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NOTE

Plasmodium falciparum AMA1 Binds a Rhoptry Neck Protein Homologous to TgRON4, a Component of the Moving Junction in *Toxoplasma gondii*

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***Plasmodium falciparum* apical membrane antigen 1 (PfAMA1) coimmunoprecipitates with the *Plasmodium* homologue of TgRON4, a secreted rhoptry neck protein of *Toxoplasma gondii* that migrates at the moving junction in association with TgAMA1 during invasion. PfRON4 also originates in the rhoptry necks, suggesting that this unusual collaboration of micronemes and rhoptries is a conserved feature of *Apicomplexa*.**

Apicomplexa is a protozoan class of obligate intracellular parasites that includes many important human and animal pathogens. Apical membrane antigen 1 (AMA1) was first identified in *Plasmodium knowlesi*, and since then homologues have been seen in all *Plasmodium* species so far examined as well as at least two other apicomplexan genera, *Toxoplasma* and *Babesia* (homologues have not been detected in organisms outside *Apicomplexa*) (9, 12, 14, 25). This essential membrane protein is stored in the micronemes of the asexual stages and transported to the parasite surface prior to and during host cell invasion (3). Antibodies to AMA1 directly interfere with invasion by *Toxoplasma* sp. tachyzoites (14) and *Plasmodium falciparum* merozoites (11, 20), suggesting a key role in the invasion process. A similar function for the *P. falciparum* AMA1 protein (PfAMA1) has been described during sporozoite invasion of hepatocytes (27), indicating PfAMA1 might be an effective vaccine target for both the preerythrocytic and the asexual blood stages (17, 28).

One of the most distinctive features of apicomplexan invasion is the moving junction (MJ) that occurs at the site where the parasite invades into the developing parasitophorous vacuole (PV) (1, 2, 21). The appearance of electron-dense structures at the MJ is consistent with the organization of a secreted parasite complex at the interface with the host membrane. In *Toxoplasma gondii*, a complex minimally composed of TgAMA1 and the rhoptry neck protein, TgRON4, specifically localizes to the ring-like MJ (2, 19). This ring marks the boundary where specific surface antigen complexes are removed from the parasite surface as it enters into the nascent PV (10). Host membrane proteins are also sorted at the MJ, and many that are found in complexes or associated with the extracellular matrix are excluded from the developing PV membrane (8). Thus, the MJ marks a site of intimate attachment by the parasite to the host and a sieve at which parasite and host surface proteins are selectively sorted, allowing some but not others to pass into the nascent vacuole.

AMA1 has been presumed to function similarly in all *Apicomplexa* organisms. Given our findings in *Toxoplasma*, we asked whether similar immunoprecipitation experiments might reveal previously undetected binding partners for PfAMA1. *P. falciparum* strains 3D7 and D10 were cultured in human erythrocytes according to standard protocols (5, 18). Synchronous cultures containing a majority of the parasites in the mature schizont stage were harvested ~40 h postinfection by lysis in 0.15% saponin (to disrupt the erythrocyte and PV membrane [4]) and stored at −80°C for further analysis. The rat monoclonal antibody (MAb) against PfAMA1, 28G2dc1 (4, 22), was coupled to protein G-Sepharose using dimethyl pimelimidate dihydrochloride (13). A total of 2.7×10^9 mature schizont-stage parasites were thawed directly into 5 ml of TEN lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA) with RIPA detergents (1% NP-40, 0.5% deoxycholate, 0.01% sodium dodecyl sulfate [SDS]) with Complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Parasites were extracted for 30 min on wet ice, and the extract was clarified by centrifugation at $10{,}000 \times g$ for 20 min at 4°C. The resulting supernatant was incubated for 6 h at 4°C with MAb-coupled protein G-Sepharose beads (~0.4 μg immunoglobulin G/μl bead) or beads alone and then washed three times (15 min each) in RIPA lysis buffer and three times (15 min each) in TEN buffer. Bound polypeptides were eluted in 0.1 M triethylamine (pH 11.5), lyophilized, and resuspended in TEN buffer.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the polypeptides eluted from the MAb 28G2-coupled beads identified three major bands on Coomassie-stained gels (Fig. 1A). Relative to the molecular mass standards, these

