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The conserved *clag* multigene family of malaria parasites: essential roles in host-pathogen interaction

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Abstract

The *clag* multigene family is strictly conserved in malaria parasites, but absent from neighboring genera of protozoan parasites. Early research pointed to roles in merozoite invasion and infected cell cytoadherence, but more recent studies have implicated channel-mediated uptake of ions and nutrients from host plasma. Here, we review the current understanding of this gene family, which appears to be central to host-parasite interactions and an important therapeutic target.

1. Introduction

Malaria parasites of the genus *Plasmodium* are successful protozoan parasites that infect humans and other primates, rodents, birds, and reptiles. In each host, *Plasmodium* species have complex life cycles that include intracellular stages within hepatocytes and circulating erythrocytes. The growth and replication within erythrocytes is responsible for most of the clinical sequelae of human malaria, as caused by 5 *Plasmodium* spp. The most virulent parasite, *P. falciparum*, remodels its host erythrocyte by exporting many proteins and generating a membranous network in the host cell cytoplasm (Bullen et al., 2012; Spielmann and Gilberger, 2010; Tilley and Hanssen, 2008). This remodeling alters many host cell properties, with two key changes being increased solute permeability and greater cytoadherence to endothelium and other cells (Desai et al., 2000; Staines et al., 2007; Leech et al., 1984; Craig and Scherf, 2001).

Much research has focused on identifying the parasite and host proteins responsible for altered cytoadherence and permeability of infected erythrocytes. Surprisingly, both of these divergent phenotypes have been linked to the *clag* multigene family (cytoadherence linked asexual gene) and also sometimes referred to as *rhophil* (Trenholme et al., 2000; Goel et al., 2010; Nguitragool et al., 2011; Sharma et al., 2013). These genes are especially interesting

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because, in contrast to established cytoadherence genes present in only one or a few species (Jemmely et al., 2010), the *clag* genes are conserved and expanded to varying numbers in all members of the genus *Plasmodium*. At the same time, these genes are absent from other protozoa, suggesting that *clag* genes mediate one or more functions essential and unique to malaria parasites. These genes encode large 120-170 kDa proteins without homology to proteins in other genera, defying attempts to identify possible functions through computational analyses. Studies using molecular and biochemical approaches have shed light on possible roles, but there are still important gaps in our understanding. Here, we critically review the history of research on the *clag* genes and the proteins they encode, examine proposed roles, and present testable models for CLAG protein trafficking and function.

2. Discovery of RhopH1/CLAG and early insights

The CLAG proteins were discovered with specific antibodies that revealed a high molecular weight complex within the rhoptry, an organelle present in the merozoites of all apicomplexan parasites. Some studies have used monoclonal antibodies raised against parasite lysates (Holder et al., 1985; Campbell et al., 1984; Kaneko et al., 2001), while others recovered specific antibodies from human subjects with acquired immunity to malaria (Coppel et al., 1987; Lustigman et al., 1988). These various studies confirmed a complex of three unrelated proteins in the virulent human pathogen, *P. falciparum*. These proteins have molecular weights of 155, 140 and 110 kDa and were referred to as RhopH1, RhopH2, and RhopH3 respectively. Based on studies described below, RhopH1 is now also known as CLAG. A few studies suggested additional interacting proteins (Kaneko et al., 2001; Ling et al., 2003; Ranjan et al., 2011), but these have not been consistently observed. Early biochemical studies revealed that the complex was partially soluble and partially membrane-associated (Cooper et al., 1988; Etzion et al., 1991); a fraction was found released from infected cells into culture supernatants (Lustigman et al., 1988; Cortes et al., 2007). Confocal immunofluorescence microscopy revealed that each member of the complex localizes to the rhoptries and to sites within the host erythrocyte after invasion (Coppel et al., 1987; Sam-Yellowe et al., 1988; Kaneko et al., 2001; Gardiner et al., 2004; Hiller et al., 2003; Ling et al., 2004; Vincensini et al., 2008; Nguitragool et al., 2011). Translation of all three proteins begins at the mid-trophozoite stage (30-34 h after invasion) and peaks during the mature schizont stage (42 h). Putting these observations together, the most parsimonious model has been that the three proteins are packaged into rhoptries of newly formed merozoites upon synthesis. Either during translation or after they reach the rhoptry, these proteins associate with each other non-covalently to form the high molecular weight complex. The complex may then be transferred to the next host erythrocyte upon invasion (Fig. 1; Lustigman et al., 1988; Coppel et al., 1987; Sam-Yellowe et al., 1988; Ling et al., 2004).

