entire MSP-1 molecule binds with low affinity to glycophorin and it was consequently proposed that MSP-1 is required for the red blood cell invasion process (Perkins and Rocco, 1988). Recent studies however, indicate that only the ultimate carboxy-terminal processing product of MSP-1 is present on the surface of invading merozoites (Blackman *et al.*, 1991a,b). The remaining MSP-1 fragments are recovered in culture medium supernatants after schizont burst (Camus *et al.*, 1987) or associated as a loose complex on the merozoite surface (McBride and Heidrich, 1987).

Apart from variable protein domains, the processed MSP-1 polypeptides contain also remarkably conserved sequences (Tanabe *et al.*, 1987). One such invariable region is localized in proximity of the amino-terminus of the mature MSP-1 protein and is included in the 80 kDa processing fragment, which is found associated with the surface of mature merozoites (Strych *et al.*, 1987). The most conserved part of this polypeptide is represented by a recombinant protein termed 190L (Gentz *et al.*, 1988). It contains an unusually elevated number of B- and T-cell epitopes (Crisanti *et al.*, 1988; Sinigaglia *et al.*, 1988; Guttinger *et al.*, 1991). In addition, immunization of susceptible monkeys with native MSP-1 gives complete protection against challenge infection and partial immunity is obtained with 190L as immunogen (Siddiqui *et al.*, 1987; Herrera *et al.*, 1990; Etlinger *et al.*, 1991; Herrera *et al.*, 1992). The outcome of immunization studies and the high degree of primary sequence conservation in *P.falciparum* and other malaria parasite MSP-1 molecules, suggested to us the possibility that 190L has a biological function, for example red blood cell recognition.

We present evidence that 190L binds with high affinity to red blood cells. Surprisingly, 190L does not interact with

external structures of the red blood cell but rather with the intracellular membrane protein complex spectrin. Based on our findings and the present knowledge concerning MSP-1 we propose that interaction of this protein with spectrin may be required *in vivo* late during the intraerythrocytic development of *P.falciparum*.

Results

Interaction of radiolabelled 190L with red blood cells

Affinity-purified recombinant 190L was labelled by radioiodination and then incubated with intact human erythrocytes. After centrifugation and seven washes in physiological buffer, the radioactivity in the supernatant and pellet was determined (intact red blood cells; Table I). 22% of the 190L input radioactivity remained bound to the cells, whereas 0.1% of a recombinant *P.falciparum* circumsporozoite protein as control (CSFM) bound under the same conditions. A similar degree of 190L binding was obtained using mouse erythrocytes (20%; data not shown). The 190L-labelled human erythrocytes were next lysed by osmotic shock, followed by high speed centrifugation (lysed red blood cells; Table I). Virtually all input radioactivity was recovered in the pellet suggesting that 190L is associated with red blood cell membranes or other insoluble constituents of the erythrocyte. The pellet was then treated with the non-ionic detergent Triton X-100 in order to disintegrate the membranes. After centrifugation, ~75% of the input material remained bound to detergent insoluble material (1% Triton X-100; Table I). Also a pH shift to 2.8 did not release 190L from the pellet (pH shift; Table I) suggesting that 190L is possibly bound to an insoluble membrane protein.

Binding of 190L to spectrin

Next, we wished to identify the red blood cell membrane target protein recognized by 190L. In particular we were interested whether the 190L target protein is an inner or outer red blood cell membrane protein. Most of the proteins constituting the red blood cell membrane are characterized (for review see Bannister and Dluzewski, 1990), a circumstance that simplifies the identification of the polypeptide recognized by 190L (Figure 1A). Thus membrane proteins of human or mouse red blood cell and human leukocytes were prepared and separated by SDS electrophoresis. After electrotransfer to a nitrocellulose matrix the blot was probed with radiolabelled 190L protein, washed and autoradiographed. In contrast to the leukocyte sample, a large protein migrating at the position of the spectrin complex is labelled in the red blood cell samples. A similar, if not the same, polypeptide is labelled when red blood cell membranes are incubated with iodinated 190L followed by denaturation, SDS electrophoresis and autoradiography (Figure 1C). The limited complexity of the red blood cell membrane protein composition made it likely that the protein detected by 190L is spectrin. Binding of 190L to a commercial spectrin preparation directly confirms this conclusion (Figure 1D).

At this point we had to consider the possibility that chemical iodination of 190L had caused partial denaturation or structural changes that may cause spectrin binding. We thus pre-incubated a blot with red blood cells, parasite or spectrin protein preparations with soluble unlabelled 190L protein. After washing the blot was probed with monkey serum against 190L. In contrast to the control, pre-incubation with 190L results in detection of the spectrin complex by the MSP-1-specific antibody probe (Figure 1G and H).

We next wished to rule out that spectrin binding of 190L is related to unique features of the recombinant protein. The 190L sequence is contained in the 80 kDa processing product of MSP-1, which is present in its native form in supernatants of *P.falciparum* cultures (Hall *et al.*, 1984b; Camus *et al.*, 1987). To demonstrate binding we incubated a blot with a red blood cell membrane protein preparation with the supernatant of a parasite culture. After washing monkey serum against 190L detects the native MSP-1 protein associated with spectrin (Figure 1I).

Association of 190L with spectrin-deficient red blood cells

In order to obtain independent evidence for the interaction of 190L with spectrin, we took advantage of spectrin-defective red blood cells that naturally occur in patients with hereditary spherocytosis. We obtained blood from two such individuals and prepared red blood cells. After incubation with radiolabelled 190L and extensive washing, binding to the spherocytes was significantly reduced compared with normal red blood cell controls (Table II). This result and the binding studies above are consistent and we conclude that 190L binds to spectrin.

