Plasmodium falciparum

Protein Kinase CK2

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Abstract

Malaria, caused by infection with intracellular protozoan parasites of the genus *Plasmodium*, is responsible for 300 to 600 million clinical cases annually (Snow et al., 2005), resulting in the deaths of up to three million people every year (Breman, 2001, Breman et al., 2004). There is a clear need for further research aimed at identifying novel drug targets (Ridley, 2002). Reversible phosphorylation of proteins is a major regulatory mechanism in most cellular processes, and protein kinases are considered promising drug targets, comprising as much as 30% of all protein targets under investigation (Cohen, 2002). The divergences between human and plasmodial protein kinases suggest that specific inhibition of the latter is an achievable goal (Doerig, 2004, Doerig and Meijer, 2007). This study investigates protein kinase CK2 of *Plasmodium falciparum*, seeking to establish by reverse genetics and biochemical approaches whether it represents a possible antimalarial drug target.

Protein-kinase CK2, formerly known as Casein Kinase II, is a dual-specificity (Serine/Threonine and Tyrosine) protein kinase ubiquitously expressed in eukaryotes. It has over 300 cellular substrates catalogued to date (Meggio and Pinna, 2003). Consistent with its multiple substrates, the enzyme plays a crucial role in many cellular processes, and is essential to viability in yeast and slime mould (Padmanabha et al., 1990, Kikkawa et al., 1992). The human CK2 holoenzyme consists of two catalytic α or α' subunits and two regulatory β subunits, and recent evidence indicates that the latter interact with several protein kinases in addition to CK2α (reviewed in (Bibby and Litchfield, 2005)), pointing to a likely role in the integration of numerous signalling pathways. A putative CK2α orthologue and two predicted CK2β subunits were identified in the *P. falciparum* genome (Ward et al., 2004, Anamika et al., 2005). Here we present the biochemical characterisation of the PfCK2α orthologue and both PfCK2β orthologues, and demonstrate by using a reverse genetics approach that each of the three subunits is essential for completion of the erythrocytic asexual cycle of the parasite, thereby validating the enzyme as a possible drug target. Recombinant PfCK2α possesses protein kinase activity, exhibits similar substrate and co-substrate preferences to those of CK2α subunits from other organisms, and interacts with both of the PfCK2β subunits *in vitro*. PfCK2α is amenable to inhibitor screening, and we report differential susceptibility between the human and *P. falciparum* CK2α enzymes to a small molecule inhibitor. Taken together, the data indicate that PfCK2α is an attractive, validated target for antimalarial chemotherapeutic intervention.

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Author's Declaration

I hereby declare that I am the sole author of this work and that I performed all of the work presented, with the following exceptions:

- DNA sequencing was performed by The Sequencing Service at the School of Life Sciences at the University of Dundee (Chapters 3, 4 and 5)

- The pET29-PfCK2a plasmid was produced in the laboratory of Debopam Chakrabarti (University of Central Florida)

- The IC_{50} calculations for the compounds on *P. falciparum* parasites were performed with the help of Audrey Sicard (Chapter 4)

- The data presented in figures 4-11, 4-13, 4-14, and 5-9, and the enzymological characterisation presented in section 4.5.1 (with the exception of the IC_{50} for NEB p6012), were jointly gathered by myself, Renaud Prudent and Claude Cochet, in the laboratory of C. Cochet in Grenoble (INSERM U873, Grenoble, France)

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Abbreviations

μg	Microgram
μl	Microlitre
μM	Micromolar
3’ UTR	Three prime untranslated region
A	Adenine
Abl	Abelson tyrosine kinase
aa	Amino acid(s)
ACT	Artemisinin-combination therapy
AMPPNP	Adenylyl imidodiphosphate
ASKA	Analogue-sensitive kinase allele
ATP	Adenosine triphosphate
AmpR	Ampicillin resistance
APS	Ammonium persulfate
BLAST	Basic local alignment search tool
BME	β-mercaptoethanol
bp	Base pairs
BSA	Bovine serum albumin
BSD	Blasticidin-S-deaminase

C	Cytosine
C-terminal	Carboxy-terminal
CAM	Calmodulin
CamR	Chloramphenicol resistance
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CDPK	Calcium-dependent protein kinase
CKIP-1	CK2-interacting protein 1
CML	Chronic myelogenous leukaemia
D-box	Destruction box
Da	Daltons
DHPS	Dihydroperoate synthase
DHFR	Dihydrofolate reductase
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DRB	5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid

EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
eIF	Eukaryotic translation initiation factor
EIR	Entomological inoculation rate
ePK	Eukaryotic protein kinase
ERK	Extracellular-signal-regulated kinase
FACT	Facilitates chromatin transcription
FAF-1	Fas-associated factor 1
FGF-2	Fibroblast growth factor 2
FKBP	FK506-binding protein
G	Guanine
g	Gram
gDNA	Genomic DNA
GMPPNP	Guanylyl imidodiphosphate
GFP	Green fluorescent protein
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
h	Hour(s)
HA	Haemaglutinin
hDHFR	Human DHFR

HDTAB	Hexadecyltrimethylammonium bromide
HEPES	4-(2-hydrozyethyl)-1-piperazineethanesulfonic acid
HIF-1	Hypoxia-inducible factor 1
HMGB	High mobility group B
HRPII	Histidine-rich protein two
Hs	*Homo sapiens*
HSP	Heat shock protein
HS1	Hematopoietic lineage cell-specific protein 1
IgG	Immunoglobulin G
IP	Immunoprecipitation
IPTG	Isopropyl-b-D-thiogalactopyranoside
KanR	Kanamycin resistance
kb	Kilobases
kDa	Kilodaltons
KIT	Receptor tyrosine protein kinase, also known as CD117
K_m	Michaelis constant
L	Litre(s)
LB	Luria Bertani
LSA-1	Liver-stage antigen 1
M	Molar/moles

MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase (MAPK) kinase
MAPKKK	Mitogen-activated protein kinase kinase (MAPKK) kinase
Mb	Megabases
MBP	Myelin basic protein
MCS	Multiple cloning site
MEK	Mitogen-activated protein kinase (MAPK) kinase
MEKK	MEK kinase
mg	Milligram(s)
ml	Millilitre(s)
mM	Millimolar
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
N-terminal	Amino terminal
NAP	Nucleosome assembly protein
Ni-NTA	Nickel-nitrilotriacetic acid
OD	Optical density
Olig2	Oligodendrocyte lineage transcription factor 2
PAGE	Polyacrylamide gel electrophoresis
Pb	*Plasmodium berghei*

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEXEL	*Plasmodium* export element
Pf	*Plasmodium falciparum*
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PK	Protein kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
PlasmoDB	*Plasmodium* database, www.plasmodb.org
PMSF	Phenyl methyl sulphonyl fluoride
rpm	Revolutions per minute
RNA	Ribonucleic acid
Sc	*Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
SL1	Promoter selectivity factor, a transcription factor for rRNA, consisting of a TATA-binding protein and three associated factors
SNP	Single nucleotide polymorphism
SP	Sulfadoxine-pyrimethamine

T	Thymine
TAE	Tris-acetate containing EDTA
TBB	3,4,5,6-Tetrabromobenzotriazole
TEMED	Tetramethylethylenediamine
Tet	Tetracycline
TFIIIB	Transcription factor IIIB
TG-SDS	Tris glycine SDS
Tris	Tris [hydrozymethyl] aminomethane
UBF	Upstream binding factor
UV	Ultraviolet
V	Volt(s)
V_{max}	Maximum velocity of a reaction
v/v	Volume per volume
WHO	World Health Organisation
WT	Wild type
w/v	Weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1
Zm	*Zea mays*
^{o}C	Degree(s) Celsius

1 Introduction

1.1 Malaria

Malaria, caused by parasitic protozoa of the genus *Plasmodium*, is responsible for the deaths of one million people every year, and more than 500 million clinical cases (Snow et al., 2005). These burdens of morbidity and mortality present a serious hindrance to the socio-economic development of the affected countries (Sachs and Malaney, 2002). Although malaria presents a health risk across large parts of the world (Fig. 1-1), the vast majority of deaths (90%) occur in Sub-Saharan Africa (Greenwood and Mutabingwa, 2002), and in children (Snow et al., 2005).

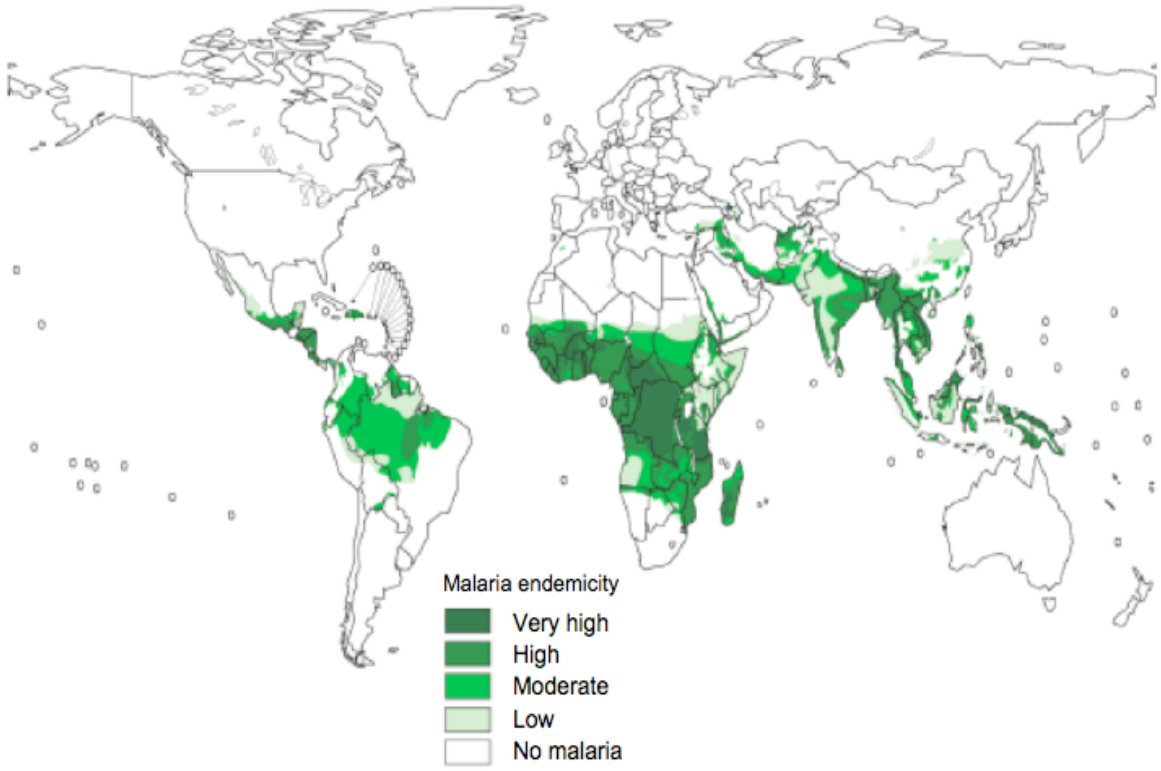

Figure 1-1 World distribution of malaria transmission risk
This image was taken from the World Health Organisation (WHO)'s World Malaria Report, 2005 (http://rbm.who.int/wmr2005/).

Malaria parasites belong to the Apicomplexan phylum, all members of which possess apical organelles required for the invasion of their host. Many vertebrate species can be infected with malaria parasites, including reptiles, birds, rodents, and primates. There are four species of *Plasmodium* parasites that cause malaria in humans: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. Recently, a fifth species has been added to the list: *P. knowlesi*, originally a malaria parasite of long-tailed macaque monkeys, which has recently jumped the species barrier and can naturally infect humans (Singh et al., 2004a). Infections

with the different species lead to differences in symptoms and clinical outcomes. *P. falciparum* is the most deadly, being responsible for the majority of malarial deaths. In part this lethality stems from the unique ability of the mature stages of this parasite to modify its host erythrocyte membrane so that it is able to adhere to endothelial surfaces and sequester in microcapillaries of major organs such as the brain. *P. vivax* is less deadly, but highly debilitating. *P. malariae* can persist for decades as an asymptomatic infection (a period of 53 years has been documented (Guazzi and Grazi, 1963)). *P. vivax* and *P. ovale* can form dormant stages in the liver, known as hypnozoites, which can reactivate and cause relapses, rendering infections with these species difficult to eradicate. Clinical symptoms of malaria include fever, shivering, joint pain, headaches, diarrhoea, vomiting, and in more severe cases, anaemia, respiratory distress, organ failure, and coma and death.

Human malaria parasites are transmitted by the bite of infected female mosquitoes of the *Anopheles* genus. Malaria parasites can be transmitted by a variety of species of anopheline mosquitoes, whose differing behavioural patterns contribute to the epidemiology of the disease (Greenwood et al., 2005). The most globally significant mosquitoes for the transmission of malaria are those of the *Anopheles gambiae* complex, which is predominant in Sub-Saharan Africa. *A. gambiae* feeds preferentially on humans, and has a long life span, making it an excellent transmitter of malaria parasites. The entomological inoculation rate (EIR), a measure of how many infectious bites an individual receives each year, is rarely above 5 in Asia and South America, but can reach over 1000 in parts of Africa (Greenwood and Mutabingwa, 2002). The parasite has obligatory life cycle stages in the mosquito, and therefore the mosquito is a target for anti-malarial intervention.

1.2 Lifecycle of *Plasmodium falciparum*

The parasite lifecycle is complex, involving sexual, and various rounds of asexual, multiplication, and metamorphoses into motile, invasive, intracellular, encysted and dormant forms. Infection of the human host begins with the injection of sporozoites during a bite from an infected female Anopheline mosquito. These motile forms rapidly locate a blood vessel and travel to the liver, where they traverse through several hepatocytes before setting up an infection in a hepatocyte (Mota et al., 2001). The liver stage of the infection is asymptomatic, and lasts roughly six days, during which the parasites undergo huge asexual replication (exoerythrocytic schizogony), with one sporozoite producing up to 30,000 merozoites (Prudencio et al., 2006). These are released into the blood stream where they invade erythrocytes, invaginating the erythrocyte membrane to form a

parasitophorous vacuole surrounding the parasite and separating it from the cytoplasm of the host cell. The parasites develop within the erythrocyte for 48 hours (or 72 hours for *P. malariae*), beginning with the ring stage, during which the parasite begins to export proteins to the host erythrocyte, modifying the membrane and allowing the adhesion to non-infected erythrocytes (rosetting) and to the lining of blood vessels (cytoadherence) (Pouvelle et al., 2000). The parasite grows into the trophozoite stage, the period of the most active feeding and erythrocyte modification (Bannister and Mitchell, 2003). During this stage, exported parasite proteins form knobs on the erythrocyte surface, and proteins such as PfEMP1 bind strongly to the endothelium of blood vessels, sequestering the infected erythrocytes away from the normal circulation and therefore avoiding parasite clearance by the spleen. Adhesion to the blood vessels of the brain can lead to a severe form of malaria known as cerebral malaria, and adhesion to the placenta can adversely affect foetal growth (Bannister and Mitchell, 2003). The parasite feeds on haemoglobin, crystallizing the toxic haem by-products into the non-toxic dark pigment haemozoin, which accumulates in the food vacuole. The parasite is termed a schizont when nuclear division occurs, generating up to 32 nuclei, which bud off from the main body of cytoplasm, and eventually become separate merozoites (erythrocytic schizogony), released on schizont rupture to invade new erythrocytes. This leads to rapid multiplication of parasites within the host, with parasite numbers reaching up to 10^{13} per infected host (roughly 30% parasitaemia). The asexual blood stages of the parasites are responsible for malaria pathogenesis, including the periodic fevers (every 48 or 72 hours) accompanying the rupture of erythrocytes, and are therefore the targets of most of the antimalarial chemotherapies. On invasion of an erythrocyte, some merozoites cease asexual multiplication and differentiate into non-pathogenic, cell cycle arrested male and female sexual forms (gametocytes). The molecular mechanisms underlying this differentiation are not understood (Dyer and Day, 2000), but situations leading to cellular stress, such as sub-lethal doses of antimalarial drugs, high parasitaemia, and host immune responses, stimulate gametocytogenesis (Dyer and Day, 2000, Talman et al., 2004).

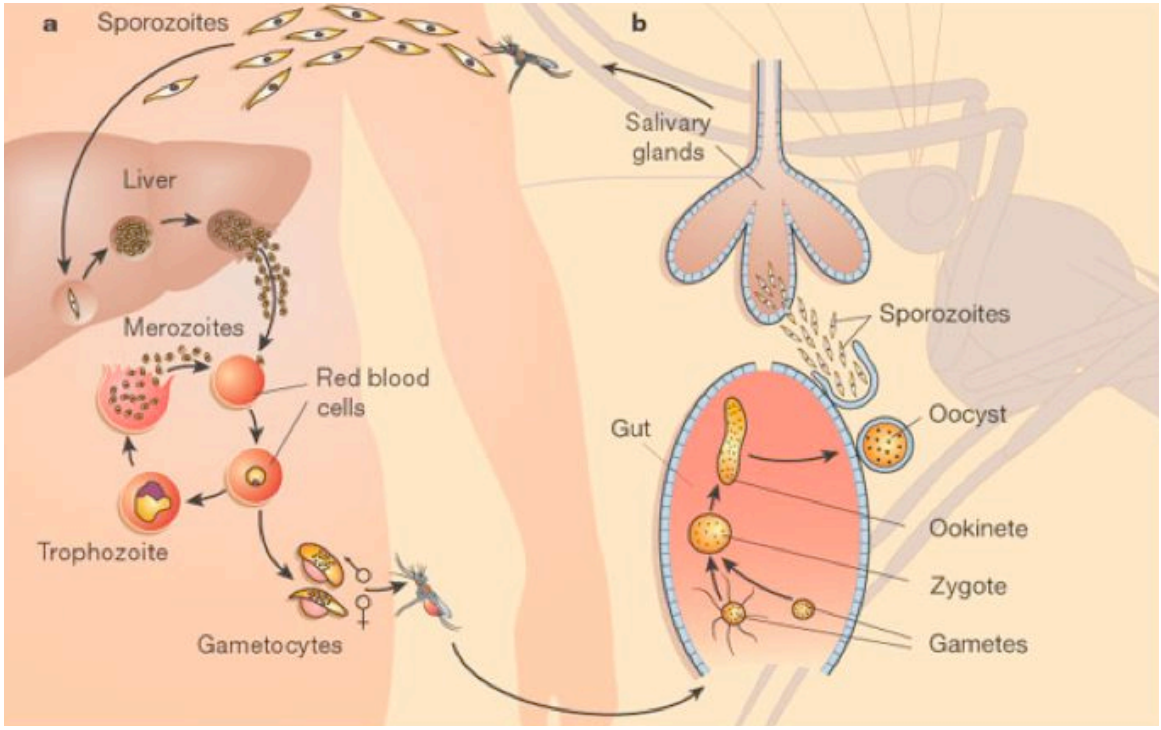

Figure 1-2 Lifecycle of *P. falciparum*
The various stages of the parasite lifecycle are illustrated. A: human host. B: Mosquito host. Reprinted by permission from Macmillan Publishers Ltd: Nature (Wirth, 2002), copyright 2002.

When a female *Anopheles* mosquito takes a blood meal from an infected person, the mature male and female gametocytes (micro- and macrogametocytes) that are taken up into the mosquito develop into gametes and initiate the mosquito infection (sporogonic cycle). The microgametocyte divides to form eight flagellated gametes, a process known as exflagellation, and the macrogametocyte escapes from the erythrocyte membrane. Fertilization of the female gamete leads to the formation of a diploid zygote (the parasite is haploid for most of its lifecycle). The zygote rapidly differentiates into a motile elongated form known as the ookinete, in which meiotic reduction occurs. The motile ookinete traverses the mosquito midgut epithelium and encysts on the outer wall, forming an oocyst. Within this structure, the parasite undergoes intense asexual replication (sporogony) to form thousands of haploid sporozoites. On oocyst rupture, the sporozoites migrate to and invade the mosquito salivary glands, where they wait to be injected into another human host when the mosquito feeds, thus completing the life cycle. The mosquito stages of the lifecycle take two weeks, from ingestion of the mature gametocytes to the presence of infectious sporozoites in the salivary glands, although this time period is influenced by external temperature, thus rendering the distribution of malaria transmission highly temperature-dependent.

1.3 *P. falciparum* biology

P. falciparum has a number of unique features, including the ability to modify the membrane of its host erythrocyte, which mediates the phenomena of rosetting and sequestration, which have already been mentioned. The parasite sequesters in a variety of organs, including the heart, lungs, kidneys, brain, liver, placenta, and subcutaneous tissues. These organs express different receptors on their endothelial cell surfaces, which are all recognised by the same molecule, *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1) (Miller et al., 2002). PfEMP-1 is an antigenically variant protein encoded by the large and diverse *var* gene family (roughly 60 members (Chookajorn et al., 2007)), and is expressed on the surface of infected erythrocytes. The extracellular domain contains multiple adhesion modules, which can recognise the different receptors on the endothelial cell surfaces. The sequestration of parasites prevents their clearance by the spleen, allowing high parasitaemia to develop. Sequestration in different organs can lead to some of the unique pathologies associated with *P. falciparum*: binding to the placenta can lead to low birth weight, premature birth, and anaemia in the mother; sequestration in the brain is related to cerebral malaria.

The parasite exports a variety of proteins to the erythrocyte cytoplasm and cell surface. These exported proteins have functions in cytoadherence, nutrient acquisition and evasion of the host immune response (Charpian and Przyborski, 2008). To reach the erythrocyte cytoplasm, parasite proteins must cross the parasite plasma membrane and the parasitophorous vacuole membrane. Short signal sequences named PEXELs (*Plasmodium* export elements) appear to be responsible for directing many soluble and membrane proteins to the infected erythrocyte (Hiller et al., 2004, Marti et al., 2004), although the mechanistic details of the translocation remain to be elucidated (Charpian and Przyborski, 2008).

The ability to culture the *P. falciparum* parasites *in vitro* (Trager and Jensen, 1976) has been an enormous advantage in the study of the parasite. The genome sequence of the laboratory strain of the human malaria parasite *P. falciparum*, 3D7, was published in 2002 (Gardner et al., 2002). The parasite was revealed to possess a genome of 22.8 Mb, roughly double the size of the *Schizosaccharomyces pombe* genome, on 14 chromosomes that varied between 0.642 and 3.29 Mb in length. One of the striking features of the genome was the extreme A+T bias, with 80.6% of the genome comprised of A or T bases, rising to 90% in the introns and intergenic regions. Gene searches revealed just fewer than 5300

putative genes, which is roughly the same number as are present in the genome of *S. pombe*. 54% of the predicted parasite genes have introns. 60% of the genes encode predicted proteins with insufficient similarity to proteins from other organisms for their functional assignment (thus they are listed as 'hypothetical proteins'), reflecting the divergence in phylogeny of the parasite from other organisms whose sequence had been investigated (Fig. 1-3).

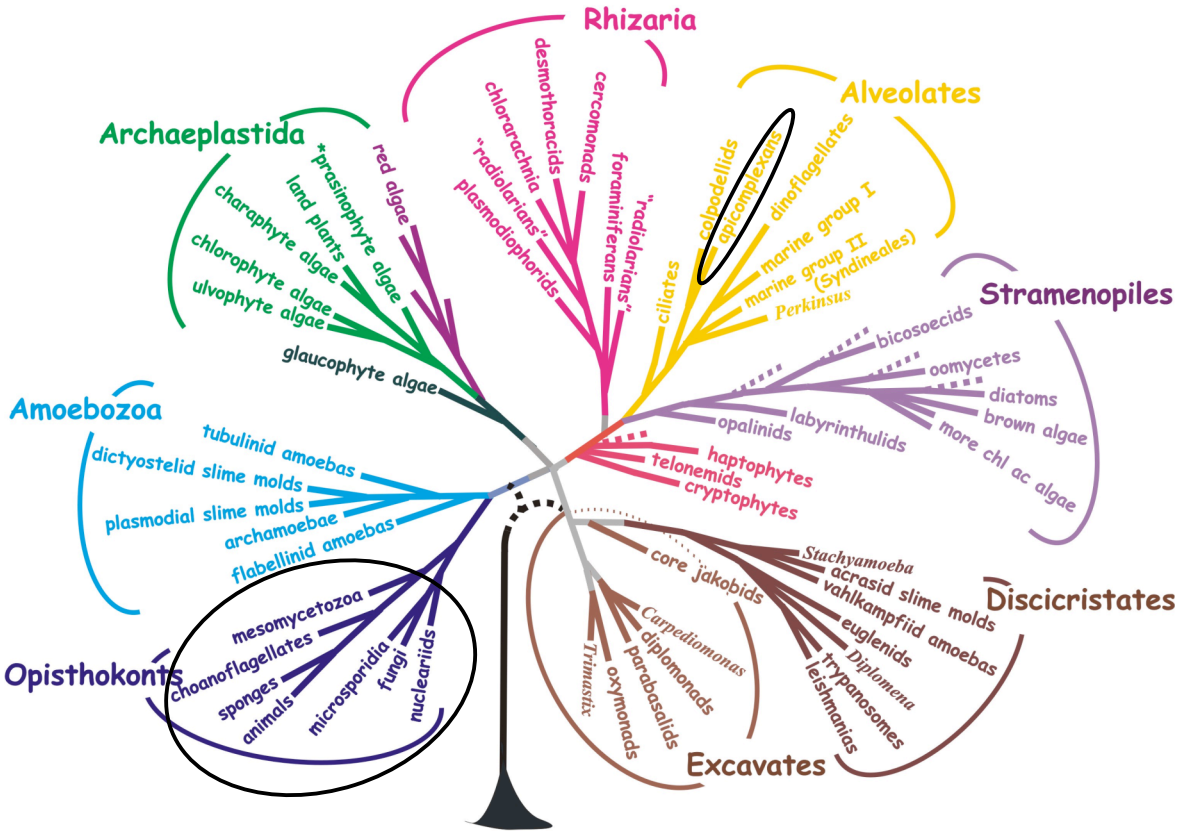

Figure 1-3 Phylogenetic tree of eukaryotes
***P. falciparum* is an apicomplexan parasite, which clusters with the alveolates. The phylogenetic distance of the Apicomplexa from the Opisthokonts (which include humans, yeast, and many other model organisms) is evident. Both groups are circled in black. Personal communication from Prof Sandie Baldauf, University of York.**

1.4 Current chemotherapies

Efforts to control malaria are decreasingly successful due to rising resistance in parasite populations to antimalarial drugs, resistance in mosquito populations to insecticides, and increasing human movements caused by conflict and travel, although "drug resistance is probably the major cause of the deterioration in Africa" (Greenwood et al., 2005). Treatment and prevention of malaria relies heavily on chemotherapy and chemoprophylaxis. There are a number of different antimalarial drugs available, although parasite resistance is becoming an increasing problem, especially for the most commonly

used antimalarials chloroquine and sulfadoxine-pyrimethamine (Fig. 1-4). Resistance arises due to overuse of the antimalarials and over diagnosis of malaria, incomplete treatment of infections, the adaptability of the parasite, and the massive proliferation rate of the parasite, which allows resistant populations to be selected fairly rapidly (Hyde, 2007). The presence on the market of fake drugs, containing sub-lethal concentrations of antimalarials, can also select for resistance. Because *P. falciparum* malaria is the most lethal species, and because it is the one that has developed resistance against multiple drugs, the following summaries of some of the major therapeutic and prophylactic drugs focus upon this species.

1.4.1 Quinolines

Quinine is a naturally-occurring compound derived from *Cinchona* bark, imported into Europe from Peru from the seventeenth century as a cure for malaria (Ridley, 2002). The elucidation of the structure of quinine allowed the synthesis of the artificial 4-aminoquinolines chloroquine and amodiaquine. Chloroquine was the antimalarial of choice for several decades from the mid-forties, being safe, cheap and effective. Resistance to chloroquine was first detected in Thailand in 1957 (Harinasuta et al., 1965), and three more independent foci of resistance arose, in Colombia and Venezuela in 1960 (Moore and Lanier, 1961), and in Papua New Guinea in 1976 (Grimmond et al., 1976). Resistance had spread over most of Africa by 1988, and now only a few places remain where chloroquine is effective (Talisuna et al., 2004). As mentioned above, haem is a toxic by-product of haemoglobin digestion, and is detoxified by the parasite by crystallisation to form inert haemozoin. Chloroquine forms a complex with haem, preventing its crystallisation and therefore maintaining its toxicity. Chloroquine-resistant parasites accumulate much less chloroquine in their digestive vacuoles than sensitive parasites, and the genetic basis for resistance has been traced to the *pfcrt* gene, encoding a putative transporter located in the membrane of the digestive vacuole (Fidock et al., 2000b). These mutations have also been linked to resistance to the related quinoline drugs mefloquine, halofantrine and lumefantrine, although many strains of *P. falciparum* that are resistant to chloroquine remain sensitive to amodiaquine (Winstanley and Ward, 2006). Amodiaquine has been limited in use since the 1980s, when it was causally linked with agranulocytosis in travellers taking the drug for prophylaxis (Ridley, 2002). However, because it remains effective against most chloroquine-resistant parasites, it has been coming back into use.

Primaquine, an 8-aminoquinoline, is used for the treatment of hypnozoites of *P. vivax* and *P. ovale*, in combination with chloroquine or quinine, which potentiate its activity (Alving

et al., 1955). The eradication of hypnozoites is necessary to prevent these dormant stages from reactivating and causing relapse of the disease.

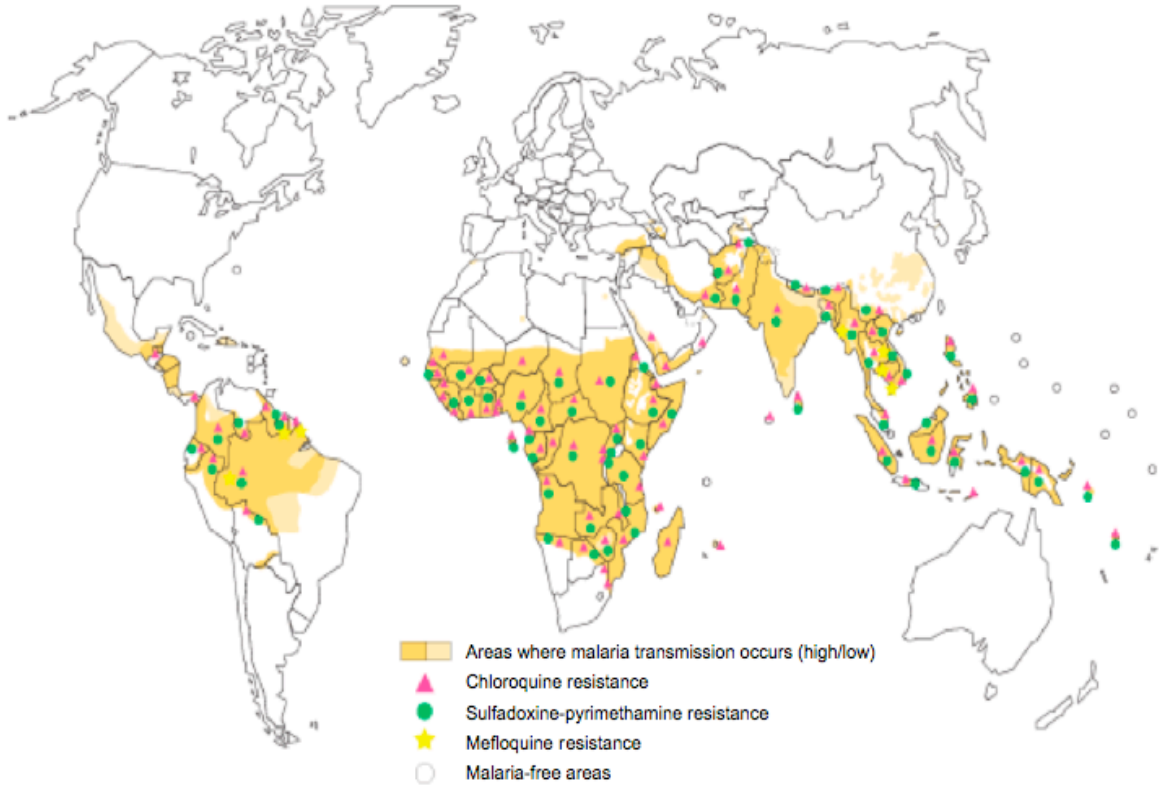

Figure 1-4 Distribution of drug resistance in *P. falciparum*
Drug resistance as monitored in sentinel sites, up to 2004. From the WHO's World Malaria Report 2005 (http://rbm.who.int/wmr2005/).

1.4.2 Antifolates

Antifolates target the folate biosynthesis pathway. The most widely used antifolate is sulfadoxine-pyrimethamine (SP), a combination of 2,4-diaminopyrimidine pyrimethamine, a dihydrofolate reductase (DHFR) inhibitor, and sulfadoxine, an inhibitor of dihydroperoate synthase (DHPS). The effect of these compounds is to block the parasite DNA synthesis (Hyde, 2007), by depleting the stocks of tetrahydrofolate, a cofactor required for DNA synthesis (Winstanley and Ward, 2006). The compounds are synergistic in activity, and are used in combination for the treatment of *P. falciparum* malaria (Hyde, 2007). Many countries adopted SP as the first line drug for treatment of malaria after resistance to chloroquine rendered the drug ineffective. Sulfadoxine and pyrimethamine both have long half-lives, thus new infections are exposed to sub-lethal concentrations of the drugs, facilitating the selection of resistant parasites. Resistance to the sulfadoxine-pyrimethamine combination is now widespread (Fig. 1-4). The genetic basis of resistance is point mutations in the target genes. In South East Asia and South America, parasites

harbouring quadrupally-mutated *dhfr* and doubly-mutated *dhps* are completely resistant to maximum tolerated levels of sulfadoxine-pyrimethamine, and there are indications that these mutation combinations have now spread to Africa (Hyde, 2007).

1.4.3 Artemisinin-Combination Therapies

Artemisinins are derived from the shrub *Artemisia annua*, which has been used in Chinese medicine for centuries in the treatment of malaria and other parasitic diseases. Several semi-synthetic derivatives of artemisinin have been developed, and are now in widespread use. Artemisinin-based drugs are fast acting, offering rapid relief from clinical symptoms, and gametocidal, reducing the carriage of the transmission stages (Talisuna et al., 2004). Recrudescence occurs if artemisinins are used in monotherapy, due to the short half-lives of the drugs (which are rapidly metabolized, with half-lives of roughly four hours), but in combination with other drugs they are now the recommended antimalarial treatment. Their use in combination therapy is recommended by WHO, as it is thought that the rapid clearance of parasites by artemisinin decreases the likelihood of resistance developing against the partner drug, and that the partner drug can eradicate the small numbers of parasites that escape the artemisinin, thus preventing recrudescence. However, if there is already resistance in the field to the partner drug, the artemisinin-combination therapy (ACT) may also be compromised, as is the case with the popular combination artemether-lumefantrine (Coartem®) (Dokomajilar et al., 2006, Duffy and Mutabingwa, 2006, Sisowath et al., 2005), and with artesunate + sulfadoxine- pyrimethamine (Rwagacondo et al., 2003, Staedke et al., 2001). ACTs are relatively expensive drug regimens, and the cost represents probably the biggest challenge to their implementation as the first line treatment in many developing countries (Mutabingwa, 2005).

1.4.4 Antibiotics

Some common antibiotics that target bacterial protein synthesis, such as clindamycin and the tetracyclines, are also effective antimalarials. They are thought to target protein synthesis in the apicoplast, an apicomplexa-specific plastid organelle thought to originate from a green algal symbiont, or the mitochondrion, due to the similarity of these organelles to bacteria (Dahl and Rosenthal, 2008, Dahl et al., 2006, Goodman et al., 2007). These antibiotics are used as prophylactics, and in combination with other antimalarials: quinine plus tetracycline or quinine plus doxycycline are common treatments in South East Asia. However, tetracycline and doxycycline are contra-indicated in children under eight years

of age, so these treatments are unlikely to be widely used in Africa, where the main burden of disease is born by the under fives (Ridley, 2002).

The emergence and spread of resistance in parasite populations highlights the need to develop new antimalarial drugs. Of the 1223 new drugs developed between 1975 and 1996, only 3 were antimalarials (Greenwood and Mutabingwa, 2002). There is an urgent need for further research in order to identify new drug targets (Ridley, 2002, Sahu et al., 2008).

1.5 Protein kinases

Reversible protein phosphorylation was discovered over fifty years ago. (Sutherland and Wosilait, 1955, Fischer and Krebs, 1955, Krebs and Fischer, 1956). This covalent modification of cellular proteins is involved in the regulation of almost all cellular functions, and up to a third of cellular proteins are thought to be phosphorylated (Ahn and Resing, 2001, Ficarro et al., 2002). The addition of phosphate groups to proteins can bring about changes in the stability, localisation, enzymatic activity, or binding properties of the proteins. Enzymes that catalyse protein phosphorylation are known as protein kinases, most of which are members of a large conserved gene family, the eukaryotic protein kinases (ePKs). EPKs are enzymes that catalyse the transfer of the γ-phosphate of ATP (or GTP) to an acceptor hydroxyl residue (serine, threonine or tyrosine) of proteins, and form the largest enzyme family encoded by the human genome, with 478 members (there are also 40 atypical protein kinases, bringing the total protein kinase complement to 518 members in 20 families) (Kostich et al., 2002, Manning et al., 2002). All members of the family share a highly conserved catalytic domain of 250 to 300 amino acids (Hanks et al., 1988), which folds into a common catalytic core structure. The catalytic domain can be divided into eleven conserved subdomains (Hanks et al., 1988), defined as regions that are never interrupted by long stretches of amino acid insertions, and that contain characteristic patterns of conserved residues (Hanks and Hunter, 1995) (Fig. 1-5). Twelve residues in the protein kinase catalytic domain are nearly invariant across the eukaryotic protein kinase family (using the cAMP-dependent protein kinase PKA as the reference): Gly50 and Gly52 in subdomain I, Lys72 in subdomain II, Glu91 in subdomain III, Asp166 and Asn171 in subdomain VIB, Asp184 and Gly186 in subdomain VII, Glu208 in subdomain VIII, Asp220 and Gly225 in subdomain IX, and Arg280 in subdomain XI (Hanks and Hunter, 1995). Some protein kinases are extremely specific, phosphorylating only one or two cellular targets (for example, wee1 and MEK (mitogen-activated protein kinase

(MAPK)/extracellular-signal-regulated kinase (ERK) kinase) (Litchfield, 2003)), whereas others have a much broader specificity.

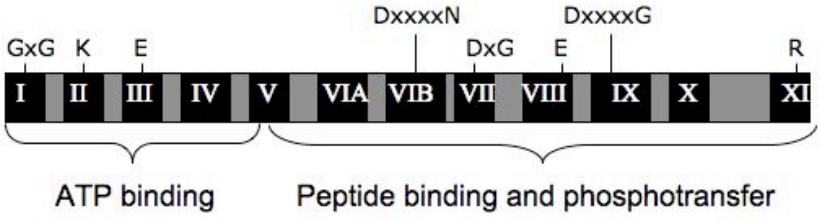

Figure 1-5 Primary structure of the ePK catalytic domain
The eleven conserved subdomains of ePK catalytic domains are shown in the central bar, and the twelve nearly invariant residues conserved across ePKs (Hanks, 2003) are indicated at the top. The two main lobes of the catalytic domain and their main functions are indicated at the bottom.

The catalytic domain of a kinase folds into a two-lobed structure (Knighton et al., 1991): the amino-terminal lobe, involved in anchoring and orientating the nucleotide, includes subdomains I-IV, and has a primarily β-sheet structure, and the C-terminal lobe, which is mainly involved in anchoring the peptide substrate and in initiating phosphotransfer, includes the subdomains VIA-XI, and is primarily α-helical in structure (see Fig. 1-6). Subdomain V residues span the two lobes. The catalytic site is located in the deep cleft between the two lobes.

Figure 1-6 Crystal structure of a protein kinase
The cAMP-dependent PK (PKA) was the first ePK crystal structure to be solved (Knighton et al., 1991). A ribbon diagram of the conserved catalytic core (PKA residues 40 – 280) shared by all protein kinases is shown. Note the bilobal structure, with the N-terminal lobe composed mainly of β-sheets and the C-terminal lobe composed mainly of α-helices. Cover reprinted with permission from AAAS.

There are two main divisions of the ePK family, based on their phosphate acceptor amino acid group: protein-serine/threonine kinases, and protein-tyrosine kinases. The ePK superfamily was divided initially into four families, based on phylogenetic tree analysis (Hanks and Quinn, 1991, Hanks and Hunter, 1995). The AGC group includes the PKA and PKG families of cyclic-nucleotide dependent kinases, the PKC family, the β-adrenergic receptor kinase family, the ribosomal S6 kinase family, and other close relatives. The CaMK group includes the family of kinases regulated by calcium/calmodulin, the Snf1/AMPK group, and other close relatives. The CMGC group includes the family of cyclin-dependent kinases, the MAP kinase family, the glycogen synthase 3 (GSK3) family, the Clk (cyclin-dependent-kinase-like kinase) family, the casein kinase II family, and close relatives. The protein-tyrosine kinase (PTK) group includes the conventional protein tyrosine kinases ('conventional' to distinguish it from other protein kinases that have been reported to exhibit dual specificity, capable of phosphorylating both tyr and ser/thr residues). Members of each family tend to share related functions, and similarities in mode of regulation and substrate specificity (Hanks and Hunter, 1995). Protein kinases of the AGC and CaMK groups tend to phosphorylate at serines or threonines in close proximity to the basic amino acids arginine and lysine. The CaMK group of kinases, as its name suggests, includes kinases activated by calcium or calmodulin. Protein kinases of the CMGC group are, in the most part, proline-directed kinases, phosphorylating residues lying in a proline-rich environment. The cyclin-dependent kinase family, for example, requires a proline residue in the n+1 position of the protein substrate. The casein kinase II family is a notable exception within the CMGC group, showing strong preferences for sites surrounded by acidic residues, with the presence of proline being a negative determinant (Meggio and Pinna, 2003). The TyrK group of kinases phosphorylates specifically on tyrosine residues, and cannot phosphorylate serine or threonine amino acids. They include many membrane-spanning receptor families. With the publication of analyses of the kinomes of increasing numbers of species, three additional major groups of ePKs have been defined (Hanks, 2003): (1) the CK1 group, which includes casein kinase 1 and related enzymes; (2) the STE group, which includes enzymes that were first described during the characterisation of yeast sterile mutants, and which function in the MAPK kinase pathway; and (3) the TKL (tyrosine-kinase like) group of enzymes phylogenetically related to the tyrosine kinases, although they function as serine-threonine kinases.

1.5.1 Protein kinases as drug targets

Reversible phosphorylation is an integral part of many cellular processes, and perturbations in its regulation have been identified in many diseases such as cancer, diabetes and rheumatoid arthritis (Cohen, 2001). Protein kinases are therefore attracting much interest as potential drug targets (Cohen, 2002, Doerig et al., 2002). Initial concerns over the suitability of protein kinases as drug targets, due to the common catalytic mechanism across the kinase family, and the high intracellular concentrations of ATP in relation to the potential concentrations of ATP-competitive inhibitors, have been assuaged by the successful clinical use of drugs based on kinase inhibition (Dancey and Sausville, 2003). The recent success of drugs whose mode of action is kinase inhibition demonstrates that kinase inhibitors can perform as drugs with appropriate selectivity, potency and pharmacokinetic properties (Giamas et al., 2007). The first kinase inhibitor in clinical trials was fasudil hydrochloride, approved in Japan in 1995 (Cohen, 2002). It was followed by imatinib mesylate (Gleevec®, Novartis), the first important drug to be developed against a specific protein kinase (Abelson tyrosine kinase, although it has subsequently been demonstrated that it targets other PKs such as KIT as well (Buchdunger et al., 2000)). Gleevec® was approved for clinical use in America in 2001 and is a successful anti-cancer drug for chronic myelogenous leukaemia. There are now over 60 drugs based on kinase inhibition in clinical development (Giamas et al., 2007), and protein kinases are rapidly becoming major drug targets, comprising 30% of all protein targets under investigation (Cohen, 2002, Giamas et al., 2007), and second only to the G-protein coupled receptors in the number of targets under investigation by the pharmaceutical industry (Doerig and Meijer, 2007). A recent investigation of the druggability of the genome revealed serine/threonine protein kinases as one of the most promising groups for future small molecule inhibitors, comprising up to 20% of the druggable genome (Hopkins and Groom, 2002).

1.5.2 *The* P. falciparum *kinome*

The sequencing of the *P. falciparum* genome (Gardner et al., 2002) allowed an analysis of the entire complement of *P. falciparum* protein kinases (Anamika et al., 2005, Ward et al., 2004). These studies revealed a set of 85 (Ward et al., 2004) – or 99 (Anamika et al., 2005), depending on stringency criteria for inclusion – ePKs, a smaller group than was expected from analogy with other organisms (Ward et al., 2004). The parasite possesses ePKs belonging to all of the major groups with the exception of the STE and TyrK groups

(Fig. 1-7). TyrK are absent from yeast and most other unicellular eukaryotes (Shiu and Li, 2004), and their function has been linked to cell-cell signalling and the multicellular mode of life, so the absence of this group of kinases from *P. falciparum* is perhaps not surprising. The STE group contains enzymes of the MAPK pathways, which are signalling pathways that link stimuli transmitted through cell surface receptors to regulatory targets within the cell. These pathways are composed of a MAPK, a MAPK kinase (MAPKK, or MEK) and a MAPKK kinase (MAPKKK, or MEKK), and are found in all other eukaryotes bar the microsporidian *Encephalitozoon cuniculi*, which has the smallest genome (2.9 Mb) and kinome (32 ePKs) in any eukaryote described to date (Miranda-Saavedra et al., 2007). The absence of the STE group of kinases in *P. falciparum* points to the lack of typical three-component MAPK pathways in the parasite (Ward et al., 2004, Dorin et al., 2005). Other divergences in signalling pathways include the absence of PKC homologues, signalling enzymes present in most other eukaryotes (Doerig and Meijer, 2007), and the presence of calcium-dependent protein kinases (CDPKs), which are found in ciliates, plants, and Apicomplexa, but not in vertebrates (Harper and Harmon, 2005, Doerig, 2004, Kappes et al., 1999).

Unusual features of the *P. falciparum* kinome include a novel group of 20 ePKs unique to the apicomplexa (the FIKK family), at least four ‘composite’ protein kinases with features from more than one ePK family, and a number of parasite ePKs that have no clear homologues in other organisms and do not fall into any of the major groupings, which have thus been classified as ‘orphan’ kinases (Ward et al., 2004). There are large N- or C-terminal extensions in many of the malarial PKs, and occasionally large insertions within the catalytic domain (Doerig, 2004). The phylogenetic distance of *Plasmodium* from vertebrates and from other model organisms (Fig. 1-3) is reflected in the structural and functional features of individual kinases, and the divergent kinase repertoire. The differences between the *P. falciparum* and mammalian kinomes lend weight to the hypothesis that specific intervention can be achieved (Doerig, 2004).

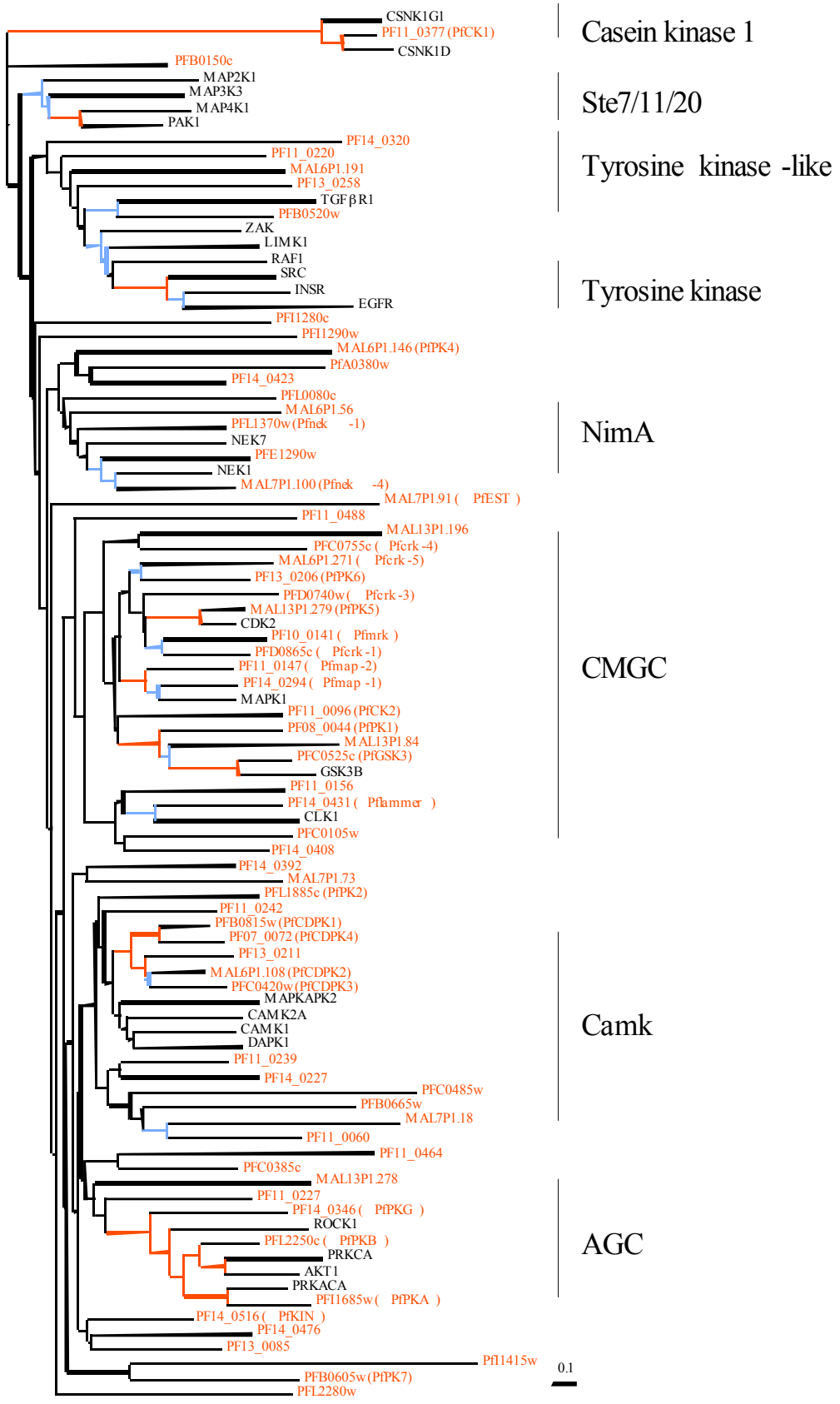

Figure 1-7 The *P. falciparum* kinome
Figure from (Ward et al., 2004). The *P. falciparum* kinome has members from each major grouping of kinases, except for the tyrosine kinases and the STE group. The 65 *P. falciparum* kinases are shown in red, and representative members of each major group of kinases from the human kinome are shown in black. Branches with bootstrap values of over 70 are shown in red, and those with bootstrap values over 40 are shown in blue. The scale bar represents 0.1 mutational changes per residue. For detailed discussion of the kinome, see (Ward et al., 2004). Figure reproduced with permission.