Somewhat after these biochemical studies, Trenholme and colleagues happened upon the genes that encode the largest member of the RhopH complex (Holt et al., 1999). They were studying a chromosome 9 deletion event associated with loss of infected erythrocyte binding to melanoma cells, which carry the CD36 receptor (Barnes et al., 1994). They identified a candidate gene within this locus and named it *clag9* (cytoadherence linked asexual gene);

molecular studies suggested several paralogs on other chromosomes in *P. falciparum*. Subsequent mass spectrometry sequencing of the RhopH complex determined that the *clag* genes encode the RhopH1 protein (Kaneko et al., 2001).

3. *Clag* gene structure and transcriptional regulation

After these early studies, genome sequencing and assembly revealed that *P. falciparum* has 5 paralogous *clag* genes (Fig. 2). In contrast, RhopH2 and RhopH3 were found to be encoded by single copy genes without homology to each other or to the *clag* genes. Each member of the complex is strictly conserved in all plasmodial species. At the same time, none have significant homology to proteins from other genera except a domain in the CLAG proteins shared with RON2, an invasion ligand also localizing to rhoptries of various apicomplexan parasites (Richard et al., 2010; Kaneko, 2007; Anantharaman et al., 2007). Interestingly, expansion of the *clag* gene family is quite variable amongst plasmodial species, with at least 2 and no more than 5 paralogs per parasite. Phylogenetic analysis suggests that the gene family was split into two distinct groups early in the plasmodial lineage (Kaneko et al., 2005; Nguitragool et al., 2011; Sharma et al., 2013). The first group has a single member in each species and is represented by *clag9* in *P. falciparum*. The second group accounts for the variable expansion in different species and includes members on chromosomes 2, 3, and 8 in *P. falciparum* (*clag2*, *clag3.1*, *clag3.2*, and *clag8*). Of these, *clag3.1* and *clag3.2* are most similar, with 95 % identity at the protein level. Despite being nearly identical proteins, there are specific polymorphisms between these two paralogs that are conserved across geographically divergent parasite lines, suggesting that there is ongoing evolution amongst the group II *clag* genes (Sharma et al., 2013).

In *P. falciparum*, the *clag* genes all have 9 exons; the genes for RhopH2 and RhopH3 are also highly cryptic with 10 and 7 exons, respectively (Fig. 2). All 5 *clag* genes are subtelomeric, lying within 150 kB of a chromosome end. Preferential subtelomeric localization suggests critical virulence functions, as these sites are known to have greater rates of diversity through recombination and more opportunity for clonally variant expression (Lopez-Rubio et al., 2009; Flueck et al., 2009; Witmer et al., 2012).

