Journal of Cell Science 111, 1831-1839 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JCS4510

Actomyosin motor in the merozoite of the malaria parasite, *Plasmodium falciparum*: implications for red cell invasion

J. C. Pinder[1], R. E. Fowler[2,3], A. R. Dluzewski[1], L. H. Bannister[2], F. M. Lavin[3], G. H. Mitchell[3], R. J. M. Wilson[4] and W. B. Gratzer[1,*]

[1]Medical Research Council Muscle and Cell Motility Unit, Randall Institute, King's College, 26-29 Drury Lane, London WC2B 5RL, UK
[2]Department of Anatomy and Cell Biology, UMDS, The Medical School, Guy's Hospital, London SE1 9RT, UK
[3]Department of Immunology, UMDS, The Medical School, Guy's Hospital, London SE1 9RT, UK
[4]National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
*Author for correspondence

Accepted 1 May; published on WWW 15 June 1998

SUMMARY

The genome of the malaria parasite, *Plasmodium falciparum*, contains a myosin gene sequence, which bears a close homology to one of the myosin genes found in another apicomplexan parasite, *Toxoplasma gondii*. A polyclonal antibody was generated against an expressed polypeptide of molecular mass 27,000, based on part of the deduced sequence of this myosin. The antibody reacted with the cognate antigen and with a component of the total parasite protein on immunoblots, but not with vertebrate striated or smooth muscle myosins. It did, however, recognise two components in the cellular protein of *Toxoplasma gondii*. The antibody was used to investigate stage-specificity of expression of the myosin (here designated Pf-myo1) in *P. falciparum*. The results showed that the protein is synthesised in mature schizonts and is present in merozoites, but vanishes after the parasite enters the red cell. Pf-myo1 was found to be largely, though not entirely, associated with the particulate parasite cell fraction and is thus presumably mainly membrane bound. It was not solubilised by media that would be expected to dissociate actomyosin or myosin filaments, or by non-ionic detergent. Immunofluorescence revealed that in the merozoite and mature schizont Pf-myo1 is predominantly located around the periphery of the cell. Immuno-gold electron microscopy also showed the presence of the myosin around almost the entire parasite periphery, and especially in the region surrounding the apical prominence. Labelling was concentrated under the plasma membrane but was not seen in the apical prominence itself. This suggests that Pf-myo1 is associated with the plasma membrane or with the outer membrane of the subplasmalemmal cisterna, which forms a lining to the plasma membrane, with a gap at the apical prominence. The results lead to a conjectural model of the invasion mechanism.

Key words: Myosin, Malaria, *Plasmodium falciparum*, Merozoite, *Toxoplasma gondii*

INTRODUCTION

The mechanism by which the malaria parasite enters the red blood cell to set in train the events that characterise the disease is poorly understood. Invasion is characterised by a sequence of separable steps, comprising weak random attachment to the red cell surface, followed by reorientation of the parasite to bring its apical prominence into contact with the host cell, and passage into the interior (see Discussion and Ward et al. (1994) for review). Tight attachment, leading to invasion, requires the interaction of parasite surface receptors with ligands on the host cell membrane. This step can be uncoupled from invasion by pretreatment of the parasites with cytochalasin B, as first shown for *P. knowlesi* (Miller et al., 1979) and later for *P. falciparum* (Field et al., 1993). Merozoites treated in this manner remain attached to the red cell and apparently secrete material from the apical organelles (rhoptries), engendering local vacuolation of the red cell membrane in what appears to be a normal feature of the invasion sequence (Dluzewski et al., 1989). Normally the merozoite then moves into the vacuole, apparently enveloping itself in a portion of host cell membrane (Bannister et al., 1986). At the same time an electron-dense annular junction moves backwards over the parasite surface until internalisation is complete (Aikawa et al., 1978).

The origin of the driving force that impels the attached merozoite into the cell is unclear, but the action of the actin capping and depolymerising agent, cytochalasin B, argues for the participation of actin and thus for the existence of an actomyosin motor. This evidence is far from conclusive, however, since many essential cellular processes are dependent on actin. Actin filaments have never been detected in the blood-stage parasite by electron microscopy, but the protein is present in high concentration in *P. falciparum*, much of it in the filamentous form (Field et al., 1993; Webb et al., 1996). The

filaments may be capped and short, and therefore elusive in electron microscopy. We have also given evidence, based on antigenic reactivity in immunoblots and on affinity for actin, for the presence of myosin in merozoites and schizonts (Webb et al., 1996). In trophozoites the actin appeared diffusely distributed, as judged by fluorescence microscopy, whereas in merozoites staining was prominent at the apical pole and the periphery. Myosin in merozoites appeared to be mainly at the pole, or at all events sparse or absent in the centre of the cell. We have now identified and partially characterised a malarial myosin, and we give evidence, based on its distribution in the parasite and the stage-specificity of its expression, that it is a merozoite invasion-linked motor protein.

MATERIALS AND METHODS

Parasites and parasite extracts

Blood-stage *P. falciparum* parasites were maintained in continuous culture (Trager and Jensen, 1976). Partial synchrony was achieved by differential sorbitol lysis (Lambros and Vanderberg, 1979). Cells containing segmenting schizonts, with parasitaemias of ~10%, were purified by centrifugation through a Percoll layer (Dluzewski et al., 1984). Merozoites were obtained from synchronised cultures by a procedure modified from that of Freeman and Holder (1983): parasites were collected at 1 hour intervals and separated from intact cells by low-speed centrifugation (375 ***g*** for 10 minutes) at room temperature. Merozoites were recovered from the supernatant by pelleting at 3,000 ***g*** for 10 minutes.

Toxoplasma gondii parasites were cultured in canine fibroblasts, as described by Roos et al. (1994).

Red cell invasion assays

P. falciparum merozoite preparations or schizont-infected cells were added to target cells, and Giemsa-stained smears were examined after 15-20 hours. Parasitaemias were determined by counting 1,000 cells.

Gel electrophoresis and immunoblots

Total cellular protein from malaria parasites at different stages and from *T. gondii* tachyzoites and *T. gondii*-infected fibroblasts was examined by polyacrylamide gel electrophoresis in SDS (Laemmli, 1970). The cells were solubilised in the sample buffer for gel electrophoresis, containing 2% SDS, 1 mM EDTA, pH 7.6, together with 1 mM phenylmethylsulphonyl fluoride and the following peptide protease inhibitors, all at 1 μg ml^{-1}: chymostatin, leupeptin, pepstatin, aprotinin, antipain and bestatin (Sigma). To prepare cell fractions of the malaria parasites the cells were homogenised in phosphate-buffered isotonic saline (PBS), containing the above protease inhibitors. The particulate fraction was separated by low-speed centrifugation (2,000 ***g*** for 10 minutes). For detergent extraction the particulate fraction was left on ice for 5 minutes with 1% $C_{12}E_8$ (octaethyleneglycol mono-*n*-dodecyl ether) or Triton X-100. The pellet and supernatant were collected after a further centrifugation at 10^5 ***g*** for 5 minutes.