1.6 CK2

Phosphorylation activity from rat liver against exogenous casein was discovered in 1954 (Burnett and Kennedy, 1954), and the kinases responsible were later named casein kinase I (CKI) and casein kinase II (CKII) (Hathaway and Traugh, 1979). It is unlikely that casein kinase II has a role in the *in vivo* phosphorylation of casein (Litchfield, 2003), therefore it was renamed CK2. CK2 is a serine/threonine protein kinase that exhibits extraordinary evolutionary conservation, and orthologues have been found in all eukaryotes whose genomes have been sequenced, including the microsporidian *Encephalitozoon cuniculi*, which possesses an extremely reduced kinome (Miranda-Saavedra et al., 2007). These facts point to a fundamental role for CK2 in eukaryotic cellular function.

CK2 has a number of unusual biochemical properties. It is the most pleiotropic kinase known, with over 300 substrates catalogued so far, most of which have been confirmed *in vivo* (Meggio and Pinna, 2003). Indeed, an analysis in yeast (Ficarro et al., 2002) lead to the suggestion that CK2 is responsible for up to a quarter of the eukaryotic phosphoproteome (Meggio and Pinna, 2003). CK2 is a dual-specificity kinase: although it has long been known as a serine/threonine kinase, many reports in recent years reveal the enzyme's ability to phosphorylate tyrosine (Marin et al., 1999b, Wilson et al., 1997, Chardot et al., 1995). CK2 also has dual-co-substrate specificity, being able to utilize both ATP and GTP as phosphoryl donors (Niefind et al., 1999). Unlike most protein kinases, CK2 phosphorylates at acidic locations, with the consensus sequence S/T-D/E-X-D/E/phosphoS/phosphoY, with the n+3 position being the most significant (Meggio and Pinna, 2003, Songyang et al., 1996, Pearson and Kemp, 1991, Kuenzel et al., 1987). Protein kinases are generally tightly controlled, with their activity turned on by extracellular ligands, second messengers, association or dissociation of regulatory subunits, or phosphorylation/dephosphorylation of residues. CK2 is highly unusual in that it is constitutively active, and not switched on or off under the control of these regulatory devices, and is therefore widely called a secondary messenger-independent kinase.

These unusual biochemical properties, and the importance of the enzyme in cancer and tumorigenesis (Pinna, 2002, Ahmed et al., 2002, Unger et al., 2004, Morales and Carpenter, 2004), have rendered CK2 a popular subject of research and drug discovery efforts. The majority of research has been undertaken on mammalian and yeast CK2. The following sections highlight some of the most significant features of CK2 under the headings of structure, function, and regulation.

1.6.1 Structure

In vivo, CK2 exists mainly as a heterotetramer composed of two catalytic (alpha) subunits and two regulatory (beta) subunits (see Fig. 1-8). Many organisms possess more than one distinct isoform of each subunit. Two isoforms of the alpha subunit have been characterised in humans: alpha and alpha prime (Lozeman et al., 1990), which share 90% identity within their catalytic domains. They are the products of two different genes. More recently, a third isoform has been identified, alpha double prime, almost identical to alpha except for the unique C-terminal domain, and thought to arise from alternative splicing (Shi et al., 2001). Tetrameric CK2 may contain identical or non-identical catalytic subunits (Chester et al., 1995, Gietz et al., 1995). Only one form of the beta subunit has been found in mammals (Bibby and Litchfield, 2005), but other organisms such as *Saccharomyces cerevisiae* and *Arabidopsis thaliana* possess multiple isoforms (Glover, 1998, Salinas et al., 2006). The beta subunit does not display extensive homology to any known protein kinase regulatory units, but is highly conserved amongst species (Allende and Allende, 1995), with the 215 amino acid sequence 100% conserved between chicken and mammals (Maridor et al., 1991).

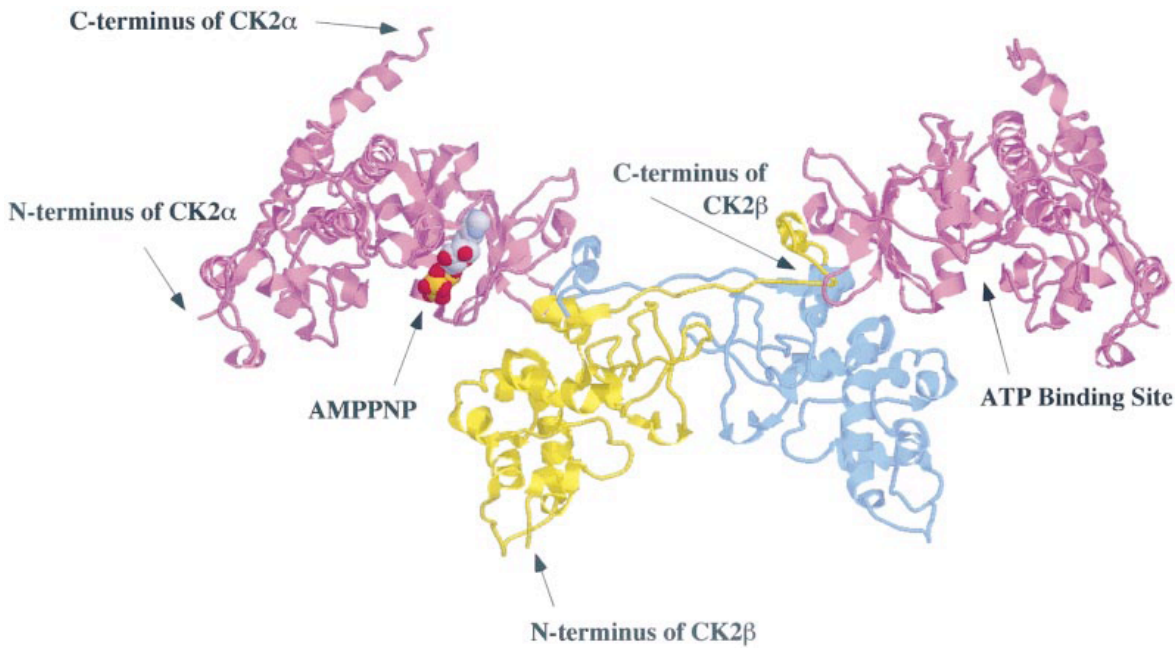

Figure 1-8 CK2 holoenzyme crystal structure
The crystal structure of the recombinant human CK2 holoenzyme (Niefind et al., 2001). Catalytic subunits are shown in pink, and the regulatory subunits in blue and yellow. The holoenzyme was co-crystallized with an ATP analogue, AMPPNP, showing where the active site is located. Figure from (Litchfield, 2003), reproduced with permission from Portland Press Ltd: Biochemistry Journal.

The crystal structure of the recombinant human CK2 holoenzyme (Niefind et al., 2001) established that the central part of the holoenzyme is the dimer of CK2β subunits (Fig. 1-8). Both CK2β subunits make contact with both of the CK2α subunits, which make no

contact with each other. This crystal structure confirms the earlier findings from yeast-two-hybrid screens that beta subunits interact with alpha, alpha' and other beta subunits, and that the CK2α and CK2α' subunits can interact with the beta subunit but not with each other (Gietz et al., 1995). Both CK2α subunits show the typical bilobal structure of ePKs, with the β-sheet-rich N-terminal lobe and the α-helical C-terminal lobe, and the catalytic cleft in between (Niefind et al., 2001).

In vitro, CK2 tetramers form spontaneously by self-assembly around the CK2β dimer, and are stable (Pinna and Meggio, 1997, Graham and Litchfield, 2000). However, when the holoenzyme structure was solved (Niefind et al., 2001) (see Fig. 1-8), it was discovered that the size of the interface of the holoenzyme (832Å^2) is in the range of the non-obligate protein-protein complexes (804Å^2) rather than that of permanent protein-protein complexes, whose average interface size is 1722Å^2 (Jones and Thornton, 1996). These findings suggest that the CK2 tetramer is a transient complex. Live cell imaging corroborates these findings, showing extensive but not exclusive overlap in the localisation of the CK2α and CK2β subunits, and independent translocation into the nucleus (Martel et al., 2001, Filhol et al., 2003). The potential role of the independent subunits is discussed further in section 1.6.2.7.

The crystal structure of the catalytic subunit (Niefind et al., 1998) revealed the structural basis for the constitutive activity of the catalytic subunit. To achieve full activation, many protein kinases require the phosphorylation of residues within their activation loop (Johnson et al., 1996), to convert the enzyme to the active conformation. In contrast, "there is no indication that phosphorylation of the activation loop of CK2α is important for activation of the enzyme" (Olsten and Litchfield, 2004). Instead, the N-terminal region of CK2α stabilizes the active conformation of the free catalytic subunit by extensive contacts with the activation region (Niefind et al., 1998, Sarno et al., 2002), similar to the way by which cyclin A stabilizes the active conformation of CDK2 (see Fig. 1-9). This interaction is essential for the activity of the isolated catalytic subunit (Sarno et al., 2002, Sarno et al., 2000).

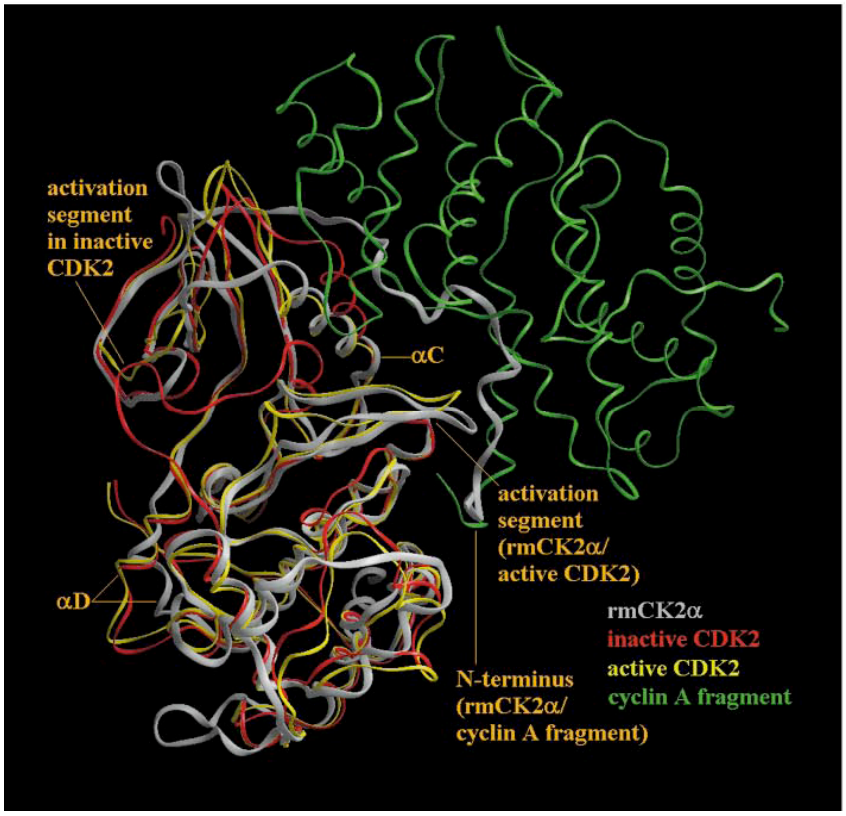

Figure 1-9 Structure of the CK2α subunit
The N-terminus of CK2α stabilizes the active conformation. Superposition of the crystal structures of recombinant maize CK2α (grey), the inactive conformation of CDK2 (red), and the partially active form of CDK2 (yellow) bound to a fragment of cyclin A (green). The N-terminus of maize CK2α forms similar interactions with the activation loop of the kinase to the interactions formed by cyclin A with CDK2, which stabilize the active conformation of the kinase. Reprinted by permission from Macmillan Publishers Ltd: EMBO J (Niefind et al., 1998), copyright 1998.

The ability of CK2 to utilize both ATP and GTP as co-substrate has also been explained by the recent crystal structure: a non-restrictive hydrogen-bonding pattern at the purine binding site allows dual co-substrate specificity (Niefind et al., 1998). Crystal structures of CK2α in complex with the ATP analogue adenylyl imidodiphosphate (AMPPNP) and with the GTP analogue guanylyl imidodiphosphate (GMPPNP) corroborate this finding, revealing that the ability of CK2 to use GTP as well as ATP is due to the provision of enough space at the nucleotide-binding site for shifts in the hydrogen bonding pattern, and to the presence of water molecules that allow the purine bases to utilize the full hydrogen bonding potential of the site (Niefind et al., 1999).

The crystal structure of the core of the regulatory subunit of CK2 (Chantalat et al., 1999) reveals that CK2β has no structural homology to any other known protein. Acidic residues contribute over 40% of the surface area of the monomer. Notable features of the CK2β subunit are shown in Fig. 1-10, and include three phosphorylation sites (see section 1.6.3.3), a destruction box, which may be related to the ubiquitination and degradation of

CK2β (Litchfield, 2003), and an acidic loop, which is responsible for downregulation of CK2 (this downregulation is reversed by the binding of polyamines) and association with the plasma membrane (Leroy et al., 1999, Meggio et al., 1994a). Zinc fingers, held in place by two pairs of cysteine residues, mediate dimerisation of the beta subunits. Mutation of two of these cysteine residues (one of the pairs) resulted in a loss of beta dimer formation (Canton et al., 2001), and these mutant betas also failed to interact with alpha subunits *in vivo* and *in vitro*. The dimerisation of beta units is a necessary precursor to tetramer formation (Graham and Litchfield, 2000). The crystal structure of the regulatory subunit in monomeric form (Chantalat et al., 1999) and in the holoenzyme (Niefind et al., 2001) reveals that the large insertion sequences seen in organisms such as *Arabidopsis thaliana* and *Saccharomyces cerevisiae* are found in areas that are distant from the core, and which form loops out from the core structure, therefore they are unlikely to interfere with the conserved structure of the CK2β subunit. *P. falciparum* also has lengthy insertion sequences in these locations in one of its two beta subunit isoforms (PfCK2β2, see Chapter 5).

The C-terminus of the regulatory subunit binds to the alpha units and is responsible for enhancing and stabilising CK2 activity (Bibby and Litchfield, 2005, Marin et al., 1997, Sarno et al., 2000) ("positive regulatory region" in Fig. 1-10). The beta chain contacts the alpha chain within the conserved kinase core, which may explain the experimental observations that free beta subunits are able to interact with other protein kinases such as A-Raf (Boldyreff and Issinger, 1997) and c-Mos (Chen et al., 1997). The crystal structure of the holoenzyme revealed that the beta C-terminal tail is involved in beta-beta dimerisation as well as in alpha-beta contacts (Niefind et al., 2001).

Polymerisation of CK2 into filaments and rings has also been observed (Glover, 1986, Valero et al., 1995), under conditions that may occur *in vivo*. Molecular modelling of these filaments (Poole et al., 2005), combined with biochemical data on autophosphorylation (Pagano et al., 2005), lead to hypotheses that the filamentous form of CK2 is inactive against exogenous substrates (although capable of autophosphorylation) (Poole et al., 2005), thus opening up another possible regulatory mechanism for CK2 in addition to those reviewed in section 1.6.3. At present, however, there is no direct evidence for the formation of such higher order CK2 structures *in vivo*.

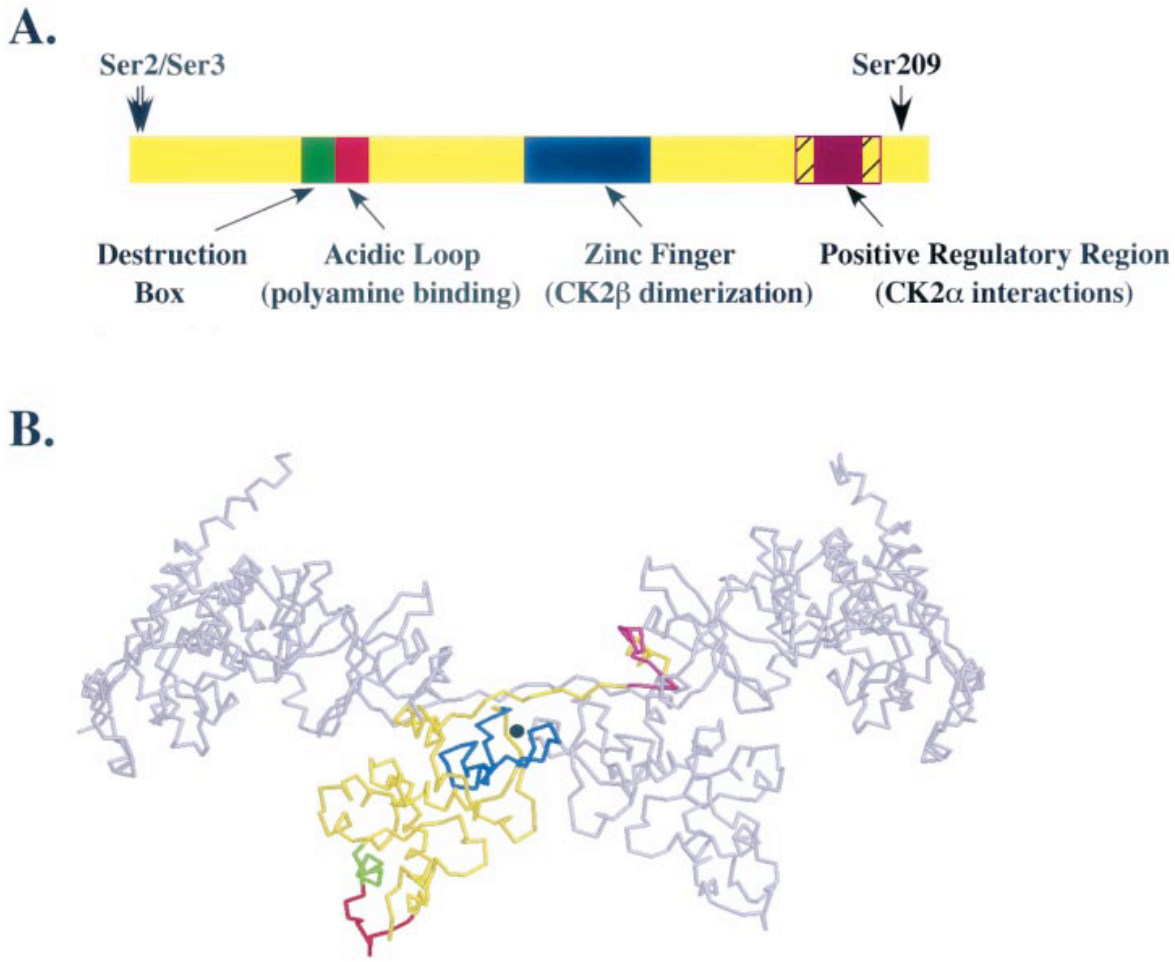

Figure 1-10 Structure of the CK2β subunit
A. Primary structure of the human CK2β subunit, showing key features mentioned in the text. B. The crystal structure of the CK2 holoenzyme with one CK2β subunit highlighted in yellow, and showing the location of the motifs from part A. The black dot represents the Zn^{2+} that is involved in the formation of the zinc finger. Figure from (Litchfield, 2003), reproduced with permission from Portland Press Ltd: Biochemistry Journal.

1.6.2 Function

CK2α has been shown to be essential for viability in all organisms in which its role has been assessed, such as *Saccharomyces cerevisiae* (Padmanabha et al., 1990) and *Dictyostelium discoideum* (Kikkawa et al., 1992). The requirement for the beta subunit of CK2 for viability varies across species, with disruption of the beta subunit being lethal in mice (Buchou et al., 2003) and *Caenorhabditis elegans* (Fraser et al., 2000), whereas disruption of either or both of the CK2β subunits in *S. cerevisiae* is not lethal (Ackermann et al., 2001, Bidwai et al., 1995, Reed et al., 1994). Disruption of the beta gene of *Schizosaccharomyces pombe* yields a cold-sensitive phenotype, slow growth, and cell shape abnormalities (Roussou and Draetta, 1994).

Studies on species possessing more than one isoform of a subunit demonstrate that the isoforms exhibit substantial but incomplete functional compensation. *Saccharomyces cerevisiae* has two catalytic subunit isoforms, CKA1 and CKA2. Disruptions in either one can be tolerated, indicating functional compensation, but disruption of both simultaneously is lethal (Chen-Wu et al., 1988, Padmanabha et al., 1990). Temperature-sensitive alleles of CKA1 and CKA2 exhibit distinct phenotypes (Glover, 1998), indicating incomplete functional overlap. Knockout of CK2α1 in mice is embryonic lethal (Lou et al., 2008), whereas knockout of CK2α2 is not lethal, although male mice with knockouts in CK2α2 exhibit spermatogenesis defects and are infertile (Escalier et al., 2003, Xu et al., 1999), indicating that CK2α1 cannot compensate entirely for the loss of CK2α2. Overexpression of a kinase-inactive form of CK2α' in human osteosarcoma U2-OS cells lead to a dramatic reduction of proliferation, which was not seen with overexpression of a kinase-inactive form of CK2α (Vilk et al., 1999). These examples show that functional overlap between the isoforms is incomplete, with the different forms of the subunits performing distinct functions.

The *Drosophila* gene encoding the CK2 alpha subunit is capable of rescuing haploid *S. cerevisiae* cells lacking both alpha genes (which would otherwise be inviable), implying that function as well as structure of these genes is highly conserved across evolution (Padmanabha et al., 1990).

CK2 is probably the most pleiotropic of all protein kinases, with over 300 substrates identified so far (Meggio and Pinna, 2003). Its pleiotropy is reflected in its myriad functions: the enzyme has been implicated in a wide array of cellular processes, including differentiation, proliferation, survival, translation, apoptosis, transformation and tumorigenesis, RNA synthesis, cell cycle progression, cell morphology and polarity, cellular responses to stress and to DNA damage, and circadian rhythms (Bibby and Litchfield, 2005, Canton and Litchfield, 2006). Some of these functions are highlighted in more detail below.

1.6.2.1 Proliferation and cell cycle regulation

CK2 plays a significant role in the control of cellular proliferation. The enzyme phosphorylates a range of DNA binding proteins, nuclear oncoproteins and transcription factors involved in cellular proliferation (topoisomerase II, c-Myb, c-Myc, Max, c-Jun, serum response factor, and the p53 tumour suppressor), and is thereby implicated in the control of cellular proliferation (Olsten and Litchfield, 2004). Other experimental

observations concur: gene disruption of CK2β in *Schizosaccharomyces pombe* produces a slow growth phenotype, indicating a role for the CK2 enzyme in proliferation (Roussou and Draetta, 1994); induced expression of catalytically inactive CK2α' in human osteosarcoma U2-OS cells resulted in a significant decrease in proliferation (Vilk et al., 1999), and growth stimuli cause a rapid translocation of CK2 to the nuclear matrix, which has been suggested to be an important feature of early growth control (Yu et al., 2001). Tumor and leukemic cells exhibit higher levels of expression of CK2 (Daya-Makin et al., 1994, Faust et al., 1996, Munstermann et al., 1990). In general, cells exhibiting higher rates of proliferation have higher levels of CK2 (Litchfield, 2003, Munstermann et al., 1990). CK2 has also been implicated in the regulation of cell division (Bosc et al., 1999). CK2 interacts with the replication checkpoint protein Pin1 (Messenger et al., 2002, Winkler et al., 2000), yeast CK2 is necessary for the G_1/S and G_2/M cell cycle transitions (Hanna et al., 1995, Glover, 1998), and CK2 is required at multiple stages of the mammalian cell cycle (Pepperkok et al., 1994). However, the precise role of CK2 in cell-cycle progression remains largely unknown (Oh et al., 2007).

1.6.2.2 Transcription

Several studies analysing the substrates or interacting proteins of CK2 have highlighted the predominance of substrates/interactors involved in transcription, chromatin structure, and gene expression (Ackermann et al., 2001) (Gyenis and Litchfield, 2008, Meggio and Pinna, 2003, Poole et al., 2005), implicating regulation of transcription as one of the major roles of the enzyme. Knockout of the gene coding for CK2α1 in mice resulted in the reduced abundance of a number of mRNAs (Lou et al., 2008). Substrates of CK2 include subunits of all three classes of RNA polymerases (Glover, 1998), for example, CK2 associates with and activates the TATA binding protein of TFIIIB, a step which is necessary for RNA polymerase III transcription (Ghavidel and Schultz, 2001), and CK2 regulates RNA polymerase I transcription re-initiation by stabilizing the interaction between the associated factors SL1 and UBF (Lin et al., 2006). Analyses of CK2 null mutants in yeast implicate CK2 in chromatin remodelling (Barz et al., 2003). CK2 associates with key molecules involved in translation initiation, such as eukaryotic translation initiation factors 5 (eIF5) (Homma et al., 2005) and 2 (eIF2) (Gil et al., 1996, Llorens et al., 2003); and with key molecules involved in elongation, such as transcription elongation factor-1 (Elf1) (Prather et al., 2005). A recent study using oligonucleotide array chips to examine the effects of functional deletion of the different subunits of CK2 on global gene transcription in yeast (Ackermann et al., 2001) demonstrated that CK2 has a distinct role in the control of gene

transcription, with 118 genes affected with a functional knockout of CK2α1, 57 with CK2α2, and 54 with a double knockout of the beta genes. Thus there are distinct functional differences in the catalytic isoforms in gene targeting, and the beta subunits also have a role in the control of transcription.

1.6.2.3 Apoptosis

CK2 has been widely reported to have a role in apoptosis (Ahmed et al., 2002, Litchfield, 2003). CK2 inhibitors have been reported to induce apoptosis (Faust et al., 2000, Ravi and Bedi, 2002, Ruzzene et al., 2002, Fan et al., 2008), and an increase in expression of CK2 is protective against drug-induced apoptosis (Guo et al., 2001). CK2 inhibits apoptosis and controls caspase activity following DNA damage (Yamane and Kinsella, 2005a). The consensus target sequences for CK2 and caspases are similar, suggesting that CK2 may achieve its antiapoptotic role by phosphorylating caspase cleavage sites and therefore inhibiting the cleavage of important cellular proteins (Ruzzene et al., 2002, Litchfield, 2003, Ahmed et al., 2002). Supporting evidence is provided by proteins such as Bid, Max and HS1, which are phosphorylated by CK2 and as a result are protected from caspase-mediated cleavage (Pinna, 2002).

1.6.2.4 Cellular response to stress

CK2 is involved in cellular responses to a number of different stresses, such as ionizing radiation, heat shock, hypoxia, and DNA damage. CK2 participates in inhibition of apoptotic responses and negatively regulates caspase activity after ionizing radiation and DNA damage (Yamane and Kinsella, 2005a, Yamane and Kinsella, 2005b).

CK2 activates heat-shock factor-1 by phosphorylation, allowing the protein to bind heat shock elements in the nucleus (Soncin et al., 2003), thus indicating that CK2 has important functions in cellular response to heat shock. CK2 is also relocated after cellular exposure to heat stress (Gerber et al., 2000), being trafficked between cytosolic and nuclear compartments in response (Davis et al., 2002).

Hypoxia increases the expression of CK2β, and induces a relocalization of this subunit to the plasma membrane (Mottet et al., 2005). It also induces the nuclear translocation of the CK2α subunit, and increases CK2 activity, which may be involved in regulating the transcriptional activity of HIF-1, a transcription factor playing a major role in cellular adaptation to hypoxia (Mottet et al., 2005).

CK2 has been implicated in cellular responses to DNA damage (Ghavidel and Schultz, 2001, Toczyski et al., 1997, Morales and Carpenter, 2004, Loizou et al., 2004). CKB2 of yeast has been implicated in adaptation and recovery from the G2 arrest caused by DNA damage (Toczyski et al., 1997). CK2 phosphorylates the scaffold protein XRCC1, enabling the formation of DNA single strand break repair complexes *in vitro*, and at sites of chromosomal damage (Loizou et al., 2004). CK2 catalytic subunits dissociate from the TFIIIB complex in response to DNA damage, repressing RNA polymerase III transcription (Ghavidel and Schultz, 2001).

1.6.2.5 CK2 in erythrocytes

It has long been known that casein-phosphorylating kinases exist in the cytosolic and membrane fractions of erythrocytes (Boivin et al., 1980, Simkowski and Tao, 1980, Tao et al., 1980). CK2 has been purified from the erythrocyte cytosol and membranes, and can phosphorylate spectrin, ankyrin and adducin, but not actin (Wei and Tao, 1993). Wei and Tao (1993) hypothesize a role for CK2 in the regulation of cytoskeletal protein interactions. This hypothesis is supported by the finding that CK2 is the major ankyrin kinase in chicken erythroid cells, and regulates the ability of ankyrin to bind to spectrin (Ghosh et al., 2002).

The COP9 signalosome is a multi-subunit conserved protein complex involved in the control of ubiquitin-proteasome-mediated protein degradation (Wei and Deng, 2003). In erythrocytes, CK2 associates with the COP9 signalosome, potentially to regulate ubiquitin-conjugate formation (Uhle et al., 2003).

1.6.2.6 Function of the CK2β subunit in the tetramer

The various roles of the CK2β subunit have been reviewed recently (Litchfield, 2003, Bibby and Litchfield, 2005). Although not absolute regulators of CK2 activity in a manner analogous to Protein Kinase A, the CK2β subunits have important roles in the assembly of CK2, in enhancing the catalytic activity and stability of the catalytic units, and in modulating the substrate specificity of CK2 (Litchfield, 2003). Dimers of beta subunits are a necessary precursor to CK2 holoenzyme formation (Luscher and Litchfield, 1994), as outlined above in section 1.6.1. CK2β can enhance the catalytic activity of alpha by between 4 and 10 fold (Romero-Oliva et al., 2003, Bidwai et al., 1994, Bodenbach et al., 1994). The activity towards the synthetic peptide RRDDDSDDD is stimulated by a factor of four by the CK2β subunit (Boldyreff et al., 1994b). The CK2β subunit can decrease the

activity of CK2α towards some substrates, for example, calmodulin (Marin et al., 1999a). However, these stimulatory and inhibitory effects of the beta subunit are strongly dependent on the salt concentration and the presence of polyamines or polybasic peptides (Pinna and Meggio, 1997), and as a result, the CK2β subunit has been termed "an environmental- and substrate-dependent modulator of CK2α activity rather than an on-off switch" (Niefind et al., 2001). The two main ways in which the CK2β subunit modulates the activity of CK2 are through its role as a substrate-docking site, and through interaction with regulatory proteins (Bibby and Litchfield, 2005). A number of CK2β-dependent interacting partners of CK2 have been identified (Bibby and Litchfield, 2005). For example, topoisomerase II and p53 depend on interactions with CK2β in order to be phosphorylated by CK2 (Appel et al., 1995, Bojanowski et al., 1993). Regulatory proteins can exert their effect on CK2 substrate specificity through their interaction with CK2β, one example being FGF-2, which stimulates the *in vitro* activity of CK2 towards nucleolin through its interaction with CK2β (Bonnet et al., 1996). The CK2β units have a role in the localisation of the kinase, mediating the association of the CK2 holoenzyme with the plasma membrane (Leroy et al., 1999, Sarrouilhe et al., 1998), and are necessary for its export as an ectokinase (Rodriguez et al., 2005, Rodriguez et al., 2008).

1.6.2.7 CK2-independent functions of the alpha and beta subunits

In addition to having many and varied roles as part of the CK2 heterotetramer, the catalytic and regulatory subunits have CK2-independent functions. Free populations of both subunits have been detected, in locations where the other subunit is absent (Filhol et al., 2003, Guerra et al., 1999, Krek et al., 1992, Salinas et al., 2006, Faust et al., 2001). Two recent studies of protein complexes in the yeast proteome found that the catalytic and regulatory CK2 subunits show incomplete overlap, with all four (yeast has two CK2α and two CK2β genes) found in some multi-protein complexes, but only one, two or three of the subunits found in other protein complexes (Bibby and Litchfield, 2005, Gavin et al., 2002, Ho et al., 2002). The independent functions of the beta subunit have been recently reviewed (Bibby and Litchfield, 2005). CK2β bound to over 30 different proteins in yeast two-hybrid studies, and in the case of the PKs c-Mos, A-Raf and Chk1, the region of CK2β responsible for interaction is the same stretch which binds CK2α (Olsten and Litchfield, 2004), indicating that only the uncomplexed beta units are able to interact with these kinases. CK2β has been shown to enhance the activity of A-Raf and Chk1, and reduce the activity of c-Mos (Chen et al., 1997, Guerra et al., 2003, Hagemann et al., 1997). It has been speculated that CK2β could be acting as a substrate docking site and transducer of

regulatory signals for these kinases in an analogous manner to the way in which it acts in the CK2 complex (Bibby and Litchfield, 2005). *In vitro*, CK2β interacts with the cell-cycle regulator PK Wee1, removing its inhibition of the kinase CDK1, which suggests that the independent CK2β subunit may function in the regulation of protein kinases involved in cell-cycle progression (Olsen and Guerra, 2008). CK2α as a free subunit is able to phosphorylate calmodulin (Benaim and Villalobo, 2002, Marin et al., 1999a, Meggio et al., 1987), but tetrameric CK2 is not, implying that CK2α may have CK2-independent substrates and functions. Interactions between CK2 and CK2-interacting proteins are often mediated by just one of the subunit types (Olsen and Guerra, 2008, Gyenis and Litchfield, 2008), and thus these interactions may also occur with the free subunits.

1.6.3 Regulation

CK2 is independent of second messenger small molecules such as calcium, lipids and cyclic nucleotides that act as activators for many cellular enzymes including some PKs (Olsten and Litchfield, 2004). The natural assumption given the pleiotropy of CK2 and its importance in myriad cellular functions is that it would be tightly regulated. The catalytic subunit of CK2 is associated with regulatory subunits, but unlike other heterooligomeric kinases such as Protein Kinase A and the cyclin-dependent kinases, both the CK2 holoenzyme and its free catalytic subunits are constitutively active *in vitro* (Olsten and Litchfield, 2004).

However, CK2 is not unregulated: there is evidence for regulation by expression and assembly, phosphorylation, interactions with small molecules and other proteins, and by localisation.

1.6.3.1 Expression and assembly

Given the importance of the interaction of the alpha and beta subunits for the function of the CK2 holoenzyme, and the emerging picture of the CK2-independent functions of the two subunits, one obvious area of regulation for the kinase is in the relative proportions of CK2α and CK2β subunits, and the assembly and disassembly of the tetramer. Studies have indicated that there may be imbalances in the levels of CK2α and CK2β subunits within certain cells (Guerra et al., 1999, Stalter et al., 1994), and that beta subunits are synthesized in excess of alpha units, and the uncomplexed units are rapidly degraded (Luscher and Litchfield, 1994). The subunits may cross-regulate: loss of CK2α1 in mouse embryos leads

to a reduction in levels of CK2β (Lou et al., 2008). This is also observed when CK2α levels in cells are reduced using siRNA (Seldin et al., 2005). However, all CK2 subunits are expressed throughout the cell cycle (Bosc et al., 1999).

CK2 substrate preferences and activity can be modulated by the presence of the beta subunit, as outlined above, and there are many reports of the independent existence of CK2 subunits (Filhol et al., 2003, Ghavidel and Schultz, 2001, Guerra et al., 1999, Krek et al., 1992, Martel et al., 2001). Together with the observations that the tetrameric form of CK2 has an area of interaction much more in keeping with that of a non-obligate complex than a permanent association, these data indicate that regulation of CK2 may occur through the regulated assembly and disassembly of the CK2 tetramer. For example, on DNA damage, there is a decrease in the amount of CK2, and the subunits dissociate (Ghavidel and Schultz, 2001). The localisation of the holoenzyme and that of the individual subunits is thought to play a large part in the regulation of the enzyme.

1.6.3.2 Localisation

Localisation is important for the function of CK2 (Lorenz et al., 1993). Although predominantly nuclear (Krek et al., 1992, Huh et al., 2003), CK2 has also been shown to be localised to the cytoplasm, and a number of organelles such as the golgi, endoplasmic reticulum and ribosomes, and in the chloroplasts of plants (Salinas et al., 2006, Faust et al., 2001, Issinger, 1977). CK2α is also exported as an ectokinase in human cells (Walter et al., 1994, Rodriguez et al., 2005, Rodriguez et al., 2008) in epithelial cells, neutrophils, platelets and endothelial cells (Seger et al., 2001), and phosphorylates extracellular Vitronectin, possibly regulating the adhesion of cells to the extracellular matrix (Seger et al., 2001, Stepanova et al., 2002), C9 complement, regulating the control of cell lysis by this factor (Bohana-Kashtan et al., 2005), and collagen XVII receptor (Zimina et al., 2007). There is abundant evidence for the regulation of CK2 via its localisation, with CK2 altering its subcellular location in response to a range of stimuli. Growth stimuli cause a rapid translocation of CK2 to the nuclear matrix, which has been suggested to be an important feature of early growth control (Yu et al., 2001); CK2 may also be targeted to the nuclear matrix in a cell-cycle dependent manner (Wang et al., 2003); CK2 is trafficked between cytosolic and nuclear compartments in response to heat shock (Davis et al., 2002, Gerber et al., 2000); and hypoxia induces a relocalization of CK2β to the plasma membrane and the nuclear translocation of the CK2α subunit (Mottet et al., 2005). Under fluorescence imaging of live cells, the different subunits appear to exhibit different nuclear localisation dynamics, which, given the alterations to CK2α activity and specificity that

CK2β can make, indicates that independent localisation of the CK2 subunits may be an important mechanism of regulation (Filhol et al., 2003, Martel et al., 2001). The subcellular location of the CK2 holoenzyme and the individual CK2 subunits will also be likely to have an impact on their availability to other forms of regulation, and it is postulated that there may be many distinct, independently-regulated subpopulations of CK2 within the cell (Litchfield, 2003, Olsten and Litchfield, 2004).

1.6.3.3 Phosphorylation

Phosphorylation is not an absolute requirement for the activation of CK2, as it is, for example, for the MAP kinases (Litchfield, 2003). The autophosphorylation of CK2β at Ser2 and Ser3 (Boldyreff et al., 1993a, Litchfield et al., 1991) serves to enhance the stability of the subunit (Zhang et al., 2002) and therefore of the tetramer. This may regulate ubiquitination and proteasome-dependent degradation of CK2β (Zhang et al., 2002), and perhaps the association of tetramers into filaments of CK2 (Olsten and Litchfield, 2004), which have been proposed as inactive forms of CK2. The human CK2β is phosphorylated at S209 in a cell-cycle dependent manner by $p34^{cdc2}$ (Litchfield et al., 1995, Litchfield et al., 1991, Meggio et al., 1995), although the function of this phosphorylation is unknown. CK2α is also phosphorylated by $p34^{cdc2}$ (but alpha prime is not) in mitotic cells, indicating that the CK2α isoforms are differentially regulated during mitosis (Bosc et al., 1999, Litchfield et al., 1992). When CK2α is phosphorylated by $p34^{cdc2}$, it interacts with Pin1, and this interaction inhibits the phosphorylation of Topoisomerase II by CK2 (Messenger et al., 2002). This is an example of how the phosphorylation of a specific target may be regulated, by a phosphorylation-specific interaction.

There is evidence that HsCK2α can undergo autophosphorylation at tyrosine 182 *in vitro* (Donella-Deana et al., 2001), but tyrosine phosphorylation of CK2 has not been detected in intact cells, and the physiological significance of this observation is unknown (Olsten and Litchfield, 2004).

1.6.3.4 Interactions with small molecules

The activity of CK2 is not absolutely regulated by calcium, lipids and cyclic nucleotides, and the enzyme has therefore traditionally been classed as a messenger-independent kinase. However, it is possible that small molecules do make a contribution to its regulation. It is well established that CK2 is inhibited by negatively charged compounds such as heparin, and stimulated by positively charged compounds such as polyamines *in*

vitro (Tuazon and Traugh, 1991, Boldyreff et al., 1993b, Meggio et al., 1992b). There is also some evidence for the role of polyamines in activating CK2 *in vivo* (Olsten and Litchfield, 2004). Recent studies indicate that phosphoinositides can activate CK2 whose activity has been lowered by a negatively charged inhibitor (Solyakov et al., 2004).

1.6.3.5 Interactions with proteins

The essential role of protein-protein interactions in many signal transduction events has been reviewed elsewhere (Pawson and Nash, 2000, Pawson and Scott, 1997). CK2 has multiple substrates in different cellular locations, but it is not clear how different subpopulations of CK2 are targeted to different subcellular locations. Protein-protein interactions might be responsible. A subset of the proteins that interact with CK2 could be potential adaptor/scaffold proteins, such as tubulin, FAF-1, and CKIP-1 (Bosc et al., 2000, Kusk et al., 1999, Meggio and Pinna, 2003, Olsten and Litchfield, 2004).

Protein partners of CK2 can be involved in several processes: (1) enzyme-substrate interactions (the majority of interactions fall into this category); (2) direct modulation of the catalytic activity of CK2, (e.g. FGF-1 and -2, HSP90, and cdc37); (3) indirect regulation of CK2 by acting as adaptors/scaffolds/targeting proteins, for example, CKIP-1 targets CK2α to the plasma membrane (Olsten et al., 2004); (4) disruption/enhancement of CK2 activity towards specific substrates (e.g. Pin1 interacts with CK2 in a phosphorylation-dependent manner to inhibit the phosphorylation of topoisomerase II (Messenger et al., 2002), and the FACT complex interacts with CK2, facilitating the phosphorylation of p53 (Keller et al., 2001)).

1.6.4 Plasmodium falciparum *CK2*

Phylogenetic analysis of plasmodial ePKs (Ward et al., 2004, Anamika et al., 2005) identified sequence PF11_0096 (hereafter called PfCK2α) as a CK2α orthologue (see Fig. 1-11), and PF11_0048 and PF13_0232 as putative CK2 beta subunits (hereafter referred to as PfCK2β1 and PfCK2β2, respectively).

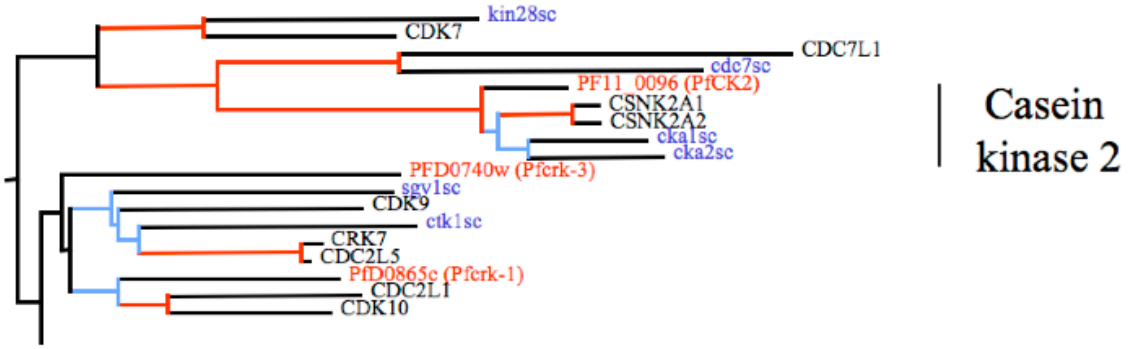

Figure 1-11 PfCK2α clusters with human and yeast CK2α on a three-species kinase tree
A portion of a three-species kinase tree of the CMGC group of kinases from *P. falciparum*, human and yeast is shown. The *P. falciparum* kinases are shown in red, the human kinases in black and the yeast kinases in blue. Branches with bootstrap values of over 70 are shown in red, and those with bootstrap values over 40 are shown in blue. Figure taken from (Ward et al., 2004); reproduced with permission.

Genome-wide analyses of transcription reveal that all three subunits are expressed throughout the asexual erythrocytic stage of the parasite lifecycle, in gametocytes and in sporozoites (i.e. in all stages for which gene expression data are available) (Bozdech et al., 2003, Le Roch et al., 2003). Proteomics data are available for PfCK2α and PfCK2β1, and indicate that both proteins are present in the asexual erythrocytic stages and gametocytes, and that the PfCK2α subunit is also present in sporozoites (Florens et al., 2004, Florens et al., 2002). No proteomics data are currently available for PfCK2β2. *PfCK2α* encodes a predicted protein of 335 amino acids, *PfCK2β1* a predicted protein of 245 amino acids, and *PfCK2β2* a predicted protein of 385 amino acids.

1.7 Aims

- Determining whether the three PfCK2 subunits are required for the completion of the parasite lifecycle, through reverse genetics approaches; a major objective is the validation of PfCK2 as a potential drug target

- Assessing whether the two PfCK2β subunits can functionally compensate for each other, by reverse genetics approaches

- Determining whether PfCK2α can be expressed in an active form as a recombinant enzyme, and assessing the impacts of the PfCK2β subunits on substrate specificity and activity of the kinase

- Characterisation of the PfCK2 subunits at the biochemical and enzymological levels

- Investigating the substrates of the enzyme and the effects of small molecule inhibitors on enzymatic activity

Three results chapters cover the data gathered during the period of study: reverse genetics studies are covered in Chapter 3, data pertaining to PfCK2α is covered in Chapter 4, and data pertaining to the PfCK2β subunits in Chapter 5. Discussion of the results will take place primarily in the context of the results chapters, with a general discussion and conclusion chapter (Chapter 6) at the end.

2 Materials and Methods

2.1 Companies from which chemicals and kits were purchased

BDH	Saponin; Giemsa stain
Biogenes	All custom antibodies
BioRad	10 X TG-SDS; Coomassie Brilliant Blue R-250; Blocking Grade Blocker Non-Fat Dry Milk; 30% Acrylamide/Bis Solution
Blood transfusion service	Human erythrocytes
BOC	5% CO_2 gas
Calbiochem	Blasticidine-S-HCl
Eurogentec	SmartLadder DNA ladder
Fermentas	PageRuler Prestained protein ladder (#5M0671)
Fisher Scientific	Ampicillin
GE Healthcare	Gene Images Random Prime DNA Labelling Kit (RPN 3520); Gene Images CDP-Star Detection Kit (RPN 3555); Hybond-N+ Positively Charged Nylon Transfer Membrane; Amersham Hyperfilm ECL; pGEX-4T-3 expression plasmid
Gibco	RPMI 1640, GlutaMAX-1
Invitrogen	Custom oligonucleotides; Ultrapure 10XTAE buffer; Luria Broth Base; Pfx Platinum polymerase; T4 DNA Ligase; Agarose (electrophoresis grade)
Jacobus pharmaceuticals	WR99210
Kodak	Medical X-Ray film
Millipore	Montage Life Science Kits DNA Gel Extraction Kit
New England Biolabs	All restriction endonucleases; Protein marker, broad range (2-212kDa) P7702S
Promega	Wizard PCR Preps DNA Purification System; pGEM-T Easy Vector System I (A1360); Kinase-GLO Luminescent Kinase Assay kit
Qiagen	QIAprep Spin Miniprep Kit; Plasmid Maxi kit; QIAamp DNA Minikit; Ni-NTA agarose; pQE30 expression vector
Roche	Complete protease inhibitor tablets
Sigma	All chemicals unless otherwise stated
TaKaRa Bio Inc.	Ex Taq polymerase

2.2 Biological and chemical reagents

2.2.1 Solutions and buffers

Electrophoresis

6x DNA loading buffer: 0.25% Bromophenol Blue, 0.25% Xylene Cyanol FF, 30% glycerol

4x Laemmli buffer: 40% glycerol, 2% SDS, 20% BME, 250mM Tris-HCl pH 6.9

1xTAE: Diluted from 10x stock (Invitrogen) in ddH_2O to give 40mM Tris-Acetate, 1mM EDTA

1xTG-SDS: Diluted from 10x stock (BioRad) in ddH_2O to give 25mM Tris, 192mM Glycine, 0.1% SDS, pH 8.3

Coomassie stain: 5g/L Coomassie Brilliant Blue stain (BioRad), 50% ethanol, 10% acetic acid

Coomassie destain: 45% Methanol, 10% Acetic Acid

Western blotting

Towbin buffer: 10x TG stock (BioRad) diluted 1/10 in methanol/ddH_2O to give 25mM Tris pH8.3, 192mM Glycine, 20% methanol

PBS: 8g/L NaCl, 40mg/L KCl, 1.15g/L Na_2HPO_4, 328mg/L KH_2PO_4, 0.1g/L $CaCl_2$, 0.1g/L $MgCl_2$, pH7.2, in ddH_2O

PBST: PBS with 0.05-0.3% Tween20 (Sigma)

Southern blotting

Buffer A: 100mM Tris/HCl, 300mM NaCl, pH 9.5

1xSSC : 15mM Tri-sodium citrate, 150mM NaCl

Depurination solution: 0.25M NaCl

Denaturation solution: 1.5M NaCl, 0.5M NaOH

Neutralisation solution: 1.5M NaCl, 0.5M Tris HCl pH7.5

Bacterial culture

LB media: 10g/L Tryptone (Oxoid L.42), 5g/L Yeast Extract (Oxoid L.21), 10g/L NaCl, pH7.5, in ddH_2O

LB agar: 15g/L Tayo agar, 10g/L Tryptone (Oxoid L.42), 5g/L Yeast Extract (Oxoid L.21), 10g/L NaCl, pH7.5, in ddH_2O

Ampicillin: 100mg/ml in ddH_2O, stored at -20^oC

Kanamycin: 10mg/ml in ddH_2O, stored at -20^oC

Tetracyclin: 5mg/ml in ethanol, stored at -20^oC

Chloramphenicol: 34mg/ml in ethanol, stored at -20^oC

***Plasmodium* culture and protein extract preparation**

Complete RPMI: 15.89g/L RPMI 1640, 0.05g/L hypoxanthine, 10ml/L GlutaMAX-1, 5g/L Albumax, 2g/L $NaHCO_3$, 0.01g/L Gentamycin sulphate, pH 7.4

Saponin solution: 1.5g/L saponin in PBS

Sorbitol solution: 5% sorbitol (w/v) in ddH_2O

Giemsa staining solution: 10% Giemsa, 0.3% Na_2HPO_4 (w/v), 0.06% KH_2PO_4 (w/v) in ddH_2O

Cytomix buffer: 120mM KCl, 0.15mM $CaCl_2$, 2mM EGTA, 5mM $MgCl_2$, 10mM K_2HPO_4/KH_2PO_4, 25mM HEPES

Freezing solution: 0.583g/L sorbitol, 0.125g/L NaCl, 28% glycerol

Defrosting solutions: A 12% NaCl (w/v); B 1.6% NaCl (w/v); C 0.2% Dextrose (w/v), 0.9% NaCl (w/v)

RIPA buffer: 30mM Tris pH8, 150mM NaCl, 20mM $MgCl_2$, 1mM EDTA, 0.5% Triton X-100, 1% NP-40, 10mM β-glycerophosphate, 10mM NaF, 1mM PMSF, 10mM benzamidine hydrate, 1x Roche Complete protease inhibitors

2.2.2 E. coli *strains*

XL10-Gold (Stratagene) Tet^{r}Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lacI*^{q}*Z*Δ*M15* Tn*10* (Tet^r) Amy Cam^r]

BL21 Gold (Stratagene) *E. coli* B F$^-$ ompT hsdS(rB$^-$ mB$^-$) dcm$^+$ Tet^r gal endA

BL21 Gold (DE3) (Stratagene) *E. coli* B F$^-$ ompT hsdS(rB$^-$ mB$^-$) dcm$^+$ Tet^r gal λ(DE3) endA Hte

BL21 Gold (DE3)pLysS (Stratagene) *E. coli* B F$^-$ ompT hsdS(rB$^-$ mB$^-$) dcm$^+$ Tet^r gal λ(DE3) endA Hte [pLysS Cam^r]

SG13009[pREP4] (Qiagen) *E. coli* B F$^-$ *his pyrD* Δ*lon-100 rpsL*

Rosetta2(DE3)pLysS (Novagen) *E. coli* B F$^-$ *ompT hsdS*B(rB$^-$ mB$^-$) *gal dcm* (DE3) pLysSRARE2

2.2.3 Oligonucleotide primers

The oligonucleotide primers used in this study (custom synthesized by Invitrogen) are listed below (Table 2-1). Oligonucleotide primers were designed for site-directed mutagenesis, colony screening PCR, and the amplification of genes for recombinant protein expression and reverse genetics studies.