Overlapping peptides define the spectrin-binding region within 190L

The results above suggest a strong and specific affinity of 190L to spectrin under native and denaturing conditions. This made it possible to map the spectrin recognition sequence of 190L using 17 overlapping peptides (Figure 2; top) in a binding competition assay. Washed red blood cell membrane preparations were first pre-incubated with four pools of peptides (Figure 2, upper panel) followed by washing and incubation with labelled 190L. The most potent

Table I. Uptake and binding of 190L to erythrocytes

Cell fraction	c.p.m. (SN; $\times 10^3$)	c.p.m. (pellet; $\times 10^3$)	D	PB (%)
Intact red blood cells	–	1770	110	22
Lysed red blood cells	402	1370	32	19
1% Triton X-100	56	1310	18	18
pH shift	10	1305	7	18

c.p.m. values are given as the average of five independent assays. D represents the deviation from the average c.p.m. value; PB indicates the percentage of the input radioactivity (8×10^7 c.p.m.) bound to the pellet fractions; SN represents supernatant. The nature of fractions is described in Materials and methods. The radioactivity uptake for the intact cells was calculated by subtraction of the counts present in the washed red blood cell cell pellet from the total input.

blockers of the 190L−spectrin assembly were the peptides of pool four, which gave 80% inhibition of binding (Figure 2, lower panel, A). When these peptides were assayed individually, two peptides turned out to be the major competitors for 190L binding (peptides 15 and 16; Figure 2, lower panel, B). This suggests that the main spectrin attachment site of 190L is contained in the 30 amino acids covered by the active peptides 15 and 16.

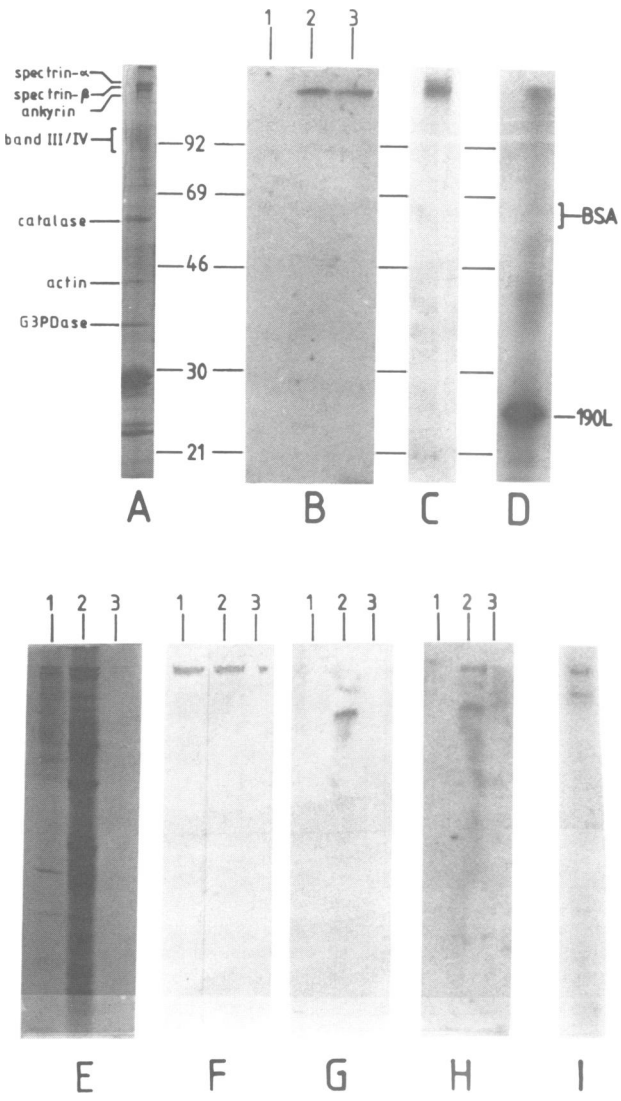

Fig. 1. Interaction of 190L and native MSP-1 with red blood cell membrane proteins. **A.** Typical Coomassie blue-stained protein preparation from non-infected red blood cells. The names of known protein bands are indicated to the left as adapted from Bannister and Dluzewski (1990). The position and mass of molecular weight markers (kDa) is given at the right side. **B.** Shows either human leukocyte (lane 1), mouse and human (lanes 2 and 3) erythrocyte proteins immobilized on a matrix were probed with radiolabelled 190L. **C.** Red blood cell membranes incubated with labelled 190L followed by boiling in SDS sample buffer, electrophoresis and autoradiography. **D.** Shows the result of the same experiment using commercial spectrin as ligand. The position of BSA contained in the incubation buffer is indicated and 190L marks unbound 190L protein. Weak shadow bands represent aggregates of 190L. **E.** Coomassie blue-stained gel displaying typical red blood cell membrane proteins (lane 1), proteins of a parasite saponine lysate (lane 2) or purified spectrin (lane 3) used for Western blotting. **F.** The protein preparation shown in panel E was blotted and probed with a monoclonal antibody to spectrin. **G.** The same as panel F, but antibody to 190N was used as probe. **H.** Preincubation with unlabelled 190L protein followed by probing with MSP-1 antibody (R551). **I.** Shows association of native MSP-1 from culture supernatants with spectrin using the same detection method. Note that the parasite saponin lysate contains significant amounts of spectrin (panel E, lane 2).

