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Integral Membrane Protein Located in the Apical Complex of *Plasmodium falciparum*

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We describe the cloning of a novel antigen of *Plasmodium falciparum* which contains a hydrophobic domain typical of an integral membrane protein. This antigen is designated apical membrane antigen 1 because it appears to be located in the apical complex. Apical membrane antigen 1 appears to be transported to the merozoite surface near the time of schizont rupture.

The molecular events that occur when a malaria merozoite invades a host erythrocyte are poorly understood. Parasite molecules contained within the apical complex of merozoites are believed to play a critical role in invasion. The major organelles that make up the apical complex are the rhoptries, membrane-bound electron-dense structures which appear to discharge their contents both at the time of schizont rupture and on the invasion of erythrocytes (1, 3, 14). Electron microscopy using fixatives containing tannic acid has revealed multilamellar membranous whorls derived from rhoptry secretions (4, 24). Thus, the rhoptries apparently contain lipid and the membrane of the parasitophorous vacuole may derive from this material (4, 24).

An important reason for dissecting this process is the evidence from animal trials and in vitro inhibition data that protective immune responses can be induced by antigenic components of the rhoptries (10, 16, 18). We describe here a blood stage antigen of *Plasmodium falciparum* that has the characteristics of an integral membrane protein and is localized to the apical complex. It appears to be transported to the merozoite surface in late schizonts and free merozoites.

Structure of Ag352 and its gene. A novel cDNA clone designated Ag352 was identified by screening a λ gt11-Amp3 cDNA library, prepared from *P. falciparum* isolate NF7, with human antimalarial antibodies (23). The DNA sequence of Ag352 is shown in Fig. 1. To complete the coding sequence, two overlapping genomic fragments of isolate FCQ27/PNG (FC27) (a 5′ partial *Sau*3A fragment and a 3′ partial *Ssp*1 fragment) were cloned and sequenced (Fig. 1).

The FC27 genomic sequence extends the 1,438-nucleotide NF7 cDNA sequence a further 514 nucleotides 3′ and 809 nucleotides 5′. A single long open reading frame is present in the FC27 genomic sequence between nucleotides 333 to 2202 encoding a polypeptide of 622 residues with a predicted molecular mass of 71,929 daltons. On the basis of the subcellular localization results presented below, this polypeptide was termed apical membrane antigen 1 (AMA-1).

The FC27 genomic sequence and the NF7 cDNA sequence are highly homologous, with nine nucleotide differences in 1,438 nucleotides giving rise to seven amino acid differences. Strikingly, only one nucleotide difference is silent and five of the amino acid differences result in charge changes. These data indicate strong selection, perhaps immunological, on the polypeptide sequence.

Comparison of the sequence of AMA-1 with those in the GenBank and National Biochemical Research Foundation databases failed to identify any similar proteins. However, the AMA-1 polypeptide sequence has a number of striking features. Unlike most other blood stage antigens of *P. falciparum* that have been sequenced, AMA-1 lacks repetitive sequences. The antigen has a structure expected for an integral membrane protein. It contains two hydrophobic stretches, one near the N terminus, which may act as a signal peptide, and a second located 55 amino acids from the C terminus. This consists of 21 predominantly hydrophobic residues and lacks charged residues. It is preceded by a lysine residue and followed by the tripeptide Lys-Arg-Lys, consistent with the notion that this region is a membrane-spanning domain.

All 17 cysteine residues precede the predicted membrane domain. The most N-terminal cysteine is presumably removed along with the signal peptide. We assume that many of the other 16 cysteines form intramolecular disulfide bonds important for maintaining the three-dimensional structure of the molecule. This is consistent with the finding that the mobility of AMA-1 was decreased by electrophoresis under reducing conditions compared with nonreducing conditions (P.E.C., unpublished data). The importance of the cysteine residues is supported by the finding that all 16 are conserved in AMA-1 from *P. chabaudi* (M.G.P., unpublished data).

AMA-1 partitions into Triton X-114. We examined the phase partitioning of AMA-1 in Triton X-114. Parasite cell pellets from isolate FC27 were solubilized in Triton X-114 and separated into an aqueous phase, a Triton X-114 phase, and an insoluble pellet (22). An immunoblot of this material was probed with rabbit antibodies affinity purified (9) on a glutathione S-transferase fusion protein (termed Ag352.24) (21) containing only the C-terminal 52 amino acids of AMA-1 (571 to 622; Fig. 1). Two proteins with M_rs of 80,000 and 62,000 were detected in variable amounts in the total parasite material, in the aqueous phase, and in the Triton X-114 phase (Fig. 2). The 62,000-M_r protein appears to be a processed fragment of the 80,000-M_r protein (P.E.C., unpublished data). Both the 80,000-M_r protein and the 62,000-M_r protein were partially soluble in Triton X-114, with some variation in different experiments.