Table 2-1 Oligonucleotide primers used in this study
Restriction endonuclease recognition sites are underlined.

CK2aForBam	GGGGGGATCCATGTCGGTTAGCTCAATTAATAAA
CK2aRevSal	GGGGGTCGACTTATGATTCCTCACGGACTTCTC
CK2b1ForEco	GGGGGAATTCATGGAAAATAGTGATTCGAATAAAGAC
CK2b1RevSal	GGGGGTCGACTTACGTTTCAGAAATTTGTAGTTCTTCC
CK2b2ForBam	GGGGGGATCCATGGAGTTTGTTTCAAACGATGAAAG
CK2b2RevSal	GGGGGTCGACTCATTGACACTCTTCAGAGGATTCCG
CK2b2RSpe	GGGGACTAGTTCATTGACACTCTTCAGAGGATTCCG
CK2b1FBgl	GGGGAGATCTATGGAAAATAGTGATTCGAATAAAGAC
CK2b2shortForBam	GGGGGGATCCATGGAAGCAACAGTGTCTTGGATTG
CK2a3primeRev	GTCTGATATATCAAAGATAAGC
CK2b13primeRev	GCATTAAAATATGAGATGTACAC
CK2b23primeRev	CAAACTATGTCAACTGTTTTGGG
CK2a5primeFor	GAGACAGGAATAATG
CK2b15primeF	CTTAAGTGTTAATCGG
CK2b25primeF	GGCATAGGAATATTTAAC
CK2aK72MFor	GTGCTATTATGGTATTAAAGCC
CK2aK72MRev	GGCTTTAATACCATAATAGCAC
I178ACK2aF	CAAATTAGATTAGCTGATTGGGGTC
I178ACK2aR	GACCCCAATCAGCTAATCTAATTTG
F117ACK2aRev	CTATATTGTTAATATATTCAGCTATTAAAGATGG
F117ACK2aFor	CCATCTTTAATAGCTGAATATATTAACAATATAG
CK2aKOForBam	GGGGGGATCCAGTGAGGTGTTTAATGG
CK2aKORevNot	GGGGGCGGCCGCACCTTTATAATATCTACT
CK2b1KOForBgl	GGGGAGATCTTAAGAAAATAGTGATTCGAATAAAGAC
CK2b1KORevNot	GGGGGCGGCCGCATATACATGAACTTTGGC
CK2b2KOForBam	GGGGGGATCCTAAGATGAAAGTGCAGATGACATAATC
CK2b2KORevNot	GGGGGCGGCCGCATATAATAATGGGCTTAAAAATTTAG
CK2aTAGForPst	GGGGCTGCAGGCATAGAGATGTTAAACC
CK2aTAGRevBam	GGGGGGATCCTGATTCCTCACGGACTTCTC
CK2b1TAGForPst	GGGGCTGCAGGCTGGGGATGCACCTGAAG
CK2b1TAGRevBglII	GGGGAGATCTCGTTTCAGAAATTTGTAG
CK2b2TAGForPst	GGGGCTGCAGGTTCCTCATTTTAAGAAGC
CK2b2TAGRevBam	GGGGGGATCCTTGACACTCTTCAGAGGATTCC
pCAMBSDFor	TATTCCTAATCATGTAAATCTTAAA
pCAMBSDRev	CAATTAACCCTCACTAAAG
T7	GCTAGTTATTGCTCAGCGG
SP6	ATTTAGGTGACACTATAG

2.2.4 Antibodies

Primary and secondary antibodies and their dilutions for western blotting are shown in Tables 2-2 to 2-4. Antibodies used in this study were bought from commercial suppliers, or commissioned from a custom antibody supplier (BioGenes). Pre-immune sera from the rabbits were used as negative controls for the custom antibodies, and used at 1:500 dilution.

Table 2-2 Custom affinity-purified primary antibodies from BioGenes (raised in rabbit)

Antibody	Peptide against which antibody was raised	Antibody stock concentration	Dilution for western blotting
Anti-PfCK2α	ADVNIHKPKEYYDY-amide	0.827 mg/ml	1:200
Anti-PfCK2β1	DSNKDLQDSKSDKS-amide	0.27 mg/ml	1:500
Anti-PfCK2β2	DEINRDSEEMYKNK-amide	0.648 mg/ml	1:750-1:1000

Table 2-3 Commercial primary antibodies

Antibody (animal species raised in)	Dilution for western blotting	Source
Anti-His-tag (rabbit)	1:1000	Santa Cruz Biotechnology
Anti-HA (mouse)	1:1000	Roche
Anti-GST-tag (rabbit)	1:10,000	Sigma

Table 2-4 Secondary antibodies

Antibody (animal species raised in)	Dilution for western blotting	Source
Anti-rabbit IgG (whole molecule)-Peroxidase (Goat)	1:10,000	Sigma
Anti-mouse IgG (whole molecule)-Peroxidase (Sheep)	1:10,000	Sigma

2.3 *Plasmodium falciparum* cell culture techniques

2.3.1 Malaria parasite culture

Cultures of the *P. falciparum* strain 3D7A (Walliker et al., 1987) were maintained according to standard methods (Trager and Jensen, 1976). Parasites were incubated at 37°C in a humidified atmosphere of 5% CO_2 in 5% (v/v) human erythrocytes in complete

RPMI 1640 medium buffered with 25mM HEPES and supplemented with 25mM sodium bicarbonate, 2mM L-glutamine, 300mM hypoxanthine, 10mg/L gentamycin and 5g/L albumax II (Sigma). Cultures were seeded at a 5% haematocrit and maintained at a parasitaemia of 1-10% with daily changes of medium. Smears were fixed in methanol then stained with Giemsa staining solution. Smears were viewed under a light microscope with an objective magnification of 100, and parasitaemia (percentage of infected erythrocytes) was determined.

2.3.2 Plasmids for parasite transfection

To produce parasite lines expressing proteins with 3' haemaglutinin (HA) tags, the plasmid pCAM-BSD-HA (as described in Dorin-Semblat 2007 (Dorin-Semblat et al., 2007), except with the Pfmap-2 sequence removed; Fig. 2-1) was used. The pCAM-BSD-HA plasmid is derived from the pCAM-BSD plasmid (see Fig. 2-2) of Sidhu and colleagues (Sidhu et al., 2005), through the addition of a sequence encoding a haemaglutinin (HA) epitope allowing the in-frame insertion of any sequence of interest, and resulting in C-terminal tagging with the HA epitope (Jean Halbert, this laboratory). The original pCAM-BSD plasmid was used for gene disruption experiments in the present study. Both plasmids (pCAM-BSD and pCAM-BSD-HA) contain a blasticidine-S-deaminase expression cassette, conferring resistance to blasticidine and allowing for selection in *P. falciparum*.

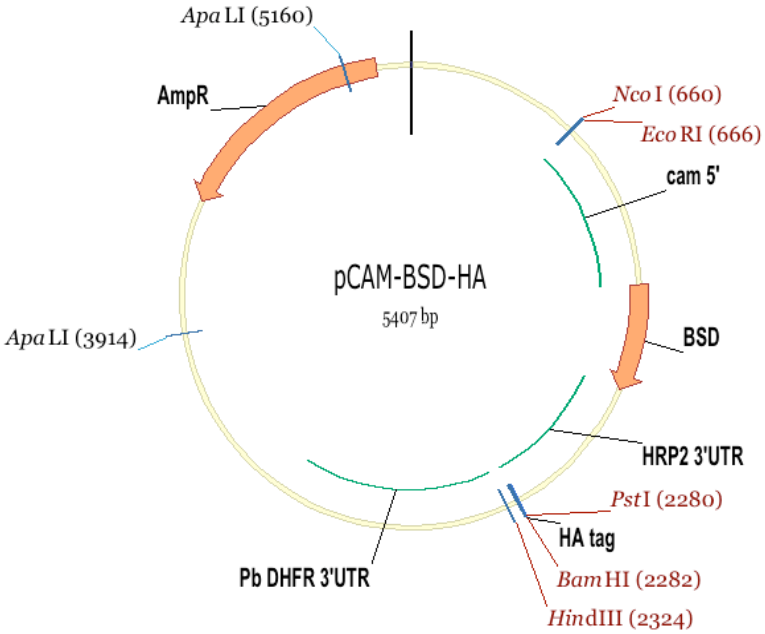

Figure 2-1 Map of the pCAM-BSD-HA plasmid
This figure shows the main features of the vector pCAM-BSD-HA. The plasmid contains an ampicillin cassette for selection in *E. coli*, and a BSD cassette for selection in *P. falciparum*. The 3' end of a gene can be inserted between the PstI and BamHI sites, in frame with a C-terminal HA tag, which is followed by the 3' untranslated region of *P. berghei* dihydrofolate reductase (Pb DHFR). Figure produced using Vector NTI software (Invitrogen).

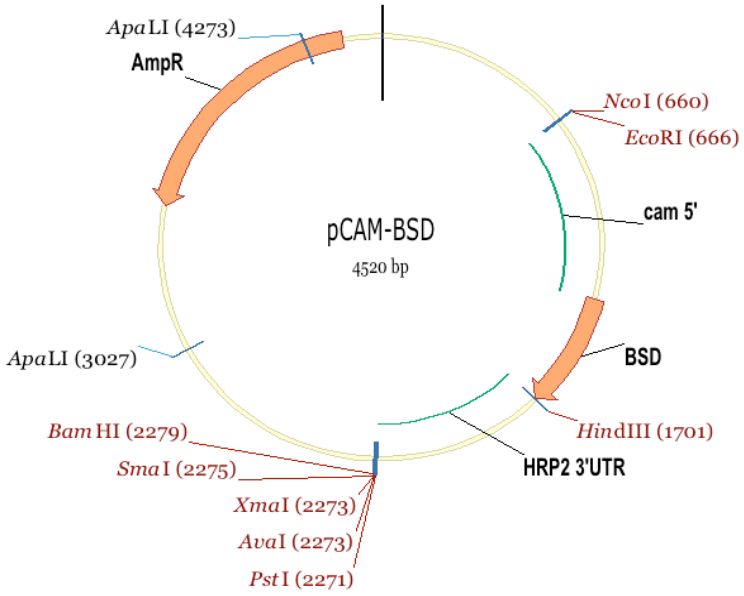

Figure 2-2 Map of the pCAM-BSD plasmid
This figure shows the main features of the vector pCAM-BSD. The plasmid contains an ampicillin cassette for selection in *E. coli*, and a BSD cassette for selection in *P. falciparum*. The truncated gene fragment can be inserted between the BamHI and NotI restriction sites. Figure produced using Vector NTI software (Invitrogen).

Complementation plasmids for use in the reverse genetics studies were produced by Gateway cloning technology (Invitrogen), which is based on the bacteriophage lambda site-specific recombination system. The complementation plasmids allow the episomal expression of a gene in the *P. falciparum* parasite. The entry plasmid pHGB and the destination plasmid pCHD-1/2 (see Fig. 2-3), with the selectable marker DHFR, were used in this study (Tonkin et al., 2004). PfCK2α, PfCK2β1, PfCK2β2 and F117AI178APfCK2α were cloned into the plasmid pHGB using the restriction endonucleases BamHI (or BglII which generates complementary sticky ends to BamHI) and NotI, placing the genes in an expression cassette under the control of the *P. falciparum* Hsp86 promoter. The expression cassette of pHGB, containing the gene of interest, was then transferred into pCHD-1/2 by site-specific recombination of the attL and attR sites, via a LR clonase reaction, carried out according to Invitrogen instructions.

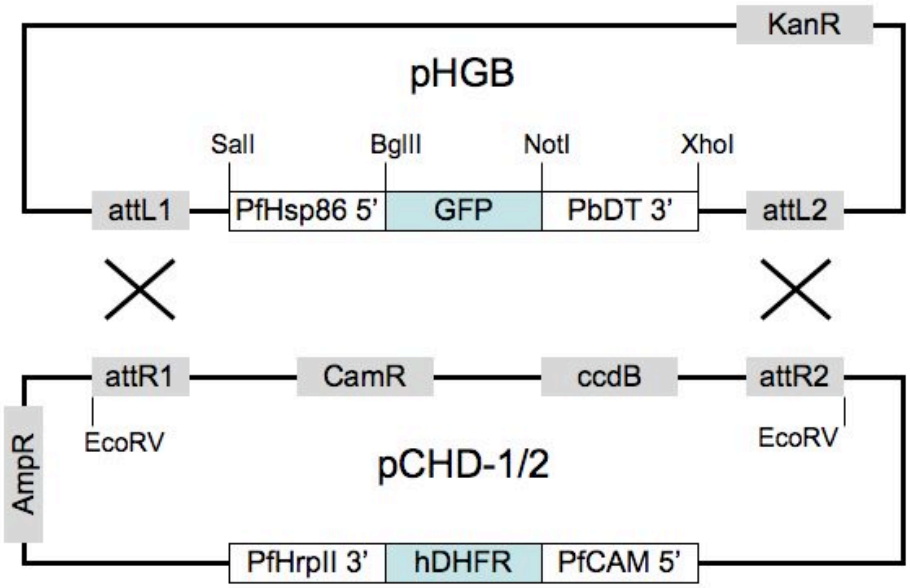

Figure 2-3 Maps of pHGB and pCHD-1/2 plasmids
This figure displays the important features of the SingleSite gateway vectors described by (Tonkin et al., 2004). The entry vector, pHGB, contains a kanamycin resistance cassette (KanR), for selection in *E. coli*, and an expression cassette flanked by the attL1 and attL2 sites. The gene of interest is cloned into the expression cassette, replacing the green fluorescent protein (GFP) coding sequence. The expression cassette is under the control of the *P. falciparum* heat shock protein 86 promoter (PfHsp86 5'), and the *P. berghei* dihydrofolate reductase/thymilidate synthase 3' untranslated region (PbDT 3'). The destination vector pCHD-1/2 contains an ampicillin resistance cassette (AmpR) for selection in *E. coli*, and the attR1 and attR2 sites. It also contains the variant form of the human dihydrofolate reductase gene (hDHFR), which confers resistance to the drug WR99210 (Fidock and Wellems, 1997), for selection in *P. falciparum*. The hDHFR gene is under the control of the *P. falciparum* calmodulin promoter (PfCAM 5') and the *P. falciparum* histidine-rich protein 2 3' untranslated region (PfHrpII 3'). The pCHD-1/2 plasmid has a selection cassette between the attR1 and attR2 sites, with a chloramphenicol resistance cassette (CamR) and a ccdB death gene, a negative selectable marker for recombination. Upon recombination, the expression cassette of pHGB replaces the selection cassette of pCHD-1/2, and the ccdB gene is lost, allowing retention of the recombined plasmid in bacterial strains susceptible to the effects of the ccdB gene. Figure adapted from (Tonkin et al., 2004).

All plasmids were sequenced (see 2.5.9) prior to use for transfection of *P. falciparum*, and produced in sufficient quantity using the Plasmid Maxi kit (Qiagen; see 2.5.6).

2.3.3 Parasite transfection

Asexual blood stage parasites were synchronized by sorbitol treatment (Lambros and Vanderberg, 1979) to obtain a majority of ring stage parasites. Forty-eight hours later, ring stage parasites were transfected by electroporation with 100μg of purified plasmid DNA (produced by maxiprep, see 2.5.6) in cytomix buffer as described (Fidock et al., 2000a, Fidock and Wellems, 1997). Briefly, parasites from a 4ml ring stage culture were washed

in sterile cytomix buffer and resuspended in cytomix buffer containing 100μg of plasmid DNA. The culture was transferred to an electroporation cuvette (0.2cm) and electroporated at 0.31kV, 950μF and infinity resistance. Complete medium was added immediately, and the contents of the cuvette transferred to a prewarmed tissue culture flask. Complete medium and blood were added to give a 5ml culture of 5% haematocrit. Lysis products were removed by a change of medium 4 hours post-transfection. Transformed parasites were selected by inclusion of blasticidine-S-HCl in the culture medium from two days post-transfection at a concentration of 2.5μg/ml, and if appropriate (i.e. if a complementation plasmid was also present, see below) 5nM WR99210 was also included in the culture medium. Parasites were maintained in this supplemented medium from two days post transfection.

Parasites from transformed populations harbouring chromosomal integration events (as determined by PCR analysis) were cloned by limiting dilution. The haematocrit of a culture at 3% parasitaemia was determined using a haemocytometer, and the culture was diluted in complete culture medium to give 10^5 cells per millilitre. A range of dilutions in complete culture medium with 1% haematocrit was performed, to give 1 infected cell per 200μl, 666μl or 2ml. 200μl of these dilutions were transferred to each well of a 96-well tissue culture plate. After 6 days' growth the medium was changed and supplemented with 1% (v/v) erythrocytes and the appropriate drugs to select for transfection. Parasites were detected by blood smears, and the contents of parasite-positive wells were bulked up to 2ml cultures (5% haematocrit) in 12 well plates. These were monitored by blood smears and transferred to 5ml cultures in flasks when the parasitaemia reached 1%.

2.3.4 Saponin lysis of P. falciparum *parasites*

Parasite cultures (25ml) to be harvested by saponin lysis were centrifuged for two minutes at 4°C and 1300g, the pellet was washed in 30ml PBS (centrifuging as before), and the erythrocytes were lysed on ice in 7ml of 0.15% saponin solution by repeated pipetting. Forty millilitres of cold PBS was added and the cells centrifuged at 5500g for five minutes at 4°C. After two additional washes with PBS, the pellets were stored at -80°C until needed.

2.3.5 DNA extraction from parasite cultures

Parasite cultures were saponin-lysed (see 2.3.4), then resuspended in 500μl of cold PBS. Proteinase K was added to a final concentration of 150mg/ml, and SDS to a final concentration of 2%, and the tubes incubated at 55°C for 2-3 hours. The DNA was extracted in an equal volume of Phenol:chloroform:isoamyl alcohol (25:24:1), saturated with 10mM Tris pH8 and 1mM EDTA (Sigma). The contents of the tube was gently mixed and then centrifuged at 16000g for 5 minutes. The top aqueous layer was transferred to a new tube. Once the phenol extraction had been repeated twice more, 0.1 volumes of 3M sodium acetate pH5.2 was added, and the DNA precipitated in 2-4 volumes of ethanol at -20°C for 30 minutes or -70°C for 15 minutes. The tubes were then centrifuged to recover the DNA pellet at 16000g for 12-15 minutes, and the pellet resuspended in sterile water.

2.3.6 Cryopreservation of parasites

A stock culture of 5-10% parasitaemia was selected, containing a high proportion of ring stages. Three to five millilitres were resuspended and centrifuged at 2000rpm (Sigma 4K15 centrifuge, rotor 11150) for 5 minutes. The supernatant was removed and the volume of the cell pellet measured. An equal volume of deep freeze solution (28% glycerol, 3% sorbitol, 0.65% NaCl) was added and a final volume of 0.2-0.5ml was placed into a cryovial and frozen immediately in liquid nitrogen.

2.3.7 Defrosting parasite stabilates

Vials of frozen parasites (‘stabilates’) were thawed at room temperature; the parasites were then transferred to a sterile 15ml falcon tube and the volume of culture measured. For each 1ml of thawed culture, 0.2ml of defrosting solution A (12% NaCl) was added. The tube was incubated at room temperature for 3 minutes, then 10ml of defrosting solution B (1.6% NaCl) were added for each 1ml of thawed culture. The solution was mixed well, then centrifuged at 2000rpm (Sigma 4K15 centrifuge, 11150 rotor) for 5 minutes. The supernatant was discarded, then for each original 1ml of thawed culture, 10ml of defrosting solution C (0.2% dextrose, 0.9%NaCl) was added and mixed by pipetting. After centrifugation (2000 rpm 5 minutes), the supernatant was discarded and the cells resuspended in 5ml complete medium in a small culture flask, and several drops of fresh red blood cells were added.

2.4 Bioinformatics

2.4.1 Multiple sequence alignments

Alignments of *P. falciparum* protein sequences and homologues were performed using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

BLAST searches were performed using the NCBI BLAST tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Searches for destruction box motifs were performed using the D box finder (http://bioinfo2.weizmann.ac.il/~danag/d-box/main.html).

2.5 Molecular biology methods

2.5.1 Polymerase Chain Reaction

The proof-reading polymerase Pfx Platinum (Invitrogen) was used to amplify DNA sequences where the fidelity of the amplified sequence to the original sequence was important (for example, when amplifying parasite genes for insertion into a bacterial expression plasmid), and the non-proof-reading polymerase ex Taq (TaKaRa) was used when the size of the amplified sequence was more important than whether any mutations might have been introduced into the amplified sequence (for example, when using PCR for reverse genetics diagnosis, or colony screening (see 2.5.1.3)).

2.5.1.1 Pfx Platinum polymerase

Reactions were set up to contain 0.3mM of each dNTP, 1μM of each oligonucleotide primer, 1.25U Pfx Platinum polymerase, 1x Pfx buffer (supplied with the polymerase), template DNA, and an oligonucleotide-specific concentration of $MgSO_4$ (1-10mM), in a final volume of 25μl. The PCR was performed under the following conditions:

Initial denaturation	94°C, 5 minutes
30 cycles of:	
Denaturation	94°C, 30 seconds
Annealing	oligonucleotide-specific temperature, usually 55°C, 30 seconds
Elongation	68°C, 1 minute per kb DNA to be amplified
Followed by:	
Final elongation	68°C, 10 minutes

2.5.1.2 ex Taq polymerase

Reactions were set up to contain 0.2mM of each dNTP, 1μM of each oligonucleotide primer, 0.75U ex Taq polymerase (TaKaRa), 1x ex Taq buffer (supplied with the polymerase; final $MgCl_2$ concentration: 2mM), and template DNA in a final volume of 25μl. The PCR was performed under the following conditions:

Initial denaturation	94°C, 5 minutes
35 cycles of:	
Denaturation	94°C, 30 seconds
Annealing	oligonucleotide-specific temperature, 30 seconds
Elongation	68°C, 1 minute per kb DNA to be amplified
Followed by:	
Final elongation	68°C, 10 minutes

2.5.1.3 Colony-screening PCR

Bacterial colonies transformed with a ligation mixture (see 2.5.5 and 2.5.2.1-2) were spread on agar plates with antibiotic selection. Those bacteria that were able to grow were screened by PCR to see which colonies contained plasmids with ligated inserts of the correct size.

Individual colonies were picked off the agar plate and resuspended in 25µl PBS. 2µl of this suspension was used as the template in an ex Taq PCR screen (see 2.5.1.2).

2.5.1.4 Site-directed mutagenesis

To obtain plasmids encoding mutated CK2α proteins, oligonucleotides were designed to perform site directed mutagenesis by overlap extension PCR (Ho et al., 1989). The template used was the PfCK2α expression plasmid, pGEX-4T-3-PfCK2a, constructed as described in section 4.4. PfCK2α sequences containing a lysine to methionine change at residue 72 (‘kinase dead mutant’) were constructed, using the CK2aForBam and CK2aK72MRev, and CK2aK72MFor and CK2aRevSal, primers for the first round of amplification, producing two DNA fragments having overlapping ends that contain the mutation. The PCR products from the first rounds of amplification were purified using the Wizard PCR Preps DNA Purification System (Promega), prior to their use as templates in the second round of PCR amplification. In the second round of PCR amplification, the overlapping ends anneal, acting as primers for the extension of the complementary strand. The CK2aForBam and CK2aRevSal oligonucleotide primers were also included in the second round PCR reaction. PfCK2α sequences containing a phenylalanine to glycine change at residue 117 and an isoleucine to alanine change at residue 178 (‘chemical genetics mutant’) were constructed in a similar manner, first producing the F117A mutation as outlined above and verifying the sequence by DNA sequencing (see 2.5.9), then producing the I178A mutation by site-directed mutagenesis using the F117A sequence as a template. The F117A mutation was produced in a similar manner to the K72M mutation above, using the primer pairs CK2aForBam and F117ACK2aRev, and F117ACK2aFor and CK2aRevSal, for the first round of amplification, and CK2aForBam and CK2aRevSal for the second round of amplification. The I178A mutation was produced using the primer pairs CK2aForBam and I178ACK2aRev, and I178ACK2aFor and CK2aRevSal, for the first round of amplification, and CK2aForBam and CK2aRevSal for the second round of amplification.

The sequences were cloned into the vector pGEM-T-Easy (Promega; see 2.5.2.1) for sequencing, to confirm that the correct sequences (and no additional mutations) were generated by PCR amplification. Vectors containing the correct insert were digested using the restriction endonucleases BamHI and SalI, and the PfCK2α mutant sequences were inserted into the expression vector pGEX-4T-3, digested by the same enzymes. The inserts were resequenced prior to expression in the *E. coli* strain BL21 Gold.

2.5.2 Cloning techniques

2.5.2.1 pGEM-T-Easy cloning

All PCR products were initially cloned into the pGEM-T-Easy vector using the pGEM-T-Easy Vector System I kit (Promega), for verification by DNA sequencing, before being subcloned into their destination vector. The TaKaRa ex Taq polymerase is a non-proofreading enzyme, and produces PCR products with adenine overhangs at the 3' ends, allowing ligation into the pGEM-T-Easy vector (Fig. 2-4), which contains thymine overhangs in the cloning site. DNA sequences amplified by Pfx Platinum, the proofreading polymerase, could also be ligated into pGEM-T-Easy if they first had a 10-minute incubation at 68^{o}C with 0.75U of TaKaRa ex Taq polymerase, to add adenosine overhangs. Ligation reactions were performed according to the manufacturer's instructions, and their products subsequently transformed into thermocompetent *E. coli* XL10 Gold cells (Stratagene) (see 2.5.5). These were plated on LB agar plates containing 100μg/ml ampicillin, and which had had 100μl of 100mM IPTG and 20μl of 50mg/ml X-Gal absorbed into the surface. The addition of IPTG and X-Gal allows visual discrimination between bacterial colonies harbouring self-ligated vectors, and those harbouring vectors with inserts of DNA. Successful cloning of PCR products into the pGEM-T-Easy plasmid results in white bacterial colonies, whereas bacteria containing self-ligated vectors are blue. The ability of β-galactosidase to hydrolyse the sugar X-Gal lies behind this colour distinction, with the hydrolysed X-Gal producing a characteristic blue colour in the colonies in which the *LacZ* gene remains intact. Insertion of a PCR product into the pGEM-T-Easy vector disrupts the *LacZ* gene, and therefore the cells are unable to produce β-galactosidase, and unable to hydrolyse the marker, resulting in white colonies. IPTG is added to the plates to induce transcription of the *LacZ* gene. Colony PCR was used to screen the white colonies for bacteria harbouring plasmids with the appropriate size of inserts (see section 2.5.1.3), using oligonucleotide primers which bind to the T7 and SP6 sequences of the pGEM-T-Easy vector (see Fig. 2-4). Single bacterial colonies containing

the correct size of insert were used to inoculate 5ml cultures of LB medium containing 100μg/ml ampicillin, and grown in a shaking incubator at 37°C overnight. Plasmid DNA was extracted from the bacteria (see section 2.5.6), and analysed by restriction endonuclease digestion (see section 2.5.8). The plasmid DNA was sequenced (see 2.5.9) using the SP6 and T7 primers, to verify that it contained the correct insert, without any unwanted mutations.

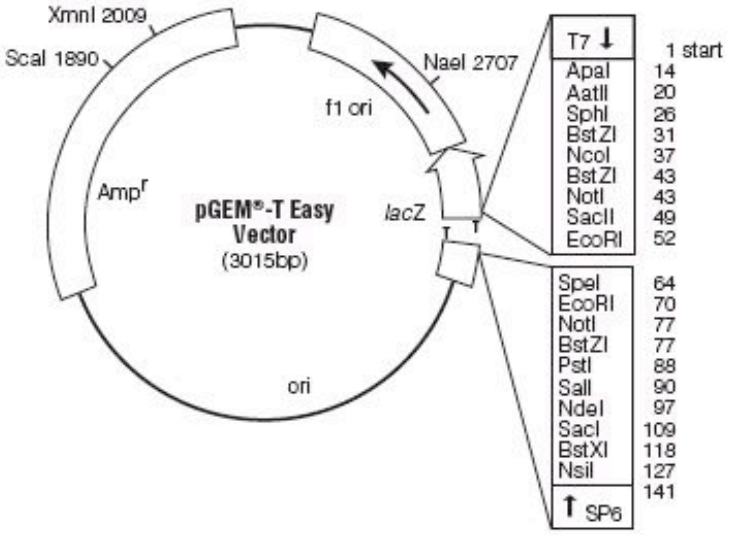

Figure 2-4 Map of the pGEM-T-Easy plasmid.
This figure shows the important features of the pGEM-T-Easy cloning plasmid (Promega). The plasmid contains an ampicillin resistance cassette for selection in *E. coli*. The plasmid has been cut in the middle of the *lacZ* gene, and has a single 3' terminal thymidine at each end, providing compatible overhangs for the insertion of PCR products amplified by Taq based polymerases, such as TaKaRa *ex Taq*, which often add single deoxyadenosines to the 3' ends of amplified sequences. Bacterial colonies containing vectors with inserts were distinguished from bacterial colonies containing self-ligated vectors by blue-white colony screening (see 2.5.1.3). Colonies identified as containing vectors with inserts were screened for the correct size insert using the primers T7 and SP6, which bind to the T7 and SP6 RNA polymerase promoters, flanking the multiple cloning region. The insertion site is in the middle of the multiple cloning region, allowing the insert to be easily removed and reinserted into destination vectors. Figure taken from the Promega website (www.promega.com).

2.5.2.2 Sub-cloning into destination vectors

Verified correct sequences in pGEM-T-Easy clones were sub-cloned into destination vectors. The inserts were released from the pGEM-T-Easy plasmid by restriction endonuclease digestion (see section 2.5.8), and the destination plasmid linearised using the same restriction endonucleases, or restriction endonucleases with complementary sticky ends (e.g. BamHI and BglII) to those used to release the insert. The digests were separated by agarose gel electrophoresis (see section 2.5.10), and the inserts and plasmids were

excised from the gel under UV illumination and purified using the Millipore Montage Life Science Kits DNA Gel Extraction Kit according to manufacturer's instructions.

The insert sequence was ligated into the destination plasmid in a 10µl ligation reaction containing 5 units of T4 DNA ligase (Invitrogen), 1x DNA ligase reaction buffer and a range of molar ratios of vector to insert. Ligated plasmids were transformed into chemically competent *E. coli* (see 2.5.5) and plated on LB agar plates containing the appropriate antibiotics. Colony PCR (see section 2.5.1.3) was used to identify colonies containing the correct insert. Plasmid DNA was produced from these colonies (see section 2.5.6), analysed by restriction endonuclease digestion (see section 2.5.8), and verified by sequencing (section 2.5.9).

2.5.2.3 Gateway cloning

The Gateway cloning system (Invitrogen) was used to generate 'complementation' plasmids, for use in reverse genetics studies, as described in section 2.3.2.

2.5.3 *Preparation of thermocompetent* E. coli

An overnight culture was used to inoculate 25ml LB medium at a 1:100 dilution. The culture was shaken at 37°C until the OD_{600} reached 0.4. The cells were centrifuged at 1600g, 4°C, for 10 minutes. The supernatant was discarded and the cell pellet resuspended in 12.5ml sterile 4°C 50mM $CaCl_2$. Cells were incubated on ice for 30 minutes, then centrifuged as previously. The supernatant was discarded, and the cell pellet resuspended in 2.5ml $CaCl_2$. Glycerol was added to a final concentration of 10%, then the cells were rapidly frozen on dry ice in 100µl aliquots, and stored at -80°C.

2.5.4 *Preparation of chemically competent* E. coli

One litre of LB medium was inoculated with an overnight culture of the appropriate bacterial strain, at a 1:100 dilution. Cells were shaken at 37°C until the OD_{600} reached between 0.5 and 0.7. The cells were chilled on ice for 15 to 30 minutes, and centrifuged at 4000g and 4°C for 15 minutes. The supernatant was discarded, the pellet resuspended in one litre of cold water, and then centrifuged again. The supernatant was discarded, the pellet resuspended in 0.5L cold water, and then centrifuged again. The supernatant was discarded, the pellet resuspended in 20ml of cold 10% glycerol, and then centrifuged again. The pellet was resuspended to a final volume of 2 to 3ml in cold 10% glycerol. The cell

concentration should be about 1-3 x 10^{10} cells/ml. The suspension was frozen in aliquots of 50µl on dry ice, and stored at -80°C.

2.5.5 E. coli *transformation*

Thermocompetent or chemically competent *E. coli* cells were used as recipients for plasmid DNA or ligation reactions. For transformation of thermocompetent *E. coli*, an aliquot of cells (100µl) was thawed on ice for 10 minutes, then the DNA or ligation product was added. Cells were incubated on ice for a further 5 minutes, then heat-shocked at 42°C for 30-40 seconds, and immediately returned to the ice for 2 minutes. LB medium at 37°C was then added (100µl). The cells were incubated at 37°C for one hour prior to plating out on agar plates containing antibiotic to allow expression of the resistance gene. The plates were incubated at 37°C overnight to allow colonies to grow.

For the transformation of chemically competent *E. coli* cells, a 50µl aliquot of cells was thawed on ice for 10 minutes. 0.5µl of plasmid DNA was added and then cells transferred to an ice-cold electroporation cuvette (0.2cm). Electroporation was performed at 2.5kV, and one millilitre of LB medium was immediately added. Cells were shaken at 37°C for 45 minutes to one hour to allow the cells to recover before being plated out onto an agar plate containing the appropriate antibiotic. Plates were incubated at 37°C overnight to allow colonies to grow.

2.5.6 Plasmid DNA isolation from E. coli

The Qiaprep Spin Miniprep kit from Qiagen was used for small-scale plasmid isolation. Four millilitres of LB medium, containing appropriate antibiotic selection, was inoculated with a single bacterial colony, and incubated overnight at 37°C in a shaking incubator. The bacteria were pelleted by centrifugation and the plasmid purified according to the manufacturer's instructions. Plasmid DNA was eluted in 35µl ddH_2O and quantified by spectrophotometry (see section 2.5.7).

The Plasmid Maxi kit from Qiagen was used for large-scale plasmid isolation of constructs to be used for transfection of parasites. A 3ml culture was inoculated with bacteria and incubated at 37°C in a shaking incubator for six to eight hours, then used to inoculate a 250ml culture of LB medium at a 1:1000 dilution. This culture was incubated at 37°C in a shaking incubator overnight, with appropriate antibiotics. The bacteria were centrifuged at

5500rpm (Beckman Coulter Avanti J-26XP, JA-14 rotor) for 15 minutes at 4°C, and the pellet treated for plasmid extraction according to the manufacturer's instructions.

2.5.7 Spectrophotometric quantification of DNA

The DNA to be quantified was diluted fifty fold in ddH_2O, and the concentration determined by measuring the UV absorbance at A_{260} using an UV spectrophotometer, and comparing it with the following standards: an absorbance of 1 indicates 50μg/ml double stranded DNA, 40μg/ml single stranded DNA/RNA, or 20μg/ml of oligonucleotide.

2.5.8 Restriction endonuclease digestion

Restriction endonuclease digestion was used to analyse plasmid DNA, to release DNA sequences from plasmids for insertion into other vectors, and to prepare vectors for the insertion of a DNA sequence. For the analysis of plasmid DNA, reactions took place in a 10μl volume, containing 1μl plasmid DNA, 1μl of the appropriate buffer (supplied with the restriction endonuclease), 0.5μl of each restriction endonuclease, and ddH_2O to complete the 10μl. For the release of an insert from a plasmid, or to prepare a plasmid for receiving an insert, larger reaction volumes were used: reactions took place in 20μl volumes, containing 15μl plasmid DNA, 2μl of the appropriate buffer (supplied with the restriction endonuclease), 0.5μl of each restriction endonuclease, and ddH_2O to complete the 20μl. Digestions took place at 37°C for 1-3 hours. An appropriate volume of 6x DNA loading buffer was added, and the reactions were analysed by agarose gel electrophoresis (see 2.5.10).

2.5.9 DNA sequencing

DNA was sequenced at The Sequencing Service at the School of Life Sciences at the University of Dundee (www.dnaseq.co.uk).

2.5.10 Agarose gel electrophoresis

Standard agarose gels were prepared by adding 1% (w/v) agarose to 1xTAE buffer and boiling the solution in a microwave to allow the agarose to dissolve. Once the mixture had cooled to 40-50°C, SYBR safe (Invitrogen, 1:10,000) or ethidium bromide (0.5μg/ml) was added and the mixture poured into a gel tray. Combs were slotted into the tray to provide

wells for the DNA samples to be loaded into. DNA samples were mixed with 6x DNA loading buffer, and loaded into the wells alongside DNA ladder (Eurogentec) to allow size determination of the bands. Electrophoresis was performed at 100-120 V in 1xTAE buffer. The bands were visualised under UV illumination in a Gel Doc system (Bio-Rad).

2.5.11 Southern blot analysis

Parasite DNA was analysed by Southern blot to detect plasmid integration. Restriction endonuclease digestions (see 2.5.8) were performed in 30µl volumes, with 3µg of parasite DNA (see 2.3.5), and incubations lasting 20-24 hours. Restriction endonucleases were chosen to provide a diagnostic pattern of bands detectable on a Southern blot. The digested DNA was mixed with an appropriate volume of 6x DNA loading buffer, and loaded into wells of a 0.8% (w/v) agarose gel. Electrophoresis was performed at 23 V, and monitored by UV illumination so that it could be stopped when the DNA ladder had migrated an appropriate length of the gel for the best distinction of bands. The gel was then incubated in depurination solution (0.25 M HCl) for 10 minutes, in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 25 minutes, and in neutralisation solution (1.5 M NaCl, 0.5 M TrisHCl pH7.5) for 30 minutes (with ddH_2O washes in between each incubation). The DNA was then transferred from the gel to a nylon membrane (Hybond-N+, GE Healthcare) in 10x SSC buffer by means of overnight wicking transfer (see Fig. 2-5). The DNA was crosslinked to the membrane by UV (1200µJ), using the UVP Crosslinker CX-2000. DNA bands on the membrane were visualised using the GE Healthcare Gene Images CDP-Star detection kit, and fluorescein-labelled probes made using the GE Healthcare Gene Images Random Prime Labelling kit, according to manufacturer's instructions. The membrane was incubated in preheated (60°C) pre-hybridisation solution (5x SSC, 0.1% SDS, 5% dextran sulphate, and a 1:20 dilution of the liquid block provided in the kit) at 60°C for over two hours. Fluorescein-labelled probe was denatured at 100°C for 5 minutes, then added to the buffer (2µg of probe per millilitre of pre-hybridisation solution) and incubated with the membrane at 60°C overnight. The membrane was washed twice for 10 minutes in 60°C 1x SSC + 0.1% SDS, twice for 10 minutes in 60°C 0.5x SSC + 0.1% SDS, then blocked at room temperature for an hour in a 1:10 dilution of Gene Images liquid block buffer in buffer A (100mM Tris HCl pH9.5, 100mM NaCl). The Gene Images anti-fluorescein alkaline phosphatase-conjugated antibody was diluted 1:5000 in buffer A with 0.5% (w/v) BSA and incubated with the membrane for one hour. Three 30-minute washes in buffer A with 0.3% Tween20 (Sigma) were performed, then the detection solution was pipetted on to the membrane and left to react for 5 minutes. Autoradiography film (Kodak, or

Hyperfilm ECL from GE Healthcare) was used to detect the light produced by the alkaline phosphatase reaction.

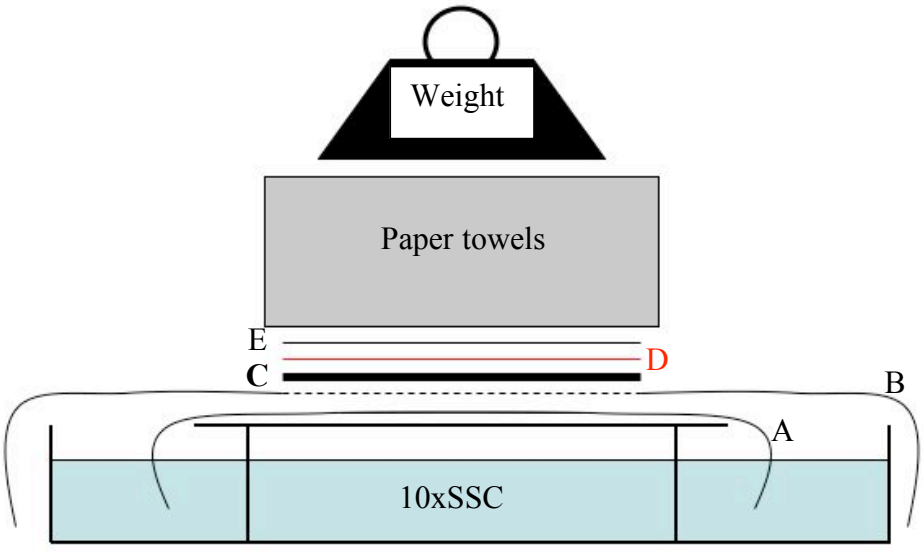

Figure 2-5 Wicking transfer apparatus
This figure shows how the wicking transfer apparatus was put together. A: Three pieces of Whatman paper, soaked in 10x SSC, and with the ends resting in the buffer reservoir. B: Saranwrap, with a hole the size of the gel cut in it. This will stop evaporation from the reservoir, and ensure that the only route for the buffer to get to the absorbent paper is through the gel and membrane. C: The gel, laid to cover precisely the hole in the Saranwrap. D: H+ Hybond nylon membrane, cut to exactly the same size as the gel. E: Three more pieces of Whatman paper, cut to the same size as the gel, and soaked in 10x SSC.

To reuse the membrane with another probe, the membrane was first stripped by washing for 2 minutes in 5x SSC buffer, and washing twice for 10 minutes in freshly boiling 0.1% SDS. The membranes could then be probed again, starting with the incubation in pre-hybridisation buffer.

2.6 Biochemistry methods

2.6.1 Expression and purification of proteins with a GST tag

The plasmid pGEX-4T-3 (GE Healthcare, Fig. 2-6) was used for the expression of recombinant proteins with an N-terminal GST tag, in BL21 Gold (Stratagene) or Rosetta2 cells (Novagen). The recombinant proteins GST-PfCK2α, GST-PfCK2β1, and GST-shPfCK2β2 (and GST alone) were expressed in *E. coli* using this plasmid.

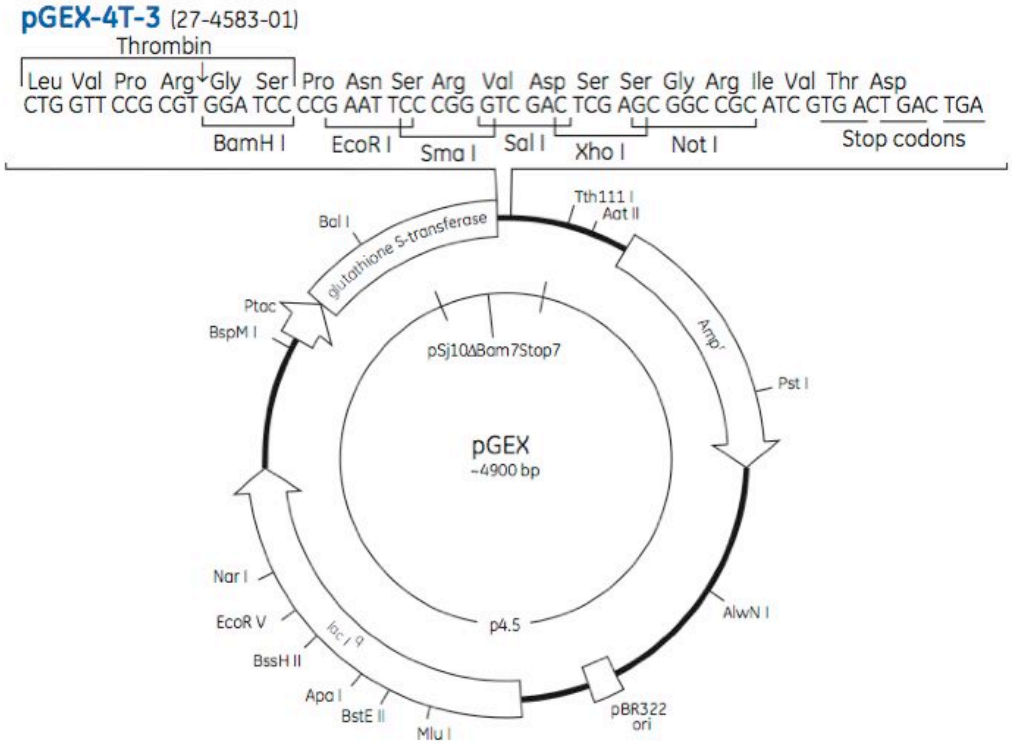

Figure 2-6 Map of the pGEX-4T-3 plasmid
The figure shows the main features of the plasmid pGEX-4T-3 (GE Healthcare) for recombinant expression of proteins with an N-terminal glutathione-S-transferase (GST) tag. The plasmid contains an ampicillin resistance cassette for selection in *E. coli*, and a multiple cloning site. The expression of the GST and any 3' in-frame coding sequence is under the control of the *tac* promoter, which is induced by the lactose analogue IPTG. Figure taken from the GE Healthcare website (www6.gelifesciences.com).

For expression of recombinant proteins, 3-5ml of an overnight culture of bacteria containing the expression plasmid was used to inoculate a 300ml flask of LB medium containing 100μg/ml ampicillin. The flask was placed in a shaking incubator at 37°C until the OD_{600} reached 0.5-0.7. The flask was then cooled to 20°C before expression of recombinant protein was induced by the addition of 0.1mM isopropyl thio-β-D-galactoside (IPTG). The proteins were induced in a shaking incubator at 20°C for 16 to 20 hours. The cells were then centrifuged at 5000g and 4°C for 30 minutes (Beckman Coulter Avanti J-26XP with the JA-14 rotor), and the pellets frozen at -20°C. When the protein was to be purified, the pellets were defrosted on ice, and resuspended in lysis buffer (PBS pH7.5, 2mM ethylenediaminetetraacetic acid (EDTA), 1mM dithiothreitol (DTT), 0.5% Triton x100). Lysozyme was added to a final concentration of 1mg/ml, and the suspension incubated on ice for 10 minutes. Protease inhibitors (1mM Phenyl Methyl Sulphonate (PMSF), 1mM benzamidine hydrate and 1x complete cocktail inhibitors (Roche)) were then added and the cells were disrupted by sonication at 20% amplitude for six 15-second pulses using a Bioblock Scientific Vibra Cell 72405 sonicator. The soluble (supernatant) and insoluble (pellet) fractions were separated by centrifugation at 8000g, 4°C, for 15 minutes (Sigma SK15 with the 12150 rotor).

Optimal expression conditions (0.1mM IPTG, 20°C, 16-20 hours) were determined by analysing samples taken before IPTG induction, after IPTG induction, and from the pellet (insoluble) and supernatant (soluble) fractions, from small-scale protein expressions at a range of temperatures (37 °C, 30 °C, 20°C) and IPTG concentrations (0.1-1mM). These samples were analysed by Coomassie-stained polyacrylamide gels and western blots, and the expression conditions that resulted in the highest levels of soluble recombinant protein production were selected for large-scale expressions.

For purification of the proteins, the supernatant from the centrifugation following sonication (soluble fraction) was transferred to a tube containing glutathione-agarose beads, which had been pre-equilibrated by washing four times in lysis buffer. The supernatant and beads were rotated at 4°C for two hours to allow the GST-tagged proteins to bind to the glutathione beads. The mixture was then centrifuged for 2 minutes to collect the beads, and washed four times in lysis buffer and once in pre-elution buffer (40mM TrisHCl pH8.7, 75mM NaCl). The recombinant proteins were eluted in elution buffer (pre-elution buffer plus 15mM reduced glutathione). Protein concentration was estimated using the Biorad reagent assay (see 2.6.3), with bovine serum albumin as a control. Glycerol was added to the eluate to a final concentration of 20%, and the protein was stored at -20°C until required.

2.6.2 Expression and purification of proteins with a His tag

The plasmid pQE-30 (Qiagen; Fig. 2-7) was used for the expression of recombinant proteins with an N-terminal 6x His tag, in SG13009 cells (Qiagen). The proteins PfCK2α and shPfCK2β2 were recombinantly expressed using this plasmid. The plasmid pET29-PfCK2a was used for the expression of recombinant PfCK2α protein with a C-terminal 6xHis tag, in BL21 Gold DE3 cells (Stratagene) or Rosetta2 DE3 cells (Novagen). The plasmid was produced in Debopam Chakrabarti's laboratory (University of Central Florida), and verified by sequencing before use; pET29 is available from Novagen.

Expression of recombinant His-tagged proteins was carried out following the same protocol as for the GST-tagged proteins, with the following modifications: the antibiotics used were 100μg/ml ampicillin and 20μg/ml kanamycin (for pQE30 expressions), or 20μg/ml kanamycin (for pET29 expressions), and the lysis buffer was 100mM TrisHCl pH 7.5, 300mM NaCl. Optimal expression conditions were deduced as outlined for the GST-tagged proteins (see 2.6.1).

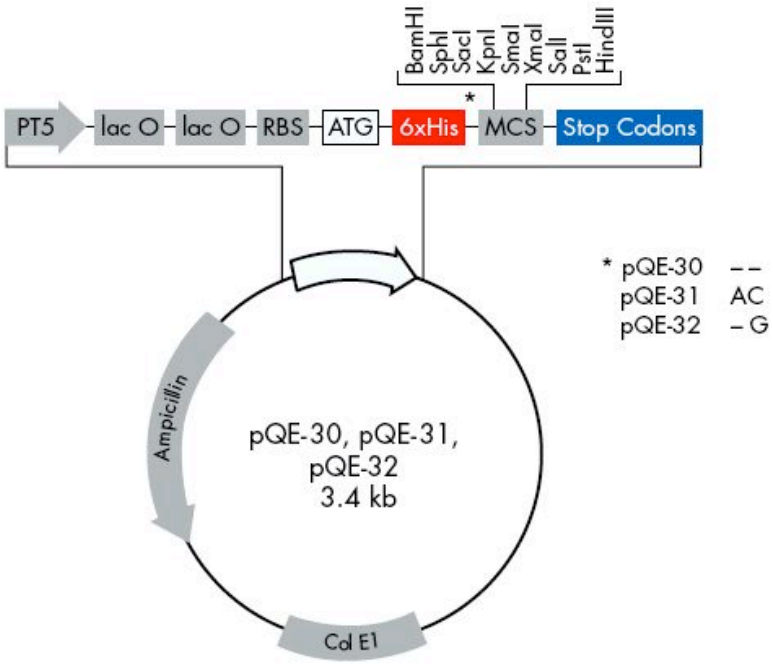

Figure 2-7 Map of the pQE-30 plasmid
This figure shows the main features of the pQE-30 plasmid (Qiagen) for recombinant expression of proteins with an N-terminal 6x His tag. The plasmid contains an ampicillin resistance cassette for selection in *E. coli*, and an expression cassette containing the phage T5 promoter (PT5), two lac operator repression modules (lac O), a ribosome binding site (RBS), a start codon (ATG), a 6xHis tag, a multiple cloning site (MCS), and stop codons. Expression is induced with IPTG. Figure taken from the Qiagen website (www1.qiagen.com).