Parasite development in spherocytes

In vitro and *in vivo* the growth of malaria parasites is markedly reduced in spectrin deficient red blood cells (Schulman *et al.*, 1990; Shear *et al.*, 1991). We thus compared parasite development in spectrin deficient spherocytes with that in normal red blood cells. In particular, we were interested at which point of the erythrocytic cycle development arrests. The cultures were inoculated with synchronized schizonts and after burst, the released merozoites invaded the spectrin defective cells with the same efficiency as in the control cultures. After 50 hours, the spherocyte cultures reached 7.2 or 4.8% parasitemia compared with 10% in the control cultures (Table III). Compared with the control cultures, the [^{3}H]hypoxanthine uptake was significantly reduced in the spherocyte cultures that indirectly indicates reduced parasite replication. Indeed, microscopic examination of the cultures showed that the number of schizont stages in the spherocyte cultures was at least 10-fold lower as in the controls (Table III). This result indicates that spectrin is required for the development of a trophozoite into a schizont during the intraerythrocytic development of *P.falciparum*.

Parasite development in the presence of peptides covering 190L

We next attempted to simulate spectrin deficiency by blocking the MSP-1-binding sites with peptides coming from the spectrin-binding region of 190L. Parasite cultures were supplemented with either the peptides covering amino acids 1−130 (control) or the peptides from the mapped spectrin-binding site. In this assay the peptides with spectrin-binding activity were the most potent inhibitors of parasite growth including peptides 15 and 16, which constitute the binding site of 190L (Figure 3). We noted that addition of the entire 190L molecule to cultures entirely inhibits parasite development including invasion. However, we noted that addition of the protein to cultures induces alterations in the shape of the red blood cells. This morphological change is probably preventing parasite invasion rather than binding of 190L to spectrin (data not shown).

Cellular localization of MSP-1 by immunoelectron microscopy

An intracellular MSP-1−spectrin interaction requires that MSP-1 is located on the outer parasite vacuole membrane. We thus examined a mixed culture of *P.falciparum* by

Table II. 190L binding to spherocytes

Ligand	red blood cells (%)	sph-1 (%)	sph-2 (%)
190 L	18.0 (± 0.7)	12.1 (± 1.1)	11.6 (± 0.9)
CSFM	<0.1	<0.1	<0.1

Spherocytes or red blood cells were incubated with radiolabelled 190L or CSFM protein at 37°C as described in Materials and methods. Spherocytes were isolated from patients with hereditary spherocytosis (sph-1, E.V.; sph-2, C.C.). The results given represent the average of three assays using blood from several independent bleedings. The experimental deviation is given in brackets.

```
   1
   |
 1 TLCDNIHGFKYLIDGYEEIN
 2           YLIDGYEEINELLYKLNFYF                                    1
 3                     ELLYKLNFYFDLLRAKLNNV
 4                               DLLRAKLNNVCANDYCQIPF
-----------------------------------------------------------------------
   51
   |
 5 CANDYCQIPFNLKIRANELD
 6           NLKIRANELDVLKKLVFGYR                                    2
 7                     VLKKLVFGYRKPLDNIKDNV
 8                               KPLDNIKDNVGKMEDYIKKN
-----------------------------------------------------------------------
   91
   |
 9 GKMEDYIKKNKKTIENINEL
10           KKTIENINELIEESKKTIDK                                    3
11                     IEESKKTIDKNKNATKEEEK
12                               NKNATKEEEKKKLYQAQYDL
-----------------------------------------------------------------------
   131
   |
13 KKLYQAQYDLFIYNKQLEEA
14           FIYNKQLEEAHNLISVLEKR                                    4
15                     HNLISVLEKRIDTLKKNENI
16                               IDTLKKNENIKELLDKINEI
17                                         KELLDKINEIKNPPP
```

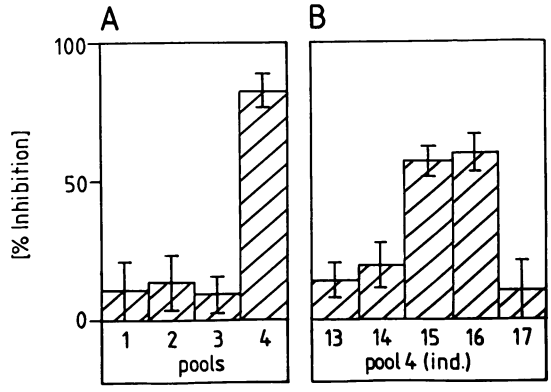

Fig. 2. Mapping of the 190L spectrin-binding site. The 190L amino acid sequence was covered by overlapping peptides as indicated on top. The peptide numbers appear at the left and the first amino acid of each pool is numbered. The inhibitory peptides are underlined. In chart A, results of the 190L binding inhibition assay using peptide pools are plotted. In chart B, the peptides of pool 4 were assayed individually. Vertical lines indicate the experimental deviations of quadruplicate assays.

Table III. *In vitro* culture of *P.falciparum* using normal red blood cells or spherocytes

Cell	Parasitemia	Schizont stages	Trophozoite stages	Ring stages	HX (c.p.m.)
red blood cell	10.0 (± 0.2)	2.3 (± 0.1)	0.3 (<0.1)	7.4 (± 0.2)	9680 (± 210)
sph-1	7.2 (± 0.2)	0.0 (n.d.)	0.3 (<0.1)	6.9 (± 0.2)	7200 (± 165)
sph-2	4.8 (± 0.2)	0.2 (<0.1)	0.0 (n.d.)	4.6 (± 0.2)	5070 (± 95)

Cultures were inoculated with synchronized schizonts and grown for 50 h (1 cycle). The average parasitemia of triplicate cultures is given for either normal red blood cells or spherocytes (sph-1, donor E.V.; sph-2, donor C.C.). HX indicates the incorporation of tritiated hypoxanthine as an independent indicator for parasite replication. Experimental deviation is indicated in brackets. n.d., not detected.

immunoelectron microscopy using a MSP-1-specific serum as a probe. Consistent with the biosynthesis scheme for MSP-1 (Lyon *et al.*, 1986), the protein is absent in ring stages (not shown) and first detectable in the trophozoite stage (Figure 4, panels 1 and 2). In schizonts, MSP-1 biosynthesis increases and the protein is mainly found associated with membrane structures including the outer parasite membrane (Figure 4, panels 3 and 4). It is important to note that the MSP-1 molecule is abundantly detected in regions where the parasite and red blood cell membrane are apparently in contact (Figure 4, panel 5).