All fractions, with freshly added EDTA and protein inhibitors as above, were mixed with an equal volume of 10% SDS, diluted fivefold with electrophoresis sample buffer, heated at 100°C for 3 minutes, clarified by centrifugation and applied immediately to 8 or 12% polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue R or used for immunoblotting. Electroblots on nitrocellulose membrane were blocked with 0.5% Tween-20, in isotonic phosphate-buffered saline and exposed to antibodies at dilutions between 1:100 and 1:6,400 in PBS, containing 0.1% Tween-20. The membranes were then washed with 0.1% Tween-20 in PBS. For the antiserum against the expressed myosin fragment an optimal dilution of 1:1,600 was subsequently used. The blots were washed and treated with peroxidase-coupled second antibody (Sigma) and developed with the SuperSignal chemiluminescent reagent (Pierce).

Myosin gene sequences

The EMBL/GenBank/DDBT databases contain only one (incomplete) malaria parasite myosin gene sequence (accession number Y09693); this was determined in one of our laboratories. We refer to it herein as Pf-myo1. A search for additional malaria myosins was undertaken by the procedure of Bement et al. (1994).

Expression and purification of *P. falciparum* myosin fragment

A 249 residue segment of the parasite myosin was selected as an antigen. This is located in the head domain and corresponds to residues 234-483 in the alignment with the *T. gondii* TgM-A myosin sequence, reported by Heintzelman and Schwartzman (1997) (accession number AF006626). Forward and reverse primers, corresponding to amino acid sequences ANPVLEA and DIFGFEVF respectively, were synthesised with an added *Nde*I restriction site in the sense-strand primer and a *Bam*HI restriction site and a stop codon in the antisense-strand primer (forward primer sequence: 5′-C TCG AGT **CAT ATG** GCA AAT CCA GTT CTT GAA GCT-3′, reverse primer sequence 5′-T CAG A**GG ATC** CTA AAA TAC TTC AAA ACC AAA AAT ATC-3′, where bold type denotes restriction sites and start/stop codons are underlined). PCR amplification was performed on a blood-stage *P. falciparum* cDNA clone and the purified product was cleaved with *Nde*I; the fragment was isolated by gel electrophoresis, and ligated into the cloning vector, pET-15b (Novagen), which had been digested with *Nde*I and *Bam*HI and dephosphorylated. The ligated product was then cyclised after digestion of the insert with *Bam*HI. The plasmid was transformed into XL1 Blue *E. coli* for amplification and, after confirmation of the recombinant sequence, was transformed into *E. coli* BL21(DE3)pLys S. A large bacterial colony was used to inoculate M9 minimal medium and a preparative culture was grown on at 37°C. Expression of the myosin fragment was induced by 0.5 mM IPTG and growth was allowed to proceed for a further 4 hours. The cells were harvested by centrifugation and stored frozen. Total bacterial protein was examined by SDS-gel electrophoresis and revealed the presence of an IPTG-induced protein of apparent molecular mass 27,000 (Fig. 1). The myosin fragment was recovered from inclusion bodies after lysis of the bacteria in 6 M guanidinium chloride and isolated by adsorption to a His-Bind chelate affinity matrix (Novagen). For the production of antibodies the polypeptide, dissolved in 6 M guanidinium chloride, was further purified by FPLC gel filtration on Superose 12 (Pharmacia) (Fig. 1).

Antibodies

Six antisera were prepared against oligopeptide sequences attached to a multifunctional peptide (MAP) backbone (Posnett and Tam, 1989). Two of the oligopeptides contained highly conserved myosin consensus sequences, viz **GESGAGKT**EAAKQI (antigen I, the residues in bold type representing the conserved sequence) and **LEAFGNAKT**IRNNN (antigen II). The remaining four sequences from the parasite myosin sequence show either no or very few homologies with any proteins in the database. They are: SKSGNMDLRIQTAIM (III), FMQLVISHEGGIRYG (IV), TMKSKFGLKGVTE (V) and YKLLNPNSTEVSGV (VI). These and the purified expressed myosin fragment were all used to raise polyclonal antibodies. The sera of the rabbits were tested before immunisation (preimmune sera) for lack of reactivity against parasites by immunofluorescence.

Immunofluorescence

For routine screening, films from schizont-rich blood cultures were fixed with methanol or ice-cold acetone, incubated with antiserum at the optimal dilution of 1:250-1:1,000, washed and labelled with

fluorescein-conjugated pig anti-rabbit (Dako) or goat anti-rabbit (Vector Laboratories) immunoglobulin. For more detailed studies schizonts isolated from Percoll layers were cultured on polylysine-coated slides for 2-3 hours, fixed with fresh 3.7% (w/v) paraformaldehyde in PBS, following the method of Read et al. (1993), permeabilised with cold acetone, incubated with antiserum and developed with goat anti-rabbit IgG (Sigma). Controls were performed with the preimmune sera and with second antibody alone. Slides were examined with a Nikon Optiphot epifluorescence or Olympus PROVIS AX70 microscope.

Immuno-electron microscopy

Schizonts from a Percoll layer were briefly washed and fixed for 20 minutes in ice-cold 0.1% double-distilled glutaraldehyde in RPMI 1640, pH 7.2, and washed three times in ice-cold RPMI 1640 by centrifugation. The pellet was dehydrated in a succession of ethanol concentrations from 0 to 95% at progressively decreasing temperatures from 0 to −20°C over a period of 2 hours, infiltrated overnight with LR White at −25°C and polymerised at room temperature for 48 hours under ultraviolet light (see Bannister and Kent, 1993). After blocking with 1% bovine serum albumin in phosphate-buffered isotonic saline, sections were immuno-stained by the indirect antibody method, using the polyclonal anti-parasite myosin antiserum at a dilution of 1:100. The same dilution of preimmune serum was used as control. The second antibody was goat anti-rabbit IgG, conjugated with 5 or 10 nm gold particles. Sections were briefly stained with 2% aqueous uranyl acetate before viewing.

RESULTS

Myosin in *P. falciparum*

A myosin gene was identified in a cDNA library of the blood-stage parasite and partially sequenced (accession number Y09693). We have carried out a search for other myosin-like sequences, but none were detected when 20 clones, obtained by PCR amplification at low stringency with a set of highly degenerate primers (Bement et al., 1994), were examined.

The failure of low-stringency screening with such primers to identify any other myosin-like sequences in *P. falciparum* might be taken to imply that no other myosin genes occur in the parasite genome. However, one would expect at least a cell-division myosin to be present and the multiplicity of myosin genes in most organisms is well-established (Sellers et al., 1996; Cope et al., 1996).

Relation to *T. gondii* myosins

A search of the DNA database yielded homologies between the partial (~50%) sequence of the *P. falciparum* myosin head domain (unusual in that it contains 69% AT) and those of several other myosins. The deduced protein sequence exhibits much the strongest homology with the recently determined sequences of three myosins from *T. gondii*, two of which (TgM-B and TgM-C) are thought to be splicing variants, derived from a single gene (Heintzelman and Schwartzman, 1997). In particular, there is 67% identity between the known part of the Pf-myo1 polypeptide sequence and that of TgM-A of *T. gondii*. The three *T. gondii* sequences, together with Pf-myo1, define a new category of myosins, designated as class XIV (Mermall et al., 1998).