Consistent with the above protein translation studies, all *clag* genes, *rhoph2*, and *rhoph3* genes exhibit concordant regulated transcription that peaks around 42 h after invasion (Fig. 1; Ling et al., 2004); some studies suggest transcription of *clag9* might be initiated somewhat earlier (Kaneko et al., 2005; Goel et al., 2010). *Rhoph2* and *rhoph3* appear to be expressed by all parasite lines in a stage-dependent manner; spontaneous deletions or gene silencing have not been found. Limited attempts to disrupt these genes have also been unsuccessful (Cowman et al., 2000). In contrast, transcription control and essentiality of *clag* genes is complicated and less well-established. Spontaneous deletions of the *clag9* locus have been known for some time (Day et al., 1993); *clag9* knockout lines have also been generated by transfection and characterized (Trenholme et al., 2000; Nacer et al., 2011). Deletions or knockouts of *clag2* or *clag8* have not been reported yet, but parasite lines with negligible expression of one or the other gene have been described (Cortes et al., 2007; Sharma et al., 2013). Studies have shown that both *clag3* genes can be simultaneously suppressed (Sharma et al., 2013; Mira-Martinez et al., 2013); one paper has also reported a

clag3 knockdown through a strategy based on integration of the *hdhfr* selectable marker cassette (Comeaux et al., 2011). At the same time, our unpublished studies suggest that this locus cannot be irreversibly disrupted. Some laboratory parasite lines carry only a single *clag3* gene (Chung et al., 2007; Iriko et al., 2008); because these lines carry the promoter of *clag3.2* and the 3' UTR of *clag3.1*, it is likely that these clones underwent reduction to a single *clag3* gene through chromosome-level recombination.

Most interestingly, parasites with two *clag3* genes do not express both genes simultaneously, but rather express only one and silence the other through an epigenetic mechanism that involves histone marks (Cortes et al., 2007; Crowley et al., 2011). For other parasite gene families, monoallelic expression typically permits immune evasion by allowing expression of a single gene for several bloodstream cycles; then, when a host immune response is elicited against that allele, the few progeny that switched to expression of a different gene family member evade immune-mediated killing. The mechanisms responsible for switching are only partially understood (Hoeijmakers et al., 2012; Guizetti and Scherf, 2013), but the kinetics must be optimized to allow persistence of the infection and protection of critical virulence gene families (Recker et al., 2011). This process accounts for the large-scale expansion of certain gene families in malaria and other parasites.

Two observations suggest that *clag3* switching serves a role other than immune evasion. First, only the two *clag* genes on chromosome 3, *clag3.1* and *clag3.2*, undergo monoallelic expression while paralogs on other chromosomes are transcribed independently; this would presumably undermine immune evasion by allowing development of a broad spectrum response against this gene family. Second, the relatively small number of paralogs in *P. falciparum* may argue against a role in immune evasion because studies with other gene families suggest a large number of paralogs may be needed for avoiding the multiple rounds of immune attack that occur during a single human infection (Recker et al., 2011). What other role could *clag3* switching serve for the parasite? One possibility is that switching between the two alleles permits fine-tuning of the encoded parasite activity. As described below, recent studies suggest that *clag3* genes encode a nutrient uptake channel at the host membrane; then, the two alleles may produce different channels that allow optimal parasite growth despite a broad range of nutritional statuses of human hosts (Mira-Martinez et al., 2013).

Yet another level of *clag* gene regulation has been identified recently using mutants resistant to blasticidin S (Sharma et al., 2013; Mira-Martinez et al., 2013). Selection with this toxin, which acts on parasite ribosomes, suppresses both *clag3* genes simultaneously; one study found it also silences *clag2* (Sharma et al., 2013), a paralog that exhibits clonally variant expression independent of the *clag3* switching program (Cortes et al., 2007). These parasites may therefore have the minimal level of *clag* gene expression tolerated by *P. falciparum*. This pattern of gene silencing yields dramatically reduced host cell permeability and confers blasticidin S resistance by reducing its uptake and access to the parasite target. These findings therefore also represent the first description of an epigenetic antimalarial drug resistance mechanism.

