UNIVERSITE DE LA MEDITERRANEE – AIX MARSEILLE II

FACULTE DE MEDECINE DE LA TIMONE

ECOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE

THESE DE DOCTORAT

pour obtenir le grade de

DOCTEUR de L'UNIVERSITÉ de la MÉDITERRANÉE

Mention Pathologies Humaines Spécialité Maladies Transmissibles et Pathologies Tropicales

Protéine kinase AMP cyclique dépendante

et cycle de *Plasmodium falciparum*

Présentée publiquement le 12 juillet 2010

Par **WURTZ Nathalie**

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Professeur MARVALDI Jacques	Président du jury et Examinateur
Professeur PARZY Daniel	Directeur de thèse

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AVANT-PROPOS

Le format de présentation de cette thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille. Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette thèse. Par ailleurs, la thèse est présentée sur article publié, accepté ou soumis associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Prof. Didier Raoult.

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RESUME

L'aggravation actuelle du risque lié au paludisme résulte du développement du phénomène de résistance de souches de *Plasmodium falciparum* aux molécules antipaludiques. Une telle situation et l'absence de vaccin efficace nécessitent le développement de nouvelles stratégies antiparasitaires. Jusqu'à présent, les mécanismes moléculaires qui contrôlent le cycle parasitaire sont méconnus. Chez la plupart des eucaryotes, les protéine kinases sont impliquées dans des fonctions cellulaires essentielles et constituent une cible privilégiée pour la conception de nouveaux médicaments. Dans ce cadre, nous nous sommes intéressés à la voie de transduction de l'AMP cyclique et en particulier à la sous-unité catalytique de la protéine kinase AMPc dépendante (PfPKAc) dont le rôle essentiel reste mal défini chez *P. falciparum*. Deux approches complémentaires ont été choisies pour étudier cette kinase :

1) **au niveau biochimique** par le clonage, l'expression, la purification et la caractérisation enzymatique de la PfPKAc. L'objectif était d'obtenir une enzyme active *in vitro* de façon à pourvoir mesurer les constantes enzymatiques de la PfPKAc et conduire les premiers essais d'inhibitions.
2) **au niveau cellulaire** en analysant les conséquences de l'inhibition par des ARN interférents spécifiques des transcrits de la PfPKAc. Le développement parasitaire mais également le transcriptome global ont été étudiés de manière à préciser les voies métaboliques liées à cette kinase plasmodiale.

L'ensemble de ces études précise la compréhension de la voie de transduction de l'AMP cyclique et de la PfPKA qui pourrait conduire au développement de nouvelles voies thérapeutiques.

Mots clés : *Plasmodium falciparum*, protéines kinases, PKA, caractérisation biochimique, ARN interférence, puces à ADN, voies métaboliques.

ABSTRACT

cAMP-dependent protein kinase and *Plasmodium falciparum* life cycle

Nowadays, the increase of risks associated with malaria results from the development of resistance of *Plasmodium falciparum* strains to antimalarial drugs. This situation and the lack of an effective vaccine require the development of new antimalarial strategies. Until now, molecular mechanisms controlling the life cycle of malaria parasites, are still poorly understood. In most eukaryotes, protein kinases are implicated in essential cellular functions and represent attractive targets for the development of new drugs. In this context, we focused on the signaling pathway implicating cAMP and particularly the catalytic subunit of cAMP-dependent protein kinase (PfPKAc), whose function is still unclear in *P. falciparum*. Two complementary strategies were chosen to study this kinase:

1) **at the biochemical level** by the cloning, expression, purification and enzymatic characterization of the PfPKAc. The objective was to obtain an *in vitro* active PfPKAc to evaluate the kinetic constants of PfPKAc and to conduct the first inhibition studies.
2) **at the cellular level** by studying the consequences of PfPKAc transcripts inhibition by specific interfering RNAs. The parasite growth but also the overall transcriptome were studied to specify the metabolic pathways associated with this plasmodial protein kinase.

All of these studies improve the understanding of cAMP transduction pathway and PfPKA, which could allow the development of new therapeutic approaches.

Keywords : *Plasmodium falciparum*, protein kinases, PKA, biochemical characterization, RNA interference, microarray, metabolic pathways.

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INTRODUCTION

Le paludisme dans le monde

Le paludisme demeure un problème majeur de santé publique dans le monde, responsable de 0,863 Millions de décès en 2008 principalement en Afrique, en Asie et en Amérique latine [1]. Deux milliards d'individus, soit environ 40% de la population mondiale sont exposés au paludisme et on estime à 258 millions le nombre de cas cliniques survenant en 2008 (**Figure 1**) [2]. 80% des cas sont enregistrés en Afrique subsaharienne, où ils concernent majoritairement les enfants de moins de cinq ans et les femmes enceintes [2]. Le paludisme représente la 5ème maladie infectieuse la plus meurtrière dans le monde après le sida, les maladies respiratoires aiguës, les maladies diarrhéiques et la tuberculose [3].

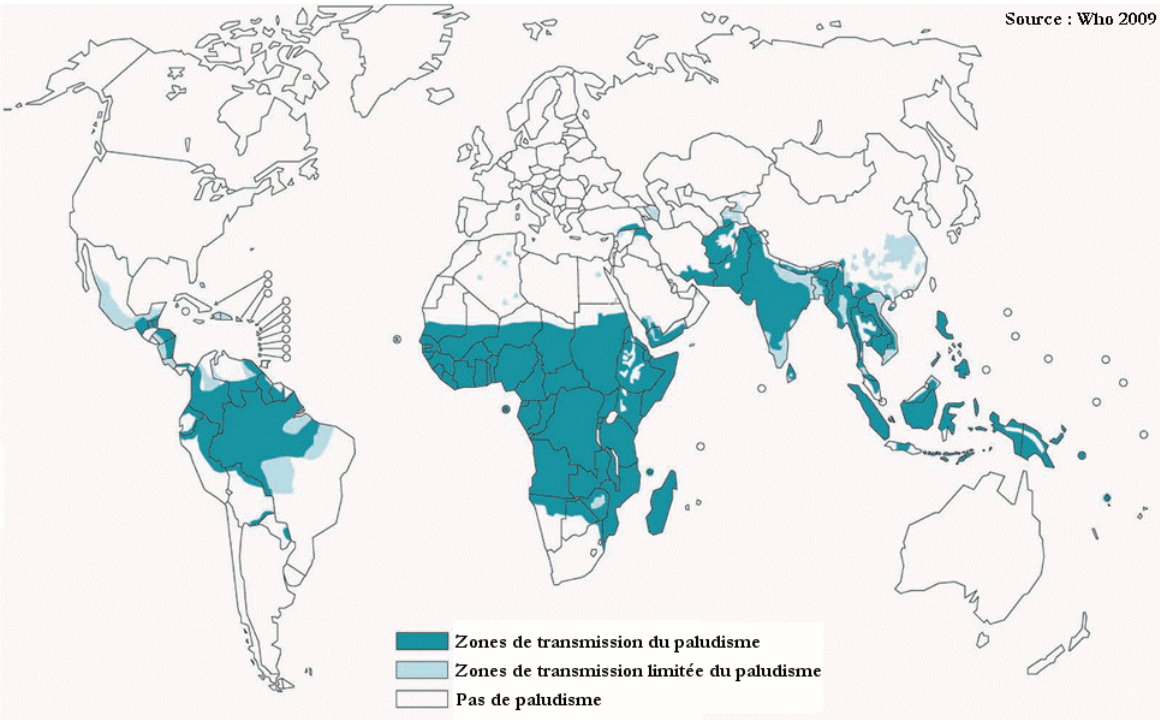


Figure 1 Répartition du paludisme dans le monde en 2009

Les différentes espèces de *Plasmodium*

Le paludisme (du latin *palus*, *paludis*, marais), appelé aussi malaria (de l'italien *mal'aria*, mauvais air), est une parasitose due à un protozoaire du genre *Plasmodium* de la famille des apicomplexes, transmis par la piqûre d'une anophèle femelle. Cinq espèces peuvent infecter l'homme :

1. *Plasmodium falciparum* : responsable de la forme la plus grave de la maladie (neuropaludisme), souvent mortelle, présent dans les régions équatoriales et subtropicales. La grande majorité des cas mortels est due à *P. falciparum* qui induit des atteintes neurologiques (neuropaludisme ou paludisme cérébral), hématologiques (anémie sévère) et/ou des complications durant la grossesse (paludisme gestationnel) [4].
2. *Plasmodium vivax*, largement répandu en Amérique du Sud et en Asie, pouvant causer des accès de reviviscence pendant 3 à 4 ans.
3. *Plasmodium ovale*, présent en Afrique intertropicale du centre et de l'ouest, ainsi que dans certaines régions du Pacifique (principalement présent dans les zones endémiques où *P. vivax* est absent), pouvant lui aussi causer des accès de reviviscence pendant 5 ans.
4. *Plasmodium malariae*, dont la répartition géographique est dispersée et qui provoque des accès palustres récurrents très tardifs pouvant avoir lieu jusqu'à 20 ans après l'infection originelle.
5. *Plasmodium knowlesi*, dont les infections chez l'homme sont récentes car cette espèce a été longtemps confondue avec *P. malariae*. [5,6].

La famille des apicomplexes et ses particularités

La famille des apicomplexes contient plusieurs milliers d'espèces parasites unicellulaires, parmi lesquelles un grand nombre sont des agents pathogènes majeurs tels que la toxoplasmose (*Toxoplasma*), la coccidiose des ovins et des gallinacés (*Eimeria*), la cryptosporidiose (*Cryptosporidium*), la babesiose (*Babesia*), la theileriose (*Theileria*) et des maladies émergentes telles que la néosporose bovine et canine (*Neospora*). La classification phylogénétique des protistes a été remaniée à de nombreuses reprises [7]. La révision la plus récente de la classification des apicomplexes tient compte des avancées de la recherche fondamentale (approche morphologique, étude des voies de synthèse biochimiques, phylogénétique moléculaire) accomplies depuis la version précédente, commandée par « l'International Society of Protistologists » [8-10] (**Figure 2**). Dans cette nouvelle classification le phylum des apicomplexes comprend uniquement des êtres parasitaires à l'exception des Colpodellida.

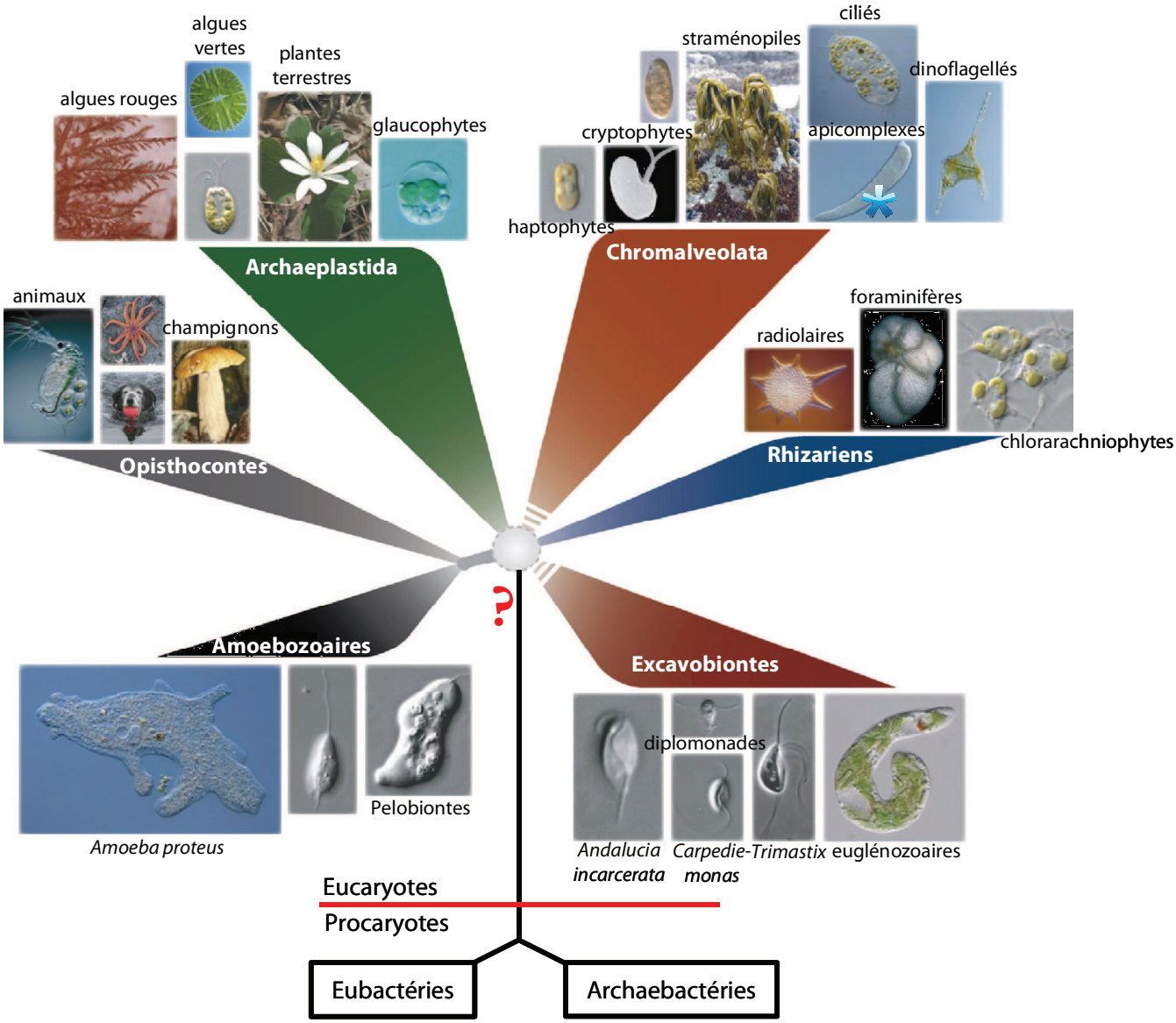


Figure 2 Représentation schématique de l'organisation des eucaryotes en 6 règles.

La classification repose sur les informations collectées grâce aux avancées des méthodes analytiques en phylogénétique moléculaire. Les relations exactes entre les six groupes et la position exacte de la racine de l'arbre demeurent non résolues. Le point d'interrogation indique la position hypothétique de la racine [10]. Le signe (*) indique le groupe dans lequel les apicomplexes sont classés.

A ce jour, la classification officielle de *Plasmodium* est la suivante: un Apicomplexa Aconoidasida (complexe apical incomplet –conoïde absent- aux stades asexués mobiles) Haemospororida (zygote mobile avec conoïde, microgamètes flagellés produits par schizogonie) [8].

Malgré la grande variété des cycles parasitaires, la famille des apicomplexes à laquelle appartient *Plasmodium* partagent des structures subcellulaires uniques (**Figure 3)**. En terme morphologique, les apicomplexes sont constitués d'un complexe apical qui regroupe un ensemble d'organites spécialisés (rhoptries, granules denses et micronèmes), outil qui leur permet de pénétrer à l'intérieur de la cellule hôte [11,12].

La majorité des apicomplexes étudiés à ce jour (*Plasmodium*, *Toxoplasma*, *Babesia*…) présentent deux organites contenant des éléments génétiques extrachromosomaux, la mitochondrie et un chloroplaste vestigial, l'apicoplaste.
Le genre *Plasmodium* comprend près de 200 espèces constituant un groupe de 9 sous-genres de parasites intracellulaires obligatoires de vertébrés, capables de se développer uniquement chez les mammifères (3 sous-genres), les oiseaux (4 sous-genres) et les lézards (2 sous-genres) [13].

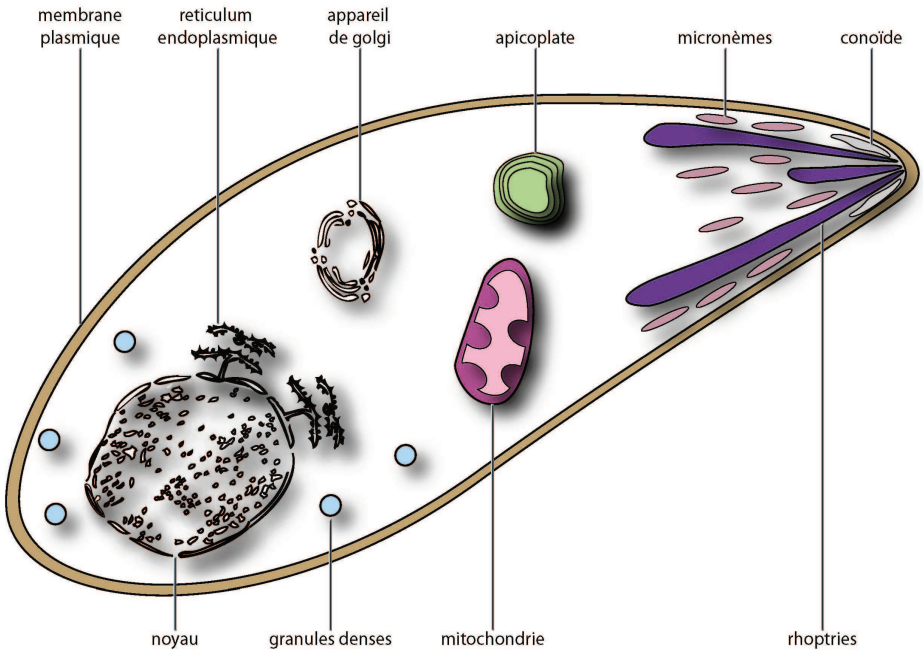


Figure 3 Caractéristiques générales d'une cellule de parasite apicomplexe

Trois organites sécrétoires vésiculaires, les rhoptries, micronèmes et granules denses participent à l'invasion et à l'élaboration de la vacuole parasitophore. Comme dans les cellules végétales, en plus du noyau et de la mitochondrie, un *plaste vestigial*, ou apicoplaste contient de l'ADN.

Le cycle de *P. falciparum*

Le cycle du *Plasmodium* est complexe et consiste en une succession de différents stades de développement, chacun adapté à une fonction particulière dans un environnement intra- ou extra-cellulaire (**Figure 4**) [14,15]. Au cours de son repas sanguin, l'anophèle femelle injecte des sporozoïtes dans le derme. Ceux-ci transitent dans la circulation sanguine et en quelques minutes envahissent les hépatocytes [16]. Dans les hépatocytes, les sporozoïtes se multiplient pour former des schizontes pré-érythrocytaires, qui contiennent chacun des milliers de mérozoïtes. Cette phase de multiplication est asymptomatique et dure environ 8 à 15 jours, selon les espèces. Contrairement à *P. vivax* et *P. ovale*, *P. falciparum* ne possède pas de formes persistantes dans les hépatocytes (hypnozoïtes) [17,18]. Les mérozoïtes sont ensuite libérés dans la circulation sanguine et vont débuter le cycle sanguin asexué en infectant les hématies ; seule cette phase sanguine est responsable des symptômes de la maladie [19]. Le mérozoïte pénètre grâce à un processus actif et se différencie au sein de la vacuole digestive en anneau, en trophozoïte (début de réplication intensive) puis en schizonte. Les globules rouges parasités par les schizontes vont éclater et libérer 8 à 32 mérozoïtes dans la circulation sanguine qui ré-infectent rapidement de nouveaux globules rouges (**Figures 4 et 5**). Au cours de leur développement au sein des érythrocytes, certains parasites se différencient en gamétocytes : macrogamétocyte (femelle) et microgamétocyte (mâle) [20] Ces gamétocytes ne poursuivent leur développement en gamètes que s'ils sont ingérés au cours d'un repas sanguin de l'anophèle femelle (**Figures 4 et 5**).

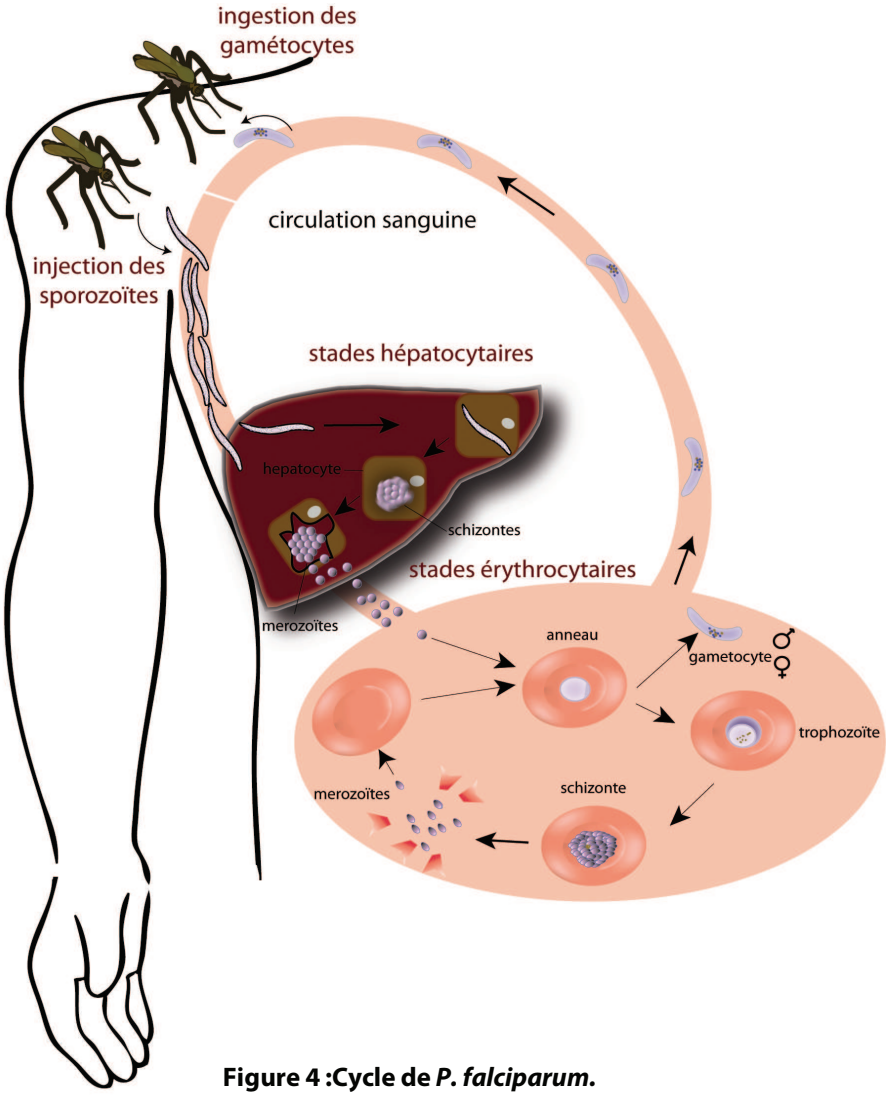
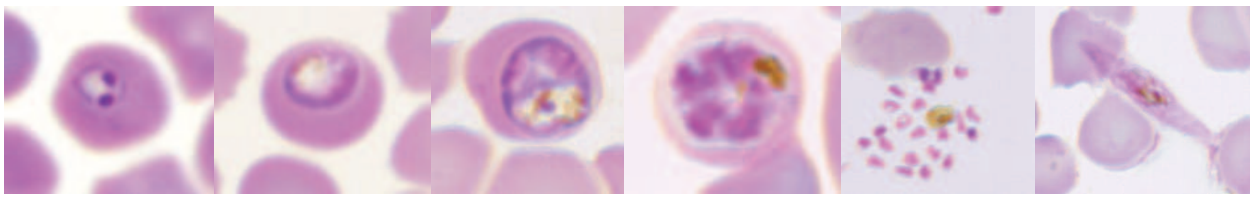


Figure 4 :Cycle de *P. falciparum*.

La transmission du parasite de l'anophèle s'effectue au cours du repas sanguin d'un moustique à l'homme. Le parasite subira un premier cycle de divisions au sein des cellules hépatiques (schizogonies exo-érythrocytaires) puis un cycle de divisions au sein des globules rouges (schizogonies érythrocytaires). Au cours de son développement érythrocytaire, certains parasites se différencient en gamétocytes, qui évolueront chez le moustique après ingestion.



anneau | trophozoïte | schizonte jeune | schizonte âgé | libération des mérozoïtes | gamétocyte

Figure 5 Stades érythrocytaires de *P. falciparum*

Frottis sanguin effectué sur une culture de *P. falciparum* (souche 3D7), observé après coloration au RAL-555. On peut différencier les stades anneaux, trophozoïtes, schizontes jeunes, schizontes âgés, mérozoïtes libérés après éclatement du globule rouge et gamétocyte (photos prises par N. Wurtz sur un microscopie optique avec objectif à immersion x100 équipé d'une caméra digitale Nikon DXM 1200 couplé au logiciel d'acquisition Lucia 4.8 Nikon).

Les symptômes d'une infection à *Plasmodium*

Les premiers symptômes d'une infection à *Plasmodium* ressemblent à ceux d'une maladie virale systémique mineure. Les manifestations cliniques du paludisme sont très diverses et la gravité dépend du type de *Plasmodium* impliqué, de la parasitémie induite et du sujet lui-même (âge, degré d'immunisation…) [21]. Après la piqûre infectante et la phase asymptomatique hépatique apparaissent les premiers parasites sanguins et les premiers symptômes, tels que fièvre, maux de tête, douleurs musculaires, affaiblissement, vomissements, diarrhées, toux [22]. Des cycles typiques alternant fièvre, tremblements avec sueurs froides et transpiration intense, peuvent alors survenir : c'est l'accès palustre simple. La périodicité de ces cycles dépend de l'espèce de parasite en cause, et coïncide avec la multiplication des parasites et l'éclatement des globules rouges, qui conduisent à l'anémie. Classiquement, *P. falciparum* provoque la fièvre tierce maligne (survenant toutes les 48h), *P. ovale* et *P. vivax* provoquent une fièvre tierce bénigne, *P. malariae* provoque la fièvre quarte (survenant toutes les 72h) et enfin *P. knowlesi* entraîne la fièvre « quotidienne » (survenant toutes les 24h). Diverses complications peuvent survenir en cas de paludisme à *P. falciparum* : neuropaludisme avec ou sans coma, anémie, insuffisance rénale aiguë, œdème pulmonaire, hypoglycémie, collapsus circulatoire, hémorragie spontanée avec trouble de la coagulation, convulsions généralisées et répétées, acidose métabolique et hémoglobinurie pour aboutir au décès [23]. Dans les régions où le paludisme est hautement endémique, les personnes régulièrement piquées par des moustiques infectés deviennent naturellement immunisées (immunité acquise) et sont alors des porteurs asymptomatiques de l'infection [21].

Les moyens de lutte contre le paludisme et les problèmes de résistance

Le développement d'un vaccin efficace reste l'un des plus grands défis pour permettre l'éradication du paludisme [24,25]. Les perspectives vaccinales restent limitées par la complexité de la biologie *de P. falciparum*, sa diversité antigénique et les essais cliniques de candidats vaccinaux menés jusqu'alors ne sont pas concluants en terme de protection et de durée d'action [26]. Cependant, des essais cliniques en phase III sont actuellement en cours dans 7 pays africains avec un candidat vaccin le RTS,S ([27], http://www.malariavaccine.org/publications-factsheets.php).

A l'heure actuelle, le seul moyen de lutter contre le paludisme est d'une part la prévention (lutte anti-vectorielle et traitement préventif pour les voyageurs), d'autre part le traitement par des molécules antipaludiques efficaces en cas d'infection (traitement curatif).

Au début des années 1960, le programme mené par l'Organisation Mondiale de la Santé (OMS) pour éradiquer le paludisme a conduit à une diminution significative des cas de paludisme. La stratégie suivie, à la fois anti-vectorielle (assèchement des marais et usage massif d'insecticides tel que le DDT) et prophylactique (antipaludique de synthèse tels que la chloroquine) a permis la disparition du paludisme en Europe, au Moyen Orient et en Amérique du Nord et une réduction de la morbidité palustre dans beaucoup de régions endémiques [28]. Plus tard, les membres de l 'assemblée de l'OMS devaient en reconnaître les limites et, dans une certaine mesure, l'échec : les facultés d'adaptation du vecteur et du parasite avaient conduit à la propagation d'anophèles résistantes aux insecticides et de souches plasmodiales résistantes aux antipaludiques [29]. L'extension de la chloroquinorésistance à la quasi-totalité des zones d'endémie palustre en limite aujourd'hui l'utilisation [30]. Les mécanismes de résistance de *P. falciparum* et les marqueurs moléculaires qui y sont associés sont actuellement plus ou moins bien documentés pour quelques molécules antipaludiques [31] : la chloroquine, la méfloquine, l'halofantrine, le cycloguanil, la pyriméthamine, la sulfadoxine et l'atovaquone.

Avec la progression de la résistance aux antipaludiques, les traitements du paludisme ont pris de nouvelles orientations, qu'il s'agisse du traitement curatif ou du traitement préventif.

Pour le traitement curatif de l'accès simple à *P. falciparum*, l'OMS recommande les combinaisons thérapeutiques contenant un dérivé de l'artémisinine (A.C.T.) dans les pays où cette espèce plasmodiale est résistante à la chloroquine, à l'amodiaquine et à la sulfadoxine (Arthemeter / Luméfantrine, Artésunate / Amodiaquine, Artésunate / Sulfadoxine-pyriméthamine, Artésunate / Méfloquine) [22,32]. Cependant, des cas de résistances à l'artémisinine et ses dérivés utilisés en association dans la thérapie de l'accès palustre sont déjà décrits et des isolats présentent *in vitro* des sensibilités diminuées à l'arthéméter et à l'artésunate [32-37].

Le traitement préventif, quant à lui, consiste en la prise de molécules antipaludiques pendant les séjours en zones endémiques. Cette dernière repose sur l'utilisation de molécules dont la prescription est conditionnée par leur efficacité, le niveau de résistance de *P. falciparum* dans le pays de destination, leurs contre-indications, leur tolérance clinique et leur coût financier (en général chloroquine, chloroquine / proguanil, méfloquine, doxycycline ou atovaquone/proguanil) [22].

Par ailleurs, le traitement préventif ou curatif n'offrant pas une protection totale, la protection contre les piqûres de moustiques et la mise en place de mesures de contrôle du vecteur est nécessaire. L'une des mesures essentielle recommandée par l'OMS et adoptée par tous les programmes nationaux de lutte contre le paludisme est l'utilisation de moustiquaires imprégnées d'insecticides à base de pyréthrinoïdes à longue durée d'efficacité [38,39]. Les autres moyens, surtout destinés à la protection individuelle**,** sont : l'utilisation d'insecticides domestiques, le port d'habits couvrant les parties du corps exposées aux piqûres du moustique, l'utilisation de grillages à mailles fines aux ouvertures des habitations, la climatisation et l'utilisation de répulsifs. Les mesures de contrôle du vecteur consistent à assainir l'environnement et le cadre de vie, ce qui permet entre autres de supprimer certains gîtes larvaires potentiels [38,39]. Des méthodes chimiques et biologiques sont aussi utilisées pour limiter la population de moustiques [39]. En effet, les larves du moustique vecteur peuvent êtres détruites par des larvicides (Téméphos) et grâce à la lutte biologique en introduisant dans les gîtes larvaires des poissons larvivores (tels que *Aphanius dispar*) ou des bactéries (telles que *Bacillus thurengiensis*) [38]. Quant à la lutte contre les moustiques adultes, elle fait surtout appel aux pulvérisations d'insecticides, soit dans les habitations, soit dans des aires géographiques données. La lutte antivectorielle s'est cependant heurtée à l'apparition de résistances des anophèles aux principaux insecticides employés [38].

Les programmes de lutte contre le paludisme

L'augmentation de la prévalence de la maladie, du fait des résistances des parasites aux molécules antipaludiques mais également des moustiques aux insecticides, souligne la nécessité de découvertes rapides de médicaments novateurs et peu coûteux.

Il devient donc urgent de rechercher de nouvelles cibles thérapeutiques, ce qui ne peut se faire sans une meilleure compréhension de la biologie du parasite.