Discussion

We have shown that the MSP-1-derived recombinant protein 190L and the parasite-derived MSP-1 protein bind to red blood cell spectrin, a red blood cell membrane protein complex. We have used overlapping peptides in a

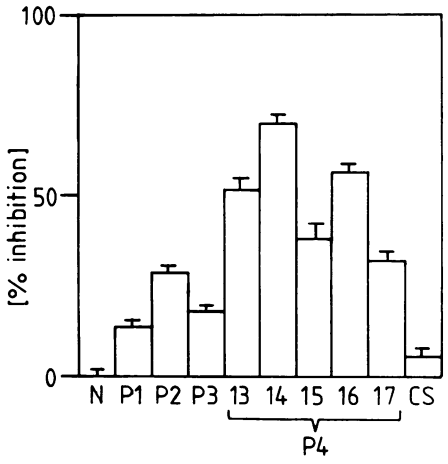

Fig. 3. Inhibition of parasite development by 190L-derived peptides. Pools of peptides covering 190L amino acids 1–130 (P1–P3) or individual peptides coming from the spectrin-binding site (P4; peptides 13–17) were added to synchronized parasite cultures. After 50 h in culture the [^{3}H]hypoxanthine incorporation was determined and the percentage of inhibition relative to controls is shown (N, no peptide added; CS, recombinant CS protein).

competition assay to map the spectrin contact region within 190L (Figure 2). The specificity of the 190L association with spectrin is indirectly shown by the reduced binding to red blood cell membrane preparations from patients with hereditary spherocytosis, a disease in which spectrin deficiency is apparently the sole defect detected at the protein level (for review see Palek and Lambert, 1990). These cells are poor host cells for intraerythrocytic parasite development (Table III; Schulman *et al.*, 1990). Parasite penetration into spherocytes appears normal, but an arrest of intracellular development occurs during the transition from the trophozoite to the early schizont stage based on the significantly reduced number of schizonts in spherocyte cultures (Table III). Interestingly, the *in vivo* requirement for spectrin coincides with the onset of MSP-1 protein synthesis *in vivo* (Figure 4; Freeman and Holder, 1983).

The demonstration of spectrin-binding may define a function of a conserved part of the 80 kDa processing product of the MSP-1 protein of *P.falciparum*. 190L is contained in the interspecies conserved block ICB-2 of known malarial MSP-1 proteins, which makes it possible that it has a similar, if not the same, function in the genus plasmodium (Lewis, 1989; del Portillo *et al.*, 1991). An *in vivo* requirement for spectrin is documented by the observation that inbred mice strains carrying homozygous mutations that affect either spectrin synthesis or assembly, are highly resistant to infections by the rodent malaria parasite *P.chabaudi* (Shear *et al.*, 1991). The marked inhibition of parasite growth in cultures supplemented with peptides that block 190L association with spectrin supports the view that MSP-1 binding to spectrin may occur late during bloodstage development of the parasite (Figure 3).

RESA, another malaria spectrin-binding protein, has previously been proposed (Foley *et al.*, 1991; Ruangjirachuporn *et al.*, 1991). During or shortly after invasion RESA is probably released into the parasitophorous vacuole by an unknown process (Aikawa *et al.*, 1990). We explored the possibility that the spectrin contact site is similar in RESA and 190L. When we aligned both polypeptide sequences, the overall homology was found to be low (8.8%). An important exception was amino acids 129–162 of 190L, which are 78% homologous with residues 800–841 in RESA (data not shown; Favarolo *et al.*, 1986). Strikingly, this region of 190L contains the spectrin-binding region defined by the peptide competition assay (Figure 2) and implies that the spectrin-binding domain of RESA resides within residues 800–841. This conclusion is consistent with mapping experiments aimed to identify the spectrin-binding domain in RESA. Synthetic peptides coming from the amino acid repeat structures that are located directly upstream of the proposed binding site do not bind to spectrin (Ruangjirachuporn *et al.*, 1991). On the other hand, binding of recombinant RESA protein is abolished if carboxy-terminal deletion mutants lack the 190L homologous spectrin contact site (M.Foley and R.F.Anders, unpublished results).

The outcome of the 190L binding studies implies that the polypeptide has to penetrate by an unknown mechanism through the red blood cell membrane, prior to spectrin binding. Under natural conditions, however, red blood cell membrane penetration of MSP-1 is not necessary, because protein synthesis occurs inside the red blood cell followed by translocation of the protein to membrane structures including the outer vacuole membrane of the parasite (Figure 4).

The results presented here may go towards the proposal that KEL, KEK or LEK tripeptide motifs are the major targets of antibodies involved in immunity to *P.falciparum* (Calvo *et al.*, 1991; Molano *et al.*, 1992). LEK is part of the spectrin recognition domain of 190L and it is conceivable that the protective antibodies, in part, mediate their action by inhibiting the MSP-1 association with spectrin. It is noteworthy that antibodies from monkeys immunized with 190L inhibit parasite invasion, but also the intracellular development *in vitro* (Herrera *et al.*, 1992).