DNA transfections to disrupt or alter individual genes may be the most powerful way to examine the roles served by CLAG proteins, but the complex regulation of this gene family requires caution in interpreting such experiments. Although successful production of a knockout line suggests that a specific *clag* is not essential (Trenholme et al., 2000; Comeaux et al., 2011; Nacer et al., 2011), other family members may compensate for the lost paralog. Another possibility is that there may be compensatory mutations, ectopic recombination, or gene conversion events that allow other *clags* to acquire functions not normally served. For example, *in vitro* selection to suppress *clag3.1* expression in the Dd2 parasite line, which is transcription-incompetent for *clag3.2* (Nguiragool et al., 2011), recently yielded a recombinant line with three copies of *clag3* (Pillai et al., 2012). Unfortunately, molecular studies to characterize transfectant lines often fail to anticipate possible escape strategies that may preserve essential functions.

4. Predicted protein structures

In *P. falciparum*, the CLAG proteins, RhopH2, and RhopH3 all have a hydrophobic stretch near their N-terminus resembling traditional signal sequences, but they lack the PEXEL/VTs motif associated with direct export to the erythrocyte cytosol (Boddey et al., 2009; van Ooij et al., 2008). DNA transfection experiments have shown that the N-terminal 24 aa leader sequence of RhopH2 is sufficient to traffic GFP to the merozoite rhoptry (Ghoneim et al., 2007); this study also made some early insights into sites of interaction between CLAG3 and other members of the RhopH complex. Similar studies are needed to define the precise targeting motifs within CLAG proteins and to map out elements required for trafficking these proteins into erythrocyte cytosol and the host membrane after invasion. A small domain with homology to RON2 may be important for either trafficking or function within the post-invasion host cell (Ghoneim et al., 2007; Nguiragool et al., 2011).

Aside from the signal sequence, there are hydrophobic regions that do not meet stringent criteria for conventional transmembrane domains (red shading, Fig. 2), but conservation in divergent plasmodia suggests that these regions render CLAG3 an integral membrane protein, as experimentally confirmed with biochemical studies (Nguiragool et al., 2011). RhopH2 and RhopH3 have similar hydrophobic regions (Etzion et al., 1991; Vincensini et al., 2008). Although Fig. 2 shows a single predicted transmembrane domain for each of these proteins, a small extracellular loop identified for CLAG3 suggests at least one other flanking transmembrane domain (Nguiragool et al., 2014). Interestingly, these studies show that each of these proteins is partially extracted by alkaline treatment using 0.1 M sodium carbonate (Fujiki et al., 1982), suggesting both peripheral and integral membrane pools for these proteins within infected erythrocytes. Clearly, more intensive biochemical and structural studies are needed for these unusual proteins.

CLAG proteins have highly conserved sequences in *P. falciparum* lines; for each paralog, there is at least 90% sequence identity amongst parasite lines harvest from around the world. Interestingly, however, each group II CLAG has a small hypervariable region of 5-30 amino acids size located at 1000-1200 aa from the N-terminus (HVR, blue shading, Fig. 2). Such an HVR is not detectable in CLAG9, RhopH2, or RhopH3, each of which is nearly identical in all *P. falciparum* lines. Biochemical studies of CLAG3 have revealed that its HVR is

exposed at the erythrocyte surface (Nguiragool et al., 2011; Nguiragool et al., 2014). This observation, along with evidence for positive selection for polymorphism at this motif in CLAG2 and CLAG3 (Iriko et al., 2008; Alexandre et al., 2011), suggests that variability at this site is driven by immune pressure against the exposed polypeptide loop.

5. What do CLAG proteins do?

A number of roles have been proposed for CLAGs and the interacting RhopH2 and RhopH3 proteins. We next critically examine the available evidence for each of these proposed roles (Fig. 1).