Anti-myosin antibodies

The six antibodies directed against synthetic peptides based on the deduced Pf-myo1 sequence all reacted strongly with their cognate peptide antigens on immunoblots. Two of the four directed against sequences within the expressed fragment of Pf-myo1 (see below) also showed good reactivity against the intact fragment. The same two antibodies, after affinity-purification, showed somewhat diffuse staining in immunofluorescence around the peripheries of the merozoites within a schizont, with brighter punctate staining at the apex of the merozoites. Neither antibody, however, proved to have sufficiently high reactivity for satisfactory labelling in immunogold electron microscopy.

To generate a more reactive antibody we used a large fragment of Pf-myo1, expressed in *E. coli*, as immunogen. As inferred from an alignment with vertebrate skeletal myosin II and comparison with its three-dimensional structure (Rayment et al., 1993) this portion of the protein comprises an outward-facing lobe of the head. The purified expressed product (Fig. 1) became insoluble when transferred from the denaturing medium used for chromatographic purification into aqueous buffer. It proved, however, to be an excellent immunogen, which elicited an antibody that reacted strongly with the parasite myosin band on immunoblots at high dilution and gave rise to prominent immunofluorescent staining. The preimmune serum showed no reactivity in any assay. The specificity of the antibody was demonstrated by the absence of any detectable reactivity on immunoblots against mammalian striated muscle myosin or smooth muscle myosin and myosin I of rat intestine, nor could any reactivity be observed in the total protein from red cells, leukocytes or the canine fibroblast host cells of *T. gondii*. The antibody reacted strongly, however, with a band in immunoblots of the total protein of homogenised *T. gondii* tachyzoites; this constituent had apparent molecular mass 93,000 (Fig. 2), and probably corresponds therefore to the myosin, TgM-A (Heintzelman and Schwartzman, 1997). Variable amounts of two bands of lower molecular mass were also generally observed, which were almost certainly

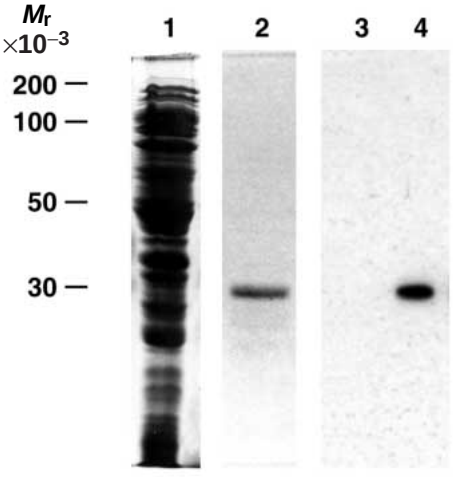

Fig. 1. Expression of *P. falciparum* myosin, Pf-myo1, fragment in *E. coli*. Electrophoresis in a 12% SDS-polyacrylamide gel of total protein from transformed *E. coli* (lane 1) and of chromatographically purified myosin fragment (lane 2), stained with Coomassie Brilliant Blue; lanes 3 and 4 show immunoblots with preimmune serum and the antiserum against the myosin fragment, respectively. Apparent molecular masses are based on marker proteins.

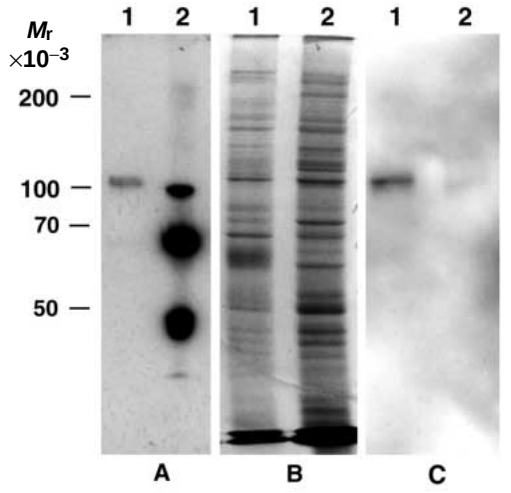

Fig. 2. Reaction of antibody against *P. falciparum* Pf-myo1 fragment with proteins from *P. falciparum* and *T. gondii*. (A) Immunoblot of *P. falciparum* merozoite and *T. gondii* tachyzoite proteins (lanes 1 and 2, respectively) after electrophoresis in 8% SDS-polyacrylamide gel. (B and C) Stage-specificity of expression of Pf-myo1: (B) an SDS-polyacrylamide gel stained with Coomassie Brilliant Blue, and (C) an immunoblot of the same gel, stained with the anti-Pf-myo1 antibody. Lane 1 in each case is of the total merozoite protein and lane 2 the total protein solubilised from schizonts. Note that the myosin is not directly detected by the Coomassie stain because of the superimposition of a prominent zone from an unidentified protein.

degradation products. *T. gondii* within its fibroblast host cell gave rise to an additional weaker component of higher molecular mass, possibly one of the other two myosins, TgM-B or TgM-C (data not shown).

Stage-dependence of myosin expression in *P. falciparum*

With the aid of the same antibody we were able to study the stage-specificity of Pf-myo1 expression during the blood-stage cycle of the parasite. Blots of electrophoretic gels of total parasite protein were exposed to the antiserum, which recognised a single band migrating at an apparent molecular mass of about 105,000. As Fig. 2 shows, myosin could not be detected in the schizont preparations, but was prominent in the merozoite. When preparations containing both very mature and younger schizonts were examined by immunofluorescence with the same antiserum (Figs 3 and 4), staining was confined to the most mature and rupturing forms and was not seen in younger parasites (including established rings). Pf-myo1 is thus expressed very late in schizogony just before the start of the invasion cycle, and vanishes soon after invasion.

Effect of actomyosin ATPase inhibitor on invasion of host cells

Butane-2,3-dione monoxime is a weak but largely specific inhibitor of the actomyosin contractile cycle (see e.g. Zhao et al., 1995). The presence of this reagent in the culture medium in in vitro red cell invasion assays, using either schizont-infected cells or merozoites, inhibited invasion. The concentration for half-inhibition was about 5 mM and inhibition was essentially complete at 25 mM. In a typical experiment with merozoites the parasitaemia of 7.2% (ring-stage parasites) in control cells fell to 1.2% at an inhibitor concentration of 12 mM and 0.2% at 25 mM.

