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Identification of the *Plasmodium falciparum* rhoptry neck protein 5 (PfRON5)

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ABSTRACT

Gathering knowledge about the proteins involved in erythrocyte invasion by *Plasmodium* merozoites is the starting point for developing new strategies to control malarial disease. Many of these proteins have been studied in *Toxoplasma gondii*, where some belonging to the Moving Junction complex have been identified. This complex allows a strong interaction between host cell and parasite membranes, required for parasite invasion. In this genus, four rhoptry proteins (RON2, RON4, RON5 and RON8) and one micronemal protein (TgAMA-1) have been found as part of the complex. In *Plasmodium falciparum*, RON2 and RON4 have been characterized. In the present study, we identify PfRON5, a ~110 kDa protein which is expressed in merozoite and schizont stages of the FCB-2 strain.

1. Introduction

Among the five parasites causing malaria in humans, *Plasmodium falciparum* is the species responsible for the highest morbidity; ~250 million cases of malaria are reported per year and a 93% of them are attributed to this parasitic species (WHO, 2009). Different strategies to eradicate this disease have been designed, such as pesticides, mosquito nets, antimalarial drugs, and different types of vaccines. However, several factors such as the global expansion of the disease (due in part to the increase of strains resistant to antimalarial drugs), the parasite's high genetic variability, the vector resistance to insecticides and the poor socioeconomic conditions of affected populations justify the search and adoption of new measures, such as the development of a fully effective vaccine (Good, 2001).

Merozoite surface proteins and some rhoptry and micronemal proteins have been considered as vaccine targets, since they are exposed to the immune system during the invasion of red blood cells (Cowman et al., 2002). This invasion begins with the merozoite reversible binding to the erythrocyte surface, mainly mediated by the *M*erozoite *S*urface *P*roteins (MSPs) (Chitnis and Blackman, 2000). Subsequently, a high affinity binding known as Tight Junction (TJ) occurs between the merozoite apical end and the erythrocyte membrane. The TJ migrates from the anterior to the posterior end of the merozoite during invasion (Moving Junction) activating the actin-myosin machinery (Alexander et al., 2005; Baum et al., 2008; Straub et al., 2009). As the parasite is moving in, the parasitophorous vacuole is formed, in which the parasite will develop and replicate for the next cell generation (Kaneko, 2007).

Recently, some authors have identified the proteins present in *Toxoplasma gondii* TJ complex (another member of the *Apicomplexa* phylum), using immunoprecipitation techniques. This TJ complex is formed by a micronemal protein, known as the apical membrane antigen 1 (AMA-1), and four rhoptry neck proteins (TgRON2, 4, 5 and 8) (Alexander et al., 2005; Baum et al., 2008; Straub et al., 2009). Besteiro and coworkers have recently proposed a model for TJ proteins organization, where TgRON2 and TgRON5 are exported to the host cell membrane, while TgRON4 and 8 are translocated to the host cell cytoplasm. In this model, TgRON2 and TgRON5 are exposed to the host cell surface and TgRON2 acts as a specific receptor for TgAMA-1, which is located on the parasite membrane (Besteiro et al., 2009).

The presence of orthologous genes which encode for RON proteins in different *Apicomplexa* members suggests that the TJ complex formation is a conserved mechanism in this phylum (Proellocks et al., 2010). TgRON2 and TgRON4 homologous proteins have been

Abbreviations: AMA-1, Apical membrane antigen-1; DAPI, 4',6-Diamidino-2-phenylindole; EGTA, Ethylene glycol tetraacetic acid; ELISA, Enzyme-linked immunosorbent assay; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; FITC, Fluorescein isothiocyanate; IFA, Indirect immunofluorescence assay; MSPs, Merozoite surface proteins; PBS, Phosphate buffered saline; RBC, Red blood cell; RON, Rhoptry neck protein; RP-HPLC, Reverse phase-high performance liquid chromatography; RT-PCR, Reverse transcription polymerase chain reaction; TMs, Transmembrane domains.

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characterized in *Plasmodium falciparum* (Alexander et al., 2006; Cao et al., 2009; Morahan et al., 2009), but TgRON8 does not share similarity to *Plasmodium* proteins (Straub et al., 2009). Additionally, PfRON6 has been characterized in *P. falciparum*, but it is not present in the TJ complex (Proellocks et al., 2009).

Although PfRON5 has been considered as a member of the RON complex (Collins et al., 2009; Richard et al., 2010) it has not been identified yet. Here we report PfRON5 sequence, localization and expression in *P. falciparum* FCB-2 strain.

