Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

Identification and Characterisation of an FK506-Binding Protein from *Plasmodium falciparum*

A thesis submitted for the degree of Doctor of Philosophy

by

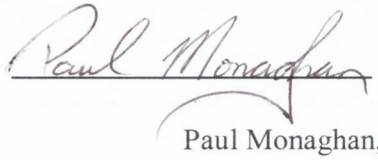
Paul Monaghan

Department of Microbiology
Moyne Institute of Preventive Medicine
Trinity College
University of Dublin

August 2004

DECLARATION

This is to certify that the experimentation recorded herein represents my own work, unless otherwise stated, and has not been submitted for higher degree at this or any other university. The thesis may be lent or copied at the discretion of the librarian, Trinity College.



Paul Monaghan,

August, 2004.

SUMMARY

Malaria remains one of the most significant diseases worldwide. The most common and severe form of the disease is caused by *Plasmodium falciparum*. The need for new anti-malarial compounds has intensified in recent years as parasites continue to develop resistance to currently used drugs.

It was previously shown that FK506 and rapamycin, drugs commonly used for their potent immunosuppressive activities, have activity against *P. falciparum* in culture. The pathway by which these drugs induce immunosuppression in humans is known to involve an FK506-binding protein (FKBP). Homologues of FKBPs have been identified in a wide variety of organisms and the fact that *P. falciparum* is susceptible to FK506 suggested that this organism possesses at least one member of the FKBP family.

This project was undertaken to identify homologues of FKBPs in *P. falciparum* in an attempt to understand the mechanism by which FK506 and rapamycin exert their anti-malarial effects. The immunosuppressive properties of these compounds exclude them from consideration as anti-malarials, but such studies may facilitate the design of non-immunosuppressive analogues that retain their activity against *P. falciparum*.

The first member of the FKBP family identified in *P. falciparum* was identified here by a genome sequence data-mining approach. Analysis of the derived amino acid sequence of this putative *FKBP* gene showed this 35-kDa protein, PfFKBP35, to be comprised of a single, N-terminal, FKBP domain and a C-terminal tripartite tetratricopeptide repeat domain. This is a strikingly similar modular structure to certain other FKBPs. However, PfFKBP35 differs from human FKBP12 at a number of key residues implicated in FK506- and rapamycin-induced immunosuppression. Analysis of the *P. falciparum* genome database suggested this to be the only FKBP present in the parasite.

A recombinant form of PfFKBP35, like most other FKBPs, displayed peptidyl-prolyl *cis-trans* isomerase activity that was inhibitable by FK506 and rapamycin. Unusually, the phosphatase activity of calcineurin, the target of the FK506-FKBP12 complex in T-lymphocytes, was inhibited by PfFKBP35 independently of FK506 binding. PfFKBP35 also inhibited the thermal aggregation *in vitro* of two model substrates, suggesting that it has general chaperone properties. A number of non-immunosuppressive analogues of FK506 were shown to be inhibitors of parasite

growth. While all compounds inhibited the chaperone activity of PfFKBP35, only some inhibited the PPIase activity, suggesting that the anti-malarial effects of this class of drug may be mediated via inhibition of the chaperone activity rather than the enzymatic activity of PfFKBP35. The potential for this class of drugs as anti-malarials was further suggested by the finding that two of the non-immunosuppressive compounds tested appear to inhibit the parasite protein while having no measurable affinity for homologous host proteins.

The function of PfFKBP35 remains unknown but it may play a role in protein folding or modulation of protein function, as suggested by the observed peptidyl-prolyl *cis-trans* isomerase and chaperone activities. The presence of tetratricopeptide repeat motifs is suggestive of a role in intracellular protein transport. The results reported here suggest that PfFKBP35 could serve as a novel anti-malarial drug target and that compounds based on FK506 or rapamycin may have potential as anti-malarial drugs.

"I have not failed, I've just found 10,000 ways that won't work."

- Thomas Edison

THE ROAD NOT TAKEN

Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveller, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black,
Oh, I kept the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a yellow wood, and I –
I took the one less travelled by,
And that has made all the difference.

- Robert Frost

For Mam and Dad

ACKNOWLEDGEMENTS

My time in the Moyne has been truly memorable, thanks in no small part to the wonderful people who have played a part in some form or other.

Firstly, I would like to thank Gus for his remarkable supervision, guidance and friendship. Your interminable confidence and respect was a constant source of inspiration. In my future career I would hope to be held with even a fraction of the amount of esteem and respect that I hold for you.

I have been fortunate to work with a group of people whose presence in the lab made the struggle worth it – Clare, Brian, Gerry, Julie, Eithne, Zenab and Kate. Thanks to Clare for helping me fit in from the very first day, but more importantly, for teaching me how to deal with women's problems! If my career as a scientist fails, at least I can fall back on my experience as a psyhcoanalyst! Brian - thanks for the countless hours of laughs, all at your expense! I know well that I'm going to get my come-uppance someday in Boston when I mispronounce 'bagel'! I'll have great difficulty in finding a coffee-buddy to replace you. Thanks to Gerry for providing the intellectual stimulus! I'll miss the daily chats - those in the coffee room, the bay, and the secret ones in the culture room! Particularly the secret ones in the culture room!! My decline from being an optimist to a realist, and ultimately a pessimist, is all your doing!

Thanks to all members, past and present, of the West Bunker Lab. Sorry for the years you've had to endure my singing, but at least I had a better array of songs than Brian's CD collection! A particular thanks to Mary Meehan for her time and patience in sharing her prodigious knowledge of molecular biology. Most of the work in this thesis would have been impossible without your advice and guidance. Thanks also to Dee for her help and advice, and also for letting me nick things (sssh, don't tell Tim!!).

Thanks to all those who started out on the road to PhD with me – Fred, Jamie, Ronan, Chris, Jenny and Sorcha. I couldn't have hoped for a better group of people to share the experience with. Thanks to you guys, the first two years of lab-work were a complete write-off! A special thanks goes to both Fred and Jamie. Your friendship made the Moyne years what it was. And Jamie, you're the best Valentine's date I ever had! I truly hope that we all keep in touch in the future.

A big thanks goes to the members of the prep-room – Paddy, Joe, Ronan, Dave, Margaret, Fionnuala and Henry. Your assistance and support has always been truly

appreciated. I would also like to express my gratitude to the various members of my committee. I would also like to thank Peter Revill and Kosan Biosciences for providing the analogues of FK506 that played such an integral role in the work presented in this thesis. Also, I would like to acknowledge the financial support I received from the Health Research Board and Dublin City Council.

A special thanks goes to my family and friends who haven't got a clue what I have spent the past five years doing! In particular, I thank Steph and Eoin for being there whenever I needed a pint and never once telling me to get a proper job! Eoin – you came close, but I lasted in college longer than you did! The support I have received from my mother has been truly incredible. I appreciate so much that, although you don't know what I do, you realise how important it is for me. It hasn't been an easy time for me, and I haven't made it an easy time for you. Thanks for understanding. This thesis is for you and Dad.

Blanca, your love and support has guided the way and provided the desire and inspiration to battle on. You, more than anyone, know what this means to me. I love you very much.

PUBLICATIONS ORIGINATING FROM THIS STUDY

Monaghan, P. and Bell, A. (2004) A *Plasmodium falciparum* FK506-binding protein (FKBP) with peptidyl-prolyl *cis-trans* isomerase and chaperone activities. *Mol Biochem Parasitol* (in press).

Monaghan, P., Fardis, M., Revill, W.P. and Bell, A. (2004) Anti-malarial effects of macrolactones related to FK520 (ascomycin) are independent of the immunosuppressive properties of the compounds. *J Infect Dis* (in press).

TABLE OF CONTENTS

List of Tables

Following Page

List of Figures

Following Page

Abbreviations

AAC ..... amylose affinity column

AHR ..... aryl hydrocarbon receptor

Amp ..... ampicillin

Amp^r ..... ampicillin-resistant

APAD ..... 3-acetyl pyridine adenine dinucleotide

bp ..... base pair(s)

BSA ..... bovine serum albumin

CDS ..... coding sequence

Chlor ..... chloramphenicol

$Chlor^r$ ..... chloramphenicol-resistant

CsA ..... cyclosporin A

Cyp ..... cyclophilin

dH_2O ..... deionised water

DMSO ..... dimethylsulphoxide

DTNB ..... 5, 5'-dithio-bis(2-nitrobenzoic acid)

EDTA ..... ethylenediaminetetraacetic acid

EST ..... expressed sequence tag

FKBP ..... FK506-binding protein

GR ..... glucocorticoid receptor

h ..... hour(s)

Hsp ..... heat shock protein

IC_{50} ..... median inhibitory concentration

IL-2 ..... interleukin-2

IP_3 ..... inositol 1,4,5- trisphosphate

IP_3R ..... inositol 1,4,5- trisphosphate receptor

IPTG ..... isopropyl-L-β-thiogalactopyranoside

kbp ..... kilobase pair

kDa ..... kilodalton

LDH ..... lactate dehydrogenase

MBP ..... maltose binding protein

MCAC ..... metal-chelate affinity chromatography

MCS..... multiple cloning site

min..... minute(s)

Mip..... macrophage infectivity potentiator

NBT..... nitroblue tetrazolium

NFAT..... nuclear factor of activated T-cells

OD..... optical density

ORF..... open reading-frame

PAGE..... polyacrylamide gel electrophoresis

PBS..... phosphate buffered saline

PCR..... polymerase chain reaction

PES..... phenazine ethosulphate

PMSF..... phenylmethylsulphonyl fluoride

PPIase..... peptidyl-prolyl *cis-trans* isomerase

PVDF..... polyvinylidene fluoride

rpm..... revolutions per minute

RT-PCR..... reverse transcriptase polymerase chain reaction

RyR..... ryanodine receptor

SDS..... sodium dodecyl sulphate

SDS-PAGE..... sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SSC..... saline sodium citrate

TAE..... tris-acetate-EDTA

TCA..... trichloroacetic acid

TE..... tris-EDTA

TEMED..... N, N, N', N'-tetramethyl-ethylenediamine

TF..... trigger factor

TGF-β..... transforming growth factor-β

TPR..... tetratricopeptide repeat

TVM..... tubovesicular membrane

TβR..... transforming growth factor-β receptor

Chapter 1

General Introduction

1.1. MALARIA

1.1.1. Malaria: an introduction

Despite over a century of efforts to control and eradicate malaria, it remains one of the most significant human diseases worldwide. It is estimated that approximately 40% of the world's population is at risk of contracting the disease (Gardner *et al.*, 2001), with 3-7% suffering clinical manifestations each year (Bremen, 2001). Malaria presents significant health problems to parts of Asia, the Pacific region and Central and South America, but the heaviest burden is borne by sub-Saharan Africa where close to 90% of global malaria cases occur. As many as 2.7 million people die each year from the disease, with most of these deaths occurring in Africa. Most of the victims are children under the age of 5. Excluded from these figures are the estimated 400,000 infants who die each year, not due to infection, but because they were born prematurely, and drastically underweight, to infected mothers (Bremen *et al.*, 2001). Recent studies predict that, unless new methods of control are implemented, the number of malaria cases in Africa will more than double by the year 2020, with worldwide cases easily exceeding 2 billion (Bremen, 2001). Aside from the impact on human health, the disease represents a major burden to the economic development of countries in which the disease is endemic, with a striking correlation between malaria and poverty (Sachs and Malaney, 2002; Gallup and Sachs, 2002).

Malaria in humans is caused by one of four species of protozoan parasite belonging to the genus *Plasmodium* (López-Antuñano and Schmunis, 1993): *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. These four organisms are obligate intracellular parasites whose life cycle is divided between two host organisms – humans and mosquitoes. Around 60 different species of mosquito belonging to the genus *Anopheles* transmit the parasites to humans (Budiansky, 2002). The insect vector is required for the natural transmission of the disease, and malaria, therefore, is restricted to areas of the world where *Anopheles* mosquitoes can breed (Fig. 1.1).

1.1.2. Life cycle of *Plasmodium* species

The life cycle of the malaria-causing *Plasmodium* species represents one of the most complex life cycles of any human infection. Both the insect and human

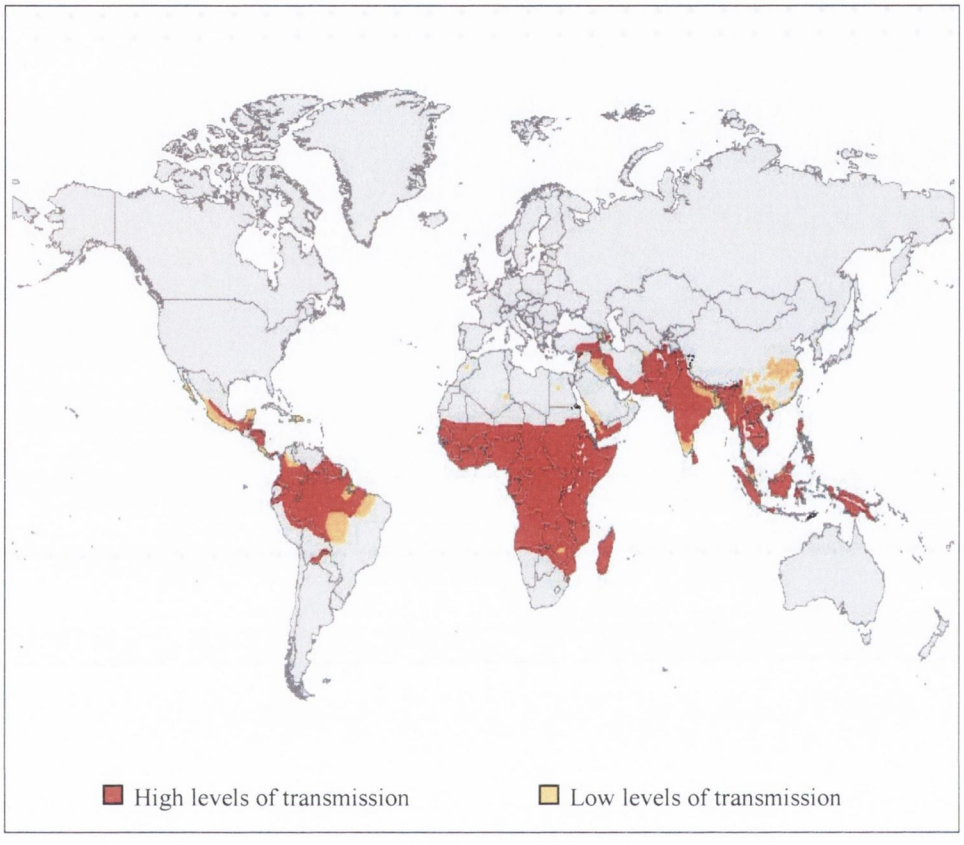


Fig. 1.1. Worldwide malaria distribution in 2002. (www.well.ox.ac.uk/ich/images/malaria_2002.jpg)

stages of the life cycle comprise a number of morphologically distinct forms of the parasite.

