A *Plasmodium falciparum* novel gene encoding a coronin-like protein which associates with actin filaments

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Abstract ***Plasmodium falciparum*, the major causative agent of human malaria, is an Apicomplexa protozoan parasite which invades in its intermediate host hepatocytes and erythrocytes. The driving force underlying internalization into the host cell is thought to involve both polymerization of parasite actin, as entry is inhibited by the cytochalasins, and an actin motor-associated protein. In the related Apicomplexa parasite, *Toxoplasma gondii*, the involvement of parasite actin during both processes of motility and host cell entry has been genetically established. In a search for molecules that can regulate actin dynamics within Apicomplexa parasites, we have identified a *P. falciparum* homologue of the actin associated protein called coronin originally described in the amoeba *Dictyostelium discoideum*. The single copy gene displays a strong homology with the amoeba sequence and with the bovine and human coronin homologues recently cloned. This homology lies not only within the N-terminus containing the five WD repeats that characterize coronin but also extends in the C-terminal part. Furthermore, using an affinity-purified mouse monoclonal antibody against *D. discoideum* coronin, we have detected in extracts of *P. falciparum* young and mature schizonts a 42-kDa polypeptide which binds this antibody and is present in a Triton insoluble fraction that also contains parasite actin filaments. In addition, the recombinant protein encoded by the homologue nucleotidic sequence of *P. falciparum* coronin is indeed recognized by the antibody against *D. discoideum* coronin. Finally, the cross-reactive polypeptide displays the ability to cosediment with exogenous F-actin, a property which fits with its involvement in actin dynamics.**

Key words: Actin dynamic; Coronin; WD motif; Apicomplexa

1. Introduction

Among protozoan parasites of the Apicomplexa phylum some members are responsible for severe diseases such as malaria, a disease developing in a broad range of vertebrates and which is caused by the genus *Plasmodium*. The two species that are responsible for most cases of human malaria are *Plasmodium falciparum* and *Plasmodium vivax*. Of the two parasites, *P. falciparum* provokes the death of about two to three million children in Africa every year, while *P. vivax* is usually not lethal but causes significant morbidity. A vaccine is not available and we are facing increasing crucial problems of drug resistant *P. falciparum* and *P. vivax* strains. In consequence, there is an urgent need for a better understanding of the processes underlying the interaction between the parasite and the host at cellular, subcellular and molecular levels. *Plasmodium* is transmitted to the vertebrate host while a blood sucking mosquito releases into the host capillaries infective *Plasmodium* sporozoites. Then, in the vertebrate host, sporozoites and their progeny called merozoites rely on an intracellular milieu provided by hepatocytes and erythrocytes, respectively. To efficiently invade its host cell, *Plasmodium* has evolved a molecular machinery the components of which are involved in a set of interactions with the host cell. Though data have been recently reported on the identity of the parasite ligands and receptor molecules controlling the interaction between *Plasmodium* sporozoites and merozoites with their respective host cell [1–4], very little is known about the signalling events that take place within the two cells and further downstream, about the nature and function of effector molecules that trigger host cell invasion.

Plasmodium and other members of Apicomplexa including *T. gondii* share a polarized apical structure comprising a set of secretory organelles the function of which is important during the genesis and the maturation of the parasitophorous vacuole shaped by the parasite [5–7]. Electron micrographs, video microscopy and pharmacological data, mainly collected from the three related generi, e.g. *Plasmodium*, *Toxoplasma* and *Eimeria*, indicate that the sequential events leading to the internalization of some parasites stages closely resemble each other [8–11]. Among the similarities, the driving force underlying parasite motility and host cell invasion has been shown to be parasite actin based. While it has been directly demonstrated in the case of *T. gondii* [12,13], it has also been strongly suggested in the cases of *Plasmodium* [14–16] and *Eimeria* [17].