2. Materials and methods

2.1. Primary structure analysis and identification of orthologues

The MAL8P1.73 gene sequence coding for PfRON5 (Bahl et al., 2003; Collins et al., 2009) was used as a starting point for bioinformatics analyses and primer design. PfRON5 homologous sequences in *Apicomplexa* were obtained using BLASTP searches in NCBI and EuPath databases (Altschul et al., 1990; Aurrecoechea et al., 2007). The Polyphobius and InterProScan predictors (Kall et al., 2005; Quevillon et al., 2005) were used to search for a signal peptide. Phobius, TMpred, TMHMM, ConpredII and Polyphobius programs were used to predict the transmembrane regions (TMs) (Hofmann and Stoffel, 1993; Krogh et al., 2001; Arai et al., 2004; Kall et al., 2004, 2005). The phylogenetic relationship between the homologous sequences were assessed with Mega 4.0 software (Tamura et al., 2007) to construct trees by Neighbor-Joining, Maximum Parsimony and Minimum-Evolution methods (Cavalli-Sforza, 1967; Saitou and Nei, 1987; Nakhleh et al., 2005). Protein alignment was carried out in Clustal W software (Thompson et al., 1994).

2.2. RNA isolation and cDNA synthesis

Synchronous *in vitro* cultures of the *P. falciparum* FCB-2 strain maintained as previously described (Trager and Jenson, 1978) were used as source of RNA and proteins. RNA (5 μL) isolated by the Trizol method (Chomczynski, 1993) was reverse-transcribed using Super-Script III (Invitrogen) and random primers in a 60 min cycle at 50 °C. The cDNA was amplified with pEXP5-F (5′-ATGTTGAAATA-CACTTTGCTCAT-3′) and pEXP5-R (5′-AGGTATTCTAGTGTGTACAA-TAA-3′) primers and the TAQXpedite™ enzyme (EPICENTRE Biotechnologies).

2.3. Cloning and sequencing

PCR products obtained from the cDNA amplification were purified and cloned into the pEXP5-CT/TOPO vector (Invitrogen), an additional reaction was necessary for adding adenines in the 3' region with Biolase enzyme (Bioline). The recombinant clones, confirmed by PCR and purified using Ultra Clean 6 Minute Plasmid prep (MOBIO Laboratories), were sequenced in an automatic sequencer (ABI PRISM 310 Genetic Analyzer, PE Applied Biosystems) using pEXP5-CT/TOPO vector primers: T7 Forward 5′-TAATACGACTCACTATAGGG-3′ and pEXP5-CT-Sec-rev 5′- CAAGGGGTTATGCTAGTTAT-3′, and the following internal primers: 1R 5′- TAAAACGACATCAGCACTAG-3′, 2F 5′-TCAAAAACGGCACAAAGAAG-3′, 2R 5′-TATCAACGGCAATTCT-TAAAAT-3′ and 3F 5′-ACAAAGATAAGTATAAGAAAAC-3′ (Fig. 1).

2.4. Antibodies

Polymeric peptides were used for rabbit immunization to obtain anti-PfRON5 polyclonal antibodies. Two New Zealand rabbits (69 and 73, previously determined to be nonreactive to *P. falciparum* lysate by Western blotting) were injected with a mixture made up of three peptides: CG-33-FKKPKPFKKNEIKKGIDKDE-52-GC (peptide 36924), CG-348-ADYDLSEYKNEFSPSKTAQR-367-GC (peptide 36923) and CG-776- RCDHFNRSKNIDNVKTKKNK-795-GC (peptide 36926), which were predicted to be B epitopes using ANTHEPROT and BepiPred tools (Deleage et al., 2001; Larsen et al., 2006). Peptides were synthesized using the t-Boc/Bzl solid-phase synthesis (Merrifield, 1963; Houghten, 1985), lyophilized and characterized by RP-HPLC and MALDI-TOF MS. Glycine and cysteine residues were added to the N and C termini to allow polymerization. The peptide mixture was inoculated (250 μg per peptide) intramuscularly emulsified in Freund's complete adjuvant (FCA) (Sigma) for the first dose. The rabbits received two additional doses of this mixture, emulsified in Freund's incomplete adjuvant (FIA) (Sigma) on days 20 and 40. Sera were collected before the first immunization (pre-immune), and 20 days after each immunization (post-first, post-second and hyper-immune, respectively).