Distribution of Pf-myo1 in the parasites

The distribution of Pf-myo1 in the merozoite was examined with the aid of the polyclonal antibody against the expressed protein fragment. By DAPI staining we were able to distinguish the nucleus in the basal region and the mitochondrion with the plastid towards the apical end, and thus to determine the orientation of the merozoite. The myosin is concentrated around the periphery of each merozoite within the schizont (Fig. 4a); in merozoites liberated from schizonts it also lies at the periphery, being most concentrated around the

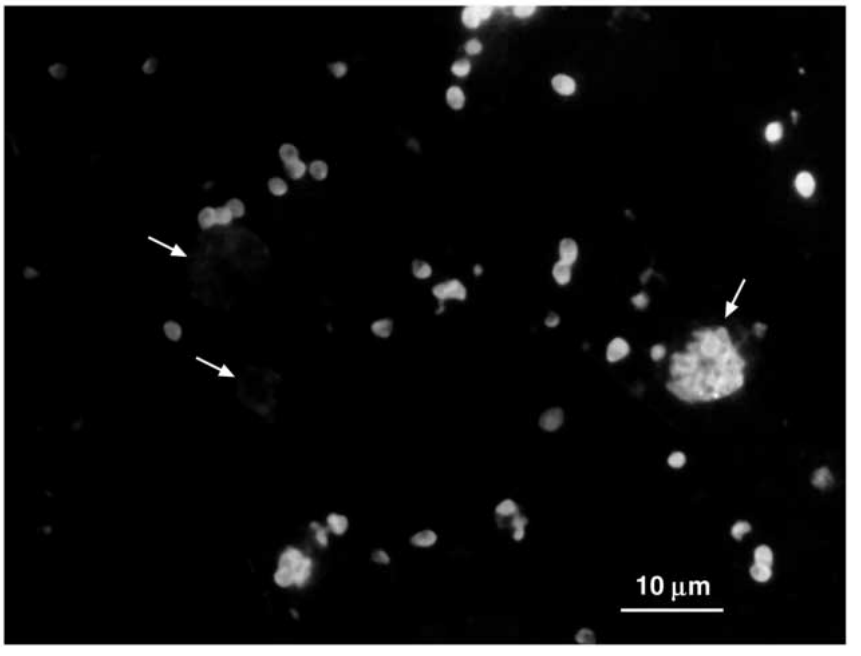

Fig. 3. Detection of Pf-myo1 by immunofluorescence. Labelling is seen in free merozoites and a rupturing schizont (arrow, right). Two immature schizonts (arrows, left) are barely stained.

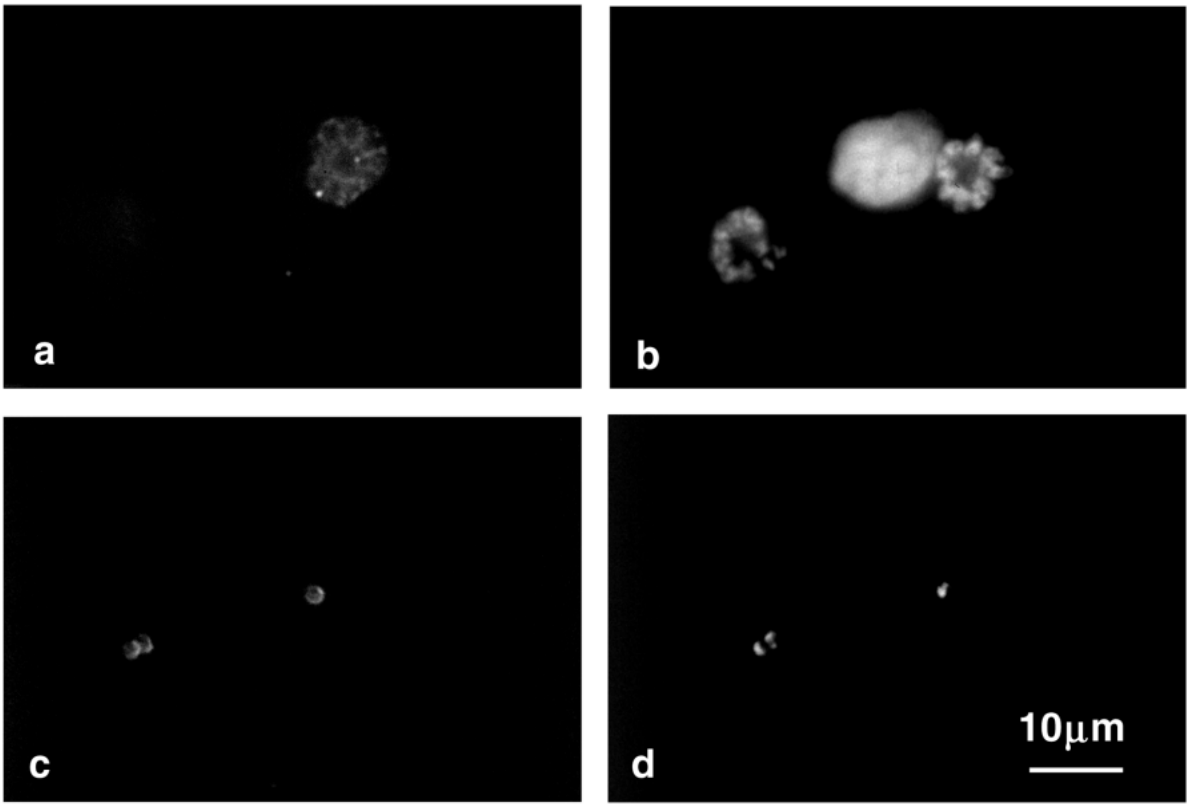

Fig. 4. Mature intraerythrocytic parasites: (a) stained with anti-myosin and fluorescent second antibody; (b) corresponding DAPI-stained image, showing the nuclei of three schizonts in the same field, only one of which (furthest right) displays antibody staining in a; (c) three free merozoites, showing myosin staining mainly at the apical pole. The orientation is determined by the DAPI staining pattern (d), which reveals the positions of the mitochondrion together with the plastid at the apical pole and the nucleus in the basal region (smaller and larger dots, respectively).

anterior half (Figs 4c, 5a). Comparable (though not significantly better) results were obtained by confocal microscopy (not shown). Fig. 5 shows myosin immunofluorescence in merozoites attached to red cells, which again indicates a predominantly apical location. The peripheral distribution of immunofluorescence suggests that Pf-myo1 may be largely membrane associated.

The antiserum also gave rise to strong immunofluorescence in tachyzoites of *T. gondii* (not shown). Strongest staining was seen at one end, but in many cases bright patches were also present throughout the cytoplasm.

To identify the distribution of the myosin between the cellular compartments of the malaria parasite we first separated the soluble cytosolic fraction from the insoluble material, which comprises organelles and membranes, together with attached cytoskeletal filaments. As Fig. 6 shows, most of the myosin was associated with the particulate fraction, but a minor proportion was also found in the cytosol. It is unlikely that the results were vitiated by proteolysis because the soluble and insoluble myosin populations migrated at the same apparent molecular mass in SDS-gel electrophoresis and no prominent additional components of smaller size were seen. The myosin was not released into the supernatant when the pellet was extracted with isotonic buffer containing the non-ionic detergent, Triton X-100. In hypertonic salt, however, in the presence of the detergent a part of the myosin was solubilised. This proportion remained unchanged when the detergent concentration was increased from 1 to 3.75% and when 2 mM magnesium-ATP or 20 mM sodium pyrophosphate were included in the medium.