5.1 Cytoadherence

Cytoadherence, the ability of infected erythrocytes to bind other cells including those that line blood vessels, is essential for parasite survival because it prevents circulation and elimination of infected cells by the spleen (Craig et al., 2012). Although PfEMP1, an unrelated protein encoded by members of the *var* gene family, is the primary cytoadherence determinant (Pasternak and Dzikowski, 2009), workers have long suspected that there may be additional parasite ligands for receptors on blood vessel endothelium. Early experimental studies suggested that CLAG proteins might be these ligands, leading to their name (cytoadherence linked asexual gene); cleverly, this is also an Australian glue brand name (Craig, 2000). The evidence stems from studies of a parasite chromosome 9 deletion event associated with loss of cytoadherence to C32 melanoma cells (Day et al., 1993). While such associations are often coincidental, this link appears to be strong because selection of cultures for melanoma cell binding enriched parasites without the chromosome deletion event. The minimal associated deletion event was ~ 55 kB in size; it lacked *var* genes encoding PfEMP1, but contained the *clag9* gene. To examine this candidate, a knockout was generated in the cytoadherent 3D7 parasite line by single crossover recombination (Trenholme et al., 2000). This knockout parasite failed to bind melanoma cells, suggesting a *clag9* role in cytoadherence. The same group then obtained reduced melanoma cell binding with RNAi knockdown experiments (Gardiner et al., 2000), but subsequent studies have failed to detect RNAi machinery in *P. falciparum* and questioned whether such experiments yield reliable results (Baum et al., 2009). In contrast to these studies, *clag9* disruption in another lab did not abolish cytoadherence, despite using the same laboratory parasite line and an essentially identical transfection strategy (Nacer et al., 2011).

A less direct role in cytoadherence has been proposed by Gowda and colleagues (Goel et al., 2010). This study reported that CLAG9 interacts with VAR2CSA, a special PfEMP1 family member that binds placental CSA (Fried and Duffy, 1996). Their co-immunoprecipitation and localization studies suggest that CLAG9 and RhopH2 may function to traffic VAR2CSA, and possibly other PfEMP1 proteins to the host membrane.

Despite these studies, whether CLAG proteins serve a role in cytoadherence remains uncertain. Direct studies using additional DNA transfections and appropriate controls will be needed for this proposal to be better supported.

5.2 Merozoite Invasion and/or Parasitophorous Vacuole Formation

Another proposal has been that CLAGs and the associated RhopH proteins function in erythrocyte invasion, an idea partly motivated by the localization of these proteins to the rhoptries (Coppel et al., 1987; Cooper et al., 1988; Etzion et al., 1991; Kaneko et al., 2001; Gardiner et al., 2004). Because these organelles are at the apical end of merozoites and are secreted onto the host cell upon invasion (Fig. 3; Kaneko, 2007), it seemed likely that most proteins and other contents of these organelles would function in invasion. For example, RON2, probably the best characterized rhoptry protein, is secreted and anchors into the host membrane early during invasion (Cao et al., 2009); it then forms specific interactions with AMA1 on the merozoite membrane to form a moving junction that envelops the merozoite (Richard et al., 2010; Srinivasan et al., 2011). With successful completion of invasion, this process yields an internalized parasite surrounded by an additional lipid bilayer known as the parasitophorous vacuolar membrane (PVM). By analogy to rhoptry proteins such as RON2, it was postulated that the RhopH complex would function either in merozoite-host cell interaction or in formation of the parasitophorous vacuole.

Early evidence supporting this role included the observation that the RhopH complex binds to erythrocyte membranes and to synthetic liposomes (Sam-Yellowe et al., 1988; Sam-Yellowe and Perkins, 1991; Rungruang et al., 2005). Workers also found that a subset of synthetic peptides based on the CLAG3 sequence bind to erythrocyte membranes with reasonable affinity (Ocampo et al., 2005). Finally, antibodies raised to RhopH components were also found to inhibit invasion (Sam-Yellowe and Perkins, 1990; Ranjan et al., 2011). While these observations are suggestive, many proteins or peptides bind nonspecifically to membranes; the interactions found may therefore be coincidental. There are also alternative explanations for reduced invasion found with addition of antibodies (Duncan et al., 2012). Thus, when compared to more established invasion ligands, a role of the RhopH complex in invasion is still only weakly supported by experimentation.

