in both subtelomeric and telomeric regions, and the chromatin structure of these regions is believed to be an important factor in the mutually exclusive gene expression (Lopez-Rubio et al., 2009). In other *Plasmodium* species, the subtelomeric regions contain other multigene families (Janssen et al., 2004), and their transcription may be regulated by similar epigenetic mechanisms that are important in *P. falciparum*. The trimethylation of Lys9 of histone H3 is involved in the regulation of telomeric length. Given that the telomeric length of L-PAC is controlled, it is entirely plausible that L-PAC has a similar chromatin modification that is present in *Plasmodium* chromosomal telomeres. Therefore, this suggested that L-PAC reporter constructs should permit us to examine the mechanism regulating epigenetically controlled gene expression of subtelomerically located multigene families.

Various mutant parasites exhibiting clinically important phenotypes (e.g., drug resistance) have been isolated from field or generated in laboratories thus far. Currently, linkage analysis using progeny clones from genetic crosses between mutant and wild-type parasites is the only way to identify the specific genes responsible for these observed phenotypes. These methods are often complicated, technically challenging, and time consuming. We propose here an alternative approach with gene libraries generated by using L-PAC: genomic DNA or cDNA libraries of mutant parasites are constructed by using L-PAC and then directly introduced into wild-type parasites. The transformed parasites acquiring the mutant phenotype can then be selected by screening (e.g., drug selection), and then target genes incorporated in the L-PAC are identified from the selected parasites. This approach to surveying target genes using the gene library will provide fresh avenues for the analysis of mutant parasites.

Genetic modification of *Plasmodium* using episomally maintained plasmids was first described about 15 years ago (van Dijk et al., 1997), and the use of this method of genetic analysis has contributed significantly to studies of the parasite's biology. However, genetic modification using episomally maintained plasmids has two limitations that make it unsuitable for a number of parasite transformation studies. First, maintenance of the plasmids in parasites requires drug selection to prevent loss of the plasmids during mitosis. Consequently, the use of this technique is mainly restricted to the blood stage and is less suited for studies on parasites in either the mosquito or the liver. Second, episomal plasmids often form undesirable concatamers, resulting in high and variable copy numbers of the plasmid. As a result, the control or estimation of transcription activity of the genes introduced into the plasmid is difficult, and therefore plasmids are not suitable for precisely assessing gene expression levels in reporter assays. These technical limitations can be overcome by using the PAC and PCEN plasmids, since they are stably maintained throughout the life cycle and do not form multimeric concatamers. For example, accurate reporter assays at the mosquito and liver stages will only be achieved by using modified PAC and the PCEN plasmids or equivalents. Indeed, we have recently reported the use of such reporter assays (Yuda et al., 2009). In this study, we generated reporter PCEN plasmids containing several promoter regions (i.e., either containing or not containing *cis*-acting elements) and were able to precisely determine the transcriptional activities of those promoter regions controlled by the ookinete-specific transcription factor.

In conclusion, we describe in this paper the functional characterization of a *Plasmodium* centromere and the generation of PAC that appears to behave like a true *Plasmodium* chromosome. The study not only will expand the number of molecular tools available to malarial research but also sets out a template that should permit the generation of *P. falciparum* PAC.

EXPERIMENTAL PROCEDURES

Construction of Plasmids Containing PCEN

The various (complete and partial) PCEN-containing constructs were cloned into the plasmid vector pbGFPcon, which contains a selectable marker cassette encoding the *Toxoplasma gondii* pyrimethamine-resistant *dhfr-ts* gene (*Tgdhfr-ts*) as well as a *gfp* expression cassette under the control of the *P. berghei eef1αa* promoter. pbGFPcon was digested with EcoRI, thereby removing its *d-ssu-rrna* sequence. Subsequently, the PCEN-containing DNA fragments were digested with EcoRI and cloned into the EcoRI-digested pbGFPcon. All PCEN-containing plasmids are schematically shown in Figure 2A.

Construction of the *Plasmodium* Artificial Chromosome

The DNA fragment containing the two telomeric sequences, oriented head to head with an ~500 bp spacer region, was digested with HindIII and then cloned into the HindIII-digested pbCEN5A/T plasmid. This resulted in a circular plasmid, termed C-PAC. When we digested the C-PAC with PmeI, removing the spacer region between the two telomeres, the remaining linearized fragment was designated L-PAC.

Assays to Determine the Efficiency of Segregation and Maintenance of PCEN Plasmids and PACs in Transfected Parasites

Transfected parasites were first maintained for a period of 1–2 weeks in Swiss mice under pyrimethamine drug pressure. After this period, when the parasitemia had reached 5%–10%, the parasites were transferred to naive mice. The parasites were then maintained in these mice and subsequent mice for 18–21 days without drug treatment. During this period, mechanical passage of the parasites was repeated three to five times once the parasitemia in the infected mice had reached 5%–10%. Mechanical passage was performed by intraperitoneal injection of 2–4 × 10^4 infected erythrocytes. At each mechanical passage, 10 μl of blood was collected from each mouse in 1 ml of culture medium (RPMI1640 medium [pH 7.3], containing 10% fetal calf serum) to determine the percentage of parasites retaining PCEN constructs. This was done by assessing the percentage of infected erythrocytes that were GFP positive using a fluorescence microscope. Specifically, to determine the percentage of GFP-positive parasites, the samples of infected blood were incubated at 37°C for 5 min in the presence of Hoechst 33258 (10 μM final concentration) to stain all parasite nuclei. According to the percentages of GFP-positive parasites at the end of the 18–21 day multiplication period, we calculated the segregation efficiencies of the PCEN-containing constructs in the blood stage based on the assumption that during blood-stage schizogony the parasite nuclei undergo four nuclear divisions, resulting in the production of 16 daughter nuclei over a 24 hr period.

To evaluate segregation and maintenance of the PCEN-containing constructs during mosquito development, transfected parasites were first maintained for a period of 1 week in Swiss mice under pyrimethamine drug pressure. To obtain mosquito-stage parasites, *Anopheles stephensi* mosquitoes (3–4 days old) were fed for 10 min on anesthetized, transgenic parasite-infected mice. Mosquitoes were maintained at 20°C and 80% humidity and fed daily on 10% sucrose. To isolate salivary gland sporozoites, salivary glands were dissected at day 20–22 p.i., collected in ice-cold saline, and homogenized in 200 μl of medium 199. The percentage of GFP-positive sporozoites was determined by fluorescence microscopy.

To analyze segregation and maintenance of the plasmids within the parasites during liver-stage development, 3 × 10^4 salivary gland sporozoites were injected intravenously into young Wistar rats (3 weeks old). Four to eight

days after infection of the rats, the development of blood-stage parasitemia was determined using Giemsa-stained blood smears. At a parasitemia of 5%–10%, tail blood was collected and stained with Hoechst as described above, and the percentage of GFP-positive to Hoechst-stained parasites was determined by fluorescence microscopy.

Southern Analysis of DNA Collected from *P. berghei* Blood Stages Transfected with PCEN Plasmids and PACs

Genomic DNA was isolated from blood-stage parasites transfected with the various PCEN plasmids and PACs and maintained for 18–21 days without pyrimethamine. Parasite DNA containing the episomal plasmid DNA was digested with HindIII, which cleaves a single site within all PCEN plasmids and two sites in both the C- and L-PAC constructs. Southern hybridization was performed using a 5′UTR DNA fragment of the *P. berghei dhfr-ts* gene as a probe, which is present in all PCEN plasmids and PACs and on chromosome 7 of the *P. berghei* genome. The hybridization intensity of the signal in each sample was quantified using Quantity One software (Bio-Rad). By comparing the intensity of the hybridization signal for the plasmid and the hybridization of the probe to the endogenous *dhfr-ts* gene in the genome, the copy number of each plasmid was determined. In addition, to examine the integration of the C-PAC, genomic DNA was digested with KpnI, which cleaves at a single site within the C-PAC. The digested DNA was processed and analyzed by Southern analysis as described above.

Genomic DNA from parasites transfected with the L-PAC was collected both from blood stages, as described above, and from blood stages after mosquito transmission. This genomic DNA from parasites transfected with the L-PAC was digested with either HindIII, which recognizes two sites within the L-PAC, or with NheI or KpnI, which each recognize a single site within the L-PAC. Hybridization was performed using a fragment of the *gfp* gene as a probe (present exclusively in the L-PAC plasmid), which specifically recognized fragments from the L-PAC vector.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chom.2010.02.010.

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Identification of a transcription factor in the mosquito-invasive stage of malaria parasites

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Summary

Gene expression in *Plasmodium* parasites undergoes significant changes in each developmental stage, but the transcription factors (TFs) regulating these changes have not been identified. We report here a *Plasmodium* TF (AP2-O) that activates gene expression in ookinetes, the mosquito-invasive form, and has a DNA-binding domain structurally related to that of a plant TF, Apetala2 (AP2). AP2-O mRNA is presynthesized by intraerythrocytic female gametocytes and translated later during ookinete development in the mosquito. The *Plasmodium* TF activates a set of genes, including all genes reported to be required for midgut invasion, by binding to specific six-base sequences on the proximal promoter. These results indicate that AP2 family TFs have important roles in stage-specific gene regulation in *Plasmodium* parasites.

Introduction

Elucidation of gene regulation mechanisms in the parasitic protozoan *Plasmodium* could provide important information for understanding parasite developmental programmes and its interactions with both host and vector during infection at each infection stage and could support the development of new antimalarial strategies. However, at present, little is known about the mechanisms of gene regulation in *Plasmodium* and only a small number of transcription factors (TFs), which may bind to specific DNA promoter sequences and control gene expression, have been predicted in the *Plasmodium* genome (Iyer *et al.*, 2008).

At present the Apetala2 (AP2) family is the only known gene family predicted to encode TF candidates in the *Plasmodium* genome (Balaji *et al.*, 2005). The AP2 family TFs were first identified in plants (Jofuku *et al.*, 1994) and then shown to form a large family in the genome of several plants (Gutterson and Reuber, 2004). In *Arabidopsis thaliana*, the AP2 family contains at least 140 members, some of which are involved in stress responses and others in regulation of reproductive and vegetative organ development (Gutterson and Reuber, 2004). Plant AP2 family TFs are named for their possession of at least one common DNA-binding domain of approximately 60 amino acids, the AP2 domain. A bioinformatic analysis of the complete *Plasmodium falciparum* genome revealed that AP2-related genes are present in apicomplexan genomes, including *Plasmodium* (Balaji *et al.*, 2005), where in the human malaria parasite *Plasmodium falciparum*, 26 AP2-related genes were predicted. The predicted *Plasmodium* AP2-related genes encode proteins with one to four AP2 domains; however, their function remains to be elucidated.

Plasmodium has three host-invasive stages: merozoites, ookinetes and sporozoites, which are erythrocyte-, mosquito midgut- and salivary gland/Kuppfer cell/hepatocyte-invasive stages respectively. These stages produce various stage-specific proteins involved in subsequent host invasion, some of which have been identified through various transcriptome and proteome analyses (Kaiser *et al.*, 2004; Hall *et al.*, 2005; Raibaud *et al.*, 2006). Each invasive form of the parasite produces a distinct repertoire of proteins that are secreted from apical secretory organelles such as micronemes and rhoptries and associated with invasion of host cells or tissues (Dubremetz *et al.*, 1998) or associated with the cell surface, suggesting that they participate in specific host–parasite interactions. However, regulatory mechanisms of individual or groups of stage-specific genes remain elusive in all these stages.

