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A NIMA-related Protein Kinase Is Essential for Completion of the Sexual Cycle of Malaria Parasites*

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The molecular mechanisms regulating the sexual development of malaria parasites from gametocytes to oocysts in their mosquito vector are still largely unexplored. In other eukaryotes, NIMA-related kinases (Neks) regulate cell cycle progression and have been implicated in the regulation of meiosis. Here, we demonstrate that Nek-4, a new *Plasmodium* member of the Nek family, is essential for completion of the sexual cycle of the parasite. Recombinant *Plasmodium falciparum* Nek-4 possesses protein kinase activity and displays substrate preferences similar to those of other Neks. Nek-4 is highly expressed in gametocytes, yet disruption of the *nek-4* gene in the rodent malaria parasite *P. berghei* has no effect on gamete formation and subsequent fertilization. However, further differentiation of zygotes into ookinetes is abolished. Measurements of nuclear DNA content indicate that zygotes lacking Nek-4 fail to undergo the genome replication to the tetraploid level that precedes meiosis. Cell cycle progression in the zygote is identified as a likely precondition for its morphological transition to the ookinete and for the successful establishment of a malaria infection in the mosquito.

Malaria remains a devastating disease in most tropical and subtropical regions. The problem has been exacerbated over the last decades of the twentieth century by the emergence and spread of resistance of the causative agents, parasitic protists of the genus *Plasmodium*, to available antimalarials (*Plasmodium falciparum* is the species responsible for the vast majority of lethal cases) (1). Infection of the human host is initiated by the bite of an infected *Anopheles* mosquito, which delivers sporozoites into the bloodstream. The sporozoites rapidly gain the liver, where they invade hepatocytes and undergo a first round of schizogony. The merozoites produced in this process are released into the bloodstream and invade erythrocytes, where they undergo recurrent and synchronized schizogony. This is the phase of the parasite's life cycle that is responsible for malaria pathogenesis. A proportion of merozoites, upon invasion of a new red blood cell, do not enter schizogony, but arrest their cell cycle and develop into male or female gametocytes, the only forms capable of infecting the mosquito vector. Ingestion of gametocytes during a blood meal triggers their further development into gametes, a process, which for the male gametocyte, involves three rounds of genome replication and generation of eight flagellated male gametes. Fertilization is followed by nuclear fusion, one round of genome replication, and meiosis, which occurs within 3 h (2, 3). The nuclear envelope remains intact throughout this process, and meiosis is not followed by nuclear division. As a consequence the ookinete, a motile form that develops from the zygote and exits the mosquito midgut lumen, is tetraploid. The ookinete establishes an oocyst at the basal lamina, which produces several thousand sporozoites. These accumulate in the insect salivary glands and render the mosquito infective for a new human host. Sexual development in the mosquito vector is thus essential for transmission of malaria. Compounds interfering with this process, "transmission-blocking drugs," would represent a useful tool in the context of malaria control, especially with respect to prevention of the escape of drug-resistant genotypes selected during schizogony in the human host (for more information on malaria, see www.malaria.org).

Protein phosphorylation plays central roles in the control of eukaryotic cell proliferation and development, and protein kinases are now considered as prime targets in a number of pathologies, including cancer and neurodegenerative diseases (4). Genome-wide analyses of the *P. falciparum* genome (5, 6) identified 65 genes encoding serine/threonine protein kinases, and one of these, a calcium-dependent protein kinase (CDPK4) has recently been identified as essential for male gametogenesis in *Plasmodium berghei*, a *Plasmodium* species infecting rodents (7).

To further investigate the molecular mechanisms regulating sexual development of the parasite in its vector, we decided to

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identify protein kinase genes expressed specifically in gametocytes, and to characterize such enzymes at the biochemical and functional levels.

The kinome analyses in *P. falciparum* detected a cluster of four structurally related serine/threonine kinases belonging to the NIMA[1] (never in mitosis/Aspergillus) family, designated Neks (NIMA-related kinases); of these four genes, three were revealed by micoarray analysis to be expressed predominantly in gametocytes (8, 9). The fourth one, Pfnek-1, is expressed in both asexual parasites and gametocytes, possesses atypical properties (such as an activation site that is much closer to those of MEK1/2 that to those of other Neks), and has been implicated in the regulation of an atypical *P. falciparum* mitogen-activated protein kinase (10).

The founding member of the NIMA family was identified in the filamentous fungus *Aspergillus nidulans* and was shown to be required for G_2/M transition (11). Overexpression of NIMA leads to premature chromatin condensation and mitotic spindle formation (12). NIMA homologues have been identified in all eukaryotes, and there is accumulating evidence that members of the extended Nek family play important roles in the control of mitosis (13). The closest relative of NIMA in human cells is NEK2, and several observations point to a role for NEK2 in regulating the organization of centrosomes (14). Other mammalian members of the NIMA/Nek family (*e.g.* Nek4) have been shown to be expressed predominantly in the testis, and may intervene in meiosis (15).

Here, we (i) present the characterization at the biochemical level of a novel member of the *P. falciparum* Nek family, Pfnek-4, that displays maximal homology to the catalytic domain of human NEK4, (ii) verify the gametocyte-specificity of its expression pattern, and (iii) demonstrate that in the rodent malaria parasite *P. berghei*, Pbnek-4 plays an essential role in zygote-to-ookinete transformation, by regulating DNA replication that precedes meiosis.

MATERIALS AND METHODS

Bioinformatics—Members of the NimA protein kinase family encoded by the human, yeast, and *P. falciparum* genomes were identified from global phylogenetic trees of the respective kinomes (5, 16, 17). Family members from *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Arabidopsis thaliana* were identified through BLAST searches of the Refseq protein data base (18). Membership of the NimA family was verified by construction of a phylogenetic tree that contained representatives of all other major protein kinase families as an outgroup (not shown). Multiple sequence alignments were constructed using the HMMalign algorithm (19, 20) and a hidden Markov model trained on a complete set of aligned human protein kinases. The alignments were polished manually. For the construction of the phylogenetic tree, the alignment was trimmed to remove all regions containing gaps or uncertainties; 194 amino acid positions were retained. The 100-replicate bootstrapped, distance matrix tree was constructed using the Protdist and Fitch algorithms from the Phylip package (21).