In many systems, actin dynamics is tightly regulated by a cohort of actin associated proteins the functions of which are often conserved: they promote or inhibit either actin nucleation, or polymer elongation from newly formed and/or preexisting actin filaments [18–20]. In order to elucidate the events underlying the reorganization of the actin cytoskeleton in Apicomplexa, we have undertaken the identification and functional characterization of parasite molecules that potentially control actin dynamics in vivo. We and others have already identified *Plasmodium* and *Toxoplasma* proteins which can inhibit actin filament elongation in vitro [21,22] or act as actin-associated motors [23,24]. Here, we report on the iden-

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tification of a single copy *P. falciparum* gene the sequence of which encodes a protein that displays strong homology with the *Dictyostelium discoideum* actin binding protein named coronin (Dd coronin) [25]. Using a mouse monoclonal antibody against Dd coronin, we have detected in extracts of *P. falciparum* young and mature schizonts a 42-kDa polypeptide which displays the ability to cosediment with exogeneous F-actin. In addition, we demonstrated that the nucleotidic sequence displaying the homology with Dd coronin encodes the polypeptide recognized by anti-Dd coronin antibody.

2. Materials and methods

2.1. Parasite culture and purification of schizont cells containing vacuoles

P. falciparum (FcB1 strain) were maintained in continuous culture in vitro according to the protocol described by Trager and Jensen [26] with human erythrocytes O^+ in RPMI 1640 medium (Gibco-BRL) supplemented with 6% human serum. At about 6–10% of 8–16 nuclei schizont parasitemia, an enrichment of schizont cells is performed using plasmagel separation. After two washes in phosphate buffered saline (pH 7.2), the infected cells are lysed in 150 mM NaCl, 15 mM Na citrate, pH 7.2 (SSC) supplemented with 0.05% saponin and 1% (v/v) protease inhibitor stocks (stock 1 contained: 5 mg/ml of AEBSF, 2 mg/ml of aprotinin, 2 mg/ml of leupeptin, and 16 mg/ml of benzamidine in H_2O; stock 2 consisted of 5 mg/ml of Pepstatin A in DMSO). The intact parasitophorous vacuoles containing parasites are pelleted at $700\times g$ for 10 min (4°C) and washed once in SSC buffer under the same conditions. Pellets were kept at −70°C until use.

2.2. Parasite extraction

5×10^9 purified schizont cells containing vacuoles were resuspended in 3 ml of calcium and magnesium free phosphate buffered saline (pH 7.2) containing 1 mM EGTA and 1% (v/v) protease inhibitor stocks. Parasites were subjected to four freeze-thaw cycles on liquid nitrogen. Unbroken cells, debris and parasite nuclei were pelleted at $800\times g$ for 15 min at 4°C. The supernatant was further centrifuged in a TL100 table top ultracentrifuge (Beckman) using the TLA100 rotor at $100\,000\times g$ for 1 h (4°C). The supernatant was concentrated 10 times using Centricon 10 (Amicon). The pellet was resuspended in 3 ml of the same saline buffer and centrifuged in similar conditions. Both pellet and supernatant were adjusted with, respectively, 1× and 5× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled for 3 min and kept frozen at −70°C. For the experiments when fractions from detergent extracted parasites were prepared, 0.5% (v/v) Triton X-100 was previously added to the buffer.

2.3. Western blotting

After separation by SDS-PAGE on a 12.5% acrylamide gel, the proteins were transferred onto ECL Hybond nitrocellulose (Amersham) using a semi-dry blot apparatus (Hoeffer) with a current of 1.5 mA/cm^2 of blot surface. The transfer was controlled by red Ponceau-S staining and the blots were then blocked for 2 h with 10% (w/v) slim milk in O.1% (v/v) Tween 20 Tris buffered saline (TTBS). The blots were incubated overnight with 1:2000 of *D. discoideum* anti-coronin antibody in TTBS. After several washes in TTBS, the blots were incubated for 1 h (23°C) with 1:2000 horseradish peroxidase-labelled anti-mouse Ig in TTBS (Amersham). The blots were washed several times in TTBS, incubated 60 s with chemiluminescence substrate (Amersham) and exposed on ECL X-ray film (Amersham). To probe parasite actin, we used anti-*T. gondii* actin antibody at 1:3000 in TTBS and to probe rabbit muscle actin in the co-sedimentation assay, we used monoclonal anti-chicken gizzard actin (N350, Amersham) at 1:5000 in TTBS. To probe the GST fusion protein, we used monoclonal anti-glutathione *S*-transferase at 1:10 000 (Euromedex).