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During asexual replication in animal host erythrocytes, some *Plasmodium* parasites differentiate into sexual forms, called gametocytes. When ingested by a mosquito with a blood meal, gametocytes generate gametes that are fertilized and form zygotes in the mosquito midgut lumen. The zygotes develop into motile ookinetes that invade the midgut epithelium and differentiate into oocysts on the basal side of the midgut. The developing ookinetes produce large amount of stage-specific proteins for subsequent midgut invasion (Vlachou *et al.*, 2006) including microneme proteins such as circumsporozoite protein thrombospondin-related anonymous protein (TRAP)-related protein (CTRP), a protein belonging to the TRAP family and essential for gliding motility (Dessens *et al.*, 1999; Yuda *et al.*, 1999a); chitinase, a chitinolytic enzyme necessary for ookinete crossing of the mosquito chitin-containing peritrophic matrix (Vinetz *et al.*, 2000); membrane attack ookinete protein (MAOP), essential for rupture of mosquito midgut epithelial cell membrane (Kadota *et al.*, 2004); a protein kinase CDPK3 (calcium-dependant protein kinase 3) that stimulates invasion (Ishino *et al.*, 2006); cell surface-associated proteins, P28 and P25, which are necessary for ookinete development and are transmission-blocking vaccine targets (Tomas *et al.*, 2001; Saul, 2007); and other secreted proteins (Yuda *et al.*, 2001; Dessens *et al.*, 2003). The expression of some ookinete-specific proteins is regulated at the translational level where the female *Plasmodium* gametocyte represses specific transcripts through a protein complex composed of DOZI (development of zygotes inhibited), a conserved DDX6-class RNA helicase, and other proteins (Mair *et al.*, 2006). After fertilization, these gene transcripts are released from the complex and enter the translation pathway. On the other hand, *de novo* mRNA synthesis of invasion-related genes also occurs after fertilization (Raibaud *et al.*, 2006), and full-scale production of invasion-related proteins begins several hours after fertilization in developing ookinetes.

During a systematic investigation of the predicted AP2 factors that are not expressed in asexual blood-stage parasites we discovered one we designate AP2-O (AP2 in ookinetes) that is essential for the formation of invasive ookinetes and the expression of ookinete invasion-related genes. Here, we report that AP2-O is the ookinete TF that directly activates the invasion-related genes by binding to a specific motif found upstream of them.

Results

Expression of AP2-O *is regulated at translational level*

Plasmodium berghei AP2-O (PB000572.01.0) encodes a protein with a single AP2 domain and has an orthologue in *P. falciparum* (PF11_0442, Fig. 1A) and other *Plasmodium* spp. AP2-O has two conserved regions which include the AP2 domain near the C-terminus. The amino acid sequences of the AP2-O AP2 domain are almost identical among *Plasmodium* spp. (Fig. S1).

To investigate *AP2-O* protein expression, a transgenic line of *P. berghei* that expressed green fluorescent protein (GFP) fused to the endogenous allele of *AP2-O* (AP2-O::GFP parasites) was generated (Fig. 1B). The fusion did not appear to affect the parasite's ability to infect mosquitoes, as the AP2-O::GFP parasite generated a normal number of oocysts in mosquitoes (Table 1). In the blood stages of AP2-O::GFP parasites, fluorescence of the GFP-fused protein was not observed (Fig. 1C). However, weak fluorescent signals were observed in retort-form ookinetes beginning 8 h after fertilization (Fig. 1C). The signals were localized in the nucleus and signal intensities increased with ookinete development.

We also compared the amount of *AP2-O* transcripts between gametocytes and ookinetes with real-time reverse transcription PCR (RT-PCR) analysis using wild-type (WT) asexual-free parasite preparations (Fig. 1D). Unexpectedly the analysis showed that *AP2-O* transcripts are most abundant in the gametocyte stage and markedly decreased in the subsequent zygote and ookinete stages. This expression profile was in contrast with that of the typical ookinete microneme protein, secreted ookinete adhesive protein (SOAP), whose transcripts are mainly synthesized in the zygote and ookinete stages (Dessens *et al.*, 2003). These results suggest that *AP2-O* expression is translationally repressed in female gametocytes and subsequently translated in ookinetes (Mair *et al.*, 2006).

We examined whether *AP2-O* transcripts form physical complexes with DOZI in gametocytes using immunoprecipitation (IP) experiments on DOZI::GFP gametocytes (Fig. 1E). *AP2-O* transcripts were co-precipitated with GFP-tagged DOZI with anti-GFP antibodies. These results demonstrated that the translation repression system of the DOZI complex controls expression of AP2-O mRNA.

AP2-O *disruption decreases expression of several mosquito invasion-related genes*

To investigate the function of *AP2-O*, we prepared *P. berghei* mutant parasites with the *AP2-O*-disrupted [*AP2-O* (–)] (Fig. 2A). The *AP2-O* (–) parasites formed morphologically normal female and male gametocytes and exhibited normal exflagellation rates (Table 1). However, they lacked the ability to infect mosquitoes as no oocysts or sporozoites were found in the mosquito midgut. In culture, *AP2-O* (–) parasites formed zygotes at normal conversion rates and differentiated into retort forms by 9 h after fertilization, as in WT parasites (Fig. 2B, top). Subtle morphological differences between WT and

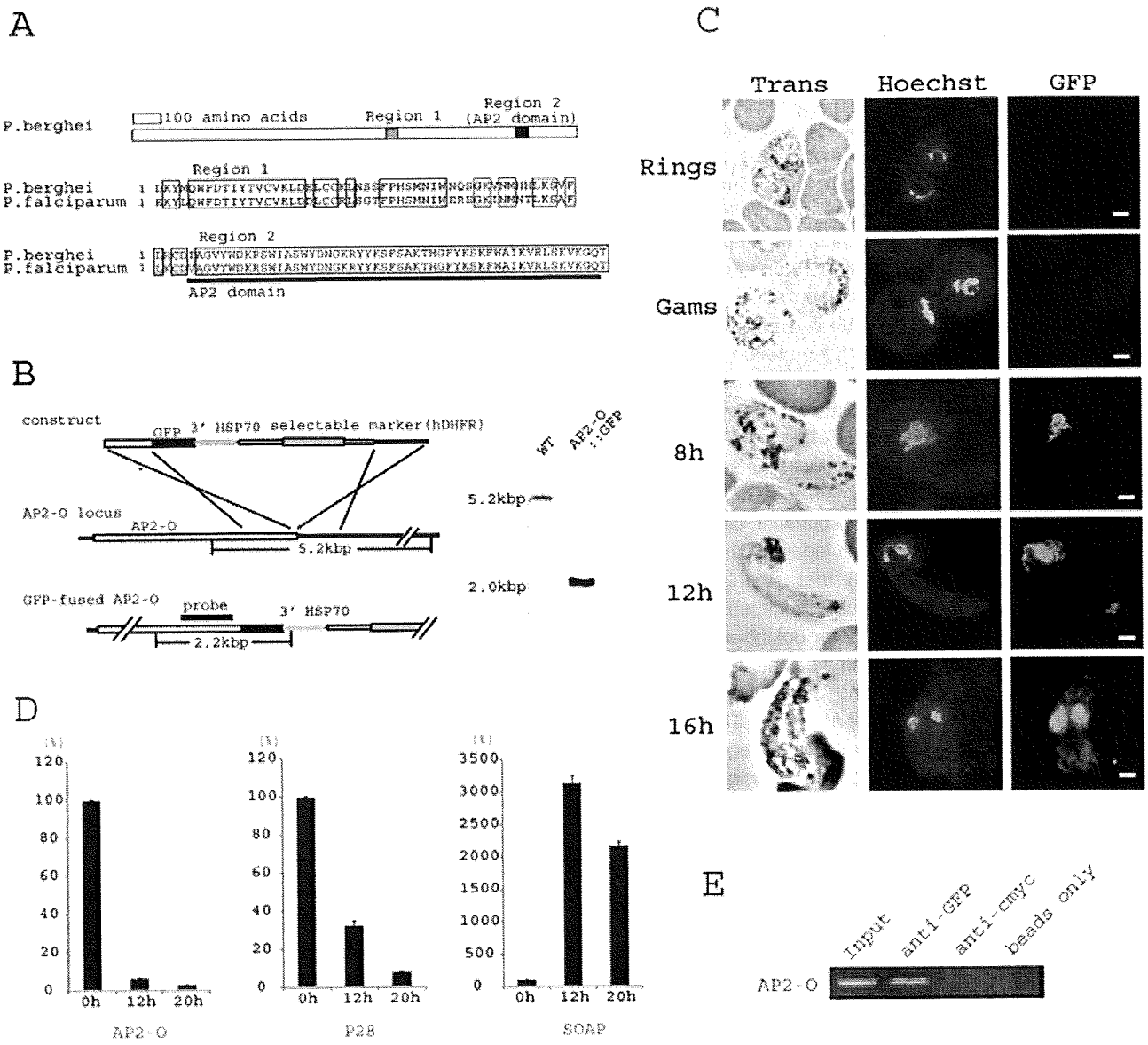


Fig. 1. Expression of AP2-O is controlled at the translational level.
A. The amino acid sequences around the single AP2 domain and another conserved region (region 1) were aligned between *Plasmodium berghei* and *P. falciparum*. Identical amino acids are highlighted by rectangles.
B. To construct AP2-O::GFP parasites, the targeting vector containing a marker gene (left top), human DHFR (hDHFR), was integrated into the *AP2-O* locus (left middle) by double-cross-over recombination, adding the GFP gene to the C-terminal portion of *AP2-O* and conferring pyrimethamine resistance to the AP2-O::GFP parasites (left bottom). For Southern hybridization (right), WT and AP2-O::GFP genomic DNA was digested with MunI and hybridized with a probe (solid bar; bottom left). Integration decreased detected fragment size from 5.2 to 2.0 kb.
C. To localize AP2-O *in vivo*, AP2-O::GFP parasites were cultured to the ookinete stage. At 8, 12 and 16 h after fertilization, nuclei were stained with Hoechst 34580. Images of blood-stage parasites were also shown. Rings, ring forms; Gams, gametocytes. Scale bars, 2 μm.
D. Total RNA was prepared from asexual-free blood-stage parasites (0 h), retort-form ookinetes (12 h after fertilization) and mature ookinetes (20 h after fertilization) cultured from the same infected blood. Quantitative RT-PCR was performed with primers for *AP2-O*, *P28* and *SOAP*. Each result is the mean of three independent experiments with standard error bars. For each gene, the amount of mRNA at each time was expressed as the per cent of mRNA relative to that at 0 h after fertilization. P28 mRNA is abundant in gametocytes, while SOAP mRNA is mainly synthesized in ookinetes. *AP2-O* showed an expression pattern different from that of *SOAP*.
E. For analysis of complex formation of *AP2-O* mRNA and the translation repression complex, IP was performed on gametocyte lysate of DOZI::GFP parasites using anti-GFP antibodies, anti-myc antibodies and protein G Sepharose beads only. RNAs recovered from IPs were analysed by RT-PCR with primers for *AP2-O*.