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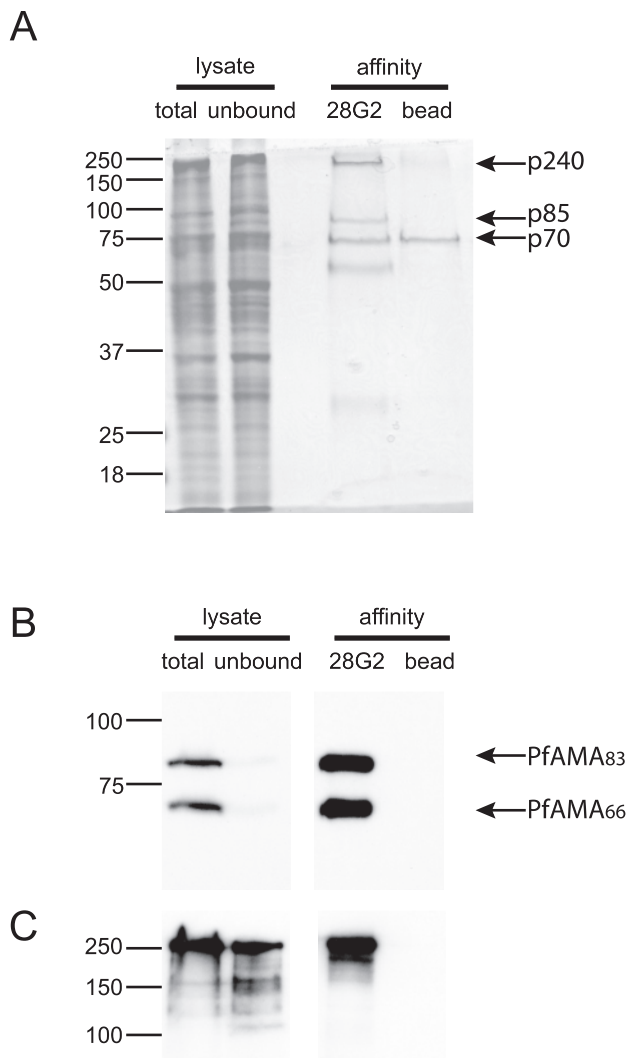

FIG. 1. Identification of PfAMA1-associated proteins. A. MAb 28G2-affinity-selected proteins from RIPA detergent extracts of segmented schizonts were separated on reducing SDS-PAGE gels and stained with Coomassie blue. Molecular mass markers (kDa) are denoted on the left, and the sizes of the major bands are indicated on the right (arrows) and are based on relative mobility. The profiles for the RIPA parasite extract (7×10^6 parasite equivalents) before (lane 1) and after (lane 2) immuno-selection are shown. MAb 28G2-immuno-selected proteins (7×10^8 parasite equivalents) are shown (lane 3) in comparison with those precipitated by protein G-Sepharose beads alone (lane 4). B. Immunoblot of parasite extract and affinity-selected proteins probed with anti-PfAMA1 MAb 28G2. Note that this is a different gel from that shown in panel A, in order to better resolve proteins in the 50- to 100-kDa range. C. Immunoblot of the lanes shown in panel B that were probed with anti-PfRON4 MAb 24C6.

migrated at ~240 kDa (p240), ~85 kDa (p85), and ~70 kDa (p70), the last being also found in control precipitations (i.e., without the coupled MAb). Two additional faint bands at ~50 kDa and ~25 kDa were determined to be the rat immunoglobulin G heavy and light chains and so were not further pursued. Immunoblotting of the affinity-selected proteins using MAb 28G2 showed binding to the p85 and p70 bands, which are consistent with the proform ($PfAMA1_{83}$) and processed form ($PfAMA1_{66}$) of PfAMA1, respectively (15) (Fig. 1B). Based on the band intensities, the majority of the PfAMA1 in the starting material was recovered from the parasite lysate.