P. falciparum Cultures—*P. falciparum* (clone 3D7) was grown in human erythrocytes as described previously (22). Gametocytes were prepared according to the protocol of Carter *et al.* (23).

Molecular Cloning, Expression, and Kinase Assays of Pfnek-4—Oligonucleotides (forward: GAGAGGGATCCATGAATAAATATGAAAAGATTAGA, reverse: GAGAGGTCGACTTAAGTATCAACAACATCCAGAAG) were designed to contain the start and stop codons (double underline) of the full-length, 7-exon Pfnek-4 open reading frame predicted by the Glimmer algorithm on PlasmoDB (gene identifier MAL7P1.100), as well as BamHI and SalI sites, respectively (single underline). The open reading frame was amplified by PCR from a gametocyte cDNA library (a gift from Pietro Alano) and the 933-bp amplified product was inserted between the BamHI and SalI sites of the pGEX-4T3 vector, yielding the plasmid pGEX-Pfnek-4. Catalytically inactive ("kinase-dead") recombinant Pfnek-4 was obtained by site-directed mutagenesis ($Lys^{32} \rightarrow$ Met) of pGEX-Pfnek-4 using the overlap extension PCR technique (24). A similar strategy was used to mutate Thr^{163} into Ala. All inserts were verified by DNA sequencing prior to expression of the recombinant proteins in *Escherichia coli* (strain BL21-CodonPlus). Expression and purification of wild-type and mutated GST-Pfnek-4 were performed following published procedures (25). Kinase assays were performed in a standard reaction (30 μl) containing 25 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$, 2 mM $MnCl_2$ 15 μM ATP/5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Biosciences) and 1 μg of substrate (α-casein, β-casein, myelin basic protein (MBP), or histone H1, purchased from Sigma). Reactions were initiated by addition 1 μg of the recombinant wild-type or mutated Pfnek-4. The reaction proceeded for 30 min at 30 °C and was stopped by the addition of Laemmli buffer, boiled for 3 min, and analyzed by electrophoresis on 12% SDS-polyacrylamide gel. The gels were dried and submitted to autoradiography.

Immunological Methods—To obtain IgYs against Pfnek-4, a synthetic peptide (SQKEKRQCLKEVE) derived from the N-terminal region (residues 40–53) was used for immunization of chickens. The internal Cys residue allowed coupling to rabbit albumin. Protocols for immunization, preparation of parasite protein extracts, isolation and affinity purification of IgYs, and Western blot analysis using an anti-IgY horseradish peroxidase-conjugated secondary antibody, were described previously (26).

P. berghei Maintenance, Culture, Transmission, and Purification—The *P. berghei* ANKA strain clone 2.34 and a CDPK4-KO parasite, clone 9.2 (7), were maintained in Theiler's original outbred mice as described. *Anopheles stephensi* mosquitoes of the SD500 strain were reared and infected by feeds on infected mice as described. To purify gametocytes for analysis by Western blot, asexual parasites were first eliminated by treating infected mice for 2 days with sulfadiazine (20 mg/liter in the drinking water), followed by gametocyte enrichment on a 48% Nycodenz gradient (see Ref. 7 for details).

Generation of Transgenic Parasites—The KO vector for *pbnek-4* was constructed in plasmid pBS-DHFR, in which polylinker sites flank a *Toxoplasma gondii dhfr/ts* expression cassette that conveys resistance to pyrimethamine. PCR primers olLR87KpnI and olLR88HindIII (see Table I for the sequences of oligonucleotides used in the *P. berghei* study) were used to generate a 481-bp fragment from the 5′-coding sequence of *pbnek-4* from genomic DNA, which was inserted into KpnI/HindIII sites upstream of the *dhfr/ts* cassette of pBS-DHFR. A 527-bp fragment generated with primers olLR89BamHI and olLR90NotI from the 3′-end of *pbnek-4*, and the downstream flanking region was inserted downstream of the *dhfr/ts* cassette. This replacement construct was then excised as a KpnI-NotI fragment and used for transfection.

The *pbnek-4* complementation construct was assembled in a C-terminal-tagging vector, pSD141, in which a double c-Myc epitope tag is followed by a STOP codon and the 3′-untranslated region of the *pbdhfr/ts* gene (7). A 2,274-bp DNA fragment beginning 667-bp upstream of the predicted start codon of *pbnek-4* and extending to the end of the last exon (but omitting the TGA stop codon) was amplified from genomic DNA as two overlapping fragments using primer pairs olLR91XhoI/olLR92EcoRV and olLR93EcoRV/olLR94ApaI. These oligonucleotides were designed in such a way that, following restriction digest of vector and PCR products, a three-fragment ligation reaction inserted the *P. berghei* genomic fragments into unique XhoI and ApaI restriction sites in pSD141, creating a unique EcoRV restriction site between the fragments, upstream of the *pbnek-4* start codon (Fig. 5*A*). The insert sequences of the resulting complementation vector pSD141nek-4 were verified by sequencing. For insertion into the 5′-flanking sequence of the genomic *pbnek-4* locus by a single crossover strategy, pSD141nek-4 was linearized at the unique EcoRV restriction site.

Transgenic *P. berghei* were generated by electroporation of cultured schizonts, selected, and cloned essentially as described (7, 27), except that $\sim 2 \times 10^7$ schizonts were electroporated with 5 μg of linearized plasmid DNA using a Nucleofector Device (Amaxa, Germany) with protocol U33 and an Amaxa T Cell Nucleofector kit (VPA-1002).