For purification of the proteins, the supernatant from the centrifugation following sonication (soluble fraction) was transferred to a tube containing Ni-NTA beads (Qiagen), which had been pre-equilibrated in lysis buffer by washing four times. The supernatant and beads were rotated at 4^{o}C for two hours to allow the His-tagged proteins to bind to the beads. The mixture was then centrifuged for 2 minutes to collect the beads, and washed twice in lysis buffer plus 10mM Imidazole, twice in lysis buffer plus 60mM Imidazole, and once in pre-elution buffer (50mM TrisHCl pH8, 400mM NaCl). The recombinant proteins were recovered by incubation of the beads in elution buffer (pre-elution buffer plus 500mM imidazole). Protein concentration was estimated using the Biorad reagent assay (see 2.6.3), with bovine serum albumin as a standard. Glycerol was added to the eluate to a final concentration of 20%, and the protein was stored at -20^{o}C until required.

2.6.3 Determining protein concentration

The protein concentration of a solution can be determined by the Bradford Assay (Bradford, 1976). The absorbance at 595nm of the protein solution of interest in Bradford reagent (Bio-Rad) was measured and the result compared with a range of BSA standards to determine the protein concentration of the solution. The calculated protein concentration was checked by SDS-PAGE analysis (see section 2.6.4).

2.6.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated by polyacrylamide gel electrophoresis with self-made 10%, 12% or 15% polyacrylamide gels, depending on the range of protein sizes at which the best separation was desired. The separating gel consisted of 10-15% Acrylamide, 375mM TrisHCl pH8.7, 0.1% SDS, 0.0008% TEMED and 0.034% APS. The separating gel was poured between glass plates (BioRad Mini-PROTEAN 3 system) and allowed to set. The stacking gel consisted of 3% Acrylamide, 120mM Tris pH6.7, 0.1% SDS, 0.0024% TEMED and 0.05% APS. This was poured on top of the separating gel, and an appropriate comb was placed in the top, to provide 10 wells or 15 wells for the loading of samples.

Samples for separation by SDS-PAGE were boiled in Laemmli buffer for 3-5 minutes (100^{o}C), and loaded into the wells of the gel, alongside protein markers (either PageRuler prestained protein marker (Fermentas) or unstained broad range protein marker (NEB p7702)). The samples were separated in 1x TG-SDS buffer at 80V until the front had left the stacking gel, then 180-200V until the front had left the separating gel. The separated proteins were then analysed by staining or western blotting.

2.6.5 Coomassie Blue staining of polyacrylamide gels

Following electrophoresis (see 2.6.4), the gels were removed from the glass plates, and incubated in Coomassie stain for 10 minutes at room temperature. The stain then was drained off and the gels incubated in destain solution for 1-3 hours at room temperature.

2.6.6 Silver staining of polyacrylamide gels

To allow Mass Spectrometry analysis of excised bands from polyacrylamide gels, samples were separated by SDS-PAGE on NuPAGE 4-12% BisTris pre-cast gels (Invitrogen), using the NuPAGE electrophoresis system and MOPS buffer (Invitrogen), to avoid the potential keratin contamination that could be present in self-made gels. The gels were stained using the GE Healthcare PlusOne Silver Staining Kit, following the mass-spectrometry compatible protocol recommended by the manufacturers.

2.6.7 Western blot analysis

Western blots were performed according to conventional protocols, using commercial antibodies, or custom-made immunoaffinity purified rabbit antibodies (see Tables 2-2 to 2-4).

Proteins previously separated in polyacrylamide gels were transferred onto nitrocellulose membrane (BioRad) in Towbin buffer in a Trans-Blot SD Semi-Dry Transfer Cell (BioRad; transferred at 20V and 150mA for 45 minutes), or in BioRad Mini Trans-Blot cells (transferred at 100V for 90 minutes), both according to manufacturer's instructions. The nitrocellulose membranes were then blocked for one hour at room temperature in PBS with 5% (w/v) non-fat dried milk and 0.05-0.3% Tween20, and the transfer efficiency checked by staining the gels after transfer with Coomassie blue (see 2.6.5). The primary antibody (for dilutions, see Tables 2-2 or 2-3) was added to the blocking solution and incubated with the membrane for one hour. After washing four times in PBS containing 0.05-0.3% Tween20 (PBST), membranes were incubated for 45 minutes to one hour with peroxidase-conjugated secondary antibody in blocking buffer. The secondary antibodies were raised against immunoglobulin from the animal species in which the primary antibody was raised, and their dilutions for western blotting are listed in Table 2-4. Membranes were washed thoroughly in PBST and developed by enhanced chemiluminescence according to the manufacturer's instructions (PerkinElmer Life Sciences). The light generated by the reaction between the reagents and the peroxidase was detected on autoradiography film (Kodak).

To reprobe a membrane using a different antibody, the membrane was stripped of antibodies by washing for four times 5 minutes in PBST, incubating for 30 minutes at 50^{o}C in stripping buffer (62.5mM TrisHCl pH6.8, 2% SDS, 100mM 2-mercaptoethanol), and washing in PBST again for six times five minutes. The membrane could then be used again, starting with the blocking step.

2.6.8 Immunoprecipitation

Immunoprecipitations (IPs) were performed in order to analyse potential protein complexes the PfCK2 subunits might be involved in. IPs using the anti-HA antibody (see Table 2-3) were performed on protein extracts from parasite lines that had integrated the HA tag at the three-prime end of one of the genes of interest. One or two pellets of parasites (see 2.3.4) from 25ml cultures of each parasite line of interest were resuspended

in 200µl RIPA buffer, and sonicated twice at 20% amplitude for 3 seconds using a Bioblock Scientific Vibra Cell 72405 sonicator. Cells were then centrifuged for 30 minutes at 4°C and 11000rpm (Sigma SK15 centrifuge, rotor 12131). The concentration of proteins was then estimated using the Bradford Assay (see 2.6.3). Parasite proteins were diluted to 1mg/ml in RIPA buffer, and 200µl were then incubated with 3µg anti-HA antibody on ice for 2 hours. Meanwhile, Protein A sepharose beads (GE Healthcare) that had been swollen for several hours in RIPA buffer were washed four times in 500µl RIPA buffer. The beads were resuspended to a 50% slurry, and 40µl were added to the protein plus antibody mix, and rotated for 1 hour 45 minutes at 4°C. The beads were then washed four times in 500µl RIPA buffer, twice in RIPA plus 0.1% SDS, and once in 50mM TrisHCl pH7.5. The beads were then used in a kinase assay, or boiled in Laemmli buffer for analysis by SDS-PAGE.

2.6.9 Kinase assays

2.6.9.1 Standard kinase assays

Standard kinase assays were performed according to published procedures (Dorin et al., 2001). Briefly, kinase reactions (30µl) occurred in a standard kinase buffer (20mM TrisHCl pH7.5, 20mM $MgCl_2$, 2mM $MnCl_2$ and 10µM ATP) containing 0.075MBq [γ-^{32}P]ATP (220TBq/mmol; GE Healthcare), 1µg of recombinant kinase, and 5µg of substrate (β-casein). After 30 minutes at 30°C, the reaction was stopped by the addition of 8µl of 4x Laemmli buffer and boiled for 3 minutes at 100°C. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% acrylamide gels, which were stained with Coomassie blue, dried, and exposed for autoradiography.

2.6.9.2 Scintillation count (or phosphocellulose) kinase assays

Kinase assays were also analysed by the phosphocellulose method (Glass et al., 1978). A final assay volume of 18µl contained 36ng of PfCK2α, 167mM of peptide substrate, 50mM TrisHCl pH7.5, 20mM $MgCl_2$, 20µM ATP, 40-150mM NaCl, and 0.037 MBq [γ-^{32}P]ATP (220 TBq/mmol; GE Healthcare). Reactions took place for 10 minutes at room temperature, and were terminated by the addition of 60µl of 4% trichloroacetic acid. Reactions were chilled on ice for 10 minutes, then centrifuged at 10000g for 15 minutes. 60µl of supernatant was spotted onto 4x4cm Whatman P81 phosphocellulose paper squares. The squares were washed three times 15 minutes in 0.5% orthophosphate then the amount of precipitable radiolabel incorporated into the peptide substrate was quantified by

scintillation counting (in microbeta vials, with Optiphase HiSafe scintillation fluid). Three peptides substrates were used in this study, the NEB peptide p6012 (RRRADDSDDDDD), the custom peptide RRREDEESDDEE, obtained from NeoMPS, and the eIF2β-derived peptide MSGDEMIFDPTMSKKKKKKKKP (Poletto et al., 2008, Salvi et al., 2006) obtained from Genecust (Evry, France).

2.6.9.3 Kinase-GLO kinase assays

The Kinase-GLO Luminescent Kinase Assay kit from Promega was used for large-scale initial screens of a library of potential inhibitor compounds against PfCK2α. This method measures kinase activity indirectly, by measuring the amount of ATP remaining in solution following a kinase reaction. The assay is luciferase-based, and the luminescent signal produced is correlated with the amount of ATP present (and therefore is inversely correlated with the amount of kinase activity). The Kinase-Glo assays were performed and according to manufacturer's instructions using 10μM ATP, 20μM compound, 60ng of kinase. Kinase reactions were performed in black multiwell plates, and quantified after one hour by adding an equal volume of Kinase-Glo Reagent to the completed reactions and measuring luminescence in a plate reader.

2.6.10 K_m calculations

Kinases assays were performed according to 2.6.9.2, with varying concentrations of ATP, GTP or peptide, to allow calculation of the K_m of CK2α for the nucleotides and substrates. Experiments were carried out in duplicate or triplicate. Scintillation count outputs were converted into moles of phosphate incorporated into the substrate, by comparison with the scintillation count output for an aliquot of the radioactive ATP/GTP mix used for the experiment. These values were converted into reaction rates by dividing the number of moles by the length of the experiment in minutes, to give an output of nmol/min. Lineweaver-Burke plots were performed by taking the reciprocals of the kinetic data. The intercepts of the lines of best fit with the x-axis give the reciprocal of the K_m values, and the intercepts with the y-axis give the reciprocal V_{max} values.

2.6.11 IC_{50} measurements (enzymatic assays)

To test the effect of human CK2α inhibitors on PfCK2α, kinase activity was tested in kinase reactions in the presence of a range of concentrations of the potential inhibitor molecules. Stock solutions of inhibitors were at 10mM in dimethyl sulphoxide (DMSO),

and each reaction contained a final concentration of 1% DMSO. Negative controls were provided by reactions containing 1% DMSO alone (no inhibitor). Kinase reactions were performed in 18µl volumes as detailed above (2.6.9.2), except that 25µM ATP was used in each reaction.

2.6.12 IC_{50} measurements (cellular assays)

To test the effect of human CK2α inhibitors on *P. falciparum* culture, 96-well plates were inoculated with cultures at 0.5% parasitaemia and 2.5% haematocrit, and a range of inhibitor concentrations (0.04-100µM). Controls were provided by wells containing chloroquine, and wells containing uninfected or infected erythrocytes without inhibitors. Plates were incubated at 37°C for 24 hours, then 0.037MBq per well of [^{3}H]-hypoxanthine were added and the culture resumed for a further 24 hours. The experiment was terminated by incubation of the plates at -20°C for 24 hours. The material was collected on a filter mat by a cell harvester, incubated with scintillation fluid, and quantified for amount of incorporated radiolabel.

2.6.13 Protein-protein interaction assay

A mixture of 5µg of each GST-tagged and His-tagged recombinant protein in reaction buffer (20mM Tris-HCl pH7.5, 0.2 M NaCl, 0.1% Nonidet-p40 (IGEPAL) and 10% glycerol) was incubated for 30 minutes at 4°C. Glutathione-agarose beads were washed three times in reaction buffer, and resuspended to a 50% slurry. 40µl of 50% beads was added to each reaction. Reactions were rotated at 4°C for one hour, and centrifuged at 500g and 4°C for 5 minutes to recover the beads. The beads were washed four times in 1ml reaction buffer, then boiled in Laemmli buffer and stored at -20°C until analysis by SDS-PAGE and western blotting using an anti-His antibody.

3 Reverse genetics

3.1 Introduction

The primary criterion for suitability for a drug or vaccine target is the essentiality of the protein for organism viability (Greenbaum, 2008). CK2α is essential in all organisms in which essentiality has been tested, including *Saccharomyces cerevisiae* (Ackermann et al., 2001, Glover, 1998, Padmanabha et al., 1990), *Dictyostelium discoideum* (Kikkawa et al., 1992), and mice (Lou et al., 2008). In contrast, CK2β is essential in mice (Buchou et al., 2003) and *Caenorhabditis elegans* (Fraser et al., 2000), and not essential in *Schizosaccharomyces pombe* (Roussou and Draetta, 1994), or *Saccharomyces cerevisiae* (Ackermann et al., 2001). This chapter describes attempts to knock out the three CK2 genes in *Plasmodium falciparum*.

We adopted the gene disruption technique to test for essentiality of the genes (Sidhu et al., 2005). A truncated portion of the gene, lacking regions coding for motifs essential to the function of the encoded protein, is inserted into a plasmid that contains a drug resistance cassette for selection in *P. falciparum*. Upon integration at the gene locus by single-cross-over homologous recombination, a pseudo-diploid locus is generated, with both truncated copies of the gene lacking essential regions. Neither copy is expected to be capable of producing functional protein, since one lacks a 3'UTR, and the other lacks a promoter and initiation codon. Even if the genes were to be expressed, neither of the truncated proteins would be expected to be functional, since both lack motifs essential for the protein activity.

The PfCK2 proteins are expressed in both the asexual and the sexual intraerythrocytic stages of the parasite (see sections 4.3 and 5.3). The proteins may be required for sexual development, the asexual cycle, or both. To distinguish between these possibilities, we transfected parasites with (i) a knockout (or disruption) plasmid alone, or (ii) a knockout plasmid and a complementation plasmid, which expresses a functional copy of the gene targeted for disruption. The parasite is haploid during the intraerythrocytic stages, so the disruption of an essential gene leads to inviable parasites. If parasite populations can be recovered in which the gene has been disrupted in the absence of the complementing plasmid, the gene is not essential for asexual growth. If, on the other hand, viable parasites harbouring gene disruptions can only be recovered when they have also been transfected with the complementation plasmid, the gene is considered to be essential for asexual growth.

We performed an additional set of transfections to test for the recombinogenicity of the gene loci. Plasmids were constructed that on integration into the genomic locus would add a sequence coding for an HA tag to the 3' end of the targeted PfCK2 gene. The integration of these tags should not interfere with the functions of the proteins, therefore if they cannot integrate into the genomic locus, it indicates that the locus is non-recombinogenic. These parasite lines will be used for protein-protein interaction studies as well as for the reverse genetics information they provide.

This chapter describes the production of parasite lines expressing HA-tagged PfCK2 subunits, and attempts to knock out the PfCK2 genes by gene disruption experiments, in the presence or absence of complementation plasmids.

3.2 Construction of plasmids for transfection

3.2.1 Plasmids for in vivo *3' tagging*

In order to (i) demonstrate locus accessibility for recombination, and (ii) generate tools allowing the isolation of protein complexes from *in vivo* contexts, and thus begin to determine binding partners of the three subunits, vectors were designed that would add sequences coding for haemaglutinin (HA) tags to the 3' ends of each of the three *PfCK2* subunit genes, without affecting protein function. The modified genes will encode HA-tagged subunits, allowing for purification of protein complexes containing any of the subunits. The pCAM-BSD-HA vector (Jean Halbert, this laboratory) is based on the pCAM-BSD plasmid (Sidhu et al., 2005), and is designed to contain PstI and BamHI sites between which to clone ~500bp of the 3'end of the gene of interest, lacking a stop codon. This is followed by the HA tag sequence, a stop codon and a 3' UTR. Single crossover homologous recombination of the tagging plasmid with the gene locus should result in the gene locus being modified to encode the PfCK2 subunit with a C-terminal HA tag (see Fig. 3-7D for a schematic of the strategy). pCAM-BSD contains the *Aspergillus terrus* blasticidine-S-deaminase gene, whose gene product mediates resistance to the drug blasticidin, allowing for selection in *P. falciparum*.

The 3' end of the *PfCK2*α gene (535bp), without the stop codon, was amplified from the pGEM-T-Easy-PfCK2a plasmid (see 4.4.1) using Pfx Platinum polymerase and the oligonucleotide primers CK2aTAGForPst and CK2aTAGRevBam, which add a PstI and BamHI site to the 5' and 3' ends of the PCR product respectively. The PCR products were ligated into the vector pGEM-T-Easy (see section 2.5.2.1) and transformed into XL10

Gold bacteria. Bacterial colonies containing plasmid with insert of the correct size were identified by colony screen PCR (section 2.5.1.3) using the primers T7 and SP6. Plasmid DNA was extracted (section 2.5.6) and the insert regions of the plasmids were sequenced (2.5.9) to verify that they contained the correct sequence (with no mutations), before the insert was subcloned (section 2.5.2.2) into pCAM-BSD-HA using the restriction enzymes PstI and BamHI. This plasmid was named pCAM-BSD-HA-PfCK2a (see Fig. 3-1), and the insert region was sequenced before use. Prior to transfection of *P. falciparum* cells, the plasmid was extracted from 250ml cultures of bacteria using the Maxiprep kit (Qiagen).

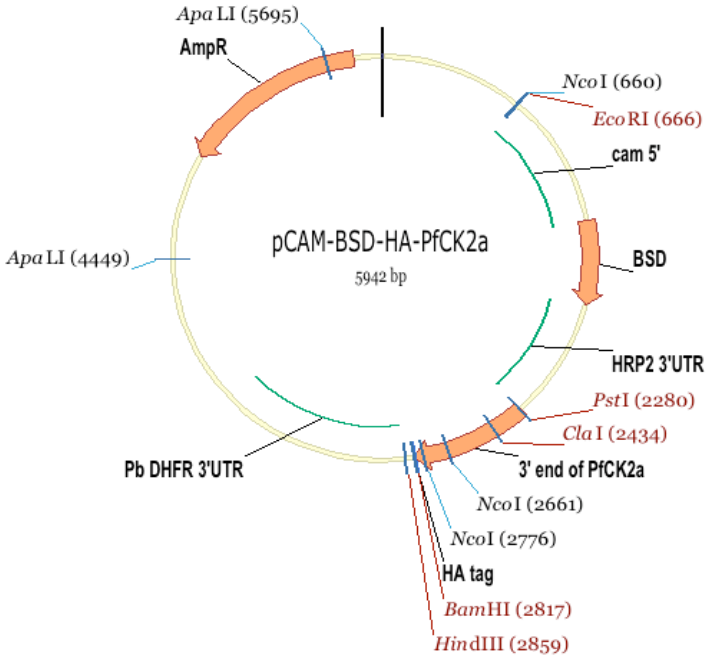

Figure 3-1 Map of the pCAM-BSD-HA-PfCK2a plasmid
The plasmid contains two selectable markers: an ampicillin resistance cassette allowing for selection in *E. coli*, and the blasticidin-S-deaminase gene under the control of the *Plasmodium* calmodulin promoter (cam 5') allowing for selection in *P. falciparum*. The 3' end of *PfCK2α* was inserted between the restriction sites PstI and BamHI, in frame with the HA tag coding sequence, which is followed by the 3' UTR of *P. berghei* dihydrofolate reductase (Pb DHFR). Restriction sites in black are for the enzymes that have multiple sites in the plasmid, and those in red are for the enzymes that will only cut the plasmid in one location. Figure produced using Vector NTI software (Invitrogen).

The plasmids pCAM-BSD-HA-PfCK2b1 and pCAM-BSD-HA-PfCK2b2 (Appendix 1) were constructed as described for pCAM-BSD-HA-PfCK2a above, with the following changes. For *PfCK2β2*, the 3' end of the second exon of the gene (573bp) was amplified from the pGEM-T-Easy-PfCK2b2 plasmid (section 5.5) using the oligonucleotide primers CK2b2TAGForPst and CK2b2TAGRevBam. For *PfCK2β1*, the 3' end of the gene (540bp) was amplified from the pGEM-T-Easy-PfCK2b1 plasmid using the oligonucleotide primers CK2b1TAGForPst and CK2b1TAGRevBgl. The restriction enzymes PstI and BglII were used to release the PCR product from pGEM-T-Easy, and PstI and BamHI were used to cut pCAM-BSD-HA. The enzymes BglII and BamHI have compatible sticky ends,

enabling successful ligation of the PCR product into the destination vector. BamHI could not be used to digest the 3' fragment of *PfCK2β1*, because it contains an internal BamHI site. Both plasmids pCAM-BSD-HA-PfCK2b1 and pCAM-BSD-HA-PfCK2b2 were confirmed as containing the correct sequences by DNA sequencing of the insert, and extracted in bulk using the Plasmid Maxi kit (Qiagen; see 2.5.6) for use in transfection of *P. falciparum* cells.

3.2.2 Gene disruption plasmids

To analyse the role of PfCK2 in the parasite lifecycle, and validate it (or not) as a potential drug target, we performed gene disruption experiments. Truncated versions of the *PfCK2* genes were inserted into plasmids with drug-selectable markers. Single crossover homologous recombination of the truncated gene in the plasmid with the parasite chromosomal gene locus should result in a pseudo-diploid locus, with two truncated and non-functional versions of the gene (see Fig. 3-8A for a schematic of the strategy).

The sequence used for homologous recombination with *PfCK2α*, KOPfCK2α, was amplified from 3D7 cDNA using primers CK2aKOForBam and CK2aKORevNot to produce a 447bp sequence of the PfCK2α gene. This truncated sequence lacks the 3' and 5' ends of the coding region, which code for the protein kinase motifs GxGxxG and APE (present as GxGxxS and GPE in PfCK2α) respectively; both motifs are necessary for the catalytic function of the encoded protein. The PCR product was cloned into the pGEM-T-Easy plasmid, verified by sequencing, and subcloned into the pCAM-BSD plasmid between the BamHI and NotI sites. The construct was named pCAM-BSD-KOPfCK2a (Fig. 3-2). This plasmid was also sent for sequencing, to verify that it contained the correct insert.

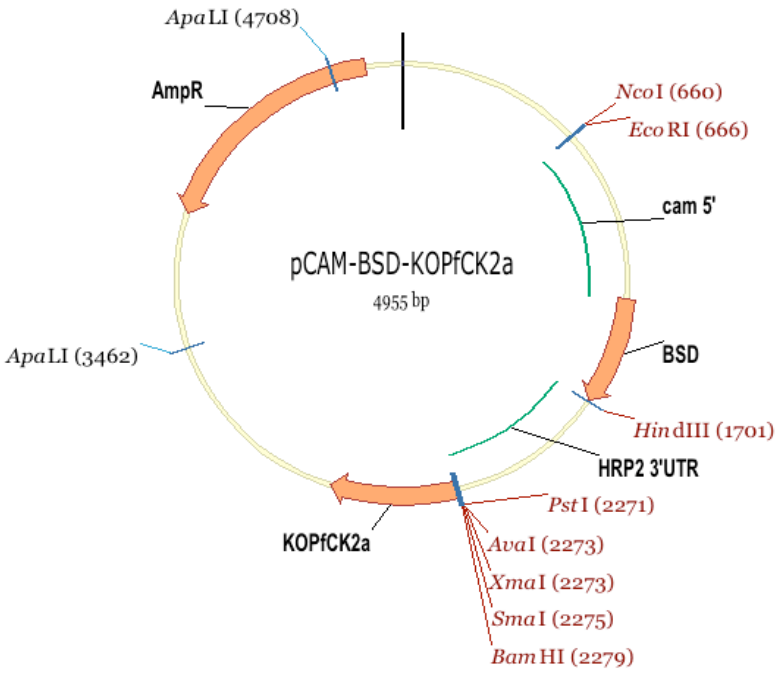

Figure 3-2 Map of the pCAM-BSD-KOPfCK2a plasmid
This figure shows the main features of the plasmid pCAM-BSD-KOPfCK2a. The plasmid contains an ampicillin resistance cassette for selection in *E. coli*, and a BSD cassette for selection in *P. falciparum*. The KOPfCK2a fragment was inserted between the BamHI and NotI sites. Several of the restriction enzyme sites are shown. Those in black are for the enzymes that have multiple sites in the plasmid, and those shown in red are for the enzymes that will only cut the plasmid in one location. Enzymes for the digestion of transfected parasite gDNA for analysis by Southern blotting were chosen out of the list of enzymes that cut the plasmid once. Figure produced using Vector NTI software (Invitrogen).

CK2β proteins possess two pairs of cysteine residues that form the base of a Zinc finger required for homo- and hetero-dimerisation of the CK2β subunits (Canton et al., 2001), which is a prerequisite for CK2 holoenzyme formation (Graham and Litchfield, 2000). Constructs for the disruption of the two beta genes were designed in such a way that after integration, neither copy of the gene will be active: one truncated copy will possess its promoter and initiation codon, but lack the C-terminal cysteine pair, a stop codon and a 3'UTR, while the other copy will possess both cysteine pairs but lack a promoter and an initiation codon, and have an artificial stop codon introduced; therefore, the target gene will be inactivated. The sequence used for homologous recombination with *PfCK2β1*, KOPfCK2b1 (429bp), was amplified using Pfx Platinum polymerase and the oligonucleotide pairs CK2b1KOForBgl and CK2b1KORevNot, and as template an amplified *PfCK2β1* construct that had been verified by sequencing (section 2.5.9). The oligonucleotide pairs CK2b2KOForBam and CK2b2KORevNot were used to amplify KOPfCK2b2, a 1061bp product from the *PfCK2β2* gene. The KOPfCK2b2 sequence extends over the intron of the *PfCK2β2* gene, so 3D7 gDNA rather than cDNA was used as the template. The KOPfCK2b1 and KOPfCK2b2 constructs were inserted between the BamHI and NotI sites of the pCAM-BSD plasmid. The final constructs were sequenced

prior to use, and named pCAM-BSD-KOPfCK2β1 and pCAM-BSD-KOPfCK2β2 (Appendix 1).

All three disruption/knockout plasmids were extracted from 250ml cultures of bacteria using the Plasmid Maxi kit (Qiagen; see 2.5.6) for transfection into *P. falciparum* cells.

3.2.3 Complementation plasmids

To provide a control for the gene disruption experiments, complementation plasmids were produced. These plasmids are designed to provide a fully functional episomal copy of a gene, such that on integration of a co-transfected knockout plasmid into the chromosomal gene locus, the protein will still be produced episomally. These studies will test the hypothesis that the gene loci can be disrupted if an additional functional copy of the gene is present.

Full-length coding sequences for PfCK2α and PfCK2β2 were digested out of the expression plasmids pGEX-4T-3-PfCK2a (see 4.4.1) and pGEX-4T-3-PfCK2b2 (see 5.5.1) using the restriction enzymes BamHI and NotI. PfCK2β1 was digested out of the plasmid pGEM-T-Easy-PfCK2b1 (see 5.4.1) using the restriction enzymes BglII and NotI. The fragments were sub-cloned into the BglII/NotI sites of the expression cassette of the plasmid pHGB (Tonkin et al., 2004) (see Fig. 2-3), between the *P. falciparum* heat shock protein 86 promoter (PfHSP86 5'UTR) and the 3' untranslated region of *P. berghei* dihydrofolate reductase/thymilidate synthase (PbDT 3'). The *P. falciparum* heat shock protein 86 displays a similar expression profile to the three PfCK2 genes (Le Roch et al., 2003), therefore the Hsp86 promoter is presumably an appropriate choice for the complementation plasmid promoter. These expression cassettes from pHGB replaced the selection cassette in the complementation plasmid pCHD-1/2 (Tonkin et al., 2004) by a clonase reaction, as described in section 2.3.2, to produce the pCHD-PfCK2a, pCHD-PfCK2b1 and pCHD-PfCK2b2 plasmids (Fig. 3-3, and Appendix 1). The pCDH-1/2-based plasmids can be selected for in *P. falciparum* under WR99210 treatment.

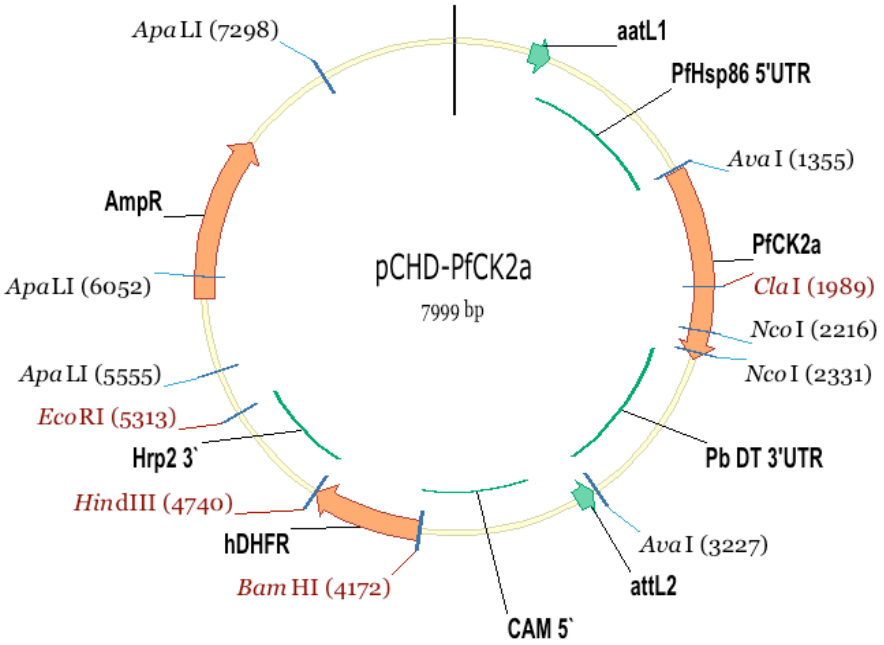

Figure 3-3 Map of the pCHD-PfCK2a plasmid
This figure shows the important features of the plasmid pCHD-PfCK2a. The plasmid encodes two selectable markers, the ampicillin resistance gene (AmpR) for selection in *E. coli*, and the human dihydrofolate reductase (hDHFR) gene, which mediates WR99210 resistance, for selection in *P. falciparum*. Some restriction enzyme recognition sites are also shown. See also Fig. 2-3. Figure produced using Vector NTI software (Invitrogen).

Schematics of the PfCK2 gene structures, and the portions of the genes used for cloning and for generation of gene-specific probes for Southern blots are shown in Figs. 3-4, 5 and 6.

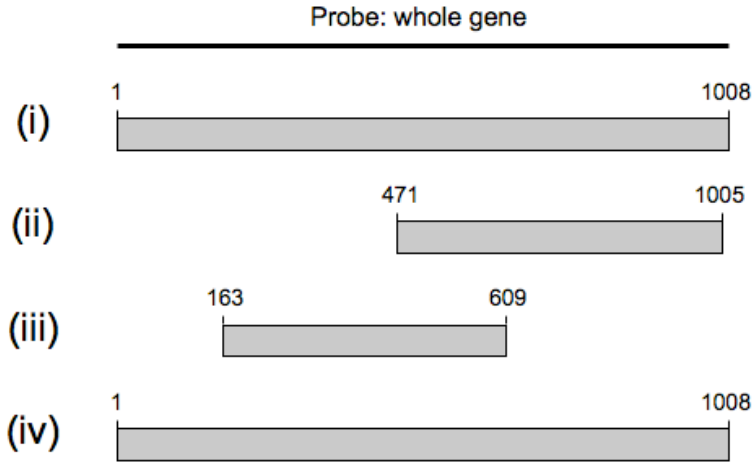

Figure 3-4 PfCK2α schematic
PfCK2α gene structure and portions of the gene used for cloning and production of the gene-specific probe are shown. i) PfCK2α gene. ii) Portion of PfCK2α gene used for generation of the 3'-tagging plasmid pCAM-BSD-HA-PfCK2a. iii) Portion of PfCK2α gene used for generation of the knockout plasmid pCAM-BSD-KOPfCK2a. iv) Portion of PfCK2α gene (whole gene) used for generation of the complementation plasmid pCHD-PfCK2a.

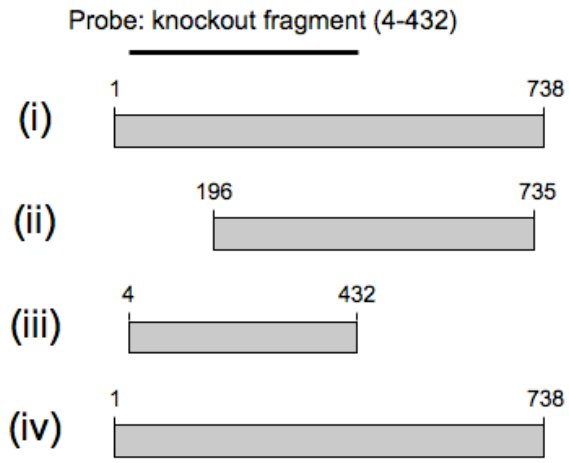

Figure 3-5 PfCK2β1 reverse genetics cloning schematic
PfCK2β1 gene structure and portions of the gene used for cloning and production of the gene-specific probe are shown. i) PfCK2β1 gene. ii) Portion of PfCK2β1 gene used for generation of the 3'-tagging plasmid pCAM-BSD-HA-PfCK2b1. iii) Portion of PfCK2β1 gene used for generation of the knockout plasmid pCAM-BSD-KOPfCK2b1. iv) Portion of PfCK2β1 gene (whole gene) used for generation of the complementation plasmid pCHD-PfCK2b1.

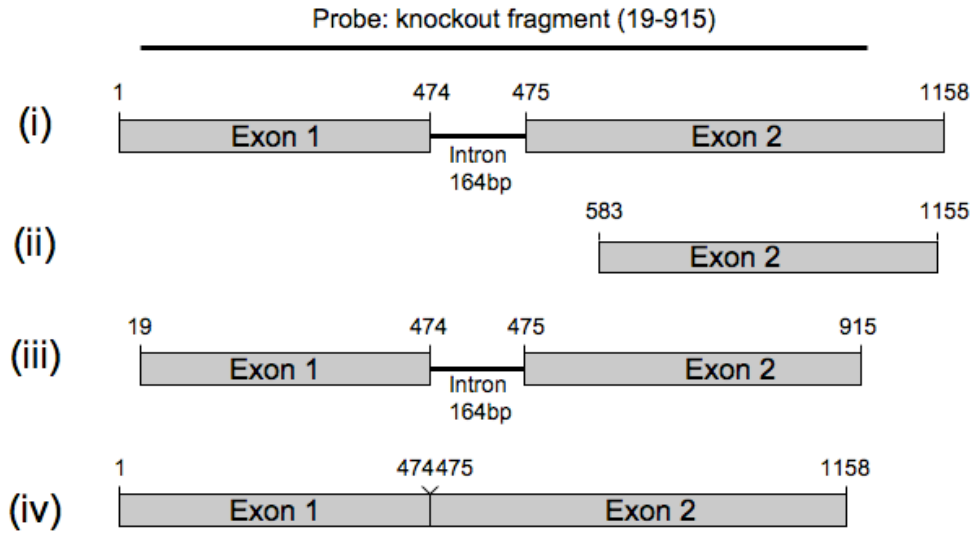

Figure 3-6 PfCK2β2 reverse genetics cloning schematic
PfCK2β2 gene structure and portions of the gene used for cloning and production of the gene-specific probe are shown. i) PfCK2β2 gene. ii) Portion of PfCK2β2 gene used for generation of the 3'-tagging plasmid pCAM-BSD-HA-PfCK2b2. iii) Portion of PfCK2β2 gene used for generation of the knockout plasmid pCAM-BSD-KOPfCK2b2. iv) Portion of PfCK2β2 gene (whole of both exons, no intron) used for generation of the complementation plasmid pCHD-PfCK2b2.

3.3 PfCK2α

3.3.1 In vivo *tagging*

P. falciparum 3D7 parasites were transfected with p-CAM-BSD-HA-PfCK2a (see sections 2.3.2&3) and maintained under blasticidin selection. Screening to test for integration was first conducted at 90 days post-transfection, by gDNA extraction and PCR screening using TaKaRa ex Taq polymerase and the diagnostic oligonucleotide pairs detailed in Table 3-1. There was clear evidence of integration (Fig. 3-7A). Twelve clonal lines were produced by limiting dilution, and the clones similarly screened for integration by PCR (two shown here, Fig. 3-7B&C).

Table 3-1 Oligonucleotide pairs for detecting integration of pCAM-BSD-HA-PfCK2a
The number in brackets after the oligonucleotide primer names is how the primers are identified in Fig. 3-7D.

Band to be amplified	Forwards oligonucleotide	Reverse oligonucleotide	Expected size of amplified band (bp)
Wild type	CK2aForBam (1)	CK2a3primeRev (2)	1176
5' integration	CK2aForBam (1)	pCAMBSDRev (4)	1973
3' integration	pCAMBSDFor (3)	CK2a3primeRev (2)	810
Plasmid	pCAMBSDFor (3)	pCAMBSDRev (4)	1577

DNA from two of the clonal lines was also analysed by Southern blotting (Fig. 3-7E). Probing the Southern blot with a *BSD* gene probe demonstrated that the plasmid had integrated, with the expected size band clearly seen (14.5kb) in the gDNA from the clonal lines (lanes 2&3), and no band seen in the gDNA from untransfected parasites (lane 1). Probing the Southern blot with *PfCK2α* showed that no wild type band (13kb) remained in the clonal cultures, and the expected sizes of bands indicative of integration were seen (14.5kb and 4.4kb). Hence, the plasmid pCAM-BSD-HA-PfCK2a is able to integrate into the *PfCK2α* genomic locus, demonstrating that the locus is amenable to recombination. The 5.9kb band corresponding to the linearised plasmid could represent linearised episome, or integrated concatemers of the plasmid. Repetition of the Southern Blot using a different restriction enzyme that does not have a recognition site in the plasmid would clarify whether there is more than one integrated copy of the plasmid in the locus.

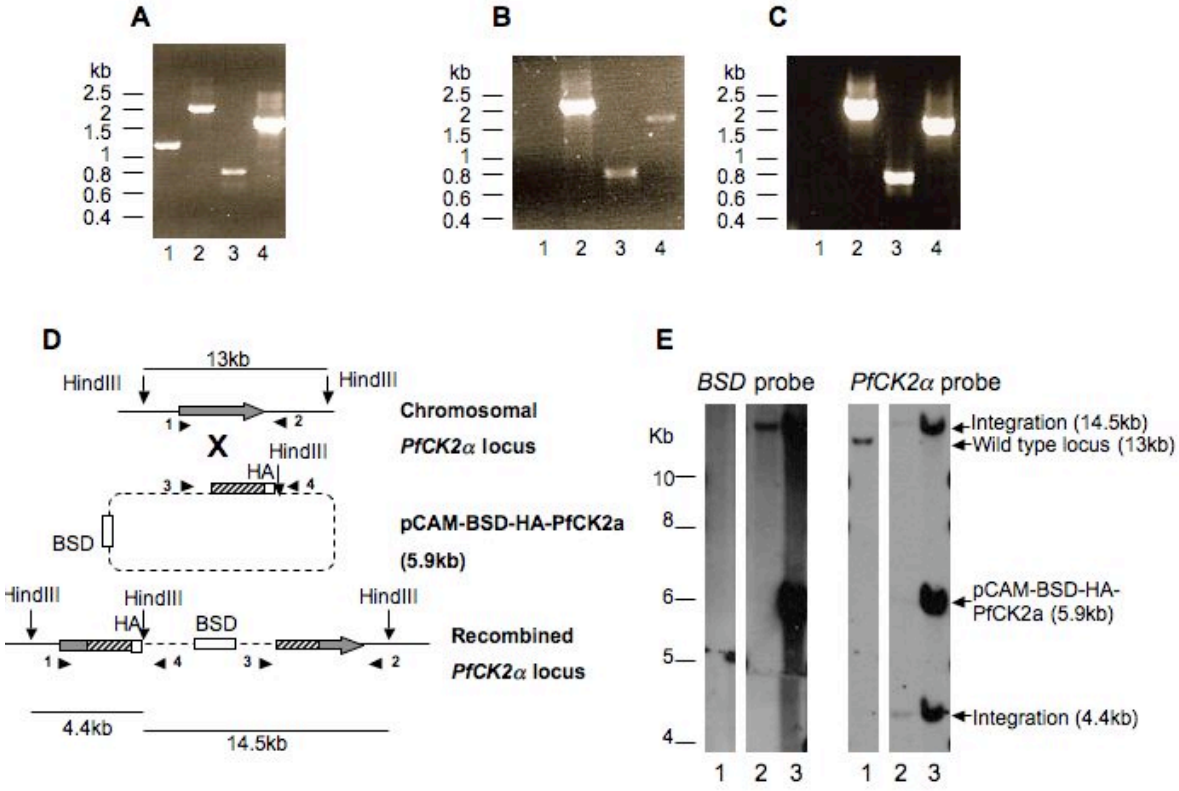

Figure 3-7 HA tagging of *PfCK2α*
Panel A: PCR screening for integration in gDNA from parasites transfected with pCAM-BSD-HA-PfCK2a revealed the presence of parasites in which integration events had occurred (lane 2: 1973 bp, lane 3: 810 bp). This culture still contained the wild type locus (lane 1: 1176bp), and therefore clonal lines were derived from the culture by limiting dilution. The plasmid was still present in the cultures (lane 4: 1577bp diagnostic band). Panels B and C: PCR screening for integration in gDNA from two of the parasite lines derived by limiting dilution (B: clone B3, C: clone E1) revealed that the wild type band had been lost (lane 1) and only the integration bands were seen (lanes 2 and 3). These clonal lines were further analysed by Southern blotting (Panel E). Panel D is a schematic of the chromosomal gene locus, the pCAM-BSD-HA-PfCK2a plasmid and the recombined locus, and shows the locations of oligonucleotide primers (indicated by numbered arrowheads) used for the PCR screens (Panels A-C). Oligonucleotide identities are listed in Table 3-1. The locations of the HindIII restriction enzyme recognition sites are depicted and the expected sizes of the fragments of gDNA after restriction digestion are shown. Fragments of gDNA resulting from restriction endonuclease digestion were analysed by Southern blotting (Panel E). 1: untransfected 3D7 parasites, 2: PfCK2αHA clone B3, 3: PfCK2αHA clone E1. The wild type locus, recognised by the *PfCK2α* probe, disappears in the clonal lines, and integration bands of the expected sizes are seen, indicating that the plasmid successfully integrated into the locus.

The modified gene should encode an HA-tagged PfCK2α subunit, allowing for purification of protein complexes containing PfCK2α. Two clonal lines were tested for the presence of HA-tagged PfCK2α subunits by anti-HA immunoprecipitations, with untransfected parasites as negative controls (see Fig. 5-7).

3.3.2 Knockout and complementation

To determine whether PfCK2α has essential functions for parasite survival, and is thus a valid (or not) potential drug target, we attempted to disrupt the *PfCK2α* gene. To generate

a plasmid able to disrupt the *PfCK2α* gene, an internal fragment of the coding sequence, excluding the DNA coding for the critical motifs Gly-x-Gly-x-x-Ser (subdomain I, involved in anchoring of the ATP molecule) and Gly-Pro-Glu (subdomain VIII, required for structural stability of the C-terminal lobe, see Figs. 1-5 and 4-1), was amplified and cloned into the transfection vector pCAM-BSD, which confers resistance to blasticidin. Integration of this construct (pCAM-BSD-KOPfCK2α) into the genomic locus by single cross-over homologous recombination is expected to result in a pseudo-diploid configuration, where both truncated copies will be unable to express a functional enzyme, since one will lack a stop codon and 3'UTR, the other will lack a promoter, and both will lack one of the essential motifs for enzyme activity (Fig. 3-8A).

After two independent transfections of pCAM-BSD-KOPfCK2α into 3D7 parasites, integration was monitored in the blasticidin-resistant populations by PCR (Fig. 3-8B), using primer combinations that allow discrimination between the episome, the wild-type locus and the disrupted locus (Table 3-2). Only the episome (610bp, lane 4) and the wild-type locus (1030bp, lane 1) were detectable, with no sign of integration even after prolonged culture (16 weeks). In contrast, we regularly observe disruption of non-essential genes 6-7 weeks post transfection (for example, see (Dorin-Semblat et al., 2007, Dorin-Semblat et al., 2008)), and integration of the PfCK2α tagging construct was readily observed 12 weeks after transfection (see section 3.3.1).

Table 3-2 Oligonucleotide pairs for detecting integration of pCAM-BSD-KOPfCK2a
The number in brackets after the oligonucleotide primer names is how the primers are identified in Fig. 3-8A.

Band to be amplified	Forwards oligonucleotide	Reverse oligonucleotide	Expected size of amplified band (bp)
Wild type	CK2a5primeFor (1)	CK2aRevSal (2A)	1030
5' integration	CK2a5primeFor (1)	pCAMBSDRev (4)	677
3' integration	pCAMBSDFor (3)	CK2a3primeRev (2B)	1060
Plasmid	pCAMBSDFor (3)	pCAMBSDRev (4)	610

Two hypotheses can account for the non-integration of the disruption plasmid: the presence of an intact gene is crucial for parasite asexual multiplication, or the locus is not amenable to recombination. We have already demonstrated that the *PfCK2α* locus is recombinogenic, by integration of the pCAM-BSD-HA-PfCK2a construct into the locus (Fig. 3-7). We nevertheless wanted to ascertain that *PfCK2α* can be disrupted if the PfCK2α protein is provided through expression of an episomal copy of the gene. To this effect, a complementation plasmid (pCHD-PfCK2a, Fig. 3-3) was constructed as described above (section 3.2.3). In parallel with the transfection of the pCAM-BSD-KOPfCK2a

plasmid alone, two further populations of parasites were co-transfected with both pCAM-BSD-KOPfCK2a and pCHD-PfCK2a (however, one of these parasite lines never recovered to a visible parasitaemia under double drug selection, and was discarded). PCR analyses (Fig. 3-8B) showed that disruption of the targeted locus occurred only in the doubly transfected, doubly resistant parasites. Integration was detected at 10 weeks post-transfection (the first time it was tested) in the doubly-transfected line, whereas even after 16 weeks post-transfection, no integration was detected in the singly-transfected lines. Southern blot analysis confirmed this result (Fig. 3-8C). The *P. falciparum PfCK2a* probe hybridised to a 13kb band that represents the wild type locus in the lanes that contained gDNA from untransfected parasites and parasites transfected with the knockout plasmid alone (lanes 1-3). The intensity of this band dramatically decreased in the gDNA from doubly-transfected parasites, and was undetectable in two clonal lines (E7 and G9) that were derived from this culture by limiting dilution, indicating that the gene can be disrupted only when an additional cassette coding for the PfCK2α enzyme is provided to the parasites. There are multiple possibilities for the recombination of the knockout and complementation plasmids with each other before or after integration, which could account for the additional bands of unexpected size observed (6kb, 14kb), and the lack of the band corresponding to the 3' end of the integration site (10kb). The most important observation is that the wild type band only disappears in the doubly-transfected parasites.

Taken together, these data provide strong evidence that PfCK2α is essential to viability of the asexual erythrocytic stage parasites. This fits with what we know of the essentiality of CK2α orthologues in other species (Kikkawa et al., 1992, Lou et al., 2008, Padmanabha et al., 1990), and validates the enzyme as a potential drug target.

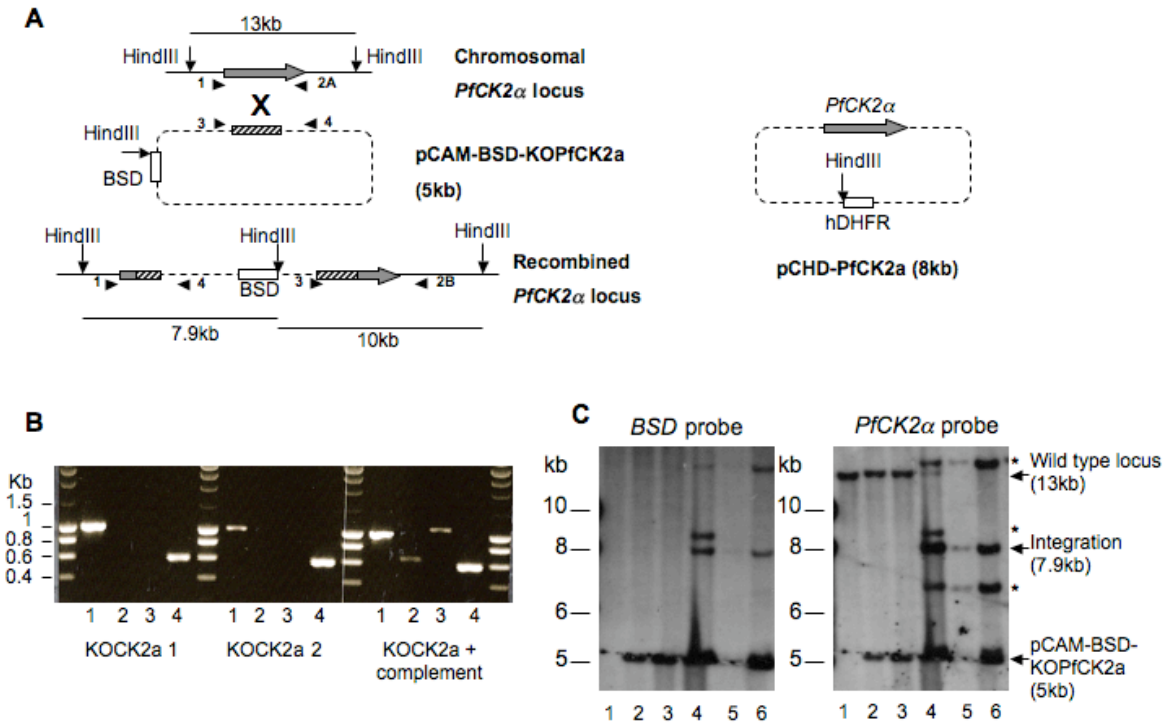

Figure 3-8 Knockout studies of *PfCK2α*
3D7 parasites transfected with pCAM-BSD-KOPfCK2a with or without pCHD-PfCK2a were analysed by PCR and Southern blotting. Panel A: Diagram showing the locations of the primers used for PCR screening (indicated by numbered arrowheads; their identities are listed in Table 3-2), and the recognition sites for the HindIII restriction enzyme used to cut the gDNA to give a diagnostic pattern of bands for analysis by Southern blotting. Panel B: PCR screening of gDNA from two separate pCAM-BSD-KOPfCK2a-transfected lines (KOCK2a 1 and KOCK2a 2), and parasites transfected with both the knockout plasmid and the complementation plasmid (KOCK2a + complement). 1: amplification of the wild type *PfCK2α* locus. 2: amplification over the 5' integration boundary. 3: amplification over the 3' integration boundary. 4: amplification of the insert in the pCAM-BSD-KOPfCK2a plasmid. Evidence of integration is seen only in gDNA from the doubly-transfected parasite culture (KOCK2a + complement). Clonal cultures were derived from this population by limiting dilution, and the gDNA analysed by Southern blotting (Panel C). The restriction enzyme HindIII was used to digest the gDNA, and the fragments were analysed by Southern blotting using *BSD* and *PfCK2α* as probes. 1: untransfected 3D7, 2: KOCK2a1, 3: KOCK2a2, 4: KOCK2a + complement, 5: KOCK2a + complement clone E7, 6: KOCK2a + complement clone G9. Stars mark unexplained bands.

3.4 PfCK2β1

3.4.1 In vivo *tagging*

P. falciparum 3D7 parasites were transfected with p-CAM-BSD-HA-PfCK2b1 (see sections 2.3.2&3) and maintained under blasticidin selection. Screening to test for integration was first conducted at 73 days post-transfection, by gDNA extraction and PCR screening using TaKaRa ex Taq polymerase and the diagnostic oligonucleotide pairs detailed in Table 3-3. Evidence of integration was clearly seen (Fig. 3-9A). Three clonal lines were produced by limiting dilution (2.3.3), and the clones screened for integration by PCR (two are shown here, Fig. 3-9B&C).