AMA-1 is localized to the apical complex and the merozoite surface. Indirect immunofluorescence microscopy with affinity-purified human antibodies (described above) stained mature asexual blood forms. Segmented schizonts showed a punctate pattern of immunofluorescence within merozoites (Fig. 3A) characteristic of antigens located in the apical

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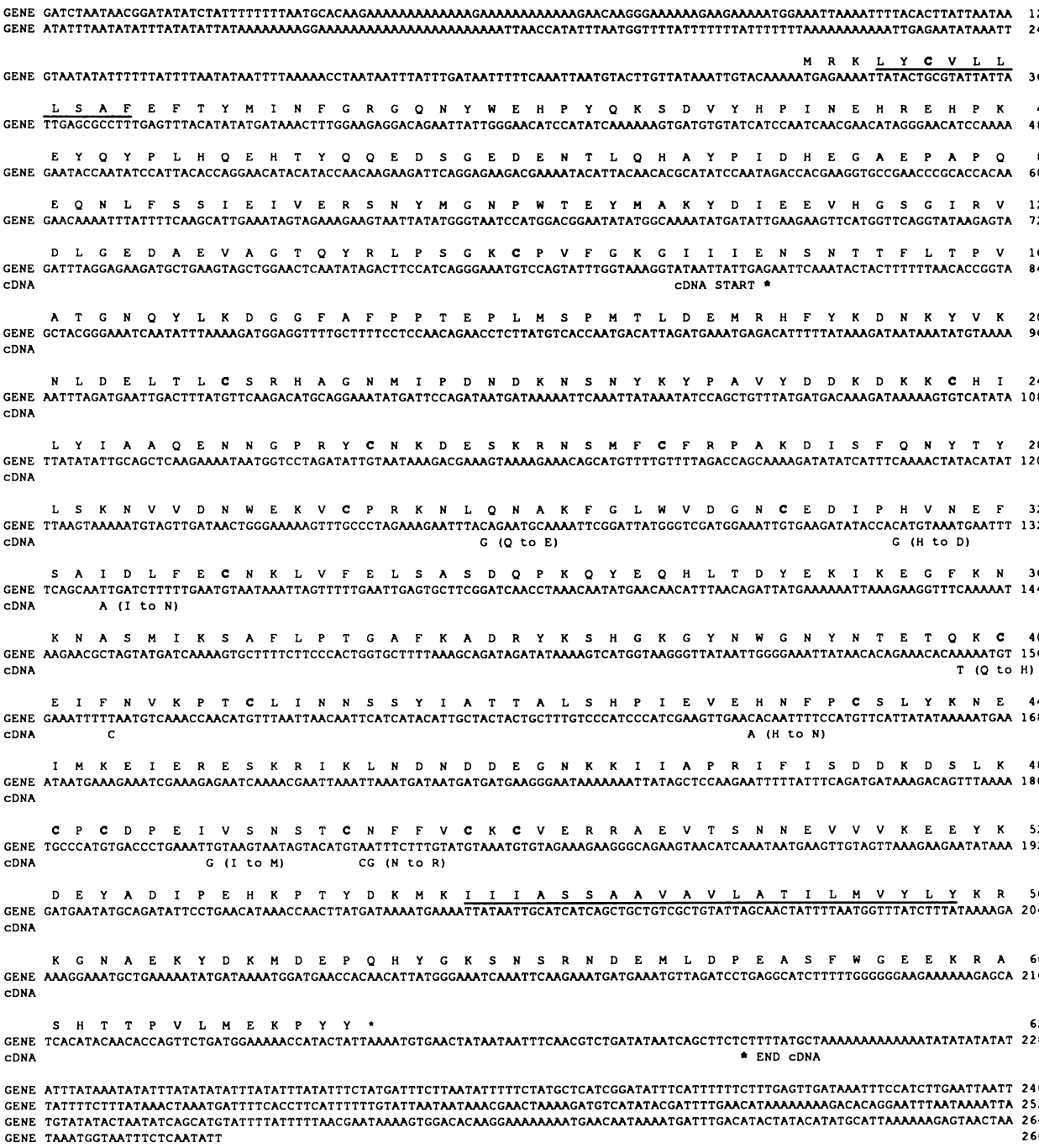

FIG. 1. Nucleotide sequences of the AMA-1-encoding gene of *P. falciparum* isolate D10 (top line) and AMA-1 cDNA from isolate NF7 (bottom line; the asterisks denote the extent of the sequence). The predicted polypeptide sequence is translated. The cDNA sequence is shown only where it differs from the genomic sequence. Where this also results in an amino acid change, the change is shown in brackets. Two stretches of hydrophobic amino acids are underlined. Cysteine residues are shown in boldface.

complex, possibly the rhoptries (12). This pattern was also evident within some merozoites of disrupted schizonts, although other clusters of merozoites gave a bunch-of-grapes fluorescence pattern characteristic of a merozoite surface location (11) (Fig. 3D). Patterns of fluorescence intermediate between these were also evident in some merozoites escaping from burst schizonts (Fig. 3B and C), in which there was merozoite surface fluorescence with increased intensity at the apex of each merozoite. Attempts to localize the antigen more precisely by electron microscopy were unsuccessful.

