The conserved plant sterility gene *HAP2* functions after attachment of fusogenic membranes in *Chlamydomonas* and *Plasmodium* gametes

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The cellular and molecular mechanisms that underlie species-specific membrane fusion between male and female gametes remain largely unknown. Here, by use of gene discovery methods in the green alga *Chlamydomonas*, gene disruption in the rodent malaria parasite *Plasmodium berghei*, and distinctive features of fertilization in both organisms, we report discovery of a mechanism that accounts for a conserved protein required for gamete fusion. A screen for fusion mutants in *Chlamydomonas* identified a homolog of *HAP2*, an *Arabidopsis* sterility gene. Moreover, *HAP2* disruption in *Plasmodium* blocked fertilization and thereby mosquito transmission of malaria. HAP2 localizes at the fusion site of *Chlamydomonas minus* gametes, yet *Chlamydomonas minus* and *Plasmodium hap2* male gametes retain the ability, using other, species-limited proteins, to form tight prefusion membrane attachments with their respective gamete partners. Membrane dye experiments show that HAP2 is essential for membrane merger. Thus, in two distantly related eukaryotes, species-limited proteins govern access to a conserved protein essential for membrane fusion.

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Fusion of gametes of opposite sex (or mating type) to form a zygote is the defining moment in the life of a eukaryote. In the first phase of gamete interactions, cell adhesion molecules displayed on the surfaces of the gametes bring the two cells together. In animals, the sperm plasma membrane binds to the extracellular matrix of the egg (the zona pellucida in mammals and the jelly coat in many invertebrates). The interacting gametes use this first-phase adhesion step not only to bind to each other, but also to initiate a signal transduction cascade that activates the sperm and exposes new, fusogenic regions of the sperm plasma membrane. In the second phase of fertilization, the membrane fusion reaction, the plasma membranes of the two gametes come into intimate contact and then fuse, bringing about cytoplasmic continuity (Primakoff and Myles 2002; Rubinstein et al. 2006). Although these two steps—prefusion attachment of the plasma membranes of gametes and merger of their lipid bilayers—have been experimentally separated using in vitro bioassays, gene disruption studies to date have failed to distinguish the two, and no genes have been identified whose disruption allows prefusion attachment and disallows membrane merger. In mice, several proteins involved in gamete membrane interactions have been described, including ADAMS family members and CRISP proteins on sperm and integrins and tetraspanin family members CD9 and CD81 on eggs (for review, see Ellerman et al. 2006; Inoue et al. 2007; Primakoff and Myles 2007). Izumo, an immunoglobulin superfamily sperm protein that appears to be limited to mammals, is gamete-specific and shown by gene disruption to be essential at a late step in fertilization. Izumo is the best

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candidate to date for a role in the membrane fusion reaction in mice. Its specific function has yet to be determined, however, and the presence of conserved Ig superfamily domains predicts a role in membrane adhesion (Inoue et al. 2005, 2007; Primakoff and Myles 2007).

In nematodes, several genes have been identified whose disruption leads to sterility. The *Caenorhabditis elegans* gene SPE-9 is essential for gamete interactions, but it is proposed to be an adhesion and signaling molecule and probably is not involved in the membrane fusion reaction (Putiri et al. 2004). Other proteins are implicated in gamete interactions in worms, including EGG-1, EGG-2, SPE-38, and SPE-42 (Chatterjee et al. 2005; Kadandale et al. 2005; Kroft et al. 2005), but their precise roles in fertilization also are unclear, in large part because of the difficulty of studying sperm–egg interactions in vitro. The protein Prm1p has been implicated in cell–cell fusion of *Saccharomyces cerevisiae*. Its disruption reduces fusion of *a* and *alpha* cells by 50%, but only when the gene is disrupted in both cell types (Heiman and Walter 2000; Aguilar et al. 2007; Heiman et al. 2007); thus, it is not essential for fusion. In invertebrates, at least two sperm proteins have emerged as candidate adhesion/fusion molecules—an 18-kDa protein in abalone (Swanson and Vacquier 1995) and the sea urchin protein bindin (Kamei and Glabe 2003). Because of the difficulty of generating mutants in these organisms, the functions of the abalone and urchin proteins in gamete interactions remain unidentified.

A recent screen for sterile mutants in *Arabidopsis* identified the male-specific sterility gene *HAP2* (Johnson et al. 2004). A *HAP2* family member called *GCS1* (for generative cell-specific) was subsequently identified in a screen for lily genes whose transcripts were up-regulated in sperm (generative cells) (Mori et al. 2006). In *Arabidopsis*, the only organism for which *HAP2* mutants are available, the gene is involved in pollen tube guidance, is expressed in sperm, and also is essential for seed formation (Johnson et al. 2004; Mori et al. 2006; von Besser et al. 2006). Although the gene acts after sperm deposition in the female gametophyte, its cellular and molecular functions in seed formation are unknown, since the cellular events subsequent to sperm deposition—sperm migration, sperm–egg attachment, and sperm–egg fusion—have not been distinguished experimentally. Interestingly, *HAP2* is conserved, and in addition to being in *Arabidopsis* and rice (Johnson et al. 2004), members were found in *Chlamydomonas*, a red alga, a slime mold, *Plasmodium falciparum*, and *Leishmania major* (Mori et al. 2006).

In contrast to fertilization in the organisms described above, fertilization in some protists is highly amenable to study. In the unicellular, biflagellated green alga *Chlamydomonas reinhardtii*, initial adhesion of the flagella of mating type *minus* and mating type *plus* gametes in the first phase of interactions triggers cilium-generated signaling (Wang et al. 2006) and gamete activation (for review, see Pan and Snell 2000; Goodenough et al. 2007). Gamete activation prepares the gametes for fusion and comprises a complex signaling pathway including a protein tyrosine kinase (Wang and Snell 2003), a cGMP-dependent protein kinase (Wang et al. 2006), a flagellar adenylyl cyclase (Saito et al. 1993; Zhang and Snell 1994), and a 10- to 20-fold increase in cAMP. Both gametes shed their glycoproteinaceous cell walls through the action of a metalloprotease released from each cell (Matsuda et al. 1985; Buchanan et al. 1989). And sites specialized for cell fusion—the mating structures—located on the apical cell membranes between the two flagella of both gametes are activated (Goodenough et al. 1982; Wilson et al. 1997). The activated *plus* mating structure is a microvillus-like cellular extension ~3 µm in length and ~0.15 µm in diameter. The activated *minus* mating structure lacks actin filaments and is shorter and more bulbous. Continued flagellar adhesion brings the activated mating structures into intimate contact, and within seconds after contact, the membranes of the two mating structure fuse, followed by complete coalescence of the two gametes into a single, quadriflagellated zygote. The entire process of fertilization, from initial flagellar adhesion of gametes through fusion, can occur within 30 sec or less.

Previously, we showed that the *plus* gamete-specific protein FUS1, which is not found in other species, is present on the plasma membrane of the mating structure, the fertilization tubule. Furthermore, in experiments with flagellar adhesion mutants, we demonstrated that wild-type *plus* gametes, but not *fus1 plus* gametes, were capable of adhering to *minus* gametes solely via their activated mating structures. Thus, during the membrane fusion reaction, *plus*-specific FUS1 is essential for prefusion attachment between the *plus* and *minus* mating structures (Ferris et al. 1996; Misamore et al. 2003).

Fertilization in the rodent malaria organism, *Plasmodium berghei*, is also highly amenable to study. Sexual precursor stages, the gametocytes, form in the vertebrate host inside infected erythrocytes, but remain quiescent until ingested by a susceptible *Anopheles* mosquito. In the bloodmeal, gametocytes emerge from their host cells and within minutes differentiate into gametes. Each female (macro) gametocyte gives rise to a single immotile female gamete, while male (micro) gametocytes generate up to eight flagellated male gametes in a process termed "exflagellation"; within minutes after release, the gametes meet, adhere for a few seconds, and then fuse to form a zygote (Sinden 1983). Male gamete adhesion to a female gamete requires the species-limited surface protein and transmission-blocking vaccine candidate P48/45 (van Dijk et al. 2001). P48/45 interacts physically with at least one other gametocyte protein, P230 (Kumar 1987), and in *P. falciparum* is required to retain the complex on the cell surface once gametes have emerged from their host cells (Eksi et al. 2006). The male-specific function of the P48/45–P230 complex is in contrast with its expression in both male and female gametes, and whether either protein on male gametes binds directly to a receptor on female gametes is unknown.

Within 15–20 h, the zygote transforms into a motile ookinete, which penetrates the midgut epithelium and

establishes the infection in the mosquito by forming an oocyst between the midgut epithelial cells and their underlying basal lamina. Thus, gamete adhesion and fusion are obligate steps in mosquito transmission of malaria and attractive targets for transmission-blocking vaccines. In the rodent malaria parasite *P. berghei*, gametocytes respond efficiently to well-characterized developmental triggers (Billker et al. 1998) in vitro, and gametogenesis, fertilization, and ookinete formation are accessible to analysis in culture. Moreover, targeted gene disruption is now a routinely used method in *Plasmodium* (Janse et al. 2006).

To date, no widely conserved mechanism of gamete fusion has been identified (Chen and Olson 2005; Rubinstein et al. 2006; Primakoff and Myles 2007). Thus, it remains unknown for any organism whether adhesion of gamete membranes and fusion of the membranes are together accomplished by a single set of proteins, as with fusion of many viruses (Earp et al. 2005), or if the two functions are allocated to distinct sets of proteins. We also do not understand the molecular basis for the species specificity of gamete fusion in many organisms (Ferris et al. 1997; Swanson and Vacquier 2002; Vieira and Miller 2006).

Here, in coupled studies of fertilization in *Chlamydomonas* and *Plasmodium*, we show that gamete fusion requires the plant sterility gene *HAP2*. We genetically distinguish attachment of gamete fusogenic membranes from membrane merger, and show that the membrane fusion reaction is governed by species-limited proteins required for prefusion attachment. In both species, post-adhesion events resulting in membrane fusion depend on the conserved HAP2 protein.