To identify the immuno-selected polypeptides, individual bands from Coomassie-stained SDS-PAGE gels were excised, treated with trypsin, and extracted for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis at the Stanford University mass spectrometry facility (http://mass-spec.stanford.edu). Matching peptides were identified by a correlative search of the annotated and predicted amino acid databases from Plasmodb (http://v4-4.plasmodb.org) using the MS/MS ion search programs Sequest (http://thermo.com) and X!Tandem (www.proteomesoftware.com). For trypsin-constrained analysis (two missed cleavages), cross-correlation (X_{corr}) values of 1.5 for charge state 1, 2.5 for charge state 2, and 3 for charge state 3 were used to rank peptides identified by Sequest (16). A 95% expectation value was used as a benchmark in the X!Tandem analysis. Unassigned spectra were reanalyzed unconstrained (no enzyme) against a subset Plasmodb database comprised of the top 10 proteins identified in the first analysis. This second analysis allowed identification of peptides with a single tryptic cleavage as well as nontryptic peptides, which can be the result of poor fragmentation or other degradation of terminal amino acids (23).

As expected, LC-MS/MS analysis of p85 identified a single protein, PfAMA1, with 32% peptide coverage (Table 1). No peptides were identified for any other protein in this band. One nontryptic peptide matched the predicted N terminus of the proform $PfAMA1_{83}$.

Analysis of p70 from both the control and the MAb 28G2 precipitations yielded peptide profiles for the 70-kDa heat shock protein (HSP70) family (data not shown). Among the hits were some that unambiguously indicated that a *P. falciparum* HSP70 family member predicted by chr11.glimmerm_1089A was present. Because several stretches within the various members of this highly conserved protein family are identical, many of the peptides observed could have been derived from more than one HSP70 family member, and so more than just chr11.glimmerm_1089A may be present within the p70 band; no peptides unambiguously indicated this, however. Heat shock proteins are often found in association with secreted surface proteins (7), but the similar profile of HSP70-derived peptides in the p70 band from control precipitations clearly indicates a nonspecific interaction with the affinity matrix and does not suggest a specific association with PfAMA1.

LC-MS/MS analysis of p70 from the MAb 28G2-immunoprecipitated material also identified peptides for PfAMA1, whereas no such peptides were identified in the p70 band precipitated with protein G beads alone. This is consistent with the immunoblotting data that showed a form of PfAMA1 (presumably the previously described $PfAMA1_{66}$ [22]) was present in the MAb 28G2-selected material at about 70 kDa (Fig. 1B).

Analysis of p240 identified tryptic peptides corresponding to a single predicted protein, chr11.genefinder_174r (Table 1). BLAST analysis of this predicted protein sequence revealed it to be most similar (e value of 10^{-9}) to a recently described rhoptry neck protein in *Toxoplasma gondii*, TgRON4, that has been shown to form a strong and stable complex with TgAMA1 in invading *Toxoplasma* tachyzoites (2); this, plus the data presented here, strongly indicates that the interaction

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TABLE 1. Correlative peptide matches for proteins immunoprecipitated with MAb 28G2 and analyzed by LC-MS/MS[a]

Gel band	Protein prediction or name	Peptide	Position
p240	Chr11.genfinder_174r	K.NPIDNSNISNLDK	121–133
		K.NVPHLDQSAMSNEK	220–233
		K.LALVPFQGIK	560–569
		K.EGPIITPLEGEQAGTAHK	612–629
		R.IIIEIMESAK	768–777
		K.DKNLISLEVYDK	813–824
		K.ILTEMSFYEDSK	832–843
		K.FYETLGIK	844–851
		K.YYQNMLGFEEDK	947–958
		K.VSSVPFNYENVK	1008–1019
		K.PSSSIIGSLGNLIK	1021–1034
		R.INSYFHYTEK	1048–1057
		K.IISVCTLLHLTDMLYK	1067–1082
		K.MVLQYLVHLK	1108–1117
		K.EICEPQNGLIDETLTK	1129–1144
		K.MLILLSTDSHELLSHELENK	1145–1164
		K.GFDEDYIQDEIK	1165–1176
p85	PfAMA1	G.QNYWEHPYQNSDVY*	25–38
		R.SNYMGNPWTEYMAK	103–116
		K.YDIEEVHGSGIR	117–128
		R.VDLGEDAEVAGTQYR	129–143
		K.GIIIENSNTTFLTPVATGNQYLK	155–177
		K.NLDELTLCSR	210–219
		R.HAGNMIPDNDKNSNYK	220–235
		K.YPAVYDDKDK	236–245
		K.DISFQNYTYLSK	281–292
		K.LVFELSASDQPK	340–351
		R.AEVTSNNEVVVK	514–525
		K.DEYADIPEHKPTYDK	530–544
		I.ASSAAVAVLATILMVYLYK**	550–568
		R.NDEMLDPEASFWGEEK	592–607
p70	PfAMA1	R.SNYMGNPWTEYMAK	103–116
		R.VDLGEDAEVAGTQYR	129–143
		K.DISFQNYTYLSK	281–292
		R.NDEMLDPEASFWGEEK	592–607