Conclusions. Most of the antigens located in the apical organelles of *P. falciparum* merozoites appear to be components of two antigenic complexes in the rhoptries (5, 8, 12, 13, 15, 20, 25). Detailed studies using anti-AMA-1 antibodies indicated that AMA-1 is not part of either of these complexes (P.E.C., unpublished data). Furthermore, AMA-1 clearly cannot be the 76-kilodalton serine protease with a phosphatidyl inositol lipid anchor (5), since AMA-1 has a hydrophobic transmembrane region. Two other rhoptry antigens have been described, one with an M_r of 55,000 which, like

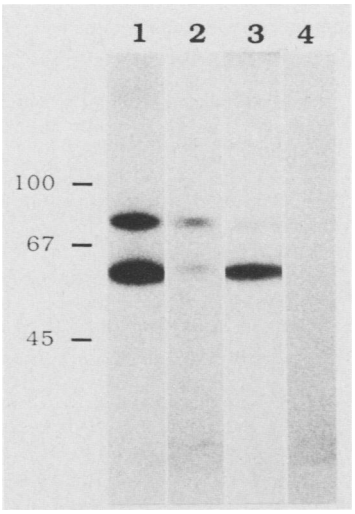

FIG. 2. Immunoblot of AMA-1 from *P. falciparum* isolate FC27 with affinity-purified rabbit anti-Ag352.24 antibodies. Infected erythrocytes (lane 1) were fractionated by temperature-dependent phase partitioning with the detergent Triton X-114 to generate three fractions: an aqueous phase (lane 2), a Triton X-114 phase (lane 3), and an insoluble pellet (lane 4). The numbers on the left indicate molecular weights in thousands.

AMA-1, has the solubility characteristics of an integral membrane protein and a 240,000-M_r molecule which is processed to a 225,000-M_r form (19, 22). The characteristics of AMA-1 are also distinct from those of these antigens (P.E.C., unpublished data). The only antigen known to be located in the micronemes is the ring-infected erythrocyte surface antigen (6). Thus, AMA-1 represents a previously uncharacterized antigen.

Strikingly, AMA-1 appears first to be located in the apical complex and then to be exported to the merozoite surface at around the time of schizont rupture, although this form of analysis is not capable of precisely defining the timing of events. This relocation could result from its being released from the merozoite at the time of schizont rupture and passively acquired on the merozoite surface or integrated into the merozoite surface membrane. A number of antigens appear to be secreted into the parasitophorous vacuole and then passively acquired in small quantities on the merozoite surface, but none of these are integral membrane proteins (2, 7, 17, 26). Alternatively, AMA-1 may be anchored in the structural membrane of the rhoptry and relocated by lateral movement in the membrane out onto the merozoite surface, since these membranes appear by electron microscopy to be contiguous (4). AMA-1 is lost during invasion, since it cannot be detected by immunofluorescence in rings, suggesting that it functions in the process of invasion.

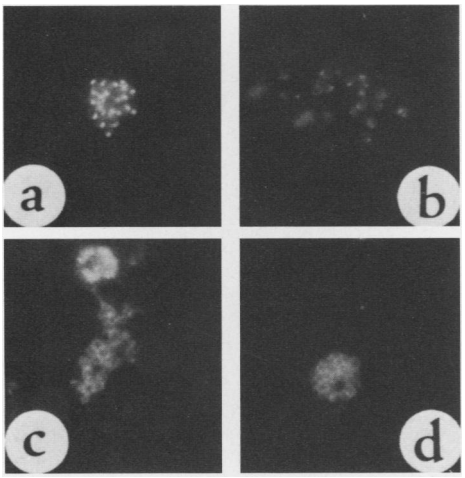

FIG. 3. Indirect immunofluorescence microscopy of AMA-1 from *P. falciparum* isolate FC27 with affinity-purified rabbit anti-Ag352.24 antibodies. Phase-contrast microscopy was used to determine the stage specificity of the parasites. Panels: A, rhoptry fluorescence on a mature schizont; B and C, rhoptry and merozoite surface labeling on merozoites escaping from a burst schizont; D, surface labeling on merozoites.

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