Results

A screen for zygote formation mutants in Chlamydomonas *identifies a homolog of an* Arabidopsis *male sterility gene*

To identify proteins in *Chlamydomonas minus* gametes that are essential at a late stage of gamete interactions, we generated insertional mutants in *Chlamydomonas minus* cells (strain *B215*, mt^-) by transformation with a paromomycin resistance gene (Sizova et al. 2001). Colonies that grew on paromomycin were induced to undergo gametogenesis, and the gametes were mixed with wild-type *plus* gametes and screened for their ability to undergo flagellar adhesion within minutes after mixing and to form the zygote aggregates that appear ~4 h after mixing. After screening ~2500 insertional mutants, we identified one transformant with the desired phenotype, *63B10*. Gametes of *63B10* adhered via their flagella to wild-type *plus* gametes and formed small clusters visible at 10 min after mixing similarly to wild-type *minus* gametes (Fig. 1A, top panels). On the other hand, the *63B10 minus*/wild-type *plus* mixtures failed to form the large zygote aggregates characteristic of wild-type/wild-type gamete mixtures at 4 h and remained in the small clusters (Fig. 1A, bottom panels). In contrast to this easily observed phenotype when they were gametes, the growth, motility, phototaxis, and morphology of *63B10* vegetative cells were indistinguishable from those of wild-type vegetative cells. When we examined wild-type *minus*/wild-type *plus* gamete mixtures and *63B10 minus*/wild-type *plus* gamete mixtures soon after mixing, we found that, as expected, most wild-type cells had fused to form quadriflagellated zygotes, but *63B10 minus*/wild-type *plus* mixtures failed to form quadriflagellated cells. Instead, the *63B10 minus* gametes continued flagellar adhesion with the wild-type *plus* gametes, many visible as pairs of cells with their flagella entwined and their apical ends closely apposed (Fig. 1B).

After confirming by Southern blotting (Fig. 1C) that *63B10* contained a single insertion of the paromomycin resistance gene, we used thermal asymmetric interlaced PCR (TAIL–PCR) to identify 180 base pairs (bp) of genomic sequence adjacent to the inserted plasmid. Searches of version 2.0 of the *Chlamydomonas reinhardtii* genome sequence from the DOE Joint Genome Institute identified C_530033 as the adjacent gene (Fig. 1D). Transformation of *63B10* cells with BAC 20L3, which contained several putative genes in addition to C_530033, restored their ability to form zygotes (data not shown), thus confirming that we had identified the genomic region containing the disrupted gene. To confirm that C_530033 indeed was sufficient for rescue of zygote formation, we transformed *63B10* cells with a 20L3 restriction fragment whose only full-length gene was C_530033. As shown in the diagnostic PCR in Figure 1E, the transformants in which formation of quadriflagellated zygotes was restored contained the rescuing wild-type gene as well as the disrupted gene. Moreover, only wild-type gametes and rescued *63B10* gametes formed the macroscopic aggregates characteristic of zygotes 4 h after mixing wild-type *minus* and *plus* gametes (Fig. 1F). Thus, C_530033 was essential for gamete fusion. To our surprise, BLAST searches of NCBI databases (Altschul et al. 1997) showed that C_530033 encodes the *Chlamydomonas* homolog of *HAP2*, the *Arabidopsis* pollen tube guidance and male sterility gene first reported by Johnson et al. (2004), Mori et al. (2006), and von Besser et al. (2006).

HAP2 is present in multicellular animals and in P. berghei

Using PSI-BLAST (Altschul et al. 1997), we extended previous results (Johnson et al. 2004; Mori et al. 2006) on the species distribution of *HAP2*, and found *HAP2* family members in many higher plants whose genome sequences are available including maize, wheat, and tomato (data not shown), as well as in many other non-pathogenic and pathogenic protists including *Toxoplasma gondii*, *Cryptosporidium hominis*, *Theileria parva*, *Naegleria gruberi*, and *Trypanosoma brucei* (Fig. 1G). The *HAP2* gene is also present in a choanoflagellate (*Monosiga brevicollis*), one of the closest unicellular relatives of animals. Consistent with its presence in *Monosiga*, *HAP2* family members were also present in multicellular animals including *Hydra magnipapillata*, the starlet sea anemone *Nematostella vectensis*, and in-

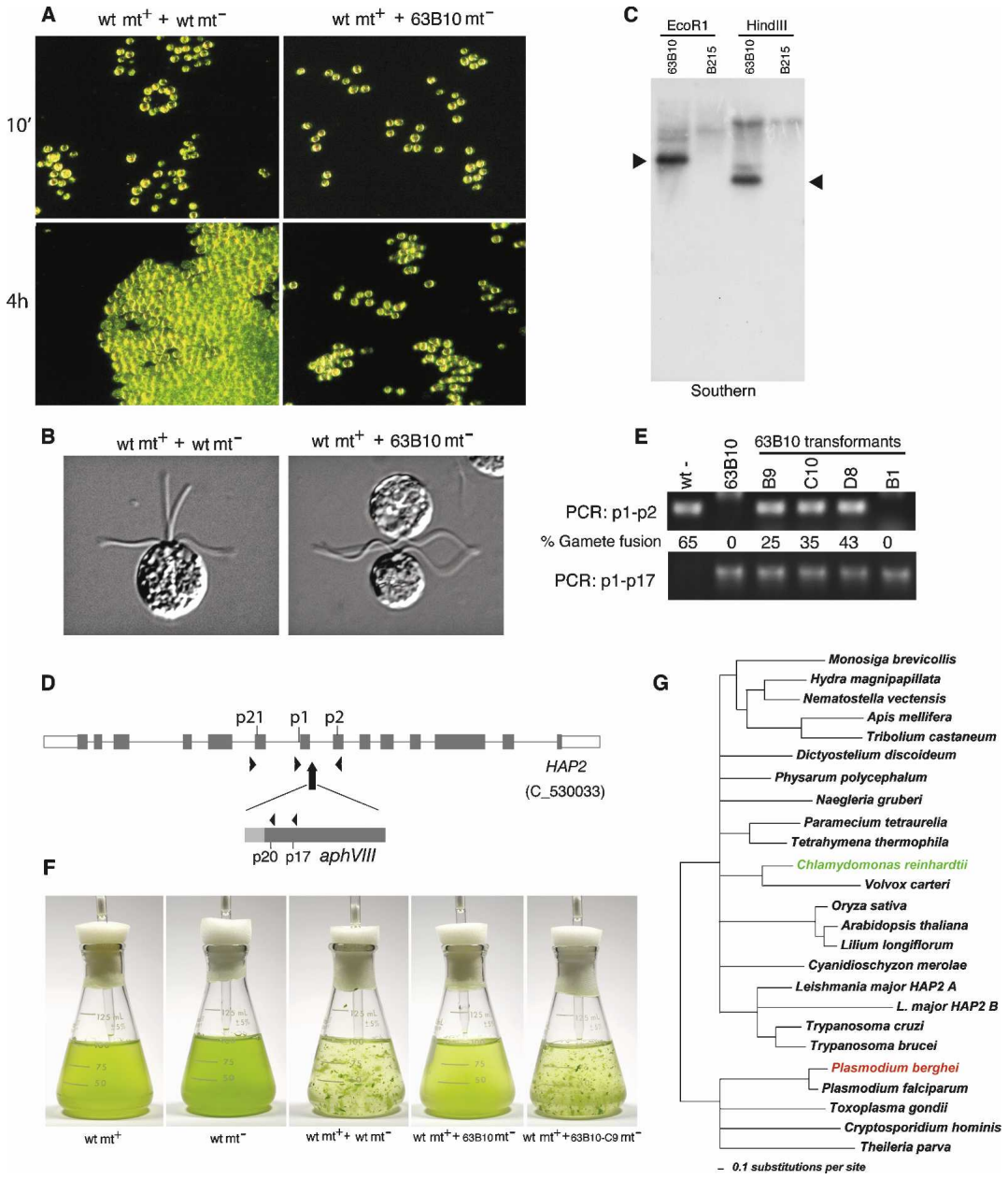

Figure 1. *HAP2* is required for fertilization in *Chlamydomonas* and is phylogenetically conserved in many eukaryotes. (*A*) Dark-field images of wild-type *plus* gametes 10 min and 4 h after mixing with wild-type or *63B10 minus* gametes. Clusters of gametes undergoing flagellar adhesion are present in both samples at 10 min. A large aggregate of zygotes is visible in the wild-type mixture at 4 h, whereas the *63B10 minus* gametes failed to form zygote aggregates with wild-type *plus* gametes, and the small clusters persisted. (*B*) Differential interference contrast (DIC) microscopy images of a quadriflagellated zygote formed from fusion of a wild-type *plus* gamete with a wild-type *minus* gamete (*left* panel) and a wild-type *plus* gamete undergoing flagellar adhesion with a *63B10 minus* gamete, but failing to fuse (*right* panel). (*C*) Southern blotting of EcoRI- and HindIII-digested *Chlamydomonas* genomic DNA from *63B10* and wild-type (*B215*) strains with a pSI103/PvuII DNA fragment as a probe. Arrowheads indicate the pSI103 plasmid in both samples. (*D*) Structure of the *HAP2* gene and location of the *aphVIII* gene pSI103 plasmid. (*E*) Diagnostic PCR on genomic DNA showing the presence of disrupted *HAP2* (primers p1–p17) in *63B10* gametes and the absence of intact *HAP2* in *63B10* gametes and its reappearance in those *63B10* gametes that were rescued for fusion with the wild-type *HAP2* gene (primers p1/p2). (*F*) Large aggregates of zygotes were present only in mixtures of wild-type *plus* and *minus* gametes and wild-type *plus* and *63B10 minus* gametes rescued with the *HAP2-HA* construct (*63B10-C9*). (*G*) Phylogenetic tree based on trees generated using MOLPHY and TREE-PUZZLE illustrating the relationships of *HAP2* proteins from several species (see Supplemental Material for accession numbers and methods).

sects (the honeybee and the flower beetle) (Fig. 1G). An alignment of predicted HAP2 proteins generated by PROMALS (Pei and Grishin 2007) and phylogenetic trees of HAP2 proteins generated by MOLPHY (Adachi and Hasegawa 1996) and TREE-PUZZLE (Jones et al. 1992) are shown in the Supplemental Material.

Previous studies on *HAP2* in *Arabidopsis* showed that it was involved in pollen tube guidance (Johnson et al. 2004; von Besser et al. 2006) and that it had a second role in seed formation after release of sperm from the pollen tube (Johnson et al. 2004; Mori et al. 2006; von Besser et al. 2006). Our results showing that *Chlamydomonas HAP2* mutants were fully motile and fully capable of flagellar adhesion demonstrated that the protein functions directly in the interactions between *minus* and *plus* gametes at a step in fertilization after initial gamete recognition. Moreover, we found that in addition to being present in the human malaria parasite *P. falciparum* (Mori et al. 2006), *HAP2* was also present in the rodent malaria parasite *P. berghei* (Fig. 1G), in which sexual development is most amenable to experimentation. We therefore chose this species to ask if *HAP2* functioned directly in gamete interactions in an organism that is only very distantly related to plants and green algae.

Plasmodium *HAP2 is essential for mosquito transmission of malaria*

To test for a function of *HAP2* in *P. berghei*, we used targeted gene disruption. Cultured *Plasmodium* schizonts were electroporated with a targeting vector that contained an expression cassette conveying resistance to pyrimethamine and designed to replace all of the protein-coding sequence of the *P. berghei HAP2* gene (GenBank accession no. XM_671808) (Fig. 2A). Following dilution cloning of drug-resistant parasites, genotyping by Southern blotting (Fig. 2B) of two independently produced *hap2* clones and diagnostic PCR (Fig. 2C) documented that the replacement construct had integrated at the targeted site.