The results presented here strongly suggest a requirement of an association between MSP-1 and spectrin during the late trophozoite or early schizont development *in vivo*. Based on the electron microscopic examination of parasitized cells it is possible that a MSP-1–spectrin interaction fuses the outer parasitophorous membrane with the inner surface of the red blood cell membrane. Following such a membrane contact, which is evident in the so called 'accolé' position of *P.falciparum*, one could have enhanced diffusion of nutrients from the plasma into the parasite. This could be important during the schizont stage where energy consuming cell divisions occur and an increase in uptake of metabolites may be crucial. Furthermore, pulling the spectrin meshwork towards the parasite may be connected to the disintegration process of the red blood cell membrane skeleton prior to cell burst. This process would make the spectrin meshwork more accessible for a parasite-derived spectrinase that is synthesized late in the trophozoite stage and during schizogony (Deguercy *et al.*, 1990). It may be relevant that the onset of MSP-1 synthesis and the *in vivo* requirement for spectrin occur in the same phase of the life cycle. In addition, attachment of the parasite to spectrin may support a controlled execution of cellular processes such as the cell divisions during schizont development. Once genetic manipulation of malaria parasites becomes available, it can

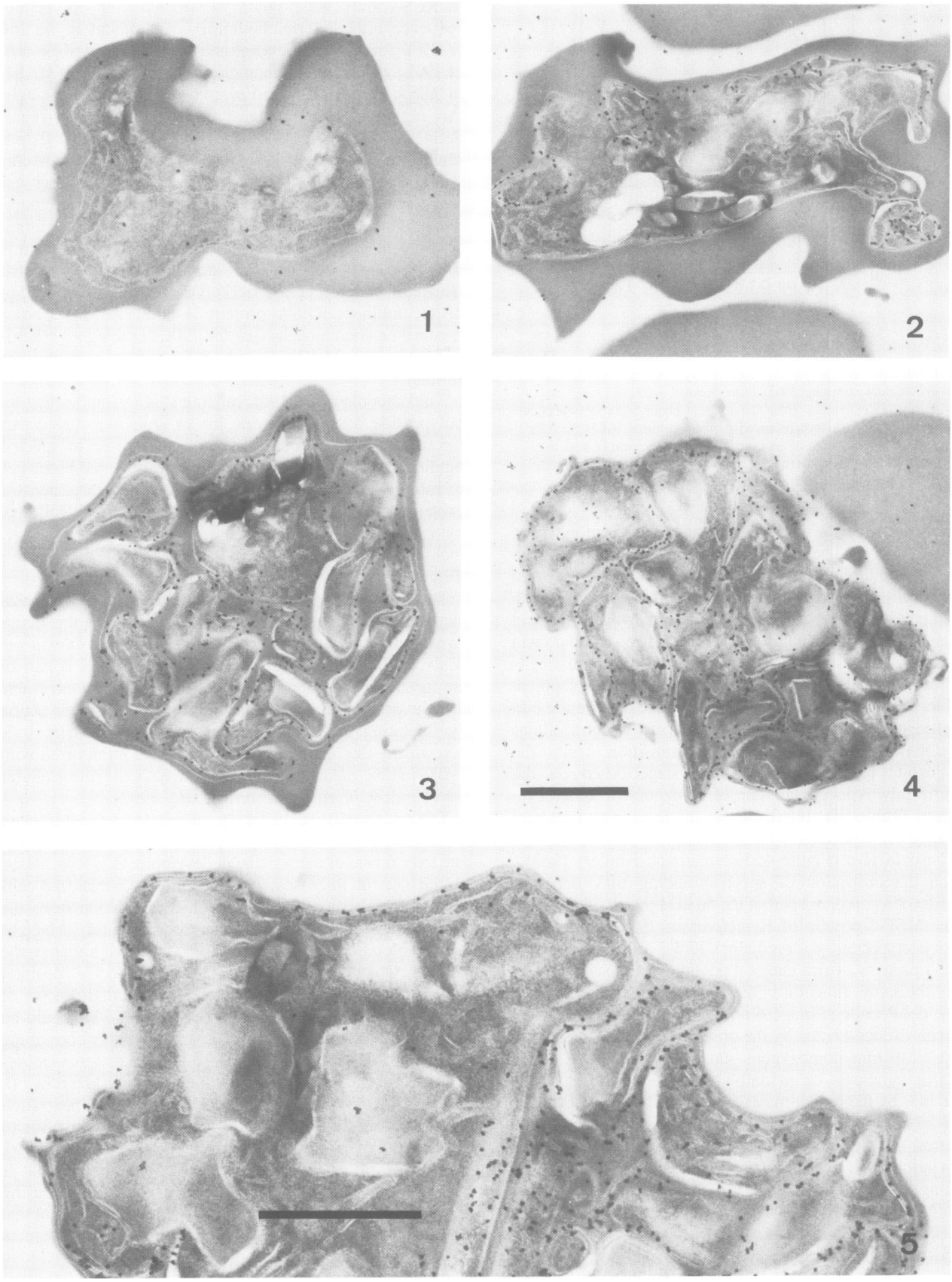

Fig. 4. Expression and cellular localization of MSP-1 during bloodstage development. Sections of bloodstage forms of the parasite were labelled with monospecific MSP-1 serum followed by protein A gold labelling and silver enhancement. Panel 1, young trophozoite; panel 2, mature trophozoite; panel 3, young schizont; panel 4, mature schizont; panel 5, a typical contact zone of the parasite with the red blood cell membrane at higher magnification. Note the increase in MSP-1 synthesis during development and the localization of MSP-1 on the outer parasitophorous membrane (bars indicate 1 μm).

be tested in detail whether one of the proposed models for the interaction between MSP-1 and spectrin is correct. Due to the conserved nature of the 190L sequence and its potential function, it seems unlikely to recover parasites lacking the spectrin-binding site in natural populations of *P.falciparum*.

Finally, the structure of the inhibitory 190L peptides may be exploited to develop macromolecular inhibitors that would block spectrin association and as a consequence the intracellular development of this pathogenic organism.