[a] Column 1 is the designation of the Coomassie-stained band as indicated in Fig. 1A. Column 2 indicates the protein to which the identified peptides map. Column 3 lists the individual peptide sequences identified. All of the spectra were manually examined for ion coverage and signal-to-noise ratios. The predicted upstream amino acid is shown to the left of the period. Column 4 indicates the location of the peptide in each of the protein sequences. A single asterisk indicates the predicted N terminus of $PfAMA1_{83}$, and double asterisks indicate a peptide consistent with a tryptic fragment where not all residues were identified.

between PfAMA1 and p240, which we will henceforth refer to as PfRON4, is a real and evolutionarily conserved association.

The TgAMA1/TgRON4 complex is unusual in that it is derived from two distinct secretory compartments: the micronemes and the rhoptry necks (2). A *P. falciparum* rhoptry antigen migrating in the 225- to 240-kDa range has previously been described based on binding of MAb 24C6 (26). The identity of the antigen was not determined, but immuno-electron microscopy showed it discretely localizes within the apical neck region of rhoptries in segmented schizonts (Fig. 2) (26). These properties are consistent with the SDS-PAGE mobility and predicted location of PfRON4, and so we asked whether the antigen seen by MAb 24C6 might be PfRON4. Immunoblots of the PfAMA1-coprecipitating material probed with MAb 24C6 showed a specific, strongly reacting band that was recognized at ~240 kDa (Fig. 1C). Given that mass spectrometry indicated that this band is comprised exclusively of PfRON4, this result confirms that PfRON4 and the rhoptry neck antigen seen by MAb 24C6 are one and the same. Immunoprecipitation with anti-PfAMA1 did not substantially deplete PfRON4 (Fig. 1C) from RIPA extracts (even though the bulk of the PfAMA1 was removed [Fig. 1B]), and given that the Coomassie staining indicates there is a rough equivalence of the two proteins within the PfAMA1-immunoprecipitated material, it appears that PfRON4 protein is in considerable excess over PfAMA1. This is similar to our findings in *Toxoplasma*, where only a fraction of the total TgRON4 in parasite extracts associates with TgAMA1 (2). Also consistent with our *Toxoplasma* findings is that the immuno-electron microscopic localization of PfRON4 did not show localization in the micronemes, suggesting the PfAMA1/RON4 complex is formed following the secretion of these two proteins from the microneme and rhoptry neck compartments.

The GeneFinder prediction corresponding to PfRON4 is a single exon with a clearly predicted N-terminal signal peptide and a predicted mass of ~134 kDa (minus the signal peptide). The discrepancy between the predicted size and observed mobility on SDS-PAGE (comigrating with markers in the range of ~240 kDa) is explained by the fact that the predicted PfRON4 protein has an extended N-terminal domain consisting of 22 tandem iterations of a near-perfect, 13-amino-acid repeat that is rich in proline and glutamic acid (the consensus motif is