Table 1. *AP2* (–) malaria parasites lack the ability to infect mosquitoes.[a]

Genotype	Exflagellation rate	Conversion rate[b]	Number of oocysts per mosquito[c]	Number of sporozoites per mosquito[d]
WT	44.6	63.8 (6.8)	76.1 (14.73)	40 604 (6957)
AP2 (–) 1	37.5	56.8 (11.9)	0	0
AP2 (–) 2	24.3	71.0 (2.3)	0	0
AP2-O::GFP	35	70.5 (1.12)	71.6 (20.85)	42 940 (6797)

a. Infected mice were subjected to mosquito bites when exflagellation rates became more than 20 per 10^5 red blood cells. Fully engorged mosquitoes were dissected after 14 days, and the numbers of oocysts and sporozoites in the midgut were counted.
b. Conversion rates (%) of female gametocytes to retort-form ookinetes (standard errors in parentheses).
c. Twenty mosquitoes were dissected, and the number per mosquito was calculated (standard errors in parentheses).
d. Ten mosquitoes were dissected, and the number per mosquito was calculated (standard errors in parentheses).

AP2-O (–) parasites, however, became visible 12 h after fertilization with a swelling protrusion in the retort forms (Fig. 2B, centre). Final development was aberrant ookinetes with an pear-shaped form, different from the sword-like form of WT ookinetes (Fig. 2B, bottom).

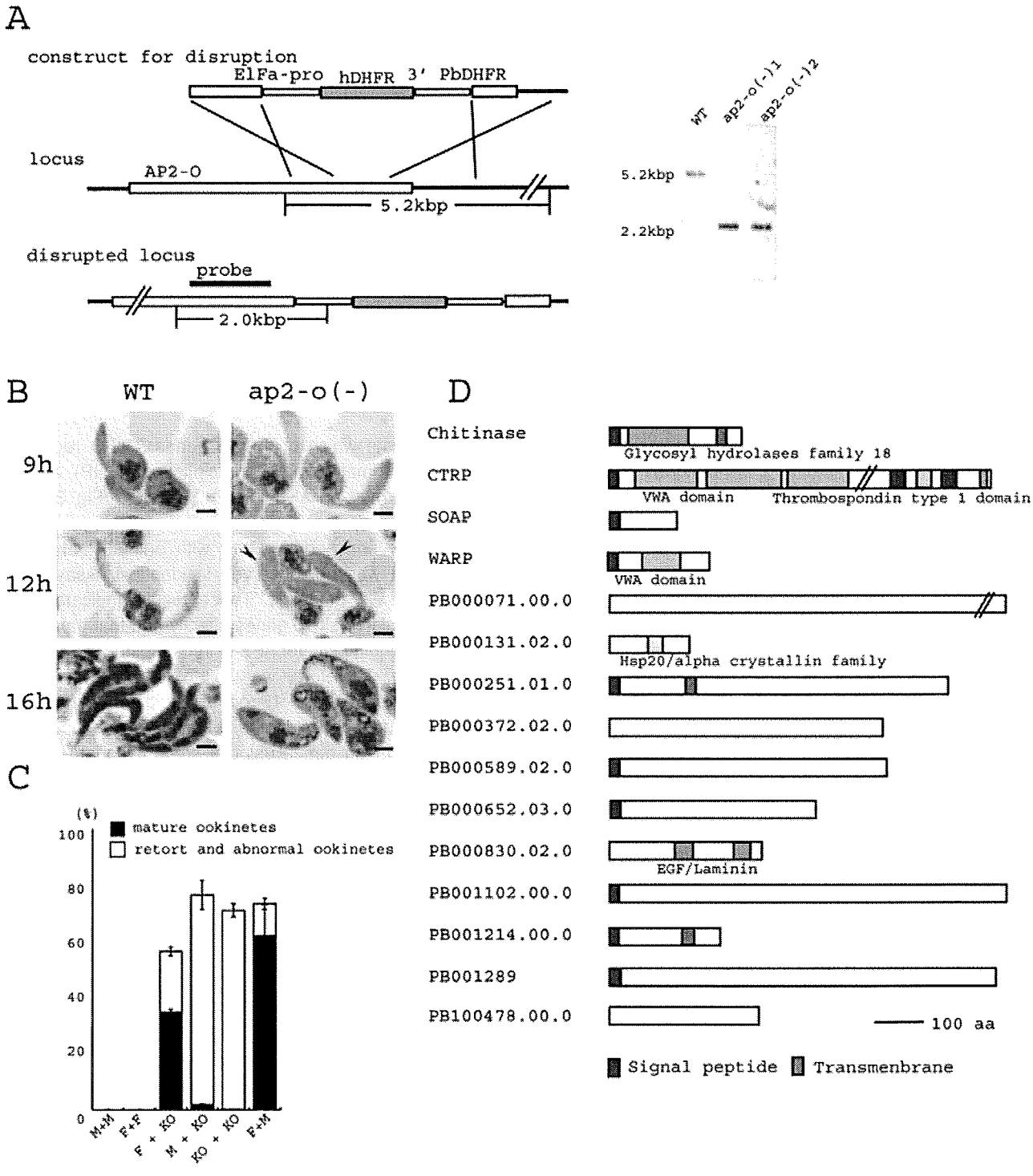
Cross-fertilization experiments mating *AP2-O* (–) mutant gametocytes with those from either female or male gametocyte-defective lines indicated that the *AP2-O* defect is inherited from the female line (Fig. 2C). *AP2-O* (–) females when fertilized with WT males produced deformed ookinetes. However, WT females when fertilized by *AP2-O* (–) males produced normal ookinetes. These results suggest that *AP2-O* transcripts in zygotes/ookinetes are derived from female gametocytes and essential for zygote development into normal infective ookinetes.

DNA microarray analyses were performed to investigate whether *AP2-O* disruption affects gene expression in ookinetes. Samples were obtained from WT and *AP2-O* (–) retort-form ookinetes at 12 h after fertilization, when slight morphological differences between them started to appear (Fig. 2B, centre). Genes were selected whose expression was decreased at least fivefold (Fig. 2D). A total of 15 genes were identified with this analysis, of which four were ookinete-specific genes (all encoding microneme proteins) (Yuda *et al.*, 1999b; 2001; Vinetz *et al.*, 2000; Dessens *et al.*, 2003), six were genes reported to be transcribed during ookinete development after zygote formation (Raibaud *et al.*, 2006) and five were novel genes not ascribed to. We performed real-time RT-PCR analysis in the five novel genes and showed that they were transcribed in the zygote/ookinete stage (Fig. S2). Very recently two of them (PB000652.03.0 and PB001214.00.0) were reported to be involved in ookinete midgut invasion (Ecker *et al.*, 2008). As shown in Table S1 and Fig. 2D, 14 of these 15 proteins have orthologous genes in *P. falciparum* and *P. vivax*, and 10 have a putative N-terminal signal peptide sequence, suggesting that they are microneme or cell surface-associated proteins that are available for subsequent midgut invasion.

AP2-O directly activates several genes by binding to a six-base sequence, TAGCTA

To investigate which of the 15 genes AP2-O regulates directly, chromatin immunoprecipitation (ChIP) assays were performed on AP2-O::GFP parasites (Fig. 3A and Fig. S3). The upstream promoter region of all genes was significantly enriched by ChIP with anti-GFP antibody. Enrichment was not observed in identical assays performed on WT parasites. These results indicated that AP2-O regulates all 15 genes directly and that there is at least one binding site for AP2-O in the upstream region of each gene. Therefore, we looked for putative AP2-O binding sites in these 15 genes by determining high-frequency sequences in the 1 kb immediate upstream regions of these genes using a computational analysis (Table S2). The sequence conservation pattern (Fig. 3B) shows a 6 bp sequence, TAGCTA, as the most frequently occurring sequence. This sequence was present in the upstream region of 14 of the 15 genes, with two or more TAGCTA sequences observed in 13 genes (Fig. 3C). In addition to TAGCTA, four other closely related sequences, TGGCTA/TAGCCA and CAGCTA/TAGCTG, are possible binding sites for AP2-O (see Fig. 3E). Including the less frequent binding elements, TGGCTA/TAGCCA, two or more binding sites were present in all 15 genes. Frequencies of CAGCTA/TAGCTG were significantly low compared with the other three binding sequences (Table S2). The majority of the binding sequences were adjacent (approximately 100–400 bp upstream) to the putative transcription start site as estimated from the most upstream expressed sequence tags (ESTs) of each gene (Fig. 3C).

Electrophoretic mobility shift assays (EMSA) were performed to examine whether the 6 bp sequences act as binding sites for AP2-O. The ~300 bp *SOAP* upstream region, which contains two TAGCTA sites, was used as the probe (Fig. 3D). The AP2 domain of AP2-O bound to the probe and produced a single band shift. Addition of a point mutation (TAGCTA to TAGGTA) in one of the two TAGCTA sites significantly reduced the intensity of the



shifted band, and addition of the mutation in both sites eliminated the band shift. The same results were obtained using the ~300 bp upstream region of the chitinase gene (Fig. S4A).

To further analyse the binding specificity of the AP2 domain, we performed EMSA using probes with various point mutations in the TAGCTA sequence. Of the 18 mutations examined, 14 nearly eliminated binding and 4 (TGGCTA/TAGCCA and CAGCTA/TAGCTG) moderately reduced band shift intensity (Fig. 3E). These results demonstrated that AP2-O preferentially binds to TAGCTA, but also binds with weaker affinity to the similar sequences.

Fig. 2. AP2-O is necessary for normal development of ookinetes and expression of invasion-related genes.
A. To construct *AP2-O* (–) parasites, a targeting vector (left top) containing a selectable marker gene was integrated into the *AP2-O* locus (left middle) by double-cross-over recombination resulting in disruption of the *AP2-O* gene and conferring pyrimethamine resistance to the *AP2-O* (–) mutants (left bottom). For Southern hybridization (right), WT and *AP2-O* (–) genomic DNA was digested with MunI and hybridized with a probe (solid bar; left bottom). Integration decreased fragment size from 5.2 to 2.2 kb. Independently prepared *AP2-O* (–) mutants, *AP2-O* (–) 1 and *AP2-O* (–) 2, exhibited the same phenotype.
B. Phenotype of *AP2-O* (–) parasites in Giemsa-stained cultured ookinetes at different times after fertilization. A morphological difference between WT and *AP2-O* (–) parasites apparent 12 h after fertilization was the swelling protrusion (arrowheads) in *AP2-O* (–) retort-form ookinetes. *AP2-O* (–) parasites developed into pear-shaped ookinetes 16 h after fertilization (lower right). Scale bars, 2 μm.
C. Cross-fertilization experiments were performed with *AP2-O* (–) parasites, a male-deficient line (*P48/45*$^-$) and a female-deficient line (*P47*$^-$). Conversion rates of female gametocytes to deformed ookinetes and mature normal ookinetes are shown. The abnormal ookinetes observed in the combination of *AP2-O* (–) mutants and a male-defective line (*P48/45*$^-$) may have derived from self-fertilization of *AP2-O* (–) mutants. M, female-deficient line (*P47*$^-$); F, a male-deficient line (P48/45$^-$); KO, *AP2-O* (–) parasites.
D. Names or IDs of 15 *P. berghei* genes whose expression was decreased at least fivefold in *AP2-O* (–) ookinetes compared with WT (left), and the structure of their encoded proteins (right).