Materials and methods

Parasite cultures

The FVO isolate of *P.falciparum* was used for all experiments (Herrera *et al.*, 1990). Parasites were synchronized to schizonts as described by Lambros and Vanderberg (1979). 100 μl triplicate micro-cultures of normal red blood cells or spherocytes were inoculated with 50 μl of a synchronized normal red blood cell parasite culture with 6% parasitemia. Parasite development was monitored either microscopically on Giemsa-stained smears or by [^{3}H]hypoxanthine incorporation after 50 h in culture (Herrera *et al.*, 1992). Red blood cells were either obtained from normal blood donors or from two spherocytosis patients (E.V. and C.C.) with confirmed clinical symptoms and history.

For inhibition of parasite growth in the presence of 190L peptides (see below), synchronized schizonts at 1.5% parasitemia were used to inoculate triplicate cultures supplemented with 20 μg/ml of test peptide (see below). The peptide-containing culture medium was changed every 12 h in order to reduce the risk of peptide degradation. After 48 h the cultures were pulse-labelled for 12 h with 50 nCi of [^{3}H]hypoxanthine followed by the determination of tritium incorporation and microscopic examination. Control cultures contained either no peptide or 20 μg/ml recombinant CSFM protein.

Antibodies

For blotting experiments pre-challenge serum from a monkey immunized with 190L was used (animal C9; Herrera *et al.*, 1992). For immunoelectron microscopy, a rabbit serum raised against a recombinant MSP-1 protein covering amino acids 34–595 of the MAD-20 isolate of *P.falciparum* was used (serum R551; H.Matile, unpublished results). Prior to use the specificity of the sera was confirmed by immune fluorescence and Western blotting (data not shown).

Protein purification and labelling

The *P.falciparum* recombinant proteins 190L and CSFM were produced in *Escherichia coli* cells harbouring the plasmid p190L-6H (Herrera *et al.*, 1992) or pCSFM-6xHIS, respectively (P.Caspers, unpublished results). The CSFM recombinant polypeptide is an engineered fusion protein containing amino acids 103–191 and 248–396 of the T4 isolate of *P.falciparum* (numbered according to Caspers *et al.*, 1989). Recombinant proteins (190L and CSFM) were purified by affinity chromatography followed by overnight dialysis against 1×PBS buffer (Herrera *et al.*, 1990). The amount of *E.coli* protein contaminants in both protein preparations was estimated to be <10% according to Coomassie blue-stained SDS gels (data not shown). After dialysis, centrifugation (5600 *g* for 10 min at 4°C) and filtration through a 0.2 μm filter the proteins were stored frozen in 20 μg aliquots. Each protein was labelled with 1 mCi of [^{125}I]sodium iodide (Amersham) using the 'Iodogen' enzymatic method following the instructions of the supplier (Pierce Chemicals). Fractions containing 8×10^7 c.p.m. were stored frozen in 1×PBS buffer containing 2% bovine serum albumin (BSA). Prior to use, the aliquots were centrifuged for 10 min at 5600 *g* followed by filtration through a 0.2 μm microfilter (Millipore).

Erythrocyte and membrane preparations

Erythrocytes were isolated from fresh human or mouse blood by conventional Ficoll gradient fractionation. The human blood came either from normal blood bank donors, from two spherocytosis patients (E.V. and C.C.) or laboratory mice. The red blood cells were washed three times in 10 vol of 1×PBS buffer and used directly for binding assays. Leukocytes were recovered from the same gradient. Crude red blood cell membrane protein preparations were obtained by lysing the washed red blood cells in distilled water in the presence of a protease inhibitor cocktail (Boehringer Mannheim). The lysate was centrifuged for 10 min at 5600 *g* and 4°C. The crude membrane pellet was washed three times in 1×PBS and stored frozen until use. A typical red blood cell membrane protein preparation is shown in Figure 1A.

Protein binding assay to erythrocytes or fractions

10^8 cells were incubated for 30 min at 37°C with either 10^8 c.p.m. labelled 190L or CSFM in 500 μl of 1×PBS buffer. After seven washes in 1×PBS, the radioactivity in the pellet was determined (intact red blood cells; Table I). The red blood cell pellet was lysed by addition of 500 μl of distilled water and a 10 min incubation at ambient temperature. After centrifugation for 15 min at 5600 *g* and 4°C the radioactivity in the pellet and supernatant was measured. The pellet was then dissolved in 1×PBS containing 1% Triton X-100 and incubated for 10 min at room temperature. Radioactivity was determined as above (Triton X-100; Table I). The Triton X-100 insoluble pellet was suspended in 500 μl 0.5 M aminoacetic acid (pH 2.7) followed by a 10 min incubation at room temperature. The radioactivity in the pellet and supernatant was determined as above (pH shift; Table I). The final pellet was dissolved in SDS sample buffer, boiled for 3 min and loaded onto a 5% SDS–PAGE gel (Laemmli, 1970). The gel was fixed, dried and exposed overnight to X-ray film.

Binding of 190L to spectrin

2 μg of a commercial spectrin preparation (Sigma; cat. no. S 3644) were incubated for 30 min at 37°C with 10^6 c.p.m. of radioiodinated 190L or CSFM in 1×TBSA (50 mM Tris–HCl pH 7.4, 140 mM NaCl and 0.2% bovine serum albumin). The sample was dissolved in SDS sample buffer and analysed on a 5% SDS gel followed by autoradiography.

Ligand blotting

Human or mouse red blood cell and human leukocyte membrane proteins were prepared as described above and separated by 5% SDS–PAGE (Laemmli, 1970), followed by electroblotting onto a nylon supported nitrocellulose membrane (Nitroplus 2000, Micron Separations, Inc.). The blot was washed twice for 10 min in 1×PBS, followed by a 1 h blocking step in 1×PBS containing 0.2% BSA. In order to reduce the background on the autoradiographs, it was essential to pass the labelled 190L or CSFM proteins (10^8 c.p.m. each) through a 0.2 μm membrane filter. The blots were then incubated for 3 h at room temperature with gentle shaking with the labelled proteins dissolved in 1×TBSA. The filter was washed in 1×PBS, 0.1% Triton X-100 until the washing buffer was essentially depleted of radioactivity. The filter was dried and exposed overnight to X-ray film.