Analysis by RT–PCR showed that *HAP2* transcripts were present in wild-type gametocytes, but not in *hap2* gametocytes or in wild-type asexual erythrocytic stages of a gametocyte-deficient parasite strain (Fig. 2D). Consistent with this sexual stage-specific transcription, examination of mice infected with *hap2* clones showed that the parasites underwent normal asexual development in erythrocytes. Neither the rate of gametocyte formation nor the sex ratio was affected, and gametocytes were able to emerge from their host cells and differentiate into gametes when exposed to activating conditions (data not shown). To test for a role of *HAP2* in fertilization, we first allowed female *Anopheles* mosquitoes to feed on mice infected with *hap2* parasites and 10

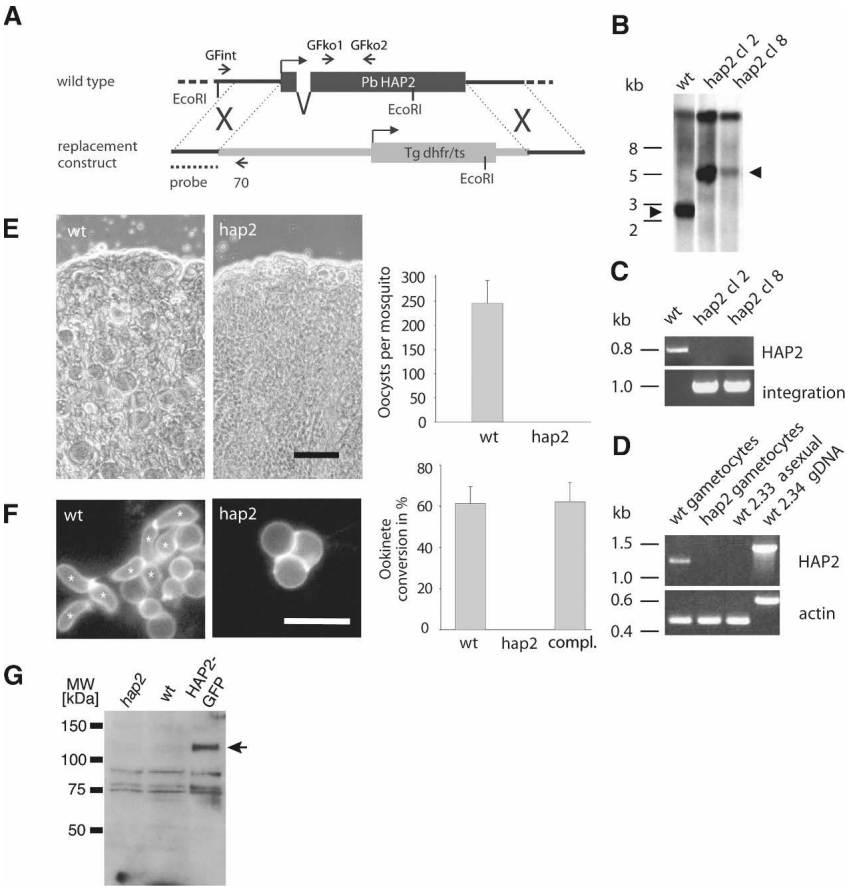

Figure 2. *HAP2* is essential for sexual development and mosquito transmission of *P. berghei*. (*A*) Structure of the *Plasmodium HAP2* gene and gene replacement construct. Short arrows indicate oligonucleotides used for PCR genotyping. (*B*) Southern hybridization of EcoRI-digested genomic DNA using the 5′ targeting sequence as a probe. Arrowheads indicate diagnostic 2.8-kb (wild-type) and 5.0-kb (*HAP2*) bands. (*C*) Diagnostic PCR with genomic DNA templates and primers GFko1/GFko2 to test for the presence of *HAP2*, and primers GFint/70 to detect a unique 1-kb product across the integration site. (*D*) RT–PCR detection of *HAP2* transcript in parasite lines and stages (the expected larger product from genomic DNA includes one intron). (*E*) Representative images of midguts from *A. stephensi* mosquitoes 10 d after feeding on wild-type and *hap2*-infected mice (bar, 100 µm) and bar chart showing average numbers of oocysts per gut (error bar indicates SEM; $n = 47$ wild-type or *hap2*-exposed mosquitoes from three independent experiments). The overall prevalence of infection was 87% for wild type, and 0% for *hap2*. (*F*) Immunofluorescence images of live 20-h *Plasmodium* cultures immunostained for the female gamete/zygote marker P28. Elongate ookinetes (asterisks) were absent from the *hap2* mutant (bar, 10 µm), which possessed only round female gametes. The bar chart shows ookinete conversion rates for wild type, *hap2* clone 8, and the *hap2* mutant that was complemented with *HAP2-GFP*. Conversion rate is expressed as the percentage of P28-positive parasites that had progressed to the ookinete stage (error bar indicates SD; $n = 3$). (*G*) Western blot analysis with anti-GFP, showing expression of a HAP2-GFP fusion protein (arrow) of the expected motility (120 kDa predicted) only in the complemented parasite line. Nonspecifically recognized bands confirm equal loading.

d later used phase contrast microscopy to examine the walls of midguts from the mosquitoes for the presence of oocysts. As shown in Figure 2E, whereas oocysts were plentiful in midguts of control mosquitoes allowed to feed on mice infected with wild-type *P. berghei* (Fig. 2E, left panel of photomicrograph and bar graph), we failed to detect oocysts in the mosquitoes that were fed on mice infected with *hap2* parasites (Fig. 2E, right panel of photomicrograph and bar graph). Thus, *HAP2* is required for transmission of *P. berghei* to mosquitoes.

The complete block of oocyst formation and thus of malaria transmission in vivo was similar to our finding that *Chlamydomonas hap2* mutants failed to form zygotes. To determine if zygote formation per se was also blocked in *P. berghei hap2* mutants, we examined ookinete formation in blood collected from mice infected with *hap2* parasites. Ookinetes are differentiated, motile forms of *Plasmodium* zygotes. They are capable of invading the midgut epithelium, passing through the midgut cells, and taking up residence beneath the basement membrane, where they become static oocysts. Gametocytes in the infected blood were induced to escape from blood erythrocytes and allowed to undergo gametogenesis and gamete fusion by incubation in ookinete medium at 19°C. After ~24 h, the cultures were stained with an antibody against P28, a surface protein of activated female gametes, zygotes, and ookinetes. As shown in Figure 2F, in blood cultures from mice infected with wild-type parasites, most activated female gametes were fertilized and thus transformed into characteristically banana-shaped ookinetes. Blood cultures from mice infected with *hap2* parasites failed to produce ookinetes (Fig. 2F), a finding we confirmed with another *hap2* mutant clone from an independent transfection (data not shown). When we reintroduced a wild-type *HAP2* gene fused to a coding sequence for green fluorescent protein (GFP) into the disrupted *hap2* locus of the mutant, gametocytes expressed a fusion protein of the predicted size (Fig. 2G), ookinete formation was fully restored (Fig. 2F, bar graph), and the complemented parasites were able to complete their life cycle through the mosquito (data not shown). These results are consistent with a key role for *HAP2* upstream of zygote formation during fertilization in *Plasmodium*.

HAP2 is a sex- and gamete-specific protein in Chlamydomonas *and* P. berghei *and functions after gamete activation*

To dissect the function of *HAP2* in *Chlamydomonas* fertilization, we asked whether it was required in *minus* or *plus* gametes or both. Studies in *Arabidopsis* were consistent with a male-specific role of the protein in seed formation (Johnson et al. 2004; Mori et al. 2006; von Besser et al. 2006), whereas RT–PCR studies by Mori et al. (2006) indicated that *HAP2* was expressed in mt^+ and mt^- gametes of a slime mold (*Physarum polycephalum*) and in *Chlamydomonas minus* and *plus* gametes, although expression was stronger in *Chlamydomonas minus* than *plus*. To generate *plus* gametes containing only the *hap2* allele, we crossed wild-type *plus* gametes with *63B10 minus* gametes that had been rendered fusion-competent by transformation with the wild-type *HAP2* gene and selected *plus* progeny that contained only the mutant allele. Southern blotting confirmed that the *plus* cells contained only the *hap2* allele (Fig. 3A, top panel). *Plus* strain cells that lacked a functional *HAP2* gene exhibited no detectable mutant phenotype during vegetative growth or gametogenesis, and the *hap2 plus* gametes underwent gamete fusion to form quadriflagellated zygotes similarly to wild type (Fig. 3A, bottom panel). Thus, HAP2 protein is essential in fusion of *Chlamydomonas minus* gametes only.

Malaria parasites always produce gametocytes of both sexes at the same time, and fertilization experiments with pure male or female gamete populations are not practical. Cross-fertilization experiments, however, with known gender-specific sexual development mutants, such as the male-deficient *cdpk4* or the female-defective *nek4* mutant (Billker et al. 2004; Reininger et al. 2005), make it possible to detect gender-specific sterility phenotypes. As shown in Figure 3B, neither *cdpk4* nor *nek4* strains produced ookinetes when cultured on their own, but when cultures containing both mutants were mixed, *nek4* male gametes productively fertilized *cdpk4* female

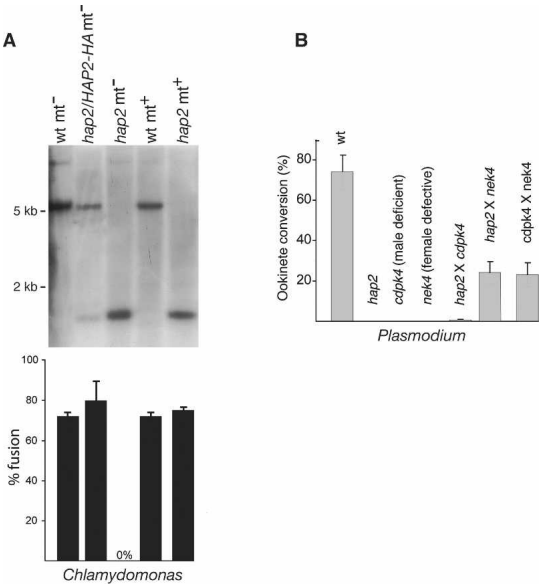

Figure 3. *HAP2* has a sex-restricted function in both *Chlamydomonas* and *Plasmodium*. (*A*) *Chlamydomonas hap2 plus* gametes can fuse with wild-type *minus* gametes. (*Top* panel) Genotyping by Southern hybridization of one mating partner. The second mating partner was always wild type (not shown). The 5.3 kb Not1 fragment is diagnostic of wild-type *HAP2* and the 1.3-kb fragment is diagnostic of the disrupted allele. (*Bottom* panel) Percentage of the indicated gametes that fused when mixed with wild-type gametes of the opposite sex. (*B*) In vitro malaria ookinete conversion analysis demonstrates that the *Plasmodium hap2* mutant shows productive cross-fertilization with the *nek4* sterility mutant, which produces functional males only, and not with *cdpk4*, which produces functional females only (error bar indicates SD; $n = 3$).

gametes, restoring the capacity to form ookinetes (Fig. 3B). The reduced conversion rate compared with wild type was expected, because cultures also contained *nek4* female gametes, which are unable to convert to ookinetes. In *hap2*/*nek4* crosses the *hap2* female gametes were productively fertilized by *nek4* male gametes, but *cdpk4* females remained unable to differentiate into ookinetes in *cdpk4*/*hap2* crosses (Fig. 3B), showing that *hap2* males are sterile. Thus, our results demonstrate that during fertilization in *Chlamydomonas* and *Plasmodium*, *HAP2* is essential in gametes of only one sex, *minus* in *Chlamydomonas* and male in *Plasmodium*.