2.4. Co-sedimentation assay

Actin was extracted from rabbit muscle acetone powder (Sigma) as described by Pardee and Spudich [27]. Magnesium bound monomeric actin (Mg-G-actin) was diluted to 2.25 mg and added to the parasite extract. This extract was prepared from 5×10^9 mature schizonts in a low salt buffer (2 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.1 mM 2-mercaptoethanol) supplemented with 0.5% (v/v) Triton X and protease inhibitors. The final concentration of rabbit actin was 22.5 μg/ml and polymerizing conditions were added to the mixture (50 mM NaCl, 2 mM $MgCl_2$, 1 mM ATP). After an overnight incubation at 4°C, it was centrifuged (4°C) for 3 h at $80\,000\times g$ in the TL100 ultracentrifuge with a TL100.A rotor (Beckman). The supernatant and pellet were analyzed by SDS-PAGE. Bovine serum albumin (BSA fraction V, Sigma) was used as a negative control.

2.5. Library screening and sequencing

A search of a random collection of *P. falciparum* cDNA sequences identified two clones showing homology to Dd coronin. One sequence corresponds to an ETS (1019c3) generated as part of the *P. falciparum* genome project (see http://parasite.arf.ufl.edu/malaria.html). These partial sequences were used to generate PCR products which allowed to screen the *P. falciparum* cDNA library [28]. Five independent overlapping clones were isolated and their nucleotidic sequence was determined using both vector and *P. falciparum* (Pf) coronin specific oligonucleotides bought from Genset (France). The complete cDNA is deposited in the nucleotide data base under the accession number AJ002197.

2.6. Expression of glutathione S-transferase (GST)-Pf coronin fusion protein

The expression vector of Pf coronin was prepared by restrictional endonuclease digestion and ligation. The fragment for expression of Pf coronin was prepared by amplification of the region devoid of the WD repeats, using primers containing restriction sites for, respectively, *Bam*HI and *Sal*I, as follows: upper strand: 5′-GGCCGGATCCTATTATCAATATTCACAAGGTTC-3′ and for the lower strand: 5′-GGCCGTCGACTCAATCGAAGATTGGTTC-3′. The amplified fragment was cloned into expression vector pGEX6-P3 (Pharmacia) after digestion with *Bam*HI and *Sal*I of both fragment and vector. From transformed *E. coli* (Strain Sure), a plasmid preparation was performed (QUIAGEN) and followed by transformation of *E. coli* (BL21 strain) according to the protocols of the manufacturer. A transformed BL21 clone was cultured overnight and then diluted 100-fold with fresh LBGA medium (LB supplemented with 20 mM glucose and 0.3 mM ampicillin), grown to DO = 1 and then induced with 1 mM isopropylthio-β-D-galactoside for 3 h at 37°C. After the bacteria were pelleted, they were lysed in 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM ethylenediaminetetraacetate (EDTA) supplemented with 1% Triton X-100 and 1.5% *N*-lauryl sarcosyl (Sigma) at 4°C. After centrifugation ($15\,000\times g$ for 15 min at 4°C), the supernatant was incubated with glutathione Sepharose (Pharmacia) overnight at 4°C and beads were washed with 10 bed volumes of 50 mM

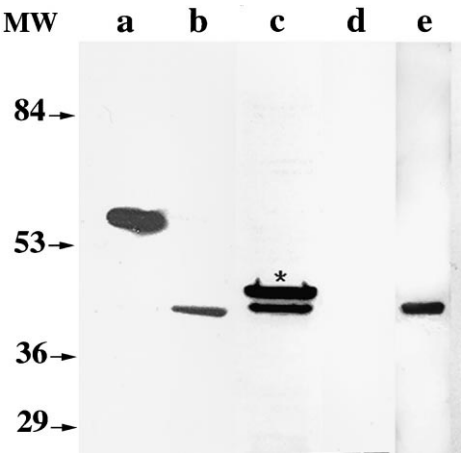

Fig. 1. An actin-associated *P. falciparum* protein homologous to *D. discoideum* coronin. Western blot analysis of protein extracts incubated with affinity purified anti-Dd coronin antibodies. Track a: *D. discoideum*; track b: *P. falciparum* cytosolic fraction; track c: *P. falciparum* Triton X-100 insoluble fraction; track d: blood extract; track e: *T. gondii* cytosolic fraction. The additional signal obtained when the blot was probed with anti-parasite actin is marked by a star on track c.