De ce fait, de nombreux programmes de prévention et de traitement du paludisme associant divers acteurs ont été mis en place (Roll Back Malaria RBC, Malaria Vaccine Initiave MVI, Mapping Risk in Africa MARA/AMRA, Medicines for Malaria Venture MMV, Multilateral Initiative on Malaria IMI, *P. falciparum* Genome Sequencing Consortium, The malaria Research and Reference Reagent Resource Center MR4, The malaria Research and Development Alliance 2004…). On peut citer par exemple, l'initiative « Faire reculer le paludisme » (Roll Back Malaria – RBM) qui a été lancée conjointement par l'OMS, la Banque Mondiale, le Fond des Nations Unies pour l'Enfance (UNICEF) et le Programme des Nations Unies pour le développement (PNUD) en novembre 1998. Son objectif était de réduire de moitié le taux de mortalité de la maladie d'ici à 2010, de réduire la pauvreté, d'améliorer la fréquentation scolaire et les conditions de vie de millions de personnes dans les zones les plus exposées, en particulier en Afrique. Le 18 mars 2010, un nouveau rapport du Partenariat RBM confirme que les investissements actuels en matière de lutte contre le paludisme ont permis de sauver des vies et sont très bénéfiques pour les pays (384 000 cas en moins dans 12 pays africains entre 2000 et 2009) [1]. Cependant, ce rapport met également en avant que sans un financement prévisible sur le long terme, l'importante contribution de la lutte contre le paludisme vers la réalisation des Objectifs du Millénaire pour le Développement (OMD) pourrait s'inverser (Objectif 6 : combattre le VIH/SIDA, le paludisme et les autres maladies) (www.un.org/fr/millenniumgoals). En octobre 2010 se tiendra la troisième session de reconstitution des ressources du Fonds Mondial au cours de laquelle les gouvernements feront des promesses de financements pour les années 2011 à 2013, période charnière qui déterminera si les OMD relatifs à la santé peuvent être atteints d'ici à 2015. Bien que l'incidence du paludisme ait diminué au cours des dernières années, la maladie continue d'ôter la vie à près de 860.000 personnes chaque année, notamment des femmes et des enfants en Afrique. Devant la nécessité de développer de nouveaux traitements anti-parasitaires (vaccins ou médicaments), des organisations gouvernementales et internationales (OMS, banque mondiale), ou des fonds de type fondations privées (Bill et Melinda Gates Foundation), industriels (Sanofi, Impact Malaria) ont mis en place des programmes intégrant la recherche pour de nouveaux traitements, leur développement, la modernisation des pratiques médicales et l'harmonisation des politiques de prévention.

La recherche de nouvelles cibles biologiques

En 1996, un effort international a permis de mettre en place un projet de séquençage et d'annotation du génome du clone 3D7 de *P. falciparum* [40], avec l'espoir d'obtenir des données utiles à toute la communauté scientifique. Entre 1998 et 1999, les chromosomes 2 et 3 ont été entièrement séquencés [41,42] et il faudra attendre fin 2002 pour avoir accès à la totalité des séquences du génome [43]. La banque de données PlasmoDB ainsi créée, librement accessible, permet à la communauté scientifique d'avoir accès à un ensemble d'informations génétiques, moléculaire, génomiques, protéomiques des différentes espèces plasmodiales (www.plasmodb.org) [44].

Le génome nucléaire du clone 3D7 de *P. falciparum* est composé de 23.26 mégabases (Mb) réparties en 14 chromosomes dont la taille varie de 0,643 à 3,29 Mb. La composition en nucléotides est largement dominée par les adénines et les thymines qui représentent en moyenne 80.1% du génome avec des régions intergéniques et des introns pouvant atteindre 90% ou plus [43,45]. Le génome de *P. falciparum* se compose de 5560 gènes et code pour 5446 protéines (www.plasmodb.org). Environ 40% des protéines prédites (2119 protéines hypothétiques ou ayant une fonction inconnue) ne présentent soit aucune soit trop peu de similarité avec des protéines d'autres eucaryotes pour qu'une fonction leur soit assignée. En plus de son génome nucléaire, le parasite possède un génome mitochondrial de 5,957 kb ainsi qu'un génome apicoplastique de 34,242 kb. L'étude du génome de *P. falciparum* a permis d'ouvrir de nouvelles voies de recherche visant à annoter les gènes séquencés mais non identifiés, à en caractériser l'expression par des approches transcriptomiques et les protéines qui en découlent par des études protéomiques [46-53], le but ultime étant l'identification de nouvelles cibles thérapeutiques pour le développement de molécules antipaludiques [54-60]. De plus, l'étude du génome/transcriptome/protéome reste un outil puissant pour mieux comprendre les mécanismes biologiques utilisés par *P. falciparum* pour s'adapter à la complexité des environnements rencontrés chez les différents hôtes. En effet, le cycle du *Plasmodium* consiste en une succession de différents stades de développement, chacun adapté à une fonction particulière dans un environnement intra- ou extra-cellulaire.

Coordonner les divisions cellulaires lors de chacun des stades de différenciation nécessite de la part du parasite l'intégration des changements environnementaux et un contrôle strict de la machinerie du cycle cellulaire assurés par les cascades de transduction. Ces processus impliquent probablement l'interaction de diverses molécules de signalisation dans le parasite et avec l'hôte, et représentent ainsi des cibles thérapeutiques potentielles [56].

Les protéines kinases, des cibles thérapeutiques antipaludiques prometteuses

Un des principaux processus biochimiques utilisés par les cellules eucaryotes pour transmettre des signaux est la modulation de la phosphorylation des protéines par des protéines kinases ou phosphatases [61]. Les protéines kinases jouent un rôle crucial dans le contrôle de la prolifération et de la différenciation des cellules eucaryotes [62]. Ces dernières années, la recherche sur les mécanismes de phosphorylation s'est accrue et de fait les protéines kinases représentent des cibles médicamenteuses attractives dans de nombreuses maladies [61,63-66]. Plus de 500 protéines kinases sont codées par le génome humain ce qui représente environ 2% par rapport au nombre total de gènes et on estime qu'approximativement 30% des protéines sont directement concernées par ce mécanisme de phosphorylation [64]. L'ensemble des protéines kinases dans un génome est appelé le kinome. Les protéines kinases sont des enzymes qui catalysent le transfert d'un groupe phosphate de l'adénosine triphosphate (ATP) sur l'hydroxyle (groupe -OH) des acides aminés alcool : sérine, thréonine et tyrosine. De plus, des phosphorylations au niveau des résidus histidine et aspartate sont rencontrées chez les organismes procaryotes, les archées mais aussi chez les eucaryotes comme les plantes, champignons et levures [67].
Les protéines kinases se caractérisent par la présence d'un domaine catalytique d'environ 250 à 300 acides aminés qui contient des résidus conservés importants notamment dans la liaison avec les nucléotides, la fixation avec les substrats ou encore les réactions de transfert de phosphate [68,69]. Selon la nature du groupe phosphorylé sur la protéine, ces enzymes sont classées en protéines sérine/thréonine kinases et en protéines tyrosine kinases. Selon la classification actuelle en vigueur de la superfamille des protéines kinases, il existe huit groupes de protéines kinases « classiques » (ePKs) et quatre groupes de protéines kinases « atypiques » (aPKs) [64,70,71].

Les principaux groupes de ePKs sont les suivants (**Figure 6**) :

- **AGC** : kinases dépendantes des nucléotides cycliques, des phospholipides et du calcium (protéine kinase dépendant de l'AMP cyclique (PKA), protéine kinase dépendante du GMP cyclique (PKG), protéine kinase dépendante du calcium et des phospholipides (PKC)).
- **CAMK** : kinases dépendantes du calcium et de la calmoduline
- **CK1** : caséine kinase 1 (et homologues)
- **CMGC** : contient les kinases de type **C**DK (kinase dépendante des cyclines), **M**APK (kinase activée par des mitogènes), **G**SK3 (glycogène synthase kinase), et **C**LK (kinase apparentée au CDK).
- **STE** : ce groupe contient les activateurs de MAPK (STE pour homologues des kinases **sté**riles 7, 11 et 20 de la levure).
- **TK** : tyrosines kinases
- **TKL** : homologues de tyrosine kinases (groupe varié contenant à la fois à des tyrosine et à des sérine-thréonine kinases)
- **RCG** : récepteur guanylate cyclase
- **Autres** : kinases qui possèdent des similarités avec les groupes ci-dessus mais qui ne peuvent être classées

Les aPKs sont un petit ensemble de protéines kinases qui ne présentent pas de similitudes de séquences avec les ePKs, mais pour lesquelles une activité kinase a été démontrée expérimentalement (les alpha-kinases, les kinases apparentées aux phosphatidylinositol kinase (PIKK), les kinases de biogénèse des ribosomes et progression du cycle cellulaire (RIO), les pyruvate kinases déshydrogénase (PHDK)) [70].

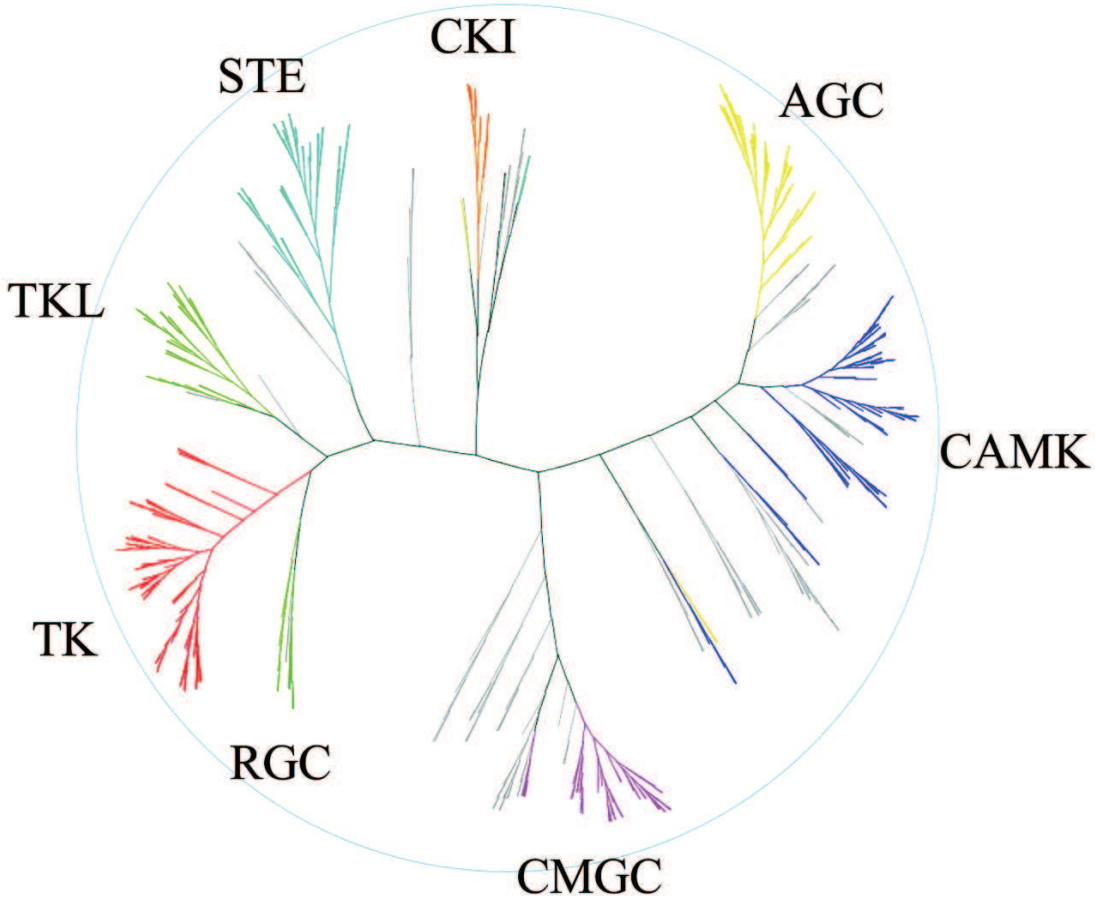


Figure 6 : Dendogramme des protéines kinases eucaryotes [64]

Les grands groupes des ePKs sont représentés d'une couleur différente

En raison de leur grande diversité d'action, les protéines kinases doivent être rigoureusement régulées : l'activité aberrante de ces enzymes génère des maladies comme le cancer ou le diabète, mais aussi des dysfonctionnement nerveux, cardiovasculaire, inflammatoires ou auto-immunitaires [64]. Ainsi, ces enzymes représentent des cibles thérapeutiques très pertinentes [63,65]. Cette situation a incité la recherche de médicaments ciblant spécifiquement les protéines kinases et a conduit à la commercialisation en 2002 du Gleevec® (imatinib mesylate), le premier inhibiteur de la catégorie des anti-kinases utilisé dans le traitement de certaines leucémies myéloïdes chroniques [72]. Le kinome *de P. falciparum* est beaucoup plus petit que celui des métazoaires, avec environ 86 à 99 protéines kinases dont les fonctions, les mécanismes de régulation et les cibles cellulaires sont encore très peu connus [43,73,74].

L'analyse des kinases de *P. falciparum* a permis de mettre en évidence certaines divergences par rapport au kinome de l'homme [74,75] :

- Absence des membres des groupes TK et STE
- Présence d'un groupe de kinases « orphelines » n'ayant aucune similitude avec les kinases humaines. Certaines de ces enzymes présentent des caractéristiques de plusieurs ePKs
- Présence de kinases dépendantes du calcium appartenant au groupe des CAMK, dont la structure est retrouvée chez les plantes et les alvéolés mais pas chez les métazoaires [76].
- Certaines protéines kinases ne peuvent être classées dans aucun groupe d'ePKs (trop peu de similitudes).

Les différences observées entre les voies de signalisation parasitaire et humaine suggèrent la possibilité d'inhiber spécifiquement le développement de *P. falciparum* en ciblant les protéines kinases plasmodiales [57,59,77,78].

Thème de notre étude : La protéine kinase AMP cyclique dépendante de *P. falciparum* :

Dans ce contexte, nous avons décidé d'étudier plus particulièrement la protéine kinase AMP cyclique dépendante de *P. falciparum* (PfPKA), qui joue chez les eucaryotes un rôle fondamental dans la prolifération et le développement cellulaire [79-81]. Les données de la littérature disponibles sur la voie de l'AMPc et plus particulièrement la PfPKA sont présentées dans notre revue intitulée « cAMP-dependent protein kinase from *Plasmodium falciparum*: an update ».

L'objectif de ce travail de thèse était de préciser la fonction biochimique et biologique de la sous-unité catalytique de la PfPKA (PfPKAc) de *P. falciparum* avec pour but ultime l'identification de nouvelles cibles thérapeutiques et/ou de nouveaux inhibiteurs ciblant spécifiquement le parasite.

Ainsi, une stratégie avec deux approches complémentaires a été mise en place :

1) La première approche a consisté à la caractérisation biochimique de la PfPKAc. Cette protéine kinase plasmodiale a été clonée et exprimée dans un système bactérien puis purifiée par deux étapes de chromatographie. Finalement, des essais biochimiques ont pu être réalisés grâce à la mise en place d'un test de mesure de l'activité enzymatique utilisant des substrats fluorescents, qui nous servira par la suite à réaliser du criblage d'inhibiteurs spécifiques de la kinase plasmodiale. Nos résultats sont présentés dans le premier chapitre de cette thèse « APPROCHE BIOCHIMIQUE DANS L'ETUDE DE LA PROTEINE KINASE AMP CYCLIQUE DE *PLASMODIUM FALCIPARUM* ».

2) La seconde approche vise à préciser la fonction biologique de la PfPKAc au cours du cycle érythrocytaire de *P. falciparum* et plus particulièrement les voies métaboliques qui sont en relation avec cette kinase plasmodiale. Dans cette étude, les conséquences de l'inhibition des transcrits de la PfPKAc par l'utilisation d'ARN interférents spécifiques ont été étudiées sur *i)* sur le développement parasitaire, *ii)* le transcriptome global par à l'aide de puces à ADN afin d'identifier les voies métaboliques en relation avec la PfPKAc. Nos résultats sont exposés dans le deuxième chapitre de cette thèse « APPROCHE CELLULAIRE DANS L'ETUDE DE LA PROTEINE KINASE AMP CYCLIQUE DE *PLASMODIUM FALCIPARUM* ».

REVUE DE LA LITTERATURE

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REVIEW ARTICLE

cAMP-dependent protein kinase from *Plasmodium falciparum*: an update

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SUMMARY

One of the most important public health problems in the world today is the emergence and dissemination of drug-resistant malaria parasites. *Plasmodium falciparum* is the causative agent of the most lethal form of human malaria. New anti-malarial strategies are urgently required, and their design and development require the identification of potential therapeutic targets. However, the molecular mechanisms controlling the life cycle of the malaria parasite are still poorly understood. The published genome sequence of *P. falciparum* and previous studies have revealed that several homologues of eukaryotic signalling proteins, such as protein kinases, are relatively conserved. Proteins kinases are now widely recognized as important drug targets in protozoan parasites. Cyclic AMP-dependent protein kinase (PKA) is implicated in numerous processes in mammalian cells, and the regulatory mechanisms of the cAMP pathway have been characterized. *P. falciparum* cAMP-dependent protein kinase plays an important role in the parasite's life cycle and thus represents an attractive target for the development of anti-malarial drugs. In this review, we focus on the *P. falciparum* cAMP/PKA pathway to provide new insights and an improved understanding of this signalling cascade.

Key words: *Plasmodium falciparum*, malaria, cAMP-dependent protein kinase, PKA, cAMP pathway.

INTRODUCTION

Malaria is one of the most important infectious diseases affecting humans, particularly in developing countries. *Plasmodium falciparum*, an apicomplexan protozoan parasite, is the causative agent of the most lethal form of human malaria. The presence of widespread drug resistance and the lack of a proven vaccine complicate the problem, and limit the available options for effective malaria control. New anti-malarial drugs that interfere with parasite growth are urgently needed, and their rational design and development require the identification of potential therapeutic targets (Renslo and McKerrow, 2006). Detailed knowledge of the molecular mechanisms that control the life cycle of malaria parasites may provide useful information for this purpose (Doerig *et al*. 2008, 2009).

Malaria parasites have a complex life cycle. Infection of humans by *P. falciparum*, the species responsible for the lethal form of human malaria, begins with the bite of an infected *Anopheles* mosquito, which delivers sporozoites to the bloodstream. These cells rapidly gain access to the liver and invade hepatocytes, where they undergo substantial multiplication, generating several thousand merozoites (exo-erythrocytic schizogony). The merozoites invade red blood cells (erythrocytic schizogony), the process responsible for malaria pathogenesis. Some merozoites, however, arrest the cell cycle and differentiate into male or female gametocytes, which are infective to the mosquito. Only after being ingested by the insect, the gametocytes develop into gametes and fuse into a zygote (the only diploid stage). Further development in the mosquito involves a process of sporogony, producing sporozoites that accumulate in the salivary glands and are now ready to infect a new human host. The life cycle of malaria parasites is therefore composed of a succession of developmental stages that vary in their proliferative state (massive cell multiplication during schizogony and sporogony, and cell cycle arrest in sporozoites and gametocytes). These different parasite developmental stages require a high degree of adaptation and strict control of the cellular machinery as well as the coordinated modulation of distinct sets of genes. *Plasmodium* cells regulate these processes by several means, including phosphorylation, transcriptional control, post-transcriptional control and protein degradation. These mechanisms probably involve various interactions between parasite and host

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signalling molecules and may thus represent strategic targets in the fight against malaria (Doerig *et al*. 2009).

The modulation of protein phosphorylation through the antagonistic effects of protein kinases and phosphatases is a major regulatory mechanism of many eukaryotic intracellular processes (Manning *et al*. 2002*a*). The published *P. falciparum* genome sequence and previous studies have revealed that several homologues of eukaryotic signalling proteins are conserved in *P. falciparum* (Gardner *et al*. 2002; Chung *et al*. 2009; Koyama *et al*. 2009), but many of these major mediators have not been characterized for malaria parasites. There is now evidence that protein kinases are essential for the control of the parasite life cycle and that inhibition of such activities can have anti-malarial effects (Doerig *et al*. 2010). Moreover, these enzymes are widely recognized as valuable drug targets for the treatment of several diseases (Johnson, 2007; Grant, 2009). Analyses of the *P. falciparum* kinome have revealed 86 or 99 genes (depending on the study) that encode proteins containing kinase domains; however, their functions, mechanisms of regulation and cellular targets are largely unknown (Ward *et al*. 2004; Anamika *et al*. 2005).

cAMP-dependent protein kinase (protein kinase A, or PKA) is a key signal transduction element in mammalian cells, and the regulatory mechanisms of the cAMP pathway are well known (Taylor *et al*. 2008*b*). However, in *P. falciparum*, components of the cAMP pathway and the precise function of PKA, its downstream target, have not been clearly defined. The cAMP/PKA signalling pathway has attracted interest from a number of research groups, and there are reports of cAMP-dependent protein kinase homologues (PfPKA) in the *P. falciparum* genome (Li and Cox, 2000; Syin *et al*. 2001; Beraldo *et al*. 2005; Doerig *et al*. 2008; Merckx *et al*. 2008*a*; Wurtz *et al*. 2009*b*). The aim of this review is to provide a synthesis of the recent published experimental data on the cAMP/PfPKA signalling pathway, which appears to be essential for parasite growth and survival and, consequently, represents an attractive target for the development of new anti-malarial drugs.

OVERVIEW OF THE cAMP/PKA SIGNALLING PATHWAY

PKA is the best-studied protein kinase, belonging to the AGC group within the eukaryotic protein kinase superfamily (Manning *et al*. 2002*a*, *b*). It was one of the first protein kinases to be discovered, sequenced and cloned, and the resolution of its structure provided the first three-dimensional template for this family (Walsh *et al*. 1968; Shoji *et al*. 1981; Uhler *et al*. 1986; Knighton *et al*. 1991). PKA and cAMP pathways have been implicated in numerous cellular processes, including modulation of other protein kinases, regulation of intracellular calcium concentration and regulation of transcription (Shabb, 2001; Tasken and Aandahl, 2004).

In most organisms, PKA is a heterotetramer composed of 2 regulatory subunits (PKAr), which bind to and inhibit 2 catalytic subunits (PKAc). The cAMP pathway is activated by the binding of a ligand to a membrane–bound G-protein-coupled receptor (GPCR) (7 transmembrane receptor), which interacts with heterotrimeric G-proteins composed of α, β and γ subunits that are bound in the inactive state. The agonist binding triggers a conformational change in the receptor, which catalyses the exchange from GDP to GTP and the dissociation of G_α from $G_{\beta\gamma}$ subunits. Both G_α and $G_{\beta\gamma}$ subunits can modulate the activity of downstream effectors. In particular, Gα subunit proteins are divided into four subfamilies ($G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$ and $G_{\alpha 12/13}$) (Hamm, 1998) and a single GPCR can couple to 1 or more families resulting in different cellular responses (Cabrera-Vera *et al*. 2003). When coupling to $G_{\alpha s}$ or $G_{\alpha i}$ proteins, GPCRs either activate or inhibit adenylate cyclase (AC) activity resulting in an increase or decrease in cAMP formation, respectively (Cabrera-Vera *et al*. 2003). The flux through the pathway is controlled by the self-inactivating GTPase activity of the G_α subunit, which hydrolyses GTP to GDP, resulting in re-association of G_α subunit with the $G_{\beta\gamma}$ subunits and termination of the G protein signalling. The binding of 2 cAMP molecules to each PKAr alters its affinity for the catalytic subunit, resulting in release of the active PKAc. PKAc, a serine/threonine kinase, can phosphorylate many substrates, such as additional protein kinases and transcription factors (Shabb, 2001). The termination of cAMP signalling is conferred by a large superfamily of enzymes known as phosphodiesterase proteins (PDE) that catalyse the degradation of cAMP into 5′AMP (Fimia and Sassone-Corsi, 2001; Lugnier, 2006). The intracellular concentrations of cAMP are therefore regulated by the counterbalancing activities of ACs and PDEs. In addition, the signalling events induced by agonist activation of GPCRs can be counteracted in the cell by intrinsic mechanisms known as the receptor desensitization. Phosphorylation is the most rapid means of GPCR desensitization and is achieved by 2 classes of serine/threonine protein kinases: PKAc directly *via* a feedback regulation and G protein-coupled receptor kinases (GRKs). GRK-mediated receptor phosphorylation promotes the binding of β-arrestins, which not only uncouple receptors from heterotrimeric G proteins but also target many GPCRs for internalization, followed by either recycling or degradation of the receptor (Lefkowitz, 1998; Ferguson, 2001; Hendriks-Balk *et al*. 2008). In mammalian cells, PKA contains 3 catalytic subunit isoforms (Cα, Cβ and Cγ) and 4 regulatory subunit isoforms (RIα, RIβ, RIIα and RIIβ) (Doskeland *et al*. 1993). The tissue-specific expression and assembly patterns of these kinase isoforms are thought to be

responsible for the diverse cellular responses to cAMP (Taylor *et al*. 1990). PKAc activity can be regulated by binding to protein kinase inhibitor (PKI), its natural endogenous inhibitor (Dalton and Dewey, 2006). PKI inhibits the activity of PKAc by binding to free catalytic subunits of this enzyme and inhibiting the phosphorylation of PKAc substrates (Ashby and Walsh, 1972, 1973). PKI is similar to the PKAr in that both proteins contain amino acid sequences (pseudosubstrate sites) that allow them to bind to the PKAc and inhibit its activity. The functional specificity of PKA is largely dependent on the targeting of the catalytic subunit to specific substrates at precise locations in the cell. This is accomplished by the A-kinase anchoring-protein (AKAP) family, whose members are bound to subcellular structures and recruit PKA *via* interactions with the regulatory subunit (Barradeau *et al*. 2002; McConnachie *et al*. 2006).

P. falciparum cAMP-dependent protein kinase activity has been detected in cytosolic extracts of both the asexual and sexual stages of the parasite, and a putative regulatory subunit has been identified in asexual forms (Kaushal *et al*. 1980; Read and Mikkelsen, 1990; Read and Mikkelsen, 1991*b*), suggesting the existence of the cAMP pathway in the parasite (Fig. 1). The single *P. falciparum* PKA catalytic subunit (*pfpkac*) gene was first isolated and characterized using a PCR-based approach, which identified a DNA fragment that shared high sequence homology with catalytic subunits of the PKA family (Li and Cox, 2000). Subsequent studies of this kinase have illuminated much about its structure and substrate specificity (Syin *et al*. 2001; Sudo *et al*. 2008; Wurtz *et al*. 2009*b*).

The single *P. falciparum* PKA regulatory subunit (PfPKAr) was first identified using BLASTP analyses (Altschul *et al*. 1990) with PKAr subunits from various eukaryotes as queries, and the gene was later cloned and expressed (Ward *et al*. 2004; Merckx *et al*. 2008*b*).

An AC activity, biochemically distinct from that of the host was first measured in *P. falciparum* by Mikkelsen and Read (Read and Mikkelsen, 1991*b*). Two different genes with high homology to ACs (PfACα PF14_0788 and PfACβ MAL8P1.150) have been identified and characterized in *P. falciparum* (Muhia *et al*. 2003; Baker, 2004; Baker and Kelly, 2004; Weber *et al*. 2004). Four putative *P. falciparum* PDEs have now been identified, containing the class I signature motif and sharing approximately 40% amino acid identity (PfPDEα PFL0475w, PfPDEβ MAL13P1.118, PfPDEγ MAL13P1.119 and PfPDEδ PF14_0672). The cyclic nucleotide specificity of the 4 encoded enzymes cannot be predicted on the basis of primary amino acid sequence, but it seems that PfPDEα and PfPDEδ are more specific to cGMP (Yuasa *et al*. 2005; Taylor *et al*. 2008*a*; Wentzinger *et al*. 2008). In the Plasmodb database, 2 genes coding for a G-protein coupled receptor, putative (PFE1265w) and for a G-protein associated signal transduction protein, putative (PFF0365c) have been found, but no other data are available regarding the function of these two proteins. In addition, Madeira *et al*. have identified 4 putative serpentine receptors in *P. falciparum* (PF11_0321, PFL0765w, PFD1075w, MAL7P1.64), but again, their roles and their implication in cAMP pathway have not been defined (Madeira *et al*. 2008). They predicted that these receptors could be implicated in sensing extracellular signal and that elucidation of their detailed function may highlight the mechanisms used by the parasite to modulate its life cycle. Results consistent with the presence of heterotrimeric G proteins in *P. falciparum* have also been reported, and it has been suggested that they might be involved in the switch to sexual development (Dyer and Day, 2000). However, until now no gene encoding heterotrimeric G-proteins have been identified in *P. falciparum* genome. On the other hand, it has recently been proposed that the cAMP signalling system of the red blood cell could play a role in malaria infection (Harrison *et al*. 2003). Indeed, host GPCRs and $G_{\alpha s}$ appear to be associated with the parasite vacuole (Lauer *et al*. 2000) and addition of peptides that block the interaction between GPCR and $G_{\alpha s}$ led to decreased parasitaemia (Harrison *et al*. 2003). Finally, until now, only 1 AKAP was annotated in the *P. yoelii* genome (PY04627) (Carlton *et al*. 2002). In *P. falciparum*, an orthologue of this AKAP has been annotated as a conserved protein with unknown function (PFE0640w), but no study has been conducted on this topic.

The knowledge of signal-transduction pathways in *Plasmodium* is fundamental to allow the design of new strategies against malaria. According to these previous data, there is evidence that the cAMP/PKA pathway exist in the malaria parasite. However, a number of important components in this pathway have so far not yet been clearly defined and must be studied more thoroughly: the ligand-receptor complex that initiates the cAMP pathway (GPCRs), the signalling molecules that activate AC (is this mediated by heterotrimeric G proteins in *P. falciparum* or not?), the regulation of this cellular network (do GRK, PKI and feedback PKAc control the mechanisms of regulation in the parasite?), and the downstream targets of PfPKAc.

STRUCTURE OF THE cAMP-DEPENDENT PROTEIN KINASE COMPLEX

cAMP-dependent protein kinase catalytic subunit

The PFI1685w gene in the PlasmoDB database (Bahl *et al*. 2003) has 4 introns and 5 exons, and the protein product, PfPKAc, has a predicted molecular mass of

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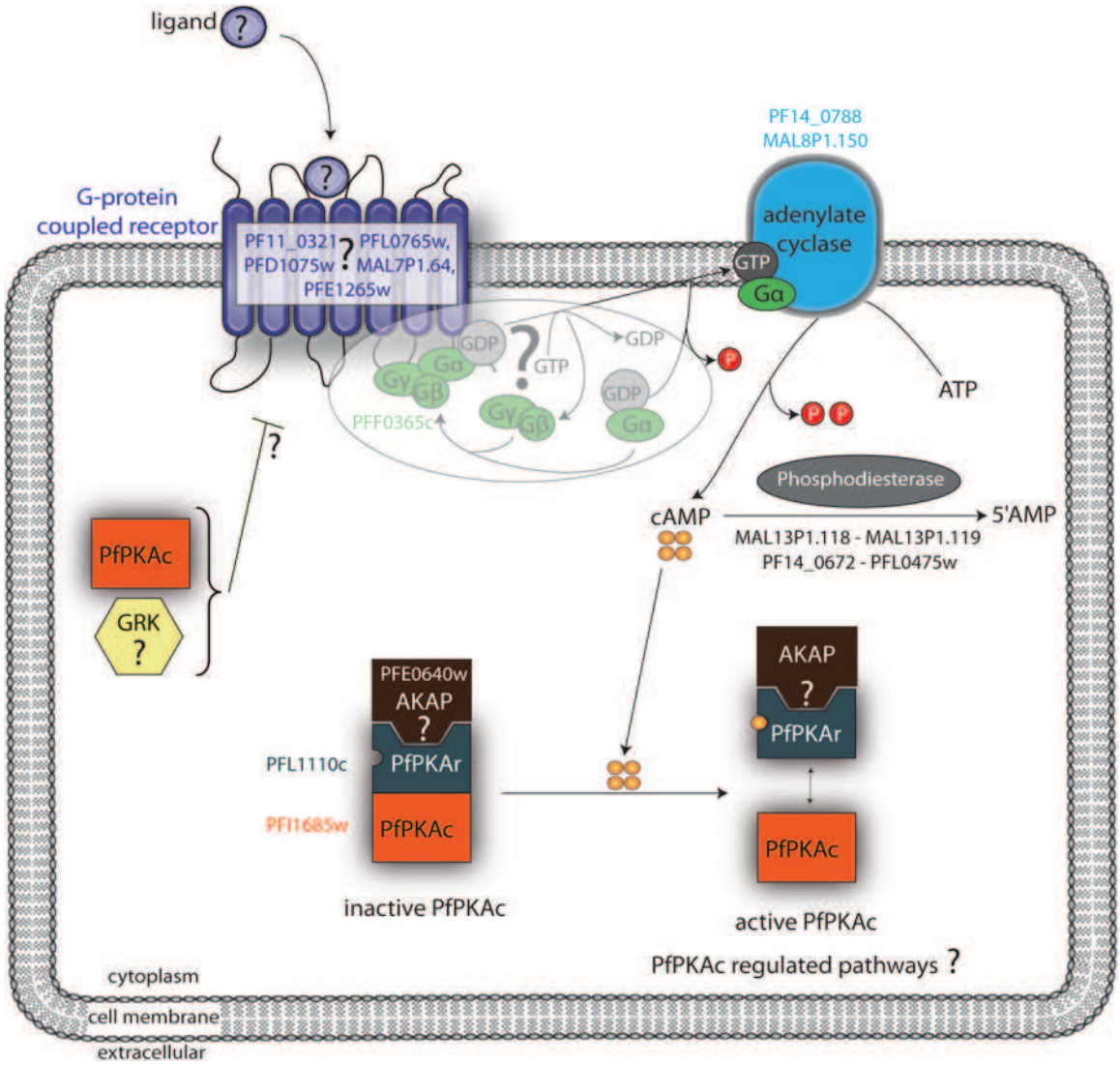

Fig. 1. Schematic model of cAMP/PKA signalling pathway in *Plasmodium falciparum*. This pathway is based on data reported in the literature. All elements that are not known or not proven are associated with a question mark. The pathway begins when an unknown ligand activates a GPCR. The transduction signal is transmitted to G-proteins (not yet identified in *P. falciparum*), which activate adenylate cyclase. The latter allows the production of cAMP from ATP. The cAMP binds to the PfPKAr, which changes its conformation and allows the release of PfPKAc. Once free, PfPKAc phosphorylates unknown substrates using ATP as the phosphate donor. PDEs degrade cAMP and thus limit PfPKAc activation. In *P. falciparum*, the existence of AKAP proteins has not yet been proven, but an orthologue of *P. yoelli* AKAP is annotated in the *P. falciparum* genome as a conserved protein with unknown function (PFE0640w). The signalling events induced by agonist activation of GPCRs can be counteracted in eukaryotes by PKAc directly or by GRKs. However, in *Plasmodium* these mechanisms, as well as GRKs, have not been identified.