The cycle is initiated by the bite of a previously infected female *Anopheles* mosquito (Fig. 1.2). This biting event is prompted by the mosquito's requirement for blood for the gestation of her eggs. Therefore, transmission of malarial parasites to humans is restricted to female mosquitoes. In the process of extracting blood from humans, the infected mosquito inadvertently injects sporozoite forms of the parasite into either the subcutaneous tissue of the victim or, less frequently, directly into the bloodstream. Some of these sporozoites find their way to the liver, where they enter hepatocytes and undergo asexual multiplication (a process known as extra-erythrocytic schizogony) to form thousands of uninucleated merozoites. Upon rupture of the hepatocyte, these merozoites are released into the bloodstream where they quickly enter circulating erythrocytes. Once inside these blood cells, each merozoite begins to mature within a membrane-bound parasitophorous vacuole. As it matures within this parasitophorous vacuole, the parasite undergoes distinct morphological changes. The first such stage, due to its microscopic appearance, is known as a ring. As the parasite matures further it progresses into a more rounded form known as a trophozoite, which represents the most metabolically active stage. Further progression into the schizont form follows, which is the stage where asexual multiplication (intra-erythrocytic schizogony) occurs. Each schizont gives rise to a number of merozoites which are released from the parasitophorous vacuole and erythrocyte by a protease-dependent process. The free merozoites are then free to re-invade fresh erythrocytes and re-initiate the intra-erythrocytic stage. Essential to the perpetuation of the cycle is the development of a small sub-population of merozoites into micro- (male) and macro- (female) gametocytes within the erythrocyte. Erythrocytes harbouring gametocytes are taken up by another mosquito during feeding, whereupon they initiate the insect stage of the cycle. On entering the gut of the insect, the macrogametocytes rapidly escape from the erythrocyte to form macrogametes. Each microgametocyte, meanwhile, undergoes a process of exflagellation in which nuclear division results in the formation of sperm-like microgametes which break away from the parent cell. These actively mobile forms fertilise macrogametes, resulting in the formation of a zygote. The zygote, in turn, develops into a motile ookinete that penetrates the gut mucosa and settles beneath the elastic outer membrane covering the gut. Here it develops into an oocyst within

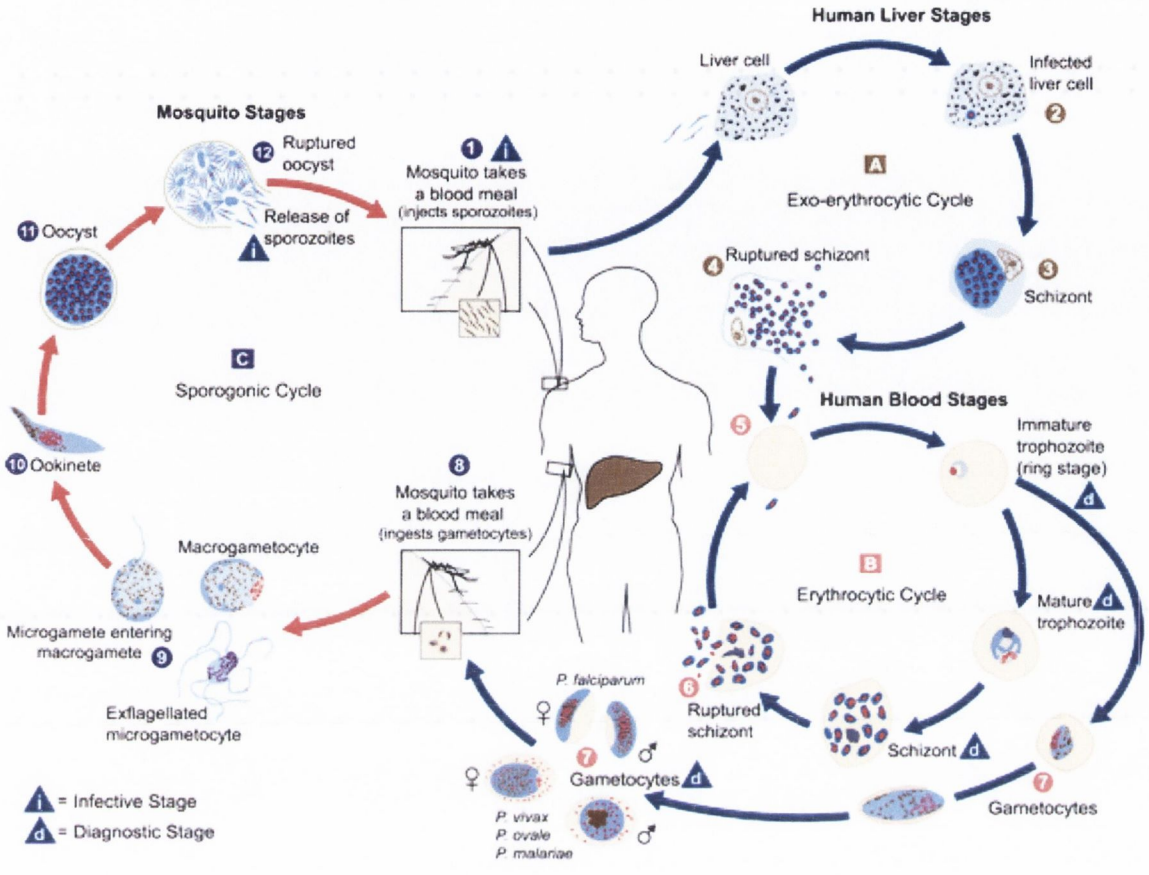


Fig. 1.2. Life cycle of *Plasmodium falciparum*. See text (section 1.1.2.) for details. Graphic obtained from http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Malaria_il.htm

a thin cyst wall. The oocyst expands and nuclear division results in the formation of thousands of sporozoites. When the oocyst bursts, sporozoites are released into the insect gut, whereupon they migrate to the salivary glands where the complete process begins again.

The clinical symptoms of malaria are associated exclusively with the intra-erythrocytic stage of the life cycle. This represents the only stage of parasite development that can be maintained easily in cell culture, and therefore, is the best studied. From hereon, only *P. falciparum* will be discussed as this is the species that has received most attention due to its greater clinical significance – not only is it the most common of the four human malarial parasites, but also almost all malaria mortalities are attributable to this organism. *P. falciparum* is a remarkable organism that has evolved a complicated life cycle. An understanding of the underlying biology is a pre-requisite for the implementation of successful control measures against this organism and the disease it causes.

1.1.3. A brief introduction to the biology of *P. falciparum*

P. falciparum is the first eukaryotic pathogen for which a complete genome sequence has been published (Gardner *et al.*, 2002) – a few gaps, however, remain to be completed. The nuclear genome is 23 Mb in size, spread amongst 14 chromosomes ranging in size from 0.6 to 3.3 Mb. With an overall A+T content of 80.6%, this is the most AT-rich genome yet sequenced. Although the genome of *P. falciparum* is about twice the size of that of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, the number of genes appears to be roughly the same in these three unicellular eukaryotes. Of the approximate 5,300 genes of *P. falciparum*, 54% contain at least one intron, a proportion similar to that in *S. pombe* (43%), but significantly higher than that in *S. cerevisiae* (5%). Based on lack of significant primary sequence similarities, approximately 60% of the predicted proteins encoded by the genome of *P. falciparum* appear to be unique to the organism.

Aside from the nuclear genome discussed above, *P. falciparum* also contains two other genomes. The mitochondrial genome of *P. falciparum*, at only 6 kbp, is the smallest yet known and encodes a mere three proteins (Wilson and Williamson, 1997). Therefore, functional mitochondria are maintained by the import of proteins encoded by the nuclear genome. The apicoplast (also referred to as the plastid), an

organelle thought to have been acquired during the course of evolution through the endosymbiosis of a unicellular alga, houses a 35-kbp genome (Wilson and Williamson, 1997). Although the apicoplast genome encodes components solely for the self-replication of the organelle (Wilson *et al.*, 1996), recent advances have shown this organelle to be the site of important biosynthetic pathways. As for mitochondria, all enzymes involved in metabolic processes inside the apicoplast are encoded by the nuclear genome and are targeted to the organelle by amino-terminal recognition sequences (Gardner *et al.*, 2002). Analysis of the nuclear genome of *P. falciparum* has identified the complete set of genes encoding proteins necessary for the tricarboxylic acid (TCA) cycle in mitochondria (Gardner *et al.*, 2002). It is known that, in erythrocytic stages at least, *P. falciparum* relies primarily on glycolysis for energy production, so it remains unclear as to whether the TCA cycle is used for energy production purposes or for the generation of intermediates for other biosynthetic pathways. Analysis of the genome sequence has also identified the genes necessary for fatty acid and isoprenoid biosynthesis, pathways known to occur within the apicoplast. While humans generate fatty acids by a type I biosynthetic pathway involving a single, multifunctional enzyme, *P. falciparum* uses the type II pathway, previously found only in plant chloroplasts and bacteria, that utilises distinct enzymes for each step (Waller *et al.*, 1998). Likewise, the mevalonate-independent pathway by which the parasite synthesise isoprenoids, chemical precursors of numerous cellular molecules, is the same as that used by plants and bacteria, but completely distinct from the mevalonate-dependent pathway of the host (Jomaa *et al.*, 1999).

The complete set of enzymes required for the metabolic pathways within the mitochondria and apicoplast need to be imported. Targeting of proteins to the apicoplast is the best-studied of these, with proteins destined for this organelle exhibiting a bipartite amino acid motif: an N-terminal signal sequence, which targets proteins into the secretory system, is followed by a specific motif directing the protein to the apicoplast (Waller *et al.*, 2000). The 'classical' secretory pathway found in most eukaryotes involves the transfer of proteins from the endoplasmic reticulum (ER) to the Golgi and onwards to their final destination within vesicles (Trombetta and Parodi, 2003). However, the precise nature of secretory pathways in *P. falciparum* remains unclear, primarily due to the absence of an obvious Golgi,

although homologues of proteins known to reside in the Golgi in other organisms have been identified (Haldar and Akompong, 2001).

The trafficking of *P. falciparum* proteins is further complicated by the fact that certain proteins are specifically targeted to distinct locations within the erythrocyte in which the parasite is contained. Based on the finding that proteins targeted within the parasite and to the parasitophorous vacuole all contain classical N-terminal signal sequences, while those destined to the erythrocyte either contain an unusual internal signal sequence or no signal sequence at all, a dichotomy in secretory mechanisms has been proposed whereby proteins destined for the erythrocyte are transported by a mechanism distinct from the classical secretory pathway (Foley and Tilley, 1998). This is supported by the finding that brefeldin A, a fungal metabolite known to inhibit vesicle formation, leads to the accumulation of parasite proteins destined for the erythrocyte in a compartment distinct from the ER (Wiser *et al*, 1999). This led to the suggestion that this compartment, located close to the parasite periphery, may be a secondary ER specialising in the transport of proteins to the erythrocyte.

The trafficking of *P. falciparum* proteins to the erythrocyte is fundamental to the survival and maturation of the parasite. Upon invasion, the parasite begins remodelling the host cell, both internally and externally. These remodelling processes are aimed at solving three basic problems faced by *P. falciparum*. First, the parasite must create a system whereby it can gain rapid access to nutrients and concomitantly dispose of waste products. This involves modifications to the host cell cytosol and membrane. This restructuring of the host cell, however, leads to the second major problem faced by the parasite. The primary function of the spleen is to remove old and damaged erythrocytes from the circulation and the remodelling of the erythrocyte by *P. falciparum* would, under normal circumstances, target the cell for removal from the circulatory system. By transporting proteins to the erythrocyte surface that have the capacity to bind to endothelia of blood vessels, *P. falciparum* may have evolved a mechanism to avoid removal by the spleen. However, this leads to the third problem faced by the parasite. Due to its intra-erythrocytic location, *P. falciparum* is essentially invisible to the host immune system for the majority of its erythrocytic life cycle. However, the insertion of proteins into the erythrocyte membrane alerts the host immune system to its presence. Accordingly, the proteins used by the parasite to evade removal from the circulation must continually be

changed to combat the host immune defences. Proteins, therefore, must be continually transported from the parasite to the host cell surface.

Microscopic studies of *P. falciparum*-infected erythrocytes have revealed the presence of a complex, interconnected network of tubovesicular membranes (TVM) that extends from the parasitophorous vacuolar membrane to the periphery of the erythrocyte (Haldar and Akompong, 2001). Disruption of TVM development with a known inhibitor does not prevent protein transport, but does retard the movement of solutes from the extracellular medium to the parasite, suggesting that the primary function of this structure is the uptake of nutrients by the parasite (Laurer *et al.*, 1997). The presence of membrane bound structures known as Maurer's clefts have also been identified in the cytosol of erythrocytes infected with *P. falciparum* (Haldar *et al.*, 2002). Reports of proteins exported to the erythrocyte associated with these structures suggest that Maurer's clefts function in protein trafficking (Hinterberg *et al.*, 1994; Das *et al.*, 1994). Several studies suggest that transport through the erythrocyte cytosol occurs in vesicles that bud from the parasitophorous vacuolar membrane (reviewed in Taraschi *et al.*, 2001). Another study suggests that, while some parasite proteins destined for the erythrocyte surface are transported in vesicles, others are transported as protein aggregates (Gormely *et al.*, 1992). Taken together, it appears that multiple mechanisms exist for the transport of *P. falciparum* proteins beyond the parasitophorous vacuolar membrane.