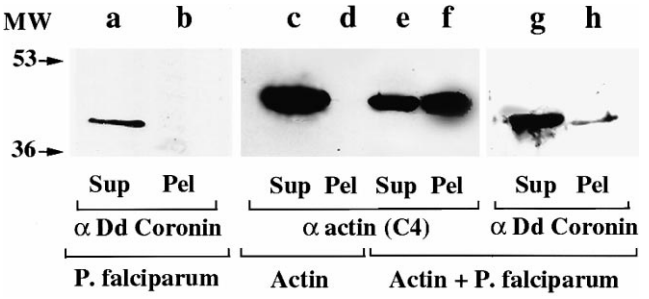

Fig. 2. F-actin cosedimentation assay using rabbit muscle actin. Sup represents a 100 000×*g* supernatant and Pel the corresponding pellet. Tracks a and b show the Triton X-100 soluble and insoluble fraction of *P. falciparum* alone. Tracks c and d: actin is prepared under depolymerizing conditions; tracks e–f: actin is prepared under polymerizing conditions. Tracks c–f are probed with anti-chicken gizzard actin, while tracks a, b, g and h are probed with anti-Dd coronin antibody. Only a fraction of Pf coronin cosediments with actin filaments.

Tris, pH 8.0, 150 mM NaCl, 1 mM ethylenediaminetetraacetate (EDTA) supplemented with 0.1% Triton X-100 3 times. The bound GST-polypeptide was eluted with 5 mM reduced glutathione in 50 mM Tris-Cl, pH 8.0.

3. Results

First, using an affinity-purified anti-Dd coronin monoclonal antibody, we detected a single 42-kDa polypeptide in *P. falciparum* schizonts extracts (Fig. 1, tracks b, c). No reactivity was observed with uninfected blood extract as illustrated in track d. A co-migrating polypeptide is also recognized in *T. gondii* tachyzoite extract, a parasite stage that can be considered as homologous to *P. falciparum* merozoites (track e). In *P. falciparum*, this protein is found in the cytosolic fraction (tracks b), but can also be detected in membrane fractions (data not shown). Importantly, it is present in the Triton X-100 insoluble fraction (track c), a fraction that also contains actin filaments as seen when after probing the blot with anti-Dd coronin, we probe the same blot with anti-parasite actin. The additional signal obtained when probed with anti-parasite actin is marked by a star on track c.

To check whether this cross-reactive polypeptide behaves as an actin-associated protein as has been shown for the amoeba coronin, we performed actin co-sedimentation assay. When exogenous actin prepared from muscle rabbit acetone powder is incubated under polymerization conditions (Fig. 2, tracks c–h), the *P. falciparum* cross-reactive polypeptide is found