2.5. Rabbit sera adsorption to sepharose coupled to either Escherichia coli sonicate, RBC lysate or SPf66

Escherichia coli (DH5a strain) protein sonicate was obtained from an overnight culture in Luria-Bertani medium, then washed, resuspended, sonicated in an Ultrasonic Homogenizer (Cole Parmer) for 2 min at 4 °C, and spun for 10 min at 4,500 g and the supernatant was resuspended in coupling buffer (1.0 M NaHCO3, 0.5 M NaCl, pH 8.3). Rabbit RBCs (60%) were lysed with saponin (Sigma) (0.2%) and resuspended in coupling buffer. The suspended lysate, sonicate and synthetic SPf66 peptide vaccine (Patarroyo et al., 1987, 1988) were collected and used individually for coupling to CNBr-activated Sepharose 4B (Amersham Biosciences) according to the manufacturer's recommendations. Rabbit sera (pre-immune and immune) were preadsorbed to *Escherichia coli,* rabbit RBC and SPf66 Sepharose affinity columns to eliminate nonspecific cross-reactivities, as previously described (Curtidor et al., 2008).

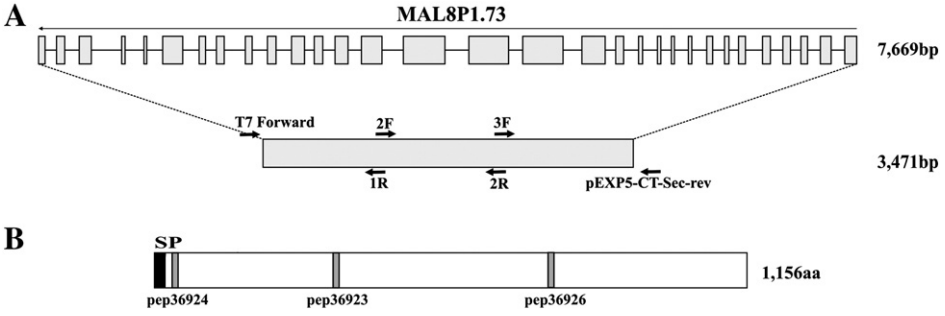

Fig. 1. Schematic representation of PfRON5. A. Genomic DNA, which consists of 31 exons, followed by a diagram of the transcript where primers used for sequencing are shown with arrows (details in the text). B. Schematic representation of the PfRON5 protein. Grey bars indicate the localization of the peptides synthesized for rabbit immunization. SP indicates the signal peptide.

2.6. Recognition of inoculated peptides

For the ELISA test, 96-well plates (Nunc) coated with 10 μg/mL of each peptide (36924, 36923 and 36926) were incubated at 37 °C for 1 h, left overnight at 4 °C, and incubated at 37 °C for an additional hour. Plates were washed five times with PBS–Tween 0.05% and incubated with 0.5% skimmed milk diluted in PBS–Tween 0.05% for 1 h at room temperature. A 1:100 sera dilution collected from immunized rabbits (69 and 73) was added by duplicate. Plates were incubated for 1 h at 37 °C and washed five times with PBS–Tween 0.05% to remove excess of unbound antibody. Wells were then loaded with 100 μL of peroxidase-coupled goat anti-rabbit IgG antibody (Vector Laboratories) diluted 1:5000 and incubated for 1 h at 37 °C. Excess of unbound antibody was removed by washing wells thrice with PBS–Tween 0.05% and immunoreactivity was revealed by using the TMB Micro-well Peroxidase Substrate System kit (KPL Laboratories), according to the manufacturer's instructions. Absorbances were read at 620 nm in an ELISA reader (Lab Systems Multiskan MS).

2.7. SDS-PAGE and Western blot analysis

Protein lysates from *P. falciparum* (FCB-2) infected RBC rings (4–8 h), early trophozoites (19–24 h), late trophozoites (26–31 h), schizonts (42–47) and free merozoites (Mz) were obtained using saponin (Sigma) (0.2%) and lysis buffer (100 mM PMSF, 20% SDS, 0.5 M EDTA pH 8.3, 100 mM iodoacetamide) and then separated by SDS-PAGE using a 5% (w/v) polyacrylamide gel and transferred to a nitrocellulose membrane (Trans-Blot, Bio-Rad) using the semidry blotting technique. All samples were separated under denaturing conditions. For Western blot analysis, the nitrocellulose membrane was blocked using 5% skimmed milk diluted in Tris-buffered saline with 0.05% Tween (TBS-T) and washed thrice with TBS-T.