The development of *P. falciparum* within the human host is heavily dependent upon the activities of a range of proteases, and members of four major classes of proteases (serine, cysteine, aspartic and metallo) have been reported in the parasite (Rosenthal, 2001). Protease activity is implicated in a diverse range of functions, from the initial invasion of the host cell, through the intracellular maturation process, up to the rupture of the host cell and release of free merozoites. The best studied of these protease-dependent mechanisms is the degradation of host cell haemoglobin. Haemoglobin constitutes approximately 95% of total erythrocyte protein, up to 75% of which is digested by *P. falciparum* (Banerjee and Goldberg, 2001). This process occurs in a specialised lysosome-like organelle known as the digestive vacuole (DV). Haemoglobin is delivered to the DV by a vesicle-mediated process, whereupon the sequential actions of proteases degrade it into its constituent amino acids and haem. It is thought that this process provides the parasite with its

primary source of amino acids for protein synthesis, as they have limited capacity for *de novo* synthesis of certain amino acids. However, recent work suggests that the parasite only utilises a fraction of the amino acids derived from this process, and is more reliant on amino acids derived from extracellular sources (Krugliak *et al.*, 2002). An alternative theory is that this process creates physical space for the parasite to grow – the parasite can increase its volume by 25-fold in one intra-erythrocytic cycle (Banerjee and Goldberg, 2001). This process is essential to parasite development and it represents an extensive metabolic undertaking. Not only does it involve the ingestion and degradation of haemoglobin, but also requires a significant detoxification process. Free haem is toxic to the parasite due to its potential to produce free oxygen radicals. As *P. falciparum* lacks the enzyme to catabolise haem, it detoxifies it by a unique polymerisation process resulting in a crystalline structure known as haemozoin, or malaria pigment. The process by which haem is detoxified by *P. falciparum* has not been fully elucidated but is the subject of intense research as this appears to be the target of a number of important anti-malarial compounds, most notably the quinoline class of drugs of which chloroquine is a member (Tilley *et al.*, 2001).

1.1.4. Clinical manifestations of malaria

The clinical manifestations of malaria are associated exclusively with the erythrocytic stage of the life cycle, with specific symptoms dependent upon the species of parasite. Common to all four forms of the disease is a characteristic fever, which coincides with the rupture of erythrocytes. Malarial fever is typified by a feeling of intense cold and shivering that is followed by a hot, dry stage, and finally a period of drenching sweats. Common ancillary symptoms include headache, muscle pain and vomiting. The severity of symptoms is dependent upon a number of factors, most notable the species of parasite, the intensity of infection (the number of erythrocytes infected, i.e. parasitemia) and the age and immune status of the patient. Aside from fever, anaemia is the primary consequence of malaria infection. This is due to a number of factors, the most obvious being loss of erythrocytes due to parasite-induced lysis. However, a reduction in erythrocyte levels is also due to removal of damaged/infected cells by the spleen, immune-mediated haemolysis and a decline in the production of erythrocytes due to a

depression in erythropoiesis. The removal of erythrocytes by the spleen results in splenic enlargement (splenomegaly).

As mentioned in section 1.1.3, *P. falciparum* remodels the surface of the host cell. An important consequence of this is that the parasitised erythrocytes can bind to various receptors on the surface of the endothelia lining the blood vessels (Craig and Scherf, 2001). In addition to cytoadherence to vascular endothelia, two other adhesive phenomena occur as a result of host cell modifications. The process by which parasitised erythrocytes adhere to each other, as opposed to adhering to endothelial cells, is termed autoagglutination. A similar phenomenon, termed rosetting, is the adherence of parasitised erythrocytes to non-infected erythrocytes. This ability of *P. falciparum* to sequester erythrocytes in the microcirculation of vital organs, an attribute unique amongst the human malaria parasites, underlies the aggressive nature of this particular form of malaria. Given the right conditions, *falciparum* malaria can progress to "severe malaria" with a range of life-threatening manifestations. Severe malaria occurs when the cytoadherence mechanisms of *P. falciparum*-infected erythrocytes obstruct the flow of blood to vital organs, resulting in ischemia. Cerebral malaria, the most lethal form of the disease refers to conditions in which blood flow to the brain is restricted. Depending on the extent of the occlusion, this can result in hallucinations, behavioural changes, generalised seizures, hearing impairment, blindness, and epilepsy. An estimated 600,000 Africans develop cerebral malaria annually, with a mortality rate in the region of 20% (Murphy and Bremen, 2001). An estimated 5-20% of those who survive cerebral malaria are left with neurological damage (Bremen *et al*, 2001). Other major complications arising directly from malaria-associated ischemic injuries are renal failure and lung damage.

1.1.5. Control of malaria

Frustratingly, malaria is usually a curable disease if promptly diagnosed and adequately treated. Although malaria represents one of the most significant diseases in the world today, its status as a disease of the poor has resulted in relative neglect by pharmaceutical companies. The development of new anti-malarial drugs since the 1970s has been largely ignored: of the 1223 new drugs developed in the period between 1975 to 1996, only 3 were anti-malarials (Trouiller and Olliaro, 1998). However, interest in the development of new anti-malarials has been rekindled in

recent years, thanks in no small part to programmes such as 'Roll Back Malaria' (http://mosquito.who.int/), the 'Multilateral Initiative on Malaria' (www.mim.su.se/) and the 'Medicines for Malaria Venture' (www.mmv.org), all of which are collaborative alliances of various groups and organisations committed to combating malaria. The recent sequencing of the *P. falciparum* genome (Gardner *et al.*, 2002) promises to bring a fresh impetus to the search for new and novel anti-malarial drugs.

The development of effective anti-malarial drugs has had notable success before. In fact, malaria was among the first diseases to be treated with a pure chemical compound when, in 1820, quinine was isolated as the active antimalarial ingredient from the bark of the *Cinchona* tree (Meshnick and Dobson, 2001). The bark of this tree had been used for the treatment of fever for generations, but quinine rapidly replaced crude bark in the 1820s. Malaria was the first disease for which a synthetic drug was used to treat humans when, in 1891, Paul Erlich successfully treated two patients with methylene blue (Wiesner *et al.*, 2003). Analogues of methylene blue were used extensively as anti-malarials in World War II. Chloroquine, a synthetic analogue of quinine, was first used in 1946 and is regarded as one of the most significant and successful drugs ever developed. However, towards the end of the 1950s, it was noted that malaria parasites were emerging that were resistant to chloroquine (Wiesner *et al.*, 2003). After over forty years of use, chloroquine is no longer fully effective against *P. falciparum* in most of Africa and South East Asia (Whitty *et al.*, 2002). It is still an important agent, particularly for the treatment of other forms of malaria throughout the world, but resistance is developing within these species too.

Since the emergence of widespread chloroquine resistance, only a small number of compounds have been found suitable for clinical treatment of malaria (Table 1.1). After chloroquine, the combination of sulfadoxine and pyrimethamine is the most common anti-malarial in use today. However, resistance to this treatment is becoming a major problem. Resistance has also been reported for the two newest combination treatments on the market, lumefantrine-artemether and atovaquone-proguanil. This development of cross-resistance between drugs is no small part due to the overall lack of chemical diversity in the anti-malarial drugs currently in use and the narrow range of pathways that are targeted by these drugs. Although the mechanisms by which current anti-malarial drugs exert their action are

Table 1.1. Major anti-malarial drugs in use and promising drugs currently in development

Drug	Principal Target	Major Advantages	Major Disadvantages
Established			
Quinine	Haem polymerisation?	Intravenous formulation for treatment of severe malaria	Side effects; resistance
Chloroquine	Haem polymerisation?	Low cost; prophylactic use	Widespread resistance
Amodiaquine	Haem polymerisation?	Low cost; prophylactic use	Side effects
Mefloquine	Haem polymerisation?	Prophylactic use	High cost; neuropsychiatric side effects; resistance
Halofantrine	Unknown	High efficacy	High cost; cardiotoxicity; resistance
(Sulphadoxine + Pyrimethamine) #	Folate metabolism	Low cost	Resistance; side effects
Proguanil (Cycloguanil) ¶	Folate metabolism + mitochondrial membrane potential?	Low cost	Low efficacy; resistance
Atovaquone	Mitochondrial electron transport	Low toxicity	High cost; recrudescence
(Atovaquone + Proguanil) #	Mitochondrial electron transport + mitochondrial membrane potential?	Active against hypnozoites	High cost
Doxycycline	Apicoplast replication	Active against hypnozoites	Slow action; photosensitising effects
(Lumefantrine + Artemether) #	Haem polymerisation?	Rapid action	High cost
Primaquine	Unknown	Active against hypnozoites and female gametocytes	Inactive against blood stage parasites; haemolysis in G6PD-deficient subjects *
In Development			
Tafenoquine	Unknown	Active against all human stages	Haemolysis in G6PD-deficient subjects *
Pyronaridine	Haem polymerisation?	Active against all human stages	Recrudescence
(Chloroproguanil + Dapsone) #	Folate metabolism	Low cost	Low efficacy
(Fosmidomycin + Clindamycin) #	Isoprenoid biosynthesis + Apicoplast replication	Low toxicity; active against multi-resistant strains	Recrudescence
Desbutylhalofantrine	Unknown	High efficacy, but without cardiotoxic effects of parent drug (halofantrine)	High cost
Artelinic Acid	Haem polymerisation?	Rapid action; intravenous formulation for treatment of severe malaria	Recrudescence
Artesunate	Haem polymerisation?	Rapid action; rectal application for treatment of severe malaria	Recrudescence

? Uncertainty about precise target

Drugs used in combination

¶ Proguanil is metabolised into cycloguanil

* The incidence of glucose 6-phosphate dehydrogenase (G6PD) deficiency is particularly high in malaria-endemic regions

Footnote: Some of the drugs mentioned are used, or proposed to be used, in combinations other than those indicated, e.g. mefloquine + artesunate

incompletely understood, most can be classified into two groups: those that interfere with detoxification of haem, and those that interfere with *de novo* pyrimidine nucleotide synthesis by inhibiting folate metabolism. The combination of atovaquone and proguanil exerts its affect, at least in part, by inhibition of mitochondrial electron transport. Therefore, there exists an urgent need to develop new classes of compounds that exert their anti-malarial effects through novel mechanisms. Of the drugs currently undergoing clinical development, only fosmidomycin represents a truly novel anti-malarial compound.

1.1.6. Recent progress in anti-malarial drug development

The past few years have seen great progress in the understanding of *Plasmodium* biology, shedding light on metabolic pathways and parasite proteins that may serve as novel drug targets. In light of the recent publication of the genomes of *P. falciparum* (Gardner *et al.*, 2002) and the model rodent malaria parasite *P. yoelii* (Carlton *et al.*, 2002), further advances in the understanding of parasite biology are promised and will no doubt play a major role in the design of the next generation of anti-malarials. A number of potential chemotherapeutic targets are discussed here.

Proteases have long been known to play important roles in malaria pathogenesis. As discussed in section 1.1.3, malarial parasites require protease activity throughout their erythrocytic development (little information is available about protease involvement during extra-erythrocytic development). In particular, the manner in which haemoglobin is broken down into its constituent amino acids by the sequential actions of various protease is quite well characterised and studies have shown the anti-malarial potential for inhibitors of such proteases (Rosenthal, 2001). The protease-dependent mechanisms by which merozoites are released from erythrocytes and subsequently invade fresh cells, remain largely uncharacterised, but are of interest due to their potential as chemotherapeutic targets (Salmon *at al.*, 2000).

Due to the large number of membranes that are maintained within parasitised erythrocytes – including the membranes of the parasite itself, the parasitophorous vacuole and the interconnected network of the TVM – the requirement for phospholipids of infected erythrocytes is considerably higher than that of normal erythrocytes, with the phospholipid content of erythrocytes increase

by up to 600% upon infection by malarial parasites (Vial *et al.*, 2003). Because erythrocytes lack any biosynthetic capability, the synthesis of the additional phospholipids must be met by the parasites themselves, and analysis of the genome sequence data has suggested that the phospholipid biosynthetic pathways of *P. falciparum* is considerably different from the host offering the potential to design selective inhibitors. Similarly, the unique nature of the anabolic pathways leading to fatty acid and isoprenoid biosynthesis by *P. falciparum* suggests that these pathways may represent potential anti-malarial targets. A data-mining approach has also played a role in the identification of the shikimate pathway in *P. falciparum* (Roberts *et al.*, 2002). This pathway is central to the production of a variety of aromatic compounds – such as folates, ubiquinone and certain amino acids – in plants, bacteria and fungi, but is completely absent in animals.

The potential of the aforementioned pathways to serve as targets for novel anti-malarial drugs stems from their unique nature when compared to pathways in the human host. This is not to say, however, that compounds targeting other biochemical pathways have no potential. For example, *P. falciparum* is almost entirely reliant on glycolysis for its energy metabolism, and while the parasite and host exhibit almost identical glycolytic pathways, that of the parasite represents a potential target due to structural differences in lactate dehydrogenase, the last enzyme involved in the pathway (Dunn *et al.*, 1996). Identification of other *P. falciparum* proteins with significant differences to their host counterparts will be greatly assisted by the availability of the parasite genome sequence.

In order to survive within erythrocytes, *P. falciparum* must gain access to nutrients and concomitantly dispose of waste products. To these ends, the parasite induces profound alterations in the permeability of the erythrocyte membrane. This appears to be achieved through a combination of parasite-induced transporters and the modulation of the activity of endogenous transporters (Krishna *et al.*, 2002). Although little is known about the precise mechanisms involved, the induction of transport pathways in parasitised erythrocytes offers the potential for the selective uptake of drugs into infected erythrocytes. In this regard, Brown and colleagues reported on the use of L-adenosine analogues that penetrate poorly into normal erythrocytes but enter readily into infected cells and subsequently into the parasite, where upon they are metabolised into toxic forms by the action of parasite adenosine deaminase (Brown *et al.*, 1999).

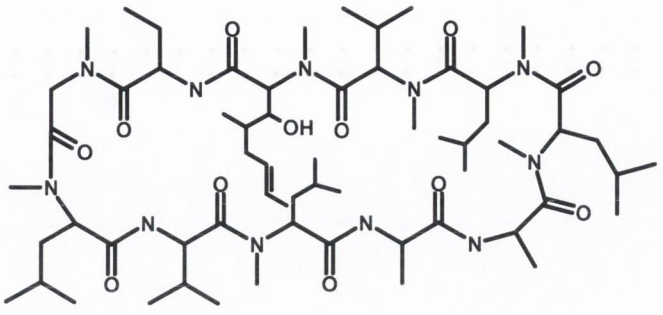
The discovery of the type II fatty acid synthesis system, the mevalonate-independent biosynthesis pathway and the shikimate pathway has demonstrated how fundamental research based on the *P. falciparum* genome sequence data can lead to the identification of new targets. Together with advances in fields such as genomics and proteomics, the identification of *P. falciparum* genes and analysis of their protein products heralds a bright future for anti-malarial drug development.