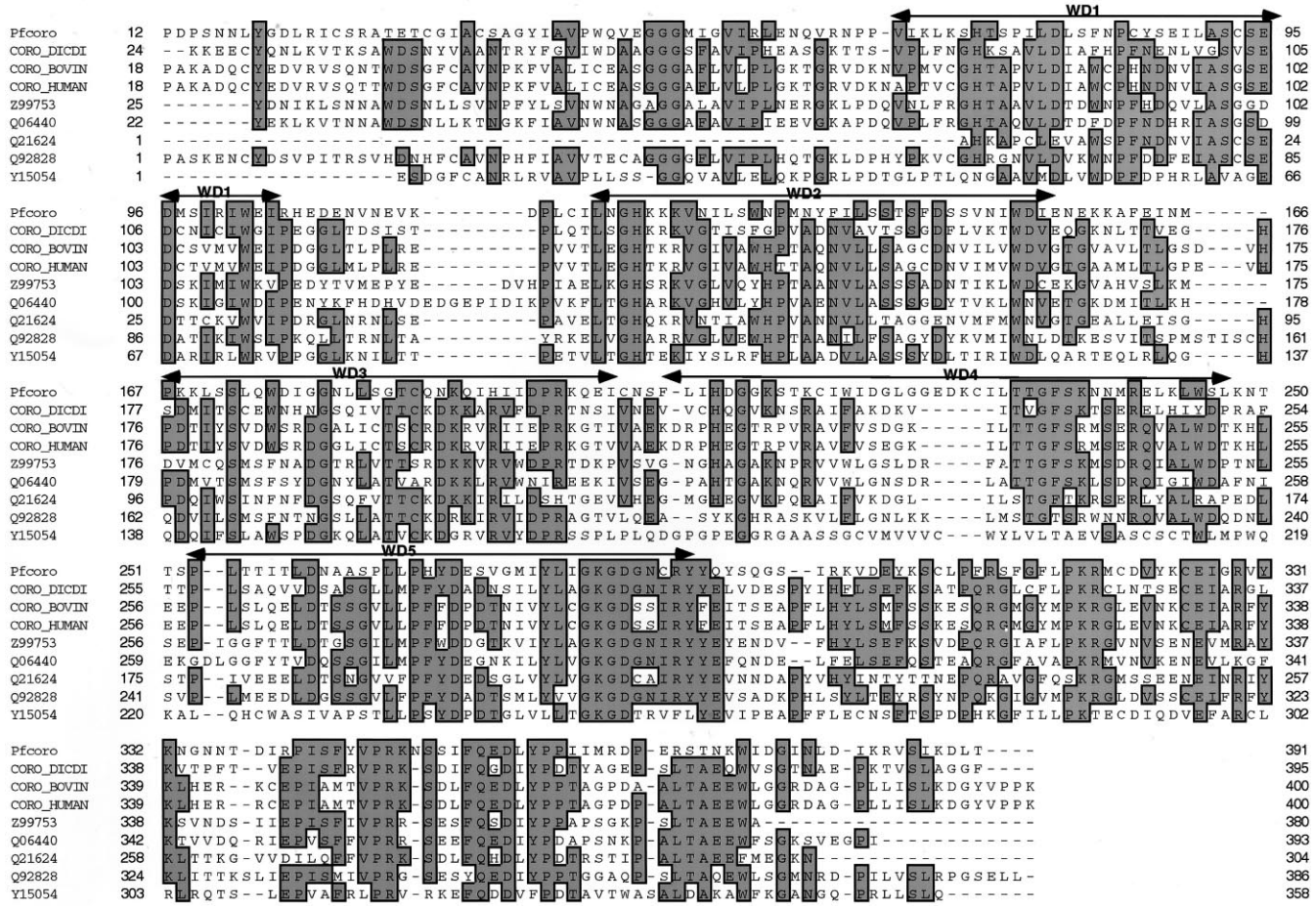

Fig. 3. Multiple alignments of Pf coronin and 8 distinct coronin related sequences. The Swissprot identification and GenBank accession numbers are given. The numbers correspond to the amino acid positions within the complete coronin sequence and the divergent N-termini are not shown. Note that three putative coronins found in the data base (accession nos. Q21624, Q92828, Y15054) correspond to partial sequences and are numbered from 1. Alignments were performed with the ClustalW program, where identical amino acids conserved in at least 50% of the sequences are shaded. The WD domains are shown arrowed and are numbered 1 to 5. Note that the identity between the different coronins extends throughout the C-termini.

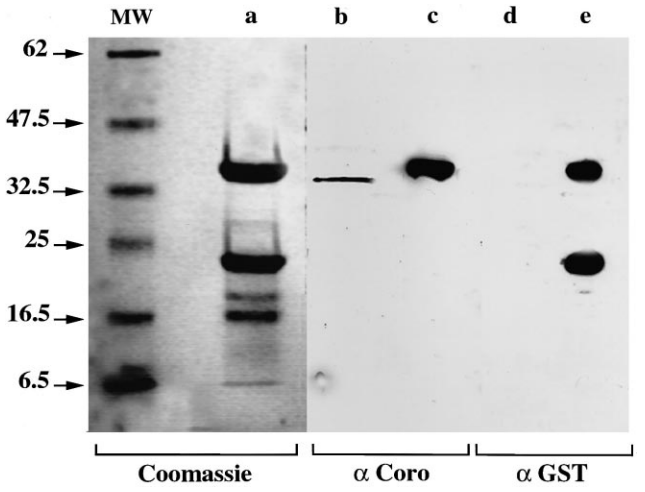

Fig. 4. The GST-Pf coronin fusion protein is recognized by the anti-Dd coronin antibody. Track a: Coomassie staining of the purified GST fusion protein eluate; tracks b and d: *P. falciparum* cytosolic fraction; tracks c and e: GST fusion protein eluate. Tracks b and c are probed with anti-Dd coronin antibodies while tracks d and e are probed with the anti-GST antibody.