Genotype Analysis of Transgenic Parasites—Genotypes of transgenic parasites were analyzed by pulsed-field gel electrophoresis (PFGE), Southern hybridization, and PCR, using standard protocols. For PFGE plugs of parasites were prepared in low melting agarose and extracted with sodium lauryl sarcosinate in the presence of proteinase K. Chromosomes were separated at 4 °C with a Amersham Biosciences LBK 2015 Pulsaphor system using 1% agarose gels in 0.5× Tris borate/EDTA (0.05 M Tris, 0.05 M boric acid, 0.125 M EDTA). The pulsing regime comprised a linear ramp of 500–700 s for 60 h, followed by a second linear ramp of 300–500 s for 25 h and a final ramp of 200–300 s for 8 h, all at 3.5 V/cm. Separated chromosomes of a selected but

[1] The abbreviations used are: NIMA, never in mitosis/Aspergillus; Nek, NIMA-related kinase; GST, glutathione *S*-transferase; KO, knockout; wt, wild type; RT, reverse transcriptase.

uncloned parasite population were blotted onto Hybond N+ nylon membrane (Amersham Biosciences) and probed either with the *pbnek-4* 5′-targeted sequence to identify the *pbnek-4* locus, or with a *tgdhfr/ts* probe to identify the chromosome, into which the KO construct had integrated. Following cloning, disruption of the *pbnek-4* gene was verified by diagnostic PCR on genomic DNA, using oligonucleotides shown in Fig. 5*A*. Primer pair olOB81/olOB84 was used to generate a 2.5-kb product characteristic of the intact pbnek-4 locus. Primer pair olOB81/olOB70 amplified across the integration site, giving rise to a 1.5-kb product only in the KO. Positive control primers olOB81/olLR88 produced a 0.6-kb fragment with template DNA from wt and KO.

Analysis of P. berghei Sexual Development—To quantify exflagellation, 2.5 μl of blood from the tail of an infected mouse on day 4–5 post-infection were taken up in a heparinized pipette tip, mixed with 7.5 μl of medium (RPMI1640 containing 25 mM HEPES, 10% fetal bovine serum, pH 7.30 or pH 8.00, supplemented or not with 50 μM xanthurenic acid), placed on a slide and covered with a Vaseline®-rimmed coverslip. To obtain a measure of exflagellation, centers of movement were then counted in 10 microscopic fields (×40 objective, ×10 ocular lens), containing ca. 15,000 RBC, by phase contrast microscopy between 15 and 25 min postinduction, when exflagellation activity is maximal (28, 29). To compare exflagellation levels between parasite clones in Fig. 6*A*, the number of centers of movement is expressed relative to microgametocyte numbers, which were determined per 15,000 RBC on Giemsa-stained blood films.

In vitro fertilization and ookinete formation assays were done by taking up 10 μl of blood from the tail of an infected mouse on day 4 postinfection into a heparinized pipette tip and mixing it immediately with 30 μl of ookinete culture medium (RPMI1640 containing 25 mM HEPES, 25% fetal bovine serum, 10 mM sodium bicarbonate, 50 μM xanthurenic acid, pH 7.6). After 1 h at 19 °C to allow fertilization to take place, cultures were diluted with 0.5 ml of ookinete medium, transferred to a 24-well plate, and cultured at 19 °C for a further 21–24 h. For direct immunolabeling cultured cells were pelleted for 2 min at 800 × *g* and then labeled for 1 h on ice in 50 μl of ookinete medium containing Cy3-conjugated mouse monoclonal antibody 13.1 specific for P28 (29) and Hoechst 33342 (Sigma) to a final concentration of 5 μg/ml. P28-positive cells were than counted in a hemocytometer or observed under Vaseline®-rimmed cover slips with a Leica DMR fluorescence microscope equipped with a Leica DC500 digital camera. To measure nuclear DNA content digital images of Hoechst-stained cells were analyzed using NIH ImageJ software version 1.33u. The relative nuclear fluorescence intensity was determined with the following formula: Area [pixel] × (average intensity [relative units] − average background intensity [relative units]). The nuclear fluorescence intensity was standardized to the haploid DNA content by measuring the fluorescence intensity of ring stage parasites and early trophozoites in parallel on the same slide and with the same microscope and camera settings.

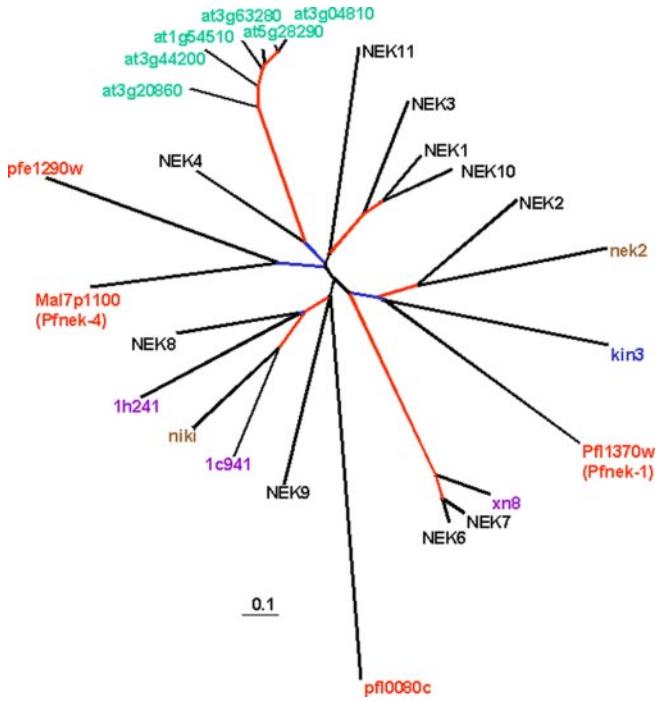

FIG. 1. **Multispecies tree of NIMA-related kinases.** Model organism sequences were extracted from the Refseq data base. Human (*black*), *D. melanogaster* (*brown*), and *C. elegans* (*purple*) proteins are labeled according to EntrezGene names. *S. cerevisiae* (*blue*) and *A. thaliana* (*green*) proteins are named according to the Refseq entries. *P. falciparum* proteins are labeled in *red*. The consensus bootstrapped tree was constructed from conserved regions of a multiple sequence alignment. Branches with bootstrap values >70 (of 100 replicates) are shown in *red* and with values >40 in *blue*.