Table 3-3 Oligonucleotide pairs for detecting integration of pCAM-BSD-HA-PfCK2b1 The number in brackets after the oligonucleotide primer names is how the primers are identified in Fig. 3-9D.

Band to be amplified	Forwards oligonucleotide	Reverse oligonucleotide	Expected size of amplified band (bp)
Wild type	CK2b1ForEco (1)	CK2b13primeRev (2)	804
5' integration	CK2b1ForEco (1)	pCAMBSDRev (4)	1673
3' integration	pCAMBSDFor (3)	CK2b13primeRev (2)	722
Plasmid	pCAMBSDFor (3)	pCAMBSDRev (4)	1582

DNA from the clonal lines was also analysed by Southern blotting (Fig. 3-9E). Probing the Southern blot with a *BSD* gene probe demonstrated that the plasmid had integrated, with the expected size band (6.2kb) seen in the gDNA from the clonal lines (lanes 2&3), and no band seen in the gDNA from untransfected parasites (lane 1). The *PfCK2β1* probe hybridised to the wild type band (4.1kb) in the untransfected parasites (Fig. 3-9E, lane 1), but no band of this size was seen in the clonal HA-tagged lines (lanes 2&3). The expected sizes of bands indicative of integration were seen in these clonal lines (3.3kb and 6.2kb). We can thus conclude that the plasmid pCAM-BSD-HA-PfCK2b1 is able to integrate into the *PfCK2β1* genomic locus, demonstrating that the locus is available for recombination. The modified gene should encode an HA-tagged PfCK2β1 subunit, allowing for purification of protein complexes containing PfCK2β1. Two clonal lines were tested for the presence of HA-tagged PfCK2β1 subunits by anti-HA immunoprecipitations, with untransfected parasites as negative controls (see Fig. 5-7).

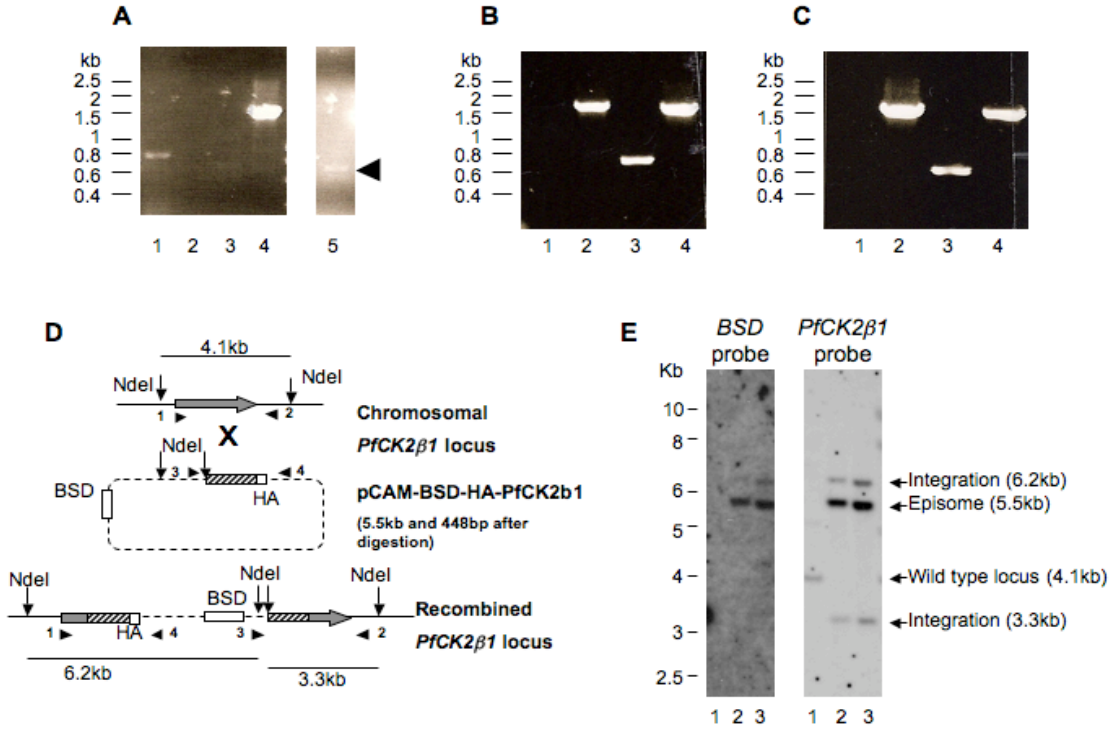

Figure 3-9 HA tagging of *PfCK2β1*
Panel A: PCR screening for integration in gDNA from parasites transfected with pCAM-BSD-HA-PfCK2b1 revealed the presence of parasites in which integration events had occurred (lane 3: 722bp. This lane is shown overexposed (5), where the 3' integration band, which is indicated with an arrowhead, can more clearly be seen). Lane 1: amplification of the wild type locus, lane 2: amplification over the 5' integration boundary, lane 3: amplification over the 3' integration boundary, lane 4: diagnostic band for the presence of the plasmid. Panels B&C: PCR screening for integration in gDNA of parasite lines derived by limiting dilution (B: clone C9, C: clone E8) revealed that the wild type band had been lost (lane 1) and only the integration bands (lane 2: 1673bp, lane 3: 722bp) were seen. These clonal lines were further analysed by Southern blotting (Panel E). Panel D is a schematic of the chromosomal gene locus, the pCAM-BSD-HA-PfCK2b1 plasmid and the recombined locus, and shows the locations of oligonucleotide primers (indicated by numbered arrowheads) used for the PCR screens (Panels A-C). Oligonucleotide identities are listed in Table 3-3. The location of the restriction enzyme recognition sites are depicted and the expected sizes of the fragments of gDNA after restriction endonuclease digestion are shown. Fragments of gDNA resulting from restriction endonuclease digestion were analysed by Southern blotting (Panel E), using *PfCK2β1* and *BSD* as probes. gDNA from wild type 3D7 parasites (lane 1), and PfCK2b1HA clonal lines C9 (lane 2) and E8 (lane 3), was digested using the restriction enzyme NdeI. The wild type locus, recognised by the *PfCK2β1* probe, disappears in the clonal lines, and integration bands appear in the clonal lines, indicating that integration has occurred.

3.4.2 Knockout and complementation

We wanted to determine whether PfCK2β1 has essential functions for parasite survival. Reverse genetics data for CK2β from other organisms gives a mixed picture: for some organisms the beta subunits are essential, for others they are not (see sections 1.6.2 and 3.1). Plasmids were constructed to achieve gene disruption by single crossover integration. Constructs for the disruption of the two beta genes were designed as detailed in section 3.2.2 above.

After two independent transfections of pCAM-BSD-KOPfCK2b1 into 3D7 parasites (parasite lines named KOCK2b1 1 and 2), integration was monitored in the blasticidin-resistant populations by PCR (Fig. 3-10B), using primer combinations that allow discrimination between the episome, the wild-type locus and the disrupted locus (Table 3-4). Only the episome (604bp diagnostic fragment amplified, lane 4) and the wild-type locus (934bp fragment amplified, lane 1) were detectable, with no sign of integration even after prolonged culture (15 weeks). We have already confirmed that the *PfCK2β1* locus is recombinogenic, since the pCAM-BSD-HA-PfCK2b1 plasmid was able to integrate into the locus (Fig. 3-9). The lack of integration of the knockout plasmid therefore indicates that the gene is essential to the survival of asexual erythrocytic stage parasites.

Table 3-4 Oligonucleotide pairs for detecting integration of pCAM-BSD-KOPfCK2b1 The number in brackets after the oligonucleotide primer names is how the primers are identified in Fig. 3-10A.

Band to be amplified	Forwards oligonucleotide	Reverse oligonucleotide	Expected size of amplified band (bp)
Wild type	CK2b15primeF (1)	CK2b13primeRev (2)	934
5' integration	CK2b15primeF (1)	pCAMBSDRev (4)	598
Plasmid	pCAMBSDFor (3)	pCAMBSDRev (4)	604

We nevertheless wanted to ascertain that *PfCK2β1* can be disrupted if the subunit is provided through expression of an episomal copy of the gene. To this effect, a complementation plasmid (pCHD-PfCK2b1, Appendix 1) was constructed, containing the full-length *PfCK2β1* gene under the control of the *PfHsp86* promoter and preceding a 3'UTR. In parallel with the transfection of the pCAM-BSD-KOPfCK2b1 plasmid alone, two further populations of parasites were transfected with both pCAM-BSD-KOPfCK2b1 and pCHD-PfCK2b1. These parasite cultures were named KOCK2b1 + comp 1 and KOCK2b1 + comp 2. PCR analyses (Fig. 3-10B) showed that disruption of the targeted locus occurred only in a doubly transfected, doubly resistant parasite culture (lanes for the KOCK2b1 + comp 1 gDNA: wild type band absent in lane 1, and weak integration band

present in lane 2 (598bp), Fig. 3-10B). Integration was detected at 10 weeks post-transfection (the first time it was tested) in one of the doubly-transfected parasite lines, whereas even after 15 weeks post-transfection, no integration was detected in the singly-transfected lines. Southern blot analysis independently confirmed that integration only occurred in a doubly-transfected parasite culture (Fig. 3-10C, with the expected sizes of DNA fragments after digestion with the restriction enzymes EcoRI and ClaI shown in Fig. 3-10A). When the membrane was probed with the *BSD* probe (Fig. 3-10C, left panel), the only bands recognised other than the linearised plasmid were in lane 4, indicating that integration only occurred in parasites transfected with both the knockout and complementation plasmids. Probing the membrane with the *PfCK2β1* probe corroborated these findings (Fig. 3-10C, right panel). The probe hybridised to a 11.1kb band that represents the wild type locus in the lane that contained gDNA from untransfected parasites and parasites transfected with the knockout plasmid alone (lanes 1-3). This band was undetectable in gDNA from parasites transfected with both the knockout and complementation plasmids (KOCK2b1 + comp 1, lane 4), indicating that the gene can be disrupted only when an additional cassette coding for the PfCK2β1 subunit is provided to the parasites. There are multiple possibilities for the recombination of the knockout and complementation plasmids with each other before or after integration, which could account for the additional bands of unexpected size observed (9, 6, 4.8kb, and just above 6kb), and the lack of the 8.1kb integration band. The lack of integration in the second doubly-transfected parasite line (KOCK2b1 + comp 2, Fig. 10B and lane 5 in Fig. 10C), contrasted with the complete integration (and disappearance of the wild type band without the need to clone the culture by limiting dilution) in the first doubly-transfected line, is intriguing and should be further investigated. The presence of an unexpected-size band in the digested KOCK2b1 + comp 2 gDNA when probed with the *PfCK2β1* probe (Fig. 10C, right panel, lane 5) may indicate recombination between the two plasmids, or spurious integration of one or both plasmids. Plasmid rescue experiments on this parasite culture should be performed to determine whether it still possesses both plasmids (although the continued survival of the parasite line when cultured in the presence of both selective drugs indicates that both plasmids are likely to be present), pulsed-field gel experiments should be performed to determine whether the plasmids have integrated in another chromosome, and all of the cultures should be propagated for a longer period of time and the Southern blots repeated.

The ease of integration of the HA-tagging plasmid, and of the knockout plasmid in the presence of the complementation plasmid (albeit only in one of the two parallel cultures)

coupled with the complete absence of integration of the knockout plasmid in the absence of the complementation plasmid, provides compelling evidence that PfCK2β1 is essential to viability of the asexual erythrocytic stage parasites.

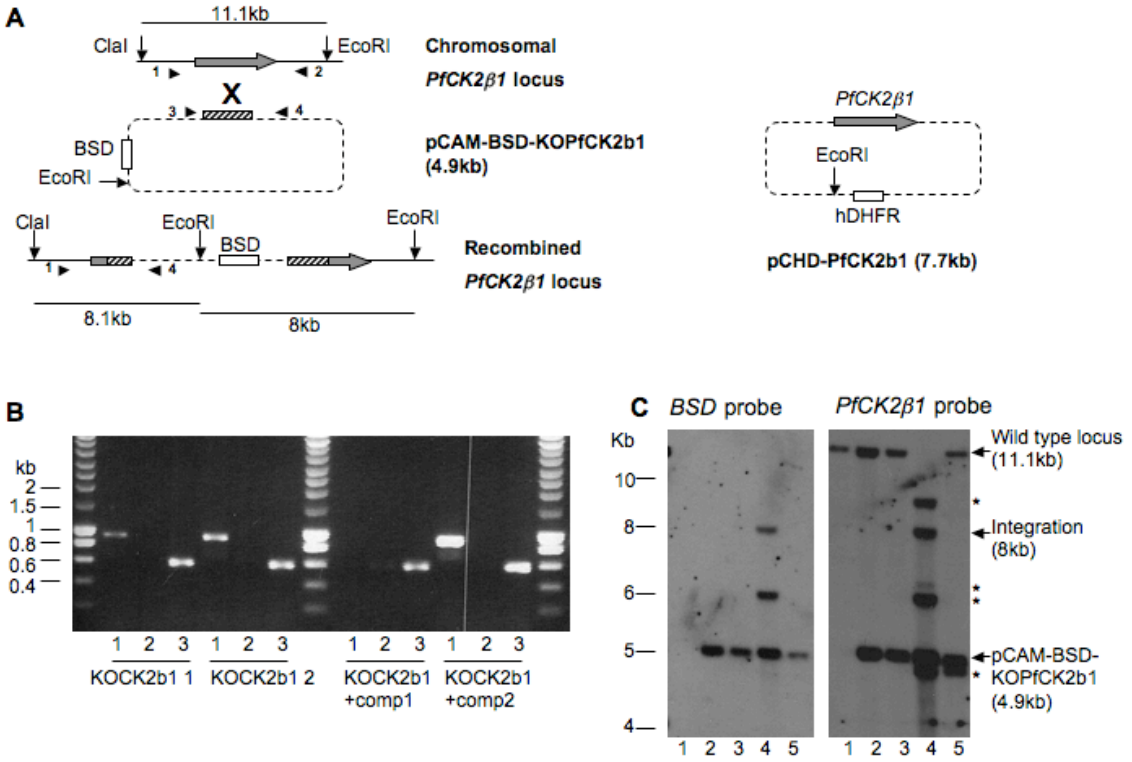

Figure 3-10 Knockout studies of *PfCK2β1*
3D7 parasites transfected with pCAM-BSD-KOPfCK2b1 with or without pCHD-PfCK2b1 were analysed by PCR and Southern blotting. Panel A: Diagram showing the locations of the primers used for PCR screening, and the restriction enzymes used to cut the gDNA to give a diagnostic pattern of bands for analysis by Southern blotting. Panel B: PCR screening of gDNA from two separate pCAM-BSD-KOPfCK2b1-transfected lines (KOCK2b1 1 and KOCK2b1 2), and parasites transfected with both the knockout plasmid and the complementation plasmid (KOCK2b1 + comp). 1: amplification of the wild type *PfCK2β1* locus. 2: amplification over the 5' integration boundary. 3: amplification of the insert in the pCAM-BSD-KOPfCK2b1 plasmid. Evidence of integration is seen only in the gDNA from the doubly-transfected parasite culture (KOCK2b1 + comp, lane 2, faint band seen at 598bp). Parasite gDNA was digested using the restriction enzymes EcoRI and ClaI, and analysed by Southern blotting (Panel C), using *BSD* and *PfCK2β1* as probes. 1: untransfected 3D7, 2: KOCK2b1 1, 3: KOCK2b1 2, 4: KOCK2b1 + complement. Stars mark unexplained bands. See text for discussion.

3.5 PfCK2β2

3.5.1 In vivo *tagging*

P. falciparum 3D7 parasites were transfected with p-CAM-BSD-HA-PfCK2b2 (see sections 2.3.2&3) and maintained under blasticidin selection. Screening for integration was first conducted at 90 days post-transfection, by gDNA extraction and PCR screening using TaKaRa ex Taq polymerase and the diagnostic oligonucleotide pairs detailed in Table 3-5. Evidence of integration was clearly seen (Fig. 3-11A, lane 2: 2255bp). Three clonal lines were produced by limiting dilution, and the clones screened for integration by PCR (two shown here, Fig. 3-11B&C). The 3' integration band was amplified (lane 3: 822bp) from these gDNA extractions, as well as the 5' integration band seen in the uncloned population.

Table 3-5 Oligonucleotide pairs for detecting integration of pCAM-BSD-HA-PfCK2b2 The number in brackets after the oligonucleotide primer names is how the primers are identified in Fig. 3-11D.

Band to be amplified	Forwards oligonucleotide	Reverse oligonucleotide	Expected size of amplified band (bp)
Wild type	CK2b2ForBam (1)	CK2b23primeRev (2)	1483
5' integration	CK2b2ForBam (1)	pCAMBSDRev (4)	2255
3' integration	pCAMBSDFor (3)	CK2b23primeRev (2)	822
Plasmid	pCAMBSDFor (3)	pCAMBSDRev (4)	1615

DNA from the clonal lines was also analysed by Southern blotting (Fig. 3-11E), alongside gDNA from untransfected 3D7 parasites. The parasite gDNA was digested using the restriction enzymes NcoI and ClaI to give a diagnostic pattern of bands when analysed by Southern blot. Panel D of Fig. 3-11 shows the expected size bands for the wild type and recombined locus. When the membrane was probed with *BSD*, bands of the expected size for the plasmid and the 3' end of the recombined locus were seen in the lanes containing digested gDNA from the clonal lines (lanes 2 and 3). No bands were seen in the untransfected parasite gDNA (lane 1). When the membrane was reprobed with *PfCK2β2*, a band corresponding to the size of the wild type locus was seen in the lane containing digested gDNA from the untransfected parasites (lane 1), and not in the gDNA from the clonal lines (lanes 2 and 3). Bands of the expected size for the 5' and 3' ends of the recombined locus, as well as the plasmid, were recognised by the *PfCK2β2* probe only in the gDNA from the clonal lines. These data indicate that the plasmid pCAM-BSD-HA-PfCK2b2 is able to integrate into the *PfCK2β2* genomic locus, demonstrating that the locus is amenable to recombination. The modified gene should encode an HA-tagged PfCK2β2 subunit, allowing for purification of protein complexes containing PfCK2β2. The two

clonal lines were tested for the presence of HA-tagged PfCK2β2 subunits by anti-HA immunoprecipitation, using untransfected parasites as negative controls (see Fig. 5-7).

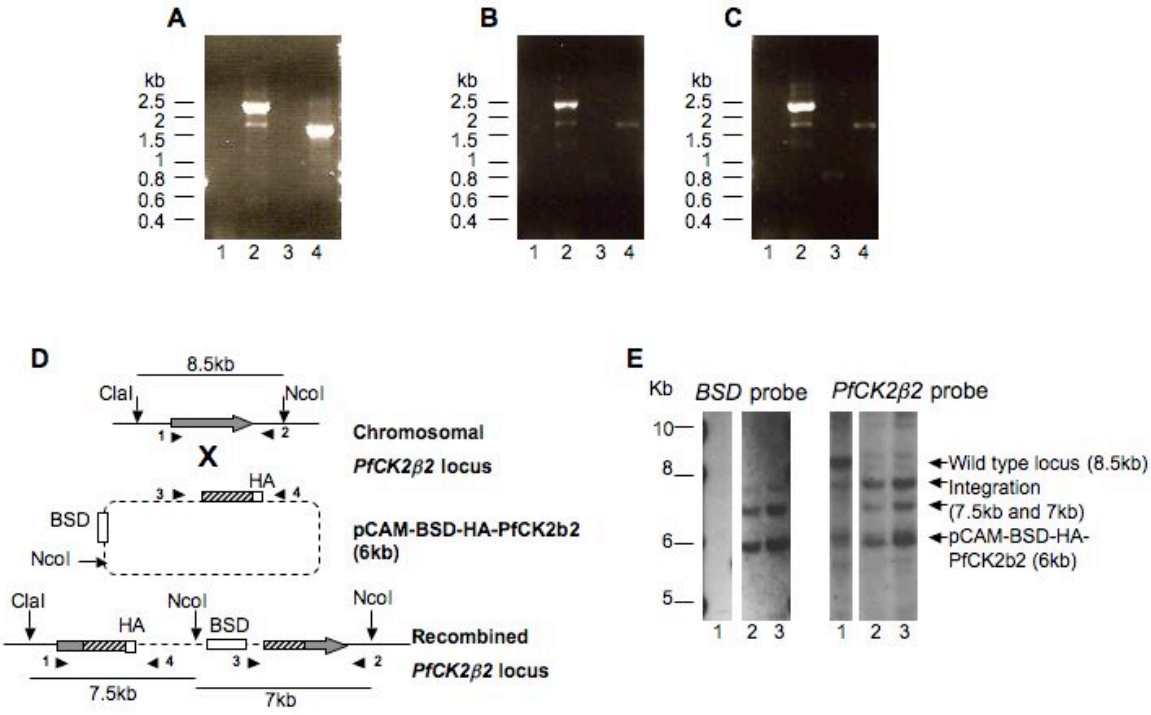

Figure 3-11 HA tagging of *PfCK2β2*
Panel A: PCR screening for integration in gDNA from parasites transfected with pCAM-BSD-HA-PfCK2b2 revealed the presence of parasites in which integration events had occurred (lane 2: 2255bp). Panels B&C: PCR screening for integration in gDNA of clonal cultures derived by limiting dilution (B: clone D5, C: clone E2) revealed that the wild type band had been lost (lane 1) and the integration bands were seen (lane 2: 2255bp, lane 3: 822bp). These two clonal lines were further analysed by Southern blotting (Panel E). Panel D is a schematic of the chromosomal gene locus, the pCAM-BSD-HA-PfCK2b2 plasmid and the recombined locus, and shows the locations of oligonucleotide primers used for the PCR screens (Panels A-C). Oligonucleotide identities are listed in Table 3-5. The location of the recognition sites for the restriction enzymes NcoI and ClaI are depicted, and the expected sizes of the fragments of gDNA after restriction digestion are shown. Fragments of gDNA resulting from restriction endonuclease digestion were analysed by Southern blotting (Panel E), using *BSD* and *PfCK2β2* as probes. gDNA from wild type 3D7 parasites (lane 1), and PfCK2β2HA clonal lines D5 and E2 (lanes 2 and 3), was digested using the restriction enzymes NcoI and ClaI. The wild type locus, recognised by the *PfCK2β2* probe, disappears in the clonal lines, and integration bands of the expected sizes are seen, indicating that the plasmid successfully integrated into the locus.

3.5.2 Knockout and complementation

The fragment of the *PfCK2β2* gene ('KOPfCK2b2') that was amplified and cloned into pCAM-BSD for transfection of parasites was chosen as detailed above (section 3.2.2). Two independent transfections of pCAM-BSD-KOPfCK2b2 into 3D7 parasites were performed, and the parasite lines named KOCK2b2 1 and 2. Integration was monitored in the blasticidin-resistant populations by PCR (Fig. 3-12B), using primer combinations that allow discrimination between the episome, the wild-type locus and the disrupted locus (Table 3-6, locations of primers shown in Fig. 3-12A). Only the episome (1224bp diagnostic fragment amplified, lanes 3) and the wild-type locus (1356bp amplified fragment, lanes 1) were detectable, with no sign of integration even after prolonged culture (15 weeks). We have already confirmed that the *PfCK2β2* locus is recombinogenic, since the pCAM-BSD-HA-PfCK2b2 plasmid was able to integrate into the locus (Fig. 3-11).

Table 3-6 Oligonucleotides for detecting integration of pCAM-BSD-KOPfCK2b2
The number in brackets after the oligonucleotide primer names is how the primers are identified in Fig. 3-12A.

Band to be amplified	Forwards oligonucleotide	Reverse oligonucleotide	Expected size of amplified band (bp)
Wild type	CK2b25primeF (1)	CK2b2RSpe (2)	1356
5' integration	CK2b25primeF (1)	pCAMBSDRev (4)	1169
Plasmid	pCAMBSDFor (3)	pCAMBSDRev (4)	1224

To assess whether *PfCK2β2* can be disrupted if the subunit is provided through expression of an episomal copy of the gene, a complementation plasmid (pCHD-PfCK2b2, Appendix 1) was constructed, containing the full-length *PfCK2β2* gene under the control of the *PfHsp86* promoter and preceding a 3'UTR. Two populations of parasites were transfected with both pCAM-BSD-KOPfCK2b2 and pCHD-PfCK2b2 ('KOCK2b2 + comp 1' and 'KOCK2b2 + comp 2'), and kept in culture for the same amount of time as the parasites transfected with the pCAM-BSD-KOPfCK2b2 plasmid alone. PCR analyses (Fig. 3-12B and C) showed that disruption of the targeted locus occurred only in the gDNA from the doubly transfected, doubly resistant parasite cultures (lanes 3 and 4 of Fig. 3-12C show the integration band (1169bp)). Integration was detected at 10 weeks post-transfection (the first time it was tested) in the doubly-transfected parasite lines, whereas even after 16 weeks post-transfection, no integration was detected in the singly-transfected lines. This result was corroborated by Southern blot analysis, which also showed that integration only occurred in the doubly-transfected parasites (Fig. 3-12D). The expected sizes of DNA fragments after digestion of gDNA using the restriction enzymes NcoI and ClaI is shown in Fig. 3-12A. When the membrane was probed with the *BSD* probe (Fig. 3-12D, left

panel), the only bands recognised other than the linearised plasmid were in lanes 4 and 5, implying that the only integration events occurred in the parasites transfected with both knockout and complementation plasmids. The membrane was stripped and reprobed with the *PfCK2β2* probe (Fig. 3-12D, right panel), which hybridised to an 8.5kb band that represents the wild type locus. This band was recognised in the lane that contained gDNA from untransfected parasites and parasites transfected with the knockout plasmid alone (lanes 1-3). The band was almost undetectable in gDNA from parasites transfected with both the knockout and complementation plasmids (lanes 4 and 5), indicating that the gene can be disrupted only when an additional cassette coding for the PfCK2β2 subunit is provided to the parasites. Equal loading of gDNA in each lane is shown in the ethidium bromide-stained agarose gel (Fig. 3-12E). The membrane was stripped and reprobed with the *PfCK2α* probe to provide another control for equal loading, but there were no visible bands, indicating that a second stripping of the membrane had removed too much DNA for any further analysis to be possible. An attempt was made to produce clonal lines from the doubly-transfected cultures by limiting dilution, but analysis of the lines by PCR showed that they were not clonal (diagnostic bands for the wild type locus, and the recombined locus, were amplified from each parasite line). The dilution cloning must be repeated in the future, and the Southern blotting repeated using gDNA extracted from the clonal lines, to ascertain whether the wild type band completely disappears. Bands of a size diagnostic for integration were observed only in the doubly-transfected parasites (Fig. 12C, lane 4). Additional bands of unexpected size were observed (several bands, 9.5kb and larger), which may be explained by recombination between the two plasmids, before or after integration. A different set of bands was seen in the second doubly-transfected parasite culture (lane 5), not corresponding to the expected sizes for integration. This could be because of recombination between the two plasmids, before or after integration, leading to different length fragments on digestion. PCR analyses showed that amplification across the integration boundary was possible (Fig. 3-12C, lane 4), so integration had clearly occurred in the locus. Taken together, these data provide strong evidence that PfCK2β2 is essential to viability of the asexual erythrocytic stage parasites.

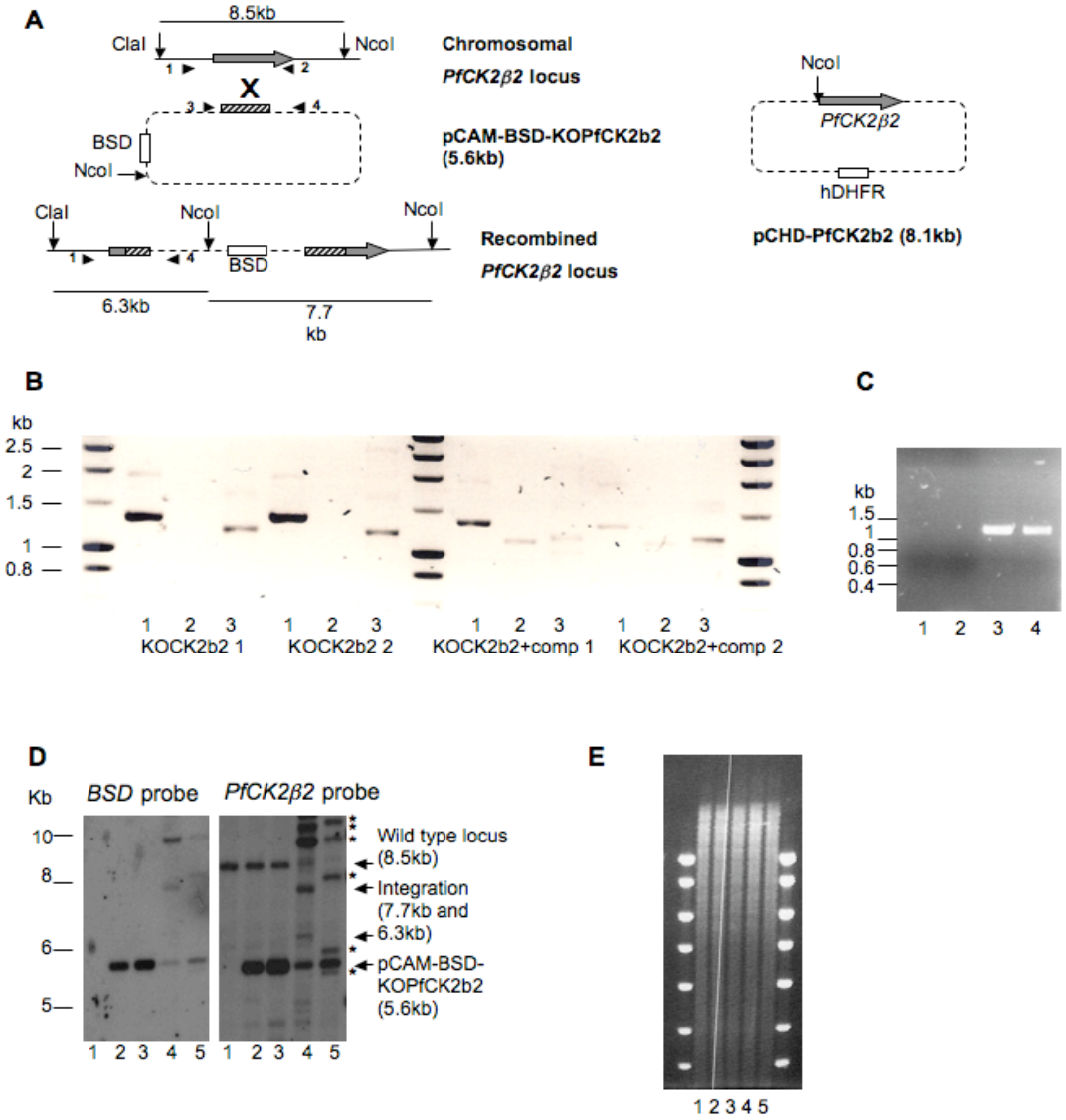

Figure 3-12 Knockout studies of *PfCK2β2*
3D7 parasites transfected with pCAM-BSD-KOPfCK2b2 alone or alongside pCHD-PfCK2b2 were analysed by PCR and Southern blotting. Panel A: Diagram showing the locations of the oligonucleotide primers used for PCR screening (indicated by numbered arrowheads), and the recognition sites for the restriction enzymes used to cut the gDNA to give a diagnostic pattern of bands for analysis by Southern blotting. Oligonucleotide identities are listed in Table 3-6. Panel B: PCR screening of gDNA from two separate pCAM-BSD-KOPfCK2b2-transfected cultures (KOCK2b2 1 and KOCK2b2 2), and two separate cultures of parasites transfected with both the knockout plasmid and the complementation plasmid (KOCK2b2 + comp 1 and 2). 1: amplification of the wild type *PfCK2β2* locus. 2: amplification over the 5' integration boundary. 3:amplification of the insert in the pCAM-BSD-KOPfCK2b2 plasmid. Evidence of integration is seen only in the doubly-transfected parasite lines (KOCK2b2 + comp 1 and 2), as confirmed by panel C, showing just the PCR products from the amplifications across the 5' integration boundary. Lane 1: KOCK2b2 1. Lane 2: KOCK2b2 2. Lane 3: KOCK2b2 + comp 1. Lane 4: KOCK2b2 + comp 2. Amplification over the integration boundary is only possible in the parasite lines cotransfected with the knockout and complementation plasmids. Parasite gDNA was also analysed by Southern blotting (Panel D). The restriction enzymes ClaI and NcoI were used to digest the gDNA, and the fragments were analysed by Southern blotting using *BSD* and *PfCK2β2* as probes. 1: untransfected 3D7, 2: KOCK2b2 1, 3: KOCK2b2 2, 4: KOCK2b2 + comp 1, 5: KOCK2b2 + comp 2. Stars mark unexplained bands. Panel E: ethidium-stained Southern blot gel showing equal loading of gDNA in each lane.

Higher organisms in which gene disruption experiments have been attempted possess a single copy of the *CK2β* gene, and it is essential in these organisms (Fraser et al., 2000, Lou et al., 2008). The yeast *Schizosaccharomyces pombe* also possesses a single copy of the *CK2β* gene, but in this organism it is dispensable for viability (Roussou and Draetta, 1994). The only other organism expressing more than one isoform of beta subunit in which gene disruption experiments have been attempted is *Saccharomyces cerevisiae*, and in this organism the different forms display incomplete functional overlap, with each version of CK2β having a particular role, and single and double *CK2β* gene knockouts have been achieved (Ackermann et al., 2001, Bidwai et al., 1995, Reed et al., 1994). This does not seem to be possible in *P. falciparum*. The two beta subunits of *P. falciparum* are distinctly different from one another (see Fig. 5-1), which, combined with the inability to produce viable parasite populations lacking either of the two Pf*CK2β* genes, leads to the hypothesis that the two PfCK2β subunits display non-redundant functions within the parasite, such that the presence of one cannot compensate for the absence of the other form. Thus the essentiality or dispensability of the beta subunit of CK2 is not related to adaptations to multicellular life.

The many and varied functions of the CK2β subunit orthologues in model organisms (see section 1.6.2) preclude firm hypotheses regarding the roles of the CK2β subunits in *P. falciparum*, and the significance of the essentiality of both of the subunits must be further investigated. The subcellular localisation and trafficking of the subunits is of particular interest, to determine whether the PfCK2β subunits exist independently of the CK2 tetramer, as has been amply proven in other systems (e.g. (Filhol et al., 2003, Salinas et al., 2006)). Immunoprecipitation studies to ascertain whether the PfCK2β subunits have distinct binding partners, and are able to associate with other protein kinases in addition to CK2α, will be particularly informative as to their roles within the parasite. We have started to address this issue (see section 5.7.1).

3.6 Summary

- Three-prime tagging plasmids were constructed for transfection into parasites, which on single crossover homologous recombination with a *PfCK2* gene would add an HA tag to the 3'end of the locus, but otherwise leave the locus intact. PCR and Southern blot analysis of gDNA from transfected 3D7 parasites indicated that the plasmids were able to integrate at each of the gene loci encoding PfCK2 subunits. The gene loci are thus not refractory to recombination.

- The HA tagged lines were cloned by limiting dilution.

- Disruption, or knockout, plasmids were constructed for transfection into parasites, which on single crossover homologous recombination with a *PfCK2* gene would disrupt the coding region, resulting in a pseudo-diploid locus, neither half of which would be functional.

- We are unable to detect integration of the knockout plasmids targeting *PfCK2α*, *PfCK2β1*, or *PfCK2β2* in the absence of a complementation plasmid. This is strong evidence that each of the *PfCK2* genes is essential for parasite survival.

- Complementation plasmids were transfected into 3D7 parasites alongside the knockout plasmids. The complementation plasmids contain PfCK2α/β1/β2 expression cassettes under the control of a *Plasmodium* promoter, and thus provide an extra functional copy of the gene to the parasites. PCR and Southern blot analysis of gDNA extracted from transfected parasite cultures showed that the knockout plasmid is able to integrate into the genome in the presence of the episomally expressed PfCK2, and not in its absence. The parasite cultures transfected with the knockout plasmid alone showed no evidence of integration, even after the same amount of time in culture (or longer) as the doubly-transfected parasites.

- These results strongly suggest that the three PfCK2 subunits are essential to viability of the erythrocytic stage parasites.

4 Biochemical characterisation of PfCK2α

4.1 Introduction

Protein kinase CK2 has been found in all eukaryotic organisms whose genome has been fully sequenced, including the microsporidian *Encephalitozoon cuniculi*, which has an extremely reduced kinome of only 29 typical eukaryotic protein kinases (Miranda-Saavedra et al., 2007). The number of isoforms for the alpha and beta subunits varies between species, usually ranging between 1 and 3 isoforms for each subunit (with some exceptions e.g. *Arabidopsis thaliana* possesses four isoforms of CK2α and four of CK2β (Salinas et al., 2006)). One gene encoding a CK2α subunit and two genes encoding CK2β subunits were found in the *Plasmodium falciparum* genome (Anamika et al., 2005, Ward et al., 2004).

In this chapter we discuss the putative *P. falciparum* CK2α subunit, PF11_0096, its sequence, cloning and expression, characteristics it holds in common with CK2α of other organisms, biochemical characterisation, and susceptibility to small molecule inhibitors. We discuss the two PfCK2β subunits in the following chapter.

4.2 Identification of a CK2α subunit in *P. falciparum*

Phylogenetic analysis of *P. falciparum* protein kinases identified the PlasmoDB (*Plasmodium* database, www.plasmodb.org (Bahl et al., 2003)) sequence PF11_0096 as that of a CK2α orthologue (Anamika et al., 2005, Ward et al., 2004). PF11_0096 clearly clustered with CK2α from humans and yeast in a three-species phylogenetic tree of CMGC kinases (Ward et al., 2004). The top hits from a BlastP search using PF11_0096 as the query were CK2α orthologues, with CK2α from the green plants *Lolium perenne* (70% identical) and *Oryza sativa* (69% identical) as the closest related sequences (excluding other Apicomplexa). PF11_0096 was therefore named PfCK2α. All four gene-prediction algorithms available on PlasmoDB predict a one-exon structure for PfCK2α, encoding a predicted protein of 335 amino acids (calculated molecular weight 39.9kDa). An alignment of PfCK2α with CK2α subunits from *Homo sapiens* and *Zea mays* (Fig. 4-1) reveals that PfCK2α possesses all 11 of the subdomains conserved across eukaryotic protein kinases (Hanks and Quinn, 1991, Hanks and Hunter, 1995), all twelve of the conserved residues across eukaryotic protein kinases (listed in section 1.5), and the majority of the conserved

features of CK2α subunits (Allende and Allende, 1995). Just downstream from subdomain II is a putative nuclear localisation signal ProValLysLysLysLysIle, conserved across CK2α homologues. PfCK2α also possesses three invariant residues common to CK2 family members: the ATP binding motif present in most other protein kinases is Gly-x-Gly-x-x-Gly, whereas in the CK2 family the motif is Gly-x-Gly-x-x-Ser (PfCK2α Gly50-Ser55). The most highly conserved amino acid motif specific to members of the CK2 family is Asp179-Trp-Gly181 (notation from PfCK2α; most protein kinases display Asp-Phe-Gly at this position). Likewise, Gly203-Pro-Glu205 (notation from PfCK2α) is a common feature of the family, which diverge from the Ala-Pro-Glu motif present in the vast majority of other protein kinases; thus all three CK2-specific motifs are present in PfCK2α (indicated by boxes in Fig. 4-1).

```
                                                                      I
ZmCK2α   MS---------KARVYADVNVLRPKEYWDYEALTVQWGEQDDYEVVRKVGRGKYSEVFEG 51
HsCK2α   MSGPVP----SRARVYTDVNTHRPREYWDYESHVVEWGNQDDYQLVRKIGRGKYSEVFEA 56
PfCK2α   MSVSSINKKIYIPKFYADVNIHKPKEYYDYDNLELQWNKPNRYEIMKKIGRGKYSEVFNG 60

                    II           III              IV
ZmCK2α   INVNNNEKCIIKILKPVKKKKIKREIKILQNLCGGPNIVKLLDIVRDQHSKTPSLIFEYV 111
HsCK2α   INITNNEKVVVKILKPVKKKKIKREIKILENLRGGPNIITLADIVKDPVSRTPALVFEHV 116
PfCK2α   YDTECNRPCAIKVLKPVKKKKIKREIKILQNLNGGPNIIKLLDIVKDPVTKTPSLIFEYI 120

          V                    VIa               VIb              VII
ZmCK2α   NNTDFKVLYPTLTDYDIRYYIYELLKALDYCHSQGIMHRDVKPHNVMIDHELRKLRLIDW 171
HsCK2α   NNTDFKQLYQTLTDYDIRFYMYEILKALDYCHSMGIMHRDVKPHNVMIDHEHRKLRLIDW 176
PfCK2α   NNIDFKTLYPKFTDKDIRYYIYQILKALDYCHSQGIMHRDVKPHNIMIDHENRQIRLIDW 180

                         VIII                        IX
ZmCK2α   GLAEFYHPGKEYNVRVASRYFKGPELLVDLQDYDYSLDMWSLGCMFAGKIFRKEPFFYGH 231
HsCK2α   GLAEFYHPGQEYNVRVASRYFKGPELLVDYQMYDYSLDMWSLGCMLASMIFRKEPFFHGH 236
PfCK2α   GLAEFYHPGQEYNVRVASRYYKGPELLIDLQLYDYSLDIWSLGCMLAGMIFKKEPFFCGH 240

                  X
ZmCK2α   DNHDQLVKIAKVLGTDGLNVYLNKYRIELDPQLEALVGRHRRKPWLKFMNADNQHLVSPE 291
HsCK2α   DNYDQLVRIAKVLGTEDLYDYIDKYNIELDPRFNDILGRHSRKRWERFVHSENQHLVSPE 296
PfCK2α   DNYDQLVKIAKVLGTEDLHAYLKKYNIKLKPHYLNILGEYERKPWSHFLTQSNIDIAKDE 300

                     XI
ZmCK2α   AIDFLDKLLRYDHQERLTALEAKTHPYF-----QQVRAAENSRTRA-------------- 332
HsCK2α   ALDFLDKLLRYDHQSRLTAREAMEHPYFYTVVKDQARMGSSSMPGGSTPVSSANMMSGIS 356
PfCK2α   VIDLIDKMLIYDHAKRIAPKEAMEHPYF-----REVREES------------------- 335


ZmCK2α   ----------------------------------
HsCK2α   SVPTPSPLGPLAGSPVIAAANPLGMPVPAAAGAQQ 391
PfCK2α   ----------------------------------
```

Figure 4-1 Alignment of CK2α sequences from *Zea mays*, *Homo sapiens* and *Plasmodium falciparum*.
Protein sequences were aligned using ClustalW. Roman numerals above the alignments indicate the eleven subdomains conserved across protein kinases (Hanks and Quinn, 1991, Hanks and Hunter, 1995). The three CK2-specific family motifs (GxGxxS, DWG and GPE) are boxed. The putative nuclear localisation sequence PVKKKKI is underlined. Residues thought to be important for the utilisation of GTP as a co-substrate are highlighted in bold. The peptide used to raise anti-CK2α antibodies is underlined in light blue. Highlights indicate conserved amino acids.

HsCK2α is phosphorylated at four positions within a C-terminal stretch that is also present in chicken and rat CK2α, but not in the alpha prime isoforms, or in the alpha subunits of CK2 from *Xenopus*, *Drosophila*, plants or yeast (Allende and Allende, 1995). The C-terminal region containing these sites is only present in mammalian and avian CK2α (Blanquet, 2000), suggesting that this region may be involved in some specialised function in a subset of Metazoans.

4.3 Transcription and expression

Microarray data reveals that *PfCK2α* mRNA is present at all stages of the parasite lifecycle that were examined (Le Roch et al., 2003, Bozdech et al., 2003). Proteomics data from various stages of the parasite lifecycle confirm that the PfCK2α subunit is expressed as protein in sporozoites and all blood stages, including gametocytes (Florens et al., 2004, Florens et al., 2002). Proteomic studies of *P. yoelii* liver-stage parasite proteins revealed that the putative *P. yoelii* CK2α orthologue (PY05048) is present in the liver stage parasites (Tarun et al., 2008). In other organisms CK2 is involved in basic cellular functions (see section 1.6.2), and therefore to find it to be expressed throughout the *Plasmodium* lifecycle was not unexpected.

We confirmed by western blot analysis of mixed erythrocytic stage parasite extract that the PfCK2α protein is expressed during the erythrocytic cycle (see section 5.3 for the PfCK2β subunits). Western blot analyses of mixed blood-stage parasite extracts were performed with affinity purified rabbit anti-PfCK2α antibodies directed against the PfCK2α-derived peptide ADVNIHKPKEYYDY, and pre-immune serum as a control (Fig. 4-2). The antibodies were ordered from BioGenes (Berlin). The antibodies were purified against the immobilised peptide that was used to raise the antibodies. We used recombinant GST-PfCK2α (see below, section 4.4) as a positive control. The band in lane 5 between the 55 and 72kDa markers has the expected size of the GST-tagged PfCK2α subunit, and although there is a small amount of background binding to this protein in the pre-immune serum control, there is much greater binding with the anti-PfCK2α antibodies. The smaller bands seen in this lane probably represent degraded recombinant protein. A band of roughly the expected size for PfCK2α (40kDa) is recognised in wild-type 3D7 extract by the anti-PfCK2α antibodies and not by the pre-immune serum. The band in the wild-type 3D7 parasite extract recognised by the specific antibodies runs at a slightly lower molecular weight than that in protein extracts from parasites that had incorporated an HA tag at the 3' end of the *PfCK2α* gene (see Chapter 3), as expected, thus providing

supporting evidence that this band is indeed PfCK2α. No bands of these sizes are recognised in the red blood cell extract (lane 1). Pre-immune serum dilutions of 1 in 500 were used for western blotting controls. The bands recognised by the specific antibodies were not recognised by the pre-immune serum (Fig. 4-2, left panel).

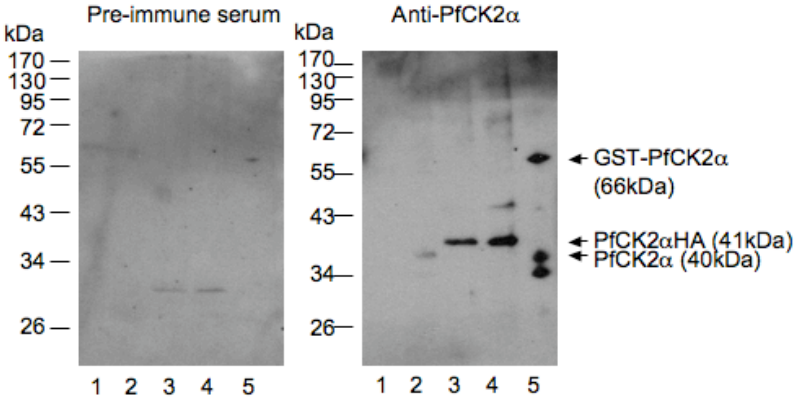

Figure 4-2 Western blot showing PfCK2α expression in erythrocytic stage parasites
Protein extract from unsynchronised erythrocytic stage *P. falciparum* parasites was prepared from wild-type 3D7 parasites (lane 2), and from parasites with a sequence encoding an HA-tag incorporated at the 3' end of the *PfCK2α* gene locus (lane 3: clone B3, lane 4: clone E1; see Chapter 3 for details of these parasite lines). Protein extract from unparasitized red blood cells (lane 1), and recombinant GST-PfCK2α (lane 5, see section 4.4), were included as negative and positive controls. Two identical acrylamide gels were run, the proteins transferred to membrane, and western blots performed with immunopurified rabbit anti-PfCK2α antibodies (right panel), or with pre-immune serum from the same rabbit as a negative control (left panel). The expected sizes of the proteins mentioned in the text are indicated with arrows.

4.4 Cloning and expression of the PfCK2α subunit

4.4.1 Cloning

The coding sequence for the putative *PfCK2α* gene (1008bp, one exon) was amplified from *P. falciparum* 3D7 cDNA by the polymerase chain reaction, using Pfx Platinum polymerase and the oligonucleotide primers CK2aForBam and CK2aRevSal, which introduced an N-terminal BamHI site and a C-terminal SalI site to the PCR product. After cloning into the vector pGEM-T-Easy (see section 2.5.2.1) for sequence verification, the insert was subcloned (see section 2.5.2.2) into pGEX-4T-3 between the BamHI and SalI sites. pGEX-4T-3 attaches an N-terminal GST tag to the recombinant protein. This plasmid was named pGEX-4T-3-PfCK2a, and its insert region was sequenced prior to use in recombinant protein expression.

Catalytically inactive recombinant PfCK2α was obtained by site-directed mutagenesis (Lys72→Met) of pGEX-4T-3-PfCK2a by overlap extension PCR (Ho et al., 1989), as described in section 2.5.1.4. The lysine residue of subdomain II that was targeted for mutagenesis is involved in the correct anchoring and orientation of the ATP molecule and is conserved across all known protein kinases (Hanks and Hunter, 1995). For the first round of amplification by PCR, the oligonucleotide primers CK2aForBam and CK2aK72MRev, and CK2aK72MFor and CK2aRevSal, were used to generate two DNA fragments (237bp and 813bp) having one overlapping end containing the mutation. These were used as templates in the second round of PCR amplification, with the oligonucleotide primers CK2aForBam and CK2aRevSal. The PCR product, *K72MPfCK2α*, was cloned into pGEM-T-Easy, sequenced to verify the presence of the Lys72→Met mutation and the absence of other mutations, then subcloned into pGEX-4T-3 as described for *PfCK2α* above. This plasmid was named pGEX-4T-3-K72MPfCK2a. The insert region of the plasmid was sequenced prior to use in recombinant protein expression.

The pET29-PfCK2a plasmid was constructed in Debopam Chakrabarti's laboratory (University of Central Florida). The insert region of the plasmid was sequenced prior to use, and contained the full *PfCK2α* sequence, between the NdeI and XhoI sites of the plasmid (see Appendix 1 for plasmid map), in frame with a sequence coding for a C-terminal 6x His tag.

4.4.2 Expression and purification

Test expressions of PfCK2α (in pET29 and pGEX-4T-3) and K72MPfCK2α (in pGEX-4T-3) were carried out to find the optimum expression conditions. Expression from pGEX-4T-3-PfCK2a and pGEX-4T-3-K72MPfCK2a was tested in *E. coli* BL21 cells (Stratagene), and from pET29-PfCK2a in *E. coli* Rosetta 2 DE3 cells (Novagen), at a range of temperatures (37°C, 30°C and 20°C) and IPTG concentrations (0.1-1mM). Samples were taken before induction, after induction, from the soluble and insoluble protein fractions after lysis, and of the eluted proteins (see sections 2.6.1&2). Analysis of the samples by SDS-PAGE indicated that the conditions that yielded the most recombinant protein were the same for all three plasmids: induction at 20°C overnight with 0.1mM IPTG. Large-scale expressions were performed using these conditions.

Recombinant proteins were purified using batch glutathione affinity chromatography (see 2.6.1) of the GST-tagged PfCK2α and K72MPfCK2α, and batch nickel affinity

chromatography (see 2.6.2) of the His-tagged PfCK2α. 250ml cultures were used for expression of recombinant proteins, and produced roughly 0.5mg of GST-PfCK2α, 0.4mg of K72MPfCK2α, and 0.3mg of PfCK2α-His. Samples taken during expression and purification of the three recombinant proteins were separated on 12 % acrylamide gels and visualised using Coomassie blue stain (Figs 4-3&4).