Unlike many organisms whose gametes possess an extracellular matrix that must be removed before fusion, *Plasmodium*'s gametes are "naked" (Sinden 1983). We reasoned, therefore, that HAP2 also would function at a step in *Chlamydomonas* fertilization when the gametes are "naked"; that is, after gamete activation and degradation of their glycoproteinaceous cell walls. One of the first events in the cilium-generated signaling pathway triggered by flagellar adhesion is release into the medium of an active metalloprotease that degrades the gamete cell walls. The experiments shown in Figure 1 demonstrated that HAP2 functioned after flagellar adhesion, but they did not address whether the *hap2* gametes were capable of being activated when mixed with wild-type *plus* gametes, nor did they address whether *hap2 minus* gametes were capable of activating wild-type *plus* gametes during flagellar adhesion. To test whether *hap2* mutants possessed the signaling pathway essential for cell wall degradation, we incubated them in dibutyrlyl cAMP, a cell-permeable analog of the second messenger that activates *Chlamydomonas* gametes (Pasquale and Goodenough 1987) and used a detergent sensitivity assay to assess cell wall loss (Snell 1982). To determine if *hap2* cells responded to more physiological triggers of gamete activation, we also assessed wall loss in mixtures of *hap2* gametes with wild-type *plus* gametes and with flagella isolated from wild-type *plus* gametes. As shown in Figure 4A, all of the gametes in such mixtures lost their cell walls in each of the three experimental conditions. The *hap2 minus* gametes released their walls when mixed with wild-type *plus* cells, indicating that at least one of the gametes was activated by the interaction; they released their walls when incubated with cAMP, indicating that the signaling pathway was intact in the absence of flagellar adhesion; and they released their cell walls when undergoing flagellar adhesion with isolated *plus* flagella (Snell 1982), indicating that they were capable of responding to flagellar adhesion per se and releasing their own metalloprotease. Thus, the flagellar adhesion-induced gamete activation pathway was fully functional in *hap2* gametes.

The above experiments, nevertheless, would not have indicated whether the defect in *hap2 minus* gametes abrogated their ability to activate *plus* gametes, since it was possible, for example, that the wall loss we observed in the mixture of *hap2* and wild-type *plus* gametes was due to release of the metalloprotease from the *minus* gametes only. When we examined the *plus* cells in such mixtures, however, we found that they formed the typical, actin-filled fertilization tubules that characterize activated *plus* gametes (Fig. 4B). In the electron micrograph (Fig. 4B, right panel), the actin filaments within the fertilization tubule are visible and can be seen arising from the submembranous doublet zone (Fig. 4B, arrowheads) at the base of the process. The fringe material (Fig. 4B, arrow) on the outer surface of this wild-type *plus* mating structure is reported to be a manifestation of the FUS1 protein and is absent in *fus1* mutants (Goodenough et al. 1982). In an independent approach to confirm that the defect in the *hap2* gametes was downstream from cAMP signaling, we found that addition of db-cAMP to samples of adhering wild-type *plus* gametes and *63B10 minus* gametes failed to rescue the formation of zygote aggregates (data not shown). Thus, *HAP2* is not required for activation of *minus* gametes, nor is it required for *minus* gametes to activate *plus* gametes to undergo the cytoskeletal reorganization required for formation of the fertilization tubule.

HAP2 is exposed on the external surface of the plasma membrane of Chlamydomonas *and localized at the minus mating structure, a site specialized for cell fusion*

To dissect further the function of HAP2 in gamete interactions, we introduced an *HAP2-HA* into *63B10* gametes. Immunoblotting with an anti-hemagglutinin (HA) antibody showed that HAP2-HA, which exhibited the predicted molecular mass of ~120 kDa, was expressed only in gametes and was not detectable in vegetative cells (Fig. 4C). The presence of two closely spaced isoforms of HAP2-HA in SDS-PAGE (Fig. 4D) suggested that the protein undergoes post-translational modification. If HAP2 were exposed on the external surface of the cell, as expected from the presence of a signal peptide and a single transmembrane domain near the C terminus of the predicted protein, then it should be sensitive to protease treatment of live cells. To determine if HAP2 is exposed at the cell surface, HAP2-HA-expressing *minus* gametes were incubated for 20 min at room temperature with 0.01% trypsin. Examination by phase contrast microscopy of the trypsin-treated gametes showed that the cells remained fully viable and motile, indicating that the protease treatment was not toxic to the cells. Analysis by immunoblotting indicated that the staining profile of HAP2-HA was modified by the trypsin treatment. The upper form was trypsin-sesitive and only a more rapidly migrating form remained. Although further experiments would be required to determine the relationships between the HAP2-HA isoforms present before and after trypsin treatment, this result demonstrated that at least one of the isoforms is exposed on the external surface of gametes (Fig. 4D). The presence on the immunoblots of equal amounts of cGMP-dependent protein kinase (PKG) (Wang et al. 2006), which is not exposed on the external cell surface, confirmed equal loading on the control and trypsin-treated lanes.

Immunofluorescence imaging of *minus* gametes ex-

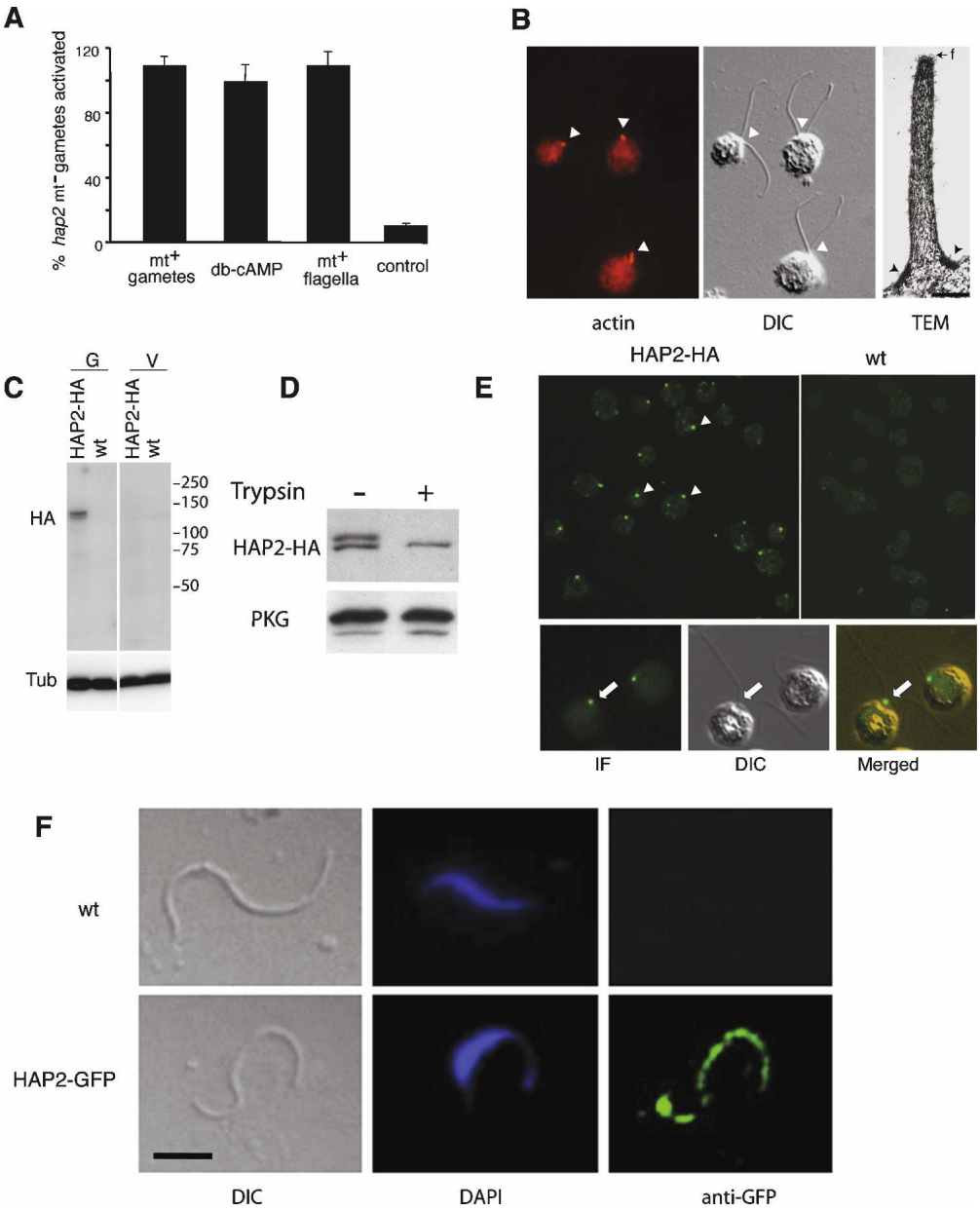

Figure 4. HAP2 functions after gamete activation, is present at the surface of the *minus* mating structure in *Chlamydomonas* (*A–E*), and is distributed along the length of the male gamete in *Plasmodium* (*F*). (*A*) *63B10* gametes were activated by incubation with wild-type *plus* gametes, flagella isolated from wild-type *plus* gametes, or db-cAMP. The percentage of cells that was activated was determined by measuring cell wall loss. (*B*, *left* panel) Fluorescence image of activated *plus* gametes in a mixture of wild-type *plus* gametes and *63B10 minus* gametes. The arrowheads in the fluorescence (*left* panel) and DIC (*middle* panel) images indicate the site of the actin-filled mating structures. The *right* panel is an electron micrograph of an activated mating structure (fertilization tubule) on a wild-type *plus* that had been mixed with *hap2 minus* gametes. The actin filaments within the fertilization tubule originate from the cone-shaped, electron-dense doublet zone (arrowheads) at the base of the process (Detmers et al. 1983). Fringe (f; arrow) is visible on the surface of the fertilization tubule. Bar, 200 nm. (*C*) Immunoblotting with an anti-HA antibody documents that *63B10* cells rescued with HA-tagged *HAP2* expressed HAP2-HA protein only in the gamete phase of their life cycle. (*D*) Immunoblotting with anti-HA antibody indicates that only a single form of HAP2-HA remained after treatment with 0.01% trypsin for 20 min at room temperature. (*E*) Anti-HA immunostaining combined with DIC microscopy of *HAP2-HA* gametes demonstrates that HAP2-HA is localized between the two flagella at the site of the *minus* mating structure. (*F*) Anti-GFP immunostaining and DIC microscopy of HAP2-GFP gametes of *P. berghei* shows HAP2-GFP localized along the length of the male gamete (bar, 2 μm).

pressing HAP2-HA showed that the protein was present at a single spot on each cell near the bases of the two flagella, the location of the mating structure (Fig. 4E). Control wild-type *minus* gametes did not stain with the antibody. Thus, the topology and the location of HAP2 were consistent with the cell biological properties of the

protein, all of which pointed to a function of the protein in the membrane fusion reaction, either in prefusion attachment of the fusogenic membranes or in the membrane merger events that are downstream from attachment.