Immuno-electron microscopy

During the terminal period of the intraerythrocytic stage the 16 or so merozoites, which are budded off from the parent schizont, come to lie in close contact with each other within the confines of the host cell membrane. An elevation at one end of each ellipsoidal merozoite forms the apical prominence. A flat membranous cisterna underlies the whole parasite plasma membrane, except at the apical prominence, so that most of the periphery is enclosed by three membranes, termed the pellicle (see Fig. 8D). As Fig. 7 shows, the myosin is localised in the region between the plasma membrane and the underlying cisterna around almost the entire circumference of the cell. In some cases the labelling was stronger towards the apical prominence (Fig. 7b), corresponding to the bright apical labelling seen in immunofluorescence. There was, by contrast, no labelling in the most anterior part of the apical prominence, where there is a gap in the pellicular cisterna beneath the plasma membrane. There was a small amount of random labelling in the nucleus and cytoplasm of the merozoites. The preimmune serum showed no significant labelling (Fig. 7c). The antiserum showed some promiscuous

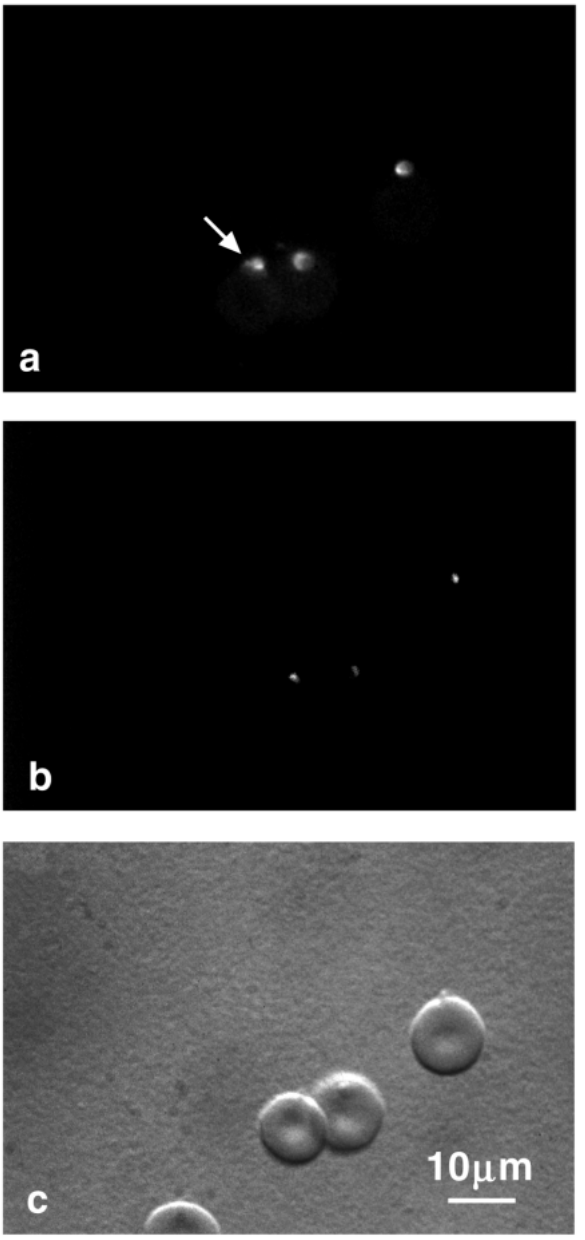

Fig. 5. Merozoites attached to red cells, showing (a) apical myosin staining. One of these (arrowhead), may be in the process of invading a red cell. (b) The same stained with DAPI. (c) The corresponding differential interference contrast image.

cross-reactive staining of the cytosol of red cells, but, as with the preimmune serum, there was no significant labelling of immature parasites.

Immunogold labelling was again tightly linked to development, peripheral antigen being first observed in merozoites in the final stages of budding from mature schizonts. No label was detected on any other organelle than the plasma membrane (including the cytostome) at any stage of the parasite's asexual erythrocytic development, nor was more prominent labelling seen in the narrow stalk-like regions where the budding merozoites detach from the residual body of the parent schizont.

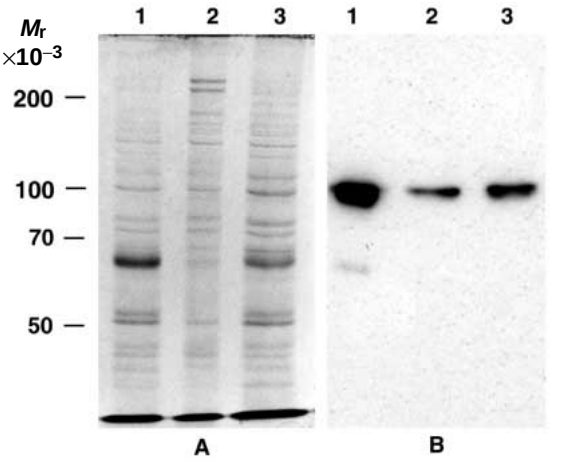

Fig. 6. Distribution of Pf-myo1 between different cellular fractions. SDS-polyacrylamide gel electrophoresis of parasite protein fractions (A) Coomassie Brilliant Blue stain, showing: lane 1, supernatant of low-speed centrifugation of lysed merozoites; lane 2, detergent-insoluble fraction of pellet from above; lane 3, detergent-soluble fraction of same. (B) Immunoblots with antiserum against expressed Pf-myo1 fragment, showing: lane 1, Triton-insoluble fraction of pellet from low-speed centrifugation of lysed merozoites (cf sample A2); lane 2, Triton-soluble fraction of same (cf A3); lane 3, supernatant fraction from low-speed centrifugation of lysed merozoites (cf A1).

DISCUSSION

Pf-myo1 and the myosins of *T. gondii* are the first members of a new category (class XIV) of myosin (Mermall et al., 1998). In general micro-organisms express several myosins and it was therefore unexpected that more myosin genes were not detected in *P. falciparum.* We are unable to account for this observation, since the search for myosin genes was carried out at low stringency and with degenerate probes (Bement et al., 1994), based on highly conserved sequences. In our initial immunoblots of parasite protein we observed three myosin-reactive electrophoretic components, the largest corresponding in molecular mass to the myosin described here; the proportion of the two smaller polypeptides is reduced by measures taken to limit proteolysis and they are almost certainly degradation products.

The *T. gondii* myosin, TgM-A, which bears a close sequence similarity (67% identity) to Pf-myo1, is characterised by unusual C-terminal (tail) sequences, an N-terminal element, not found in other known myosins, and no obvious neck (Heintzelman and Schwartzman, 1997). The presumptive tail domain is vestigial and devoid of any readily definable heptad hydrophobic repeat. This would imply that it is incapable of forming filaments. If the homology between the two myosins extends to the C-terminal side of the known part of the Pf-myo1 sequence, the same conclusion will apply.