associated with the actin filaments (track h) as well as with the supernatant (track g) after ultracentrifugation; a property consistent with its role in actin dynamics. Using the anti-Dd coronin antibody to detect it, we have named the 42-kDa protein Pf coronin. As controls, BSA was not co-pelleting with F-actin (data not shown) and rabbit muscle actin incubated in low salt buffer to prevent polymerization is not pelleting (track d) and remains totally in the supernatant (track c) where rabbit actin is detected with the cross-reactive anti-chicken gizzard actin antibody (clone N350). The presence of a small amount of parasite coronin associated with exogenous F-actin is due to the sedimentation of exogenous actin filaments since the high speed pellet of the parasite extract, run without adding actin, while containing proteins as seen by Ponceau staining (not shown), does not contain any detectable Pf coronin (lane b). Thus, in our conditions, following extraction in low salt buffer which promotes F-actin depolymerization, parasite coronin is not detectable as an aggregate or as a cellular complex.

Having suggested that *P. falciparum* has a protein homologous to Dd coronin, we sought the corresponding parasite gene. Five independent cDNA clones were isolated and Southern blot analysis indicated that they were derived from a single gene (data not shown). The nucleotide sequence of all five clones was determined and the predicted amino acid sequence encoded by the single open reading frame shows a remarkable degree of similarity to the three previously known coronin proteins, establishing the *P. falciparum* sequence as parasite coronin (Fig. 3). This homology extends outside the five WD repeats (shown arrowed) known to characterize these proteins [29]. Pf coronin displays 35.2% identity with Dd coronin throughout a 372 amino acid overlap while there is 27–32% identity between Pf coronin and the bovine and human coronin homologues throughout more than 80% of the protein.

To demonstrate that this *P. falciparum* sequence was indeed encoding the polypeptide recognized by the anti-Dd coronin, we made a GST-Pf coronin fusion protein using the pGEX expression vector. We used successfully the part of the nucleotidic sequence devoid of the 5 WD repeats at the N-terminal end of the protein, the length of which is 499 bp. Fig. 4 shows (track a) a Coomassie stained protein profile of the eluate after purification of GST fusion proteins. It displays two bands: one corresponding to GST and migrating around 26 kDa and an additional one migrating around 44 kDa (26+18) corresponding to the GST-Pf coronin fusion protein. Western blotting with anti-GST antibody confirms that, while there is no cross-reactivity with polypeptides from *P. falciparum* lysate (track b), the antibody recognizes GST and the GST-Pf coronin fusion protein (track e). When the same blot is probed with anti-Dd coronin, the GST-Pf fusion protein is recognized by the antibody (track c) as well as *P. falciparum* extracts (track b).

In addition, we identified in the data bases four additional WD-containing sequences (*Saccharomyces* spp., *C. elegans*) not previously recognized as coronin domains or motifs. Our classification of these sequences as coronins, rather than just members of the WD family of proteins, is based on the shared homology of their C-termini (Fig. 2). Bovine coronin, which has 31.4% identity in a 388 amino acid overlap with Pf coronin, also contains a C-terminal leucine zipper type motif [30]. We found a similar motif in both the human coronin and *S. cerevisiae* C-termini (data not shown). This motif is absent from the *P. falciparum* and *S. pombe* coronins and is poorly conserved in Dd coronin. However, in Dd coronin a DEAD box motif can be detected (data not shown), implying that in addition to the WD domains, the C-termini of coronins may also be involved in protein-protein interactions.

4. Discussion

Understanding actin dynamics in Apicomplexa parasites is an emerging area which first depends on the identification and the functional characterization of parasite actin associated proteins. Interestingly, in Apicomplexa parasites, one can assume that actin dynamics is tightly regulated, since filament assembly and disassembly have to occur very rapidly at least during locomotion and host cell entry. Host cell invasion occurs within 15–40 s for *T. gondii* tachyzoites [31] and *Plasmodium knowlesi* merozoites [2]. We have recently purified from *P. knowlesi* invasive merozoites a complex of three proteins capable of regulating actin filament elongation which could act as barbed end capping proteins [22]. In *Toxoplasma*, Allen et al. [21] have cloned and characterized a member of the actin depolymerizing factor family, a family of proteins which promotes rapid actin filament turnover [32]. As mentioned, a rapid turnover of actin filaments is likely to occur in vivo during parasite invasion of its host cell.