Pre-immune and immune preadsorbed serum samples were diluted 1:40 in blocking solution and incubated individually with membrane strips. After five washes with TBS-T, strips were incubated for 1 h with a 1:5000 alkaline phosphatase-conjugated anti-rabbit IgG antibody (ICN Biomedicals). The reaction was detected using NBT/BCIP (Promega).

2.8. Indirect immunofluorescence assay (IFA)

Blood smears from parasite culture with 5% parasitemia (mature schizonts) were used for IFA, fixed with 4% formaldehyde, permeated with 0.1% Triton X-100 (Sigma) in PBS and blocked with PBS–BSA 1% for 30 min at 37 °C. Preadsorbed rabbit polyclonal sera directed against the mixture of the three synthetic peptides were used as primary antibody at a 1:40 dilution in PBS–BSA 1%. Fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG (Vector Laboratories) was used as secondary antibody at a 1:30 dilution in PBS–BSA 1%, followed by the addition of DAPI (Sigma) 2 μg/mL. The smears were analyzed with an Olympus BX51 fluorescence microscope using an Olympus DP2 camera and Volocity software (Perkin Elmer).

3. Results

3.1. PfRON5 sequence analysis

The MAL8P1.73 gene coding for *Pf*RON5 is located in chromosome 8 and has a length of ~7669 bp with 31 predicted exons (Fig. 1) (Wheelan et al., 2001; Gardner et al., 2002; Bahl et al., 2003). The signal peptide was here predicted using Polyphobius (aa 1–19) (Kall et al., 2005) and InterProScan (aa 1–21) (Quevillon et al., 2005) web servers. Richard and coworkers had predicted six transmembrane regions (TMs) for the hypothetical protein based on a hydrophobicity profile analysis (Richard et al., 2010). Several bioinformatics tools were here used aiming at building a consensus on the number of these regions. However, results obtained from the predictors used are quite different, ranging from 0 to 5 TMs (Table 1).

The sequence of MAL8P1.73 was used as a BLASTP query in NCBI and EuPath databases (Altschul et al., 1990; Aurrecoechea et al., 2007). We found RON5 orthologues in *P. vivax* Sal-1 strain (PVX_089530, PlasmoDB), *P. knowlesi* H strain (PKH_051420, PlasmoDB), *P. berghei* ANKA strain (PBANKA_071310, PlasmoDB), *P. yoelii* 17XNL strain (PY02282, PlasmoDB), *P. chabaudii* (PCHAS_072220, PlasmoDB), *Babesia bovis* (XP_001611063.1, NCBI), *Theileria annulata* (XP_953613.1, NCBI), *Theileria parva* Muguga strain (XP_766682.1, NCBI), *Toxoplasma gondii* (ACY08774.1, NCBI), and *Neospora caninum* (NCLIV_055360, ToxoDB). A phylogenetic tree was constructed with the orthologue sequences found using the Neighbor-Joining method (Saitou and Nei, 1987) in the Mega 4.0 software (Tamura et al., 2007) (Fig. 2A). Maximum Parsimony and Minimum-Evolution methods also displayed a similar tree topology (not shown) (Cavalli-Sforza, 1967; Nakhleh et al., 2005). The phylogenetic tree shows three clades and within one of them, *Plasmodium* species infecting primates (*P. vivax*, *P. knowlesi* and *P. falciparum*) cluster together while species infecting rodents (*P. yoelii*, *P. chabaudi* and *P. berghei*) also cluster in a separate branch. As can be observed, RON5 has been maintained across the *Apicomplexa* phylum (Fig. 2A).

The alignment analysis of RON5 orthologues in *Plasmodium*, carried out with Clustal W (Thompson et al., 1994), shows that PfRON5 displays a greater similarity to its homologue in *P. vivax* (identity 68.7% and similarity 84.8%). The alignment with other members of the phylum *Apicomplexa* shows that this rhoptry protein is more similar to its homologue in *Babesia bovis* (identity 18.6% and similarity 26.5%).

3.2. PfRON5 transcription and sequence in the FCB-2 strain

RNA was extracted from *P. falciparum* FCB-2 strain synchronized schizonts and amplified by one-step RT-PCR to assess PfRON5 transcription. In the cDNA amplification, a ~3470 bp PCR product was obtained, coding for a 1,156 amino acid long protein (Fig. 2B). Recombinant clone sequences were analyzed using CLC DNA Workbench (CLC bio) and the consensus sequence was deposited in the GenBank database (accession number HQ424431). PfRON5 sequence alignment from FCB-2 and 3D7 strains shows three nonsynonymous nucleotide substitutions producing the following amino acid replacements: Pro92Ser, Phe266Leu and Glu1015Asp, and one synonymous substitution in nucleotide 2058 (A2058C), indicating that this protein is highly conserved among *P. falciparum* strains (data not shown). Interestingly, similar results were reported for TgRON5 (Straub et al., 2009). The alignment analysis carried out with Clustal W (Thompson et al., 1994) also shows that this protein is conserved among the different *Plasmodium* species (Fig. 3).