40·2 kDa and a calculated pI of 9·11. Comparative analyses using BLASTP revealed that the amino acid sequence of PfPKAc shares about 50% of identity with PKAc from *Homo sapiens* and *Mus musculus* and high homology (88%) with *P. yoelii* PKAc (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A multiple sequence alignment of the amino acid sequences of PKAc from various organisms using T-Coffee method (Notredame *et al*. 2000; Poirot *et al*. 2003) showed strong conservation of the 11 specific kinase subdomains (I-XI) among the different organisms. Several residues required for catalytic activity are also highly conserved (Hanks *et al*. 1988) (Fig. 2A and B and Table 1). A three-dimensional model of PfPKAc was created based on the crystal structure of *Homo sapiens* PKAc alpha subunit (HsPKAc; PDB accession number 2GU8) using the Swiss-Model ternary structure prediction tool (Arnold *et al*. 2006) and Pymol 0.99 software (DeLano, 2008) (Fig. 2B). As expected, and due to a high degree of primary sequence similarity (50% identity) between the two proteins, the PfPKAc model showed structural homology to HsPKAc. The PKAc subunits are bilobal enzymes with 2 major domains (the small N-terminal lobe and the large C-terminal lobe) that are conserved throughout the protein kinase family (Hanks *et al*. 1988; Taylor *et al*. 1990; Hanks and Hunter, 1995; Smith *et al*. 1999; Johnson *et al*. 2001;

Taylor *et al*. 2008*b*). The smaller N-terminal lobe, which includes subdomains I–IV, is primarily involved in the anchoring and orientation of ATP. This lobe has a predominantly anti-parallel β-sheet structure. The larger C-terminal lobe, which comprises subdomains VI to XI, is mainly composed of α-helices. It serves as a framework for the catalytic machinery and also as docking scaffold for binding to protein partners that act as substrates or inhibitors. Moreover, the N-terminal lobe can also be involved in docking with some proteins and partners, for example, the A Kinase Interacting Protein (AKIP 1) binds to the N-terminus of PKAc and helps to traffic it into the nucleus (Sastri *et al*. 2005; Taylor *et al*. 2008*b*; Kornev and Taylor, 2010). Table 1 summarizes the important residues, secondary structure and function of each subdomain for both HsPKAc and PfPKAc. We are particularly interested in the sequence and structural differences between HsPKAc and PfPKAc, as the ultimate goal of this study is to design molecules that specifically target the parasite enzyme (Fig. 2B and Table 1). The first divergence concerns subdomain I, which is composed of 2 β-strands and 2 α-helices in PfPKAc but consists of 2 β-strands only in HsPKAc. As shown in Table 1, this subdomain participates in ATP anchoring. Next, in subdomain VI, a β-strand and an α-helix are missing from the PfPKAc model. This domain contains the putative catalytic loop sequence HRDLK*XX*N, which includes an aspartate identified as the candidate catalytic base (Hanks and Hunter, 1995). The last notable divergence concerns subdomain VIII, where the α-helix in HsPKAc is divided into 2 α-helices in PfPKAc. In the same domain, a β-strand was also absent in PfPKAc model as compared to the HsPKAc structure. Moreover, a tryptophan (W^{196}) previously identified as essential for mammalian PKAr binding is not conserved in the PfPKAc subdomain VIII sequence (Y^{187}) (Gibson and Taylor, 1997; Kim *et al*. 2005). Other differences between PfPKAc and HsPKAc were also observed, but these were not located in known functional domains (Table 1).

Despite its high sequence identity and strong structural homology with HsPKAc, PfPKAc presents some interesting differences in essential domains involved in the following functions: (i) ATP anchoring, (ii) mechanisms of substrate phosphorylation and (iii) substrate recognition and/or inhibitor sensitivity. The development of new anti-malarial compounds targeting these domains could allow for the inhibition of the parasite enzyme with low levels of host protein interaction.

cAMP-dependent protein kinase regulatory subunit

The PFL1110c gene in the PlasmoDB database has 2 introns and 3 exons, and the protein product, PfPKAr, has a predicted molecular mass of 50·8 kDa and a calculated pI of 7·49. Comparative analyses using BLASTP revealed that the amino acid sequence of PfPKAr shares ~41% identity with PKAr from *Homo sapiens, Bos taurus* and *Mus musculus* and high homology with *P. knowlesi* strain H PKAr (73%) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A multiple sequence alignment of the amino acid sequences of PKAr from various organisms using the T-coffee program (Notredame *et al*. 2000; Poirot *et al*. 2003) revealed a similar general architectural organization among the various organisms and several residues required for regulatory activity, as well as some interesting differences (Taylor *et al*. 1990) (Fig. 3A and B and Table 2). The nuclear magnetic resonance (NMR) structures of mammalian type I and type II regulatory subunit D/D domains have been solved (Banky *et al*. 1998; Newlon *et al*. 2001), as have the crystal structures of the isolated CBD-A and CBD-B (Diller *et al*. 2001; Su *et al*. 1995). However, no high-resolution structures are available for the full-length regulatory subunit, linker regions or cAMP-free regulatory subunits. Thus, we created a three-dimensional model of PfPKAr based on the crystal structure of a 1–91 deletion mutant of the type I alpha regulatory subunit from *B. taurus* (BtPKAr; PDB Accession number 1RGS) using the Swiss-Model ternary structure prediction tool and Pymol software. The BtPKAr sequence is very close to that of *H. sapiens* PKAr (97% identity), which does not have a described structure.

In most cells, the regulatory subunit is typically a highly asymmetric dimer composed of different domains. The amino-terminal region of the regulatory subunit (1–140) corresponds to the dimerisation/docking domain (D/D) responsible for homodimerization. Once dimerized, this region also provides a binding surface for the AKAPs (Banky *et al*. 1998; Newlon *et al*. 2001). The D/D domain of the type I regulatory subunit contains a number of conserved residues that are critical for dimerisation and AKAP binding (C^{16}, Y^{19}, V^{20}, N^{24}, I^{25}, L^{36}, C^{37}, L^{48}, Y^{51}, F^{52} and A^{60} in BtPKAr) (Leon *et al*. 1997; Gibson *et al*. 2006) (Fig. 3A and Table 2). This N-terminal sequence, which encodes the regulatory D/D domain found in most regulatory subunits, is not present in the parasite protein, suggesting that it does not undergo regulatory subunit dimer formation as previously reported for *P. falciparum* (Syin *et al*. 2001; Merckx *et al*. 2008*b*) and several other organisms (Mutzel *et al*. 1987; Carlson and Nelson, 1996). While an orthologue of *P. yoelii* AKAP is found in *P. falciparum* genome, no consensus AKAP-binding domain was present in the *P. falciparum* regulatory subunit. This suggests that the parasite uses a mode of binding between AKAP and PfPKAr that is distinct from that of other species (Barradeau *et al*. 2002; McConnachie *et al*. 2006).

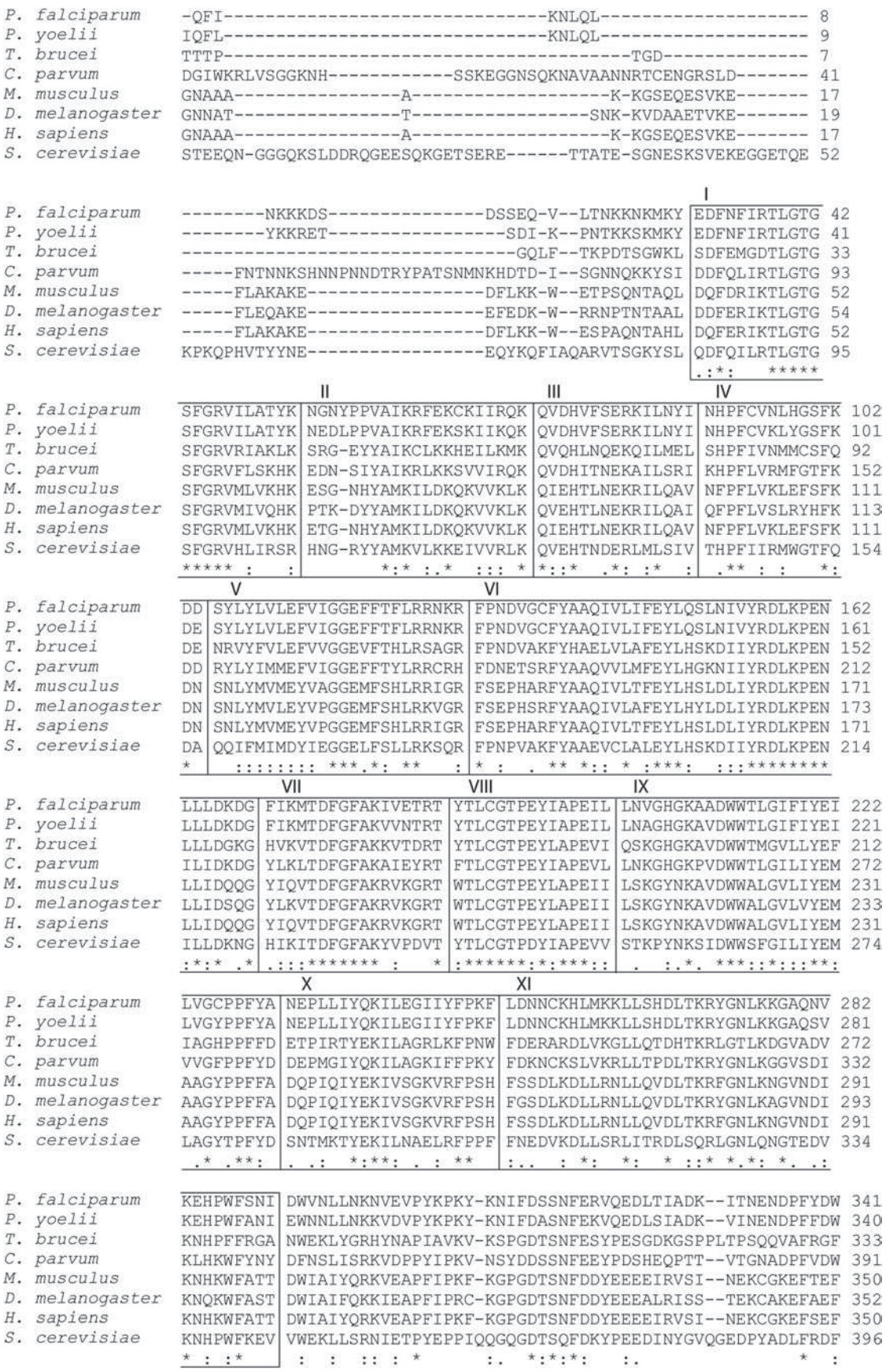
P. falciparum -QFI----------KNLQL---------- 8
P. yoelii IQFL----------KNLQL---------- 9
T. brucei TTTP----------TGD---------- 7
C. parvum DGIWKRLVSGGKNH----------SSKEGGNSQKNAVAANNRTCENGRSLD------- 41
M. musculus GNAAA----------A----------K-KGSEQESVKE------- 17
D. melanogaster GNNAT----------T----------SNK-KVDAAETVKE------- 19
H. sapiens GNAAA----------A----------K-KGSEQESVKE------- 17
S. cerevisiae STEEQN-GGGQKSLDDRQGEESQKGETSERE------TTATE-SGNESKSVEKEGGETQE 52
I
P. falciparum --------NKKKDS----------DSSEQ-V--LTNKKNKMKY EDFNFIRTLGTG 42
P. yoelii --------YKKRET----------SDI-K--PNTKKSKMKY EDFNFIRTLGTG 41
T. brucei ----------GQLF--TKPDTSGWKL SDFEMGDTLGTG 33
C. parvum -----FNTNNKSHNNPNNDTRYPATSNMNKHDTD-I--SGNNQKKYSI DDFQLIRTLGTG 93
M. musculus -----FLAKAKE----------DFLKK-W--ETPSQNTAQL DQFDRIKTLGTG 52
D. melanogaster -----FLEQAKE----------EFEDK-W--RRNPTNTAAL DDFERIKTLGTG 54
H. sapiens -----FLAKAKE----------DFLKK-W--ESPAQNTAHL DQFERIKTLGTG 52
S. cerevisiae KPKQPHVTYYNE----------EQYKQFIAQARVTSGKYSL QDFQILRTLGTG 95
II III IV
P. falciparum SFGRVILATYK NGNYPPVAIKRFEKCKIIRQK QVDHVFSERKILNYI NHPFCVNLHGSFK 102
P. yoelii SFGRVILATYK NEDLPPVAIKRFEKSKIIKQK QVDHVFSERKILNYI NHPFCVKLYGSFK 101
T. brucei SFGRVRIAKLK SRG-EYYAIKCLKKHEILKMK QVQHLNQEKQILMEL SHPFIVNMMCSFQ 92
C. parvum SFGRVFLSKHK EDN-SIYAIKRLKKSVVIRQK QVDHITNEKAILSRI KHPFLVRMFGTFK 152
M. musculus SFGRVMLVKHK ESG-NHYAMKILDKQKVVKLK QIEHTLNEKRILQAV NFPFLVKLEFSFK 111
D. melanogaster SFGRVMIVQHK PTK-DYYAMKILDKQKVVKLK QVEHTLNEKRILQAI QFPFLVSLRYHFK 113
H. sapiens SFGRVMLVKHK ETG-NHYAMKILDKQKVVKLK QIEHTLNEKRILQAV NFPFLVKLEFSFK 111
S. cerevisiae SFGRVHLIRSR HNG-RYYAMKVLKKEIVVRLK QVEHTNDERLMLSIV THPFIIRMWGTFQ 154
V VI
P. falciparum DD SYLYLVLEFVIGGEFFTFLRRNKR FPNDVGCFYAAQIVLIFEYLQSLNIVYRDLKPEN 162
P. yoelii DE SYLYLVLEFVIGGEFFTFLRRNKR FPNDVGCFYAAQIVLIFEYLQSLNIVYRDLKPEN 161
T. brucei DE NRVYFVLEFVVGGEVFTHLRSAGR FPNDVAKFYHAELVLAFEYLHSKDIIYRDLKPEN 152
C. parvum DD RYLYIMMEFVIGGEFFTYLRRCRH FDNETSRFYAAQVVLMFEYLHGKNIIYRDLKPEN 212
M. musculus DN SNLYMVMEYVAGGEMFSHLRRIGR FSEPHARFYAAQIVLTFEYLHSLDLIYRDLKPEN 171
D. melanogaster DN SNLYMVLEYVPGGEMFSHLRKVGR FSEPHSRFYAAQIVLAFEYLHYLDLIYRDLKPEN 173
H. sapiens DN SNLYMVMEYVPGGEMFSHLRRIGR FSEPHARFYAAQIVLTFEYLHSLDLIYRDLKPEN 171
S. cerevisiae DA QQIFMIMDYIEGGELFSLLRKSQR FPNPVAKFYAAEVCLALEYLHSKDIIYRDLKPEN 214
VII VIII IX
P. falciparum LLLDKDG FIKMTDFGFAKIVETRT YTLCGTPEYIAPEIL LNVGHGKAADWWTLGIFIYEI 222
P. yoelii LLLDKDG FIKMTDFGFAKVVNTRT YTLCGTPEYIAPEIL LNAGHGKAVDWWTLGIFIYEI 221
T. brucei LLLDGKG HVKVTDFGFAKKVTDRT YTLCGTPEYLAPEVI QSKGHGKAVDWWTMGVLLYEF 212
C. parvum ILIDKDG YLKLTDFGFAKAIEYRT FTLCGTPEYIAPEVL LNKGHGKPVDWWTLGILIYEM 272
M. musculus LLIDQQG YIQVTDFGFAKRVKGRT WTLCGTPEYLAPEII LSKGYNKAVDWWALGVLIYEM 231
D. melanogaster LLIDSQG YLKVTDFGFAKRVKGRT WTLCGTPEYLAPEII LSKGYNKAVDWWALGVLVYEM 233
H. sapiens LLIDQQG YIQVTDFGFAKRVKGRT WTLCGTPEYLAPEII LSKGYNKAVDWWALGVLIYEM 231
S. cerevisiae ILLDKNG HIKITDFGFAKYVPDVT YTLCGTPDYIAPEVV STKPYNKSIDWWSFGILIYEM 274
X XI
P. falciparum LVGCPPFYA NEPLLIYQKILEGIIYFPKF LDNNCKHLMKKLLSHDLTKRYGNLKKGAQNV 282
P. yoelii LVGYPPFYA NEPLLIYQKILEGIIYFPKF LDNNCKHLMKKLLSHDLTKRYGNLKKGAQSV 281
T. brucei IAGHPPFFD ETPIRTYEKILAGRLKFPNW FDERARDLVKGLLQTDHTKRLGTLKDGVADV 272
C. parvum VVGFPPFYD DEPMGIYQKILAGKIFFPKY FDKNCKSLVKRLLTPDLTKRYGNLKGGVSDI 332
M. musculus AAGYPPFFA DQPIQIYEKIVSGKVRFPSH FSSDLKDLLRNLLQVDLTKRFGNLKNGVNDI 291
D. melanogaster AAGYPPFFA DQPIQIYEKIVSGKVRFPSH FGSDLKDLLRNLLQVDLTKRYGNLKAGVNDI 293
H. sapiens AAGYPPFFA DQPIQIYEKIVSGKVRFPSH FSSDLKDLLRNLLQVDLTKRFGNLKNGVNDI 291
S. cerevisiae LAGYTPFYD SNTMKTYEKILNAELRFPPF FNEDVKDLLSRLITRDLSQRLGNLQNGTEDV 334
P. falciparum KEHPWFSNI DWVNLLNKNVEVPYKPKY-KNIFDSSNFERVQEDLTIADK--ITNENDPFYDW 341
P. yoelii KEHPWFANI EWNNLLNKKVDVPYKPKY-KNIFDASNFEKVQEDLSIADK--VINENDPFFDW 340
T. brucei KNHPFFRGA NWEKLYGRHYNAPIAVKV-KSPGDTSNFESYPESGDKGSPPLTPSQQVAFRGF 333
C. parvum KLHKWFYNY DFNSLISRKVDPPYIPKV-NSYDDSSNFEEYPDSHEQPTT--VTGNADPFVDW 391
M. musculus KNHKWFATT DWIAIYQRKVEAPFIPKF-KGPGDTSNFDDYEEEEIRVSI--NEKCGKEFTEF 350
D. melanogaster KNQKWFAST DWIAIFQKKIEAPFIPRC-KGPGDTSNFDDYEEEALRISS--TEKCAKEFAEF 352
H. sapiens KNHKWFATT DWIAIYQRKVEAPFIPKF-KGPGDTSNFDDYEEEEIRVSI--NEKCGKEFSEF 350
S. cerevisiae KNHPWFKEV VWEKLLSRNIETPYEPPIQQGQGDTSQFDKYPEEDINYGVQGEDPYADLFRDF 396

Fig. 2. (Cont.)

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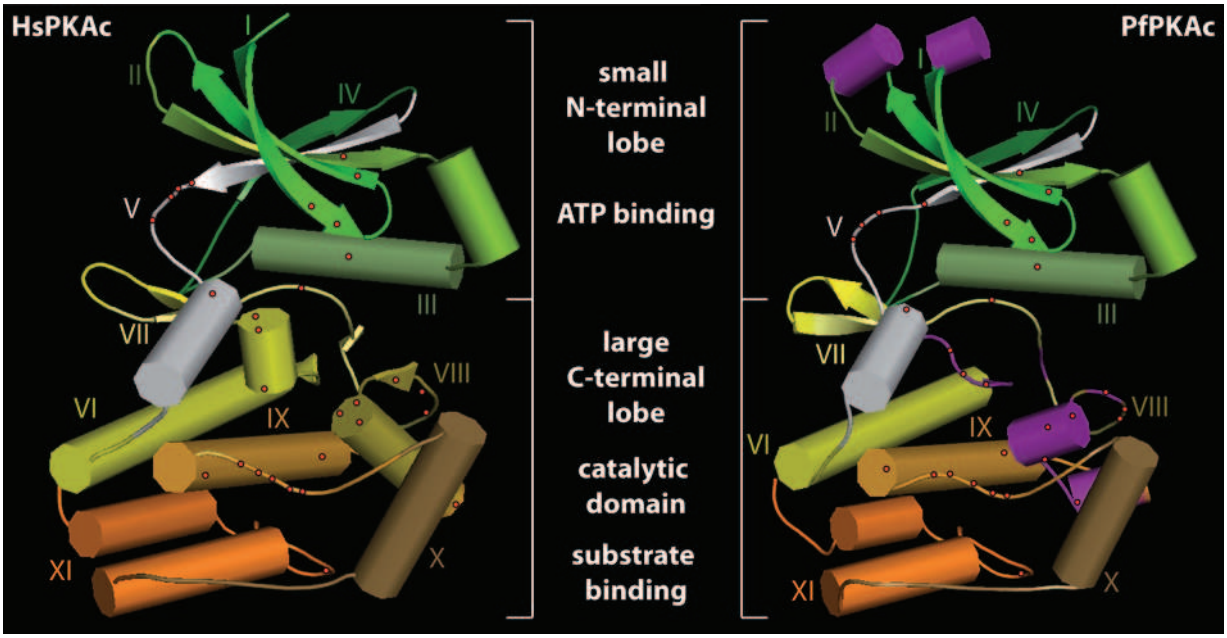

Fig. 2. Amino acid sequence alignments and structural modelling of PfPKAc. (A) Comparison of the PfPKAc amino acid sequence with those of other protein kinase catalytic subunits. *Plasmodium falciparum* (Uniprot Accession number Q7K6A0), *Homo sapiens* (P17612), *Mus musculus* (P05132), *Plasmodium yoelii* (Q7RE33), *Cryptosporidium parvum* (A3FQ39), *Trypanosoma brucei* (Q38DR5), *Saccharomyces cerevisae* (P06244) and *Drosophila melanogaster* (P12370) were aligned using T-coffee 7.38 and CLUSTAL format. The 11 typical subdomains are indicated with boxed regions and Roman numerals. Identical residues (asterisks), conservative substitutions (colons) and semi-conservative substitutions (dots) are also indicated. (B) Structural model of PfPKAc. The PfPKAc structure was modelled on the resolved structure of human PKAc alpha. The different colours represent the 11 subdomains characteristic of protein kinases. The small N-terminal lobe, which is involved in ATP binding, and the large C-terminal lobe, which comprises the catalytic regions and substrate-binding domain, are represented in the figure. α-helices (cylinders), β-sheets (arrows) and turns (lines) are indicated, as are secondary structure differences in PfPKAc (purple) and essential residues in both structures (red dots).

In mammals, the D/D domain is followed by a flexible linker region that contains an inhibitory site. This site, generally located between residues 90 and 100 in the regulatory subunit, binds to and inhibits the active site of the catalytic subunit (Li *et al*. 2000). The amino acid sequence of this region is similar to that of its catalytic subunit substrates and thus can be used to differentiate between type I and type II regulatory subunits. This region includes either a pseudosubstrate site (RR*X*A or RR*X*G in type I regulatory subunits) or an autophosphorylation region with a serine at the phosphorylation site (RR*X*S in type II regulatory subunits) (Taylor *et al*. 1990). The overall architecture of PfPKAr is closer to type I BtPKAr, but it presents a degenerated inhibitory sequence (KR*X*S) containing 1 of 2 important arginines and a serine autophosphorylated site similar to that of type II BtPKAr. Thus, PfPKAr seems to share characteristics with both type I and type II BtPKAr.

The C-terminal end of the mammalian regulatory subunit contains 2 tandem cAMP-binding domains (CBDs), named A and B (Takio *et al*. 1984). Binding of cAMP to the CBD of the regulatory subunit dissociates the catalytic subunit, which becomes catalytically active. These CBDs, which probably resulted from domain duplication, show a strong sequence homology among the different organisms. Comparison of PfPKAr with other mammalian PKAr reveals the existence of these two highly conserved CBDs, which contain essential residues necessary for cAMP binding: a conserved glutamate that binds to the 2′OH of the ribose (E^{200} and E^{324} in BtCBD-A and BtCBD-B, respectively) and a conserved arginine that interacts with the phosphate of cAMP (R^{209} and R^{333} in BtCBD-A and BtCBD-B, respectively) (Taylor *et al*. 1990; Su *et al*. 1995; Berman *et al*. 2005) (Fig. 3A and Table 2). These residues are part of an important motif called the phosphate-binding cassette (PBC) that is present in each of the CBDs (PBC-A and PBC-B). The structure of the truncated PfPKAr revealed strong structural homology with BtPKAr (Fig. 3B). The CBD is a small module, about 120 amino acids in length, which consists of helical domains and an 8-stranded β-barrel where cAMP binds. The essential feature of the β-barrel is the conserved PBC that anchors cAMP and shields it from solvent interactions (Canaves and Taylor, 2002). The PBCs comprise β-strand 6, a short turn of α-helix and β-strand 7. Most of the variability in the CBDs corresponds to the loop between β-strand 4 and β-strand 5 and the C-terminal region of each A

Table 1. Major structural features of the catalytic domain of PKAc from *Homo sapiens* and *Plasmodium falciparum*

Subdomains	PfPKAc	HsPKAc	Functions
I	$G^{40}G^{42}G^{45}$ 2 β-strands, 2 α-helices	$G^{50}G^{52}G^{55}$ 2 β-strands	ATP fixation
II	K^{63} 1 β-strand, 1 α-helix	K^{72} 1 β-strand, 1 α-helix	essential for optimal kinase activity
III	E^{82} 1 α-helix	E^{91} 1 α-helix	ATP stabilisation
IV	ND 1 β-strand	ND 1 β-strand	ND
V	$L^{111}E^{112}F^{113}V^{114}E^{118}$ 1 β-strand, 1 α-helix	$M^{120}E^{121}Y^{122}V^{123}E^{127}$ 1 β-strand, 1 α-helix	linker between the two lobes ATP stabilisation / substrate recognition
VI	$D^{157}N^{162}$ E^{161} 1 α-helice, 1 β-strands	$D^{166}N^{171}$ E^{170} 2 α-helices, 2 β-strands	catalytic loop substrate recognition
VII	D^{175} 1 β-strand	D^{184} 2 β-strands	orienting the phosphate of ATP
VIII	E^{199} $Y^{187}L^{189}C^{190}P^{193}I^{196}$ T^{188} 2 α-helices	E^{208} $W^{196}L^{198}C^{199}P^{202}L^{205}$ T^{197} 1 β-strand, 1 α-helix	catalytic loop substrate recognition essential for optimal kinase activity
IX	D^{211} $E^{221}C^{226}P^{227}P^{228}F^{229}Y^{230}$ 1 α-helix	D^{220} $E^{230}Y^{235}P^{236}P^{237}F^{238}F^{239}$ 1 α-helix	stabilisation of the catalytic loop substrate recognition
X	ND 1 α-helix	ND 1 α-helix	ND
XI	$R^{271}2$ 2 α-helices	R^{280} 2 α-helices	subdomain stabilisation

ND, Not determined.

and B domain. In *P. falciparum*, the 2 CBDs are well conserved; however, there are slight differences between them, especially in the C-terminal area of each CBD, where the α-helices 7 and 8 present in BtPKAr structure are grouped into a single helix in PfPKAr (Fig. 3B).

Analysis of the amino acid sequence and structure of PfPKAr indicates that it shares a number of conserved features with other PKAr proteins. However, there are also several interesting differences, including the degenerate D/D domain and the inhibitory sequence, which is a mixture between type I and type II regulatory subunits.

PHYLOGENETIC ANALYSIS OF PFPKA

Phylogenetic analyses of PKAc and PKAr were performed with sequences from 18 different species, including representatives of Apicomplexa, Euglenozoa and Metazoa phyla. The results are shown in Fig. 4. The methods used to infer the phylogenetic trees are presented in the legend of the figure. The Metazoa clade is fully supported, with 100% bootstrap values in the phylogenetic analyses of both PKAr and PKAc sequences. Within the Metazoa, mammals (*B. taurus, Rattus norvegicus, Mus musculus* and *H. sapiens*) are grouped together (100% bootstrap), and the mammalian relationships are congruent between the two datasets, although with weaker support in the PKAr analysis. Both datasets group the Euglenozoa as a monophyletic clade, with 87% and 100% bootstrap values for PKAc and PKAr, respectively. The Apicomplexa are monophyletic in the PKAr tree and paraphyletic in the PKAc tree. This paraphyly could be explained by the low support for the positions of Apicomplexans *Toxoplasma gondii, Cryptosporidium parvum* and *Babesia bovis* and their long respective branches in both analyses. With both genes, the *Plasmodium* species are grouped in a strongly supported clade (100% bootstrap). The 3 rodent malaria parasites, *P. berghei, P. yoelii* and *P. chabaudi,* form a group with a high bootstrap value that is separated from the human malaria species. Concerning the human parasites, *P. vivax* and *P. knowlesi* are clustered together but *P. falciparum* is either at the root of these species (PKAc) or at the root of the rodent ones (PKAr). The *P. falciparum* branches are the longest among the *Plasmodium* species. The phylogenetic distance between *Plasmodium* species and their vertebrate host (*H. sapiens*) is considerable for both PKAc and PKAr. This information, together with the differences observed in sequences and structures, supports the idea that PfPKA can be specifically inhibited, further establishing this

protein as an interesting target for antimalarial compounds.

METHODS TO STUDY THE PKA AND CAMP PATHWAY IN *P. FALCIPARUM*

The biochemical and biological studies of PfPKA has been dominated by the use of either pharmacological inhibitors/activators of members of the cAMP pathway or by molecular strategies.

Biochemical strategies

Two compounds in particular have been widely used to study PKAc function in eukaryotic cells: H89 and KT5720. H89 is an isoquinoline derivative developed from inhibitor H8 (Hidaka *et al*. 1984), while KT5720 belongs to a family of compounds synthesized by the fungus *Nocardiopsis* sp. (Kase *et al*. 1987). Both inhibitors act through similar mechanisms as competitive antagonists of ATP at its PKAc binding site, thus preventing cAMP-dependent phosphorylation of PKAc substrates. However, these two compounds seem to have various non-specific effects, such as inhibiting other protein kinases, sometimes more potently than their intended target (Davies *et al*. 2000; Murray, 2008).

Rp-cAMP, another PKAc inhibitor, acts as a competitive antagonist of cAMP by binding to PKAr (on CBD domain) without dissociating the kinase holoenzyme (Gjertsen *et al*. 1995). In contrast to H89 and KT5720, this compound may not have effects outside the cAMP signalling pathways (Murray, 2008).

PKI peptide, which is an endogenous molecule involved in the regulation of PKA activity, binds to free PKAc and prevents the phosphorylation of PKAc targets. PKI seems to be a more specific PKAc inhibitor than H89 or KT5720, but all three have been widely used to study PfPKA and cAMP signalling in *P. falciparum*. Other inhibitors of the cAMP pathway that specifically target AC, such as MDL-12, SQ22536 and dideoxyadenosine were also used and lead to cAMP depletion, impeding PKAc activation.