TAGCTA as a cis*-acting element in the ookinete stage*

Reporter assays were carried out to investigate whether the TAGCTA sequence functions as a *cis*-acting element *in vivo*. For these assays a plasmid containing a *P. berghei* centromere was used (Fig. S4B). This plasmid acts as an artificial chromosome and is maintained stably through several cell divisions in transfected parasites (S. Iwanaga *et al.*, unpubl. results). The upstream region of the SOAP gene, containing three TAGCTA sequences, was inserted upstream of the GFP reporter gene, and *P. berghei* blood-stage parasites were transfected with this construct (Fig. 4). GFP signals were not observed in the intraerythrocytic stages, including gametocytes. In ookinete cultures, GFP signals were detected 8 h after fertilization and signal intensity increased with ookinete development (data not shown), indicating that the upstream region acts as a stage-specific promoter. When one of the three TAGCTA sequences was disrupted by addition of a point mutation (TAGCTA to TAGGTA), promoter activity reduced to 40% of that of the non-mutated sequence; mutations in two of the TAGCTA sequences reduced promoter activity to < 10% of the non-mutated sequences. Similar results were obtained with the ~300 bp upstream region of the chitinase gene (Fig. S4C). These results indicate that the TAGCTA sequence functions as a *cis*-acting element *in vivo* and that the number of elements is an important factor in determining promoter activity.

cis*-acting elements are conserved among* Plasmodium *spp.*

The amino acid sequence of AP2 domain of AP2-O is highly conserved among several *Plasmodium* spp. (Fig. S1). Conservation of the amino acid sequence in the AP2 domain suggests that sequences recognized by this domain would be conserved among *Plasmodium* spp., and thus the same *cis*-acting elements would be observed in the orthologues of the 15 genes. To examine this, we performed computational analysis on *P. falciparum* and *P. vivax* orthologues (Tables S3 and S4, and Fig. 5A; 14 orthologues were used because orthologues of one *P. berghei* gene, PB100478.00, were not found in *P. falciparum* and *P. vivax*). In all three parasites, TAGCTA was conserved as the most frequently occurring sequence. The great majority of the *P. falciparum* and *P. vivax* orthologues had binding sequences for AP2-O in the upstream promoter region (11 of 14 and 13 of 14 respectively; Fig. 5B). Distribution of the binding sequences on each upstream region was also conserved among orthologues.

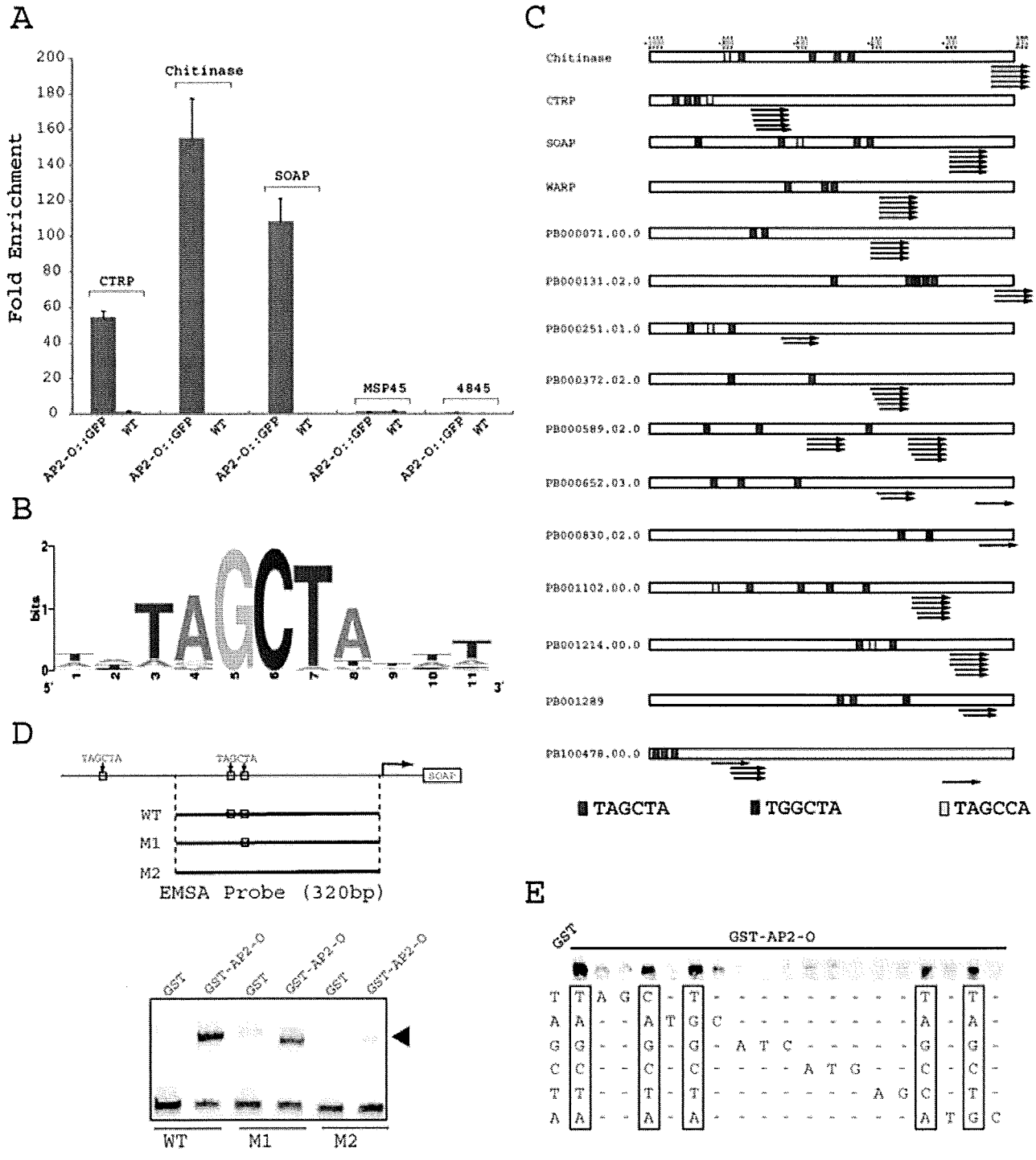
AP2-O activates all reported ookinete stage-specific genes

We analysed 15 genes whose expression significantly decreased in *AP2-O* (–) mutants. However, other genes, demonstrated or suggested to be involved in midgut invasion, *P25*, *P28*, *CDPK* and *MAOP* (Tomas *et al.*, 2001; Kadota *et al.*, 2004; Ishino *et al.*, 2006), were not in that initial group. To investigate whether these genes are regulated by AP2-O in the ookinete stage, we performed ChIP assays (Fig. 6A). The results demonstrated that AP2-O is associated with the upstream region in each of the four genes. Furthermore, two or more AP2-O binding sites were identified in the upstream region of each gene (Fig. 6B). These results indicate that these genes are activated by AP2-O in the ookinete stage.

Discussion

Global surveys of gene expression indicated that a high proportion of *Plasmodium* genes are expressed in a stage-specific manner (Le Roch *et al.*, 2003). However, how these changes are regulated in the life cycle remains elusive. In this study, we showed that specific gene expression in the ookinete stage is regulated by a sequence-specific TF belonging to the AP2 family, AP2-O. This study provided the first experimental evidence that gene expression in plasmodium is regulated by specific TF.

Ookinetes express several genes necessary for midgut invasion during development in mosquito midgut lumen.



Gene expression begins at 8–10 h after fertilization and continues for approximately 10 h until midgut invasion starts (Yuda *et al.*, 1999b; 2001). Our results showed that AP2-O is expressed during this period and activates several genes by directly binding to their upstream region. The activated genes encode different types of proteins including secretory proteins, surface-associated proteins and cytoplasmic proteins and include all known ookinete-specific genes involved in midgut invasion. Therefore, AP2-O would be a master activator that induces a cluster of different genes necessary for midgut invasion.

Our reporter assays indicated that the number of binding sites for AP2-O on the upstream promoter may be a factor defining promoter activities in the ookinete stage.

Fig. 3. AP2-O binds specifically to six-base sequences on the proximal promoter region of ookinete stage-specific genes.
A. For analysis of AP2-O complexes with the genes in Fig. 2D, ChIP was performed in AP2-O::GFP ookinetes. Of the 15 genes listed in Fig. 2D, the results for *CTRP*, chitinase and *SOAP* and for the negative controls, *P48/45* and *MSP4/5*, which are not expressed in ookinetes, are shown. Values are fold enrichments relative to IPs with control antibodies. Control experiments with wild-type parasites (WT) were also performed. Three biologically independent ChIP experiments were performed, and essentially the same results were obtained. The results of one of these experiments were shown here. Each result is the mean of three independent quantitative PCR experiments with standard error bars. The results for several other *Plasmodium* spp. genes are shown in Fig. S2.
B. WebLogo diagram of sequences occurring at high frequency in the 1 kb upstream regions of the *P. berghei* genes listed in Fig. 2D. Graphical representations created by WebLogo (Crooks *et al.*, 2004).
C. AP2-O binding sequences and the furthest upstream five 5′-ESTs (arrows) in the 1 kb upstream regions of the *P. berghei* genes listed in Fig. 2D. In each gene, more than two AP2-O binding sequences are present 100–400 bp upstream from the most upstream EST cluster. Coloured rectangles indicate binding sequence locations.
D. Sequence specificity of AP2-O binding was detected by EMSA using a GST-fused AP2-O AP2 domain. The *SOAP* upstream region containing two TAGCTA sequences was used as the probe. Probes with one (M1) or two (M2) mutations in their TAGCTA sequence (TAGCTA to TAGGTA) were also used. TAGCTA sequences in the upstream region of WT (no mutation) and the two mutations are illustrated (top). GST was used as a negative control. In the EMSA gel (bottom), the arrowhead indicates the band shift.
E. Other sequence possibilities in the AP2 domain binding site. Various point mutations were added to the TAGCTA sequence, and binding to AP2-O was determined by EMSA. The added point mutations are indicated under each lane. Binding ability was lost or greatly reduced in most mutated probes. Those mutations retaining binding activity are highlighted by a rectangle.

The AP2-O binding sites are mainly located within a short 100–400 bp region from the transcription start site (Fig. 3C), suggesting that to activate target genes, AP2-O must bind to the region just upstream of the transcription initiation site.