The stage-specificity of expression makes it highly probable that this myosin is the motor in the invasion mechanism. It is eliminated after invasion, and it is conceivable that its degradation limits the duration of the parasite's viability after release of the merozoites from the ruptured red cell. This inference is also consistent with the inhibitory effect of BDM

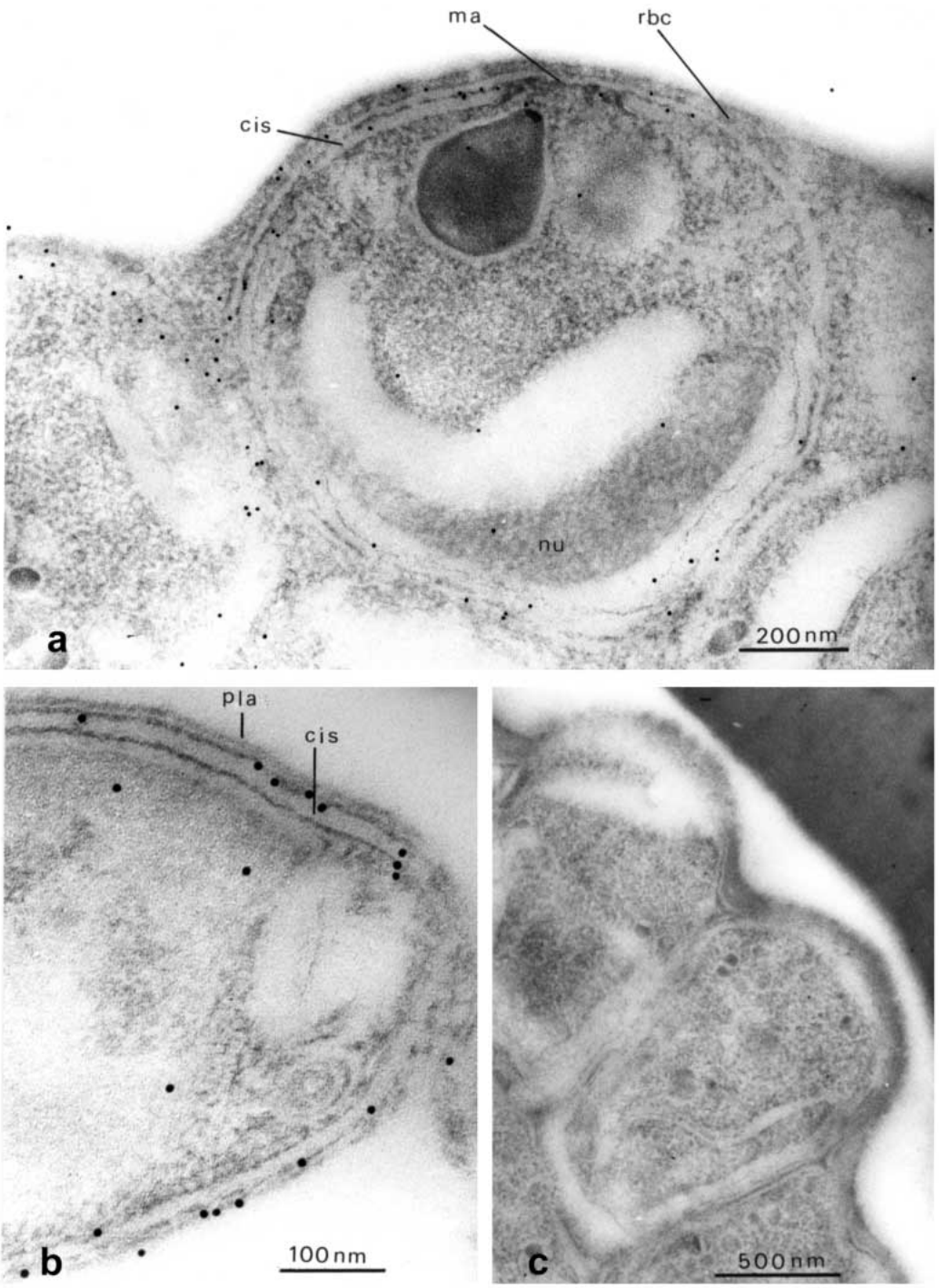

Fig. 7. Immuno-electron microscopy, showing distribution of Pf-myo1 in the malaria parasite. (a) Immunogold-labelled merozoites before their release from the red cell. The parasite was labelled with anti-Pf-myo1 and second antibody, conjugated with 10 nm gold particles. The myosin is located around the periphery of the merozoite. In the centre the merozoite plasma membrane overlies the apical prominence (ma) close to the large pear-shaped secretory rhoptry, and elsewhere in the parasite a flat cisterna (cis) underlies the plasma membrane. The curved nucleus (nu) is visible at the base of the merozoite. Note absence of label from the flat tip of the apical prominence, and the greater frequency of labelling around the apical prominence than elsewhere. (b) Longitudinal/oblique section through the apical end of a free merozoite. Labelling is associated with the cortical cytoplasm between the plasma membrane (pla) and the outer membrane of the subplasmalemmal cisterna (cis). (c) Control section through a group of mature merozoites at the periphery of a late-stage schizont, incubated with preimmune serum before gold labelling. No significant labelling can be observed.

on invasion: this occurs at a lower inhibitor concentration than is required to inhibit gliding motility in *T. gondii* tachyzoites (Dobrowolski et al., 1997). However, although BDM is generally taken to act by inhibiting actomyosin ATPase, an effect on calcium release from the sarcoplasmic reticulum has also been observed (Phillips and Altschuld, 1996). A secondary effect of BDM on invasion cannot be excluded.

The partition of Pf-myo1 between the cytosolic and insoluble fractions may imply that attachment to a membrane is regulated by a covalent modification, in particular phosphorylation: a potential phosphorylation site (a threonine residue, 16 residues upstream of a homologue of the consensus DALAK motif; Bement and Mooseker, 1995) occurs in the Pf-myo1 sequence (though not in that of TgM-A of *T. gondii*).