1.2. Immunosuppressive Drugs and Malaria

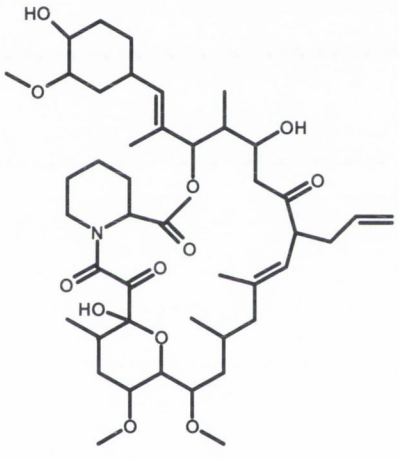
1.2.1. Anti-malarial properties of immunosuppressive drugs

The immunosuppressive drug, cyclosporin A (CsA), was shown in 1981 to have anti-malarial properties when an inhibitory effect of this drug on *P. berghei* and *P. chabaudi* infections of mice was reported (Thommen-Scott, 1981). This was a rather unexpected discovery as the immunosuppressive properties of CsA were expected to enhance the susceptibility of mice to *P. berghei* and *P. chabaudi*. Nickell and co-workers extended this work when they showed that the development of murine malaria (*P. berghei* and *P. yoelii* infections) was markedly affected by both prophylactic and therapeutic treatment with CsA (Nickell *et al.*, 1982). They ruled out the possibility that the drug was exerting an indirect effect through the host's immune system by using sub-immunosuppressive concentrations and also by testing the drug's effect against mice whose leucocytes were destroyed by irradiation prior to infection and drug treatment. In the same report, they showed that CsA had potent activity against *P. falciparum* in culture. Activity against *P. falciparum* was further reported when the drug was shown to reduce the severity of malaria in experimental infections of owl monkeys (Cole *et al.*, 1983).

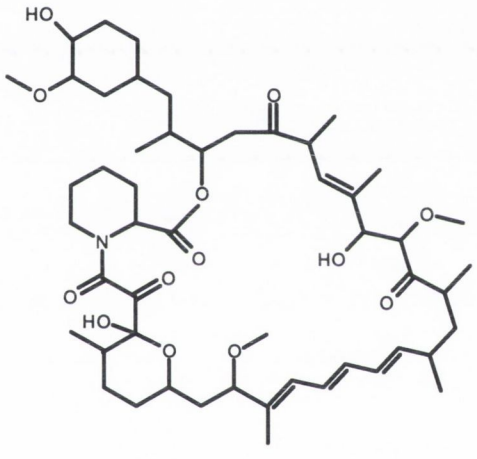
CsA is a cyclic undecapeptide (Fig. 1.3) produced as a natural metabolite from the soil fungus *Tolypocladium inflatum* (Borel *et al*, 1976). The potent immunosuppressive properties of CsA prompted its development as an immunosuppressant drug by Sandoz Pharmaceuticals (now Novartis) and since its approval for clinical use in the early 1980s, CsA has played a major role in the prevention of allograft rejection (Allison, 2000). Further advances in the prevention of allograft rejection were made in the late 1980s and 1990s when the immunosuppressive properties of two other compounds, FK506 (tacrolimus) and



Cyclosporin A



FK506



Rapamycin

Fig. 1.3. Chemical structures of cyclosporin A, FK506, and rapamycin.

rapamycin (Fig. 1.3), led to their development as immunosuppressive drugs by Fujisawa Pharmaceuticals and Wyeth-Ayerst, respectively. Like CsA, both FK506 and rapamycin are natural metabolites from soil microorganisms: FK506 is produced by the filamentous bacterium *Streptomyces tsukabiensis*; while the related organism, *S. hygrosopicus*, produces rapamycin (Kino *et al.*, 1987; Seghal *et al.*, 1975). While they share no structural similarity with CsA, FK506 and rapamycin are structurally similar to each other and, due the macrolactone ring structure, are classified as macrolides (Fig. 1.3).

Prompted by the anti-malarial properties of CsA, Bell and co-workers showed both FK506 and rapamycin to have significant inhibitory effects against *P. falciparum* in culture (Bell *et al.*, 1994). This was not surprising in light of the broad antimicrobial activity exhibited by all three drugs. Aside from activity against malarial parasites, CsA, FK506 and rapamycin are active against a range of protozoa, bacteria and fungi (High, 1994). Indeed, these three compounds were originally isolated as part of screening programmes to identify novel antimicrobial compounds (Borel *et al.*, 1976; Kino *et al.*, 1987; Seghal *et al.*, 1975). Immunosuppressive effects have limited their development as antimicrobial agents, so little is known about the antimicrobial mechanisms of CsA, FK506 and rapamycin. Rather, most research has focused on the mechanisms by which these drugs exert their immunosuppressive activities. The late 1980s and early 1990s saw an intense and fervent effort to elucidate these immunosuppressive mechanisms.

1.2.2. Mechanisms of immunosuppression by CsA, FK506 and rapamycin

CsA, FK506 and rapamycin exert their immunosuppressive effects primarily through effects on T-helper lymphocytes. Upon antigen presentation, T-helper lymphocytes produce the cytokine interleukin-2 (IL-2), and expression of its cognate receptor is induced in all classes of T-lymphocytes (Fig. 1.4). The interaction of IL-2 with its high affinity receptor, through either a paracrine or autocrine response, provides critical signals for T-lymphocyte activation and proliferation. IL-2 also induces the growth and differentiation of B-lymphocytes into antibody-secreting cells, and has a wide range of effects upon other lymphoid cells that express IL-2 receptor, such as natural killer cells and monocytes. Therefore, it is not surprising that the inability to either produce or respond to IL-2 can lead to profound immune dysfunction. Both CsA and FK506 exert their

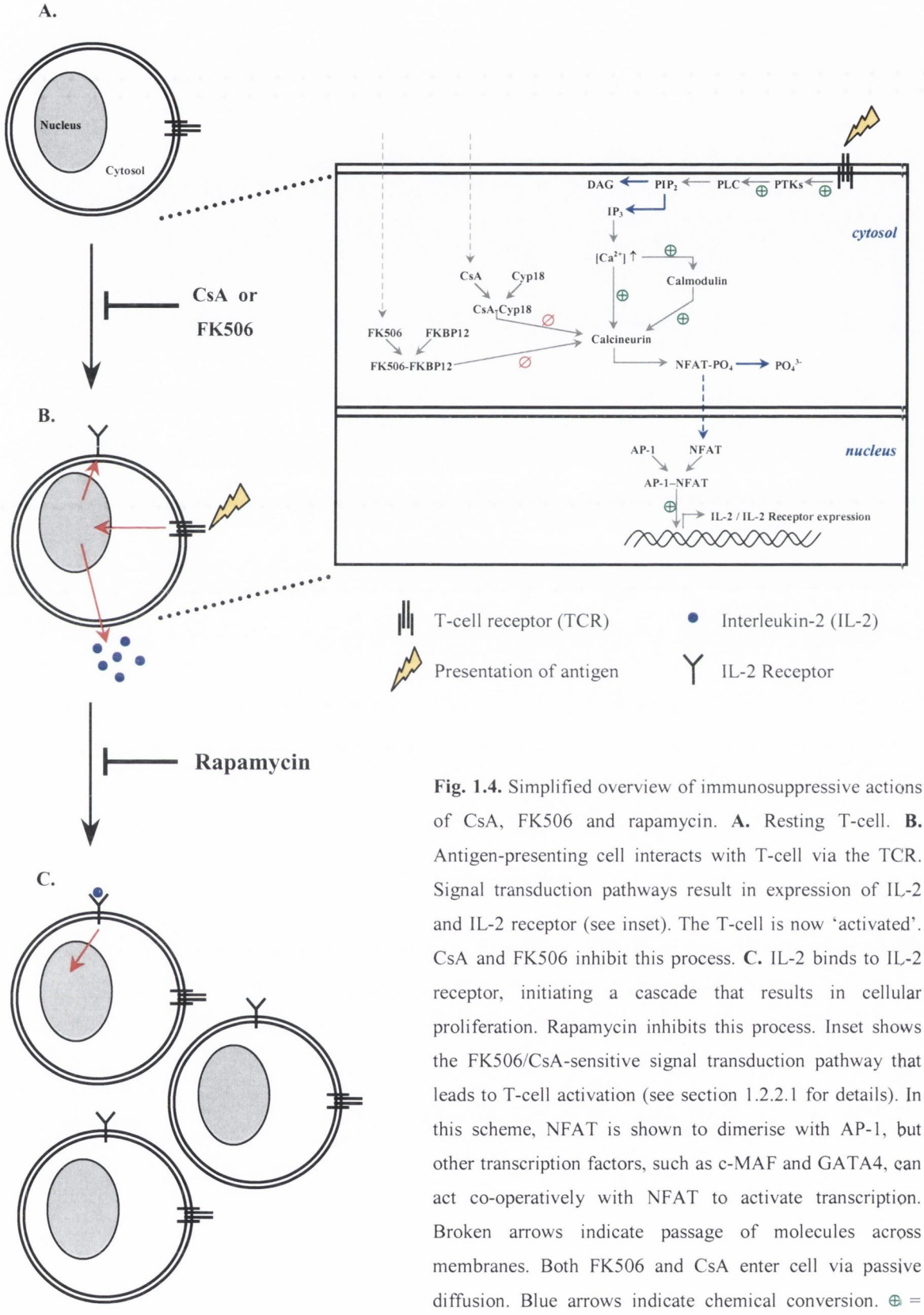


Fig. 1.4. Simplified overview of immunosuppressive actions of CsA, FK506 and rapamycin. **A.** Resting T-cell. **B.** Antigen-presenting cell interacts with T-cell via the TCR. Signal transduction pathways result in expression of IL-2 and IL-2 receptor (see inset). The T-cell is now 'activated'. CsA and FK506 inhibit this process. **C.** IL-2 binds to IL-2 receptor, initiating a cascade that results in cellular proliferation. Rapamycin inhibits this process. Inset shows the FK506/CsA-sensitive signal transduction pathway that leads to T-cell activation (see section 1.2.2.1 for details). In this scheme, NFAT is shown to dimerise with AP-1, but other transcription factors, such as c-MAF and GATA4, can act co-operatively with NFAT to activate transcription. Broken arrows indicate passage of molecules across membranes. Both FK506 and CsA enter cell via passive diffusion. Blue arrows indicate chemical conversion. ⊕ = activation; ∅ = inhibition.

immunosuppressive effects by suppressing IL-2 expression, thereby inhibiting T-lymphocyte activation (i.e. G_0 to G_1 progression), while rapamycin inhibits T-lymphocyte proliferation (i.e. G_1 to S) by interfering with the cellular response to IL-2 signalling mediated through the IL-2 receptor.

1.2.2.1. Immunosuppressive mechanisms of CsA and FK506

The transient expression and secretion of IL-2 by T-helper lymphocytes is induced by a combination of immune stimuli, the most important of which is the interaction of the T cell receptor (TCR) with a specific antigen. This antigen is presented to the T lymphocyte by the major histocompatibility complex (MHC) of an antigen-presenting cell. This interaction initiates signal transduction cascades that activate an array of transcription factors that, upon translocating to the nucleus, bind to recognition elements of the transcriptional enhancer of the IL-2 gene. A number of distinct transcription factors function co-operatively to activate transcription of the IL-2 gene (Riegel *et al.*, 1992; Schreiber and Crabtree, 1992; Galat and Rivière, 1998). Such tight regulation of IL-2 production is necessary to avoid uncontrolled T-lymphocyte growth and tumorigenesis. By inhibiting a combination of these transcription factors, CsA and FK506 suppress the transcriptional activation of the IL-2 gene (Mattila *et al.*, 1990; Matsuda *et al.*, 2000). Of these, NFAT (*n*uclear *f*actor of *a*ctivated *T*-cells) appears to be the most sensitive to the drugs and is the only pathway for which the molecular mechanism of CsA- and FK506-immunosuppression is well characterised.

Neither CsA nor FK506 affects NFAT directly, but rather act through a pivotal cellular activity that modulates its activity (Fig. 1.4). NFAT forms a dimeric complex in the T-lymphocyte nucleus with one of a number of other transcription factors, such as AP-1, c-MAF and GATA4, that co-operatively bind to DNA (Crabtree, 1999). NFAT is confined to the cytosol of resting T-lymphocytes but upon antigen presentation it translocates to the nucleus where it forms the active transcriptional dimer. The transport of NFAT to the nucleus is dependent upon a dephosphorylation event mediated by calcineurin, a serine/threonine protein phosphatase. The phosphatase activity of calcineurin is modulated by calmodulin and Ca^{2+}. Upon antigen presentation to T-lymphocytes, the T-cell receptor/CD3 complex induces activation of various membrane-bound protein tyrosine kinases (PTKs), which in turn activate phospholipase C (PLC), resulting in the cleavage of

the inositol 1,4,5-trisphosphate (IP_3) moiety from phosphatidylinositol-4,5-bisphosphate (PIP_2). IP_3 binds to its cognate receptor on the endoplasmic reticulum membrane, resulting in the liberation of stored Ca^{2+} into the cytosol. This increase in cytosolic Ca^{2+} levels activates calmodulin, which in turn activates calcineurin (calcineurin also binds Ca^{2+} directly), resulting in the dephosphorylation of NFAT. It is this dephosphorylation event that is inhibited by CsA and FK506.

Neither drug, however, can bind to calcineurin with significant affinity in the absence of other proteins. Rather, the ability of both CsA and FK506 to inhibit calcineurin is dependent upon the prior formation of binary complexes with distinct protein targets. The major cytosolic receptor for CsA is an 18-kDa protein termed cyclophilin 18 (Cyp18 – also known as CypA) (Handschumacher *et al.* 1984), while the 12-kDa FK506-binding protein (FKBP12) is the major receptor for FK506 (Siekierka *et al.*, 1989; Harding *et al.*, 1989). It is these binary complexes (CsA-Cyp18 or FK506-FKBP12) that bind to and inhibit calcineurin, thereby inducing immunosuppression.