In contrast to studies in *Chlamydomonas*, ultrastructural studies from *Plasmodium* do not suggest that gametes possess mating structures or otherwise specialized cell surface areas to serve as preferred sites for adhesion and fusion (Sinden et al. 1976). We therefore hypothesized that in *Plasmodium* male gametes HAP2 might be more evenly distributed. Consistent with this notion, we detected a faint signal along the length of male gametes by fluorescence microscopy of live HAP2-GFP male gametes (data not shown), a result that was confirmed when fixed cells were stained with anti-GFP antibody (Fig. 4F). Fluorescence microscopy of live HAP2-GFP microgametocytes detected HAP2 on the cell periphery and additionally in one or a few dot-like intracellular locations of unknown identity (data not shown). Consistent with its stage- and gender-specific function in *Plasmodium*, HAP2-GFP expression from the endogenous *HAP2* promoter occurred exclusively in *Plasmodium* male gametocytes and gametes, but neither in female or asexual erythrocytic stages, ookinetes, oocysts, nor salivary gland sporozoites (data not shown).

HAP2 functions downstream from gamete membrane attachment in Chlamydomonas *and* Plasmodium

Previously, we showed that the *plus* gamete-specific *Chlamydomonas* protein FUS1 is localized on the surface of the *plus* mating structure and is essential for prefusion attachment between the mating structures of activated *minus* and *plus* gametes. A simple model for mating structure interactions would be that HAP2 is a binding partner for FUS1, and that this interaction is required for prefusion attachment of the mating structures. To test this model, we examined whether HAP2 was essential for mating structure adhesion. In our initial characterization of the *hap2* mutant, we found that *hap2 minus* gametes bound firmly to wild-type *plus* gametes, but we were unable to establish whether the overall cell adhesion we observed was due to adhesion of the mating structures or of the flagella, or if both were involved in binding the two cells together (Fig. 1). In previously published work, we had also determined that the FUS1 on unactivated *plus* gametes was nonfunctional in adhesion and that *plus* gamete activation was required to generate active FUS1 (Misamore et al. 2003).

To examine mating structure adhesion between *plus* gametes and *hap2 minus* gametes without the interference of flagellar adhesion, we used a *plus* mutant, *sag1-1*, which does not express SAG1, the *plus* flagellar agglutinin (Ferris et al. 2005). Furthermore, since flagellar adhesion is essential for gamete activation, we activated the *hap2* and *sag1-1* gametes for these experiments with dibutyryl cAMP before we mixed them. Such treatment ensured that the cell walls were removed and that the mating structures were exposed and in their activated states. The activated *sag1-1 plus* gametes were subsequently fixed and tagged with a fluorescent marker. To our surprise, when we mixed the activated *hap2* and *sag1-1* gametes together, they formed prefusion attachments with each other at their mating structures (Fig. 5A, right two sets of panels, arrowheads indicate the *sag1-1 plus* gametes), which were indistinguishable from the mating structure adhesions of wild-type *minus* gametes and *sag1-1 plus* gametes (Fig. 5A, left two sets of panels; Misamore et al. 2003). Even though the mating structure interactions were brought about by random collisions of the cells and not by flagellar adhesion, ~20% of the *sag1-1 plus* gametes formed pairs with both the wild-type and *hap2 minus* gametes (Fig. 5A). That the gametes remained as pairs as they were propelled through the medium by flagellar motility indicated that mating structures were tightly attached to each other. Thus, these experiments demonstrated that in the absence of HAP2, FUS1-dependent mating structure adhesion was not perturbed, and that unlike FUS1, HAP2 is

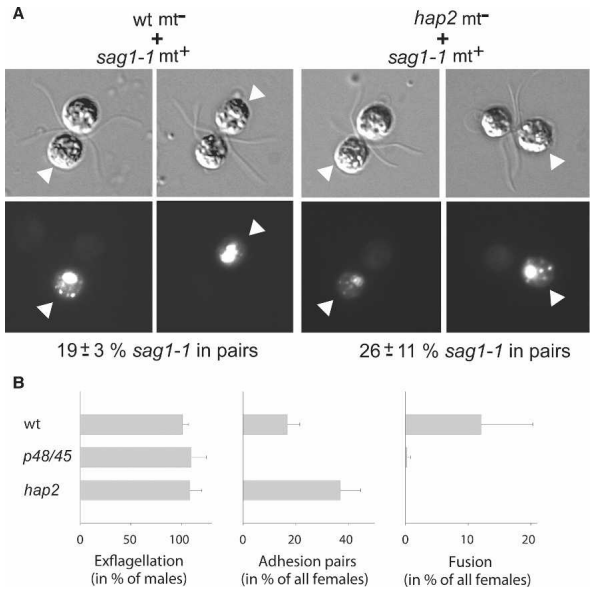

Figure 5. HAP2 functions in the gamete fusion reaction downstream from gamete membrane adhesion. (*A*) Activated live *63B10* gametes, like activated live wild-type *minus* gametes, adhered via their mating structures to activated, fixed, fluorescently tagged *sag1-1 plus* gametes, which are incapable of flagellar adhesion (*top* panel shows differential interference microscopy; *bottom* panel shows fluorescence; arrowheads indicate the *sag1-1 plus* gametes). The percent (±SEM) of *sag1-1* gametes forming pairs when mixed with an excess of activated *63B10* or wild-type *minus* gametes is shown *below* the figure (average from two independent experiments; $n = 150–200$ *sag1-1* cells examined in each). Similar results were obtained when the agglutinin mutant *sag1-2* was used (not shown). Between 0 and 6% pairs were detected in controls in which activated live *sag1-1* gametes were mixed with the fixed *sag1-1* gametes (not shown). (*B*) Efficiency of exflagellation, gamete adhesion, and gamete fusion in wild-type, *p48/45,* and *hap2* clones of *Plasmodium* (error bar indicates SD; $n = 3$ experiments, each examining 100 gametocytes).

dispensable for tight prefusion attachment during the membrane fusion reaction. These data indicate that gamete membrane adhesion and fusion are separate molecular events in which FUS1 and HAP2 function sequentially, rather than through direct interaction.

To determine whether HAP2 functioned at a similar step in the membrane fusion reaction of *Plasmodium* gametes, we used phase contrast microscopy to examine the interactions of *hap2* male gametes with female gametes. As with *Chlamydomonas*, binding of wild-type male gametes to wild-type females is followed within seconds by fusion. Microscopic examination of fertilization in vitro showed that in the absence of *HAP2* or in the absence of *P48/45*, exflagellation occurred normally as expected. On the other hand, the incidence of male/female *Plasmodium* gamete pairs in the *hap2* mutant was approximately doubled compared with wild type (Fig. 5B); the failure to detect fertilization indicated that *hap2* pairs formed and persisted but failed to progress from adhesion to membrane fusion. In marked contrast, in fertilization experiments with a *p48/45* mutant, a complete lack of gamete binding explained fully the absence of fertilization (Fig. 5B), confirming the importance of the *Plasmodium*-specific P48/45-containing complex in gamete adhesion (van Dijk et al. 2001). Thus, as with *Chlamydomonas*, fertilization in *Plasmodium* can be experimentally divided into an adhesion step that relies on at least one species-limited membrane molecule, here P48/45, and a subsequent fusion step requiring HAP2.

Post-attachment membrane merger in Chlamydomonas *requires HAP2*

Although the above experiments pointed to a role for HAP2 in membrane merger per se, it was possible that HAP2 functioned downstream from membrane merger. For example, even though in *Chlamydomonas* the *hap2 minus* gametes did not form quadriflagellated zygotes, membrane merger could have occurred, and HAP2 could have been required to bring about the cellular events that accomplish complete coalescence of the two cells into a single quadriflagellated zygote after cytoplasmic continuity was established. Studies of membrane fusion reactions in other systems have established that fusion can initiate through opening of a proteinaceous pore followed by lipid merger (Han et al. 2004) or, alternatively, with the merger of the outer leaflets of the interacting membranes to form a hemifusion connection, followed by formation of a pore lined by the fused lipid bilayers (Chernomordik and Kozlov 2005; Lu et al. 2005; Reese and Mayer 2005; Xu et al. 2005). To test whether HAP2 functioned downstream from either partial or complete membrane merger in *Chlamydomonas*, we tested for redistribution of a membrane dye (PKH26) (Podbilewicz et al. 2006) from the plasma membranes of labeled wild-type *plus* gametes to wild-type and *hap2 minus* gametes. When the labeled wild-type *plus* gametes (Fig. 6A, top panel, arrowheads) were mixed with unlabeled wild-type *minus* gametes, we detected initiation of redistribution of dye from the *plus* gametes to the *minus* gametes within seconds after their mating structures came into close contact, and complete redistribution occurred immediately thereafter (Fig. 6A, top panels). Interestingly, even the flagellar membranes of the *minus* gametes acquired the dye very quickly (Hunnicutt et al. 1990). On the other hand, when labeled wild-type *plus* gametes were mixed with *hap2 minus* gametes, redistribution of label was never detected in the hundreds of wild-type *plus*/*63B10* pairs examined in several independent experiments (Fig. 6A, bottom panels; data not shown).

Transmission electron microscopy confirmed the absence of fusion in the wild-type/*hap2* mixtures. The top panels of Figure 6B show a wild-type *plus* (cell on right) and wild-type *minus* gamete whose mating structures have just fused (bars in left panel, 200 nm; bar in right panel, 50 nm). The actin filaments within the foreshortened *plus* mating structure were still attached to the doublet zone and had penetrated into the cytoplasm of the *minus* gamete as fusion progressed (see also Detmers et al. 1983). Attesting to the rapidity of the fusion process, even after examining 500–600 wild-type gametes fixed 3 min after mixing, we were unable to capture wild-type gametes in the attachment stage of mating structure interactions or at a stage in fusion earlier than that shown in these images. On the other hand, and consistent with the absence of dye transfer described above, we never detected mating structure fusion in mixtures of wild-type *plus* and *hap2 minus* gametes. Instead, we observed many pairs in which the tip of the mating structure of the wild-type *plus* gamete was closely apposed to the apex of the shorter *minus* mating structure. Figure 6C shows one such pair at low magnification (left panel, bar, 200 nm; arrowheads point to the doublet zone in the *plus*) and higher magnification (right panel, bar, 50 nm). The membranes of the mating structures appeared to be adherent to each other by the FUS1 fringe and were separated by ~10 nm. Taken together, these results demonstrated that HAP2 is essential at a step in the gamete membrane fusion reaction that occurs within seconds after species-specific, tight, prefusion attachment of the fusogenic membranes and that is concomitant with or immediately precedes membrane fusion.