Immunofluorescence has recently been reported in *T. gondii* tachyzoites by Dobrowolski et al. (1997), using an anti-peptide antiserum directed against a conserved element (LEAF motif) found in all three *T. gondii* sequences and in many other myosins. In some but by no means all *T. gondii* parasites that we examined staining with our antibody was distributed in

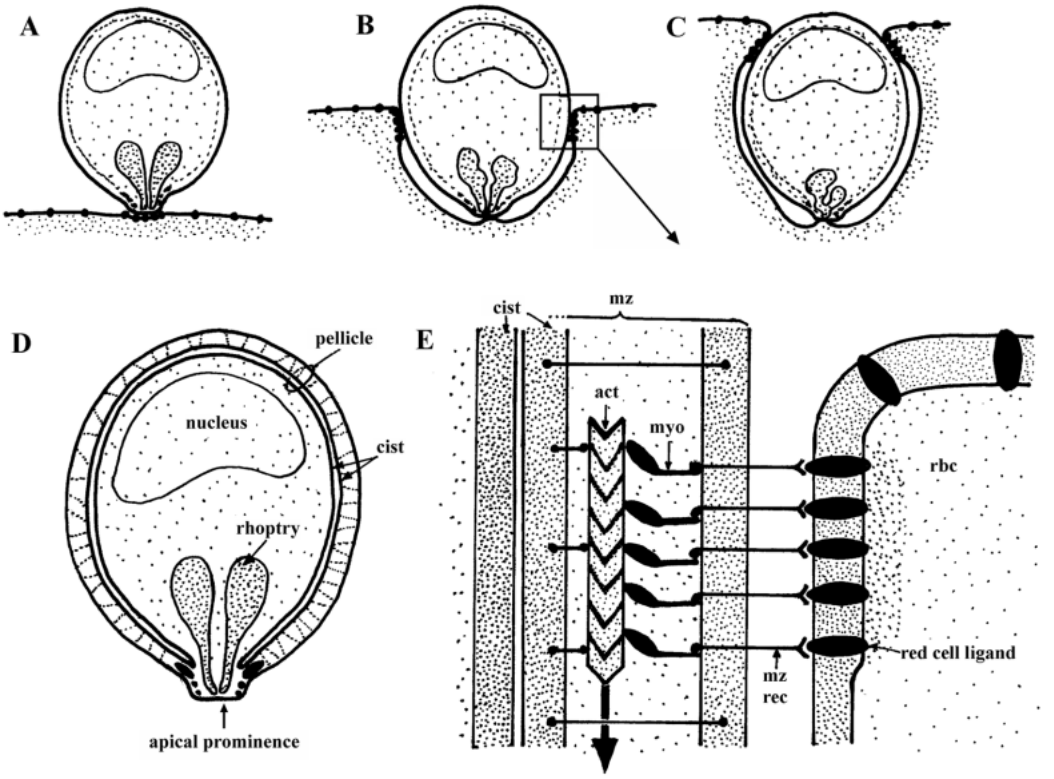

Fig. 8. Schematic view of possible invasion mechanism. (A,B,C) Successive stages in the passage of the attached merozoite into the red cell. Note host cell ligands bound to receptors on the merozoite surface. As invasion begins, the contents of the paired apical organelles, the rhoptries, are ejected. The internalised merozoite is encapsulated in an outer membrane, the parasitophorous vacuole membrane. (D) The schematic organisation of the merozoite. The two membranes of the subplasmalemmal cisterna (cist) show filamentous attachments to the plasma membrane, except at the apical prominence. (E) A schematic view of the electron-dense junction zone (enlarged from b), which is translated over the merozoite (mz) surface as it enters the red blood cell (rbc). The actin filament (act) is envisaged to lie parallel to the merozoite plasma membrane and the force exerted by the myosin (myo) is transmitted by way of transmembrane links through the merozoite receptors (mz rec) to the apposed red cell surface. In this model the actin filaments are attached to rigid elements within the merozoite. An alternative model would apply if the myosin is associated with the outer cisternal, rather than the plasma membrane (as the absence of gold particles around the apical prominence may suggest. Here the relative positions of the actin and the myosin would be reversed, so that the transmembrane linkages between the merozoite and the red cell are attached to the actin rather than the myosin.

patches. Dobrowolski et al. also did not report the prominent fluorescence that we observed at one end of the tachyzoite. A likely reason is that our antibody (unlike the anti-LEAF antibody) would have responded only to TgM-A, with which it reacted on immunoblots. The tachyzoite exhibits gliding motility and any or all the three known myosins could therefore be required for this function, rather than for invasion. On the other hand, the close sequence homology between Pf-myo1 and TgM-A, the analogous locations in the cell and the fact that TgM-A is the only one of the three myosins present in the extracellular parasite argue for a related function.

The bulk of the Pf-myo1 was recovered in the insoluble fraction of the parasite homogenate and, considering especially its location in immuno-electron microscopy, the probability is that it exerts its function at a membrane, as several similar unconventional myosins are thought to do (Mooseker and Cheney, 1995). Association of such myosins with the membrane seems in general to be dependent on interaction with phosphatidylserine, or at all events anionic phospholipid (Adams and Pollard, 1989; Hayden et al., 1990) (as well as with proteins).

The immuno-electron microscopy results indicate that the insoluble myosin is located just beneath the plasma membrane. Because the indirect antibody labelling technique affords only limited spatial resolution we cannot be sure whether the protein is associated with the inner surface of the plasma membrane itself or with the closely underlying outer cisternal membrane. Its absence from the centre of the apical prominence argues for an attachment to the outer cisternal membrane, but further work is required to place the issue beyond doubt.

Conjectural models have been put forward in the past for the function of a motor system in the invasion process (King, 1988; Mitchell and Bannister, 1988; Bannister and Dluzewski, 1990). These envisage the formation of a link extending from the apical junction zone between the parasite and the red cell, through merozoite transmembrane receptors, to an actomyosin motor within the parasite. The zone is then translocated as a ring over the merozoite surface (Aikawa et al., 1978). The ‘merozoite capping protein’, MCP-1 (Klotz et al., 1995), which collects in the junction zone, may interact with cytoskeletal components of the parasite (Hudson-Taylor et al., 1995), and this and/or other proteins may form the required non-compliant

link. Thus one may envisage the myosin as exerting force against actin filaments parallel to the membrane plane, while its C-terminal (and presumably membrane-associated) domain forms a coupling to a transmembrane protein, attached at the outer surface to the host cell contact zone. This conjectural scheme is illustrated in Fig. 8. If, on the other hand, the myosin is associated with the outer cisternal membrane the model is reversed, with the actin filaments close to the plasma membrane and a link from these to the host cell through the transmembrane connector.

Despite evidence for a high concentration of F-actin in the parasite (Field et al., 1993) no actin filaments have ever been observed by electron microscopy; it is possible that short filaments at the site of force generation are in a steady state of 'treadmill' polymerisation and depolymerisation. To develop a more securely based invasion model we need next to establish the distribution of the parasite actin around the invasion zone.

We thank the Wellcome Trust for support under grant nos 045828 and 048244, and R. M. Simmons for support and encouragement. We are indebted to A. A. Holder and I. T. Ling for genomic DNA, to W. Stafford for the *P. falciparum* cDNA library, to C. Forero, L. Collins and P. J. Marsh for discussion and advice and to K. Kirwan for preparing the figures.

REFERENCES

Adams, R. J. and Pollard, T. D. (1989). Binding of myosin I to membrane lipids. *Nature* **340**, 565-568.

Aikawa, M., Miller, L. H., Johnson, J. and Rabbege, J. (1978). Erythrocyte entry by malaria parasites. A moving junction between erythrocyte and parasite. *J. Cell Biol.* **77**, 72-82.

Bannister, L. H., Mitchell, G. H., Butcher, G. A., Dennis, E. D. and Cohen, S. (1986). Structure and development of the surface coat of erythrocyte merozoites of *Plasmodium knowlesi*. *Cell Tissue Res.* **245**, 281-290.