Another hypothesis that derived from rhoptry localization is that RhopH proteins may be involved in formation of the parasitophorous vacuole. Electron micrographs of rhoptries suggest whorls of lipid that may contribute to vacuole formation (Fig. 3; Bannister et al., 1986; Stewart et al., 1986). Although fluorescent lipid trafficking studies suggest that the lipid required for PVM formation comes from invagination of the erythrocyte surface (Ward et al., 1993), measured changes in erythrocyte surface area upon invasion suggest that rhoptry lipids may also contribute to the nascent PVM (Dluzewski et al., 1995). Although there is no experimental evidence linking the CLAGs or associated RhopH proteins to vacuole formation, imaging studies have demonstrated that they are present at the PVM both early after invasion and later in the erythrocytic cycle (Hiller et al., 2003; Ling et al., 2004; Vincensini et al., 2008; Nguitragool et al., 2011). Because localization to this site may only reflect protein in transit to the host membrane (Fig. 3B), these findings are not adequate to support a specific role in vacuole formation.

5.3 Increased erythrocyte permeability and nutrient uptake

A surprising and quite different role for CLAG proteins has more recently been proposed through focused studies of the plasmodial surface anion channel, PSAC (Nguitragool et al.,

2011). This nutrient and ion channel accounts for the marked increase in host erythrocyte permeability to diverse solutes, as characterized with macroscopic solute uptake studies by multiple groups over several decades (Overman, 1948; Neame and Homewood, 1975; Kutner et al., 1985). The mechanistic basis was subsequently identified through patch-clamp studies of infected cells, revealing that PSAC is an unusual broad selectivity channel with properties that distinguish it from known mammalian channels (Desai et al., 2000; Cohn et al., 2003; Kang et al., 2005; Lisk et al., 2007). Our group identified differences in channel behavior when erythrocytes from a single donor are infected with divergent parasite lines (Alkhalil et al., 2004; Alkhalil et al., 2009), suggesting the involvement of parasite proteins in channel formation. Based on these findings, high-throughput screens for PSAC inhibitors that may be useful research tools and possible therapeutics were executed (Pillai et al., 2010). One inhibitor, known as ISPA-28, was a potent inhibitor of channels from the Dd2 parasite, but ineffective against those of the HB3 parasite. Genetic mapping studies using this compound and an available Dd2 $\times$ HB3 genetic cross implicated the *clag3* chromosomal locus with high confidence (Nguitragool et al., 2011). DNA transfection to complement Dd2 parasites with the HB3 allele for each of the 15 genes in the mapped locus produced a significant change in ISPA-28 sensitivity with both *clag3.1* and *clag3.2*; because none of the other transfected genes produced a measurable change in PSAC activity, this result strongly implicated CLAG3 proteins. This study also used allelic exchange transfection of HB3 parasites to confer high affinity ISPA-28 block quantitatively matching that seen with Dd2 parasites. Consistent with expression switching between *clag3.1* and *clag3.2*, selection with two different PSAC inhibitors produced predictable effects on transcription of these two paralogs. Finally, CLAG3 was shown to be an integral membrane protein at the host erythrocyte membrane, consistent with functional studies that found the channel at this membrane. A role of CLAGs in host cell channels was also consistent with conservation of these genes and PSAC activity in all malaria parasites, while both are absent from other genera (Lisk and Desai, 2005; Alkhalil et al., 2007).

Genetic mapping, as used to implicate a CLAG3 role in PSAC activity, is not vulnerable to assumptions about properties or localization of proteins; it therefore overcomes some of the concerns raised above for other proposed roles. The genetic mapping strategy was subsequently used with two additional and independent phenotypes—growth inhibition by ISPA-28 and functional proteolysis of channel activity—to examine how the CLAG3 proteins may contribute to transport and to determine that the channels function in nutrient uptake (Nguitragool et al., 2014; Pillai et al., 2012).