1.2.2.2. Immunosuppressive mechanism of rapamycin

Unlike CsA and FK506, rapamycin has no effect on IL-2 expression. Rather, its immunosuppressive effect inhibits the IL-2-induced mitogenic response (Fig. 1.4). The molecular mechanisms underlying the immunosuppressive effects of rapamycin are not well understood, but two major biochemical pathways appear to be disrupted by the drug. One such pathway normally results in the translation of a specific class of mRNA transcripts by the ribosomal protein S6 (Dumont and Su, 1996), while the second pathway, involving the sequential activation of cyclin dependent kinases (cdks), is crucial in the regulation of cell cycle progression (Dumont and Su, 1996; Hleb *et al.*, 2004). Although these two pathways are distinct, they are linked upstream by a common molecular switch. It is this protein, mTOR (*m*ammalian *t*arget *o*f *r*apamycin), that represents the direct target of rapamycin.

Just as calcineurin inhibition by CsA and FK506 are dependent upon the prior formation of CsA-Cyp18 and FK506-FKBP12 binary complexes, rapamycin cannot inhibit the kinase activity of mTOR until it forms a binary complex with its cytosolic ligand. Intriguingly, the major cellular ligand for this drug is FKBP12 (Bierer *et al.*, 1990). The fact that both FK506 and rapamycin share the same

cellular ligand is not surprising, considering the structural similarity of both drugs. What is surprising, however, is the widely different cellular effects mediated by the FK506-FKBP12 and rapamycin-FKBP12 binary complexes.

1.3. IMMUNOPHILINS

1.3.1. Cyclophilins and FK506-binding proteins: an introduction

Central to the immunosuppressive effects of CsA, FK506 and rapamycin are the cognate cytosolic receptors: Cyp18 in the case of CsA and FKBP12 in the cases of both FK506 and rapamycin. Although Cyp18 and FKBP12 are distinct proteins, sharing no sequence or structural similarity, the collective term immunophilin was coined because of their ability to bind distinct immunosuppressive drugs (Harding *et al.*, 1989). Both are abundant proteins, each constituting up to 0.4% of total cytosolic protein in T-cells (Harding *et al.*, 1986; Siekierka *et al.*, 1989). However, neither Cyp18 nor FKBP12 are confined to lymphoid tissue, with both being distributed in all tissues throughout the body (Kay, 1996).

Both immunophilins were originally identified due to their involvement in the immunosuppressive effects of CsA and FK506. However, exposure of cells to such drugs is not a natural occurrence. An important advance in the understanding of the role of immunophilins in their natural drug-free state came with the discovery that Cyp18 acts catalytically in accelerating the *cis-trans* isomerisation of peptidyl-prolyl bonds in proteins and peptides (Takahashi *et al.*, 1989; Fischer *et al.*, 1989). This peptidyl-prolyl *cis-trans* isomerase (PPIase - EC 5.2.1.8) activity was subsequently reported for FKBP12 (Siekierka *et al.*, 1989; Harding *et al.*, 1989). PPIase activity had previously been identified in cellular extracts (Fischer and Bang, 1985; Fischer *et al.*, 1984), but Cyp18 and FKBP12 were the first proteins to which this activity was attributed. This discovery was strongly suggestive of a role for both these proteins in protein folding.

1.3.2. Peptidyl-prolyl *cis-trans* isomerase activity

Due to their partial double bond character, peptide bonds can only exist in one of two conformations, *cis* or *trans* (Fig.1.5; Galat and Metcalfe, 1995; Pal and Chakrabarti, 1999). The torsion angles along the polypeptide chain are called phi (ϕ

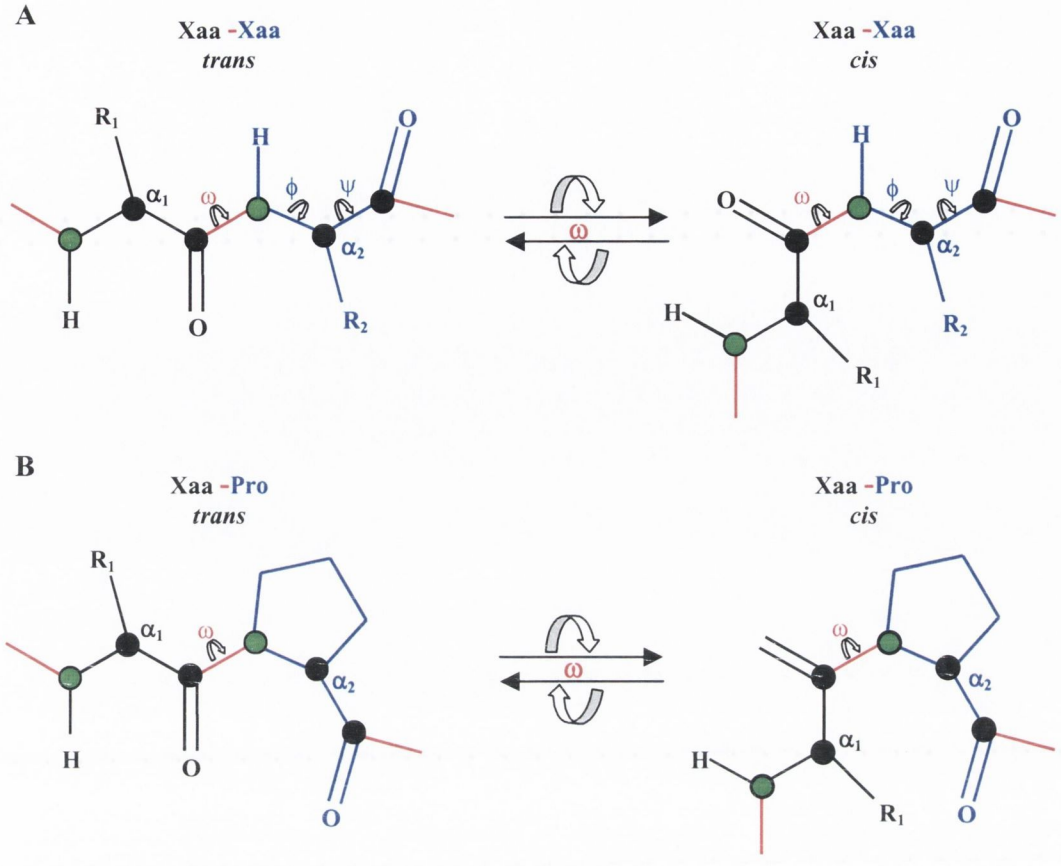


Fig. 1.5. Restriction of rotation around the peptide bond. The torsion angles along the polypeptide chain, parameters that dictate the conformation of the whole protein, are designated omega (ω), phi (ϕ) and psi (ψ). While there is considerable rotational freedom around both ϕ and ψ angles, the partial double-bonded nature of the peptide bond imparts significant restraints on the ω angle. Accordingly, dipeptide units along the length of a polypeptide can adopt one of only two conformations around the peptide bond – *trans* or *cis*. **A:** A Xaa-Xaa dipeptide unit (Xaa_1 = black; Xaa_2 = blue) joined by a peptide bond (red). The peptide backbone is designated by circles (C = black; N = green). The H atom attached to each C_α atom is not shown. In the *trans* conformation, the C_α atoms of Xaa_1 and Xaa_2 (α_1 and α_2 respectively) are 180° with respect to each other (ω = 180°), which prevents the side chains (R_1 and R_2) from approaching each other. In the alternative *cis* conformation, the C_α atoms are 0° with respect to each other (ω = 0°). Due to steric hindrance between the functional groups attached to the C_α atoms in the *cis* conformation, the *trans* conformation is more energetically favoured. For most dipeptide units, the *trans*-conformer is about 1000 times more stable than the *cis*-conformer. **B:** When the second residue of a dipeptide unit is proline (Xaa-Pro), the *trans*-conformer is only about four times more stable than the *cis*-form. The unique cyclic nature of the proline "side chain" lessens steric interference between functional groups when ω = 0°.

- between the nitrogen atom and the alpha carbon [C_α] atom of an amino acid [N-C_α]), psi (ψ - between the C_α atom and the carbonyl C (C′) atom [C_α-C']), and omega (ω - between the C′ atom of one amino acid and the N atom of another [C'-N], i.e. the peptide bond). While there is considerable rotational freedom around both ϕ and ψ angles, the partial double bonded nature of the peptide bond imparts significant restraints on the ω angle. This is a rigid bond, with all atoms directly associated with C' or N being restricted to the same plane. Accordingly, the ω angle is restricted to two possibilities – the C=O group immediately preceding the peptide bond can be either 0° (*cis*-isomer) or 180° (*trans*-isomer) with respect to the N-H group immediately following the peptide bond. Due to the planar nature of the peptide bond, when these groups are 0° with respect to each other, so too are the respective C_α atoms. This conformation results in a sterically disfavoured approach of the side chains attached to the C_α atoms. As a result, the *trans* conformation is energetically favoured.

Analyses of known protein structures show that an estimated 5-7% of peptidyl-prolyl bonds (i.e. peptide bonds immediately preceding a proline residue [Xaa-Pro]) are in the *cis* conformation, while less than 0.05% of Xaa-nonPro peptide bonds exhibit this isomeric state (Pal and Chakrabarti, 1999). The preponderance of *cis* isomers at peptidyl-prolyl bonds is due to the unique cyclic structure of proline, where the amino N atom is cyclised with the side chain terminal C atom – indeed, proline is more correctly designated as an imino acid. The *cis* conformations are typically around 1000 times less stable than the *trans* conformers in Xaa-nonPro bonds, but only around 4 times less stable in Xaa-Pro bonds. Thus, the *cis*-conformation occurs more frequently at the N-terminal side of proline residues than any other amino acid.

During protein synthesis, all peptide bonds are inserted into the growing polypeptide chain in the *trans* conformation. Therefore, many proteins require certain peptidyl-prolyl bonds to be isomerised from the *trans* to the *cis* conformation in order to achieve their correct three-dimensional structures. Due to the double bond character of the peptide bond, however, an appreciable barrier exists for the rotation around the C'-N bond. This isomerisation of specific peptidyl-prolyl bonds is, like the formation of disulphide bonds between cysteine residues, one of the slowest steps in protein folding (Kay, 1996).

Although the PPIase activities of both Cyp18 and FKBP12 are inhibited upon binding to CsA and FK506, respectively (Fischer *et al.*, 1989; Harding *et al.*, 1989; Siekierka *et al.*, 1989), this does not explain the immunosuppressive effects of the drugs as certain non-immunosuppressive derivatives of both these drugs have been shown to inhibit the PPIase activities of their receptors. Neither drug nor immunophilin alone can interact with calcineurin. Rather, the interactions with calcineurin are dependent upon combined structural characteristics of the drug-immunophilin complexes, with both drug and immunophilin contributing to a unique composite binding surface for calcineurin. The same is true for the inhibition of mTOR by rapamycin. Indeed, this explains the difference in immunosuppressive actions of FK506 and rapamycin. The regions of these drugs that bind to FKBP12 are almost identical, while the regions exposed to solvent are very different (Fig. 1.6A). These solvent-exposed regions, together with residues of the immunophilin, constitute the composite binding surface for calcineurin (in the case of FK506-FKBP12) or mTOR (rapamycin-FKBP12). Conversely, although CsA and FK506 are completely distinct compounds, and their respective immunophilins show no sequence or structural similarities, remarkably the composite binding surfaces of CsA-Cyp18 and FK506-FKBP12 allow both binary complexes to interact with calcineurin (Fig. 1.4 and 1.6B).

Since the initial discovery of these two proteins in mammalian cells, a plethora of homologues of each have been identified in a myriad of different cell types, leading to the designation of two distinct immunophilin families: the cyclophilin family, of which Cyp18 is the archetypal member; and the FKBP family, typified by FKBP12. It is important to note, however, that not all immunophilins are capable of binding an immunosuppressant, so the term immunophilin is somewhat misleading. Most cell types exhibit a range of different cyclophilins and FKBPs. For example, the human repertoire is so far known to include sixteen distinct cyclophilins, ranging in size from 18 kDa right up to 358 kDa, and sixteen different FKBPs, ranging from 12 to 135 kDa (Fischer and Aumüller, 2003). The proteome of the nematode, *Caenorhabditis elegans,* includes thirteen cyclophilins (Galat, 2003) and nine FKBPs (Galat, 2004), the yeast, *Saccharomyces cerevisiae,* includes eight cyclophilins and four FKBPs, and the plant, *Arabidodpsis thaliana*, includes eighteen cyclophilins and twenty-two FKBPs (Galat, 2003). The parvulin family of proteins, members of which have been