RESULTS

Phylogenetic Analysis of P. falciparum NIMA-related Kinases—Two independent genome-wide analyses of the protein kinase-encoding genes in *P. falciparum* identified four members of the NIMA/Nek family (5, 6). In order to gain some predictive insight into the putative function of these enzymes, we performed a multispecies phylogenetic analysis including the exhaustive complement of NIMA-related kinases of *P. falciparum*, *Saccharomyces cerevisiae*, *C. elegans, D. melanogaster*, *Homo sapiens* and *A. thaliana* (Fig. 1). None of the plasmodial sequences can be assigned clear orthology to the enzymes of other eukaryotes, which likely reflects the phylogenetic distance between malaria parasites and plants or Opis-

FIG. 2. **Alignment of Pfnek-4 with Pbnek-4 and the catalytic domain of human NEK4.** Residues in Pbnek-4 (PlasmoDB identifier Pb_111f03p1c) and human NEK4 (EMBL accession number BC063044) that are identical to those in Pfnek-4 are indicated with a (−), and gaps introduced to optimize alignment are indicated by a (.). Mutagenized residues (Lys^{32} and Thr^{163}) are *boxed*. The sequence of the peptide used for immunization is shaded in *gray*. The *bar* near the C terminus indicates the putative coiled-coil domain. Residues that are largely conserved in Ser/Thr protein kinases are indicated *above* the Pfnek-4 sequence. The *vertical bar* at residue 256 indicates the C-terminal boundary of the catalytic domain.

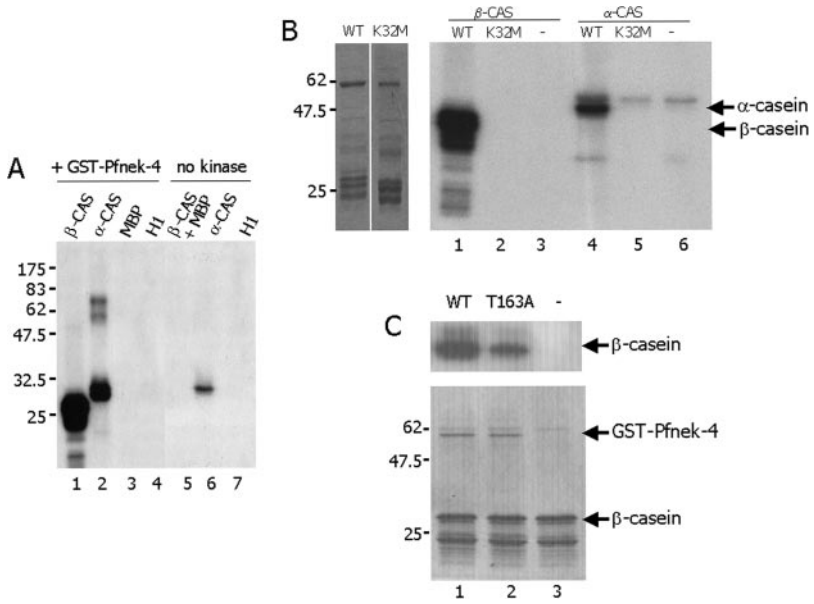

FIG. 3. **Kinase assays.** *A*, kinase activity of GST-Pfnek-4 on various protein substrates. The figure depicts the autoradiogram of an SDS-PAGE gel on which the kinase reaction was analyzed. Kinase assays were set up with α-casein, β-casein, myelin basic protein, and histone H1 as potential substrates. 1 μg of GST-Pfnek-4 was added to the reactions run in *lanes 1–4*, whereas the reactions in *lanes 3–7* did not contain recombinant kinase, as negative control. The α-casein preparation contains some endogenous kinase activity. *B*, kinase-dead control. Kinase reactions were performed with wild-type GST-Pfnek-4 (*lanes 1* and *4*), the K32M dead mutant (*lanes 2* and *5*), or without exogenous kinase (*lanes 3* and *6*), using β-casein (*lanes 1–3*), and α-casein (*lanes 4–6*) as substrates. Panels to the *left* are Coomassie-stained gels showing that both the wild-type and K32M mutant proteins were present at similar levels in the reaction. *C*, effect of the T163A mutation. Kinase reactions were performed with wild-type GST-Pfnek-4 (*lane 1*), the T163A mutant (*lane 2*), or no exogenous kinase (*lane 3*), using β-casein as a substrate. The *top panel* is an autoradiogram showing β-casein phosphorylation, and the *bottom panel* is a Coomassie Blue stain of the gel, showing that wild-type and mutant GST-Pfnek-4 were present in the same amount in the reactions. The sizes of comigrating molecular mass markers are indicated to the *left* in kDa.

thokonta, the phylogenetic lineage that regroups fungi and Metazoans (5, 30). Nevertheless, the previously described Pfnek-1 (PFL1370w) clusters within the NEK2/Kin3 branch, which confirms earlier BLASTP data suggesting relatedness to NEK2 homologues (10). PFL0080c appears to be relatively distant from other NIMA kinases, although its position on the tree may reflect some relatedness to NEK8, the overexpression of which is associated with breast tumors (31), and to NEK9, which has been implicated in the control of progression through the G_1 and S phases of the cell cycle (32). The remaining two NIMA-related kinases, PFE1290w and MAL7P1.100, appear as loosely associated with human NEK4. By analogy with the proposed function of the latter enzyme in human germ cell production (15), these two plasmodial enzymes may be involved in sexual development of the parasite, which would be consistent with the gametocyte specificity of their expression revealed by microarray analysis (8, 9). We therefore decided to investigate the properties and function of these NIMA kinases, starting with MAL7P1.100, which we called Pfnek-4.

Primary Structure of Pfnek-4—The various gene prediction algorithms on the PlasmoDB data base (33) proposed conflicting intron/exon configurations. To resolve this issue, we amplified the coding region from a gametocyte cDNA library using primers pairs designed according to all predictions. Cloning and sequencing the amplicons allowed us to conclude that the GeneFinder prediction was correct, and that the coding region is composed of seven exons. The predicted polypeptide has a molecular mass of 34.1 kDa, and a high isoelectric point of 9.3, in line with mammalian NIMA-related kinases. Both protein kinase signatures (34) are present in the sequence, as are the 15 invariant residues found in protein kinases (35). Alignment with human NEK4 (43% identity) shows that regulatory sites are conserved. Similar to several Neks from other organisms, a predicted coiled-coil domain is present at the C terminus of Pfnek-4, as detected