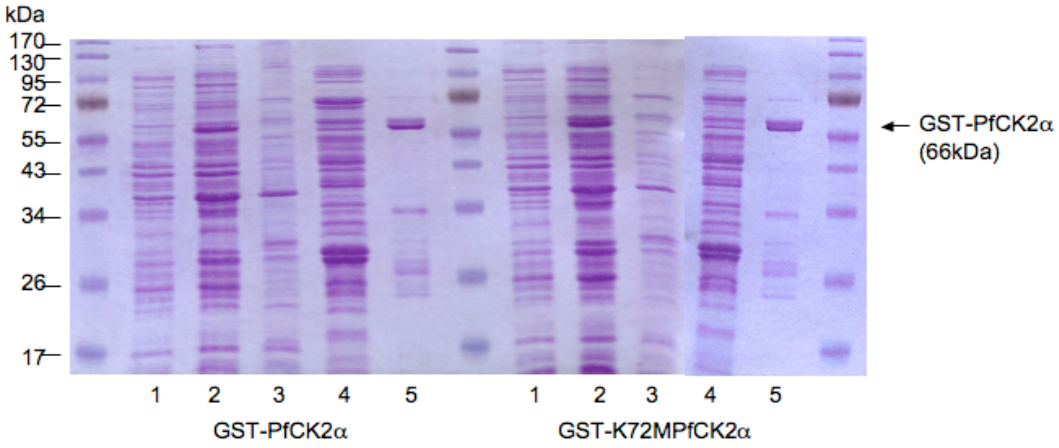

Figure 4-3 Expression and purification of GST-PfCK2α and GST-K72MPfCK2α
Samples taken during the expression and purification of GST-PfCK2α and GST-K72MPfCK2α were separated by SDS-PAGE on 12% acrylamide gels, which were then stained with Coomassie Brilliant Blue stain. 1: Sample of bacteria before induction of expression. 2: Sample of bacteria after expression was induced. 3: Insoluble protein fraction. 4: Soluble protein fraction. 5: Eluted proteins.

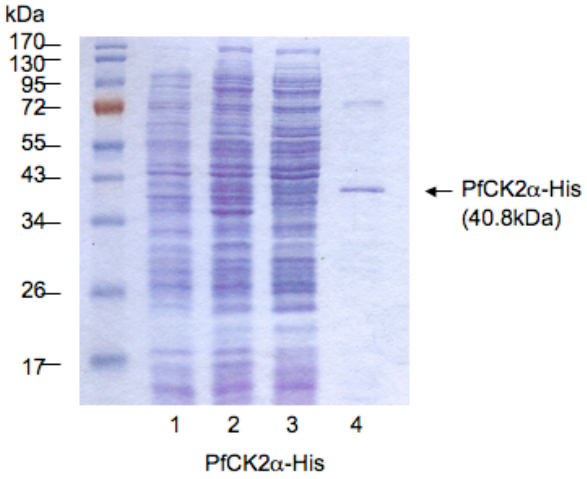

Figure 4-4 Expression and purification of PfCK2α-His
Samples taken during the expression and purification of PfCK2α-His were separated by SDS-PAGE on a 12% acrylamide gel, and stained with Coomassie Brilliant Blue. 1: Sample of bacteria before induction of expression. 2: Sample of bacteria after expression was induced. 3: Soluble protein fraction. 4: Proteins present on the Ni-NTA beads after washing.

4.5 Kinase activity of PfCK2α

4.5.1 Kinase activity against artificial substrates

Although PfCK2α contains all of the conserved kinase subdomains and catalytic residues (see section 4.2), we wanted to confirm that the recombinant protein does in fact possess kinase activity. Standard kinase assays (section 2.6.9.1) were performed using GST-PfCK2α (or GST-K72MPfCK2α as a negative control) and a variety of substrates.

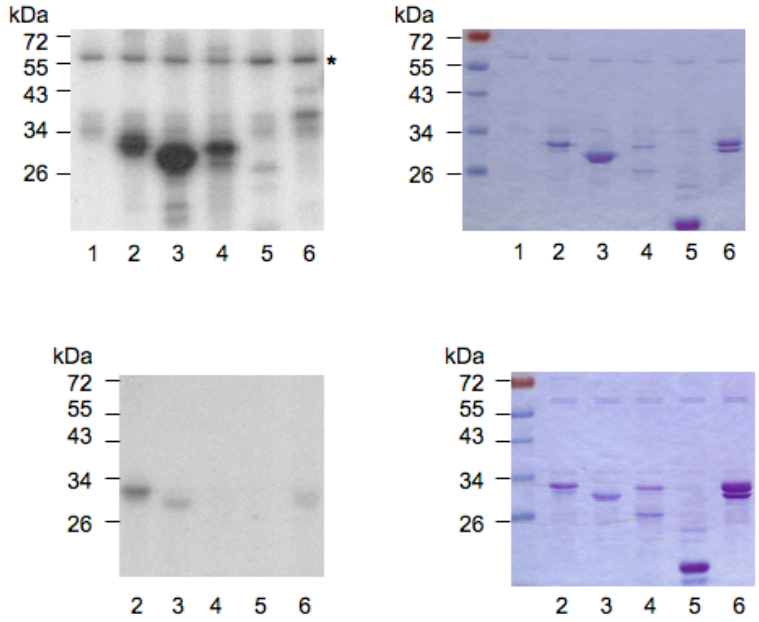

Figure 4-5 Kinase activity of GST-PfCK2α against various substrates
The left hand panels in this figure are the photographic film exposed to the Coomassie-stained gels (right hand panels) of the kinase assays. Top panels: kinase assays with GST-PfCK2α. Lower panels: kinase assays with GST-K72MPfCK2α. Substrates: lane 1: No substrate; lane 2: α-casein; lane 3: β-casein; lane 4: mixed caseins; lane 5: MBP; lane 6: histone H1. The autophosphorylation band is marked with a star.

Recombinant GST-PfCK2α showed kinase activity against a range of substrates (see Fig. 4-5; top panels are kinase assays using GST-PfCK2α, lower panels are control assays using GST-K72MPfCK2α instead of GST-PfCK2α). HsCK2α is known to autophosphorylate (Boldyreff et al., 1994a), on Tyr182 (Donella-Deana et al., 2001), a residue that is conserved in PfCK2α. The function of this autophosphorylation in HsCK2α is unknown. As can be seen from this kinase assay, GST-PfCK2α is also able to autophosphorylate: the band at approximately 66kDa, present in each lane of the kinase assay, corresponds with the GST-PfCK2α band in the Coomassie-stained gel. The strongest GST-PfCK2α kinase activity was towards the caseins (lanes 2-4). CK2 substrates generally have highly acidic phosphoacceptor sites, often with 5 or more acidic residues in

the region immediately surrounding the serine or threonine (or tyrosine) phosphoacceptor residue, with the most important determinants being an acidic residue at the n+1 or n+3 positions (Pinna, 2002, Meggio and Pinna, 2003). Casein is an acidic protein. Parallel assays performed with the K72M mutant version of PfCK2α (GST-K72MPfCK2α) were negative for autophosphorylation and kinase activity towards exogenous substrates (Fig. 4-5, lower panels), demonstrating that the activity we see with GST-PfCK2α is really due to the recombinant PfCK2α subunit and not a co-purified bacterial contaminant.

PfCK2α can also phosphorylate synthetic peptide substrates that mimic the consensus sequence for CK2 phosphorylation. The consensus sequence for CK2 phosphorylation has been the subject of a number of studies (Songyang et al., 1996, Pearson and Kemp, 1991, Kuenzel et al., 1987, Meggio et al., 1994b, Meggio and Pinna, 2003). CK2 phosphorylates acidic sequences, with the minimum consensus sequence S/T-D/E-X-E/D (phosphoserine can efficiently replace the aspartic acid and glutamic acid residues (Meggio and Pinna, 2003)). A variety of peptide substrates that mimic the consensus sequence have been synthesised. Two such peptides were used in this study: the NEB peptide p6012 (RRRADDSDDDDD) was used in the experiments performed in Glasgow, and the custom peptide RRREDEESDDEE ('peptide 29'), obtained from NeoMPS, was used in the experiments I performed while visiting the laboratory of Claude Cochet (INSERM U873, Grenoble, France). The recombinant PfCK2α was able to phosphorylate both peptides: NEB p6012 with a K_m of 135.4 μM, and peptide 29 with a K_m of 115.4 μM (see section 4.5.4, below).

4.5.2 Kinase activity against P. falciparum *substrates*

As a first step towards the identification of *P. falciparum* protein substrates for PfCK2α, *P. falciparum* 3D7 protein extract was heated to 55^{o}C for 10 minutes to inactivate any kinases and phosphatases present, then used as substrate in a GST-PfCK2α kinase assay (Fig. 4-6). Parasite cultures were synchronized by sorbitol, and extracts prepared from synchronous ring stage and trophozoite cultures. Extracts were also prepared from unsynchronized cultures. The recombinant GST-PfCK2α was able to phosphorylate several proteins in the extract, from different parasite life stages. Control reactions contained GST-K72MPfCK2α instead of GST-PfCK2α, to ascertain whether the endogenous kinases had been fully inactivated. As can be seen from lanes 5-7, this was largely the case, with only a small amount of residual activity in the extract from mixed erythrocytic stage parasites (lane 7). It is likely that the number of parasite proteins that GST-PfCK2α is capable of

phosphorylating is much greater than the number observed in the kinase assay, because proteins present in the parasite extract would have been exposed to the endogenous parasite PfCK2 prior to heat-inactivation, and therefore many of the proteins in the extract capable of being phosphorylated by GST-PfCK2α would already be phosphorylated. Conversely, the bands seen in the assay may not be true *in vivo* substrates, because within the cell they may be located in compartments where they would not naturally come into contact with PfCK2. Additionally, the process of heat-inactivation may have altered the conformation of various proteins in the extract, rendering them unnaturally compliant with GST-PfCK2α phosphorylation.

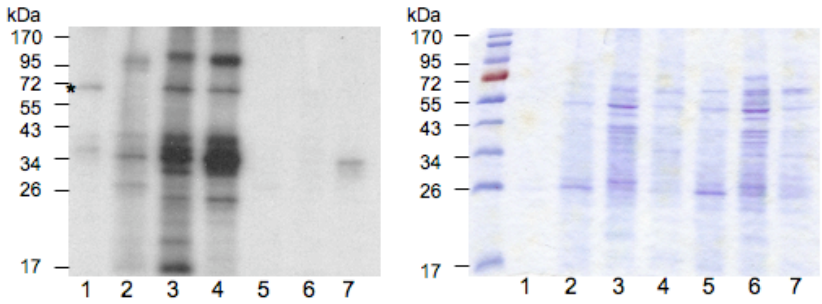

Figure 4-6 Phosphorylation of *P. falciparum* protein extract by PfCK2α
Standard kinase assays were performed with 5μg of protein extract from synchronous or asynchronous parasite cultures as substrate. Lanes 1-4 contained GST-PfCK2α, lanes 5-7 contained GST-K72MPfCK2α as negative controls. Substrates were: lane 1: no substrate (GST-PfCK2α alone), lanes 2&5: ring-stage parasite extract, lanes 3&6: trophozoite-stage parasite extract, lanes 4&7: mixed erythrocytic stage parasite extract. Left panel: autoradiogram, right panel: corresponding Coomassie-stained gel of the kinase assay. The band marked with a star, just below the 72 kDa marker, is the autophosphorylation band (expected at 66kDa). There is a small amount of background phosphorylation activity in the mixed erythrocytic stage parasite extract (lane 7).

A number of recombinant proteins from *Plasmodium falciparum*, including recombinant PfCK2α itself (Fig. 4-7A), shPfCK2β2 (the short version of the PfCK2β2 subunit, lacking the N-terminal extension, see Chapter 5; Fig. 4-7B), PfLSA-1 (a kind gift from David Lanar's laboratory, Walter Reed Army Institute of Research, Maryland, USA), PfMyb1, PfHMGB1, and PfB7-NAP (kind gifts from Catherine Vaquero's laboratory, INSERM U511, Paris, France) (Fig. 4-7C&D) function as *in vitro* substrates of recombinant PfCK2α.

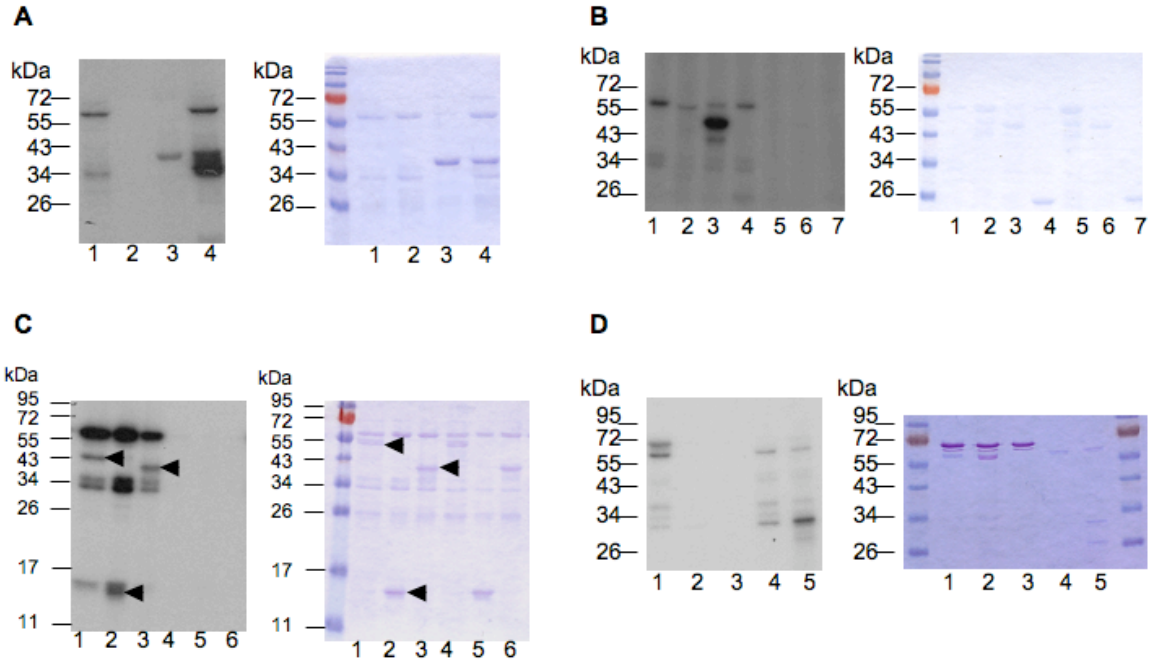

Figure 4-7 *In vitro* recombinant *Plasmodium* protein substrates of PfCK2α
For each of A-D, the left panel is the autoradiogram, and the right panel is the corresponding Coomassie-blue-stained gel of the kinase assay. A: Autophosphorylation can occur by a trans-reaction. 1: GST-PfCK2α. 2: GST-K72MPfCK2α. 3: PfCK2αHis. 4: PfCK2αHis and GST-K72MPfCK2α. B: Phosphorylation of PfCK2β2 by PfCK2α. Lanes 1-4 are kinase assays with GST-PfCK2α, lanes 5-7 are the corresponding kinase assays containing GST-K72MPfCK2α. The substrates were as follows lane 1: No substrate; lanes 2&5: GST-PfCK2β1; lanes 3&6: GST-shPfCK2β2; lanes 4&7: GST. C: Lanes 1-3 are kinase assays with GST-PfCK2α, lanes 4-6 are the corresponding kinase assays containing GST-K72MPfCK2α. The substrates were as follows lanes 1&4: PfMyb1; lanes 2&5: PfHMGB1; lanes 3&6: PfNAP-B7. The substrate locations are indicated by arrowheads (see text for further discussion). D: Phosphorylation of LSA-1 by PfCK2α. 1: GST-PfCK2α and LSA-1 2: GST-K72MPfCK2α and LSA-1. 3: LSA-1 alone. 4: GST-PfCK2α alone. 5: GST-PfCK2α and casein.

PfCK2α can autophosphorylate by a trans-reaction (Fig. 4-7A). GST-PfCK2α and PfCK2αHis autophosphorylate (lanes 1 and 3), GST-K72MPfCK2α does not (lane 2), but is phosphorylated in the presence of PfCK2αHis, indicating that the autophosphorylation of PfCK2α can occur by an intermolecular reaction.

As mentioned above, CK2α has a preference for acidic substrates. shPfCK2β2, a good substrate for PfCK2α (Fig. 4-7B), possesses several serines and threonines with acidic residues at n+1 and/or n+3 positions (see Fig. 5-1). The function of the phosphorylation on the CK2β subunit remains unknown (Bibby and Litchfield, 2005), although mutant versions of human CK2β lacking the autophosphorylation sites were able to form a comparable holoenzyme to that of wild type CK2β (Bodenbach et al., 1994, Boldyreff et al., 1992, Meggio et al., 1993). We detected no apparent kinase activity towards the PfCK2β1 subunit.

The nucleosome assembly proteins (NAPs) have roles in the maintenance and remodelling of chromatin, gene expression, and histone shuttling. CK2 has been shown to phosphorylate several of these proteins, an event which modulates the transport of these proteins between the cytoplasm and the nucleus (Rodriguez et al., 2000, Krick et al., 2006).

The Myb proteins are a family of transcription factors. CK2 has been shown to phosphorylate A- B- and c-Myb (Bergholtz et al., 2001), amongst others. The phosphorylation of c-Myb by CK2 prevents it from binding DNA, providing crucial regulatory control of this protein (Luscher et al., 1990, Oelgeschlager et al., 1995). CK2 phosphorylation of SNAP(C) on the SNAP190 subunit (which has a Myb DNA binding domain) prevents the binding of SNAP(C) to DNA, which is required for U6 transcription activity of RNA polymerase III (Gu et al., 2007).

High-mobility-group B (HMGB) proteins are chromosomal proteins that contain an HMG-box domain, which binds bent, kinked or unwound DNA with high affinity (Stros et al., 2007). The HMGB proteins play important architectural roles in the formation of complexes of nucleoproteins involved in transcription, and DNA replication and repair (Thomas, 2001, Travers, 2003). HMGB1 and HMGB2/3 are phosphorylated by CK2, which increases their thermal stability, and reduces their affinity for linear DNA (Stemmer et al., 2002). Phosphorylation of HMGB1 by CK2 abolishes its interaction with the transcription factor Dof2, and hence the binding of Dof2 to DNA (Krohn et al., 2002). Thus we had reason to believe that the PfMyb1, PfB7-NAP and PfHMGB1 proteins were good candidates for potential PfCK2 substrates.

PfMyb1 (PF11_0088; 50kDa protein) and PfB7-NAP (PFI0930c; 32kDa protein) are proteins with several serines and threonines whose n+1 or n+3 sites are D/E residues (i.e. conform to the minimal consensus sequence for CK2 phosphorylation). PfHMGB1 (PFL0145c) is an 11kDa protein with a single threonine that may be phosphorylated by CK2 (with an n+3 glutamic acid residue). Kinase assays were performed with all three proteins as substrates (Fig. 4-7C). We observed a unique band in the lane containing recombinant PfMyb1, of roughly 45kDa (indicated by an arrowhead in the left panel, lane 1), but this is not quite consistent with the expected size of the protein (50kDa) or the size of the major band from the PfMyb1 preparation on the Coomassie-stained gel (roughly 50kDa, indicated by arrowhead in the right panel, lane 1). Thus we remain cautious about the interpretation of these results. PfHMGB1 and PfNAP-B7 are weakly phosphorylated by PfCK2 *in vitro* (lanes 2 and 3), compared with β-casein or the shPfCK2β2 subunit (see Fig. 4-5 and Fig. 4-7B). The other bands seen in lanes 1-3 are from the recombinant GST-

PfCK2α protein preparation. Although the proteins contain the minimal consensus sequence, its presence does not guarantee efficient phosphorylation, nor does its absence guarantee that CK2 does not phosphorylate the substrate. Some sequences are efficiently phosphorylated even though they do not conform to the consensus sequence (for example, Ser392 of the p53 tumour suppressor (Meek et al., 1990)), and the presence of the minimal consensus sequence does not guarantee that there will be efficient phosphorylation, which may depend on overall protein conformation (Meggio et al., 1994b). Thus with an inefficient *in vitro* phosphorylation such as that seen for the PfMyb1, PfB7-NAP and PfHMGB1 proteins, we cannot conclude whether or not they are efficiently phosphorylated *in vivo*: there may be other determinants such as priming kinases *in vivo* that would make them better substrates, or, on the other hand, that they are phosphorylated at all *in vitro* may be due to the high concentration of kinase and substrate, and they may not function as *in vivo* substrates.

LSA-1 is a 230.1 kDa protein expressed in the liver stages of the parasite lifecycle in the parasitophorous vacuole (Fidock et al., 1994). The protein contains a central repeat region of 86 repeats of the 17-residue sequence EQQSDLEQERLAKEKLQ or minor variations (Zhu and Hollingdale, 1991). This repeat sequence contains a CK2-phosphorylation motif (underlined). High levels and prevalences of antibodies directed against the repeat region of LSA-1 are found in individuals from malaria-endemic areas, leading to suggestions that immune responses to LSA-1 might be involved in protection against pre-erythrocytic stages of malaria (Fidock et al., 1994). The biochemistry of this molecule is therefore of interest, and we were contacted by Dr Nicoll from Dr Lanar's laboratory to test if it was indeed a PfCK2α substrate. The recombinant LSA-1 used in this study contained the N- and C-terminal regions and 2 of the 17-residue central repeats, and was phosphorylated by recombinant PfCK2α in *in vitro* kinase assays (Fig. 4-7D). However, questions remain about whether this phosphorylation occurs *in vivo*, and if it does, how it occurs, since LSA-1 is localised to the parasitophorous vacuole and CK2 (in other organisms) has been shown to be predominantly nuclear in localisation. It is possible that some PfCK2 is exported as an ectokinase. Alternatively, LSA-1 could be phosphorylated by host CK2.

The substrates identified in this section and in the previous section fulfil the first criterion for substrate identification: they are phosphorylated *in vitro* by the recombinant kinase. However, no attempt has yet been made to assess whether they fulfil the other five criteria for formal identification of a novel protein kinase substrate listed by Berwick and Tavare (Berwick and Tavare, 2004): i) significant stochiometry of *in vitro* phosphorylation (approaching 1mol phosphate per 1mol of phosphorylation sites), ii) the substrate should

be phosphorylated *in vivo* in response to stimuli that activate the kinase, iii) the site of phosphorylation on the protein substrate must be the same *in vitro* as *in vivo* (under criterion ii)), iv) the substrate must be phosphorylated by constitutively active mutants of the kinase, and v) *in vivo* phosphorylation of the substrate should be inhibited by dominant-negative mutants of the kinase, or by depletion of the kinase such as through RNAi, or should be blocked by small-molecule inhibitors of the kinase.

4.5.3 Ability to utilise GTP and ATP as phosphoryl donors

A feature often cited as characteristic of CK2 enzymes is that they have similar affinities for GTP and ATP. CK2 enzymes possess two amino acid differences from most protein kinases, A71→V and F187→W (notation from the reference kinase, PKA), which have been hypothesized to be responsible for their ability to use ATP and GTP equally efficiently as a phosphoryl donor (Blanquet, 2000, Taylor et al., 1993, Jakobi and Traugh, 1992). PfCK2α possesses only the second of these two substitutions (W180, see Fig. 4-1). Another motif suggested to be important in the ability of CK2 to utilize GTP is the diasparagine (Srinivasan et al., 1999) indicated in Fig. 4-1. This motif is present in PfCK2α (amino acids 121 and 122). To assess whether PfCK2α was able to utilize both ATP and GTP as phosphoryl donors, we calculated K_ms for both nucleotides (see Fig. 4-8 and Table 4-1). The K_ms of PfCK2α for ATP and GTP were determined by performing kinase assays with ATP concentrations of 100μM, 25μM, 6.25μM and 1.5625μM. Reactions were carried out in triplicate. The [γ-^{32}P]ATP/GTP was added to the unlabelled ATP/GTP and diluted serially, to ensure a constant ratio of labelled to unlabelled ATP/GTP. The NEB peptide RRADDSDDDDD (100μM) was used as the substrate. The lack of the A71→V substitution does not render PfCK2α unable to utilize GTP: both ATP and GTP are able to be used in *in vitro* assays as co-substrates. ATP is the preferred cosubstrate under these conditions, with a K_m value roughly half that for GTP (Table 4-1).

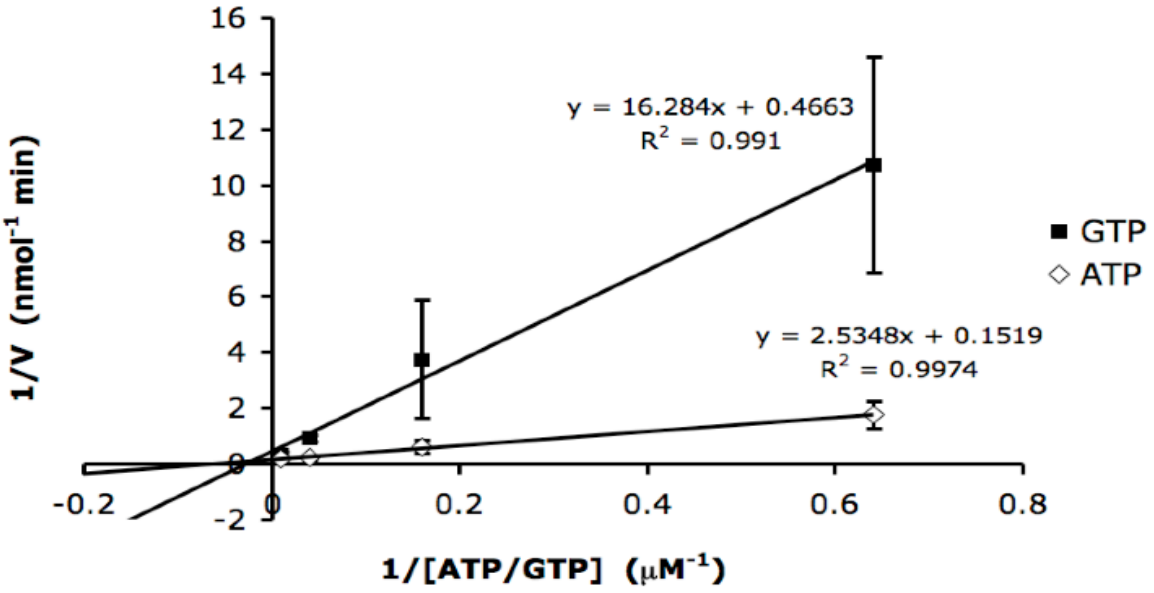

Figure 4-8 Ability of PfCK2α to utilize GTP and ATP as co-substrates
The enzyme kinetics of PfCK2α in Lineweaver-Burke presentation. The experiment was performed in triplicate. The data points represent the means, and the error bars represent three standard deviations. The graph was obtained by linear regression of the enzyme kinetic data for ATP and GTP. The intercepts on the x-axis give the negative reciprocal of the K_m, and the intercepts on the y-axis give the reciprocal of the V_{max}.

Table 4-1 K_m and V_{max} of PfCK2α for ATP and GTP

	K_m (μM)	V_{max} (nmol/min)
ATP	16.7	6.6
GTP	34.9	2.1

Few other protein kinases are able to use GTP as a co-substrate: to my knowledge, the delta isoform of PKC (Gschwendt et al., 1995), a mammalian STE20-like kinase (Schinkmann and Blenis, 1997), EGF-receptor kinase (Carpenter et al., 1979), and *Pseudomonas aeruginosa* AlgR2 (Roychoudhury et al., 1992) are the only others with reported ability to use GTP, and their dual-co-substrate specificity "is not as obvious as it is for CK2" (Niefind et al., 1999). Niefind and colleagues (Niefind et al., 1999) highlight several important potential consequences of the use of GTP by CK2: (1) rapidly proliferating cells have a higher GTP/ATP ratio, and higher levels of GTP, than normal cells, thus favouring CK2 above other kinases, which may have important implications for proliferation (see section 1.6.2.1 for roles of CK2 in proliferation). (2) Some phosphorylation events may be favoured by GTP above ATP, providing a means for the regulation of specific signalling events. For example, PKCδ autophosphorylation is "much more effective with GTP than with ATP" (Niefind et al., 1999), and the autophosphorylation with GTP occurs at some different sites from that using ATP. (3) The

use of GTP in conjunction with Mn^{2+} may be important in the phosphorylation of tyrosine residues. CK2 prefers GTP as a co-substrate when in the presence of Mn^{2+}, but ATP in the presence of Mg^{2+}. Phosphorylase kinase is a dual-specificity kinase (like CK2), whose tyrosine phosphorylation activity is activated by Mn^{2+}.

Other consequences of the ability of CK2 to utilize GTP include the use of GTP as a methodological tool: not many kinases can utilize GTP, so it could be used in functional studies in support of data showing that CK2 is responsible for a specific phosphorylation event. A common criticism of targeting protein kinases for inhibition is the potential for off-target effects, given the remarkable similarity between kinase active sites. The divergent features of the catalytic cleft that allow CK2 to use GTP as well as ATP could be targeted as part of the drug discovery process, either to inhibit all activity of the kinase, or to inhibit in a specific manner the GTP-catalysed phosphorylation events.

4.5.4 Enzymological characterisation

Kinase assays (using the scintillation counting method, section 2.6.9.2) were performed with peptide 29 (RRREDEESDDEE) and a range of enzyme concentrations (15-120ng total enzyme in 18μl reactions) to determine the linear range of the enzyme. Since comparisons between PfCK2α and HsCK2α were to be carried out (see section 4.6, below), the linear range of HsCK2α was also calculated. HsCK2α was produced in the laboratory of C. Cochet (INSERM U873, Grenoble, France). The linear range for both PfCK2α and HsCK2α is between 15 and 60ng of enzyme. In subsequent reactions, 36ng of enzyme per 18μl reaction was used.

The K_m of PfCK2α for ATP was determined using ATP concentrations of 100μM, 50μM, and 25μM. Reactions were carried out in triplicate. The [γ-^{32}P]ATP was added to the unlabelled ATP and diluted serially, to ensure a constant ratio of labelled to unlabelled ATP. Peptide 29 (111μM) was used as the substrate. K_m values are given in Table 4-2.

Table 4-2 Kinetic parameters of PfCK2α

	K_m (μM)
ATP	17.5
Peptide 29	115
NEB p6012	135.4

The K_m values of PfCK2α for peptide 29 and NEB p6012 were also calculated, by varying the concentration of peptide used in the assay. Peptide concentrations used in each reaction were (μM): 666.7, 333.3, 166.7, 88.3, 41.7, 20.8, 10.4, and 0. PfCK2α was used at 36ng per reaction, and the reactions carried out in duplicate. Since comparative studies with PfCK2α and HsCK2α were to be carried out using peptide 29 as the substrate (IC_{50} values, section 4.6), the K_m of HsCK2α for Peptide 29 was also calculated: HsCK2α had a K_m of 148 for peptide 29.

4.6 Susceptibility of PfCK2α to CK2α inhibitors

4.6.1 First inhibitor screen

The first inhibitor screen was carried out using a set of compounds from Claude Cochet's laboratory that act as HsCK2 inhibitors and their controls. Kinase assays were performed under linear kinetic conditions in 18μl volumes with 36ng PfCK2α, 25μM ATP, 20μM of compound, and the NEB peptide as a substrate. The experiment was repeated twice, and the resulting mean and standard deviations of activity incorporated into the peptide substrate are shown in Fig. 4-9. IC_{50} values were calculated for the best three inhibitors, 3,4, and 11 (see section 4.6.1.1). These data indicate that PfCK2α is amenable to inhibition by small molecules.

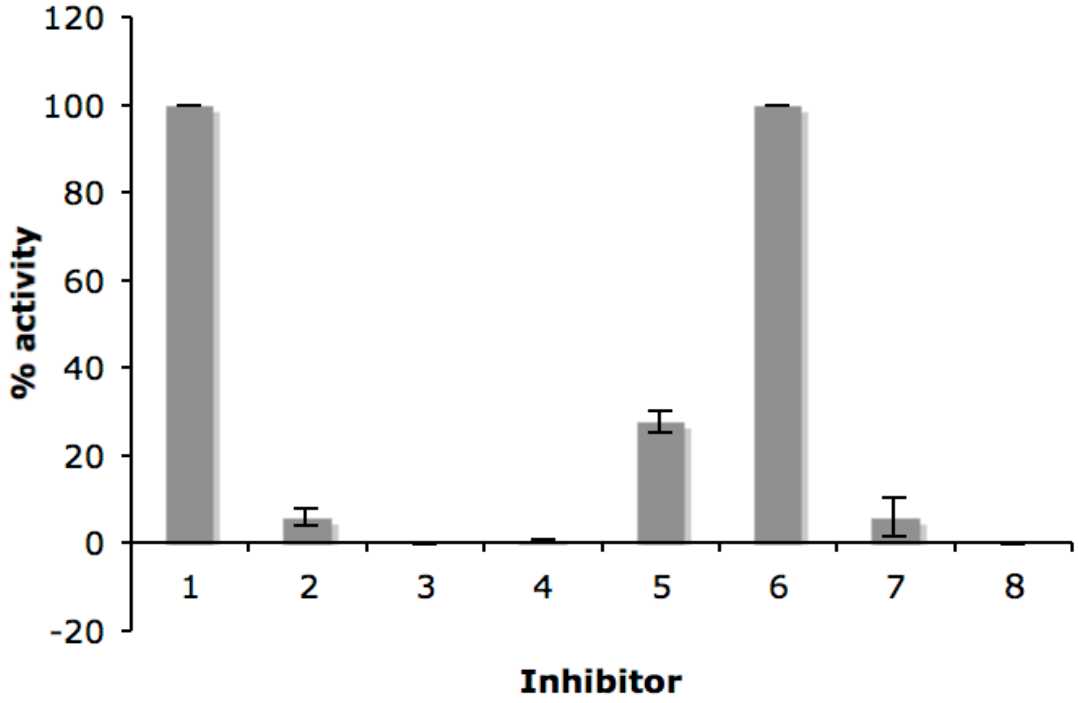

Figure 4-9 Kinase assay screen of HsCK2α inhibitors with PfCK2α
Several compounds that inhibit the activity of HsCK2α were screened against PfCK2α in a kinase assay screen using the NEB peptide as the substrate, 25μM ATP and 20μM of the following compounds (from stock solutions diluted in DMSO). 1: DMSO control, no compound. 2: TBB (3,4,5,6-Tetrabromobenzotriazole). 3: Non-competitive inhibitor A. 4: Non-competitive inhibitor B. 5: ATP-competitive inhibitor A. 6: EtOH as a control for Quercetine (number 7), which was diluted in EtOH instead of DMSO. 7: Quercetine. 8: ATP-competitive inhibitor B. The amount of radiolabel incorporated into the peptide was measured by scintillation counting, and the results plotted as a percentage activity compared with the controls (lanes 1 and 6). The mean values for three experiments are plotted, with the error bars representing the standard deviations.

4.6.1.1 IC_{50}s of HsCK2α inhibitors on recombinant PfCK2α

IC_{50} values for the three compounds that reduced the activity of PfCK2α to the lowest level (compounds 3, 4 and 11; Fig. 4-9) were calculated. For each compound, two separate kinase assays were performed using the scintillation counting method, with the NEB peptide as a substrate, 36ng of PfCK2αHis, 25μM ATP and varying concentrations of the compound (0-42μM). Results are plotted in Fig. 4-10, and the IC_{50} values given in Table 4-3.

Table 4-3 IC_{50} values for compounds 3, 4 and 11 on PfCK2α and HsCK2α
The values in brackets for PfCK2α are the IC_{50}s plus or minus one standard deviation. The HsCK2α experiments are quoted for comparison, and were performed by the laboratory of C. Cochet.

Compound	IC_{50} for PfCK2α (-SD, +SD)	IC_{50} (HsCK2α, C. Cochet)
3	0.40μM (0.38, 0.42)	10nM
4	2.5μM (2.36, 2.64)	0.2μM
11	0.45μM (0.38, 0.52)	80nM

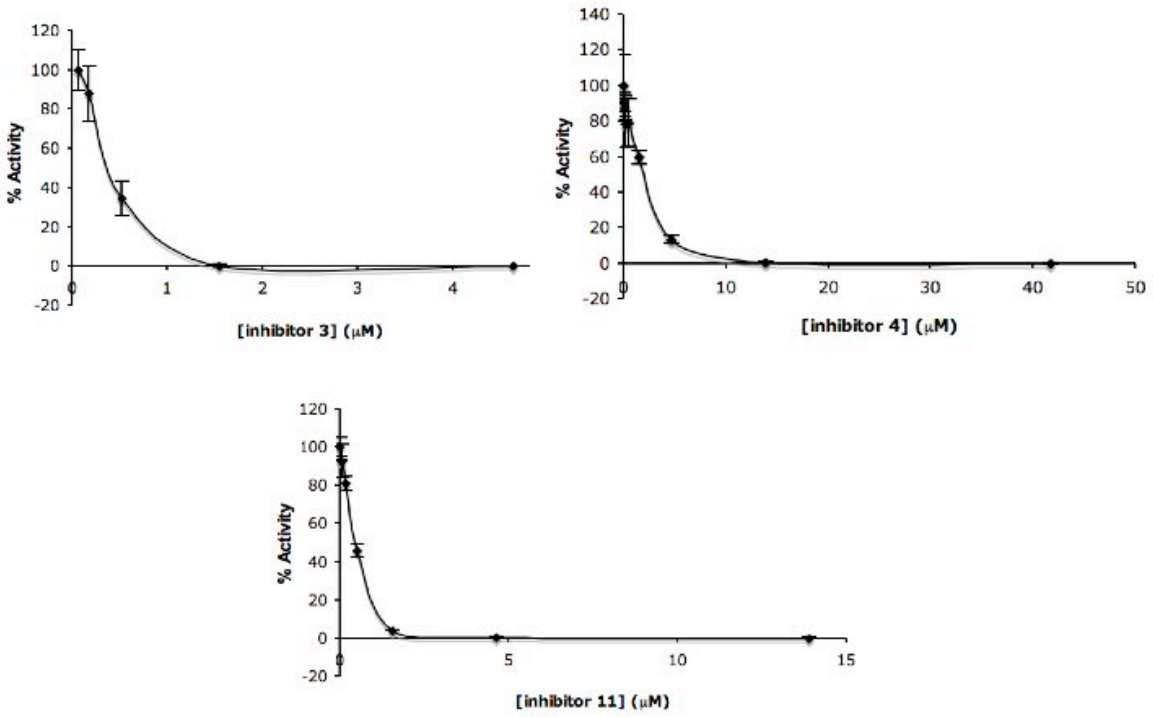

Figure 4-10 Inhibition curves for compounds 3, 4 and 11 on PfCK2α activity
The amount of radiolabel incorporated into the peptide was measured by scintillation counting, and the results plotted as a percentage activity compared with the control (no inhibitor). Data points represent the mean of the two experiments; error bars are the standard deviations. For clarity, only a subset of data points is displayed for compounds 3 and 11.

Inhibition experiments were conducted using the classic CK2 inhibitor TBB (compound 2 in Fig. 4-9) on PfCK2α and HsCK2α, to compare the effect of the inhibitor on the two enzymes (Fig. 4-11). The IC_{50} curves plotted from the data are very similar, with the IC_{50} of TBB on HsCK2α being 1.5μM, and on PfCK2α, 2μM.

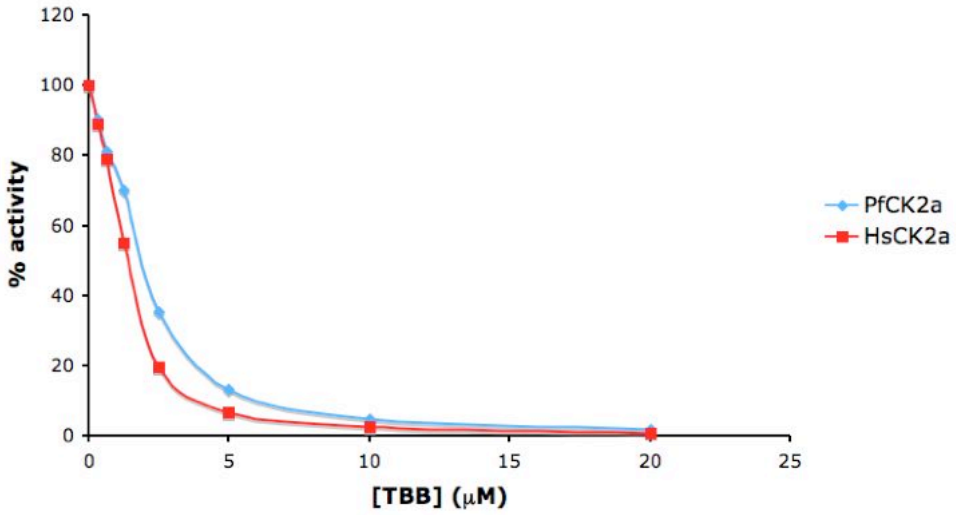

Figure 4-11 Effect of TBB on PfCK2α and HsCK2α activity
The inhibitor 3,4,5,6-Tetrabromobenzotriazole (TBB) was included in increasing concentrations in kinase assays with 25μM ATP, 36ng of enzyme, and peptide 29 as substrate. Activity was measured using the scintillation counting method, and results scored as a percentage of the control (no inhibitor). There was little difference in inhibition curves between the two enzymes. This experiment was carried out twice; this graph is representative.

The differences in IC_{50} values between PfCK2α and HsCK2α for the inhibitors in Table 4-2 may reflect differential inhibition between the two enzymes, or may just be an artefact, since the experiments were performed in separate laboratories, although under as similar conditions as was possible. I went on to perform subsequent experiments simultaneously on the two enzymes side by side, in C. Cochet's laboratory in Grenoble.

The classic CK2 inhibitor TBB inhibited PfCK2α to the same extent as HsCK2α (see Fig. 4-11), indicating that TBB could potentially be used as a tool for functional studies of PfCK2α, as it has been used in many informative CK2 studies in the past. Interpretation of the effect of TBB on *Plasmodium falciparum* parasite cultures is, however, complicated by the presence of HsCK2 in the host erythrocytes (Wei and Tao, 1993), as one cannot exclude the possibility that the activity of the human enzyme in the erythrocyte might be important for parasite viability.

4.6.1.2 IC_{50}s of HsCK2α inhibitors on malaria parasites

The three compounds that were the most effective inhibitors of recombinant PfCK2α were tested for efficacy in inhibiting *P. falciparum* parasite growth using the [^{3}H] hypoxanthine assay (Desjardins et al., 1979). Results are plotted in Fig. 4-12, and the IC_{50} values given in Table 4-3.

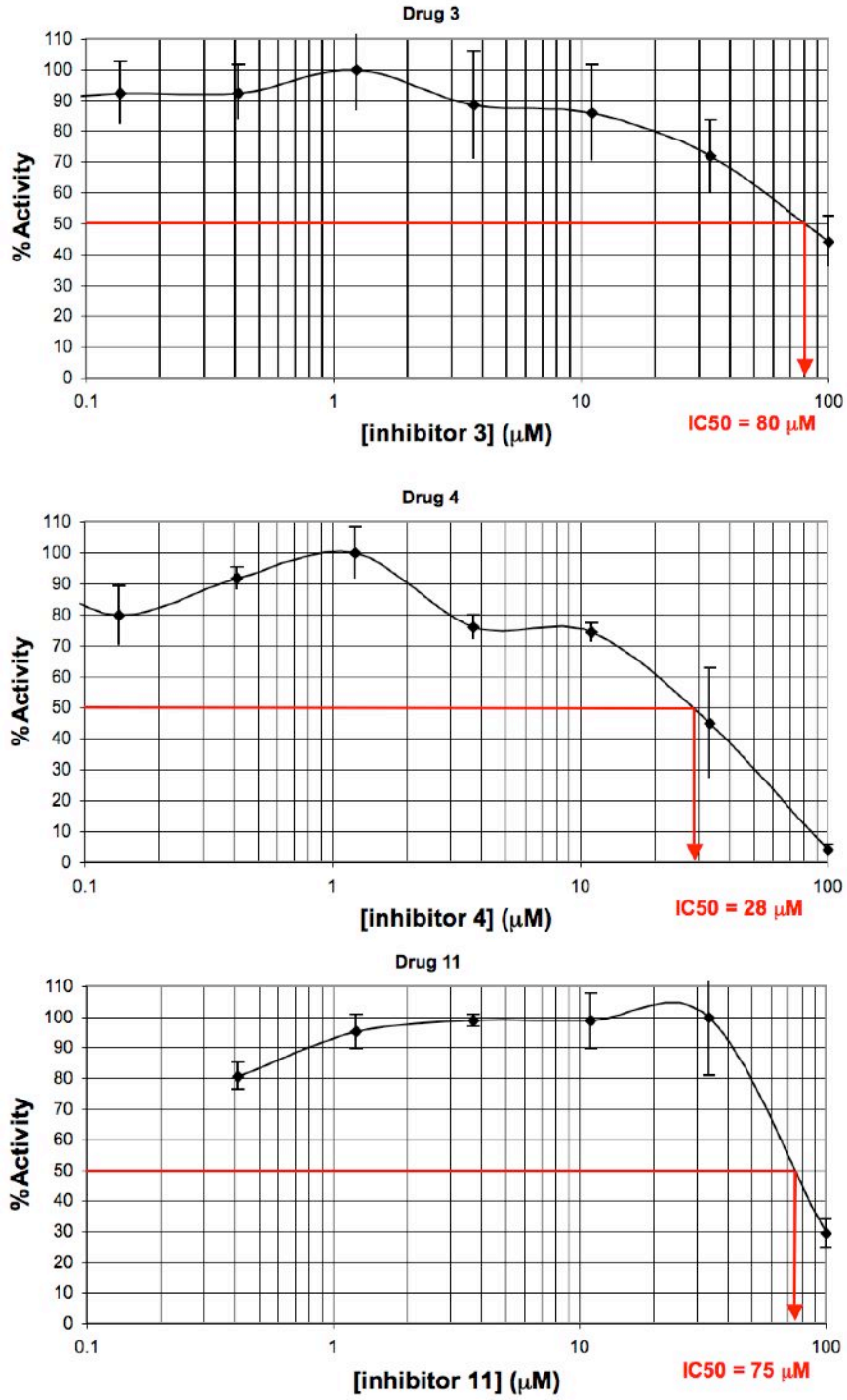

Figure 4-12 IC_{50} values for compounds 3, 4 and 11 on *P. falciparum* 3D7
***P. falciparum* parasites were incubated for 48 hours at 37°C in 96 well plates with [^{3}H] hypoxanthine (37Bq/well) and a range of inhibitor concentrations. The activity is plotted as a percentage of that of uninhibited controls. The experiment was performed in triplicate, and the values plotted are means, with the error bars being the standard deviations of the mean.**

Table 4-4 IC_{50} values for compounds 3, 4, and 11 on *P. falciparum* 3D7 growth

Compound	IC_{50} (μM) on *P. falciparum* parasites
3	80
4	28
11	75

Because these compounds are known HsCK2α inhibitors (See Table 4-2 for IC_{50} values), we cannot draw conclusions about whether it is the inhibition of the parasite CK2 or the erythrocyte CK2 that results in the inhibition of parasite growth. Indeed, pharmacological experiments imply that interfering with erythrocyte kinases can result in parasite death (Doerig and Meijer, 2007). The IC_{50} values for compounds 3, 4, and 11 are high compared with those for compounds with proven effectiveness in clearing parasitic infections, such as Chloroquine, which had an IC_{50} of 5.5μM in our cellular tests. The IC_{50} test for Chloroquine was conducted in parallel with those reported in Table 4-3 above and is therefore directly comparable.

4.6.2 Second inhibitor screen

The first screen was carried out using compounds known to inhibit HsCK2α. We wished to discover whether we are able to differentially inhibit the human and *Plasmodium* CK2α, so performed an inhibitor screen against PfCK2α using the BioMol Enzyme Inhibitor Library: Kinase Inhibitors, Catalog No 2832A (V2), in an attempt to identify compounds that were more efficacious in inhibiting PfCK2α than HsCK2α. The screen was performed using the Kinase-GLO Luminescent Kinase Assay kit (Promega, see section 2.6.9.3), with 20μM compound and 60ng of PfCK2α added to each assay point. The assay is luciferase-based, and the luminescent signal produced is correlated with the amount of ATP present (and therefore is inversely correlated with the amount of kinase activity). The unusually high number of hits recovered by this screen led us to suspect that the Kinase-GLO system was giving false positives. We retested 38 of the presumptive hits in a conventional radiometric kinase assay, using 20μM compound, and peptide 29 as the substrate (see Fig. 4-13).

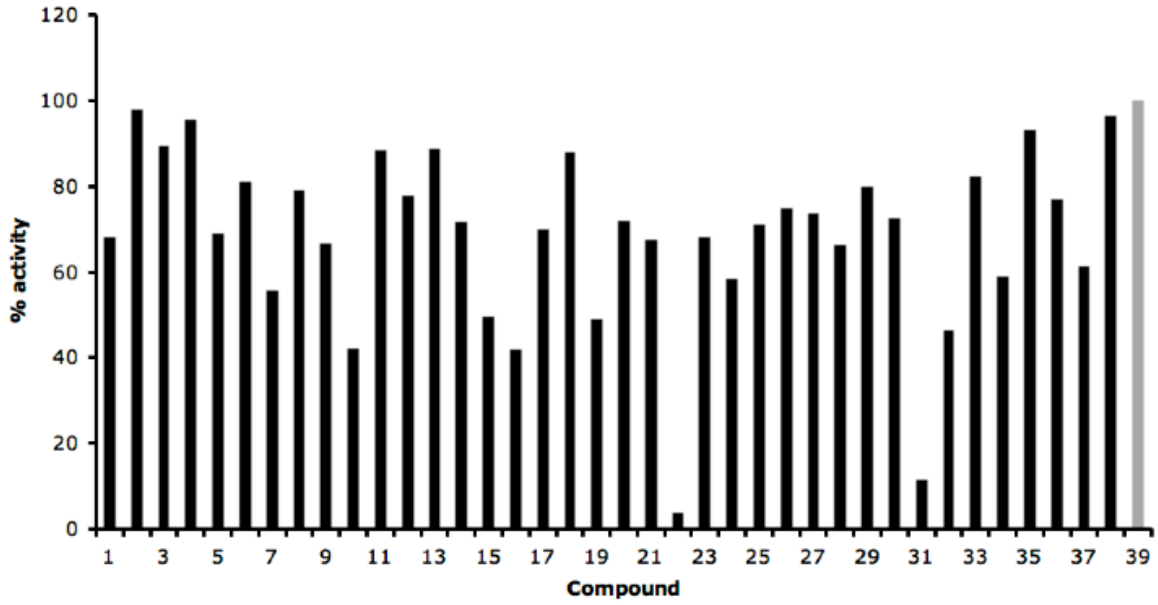

Figure 4-13 Validation of hits from BioMol library of compounds
Compounds 1-38 are the putative hits from the BioMol library, identified in the first screen using the Kinase-GLO system. These hits were then validated using phosphocellulose kinase assays under linear kinetic conditions. 39: control (no compound, 100% activity, highlighted in grey for clarity). Two of the putative hits (compounds 22 and 31, which are BioMol compounds E6 and F10) reduce the activity of PfCK2α to below 20% of the control.