Activation of cAMP pathway elements is another way to study the function of PfPKAc and, more generally, the organization of cAMP pathway in *P. falciparum*. Different molecules targeting PKAc directly or indirectly can be employed. Forskolin, a labdane diterpene produced by the *Coleus forskohlii* plant (Takeda *et al*. 1983), acts by activating the AC enzyme, resulting in an increase of cAMP level and thus allowing PKAc activation. It is interesting to note that AC activity is insensitive to forskolin in asexual blood stages (Read and Mikkelsen, 1991*b*), while ACα in sporozoites seems to be sensitive to forskolin stimulation (Ono *et al*. 2008). Many pathogenic bacteria secrete toxins, such as the cholera and pertussis toxins, that alter the intracellular concentration of cAMP. These toxins disrupt the normal regulation of the cAMP pathway by catalysing the ADP-ribosylation of the heterotrimeric G proteins, which prevents AC inactivation. Thus, AC remains inappropriately active, leading to an increase in cAMP concentration that activates PKAc. Another way to stimulate PKAc activity is to add cAMP analogues such as 8-Br-cAMP or 6-Bz-cAMP directly to the cell. PKAc activity can also be induced by preventing the destruction of cAMP by phosphodiesterases. Some molecules, including IBMX and caffeine, inhibit the action of these enzymes, allowing cAMP to accumulate in the cell thereby leading to the activation of PKAc. It should be noted, however, that these molecules had little or no effect when tested on native PfPDE enzyme activity (McRobert *et al*. 2008), but can be used to clarify and to understand the pathway connections in the parasite.

Molecular strategies

Other molecular techniques have been developed to study the cAMP-signalling pathway, such as RNA interference (RNAi). Briefly, double-stranded RNAs (dsRNA) targeting a specific cellular mRNA can be introduced to the cell to knock down the encoded proteins. To date, RNAi-related genes have not been identified in the *P. falciparum* genome (Ullu *et al*. 2004; Baum *et al*. 2009), it has been suggested that the inhibitory effect of dsRNA might be due to antisense effect rather than a classical RNAi mechanism (McRobert and McConkey, 2002; Noonpakdee *et al*. 2003; Gunasekera *et al*. 2004; Gissot *et al*. 2005; Rathjen *et al*. 2006). Another possibility is that the proteins involved in RNAi processes (Dicer, RNA-Induced Silencing Complex…) could be transported into the parasite from the human host cell (Rathjen *et al*. 2006). Although there has been some controversy surrounding the utility and effectiveness of RNAi in *P. falciparum* (Ullu *et al*. 2004; Baum *et al*. 2009), dsRNAs have been employed to explore the biological function of some *P. falciparum* proteins (Kumar *et al*. 2002; Malhotra *et al*. 2002; McRobert and McConkey, 2002; Pradhan and Tuteja, 2007; Sriwilaijaroen *et al*. 2009).

When the level of *pfpkac* mRNAs is down-regulated using dsRNA, the cAMP pathway is inhibited (Wurtz *et al*. 2009*a*). Moreover, introduction of a non-functioning mutant or over-expression of some proteins using plasmid transfection can be used to specifically perturb signalling pathways (Merckx *et al*. 2008*b*). The major drawbacks to these strategies are the low transfection efficiencies and the complexity of implementing these techniques (Meissner *et al*. 2007). However, given the high specificity of such techniques relative to

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Fig. 3. (Cont.)

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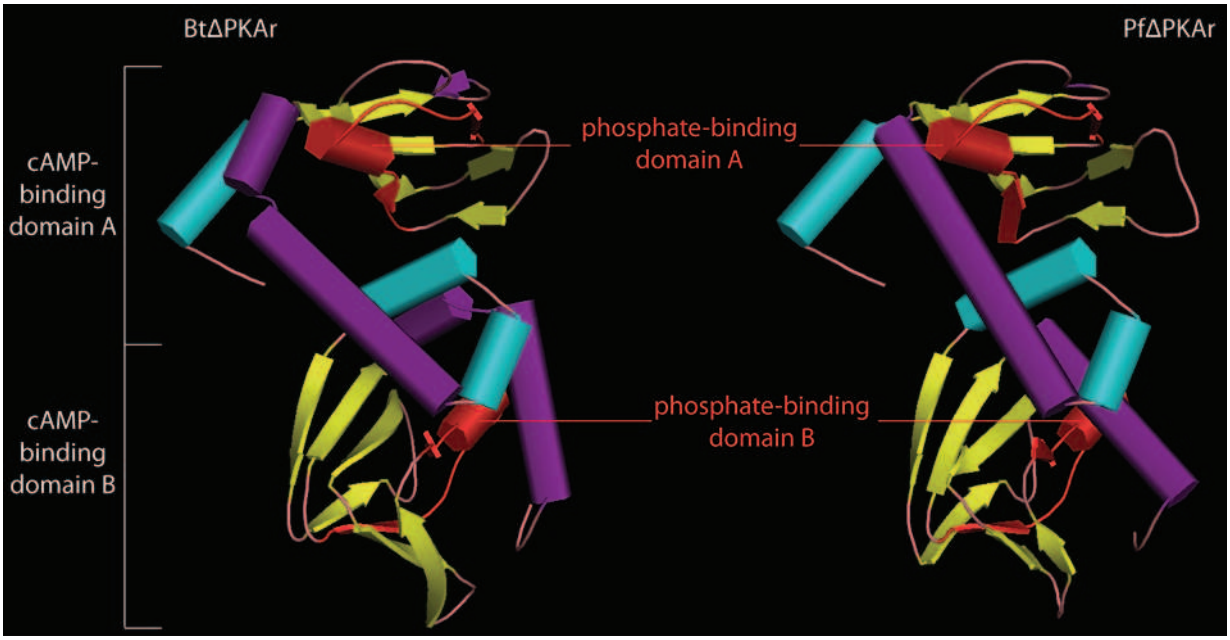

Fig. 3. Amino acid sequence alignments and partial structural modelling of PfPKAr. (A) Comparison of PfPKAr amino acid sequence with other protein kinase regulatory subunits. *Plasmodium falciparum* (Uniprot Accession number Q7KQK0), *H. sapiens* (P10644), *B. taurus* (P00514), *M. musculus* (Q9DBC7), *C. parvum* (A3FPL6), *T. brucei* (Q9GU80), *S. cerevisae* (P07278), *P. knowlesi* (B3LCL9) and *D. melanogaster* (Q9VPA) were aligned using T-coffee 7.38 and CLUSTAL format. The AKAP binding and dimerisation domain, inhibitory sequence and cAMP-binding domains A and B are indicated (black boxed regions). Phosphate-binding cassettes A and B (red rectangles), β-sheets (yellow) and α-helixes (turquoise blue) are depicted as in Fig. 2B. Secondary structural element differences between *B. taurus* PKAr and PfPKAr are shown in purple. Identical residues (asterisks), conservative (colons) and semi-conservative substitutions (dots) are also indicated. (B) Structural model of partial PfPKAr. The partial PfPKAr structure was modelled on the resolved structure of *B. taurus* 1-91 deletion mutant type I alpha PKAr. α-helices (blue cylinders), β-sheets (yellow arrows) and turns (lines) are illustrated. Secondary structural differences between PfPKAr and PfPKAc are shown in purple. Phosphate-binding cassettes A and B are shown in red.

pharmacological agents, it is likely that molecular strategies will further improve the understanding of signalling pathways in *Plasmodium*.

BIOLOGICAL FUNCTIONS OF PFPKA

While the downstream targets of PfPKAc have not yet been clearly identified, several reports have suggested that this enzyme seems to play a pleiotropic role during the different stages of the parasite life cycle. First, PfPKAc seems to be involved in asexual parasite development, including erythrocyte invasion and induction of gametocytogenesis (Kaushal *et al*. 1980; McColm *et al*. 1980; Brockelman, 1982; Rangachari *et al*. 1986; Trager and Gill, 1989; Syin *et al*. 2001; Wurtz *et al*. 2009*a*). More recently, several studies have implicated PfPKAc in the following processes: (i) activation of a Ca^{2+} influx (Beraldo *et al*. 2005; Wurtz *et al*. 2009*a*), (ii) regulation of anion transport through the erythrocyte membrane (Merckx *et al*. 2008*b*, 2009), (iii) regulation of apical exocytosis and motility of sporozoites (Ono *et al*. 2008; Kebaier and Vanderberg, 2009) and (iv) mitochondrial protein traffic (Wurtz *et al*. 2009*a*). Thus, PfPKA seems to be a key regulator of *P. falciparum* development and consequently represents an attractive target for the development of anti-malarial compounds.

PfPKA has a key role in the P. falciparum *asexual life cycle*

The catalytic and regulatory subunits of PfPKA are expressed weakly during the ring and trophozoite stages compared to the schizont stage, and *pfpkac* mRNA levels are lower in gametocytes and gametes (Syin *et al*. 2001; Bozdech *et al*. 2003; Ward *et al*. 2004; Wurtz *et al*. 2009*a*). Indeed, PfPKA seems to be essential for parasite growth and survival, as already described in previous studies: (a) H89, which inhibits PfPKAc activity *in vitro*, leads to parasite growth arrest and morphological alteration (Syin *et al*. 2001); (b) the parasite cell cycle is altered after treatment with an activator of PfPKAc (6-Bz-cAMP) (Beraldo *et al*. 2005); (c) transgenic parasites that overexpress PfPKAr have growth defects that can be restored by increasing the levels of intracellular cAMP (Merckx *et al*. 2008*b*) and (d) down-regulation of *pfpkac* mRNA using gene silencing leads to morphological changes in schizont stages and cell cycle arrest (Wurtz *et al*. 2009*a*). Down-regulation of

Table 2. Major structural features of the regulatory domain of PKAr from *Bos taurus* and *Plasmodium falciparum*

Domains	PfPKAr residues	BtPKAr type I conserved residues	Functions
D/D	None	$C^{16}Y^{19}V^{20}N^{24}I^{25}L^{36}C^{37}L^{48}Y^{51}F^{52}A^{60}$	Dimerization and AKAP binding
Inhibitory sequence	$K^{145}R^{146}X^{147}S^{148}$	$R^{94}R^{95}X^{96}A^{97}$	Binding and inhibition of PKAc
CBD-A	E^{258} R^{267}	E^{200} R^{209}	cAMP binding
CBD-B	E^{376} R^{385}	E^{324} R^{333}	cAMP binding

D/D, Dimerization/docking domain.
CBD, cAMP-binding domain.

pfpkac mRNA using gene silencing is also associated with a compensatory decrease in *pfpkar* mRNA levels, suggesting a transcriptional self-regulation of the PfPKA signalling network. The parasites appear to have tightly controlled mechanisms for self-regulating PfPKA levels to maintain appropriate PKA signalling. This type of self-regulation has also been described in mammalian cells, where, for example, knocking down the regulatory subunit causes a subsequent decrease in levels of the catalytic subunit (Duncan *et al*. 2006). This phenomenon was already proposed for *P. falciparum* because over-expression of the regulatory subunit leads to an increase in *pfpkac* transcript levels (Merckx *et al*. 2008*b*). This PfPKA self-regulation mechanism might exist to counteract the adverse effects caused by changes in the expression levels of either PfPKAc or PfPKAr.

Despite the established importance of PfPKA during parasite growth, its substrates have not been clearly identified. However, several studies presented below have shown that PfPKA is involved in many signal transduction pathways.

Putative role for PfPKA in the induction of gametocytogenesis

Several previous reports have shown that cAMP levels appear to be important for the induction of gametocytogenesis (without the direct involvement of PfPKAc). Kaushal *et al*. (1980) have shown that static cultures of ring stages develop into gametocytes following the addition of 1 mM cAMP. Treatment of *P. falciparum* cultures with cAMP agonists or with phosphodiesterase inhibitors such as caffeine and 8-Br-cAMP results in an increase in gametocyte induction (Brockelman, 1982; Trager and Gill, 1989). Moreover, AC activity and cAMP levels have been correlated with the parasite's ability to produce gametocytes (Read and Mikkelsen, 1991*a*). More recently, Dyer and Day (2000) have shown that the addition of cholera toxin, which prevents AC inactivation, causes an increase in conversion to sexual development. However, to date, no other studies have been conducted to evaluate whether or not PfPKA is involved in the induction of gametocytogenesis.

PfPKA is implicated in sporozoite motility and hepatocyte invasion

Recently, the cAMP pathway was shown to regulate sporozoite motility and hepatic cell invasion by *P. falciparum* sporozoites (Ono *et al*. 2008; Kebaier and Vanderberg, 2009). Sporozoites are deposited in the host's skin by infected mosquitoes and must penetrate cell barriers in the skin and liver sinusoid to reach their target cell, the hepatocyte; once there, they enter in a vacuole and begin the next stage of their life cycle (Fig. 5) (Mota *et al*. 2002; Mota and Rodriguez, 2002; Ejigiri and Sinnis, 2009).

Cell invasion and gliding motility of sporozoites are active processes that are tightly associated with exocytosis of apical organelles in different Apicomplexans (Carruthers *et al*. 1999). Sporozoites possess 2 types of secretory apical organelles: micronemes and rhoptries. Migration through cells activates the exocytosis of sporozoite apical organelles (Mota *et al*. 2002), which results in the release of parasite molecules that are essential for invasion. Two *Plasmodium* sporozoite microneme proteins that have been shown to be released during motility and invasion are circumsporozoite protein (CSP) and thrombospondin-related anonymous protein (TRAP) (Khan *et al*. 1992; Spaccapelo *et al*. 1997). Ono *et al*. (2008) have shown that apical exocytosis is induced by increases in cAMP level in the sporozoites of rodent and human *Plasmodium* species. Increasing the cytosolic cAMP level in *Plasmodium* sporozoites with the addition of the cell-permeable compounds 8-Br-cAMP or forskolin induces apical exocytosis *in vitro*, as measured by an increase in the accumulation of extracellular TRAP at the apical end of sporozoites. Moreover, incubation of sporozoites with AC inhibitors (MDL12330A, SQ22536) prevented sporozoite exocytosis. Thus, it is suggested that the synthesis of cAMP by AC increases sporozoite exocytosis. Indeed, treatment of *P. yoelii* sporozoites with 8Br-cAMP or forskolin was shown to decrease their ability to migrate through monolayers of the hepatoma cell line Hepa 1–6 and increase their ability to invade. The major downstream effector of cAMP is PfPKA, and direct inhibition of its activity by H89 or Rp-cAMP was also able to significantly reduce sporozoite exocytosis, suggesting

that this process is mediated by PfPKA activation (Ono *et al*. 2008). Pre-treatment of sporozoites with H89 followed by induction of cAMP synthesis by 8-Br-cAMP completely inhibited exocytosis, suggesting that PKA activation occurs after cAMP generation. In the *P. falciparum* genome, 2 genes coding for AC have been identified (ACα and ACβ) (Baker and Kelly, 2004) and their expression demonstrated in sporozoites (Le Roch *et al*. 2003). Deletion of *P. berghei* ACα does not alter parasite growth during blood stages or in the mosquito, but mutant sporozoites are not able to expose the adhesive proteins and their infectivity is reduced by 50% (Ono *et al*. 2008). Re-introduction of ACα in deficient parasites resulted in a complete recovery of exocytosis and infection. These data reveal the importance of cAMP and PfPKA in sporozoite apical-regulated exocytosis, which is involved in hepatocyte infection by sporozoites.

Kebaier and Vanderberg (2009) subsequently focused on motility rather than invasion by using albumin, which triggers motility in *Plasmodium* sporozoites, and evaluated the link between sporozoite exposure to albumin and intracellular signalling pathways, especially cAMP and calcium networks. First, they have shown that parasite intracellular calcium is necessary for sporozoite motility. Use of a calcium chelator (BAPTA-AM) inhibits sporozoite motility, and these effects are reversed by the addition of exogenous calcium (calcium ionophore A23187). Moreover, they have demonstrated that suppression of cAMP synthesis by an AC inhibitor (SQ22536) also leads to a decrease in sporozoite motility. The same results were obtained when the sporozoites were treated with PKA inhibitor H89 or Rottlerin (a non-specific kinase inhibitor). Thus, elevating the concentration of cAMP (by addition of forskolin, IBMX or cAMP analogues) allows for sporozoite motility without the addition of albumin in the medium. This study revealed that calcium and cAMP/PKA pathways act together to promote sporozoite motility.

A highly complex relationship between cAMP/PfPKA and calcium pathways exists in the P. falciparum *asexual life cycle*

Beraldo *et al*. (2005) studied in detail the involvement of cAMP and its target PfPKAc in calcium signalling mechanisms during *P. falciparum* intraerythrocytic development. They demonstrated that melatonin, by activating specific receptors coupled to phospholipase C activation, causes the release of calcium from *P. falciparum* intracellular compartments *in vitro* (Hotta *et al*. 2000). Subsequently, they observed that melatonin increases cAMP levels and PfPKAc activity, suggesting an important role for calcium in the control of cAMP production. When iRBCs were treated with 6-Bz-cAMP or IBMX (PfPKAc activators), there was a change in the parasite cell cycle and an increase in the schizont population, similar to that observed when parasites were treated with melatonin. The effects of melatonin were abolished by adding PfPKAc inhibitors (PKI, H89, Rp-cAMP). These data suggest that cAMP and PKA are key modulators of the *P. falciparum* cell cycle (Fig. 5). Inhibition of phospholipase C by U73122 blocks the melatonin-induced cAMP increase. On the other hand, the addition of exogenous calcium leads to an augmentation of cAMP levels. These data suggest that the increase in cAMP levels is directly caused by an increase in calcium concentration caused by melatonin, rather than by a coupling of the melatonin receptors to AC (Beraldo *et al*. 2005). However, the interplay between cAMP and calcium is not limited to the calcium-dependent activation of cAMP production. In fact, PKA activator 6BZcAMP is able to induce calcium level increases in the parasite, either by mobilizing calcium from intracellular compartments or by stimulating calcium entry from the medium. Beraldo *et al*. (2005) concluded that the modulation of the *P. falciparum* cell cycle by the host hormone melatonin is mediated by 2 second messengers acting together. (1) Melatonin is directly coupled to a classical calcium signalling pathway *via* phospholipase C, which cleaves phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-triphosphate (IP3). IP3 then diffuses through the cytosol to bind to IP3 receptors on calcium channels in the endoplasmic reticulum, which causes an increase in cytoplasmic calcium concentration. (2) The elevation of calcium concentration initiates an amplification cycle *via* cAMP and PKA, which in turn leads to an increase in cytoplasmic calcium either by calcium release from the endoplasmic reticulum or by stimulating calcium entry from the outside. Thus, there is a highly complex relationship between the calcium and cAMP signalling networks in *Plasmodium*. These data are consistent with another study showing that gene silencing of PfPKAc leads to a down-regulation of members of the calcium/calmodulin pathway, again suggesting an interplay between the cAMP and calcium networks (Wurtz *et al*. 2009*a*).

PfPKA plays a key role in anion transport across the erythrocyte membrane during the P. falciparum *asexual life cycle*

While replicating in red blood cells (RBCs), *P. falciparum* is able to modify the host cell by increasing its permeability to facilitate nutrient uptake and to evacuate potentially dangerous metabolites for the cell (Kirk, 2001). The parasites can alter permeability of the erythrocyte membrane either by over-expressing existing carriers or by creating new permeation pathways (NPP). Egee *et al*. (2002) found

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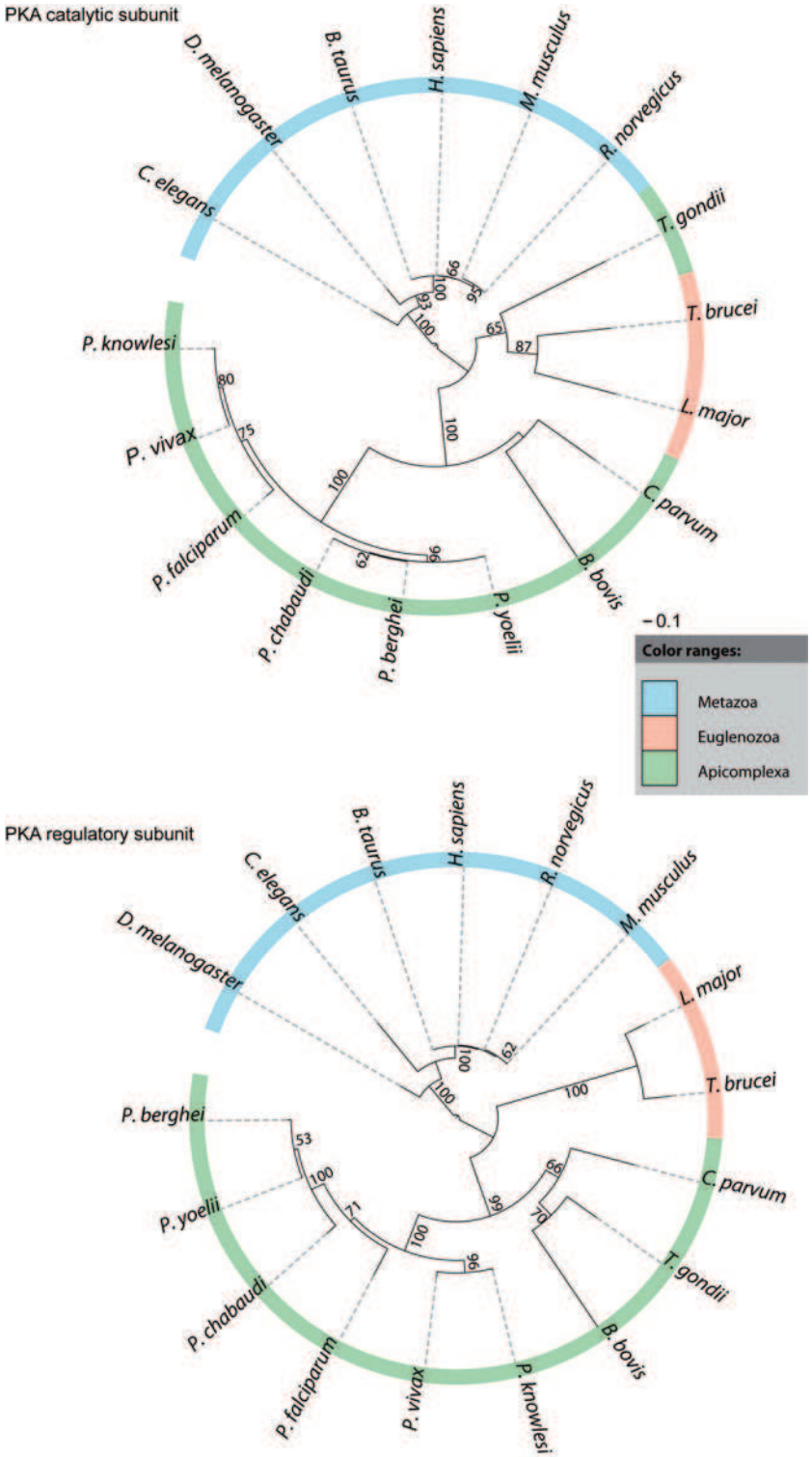

Fig. 4. Phylogenetic tree of cAMP-dependent protein kinase catalytic (A) and regulatory (B) subunits. The PKA protein sequences of 17 species have been obtained from UniProt Knowledgebase (http://www.ebi.ac.uk/uniprot/): *Plasmodium falciparum, P. vivax* (Accession number PKAc A5KE97-PKAr A5K031), *P. yoelii* (PKAr Q7RJ15), *P. knowlesi* (B3L322-B3LCL9), *P. chabaudi* (Q4XMV3-Q4Y575), *P. berghei* (Q4YW20-Q4YNR9), *Babesia bovis* (A7ANK3-A7AU83), *Cryptosporidium parvum, Toxoplasma gondii* (B6KN50-Q9BMY7), *Trypanosoma brucei, Leishmania major* (Q27687-Q4QGD8), *Homo sapiens, Mus musculus, Rattus norvegicus* (A1L1M0-P09456), *Bos taurus* (PKAc P00517), *Drosophila melanogaster* and *Caenorhabditis elegans* (P21137-P30625). Multiple alignments of these sequences were performed using T-coffee (default parameters) (Notredame *et al*. 2000). Selection of the best-fit substitution model was done by a test run with ProTest software on each of the protein alignments (Abascal *et al*. 2005). Phylogenetic relationships among sequences were determined using the maximum likelihood (ML) method implemented in PhyML (Guindon and Gascuel, 2003) using the LG + G model. The additional parameters used in PhyML analyses were as follows: gamma distribution parameter estimated with 4 rates categories, and subtree pruning

that the anion channels of uninfected erythrocytes could be activated by the addition of bovine PKA and ATP, producing a membrane current similar to that observed in *P. falciparum*-infected red blood cells (iRBCs) and suggesting that the mechanism of regulation used by the parasite may involve phosphorylation steps (Egee *et al*. 2002). Additionally, Merckx *et al*. (2008*b*) recently reported that the addition of exogenous PfPKAr protein to *P. falciparum* iRBCs leads to a down-regulation of whole-cell membrane conductance, probably regulated by cAMP. Thus, exogenous PfPKAr seems to interfere with the parasite-dependent activation of iRBC membrane conductance. Moreover, these authors generated a *P. falciparum* strain over-expressing PfPKAr and observed that the transgenic parasite produces a clearly reduced membrane current, similar to that observed after the addition of exogenous PfPKAr. They also tested whether the over-expression of PfPKAr interferes with NPP activity by using semi-quantitative haemolysis experiments with sorbitol as permeating substrate. The transgenic parasites displayed decreased membrane permeability to sorbitol since the lysis duration was significantly increased compared with control cultures. Thus, the delay observed in haemolysis for the parasites over-expressing PfPKAr suggests that some NPP activity is under the dependence of phosphorylation *via* cAMP dependent protein kinases. In addition, several mechanisms have been proposed regarding the function of PfPKAc during anion conductance regulation (Merckx *et al*. 2009). It seems that PfPKAc regulates anionic conductance, either *via* direct phosphorylation of the channels or indirectly, through phosphorylation of accessory or associated proteins that can be of human or parasite origin (Fig. 5).

Putative role for PfPKA in erythrocyte invasion by merozoites

Several studies have demonstrated roles for the cAMP/PKA and calcium/calmodulin pathways in *P. falciparum* erythrocyte invasion by merozoites (Rangachari *et al*. 1986; Green *et al*. 2008; Wurtz *et al*. 2009*a*). The expression of 6 reticulocyte-binding-like (RBL) homologue genes was found to be down-regulated in response to *pfpkac* mRNA inhibition (Wurtz *et al*. 2009*a*). These genes belong to the invasion/motility pathway and seem to be important for *P. falciparum* invasion of human erythrocytes (Tham *et al*. 2009). To date, only indirect data have demonstrated the potential role of PfPKAc in this specific pathway (McColm *et al*. 1980; Rangachari *et al*. 1986; Syin *et al*. 2001). While PfPKAc seems to be involved *via* the RBL genes in RBC invasion by the parasite, the precise roles of the cAMP/PKA and calcium/calmodulin pathways in this process still need to be elucidated.

Putative role for PfPKA in the regulation of mitochondrial protein traffic

Another unexpected pathway that could involve the cAMP network concerns genes related to mitochondrial functions (Wurtz *et al*. 2009*a*). Many nuclear genes with mitochondrial signal sequences were found to be induced when *pfpkac* expression was inhibited (Wurtz *et al*. 2009*a*). It is well known that parasite mitochondrial activity requires the import of many proteins (van Dooren *et al*. 2006; Torrentino-Madamet *et al*. 2009). PfPKAc may thus regulate part of the mitochondrial protein traffic, as has already been described for mammalian cells (De Rasmo *et al*. 2008).

PROTEIN KINASE CELLULAR SIGNALLING AS A POTENTIAL TARGET FOR THERAPEUTIC INTERVENTION

ATP binding, substrate binding and/or kinase activity are all potential targets for inhibition by drugs designed to block protein kinases. In 2008, 10 protein kinase inhibitors had been approved for clinic use, and there are many more in clinical trials (Johnson, 2009). *P. falciparum* contains members of most of the established protein kinase families (Ward *et al*. 2004). However, the differences between the host and parasite phosphosignalling pathways suggest that specific inhibition of the latter can be accomplished (Leroy and Doerig, 2008). This notion was confirmed by 2 structural studies demonstrating exploitable divergences between host and parasite protein kinases (Holton *et al*. 2003; Merckx *et al*. 2008*a*). In the case of PfPKA, we have shown here that it presents interesting differences in terms of sequence and structure compared to human PKA. These differences involve ATP anchoring, PfPKAc substrate recognition and phosphorylation, sensitivity of PfPKAc to kinase inhibitors and the D/D domain and inhibitory sequence of PfPKAr. Thus, specifically targeting these domains together or separately could be an effective strategy for inhibiting

and regrafting (SPR) tree search method from 5 random starting trees (Hordijk and Gascuel, 2005). Significances of internal branches are indicated as percentages based on 100 bootstrap replications (only bootstrap values >50% are shown). The online tool 'Interactive Tree Of Life' was used for the display and manipulation of the phylogenetic trees (Letunic and Bork, 2007). The phylogenetic analysis included 17 eukaryotes belonging to 3 groups: Metozoa (blue), Euglenozoa (pink) and Apicomplexa (green). The Metazoa group was used as an outgroup in the analyses.

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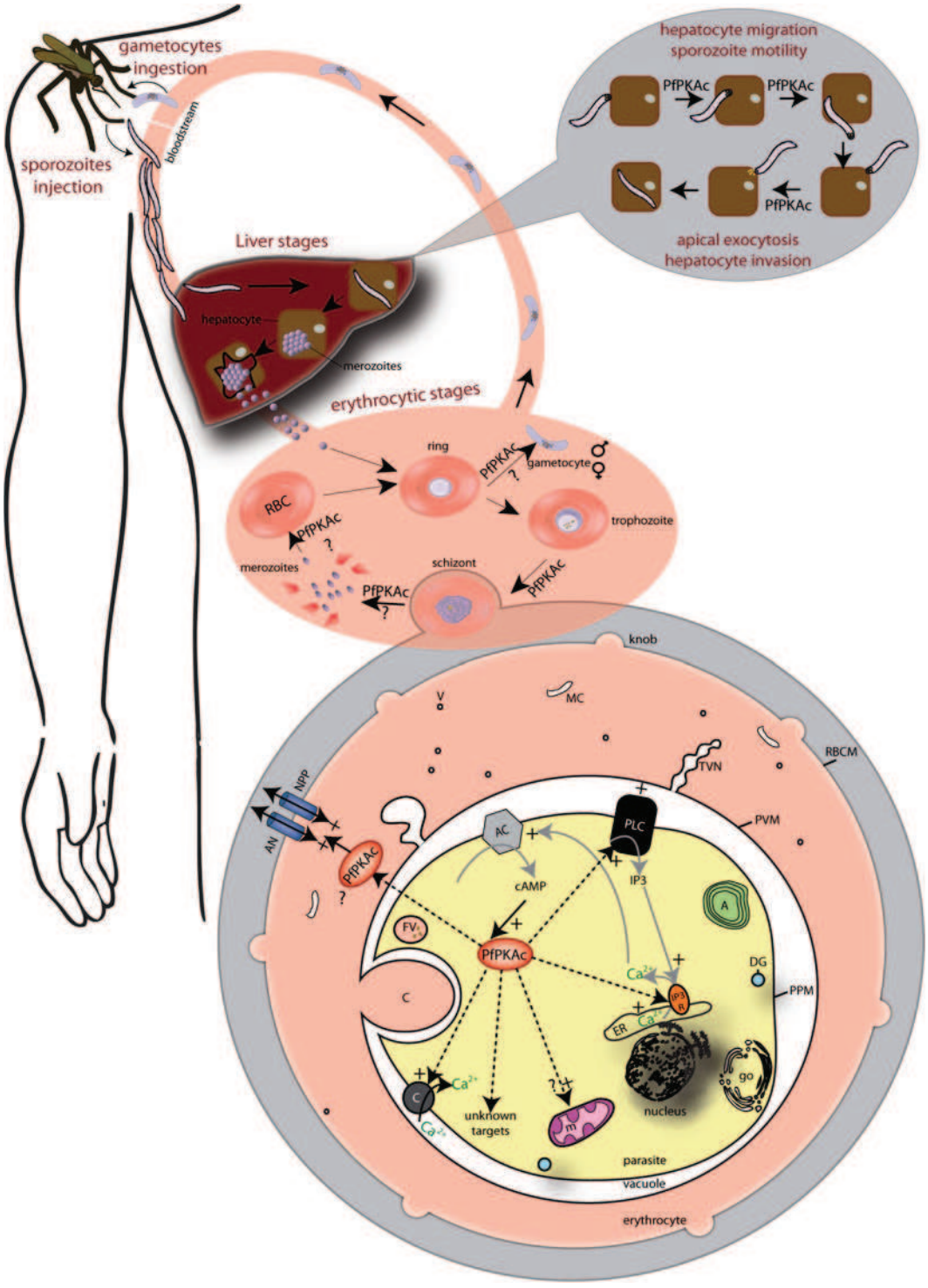

Fig. 5. Schematic model of signalling events including PfPKAc during the *Plasmodium falciparum* life cycle. The *P. falciparum* life cycle requires 2 different hosts, an *Anopheles* mosquito and a human. When an infected mosquito takes a bloodmeal, it injects sporozoites that rapidly move to the liver through the bloodstream. They then progress through the liver cells and ultimately invade a single hepatocyte, where they develop into thousands of merozoites. The cAMP/PfPKAc pathway has been shown to regulate sporozoite motility and the apical exocytosis steps necessary for the invasion of hepatic cells. Next, the hepatocyte bursts and releases the merozoites into the bloodstream, where they invade RBCs. Asexual replication progresses through a series of stages (ring, trophozoite and schizont) that ends with the rupture of the RBC, releasing merozoites that can then reinvade new RBCs. During the asexual life cycle, PfPKAc has been shown to play a role in schizont maturation, merozoite release and in the reinvasion of new RBCs. More specifically, PfPKAc might have different functions in signal transduction events, as follows. (1) A highly complex interplay exists between the cAMP/PfPKAc and Ca^{2+} signalling pathways. First, activation of phospholipase C (PLC) generates inositol 1,4,5-triphosphate (IP3), which binds to its receptor (IP3R) located on the membrane of the endoplasmic reticulum (ER). This leads to calcium release into the cytoplasm that in turns activates AC and induces

the parasite enzyme without interfering with the equivalent host protein.