Discussion

By taking advantage of gene discovery methods in *Chlamydomonas*, targeted gene disruption in *P. berghei*, and distinctive features of fertilization in both organisms, we uncovered a novel mechanism for gamete membrane fusion in which species-limited proteins are essential for binding the male and female gamete membranes together and a broadly conserved protein is essential—either directly or indirectly—for fusion per se. Each of these distantly related organisms depends on a species-limited protein—FUS1 in *Chlamydomonas* and a P48/45-containing protein complex in *P. berghei*—for attachment of the plasma membranes of gametes of opposite sex. And each requires a member of the broadly conserved HAP2 protein family to accomplish the subse-

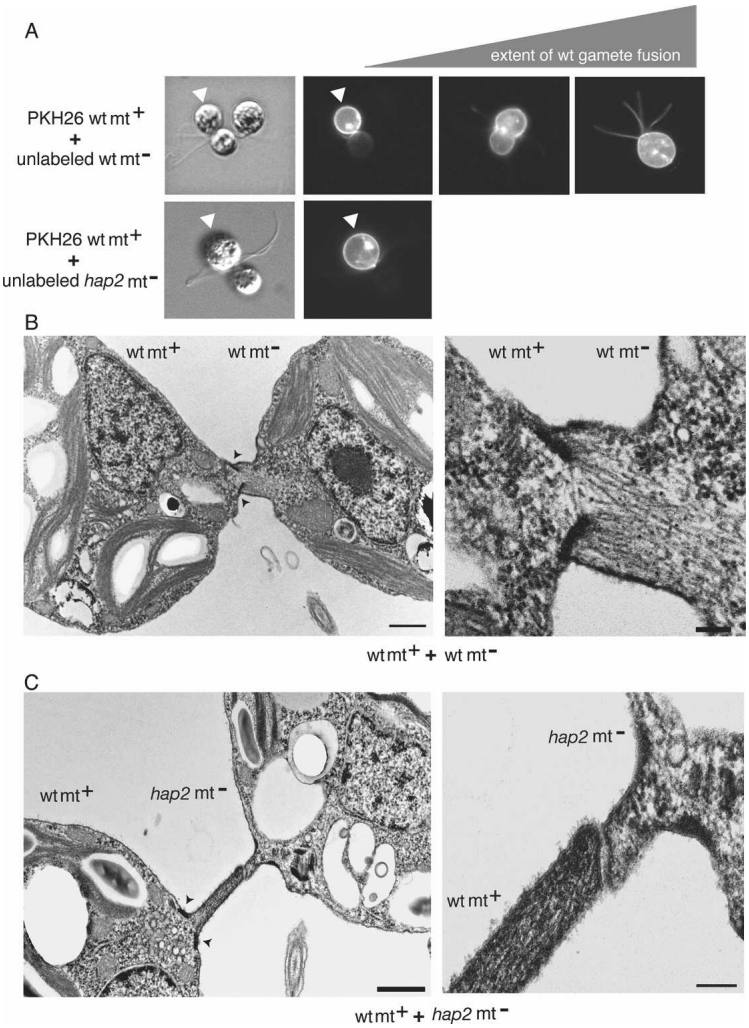

Figure 6. HAP2 is essential for membrane merger in *Chlamydomonas*. (*A*) The plasma membranes of activated *plus* gametes were labeled with the fluorescent lipid PKH26 (arrowheads), mixed with wild-type *minus* gametes (*top* panels) or *63B10 minus* gametes (*bottom* panels), and the live cells were examined by DIC (*left top* and *bottom* panels) and epifluorescence microscopy. (*B*) A pair of wild-type *plus* and *minus* gametes just after fusion is shown in transmission electron microscopy at low magnification (*left* panel; bar, 200 nm) and at higher magnification (*right* panel; bar, 50 nm). The actin filaments of the fertilization tubule of the *plus* gamete (cell on the *left* in both images) have become incorporated into the *minus* gamete as the two cells merge. (*C*, *left* panel) In a mixture of wild-type *plus* gametes and *hap2 minus* gametes, the tip of the fertilization tubule on a wild-type *plus* gamete (cell on the *left*) is tightly associated with the apex of the *minus* mating structure (bar, 200 nm; arrowheads show the doublet zone). (*Right* panel) A higher-magnification view (bar, 50 nm) shows that the membranes of the two mating structures are separated from each other by ~10 nm.

quent membrane merger step of the membrane fusion reaction. *Chlamydomonas* is particularly amenable to use of gene discovery methods to identify fertilization mutants because it is haploid for most of its life, and thus mutant phenotypes are detectable in the immediate progeny of the cell that received the mutagenizing plasmid (in these experiments, an antibiotic resistance gene), and zygotes form aggregates of a distinctive morphology that allows for rapid screening of mutants. *Plasmodium* offers the power of targeted gene disruption methods. The many steps that compose fertilization in both organisms are experimentally accessible, and genes essential at several steps in fertilization have already been identified.

HAP2 is not required for gamete migration or species-limited gamete recognition and signaling

The ability to use mutants and cell bioassays to dissect several steps in fertilization in both organisms allowed us to position HAP2 in the gamete interaction pathway with much more precision than is possible in plants. In vitro fertilization between isolated sperm and eggs has been achieved in rice and maize (Faure et al. 1994; Kranz and Lorz 1994; Khalequzzaman and Haq 2005; Peng et al. 2005), but not yet in *Arabidopsis*. Previous studies had established that in addition to its role in pollen tube guidance in *Arabidopsis*, *HAP2* also was essential in seed formation at a step after deposition of sperm in the synergid cell in the general region of the egg (Johnson et al. 2004; Mori et al. 2006; von Besser et al. 2006). Unknown, however, was the step in fertilization that required HAP2. Mori et al. (2006) proposed that HAP2 might play a role in gamete recognition. It was also possible that the protein was essential for chemotaxis or for motility to bring the sperm and egg together (von Besser et al. 2006).

In our studies, we established that HAP2 in *Chlamydomonas* is dispensable for the flagellar adhesion and signaling necessary for release of cell walls and activation of the fusogenic membranes of gametes. Presumably, these events are not required for the "naked" gametes of *Arabidopsis* or *P. berghei*. Furthermore, the results that the *hap2* mutants in both *Chlamydomonas*

and *P. berghei* were fully capable of motility ruled out the possibility that *HAP2* functioned in migration. Finally, our observations that *hap2* gametes in both *Chlamydomonas* and *Plasmodium* recognized and adhered to gametes of the opposite sex indicated that HAP2 is not essential for species-limited membrane recognition or the tight, prefusion attachment of the fusogenic membranes that precedes membrane fusion.

Although the working model for gamete fusion has been that prefusion attachment and membrane fusion per se depend on separate sets of gene products, the model was not supported by genetic evidence because no mutants were available that allowed adhesion and blocked fusion in any organism. Our results assigning HAP2 function to a step in the gamete membrane fusion reaction after close (10-nm) prefusion attachment is the first gene disruption-based evidence that the gamete membrane fusion reaction depends on at least two separate sets of proteins that function at discrete steps in the reaction. The 10-nm gap between the attached membranes is similar to the gap between *Drosophila* myoblast membranes at the prefusion attachment step of myoblast fusion (Doberstein et al. 1997) and similar to that between *C. elegans* epithelial cells just before EFF-1-dependent cell fusion (Shemer et al. 2004). Presumably, the two membranes are close enough for a fusion protein or protein complex to bridge the gap and cause membranes to merge.

Mechanisms of gamete fusion

Molecular mechanisms of membrane fusion have been best studied in viral fusion and fusion of intracellular vesicles. A complex series of protein-binding and -folding reactions drives both sets of reactions. After an initial adhesion step, the fusion proteins link the opposing membranes, followed by protein folding that pulls the membranes in close proximity. Fusion pores form, and the process culminates in the complete merging of the lipid bilayers (Sollner 2004; Earp et al. 2005). Viruses use a single protein for both specific contact and for fusion itself, and the several classes of viral fusion proteins apparently evolved independently (Earp et al. 2005). Intracellular vesicle fusion employs distinct sets of conserved protein families for each step—rabs and their effectors for specific adhesion and SNARES for membrane merger (Jahn et al. 2003). Examination of the HAP2 protein sequence fails to offer any hints as to its possible role in the membrane fusion reaction. The HAP2 sequence predicts a single-pass transmembrane protein (Mori et al. 2006), and our results document that it is present on the external surface of the plasma membrane of *Chlamydomonas*. On the other hand, HAP2 lacks a predicted fusion peptide and the coiled-coil domains present in many viral fusion proteins.

Formation of syncytia in *C. elegans* depends on a single protein (EFF-1 or AFF-1) (Mohler et al. 2002; del Campo et al. 2005; Sapir et al. 2007), but the fusogen must be present on both of the fusing membranes (Podbilewicz et al. 2006). And myoblast fusion requires a mix of conserved and species-limited proteins for prefusion attachment, signaling, and cytoskeletal rearrangements (Chen and Olson 2005). The proteins in myoblasts that mediate the membrane merger step per se have yet to be identified, although recent studies on the MARVEL domain protein, Singles Bar, showed that it is required for progression past the prefusion attachment stage of myoblast fusion (Estrada et al. 2007).

Whether HAP2 functions directly as a fusogen or has a more indirect, broadly conserved role in hypothetical events after prefusion attachment and before union of the two lipid bilayers remains to be investigated. Membrane fusion reaction mechanisms were invented only infrequently during evolution (Jahn et al. 2003), and the conserved function of HAP2 in the gamete membrane fusion reaction in two widely disparate organisms would thus be consistent with a direct role for *HAP2* in the final event of fertilization.

HAP2 is located specifically at the mating structure of *Chlamydomonas minus* gametes, yet in *P. berghei* it is distributed along the length of the microgamete, suggesting that in the *Plasmodium* microgametes, no organelle or membrane area specialized for gamete fusion may exist. HAP2-GFP was notably not concentrated at the anterior end of the microgamete, where a juxta-kinetosomal sphere and granule of unknown function are located (Sinden et al. 1976). Future work will have to examine the expression and cellular localization of HAP2 on the microgametes of human malaria species, as well as its accessibility to antibodies, which may be able to block malaria transmission by interfering with gamete fusion within the infected blood meal. Consistent with its mosquito stage-specific expression in *P. berghei*, a low frequency of single nucleotide polymorphisms in *HAP2* sequences from different *P. falciparum* isolates (Jeffares et al. 2007; Mu et al. 2007; Volkman et al. 2007) suggests that *PfHAP2* (accession no. PF10_0139) is under no diversifying immune selection, an important property it shares with current transmission-blocking vaccine candidates (Saul 2007).