The merozoite and sporozoite stages also produce large amounts of invasion-related proteins during the development to mature invasive forms. Our results suggest that AP2 family TFs regulate invasion-related genes in these stages of *Plasmodium* spp. In fact, this possibility is supported by some preceding studies. For example, in the asexual blood stages, genes for erythrocyte invasion are mainly expressed in the mid- to late-schizont stage, i.e. the stage preceding merozoites (Bozdech *et al.*, 2003). The expressed genes encode proteins necessary for erythrocyte invasion such as microneme and rhoptry proteins as well as several surface-associated proteins. Because at this period some AP2 family genes exhibit peak expression, these AP2 family genes might be TFs inducing these invasion-related genes (Balaji *et al.*, 2005). Recent study showed that one of such AP2-related protein binds to a specific sequence and that many invasion-related genes have this motif in the upstream (De Silva *et al.*, 2008), suggesting that the AP2-related gene encodes an activator of these genes. Although this suggestion should be tested further, it is compatible with our present finding that an AP2-related protein directly controls stage-specific expression of multiple genes in ookinetes. Also in sporozoites, several invasion-related genes are expressed and some of them are homologous to genes regulated by AP2-O (Yuda and Ishino, 2004). A recent *in silico* analysis reported that sporozoite-specific genes have a common six-base motif CATGCA in the upstream (Young *et al.*, 2008). Therefore, it is possible that this motif is a binding sequence for another activator belonging to the AP2 family. It remains for further study to explore which AP2 family genes are expressed during the formation of infective sporozoites.

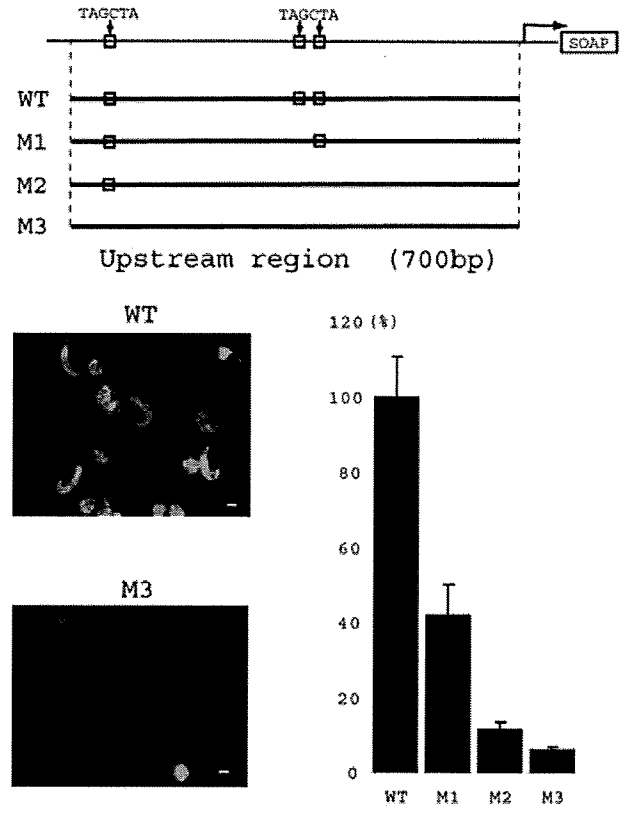


Fig. 4. The TAGCTA sequence as a *cis*-acting element in the ookinete stage. Promoter activity of the TAGCTA sequence in the ookinete stage *in vivo* was analysed using a reporter system with the *P. berghei* centromere plasmid Pcen (see also Fig. S3B). Mutations were added to three TAGCTA motifs in the SOAP upstream region (top). Mutations in M1 and M2 are the same as in M1 and M2 in Fig. 3D. M3 contains mutations in all three TAGCTA sequences. Each result is the mean of three independent experiments with standard error bars and is shown as the activity relative to that of the non-mutated WT. Representative images of GFP expression in ookinetes transfected with WT and M3 Pcen are shown at the bottom left. Round cells with strong fluorescence are mouse reticulocytes. Scale bars, 2 μm.

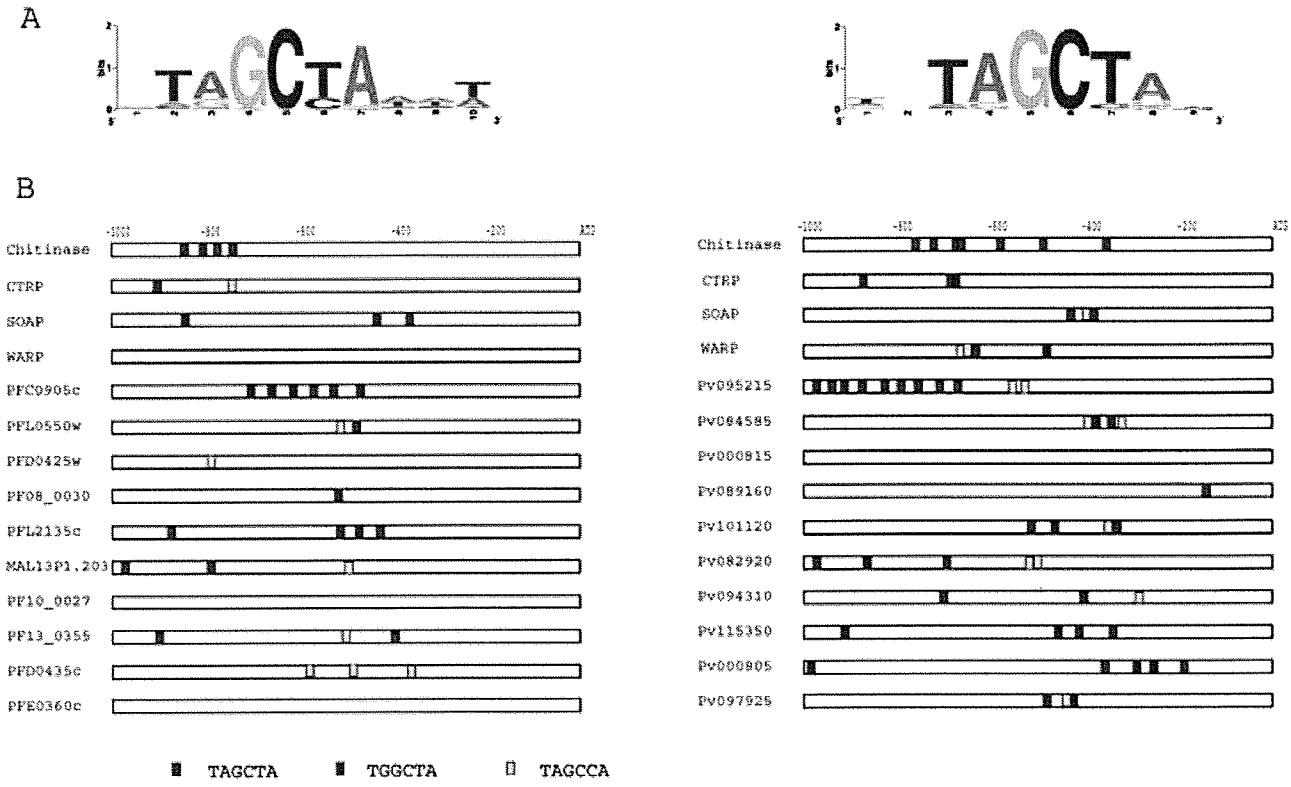


Fig. 5. Binding sequences for *Plasmodium berghei* AP2-O are conserved in *P. falciparum* and *P. vivax*.
A. WebLogo diagrams of sequences occurring at high frequency in the 1 kb upstream regions of the 14 orthologues in Fig. 2D in *P. falciparum* (left) and *P. vivax* (right). Graphical representations created by WebLogo (Crooks *et al.*, 2004).
B. AP2-O binding sequences in the 1 kb upstream regions of the 14 orthologues in Fig. 2D in *P. falciparum* (left) and *P. vivax* (right). Coloured rectangles indicate binding sequence locations.

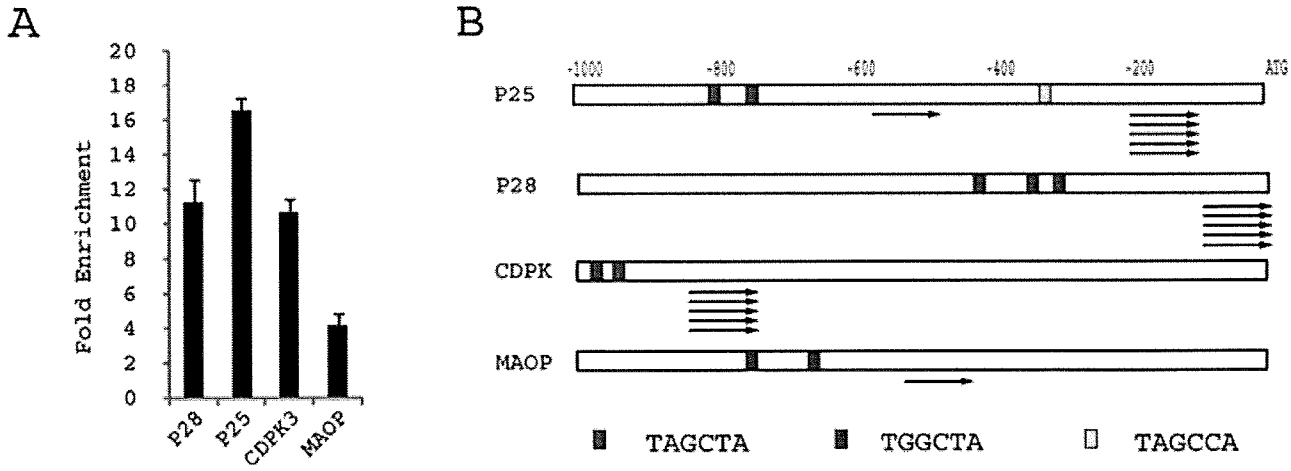


Fig. 6. Four *P. berghei* genes involved in ookinete invasion that are regulated by AP2-O.
A. ChIP was performed in *P28*, *P25*, *CDPK3* and *MAOP*, and DNA fragments were subjected to quantitative PCR with primers for upstream regions of the respective genes. Values are fold enrichments relative to IPs with control antibodies. Each result is the mean of three independent experiments with standard error bars.
B. AP2-O binding sequences and the most upstream 5′-ESTs (arrows) in the 1 kb upstream regions of four *P. berghei* genes are shown here. Each gene had more than two AP2-O binding sequences present 100–300 bp upstream from the most upstream EST cluster. Coloured rectangles indicate binding sequence locations.

In this study, genes regulated by AP2-O were first screened by comparison of gene expression between WT and *AP2-O* (–) parasites, and 15 genes were identified as being directly regulated by AP2-O. However, four genes reported so far were difficult to identify with this screening method. Of these genes, *P25* and *P28* were transcribed abundantly in female gametocytes (Paton *et al.*, 1993). Therefore, it seems that for these genes abundant transcripts had been synthesized in female gametocytes and masked the defect of transcription due to *AP2-O* disruption later in the ookinete stage. If so, many genes regulated by this TF could still remain to be identified.