Targeting the kinase activity directly

Most of the protein kinase inhibitors currently in clinical trials for cancer therapy are small molecules that compete for the ATP-binding site. A major success in this category of inhibitors is the tyrosine kinase inhibitor imatinib (Gleevec®, Novartis), a potent inhibitor of the constitutively active BCR-ABL fusion protein that is used for the treatment of leukaemia and gastrointestinal stromal tumours (Druker, 2002; Tibes *et al*. 2005). This has been followed by other small molecules such as the epidermal growth factor receptor inhibitors erlotinib (Tarceva™ Genentech) and gefitinib (Iressa™ AstraZeneca), both of which received approval for the treatment of non-small cell lung carcinoma (Modjtahedi and Essapen, 2009). As mentioned above, the ATP-binding site of PfPKAc presents some differences when compared to that of human PKAc, suggesting that the design of molecules specifically targeting the parasite domain may be feasible.

Another strategy to inhibit the activity of a protein kinase is to prevent the translation of its transcripts. This can be achieved using ribozymes, which are modified RNA molecules that can cut other RNA. Ribozymes consist of a central catalytic domain with RNA-degrading activity, flanked by RNA sequences that are complementary to the target mRNA. RPI.4610 (Angiozyme) was the first synthesized ribozyme to be studied in human trials. It is designed to prevent the process of angiogenesis by cleaving the mRNA encoding the VEGF1 receptor (Perabo and Muller, 2007). LErafAON, an antisense oligonucleotide targeting the serine/threonine kinase c-Raf that has been implicated in many cancers, has been tested in phase I clinical trials (Wellbrock *et al*. 2004; Zhang *et al*. 2009). This type of approach could be used to inhibit the expression of PfPKAc by designing ribozymes that specifically target the PfPKAc mRNA without altering human PKAc expression.

Targeting partners of kinases or effectors of the pathway

Rather than directly inhibiting the active enzyme, other strategies can be considered that target either the partners or the upstream and/or downstream effectors of the pathway. One possibility is to target the regulatory subunit of PKA, especially for cancer therapy, as its protein and mRNA levels have been found to be upregulated in a series of transformed cell lines and human neoplasms (Bradbury *et al*. 1994; Miller, 2002). GEM®231 (HYB165, Hybridon) is an 18-mer antisense oligonucleotide targeted against human PKA RIα. Used alone or in combination with other agents, GEM®231 has demonstrated anti-tumour activity in a variety of *in vitro* cancer cells and *in vivo* human tumour xenograft models (Tortora and Ciardiello, 2002; Wang *et al*. 2002). We can imagine designing an antisense oligonucleotide against PfPKAr targeting the C-terminal end of the sequence, as we have demonstrated above a significant difference between PfPKAr and human PKAr in this region.

The AKAPs introduce another level of complexity to PKA signalling and have emerged as key regulators of PKA function. To date, these partners have not yet been well studied but appear as interesting therapeutic targets, particularly in the treatment of severe cardiac pathologies and cancers (Diviani, 2008; Naviglio *et al*. 2009). In the case of *P. falciparum*, the hypothetical AKAP identified by orthology presents only 22% identity with human AKAP18. Thus, we propose specifically targeting either the parasite AKAP or the interaction between AKAP and PfPKAr because, as noted above, the mechanism of interaction between these two partners seems to be different from that of their human homologues.

cAMP synthesis. cAMP binds to the PKA regulatory subunit and leads to the release and activation of the catalytic subunit. The increased calcium concentration initiates an amplification loop *via* cAMP and PKA, which in turns leads to a further increase in cytoplasmic calcium either by stimulating PLC or IP3R directly, or by calcium entry from outside of the cell. (2) cAMP-pathway components are involved in the regulation of anion conductance at the erythrocyte membrane. Despite lacking a *Plasmodium* export element/host targeting motif (PEXEL/HT), PfPKAc can be exported to the host cytosol, where it directly phosphorylates anion channels (AN) and/or eventually participates in NPP (new permeation pathway) formation. Alternatively, PfPKAc could either be exported to the erythrocyte cytosol where it phosphorylates proteins of human or parasite origin, or could phosphorylate an unidentified parasite substrate that is then exported to the host cytosol. Furthermore, if human PKAc is activated by parasite cAMP, it could also contribute to the induction of anion conductance. (3) PfPKAc may regulate some mitochondrial protein traffic. (4) PfPKAc certainly has many other cellular targets that are still unknown. In each erythrocyte cycle, some merozoites arrest their cell cycle and develop into sexual forms (gametocytes). A role for PfPKAc in gametocytogenesis induction has been proposed. The cycle is completed when a mosquito takes his bloodmeal and ingests male (♂) and female (♀) gametocytes. Question marks represent the suggested role of PfPKAc during the various events cited above. Maurer's clefts (MC), vesicles (V), tubovesicular network (TVN), red blood cell membrane (RBCM), parasitophorous vacuolar membrane (PVM), parasite plasma membrane (PPM), dense granule (DG), apicoplast (A), food vacuole (FV), cytostome (C), golgi (go), mitochondria (m), channel (C).

Interfering with the function of protein kinases can be achieved with substrate-mimicking molecules that compete with the original substrate. By occupying the natural binding site of the substrate, these molecules end the signal transduction events that help maintain the pathogenic state of the cell. The substrate mimic Thymectacin™ (NB1011), which targets thymidylate synthase, an enzyme over-expressed in tumours, has entered clinical trials (Congiatu *et al*. 2005). Therefore, this strategy of inhibiting enzymatic activity has potential clinical applications. However, substrate competitive inhibitors targeting protein kinases have not yet entered clinical trials, and the substrates of PfPKAc have not yet been clearly defined, hampering the development of such molecules.

A different way to restrain protein kinase activity is to block the upstream activation of the pathway using small molecules or antibodies that prevent receptor-mediated signalling. In the case of the PKA signalling pathway, several upstream partners can be targeted to alter kinase function, including G-protein coupled receptors, adenylate cyclase and phosphodiesterase (Wise *et al*. 2002; Zhang *et al*. 2005; Pavan *et al*. 2009). In 2000, 26 of the top 100 pharmaceutical products were compounds that target GPCRs, including salmeterol (asthma), sumatriptan (migraine), ibuprofen (inflammation, pain), rimonabant (obesity), haloperidol (schizophrenia), cabergoline (Parkinson's disease) and many more (Wise *et al*. 2002). Phosphodiesterases play crucial roles in cell signalling and has therefore been the target of clinical drug development for indications ranging from anti-inflammation to memory enhancement. Many drugs targeting such proteins have been developed, including theophylline (asthma), anagrelide (thrombocytosis), milrinone (cardiac failure) and dipyridamole (inhibitor of platelet aggregation) (Wise *et al*. 2002; Zhang *et al*. 2005; Denault *et al*. 2006; Chakrabarti and Freedman, 2008; Emadi and Spivak, 2009). Although many drugs target the cAMP signalling pathway through G-protein coupled receptors or phosphodiesterases, adenylate cyclases have not yet been considered as drug targets. However, the generation of knockout and transgenic animals has revealed that these proteins have crucial roles in numerous biological processes (Pierre *et al*. 2009). Mammalian adenylate cyclases are currently under investigation as potential drug targets, with some compounds already being used in clinics. For example, corlforsin has been approved in Japan for the treatment of heart failure (Ogata *et al*. 2007). These PfPKAc partners could be considered as potential drug targets; however, more work is needed on these genes, which have not yet been well characterized.

Another approach to inhibiting the PKA pathway is the use of cAMP analogues. Between 1960 and 1980, numerous analogues of cAMP were synthesized and screened for their therapeutic potential, especially against diabetes, asthma and cardiovascular diseases. A good illustration is 8-Cl-cAMP which inhibits cancer cell growth through both anti-proliferation and pro-apoptotic mechanisms (Cho-Chung and Nesterova, 2005). This molecule has completed several phase I clinical studies and recently entered phase II clinical trials as an anti-cancer agent (Tortora and Ciardiello, 2002). However, the strategy of using cAMP analogues does not seem feasible in the case of *P. falciparum* because the interactions between cAMP and PfPKAr appear similar to those in humans; therefore, these compounds would not specifically target the parasite. In conclusion, PfPKA and the *P. falciparum* cAMP pathways appear to be relevant biological targets for the therapy and management of malaria.

CONCLUSIONS AND PERSPECTIVES

In spite of its established importance during parasite growth and the different pathways that are associated with it, the substrates of PfPKA have not yet been clearly identified. Merckx *et al*. (2009) identified several PfPKAc substrates in human RBCs and in the *P. falciparum* proteome by using an *in silico* search for phosphorylation sites using PkaPS (Prediction of protein kinase A Phosphorylation Sites, http://mendel.imp.ac.at/sat/pkaPS/pkaPS.html) (Neuberger *et al*. 2007). As the recombinant PfPKAc and PfPKAr proteins are available, different strategies could be employed to identify both the upstream targets and specific inhibitors of this enzyme. First, recombinant PfPKAc could be used to test the protein's capacity to phosphorylate potential parasite/human substrates using the KESTREL (kinase substrate tracking and elucidation) approach (Cohen and Knebel, 2006; Peng *et al*. 2007; Philip and Haystead, 2007). This strategy has been applied successfully to *P. falciparum* to identify the substrates of serine / threonine kinase PfPK9 (Philip and Haystead, 2007). To avoid the use of radioactive molecules, detection of phosphorylated proteins can be achieved using specific fluorescent staining allowing direct, in-gel detection of phosphate groups attached to tyrosine, serine or threonine residues (e.g., ProQ® Diamond, Invitrogen) (Schulenberg *et al*. 2004; Orsatti *et al*. 2009). In addition, the use of protein kinase inhibitors, such as H89, on *P. falciparum* could reveal novel substrates, potential new pathway interconnections and inhibitor specificity by monitoring differences in protein levels and phosphorylation (Davis *et al*. 2006).

Thus recombinant proteins obtained can also be used (i) to produce specific anti-PfPKAc and anti-PfPKAr antibodies to determine the cellular locations of PfPKAc, PfPKAr and their partners (Murtaugh *et al*. 1982; Ray *et al*. 2001), and (ii) to perform high-throughput screening of PfPKAc

inhibitors and interaction studies with PfPKAr (Merckx *et al*. 2008*b*; Blackwell *et al*. 2009). Clearly, the identification of the upstream signals/molecular events that regulate PfPKAc activity and their downstream targets will be crucial for a more precise understanding of their cellular function and should also provide new targets for drug design.

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TRAVAUX ORIGINAUX

1. APPROCHE BIOCHIMIQUE DANS L'ETUDE DE LA PROTEINE KINASE AMP CYCLIQUE DE *PLASMODIUM FALCIPARUM*

Article n°1 :

Expression and biochemical characterization of *Plasmodium falciparum* protein kinase A catalytic subunit

Wurtz N, Pastorino B, Almeras L, Briolant S, Villard C, Parzy D

« Parasitology Research » 2009 ; 104(6) :1299-305.

La première étape de notre travail a donc été de caractériser les propriétés biochimiques de cette kinase plasmodiale.

Dans un premier temps, la séquence protéique de la PfPKAc a été comparé à des PKAc d'autres organismes (*Homo sapiens*, *Mus musculus* et *Plasmodium yoelli*) et un modèle de structure tridimensionnelle de la PfPKAc a été construit en se basant sur la structure tridimensionnelle disponible de la PKAc humaine (numéro d'accession PDB 2GU8). Cette comparaison nous a permis de constater qu'il existait une forte homologie entre les deux protéines (51% d'identité) et que la kinase plasmodiale possédait les onze domaines spécifiques des protéines kinases [69] ainsi que certains éléments indispensables à son activité notamment au niveau de la fixation de l'ATP, de l'activité catalytique, de stabilisation ainsi que les domaines d'interaction avec certains inhibiteurs ou encore la PKAr [69,82]. En revanche, certaines différences situées dans des domaines essentiels ont pu être constatées : *i)* dans le domaine VIB, un feuillet- β et une hélice-α sont absents dans la PfPKAc ; ce domaine est important puisqu'il contient la poche catalytique DLKPEN [68] ; *ii)* dans le domaine VIII où l'hélice-α présente dans la protéine humaine est divisée en deux hélices α dans la PKA plasmodiale et un feuillet-β est également absent dans la protéine plasmodiale. Ce domaine contient un résidu W^{196} intervenant dans la fixation de la kinase avec la sous-unité régulatrice, qui n'est pas conservé dans la protéine plasmodiale (Y^{196}) [79,83]. Finalement, cette première étape nous a permis de constater que malgré une forte homologie de séquence et de structure avec la PKAc humaine, la PfPKAc présente certaines différences dans des domaines essentiels. Ce résultat est encourageant dans la perspective de développement de nouvelles molécules antipaludiques ciblant spécifiquement la PfPKAc.

Dans un second temps, nous nous sommes focalisés sur le clonage, l'expression et la purification de la kinase plasmodiale. Pour cela, nous avons choisi d'utiliser le système Gateway® (Invitrogen) associé au vecteur d'expression pET-DEST42 avec une production en système bactérien (*E.coli* souche Rosetta™(DE3) pLysS, Merck). Pour purifier la PfPKAc recombinante soluble, nous avons optimisé un procédé de purification original combinant une première étape de chromatographie d'affinité au cobalt suivie d'une seconde étape de chromatographie d'exclusion en présence d'arginine, un agent dissociant non dénaturant [84].

Ce procédé nous a permis d'éliminer la plupart des contaminants bactériens fortement associés à la PfPKAc recombinante que nous avons identifiés en spectrométrie de masse. Seule la chaperonne bactérienne Hsp 60 ne présentant aucune activité kinase n'a pu être totalement éliminée. Au final, nous avons réussi à obtenir une PfPKAc recombinante soluble non contaminée par des kinases bactériennes avec un taux de purification suffisant (70%) pour pouvoir initier des essais enzymatiques.

Dans un troisième temps, il a fallu tester et caractériser l'activité spécifique de la kinase recombinante purifiée. Pour cela nous avons utilisé un test original d'activité kinase avec des substrats fluorescents spécifiques (kit Omnia® Ser/Thr, Invitrogen) [85]. Après optimisation, ces essais enzymatiques nous ont permis *i)* de vérifier que la PfPKAc recombinante purifiée était active, *ii)* de déterminer les constantes enzymatiques en utilisant une gamme spécifique de substrat (K_m = 240 µM, K_{cat} = 0.0092 s^{-1}), *iii)* de réaliser les premiers essais d'inhibitions à l'aide d'inhibiteurs non spécifiques de PKAc (Ro-31 et MEKI) ou d'inhibiteurs de PKAc eucaryotes (H-89, KT-5720, PKI).

Finalement, ces essais nous ont permis de valider l'activité Ser/Thr kinase de type PKAc de la protéine recombinante obtenue par notre système d'expression et de purification. A ce jour, il s'agit du premier test *in vitro* optimisé de mesure de l'activité enzymatique de la PfPKAc qui pourra par la suite servir au criblage d'un grand nombre de molécules chimiques ou de composés susceptibles d'interférer avec la kinase plasmodiale uniquement.

Nos résultats sont présentés dans l'article suivant :

Article n°1 : Wurtz N, Pastorino B, Almeras L, Briolant S, Villard C, Parzy D. Expression and biochemical characterization of the *Plasmodium falciparum* protein kinase A catalytic subunit. Parasitology Research 2009 ;104(6):1299-305.

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ORIGINAL PAPER

Expression and biochemical characterization of the *Plasmodium falciparum* protein kinase A catalytic subunit

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Abstract Dissemination of drug-resistant malaria parasites represents one of the most important public health problems; therefore, the development of new antimalarial compounds is required. Cyclic AMP-dependent protein kinase is implicated in numerous cellular processes and an essential role for this enzyme has also been reported in the intraerythrocytic growth of the malaria parasite. The cAMP-dependent protein kinase from *Plasmodium falciparum* (PfPKA) plays an important role in the parasite life cycle and represents an attractive target for the development of antimalarial drugs. In this work, a recombinant PfPKA catalytic subunit (PfPKAc) was over-expressed in *Escherichia coli* and successfully purified using a two-step chromatographic process. The enzymatic properties of the recombinant PfPKAc were then determined using a sensitive fluorogenic assay suitable for biochemical characterization and inhibitor screening. This work provides new insights on the study of PfPKAc that will contribute to future investigations of the parasite cAMP signaling pathway and to high-throughput screening of specific malarial PKA inhibitors.

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Introduction

Plasmodium falciparum, an apicomplexan protozoan parasite, is the causative agent of the most lethal form of human malaria. The lack of vaccines and the emergence of drug-resistant malaria parasites represent one of the most important public health problems (Ridley 2002). Currently, *P. falciparum* signaling pathways are largely uncharacterized, which is slowing the development of new chemotherapeutics. A recently published genome sequence (Gardner et al. 2002) and earlier studies suggest that several homologues of eukaryotic signaling proteins are conserved in *P. falciparum* (Merckx et al. 2008a; Syin et al. 2001). However, many of these major signaling mediators remained uncharacterized in malaria parasites. Among these mediators, there is evidence that protein kinases are essential for the control of the parasite life cycle and that inhibition of such activities can yield antimalarial effects (Dluzewski and

Garcia 2006). Moreover, these enzymes are widely recognized as valuable drug targets for the treatment of several diseases (Johnson 2007). Although the regulatory mechanisms of cyclic AMP-dependent protein kinase (PKA) are well known in mammalian cells (Taylor et al. 2005), the role of *P. falciparum* PKA (PfPKA) is still unclear, in part due to the lack of molecular models. Until now, only indirect data were available to evaluate the molecular functions of PfPKA, with previous reports suggesting a role for cAMP in *P. falciparum* asexual stage development, erythrocyte invasion, and the induction of gametocytogenesis (Brockelman 1982; Syin et al. 2001). Merck et al. demonstrate that the cAMP-dependent protein kinase regulatory subunit interacts with a kinase activity in parasite extracts with downregulation of the anion membrane conductance of infected erythrocytes (Merckx et al. 2008b). Recently, a study described the susceptibility of a cAMP-dependent protein kinase catalytic subunit (PfPKAc) to mammalian inhibitors (Sudo et al. 2008), but the developed in vitro PKA assay was not adapted to interaction experiments or high-throughput screening of chemical libraries. In this work, we focused on the molecular characterization of a recombinant PfPKAc, which was expressed as a soluble His-Tag protein in *Escherichia coli* and then purified via two-step chromatography. The enzymatic properties of the recombinant kinase were determined using a specific fluorogenic peptide substrate-based assay allowing kinetic constant determination and inhibitor susceptibility. This study constitutes the first enzymatic characterization of a recombinant PfPKAc useful for future substrate specificity determination and malaria drug screening.

Materials and methods

Sequence alignment and three-dimensional modeling of PfPKAc

The complete amino acid sequence of PfPKAc (strain 3D7) was obtained from the *Plasmodium* Genome Resource database (http://www.plasmodb.org, **PFI1685w** or Swissprot accession number **Q7K6A0**) and aligned with cAMP-dependent protein kinase catalytic subunit (PKAc) sequences from *Homo sapiens*, *Mus musculus*, and *Plasmodium yoelii* (Swissprot accession numbers **P17612**, **P05132**, and **Q26217**, respectively) using the CLUSTAL W 2.0.10 multiple sequence alignment program (http://www.ebi.ac.uk/clutalw). The PfPKAc structural model was based on the published structure of human PKAc alpha (PDB accession number **2GU8**) using the Swiss-model ternary structure prediction tool (Arnold et al. 2006) and Pymol 0.99 software (http://www.pymol.org).

Parasite cultures, RT-PCR, and cDNA preparation

P. falciparum strain 3D7 was obtained from the Malaria Research and Reference Reagent Resource Center (MR4, Virginia, USA) and grown in vitro as an asynchronous culture according to the Trager and Jensen method (Trager and Jensen 1976). Total RNA was purified using the TRIZOL™ reagent following manufacturer recommendations (Invitrogen, Cergy Pontoise, France). Specific primers were designed and used for the amplification of the complete PfPKAc gene. RT-PCR was performed in a T gradient thermocycler (Biometra, Goettingen, Germany) using the ThermoScript RT-PCR system kit (Invitrogen) and the following oligonucleotide primers: 5'-CACCATG CAGTTTATTAAA-3' (forward) and 5'-CCAATCA TAAAATGGATCATTTTC-3' (reverse) (Eurogentec, Angers, France). This was followed by a PCR step using Pfu DNA polymerase (Promega, Charbonnières-les-Bains, France) with the same primer pair. The amplified gene products were subjected to agarose gel electrophoresis and purified using the High Pure PCR Product Purification kit (Roche, Meylan, France).

PfPKAc cloning

Gateway Cloning Technology (Invitrogen) was used to construct the plasmid for protein expression as described by the manufacturer. Briefly, the purified cDNA fragment was inserted into the entry vector pENTR™/SD-TOPO® and transferred into the destination vector pET-DEST42 to create the expression clone. This vector is designed to add a C-terminal V5 epitope and hexahistidine tag. All constructs were verified by automated sequencing (3100 Genetic Analyser, Applied Biosystem, Courtaboeuf, France).

Fig. 1 Alignment of the amino acid sequence and structural modeling of the PfPKAc. **a** Comparison of PfPKAc amino acid sequences with other protein kinase catalytic subunit. *P. falciparum* (Pf), *Homo sapiens* (Hs), *Mus musculus* (Mm), and *Plasmodium yoelii* (Py) were aligned using CLUSTAL W (2.0.10). The 11 subdomains of protein kinases are indicated with *boxed regions* and with *roman numerals*. Identical residues are indicated with *asterisks* and conservative changes with *colons*. Residues highlighted in *purple* and those in *turquoise blue* are predicted to form β-sheets and α-helixes, respectively, as visualized in (**b**). Amino acids colored *red* belong to secondary structure elements that differ between human PKAc and PfPKAc. **b** Structural model of PfPKAc. The PfPKAc structure was modeled on the resolved structure of human PKAc alpha. β-sheets are colored *purple* and the α-helix are *turquoise blue-colored*. Secondary structure differences are shown in *red*

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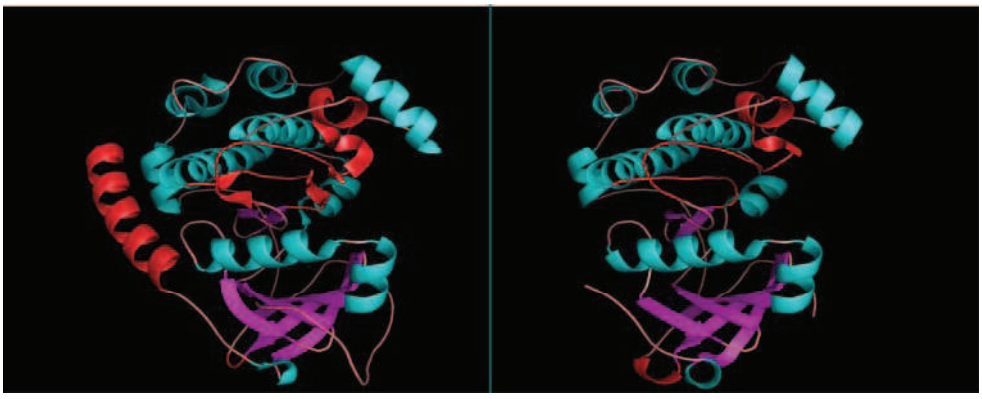
a

```
                                                                                I
Mm  MGNAAAAKKGSEQESVKEFLAKAKEDFLKKWETPSQNTAQL DQFDRIKTLGTGSFGRVML  60
Hs  MGNAAAAKKGSEQESVKEFLAKAKEDFLKKWESPAQNTAHL DQFERIKTLGTGSFGRVML  60
Pf  ---MQFIKN-------LQLNKKKDSDSSEQVLTNKKNKMKY EDFNFIRTLGTGSFGRVIL  50
Py  --MIQFLKN-------LQLYKKR--ETSDIKPNTKKSKMKY EDFNFIRTLGTGSFGRVIL  49
          *:         ::  *   :         :   :  ::*: *:**********:*
                    II              III              IV
Mm  VKHKE SGN-HYAMKILDKQKVVKLK QIEHTLNEKRILQAV NFPFLVKLEFSFKDN SNLYM 119
Hs  VKHKE TGN-HYAMKILDKQKVVKLK QIEHTLNEKRILQAV NFPFLVKLEFSFKDN SNLYM 119
Pf  ATYKN GNYPPVAIKRFEKCKIIRQK QVDHVFSERKILNYI NHPFCVNLHGSFKDD SYLYL 110
Py  ATYKN EDLPPVAIKRFEKSKIIKQK QVDHVFSERKILNYI NHPFCVKLYGSFKDE SYLYL 109
      :*:       *:* ::* *::: * *::* : *::**: : * ** *:*  ****: * **:
           V                                VI
Mm  VMEYVAGGEMFSHLRRIGR FSEPHARFYAAQIVLTFEYLHSLDLIYRDLKPENLLIDQQG 179
Hs  VMEYVPGGEMFSHLRRIGR FSEPHARFYAAQIVLTFEYLHSLDLIYRDLKPENLLIDQQG 179
Pf  VLEFVIGGEFFTFLRRNKR FPNDVGCFYAAQIVLIFEYLQSLNIVYRDLKPENLLLDKDG 170
Py  VLEFVIGGEFFTFLRRNKR FPNDVGCFYAAQIVLIFEYLQSLNIVYRDLKPENLLLDKDG 169
    *:*:* ***:*: ***  * *  :    ******** ****:**:::**********:*::*
          VII              VIII                IX
Mm  YIQVTDFGFAKRVKGRT WTLCGTPEYLAPEII LSKGYNKAVDWWALGVLIYEMAAGYPPF 239
Hs  YIQVTDFGFAKRVKGRT WTLCGTPEYLAPEII LSKGYNKAVDWWALGVLIYEMAAGYPPF 239
Pf  FIKMTDFGFAKIVETRT YTLCGTPEYIAPEIL LNVGHGKAADWWTLGIFIYEILVGCPPF 230
Py  FIKMTDFGFAKVVNTRT YTLCGTPEYIAPEIL LNAGHGKAVDWWTLGIFIYEILVGYPPF 229
    :*:*:******* *: ** :*********:****: *  *: ** ***:**:***:  * ***
               X                  XI
Mm  FA DQPIQIYEKIVSGKVRFPSH FSSDLKDLLRNLLQVDLTKRFGNLKNGVNDIKNHKWFA 299
Hs  FA DQPIQIYEKIVSGKVRFPSH FSSDLKDLLRNLLQVDLTKRFGNLKNGVNDIKNHKWFA 299
Pf  YA NEPLLIYQKILEGIIYFPKF LDNNCKHLMKKLLSHDLTKRYGNLKKGAQNVKEHPWFS 290
Py  YA NEPLLIYQKILEGIIYFPKF LDNNCKHLMKKLLSHDLTKRYGNLKKGAQSVKEHPWFA 289
    :* ::*: **:**: * : **  :  : * *:::**  *****:****:* : :*:* **:

Mm  TT DWIAIYQRKVEAPFIPKFKGPGDTSNFDDYEEEEIRVSINEKCGKEFTEF---  351
Hs  TT DWIAIYQRKVEAPFIPKFKGPGDTSNFDDYEEEEIRVSINEKCGKEFSEF---  351
Pf  NI DWVNLLNKNVEVPYKPKYKNIFDSSNFERVQED---LTIADKITNENDPFYDW  342
Py  NI EWNNLLNKKVDVPYKPKYKNIFDASNFEKVQED---LSIADKVINENDPFFDW  341
    :*  : :::*: *: **:*   *:***:  :*:   ::* :*  :*   *
```

b



human PKAc alpha PfPKAc

Expression and purification of the recombinant PfPKAc

Competent *E. coli* Rosetta (DE3) pLysS Strain (Novagen, Nottingham, UK) transformed with the expression plasmid was grown in TB medium containing 34 μg mL^{-1} chloramphenicol and 100 μg mL^{-1} ampicillin at 37°C until the OD_{600} reached 0.6–0.8 (Sigma Aldrich, Lyon, France). Recombinant protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (Sigma Aldrich) for 6 h at 22°C (Pastorino et al. 2008). Cells were harvested by centrifugation at 4,000×*g* for 30 min and stored at −80°C until use. For protein purification, cell pellets were thawed and resuspended in 1 mL of lysis buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.0) per 30 mL of original culture. Cells were disrupted by sonication (36×5 s pulses at 50 W) on ice and then centrifuged for 30 min at 27,000×*g*. The supernatant corresponding to the soluble fraction was mixed with Talon® superflow resin (Clontech, St-Germain-en-Laye, France), previously equilibrated with lysis buffer, on a rotary shaker for 30 min at room temperature. The lysate–cobalt was loaded onto a XK16/20 column (Amersham Biosciences, Velizy, France) connected to an $ÄKTA_{FPLC}$ system (Amersham Biosciences). An imidazole gradient was made by mixing two separate buffers: 50 mM sodium phosphate at pH 7.0 and 300 mM NaCl (buffer A) and 50 mM sodium phosphate pH 7.0, 300 mM NaCl and 150 mM imidazole (buffer B). The column was washed at a flow rate of 2 mL min^{-1} with buffer containing 20 mM imidazole (87% buffer A, 13% buffer B) until the OD_{280} was stable. The protein was eluted with buffer containing 150 mM imidazole (100 % buffer B). Purified protein samples were then concentrated up to 3 mL using vivaspin concentrators (Sartorius, Aubagne, France) with a 10 kDa pore-size membrane and stored at −80°C in 5% (*v*/*v*) glycerol for further purification. Next, size-exclusion chromatography (SEC) was carried out on a Superdex-75 prep grade column (XK 26/60, Amersham Biosciences) as previously described (Pastorino et al. 2008).

SDS-PAGE, western blot analysis, and protein quantification

Collected fractions were analyzed by Coomassie blue staining after SDS-PAGE. For western blot analysis, gels were transferred to a nitrocellulose membrane (Amersham Biosciences) by semi-dry blotting. Anti-His (C-term)-HRP monoclonal antibodies (Invitrogen) diluted to 1/5,000 were used to detect the recombinant PfPKAc protein after adding a 3,3',5,5'-tetramethylbenzidine substrate (Promega). Crude protein concentrations were measured using a BCA protein assay kit (Perbio, Brebières, France). The protein pattern was then analyzed using ImageQuant TL software (GE Healthcare, Velizy, France). The relative abundances of each protein were estimated by the area under the curve of a band divided by the summed areas under the curves over all bands.

Mass spectrometry analysis, database search, and analysis of tandem mass spectra

For identification, proteins were spotted, excised from the gel and digested overnight at 37°C with sequencing-grade trypsin (12.5 μg mL^{-1}; Promega) in 50 mM NH_4HCO_3. The resulting extracted peptides were then stored at −20°C until analysis. Mass spectrometry (MS) measurements were done as previously described (Orlandi-Pradines et al. 2007). MS/MS spectra were searched against the non-redundant *P. falciparum* and *E. coli* databases.