Gamete fusion, species specificity, and a conserved gene family

Because gamete fusion in many organisms is a species-specific event, it has been unclear whether the elucidation of mechanisms of the membrane fusion reaction in one species will be broadly applicable. The functional conservation of a role for HAP2 at the membrane merger step of fertilization in *Chlamydomonas* and *P. berghei* makes it likely that close to the unresolved root of eukaryote evolutionary history the last common ancestor of both species and of the higher plants would have used HAP2 for gamete fusion. The presence of HAP2 homologs in choanoflagellates and other protists, cnidarians, and some bilaterian animals further suggests that dissecting the molecular function of HAP2 could offer insights into fundamental mechanisms of gamete fusion for a large portion of the Earth's species.

The model that species-specific proteins govern access to a conserved, HAP2-dependent process of membrane

fusion is attractive because it provides an explanation for the observation that many genes involved in gamete recognition diverge rapidly (Ferris et al. 1997; Swanson and Vacquier 2002; Vieira and Miller 2006), although the process of fertilization is fundamental to all eukaryotes. Divergence of the prefusion attachment genes could contribute to establishment of barriers to fertilization that might lead to speciation. The functional separation of membrane adhesion and subsequent events resulting in fusion between two different membranes may thus be the way in which many eukaryotes reconcile two opposite evolutionary needs, on the one hand, to ensure reproductive isolation through rapidly changing gamete recognition mechanisms, and, on the other hand, to preserve the machinery for the biophysically complex process of membrane fusion.

Materials and methods

Chlamydomonas

Unless stated otherwise, strains were from the *Chlamydomonas* Genetics Center, Duke University, Durham, NC. Cell culture, induction of gametogenesis, flagellar isolation, gamete activation by db-cAMP, measurement of cell wall loss, SDS-PAGE, and immunoblotting were as described previously (Wang et al. 2006).

Insertional mutagenesis: We screened 2500 insertional mutants generated in the nitrate reductase-deficient *B215* mt^- strain (Greg Pazour, University of Massachusetts) for clones whose gametes underwent flagellar adhesion for 12–18 h but failed to fuse. Insertional mutants were generated using the plasmid pSI103 linearized with PvuII and transformed into *B215* cells using the glass bead method with selection on agar plates containing 10 µg/mL paromomycin (Sigma) in M medium (Kindle et al. 1989; Fang et al. 2006). Transformed colonies were induced to undergo gametogenesis by transferring them into 96-well plates containing M-N medium. After agitation on a reciprocal shaker for 2 h, 5 µL from each well were transferred into a duplicate 96-well plate containing M media to maintain a stock of the cells in vegetative growth. After continued agitation overnight, samples from each well of the plate with M-N were mixed with wild-type *plus* gametes. Each well was scored on an inverted microscope for flagellar agglutination at 10 min and 4 h. Zygote formation, as determined by the presence of large aggregates of zygotes visible in the inverted microscope, was assessed at 4 h. To confirm a single insertion of plasmid pSI103 in *63B10* cells, Southern blotting was carried out with genomic DNA from wild-type and *63B10* cells digested separately with EcoRI and HindIII. The probe was a pSI103/PvuII DNA fragment labeled using a Random Primed DNA labeling kit (Roche Applied Science).

PCR and TAIL–PCR: We used TAIL–PCR (Liu et al. 2005) to determine that gene model C_530033 (Protein ID 166688; version 2.0 of the *Chlamydomonas* genome sequence at http://genome.jgi-psf.org/cgi-bin/dispGeneModel?db=chlre2&tid=166688) (Grossman et al. 2003) had been disrupted by insertion of the *aphVIII* plasmid pSI103 mutagen (a paromomycin resistance gene) (Sizova et al. 2001) in mutant *63B10*. The specific, nested primers to identify genomic sequence in the 5′-flanking region of the inserted *aphVIII* plasmid in clone *63B10* cells were the following: primary, Aph.p22 (5′-GCGCCCTCATAGCCCGCCAAATC-3′); secondary, Aph.p21 (5′-CCGCCAAATCAGTCCTGTAGCTTC-3′); and tertiary, Aph.p20 (5′-TGCGCGCTTGGCGTAATCATGGTC-3′). The arbitrary degenerate primer was Ad.p24 [(G/C)TAGA(G/C)T(G/C)A(G/C)C(A/T)CA(G/C)] (C. Silflow, pers. comm.). The PCR product from the tertiary reaction, which was cloned and sequenced, is the following (the underlined sequence is C_530033, the lowercase sequence is an *Escherichia coli* cytosine methylase presumably from the plasmid host bacterium, and the nonunderlined sequence is from the *aphVIII* plasmid): (5′-CCGCCAAATCAGTCCTGTAGCTTCCATATCTGATTCGCAATCTTGCCTTGCACCTGCCTGCCACGCTCATACCATGTCGCCGTGACCCCAAAACAGGCCTGTCTGTCCGGCCAGCTCAAGGACCTGTGGGAGGCGGACCTGGCGCGTACCGCGGACGGCCGGGTGCCGCTGTACATGATCACCAGGTTCACTGGCGGCAGCGAGGGCTAATCGCGCCGGAAAATATATCAGTAACcgattcatacagcaccgggaatgccgcacaggcaatgctggagaaactgctgcaaatttatgatgttaaaacgttggtggcgcagcttaatggtgtaggtgagaatcactggagcgcggcaattttaaaacgtgcgctggcgaatgactcggcatggcaccgtttaagtgagaaagagttcgcccatctgcaaacgttattacccaaaccaccggcacatcatccgcattatgcgtttcgcttatcgatctattcgccggaattggcggcatccgtcgcggttttgaatcgattggcggacagtgcgtgtttccagcgaatggaacaaacatgcggtacgcacttataaagccaaccattattgcgatccggcgacgcatcattttaatgaagatatccgcgacatcaccctcagccataaagaaggcgtgagtgatgaggcggcggcggaacatattcgtcAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCGCGCA-3′). Other primers used for PCR were the following: Hap2.p1 (5′-ATGTCGCCGTGACCCCAAAACAG-3′); Hap2.p2 (5′-CTGGCTGGTGACAGGCAGCGCGAA-3′); and Aph.p17 (5′-TTGGCTGCGCTCCTTCTGGCGC-3′).

Transformation of Chlamydomonas *with HAP2 constructs: HAP2-HA*: The 8.3-kb SstI fragment from DNA BAC clone 20L3 obtained from the Clemson University Genomics Institute, Clemson University, containing gene model C_530033 was inserted into the SstI site of pUC119 to generate pYJ36. Standard methods were used to insert a PCR product encoding three copies of the 9-amino-acid HA epitope (Silflow et al. 2001) into the NheI site of pYJ36 to generate pYJ58. To obtain *63B10* cells containing the *HAP2-HA* construct, we carried out cotransformation with the glass bead method using pYJ58 and plasmid pmn56 encoding the nitrate reductase gene (Tam and Lefebvre 1993). For the experiment shown in Figure 1B, *63B10* cells were cotransformed with the gel-purified 8.3-kb Sst1 fragment of BAC clone 20L3 and pmn56. Transformants were selected for their ability to undergo fusion with wild-type *plus* gametes.

Generation of a plus strain containing only disrupted HAP2: *63B10 minus* gametes rescued for fusion with transgenic *HAP2-HA* were crossed with *21gr plus* gametes, and the progeny were grown using standard procedures (Harris 1988). Colonies formed by germinated zygotes on 2% agar plates were pooled and inoculated into a growth flask containing M media. Progeny cells were subcloned on agar selection plates containing 10 µg/mL paromomycin and screened for *plus* progeny that contained the disrupted *hap2* allele from the *63B10* cells and lacked both the wild-type *HAP* allele and the *HAP2-HA* insert. To confirm the genotype of the transformant, Southern blotting was carried out with genomic DNA digested with NotI. The probe was a cloned PCR product generated using Hap2.p21 (CTGATGGTGCCGCAGCCGCCAGC) and Aph.p20 primers with *63B10* genomic DNA as template and labeled using a Random Primed DNA labeling kit (Roche Applied Science).

Indirect immunofluorescence: Gametes were washed with MT buffer (30 mM Tris acetate at pH 7.3, 5 mM $MgSO_4$, 5 mM EDTA, 25 mM KCl, 1 mM dithiothreitol) and loaded onto eight-well slides coated with 0.1% polyethylenimine for 10 min (Mahjoub et al. 2004). Cells were fixed in 100% ice-cold methanol for 20 min at −20°C, washed three times for 5 min in PBS, and blocked for 30 min with blocking serum (1% cold-water fish gelatin, 0.1% bovine serum albumin [BSA], 5% goat serum in PBS). The slides with fixed cells were incubated with rat monoclonal anti-HA antibody (Roche Applied Science; diluted 100-fold) for 2 h, rinsed three times in PBS, and then incubated for 1

h with fluorescein-conjugated goat anti-rat IgG (ICN/CAPPEL; 1:400 dilution) in blocking serum. The slides were rinsed in PBS and mounted in Fluoromount-G (Southern Biotech). Fluorescence microscopy was performed using an Ultraview ERS spinning disk confocal microscope (Perkin Elmer). Final composite images were constructed using Image J (NIH) and Adobe Photoshop (Adobe Systems).

Assessing gamete activation: To test whether *63B10* gametes were capable of gamete activation, 250 µL of *63B10* gametes at 1.6×10^7 cells per milliliter were mixed for 30 min with an equal number of *21gr plus* gametes, with dibutyryl cAMP, or with flagella isolated from *plus* gametes. For the experiment with isolated flagella, 10 cell equivalents of flagella were added at 5-min intervals, and cell wall loss was determined as described previously (Snell 1982). The data shown are averages from three independent experiments, each done in duplicate, and the error bars are SEM. To evaluate activation of *plus* gametes by *63B10 minus* gametes, the two types of gametes were mixed together for 30 min, and fertilization tubules, an indicator of *plus* gamete activation, were visualized with the actin-specific fluorochrome Alexa 546-phalloidin (Molecular Probes) as described previously (Wilson et al. 1997).

Prefusion attachment assay: Activated *sag1-1* gametes were incubated in 2.5% glutaraldehyde in N-free medium for 10 min, washed twice in 1% glycine in 10 mM phosphate-buffered M-N medium for 5 min, incubated with the live-cell impermeant, nucleic acid fluorochrome SYTOX-green (1 mM in M-N medium) for 10 min, and washed twice in M-N medium. To carry out the attachment assay, fixed, SYTOX-labeled *sag1-1 plus* gametes were mixed with live, activated *63B10* or wild-type *minus* gametes and allowed to interact for 30 min. The mixed samples were placed on a microscope slide, fixed in 0.1% paraformaldehyde, covered with a coverslip supported by Vaseline posts, and viewed by fluorescence and differential interference contrast (DIC) microscopy.