Table 1
Comparison between transmembranal region predictors.

Predictor	Number of TM regions	Position	Reference
TMHMM2.0	None		Krogh et al. (2001)
Phobius	2	6–24 640–663	Kall et al. (2004)
TMpred	5	1–19 250–270 383–403 555–573 645–663	Hofmann and Stoffel (1993)
ConpredII	1	554–574	Arai et al. (2004)
Polyphobius	2	554–573 642–663	Kall et al. (2005)

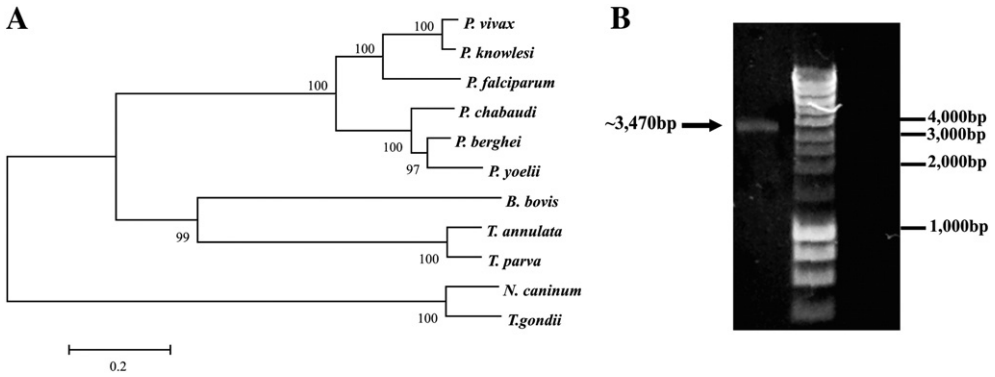

Fig. 2. A. PfRON5 orthologues. Neighbor-Joining tree of RON5 amino acid sequences from different genera and species. Numbers represent bootstrap values with 1000 replicates. The scale indicates 0.2 substitutions per site. B. *pfron5* gene is transcribed. Line 1 shows a ~3470 bp amplification product of *pfron5* from a cDNA sample; line 2 shows the molecular weight marker and line 3 corresponds to the negative control.

3.3. PfRON5 expression peaks in schizont and merozoite stages

Anti-PfRON5 sera were generated by immunizing rabbits with three synthetic peptides based on the primary PfRON5 sequence. Adsorbed rabbit sera were then tested by Western blot against *P. falciparum* lysates from different intra-erythrocytic parasite stages. No parasite proteins were recognized when pre-immune rabbit sera were tested, while hyper-immune sera elicited antibody responses specifically recognizing a ~110 kDa protein, being this pattern only observed in schizonts (42–47 h) and free merozoite (Mz) stages (Fig. 4A). No bands were observed in rings (4–8 h), early trophozoites (19–24 h) or late trophozoites (26–31 h), which suggests that PfRON5 is not expressed in these stages.

3.4. IFA revealed PfRON5 localization within the parasite

An indirect immunofluorescent assay was performed using preadsorbed anti-PfRON5 sera to determine the subcellular localization of this protein. A punctate fluorescence pattern was observed in mature schizonts when a hyper-immune serum sample was assessed; no fluorescence was observed when a pre-immune sample of serum from the same rabbit (negative control) was tested. This punctate staining pattern is typical of rhoptry proteins (Fig. 4B) (Topolska et al., 2004).

4. Discussion

In the 1970s, Aikawa described the formation of a junction between RBC and merozoite membranes as a crucial step in the invasion process. Since the junction moves over the merozoite to allow its invagination, it was then called Moving Junction (Aikawa et al., 1978). Today, 40 years later and with *T. gondii* as a model, some rhoptry and microneme-derived proteins have been identified and associated with the moving junction formation (Alexander et al., 2005; Besteiro et al., 2009; Straub et al., 2009). Additionally, it has been described that the specific interaction between plasmodial AMA-1 (from micronemes) and rhoptry neck proteins is necessary to allow the invasion (Richard et al., 2010).