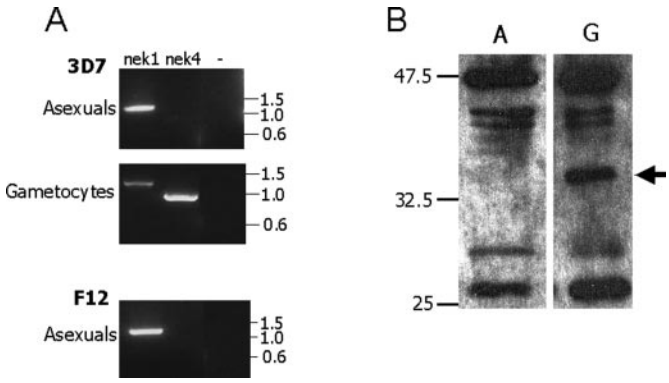

FIG. 4. **Pfnek-4 is expressed in gametocytes, but not in asexual parasites.** *A*, RT-PCR. Total RNA from clones 3D7 (asexual parasites and gametocytes) and F12 (asexual parasites) was subjected to RT-PCR using primers specific for Pfnek-1 (positive control, expressed in both asexual parasites and gametocytes) or Pfnek-4. The lanes labeled (−) are Pfnek-4 reactions performed in the absence of reverse transcriptase. *B*, Western blot of extracts from asexual parasites (*A*) and gametocytes (*G*) using the anti-Pfnek-4 IgY. Parasite pellets were resuspended in Laemmli loading buffer, and the protein run on a 12% acrylamide gel. The *arrow* indicates the position of Pfnek-4. The sizes of comigrating molecular mass markers are indicated to the *left*.

by the algorithm on www.ch.embnet.org/cgi/bin/COILS_form.html (prob. 0.64) (Fig. 2). We also identified the *P. berghei* orthologue in the PlasmoDB data base (identifier Pb_111f03p1c); Pbnek-4 displays 96.5% identity to Pfnek-4 over the full-length predicted protein, and 98% identity over the catalytic domain.

Protein Kinase Activity of Recombinant Pfnek-4—The entire Pfnek-4 coding region was amplified and inserted into the pGEX-4T3 bacterial expression vector. The purified recombinant GST-Pfnek-4 fusion protein was used in kinase assays, using a variety of protein substrates (Fig. 3*A*). The enzyme was able to phosphorylate α- and β-casein (*lanes 1* and *2*), but

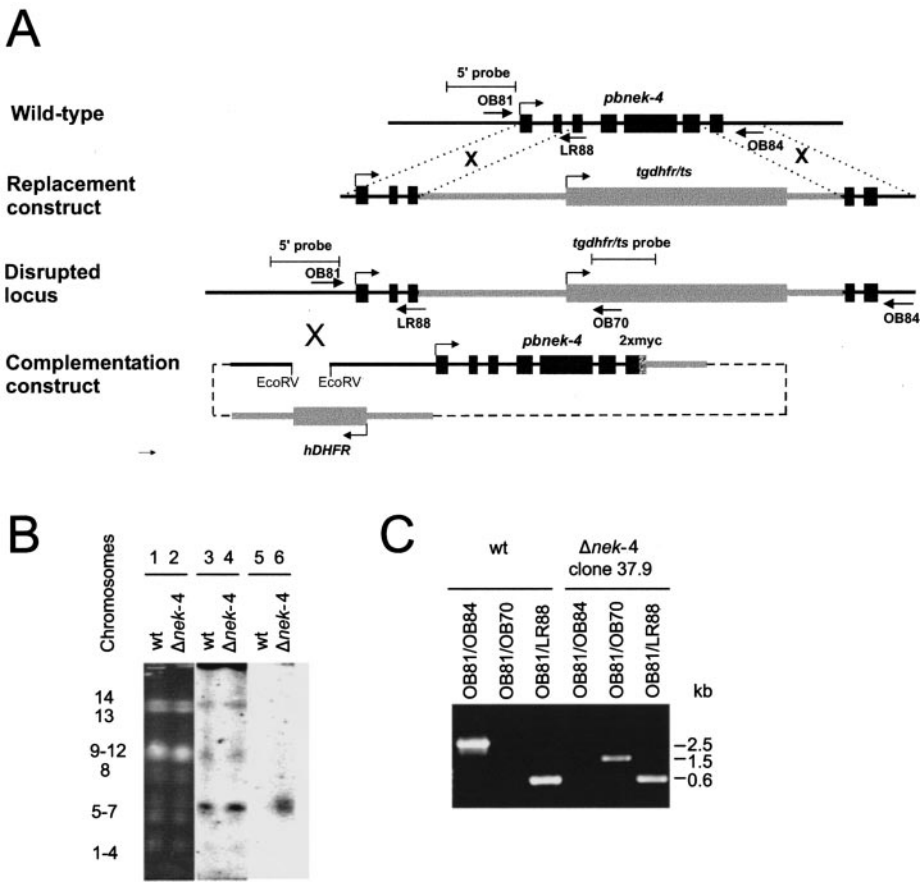

FIG. 5. **Targeted disruption of the *nek-4* gene in *P. berghei*.** *A*, schematic representation of the *pbnek-4* locus, the gene-targeting construct used for gene replacement by double homologous recombination, and a complementation vector used to reconstitute *pbnek-4* by a single cross-over strategy, integrating the vector into the non-disrupted 5′-upstream sequence. *B*, pulsed field gel electrophoresis showing chromosome separation in the agarose gel (*lanes 1* and *2*), localization of the *pbnek-4* locus by a 5′-upstream probe to within a group of chromosomes probably containing numbers 5–7 (*lanes 3* and *4*), integration of the targeting vector into the same chromosome in the *pbnek-4*-KO population (*lanes 5* and *6*). *C*, diagnostic PCR verifying disruption of the *pbnek-4* gene in clone 37.9. Genomic DNA from this clone fails to give rise to the *pbnek-4* product, but instead is positive for a specific product amplified across the predicted integration site of the targeting construct.

neither myelin basic protein nor histone H1 (*lanes 3* and *4*) were used as substrates by GST-Pfnek-4. This substrate preference is consistent with that observed for mammalian NIMA/Nek family members.

Tools are available to identify preferred substrate peptides; in conjunction with the availability of a *Plasmodium* genomic data base, this should allow the identification of potential plasmodial substrates for this enzyme, which can then be investigated experimentally.

To verify that the activity was indeed due to GST-Pfnek-4 and not to bacterial contaminants present in the recombinant protein preparation, we repeated the experiment using a kinase-dead mutant Pfnek-4, in which the Lys^{32} residue had been substituted by Met. This Lys residue is required for proper orientation of the ATP molecule and is therefore conserved in protein kinases. Its replacement by Met abolishes the activity. As can be seen on Fig. 3*B*, the K32M-mutated kinase is devoid of activity, confirming that the phosphorylation in Fig. 3*A* is caused by GST-Pfnek-4 itself.