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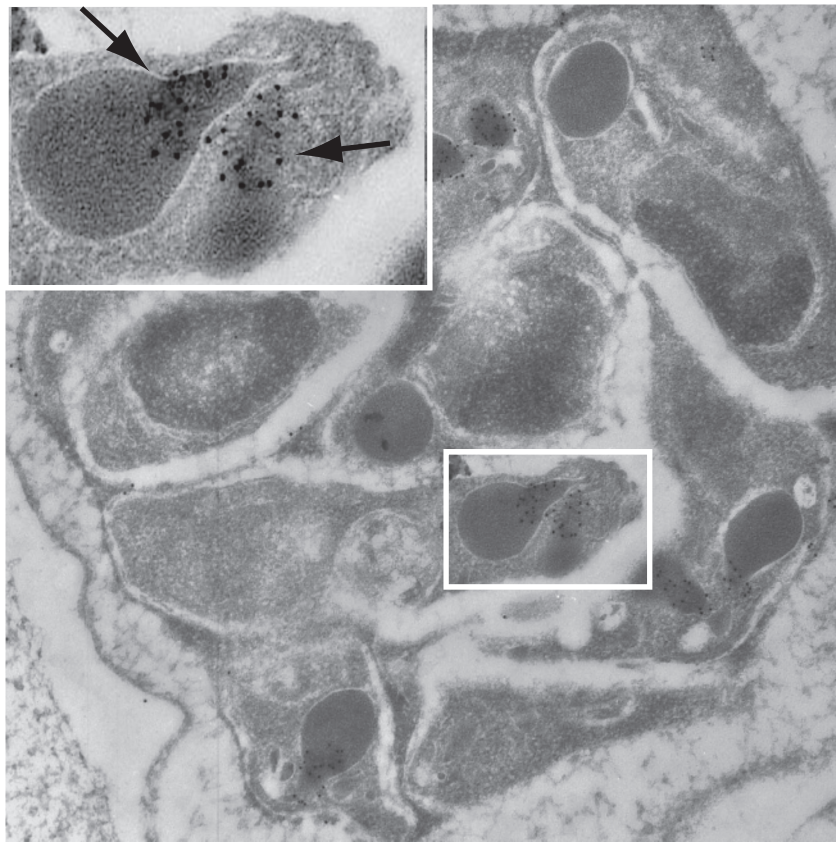

FIG. 2. PfRON4 localizes to the rhoptry necks. Immuno-electron microscopy with MAb 24C6 and protein A-gold shows specific labeling in the rhoptry necks of *P. falciparum* schizonts. Infected human erythrocytes were embedded in LRWhite and stained with MAb 24C6 as previously described (26). The enlarged area shows a longitudinally sectioned rhoptry with PfRON4 labeling restricted to the narrow apical end of the rhoptries in a maturing merozoite (arrows).

NEPIHNEHATTPT). Such repeats are known to significantly retard the mobility of proteins on SDS-PAGE (as seen, for example, with TgROP1 [24]), thus easily explaining the discrepancy in size versus mobility. All of this, plus the MS/MS peptide coverage and the fact that no peptides were seen from any adjacent predicted open reading frames, makes it extremely likely that the chr11.genefinder_174r prediction is correct and the protein is indeed ~134 kDa in size.

TgRON4 has an N-terminal stretch of 56 amino acids, in this case repeated just twice, but this bears no resemblance to the 13-mer repeat of PfRON4; all of the homology (including all five cysteines present in them both) falls within the C-terminal two-thirds of the two proteins. Given that they both associate with AMA1 from their respective cells, this strongly suggests that it is the C-terminal domain of RON4 that is responsible for interacting with AMA1.

The results presented here differ in one important respect from those for *Toxoplasma*, where two additional proteins (TgRON2 and an as-yet-uncharacterized, third rhoptry protein [Twinscan 4705]) coprecipitate with TgAMA1 (2, 19). Within the *Plasmodium* genome database, PF14_0495 and MAL8P1.73 are predicted homologues of RON2 and Twinscan_4705, respectively (6). We were surprised, therefore, to find neither of these proteins coprecipitating with PfAMA1. We cannot currently distinguish between the possibility that the four-molecule complex exists in *Plasmodium*, but without the stability needed for the detergent conditions used here, and the possibility that there is a major difference between the two genera, with only two proteins, PfAMA1 and PfRON4, comprising the complex in *Plasmodium*.

The similarity between TgRON4 and PfRON4 is relatively modest. This contrasts with the strong conservation of AMA1 across the entire phylum (e.g., TgAMA1 and PfAMA1 share 29% identity, with a BLAST e value of 10^{-33}), suggesting that the role of AMA1 requires a very particular structure, whereas the RONs may have drifted to accommodate differences in the host cells that these various parasites invade. A full understanding of how the AMA1/RON4 complex functions will require identification of all of their binding partners, both on the host and parasite, but knowing that the AMA1/RON4 collaboration is conserved throughout the *Apicomplexa* provides an important advance in dissecting the role of these unusual proteins in the invasion process.

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//www.Toxodb.org, http://v4-4.plasmodb.org, and http://www.tigr.org/tdb/e2k1/pfa1/. Genomic data were provided by the Institute for Genomic Research (supported by NIH grant AI05093), the Sanger Center (Wellcome Trust), and Stanford University (Burroughs Wellcome Fund).

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