Although coronin homologues have already been reported in human immunocompetent cells and bovine brain [30], to our knowledge, Pf coronin is the first parasite homologue identified at the genomic level. Our data indicate that the predicted amino acid sequence of Pf coronin has a mass (52 kDa) close to Dd coronin (49 kDa). However, each protein recognized by the anti-Dd coronin antibodies migrates abnormally, since Dd coronin is detected at 55 kDa and Pf coronin at 42 kDa. Since coronin is not known to be posttranslationally modified and there is little or no *N*-glycosylation in *P. falciparum*, one reasonable explanation could be the quite different charge of each protein, since Dd coronin has a predicted p*I* of 7.6, whereas the p*I* predicted for Pf coronin is 8.8.

The further characterization of Pf coronin, especially the use of recombinant protein, should clarify the molecular basis of the interaction between the parasite and its host cell. Indeed, using null mutants for Dd coronin, several studies have pointed to its key role in the reorganization of the actin network that is involved in several fundamental functions of the amoeba. According to Gerish et al. [33], Dd coronin could speed up the disassembly of the actin network and balance the actin nucleating activity which occurs at the time of cell locomotion and cytokinesis [34]. In addition, phagocytic uptake levels are decreased by 60% [35]. Fluid phase uptake that has been studied in cells capable of axenic growth is also markedly affected when lacking coronin [34]. Finally, Hacker et al. [36] have brought evidence for the participation of coronin during macropinocytosis by showing GFP-tagged coronin at the cytoplasmic side of a nascent fluid filled vesicle.

Coronin has not been shown to directly control the competence of actin to assemble and disassemble in amoebae or in vertebrates. However, it does associate with F-actin, copurifies with the actin-myosin complex and localizes to actin dynamic regions of the amoeba cell cortex. In addition, it belongs to the WD-repeat family of proteins [29] and contains in its amino-terminal region five WD repeats [25]. More than 20 proteins are known to contain WD repeats and they participate in large protein complexes such as G-protein. These WD repeating units have a region of variable length followed by a core of more or less constant length bracketed by two characteristic pattern elements, GH (Gly-His) and WD (Trp-Asp) [29]. This conserved core structure is thought to promote pairwise or multimeric interactions as well as to couple regulatory proteins to the actin cytoskeleton, in particular to the actin-myosin system. These WD repeats are present in Pf coronin. It is of interest to note that isoforms from a novel class of unconventional myosins have been recently identified in *T. gondii* [37] and there are data that the actin-myosin system is expressed in the blood stage of *P. falciparum* [24,38]. Furthermore, inhibitors of myosin block both *T. gondii* motility and host cell invasion, both processes being controlled by a common molecular mechanism in *T. gondii* [23] and in rodent *Plasmodium*, where it has been demonstrated using a gene targeting approach [39]. Recently, an actin-myosin has been identified in *P. falciparum* and an actin-myosin ATPase inhibitor has been shown to affect host cell invasion [24]. Taking these data altogether, it would be relevant to check whether coronin homologues could couple regulatory proteins to the actin myosin system in Apicomplexa, as suggested for *D. discoideum*, and thus play a role in controlling host cell invasion by parasites. In this respect, the thrombospondin-related anonymous protein (TRAP) of *Plasmodium* has recently been shown to be a transmembrane protein that is involved in motility and host cell invasion [39]. A similar function has been proposed for its homologue in *Toxoplasma* (MIC2) [23]. Thus, these proteins could connect to the actin-myosin system through coronin, or coronin binding protein(s). In another amoeba, *Entamoeba histolytica*, Ebert et al. [40] have reported a gene encoding an unusual polypeptide the amino-terminal half of which revealed homology to coronin, whereas the C-terminus revealed homology to other actin binding proteins namely gelsolin, villin, as well as severin and fragmin.

Apart from host cell invasion, one can also reasonably assume that actin dynamics within the parasite is involved in other fundamental processes such as secretion, division, and differentiation, as has been widely demonstrated for non-parasitic organisms ranging from yeast to mammals [41–43]. As an example, endogeny characterizes the asexual multiplication of Apicomplexa and is likely to require both intact microtubule and intact actin networks [44]. In particular, it has been suggested that actin dynamics might be involved during the 'budding' process which gives rise to individualized progeny within a host cell [45]. In this context, Dd coronin null mutants are largely impaired for cell cytokinesis [34]. It is clear that identifying the molecules interacting with coronin, as well as searching for other actin associated proteins, will contribute to the understanding of actin dynamics in medically relevant parasites and consequently may identify molecules critically involved in parasite survival.

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