In vitro kinase assay

Enzymatic assays were performed using an Omnia® Ser/Thr recombinant kit (Invitrogen). For K_m and k_{cat} determination, assays were performed using various concentrations of substrate (S/T peptide 5), ranging from 3 to 50 μM (3, 6, 12, 25, and 50 μM), and 2.5 μM of the recombinant PfPKAc protein. Assays were carried out in a final volume of 50 μL in a 96-well plate at 30°C, with fluorescence measurements ($\lambda_{excitation}$=360 nm, $\lambda_{emission}$=485 nm) taken every 30 s for 2 h in triplicate using a SpectraMax Gemini EM fluorometer (Molecular Devices, St. Grégoire, France). Experimental fluorescence variations, expressed in relative fluorescence units, were determined using SoftMax Pro software (Molecular Devices) and converted into initial velocities (Vi) using standard curves obtained by fluorescence measurements of a Sox-modified phosphopeptide control. K_m and V_{max} evaluations were performed by fitting the data to the double reciprocal Lineweaver–Burk equation $\left(\frac{1}{Vi} = \frac{K_m}{S_0 \times V_{max}} + \frac{1}{V_{max}}\right)$ using a least-squares regression analysis. The turnover number (k_{cat}) was calculated on the assumption that 100% of the kinase was enzymatically active $\left(k_{cat} = \frac{V_{max}}{[E]}\right)$.

Inhibition assays

H89 (Calbiochem, San Diego, USA), KT5720 (Calbiochem), PKI (Sigma Aldrich), Ro-31 (Calbiochem) and MEKI (Promega) inhibitors were tested at 1 μM for their activity against the recombinant PfPKAc. Compounds were preincubated with 2.5 μM of the recombinant PfPKAc protein, after which time, the kinase activity was assayed as previously described using 50 μM of the S/T peptide 5 substrate. Triplicate measurements were performed for each data point.

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Table 1 Identification of proteins by mass spectrometry analysis after purification (lane 2–3, Fig. 2a)

No.	Name	Gi number	MW (kDa)	Score	Coverage (%)	Peptide matched
a	Chaperonin GroEL (*E. coli*)	2624772	57.3	1,681	80	28
b	UDP-*N*-acetylmuramate (*E. coli*)	16132055	50.3	221	23	6
c	PKAc (*P. falciparum* 3D7)	124507233	40.5	565	43	14
d	50S ribosomal protein L2 (*E. coli*)	15803844	29.9	510	49	13
e	FKBP-type isomerase (*E. coli*)	15803862	21.2	212	18	4

Results and discussion

Sequence analysis and PfPKAc structural model

The PfPKAc protein contains 342 amino acids with a predicted molecular mass of approximately 40.197 kDa and a calculated isoelectric point of 9.11. Database searches revealed that the amino acid sequence of PfPKAc shares 49–92% identity with PKA kinase families and high homology with *Plasmodium yoelii* catalytic subunit. A multiple alignment of the amino acid sequence of PfPKAc (Fig. 1a) showed strong conservation of the 11 specific kinase subdomains (I-XI) (Hanks et al. 1988) as well as several features that are critical for kinase function: ATP binding, catalysis, and stabilization of protein kinases (Hanks et al. 1988; Knighton et al. 1991). Some amino acid residues required for recognition of the PKA regulatory subunit (PKAr) and/or the heat-stable inhibitor protein (PKI) have been previously identified in mammalian PKAc (Bossemeyer et al. 1993; Narayana et al. 1997). Notably, most of these residues are conserved in PfPKAc (Fig. 1a), suggesting a probable PKI inhibition, as has been previously reported (Beraldo et al. 2005; Syin et al. 2001; Merckx et al. 2008b). The three-dimensional model of PfPKAc is compared to the *Homo sapiens* PKAc alpha subunit crystal structure (PDB accession number 2GU8). As expected, and due to the high degree of primary sequence similarity (50% identity), the PfPKAc model showed strong structural homology with human PKAc. However, slight differences between the two structures are observed in some secondary though important elements (Fig. 1a, b). The first divergence is located in subdomain VI, where a β-strand and an α-helix are missing from the PfPKAc model. This domain contains the putative catalytic loop RDLKPEN (residues 166 to 171), with D_{166} identified as the candidate for the catalytic base (Hanks and Hunter 1995). The second notable divergence concerns subdomain VIII, where the α-helix in human PKAc is divided in PfPKAc into two α-helixes. In the same subdomain, a β-strand was also absent in the PfPKAc model as compared to the human PKAc structure. Moreover, a residue (W_{196}) previously identified as essential for mammalian PKAr binding was not conserved in the PfPKAc subdomain VIII sequence (Y_{196}) (Kim et al. 2005; Taylor et al. 2008). Other structural variations were observed in the PfPKAc model, but were not localized in known functional domains.

Despite high sequence identity and strong structural homology with human PKAc, the PfPKAc enzyme presents some interesting differences in essential catalytic domains. The development of new antimalarial compounds targeting either the catalytic site or the PKAr interaction domain would allow specific inhibition of the parasite enzyme with low cytotoxicity.

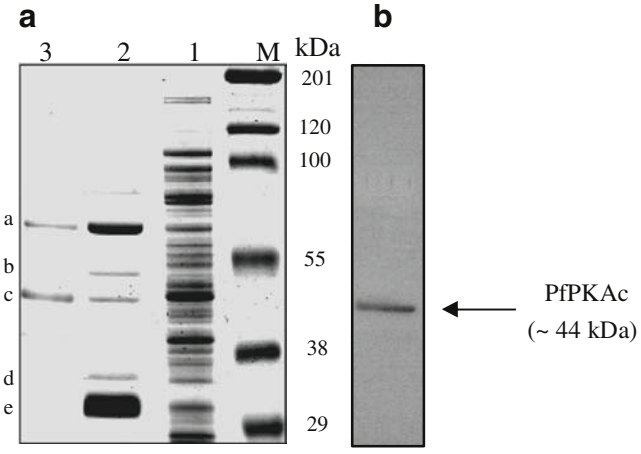

Fig. 2 Purification steps analysis of PfPKAc expressed in *E. coli* by SDS-PAGE and western blot. **a** SDS-PAGE analysis after Coomassie blue staining—lane *M* molecular weight markers, lane *1* crude lysate of *E. coli* after induction with IPTG, lane *2* eluted fraction from cobalt column, lane *3* eluted fraction after cobalt column and size exclusion chromatography. *Lower-case roman letters* on the left represent proteins analyzed by mass spectrometry (see Table 1). **b** Western blot analysis of the eluted fraction after SEC using anti-His (C-term)-HRP monoclonal antibodies for the detection of PfPKAc

Table 2 Kinetic parameters of PfPKAc

Substrate	K_m (μM)	V_{max} (μM s^{-1})	K_{cat} (s^{-1})	K_{cat} / K_m (s^{-1} M^{-1})
Ser/Thr peptide 5	240	0.024	0.0092	38.2

Kinase activity was assayed using Ser/Thr peptide 5 ranging from 3 to 50 μM (Omnia® Ser/Thr recombinant kit)

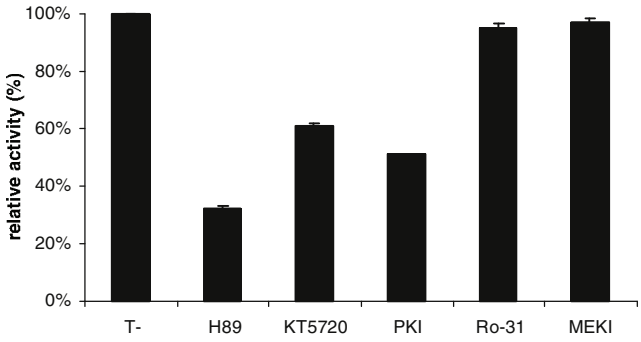

Fig. 3 In vitro inhibition assays of PfPKAc. A range of inhibitors (1 μM) were tested for their activity against PfPKAc. Kinase assays were performed using the Omnia® Ser/Thr recombinant kit. H89, KT5720, PKI are specific inhibitors of eukaryotic PKAc. Ro-31 and MEKI are inhibitors of PKC and MEK, respectively. *T-* no inhibitor

Expression and purification of PfPKAc

In our optimized expression conditions, particularly at low temperature, PfPKAc was produced as a soluble hexahistidine recombinant protein (~44 kDa) which represents about 50% of the entire soluble bacterial fraction. PfPKAc purification was first performed using Ni^{2+} affinity chromatography (Qiagen, Courtaboeuf, France), but this method proved to be inefficient due to a low purity rate and substantial loss of the targeted recombinant kinase (data not shown). The use of a Co^{2+} matrix and an increase in the stringency of the wash steps improved the PfPKAc purification. After cobalt affinity chromatography, the Coomassie-stained SDS-PAGE revealed additional bands accompanying the recombinant protein (Fig. 2a, lane 2). The main contaminating proteins were identified by mass spectrometry as members of the chaperonin complex and/or protein biosynthesis pathway (Table 1). To improve the purification, the use of SEC with addition of arginine in the mobile phase was investigated (Pastorino et al. 2008) (Fig. 2a, lane 3). Only a ~60 kDa protein was still present in purified PfPKAc fractions after this two-step purification method. This protein was identified by mass spectrometry analysis as a bacterial molecular chaperonin GroEL with no kinase activity (Table 1). The identity of the purified recombinant PfPKAc ~44 kDa was verified by immunoblotting using Anti-His (C-term)-HRP monoclonal antibodies (Fig. 2b) and mass spectrometry analysis (Table 1).

Kinetic characteristics of overexpressed PfPKAc and inhibitor studies

Various in vitro methods have been designed to measure kinase activity and to develop drug-screening assays (Li et al. 2008). The most common assay is based on the radiolabeling of target peptides or proteins using γ-^{32}P-ATP (Roskoski 1983). This method is sensitive, but radioisotopes present a potential risk for both human health and environment and are not suitable for high-throughput screening of chemical libraries. To overcome these drawbacks, a non-radioactive technique was assayed in this work (Omnia® Ser/Thr recombinant kit, Invitrogen). This method utilizes a fluorescent peptide substrate-based technology (Ser/Thr peptide 5) allowing kinase enzymatic characterization and inhibitor screening (Shults et al. 2006). In our in vitro kinase assay, PfPKAc activity was detected and characterized using the Ser/Thr peptide 5 (Table 2) and the effect of different kinase inhibitors on the PfPKAc activity was investigated. As shown in Fig. 3, the recombinant kinase was strongly inhibited in the presence of 1 μM of eukaryotic PKAc inhibitors such as H-89, KT5720, or PKI. This response might be specific since Ro-31 and MEKI, which are inhibitors of PKC and MEK respectively, do not inhibit PfPKAc. These results are consistent with the classification of PfPKAc as a Ser/Thr kinase and with previously described data (Beraldo et al. 2005; Syin et al 2001). However, our results differed from the in vitro PfPKAc inhibition profile reported by Sudo et al. (Sudo et al. 2008) where the parasite enzyme was weakly inhibited by H89 and PKI when compared to the mammalian kinase. In this previous study, kinase assays were carried out using a recombinant purified GST-PfPKAc without removal of the GST tag, that can affect enzyme conformation and accessibility (Arnau et al. 2006). Our recombinant PfPKAc was fused to a hexahistine tag commonly used for recombinant protein purification and detection. This small tag rarely interfered with recombinant enzymatic activity and there is no need to remove it for various applications like antibody production and crystallization experiments.

In conclusion, this functional in vitro assay will allow high-throughput PfPKAc inhibitor screening, interaction study with the PfPKAr (Merckx et al. 2008b) as well as better understanding of the cAMP pathway in *P. falciparum*.

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TRAVAUX ORIGINAUX

2. APPROCHE CELLULAIRE DANS L'ETUDE DE LA PROTEINE KINASE AMP CYCLIQUE DE *PLASMODIUM FALCIPARUM*

Article n°2 :

Phenotypic and transcriptomic analyses of *Plasmodium falciparum* protein kinase A catalytic subunit inhibition

Wurtz N, Desplans J, Parzy D

« Parasitology Research » 2009 ; 105(6) :1691-1699.

Parallèlement à la caractérisation biochimique de la PfPKAc, nous nous sommes également intéressés au rôle métabolique méconnu de cette kinase chez *P. falciparum*. Quelques études ont montré que la PfPKAc pourrait intervenir dans le développement des formes asexuées, dans l'invasion des érythrocytes par les mérozoïtes mais également dans l'induction de la gamétocytogénèse [86-90]. Plus récemment, d'autres travaux ont impliqué la PfPKAc dans l'activation des influx calciques, dans la régulation du transport anionique à travers la membrane du globule rouge et dans la régulation de la migration et de l'invasion des sporozoïtes lors de l'infection hépatique [91-94]. Quoiqu'il en soit, l'ensemble de ces résultats renforce l'idée que la PfPKAc joue un rôle primordial pour le développement du parasite, et par la même représente une cible attractive pour le développement de nouvelles molécules antipaludiques. La seconde grande partie de notre projet de recherche a donc été de préciser la fonction biologique de cette kinase plasmodiale au cours du cycle érythrocytaire de *P. falciparum*. Pour cela, nous avons choisi d'utiliser la technique d'ARN interférence (ARNi) [95], technique qui avait déjà donné des résultats intéressants dans des travaux antérieurs [96-99]. Dans notre étude, les effets de l'inhibition de l'expression du gène codant pour la PfPKAc ont été interprétés après observation microscopique des modifications phénotypiques induites et par une analyse comparative du transcriptome plasmodial.

Afin de déterminer la fenêtre d'introduction des ARN interférents (dsRNA), il a été nécessaire dans un premier temps, d'analyser la cinétique d'expression des transcrits de la PfPKAc et PfPKAr au cours du cycle érythrocytaire du parasite. Les mesures effectuées en RT-PCR quantitative ont montré que l'expression des transcrits augmente à partir du stade trophozoïte pour atteindre un maximum au stade schizonte, résultat en corrélation avec des études précédentes [46,90]. Ces données suggèrent que l'activité de la PfPKA pourrait être nécessaire pour l'achèvement du cycle asexué ou dans les mécanismes de ré-invasion des érythrocytes. Dans la suite de notre étude, nous avons donc récupéré et analysé les parasites traités au maximum d'expression de la PfPKA situé au stade schizonte [100,101].

Parallèlement, différentes stratégies de transfection de *P. falciparum* ont été évaluées : *i)* incubation directe de dsRNA dans le milieu de culture [97], *ii)* électroporation de globules rouges sains avec des dsRNA et invasion de ceux-ci par des parasites [102], *iii)* électroporation de schizontes âgées [103] et *iv)* électroporation directe de globules rouges parasités par des formes anneaux [104]. Dans nos conditions expérimentales, cette dernière technique s'est avérée être la plus efficace et reproductible pour la transfection de *P. falciparum* avec des dsRNA.

Après avoir optimisé les conditions expérimentales, les effets de l'ARN interférence sur le phénotype et le transcriptome parasitaire ont été étudiés 24h après électroporation de cultures synchronisées au stade anneau en présence soit de dsRNA dirigés contre la PfPKAc, soit de dsRNA témoin (séquence exogène à *P. falciparum*), soit de milieu de culture (culture témoin non traitée). L'analyse des résultats obtenus démontre que : 1) Les parasites témoins électroporés ainsi que les parasites traités avec les dsRNA témoins atteignent le stade schizonte sans apparition de formes dégénérées ou de gamétocytes indiquant un développement classique ; 2) Les cultures traitées avec les dsRNA dirigés contre la PfPKAc présentent des modifications morphologiques dans environ 40% des parasites avec un développement anormal des formes schizontes et l'apparition de noyaux pycnotiques. Durant le second cycle parasitaire (environ 15h après ré-invasion), les parasites des cultures témoins et ceux traités avec les dsRNA témoins se développent de façon normale et ont atteint en majorité le stade anneau. Cependant, 35% des parasites traités avec les dsRNA dirigés contre le PfPKAc sont toujours au stade schizonte indiquant un arrêt du cycle parasitaire au stade schizonte. L'ensemble de ces données confirme le rôle important de la PfPKAc dans la croissance et la survie du parasite, plus particulièrement dans les phases tardives de la schizogonie érythrocytaire [90,91,93].

En complément des analyses phénotypiques, les transcrits de la PfPKAc et PfPKAr ont été quantifiés dans chacune des cultures par RT-PCR quantitative. Les mesures effectuées montrent que chez les parasites témoins et traités avec les dsRNA témoins, les transcrits de la PfPKAc et PfPKAr ne varient pas. Cependant, une inhibition des transcrits de la PfPKAc de l'ordre de 70% est observée dans les cultures traitées avec les dsRNA dirigés contre la PfPKAc.

De façon inattendue, un profil d'inhibition similaire est obtenu pour les transcrits de la PfPKAr, suggérant l'existence d'un mécanisme de co-régulation entre les deux sous-unités de la PfPKA [93,105].

Dans la dernière étape de ce travail, nous avons étudié l'effet des différents traitements sur le transcriptome parasitaire en utilisant des puces à ADN pangénomique dédiées à *P. falciparum*. Pour les parasite traités avec les dsRNA témoins, aucune variation significative de l'expression des gènes n'a été constatée par rapport aux cultures témoins. Par contre, l'expression de 329 gènes est modulée après traitement des parasites par les dsRNA dirigés contre la PfPKAc. Parmi ces gènes, 57% n'ont pas eu de fonctions assignées tandis que 43% ont pu être classés dans différentes voies métaboliques (Malaria Parasite Metabolic Pathways, Ginsburg, Hagai, **http://sites.huji.ac.il/malaria/**). L'analyse de la liste des gènes a permis d'identifier au moins trois groupes importants de gènes dont l'expression pourrait être régulée directement ou indirectement par la PfPKAc :

- des gènes codant pour des protéines de liaison aux réticulocytes (Rh) et ayant un rôle important dans les mécanismes de ré-invasion des érythrocytes par les mérozoites [106] sont sous-exprimés, laissant supposer que la PfPKAc pourrait avoir un rôle dans la ré-invasion. Très récemment, il a été montré que la PfPKAc phosphoryle une protéine des micronèmes AMA-1 sur la sérine 610 [87]. La mutation de ce résidu empêche la phosphorylation par PfPKAc et entrave considérablement l'invasion, confirmant ainsi l'hypothèse que PfPKAc est impliquée dans les mécanismes de ré-invasion.
- des gènes codant pour des protéines et kinases de la voie calcium/calmoduline sont sous-exprimés. Ces données sont en corrélation avec une publication antérieure qui a révélé l'existence chez *P. falciparum* d'un lien très étroit entre la voie de l'AMP cyclique / PfPKAc et la voie calcium/calmoduline [91].
- des gènes codant pour des protéines possédant un signal d'adressage à la mitochondrie sont sur-exprimés, suggérant l'implication de la PfPKAc dans la régulation mitochondriale, comme préalablement décrit chez les mammifères [107].

Nos résultats sont présentés dans l'article suivant :

Article n°2 : Wurtz N, Desplans J, Parzy D. Phenotypic and transcriptomic analyses of *Plasmodium falciparum* protein kinase A catalytic subunit inhibition. Parasitology Research 2009 ;105(6): 1691-1699.

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ORIGINAL PAPER

Phenotypic and transcriptomic analyses of *Plasmodium falciparum* protein kinase A catalytic subunit inhibition

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Abstract The emergence and dissemination of drug-resistant malaria parasites represent one of the most important problems in malaria case management. *Plasmodium falciparum* is the causative agent of the most lethal form of human malaria. The molecular mechanisms that control the life cycle of the malaria parasite are still poorly understood. The published genome sequence (*P. falciparum* strain 3D7) reveals that several homologs of eukaryotic signaling proteins, such as protein kinases and phosphatases, are conserved in *P. falciparum*. Proteins kinases are now widely recognized as valuable drug targets in protozoan parasites. In this study, gene silencing with double-stranded RNA (dsRNA) and microarray techniques were used to study the biological function of the cAMP-dependent protein kinase catalytic subunit (PfPKAc) in the parasite erythrocytic life cycle. Treatment of parasites with PfPKAc dsRNA resulted in a marked reduction of endogenous PfPKAc mRNA associated with a compensatory decrease of PfPKAr mRNA followed by morphological changes in schizont stages and cell cycle arrest. The global effects of gene silencing were also investigated using a *P. falciparum* pan-genomic microarray. Transcriptomic analysis showed that the expression of 329 genes was altered in response to down-regulation of PfPKAc mRNA particularly genes in specific metabolic pathways linked with merozoite invasion processes, the calcium/calmodulin signaling, and kinases network and mitochondrial functions.

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Introduction

Malaria is one of the most important infectious diseases affecting humans particularly in developing countries. *Plasmodium falciparum* (*P. falciparum*), an apicomplexan protozoan parasite, is the causative agent of the most lethal form of human malaria. The lack of a proven vaccine and the widespread resistance of *P. falciparum* to most available antimalarial compounds have created an urgent need to identify new targets and/or chemotherapeutics (Renslo and McKerrow 2006).

The *P. falciparum* life cycle consists of sexual and asexual stages that imply a high degree of adaptation and strict control of the cellular machinery. These processes probably involve various interactions between parasite and host-signaling molecules and may represent critical points for therapeutic intervention. Many eukaryotic intracellular functions employ modulation of protein phosphorylation by protein kinases and phosphatases as a form of regulation (Manning et al. 2002). A published *P. falciparum* genome sequence and previous studies reveal that several homologs of eukaryotic signaling proteins are conserved in *P. falciparum* (Gardner et al. 2002; Merckx et al. 2008a; McRobert et al. 2008; Wurtz et al. 2009). Among these mediators, there is evidence that protein kinases are essential for parasite life cycle control and that inhibition of kinases can have antimalarial effects (Dluzewski and Garcia 2006). Moreover, these enzymes are widely recognized as valuable drug targets for the treatment of several diseases (Johnson 2007). Analyses of the *P. falciparum*

kinome have revealed that 99 genes encode proteins containing kinase domains with functions, mechanisms of regulation, and cellular targets that are largely unknown (Gardner et al. 2002; Anamika and Krupa 2005).

Cyclic AMP-dependent protein kinase (PKA) is implicated in numerous processes in mammalian cells, and the regulatory mechanisms of the cAMP pathway are characterized (Taylor et al. 2008). PKA is a tetrameric holoenzyme composed of two regulatory subunits (PKAr) that bind and inhibit two catalytic subunits (PKAc). The binding of two cAMP molecules to PKAr alters its affinity for PKAc resulting in the release of active PKAc, which can phosphorylate many substrates. The functional specificity of PKA is largely dependent on targeting the catalytic subunit to the proximity of specific substrate at precise locations in the cells. This is accomplished by A-kinase anchoring-protein (AKAP) family, which are bound to subcellular structures and recruit PKA through interaction with the regulatory subunit (Barradeau et al. 2002).

In the *P. falciparum* genome, gene coding for PKAc (PfPKAc) and PKAr (PfPKAr) are present in a single copy. Nucleotide cyclases and phosphodiesterases have also been identified in the parasite genome, and so far, only one AKAP was annotated in the *Plasmodium yoelii* genome (Baker 2004; Yuasa et al. 2005). The role of *P. falciparum* PKA (PfPKA) is still unclear, and until now, only indirect data were available to evaluate the molecular functions of PfPKA. Previous reports have shown roles for PfPKA in *P. falciparum* asexual stage development, erythrocyte invasion, and the induction of gametocytogenesis (McColm et al. 1980; Brockelman 1982; Rangachari et al. 1986; Syin et al. 2001). More recently, several studies have implicated PfPKA in the activation of a Ca^{2+} influx pathway, the regulation of anion transport through the erythrocyte membrane, and the regulation of apical organelle exocytosis in *P. falciparum* sporozoites (Beraldo et al. 2005; Merckx et al. 2008b; Ono et al. 2008). In fact, PfPKA seems to be a key regulator of *P. falciparum* development and consequently, represents an attractive target for the development of antimalarial drugs.

In a previous work, we focused on the enzymatic characterization of a recombinant PfPKAc, which was expressed as a soluble His-Tag protein in *Escherichia coli* and then purified via two-step chromatography (Wurtz et al. 2009). To complete the picture of the PfPKAc, we investigated in this study its biological role in the *P. falciparum* life cycle using mRNA silencing (PfPKAc dsRNA). We show that downregulation of PfPKAc mRNA inhibits parasite cell development, confirming the key role of this enzyme in the intraerythrocytic stages and leads to a subsequent decrease of PfPKAr mRNA. Moreover, we used microarray technology to evaluate the transcriptional effects of silencing PfPKAc mRNA. The analysis identified 329 genes whose expression is altered in response to the downregulation of PfPKAc mRNA including many genes involved in specific metabolic pathways in relation with merozoites invasion, calcium/calmodulin, and kinases network and mitochondrial functions.

Materials and methods

Parasite culture and synchronization

P. falciparum strain 3D7 was obtained from the Malaria Research and Reference Reagent Resource Center (MR4, Manassas, United States) and grown in vitro according to the method of Trager and Jensen (Trager and Jensen 1976). Parasite cultures were synchronized in two steps: (1) late trophozoite and the schizont stages were purified using a Variomacs system (Miltenyi Biotec, Bergisch Gladbach, Germany) followed by incubation with fresh O^+ erythrocytes (Ribaut et al. 2008); (2) at the ring stage, cultures were treated with 5% D-sorbitol for 15 min at 37°C to obtain tightly synchronized parasites in the early phase (0–5 h after parasite invasion of the erythrocyte) (Lambros and Vanderberg 1979). Morphology and parasitemia were estimated by microscopic observation (at ×100) of thin blood smears stained with RAL®555 (REACTIFS RAL, Martillac, France).

Analysis of endogenous PfPKA transcripts

Synchronized cultures were harvested at 15–20 h, 25–30 h, and 35–40 h postsynchronization corresponding to the three main stages of the intraerythrocytic life cycle (ring, trophozoite, and schizont stages). Two independent experiments were performed in triplicate for each time point. Total RNA was extracted with TRIZOL™ reagent following the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA) and treated with Dnase (DNAfree™, Ambion, Foster City, CA, USA). Samples were then quantified with a NanoDrop ND-1000 (Labtech, Palaiseau, France) followed by quality assessment with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. One microgram of total RNA was reverse-transcribed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Carlsbad, CA, USA). Transcript quantification was performed using a 7900HT Fast Real-Time PCR system (Applied Biosystems). Amplification reactions were done in a final volume of 25 μL containing 1X Power SYBR® Green master mix, 900 nM of each primers, and 5 μL of the 1/10 diluted cDNA. The primers used for PfPKA amplification were (Eurogentec, Seraing, Belgium): forward-5'-AGA AAT TTT ATT GAA CGT CGG ACA T-3' and

reverse-5'-AGG GTT CAT TCG CAT AAA AAG G–3' for PfPKAc and forward-5'-GGA GAA TTA GCT CTT CTC AAA AAT AAA CC-3' and reverse-5'-GTC CTA ATA ATC TTT TGA AAC TTT TTC TAT CTA AAT A-3'. The primers used for the amplification of the endogenous control 18S rRNA were: forward-5'-GCT GAC TAC GTC CCT GCC C–3' and reverse-5'-ACA ATT CAT CAT ATC TTT CAA TCG GTA-3' (Blair et al. 2002). The reaction mixtures were prepared at 4°C in a 96-well optical reaction plate covered with optical adhesive covers (Applied Biosystems). The thermal cycling conditions were 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was assayed in triplicate and analyzed with SDS 2.2.1 software (Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method of relative quantification was used to estimate relative expression of PfPKAc and PfPKAr mRNA (targets; Livak and Schmittgen 2001) where $\Delta\Delta Ct=(Ct_{target}-Ct_{18SrRNA})_{Time\ x}-(Ct_{target}-Ct_{18SrRNA})_{calibrator}$. Time *x* represents one of the three time points of the parasite life cycle. The ring stage was chosen as the calibration sample. All data are expressed as mean ±SD.

dsRNA transfection

The following 21-mer RNA molecules, corresponding to a fragment of PfPKAc (PfPKAc dsRNA), were designed and synthesized according to the manufacturer's protocol (Eurogentec): sense-5'-AGC CGC UGA UUG GUG GAC U99-3' and antisense-5'-AGU CCA CCA AUC AGC GGC U99-3'. The following negative control, corresponding to a 21-mer RNA molecule different than *P. falciparum* RNA (unrelated dsRNA), was also synthesized: sense-5'-AUU UCG GAG UGG CAA CCG G99-3' and antisense-5'-CCG GUU GCC ACU CCG AAA U99-3'. Synchronized parasites (10% parasitemia) were transfected by electroporation at the late ring stage (approximately 15 h postinvasion) as previously described (Wu et al. 1995; Kumar et al. 2002). In brief, 40 μg of dsRNA (PfPKAc or unrelated dsRNA) was added to the infected red blood cells in 800 μl of incomplete cytomix (120 mM KCl, 0.15 mM CaCl, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4, 10 mM KH_2PO_4, 25 mM Hepes, pH 7.6). Cells were then transferred to a 4-mm-gap cuvette (Eurogentec) and electroporated with a Gene Pulser II system (Bio-Rad, Hercules, CA, USA) with the charging voltage set to 2.5 kV, the resistance set at 200 Ω, and the capacitance set to 25 μF. Time constants were 0.7–0.9 ms. Control cultures were also assayed as described above and electroporated using an equivalent volume of dsRNA buffer added to the medium. Electroporated samples were immediately mixed with 15 mL of culture medium placed into 75 cm^2 culture flasks and incubated at 37°C.

Cultures were harvested 24 h after treatment at the schizont stage and washed three times with PBS, and total RNA extraction was performed for real-time polymerase chain reaction (PCR) and microarray experiments. For each sample, smears were also made to examine the morphology and parasitemia of the treated parasite. In parallel, a small sample of parasites was subcultured into fresh medium and erythrocytes, and smears were made 48 h posttreatment in the following cycle. Experiments were repeated two times in quadruplicate.

PfPKAc and PfPKAr RNA transcript analysis after dsRNA treatment

Real-time PCR was carried out to determine the levels of endogenous PfPKAc and PfPKAr transcripts after PfPKAc dsRNA treatment. RNA extraction, reverse transcription, and real-time PCR were performed as previously described. For each sample and each gene, measurements were performed in triplicate. The $2^{-\Delta\Delta Ct}$ method was used to normalize the detected fluorescent signal to an endogenous reference gene (18S rRNA) and to compare target signals from different samples with control cultures as calibrator ($\Delta\Delta Ct=(Ct_{target}-Ct_{18SrRNA})_{dsRNA\ treated}-(Ct_{target}-Ct_{18SrRNA})_{control\ cultures}$).

Microarray manufacturing, hybridization, and data analysis

A custom microarray containing all of the *P. falciparum* 3D7 strain coding sequences (CDS) was designed and manufactured using the SurePrint Inkjet technology® (Agilent Technologies). In brief, the microarray was composed of 10,128 60-mer oligonucleotides representing 5,364 CDS located on the chromosomes, the apicoplastic, or mitochondrial genome. Additional Agilent standard control probes were added to facilitate microarray use and for experiment quality assessment. Positive control probes were designed to hybridize with RNA spike-in (RNA Spike-In Kit, Agilent Technologies) in order to monitor the microarray workflow from sample amplification and labeling to microarray processing. Microarray support was a poly-L-lysine glass slide (25×75 mm) containing two arrays (spotting area 20×22 mm). For the comparison of dsRNA treatment versus no treatment, dye swaps were performed to compensate the dye effects and to assess the technical and biological reproducibility. Sample amplification and labeling procedures were carried with a low RNA input fluorescent amplification kit (Agilent Technologies) using 300 ng of total RNA. Microarray hybridization was performed in an ozone-controlled environment ($[O_3]<$ 2 ppb) at 60°C for 17 h followed by washing and drying steps according to the Agilent microarray processing protocol (In situ Hybridization Kit-plus, Agilent Technologies). Microarrays were then scanned in two colors with the Agilent G2505B at a resolution of 10 μm. After

extraction (Feature Extraction Software v9.5.1, Agilent Technologies), the data were processed, analyzed, and visualized using the Resolver 7.2 software (Rosetta Biosoftware, Seattle, WA, USA). A statistical analysis was performed using a Student's *t* test and Benjamini–Hochberg false discovery rate correction. Genes that were expressed differently between two experimental groups were selected for further analysis ($|\text{ratio}| \geq 1.2$, $p < 0.05$).