In conclusion, our results demonstrate an AP2-related protein as a *Plasmodium* TF. At present, the *Plasmodium* AP2 family is the sole lineage-specific TF in *Plasmodium* spp. (Iyer *et al.*, 2008). Therefore, it is possible that these AP2 TFs participate in stage-specific gene regulations of *Plasmodium* spp. through their life cycle. Thus far, 26 AP2-related genes have been identified in the *Plasmodium* genome, but their roles in the life cycle, except for *AP2-O*, remain undefined. Further studies on AP2-related TFs are necessary to elucidate gene regulation mechanisms of *Plasmodium* parasites.

Experimental procedures

Parasite preparations

Female BALB/c mice infected (6–10 weeks old, Japan SLC, Hamamatsu, Japan) were prepared by peritoneal injection of *P. berghei* ANKA strain-infected blood that had been stored at –70°C. For ookinete culture, stored infected blood was injected intraperitoneally into mice that were made anaemic by phenyl-hydrazine treatment. After checking parasite exflagellation, infected blood was collected from the mice, passed through a CF11 column to deplete WBC, and diluted 10-fold with pH 8.0 RPMI1600 medium (Gibco, Gaithersburg, MD, USA), pH 8.0, containing 20% fetal calf serum and penicillin/streptomycin, and incubated at 20°C for 20–24 h. To prepare asexual-free gametocytes and ookinetes, infected anaemic mice were treated for 2 days with sulphadiazine (Sigma, St Louis, MO, USA) in their drinking water (10 μg ml^{-1}) to kill asexual stage parasites. Parasite infectivity in mosquitoes was evaluated as follows. When exflagellation rates were increased to over $20/10^5$ red blood cells, infected mice were subjected to bites of *Anopheles stephensi* mosquitoes. Fully engorged mosquitoes were selected and maintained at 20°C. After 2 week, the mosquitoes were dissected and the numbers of oocysts and sporozoites in their midguts were counted.

Cross-fertilization experiments

Cross-fertilization experiments were performed using a similar procedure to that described by Khan *et al.* (2005). *AP2-O* (–) parasites were fertilized with lines that have either defective male gametes (*P48/45*$^-$) or defective female gametes (*P47*$^-$). The numbers of unfertilized female gametes, deformed ookinetes and mature normal ookinetes were counted 20 h after fertilization, and conversion rates from female gametocytes to deformed ookinetes and mature normal ookinetes were calculated.

Genomic Southern hybridization

A 2 μg sample of *P. berghei* genomic DNA was digested with restriction enzymes, separated on a 1.5% agarose gel and transferred onto a nylon membrane. DNA fragments for Southern hybridization probes were amplified by PCR using genomic DNA as a template. The PCR primer pairs for AP2-O disruption were 5′-GCTGGAACTCCTTATTATGTTGCC-3′ and 5′-GCTTCAATCCACTATTTTCCAAACC-3′, and for the GFP-fused AP2-O-expressing mutants, the primers were 5′-GCTGGAACTCCTTATTATGTTGCC-3′ and 5′-GCT TCAATCCACTATTTTCCAAACC-3′. Amplified DNAs were labelled with alkaline phosphatase using AlkPhos Direct Labelling Reagents (Amersham Bioscience, Piscataway, NJ, USA) and subsequently detected using a CDP-Star chemiluminescent detection reagent (Amersham Bioscience).

Electrophoretic mobility shift assays (EMSA)

The *P. berghei* TF AP2 domain (amino acid residues 488–554) was produced as a glutathione S-transferase fusion protein using the GST Gene Fusion System with a pGEX 6p-1 vector (Amersham Bioscience). The AP2 domain coding region was amplified from *P. berghei* genomic DNA with the primer set: 5′-CGGGATCCGCCTTTAGGGTATTTGATGTAG AC-3′ and 5′-CCGCTCGAGTTAATACTTTAGTTTCATCATTT CGC-3′. The recombinant protein was purified with a glutathione Sepharose column (Amersham Bioscience). Longer probes were prepared by PCR with 5′-biotinylated primers using the cloned promoter region of *chitinase* or *SOAP* as templates. The primer pairs used were 5′-GGAGAGTTTT ATATTTTCAATTTTTATACTTAAAC-3′ and 5′-GAAAACGAA AAAAAGACAAATAAAAGAAC-3′ for *SOAP*, and 5′-ATTATT AATCACTATTTTATGGATGTAC-3′ and 5′-CCAAAAAATGG TGATATAGAAAAAGGC-3′ for the chitinase gene. Probes with a mutation in the TAGCTA sequence (TAGCTA to TAGGTA) were generated by PCR-mediated site-directed mutagenesis. Short oligonucleotide probes were prepared as follows. A synthetic 5′-biotinylated oligonucleotide was annealed with a complementary oligonucleotide to generate a double-strand probe. The oligonucleotide sequence was 5′-GTACATATTTTTTTGAATAGCTACCTTATTTTCCTTTGG-3′. Probes with various point mutations in the TAGCTA sequence were also prepared. EMSA was performed using a LightShift Chemiluminescence EMSA Kit (Pierce, Rockford, IL, USA). Briefly, GST-tagged protein (45 μg ml^{-1} final concentration) or GST as a control (30 μg ml^{-1} final concentration) was pre-incubated with a probe in EMSA buffer containing 50 ng μl^{-1} poly(dI-dC) for 20 min at room temperature. Gel electrophoresis was performed according to the manufacturer's protocol.

Targeted disruption of the AP2-O *gene*

The targeting vector was prepared by PCR according to Ecker *et al.* (2006). In brief, two fragments of the *AP2-O* gene were amplified by PCR using genomic DNA as the template with the primer pairs 5′-CGCGAGCTCGCAATATGGTATTA AATTTTGGGCTAGCCA-3′ (primer 1) and 5′-CGCGGATCC

GGTATTTTCATTGTGTTAAACGATATGTGA-3′ (primer 2), and 5′-CCGCTCGAGGTCCTATTTATCATTTTAAAATGTGT TTTATC-3′ (primer 3) and 5′-CGGGGTACCAATCGTCATA AATAGGAGTTATGAAGT-3′ (primer 4). The fragments were annealed to either side of the selectable marker gene (human DHFR) by PCR with primers 1 and 4. Gene targeting was performed as described previously (Yuda *et al.*, 1999a).

ChIP-quantitative PCR assays

ChIP was performed using a Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology, Lake Placid, NY, USA) according to the manufacturer's protocol. In brief, BALB/c mice (Japan SLC) were infected with malaria parasites expressing GFP-fused AP2-O (or WT parasites as a negative control) and treated with sulphadiazine to kill the parasite asexual stages. Blood was harvested and cultured to obtain ookinetes. After 20 h, each culture was fixed with 1% paraformaldehyde for 20 min at 30°C, washed with cold PBS, and incubated two or three times with NH_4Cl on ice for 30 min to lyse the erythrocytes. The ookinete-containing samples were sonicated in 250 µl of the lysis solution with a Bioruptor (Cosmo Bio, Tokyo, Japan) 10 times with a 30 s pulse and a 30 s interval. DNA fragmentation was checked by electrophoresis. IP was performed with anti-GFP polyclonal antibody (Clontech, Palo Alto, CA, USA) and pre-immune rabbit serum (negative control). DNA fragments obtained by IP were analysed by Real Time PCR using an iCycler iQ Real-Time Detection System (Bio-Rad, Hercules, CA, USA) with the primers listed in Table S5. Data were also presented as per cent input in Table S6.

Reporter assays with P. berghei *centromere plasmids*

Reporter assays were performed with the *P. berghei* centromere plasmid, Pcen (Fig. S3B). The upstream promoter region of the chitinase or SOAP gene (600 bp) was inserted into the EcoRV/BamHI site upstream of the GFP gene. This construct was transfected into cultured *P. berghei* shizonts by electroporation. Transfected parasites were selected by pyrimethamine to obtain parasites with the reporter construct. Ookinetes were cultured as described above and images were obtained with a fluorescent microscope equipped with a digital CCD camera system (Nikon, Kawasaki, Japan). Total fluorescent signal from each ookinete was analysed with Aqua Cosmos software (Hamamatsu Photonics, Hamamatsu, Japan). Background correction was performed by subtracting the intensity of a nearby cell-free region from the signal of the ookinete. Total of 30 ookinetes were used to determine promoter activity in each assay.

Construction of GFP-fused AP2-O-expressing parasites

GFP-fused AP2-O-expressing parasites (AP2-O::GFP) were constructed as follows. The targeted insertion vector was constructed in a pBluescript plasmid (Stratagene, La Jolla, CA, USA) (Fig. 1B). For the targeted insertion construct, a DNA fragment containing the 3′ part of the AP2-O coding region was amplified by PCR and inserted into the pBluescript XhoI/NheI site in frame with the GFP gene. The downstream region of the AP2-O gene was also amplified by PCR and inserted into the pBluescript BamHI/NotI site. Plasmids containing the construct were separated from plasmids without the construct by digestion with XhoI and NotI. AP2-O::GFP parasites were obtained by inserting the construct into the *AP2-O* locus by homologous recombination. Finally, AP2-O::GFP parasites were separated from WT by limiting dilution. GFP-fused protein fluorescence was observed with a microscope equipped with a filter set for GFP. For nuclear staining, Hoechst 34580 (Molecular Probes, Eugene, OR, USA) (0.02 µg ml^{-1} final concentration) was added to the medium, and the culture was incubated at room temperature for 10 min before fluorescence microscopy.

DNA microarray analysis

A custom 60-mer microarray (Agilent Technologies, Palo Alto, CA, USA) was designed based on *P. berghei* EST contigs (assembled from approximately 1×10^5 ESTs from different developmental stages, including blood stages, ookinetes, midgut sporozoites, salivary gland sporozoites and exoerythrocytic forms) and *P. berghei* genes in the NCBI Reference Sequence (RefSeq) Database (Pruitt *et al.*, 2007). This microarray contained approximately 21 000 probes and covered most *P. berghei* genes. Total RNA was prepared from malaria parasite blood stages and 12 h cultured ookinetes using RNAgents (Promega, Madison, WI, USA). Before RNA extraction, erythrocytes were lysed in 0.83% NH_4Cl. An RNA probe for one-colour analysis was synthesized using a Low RNA Fluorescent Linear Amplification Kit (Agilent Technologies) and fragmentated using a Gene Expression Hybridization Kit (Agilent Technologies) according to manufacturer's protocols. Hybridization was performed at 65°C. Three biologically independent experiments were performed in both *AP2-O* (–) and WT parasites. The data were analysed with Genespring software (Agilent Technologies). The data were normalized as follows. First, signal intensities less than 0.01 were set to 0.01. Then each chip was normalized to the 60th percentile of the measurements taken from the chip. Genes with significant differences between WT and *AP2-O* disruptants were selected by Student's *t*-test using *P*-value cut-off 0.05 (False discovery rate) and those whose expression was decreased at least fivefold in *AP2-O* (–) parasites were selected as candidates regulated by AP2-O. From the selected genes, those belonging to multigene families and those whose upstream sequences were not present in the *P. berghei* genome database were excluded. We have deposited the microarray data to Gene Expression Omnibus (GEO) DataSets with the Accession No. GSM359409–GSM359412, GSM359415 and GSM359428.