Bannister, L. H. and Dluzewski, A. R. (1990). The ultrastructure of red cell invasion in malaria infections: a review. *Blood Cells* **16**, 257-292.

Bannister, L. H. and Kent, A. P. (1993). Immunochemical microscopic localization of antigens in malaria parasites. In *Methods in Molecular Biology*, vol. 21 (ed. J. E. Hyde), pp. 415-429. Humana Press, Totowa, NJ.

Bement, W. M., Hasson, T., Wirth, J. A., Cheney, R. E. and Mooseker, M. S. (1994). Identification and overlapping expression of multiple unconventional myosin genes in vertebrate cell types. *Proc. Nat. Acad. Sci. USA* **91**, 6549-6553.

Bement, W. M. and Mooseker, M. S. (1995). TEDS rule: a molecular rationale for differential regulation of myosins by phosphorylation of the heavy chain head. *Cell Motil. Cytoskel.* **31**, 87-92.

Cope, M. J. T. V., Whisstock, J., Rayment, I. and Kendrick-Jones, J. (1996). Conservation within the myosin motor domain: implication for structure and function. *Structure* **4**, 969-987.

Dluzewski, A. R., Ling, I. T., Rangachari, K., Bates, P. A. and Wilson, R. J. M. (1984). A simple method for isolating viable parasites of *Plasmodium falciparum* from cultures. *Trans. Roy. Soc. Trop. Med. Hyg.* **78**, 622-624.

Dluzewski, A. R., Fryer, P. R., Griffiths, S., Wilson, R. J. M. and Gratzer, W. B. (1989). Red cell membrane protein distribution during malarial invasion. *J. Cell Sci.* **92**, 691-699.

Dobrowolski, J. M., Carruthers, V. B. and Sibley, L. D. (1997). Participation of myosin in gliding motility and host cell invasion by *Toxoplasma gondii*. *Mol. Microbiol.* **26**, 163-173.

Field, S. J., Pinder, J. C., Clough, B., Dluzewski, A. R., Wilson, R. J. M. and Gratzer, W. B. (1993). Actin in the merozoite of the malaria parasite, *Plasmodium falciparum*. *Cell Motil. Cytoskel.* **25**, 43-48.

Freeman, R. F. and Holder, A. A. (1983). Surface antigens of malaria merozoites. A high molecular weight precursor is processed to 83000 mol. wt. form expressed on the surface of *Plasmodium falciparum* merozoites. *J. Exp. Med.* **158**, 1647-1653.

Hayden, S. M., Wolenski, J. S. and Mooseker, M. S. (1990). Binding of brush-border myosin-I to phospholipid vesicles. *J. Cell Biol.* **111**, 443-451.

Heintzelman, M. R. and Schwartzman, J. R. (1997). A novel class of unconventional myosins from *Toxoplasma gondii*. *J. Mol. Biol.* **271**, 139-146.

Hudson-Taylor, D. E., Dolan, S. A., Klotz, F. W., Fujioka, H., Aikawa, M., Koonin, E. V. and Miller, L. H. (1995). *Plasmodium-falciparum* protein associated with the invasion junction contains a conserved oxidoreductase domain. *Mol. Microbiol.* **15**, 463-471.

King, C. A. (1988). Cell motility of sporozoan protozoa. *Parasitol. Today* **4**, 315-319.

Klotz, F. W., Hadley, T. J., Aikawa, M., Leech, J., Howard, R. J. and Miller, L. H. (1995). A 60-kDa *Plasmodium-falciparum* protein at the moving junction formed between merozoite and erythrocyte during invasion. *Mol. Biochem. Parasitol.* **36**, 177-186.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lambros, C. and Vanderberg, J. P. (1979). Synchronization of *Plasmodium falciparum* intraerythrocytic stages in culture. *J. Parasitol.* **65**, 418-420.

Mermall, V., Post, P. L. and Mooseker, M. S. (1998). Unconventional myosins in cell movement, membrane traffic, and signal transduction. *Science* **279**, 527-533.

Miller, L. H., Aikawa, M., Johnson, J. G. and Shiroishi, T. (1979). Interaction between cytochalasin B-treated malarial parasites and erythrocytes. Attachment and junction formation. *J. Exp. Med.* **149**, 172-184.

Mitchell, G. H. and Bannister, L. H. (1988). Malaria parasite invasion: interactions with the red cell membrane. *CRC Crit. Rev. Hematol./Oncol.* **8**, 255-310.

Mooseker, M. S. and Cheney, R. E. (1995). Unconventional myosins. *Annu. Rev. Cell Dev. Biol.* **11**, 633-675.

Posnett, D. N. and Tam, J. P. (1989). Multiple antigenic peptide method for producing antipeptide site-specific antibodies. *Meth. Enzymol.* **178**, 739-746.

Phillips, R. M. and Altschuld, R. A. (1996). 2, 3-Butanedione 2-monoxime (BMD) induces calcium release from canine cardiac sarcoplasmic reticulum. *Biochem. Biophys. Res. Commun.* **229**, 154-157.

Rayment, I., Rypniewski, W. R., Schmidt-Base, K., Smith, R. Tomchick, D. R., Benning, M. M., Winkelmann, D. A., Wesenberg, G. and Holden, H. M. (1993). Three-dimensional structure of myosin subfragment-1 - A molecular motor. *Science* **261**, 50-58.

Read, M., Sherwin, T., Holloway, S. P., Gull, K. and Hyde, J. E. (1993). Microtubular organisation visualised by immunofluorescence microscopy during erythrocytic schizogony in *Plasmodium falciparum* and investigations of post translational modifications of parasite tubulin. *Parasitology* **106**, 223-232.

Roos, D. S., Donald, R. G. K., Morrisette, N. S. and Moulton, T. L. C. (1994). Molecular tools for genetic dissection of the protozoan parasite *Toxoplasma gondii*. *Meth. Cell Biol.* **45**, 27-63.

Sellers, J. R., Goodson, H. V. and Wang, F. (1996). A myosin family reunion. *J. Muscle Res. Cell Motil.* **17**, 7-22.

Trager, W. and Jensen, J. B. (1976). Human malaria parasites in continuous culture. *Science* **193**, 673-675.

Ward, G. E., Chitnis, C. E. and Miller, L. H. (1994). The invasion of erythrocytes by malarial merozoites. *Baillière's Clin. Infect. Dis.* **1**, 155-190.

Webb, S. E., Fowler, R. E., O'Shaughnessy, C., Pinder, J. C., Dluzewski, A. R., Gratzer, W. B., Bannister, L. H. and Mitchell, G. H. (1996). Contractile protein system in the asexual stages of the malaria parasite *Plasmodium falciparum*. *Parasitology* **112**, 451-457.

Zhao, L., Naber, N. and Cooke, R. (1995). Muscle cross-bridges bound to actin are disordered in the presence of 2, 3-butanedione monoxime. *Biophys. J.* **68**, 1980-1990.