The two confirmed hits (compounds 22 and 31 in the secondary screen) were ML-7 and Rottlerin. Inhibition curves for these compounds on both HsCK2α and PfCK2α were plotted (see Fig. 4-14; IC_{50} values of ML-7 were roughly 4μM for both enzymes, IC_{50} values of Rottlerin on PfCK2α were 7μM, and on HsCK2α, >>20μM). Compound 36, DRB (5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole), is a CK2 inhibitor, yet the activity of PfCK2α in the presence of 20μM of this compound is still 77% of the control. However, DRB has a relatively high reported IC_{50} on CK2 of around 20μM (Meggio et al., 2004), and therefore the low level of inhibition of PfCK2α is not unexpected.

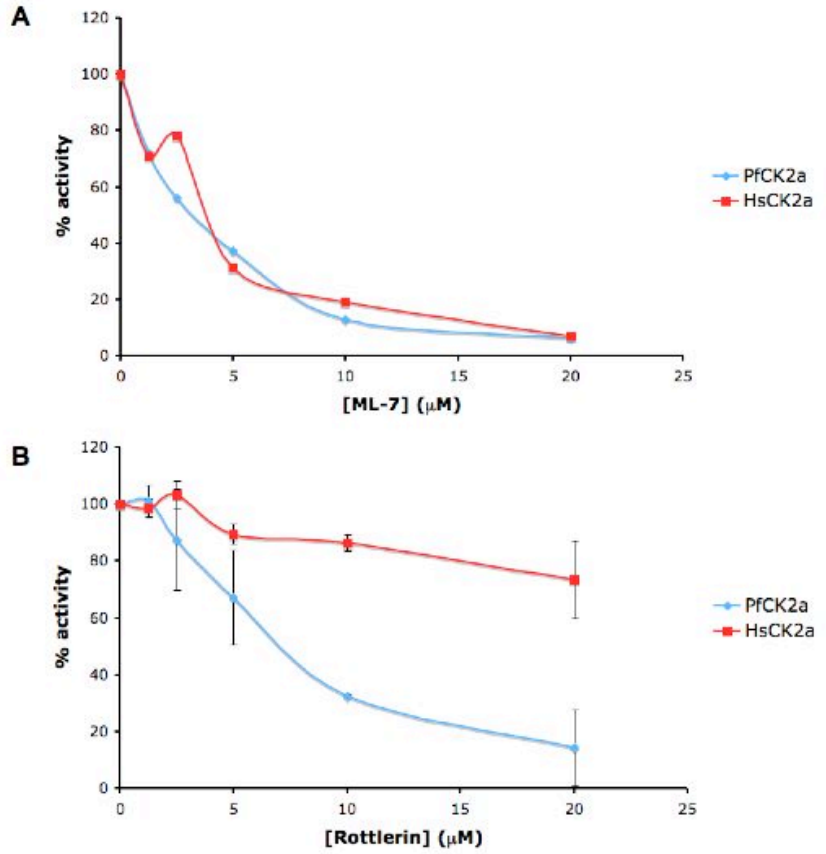

Figure 4-14 Inhibition of PfCK2α and HsCK2α by ML-7 and Rottlerin
The inhibitors ML-7 and Rottlerin were included in increasing concentrations in kinase assays with 25μM ATP, 36ng of enzyme, and peptide 29 as substrate. Activity was assessed using the scintillation counting method (2.6.9.2), and results scored as a percentage of the control (no inhibitor). A: For ML-7, there was little difference in inhibition curves between the two enzymes. B: Separation of the inhibition curves was observed with Rottlerin, and the experiment was repeated. Mean values from the two experiments are shown, with the error bars representing the standard deviations.

Although we have identified in Rottlerin a compound that can distinguish between the human and *Plasmodial* CK2α enzymes, it is unlikely to represent a suitable starting point for antimalarial drug discovery, since Rottlerin has multiple targets (Davies et al., 2000). It is too weak and non-specific an inhibitor even to be used in cellular assays (Bain et al., 2007), as a tool for understanding PfCK2 function. However, we have established that differential inhibition is possible, despite the 65% identity between the primary sequences of PfCK2α and HsCK2α, which is an encouraging starting point for future drug screening efforts.

4.7 Summary

- We confirmed the identity of the PlasmoDB sequence PF11_0096 as the *Plasmodium falciparum* CK2α subunit: The most similar sequences to the predicted protein sequence PfCK2α, revealed by a BlastP search of the NCBI non-redundant protein database, are CK2 alpha subunits, and the primary sequence of PfCK2α contains the eleven subdomains of protein kinases, and the conserved features of CK2 alpha subunits.

- Recombinant PfCK2α possesses kinase activity, and can phosphorylate a range of proteins *in vitro*, including the recombinant *P. falciparum* proteins LSA1, PfHMGB1, PfB7-NAP, shPfCK2β2, and itself, in a trans-reaction.

- PfCK2α exhibits features common to CK2 enzymes, such as constitutive activity, preference for acidic substrates, the ability to autophosphorylate, the ability to utilize GTP as well as ATP as the phosphoryl donor, and inhibition by the CK2-specific inhibitor TBB.

- PfCK2α can phosphorylate a number of proteins within heat-denatured *P. falciparum* protein extract.

- PfCK2α is amenable to inhibition, and can be distinguished from HsCK2α by the inhibitor Rottlerin.

5 Biochemical characterisation of PfCK2β1 and PfCK2β2

5.1 Introduction

The catalytic subunit of CK2 is active independently of secondary messengers and phosphorylation events (Meggio and Pinna, 2003). CK2 falls in the CMGC group of protein kinases, which contains the cyclin-dependent kinases. These PKs are inactive as free catalytic subunits, and require heterodimeric association with a cyclin for activation. Full activation also requires phosphorylation of the activation loop and dephosphorylation of the glycine-rich loop. Unlike cyclin-dependent kinases, the CK2 catalytic subunit is active without such modifications or associations. Protein Kinase A (or cAMP-dependent protein kinase) has a similar quaternary structure to CK2, with two catalytic subunits (PKA-C) and two regulatory subunits (PKA-R). The PKA holoenzyme is inactive, whereas the free catalytic subunits are active; the association of the catalytic subunits with the regulatory subunits abolishes kinase activity. Unlike PKA, the CK2 holoenzyme, as well as the free catalytic subunit, is active. Thus CK2α subunits do not require the CK2β subunits for activity. Yet in every organism in which CK2 is found, the beta subunits are present alongside the alpha subunits. They do not function as absolute regulators of activity in a manner analogous to the activating role of cyclins towards CDKs or the inhibitory role of PKA-R towards PKA-C, but they do possess a variety of functions, including roles in the regulation of CK2 (reviewed in sections 1.6.2.6&7).

In this chapter, we confirm the exon structure of the two PfCK2β subunits, describe their cloning and expression as recombinant proteins, show that the proteins are expressed in the erythrocytic stages of the parasite lifecycle, demonstrate that they interact with the PfCK2α subunit *in vitro*, elucidate some of the functional consequences of this interaction, and provide preliminary evidence that the PfCK2 subunits may interact *in vivo*.

5.2 Identification of two CK2β subunits in *P. falciparum*

Two putative CK2β subunits were identified in *P. falciparum* (Ward et al., 2004, Anamika et al., 2005), PfCK2β1 and PfCK2β2 (PlasmoDB identifiers PF11_0048 and PF13_0232 respectively). BlastP searches using the putative PfCK2β1/β2 amino acid sequences as

queries confirmed their identity as CK2β orthologues with the top hits being CK2β polypeptides from other apicomplexan species (see Table 5-1).

Table 5-1 Percentage identities of PfCK2β1 and 2 to CK2β from other species
The conserved CK2β domains of PfCK2β1 and 2 were used in BLASTP searches of the databases for various species. The best hits were CK2β proteins. The identities of the closest matches for each species are given in brackets. Where more than one CK2β protein record exists for a species, and the closest match differs between the *P. falciparum* CK2βs, the identities are given under each column.

Species (NCBI protein identity)	PfCK2β1 (AAN35637)	PfCK2β2 (CAD52554)
H. sapiens (CAI18394)	40%	40%
M. musculus (NP_034105)	40%	40%
X. tropicalis (CAJ83806)	40%	40%
D. rerio (NP_571262)	40%	40%
S. cerevisiae CKB1 (CAA96719)	31%	35%
S. cerevisiae CKB2 (CAA99229)	40%	33%
S. pombe (CAB62429)	41%	41%
A. thaliana	40% (CAB10544)	39% (CAB87862)
O. sativa (AAG60201)	37%	38%
D. discoidium (EAL65139)	41%	37%
C. parvum	44% (EAK89111)	43% (EAK88980)
T. parva	42% (EAN32867)	40% (EAN34119)
T. brucei (EAN80034)	38%	37%
L. major (CAJ09481)	40%	39%
T. gondii	47% (EEA98732)	40% (EEA98433)
P. berghei	83% (CAH97991)	89% (CAH99986)
P. yoelii	85% (EAA21344)	88% (EAA20924)
P. vivax	88% (EDL45440)	86% (EDL44476)
P. knowlesi	88% (CAQ39810)	85% (CAQ41198)

All gene-prediction programmes on the *Plasmodium* database PlasmoDB predicted a one-exon structure for PfCK2β1, encoding a predicted protein of 245 amino acids (calculated molecular weight 28.3kDa), and a two-exon structure for PfCK2β2, with the length of the predicted protein differing slightly between prediction programmes. We confirmed these exon structures and the length of the coding regions by PCR amplification from *P. falciparum* 3D7 cDNA. As expected from the homogeneity of the gene predictions for PfCK2β1, our experimental data were in agreement with the one-exon gene structure. The experimentally determined gene-structure for PfCK2β2 follows the majority prediction, which is the only prediction retained on the latest version of PlasmoDB (version 5.4). PfCK2β2 has an open reading frame of 1158bp, encoding a predicted protein of 385 amino acids (calculated molecular weight 45.3kDa). The PfCK2β2 sequence is composed of two exons, with the intron falling between the N-terminal extension and the beginning of the conserved CK2β coding region (bases coding for amino acids M1-T158 are in exon 1,

bases coding for amino acids V159-Q385 are in exon 2). Verifying the exon structure of PfCK2β2 by cDNA analysis also allowed us to confirm that the first exon – and therefore the N-terminal extension – of PfCK2β2 is actually transcribed. The full-length sequence was amplified and confirmed by DNA sequencing. There were some differences in the number of repeat codons coding for glutamic acid residues between the predicted and amplified sequence, but such length polymorphism can be expected in long repetitive sequences. Indeed, this N-terminal region of PfCK2β2 is the only region to possess SNPs as displayed on PlasmoDB (Bahl et al., 2003, Jeffares et al., 2007, Mu et al., 2007, Volkman et al., 2007).

An alignment of the PfCK2β sequences with the human CK2β sequence (HsCK2β; Fig. 5-1) reveals that many of the conserved features of CK2β subunits, including the four cysteine residues responsible for zinc-finger formation (Chantalat et al., 1999), are present in PfCK2β1 and PfCK2β2 (e.g. Cys117, 122, 145, 148 for PfCK2β1; indicated by arrows in Fig. 5-1). The human CK2β sequence has a well-documented CK2 phosphorylation site at the N-terminus (SSEE). The phosphorylatable residues of PfCK2β1 in this region, with the exception of Ser4, have basic residues in the n+1 to n+3 region, which are negative determinants for phosphorylation by CK2, or do not have acidic residues in either of the n+1 or n+3 positions, which is an absolute requirement for CK2-dependent phosphorylation (Meggio and Pinna, 2003). In contrast, PfCK2β2 possesses several phosphorylatable residues in the N-terminal region that are surrounded by a number of acidic residues, and could therefore be phosphorylated by CK2, and a TESSEE sequence at the C-terminus reminiscent of the HsCK2β N-terminal phosphorylation site (MSSEE). This lead to the hypothesis that PfCK2β2 is more likely than PfCK2β1 to be a substrate of PfCK2, a hypothesis supported by *in vitro* experimental data using recombinant proteins (see Fig. 4-7B). The physiological occurrence, and if so relevance, of this phosphorylation remains to be investigated.

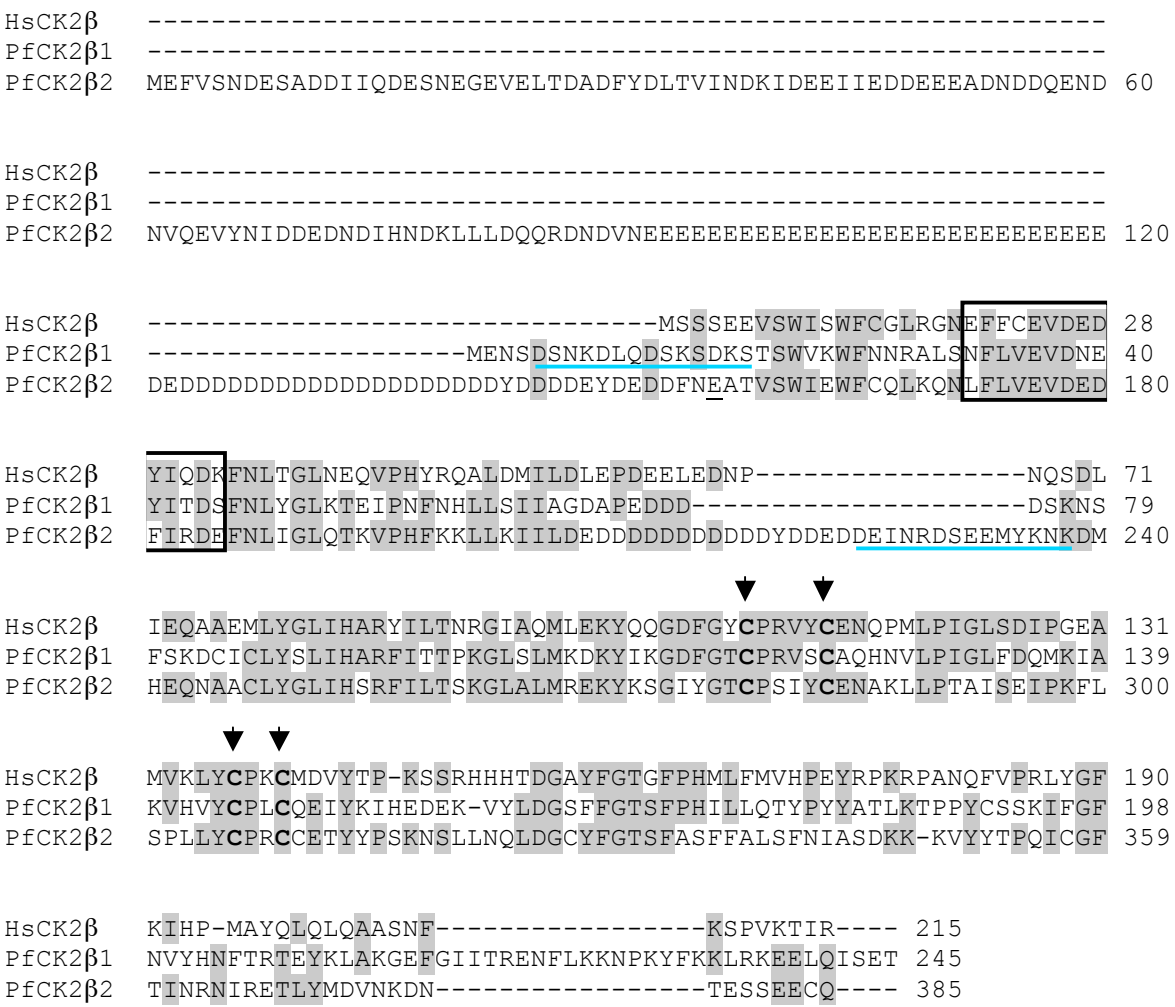

Figure 5-1 Alignment of CK2β protein sequences from *Homo sapiens* and *P. falciparum* Protein sequences were aligned using ClustalW, and by hand. PfCK2β1 has a C-terminal extension; PfCK2β2 has a long N-terminal extension and an insertion. Arrowheads and bold type indicate the conserved cysteine residues that form the base of the zinc finger. The residue that follows the artificially inserted initiating methionine in the shPfCK2β2 sequence is underlined (E156; see text for details). Amino acids thought to be important for the export of CK2 as an ectokinase are boxed. Peptides used to raise anti-CK2β1/β2 antibodies are underlined in light blue. Highlights indicate conserved residues.

The N-terminal extension of PfCK2β2 is unusually long for CK2β proteins, with 160 amino acids before the first conserved residue (Trp161 in PfCK2β2). Most CK2β subunits from vertebrates have only eight amino acids prior to this conserved residue (*Homo sapiens, Gallus gallus*, *Mus musculus*, *Xenopus tropicalis*, *Bos taurus*, *Danio rerio*); this N-terminal extension is expanded in yeast (*Saccharomyces cerevisiae*: 37 residues), discicristates (*Trypanosoma brucei*: 27, *Leishmania major*: 21), plants (*Arabidopsis thaliana*: 100, *Oryza sativa*: 92) and alveolates (*Cryptosporidium parvum*: 27, *Theileria parva*: 34). Within the alveolates, *P. yoelii yoelii* (125) and *P. vivax* (157) also have long extensions, but that of *P. falciparum* is the longest identified to date. Homorepeat-containing proteins make up 35.7% of the proteome of *P. falciparum*, although the majority of these homorepeats are asparagines and lysines (Singh et al., 2004b), unlike the polymers of acidic residues present in PfCK2β2. One hypothesis for the function of this extension is the downregulation of the CK2α subunit. Polyglutamate is a potent CK2

inhibitor (Tellez et al., 1990), and the N-terminal extension of PfCK2β2 is rich in polyglutamate and polyaspartate. We have not been able to purify PfCK2β2 with the N-terminal extension (see section 5.5.2), and therefore this hypothesis remains to be tested.

The stretch of amino acids found to be necessary for the export of CK2 as an ectokinase (aa 20-33) (Rodriguez et al., 2008) is largely conserved in the PfCK2β sequences, leading to the intriguing possibility that PfCK2 may be exported from the parasite, to the parasitophorous vacuole or to the host erythrocyte. Although a destruction box motif has been found in HsCK2β (R47-D55 (Bibby and Litchfield, 2005, Glotzer et al., 1991)), this sequence is not conserved in the PfCK2β subunits, and a search of the PfCK2β sequences using the D-box finder (http://bioinfo2.weizmann.ac.il/~danag/d-box/main.html) revealed no destruction box motif sequence in either of the PfCK2β subunits. No typical D-box motifs were found in the *P. falciparum* cyclins either (Merckx et al., 2003), so perhaps *P. falciparum* possesses a divergent D-box motif. The acidic stretch responsible for downregulation of CK2 and association with the plasma membrane (HsCK2β D55-D64; (Leroy et al., 1999, Meggio et al., 1994a)) is present in PfCK2β1 (D68-D75), and extended in PfCK2β2 (D207-E226). *Saccharomyces cerevisiae* CK2β also has an insertion sequence of 30 amino acids in this location. The insertion occurs in a region looping out from the main protein structure (Chantalat et al., 1999, Niefind et al., 2001) (see Fig. 1-10). The human CK2β is phosphorylated on S209 in a cell-cycle dependent manner by $p34^{cdc2}$ (Litchfield et al., 1995, Litchfield et al., 1991, Meggio et al., 1995), although the function of this phosphorylation is unknown. *P. falciparum* possesses a $p34^{cdc2}$ orthologue (Ward et al., 2004), and both PfCK2β subunits possess serine residues near the C-terminus that could be phosphorylated. However, $p34^{cdc2}$ is a proline-directed kinase, and there are no SP motifs in the C-terminus of either PfCK2β subunit, though PfCK2β2 does possess an SP within the zinc finger (S301P302). The *in vivo* phosphorylation status of this serine residue remains to be investigated.

5.3 Transcription and expression

Microarray experiments reveal that the mRNAs encoding all three PfCK2 subunits are detectable throughout the parasite lifecycle (Le Roch et al., 2003, Bozdech et al., 2003). Proteomics data from the erythrocytic stages of the parasite lifecycle confirm that the PfCK2β1 subunit is expressed as protein in the asexual blood cycle and in gametocytes (Florens et al., 2004, Florens et al., 2002), but there is currently no available proteomic data for the PfCK2β2 subunit. Proteomic studies of *P. yoelii* liver-stage parasite proteins

revealed that a putative *P. yoelii* CK2β orthologue (PY01577) is present in the liver stage parasites (Tarun et al., 2008).

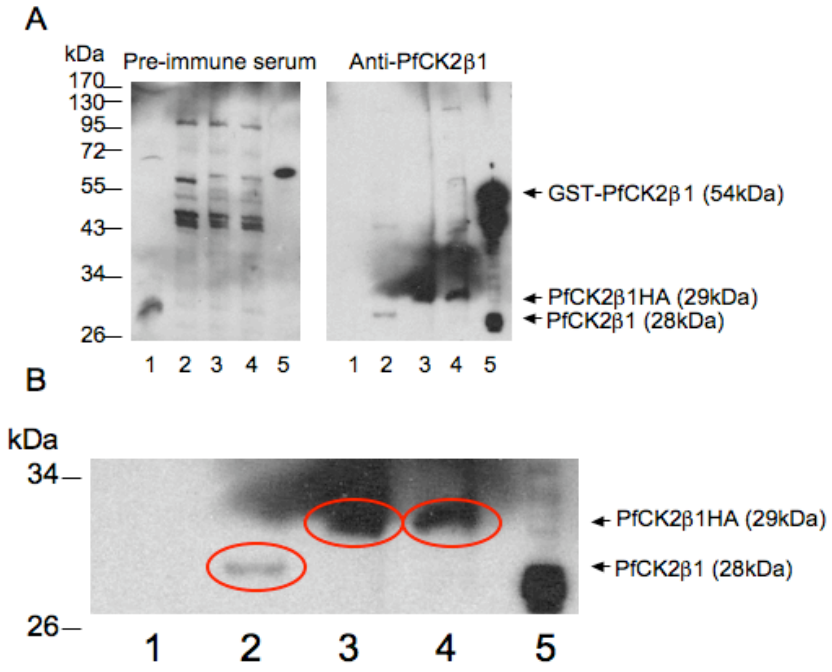

Figure 5-2 Western blot showing PfCK2β1 expression in erythrocytic stage parasites Protein extract from unsynchronised erythrocytic stage *P. falciparum* parasites was prepared from 3D7 parasites (lane 2), and from parasites with a sequence encoding an HA-tag incorporated at the 3' end of the *PfCK2β1* gene locus (lane 3: clone C9, lane 4: clone E8; see 3.4.1 for details of these parasite lines). Recombinant GST-PfKC2β1 (lane 5, see section 5.4) and protein extract from unparasitized red blood cells (lane 1) were included as positive and negative controls. A. Two identical acrylamide gels were run, the proteins transferred to membrane, and western blots performed with affinity-purified rabbit anti-PfCK2β1 antibodies (right panel), or with pre-immune serum from the same rabbit as a negative control (left panel). B. Close-up of part of anti-PfCK2β1 western blot, with relevant bands circled in red.

Western blot analysis of mixed erythrocytic stage parasite extract confirmed that the PfCK2β proteins are expressed during the erythrocytic cycle (Figs 5-2 and 5-3). Affinity-purified rabbit anti-PfCK2β-peptide antibodies were ordered from BioGenes (Berlin). The anti-peptide antibodies recognise the amino acid sequence DSNKDLQDSKSDKS from the N-terminus of PfCK2β1, and the sequence DEINRDSEEMYKNK from the insertion sequence of PfCK2β2. Two animals were immunised using each peptide, giving two independent antibody sera. The antibodies were purified against immobilised peptide. Western blots performed on parasite extract (and red blood cell extract and recombinant protein as controls) imply that the antibodies directed against the PfCK2β1 peptide specifically recognise PfCK2β1 (Fig. 5-2). The band in lane 5 at roughly the 55kDa marker has the expected size of the GST-tagged PfCK2β1 subunit. This band is not recognised by the pre-immune serum (although a larger band from the recombinant protein preparation is recognised). The affinity purified anti-PfCK2β1 antibodies specifically recognise a band of

roughly 28kDa, which is the expected size of PfCK2β1, in protein extract from 3D7 parasites (lane 2). This band runs at a slightly higher molecular weight in protein extract from parasites that had incorporated an HA tag at the 3' end of the PfCK2β1 gene (lanes 3 and 4; see section 3.4.1), which is expected, and strengthens the evidence that this band is indeed PfCK2β1. Pre-immune serum dilutions of 1 in 500 were used for western blotting. The bands mentioned above were not recognised by the pre-immune serum (Fig. 5-2, left panel).

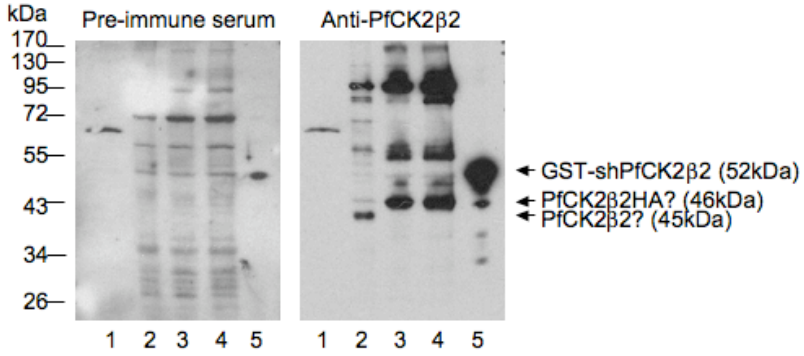

Figure 5-3 Western blot showing PfCK2β2 expression in erythrocytic stage parasites
Protein extract from unsynchronised erythrocytic stage *P. falciparum* parasites was prepared from 3D7 parasites (lane 2), and from parasites with a sequence encoding a HA-tag incorporated at the 3' end of the *PfCK2β2* gene locus (lane 3: clone D5, lane 4: clone E2; see 3.5.1 for details of these parasite lines). Protein extract from unparasitized red blood cells (lane 1), and recombinant GST-shPfKC2β2 (lane 5, see section 5.6), were included as negative and positive controls. Two identical acrylamide gels were run, the proteins transferred to membrane, and western blots performed with affinity-purified anti-PfCK2β2 antibodies from a rabbit, or with pre-immune serum from the same rabbit, as a negative control.

Similar western blots were performed using affinity-purified rabbit anti-PfCK2β2 antibodies and the corresponding pre-immune serum (Fig. 5-3). The band in lane 5 between the 43 and 55kDa markers has the expected size of the GST-tagged shPfCK2β2 subunit, and although there is a small amount of background binding to this protein in the pre-immune serum control, a much stronger signal is seen with the anti-PfCK2β2 antibodies. Several bands from wild type 3D7 parasite protein extract were recognised by the affinity purified anti-PfCK2β2 antibodies (right panel, lane 2). The lowest band in the 3D7 parasite extract recognised by the specific antibodies (just below the 43kDa marker) runs at a slightly higher molecular weight in protein extract from parasites that had incorporated an HA tag at the 3' end of the PfCK2β2 gene (lanes 3 and 4; the bands are at the 43kDa marker), indicating that this band is likely to be PfCK2β2. Pre-immune serum dilutions of 1 in 500 were used for western blotting. These antibodies did not recognise the PfCK2β2 band (Fig. 5-3, left panel). Were the full length coding sequence for PfCK2β2 to be expressed, the expected size of the protein would be just over 45kDa. The protein may

run slightly aberrantly on the acrylamide gel, or the protein may be cleaved. Mass-spectrometry analysis of immunoprecipitated PfCK2β2 would tell us whether the protein was expressed as full length or cleaved product. The larger bands recognised are presumably due to non-specific binding of the antibody. BLAST searches for other proteins containing the sequence of the peptide used for antibody generation returned one hit: PF13_0089, a 192 kDa protein with several amino acid sequences bearing some similarity to the PfCK2β2 peptide sequence. However, this protein is too large to be responsible for the additional bands seen in the western blot (the most significant bands being 55 kDa and 95 kDa), unless it is degraded or processed.

5.4 Cloning and expression of the PfCK2β1 subunit

5.4.1 Cloning

The coding sequence for the putative *PfCK2β1* gene (738bp, one exon) was amplified from *P. falciparum* 3D7 cDNA by the polymerase chain reaction, using Pfx Platinum polymerase and the oligonucleotide primers CK2b1ForBgl and CK2b1RevSal, which introduced an N-terminal BglII site and a C-terminal SalI site to the PCR product. BglII was used for cloning because PfCK2β1 has an internal BamHI site. BglII and BamHI generate compatible overhangs, so the BglII-digested PfCK2β1 fragment can be successfully ligated into the BamHI-cut pGEX-4T-3 and pQE-30 plasmids. The PCR products were first ligated into the vector pGEM-T-Easy for sequence verification, and subcloned into the BamHI and SalI sites of the pGEX-4T-3 and pQE-30 vectors. The pGEX-4T-3 plasmid attaches an N-terminal GST tag to the recombinant protein, and the pQE-30 plasmid attaches an N-terminal 6xHis tag to the recombinant protein. The insert regions of these plasmids (named pGEX-4T-3-PfCK2b1 and pQE-30-PfCK2b1) were sequenced prior to use.

5.4.2 Expression and purification

Test expressions of GST- and His-tagged PfCK2β1 were carried out to find the optimum expression conditions. Expression from pGEX-4T-3-PfCK2b1 was tested in *E. coli* BL21 cells (Stratagene), and expression from pQE30-PfCK2b1 was tested in SG13009 *E. coli* (Qiagen), both at a range of temperatures (37^{o}C, 30^{o}C and 20^{o}C) and IPTG concentrations (0.1-1mM). Recombinant proteins were purified using batch glutathione affinity chromatography (see 2.6.1) of the GST-tagged PfCK2β1, and batch nickel affinity

chromatography (see 2.6.2) of the His-tagged PfCK2β1. Samples of bacteria were taken before and after induction of protein expression, and samples were also taken from the soluble and insoluble protein fractions after lysis, and of the eluted proteins (see sections 2.6.1&2). The samples were separated by SDS-PAGE on 12% acrylamide gels, and stained with Coomassie blue stain (Fig. 5-4).

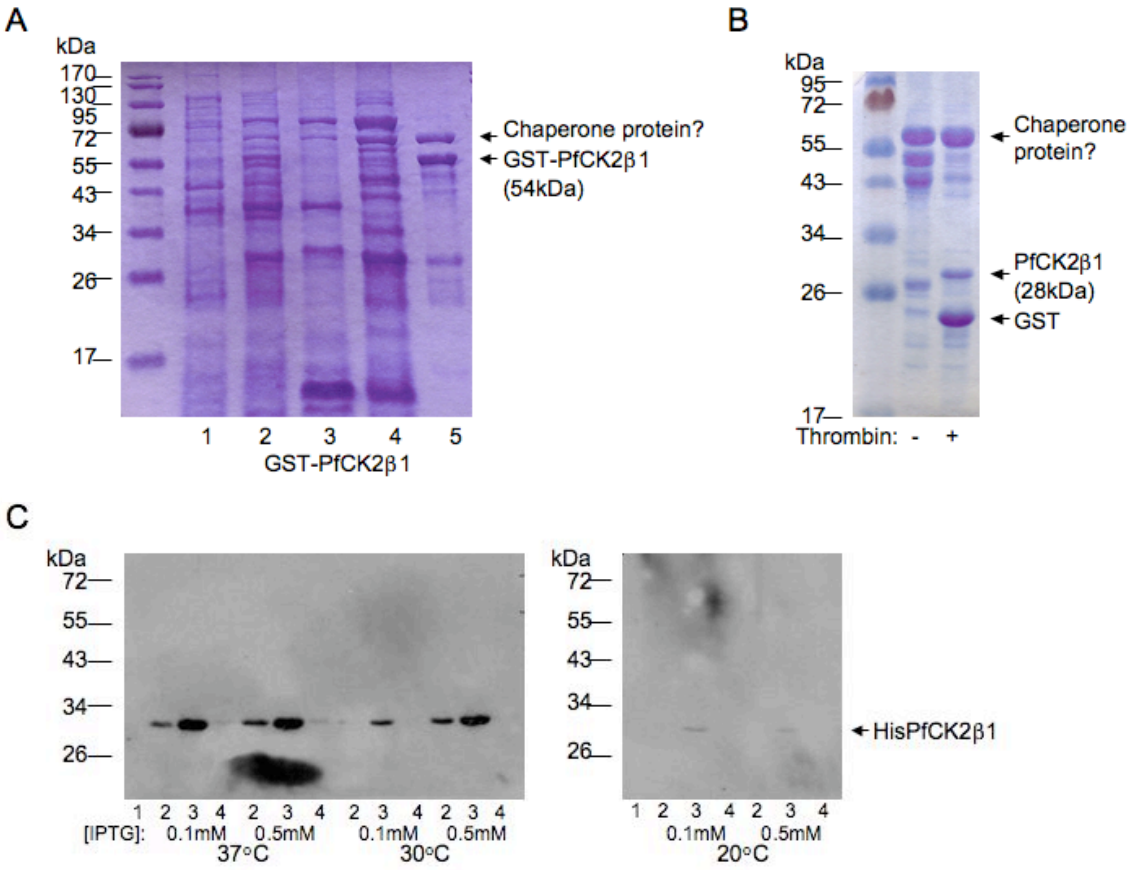

Figure 5-4 Expression and purification of tagged recombinant PfCK2β1
Panel A: Samples taken during the expression and purification of GST-PfCK2β1 were separated by SDS-PAGE on 12% acrylamide gels, which were then stained with Coomassie blue stain. 1: Sample of bacteria before induction of expression. 2: Sample of bacteria after expression was induced. 3: Insoluble fraction. 4: Soluble fraction. 5: Eluted proteins. Panel B: Thrombin cleavage of purified GST-PfCK2β1. Panel C: Anti-His western blot of samples taken during test expressions of His-PfCK2β1. 1: Before induction, 2: After induction, 3: Insoluble fraction, 4: Soluble fraction. None of the conditions produced soluble recombinant His-PfCK2β1.

For GST-PfCK2β1, the expression conditions that produced the most recombinant protein were: induction at 20°C overnight with 0.1mM IPTG. Large-scale expressions (250ml cultures) were performed using these conditions, which produced roughly 0.2mg of purified protein. The protein was purified along with another protein (larger band, between 55kDa and 72kDa markers, Fig. 5-4A), which we hypothesize to be a chaperone protein. The GST-PfCK2β1 was identified by thrombin-cleavage of the GST moiety (Fig. 5-4B); the putative chaperone protein was not affected by the thrombin, indicating that the pattern of bands was not an aberrantly running PfCK2β1 with its degradation products. The

putative chaperone protein was not recognised by the antibodies raised against CK2β1, but was recognised by the pre-immune serum (see Fig. 5-2). None of the conditions tested produced significant quantities of soluble His-PfCK2β1 (Fig. 5-4C).

5.5 Cloning and expression of the PfCK2β2 subunit

5.5.1 Cloning

The whole coding sequence for the putative *PfCK2β2* gene (two exons, 1158bp excluding the intron) was amplified from *P. falciparum* 3D7 cDNA by the polymerase chain reaction, using the Pfx Platinum polymerase oligonucleotide primers CK2b2ForBam and CK2b2RevSal, which introduced an N-terminal BamHI site and a C-terminal SalI site to the PCR product. The PCR products were ligated into the vector pGEM-T-Easy for sequence verification, and then subcloned between the BamHI and SalI sites of the vectors pGEX-4T-3 and pQE-30. The insert regions of these plasmids (named pGEX-4T-3-PfCK2b2, and pQE-30-PfCK2b2) were sequenced prior to use.

5.5.2 Expression and purification

Test expressions of GST- and His-tagged PfCK2β2 were carried out to find the optimum expression conditions. Expression from pGEX-4T-3-PfCK2b2 was tested in *E. coli* BL21 cells (Stratagene), and expression from pQE30-PfCK2b2 was tested in SG13009 *E. coli* (Qiagen), both at a range of temperatures (37^{o}C, 30^{o}C and 20^{o}C) and IPTG concentrations (0.1-1mM). Recombinant proteins were purified using batch glutathione affinity chromatography (see 2.6.1) of the GST-tagged PfCK2β2, and batch nickel affinity chromatography (see 2.6.2) of the His-tagged PfCK2β2. Samples were taken before induction, after induction, from the soluble and insoluble protein fractions after lysis, and of the eluted proteins (see sections 2.6.1&2). These samples were separated on 12 % acrylamide gels and visualised using Coomassie blue stain and western blotting. None of the expression conditions produced soluble recombinant protein (data not shown).

5.6 Cloning and expression of the shPfCK2β2 subunit

5.6.1 Cloning

Because the long N-terminal acidic repeat sequences of PfCK2β2 might have been responsible for the expression problems in *E. coli*, a sequence coding for the PfCK2β2 subunit without the N-terminal extension (named shPfCK2β2) was constructed. This sequence began with an initiating methionine, then continued with E156 of the PfCK2β2 sequence (see Fig. 5-1), which lies just N-terminal to the conserved CK2β domain. The sequence was amplified from the pGEX-4T-3-PfCK2b2 plasmid using Pfx Platinum polymerase and the oligonucleotide primers CK2b2shortForBam and CK2b2RevSal, which introduced an N-terminal BamHI site and a C-terminal SalI site to the PCR product. The sequence was ligated into pGEM-T-easy and subcloned into pGEX-4T-3 and pQE30 as described above for full-length PfCK2β2. The insert regions of these plasmids (named pGEX-4T-3-shPfCK2b2 and pQE-30-shPfCK2b2) were sequenced prior to use.

5.6.2 Expression and purification

Test expressions of GST- and His-tagged shPfCK2β2 were carried out as detailed above for PfCK2β1 (section 5.4.2). The expression conditions that produced the most recombinant protein were the same for both tags: induction at 20^{o}C overnight with 0.1mM IPTG. Large-scale expressions were performed using these conditions.

Recombinant proteins were purified using batch glutathione affinity chromatography (see 2.6.1) of the GST-tagged shPfCK2β2, and batch nickel affinity chromatography (see 2.6.2) of the His-tagged shPfCK2β2. 250ml cultures were used for expression of recombinant proteins, and produced roughly 0.8mg of GST-shPfCK2β2, and 0.8mg of His-shPfCK2β2. Samples taken during expression and purification of the recombinant proteins were separated on 12 % acrylamide gels and visualised using Coomassie blue stain (Fig. 5-5).

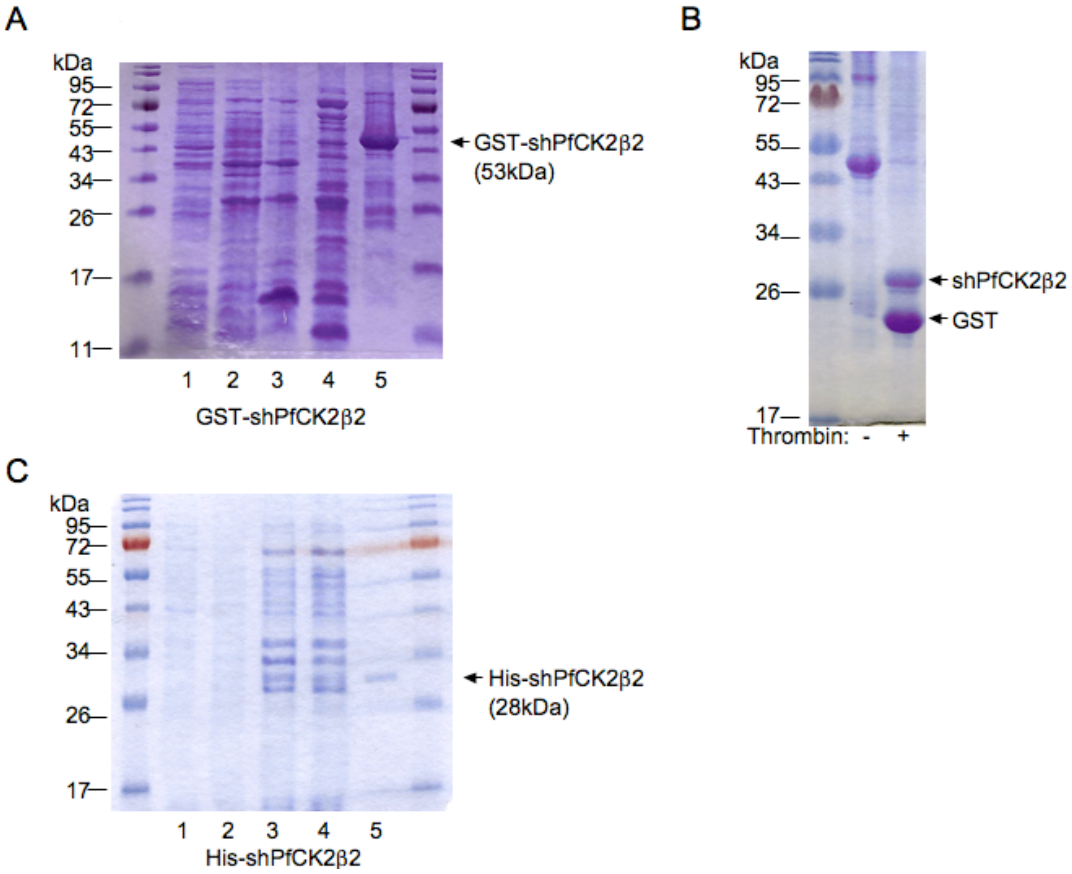

Figure 5-5 Expression and purification of tagged recombinant shPfCK2β2
Samples taken during the expression and purification of GST-shPfCK2β2 (Panel A) and His-shPfCK2β2 (Panel C) were separated by SDS-PAGE on 12% acrylamide gels, which were then stained with Coomassie blue stain. 1: Sample of bacteria before induction of expression. 2: Sample of bacteria after expression was induced. 3: Insoluble fraction. 4: Soluble fraction. 5: Eluted proteins (expected sizes: GST-shPfCK2β2: 53kDa, His-shPfCK2β2: 28kDa). Panel B: GST-shPfCK2β2 is amenable to thrombin cleavage.

5.7 Subunit interactions

5.7.1 Pulldowns and co-immunoprecipitations

To assess whether the two PfCK2 beta subunits are able to associate with PfCK2α *in vitro*, mixtures of His- and GST-tagged subunits were prepared, from which protein complexes containing GST-tagged subunits were purified using glutathione-agarose beads. The purified proteins were then subjected to western blot analysis using an anti-His antibody, to detect any bound His-tagged protein that was co-purified with the GST-tagged proteins. PfCK2α-His was co-purified with GST-PfCK2β1 and GST-shPfCK2β2, but not with GST alone (Fig. 5-6A). This reveals that the PfCK2 alpha subunit is able to interact with both of the PfCK2 beta subunits, at least *in vitro*. The amount of GST-PfCK2β1 purified from the protein mixture was lower than that of GST-shPfCK2β2 (see Fig. 5-6A, right panel,

Coomassie-stained gel), thus it is unsurprising that there was also less PfCK2α-His labelled in the western blot in the lane containing proteins purified from the GST-PfCK2β1/PfCK2α-His mix than in the lane containing proteins from the GST-shPfCK2β2/PfCK2α-His mix (Fig. 5-6A, left panel).

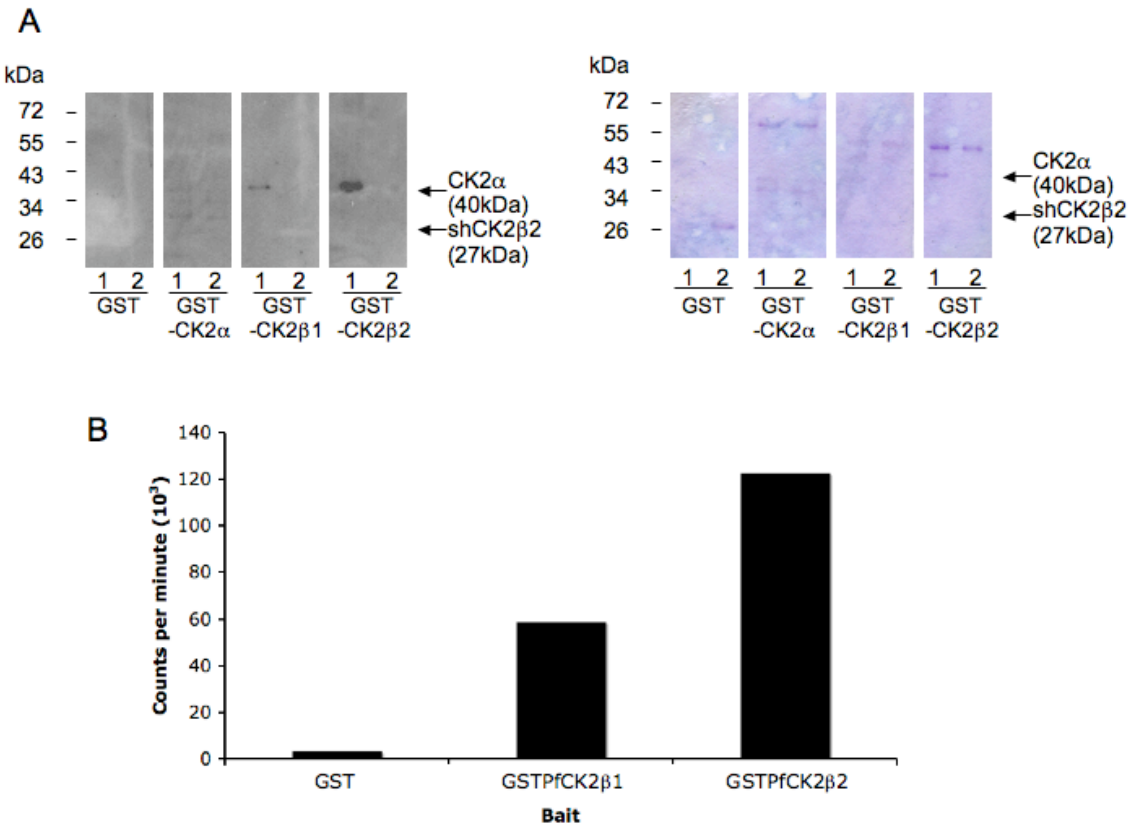

Figure 5-6 Interaction of the PfCK2β subunits with PfCK2α
Panel A: Anti-His western blot of samples from pulldown experiments in which GST-tagged and His-tagged PfCK2 subunits were mixed, and complexes containing GST-tagged proteins were purified on glutathione-agarose beads. 1: PfCK2α-His. 2: His-shPfCK2β2. Left panel: Photographic film exposed to western blot membrane. Right panel: corresponding Coomassie-stained gel of pulldown samples. Panel B: Kinase assays after pulldowns using GST-tagged proteins as bait and PfCK2αHis as prey. The NEB peptide p6012 was used as the substrate in a scintillation count kinase assay. This experiment was repeated, with similar results.

Pulldown experiments as described above were followed by kinase assays using the NEB p6012 peptide as a substrate. There was only background radioactivity incorporated in the assay containing proteins that had been pulled-down with GST, but much higher levels of radioactivity incorporated in the assays containing proteins that had been pulled-down with GST-PfCK2β1 or GST-shPfCK2β2. This confirms that the beta subunits are able to associate with the catalytic alpha subunit *in vitro*. The amount of radioactivity incorporated into the peptide substrate in the kinase reaction containing proteins purified from GST-PfCK2β1/PfCK2α-His mixtures was lower than that in the kinase reaction containing proteins purified from GST-shPfCK2β2/PfCK2α-His mixtures. This is likely to be due to

unequal amounts of PfCK2α present (due to unequal amounts of beta protein present in the samples), as shown in the western blots and Coomassie gels from the pulldowns (Fig. 5-6A&B), rather than different levels of stimulation of PfCK2α activity. Parallel experiments were performed with HsCK2α, with similar results, showing that the PfCK2β subunits are also able to associate with HsCK2α *in vitro* (data not shown; experiment performed in Grenoble).

Co-immunoprecipitation experiments were performed to determine whether the native subunits interact in parasite extracts. Clonal parasite lines were produced (sections 3.3.1, 3.4.1 and 3.5.1) that have integrated an HA tag at the 3'end of the PfCK2 genes. Protein A Sepharose beads coated in anti-HA antibodies were used to purify complexes containing the HA tags (and therefore the HA-tagged PfCK2 subunits) from protein extracts derived from these clonal parasite lines. Immunoprecipitations were also performed on wild type (untransfected) 3D7 parasite extract, to provide a negative control. Protein extract concentrations were standardized to 1mg/ml using the Bradford assay, and 200μg was used for each immunoprecipitation. Standard kinase assays were performed on the immunoprecipitated material, using a variety of potential substrates (mixed casein, MBP and histone H1; Fig. 5-7). The control reaction containing recombinant PfCK2α (lane 8) reveals the preferences of the catalytic subunit for the casein proteins (between the 26 and 34 kDa markers). No kinase activity was seen in the reaction containing immunoprecipitated material from wild type 3D7 parasite extract (lane 1). Strong phosphorylation is seen in the lanes containing immunoprecipitated proteins from parasites harbouring HA-tagged PfCK2α subunits (lanes 2 and 3), with the banding pattern being similar to that for recombinant PfCK2α (lane 8). The same pattern of bands, although with a weaker signal intensity, was seen in the reactions containing material immunoprecipitated from PfCK2β1HA and PfCK2β2HA parasites lines (lanes 4-7), suggesting that the same kinase might be responsible for the activity seen in each lane, and therefore that the PfCK2α subunit may have been co-immunoprecipitated with the HA-tagged PfCK2β subunits. We demonstrated in Chapter 3 that the HA tag plasmid integrated in the expected place in each parasite line (i.e. at the 3' end of the *PfCK2* gene loci), and therefore we can conclude that the phosphorylation activity seen is due to the presence of complexes containing the PfCK2 subunits. To confirm this result, we would need to perform anti-PfCK2-subunit western blots on the immunoprecipitated samples. These experiments were attempted, but cross-reactive bands caused by the presence of the anti-HA antibody in the samples obscured any meaningful bands that may have been present. These experiments should be repeated using an immobilized anti-HA antibody, allowing

subsequent separation of the anti-HA antibody from the immunoprecipitated material. On Coomassie-stained gels it was not possible to identify bands that were visible in lanes 2-7 but not in lane 1 (see Fig. 5-7, left panel). There were extremely faint bands on silver-stained gels (data not shown, bands do not show up in scanned photographs), and mass-spectrometry was attempted, but identification of the proteins was not possible. The phosphorylated band observed in lanes 2-7 and not lane 8 (roughly 43 kDa) may be a co-immunoprecipitated substrate of PfCK2. It was not possible to see this band on silver-stained gels, and therefore not possible to identify this band by mass-spectrometry.

However, as stated above, the banding patterns seen in the kinase assay are suggestive of an *in vivo* association of the PfCK2 subunits, or at least that the PfCK2 beta subunits complex with a kinase (or kinases) with similar substrate preferences to those of PfCK2α.