Quantitative RT-PCR

Quantitative analysis of *P. berghei AP2-O* expression was performed by quantitative RT-PCR. In brief, total RNA was extracted from asexual-free gametocytes and ookinetes. RT (1 µg of total RNA) was performed with random primers and Superscript II Reverse Transcriptase (Gibco BRL, Gaithersburg, MD, USA). SYBR Green PCR amplifications were performed using an iCycler iQ Real-Time Detection System (Bio-Rad Corporation) with the primer set: 5′-TTGGATT GCATCATGGTATG-3′ and 5′-TTCGGGGTTATTATTTTTAG GTTTTC-3′.

Identification of frequently occurring sequences in promoter regions

The frequency of occurrence of all possible 6 bp sequences in a gene's 1 kb upstream region was compared for the 15 genes in Fig. 2C and all annotated genes in the *P. berghei* genome (Table S2). Three 6 bp sequences, which had the five-base sequence TAGCT in common, were identified with significantly high frequencies (false discovery rate < 0.05) in the former group, and 10 of the 14 sequences with the highest frequency (false discovery rate < 0.5) could be aligned so that at least continuous 3 bp sequences overlap with each other. Based on the aligned sequences, the sequence conservation pattern in the 1 kb upstream regions of the 15 genes was generated using the fuzznuc and WebLogo programs (Olson, 2002; Crooks *et al.*, 2004).

Accession numbers

The PlasmoDB (http://www.plasmodb.org/plasmo/) accession number for *P. berghei AP2*-O is PB000572.01.0 and for *P. falciparum AP2*-O is PF11_0442. The reference numbers of blood-stage, ookinete and sporozoite ESTs are DC218765–DC224499, BB970378–BB981955, DC224500–DC235-241 respectively.

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LISP1 is important for the egress of *Plasmodium berghei* parasites from liver cells

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Summary

Most Apicomplexa are obligatory intracellular parasites that multiply inside a so-called parasitophorous vacuole (PV) formed upon parasite entry into the host cell. *Plasmodium*, the agent of malaria and the Apicomplexa most deadly to humans, multiplies in both hepatocytes and erythrocytes in the mammalian host. Although much has been learned on how Apicomplexa parasites invade host cells inside a PV, little is known of how they rupture the PV membrane and egress host cells. Here, we characterize a *Plasmodium* protein, called LISP1 (liver-specific protein 1), which is specifically involved in parasite egress from hepatocytes. LISP1 is expressed late during parasite development inside hepatocytes and locates at the PV membrane. Intracellular parasites deficient in LISP1 develop into hepatic merozoites, which display normal infectivity to erythrocytes. However, LISP1-deficient liver-stage parasites do not rupture the membrane of the PV and remain trapped inside hepatocytes. LISP1 is the first *Plasmodium* protein shown by gene targeting to be involved in the lysis of the PV membrane.

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Introduction

Plasmodium, the agent of malaria, is an Apicomplexan parasite that cycles through a mosquito and a mammalian host. In the mammalian host, it multiplies by schizogony inside hepatocytes and erythrocytes. The parasite form inoculated by the mosquito, the sporozoite, invades hepatocytes where it transforms into thousands of merozoites, which in turn invade erythrocytes. The successive cycles of merozoite multiplication inside erythrocytes then cause the disease pathology (Miller *et al.*, 2002a,b).

The two cell invasive stages of *Plasmodium*, the sporozoite and the merozoite, differentiate intracellularly within a parasitophorous vacuole (PV). The PV membrane (PVM), which is formed upon zoite entry and is mainly derived from the host cell plasma membrane, is devoid of host cell integral proteins and thus prevents PV fusion to endosomal compartments (Lingelbach and Joiner, 1998; Bano *et al.*, 2007). The PVM thus provides a safe niche for the maturing parasite and allows passage of vital nutrients and signals via channels and transporters (see review Charpian and Przyborski, 2008). Among the parasite proteins known to be present in the PVM of infected erythrocytes are exported protein 1 (EXP1) (Simmons *et al.*, 1987) and several members of the early transcribed membrane proteins (ETRAMP) family, which are integral PVM proteins organized in oligomeric arrays (Spielmann *et al.*, 2006). In hepatocytes, the PVM also contains EXP1 (Doolan *et al.*, 1996) and the stage-specific ETRAMPs known as UIS3 and UIS4 (Matuschewski *et al.*, 2002). The latter two proteins are expressed in sporozoites and early liver stages (LS) and are essential for their growth (Mueller *et al.*, 2005a,b; Tarun *et al.*, 2007).

Once the parasite has multiplied inside the PV, how the progeny exits the PV and the host cell remains largely unknown (Blackman, 2008). Electron (Aikawa, 1971) and live (Wickham *et al.*, 2003) microscopy have provided evidence for two distinct stages in the release of merozoites from erythrocytes, with the successive rupture of the

PVM and of the erythrocyte membrane. The two-step model applies to the release of merozoites from hepatocytes (Meis and Verhave, 1988; Sturm *et al.*, 2006; 2009). After disruption of the PVM, merozoite-filled membrane-bound extrusions, called merosomes (Sturm *et al.*, 2006; 2009; Baer *et al.*, 2007; Thiberge *et al.*, 2007), detach from the infected hepatocyte before releasing free merozoites into the blood circulation.

Here, we identify and characterize a *Plasmodium* protein, called LISP1 (liver-specific protein 1), which is specifically expressed by the LS and is expressed at high levels late during its development. Inactivation of *Lisp1* in *Plasmodium berghei* indicates that LISP1 is important for the destruction of the PVM surrounding the LS.

Results

Identification of Lisp1

We previously constructed expressed sequence tag (EST) libraries from various stages of *P. berghei* ANKA parasites, including ookinetes, midgut sporozoites, salivary gland sporozoites, LS isolated from a rat liver 31 h post infection with sporozoites, and merozoites. *Lisp1* was identified as a transcript present only in the LS library and it aligned to four annotated *P. berghei* genes: PB000708.00.0, PB001247.00.0, PB000682.00.0 and PB000250.00.0. An independent *in silico* analysis also selected PB000708.00.0 and PB000682.00.0 as candidate liver-specific genes and real-time PCR analysis confirmed that they were highly expressed in LS. The *Lisp1* transcript is predicted to encode a protein of 3249 amino acids (Accession No. AB231328) with a signal peptide sequence and a potential EF-hand but no other recognizable functional domain (http://www.plasmodb.org). The *Plasmodium yoelii Lisp1* orthologue (PY04499) has recently been detected in a proteomic analysis of infected hepatocytes (Tarun *et al.*, 2008). Orthologues of *P. berghei Lisp1* are also found in *Plasmodium falciparum* (PF14_0179) and *Plasmodium vivax* (PVX_085550), but not in other Apicomplexa parasites such as *Cryptosporidium hominis*, *Toxoplasma gondii*, *Theileria annulata* or *Eimeria tenella*.

Lisp1 *is expressed specifically in LS*

To confirm the LS-specific expression of *Lisp1*, real-time PCR analysis was performed on RNA isolated from NK65 blood stages and salivary gland sporozoites, as well as infected HepG2 cells at different time points (Fig. 1A). *Lisp1* messenger RNA (mRNA) was barely detectable in blood stages, present at low levels in sporozoites, and its quantity increased dramatically during parasite development in HepG2 cells with a peak at 40 h (10^4-fold increase compared with blood stages). Similar results were obtained with RNA isolated from the liver of rats infected with *P. berghei* ANKA, with *Lisp1* expression peaking at 48 h (Fig. S1). Thus *Lisp1* appeared to be most highly expressed at late stages of intrahepatocytic parasite development, when merozoites are formed.

Next, the subcellular localization of LISP1 was addressed by immunofluorescence analysis (IFA) with an anti-LISP1 polyclonal antibody. While no signal was detected in midgut or salivary gland sporozoites (not shown) or purified blood-stage schizonts, parasites developing in the liver of rat 48 h after sporozoite inoculation were brightly stained (Fig. 1B). LISP1 was also detected in infected HepG2 cells at 36, 48 and 65 h post infection (Fig. 1C) where it appeared to be associated with the PVM surrounding the developing LS. To better define the localization of LISP1, IFA was performed with anti-LISP1 and anti-EXP1 antibodies. As shown in Fig. 1D, the two proteins colocalized confirming that LISP1 is present in the PVM and in agreement with the localization of PY04499 in *P. yoelii*-infected hepatocytes (Tarun *et al.*, 2008).

LISP1-deficient parasites have a decreased infectivity to the mammalian host

To address the *in vivo* function of LISP1, we inactivated the gene in both wild-type (WT) ANKA and NK65 strains of *P. berghei* by double-cross-over recombination. The endogenous *Lisp1* was either interrupted by the selectable marker (*Lisp1I*) (Fig. 2A) or replaced by the marker (*Lisp1Δ*) (Fig. 2B). The *Lisp1I* modification was introduced in the WT ANKA strain, generating clone Lisp1I. The *Lisp1Δ* modification was introduced in WT NK65, generating the clone NK65Lisp1Δ, and in an ANKA strain expressing green fluorescent protein (GFP) (Janse *et al.*, 2006a), generating clone Lisp1ΔGreen. Southern blot analysis confirmed the expected structure of all recombinant loci (Fig. S2). The absence of LISP1 protein in Lisp1ΔGreen parasites was confirmed by IFA of infected HepG2 cells with an anti-LISP1 antibody (Fig. 2C). Using the same antibody, Western blot analysis of extracts from HepG2 cells 48 h post infection with WT ANKA sporozoites detected the expected 380 kDa band, whereas no band was observed upon infection with Lisp1Δ parasites (Fig. 2D).