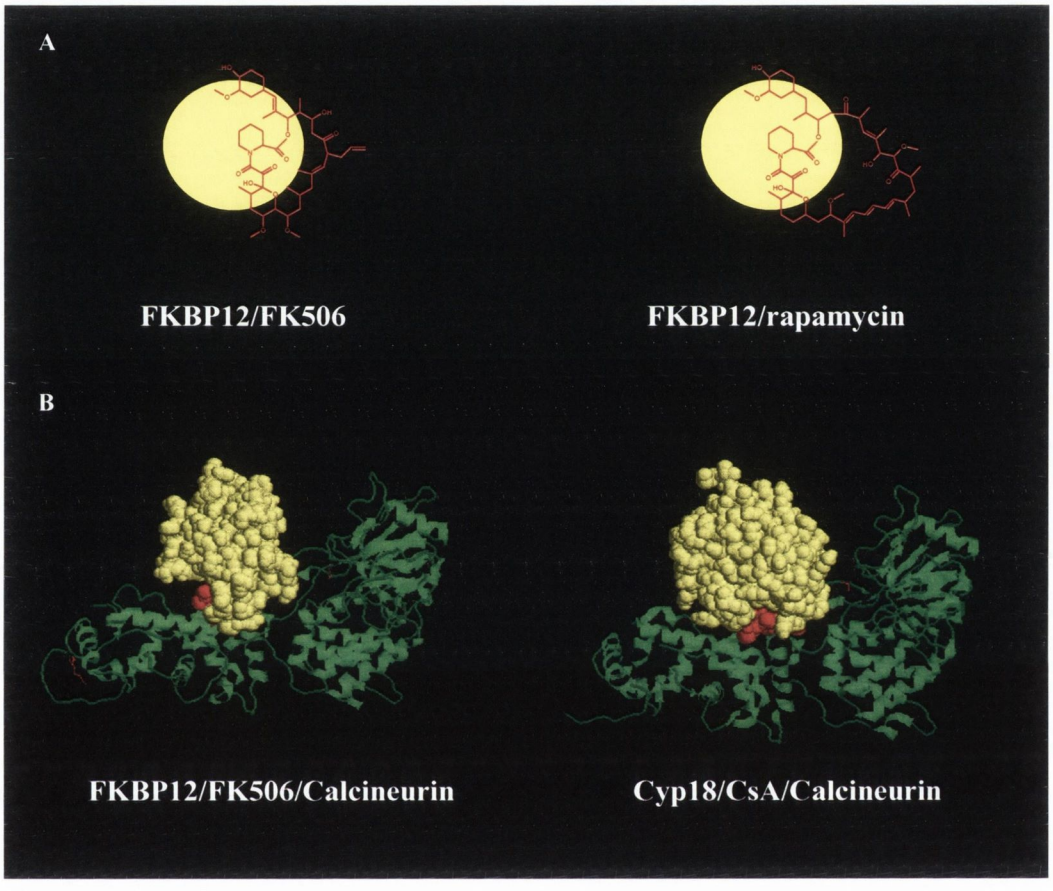


Fig. 1.6. Drug–immunophilin complexes. **A.** Two-dimensional representation of binary complexes (not to scale) formed between hFKBP12 (yellow) and either FK506 or rapamycin (red), showing the regions of both drugs that are bound by hFKBP12. **B.** Three-dimensional structure of FKBP12/FK506/calcineurin ternary complex (PDB accession # 1TC0) and Cyp18/CsA/calcineurin (PDB accession # 1MF8). Calcineurin is depicted as green ribbon, and the immunosuppressive drugs (FK506 and CsA) and their cognate receptor proteins (FKBP12 and Cyp18) are depicted as red and yellow spacefill models respectively. FK506 and CsA are shown in red, and FKBP12 and Cyp18 are shown in yellow.

identified in all eukaryotic organisms where examined, represents the third family of PPIases (Kay, 1996). Two members of this family, Pin1 and Pin2, have been identified in humans (Fischer and Aumüller, 2003). Parvulins show no sequence similarity to either FKBPs or cyclophilins, and because they do not bind immunosuppressants, they are not classified as immunophilins.

Most, but not all, immunophilins exhibit PPIase activity to some extent, with cyclophilins in general being more catalytically active in this regard than FKBPs. Whether this represents the natural function of immunophilins, however, remains a matter of debate. There is no doubt that most immunophilins catalyse the *cis-trans* isomerisation of peptidyl-prolyl bonds of short peptides *in vitro*, but there is limited evidence for such a function in intact cells. The PPIase debate centres on whether immunophilins directly or indirectly isomerise peptidyl-prolyl bonds. It has been proposed that immunophilins do not enzymatically alter partner proteins, but merely bind them at peptidyl-prolyl dipeptide motifs to fulfil other functions, such as stabilisation of non-native conformations (Schreiber and Crabtree, 1992; Kay, 1996). The observed isomerisation of the peptidyl-prolyl peptide bond of the partner protein could result from it being randomly released in either of the two possible conformations.

Many researchers in the field remain convinced that protein folding, mediated through isomerisation of peptidyl-prolyl peptide bonds, represents the primary function of immunophilins. However, there is growing evidence that immunophilins play other important roles in various aspects of protein dynamics in cells. From herein, only FKBPs will be discussed, but many of the proposed functions also hold true for cyclophilins.

1.4. FK506-BINDING PROTEINS (FKBPs)

1.4.1. Structure of FKBPs

Before discussing the various proposed functions of FKBPs, it is important to summarise what is known about the three dimensional structures of members of this protein family. NMR-elucidated structures of human FKBP12 (hFKBP12) show the protein to consist of an anti-parallel, five-stranded β-sheet wrapped around a central α-helix (Fig. 1.7A; Michnick *et al.*, 1991; Rosen *et al.*, 1991). The fifth β-

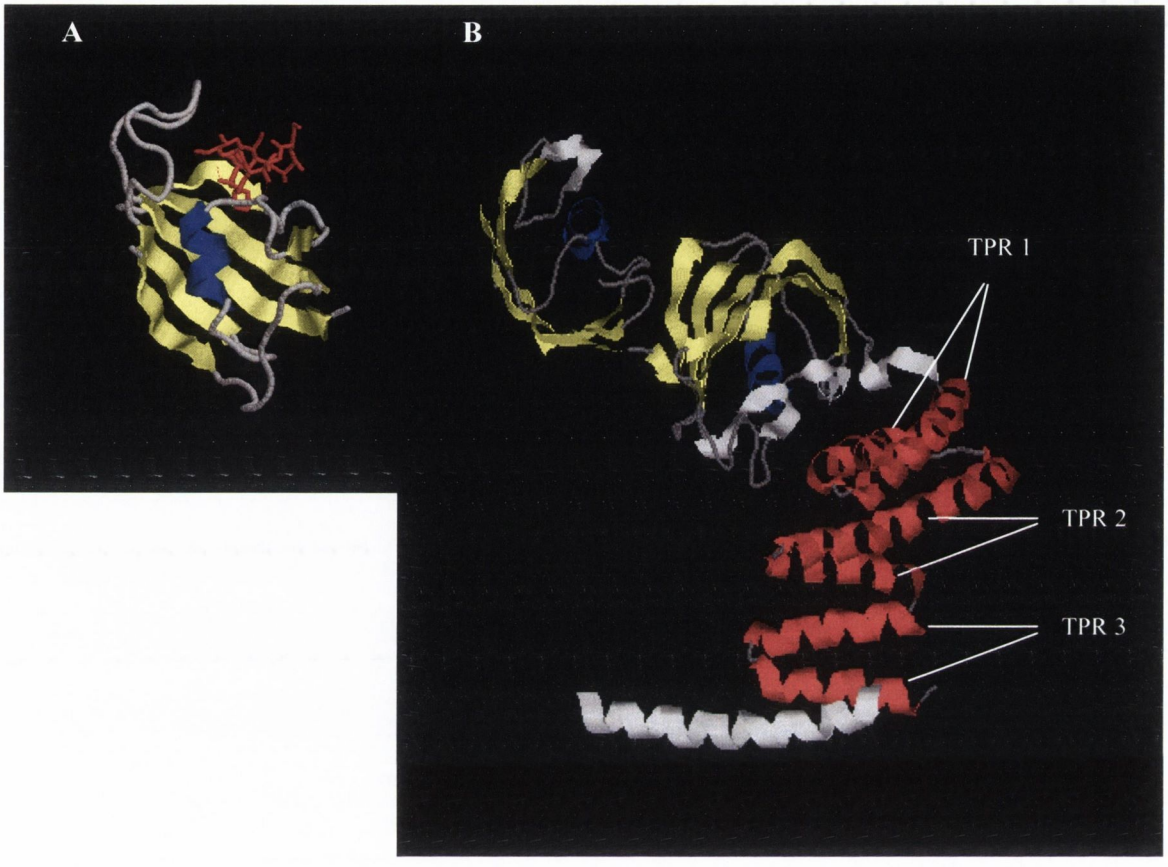


Fig. 1.7. X-ray crystal structures of hFKBP12 and hFKBP51. **A.** X-ray crystal structure of hFKBP12 complexed to FK506 (accession # 1FKF). The five-stranded anti-parallel β-sheet (depicted in yellow) wraps around a central α-helix (coloured blue). Bound FK506 is depicted in red. **B.** X-ray crystal structure of hFKBP51 (PDB accession # 1KT0). hFKBP51 incorporates two FKBP domains, similar in structure to hFKBP12, in the N-terminal half of the molecule (colour of β-sheet and central α-helix of each FKBP domain as per panel A). The C-terminal region, which includes three TPR motifs (red), is comprised of mainly α-helical structure. Each TPR motif comprises two anti-parallel α-helices.

strand is split by an eight-residue insertion that forms a large bulge in the sheet. The +3, +1, -3, +1 topology of the β-sheet (i.e. the first β-strand connects to β-strand 4, which in turn connects to β-strand 5, looping around to connect to β-strand 2 and on to β-strand 3) requires a highly unusual crossing of two intervening loops previously thought to be forbidden in anti-parallel β-sheets due to structural constraints (Richardson, 1977; Ptitsyn and Finkelstein, 1980). This structure of FKBP12 has been confirmed by X-ray crystallography of hFKBP12 bound to FK506 or rapamycin (Van Duyne *et al.*, 1991 and 1993), hFKBP12 bound to a non-immunosuppressive FK506 analogue (Becker *et al.*, 1993), and the ternary hFKBP12-FK506-calcineurin complex (Kissinger *et al.*, 1995; Griffith *et al.*, 1995). These structures reveal that the drugs (FK506, rapamycin, or 18-OH FK520, a non-immunosuppressive synthetic FK506 analogue) bind in a hydrophobic cavity between the α-helix and β-sheet that is flanked by three loop regions (Fig. 1.7A).

Because hFKBP12 was the first family member to be discovered, and remains the smallest yet identified, the full hFKBP12 sequence constitutes what is now referred to as the FKBP domain. This FKBP domain is characteristic of all FKBP family members, with the degree of similarity varying. Some FKBPs, for example, are catalytically inactive due to substitutions of key residues in the active site. FKBPs larger than FKBP12 include additional sequence outside the core FKBP domain (Fig. 1.8). Many family members, such as hFKBP52 and hFKBP65, comprise repeat units of the FKBP domain. However, only the first of these FKBP domains is catalytically active in these proteins. In many cases other motifs are present, such as tetratricopeptide repeat (TPR) motifs or calmodulin-binding motifs (Fig. 1.7B and Fig. 1.8).

TPR motifs are common to many of the larger molecular weight FKBPs. TPRs are 34-amino acid repeat motifs that have been identified in a range of proteins of diverse biological function (Blatch and Lässle, 1999; D'Andrea and Regan, 2003). These motifs exhibit limited identity at the primary sequence level, but at the secondary structure level they are similar: each repeat adopts a helix-turn-helix arrangement thereby forming two anti-parallel α-helices, with adjacent repeats packing in a parallel fashion, resulting in a spiral of repeating anti-parallel α-helices (Fig. 1.7B). Although the precise physiological function of such motifs remains to be fully elucidated, they are strongly implicated in mediating protein-protein

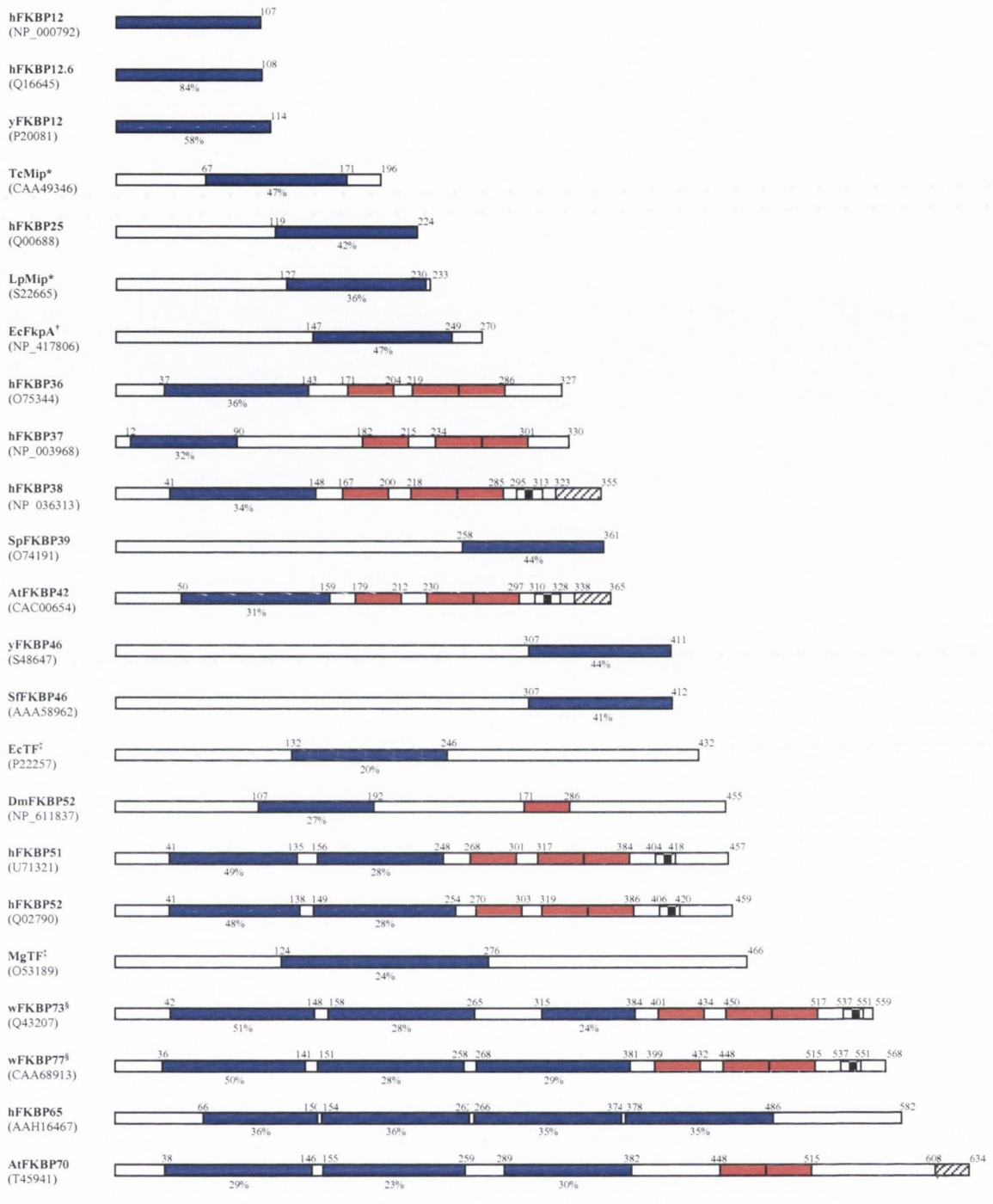


Fig. 1.8. Comparison of the modular structure of representative FKBP domain proteins. Most proteins are named according to the organism in which it is found (prefix: h, human; y, yeast (*Saccharomyces cerevisiae*); Tc, *Trypanosoma cruzi*; Lp, *Legionella pneumophila*; Ec, *Escherichia coli*; Sp, *Schizosaccharomyces pombe*; At, *Arabidopsis thaliana*; Sf, *Spodoptera frugiperda*; Dm, *Drosophila melanogaster*; Mg, *Mycoplasma genitalium*; w, wheat (*Triticum aestivum*) and its molecular weight in kilodaltons (numerical suffix). Blue boxes represent FKBP domains, red boxes symbolise TPR motifs, checkered boxes indicate calmodulin-binding motifs, and hatched boxes represent transmembrane segments. Residue numbers above each protein designate the positioning of domains in relation to primary sequence. The percentage values beneath the FKBP domains refer to their percentage identity to the primary sequence of hFKBP12. Genbank accession numbers are given in parenthesis. * Mip = Macrophage infectivity potentiator. † The 26-kDa *E. coli* FKBP homologue is termed FkpA. ‡ TF = trigger factor. § The accepted designation of wFKBP73 and wFKBP77 is retained here, although their respective molecular weights are only 62 kDa and 64 kDa.

interactions. The most common arrangement of TPRs in proteins is a tandem arrangement of three consecutive motifs, although as many as 16 repeats dispersed as various tandem units have been described.