Evidence that does not depend on genetic mapping has come from studies of PSAC mutants (Hill et al., 2007; Lisk et al., 2010; Lisk et al., 2008). These mutants were obtained through selection with water-soluble toxins that require channel-mediated uptake to reach their intracellular parasite targets. One mutant, generated by selection with leupeptin, carries a mutation at a conserved site within a predicted transmembrane domain (Nguitragool et al., 2011). A distinct blasticidin S-resistant mutant uses silencing of both *clag3* genes to achieve reduced uptake of the toxin (Sharma et al., 2013; Mira-Martinez et al., 2013). One study found that resistance is additionally associated with silencing of *clag2*, suggesting that this paralog also contributes to PSAC formation (Sharma et al., 2013).

6. Major questions for future research

In light of high confidence afforded by linkage analysis, multiple DNA transfections, and genome-wide expression studies (Nguiragool et al., 2011; Pillai et al., 2012; Sharma et al., 2013; Mira-Martinez et al., 2013), it is now clear that CLAG3 proteins contribute to PSAC-mediated solute transport at the host membrane. At the same time, these studies provide only limited insight into the structural basis of transport and raise new questions.

For example, although CLAG3 localizes to the host membrane, it is possible that the protein contributes only indirectly to solute transport, *e.g.* by functioning as an enzymatic activator of host membrane ion channels (Staines et al., 2007; Desai, 2012). A recent study using externally applied proteases with distinct substrate specificities determined that CLAG3 must be at the host membrane to contribute to transport (Nguiragool et al., 2014); this finding excludes certain models of indirect action. Because the study also implicated a polymorphic residue in functional proteolysis by chymotrypsin (L1115F position in a variant extracellular loop), it is suggestive of a direct contribution to formation of the channel pore. Additional studies will be needed to develop a better picture of channel structure and precise contribution of CLAG3.

Another important question is why the *clag* gene family has undergone variable expansion in malaria parasites. A conservative model is that these multiple paralogs all function in solute transport, yielding a collection of slightly different channels at the host membrane. These distinct isoforms may protect essential transport activities from host immune responses or may allow fine-tuned regulation of nutrient and ion transport. Epigenetic regulation of specific paralogs might then be used to modulate the relative permeabilities of key nutrients at critical times in parasite development. To date, there is only early evidence that CLAG2 functions in solute transport (Sharma et al., 2013). Transport studies with knockout parasite lines or with plasmodial species having different *clag* copy numbers should help shed light on the roles served; fortunately, there are multiple low- and high-throughput methodologies available for studying transport at the host membrane (Ginsburg and Stein, 1987; Wagner et al., 2003; Desai, 2005; Pillai et al., 2010).

It is also not clear whether the RhopH2 and RhopH3 antigens also function in solute transport; alternatively, they may function as chaperones that deliver CLAG proteins to the host membrane. In either case, their strict conservation and refractoriness to disruption in directed transfections suggest essential roles.

Do these proteins also serve additional unrelated roles in cytoadherence or invasion, as proposed (Trenholme et al., 2000; Kaneko, 2007; Goel et al., 2010)? Because these are large proteins, it is indeed possible that distinct conserved domains on CLAG proteins serve divergent functions at key time points in the parasite cycle. Better evidence is needed, especially since some studies have argued against these accessory roles (Nacer et al., 2011).