All the *Lisp1* mutant parasite clones displayed similar growth rates in mouse blood stages and similar infectivity in mosquitoes, and yielded similar numbers of salivary gland sporozoites when compared with WT parasites (not shown). This indicated that *Lisp1* has no essential role in erythrocytic and mosquito stages. The infectivity of mutant sporozoites was measured by checking the emergence of blood-stage parasites in rats injected intravenously with Lisp1I salivary gland sporozoites. The parasitaemias

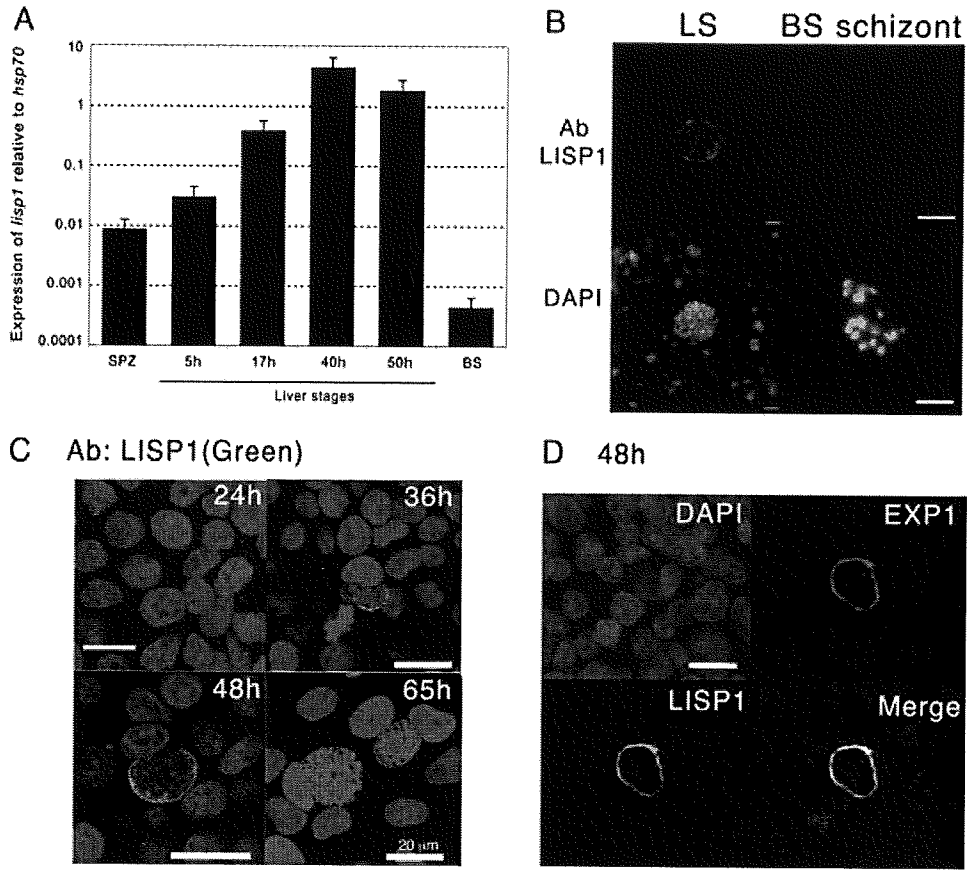

Fig. 1. *Pblisp1* is specifically expressed in late liver stages and localizes to the PVM.
A. Histogram representation of real-time RT-PCR analysis of *lisp1* relative gene expression in *P. berghei* sporozoites (SPZ), HepG2 cells 5, 17, 40 and 50 h post infection and mixed blood stages (BS). The value was normalized to the expression of *hsp70* mRNA in each sample. Error bars are standard deviation.
B. Immunofluorescence analysis of frozen sections of rat liver 48 h post infection (LS) and purified BS schizonts. Samples were incubated with an anti-LISP1 antibody followed by FITC-conjugated secondary antibody and nuclei were stained with DAPI (scale bar 5 µm).
C. Micrograph of confocal section of LS. HepG2 cells were fixed at 24, 36, 48 and 65 h post infection with WT ANKA sporozoites, incubated with an anti-LISP1 antibody followed by Alexa 488-conjugated secondary antibody and nuclei stained with DAPI (scale bars 20 µm).
D. Micrograph of confocal section of LS. HepG2 cells were fixed 48 h post infection with WT ANKA sporozoites, incubated with anti-LISP1 and anti-EXP1 antibodies followed by Alexa 488-conjugated secondary antibodies and nuclei stained with DAPI (scale bar 20 µm).

obtained with mutant sporozoites were significantly different ($P = 1.4 \times 10^{-7}$) from those obtained with WT sporozoites (Fig. 2E) and indicated a 15-fold decrease in infectivity. Similar results were obtained with all *Lisp1* mutants (Fig. S3), so the two ANKA-derived mutants, Lisp1l and Lisp1ΔGreen, were used for further analysis.

Mutant LS parasites are impaired in merozoite release

We then examined the intrahepatocytic development of mutant parasites. First, 300 000 ANKA or Lisp1l sporozoites were injected intravenously into rats and 48 h later the number of LS were counted in liver sections immunostained with anti-CS antibodies. No significant difference was observed between the numbers of ANKA and Lisp1l LS parasites (Fig. 3A). Second, the development of WT and Lisp1ΔGreen LS was assessed with an anti-UIS4 antibody. The presence of a PVM was detected until 48 h in both WT and mutant parasites (Fig. 3B). Furthermore, when WT and Lisp1l (or Lisp1ΔGreen) sporozoites were incubated with HepG2 cells there was no significant difference in the number of LS parasites at 48 or 64 h (data not shown). Finally, we assessed by real-time imaging the development of WT and mutant LS at 24, 36 and 48 h post infection of HepG2 cells. Again there was no significant difference in the size of WT compared with mutant LS over this period (Fig. S4).

To examine hepatic merozoite development, the numbers of merozoites released from HepG2 cells 65 h post infection were compared between the WT and mutant parasites. To internally control experiments, mixed infections were performed with a 1:1 ratio of sporozoites of Lisp1ΔGreen and of WTRed, a WT ANKA derivative that expresses red fluorescent protein (RFP) from the

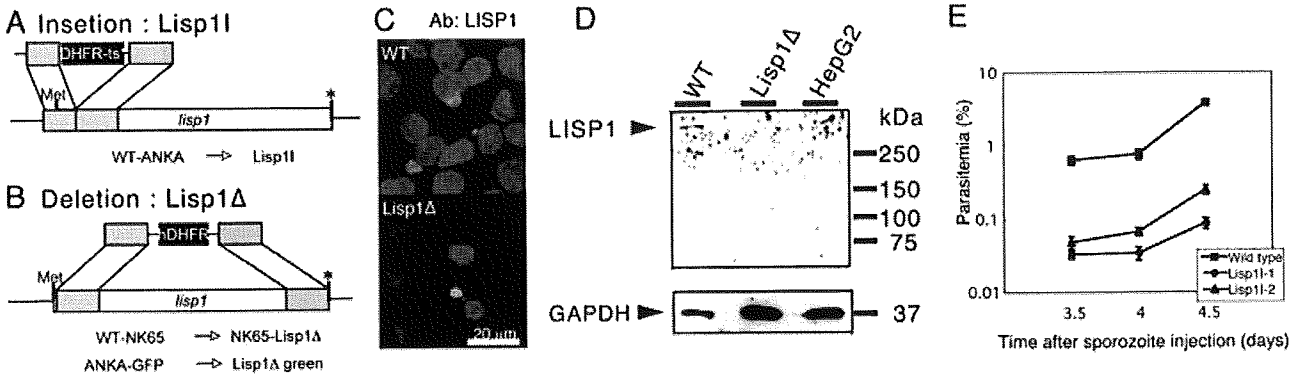

Fig. 2. Targeted gene disruption of *lisp1*.
A. Schematic representation of *Lisp1l* gene disruption.
B. Schema of *Lisp1Δ* gene disruption. Shaded boxes indicate the regions of homology used for double-cross-over recombination; black boxes indicate the selectable marker; Met and the asterisk (*) indicate the initiation and stop codons respectively.
C. Absence of LISP1 protein in Lisp1Δ parasites. Microscopy image of HepG2 cells 48 h post infection with WTGreen or Lisp1ΔGreen. LS were labelled with anti-LISP1 antibody and Alexa 647-conjugated secondary antibody (red); nuclei stained with DAPI (blue).
D. Western blot analysis of extracts of HepG2 cells 48 h post infection with ANKA (WT) or Lisp1ΔGreen sporozoites. Arrowheads indicate the bands of LISP1 and human GAPDH.
E. Lisp1l sporozoites have a decreased infectivity in the mammalian host. A total of 30 000 wild-type or Lisp1l sporozoites, from two independent Lisp1l clones, were injected intravenously into rats and blood parasitaemias were examined by Geimsa staining. The error bars show the standard errors from three rats.

eef1α promoter (Sturm *et al.*, 2009) and the ratio of green versus red merozoites (G/R) released in the culture supernatant was calculated (see Table 1). To verify that green and red merozoites were detected with similar efficiency, cells were co-infected with a 1:1 ratio of WTGreen and WTRed sporozoites. An average G/R merozoite ratio of 1 was found, confirming that green- and red-fluorescent merozoites were detected with similar efficiency. In contrast, co-infections with Lisp1ΔGreen and WTRed parasites gave an average G/R merozoite ratio of 0.1:1. The 10-fold reduction in the number of merozoites released by mutant LS suggested that LISP1 might be important for merozoite formation within, or escape from infected hepatocytes.

To evaluate the number of hepatic merozoites formed in *lisp1* mutant LS, adherent HepG2 cells, co-infected with Lisp1ΔGreen and WTRed sporozoites, were recovered after 65 h and mechanically disrupted to release the merozoites. The ratio of Lisp1ΔGreen to WTRed merozoites was ~1 (Fig. 4, left panel cells, hMZ), indicating that mutant parasites generated normal numbers of merozoites. Next, the infectivity of mutant merozoites was tested. When the approximately 1:1 ratio of Lisp1ΔGreen to WTRed merozoites collected from the disrupted HepG2 cells was injected into mice, the same ratio was found in the ensuing blood stages (Fig. 4, left panel cells, iRBC). Further, when supernatants from the mixed infections collected at 65 h were injected intravenously into mice, the ratio of Lisp1ΔGreen : WTRed blood-stage parasites (Fig. 4, right, supernatant) was the same as the ratio of injected hepatic merozoites and was maintained after several multiplication cycles, showing that the merozoites released from *lisp1* mutant LS were indeed infectious (see also Fig. S5) and had similar infectivity to red blood cells compared with WT.

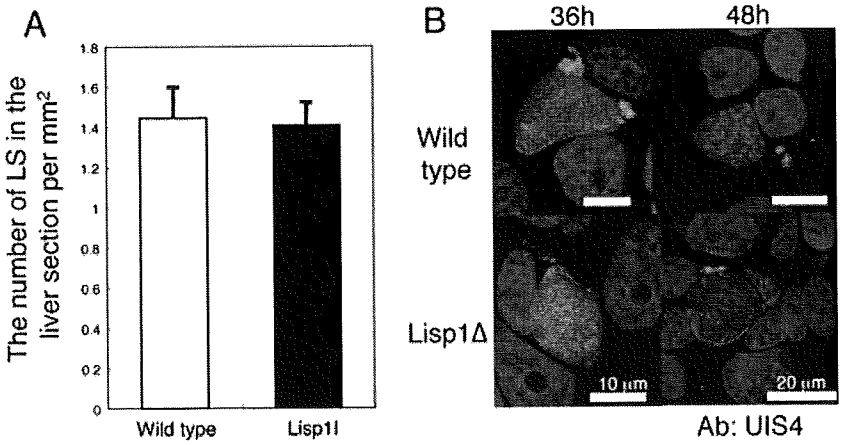

Fig. 3. *Lisp1*-defective parasites develop normally, within a PVM, into late-stage LS.
A. Histogram representation of the number of LS *in vivo*. A total of 300 000 wild-type or Lisp1l sporozoites were injected intravenously into rats and 48 h later the number of WT (white box) and Lisp1l (black box) LS were counted in the liver sections. Bars indicate the standard deviation from three rats.
B. Confocal micrograph of PVM in *Lisp1*-defective parasites. HepG2 cells 36 and 48 h post infection with WT (Wild type) or Lisp1ΔGreen sporozoites. LS parasites (green) labelled with an anti-UIS4 antibody (red) and nuclei stained with DAPI (blue).