1.4.2. Functions of FKBPs

This multiplicity of FKBPs with varying domain architectures argues in favour of these proteins having numerous, specialised cellular functions rather than acting as general folding catalysts. Indeed, although no definitive cellular function has yet been described, the list of proposed functions of FKBPs is diverse and continues to grow. The modular structures of the various FKBPs described in the following sections are depicted in Fig. 1.8.

1.4.2.1. Modulation of membrane protein function

One physiological role of FKBP12 appears to be in modulating the function of large membrane proteins. hFKBP12 is known to interact physiologically with the two major intracellular Ca^{2+}-release channels, the type-1 ryanodine receptor (RyR-1) and the type-1 inositol 1,4,5-trisphophate receptor (IP_3R-1) (Jayaraman *et al.*, 1992; Brilliantes *et al.*, 1994; Mackrill *et al.*, 2001; Cameron *et al.*, 1995a; Dargan *et al.*, 2002). These two are structurally-related large tetramers located in the sarcoplasmic reticulum membrane of skeletal muscle and the endoplasmic reticulum membranes, respectively, and participate in a vast array of signalling pathways by modulating intracellular Ca^{2+} levels. In both cases, FKBP12 is tightly associated with the channel and appears to be a physiological subunit of the channel protein complex. Dissociating FKBP12 from either channel perturbs the physiologic calcium flux of the channel. FKBP12 is not absolutely necessary for channel function, but rather exerts a stabilising function. Without the associated FKBP, channels do not open fully, and remain open for longer periods of time. FKBP12 makes the channels harder to open, but more stable and responsive once open, thereby optimising Ca^{2+} release into the cytosol. The 12.6-kDa homologue, hFKBP12.6, selectively interacts with type-II ryanodine receptor (RyR-II), the form found in cardiac muscle (Lam *et al.*, 1995; Ono *et al.*, 2000; Marks, 2002). Intriguingly, cardiac muscle from failing heart shows a tremendous decrease in hFKBP12.6 levels associated with the receptor (Yano *et al.*, 2000; Marx *et al.*, 2000).

The mechanism by which hFKBP12 and hFKBP12.6 stabilise these Ca^{2+}-release channels remains unknown. Although hFKBP12 dissociates from the complex upon treatment with FK506 and rapamycin, the intrinsic PPIase activity of hFKBP12 is not implicated as it has been shown that hFKBP12 mutants that lack PPIase activity can still bind to and modulate the function of both receptors (Timerman *et al.*, 1995; Cameron *et al.*, 1997). The latest hypothesis is that hFKBP12 serves as a scaffolding molecule to link the receptor with another regulatory protein. Intriguingly, the most likely candidate for this regulatory protein is calcineurin. Cameron and colleagues were the first to report co-purification of calcineurin with both RyR-1 and IP_3R-1 (Cameron *et al.*, 1995b). These interactions were confirmed by co-immunoprecipitation experiments, and were inhibited by FK506 and rapamycin, but not CsA. The calcineurin associated with the IP_3R-FKBP12 was shown to be catalytically active towards a peptide substrate. Within the complex, the calcineurin component reversed protein kinase C-mediated phosphorylation of IP_3R. These findings led them to the hypothesis that the Ca^{2+} flux through IP_3R is modulated by the phosphorylation status of the receptor, with calcineurin playing an active role in the dynamic regulation through a dephosphorylation mechanism.

Subsequent work by the same group used the yeast-2-hybrid system to identify the site in IP_3R that binds FKBP12 (Cameron *et al.*, 1997). This was localised to a leucyl-prolyl dipeptide motif. Yeast-3-hybrid analysis showed that binding at this site allowed FKBP12 to interact with calcineurin, thereby providing further evidence that FKBP12 modulates IP_3R activity by anchoring calcineurin to the complex. Intriguingly, this leucyl-prolyl dipeptide epitope structurally resembles a specific region of both FK506 and rapamycin that is bound by FKBP12. It had long been speculated that the gain of function of FKBP12 upon binding these two drugs resulted from peptide mimicry. This finding by Cameron and colleagues provided physiological evidence that FK506 promoted an FKBP12-calcineurin interaction by mimicking structurally similar dipeptide epitopes present within physiological substrates that use FKBP12 to anchor calcineurin.

This mechanism of functional modulation by FKBP12 through calcineurin is also proposed for transforming growth factor-β (TGF-β) type-I receptors (TβR-I). The TGF-β family consists of many structurally-related small peptides that regulate

a wide range of crucial cell growth and differentiation events. The cellular response to TGF-β is elicited through signal transduction cascades initiated by binding of TGF-β to its heterodimeric receptor on the cell surface. This receptor is composed of two serine/threonine kinase subunits. TβR-II is responsible for the actual binding, but TβR-I is responsible for initiating the signal pathway upon phosphorylation by the intrinsic kinase activity of TβR-II. The first report of an interaction of hFKBP12 with TβR-I came from yeast-2-hybrid and co-immunoprecipitation studies (Wang *et al.*, 1994). Subsequently it was shown that hFKBP12 is associated with TβR-I only in the absence of TGF-β (Wang *et al.*, 1994). Once TGF-β binds to TβR-II, the phosphorylation of TβR-I results in the dissociation of hFKBP12, thereby facilitating the propagation of the signal. The authors proposed that hFKBP12 functions as an inherent inhibitor of signalling pathways mediated by TβR-I. This inhibitory effect of hFKBP12 was abolished by mutations known to disrupt binding to calcineurin while maintaining PPIase activity. This led to the conclusion that the inhibitory effect of hFKBP12 on TβR-I is not mediated by its PPIase activity, but more likely by its ability to anchor calcineurin to TβR-I. Because TβR-I is activated by phosphorylation, the authors suggested that calcineurin acts as a natural inhibitor of TβR signalling. This mechanism of regulation was further supported by the finding that a leucyl-prolyl dipeptide motif in TβR-I is the site of interaction with FKBP12 (Charng *et al.*, 1996; Chen *et al.*, 1997).

A similar role for hFKBP12 in regulating the receptor for epidermal growth factor (EGF) has been reported (Lopez-Ilasaca *et al.*, 1998). hFKBP12 appears to inhibit the autophosphorylation of the EGF receptor, thereby preventing the receptor from initiating signalling cascades. It is known that the protein tyrosine phosphatase SHP1 is tightly associated with the EGF receptor, leading the authors to conclude that the inhibitory actions of hFKBP12 on EGF receptor autophosphorylation is mediated through SHP1 rather than a direct suppression of the receptor's inherent tyrosine kinase activity. However, unlike the regulation TβR-I, the PPIase activity of FKBP12 appears to be essential for EGF receptor regulation as hFKBP12 mutants devoid of PPIase activity lost their ability to inhibit autophosphorylation of the EGF receptor.

FKBP12 has also been implicated in regulating P-glycoprotein, a large membrane ATPase conserved from bacteria to humans. Little is known about the

natural functions of P-glycoproteins except for their involvement in multidrug resistant phenotypes when they act as efflux pumps that reduce the intracellular concentration of small hydrophobic molecules. This phenomenon underlies the growing problem of chemotherapy-resistant tumours. The first hint that FKBP12 may be involved in P-glycoprotein function came from reports that both FK506 and rapamycin can partially overcome multidrug resistance *in vitro* (Epand and Epand, 1991; Pourtier-Manzanedo *et al.*, 1991; Arceci *et al.*, 1992; Naito *et al.*, 1992; Jachez *et al.*, 1993). Expression of the murine P-glycoprotein homologue, mdr3, in *S. cerevisiae* rendered yeast resistant to dactinomycin, a cytotoxic compound and a known P-glycoprotein substrate (Hemenway and Heitman, 1996). However, when expressed in a yeast strain lacking yFKBP12, the ability of mdr3 to confer resistance to this cytotoxin was severely compromised. Episomal expression of wild type yFKBP12 in this strain restored mdr3 function, as did expression of yFKBP12 mutated to abolish PPIase function. The finding that mdr3 conferred drug resistance in a yeast strain in which the gene for the regulatory subunit of calcineurin was disrupted ruled out a role for calcineurin in mdr3 regulation. This suggests that yFKBP12 plays a direct physiological role in mdr3 function, but this is independent of PPIase activity. The ability of FK506 and rapamycin to reverse multidrug resistance could, therefore, be a direct consequence of these drugs disrupting FKBP12–P-glycoprotein complexes.

A role in the regulation of plant P-glycoprotein homologues has been proposed for the 42-kDa FKBP from *Arabidopsis thaliana* (AtFKBP42) (Geisler *et al.*, 2003). *A. thaliana* exhibits numerous P-glycoprotein homologues, and AtFKBP42 was shown to specifically interact with two of these – AtPGP1 and AtPGP19. AtFKBP42 was shown to be an integral membrane protein, anchored to the membrane by its extreme C-terminal hydrophobic helical region (Kamphausen *et al.*, 2002; Geisler *et al.*, 2003). Fractionation and co-immunoprecipitation studies suggest that AtFKBP42 and AtPGP1 interact with each other at the plasma membrane (Geisler *et al.*, 2003). It was subsequently shown that AtFKBP42 also interacts with two other membrane ATPases distinct from P-glycoproteins. AtMRP-1 and AtMRP2 colocalise with AtFKBP42 to the vacuolar membrane (Geisler *et al.*, 2004). Interestingly, the interactions of the immunophilin with AtPGP1 and AtPGP19 are mediated through the N-terminal FKBP domain of

AtFKBP42 (Geisler *et al.*, 2003), while the C-terminal TPR domain mediates the interaction with both AtMPR1 and AtMRP2 (Geisler *et al.*, 2004). As recombinant AtFKBP42 is devoid of PPIase activity (Kamphausen *et al.*, 2002), the involvement of such catalytic activity in the regulation of these membrane transporters seems unlikely.

1.4.2.2. Transport of cytosolic receptor complexes

Perhaps the best-studied physiological role for FKBPs is their involvement in cytosolic hetero-oligomeric receptor complexes. Steroid hormones (glucocorticoids, mineralocorticoids, estrogens, androgens and progestins) mediate their action by binding to specific receptors (Zubay, 1993). The glucocorticoid receptor (GR) is predominantly cytolsolic, but upon hormone-binding the hormone-receptor complex translocates across the nuclear membrane. Once in the nucleus, the complex forms a functional transcription factor by dimerising with an identical hormone-receptor complex and binds to enhancer regions of specific genes to initiate transcription. In the absence of hormone, GR requires continuous interactions with molecular chaperones to establish and maintain the receptor in a conformation competent for ligand binding and for intracellular trafficking. The involvement of major chaperone components, most notably heat shock protein 90 (Hsp90), Hsp70, Hsp40 and heat shock organising protein (Hop), along with various co-chaperones, are well established (reviewed in Pratt and Toft, 2003; Pratt *et al.*, 1999).

The discovery that an FKBP was a component of steroid receptor complexes was made independently by two groups who showed that an unknown protein, previously shown to be a component of several steroid receptor complexes through an interaction with Hsp90 (Renoir *et al.*, 1990; Rexin *et al.*, 1991), was a novel member of the FKBP family (Lebeau *et al.*, 1992; Yem *et al.*, 1992). This protein was later shown to contain two FKBP-type PPIase domains, three TPR motifs, and a calmodulin-binding domain, and was termed hFKBP52 (Callebaut *et al.*, 1992; Peattie *et al.*, 1992; Kieffer *et al.*, 1993; Ratajczak *et al.*, 1993). An identical modular structure was described for hFKBP51, a protein with ~75% similarity to hFKBP52, and this was also shown to be a component of steroid receptor complexes (Smith *et al*, 1993a and 1993b). Indeed, the modular structure of hCyp40, which can compete with both hFKBP51 and hFKBP52 for binding to

Hsp90 in the steroid receptor complex, is remarkably similar (see Fig 3.9; Ratajczak and Carrello, 1995). The TPR domain of hFKBP52 (and presumably hFKBP51 and hCyp40) facilitates the interaction with Hsp90, and therefore with the entire complex (Radanyi *et al.*, 1994). The PPIase domain of hFKBP52 was shown to bind to cytoplasmic dynein, the motor protein that shuttles cellular cargo along microtubules towards the nucleus (Silverstein *et al.*, 1999; Galigniana *et al.*, 2002). This interaction is independent of the intrinsic catalytic activity, however, as FK506 has no effect on the interaction (Silverstein *et al.*, 1997). Together with the previous observations that hFKBP52 associates with microtubules, as judged by immunofluorescene techniques (Czar *et al.*, 1994; Perrot-Applanat *et al*, 1995), this suggests that hFKBP52 targets the receptor complex to the retrograde transport system.