To date, RON2 and RON4 have been characterized in both *T. gondii* and *P. falciparum*. The identification of RON proteins started with the *Toxoplasma* rhoptry proteome work carried out by Bradley and coworkers, in which four rhoptry neck proteins (RON1, RON2, RON3 and RON4), as well as a big number of other rhoptry proteins were identified (Bradley et al., 2005). Most RON protein studies have been focused on TgRON4, since this protein has been shown to be localized in the moving junction (Leriche and Dubremetz, 1991; Lebrun et al., 2005). Two additional proteins are recovered when the monoclonal antibody directed towards TgRON4 is used to purify this protein by immunoaffinity chromatography: TgTwinScan_0698 and TgTwinScan_4705, the first protein corresponds to RON2 and second protein was going to be known years later as RON5 (Lebrun et al., 2005; Straub et al., 2009). Taking into account that proteins present in the moving junction are conserved in various members of the phylum Apicomplexa, a similar mechanism for host cell invasion has been proposed, and thus, *Toxoplasma* has been taken as a model for characterizing homologous proteins in other parasites such as plasmodia (Besteiro et al., 2009; Straub et al., 2009; Proellocks et al., 2010).

In this study, the gene codifying for RON5 was sequenced and the expression and subcellular localization of the encoded protein were assessed in the *P. falciparum* FCB-2 strain. The PfRON5 gene corresponds to that designated as MAL8P1.73 in the PlasmoDB database (Bahl et al., 2003). No functional domains previously described were detected when the sequence was submitted to some predictors from the Swiss Institute of Bioinformatics (http://www.expasy.org/tools/), such as Prosite and interPro Scan.

Based on the hypothetical PfRON5 sequence from the 3D7 strain, Richard and coworkers suggested that PfRON5 has six potential transmembrane regions according to its hydrophobicity profile (Richard et al., 2010). Since this method only identifies likely transmembrane segments, but it is unable to predict the inside–outside phasing of the segments relative to the cytoplasm (Jones, 2007), we decided to use several bioinformatics tools with the aim of building a consensus on the number of TMs. However, major discrepancies were found (Table 1) probably due to the different approaches used by the tools. 1) TMpred, is based on a statistical analysis of query results in the TMbase database, where the prediction is made using a combination of several weight-matrices for scoring (Hofmann and Stoffel, 1993); 2) TMHMM2.0, Phobius and Polyphobius use machine learning, mainly Hidden Markov Models (HMM), but Phobius also uses Artificial Neural Networks for predicting both TM topology and the presence of a signal peptide in the protein. In Polyphobius, a multiple-sequence alignment is used to calculate the best 'average' path through the states of the HMM (Krogh et al., 2001; Kall et al., 2004, 2005; Jones, 2007). 3) ConpredII is a consensus prediction server, which gives a result based on the average obtained from several predictors (KKD, TMpred, TopPred II, DAS, TMAP, MEMSAT 1.8, SOSUI, TMHMM 2.0 and HMMTOP 2.0) (Arai et al., 2004). In addition to the different approaches followed by the predictors, the dissimilar results could have also been because protozoan sequences are underrepresented in the training data sets of most of these tools in relation to other eukaryotic sequences, which difficults the prediction of TMs in these organisms.

BLAST and phylogenetic analyses show that RON5 is present in several *Plasmodium* species and other members of the phylum *Apicomplexa* (Fig. 2A), suggesting that this protein might have an