We also mutated Thr^{163}, a conserved residue that in other mammalian NIMA kinases is the target of activating phosphorylation. Replacement of this Thr with Ala reduced the activity of GST-Pfnek-4 by ~3-fold (Fig. 3*C*), suggesting that the amino acid at this position can modulate enzymatic activity.

Stage Specificity of mRNA and Protein Expression—To independently verify the microarray data available on PlasmoDB, we performed RT-PCR analysis of total RNA obtained from unsynchronized asexual parasites and gametocytes of the 3D7 clone (Fig. 4*A*). Although the signal was much stronger in the reactions using gametocyte RNA than in those using RNA from asexual parasites, a faint signal was detected in the latter. This could be due either to low level expression of Pfnek-4 in asexual parasites, or to contamination of the asexual parasite preparation by small numbers of gametocytes. To unambiguously solve this question, we repeated the experiment using the F12 parasite clone, which is derived from 3D7 through long term passage and has lost the ability to undergo gametocytogenesis (36). Clearly, there was no detectable Pfnek-4 signal from F12 RNA, but the Pfnek-1 RT-PCR included as positive control (Pfnek-1 is expressed in both asexual parasites and gametocytes) yielded the expected signal from the F12 sample. This conclusively demonstrates that Pfnek-4 mRNA accumulates with stringent specificity in gametocytes.

There are documented instances of *Plasmodium* genes being expressed at the mRNA level in gametocytes, but whose mRNA is not translated until gametogenesis has been initiated (37). The presence of Pfnek-4 protein in gametocytes was therefore monitored by Western blotting (Fig. 4*B*), using an immunopurified antibody directed against a Pfnek-4-derived peptide (see "Materials and Methods"). As a negative control, we used extracts from asexual parasites. The antibody cross-reacted with several bands in extracts from both asexual parasites and gametocytes, which provides a useful control for equal loading; Coomassie Blue staining of the gel also confirmed that total protein amounts were similar in both lanes (not shown). A band of the expected size (34 kDa) was observed only in gametocytes, demonstrating that gametocyte-specific Pfnek-4 mRNA accumulation is accompanied by translation.

Pbnek-4 Is Essential for Parasite Transmission to the Mosquito—To characterize the function of Nek-4 we turned to *P. berghei*, a malaria parasite of rodents which is more amenable to reverse genetics approaches than *P. falciparum* (27), and in which sexual development is readily accessible to analysis both *in vitro* and *in vivo*. To disrupt *pbnek-4*, most of the kinase domain (residues 84–247) was replaced with a pyrimethamine-resistant allele of the *dhfr/ts* gene from *T. gondii* (Fig. 5*A*).

TABLE I
Oligonucleotides used in the P. berghei KO study

Name	Sequence[a]	Use
olLR87KpnI	CCCCGGTACCAGGGATATAGGAAAAGG	*pbnek-4*-KO 5′ target
olLR88HindIII	CCCCAAGCTTCTTCACCTTTACAATGC	*pbnek-4*-KO 5′ target
olLR89BamHI	CCCCGGATCCATCGAGTTACTGTTCAG	*pbnek-4*-KO 3′ target
olLR90NotI	CCCCGCGGCCGCGTACATGCTGTATATGGGAAC	*pbnek-4*-KO 3′ target
olLR91XhoI	CCCCCCTCGAGGAAAGCCGAGCAAAAGTG	Complementation
olLR92EcoRV	GATATCGAATGAGCAATACACAATCC	Complementation
olLR93EcoRV	GTATTGCTCATTCGATATCATACATATTCTCACAAAAT	Complementation
olLR94ApaI	CCCGGGCCCAACATCAACAATATCCAATAATAATG	Complementation
olOB70	ACCTGCAGGCATGCAAGCTT	Diagnostic PCR
olOB72	AGAGGGGCATCGGCATCA	*tgdhfr/ts* probe
olOB73	TTGAAAGAATGTCATCTC	*tgdhfr/ts* probe
olOB81	CGAGCAAAAGTGTTAAAATTG	Diagnostic PCR
olOB84	TGCAAATTTGGAAATCTATACTG	Diagnostic PCR

[a] Restriction sites underlined.

Following drug selection, the targeting construct had integrated into the chromosome on which *pbnek-4* is located (Fig. 5*B*). Following dilution cloning, disruption of the *pbnek-4* gene was confirmed for two clones (designated Pb37.7 and Pb37.9) by a diagnostic PCR across the junction of the expected integration site of the targeting construct (Fig. 5*C*). We first characterized the two *pbnek-4*-KO clones during their development in the mouse. Both were not affected in their ability to replicate asexually after blood-induced infections and gave rise to normal numbers of gametocytes when compared with wild type (data not shown). In marked contrast, mosquito transmission of the *pbnek-4*-KO was blocked completely (Table II), and no oocysts were found on midguts of mosquitoes examined 12–14 days after feeding on infected mice. These results suggest an essential function for Nek-4 after parasite differentiation into morphologically mature gametocytes, but before the formation of oocysts.

Pbnek-4-KO Macrogametes Become Activated but Fail to Develop into Ookinetes—We then asked whether KO gametocytes were able to respond to conditions that trigger gamete formation *in vitro* (28, 38). *pbnek-4*-KO gametocytes of either sex responded to stimulation by pH shift or xanthurenic acid by emerging from their host cells (not shown), microgametocytes exflagellated as in the wild-type clone (Fig. 6*A*), and occasional fertilization events could be observed by phase contrast microscopy. To confirm that *pbnek-4*-KO macrogametocytes were efficiently activated, we examined the expression of the macrogamete/zygote/ookinete surface antigen P28. The *p28* mRNA accumulates in macrogametocytes, but is translationally repressed until the cells become activated by conditions in the mosquito midgut (39). The detection of P28 by a monoclonal antibody on the surface of a live cell 24 h after activation indicates emergence of the macrogametocyte from its host cell and the translational de-repression of the *p28* mRNA. It also allows the conversion rate from P28-positive round parasites (macrogametes or zygotes) into elongated ookinetes to be determined. The total number of P28-expressing parasite stages was comparable with wild type, indicating normal macrogametocyte activation in the *pbnek-4*-KO (not shown). However, the conversion of female *pbnek-4*-KO parasites into ookinetes was completely blocked (Fig. 6*B*). This was not caused by a defect in the male gametes, since in genetic crosses (Fig. 6*B*) these were fully capable of cross-fertilizing macrogametes of the *cdpk4*-KO clone, which on their own do not differentiate into ookinetes because of a specific defect in male gamete formation that prevents fertilization (7). Taken together these results show that the development of macrogametes into ookinetes is blocked in the *pbnek-4*-KO parasites.