For binding of unlabelled 190L protein, red blood cell membrane proteins, a parasite lysate and spectrin were electrophoresed and transferred to nitrocellulose. After blocking, the filter was shaken for 3 h with 190L protein (100 μg/ml). After three washes with TBSA the blot was incubated overnight with a 1:1000 dilution of MSP-1-specific rabbit serum (Gentz *et al.*, 1988). In controls using blots with the same samples, 190L pre-incubation was omitted or the blot was incubated with a commercial monoclonal antibody to spectrin. After washing in TBS, antibody–protein complexes were detected either with iodinated protein G (Amersham) or horse-radish peroxidase-conjugated goat serum against mouse IgG (Bio-Rad).

For binding of native MSP-1 to spectrin a filter with a red blood cell membrane was incubated overnight with a supernatant of a parasite culture with 12% parasitemia. The presence of the MSP-1 protein and its processed fragments in the supernatant was confirmed by Western blotting using 190L antibody (data not shown).

Peptide binding competition assay

17 overlapping peptides with 20 or 15 (peptide 17) residues covering the entire 190L sequence were synthesized using a solid phase coupling technique (Merrifield., 1963; Tam *et al.*, 1983; Figure 2). Red blood cell membranes were prepared as described above and suspended in 1×PBS. A 20 μl aliquot of this suspension was transferred to a microfuge tube followed by addition of 50 μg test peptide(s) dissolved in 500 μl 1×TBSA. All assays were performed in quadruplicate. After a 30 min incubation at room temperature the samples were centrifuged for 10 min at 5600 *g* and 4°C. The supernatant was withdrawn and the pellet was suspended in 500 μl 1×TBSA containing 10^6 c.p.m. of labelled 190L. After centrifugation the pellet was washed three times in 1×PBS, dried under vacuum and the 190L incorporation was determined. 190L binding after incubation with unlabelled CSFM protein was set to 0% inhibition and maximum inhibition is defined as the value obtained by pre-incubation of the membranes with unlabelled 190L.

Immunoelectron microscopy

Human erythrocytes from a *P.falciparum* culture with 10% parasitemia were fixed in 1% glutaraldehyde in 0.2 M PIPES (piperazine-1,4-*bis*-2-ethansulfonic acid) buffer (pH 7.2) for 1 h at room temperature. After blocking free aldehyde groups with 0.5 M ammonium chloride in PIPES, the cells were washed with buffer, pelleted and enclosed in 1.5% agarose dissolved in PIPES. The agar blocks were dissected into smaller pieces, blocked with ammonium chloride (30 min) and washed in PIPES buffer for several hours.

After dehydration with graded ethanol at decreasing temperature, the agarose particles were embedded in Lowicryl K4M according to the protocol supplied (Chemische Werke Lowi, FRG). 70 nm sections were obtained by cutting with a diamond knife followed by mounting on carbon-coated, parlodion-supported nickel grids.

The specimens were blocked for 15 min in 1×PBS buffer containing 5% non-fat dry milk powder and 0.01% Tween 20. The grids were then incubated for 2.5 h with serial dilutions (1:50 to 1:800) of rabbit serum against recombinant MSP-1 (serum R551) followed by washing in 1×PBS containing 1% BSA and 0.01% Tween 20. The antigen–antibody complexes were labelled for 1 h with protein A-coupled gold particles (0.5 OD at 525 nm) suspended in 1×PBS containing 0.01% Tween 20. After washing the samples were fixed for 10 min in 2.5% glutaraldehyde in 1×PBS followed by several washes in double distilled water. For visualization of the reaction sites at low magnification, the size of the gold particles was amplified by silver enhancement for 1 min using a commercial kit (BioCell SE). After staining with 2% uranylacetate (5 min) and 0.4% lead citrate (1 min) the samples were examined by using an electron microscope.

Acknowledgements

We are grateful to Dr P.Caspers for the gift of affinity-purified 190L and CSFM protein, Drs M.Foley and R.Anders for sharing unpublished results and the spherocytosis patients E.Vargas and C.Corredor for collaboration and repeated blood donations. We thank Dr G.Langsley for critical reading of the manuscript and substantial improvements and Drs P.Romero and J.Baron for comments. P.C. and U.C were supported as visiting professors through a grant of the Instituto Colombiano para Fomento de la Educacion Superior (Convenio ICFES/BID). Parts of the this work received support from a WHO-TDR training grant.

References

Aikawa,M., Torii,M., Sjölander,A., Berzins,K., Perlmann,P. and Miller,L.H. (1990) *Exp. Parasitol.*, **71**, 326–329.

Bannister,L.H. and Dluzewski,A.R. (1990) *Blood Cells*, **16**, 257–292.

Blackman,M.J., Ling,I.T., Nicholls,S.C. and Holder,A.A. (1991a) *Mol. Biochem. Parasitol.*, **49**, 29–34.

Blackman,M.J., Whittle,H. and Holder,A.A. (1991b) *Mol. Biochem. Parasitol.*, **49**, 35–44.

Calvo,M., Guzman,F., Perez,E., Segura,C.H., Molano,A. and Patarroyo,M.E. (1991) *Peptide Res.*, **4**, 324–332.

Camus,D., Lyon,J.A., Reaud-Jareed,T., Haynes,J.D. and Diggs,C.L. (1987) *Mol. Biochem. Parasitol.*, **26**, 21–17.