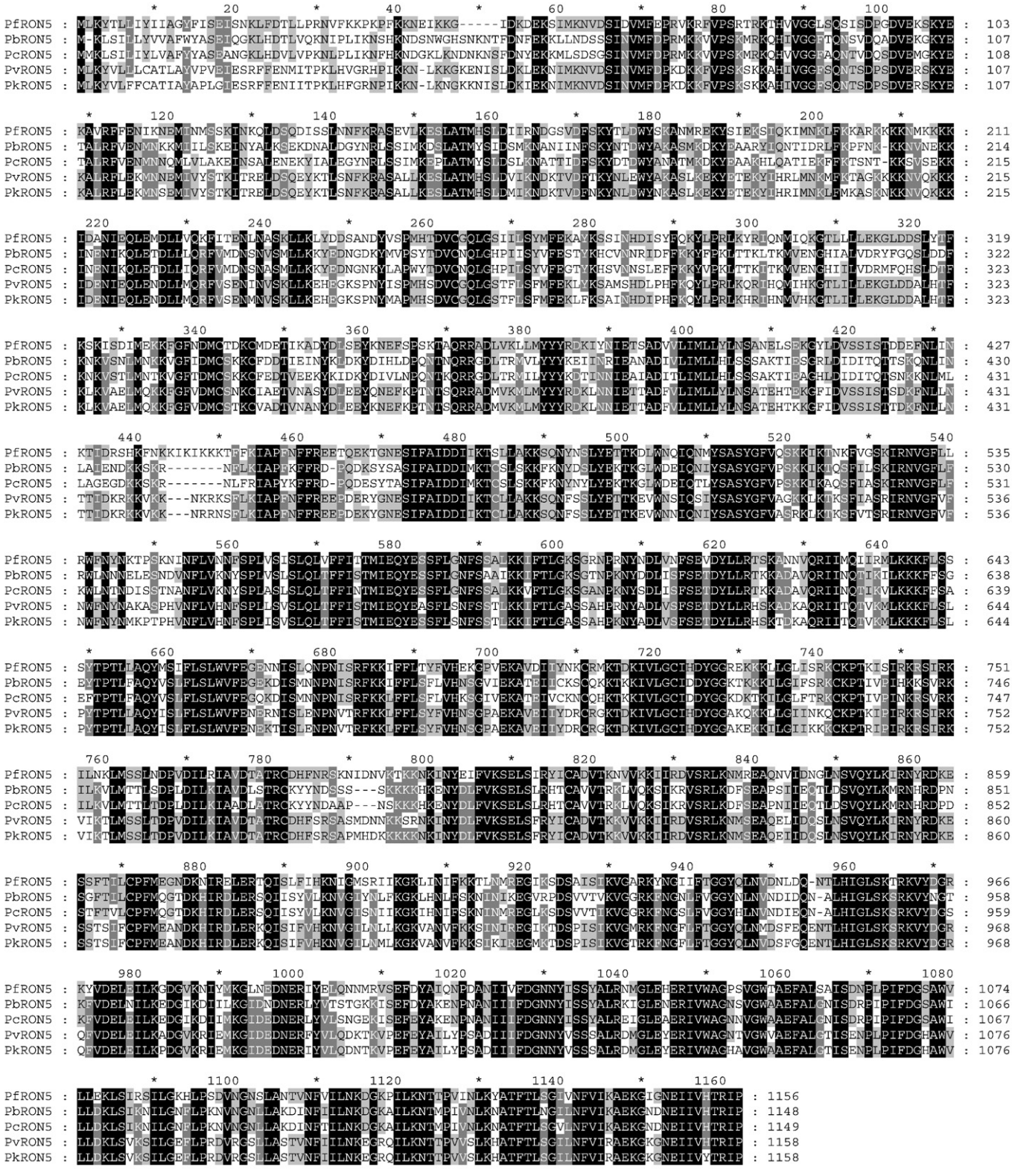

Fig. 3. Amino acid alignment of RON5 proteins from different *Plasmodium* species. The shading indicates levels of identity, among sequences. White letters on a black background represent amino acid residues that were 100% identical; white letters on a grey background represent amino acid residues that were 80% identical; and black letters on a grey background represent amino acid residues that were 60% identical. Pb, *Plasmodium berghei*; Pc, *Plasmodium chabaudi*; Pv, *Plasmodium vivax*; Pk, *Plasmodium knowlesi*; Pf, *Plasmodium falciparum*.

important biological role in this phylum, which has been maintained along parasite evolution.

Similar to what has been previously described for other *Plasmodium falciparum* rhoptry proteins such as PfRAP-1 (Howard and Peterson, 1996), PfRAP-2 (Saul et al., 1992) and PfRON2 (Cao et al., 2009), a very limited genetic polymorphism between the two sequenced strains (3D7 and FCB-2) was here found for PfRON5. This conservation gains further importance if the origin of the two strains