Complementation with c-Myc Epitope-tagged Pbnek-4 Restores Ookinete Formation—The facts (i) that parasites lacking

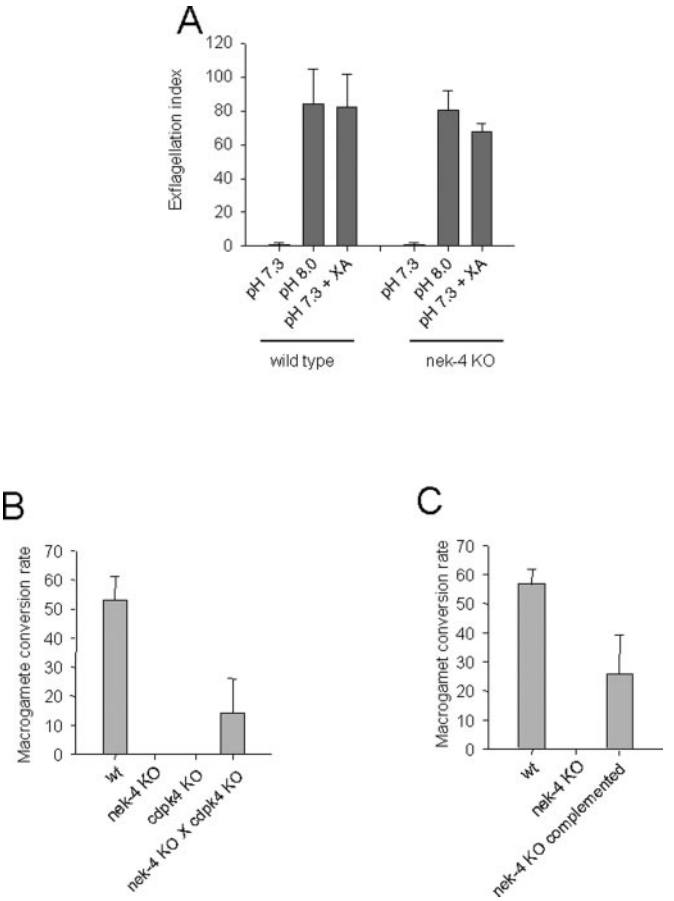

FIG. 6. **PbNek-4 is essential for ookinete formation.** *A*, microgametogenesis induced either at pH 8.0, or by xanthurenic acid at the otherwise non-permissive pH of 7.3, is not affected in the *pbnek-4*-KO clone 37.9 *in vitro*. The exflagellation index is defined as the number of exflagellation centers per 100 microgametocytes. Arithmetic means ± S.D. from three infected mice per parasite clone are shown. *B*, macrogamete conversion rate of wt and KO parasite clones and a genetic cross is shown as the percentage of P28-positive parasites in 24-h ookinete cultures that have assumed the typical ookinete shape. Arithmetic means ± S.D. from three experiments with parasites from different infected mice are shown. Loss of ookinete formation was independently confirmed with another *pbnek-4*-KO clone, 37.7 (not shown). *C*, complementation of the *pbnek-4* KO clone 37.9 with the *pbnek-4* gene restores ookinete formation in a population of KO parasites.

Pbnek-4 are able to complete asexual cycles, gametocytogenesis, and gametogenesis, and (ii) that two independent *pbnek-4*-KO clones display the same block in ookinete differentiation, argue strongly against the possibility that the observed phenotype is caused by a pleiotropic or nonspecific effect. Neverthe-

TABLE II
PbNek-4 is essential for parasite transmission to the mosquito
Geometric mean oocyst numbers and prevalence of *P. berghei* infections in *A. stephensi* mosquitoes fed directly on mice infected with wt (clone 2.34) or *pbnek-4*-KO (clones 8.2) parasites.

	Geometric mean	Prevalence	*n*
	oocyst number	%	
Experiment 1			
Wt	207.16	100.00	20
pbnek-4-KO	0.00	0.00	20
Experiment 2			
Wt	158.37	90.00	20
pbnek-4-KO	0.00	0.00	20
Experiment 3			
Wt	155.50	85.00	20
pbnek-4-KO	0.00	0.00	20

less, to verify that the phenotype of the *pbnek-4* KO clones is caused by the lack of Pbnek-4 expression, a complementation construct was designed to reintroduce the *pbnek-4* gene into the 5′-upstream region that had remained intact in the KO (Fig. 5*A*). Following transfection of the complementation construct, parasites were subjected to intermittent drug selection for two passages to reduce the proportion of parasites that had failed to integrate the construct (Table II). Gametocytes from the uncloned transfected population were then assayed for their ability to differentiate into ookinetes *in vitro*. Complementation succeeded in restoring ookinete formation, although not to the level of the wild type (Fig. 6*C*). Incomplete functional complementation could be due to not all gametocytes in the population re-expressing Pbnek-4, or to detrimental effects of the extrinsic 3′-untranslated region on expression levels.

Pbnek-4 Is Required for the Diploid Genome of the Zygote to Replicate Prior to Meiosis—We then determined more accurately the point at which sexual development in the *pbnek-4*-KO fails. Using surface expression of P28 as a stage-specific marker, we quantified the DNA content of macrogametes and subsequent parasite stages 24 h after gametocytes had been activated *in vitro*. Fluorometry on Hoechst 33342-stained nuclei confirmed that wild-type ookinetes were tetraploid, when compared with ring stage parasites in the same cultures. Unfertilized macrogametes of two different exflagellation-deficient KO clones, one lacking CDPK4 (7), and the other lacking the mitogen-activated protein kinase Pbmap-2,[3] maintained a fluorescence level between the haploid and diploid value that is typical of gametocytes (40). In the *pbnek-4*-KO clones nuclear fluorescence was elevated by 1 unit above that of the macrogamete, indicating that fertilization had occurred. Nuclear fusion also seemed to occur, as judged by the presence of single large nuclei in most zygotes. In a very small number of zygotes male and female nuclei had remained separate (*arrows* in Fig. 7*A*), a phenomenon also occasionally observed with wild-type parasites (not shown). The nuclear DNA content of *pbnek-4*-KO zygotes remained significantly below the tetraploid value of the ookinete (Fig. 7*B*), indicating a developmental arrest before or early during S-phase that precedes meiosis.