Assessing membrane fusion: The plasma membranes of activated *plus* gametes (2×10^7 cells per milliliter in M-N medium) were labeled by mixing the cells in M-N with an equal volume of Staining Solution containing PKH26 red fluorescent dye (Sigma; 4 µM in M-N) for 10 min at 23°C. The reaction was stopped by addition of BSA to a final concentration of 1% for 1 min, and cells were washed three times with M-N medium by centrifugation. The labeled gametes were mixed with unlabeled wild-type or *63B10 minus* gametes and examined by epifluorescence and DIC microscopy.

Electron microscopy: *Plus* wild-type gametes were mixed with wild-type or *hap2 minus* gametes for 3 min (wild-type *plus* gametes and wild-type *minus* gametes) or 30 min (wild-type *plus* gametes and *hap2 minus* gametes) and fixed in 1% glutaraldehyde and 0.2% tannic acid following the procedure of Goodenough et al. (1982) with some modifications (Begg et al. 1978; Detmers et al. 1983). Concentrated fixative (1.5 mL of 5% glutaraldehyde, 1% tannic acid in M-N medium) was added, one drop at a time with gentle mixing, to 6 mL of cells in M-N medium. Cells were fixed for 2 h and were then allowed to settle onto poly-L-lysine-coated coverglasses. Cells on coverglasses were rinsed in M-N medium, post-fixed in 0.5% osmium tetroxide, en bloc stained in 2% uranyl acetate, dehydrated in a graded ethanol series, and transferred through propylene oxide into EMbed-812 resin. After overnight polymerization, coverglasses were removed by immersion in liquid nitrogen. Thin sections (55 nm) were cut on a Leica EM UC6 and post-stained with 2% uranyl acetate and lead citrate. Sections were viewed in a FEI Tecnai G2 Spirit electron microscope operating at 120 kV. Images were acquired with a Gatan UltraScan 1000 camera and DigitalMicrograph software.

Plasmodium

The *P. berghei* ANKA wild-type strain 2.34 was used throughout this study. Strain 2.33 is a gametocyte-deficient strain derived from 2.34 by continued blood passage. *cdpk4*, *nek4*, and *p48/45* mutant clones were described previously (van Dijk et al. 2001; Billker et al. 2004; Reininger et al. 2005). *P. berghei* was maintained in Theiler's Original outbred mice and transmitted to *Anopheles stephensi*, strain SDA 500, as described previously (Sinden et al. 2002). Mice were infected by intraperitoneal injection of infected blood containing ~10^6 parasites. The course of infections and gametocyte production were monitored on Giemsa-stained blood films. To trigger exflagellation, 5 µL of gametocyte-infected blood was mixed with 400 µL of exflagellation medium (RPMI1640 containing 25 mM HEPES, 20% fetal bovine serum [FBS], 10 mM sodium bicarbonate, 50 µM xanthurenic acid at pH 7.6). Between 15 and 25 min later, exflagellation centers per 10^4 red blood cells (RBCs) were counted in a hemocytometer. To determine the relative ability of microgametocytes to exflagellate, numbers of exflagellation centers were compared with numbers of morphologically mature microgametocytes per 10^4 RBCs on Giemsa-stained blood films prepared in parallel from the same infected mouse. The proportion of female gametes undergoing either adhesion or fusion with a male gamete was quantified by phase contrast microscopy in cultures undergoing gametogenesis under Vaseline-rimmed coverslips on glass microscope slides. Starting 15 min after activation, when mature microgametocytes had started to release male gametes, ~100 female gametes per experiment were observed and scored. Gamete adhesion was recorded when one or more male gametes were persistently associated with the surface of the female gamete, but when there was no sign of gamete fusion. Gamete fusion was recorded when adhesion resulted in the male gamete entering the female, or when characteristic motions in the zygote's cytoplasm caused by the continued intermittent beating of the axoneme of the male gamete (Sinden and Croll 1975) indicated that fertilization had occurred. Since axoneme motility ceases ~1 min after fusion, our analysis underestimates the true incidence of fusion in wild type, as evidenced by the higher efficiency of ookinete formation in the same cultures (see Fig. 2). Ookinete conversion rates for different mutants and genetic crosses were determined in 20-h in vitro cultures of infected blood by immunolabeling the macrogamete/zygote/ookinete marker P28, as described previously (Reininger et al. 2005). For mosquito transmission experiments, batches of 50 mosquitoes were allowed to feed on anaesthetized mice on day 3–4 of a blood-induced infection for 20 min at 19°C. Unfed mosquitoes were removed the following day, and oocysts were counted on dissected midguts on day 10 after feeding.

Deletion of the HAP2 gene: To replace all protein-coding sequence of the *HAP2* gene (GenBank accession no. XM_671808) with a *T. gondii dhfr/ts* expression cassette conveying resistance to pyrimethamine, a targeting vector was constructed in plasmid pBS-DHFR. A 736-bp fragment comprising 5′-flanking sequence immediately upstream of the start codon was amplified from *P. berghei* genomic DNA using primers GF-1 (5′-CCCCGGGCCCGCGCGTTATTATTATTCGGGC-3′, restriction site underlined) and GF-2 (5′-GGGGAAGCTTTTTTTC TAAATGAAATATTAAAGAATGGC-3′) and inserted into ApaI and HindIII restriction sites upstream of the *dhfr/ts* cassette of pBS-DHFR. A 967-bp fragment of 3′-flanking sequence was then generated using primers GF-3 (5′-CCCCGAATTCAT TACATGGAATAGTATTTGCAAATTTG-3′) and GF-4 (5′-GGGGTCTAGACAATATACATGCTGATAACCTCC-3′) and inserted downstream from the *dhfr/ts* cassette using EcoRI and

XbaI restriction sites. The replacement construct was excised as an ApaI/XbaI fragment and used for the electroporation of cultured *P. berghei* schizonts as described (Janse et al. 2006). Following dilution cloning of drug-resistant parasites, genotyping of two *hap2* clones was done by Southern blot hybridization on EcoRI-digested genomic DNA using the ApaI/HindIII fragment of 5′ targeting sequence as a probe. Diagnostic PCR analysis used primers GFko1 (5′-CTCGAATATGTAGATATATCCA GATG-3′) and GFko2 (5′-CAGAGATGTTATAGCTAGT GATATAAC-3′) specific for *HAP2*, and primers GFint (5′-CTAAGTAGCAACTATTTTGTAAAATTATATC-3′) and 70 (*1*) to span the predicted 5′ integration site.

Complementation of a hap2 mutant: A 4.2-kb fragment comprising 1.5 kb of upstream sequence and the complete *PbHAP2* genomic sequence, except the stop codon, was amplified by PCR from genomic DNA using primers 485 (5′-ATATGGTAC CACGCTACTTATATATAGTGATAACC-3′) and 482 (5′-ATA TGGGCCCTCGCAATGGGGGTATTTTACTTTTAC-3′), inserted into KpnI and ApaI restriction sites of vector p277, upstream of and in frame with an EGFP gene, which was followed by 0.5 kb of 3′ untranslated region (UTR) derived from the *PbDHFR/TS* gene. The protein-coding sequence of the complementation construct was confirmed by sequencing. The vector was linearized in a unique EcoRV site in the putative *HAP2* promoter and introduced into a *hap2* knockout clone, and resistant parasites were selected with WR99210. Integration of the complementation construct into the disrupted *hap2* locus was verified by PFGE and Southern blot analysis. HAP2-GFP expression was verified by Western blot analysis of purified wild-type and *HAP2-GFP* gametocytes, which were lysed by the addition of SDS-PAGE sample buffer (3% SDS, 3% mercaptoethanol, 20% glycerol, 60 mM Tris at pH 8, 0.1% bromphenol blue) and thereafter separated on a 10% SDS-PAGE gel. Proteins were immunoblotted onto PVDF filters (Millipore) and incubated with anti-GFP antibody (Molecular Probes; diluted 1:5000) followed by incubation with HRP-conjugated anti-rabbit antibodies (GE healthcare; diluted 1:20,000). Detection was enabled with Immobilon Western chemiluminescent HRP substrate (Millipore).

RT–PCR analysis of HAP2 expression: *P. berghei* RNA was isolated from equivalent numbers of purified wild-type and *hap2* gametocytes and strain 233 asexual parasites using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Any residual gDNA was removed by treatment with RQ1 RNase-free DNase (Promega), and the resulting RNA was extracted with phenol/chloroform, precipitated with ethanol, resuspended in DEPC-treated water, and quantified by 0.8% agarose gel electrophoresis. First-strand cDNA synthesis from 1 µg of total RNA was done with M-MLV Reverse Transcriptase (Invitrogen) for 50 min at 37°C. Following heat inactivation for 15 min at 70°C, 2 µL of cDNAs were used per PCR reaction. Primers selected to amplify sections of the HAP2 ORF (spanning the 209-bp intron) were Forward, 5′-GCATAAGATTCACAAATA CAAAAAGG-3′; and Reverse, 5′-GGTCTTCCTCTAAGTAT T-3′. The expected RT amplicon was 1203 bp, whereas the gDNA amplicon was 1412 bp. The ubiquitously expressed α-tubulin gene *PB300720.00.0* was amplified for each sample to ensure amplifiability of cDNA from respective RNA samples (Forward, 5′-CCAGATGGTCAAATGCCC-3′; Reverse, 5′-CT GTGGTGATGGCCATGAAC-3′). The expected products were 432 bp (cDNA) and 592 bp (gDNA). Thirty RT–PCR cycles were carried out with denaturation for 1 min at 94°C, annealing for 45 sec at 50°C, and extension for 1.5 min at 68°C, and products were visualized on a 0.8% agarose gel.

Indirect immunofluorescence: Immunofluorescence analysis was done with enriched male gametes prepared essentially as described by Carter and Chen (1976). Blood containing wild-type or HAP2-GFP gametocytes was activated by the addition of exflagellation media for 30 min at 19°C and thereafter centrifuged twice at 500*g* for 10 min. The resulting supernatant was then centrifuged at 13,000*g* for 10 min and left for 15 min at room temperature. The supernatant was again centrifuged at 20,000*g* for 10 min. Thick smears from the pellet, containing mainly male gametes, were fixed in ice-cold methanol, blocked in 10% FBS in PBS, and incubated with rabbit anti-GFP antibodies (Molecular Probes; diluted 1:1000) followed by incubation with Alexa 488-conjugated anti-rabbit antibodies (Molecular Probes; diluted 1:2000). Fluorescence microscopy was performed using a Leica DMR microscope. Images were constructed using AxioVision (Carl Zeiss imaging solutions) and Microsoft Office Picture Manager (Microsoft).

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