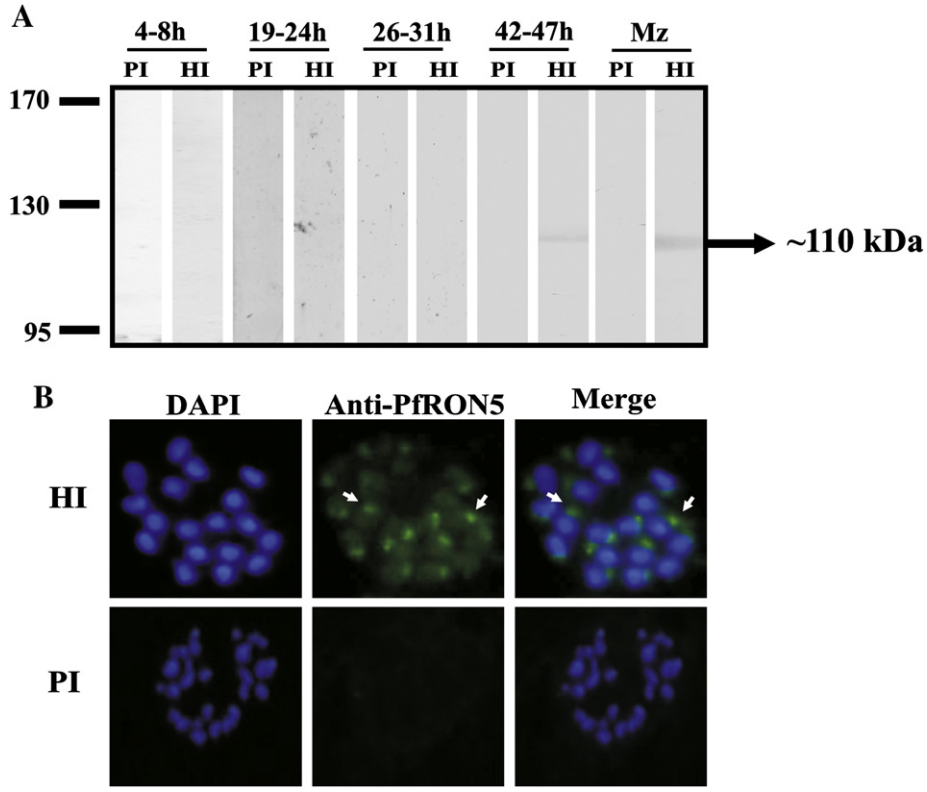

Fig. 4. PfRON5 protein expression time and localization. A. Western blot carried out with parasite extracts from synchronized parasite samples at different post-invasion times: rings (4–8 h), early trophozoites (19–24 h), late trophozoites (26–31 h), schizonts (42–47) and free merozoites (Mz). PfRON5 is expressed only in schizonts and free merozoites (Mz) (~110 kDa band). PI and HI indicate pre-immune and hyper-immune sera, respectively. B. PfRON5 displays a rhoptry localization pattern on the IFA (arrows show the punctate pattern in the hyper-immune serum sample). Hyper-immune serum (HI). Pre-immune serum (PI).

analyzed is taken into account, since 3D7 is an airport isolate found in the Netherlands, while FCB-2 is of Colombian origin. This polymorphism pattern sharply contrasts with that observed for some MSPs such as PfMSP-1 (Miller et al., 1993) or PfMSP-2 (Fenton et al., 1991), where a very high number of substitutions are present. This different behavior might, at least in part, be attributable to the degree of exposure to the immune system that each group of proteins is under, since surface proteins are more exposed and thus are subjected to a greater positive selective pressure. Taking into account that a major problem in developing an effective vaccine is the high degree of genetic diversity and antigenic variation found in target antigens, the use of conserved proteins as vaccine candidates is important for avoiding the polymorphism that the parasite displays (Hisaeda et al., 2005; Casares and Richie, 2009).

In addition to its highly conserved sequence, PfRON5 was here detected by Western blot both in schizonts and free merozoites (stages related to the RBC invasion process) (Fig. 4A), which is consistent with previous microarray data that show that PfRON5 transcription begins during the early schizont stage (Bozdech et al., 2003; Le Roch et al., 2003).

Taken together, PfRON5 expression during parasite stages related to invasion of RBCs, its highly conserved sequence and its previously reported involvement in forming protein complexes with PfAMA-1, turn this into an attractive molecule to be included in further studies aimed at assessing its potential as a vaccine candidate. The *Aotus* monkey model has been considered for many years as ideal for testing vaccine candidates against malaria, since these animals and those belonging to the *Saimiri* genus are among the few that can develop the disease when inoculated with human malaria parasites (Stewart, 2003; Collins et al., 2006). Our group has been working for more than three decades in figuring out a rational methodology for vaccine development which includes functional assays to determine those peptide regions involved in target cell binding and then performing specific modifications to them to allow a better fit into immune system molecules. This approach has allowed us to turn non-immunogenic conserved binding regions to target cells into immunogenic and protection-inducing ones, when they are tested in the *Aotus* monkey model (Patarroyo et al., 2004, 2008; Cifuentes et al., 2008; Patarroyo and Patarroyo, 2008).

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