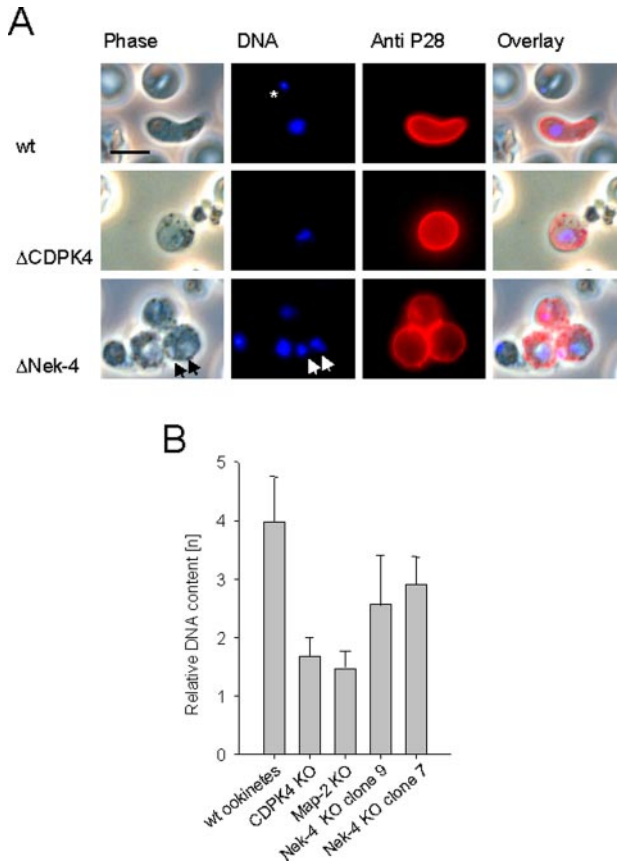

FIG. 7. **PbNek-4 is required for DNA replication in the zygote.** *A* shows the nuclear staining pattern by Hoechst 33342, and P28 expression in live cells in 24 h ookinete culture. Note the intense nuclear fluorescence of the tetraploid ookinete nucleus if compared with that of a young asexual parasite (*asterisk*). The *bottom row* of images show three zygotes, in one of which male and female pronuclei (*arrows*) have exceptionally failed to undergo fusion. The nucleus of the third zygote in the same image is out of focus. The *scale bar* corresponds to 5 μm. *B*, nuclear DNA content of parasites from the same cultures shown in *A* was determined fluorometrically in wt ookinetes, macrogametes of two KO strains that fail to fertilize (CDPK4-KO and Map-2-KO) and two Nek-4-KO clones.

DISCUSSION

Assigning clear orthology to any human NEK family member is not possible, presumably because of the phylogenetic distance between mammals and Apicomplexa. However, the fact that the closest human enzyme is NEK4, both in BLASTP searches and in the more refined analysis presented in Fig. 1, may suggest a similarity in function. NEK4 has been detected in testis, and a role for this enzyme in meiotic division has been proposed. We show that malaria parasites lacking the nek-4 enzyme are unimpaired in asexual growth, gametocytogenesis, and gametogenesis, but following fertilization are unable to initiate DNA replication in the zygote. This is consistent with *Plasmodium* Nek-4 and human NEK4 belonging to the same subgroup of the Nek family. Failure of Nek-4 KO zygotes to differentiate into ookinetes provides genetic evidence that cell cycle progression in the zygote is a requirement for ookinete morphogenesis. We note however, that preliminary observations by Janse *et al.* (41) indicated that blocking DNA synthesis in *P. berghei* zygotes with a DNA polymerase-α inhibitor, aphidicolin, did not prevent the formation of morphologically mature ookinetes. We conclude that either the Nek-4-dependent entry into S-phase (but not its aphidicolin-sensitive completion) are required for ookinete formation, or that Nek-4 has a separate and additional function in ookinete morphogenesis. In view of recent evidence for a role of a Nek family kinase in ciliary function during interphase in *Chlamydomonas* (42), we cannot currently exclude such an additional function, for instance in organizing the longitudinal subpellicular microtubules that give the ookinete its characteristic shape (43).

The observation that the competence to produce ookinetes is restored by crossing the *pbnek-4*$^-$ clone with a *PbCDPK4*$^-$ clone defective in male gametocytogenesis confirms that male *pbnek-4*$^-$ parasites are competent for fertilization, and that the

[3] Tewari, R., Dorin, D., Moon, R., Doerig, C., and Billker, O. (2005) *Mol. Microbiol.*, in press.

phenotype is likely to be linked to macrogametes. Because Nek-4 is already synthesized in gametocytes, we suggest it is the macrogamete that introduces the critical amount of Nek-4 protein into the zygote, where it performs its essential function.

This work identified the Nek-4 enzyme as essential for completion of the sexual cycle of a malaria parasite, and hence as a valid target for transmission-blocking drugs. Interference of transmission through a small molecule kinase inhibitor present in the blood meal has been documented in another study, where ookinete formation was inhibited by KN-93, an inhibitor of calcium/calmodulin-dependent kinases (44). This indicates that this approach is implementable. The strong kinase activity of recombinant Pfnek-4 makes it amenable to medium- or high-throughput screening of chemical libraries, as a first step toward the identification of compounds with transmission-blocking potential (45).

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Addendum—While this report was under review, an article was published that included the description of an independent *pbnek-4*-KO parasite (Khan, S. M., Franke-Fayard, B., Mair, G. R., Lasonder, E., Janse, C. J., Mann, M., and Waters, A. P. (2005) *Cell* **121,** 675–687). The latter study and the present report concur in their conclusions regarding the phenotype of *P. berghei* parasites lacking a functional *pbnek-4* gene.

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