Validation of microarray data using real-time PCR

Seventeen identified transcripts were quantified by real-time PCR as previously described. Primers were designed using Primer Express 2.0 software (Applied Biosystems) and are listed in Supplemental Table S1 (Eurogentec). The $2^{-\Delta\Delta Ct}$ method was used to estimate the relative expression of each gene. For each gene, measurements were done in triplicate.

Results and discussion

PfPKA transcription profile during the parasite life cycle

PfPKAc and PfPKAr mRNA expression patterns were analyzed during the parasite intraerythrocytic life cycle (Fig. 1). Both genes were weakly expressed in the ring and trophozoite parasite stages in comparison to the schizont stage. These results are consistent with previous studies using immunofluorescence and microarray assays showing that PfPKAc and PfPKAr had elevated protein and mRNA levels, respectively, during the schizont stage (Syin et al. 2001; Bozdech et al. 2003). These data suggest that PfPKA activity might be necessary for the completion of the asexual cycle, or the reinvasion of erythrocytes. For further dsRNA experiments, we decided to analyze the treated parasites at the maximum of PfPKAc expression which occurs at the schizont stage according to previous studies (Wu et al. 1995; Crooke et al. 2006).

PfPKAc dsRNA treatment alters parasite growth and leads to decreased endogenous PfPKAc and PfPKAr mRNA

In order to investigate the role of PfPKAc during the *P. falciparum* asexual life cycle, we decided to modulate its transcript expression by using a gene-silencing strategy. These mechanisms are still poorly understood in *Plasmodium*, and genes related to the RNA interference pathway were not identified yet. However, antisense RNA and dsRNA have been successfully used to study the biological function of some *P. falciparum* transcripts (Gardiner et al. 2000; Kumar et al. 2002; Noonpakdee et al. 2003; Ullu et al. 2004). In using these techniques, one important limiting factor in the modulation of parasite genes is the efficiency of

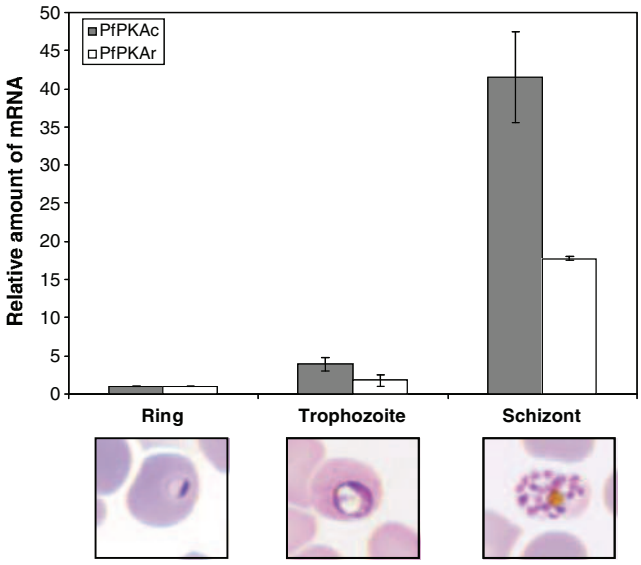

Fig. 1 PfPKAc and PfPKAr transcription profiles during the *P. falciparum* asexual life cycle using real-time PCR. Parasite cultures were tightly synchronized and harvested at three time points that covered the three main stages of the intraerythrocytic life cycle (ring, trophozoite, and schizont stages) for total RNA extraction and cDNA synthesis. Real-time quantitative PCR was performed with cDNA templates using the Power SYBR® Green master mix in triplicate. Recorded values were normalized to the amount of 18S rRNA, and the ring stage was used for calibration. The figure shows representative results of two biological independent assays. Images taken from RAL®555-stained thin blood smears show corresponding parasite stages for each time point

the transfection step. In our study, we first assayed different transfection strategies to optimize the penetration of dsRNA into the parasites: (a) direct incubation in culture medium containing dsRNA (Malhotra et al. 2002); (b) transfection of parasites by invasion of preloaded erythrocytes (Deitsch et al. 2001); (c) transfection of merozoites (van Dijk et al. 1995) and dsRNA transfection of intraerythrocytic stages by electroporation (Kumar et al. 2002). In our conditions, the latter technique using electroporation proved to be the most efficient and reproducible method for transfecting the parasite-infected red blood cells (data not shown).

After 24 h incubation, all electroporated *P. falciparum* cultures (control culture, PfPKAc dsRNA, and unrelated dsRNA) showed a 70% reduction in parasitemia, in agreement with previously reported data (Wu et al. 1995). As shown in Fig. 2a, control cultures reached the schizont stage with no pyknotic or gametocyte parasite forms indicating typical development. The same result was obtained for parasites electroporated with unrelated dsRNA (Fig. 2b). In contrast, the cultures electroporated with PfPKAc dsRNA showed clear morphological changes in about 40% of the parasites with abnormal development at the schizont stage and apparition of pyknotic forms (Fig. 2c).

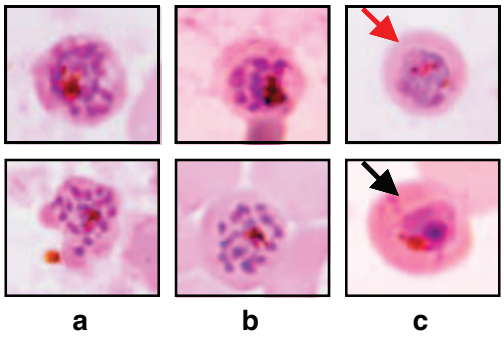

Fig. 2 Morphological examination of *P. falciparum* cultures 24 h after treatments. Parasites from different cultures were stained with RAL®555 on thin blood smears and examined by light microscopy 24 h after treatment with dsRNA buffer (**a**; control cultures) with unrelated dsRNA (**b**) or with PfPKAc dsRNA (**c**). Parasites from control cultures and those treated with unrelated dsRNA are in schizont stage. About 40% of the PfPKAc dsRNA treated parasites are altered. *Black* and *red arrows* point to pyknotic form and abnormal schizont, respectively

During the second cell cycle (approximately 15 h post reinvasion), the parasites from control cultures and cultures treated with unrelated dsRNA appeared to be morphologically normal with a majority of ring stage parasites (Fig. 3a, b). However, the cytological examination of PfPKAc dsRNA-electroporated parasites revealed that 35% of them are still at the schizont stage 48 h posttreatment indicating a possible cell cycle arrest at the schizont stage (Fig. 3c). These results confirm a possible role of PfPKAc in parasite growth and survival as already described in previous studies: (a) H-89, which inhibits PfPKAc activity in vitro, leads to parasite growth arrest and morphological alteration (Syin et al. 2001); (b) parasite cell cycle is altered after treatment with an activator of PfPKAc (3'-5'-cyclic monophosphate N6-benzoyl) (Beraldo et al. 2005); and (c) transgenic parasites that overexpress the PfPKAr have growth defect that can be restored by increasing the levels of intracellular cAMP (Merckx et al. 2008b).

In parallel to the morphological examinations, PfPKAc and PfPKAr transcripts were quantified by real-time PCR in each treated culture at the schizont stage (Fig. 4). In parasites treated with unrelated dsRNA, the level of PfPKA mRNA did not decrease significantly compared with the control cultures. However, a 70±10% PfPKAc transcript decrease was confirmed in PfPKAc dsRNA-treated cultures. Unexpectedly, the same inhibition profile was obtained for PfPKAr mRNA with a 70±11% decrease only in cultures treated with PfPKAc dsRNA (Fig. 4), supposing a transcriptional self-regulation of the PfPKA signaling system. These findings suggest that the parasites have tightly controlled mechanisms to self-regulated PfPKA levels to maintain correct PKA signaling. PKA self-regulation has been observed in studies done in mammalian cells where, for example, knocking down of the regulatory subunit causes a compensatory decrease in the catalytic subunit level (Duncan et al. 2006). Moreover, this phenomenon was already proposed in *P. falciparum*, where overexpression of PfPKAr led to an increase of PfPKAc transcript level (Merckx et al. 2008b). The mechanism of PfPKA self-regulation might be explained by the parasite's trial to correct the adverse effects caused by downregulation of the PfPKAc.

Thus, the decrease of PfPKAc and PfPKAr transcripts, combined with parasite growth and development alterations, demonstrates the key role of PfPKA in the progression of the asexual cycle and a possible regulation between the two subunits.

PfPKAc dsRNA treatment alters the parasite transcriptome

In order to identify genes in relation with PfPKAc, the effects of dsRNA treatment on the global parasite transcriptomic

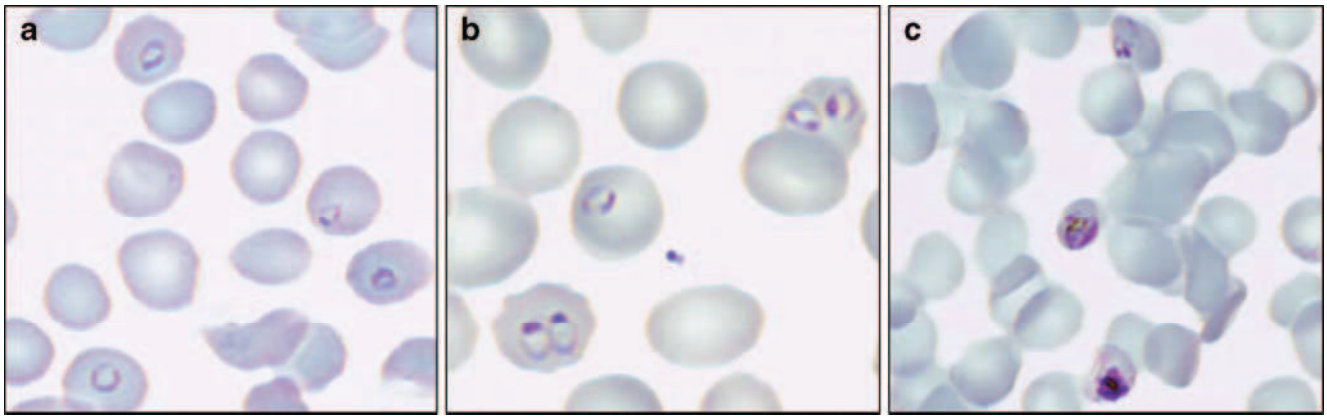

Fig. 3 Morphological examination of *P. falciparum* cultures 48 h after treatments. Cytological observations of blood smears were performed after treatment with dsRNA buffer (**a**; control cultures) with unrelated dsRNA (**b**) or with PfPKAc dsRNA (**c**) through the second erythrocytic cycle. Parasites from control cultures and those treated with unrelated dsRNA reached the ring stage, whereas 35% of parasites treated with PfPKAc dsRNA are still at the schizont stage

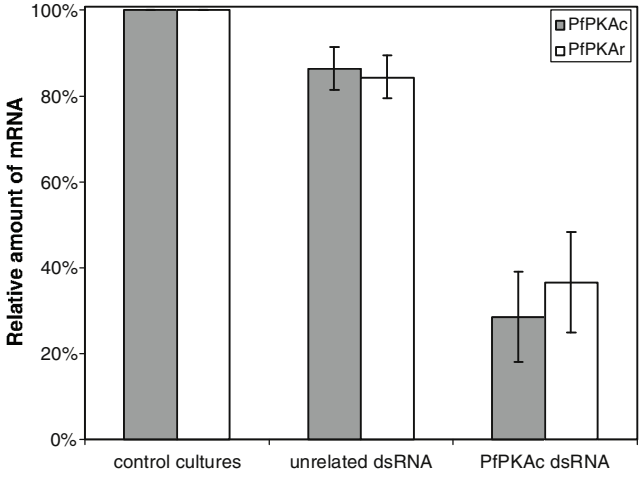

Fig. 4 PfPKA expression after the different treatments. The relative amounts of PfPKAc and PfPKAr mRNA were obtained using real-time quantitative PCR on samples from either control cultures or cultures treated with unrelated or PfPKAc dsRNA. The results are expressed as a percentage of the transcript expression in the control cultures after normalization with 18S rRNA in three independent experiments. Data are shown as mean±SD

profile were analyzed using a *P. falciparum* pan-genomic microarray. No significant difference in the expression level of any gene was observed when control cultures were compared with unrelated dsRNA-electroporated cultures. However, the expression of 329 genes (of about 5,400 *P. falciparum* genes analyzed) was up- and downregulated in response to PfPKAc dsRNA treatment (Supplemental Table S2).

To confirm the gene expression data obtained by microarray analysis, we performed real-time PCR for 17 representative genes up- or downregulated in response to PfPKAc dsRNA treatment belonging to the different pathways cited below (Fig. 5). The real-time PCR results confirmed those obtained from microarray, and the shift observed between the values was probably due to intrinsic differences in the techniques (Guerra et al. 2004).

Of the 329 selected genes, 189 are described as "hypothetical proteins" in the *P. falciparum* genome database (according to the 60% hypothetical functions found in the whole genome), and the rest of them are classified into specific metabolic pathways (Malaria Parasite Metabolic Pathways, Ginsburg, Hagai, http://sites.huji.ac.il/malaria/; Fig. 6). Several striking changes were seen across different functional classes, and we focus on three interesting pathways.

Many identified genes are involved in the posttranslational modification pathway via the calcium/calmodulin and kinase signaling network. These particular genes encode kinase catalytic and/or regulatory subunits, and analysis showed that their expression levels were down-

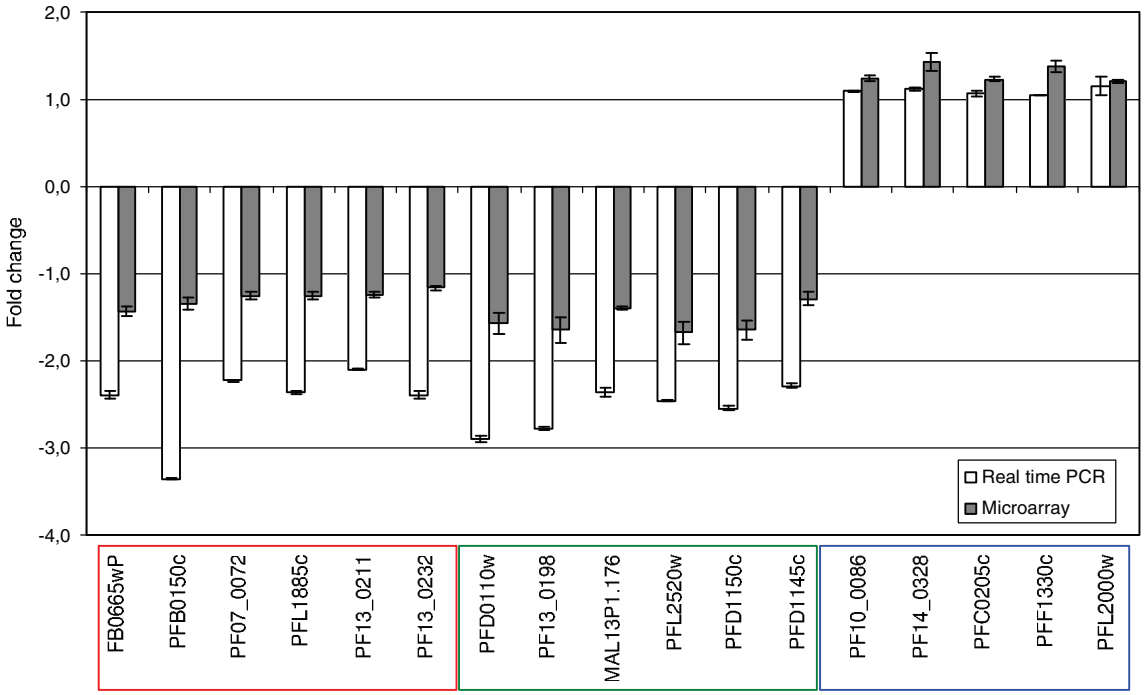

Fig. 5 Confirmation of microarray results by quantitative real-time PCR. The relative expression level of each gene in parasite cultures treated with PfPKAc dsRNA, as compared with the control cultures, are shown as fold changes for both real-time PCR (*open bars*) and microarray data (*closed bars*). The genes represented in *red boxes* belong to the posttranslational modification pathway via the calcium/calmodulin and kinases signaling network. The genes represented in *green boxes* belong to the invasion/motility pathway (RBL genes). The genes represented in *blue boxes* belong to the organellar functions (nuclear genes with mitochondrial signal sequences). Data are shown as mean±SD

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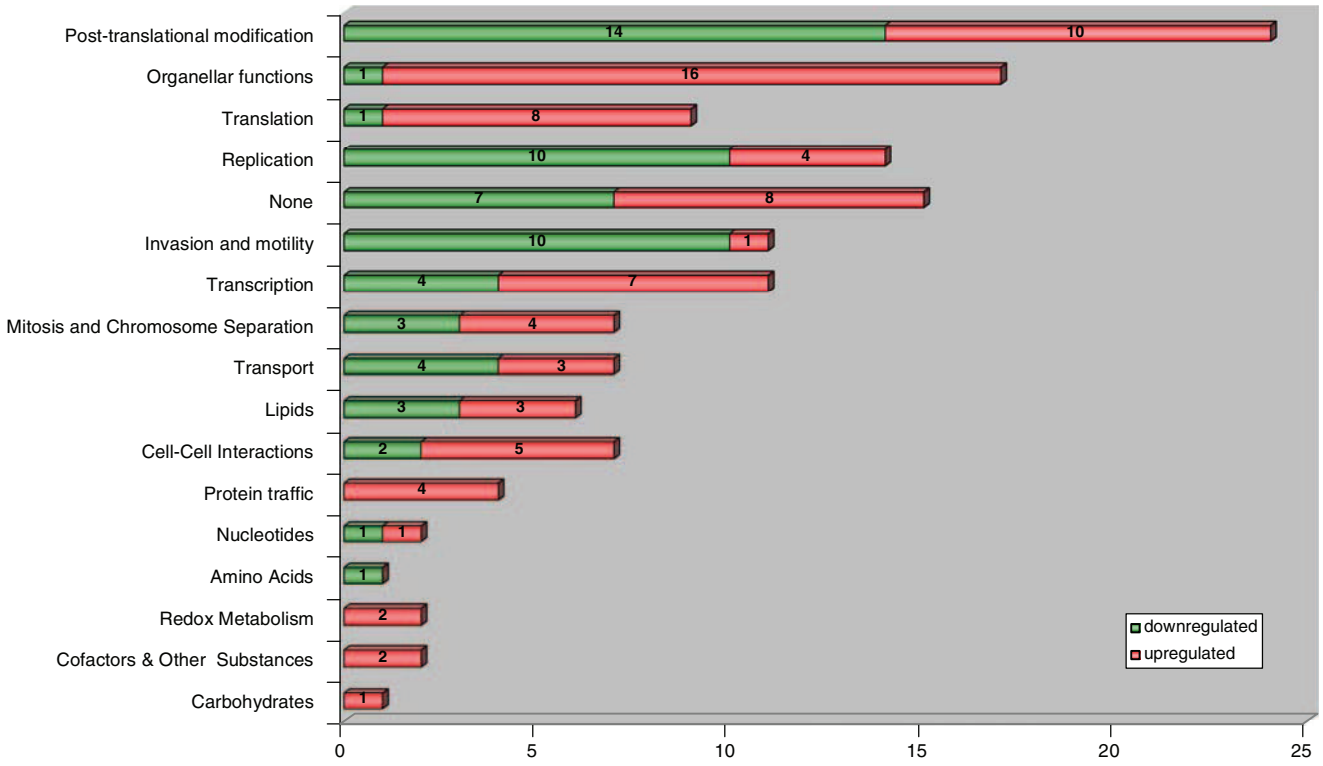

Fig. 6 Functional classification of up- and downregulated genes with known functions in PfPKAc dsRNA-treated cultures according to the Malaria Parasite Metabolic Pathways database (http://sites.huji.ac.il/malaria/). Up- and downregulated transcripts are colored *red* and *green*, respectively. The number of genes associated with each part is indicated. The calcium/calmodulin and kinase genes belong to the posttranslational modification pathway; the RBL genes belong to the invasion/motility network, and the nuclear genes with mitochondrial signal sequences belong to the organellar functions

regulated after PfPKAc dsRNA treatment (see Supplemental Table S2, genes colored red). These results are consistent with a previous work showing that the PfPKAc activation through cAMP release leads to Ca^{2+} liberation from the endoplasmic reticulum (Beraldo et al. 2005). The Ca^{2+} is able to stimulate adenylyl cyclase which, in turn, enables the production of cAMP and thus activate PfPKAc. Our study suggests the existence of a direct or indirect relation between the cAMP/PfPKA and Ca^{2+} pathways.

Several studies show a role of cAMP/PKA and calcium/calmodulin pathways in *P. falciparum* erythrocyte invasion by merozoites (Rangachari et al. 1986; Green et al. 2008). In our study, transcriptomic analysis revealed that the expression of six reticulocyte-binding-like (RBL) homolog genes was downregulated after PfPKAc dsRNA treatment (see Supplemental Table S2, genes colored green). These genes belong to the invasion/motility pathway and seem to be important partners for the process of *P. falciparum* invasion into human erythrocytes (Tham et al. 2009). Until now, only indirect data have demonstrated the potential role of PfPKAc in this specific pathway (McColm et al. 1980; Rangachari et al. 1986; Syin et al. 2001). Our results suggested the existence of a link between PfPKAc and the invasion pathway through the RBL genes. The precise network between cAMP/PKA and calcium/calmodulin pathways in relation with the invasion mechanisms needs to be further studied.

Another unexpected pathway altered by PfPKAc dsRNA treatment concerned genes related to organellar functions and, in particular, to mitochondria. The expression levels of 16 nuclear genes with mitochondrial signal sequences were upregulated after PfPKAc dsRNA electroporation (see Supplemental Table S2, genes colored blue). It is known that parasite mitochondrial activity requires the import of many specific nuclear proteins (van Dooren et al. 2006). The result obtained in our study implies that PfPKAc could regulate a part of the mitochondrial protein traffic as already described for mammalian cells (De Rasmo et al. 2008).

Genomic analysis of specific transcriptional responses to environmental perturbations have been crucial in identifying responsive regulatory factors and expression cascades in yeast and other eukaryotes (Gasch et al. 2000; Häbig et al. 2009). In *Plasmodium*, the correlation of the perturbation-specific events in its transcriptome has been limited to a few studies and is controverted (Gunasekera et al. 2007; Deitsch et al. 2007). Moreover, the study by Bozdech et al. suggested a cascade of transcriptional activation during blood-stage development where transcripts are produced in a specific

order to fulfill the demands of the cell (Bozdech et al. 2003). So, there is a possibility that any drug induces either a shift in the parasite cell cycle or a global arrest in schizogony and that the transcriptomic response observed is a mixture of specific drug-induced genes and nonspecific genes related to cell cycle modification. Furthermore, it was proposed that upon chemical stress, parasites arrest cell cycle progression to stimulate sexual development (Deitsch et al. 2007).

In our study, phenotypic observations of PfPKAc dsRNA-treated parasites did not reveal any cell cycle delay until the schizogony. In addition, analysis of genes modulated after PfPKAc dsRNA treatment has revealed some interesting genes already known to be in relation with the PfPKAc (Beraldo et al. 2005). By comparing our data with those of the normal gene expression in the intra-erythrocytic developmental cycle, the variation of expression of many genes cannot be linked to a cell-cycle delay suggesting that our results could be treatment specific (Bozdech et al. 2003). In our work, genes involved in stress or gametocytogenesis are absent as they are regularly found in transcriptomic studies (Le Roch et al. 2008).

With all these arguments, it can be assumed that the transcriptional responses observed are related specifically to the action of PfPKAc dsRNA. This specific transcriptional regulation appears to complement the posttranscriptional events observed in other studies (Le Roch et al. 2008).

In conclusion, our data suggest that PfPKAc is essential for the *P. falciparum* erythrocytic cycle and could implicate it in many essential cellular mechanisms, such as the Ca^{2+} signaling pathway, invasion processes, and mitochondrial functions. In this study, we describe the first attempt to determine the PfPKAc regulation pathway by use of specific gene silencing. While more work is needed to understand the complex cAMP/PfPKAc network, these results provide interesting information for further analysis of gene regulation involving PfPKAc. This enzyme could have a pleiotropic role during the different stages of the parasite life cycle.

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Phenotyic and transcriptomic analysis of Plasmodium falciparum protein kinase A catalytic subunit inhibition

Parasitology Research

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Supplemental Table S1

Name	Accession number[a]	Forward (F) and reverse (R) primers
reticulocyte binding protein 3 homolog	PFL2520w	F-CAACGAATCAAGCACGTTTACCTA R-TGGAAAAGGTCTTAGCAAGTTCAA
reticulocyte binding protein 4 homolog	PFD1150c	F-ATAACAGTATCTTTTGTACCTCCGAATTC R-TTTTGTCATAATAACTTCGAAATGGTGA
reticulocyte binding protein 2 homolog a	PF13_0198	F-TGAAGACCACACTATTTTGTAGCATATCT R-AGATGCTCCATGGGTATTACTTGAT
reticulocyte binding protein 1 homolog	PFD0110w	F-CACATGGGTAACGGTAGTAACTCG R-TTAATACCGTTTTCTCTTCCTCGATAG
reticulocyte binding protein 2 homolog b	MAL13P1.176	F-CAACGTGATGCTAGTAGTCATGGTAG R-TGTCAATACTATCATGAATATCAACACTATCC
reticulocyte binding protein homolog 5	PFD1145c	F-GACATTTGGATTTTGTTATAATACCTCATT R-AGCATCTACAGCTATACACTTTCCATATG
calcium-dependent protein kinase 4	PF07_0072	F-TTCTCTCAGGGTGCCCCC R-TTTCCCCGCTTCCACTTTT
calcium-dependent protein kinase	PF13_0211	F-TTGATTATACTGAATTCTTAGCAGCTTGT R-TGAAAGCATTTCTACAGATAACATCTTG
Casein kinase II regulatory subunit putative	PF13_0232	F-AAGATGATTTCAACGAAGCAACAGT R-AAGTCCTATCAAATTGAATTCGTCTCT
calcium/calmodulin-dependent protein kinase 2 putative	PFL1885c	F-TTTTGATCGCATTGGAAGCAT R-TGAGCTTGGATCATTCGGATTT
Ser/Thr protein kinase putative	PFB0665w	F-TTTCTCACAGTAAAAGTTCTCATACAACC R-TTCTTGCCCCTCCACAAAGTA
protein kinase putative	PFB0150c	F-CATACATATCTTCATAATGGTTATGGTAATGT R-CCCGTGGGCCCCTTTA
1-cys-glutaredoxin-like protein-1	PFC0205c	F-TTATGAAAGGGACCCCGGA R-TGACTACATTTGCGCTAAATCCAC
mitochondrial import inner membrane translocase subunit putative	PFF1330c	F-CCTTCTATGATCAGTGGATGTGCT R-TGGACCTTTGCCATTTTTCAAT
mitochondrial import inner membrane translocase subunit tim17 putative	PF14_0328	F-AAGAGAACCATGCCCCGATA R-CATACCAAATGCCCCACCC
adenylate kinase putative	PF10_0086	F-GGTAGATATATTTTTTTAGGTGCCCCA R-TCTTAAGGTTCAAGGATTGTGTTCC
mitochondrial carrier protein putative	PFL2000w	F-CATGTATCATTAGGCTCCCCTTTG R-TTCCTGACACTTGCATATTTTGTTTT

[a] accession number from Plasmodium Genome Resource database (www.plasmodb.org)

Phenotyic and transcriptomic analysis of Plasmodium falciparum protein kinase A catalytic subunit inhibition

Parasitology Research
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Supplemental Table S2

Accession number[a]	Description	Metabolic pathways	Fold change	Standard deviation
PF10_0322	S-adenosylmethionine decarboxylase-ornithine decarboxylase	Amino Acids	-1,3	0,01
PFL0285w	glyoxalase II family protein, putative	Carbohydrates	1,3	0,06
PFA0705c	stevor pseudogene putative	Cell-Cell Interactions	1,4	0,08
PFA0025c	erythrocyte membrane protein 1 (PfEMP1) pseudogene	Cell-Cell Interactions	1,4	0,10
PFB1005w	rifin	Cell-Cell Interactions	1,3	0,05
PF14_0767	stevor, putative	Cell-Cell Interactions	1,3	0,01
PFA0650w	surface-associated interspersed gene pseudogene, (SURFIN) pseudogene	Cell-Cell Interactions	1,2	0,04
PFA0765c	erythrocyte membrane protein 1 (PfEMP1)	Cell-Cell Interactions	-1,2	0,04
MAL13P1.405	erythrocyte membrane protein pfemp3, putative	Cell-Cell Interactions	-1,4	0,05
PFL1920c	hydroxyethylthiazole kinase, putative	Cofactors & Other Substances	1,4	0,04
PFF1105c	chorismate synthase	Cofactors & Other Substances	1,2	0,03
PF14_0218	actin-related protein homolog, arp4 homolog	Invasion and motility	1,2	0,04
PFL2460w	coronin	Invasion and motility	-1,2	0,03
PFD1145c	reticulocyte binding protein homolog 5, Rh5	Invasion and motility	-1,3	0,05
PFD0295c	apical sushi protein, ASP	Invasion and motility	-1,3	0,05
MAL13P1.176	Plasmodium falciparum reticulocyte binding protein 2, homolog b	Invasion and motility	-1,4	0,01
PFD1155w	erythrocyte binding antigen-165	Invasion and motility	-1,4	0,04
PFD0110w	normocyte-binding protein 1, pseudogene	Invasion and motility	-1,6	0,05

PFF0675c	myosin-like protein, putative	Invasion and motility	-1,6	0,02
PF13_0198	reticulocyte binding protein 2 homolog a	Invasion and motility	-1,6	0,06
PFD1150c	reticulocyte binding protein homolog 4, Rh4	Invasion and motility	-1,6	0,04
PFL2520w	Plasmodium falciparum, reticulocyte binding-like protein, homolog 3	Invasion and motility	-1,7	0,04
MAL13P1.210	dolichyl-phosphate-mannose-glygolipidalpha-mannosyltransferase	Lipids	1,4	0,10
PFD1035w	steroid dehydrogenase kik-i, putative	Lipids	1,2	0,01
PFL2270w	mannosyl transferase, putative	Lipids	1,2	0,04
MAL13P1.285	Patatin-like phospholipase, putative	Lipids	-1,3	0,04
PF14_0020	choline kinase, putative	Lipids	-1,3	0,03
PFE0485w	phosphatidylinositol 4-kinase, putative	Lipids	-1,4	0,03
PF14_0280	phosphotyrosyl phosphatase activator, putative	Mitosis and Chromosome Separation	1,3	0,07
PFI1245c	Protein phosphatase-beta	Mitosis and Chromosome Separation	1,2	0,05
PF08_0125	tubulin gamma chain	Mitosis and Chromosome Separation	1,2	0,04
PF11_0061	histone H4, putative	Mitosis and Chromosome Separation	1,2	0,04
PFE0450w	chromosome condensation protein, putative	Mitosis and Chromosome Separation	-1,4	0,04
PF10_0232	Chromodomain-helicase-DNA-binding protein 1 homolog, putative	Mitosis and Chromosome Separation	-1,5	0,04
PFF1035w	Pfs77 protein	Mitosis and Chromosome Separation	-1,5	0,04
PFL2465c	thymidylate kinase, putative	Nucleotides	1,3	0,06
PFL0475w	3,5-cyclic-nucleotide phosphodiesterase, putative	Nucleotides	-1,3	0,03
PFL0400w	50S ribosomal protein L29, putative	Organellar functions	1,7	0,11
PF13_0059	ribosomal protein S15, mitochondrial precursor, putative	Organellar functions	1,5	0,13
PF14_0328	mitochondrial import inner membrane translocase subunit tim17, putative	Organellar functions	1,4	0,10
PFF1330c	mitochondrial import inner membrane translocase subunit, putative	Organellar functions	1,4	0,07
MAL13P1.327	Ribosomal protein S17 homologue, putative	Organellar functions	1,2	0,03
PF10_0086	adenylate kinase, putative	Organellar functions	1,2	0,04
PFC0205c	PfGLP-1, 1-cys-glutaredoxin-like protein-1	Organellar functions	1,2	0,03
PFF1265w	oxidoreductase, short-chain dehydrogenase family, putative	Organellar functions	1,2	0,03
PFL0415w	acyl carrier protein, mitochondrial precursor, putative	Organellar functions	1,2	0,03
PF11_0128	coq4 homolog, putative	Organellar functions	1,2	0,04
PFL2000w	mitochondrial carrier protein, putative	Organellar functions	1,2	0,02
PF13_0121	dihydrolipoamide succinyltransferase, putative	Organellar functions	1,2	0,04
MAL13P1.335	phosphatidylserine synthase i; putative	Organellar functions	1,2	0,03