Caspers,P., Gentz,R., Matile,H., Pink,J.R. and Sinigaglia,F (1989) *Mol. Biochem. Parasitol.*, **35**, 185–190.

Crisanti,A., Müller,H.M., Hilbich,C., Sinigaglia,F., Matile,H., Mackay,M., Scaife,J., Beyreuther,K. and Bujard,H. (1988) *Science*, **240**, 1324–1326.

Deguercy,A., Hommel,M. and Schrevel,J. (1990) *Mol. Biochem. Parasitol.*, **38**, 233–244.

del Portillo,H., Longacre,S., Khouri,E. and David,P. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 4030–4034.

Etlinger,H.M., Caspers,P., Matile,H., Schoenfeld,H.J. and Stueber,D. (1991) *Infect. Immun.*, **59**, 3498–3503.

Favarolo,J.M., Coppel,R.L., Corcoran,L.M., Foote,S.J., Brown,G.V., Anders,R.F. and Kemp,D.J. (1986) *Nucleic Acids Res.*, **14**, 8265–8277.

Foley,M., Tilley,L., Sawyer,W.H. and Anders,R.F. (1991) *Mol. Biochem. Parasitol.*, **46**, 137–148.

Freeman,R.R. and Holder,A.A. (1983) *J. Exp. Med.*, **158**, 1647–1653.

Gentz,R., Certa,U., Takacs,B., Matile,H., Doebeli,H., Pink,R., Mackay,M., Bone,N. and Scaife,J.G. (1988) *EMBO J.*, **7**, 225–230.

Guttinger,M., Romagnoli,P., Vandel,L., Meloen,R., Takacs,B., Pink,J.R.L. and Sinigaglia,F. (1991) *Int. Immunol.*, **3**, 899–906.

Hall,R., McBride,J., Morgan,G., Tait.,A., Zolg,J.W., Walliker,D. and Scaife,J.G. (1983) *Mol. Biochem. Parasitol.*, **7**, 247–265.

Hall,R. *et al.* (1984a) *Nature*, **311**, 379–382.

Hall,R., Osland,A., Hyde,J.E., Simmons,D.L., Hope,I.A. and Scaife,J.G. (1984b) *Mol. Biochem. Parasitol.*, **11**, 61–80.

Herrera,S., Herrera,M.A., Perlaza,B.L., Burki,Y., Caspers,P., Döbeli,H., Rotmann,D. and Certa,U. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 4017–4021.

Herrera,M., Rosero,F., Herrera,S., Caspers,P., Rotmann,D., Sinigaglia,F. and Certa,U. (1992) *Infect. Immun.*, **60**, 154–158.

Jarolim,P., Palek,F., Amato,D., Hassan,K., Sapak,P., Nurse,G.T., Rubin,H.L., Zhai,K.E. and Liu,S.-C. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 11022–11026.

Kilejian,A., Rashid,M.A., Aikawa,M., Aji,T. and Yang,Y.F. (1991) *Mol. Biochem. Parasitol.*, **44**, 175–182.

Laemmli,U.K. (1970) *Nature*, **227**, 680–685.

Lambros,C. and Vanderberg,J.P. (1979) *J. Parasitol.*, **65**, 418–420.

Leech,J.H., Barnwell,J.W., Aikawa,M., Miller,L.H. and Howard,R.J. (1984) *J. Cell Biol.*, **98**, 1256–1264.

Lewis,A.P. (1989) *Mol. Biochem. Parasitol.*, **36**, 271–282.

Lyon,J.A., Geller,R.H., Haynes,J.D., Chulay,J.D. and Weber,J.L. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2989–2993.

McBride,J.S., Newbold,C.I. and Anand,R. (1985) *J. Exp. Med.*, **161**, 160–180.

McBride,J.S. and Heidrich,H.G. (1987) *Mol. Biochem. Parasitol.*, **23**, 71–84.

Merrifield,R.B. (1963) *J. Am. Chem. Soc.*, **85**, 2149–2154.

Molano,A., Segura,C., Guzman,F., Lozada,D. and Patarroyo,M.E. (1992) *Parasite Immunol.*, **14**, 111–124.

Palek,J. and Lambert,S. (1990) *Semin. Hematol.*, **27**, 290–332.

Perkins,M.E. (1984) *J. Exp. Med.*, **160**, 788–798.

Perkins,M.E. and Rocco,L.J. (1988) *J. Immunol.*, **141**, 3190–3196.

Ruangjirachuporn,W., Afzelius,B.A., Paulie,S., Wahlgren,M., Berzins,K. and Perlmann,P. (1991) *Parasitology*, **102**, 325–334.

Schulman,S. *et al.* (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 7339–7343.

Shear,H.L., Roth,E.F.Jr., Ng,C. and Nagel,R.L. (1991) *Br. J. Haematol.*, **78**, 555–560.

Siddiqui,W.A., Tam,L.Q., Kramer,K.J., Hui,G.S.N., Case,S.E., Yamaga,K.M., Chang,S.P., Chen,E.B.T. and Kan,S.-C. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 3014–3018.

Sinigaglia,F., Takacs,B., Jacot,H., Matile,H., Pink,J.R.L., Crisanti,A. and Bujard,H. (1988), *J. Immunol.*, **140**, 3568- 3671.

Strych,W., Miettinen-Baumann,A., Lottspeich,F. and Heidrich,H.G. (1987) *Parasitol. Res.*, **73**, 435–441.

Tam,J.P., Heath,W.F. and Merrifield,R.B. (1983) *J. Am. Chem. Soc.*, **109**, 6442–6444.

Tanabe,K., Mackay,M., Goman,M. and Scaife,J.G. (1987) *J. Mol. Biol.*, **196**, 273–287.

Received on July 8, 1992; revised on December 17, 1992