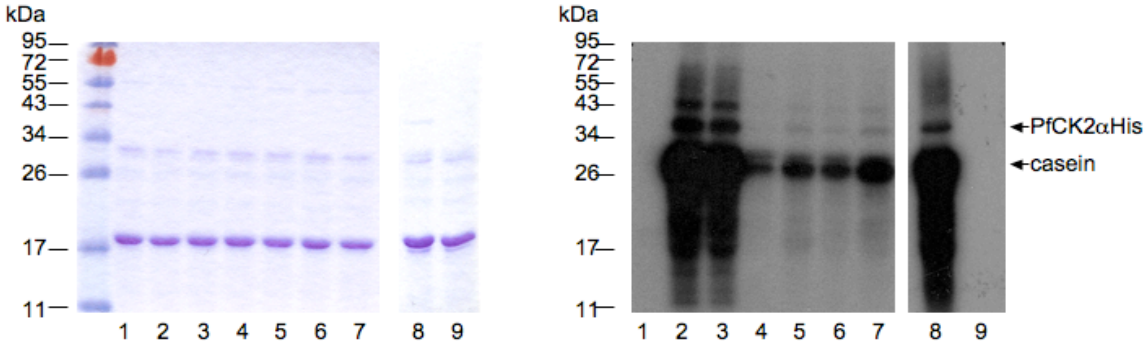

Figure 5-7 Co-immunoprecipitation of protein complexes containing HA-tagged PfCK2 subunits
Immunoprecipitations were carried out using the anti-HA antibody on 200μg of protein extract prepared from the following parasite lines: lane 1: Wild type 3D7; lane 2: HA-tagged PfCK2α, clone B3; lane 3: HA-tagged PfCK2α, clone E1; lane 4: HA-tagged PfCK2β1, clone C9; lane 5: HA-tagged PfCK2β1, clone E8; lane 6: HA-tagged PfCK2β2, clone D5; lane 7: HA-tagged PfCK2β2, clone E2. Kinase assays were performed on the resulting beads, using casein, MBP and histone H1 as substrates. Lanes 8 and 9 contain the control reactions for the kinase assay. These control reactions contained the same set of substrates as lanes 1-7, and recombinant PfCK2αHis (lane 8) or buffer (lane 9). Left panel: Coomassie-stained gel of the kinase reactions. Right panel: autoradiogram of the kinase assays.

5.7.2 Functional significance of the subunit interactions

One of the proposed functions of the CK2β subunit is alteration of the substrate specificity of CK2. To investigate whether the PfCK2β subunits can alter the substrate specificity of the catalytic subunit, we performed kinase assays with calmodulin and β-casein. Calmodulin is a confirmed *in vivo* substrate for CK2 (Meggio and Pinna, 2003, Pinna, 1990). It is a substrate for the free catalytic subunit, but there is little or no phosphorylation

of calmodulin by the CK2 holoenzyme (Benaim and Villalobo, 2002, Marin et al., 1999a, Meggio et al., 1987, Meggio et al., 1992a). It is the N-terminal acidic region of the CK2β subunit (residues 55-70 of HsCK2β) that hinders the phosphorylation of calmodulin by the holoenzyme (Meggio et al., 1994a). It had been thought that this was due to an interaction with a basic stretch of HsCK2α (residues 66-86), but the holoenzyme structure seems to refute this hypothesis (Meggio et al., 1994a, Niefind et al., 2001). The basic stretch is conserved in PfCK2α (see Fig. 4-1), and there are equivalent acidic stretches in both PfCK2β subunits, although the actual sequence conservation is not high (Fig. 5-1). Casein is the classic substrate for CK2, and gave the enzyme its name (CK2 is an acronym for 'casein kinase two'). We performed kinase assays (i) to determine whether calmodulin and casein are substrates for PfCK2α, and (ii) if so, ascertain whether the presence of the PfCK2β subunits significantly alters the phosphorylation of calmodulin and casein by PfCK2α. This was not found to be the case for calmodulin (Fig. 5-8A). Each time this experiment was repeated, the amount of radiolabel incorporated into calmodulin in the presence of the beta subunits was the same as, or slightly less than, the amount incorporated into calmodulin in the absence of the beta subunits. However, the presence of either PfCK2β subunit reduces the activity of PfCK2α towards β-casein (Fig. 5-8B). Such modulation of CK2 activity has been seen for other CK2α/β subunits, often in a substrate-dependent fashion; for example CK2β stimulates HsCK2 activity towards topoisomerase II and p53, and as mentioned above, inhibits activity towards calmodulin (Bibby and Litchfield, 2005). The presence of two CK2β subunits in *P. falciparum* (whereas there is only one in human cells) is likely to allow fine control of the activity of the catalytic subunit. Experiments on a wider range of substrates are needed to show whether the two PfCK2β subunits differentially affect the activity of PfCK2α towards certain substrates.

The wild-type human CK2α is stimulated four- to ten-fold towards the majority of substrates by the interaction with CK2β (Romero-Oliva et al., 2003), but under our conditions, and with these substrates, PfCK2α activity is not stimulated by the interaction with the beta subunits (Fig. 5-8). This could be explained by differences in the primary sequences of human and *P. falciparum* CK2α: a Val66Ala HsCK2α mutant was not stimulated by interaction with CK2β (Jakobi and Traugh, 1992), and PfCK2α has an alanine at the equivalent position.

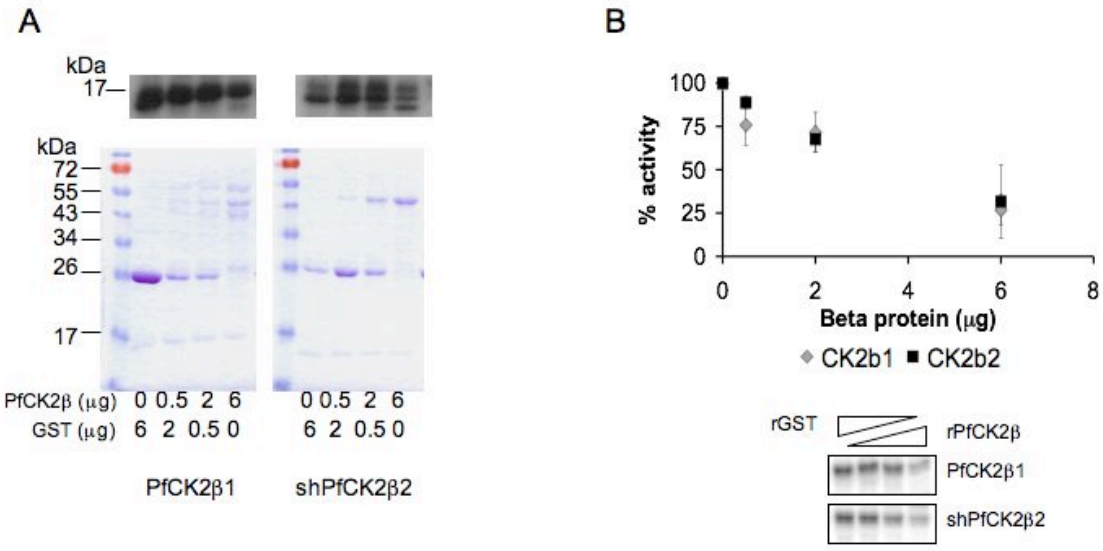

Figure 5-8 Functional significance of the subunit interactions
Panel A: Phosphorylation of calmodulin by PfCK2αHis is not greatly altered in the presence of increasing concentrations of GST-PfCK2β1 or GST-shPfCK2β2. Top panel: autoradiogram, bottom panel: corresponding Coomassie-stained gel of the kinase assay. Standard kinase assays were performed with 5μg of calmodulin, 1μg of PfCK2αHis, and 6μg of GST/GST-PfCK2β, in varying proportions. Panel B: Phosphorylation of β-casein by PfCK2αHis decreases in the presence of increasing concentrations of GST-PfCK2β1 or GST-shPfCK2β2. Top panel: Combined results of three experiments (means and standard deviations shown). Phosphorylation of β-casein by PfCK2αHis in the presence of increasing concentrations of GST-tagged PfCK2β subunits (and concomitant decreases in the concentration of GST) was quantified by phosphorimaging. Bottom panel: Autoradiograms from one of the kinase assays, showing decreased incorporation of the radiolabel into β-casein with increasing ratio of GST-PfCK2β:GST present in the reaction. Standard kinase assays were performed with 1μg of β-casein, 1μg of PfCK2αHis, and 6μg of GST/GST-PfCK2β, in varying proportions.

The association of the PfCK2β subunits with the PfCK2α subunit does not affect the K_m of the enzyme for ATP (PfCK2α alone: 17.5 μM; PfCK2α/PfCK2β1: 21.3 μM; PfCK2α/shPfCK2β2: 14.1 μM). Although the rat CK2 holoenzyme was over 5 fold more active against the peptide RRRDDDSDDD than the catalytic subunit alone (Meggio et al., 1992a), the PfCK2β subunits did not have a significant effect on the phosphorylation by PfCK2α of the similar NEB peptide RRRADDSDDDDD (data not shown). An important point to note, however, is that we do not know whether the recombinant PfCK2β subunits are correctly folded. Circular dichroism experiments, with the HsCK2β subunit for comparison, may be informative in respect to this question.

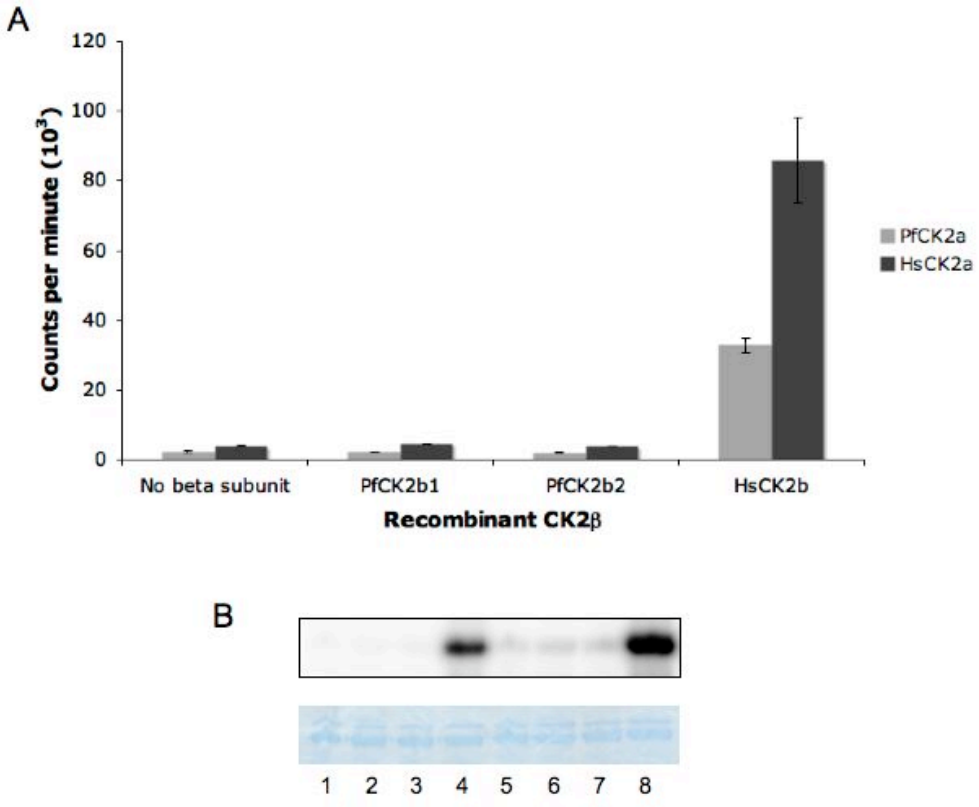

Figure 5-9 CK2β-dependent phosphorylation of eIF2β[1-22] and Olig2[1-177]
Panel A: Phosphorylation of the eIF2β[1-22] peptide (Salvi et al., 2006) by PfCK2αHis or HsCK2α in the presence and absence of GST-PfCK2β1, GST-shPfCK2β2, or HsCK2β was analysed by kinase assay, and the amount of radiolabel incorporated into the peptide counted by scintillation. Results are shown as means of two experiments, with the error bars representing the standard deviations. Panel B: Phosphorylation of the GST-Olig2[1-177] protein (Laudet et al., 2007) by PfCK2αHis (lanes 1-4) or HsCK2α (lanes 5-8) alone (1,5) or in the presence of GST-PfCK2β1 (2,6), GST-shPfCK2β2 (3,7), or HsCK2β (4,8). Top panel: autoradiogram. Bottom panel: corresponding Coomassie-stained gel of the kinase assay.

A new tool has been developed for distinguishing the activity of the catalytic subunit of CK2 from that of the holoenzyme. In contrast to what is observed with calmodulin, the eukaryotic translation-initiation factor 2 beta (eIF2β) is phosphorylated by the CK2 holoenzyme, but not by the free catalytic subunit (Llorens et al., 2003). A peptide derived from eIF2β has been produced (eIF2β[1-22], MSGDEMIFDPTMSKKKKKKKKP) that is also phosphorylated only in the presence of the beta subunit (Salvi et al., 2006). This peptide was used to assess the interaction of human and *P. falciparum* CK2 subunits (Fig. 5-9). Kinase assays were performed on the eIF2β peptide by 30ng of CK2α subunit with or without 30ng of CK2β subunit. While the HsCK2β was able to recruit CK2α from both species to the peptide for effective phosphorylation (Fig. 5-9A), neither of the PfCK2β subunits was able to do so. Since we have already shown by pulldowns (Fig. 5-6A&B) that the PfCK2 subunits interact *in vitro*, this lack of phosphorylation is likely to be due the PfCK2β subunits being unable to recognize this particular substrate. The inability of the PfCK2β subunits to recruit the CK2α subunits to the eIF2β peptide may be explained by a

recent study (Poletto et al., 2008), in which both of the HsCK2β mutants DLE_{57} to AAA and R47A had vastly reduced ability to recruit the CK2α subunit to phosphorylate the peptide. This DLE motif and arginine residue are lacking from the equivalent positions in the PfCK2β1 and PfCK2β2 subunits. The N-terminus of the *P. falciparum* eIF2β orthologue (PF10_0103) does not resemble the N-terminus of human eIF2β (which includes the peptide used in this assay, eIF2β[1-22]), and does not possess phosphorylatable residues that conform to the CK2 consensus sequence. Therefore it is unlikely that in live parasites the PfCK2β subunits bring PfCK2α to PfeIF2β in order to phosphorylate it in an analogous manner to that seen with HsCK2α and HseIF2β. However, the ability of HsCK2β to recruit PfCK2α to phosphorylate the eIF2β[1-22] peptide indicates that although the human and *P. falciparum* CK2 beta subunits are very different in primary structure, there is functional conservation in ability to form complexes with alpha subunits.

These findings are reinforced by a similar experiment involving the GST-Olig2[1-177] protein (Laudet et al., 2007), kindly provided to C. Cochet's laboratory by Thierry Buchou (iRTSV/LTS). As is the case for eIF2β, Olig2 (oligodendrocyte lineage transcription factor 2) is phosphorylated by the CK2 holoenzyme, not the free CK2α subunit (Thierry Buchou (Laudet et al., 2007)). The Olig2 protein was used in a similar experiment to that described above for the eIF2β peptide, to assess the interaction between the CK2 subunits. Standard kinase assays were performed with 2.7μg of GST-Olig2[1-177] protein, and 30ng of CK2α (alone or with 30ng of CK2β subunit). Kinase assays were performed under linear kinetic conditions, and terminated by the addition of Laemmli buffer. The proteins were separated on an acrylamide gel, which was stained with Coomassie Blue (Fig. 5-9B, lower panel) and exposed to film (Fig. 5-9B, upper panel). While the HsCK2β was able to recruit CK2α from either species to the Olig2 protein for effective phosphorylation, neither of the PfCK2β subunits was able to do so (Fig. 5-9B). Since we have shown that both CK2α can phosphorylate the protein, and that the CK2α interact with the PfCK2β subunits (Fig. 5-6, data not shown for HsCK2α, but similar to that shown for PfCK2α in Fig. 5-6C), the likely explanation for the observation that the PfCK2β subunits are unable to recruit the CK2α subunits to phosphorylate the Olig2 protein is that they are unable to recognise the substrate.

These results demonstrate that the PfCK2α subunit is a functional CK2α subunit, able to interact with an established CK2β subunit and phosphorylate known CK2 substrates.

5.8 Summary

- *Plasmodium falciparum* possesses two CK2 beta subunits, which are very different from each other in primary structure.

- One of the subunits (PfCK2β2) exhibits several unusual features: a long, repetitive and acidic N-terminal extension, and an insertion region within the conserved CK2β domain.

- Both subunits are expressed in erythrocytic stage parasites. This confirms the proteomic data available for PfCK2β1, and provides new information on PfCK2β2, for which no proteomic data were available.

- Both subunits can be expressed as tagged recombinant proteins, but the N-terminal extension of PfCK2β2 must be removed before it can be successfully expressed, and the PfCK2β1 subunit cannot be expressed with a His-tag.

- Both PfCK2β subunits are able to interact *in vitro* with PfCK2α and HsCK2α.

- The interaction of PfCK2β with PfCK2α does not alter the K_m of the enzyme for ATP, or the activity against calmodulin or the artificial NEB peptide substrate, though it does reduce the activity of PfCK2α against β-casein, suggesting a role for the PfCK2β subunits in substrate discrimination.

6 Discussion

This thesis describes the initial characterisation of the three *Plasmodium falciparum* CK2 subunit orthologues. PfCK2α looks like and behaves like a CK2α orthologue: it possesses conserved CK2 motifs (section 4.2), it is essential for parasite viability (section 3.3), it associates with both the *P. falciparum* and *H. sapiens* CK2β subunits (section 5.7), it autophosphorylates on both the PfCK2α and PfCK2β2 subunits (Fig. 4-7), it phosphorylates exogenous substrates at sites surrounded by acidic residues (section 4.5), it is able to use GTP as well as ATP as a co-substrate (Fig. 4-8), and it is inhibited by the classical CK2 inhibitor TBB (Fig. 4-11), with a similar IC_{50} value to that of TBB on HsCK2α.

PfCK2β1 and PfCK2β2 look like and behave like CK2β orthologues: they contain the conserved CK2β motifs (section 5.2), they associate with both the *P. falciparum* and *H. sapiens* CK2α subunits (section 5.7), and they are able to modulate the activity of the PfCK2α subunit (towards β-casein, section 5.7.2).

However, these conserved features and characteristics do not allow us to assume that the lists of functions and protein-protein interactions established for CK2 orthologues in other organisms will be conserved in *P. falciparum*. A number of wide-ranging studies have analysed the substrates of CK2, mainly in mammalian and yeast cells. An analysis of the published literature in 2003 identified 307 substrates for CK2 (Meggio and Pinna, 2003). The largest groupings of substrates were: transcription factors (60); nuclear proteins involved in gene expression or transcription (>40); and other signalling proteins (>80, including 10 protein kinases and 8 protein phosphatases) (Meggio and Pinna, 2003). That CK2 is highly involved in transcription is corroborated by systematic studies of yeast protein complexes (Gavin et al., 2002), which identified seven complexes in which two or more CK2 subunits were found; four of these were involved in transcription or DNA maintenance or chromatin structure (the other three were involved in RNA metabolism, protein/RNA transport, and signalling). An analysis of the protein-protein interactions of *S. cerevisiae* CK2 subunits present on the BioGRID repository also found that "the majority of the [CK2-] interacting proteins were related to nucleic acid synthesis and processing" (Gyenis and Litchfield, 2008). Many of the proteins listed in these studies have orthologues in *P. falciparum*; however, as was demonstrated for the PfMyb1, PfHMGB1 and PfNAP-B7 proteins (Fig. 4-7C), this does not necessarily mean they will be good substrates for PfCK2. *P. falciparum* has a divergent kinome (section 1.5.2), and a very

different lifecycle to the two main organisms in which CK2 is studied (*S. cerevisiae* and *H. sapiens*), and is therefore likely to organise its protein signalling pathways and mechanisms of regulation in different ways to those organisms. It would not be unexpected for PfCK2 to diverge in its list of cellular substrates from those published for HsCK2 and ScCK2.

A start has been made in identifying putative substrates for PfCK2: several proteins were phosphorylated in *P. falciparum* extract (Fig. 4-6), but these proteins have not been identified. Tools have been produced that will be invaluable in the identification of substrates: clonal parasite lines that have incorporated an HA tag at the 3' end of the PfCK2 subunit genes. Immunoprecipitations of the HA-tagged subunits (Fig. 5-7) followed by kinase assays revealed the presence of a phosphorylated band not present in the kinase assay controls, which may be a co-immunoprecipitated substrate. These experiments need to be repeated: the kinase assay following the immunoprecipitation should be repeated without the addition of exogenous substrates, to clarify whether the band mentioned above is from the immunoprecipitated material; and the immunoprecipitations should be repeated using a kit that allows immobilization of the antibody (e.g. PIERCE ProFound^{TM} Co-Immunoprecipitation kit) so that there is a minimal amount of this antibody present in the eluted samples, allowing for subsequent identification of the immunoprecipitated proteins by western blot without this antibody masking the signals. The amount of parasite material used in the immunoprecipitations should be increased, so that interacting partners can also be identified by mass-spectrometry.

A yeast-two-hybrid study of *P. falciparum* protein-protein interactions has been published (LaCount et al., 2005). This study identified one partner for PfCK2α, PFF0220w, whose function has not been investigated. However, it was mapped in an interaction network with proteins involved in chromatin metabolism and transcription (LaCount et al., 2005), which fits with the studies mentioned above that demonstrate an important role for CK2 in transcription and the structure of DNA. PfCK2β2 (base pairs 2-1024 as the bait fragment) interacted with 84 prey fragments, and was therefore excluded from the analysis as a 'promiscuous fragment'. However, these interactions might not necessarily represent false positives, given that over 300 substrates had been identified for CK2 in 2003 (with more characterised since). Therefore it is a possibility that PfCK2β2 could interact with 84 proteins, and perhaps these data should not have been excluded from the database. Another fragment of the PfCK2β2 gene (base pairs 259-617) was included in the study as a prey fragment; it interacted with PF07_0043, annotated as 60s ribosomal protein L34a

(putative) on PlasmoDB. No partners were reported for PfCK2β1, but since only a subset of the *P. falciparum* genome was examined (1,295 genes expressed in erythrocytic stage parasites), it is likely that it was not included in the analysis. This study did not find interactions between the PfCK2 subunits. However the alpha-beta interaction involves the C-terminus of the beta subunit as well as the central region (Niefind et al., 2001), and the PfCK2β2 fragment used in the study excluded the C-terminus. It was demonstrated in Chapter 5 that the PfCK2α and PfCK2β subunits can interact *in vitro*, and showed some preliminary evidence (Fig. 5-7, immunoprecipitations) that they may also interact in parasite extract. Further immunoprecipitations using immobilised antibody, and followed by western blots, should be performed to reveal whether the PfCK2α/β subunits associate in parasite extract.

The two PfCK2β subunits are both essential for parasite viability (sections 3.4&5). This is unexpected given the dispensability of the beta subunits for viability in the single celled organisms *S. cerevisiae* (which has two CK2β subunits that can be knocked out individually or simultaneously (Ackermann et al., 2001, Bidwai et al., 1995, Reed et al., 1994)) and *Schizosaccharomyces pombe* (which had a single beta subunit) (Roussou and Draetta, 1994). The single beta subunit present in *Caenorhabditis elegans* (Fraser et al., 2000) and in mice (Buchou et al., 2003) is essential. Organisms that possess more than one gene coding for a CK2α subunit display some degree of redundancy between these genes. For example, knockout of CK2α2 in mice is well tolerated, indicating that CK2α1 can functionally compensate for CK2α2 (except in male spermatogenesis) (Escalier et al., 2003, Xu et al., 1999). The two genes encoding the CK2 catalytic subunits of *Saccharomyces cerevisiae* (CKA1 and CKA2) can be individually disrupted, indicating functional compensation, but disruption of both simultaneously is lethal (Chen-Wu et al., 1988, Padmanabha et al., 1990). Putting these data together, we had hypothesized that either (i) both PfCK2β subunits would be dispensable for parasite viability, as is the case for *S. cerevisiae*, or (ii) the presence of one beta subunit may be essential to the parasite, in a manner analogous to the catalytic subunits in mice or *S. cerevisiae*, in which case at least one of the two PfCK2β subunits could be disrupted. However, our data strongly suggest that both CK2β subunits are essential for *P. falciparum* viability, indicating that they have non-redundant functions in the parasite. The differing interactomes for the CK2β subunits in *S. cerevisiae* (Gyenis and Litchfield, 2008) is consistent with functional specialisation. However, as mentioned above, the individual CK2β subunits in *S. cerevisiae* are not individually (or corporately) essential for viability, so the importance of these non-overlapping areas of function is questionable, and may just represent the limitation in our

knowledge of interaction data. The PfCK2 beta subunits cannot functionally compensate for each other: they have non-redundant functions. It is therefore of interest to investigate the different properties and functions of the CK2 holoenzymes built around PfCK2β1/β1, PfCK2β2/β2 and PfCK2β1/β2 dimers, and to clarify whether all three potential holoenzymes are present in the parasite. Potential experiments to address this issue are size-exclusion chromatography of parasite extract, followed by dot-blots of the eluted fractions, or western blots of anti-HA immunoprecipitated material from tagged parasite lines.

Many intriguing hypotheses can be constructed regarding the reasons for the essentiality of both CK2β subunits in *P. falciparum*. CK2β subunits function as docking stations for CK2 substrates, as modulators of CK2 activity, and they interact with scaffold proteins and regulatory proteins. So perhaps some substrates can only be phosphorylated by CK2 containing the PfCK2β1 subunit, and others can only be phosphorylated by CK2 containing the PfCK2β2 subunit. The PfCK2β subunits might differentially regulate PfCK2 activity towards some substrates. I did not observe differences in effects of the two PfCK2β subunits on activity towards calmodulin, β-casein, and peptide substrates, but this is a very limited set of non-physiological substrates, and the investigations were performed *in vitro*, whereas *in vivo* there may be other factors that mediate a differential regulation via the PfCK2β subunits. The PfCK2β2 subunit has an insertion of acidic amino acids in the acidic region of the conserved CK2β domain known to downregulate CK2α (Fig. 5.1) (Meggio et al., 1994a), lending weight to the differential-regulation hypothesis. The divergence in primary structure between the two PfCK2β subunits, along with the inability of the parasites to survive without either of them, leads to the conclusions that the PfCK2β subunits are likely to have different sets of interacting partners, as has been shown for the *S. cerevisiae* CK2 beta subunits (Gyenis and Litchfield, 2008). These different interacting partners could differentially localise the PfCK2β to different cellular compartments, allowing for subcellular regulation of CK2 activity. Immunofluorescence studies could be performed to support or refute this hypothesis, and would also be informative in assessing the possible functions of PfCK2: differential localisation of the alpha and beta subunits may point to CK2-independent subunit functions. There has been increasing interest in the CK2-independent functions of both CK2 subunits in recent years (see section 1.6.2.7). A very interesting hypothesis is that the PfCK2β subunits are each essential due to functions entirely independent of CK2, perhaps through their regulation of different protein kinases. Since we have a number of recombinant *P. falciparum* kinases available in the laboratory, an interesting experiment would be to determine whether the PfCK2β subunits are able to

modulate the *in vitro* activity or substrate selectivity of other *P. falciparum* kinases. Successful identification by mass spectrometry of binding partners for the PfCK2 subunits in parasite extracts (see section 5.7.1) would likewise be very informative in this respect.

Many questions remain unanswered regarding the function and protein-protein interaction partners of PfCK2. It is difficult to study the function of essential genes in *P. falciparum*, because it is a haploid parasite, and lacks the machinery required for implementation of RNAi strategies (Ullu et al., 2004). An attempt to selectively knock out the function of PfCK2α using the analogue-sensitive kinase allele (ASKA) method was initiated. The ASKA technique, otherwise known as chemical genetics, involves producing a mutant version of the kinase of interest that has a smaller-than-usual residue at the entrance to the catalytic cleft, which is normally guarded by a bulky gatekeeper residue (Bishop et al., 2001, Bishop et al., 2000). This modified kinase is introduced into the cell by gene replacement. The modified kinase alone amongst cellular kinases is sensitive to a bulky kinase inhibitor analogue, allowing selective inhibition. The mutated PfCK2α was produced as a recombinant protein, but was found to be lacking the desired biochemical properties *in vitro* (i.e. it lacked kinase activity, even when tested with high concentrations of ATP), and therefore the *in vivo* experiments were abandoned. A novel technique allowing the regulation of cellular levels of a particular protein by fusion to a destabilisation domain (Banaszynski et al., 2006) has been adapted for use in *P. falciparum* (Armstrong and Goldberg, 2007). The fusion of an engineered form of human FKBP12 to the N- or C-terminal end of a protein mediates its degradation. The protein is protected from degradation by an FKBP ligand, allowing fine control of protein levels. Armstrong and Goldberg have kindly made the plasmids developed for this technique available to our laboratory, so we now have more tools to aid the discovery of the cellular functions of PfCK2.

The first requisite of a potential antimalarial drug target is that it is essential for parasite viability/survival, so that targeting the protein with an inhibitor will lead to the death of the parasite. The PfCK2 subunits have fulfilled this criterion (Chapter 3). A potential antimalarial drug target must also be distinguishable from the human orthologue by small molecule inhibitors. One example of a small molecule inhibitor with a much lower IC_{50} for recombinant PfCK2α than for recombinant HsCK2α (Rottlerin, see Fig. 4-14) has been discovered. As discussed in Chapter 4, Rottlerin is unlikely to represent a starting molecule for drug discovery, but it provides proof of principle that differential inhibition is possible. The attractions of kinases as drug targets have already been discussed (see section 1.5.1). The peculiarities of the binding site of CK2 that allow it to use GTP as well as ATP as co-

substrate may reveal a promising way to develop inhibitors that do not interfere with ATP-dependent protein kinases (Niefind et al., 1999).

Hexadecyltrimethylammonium bromide (HDTAB) has recently been shown to be a potent inhibitor of *P. falciparum* choline kinase and of *P. falciparum* proliferation *in vitro*, while having no affect on the viability of red blood cells (Choubey et al., 2007, Leroy and Doerig, 2008). These results suggest that selectivity of small molecule inhibitors against *P. falciparum* kinases is achievable, although in this case the target is not a protein kinase. Compound 1 and *Eimeria* parasites provide an example of the ability to selectively inhibit a parasite protein kinase that has a host orthologue. *Eimeria* is an Apicomplexan parasite that infects chickens and is a major problem in the poultry industry. Compound 1 was identified as an *in vitro* and *in vivo* inhibitor of *Eimeria*, with cGMP-dependent protein kinase (PKG) being identified as the target (Gurnett et al., 2002, Donald et al., 2002). Chicken and bovine PKG are insensitive to Compound 1 (Wiersma et al., 2004). This differential effect was shown by mutagenesis to be due to T770 (Donald et al., 2002); chicken PKG has a methionine at this position, whose bulk interferes with the binding of Compound 1. A recent study of *P. falciparum* cAMP-dependent protein kinase (PfPKA) provides another example of the ability of small molecule inhibitors to distinguish between parasite and host kinase orthologues: the PfPKA catalytic subunit (PfPKA-C) was only weakly inhibited by the PKA-C inhibitor H89, compared with the inhibition of mammalian PKA-C (Sudo et al., 2008). Of course, anti-cancer drugs that target PKs face the issue of targeting of healthy cells, and this has been overcome: there are anti-cancer drugs in clinical use whose targets are PKs (see section 1.5.1). Treatment of severe malaria can only take a couple of days, therefore toxicity is not so much of a problem as for cancer chemotherapies, though of course, antimalarial prophylactics must have a very low toxicity (Doerig and Meijer, 2007).

The rise of resistance in *P. falciparum* to many of the available antimalarials (see section 1.4) means that consideration of the ease of developing resistance must be borne in mind when considering potential drug targets. Drug resistance due to single amino acid substitutions in the target protein can be a problem with drugs whose targets are protein kinases. In the Compound 1/*Eimeria* example above, mutational analysis of the parasite PKG revealed that a T770M mutation would allow the parasite to survive Compound 1 treatment (Donald et al., 2002). The drug Gleevec® is used to treat chronic myelogenous leukaemia (CML), and its target is Abelson tyrosine kinase (Abl). Point mutations in Abl give rise to Gleevec®-resistance, and cause relapse in patients with advanced CML (Gorre et al., 2001, Shah et al., 2002). Any inhibitor compound which can be rendered ineffective

by one amino acid substitution in the target protein is unlikely to last long as an effective antimalarial drug. Targeting multiple pathways by combined therapy (as is already recommended for ACTs, see section 1.4.3) is expected to hamper the emergence of resistance phenotypes (Zimmermann et al., 2007). A possible approach to targeting PfCK2 for antimalarial chemotherapy might be to target the interaction between the alpha and beta subunits, since we know that both beta subunits have essential functions in asexual blood stage parasites (Chapter 3). Targeting a region of the PfCK2β subunit that interacts with CK2α would circumvent the issue often raised with drugs based on kinase inhibition: off target effects due to the similarity of kinase active sites. Allosteric inhibition is becoming a matter of great interest, and inhibitors based on allosteric inhibition have been successfully developed (Leroy and Doerig, 2008, Pargellis et al., 2002). Small peptide inhibitors of CK2 alpha/beta subunit interaction have already been developed, which are able to disrupt the assembly of the tetrameric CK2 holoenzyme, and affect its substrate preference (Laudet et al., 2007). CK2 beta subunits interact with cellular proteins other than CK2 alpha (see section 1.6.2.7), and if PfCK2β subunits behave in an analogous fashion, targeting the beta subunits would interrupt many protein-protein interactions, and it would be far more difficult for the parasite to develop resistance. However, our data showing that the PfCK2β subunits are able to interact with the HsCK2α subunit, and that the HsCK2β subunit is able to interact with the PfCK2α subunit (section 5.7), imply that the interaction regions are conserved between humans and *P. falciparum*, which would make selectively targeting the alpha/beta interaction of *P. falciparum* more problematic.

Given that CK2 is a promising anti-cancer target, it is hoped that compounds that may be developed in the future as putative anti-cancer drugs based on CK2-inhibition, with good pharmacokinetic and safety profiles, could be used as the starting point for anti-malarial drugs, thereby hugely reducing the overall costs of the initial phases of drug discovery.

In summary, recombinant PfCK2α possesses protein kinase activity, exhibits similar substrate and co-substrate preferences to those of CK2α subunits from other organisms, and interacts with both of the PfCK2β subunits *in vitro*. Gene disruption experiments show that the presence of each of the PfCK2 subunits is crucial to asexual blood stage parasites, and thereby validate the enzyme as a possible drug target. PfCK2α is amenable to inhibitor screening, and we report differential susceptibility between the human and *P. falciparum* CK2α enzymes to a small molecule inhibitor. Taken together, my data indicate that PfCK2 is an attractive, validated target for antimalarial chemotherapeutic intervention.

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Appendix

Appendix 1: Plasmid maps for reverse genetics studies

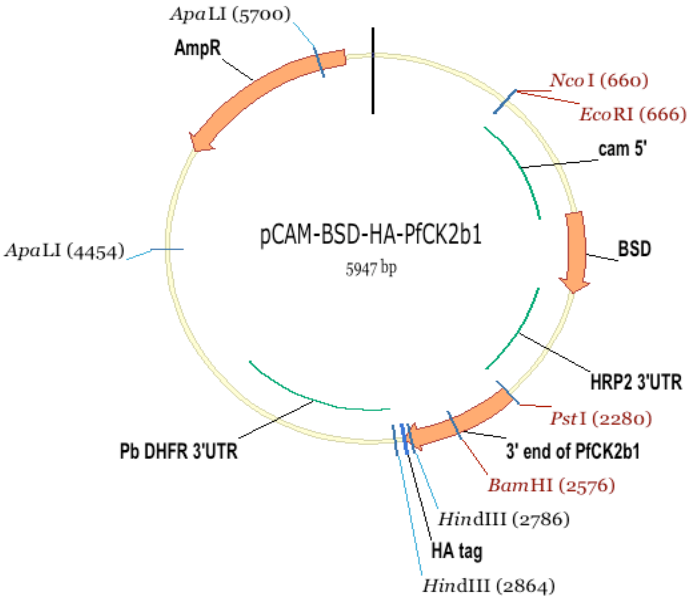

Figure A-1 Map of the pCAM-BSD-HA-PfCK2b1 plasmid.
The plasmid differs from pCAM-BSD-HA-PfCK2a (Fig 3-1) only in the inserted sequence, which is the 3' end of the *PfCK2β1* gene instead of the 3' end of the *PfCK2α* gene. Restriction enzyme sites in black are for the enzymes that have multiple sites in the plasmid, and those shown in red are for the enzymes that will only cut the plasmid in one location. Figure produced using Vector NTI software (Invitrogen).

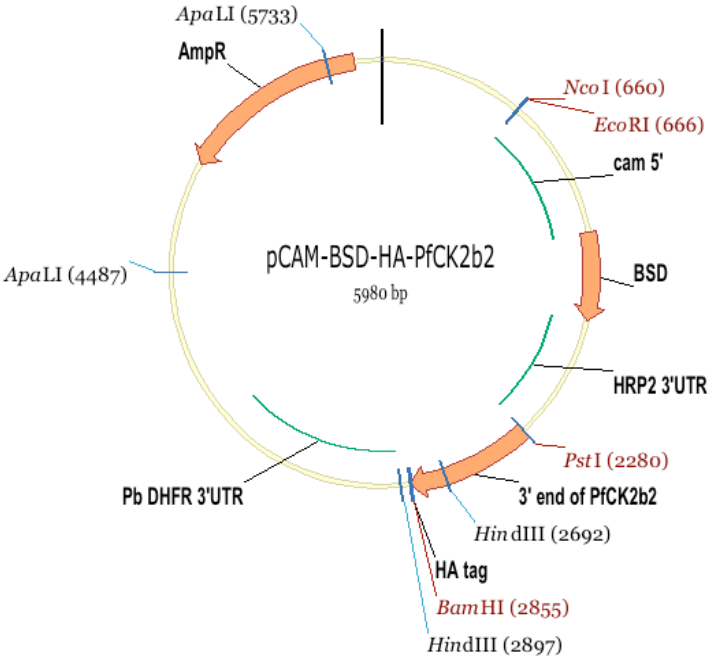

Figure A-2 Map of the pCAM-BSD-HA-PfCK2b2 plasmid.
The plasmid differs from pCAM-BSD-HA-PfCK2a (Fig 3-1) only in the inserted sequence, which is the 3' end of the *PfCK2β2* gene instead of the 3' end of the *PfCK2α* gene. Restriction sites in black are for the enzymes that have multiple sites in the plasmid, and those shown in red are for the enzymes that will only cut the plasmid in one location. Figure produced using Vector NTI software (Invitrogen).

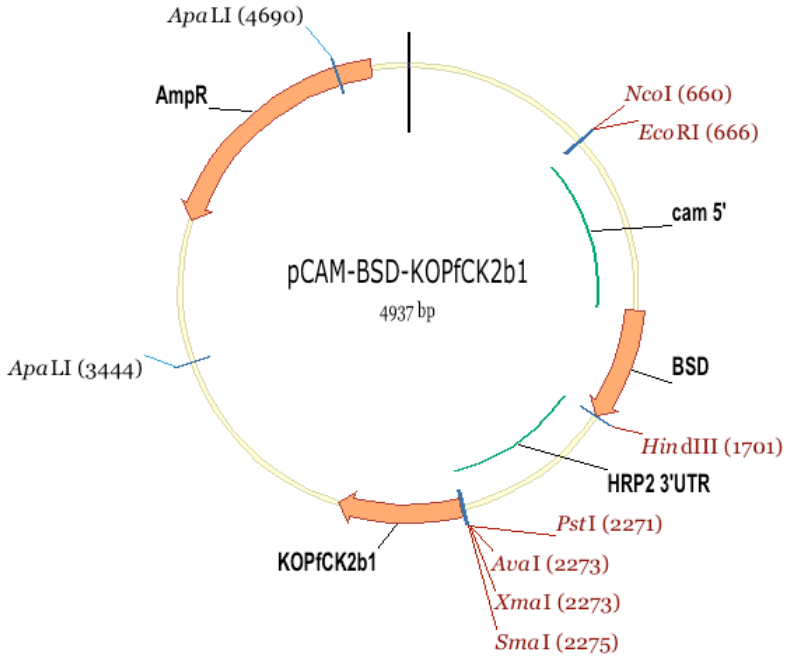

Figure A-3 Map of the pCAM-BSD-KOPfCK2b1 plasmid.
This figure shows the important features of the pCAM-BSD-KOPfCK2b1 plasmid. The KOPfCK2β1 fragment was inserted between the BamHI and NotI sites, and the plasmid lost the BamHI site in the cloning process (the fragment had a BglII-cut 5' end, which was ligated to the BamHI-cut end of the plasmid, resulting in an asymmetrical site recognised by neither BamHI or BglII). Several of the restriction enzyme sites are shown. Those in black are for the enzymes that have multiple sites in the plasmid, and those shown in red are for the enzymes that will only cut the plasmid in one location. Figure produced using Vector NTI software (Invitrogen).

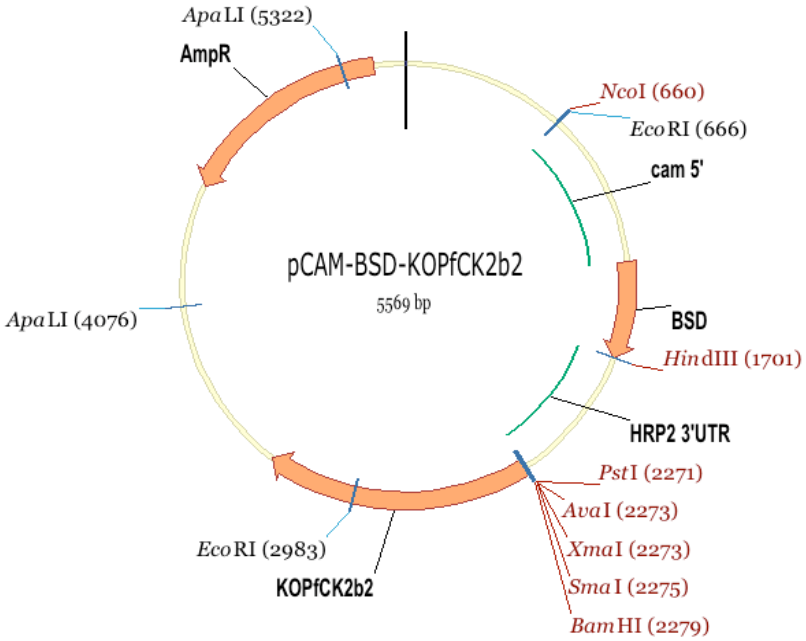

Figure A-4 Map of the pCAM-BSD-KOPfCK2b2 plasmid.
This figure shows the important features of the plasmid pCAM-BSD-KOPfCK2b2. The KOPfCK2β2 fragment was inserted between the BamHI and NotI sites. Several of the restriction enzyme sites are shown. Those in black are for the enzymes that have multiple sites in the plasmid, and those shown in red are for the enzymes that will only cut the plasmid in one location. Figure produced using Vector NTI software (Invitrogen).

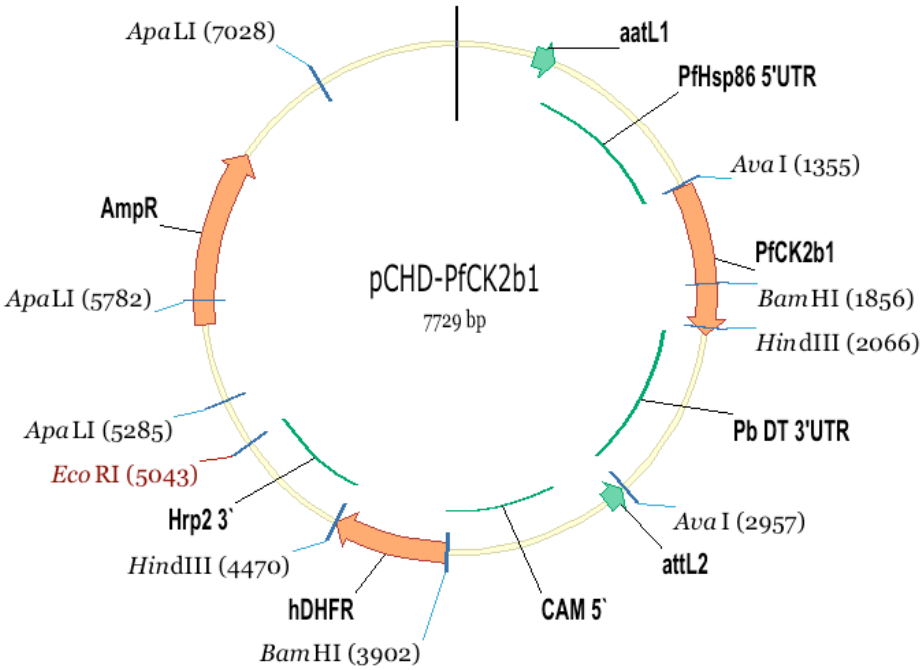

Figure A-5 Map of the pCHD-PfCK2b1 plasmid.
This figure shows the important features of the plasmid pCHD-PfCK2b1. The BamHI site of the expression cassette was lost when the NotI/BglII-cut *PfCK2β1* was cloned into the NotI/BamHI sites. Other features are as Fig 3-3. Figure produced using Vector NTI software (Invitrogen).

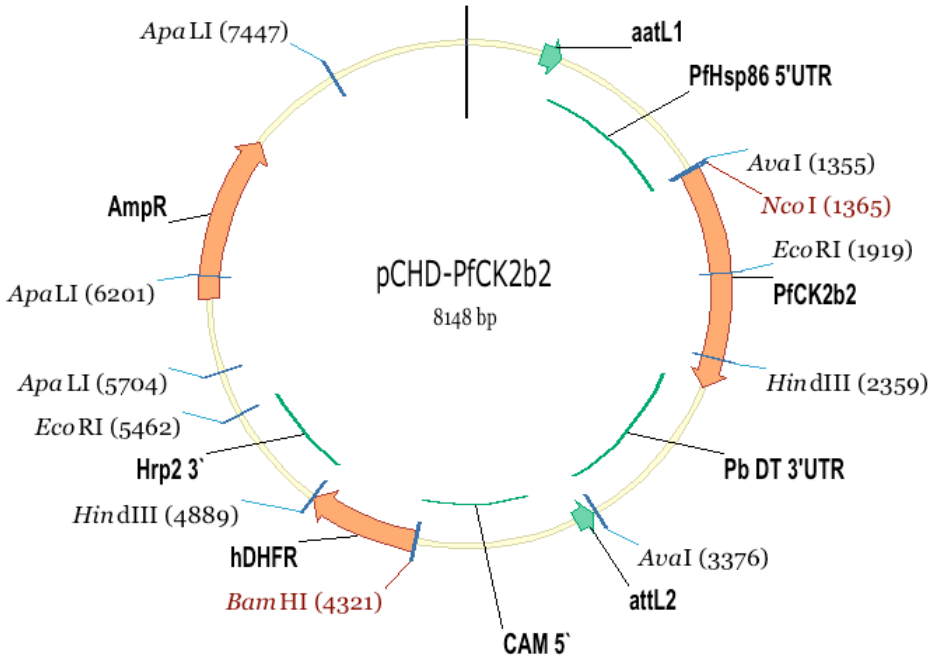

Figure A-6 Map of the pCHD-PfCK2b2 plasmid.
This figure shows the important features of the plasmid pCHD-PfCK2b2. The plasmid contains an ampicillin resistance cassette for selection in *E. coli*, and a human dihydrofolate reductase cassette for selection in *P. falciparum*. Other features as Fig 3-3. Figure produced using Vector NTI software (Invitrogen).

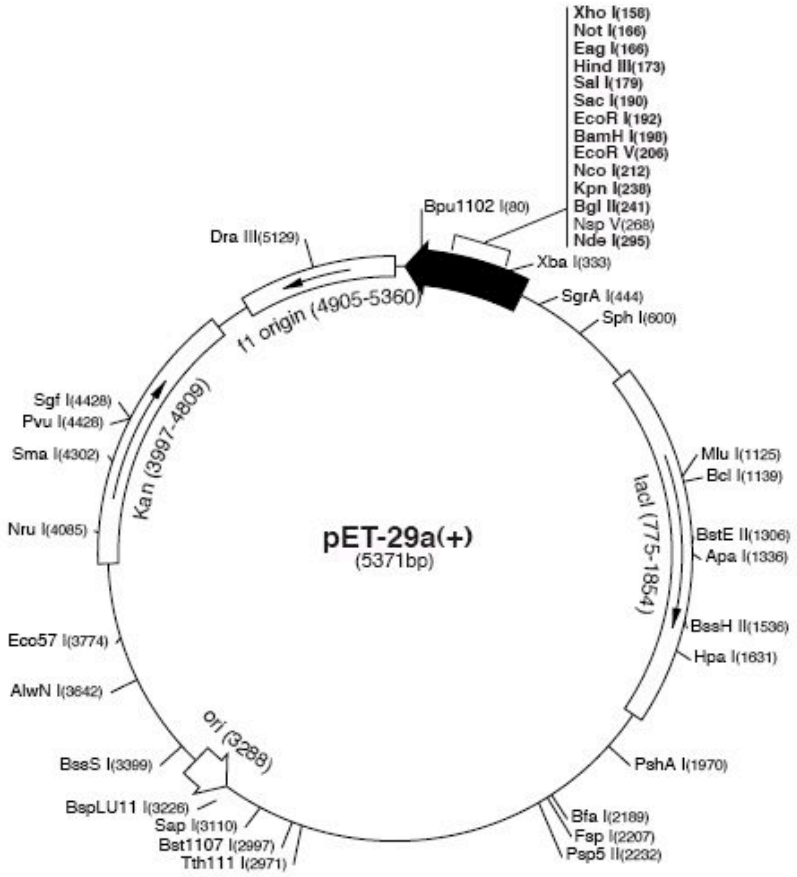

Figure A-7 Map of the pET-29 plasmid.
The plasmid contains a kanamycin resistance cassette for selection in *E. coli*. Figure from the Novagen website (www.merckbiosciences.co.uk).

Appendix 2: Compounds from BioMol library

Table A-1 Identities of compounds from Figure 4-13
The identifiers given to the compounds in Fig. 4-13 are listed in column 1, and their names or BioMol identifiers are listed in column 2. The presumptive targets of the compounds are listed in column 3.

	Compound name or ID	Target(s)
1	SB-203580	p38 MAPK
2	H-7	PKA, PKG, MLCK, and PKC
3	H-9	PKA, PKG, MLCK, and PKC
4	Staurosporine	Pan-specific
5	AG-494	EGFRK, PDGFRK
6	Lavendustin A	EGFRK
7	Tyrphostin 51	EGFRK
8	Tyrphostin 1	Negative control for tyrosine kinase inhibitors.
9	Tyrphostin AG 1288	Tyrosine kinases
10	Tyrphostin AG 1478	EGFRK
11	Tyrphostin AG 1295	Tyrosine kinases
12	Tyrphostin 9	PDGFRK
13	AG-370	PDGFRK
14	AG-879	NGFRK
15	LY 294002	PI 3-K
16	Wortmannin	PI 3-K
17	GF 109203X	PKC
18	HA-1077	PKA, PKG
19	HDBA (2-Hydroxy-5-(2,5-dihydroxybenzylamino)benzoic acid)	EGFRK, CaMK II
20	KN-62	CaMK II
21	KN-93	CaMK II
22	ML-7	MLCK
23	ML-9	MLCK
24	SB-202190	p38 MAPK
25	PP2	Src family
26	ZM 336372	cRAF
27	SU 4312	Flk1
28	AG-1296	PDGFRK
29	GW 5074	cRAF
30	Palmitoyl-DL-carnitine Cl	PKC
31	Rottlerin	PKC delta
32	Quercetin dihydrate	PI 3-K
33	SU1498	Flk1
34	ZM 449829	JAK-3
35	BAY 11-7082	IKK pathway
36	DRB (5,6-Dichloro-1-b-D-ribofuranosylbenzimidazole)	CK II
37	HBDDE (2,2',3,3',4,4'-Hexahydroxy-1,1'-biphenyl-6,6'-dimethanol dimethyl ether)	PKC alpha, PKC gamma
38	SP 600125	JNK

Appendix 3: Accepted manuscript