If CLAG proteins function exclusively in solute transport, why then do they undergo synthesis in the preceding parasite cycle, packaging within rhoptries, and trafficking via what seems to be an excessively circuitous path to the host membrane? This route is particularly surprising when one considers that increased host cell permeability is not fully

manifest until approximately 20 hours after invasion (Fig. 1; Kutner et al., 1985). The precise mechanism by which CLAG proteins reach the host membrane are unclear, but simple transfer to the host membrane surface upon invasion is unsatisfying because of the extended lag time for channel activation (route labeled “Lateral diffusion” in Fig. 3B). Packaging of CLAGs into rhoptries also contrasts with the more direct export of other parasite proteins to the host erythrocyte; many of these other proteins have a short export motif near their N-terminus and reach the host cell via a translocon on the PVM, termed the Plasmodium translocon of exported proteins (PTEX; Boddey et al., 2010; Russo et al., 2010; Bhattacharjee et al., 2012; Gruring et al., 2012). A recent study found that conditional knockdown of PTEX abolished export of nearly all other proteins but did not prevent CLAG3 from reaching the host membrane (Beck et al., 2014), further consistent with an atypical trafficking mechanism. Interestingly, PTEX knockdown interfered with induction of PSAC activity, suggesting interactions with other exported proteins may be required for channel formation.

Maybe the most important unaddressed question is whether CLAG proteins or the functions they serve can be targeted for development of therapies against malaria. A number of groups have considered these antigens for vaccine development, motivated partly by the greater conservation of CLAG proteins than other more-established cytoadherence ligands such as PfEMP1 (Ocampo et al., 2005). At the same time, specific and potent small molecule inhibitors that target PSAC activity and interfere with *in vitro* parasite development have also been found (Pillai et al., 2010). While host cation remodeling is an unnecessary byproduct of increased erythrocyte permeability (Pillai et al., 2013), nutrient acquisition via these channels is essential for intracellular development of the parasite (Pillai et al., 2012). Rational development of therapies that target this intriguing family of proteins should be facilitated by the broad array of biochemical and molecular approaches now available.

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Fig. 1.

Schematic showing synthesis, trafficking and proposed roles for members of the RhopH complex. The timeline below the schematic corresponds to the asexual cycle of *P. falciparum*. It shows peak transcription and translation of RhopH proteins at the schizont stage within a host erythrocyte (first cell at left); these proteins are packaged into rhoptries (yellow pair of organelles within each daughter merozoite). Upon merozoite egress from this cell and invasion of a new erythrocyte (0 h in the timeline), the RhopH complex is secreted into the new host cell; at this site, these proteins may serve accessory roles in invasion. As the parasite matures into a trophozoite form (20-24 h on the timeline), these proteins are trafficked to the host erythrocyte membrane, where they may contribute to nutrient uptake via PSAC and/or cytoadherence to receptors on blood vessel endothelium.

Fig. 2.

Gene structures for each of the five *clag* genes, *rhoph2*, and *rhoph3* in *P. falciparum*. For each gene, exons are shown as grey boxes separated by introns as present in the genome (black line). Coding sequences that encode putative transmembrane domains are shown in red, as predicted using the protein sequence and the Phobius algorithm (Kall et al., 2004); this analysis may miss transmembrane domains and requires further experimental evaluation. Variant domains, as identified through comparisons of coding sequences from divergent parasite lines, are shown in blue; additional small polymorphic sites are not shown here (Iriko et al., 2008). Each gene is presented to scale in a 5' to 3' orientation (left to right); scale bar represents 1000 base pairs.

Fig. 3. Schematic showing possible routes of RhopH complex trafficking. **A)** *P. falciparum* merozoite with key organelles labeled. RhopH proteins are packaged into two rhoptries (yellow); membrane whorls are detected within this organelle by electron microscopic studies. **B)** Trafficking of the RhopH complex during and after invasion of an erythrocyte. The RhopH complex, shown as a yellow bar, is secreted from rhoptries at the time of invasion. These proteins may reach their eventual destination at the host erythrocyte membrane by one of several mechanisms. They may insert directly into the host membrane

at invasion and undergo lateral diffusion. Some of the protein may cross the nascent parasitophorous vacuolar membrane (PVM). Upon completed invasion, the PVM surrounds the parasite. RhopH proteins may also be trafficked from the vacuolar space along membraneous extensions to specialized organelles known as Maurer's clefts to eventually reach the host erythrocyte membrane (RBC).