PF11_0062	histone H2B	Organellar functions	1,2	0,05
PFE1470w	cell cycle regulator protein, putative	Organellar functions	1,2	0,02
PF14_0276	ribosomal protein L15, putative	Organellar functions	1,2	0,04
PF14_0334	NAD(P)H-dependent glutamate synthase, putative	Organellar functions	-1,2	0,03
PFD0215c	pf52 protein	Post-translational modification	1,6	0,18
MAL13P1.68	peptidyl-prolyl cis-trans isomerase, putative	Post-translational modification	1,6	0,15
PF07_0112	proteasome subunit alpha type 5, putative	Post-translational modification	1,3	0,05
PFC0595c	serine/threonine protein phosphatase, putative	Post-translational modification	1,3	0,06
MAL8P1.78	small heat shock protein, putative	Post-translational modification	1,3	0,05
PFL0695c	geranylgeranyl transferase type2 beta subunit, putative	Post-translational modification	1,2	0,06
PF13_0021	small heat shock protein, putative	Post-translational modification	1,2	0,04
PFL0120c	cyclophilin, putative	Post-translational modification	1,2	0,04
PF11_0266	small nuclear ribonucleoprotein D1, putative	Post-translational modification	1,2	0,02
PFI1260c	Histone deacetylase	Post-translational modification	1,2	0,03
MAL13P1.44	protein phosphatase 2c-like protein, putative	Post-translational modification	-1,2	0,03
PF13_0232	Casein kinase II regulatory subunit, putative	Post-translational modification	-1,2	0,02
PFE0370c	subtilisin-like protease precursor, putative	Post-translational modification	-1,2	0,02
PF14_0110	rhomboid protease, putative	Post-translational modification	-1,2	0,01
PF14_0063	ATP-dependent Clp protease, putative	Post-translational modification	-1,2	0,03
PF13_0211	calcium-dependent protein kinase	Post-translational modification	-1,2	0,02
MAL8P1.157	ubiquitin-like protease 1 homolog, Ulp1 homolog, putative	Post-translational modification	-1,2	0,02
PF10_0177	erythrocyte membrane-associated antigen	Post-translational modification	-1,2	0,03
PFL1885c	calcium/calmodulin-dependent protein kinase 2, putative	Post-translational modification	-1,2	0,03
PF07_0072	calcium-dependent protein kinase 4	Post-translational modification	-1,3	0,03
PFB0150c	protein kinase, putative	Post-translational modification	-1,3	0,04
PFD0185c	peptidase	Post-translational modification	-1,4	0,03
PF11_0381	subtilisin-like protease 2	Post-translational modification	-1,4	0,02
PFB0665w	Ser/Thr protein kinase, putative	Post-translational modification	-1,4	0,03
PFD0895c	Bet3 transport protein, putative	Protein traffic	1,3	0,06
PFF0060w	Plasmodium falciparum Maurers Cleft 2 transmembrane domain protein 6	Protein traffic	1,3	0,03
PFA0065w	Plasmdoium falciparum Maurers Cleft 2 transmembrane domain protein 1.1	Protein traffic	1,3	0,06
MAL13P1.244	TBC domain protein, putative	Protein traffic	1,2	0,04

PFF0340c	glutaredoxin-like protein, putative	Redox Metabolism	1,3	0,08
PF07_0036	Cg6 protein	Redox Metabolism	1,2	0,03
PF14_0148	uracil-DNA glycosylase, putative	Replication	1,3	0,07
MAL13P1.64	ubiquitin-like protein nedd8 homologue, putative	Replication	1,2	0,04
PF14_0366	small subunit DNA primase	Replication	1,2	0,03
MAL7P1.145	mismatch repair protein pms1 homologue, putative	Replication	1,2	0,02
PFE0205w	ATP-dependent helicase, putative	Replication	-1,2	0,01
PFF0285c	DNA repair protein RAD50, putative	Replication	-1,2	0,02
PF13_0308	DNA helicase	Replication	-1,2	0,02
PF14_0437	helicase, truncated, putative	Replication	-1,2	0,02
PF14_0112	POM1, putative	Replication	-1,2	0,03
PFI1470c	leucine-rich repeat protein 8, LRR8	Replication	-1,2	0,02
PFL2440w	DNA repair protein rhp16, putative	Replication	-1,2	0,02
PFD0790c	DNA replication licensing factor, putative	Replication	-1,3	0,01
PFD0475c	replication factor a protein, putative	Replication	-1,3	0,03
PFL0150w	origin recognition complex 1 protein	Replication	-1,4	0,05
PFE1085w	DEAD-box subfamily ATP-dependent helicase, putative	Transcription	1,3	0,04
PF14_0194	spliceosome-associated protein, putative	Transcription	1,2	0,07
PFB0245c	DNA-directed RNA polymerase II 16 kDa subunit, putative	Transcription	1,2	0,04
PF14_0057	RNA binding protein, putative	Transcription	1,2	0,05
PF10_0175	tRNA pseudouridine synthase, putative	Transcription	1,2	0,02
MAL8P1.131	Gas41 homologue, putative	Transcription	1,2	0,02
PF10_0269	DNA-directed RNA polymerase II, putative	Transcription	1,2	0,02
MAL13P1.120	splicing factor, putative	Transcription	-1,2	0,03
PFF0745c	ribonuclease, putative	Transcription	-1,2	0,03
MAL13P1.14	ATP-dependent DEAD box helicase, putative	Transcription	-1,3	0,04
PF11_0477	CCAAT-box DNA binding protein subunit B	Transcription	-1,4	0,06
PFI0215c	signal peptidase, putative	Translation	1,4	0,06
MAL8P1.110	plastid 50S ribosomal protein L33, putative	Translation	1,3	0,08
PF14_0579	ribosomal protein L27, putative	Translation	1,3	0,07
PF13_0316	40S ribosomal protein S13	Translation	1,3	0,07
PFD0355c	Peptidyl-tRNA hydrolase PTH2, putative	Translation	1,2	0,05

PFD0960c	ribosomal protein L7Ae-related protein, putative	Translation	1,2	0,05
PF11_0438	Ribosomal protein, putative	Translation	1,2	0,04
MAL8P1.51	protein-transport protein sec61 beta 1 subunit, putative	Translation	1,2	0,04
PF13_0350	signal recognition particle receptor alpha subunit, putative	Translation	-1,4	0,05
PF13_0222	RNA lariat debranching enzyme, putative	Transport	1,3	0,09
PFB0210c	hexose transporter, PfHT1	Transport	1,3	0,06
PF11_0059	metabolite/drug transporter	Transport	1,2	0,06
MAL13P1.301	protein with aminophospholipid-transporting P-ATPase and guanyl cyclase domains	Transport	-1,3	0,02
PFC0135c	nuclear export receptor, crm1 homolog	Transport	-1,3	0,04
PF13_0019	sodium/hydrogen exchanger, Na+, H+ antiporter	Transport	-1,3	0,02
PF13_0271	ABC transporter, (heavy metal transporter family)	Transport	-1,3	0,05
PF11_0199	hypothetical protein	Unknown	1,8	0,23
PFI1395w	hypothetical protein, conserved	Unknown	1,6	0,07
PFF0640w	hypothetical protein, conserved	Unknown	1,5	0,14
PFE0105c	hypothetical protein, conserved	Unknown	1,5	0,12
PF14_0037	hypothetical protein	Unknown	1,5	0,05
PF14_0424	hypothetical protein	Unknown	1,5	0,12
PF11_0186	hypothetical protein	Unknown	1,4	0,06
MAL13P1.415	hypothetical protein, pseudogene	Unknown	1,4	0,08
PFE1135w	hypothetical protein, conserved	Unknown	1,4	0,04
PF11_0038	hypothetical protein	Unknown	1,4	0,07
PFC0241w	hypothetical protein, conserved	Unknown	1,4	0,09
PFL0750w	hypothetical protein, conserved	Unknown	1,4	0,10
PFD1075w	hypothetical membrane protein, conserved	Unknown	1,4	0,06
PF14_0705	hypothetical protein	Unknown	1,4	0,09
PFL1195w	hypothetical protein, conserved	Unknown	1,4	0,09
PFD0915w	hypothetical protein, conserved	Unknown	1,4	0,09
PFE1615c	hypothetical protein	Unknown	1,4	0,05
PF11_0363	hypothetical protein	Unknown	1,3	0,03
PF14_0034	hypothetical protein, conserved	Unknown	1,3	0,08
PF08_0057	hypothetical protein, conserved	Unknown	1,3	0,06
PFB0620w	hypothetical protein	Unknown	1,3	0,06

PFI1330c	hypothetical protein, conserved	Unknown	1,3	0,06
MAL8P1.138	hypothetical protein, conserved	Unknown	1,3	0,06
PFI0280c	autophagocytosis associated protein, putative	Unknown	1,3	0,07
PF14_0755	hypothetical protein	Unknown	1,3	0,06
PF13_0031	tRNA intron exonumclease, putative	Unknown	1,3	0,09
MAL7P1.173	hypothetical protein	Unknown	1,3	0,07
PF10_0276	hypothetical protein	Unknown	1,3	0,07
PFF1320c	troponin c-like protein, putative	Unknown	1,3	0,04
PF14_0662	hypothetical protein	Unknown	1,3	0,05
PF07_0109	hypothetical protein, conserved	Unknown	1,3	0,06
PFI1660w	hypothetical protein, conserved	Unknown	1,3	0,06
PFE1100w	hypothetical protein, conserved	Unknown	1,3	0,07
PF10_0007	hypothetical protein	Unknown	1,3	0,02
PFI0965w	conserved protein, putative	Unknown	1,3	0,03
PFL0515w	hypothetical protein, conserved	Unknown	1,3	0,08
PF11_0068	hypothetical protein, conserved	Unknown	1,3	0,05
PFD1065c	hypothetical protein, conserved	Unknown	1,3	0,07
MAL7P1.99	hypothetical protein, conserved	Unknown	1,3	0,05
PFF1090c	hypothetical membrane protein, conserved	Unknown	1,3	0,03
MAL13P1.50	hypothetical protein, conserved	Unknown	1,3	0,04
PF14_0317	hypothetical protein, conserved	Unknown	1,3	0,07
PFL0205w	hypothetical protein, conserved	Unknown	1,3	0,05
PFL0765w	hypothetical protein, conserved	Unknown	1,3	0,06
PFL2445c	hypothetical protein, conserved	Unknown	1,3	0,05
PFF0240c	hypothetical protein	Unknown	1,3	0,05
PFE0510c	hypothetical protein, conserved	Unknown	1,3	0,07
PFB0395w	hypothetical protein	Unknown	1,3	0,04
PF11_0088	hypothetical protein	Unknown	1,3	0,04
PF14_0200	hypothetical protein	Unknown	1,3	0,06
MAL7P1.33	hypothetical protein, conserved	Unknown	1,3	0,06
PFE1440c	hypothetical protein, conserved	Unknown	1,3	0,03
PFF0580w	hypothetical protein, conserved	Unknown	1,3	0,06

MAL13P1.129	hypothetical protein, conserved	Unknown	1,2	0,06
PFL0175c	hypothetical protein, conserved	Unknown	1,2	0,04
PF11_0453	hypothetical protein, conserved	Unknown	1,2	0,05
PFB0575c	hypothetical protein	Unknown	1,2	0,04
MAL13P1.345	hypothetical protein, conserved	Unknown	1,2	0,04
MAL8P1.122	hypothetical protein, conserved	Unknown	1,2	0,05
PFC0126c	hypothetical protein, conserved	Unknown	1,2	0,06
PF13_0260	hypothetical protein, conserved	Unknown	1,2	0,05
MAL13P1.251	hypothetical protein, conserved	Unknown	1,2	0,05
PF13_0347	hypothetical protein, conserved	Unknown	1,2	0,05
PFE1450c	hypothetical protein, conserved	Unknown	1,2	0,03
PFI0920c	Dihydrouridine synthase, putative	Unknown	1,2	0,05
PFL2435w	hypothetical protein, conserved	Unknown	1,2	0,05
PFB0880w	hypothetical protein, conserved	Unknown	1,2	0,04
PFE0560c	hypothetical protein, conserved	Unknown	1,2	0,05
MAL13P1.223	hypothetical protein	Unknown	1,2	0,03
PF10_0130	hypothetical protein	Unknown	1,2	0,03
PFB0910w	hypothetical protein	Unknown	1,2	0,04
PFB0932w	hypothetical protein	Unknown	1,2	0,03
PFC0555c	hypothetical protein, conserved	Unknown	1,2	0,04
PFB0391c	hypothetical protein	Unknown	1,2	0,04
PF13_0054	transcription factor, putative	Unknown	1,2	0,03
PF11_0431	membrane skeletal protein, putative	Unknown	1,2	0,05
MAL8P1.114	hypothetical protein, conserved	Unknown	1,2	0,02
PFE1175w	hypothetical protein, conserved	Unknown	1,2	0,02
MAL13P1.229	hypothetical protein, conserved	Unknown	1,2	0,02
PFF0545c	hypothetical protein, conserved	Unknown	1,2	0,03
PF13_0053	hypothetical protein, conserved	Unknown	1,2	0,05
PFI0150c	hypothetical protein	Unknown	1,2	0,03
PF11_0487	hypothetical protein	Unknown	1,2	0,04
PF10_0370	Enhancer of rudimentary homolog, putative	Unknown	1,2	0,05
PF13_0134	hypothetical protein, conserved	Unknown	1,2	0,03

MAL13P1.159	hypothetical protein, conserved	Unknown	1,2	0,03
PF10_0066	hypothetical protein	Unknown	1,2	0,03
PFF0105w	MYND finger domain protein	Unknown	1,2	0,02
PFL2370c	hypothetical protein, conserved	Unknown	1,2	0,04
PFL0435w	hypothetical protein, conserved	Unknown	1,2	0,04
PFL1755w	hypothetical protein, conserved	Unknown	1,2	0,03
PFB0660w	hypothetical protein	Unknown	1,2	0,03
PF11_0346	hypothetical protein	Unknown	1,2	0,03
PFI0970c	TLD domain, putative	Unknown	-1,2	0,02
PF10_0112	hypothetical protein	Unknown	-1,2	0,03
MAL13P1.262	hypothetical protein, conserved	Unknown	-1,2	0,02
PFI1335w	hypothetical protein, conserved	Unknown	-1,2	0,03
PFE1235c	hypothetical protein	Unknown	-1,2	0,03
PF14_0530	ferlin, putative	Unknown	-1,2	0,02
PFI1580c	DHHC-type zinc finger protein, putative	Unknown	-1,2	0,02
PFL1010c	hypothetical protein conserved	Unknown	-1,2	0,02
PFA0210c	hypothetical protein, conserved	Unknown	-1,2	0,02
PF14_0692	hypothetical protein	Unknown	-1,2	0,03
PF14_0363	metacaspase-like protein	Unknown	-1,2	0,03
PF10_0247	hypothetical protein	Unknown	-1,2	0,03
PFE1485w	hypothetical protein, conserved	Unknown	-1,2	0,02
MAL8P1.163	hypothetical protein, conserved in P.falciparum	Unknown	-1,2	0,03
PF11_0371	hypothetical protein	Unknown	-1,2	0,03
PF10_0286	hypothetical protein	Unknown	-1,2	0,03
PF08_0124	hypothetical protein, conserved	Unknown	-1,2	0,03
PF08_0082	hypothetical protein, conserved	Unknown	-1,2	0,02
MAL7P1.146	hypothetical protein, conserved	Unknown	-1,2	0,03
PF08_0089	hypothetical protein, conserved	Unknown	-1,2	0,02
PF11_0316	hypothetical protein	Unknown	-1,2	0,02
MAL8P1.150	hypothetical protein, conserved	Unknown	-1,2	0,02
PFA0405w	hypothetical protein, conserved	Unknown	-1,2	0,01
PFI1200w	hypothetical protein, conserved	Unknown	-1,2	0,03

PFE0440w	hypothetical protein, conserved	Unknown	-1,2	0,03
PF13_0104	hypothetical protein, conserved	Unknown	-1,2	0,02
PFD0445c	hypothetical protein, conserved	Unknown	-1,2	0,03
PFC0540w	hypothetical protein	Unknown	-1,2	0,03
PF10_0168	hypothetical protein	Unknown	-1,2	0,03
PFE0495w	hypothetical protein, conserved	Unknown	-1,2	0,04
PFL0470w	hypothetical protein, conserved	Unknown	-1,2	0,03
PF10_0163	hypothetical protein	Unknown	-1,2	0,00
PF11_0116	hypothetical protein	Unknown	-1,2	0,04
PF14_0386	hypothetical protein	Unknown	-1,2	0,02
PFB0279w	hypothetical protein	Unknown	-1,2	0,02
PFD0380c	hypothetical protein, conserved	Unknown	-1,2	0,00
PF10_0140	hypothetical protein	Unknown	-1,2	0,04
PF11_0342	hypothetical protein	Unknown	-1,2	0,04
PF14_0470	hypothetical protein	Unknown	-1,2	0,04
PF11_0168	hypothetical protein	Unknown	-1,2	0,03
PF14_0333	hypothetical protein	Unknown	-1,2	0,02
PFA0180w	hypothetical protein, conserved	Unknown	-1,2	0,04
PF10_0083	hypothetical protein	Unknown	-1,2	0,04
PF11_0193	hypothetical protein	Unknown	-1,2	0,03
MAL7P1.132	hypothetical protein, conserved	Unknown	-1,3	0,03
MAL13P1.269	hypothetical protein	Unknown	-1,3	0,03
PF14_0440	hypothetical protein	Unknown	-1,3	0,04
MAL8P1.70	hypothetical protein, conserved	Unknown	-1,3	0,04
PF10_0281	hypothetical protein	Unknown	-1,3	0,02
PF11_0210	hypothetical protein	Unknown	-1,3	0,03
PF13_0047	hypothetical protein, conserved	Unknown	-1,3	0,04
PF14_0552	hypothetical protein	Unknown	-1,3	0,04
PF11_0185	hypothetical protein	Unknown	-1,3	0,02
PF14_0196	hypothetical protein	Unknown	-1,3	0,05
MAL13P1.260	hypothetical protein, conserved	Unknown	-1,3	0,02
PF11_0241	hypothetical protein	Unknown	-1,3	0,04

PF14_0583	hypothetical protein	Unknown	-1,3	0,04
PF08_0074	DNA/RNA-binding protein Alba, putative	Unknown	-1,3	0,04
PFL1045w	hypothetical protein, conserved	Unknown	-1,3	0,04
PFB0680w	hypothetical protein	Unknown	-1,3	0,03
PFL1650w	hypothetical protein, conserved	Unknown	-1,3	0,04
PFF0965c	hypothetical membrane protein, conserved	Unknown	-1,3	0,04
PFD0390c	AAA family ATPase, putative	Unknown	-1,3	0,03
PFB0190c	hypothetical protein	Unknown	-1,3	0,02
PFE1285w	hypothetical protein, conserved	Unknown	-1,3	0,03
PF14_0135	hypothetical protein	Unknown	-1,3	0,05
PF11_0480	hypothetical protein	Unknown	-1,3	0,02
PF11_0277	hypothetical protein	Unknown	-1,3	0,02
PF11_0268	hypothetical protein	Unknown	-1,3	0,04
MAL13P1.29	hypothetical protein, conserved	Unknown	-1,3	0,04
PFE0385w	hypothetical protein, conserved	Unknown	-1,3	0,04
PF14_0383	hypothetical protein	Unknown	-1,3	0,03
PFF0990c	hypothetical protein, conserved	Unknown	-1,3	0,03
MAL13P1.102	hypothetical protein, conserved	Unknown	-1,3	0,04
PF13_0210	hypothetical protein, conserved	Unknown	-1,3	0,03
PF11_0213	hypothetical protein	Unknown	-1,3	0,04
PFD0320c	hypothetical protein, conserved	Unknown	-1,3	0,05
PFD0885c	hypothetical protein, conserved	Unknown	-1,3	0,05
PFB0765w	hypothetical protein	Unknown	-1,3	0,04
PF14_0120	hypothetical protein	Unknown	-1,3	0,03
MAL13P1.145	hypothetical protein, conserved	Unknown	-1,3	0,05
MAL13P1.112	hypothetical protein,conserved	Unknown	-1,3	0,04
PFE0070w	interspersed repeat antigen, putative	Unknown	-1,4	0,05
PFC0965w	hypothetical protein, conserved	Unknown	-1,4	0,03
PFL2335w	hypothetical protein, conserved	Unknown	-1,4	0,02
PF10_0156	hypothetical protein	Unknown	-1,4	0,05
PFI1210w	hypothetical protein, conserved	Unknown	-1,4	0,05
PFL2110c	hypothetical protein, conserved	Unknown	-1,4	0,03

PF10_0072	hypothetical protein		Unknown	-1,4	0,04
PF10_0211	hypothetical protein		Unknown	-1,4	0,05
PF11_0442	hypothetical protein		Unknown	-1,4	0,04
MAL13P1.202	hypothetical protein, conserved		Unknown	-1,4	0,03
PFC1045c	hypothetical protein, conserved		Unknown	-1,4	0,03
PF08_0035	hypothetical protein, conserved		Unknown	-1,4	0,05
PFL1320w	hypothetical protein, conserved		Unknown	-1,4	0,05
PFE0700c	hypothetical protein, conserved		Unknown	-1,4	0,05
PF11_0490	hypothetical protein		Unknown	-1,4	0,02
PFF0505c	hypothetical protein, conserved		Unknown	-1,4	0,03
PFL1705w	hypothetical protein, conserved		Unknown	-1,5	0,04
PF10_0357	hypothetical protein		Unknown	-1,5	0,03
PF14_0226	hypothetical protein		Unknown	-1,5	0,06
PF10_0079	hypothetical protein		Unknown	-1,5	0,05
PF08_0008	hypothetical protein, conserved		Unknown	-1,5	0,05
PFL1165w	hypothetical protein, conserved		Unknown	-1,5	0,03
PF14_0703	hypothetical protein		Unknown	-1,6	0,04
PFL2100w	hypothetical protein, conserved		Unknown	-1,6	0,05
PF10_0262	hypothetical protein		Unknown	-1,6	0,03
PF14_0172	hypothetical protein		Unknown	-1,7	0,03
PF11_0278	hypothetical protein		Unknown	-1,7	0,07
PFI0540w	hypothetical protein, conserved		Unknown	-1,8	0,02
PFE0360c	hypothetical protein, conserved		Unknown	-2,0	0,04

[a]accession number from Plasmodium Genome Resource database (www.plasmodb.org)

The genes colored in red belong to the calcium/calmodulin and kinases signaling network
The genes colored in green belong to the invasion/motility pathway (RBL genes)
The genes colored in blue belong to nuclear genes with mitochondrial signal sequence

CONCLUSIONS ET PERSPECTIVES

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Au cours de ce travail de doctorat, nous avons étudie la sous-unité catalytique de la PKAc de *P. falciparum* selon 2 approches complémentaires :

- Une approche *in vitro* a permis la caractérisation biochimique de la PfPKAc, qui a été exprimée sous forme soluble dans *E. coli* et purifiée par le biais d'une chromatographie en deux étapes. Les paramètres enzymatiques de cette kinase recombinante ont été déterminés à l'aide d'un test de mesure en fluorescence permettant la détermination des constantes cinétiques et l'établissement du profil d'inhibition de cette protéine.

- Parallèlement, une seconde approche visant à déterminer les fonctions biologiques de la PfPKAc a été entreprise en analysant l'effet de son inhibition par ARN interférence au niveau phénotypique et transcriptionnel. Le traitement de parasites avec des dsRNA dirigés contre la PfPKAc provoque une dégénerescence parasitaire (40%) et s'accompagne d'un blocage des parasites au stade schizonte, suggérant que la PfPKAc semble indispensable à l'achèvement du cycle cellulaire. Outre l'inhibition des transcrits de la PfPKAc, une diminution de ceux de la PfPKAr est observé, ce qui suggère l'existence d'un phénomène de co-régulation entre les deux sous-unités de la PfPKA. Enfin, la modification de l'expression de 329 gènes a pu être constatée après inhibition des transcrits de la PfPKAc. L'analyse de ces gènes a permis la mise en évidence de voies métaboliques en relation avec la PfPKAc notamment au niveau de la régulation mitochondriale, des mécanismes de ré-invasion ou encore de la voie calcium/calmoduline.

Finalement, l'ensemble des résultats obtenus au cours de ce travail, nous a permis d'améliorer la compréhension de la voie de l'AMPc/PKA chez *P. falciparum* tout en développant un certain nombre d'outils qui seront utiles aux nombreuses perspectives envisagées :

Les voies métaboliques en relation avec la PfPKAc identifiés lors de nos études transcriptomiques pourraient être approfondies par des études complémentaires :

1) L'effet d'inhibiteurs de PKAc, tel que H-89, sur le transcriptome parasitaire pourrait nous permettre de réaliser un comparatif par rapport aux données obtenues sur des parasites traitées avec les dsRNA diriges contre la PfPKAc ;
2) La même approche transcriptomique pourrait également être envisagée en utilisant cette fois-ci des dsRNA dirigés contre la PfPKAr ;
3) En comparant l'activité mitochondriale de parasites témoins et de parasites traités avec des inhibiteurs de PfPKAc, on pourrait mettre en évidence l'existence d'un lien entre la PfPKAc et la mitochondrie (MitoTracker®, Invitrogen) [124, 125]. Parallèlement, la comparaison de protéomes mitochondriaux témoins et traités avec des inhibiteurs de la PfPKAc par des techniques d'électrophorèse bidimensionnelle en fluorescence permettrait d'améliorer la connaissance sur la relation entre mitochondrie et PfPKAc [108,109]. Cette méthode a été réalisée avec succès pour comparer les protéomes mitochondriaux de *Dictyostelium discoideum* à différentes étapes de son cycle cellulaire [110] ;
4) En mesurant le taux de ré-invasion des mérozoïtes dans de nouveaux globules rouges, nous pourrions confirmer l'effet d'inhibiteurs de PfPKAc sur cette étape du cycle parasitaire. [111-115].

Le test *in vitro* de mesure de l'activité kinase en fluorescence développé au cours de ce travail pourra nous permettre d'effectuer un criblage rapide et efficace d'inhibiteurs spécifiques de cette kinase plasmodiale. Il s'agirait de cribler « en aveugle » une grande quantité de molécules issues de différentes chimiothèques. Cette méthodologie a d'ailleurs été employée avec succès pour identifier des inhibiteurs de la dihydroorotate déhydrogénase de *P. falciparum* [116,117]. La validation des molécules inhibitrices de l'activité kinase *in vitro* pourra s'effectuer à l'aide de système cellulaire sur différentes souches de *P. falciparum* [117-119].
En améliorant la quantité et la pureté de la PfPKAc recombinante, nous pourrions envisager la résolution de sa structure tridimensionnelle qui nous permettrait une conception rationnelle de molécules potentiellement inhibitrices. Cette optimisation serait envisageable en testant d'autres systèmes d'expression (BaculoDirect™ C-Term Transfection Kit, Invitrogen). L'obtention d'une PfPKAc avec un taux de pureté élevé permettrait également la production d'anticorps spécifiques qui serait très utiles pour des études de localisation cellulaire mais aussi pour l'identification de partenaires cellulaires par des techniques d'immuno-précipitation [120,121].

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Malgré la mise en évidence de l'importance de la PfPKAc au cours du cycle parasitaire et les voies de transduction qui sont en relation avec cette kinase, ses substrats n'ont pas encore été identifiés [90-94]. Ceci pourrait être envisagé selon la stratégie KESTREL (kinase substrate tracking and elucidation) qui permet d'identifier les substrats de kinase par une étude du phosphoprotéome [122,123]. Le phosphoprotéome différentiel entre des échantillons traités et non traités par la kinase recombinante nous permettrons d'identifier d'éventuels substrats cellulaires. Cette méthode a été employée avec succès chez *P. falciparum* pour identifier les substrats d'une sérine / thréonine kinase PfPK9 [124]. Pour éviter l'utilisation de molécules radioactives, la détection des protéines phosphorylées peut être réalisée à l'aide de colorants spécifiques qui fixent les phosphates ajoutés sur les résidus sérine, thréonine et tyrosine directement sur gel (ProQ®-Diamond, Molecular Probes) [125,126]. Parallèlement, la comparaison de protéome / phosphoprotéome de parasites traités avec des inhibiteurs de kinase tels que H-89 et de parasites témoins pourrait nous permettre de mettre en évidence des nouveaux substrats de la PfPKAc et les voies métaboliques qui les impliquent [127].

Enfin, il serait également intéressant de cloner, d'exprimer et de purifier la PfPKAr afin de réaliser des essais d'interaction entre les deux sous-unités de la PfPKA et d'identifier des domaines importants de cette interaction par des stratégies de mutagénèse dirigée [128-130].

La connaissance des voies de transduction de *P. falciparum* est fondamentale pour le développement de nouvelles stratégies thérapeutiques pour lutter contre le paludisme mais aussi pour une meilleure compréhension des mécanismes intimes de la multiplication/division des *Plasmodium* [56,75]. La phosphorylation des protéines est un des mécanismes les plus communs dans les cellules, et les protéines kinases représentent aujourd'hui des cibles thérapeutiques importantes [66,131]. Des différences subtiles en terme de structure et de fonction existent entre les protéines kinase humaines et plasmodiales [59], suggérant la possibilité l'inhiber spécifiquement les enzymes du parasite [135]. L'ensemble de ces études devrait permettre de dessiner un modèle plus complet de la voie de transduction de l'AMP cyclique et de la PfPKA et déboucher sur le développement de nouvelles voies thérapeutiques.

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RESUME

L'aggravation actuelle du risque lié au paludisme résulte du développement du phénomène de résistance de souches de Plasmodium falciparum aux molécules antipaludiques. Une telle situation et l'absence de vaccin efficace nécessitent le développement de nouvelles stratégies antiparasitaires. Jusqu'à présent, les mécanismes moléculaires qui contrôlent le cycle parasitaire sont méconnus. Chez la plupart des eucaryotes, les protéine kinases sont impliquées dans des fonctions cellulaires essentielles et constituent une cible privilégiée pour la conception de nouveaux médicaments. Dans ce cadre, nous nous sommes intéressés à la voie de transduction de l'AMP cyclique et en particulier à la sous-unité catalytique de la protéine kinase AMPc dépendante (PfPKAc) dont le rôle essentiel reste mal défini chez *P. falciparum*. Deux approches complémentaires ont été choisies pour étudier cette kinase :

1) **au niveau biochimique** par le clonage, l'expression, la purification et la caractérisation enzymatique de la PfPKAc. L'objectif était d'obtenir une enzyme active *in vitro* de façon à pourvoir mesurer les constantes enzymatiques de la PfPKAc et conduire les premiers essais d'inhibitions.

2) **au niveau cellulaire** en analysant les conséquences de l'inhibition par des ARN interférents spécifiques des transcrits de la PfPKAc. Le développement parasitaire mais également le transcriptome global ont été étudiés de manière à préciser les voies métaboliques liées à cette kinase plasmodiale.

L'ensemble de ces études précise la compréhension de la voie de transduction de l'AMP cyclique et de la PfPKA qui pourrait conduire au développement de nouvelles voies thérapeutiques.

Mots clés : *Plasmodium falciparum*, protéines kinases, PKA, caractérisation biochimique, ARN interférence, puces à ADN, voies métaboliques.

ABSTRACT

Nowadays, the increase of risks associated with malaria results from the development of resistance of Plasmodium falciparum strains to antimalarial drugs. This situation and the lack of an effective vaccine require the development of new antimalarial strategies. Until now, molecular mechanisms controlling the life cycle of malaria parasites, are still poorly understood. In most eukaryotes, protein kinases are implicated in essential cellular functions and represent attractive targets for the development of new drugs. In this context, we focused on the signaling pathway implicating cAMP and particularly the catalytic subunit of cAMP-dependent protein kinase (PfPKAc), whose function is still unclear in P. falciparum. Two complementary strategies were chosen to study this kinase:

1) at the biochemical level by the cloning, expression, purification and enzymatic characterization of the PfPKAc. The objective was to obtain an in vitro active PfPKAc to evaluate the kinetic constants of PfPKAc and to conduct the first inhibition studies.

2) at the cellular level by studying the consequences of PfPKAc transcripts inhibition by specific interfering RNAs. The parasite growth but also the overall transcriptome were studied to specify the metabolic pathways associated with this plasmodial protein kinase.

All of these studies improve the understanding of cAMP transduction pathway and PfPKA, which could allow the development of new therapeutic approaches.

Keywords: *Plasmodium falciparum*, protein kinases, PKA, biochemical characterization, RNA interference, microarray, metabolic pathways.