Although the involvement of hFKBP51 and hFKBP52 in GR complexes is not in doubt, the precise function they play in the complex remains uncertain. The role of hFKBP52 in these complexes has been studied more intensely than that of hFKBP51, but recent work is shedding light on important differences between the two interactions. A recent study in *S. cerevisiae,* which lacks FKBP51 or FKBP52 homologues, showed that expression of hFKBP52, but not hFKBP51, significantly increased the activity of a vertebrate GR reporter system (Riggs *et al.*, 2003). hFKBP51 blocked this hFKBP52-mediated potentiation. Furthermore, it was shown that hFKBP52 increased the affinity of GR for hormone, while hFKBP51 resulted in a lowered affinity, suggesting that the two FKBPs serve to differentially regulate the hormone-binding state of the receptor. This correlates with the observation that squirrel monkeys, which show extremely high levels of FKBP51, are insensitive to cortisol (Reynolds *et al.*, 1999; Denny *et al.*, 2000). Indeed, an *in vitro* assay based on measuring hFKBP51 levels in blood cells is currently being developed for identifying patients that are hypo- or hypersensitive to glucocorticoids (Vermeer *et al.*, 2004). Davies and colleagues argue that hormone binding to the receptor complex induces a substitution of hFKBP51 for hFKBP52, an event that facilitates transport of the entire complex to the nucleus via the microtubule/dynein retrograde transport system (Davies *et al.*, 2002). The rate of transport of the GR complex to the nucleus is unaffected by FK506, providing further evidence that the PPIase activity of bound immunophilins is not involved in this process (Galigniana *et al.*, 2001). The importance of hFKBP52 in providing a functional link between the

receptor complex and the retrograde transport system is supported by the nuclear translocation process being impeded by microinjection of antibody against hFKBP52 (Czar *et al.*, 1995), by coexpression of a PPIase domain fragment of hFKBP52 (Galigniana *et al.*, 2001), and by chemical inhibition of Hsp90, the protein that tethers hFKBP52 to the heterocomplex (Galigniana *et al.*, 1998).

Steroid receptors are not the only class of cytosolic receptors for which a physiological interaction with an FKBP has been described. The aryl hydrocarbon receptor (AHR) is recovered from the cytosol in AHR-Hsp90 complexes that contain a 37-kDa FKBP homologue (hFKBP37). AHR is a ligand-activated transcription factor responsible for mediating cellular responses to a variety of environmental pollutants, the most notable of which are the dioxins (reviewed in Gu *et al.*, 2000). Upon ligand binding, the AHR complex translocates to the nucleus and heterodimerises with Arnt (*AR*H *n*uclear *t*ranslocater). The resulting AHR-Arnt heterocomplex has high affinity for specific enhancer regions of DNA located in a network of genes encoding metabolic enzymes such as cytochrome P450, glutathione S-transferase and quinone oxidoreductase.

The hFKBP37 component of the AHR heterocomplex was originally identified by three laboratories as: AIP (*A*HR *I*nteracting *P*rotein – Ma and Whitlock, 1997); ARA9 (*A*HR *A*ssociated *P*rotein 9 – Carver and Bradfield, 1997); and XAP2 (*H*epatitus B virus *X*-*A*ssociated *P*rotein 2 – Meyer *et al.*, 1998). These synonyms are still widely used, but hFKBP37 will be used here. Like the three immunophilins involved in steroid receptor heterocomplexes, hFKBP37 exhibits an N-terminal PPIase domain linked to a C-terminal tripartite TPR domain. The PPIase domain of hFKBP37, which exhibits 32% identity to hFKBP12, is catalytically inactive and lacks affinity for FK506 (Carver *et al.*, 1998). The C-terminal region, incorporating the TPR domain, interacts with both Hsp90 and AHR (Kazlauskas *et al.*, 2002). The exact physiological function of hFKBP37 in the receptor heterocomplex remains largely unknown, but it appears to play an important role in stabilising the complex (Kazlauskas *et al.*, 2002; Lees *et al.*, 2003).

p53 is a transcription factor that shuttles between the cytosol and the nucleus (David-Pfeuty *et al.*, 1996; Komarova *et al.*, 1997). Co-immunoadsorption experiments performed recently by Galigniana and colleagues showed cytosolic p53 to be part of a heterocomplex remarkably similar to those discussed above for unliganded steroid receptors and the aryl hydrocarbon receptor (Galigniana *et al.*,

2004). *In vivo* experiments showed p53 to undergo dynein-dependent translocation to the nucleus, an event that was inhibited by overexpression of the PPIase fragment of hFKBP52. In rabbit reticulocyte lysate, exogenous PPIase domain fragment of hFKBP52 was shown to compete with the p53 heterocomplex for binding of dynein. Taken together, these show unequivocally that the PPIase domain of hFKBP52, at least, links the heterocomplex to the retrograde transport system.

An involvement of FKBPs in receptor heterocomplexes is not restricted to mammalian cells. Reports of the participation of FKBPs in such complexes in insect and plant cells have been made. An FKBP from the tobacco hornworm, *Manduca sexta*, is a component of the receptor for ecdysone, a steroid hormone that plays a pivotal role in insect morphogenesis (Song *et al.*, 1997; Fig. 1.8 shows FKBP46 from *Spodoptera frugiperda* [SfFKBP46]. The FKBP from *M. sexta* was identified by immunoprecipitation with antibody generated against purified SfFKBP46, but the sequence remains unpublished). It was recently shown that incubation of mouse GR with wheat germ lysate yielded GR heterocomplexes that contained wheat Hsp90 (wHsp90) and either wFKBP73 or wFKBP77 (Harrell *et al.*, 2002). Furthermore, this complex was shown to bind mammalian dynein, a process that was blocked by a competing PPIase domain fragment of wFKBP77. Although plants do not have glucocorticoid receptors, they possess homologues of all the chaperones and co-chaperones found in mammalian hetero-oligomeric complexes, such as Hsp90, Hsp70, Hop, and FKBPs. This led the authors concluded that TPR-containing FKBPs play a fundamental role in the intracellular transport of signalling proteins in eukaryotic cells by linking them to the retrograde transport system.

1.4.2.3. Regulation of transcription

By modulating the function of ligand-free steroid receptors and facilitating their transport to their site of action in the nucleus, FKBPs are indirectly involved in transcriptional regulation. However, a more direct involvement in this process has been proposed for certain FKBPs.

One of the most recently described functions of an FKBP is that of the nuclear FKBPs from *S. cerevisiae* (yFKBP46) and *Schizosaccharomyces pombe* (SpFKBP39) (Kazuhara and Horikoshi, 2004). Both these proteins were shown to act as histone chaperones *in vitro* in that they promoted nucleosome assembly in the presence of histones. Histone chaperones, together with enzymes such as histone

acetylase and histone deacetylase, play essential roles in the dynamics of chromatin structure, thereby regulating the access of transcriptional complexes to the DNA (reviewed in Schreiber and Bernstein, 2002). The chaperone function of both these proteins was shown to be independent of PPIase activity.

A role in chromatin remodelling has also been reported for hFKBP25 (Yang *et al.*, 2001). The involvement of hFKBP25 in this process, however, is a consequence of its interaction with histone deacetylase (HDAC) enzymes rather than any intrinsic activities. Co-immunoprecipitation experiments showed that hFKBP25 interacted with HDAC1 and HDAC2. Although this interaction was independent of PPIase activity, the authors speculated that the catalytic activity of hFKBP25 might be involved in the modification of proline-rich histones to facilitate chromatin achieving a compact, yet stable, state.

The same report also described an interaction of hFKBP25 with the transcription factor Yin Yang - 1 (YY1). Previous studies by the same group had shown this transcription factor to interact with hFKBP12 (Yang *et al.*, 1995). Although YY1 interacts with hFKBP12, which is comprised entirely of an FKBP domain, the FKBP domain of hFKBP25 is dispensable for its interaction with YY1. Indeed, the interaction between YY1 and hFKBP12, but not hFKBP25, was abrogated by FK506. Most transcription factors either activate or repress transcription, but YY1 is unusual in that it can either positively or negatively affect transcription of genes. It is thought that this transcriptional activation/repression effect of YY1 is dictated by its ability to interact with different cellular proteins. hFKBP25 was shown to dramatically increase the DNA-binding activity of YY1 (Yang *et al.*, 2001), and overexpression of FKBP12 enhanced YY1-mediated transcriptional repression of a reporter gene (Yang *et al.*, 1995). Taken together, these data suggest that FKBPs act as physiological regulators of YY1, thereby playing important roles in cellular transcriptional events.

Kunz and colleagues used yeast 2-hybrid analysis to demonstrate an interaction of yFKBP12 with a newly identified transcription factor, FAP1 (*F*KBP12-*a*ssociated *p*rotein-1; Kunz *et al.*, 2000). Rapamycin, or mutations in the active site of yFKBP12, abrogated this interaction. Although no functional significance of this interaction was shown, the authors speculated that yFKBP12 serves to regulate the transcriptional activity of FAP1. Intriguingly, FAP1 exhibits

10 Leu-Pro/Val-Pro dipeptide motifs within the DNA binding motif, providing a possible interaction site for yFKBP12.

1.4.2.4. Non-enzymatic protein folding

The initial discovery that FKBPs exhibited PPIase activity *in vitro* immediately suggested a role in protein folding. There is growing physiological evidence that many FKBPs do indeed perform important protein folding duties, but not mediated through their intrinsic enzymatic activity. In general, only small, single domain proteins fold spontaneously to their native conformation. The folding of larger, more complex proteins is often dependent upon assistance from molecular chaperones that prevent incorrectly or incompletely folded polypeptide chains from aggregating (Leroux and Hartl, 2001). Certain chaperones bind to unfolded, nascent polypeptides during or immediately after translation, while others assist in refolding proteins that have misfolded due to cellular stress. Another subset of chaperones keeps proteins in a transport-competent state during intracellular trafficking. Unlike PPIases, the involvement of chaperones in protein folding is non-enzymatic. Numerous FKBPs have been shown to possess non-enzymatic protein folding capabilities, leading to the suggestion that certain FKBPs are involved in general protein folding events independent of peptidyl-prolyl isomerisation.

E. coli expresses a 48-kDa cytosolic FKBP homologue known as trigger factor (TF) that isomerises peptidyl-prolyl bonds with a very high efficiency. Indeed, it is a much more effective folding catalyst than cyclophilins or other FKBPs (Stoller *et al.*, 1995). TF binds with high affinity to protein substrates, but only when the substrate is in an unfolded state (Scholz *et al.*, 1998). Furthermore, this interaction with unfolded protein chains is independent of proline residues. TF is physiologically linked to the large ribosomal subunit, near the peptide exit channel and binds to virtually all nascent polypeptides (Hesterkamp *et al.*, 1996; Kramer *et al.*, 2002; Valent *et al.*, 1995). This has led to the hypothesis that TF is a general co-translational chaperone, interacting with nascent chains as they emerge from the translational complex. Although the TF of *E. coli* is by far the best studied, this protein is conserved in many bacteria, including *Mycoplasma genitalium*, the organism with the smallest known genome, highlighting the evolutionary importance of this particular FKBP in prokaryotes (Hesterkamp and Bukau, 1996; Scholz *et al.*, 1998; Bang *et al.*, 2000).

TF exhibits a modular structure: the function of the C-terminal region remains unknown; the N-terminal region directs its interaction with the ribosome; and the central region harbours the PPIase active site (Stoller *et al.*, 1996; Hesterkamp and Bukau, 1996). The PPIase domain of TF exhibits only weak similarity to FKBP12 (Callebaut and Mornon, 1995) and FK506 has no effect on the PPIase activity (Stoller *et al.*, 1995). Indeed, recent work using *E. coli* mutants lacking TF has shown that episomally-expressed TF deficient in PPIase capability effectively complemented the deletion of the wild type gene, thereby showing that the PPIase activity of TF is not required for the folding of newly synthesised proteins (Kramer *et al.*, 2004). The PPIase domain, however, is responsible for TF's association with unfolded protein substrates, but this is independent of proline residues (Patzelt *et al.*, 2001). The function significance of the intrinsic PPIase activity of TF, therefore, remains unknown.

E. coli and other Gram-negative bacteria encode numerous proteins responsible for maintaining periplasmic proteins in the correct conformation. One of these, FkpA, is a member of the FKBP family (Horne and Young, 1995; Missiakas *et al.*, 1996). FkpA exhibits a catalytic efficiency comparable to other FKBPs, and this PPIase activity is sensitive to FK506. It had been thought that the PPIase activity of FkpA played a role in restructuring proteins in the periplasm, but recent work disputes this, suggesting instead that FkpA has a more general role in protein folding. This was first suggested by Botham and Plückthun when they showed that FkpA assisted in the folding of antibody fragments devoid of *cis*-prolines (Botham and Plückthun, 2000). Indeed, FkpA folded antibody fragments devoid of any proline residues. Similar results were found for the folding of antibody fragments in an *in vitro* system (Ramm and Plückthun, 2000). Work in Betton's lab using active-site and deletion variants of FkpA further demonstrated the PPIase-independent folding capabilities (Arié *et al.*, 2001). Functional FkpA is a homodimeric protein comprised of ~26 kDa subunits (Ramm and Plückthun, 2001). The monomer exhibits two distinct domains: the PPIase domain is located in the C-terminal region (Arié *et al.*, 2001), while the N-terminus is responsible for the chaperone function (Saul *et al.*, 2004). The functional significance of the PPIase activity remains unclear.

1.4.2.5. Development

A growing body of evidence performed in various model systems suggests that FKBPs play important roles in developmental processes. A proposed role for FKBP65 was suggested by work performed on embryonic chick gut (Fukuda *et al.*, 1998). This work showed that FKBP65 was expressed in smooth muscle precursor cells, and upon differentiation, this expression was restricted to smooth muscle cells. By overexpressing FKBP65 in cultured mesenchymal cells that had become dissociated from the embryonic gut lining – such dissociated cells fail to differentiate into smooth muscle cells under normal conditions – successful differentiation into smooth muscle cells was achieved, leading the authors to conclude that FKBP65 plays a vital role in smooth muscle differentiation. Moreover, this differentiation process was inhibited by FK506.

A role in development has also been suggested for a 70-kDa FKBP from the plant *A. thaliana* (AtFKBP70; also referred to as PAS1; Vittorioso *et al.*, 1998). This protein is upregulated by cytokinins, a class of plant hormones involved in cell division and differentiation, suggesting a role for AtFKBP70 in the cytokinin signalling pathway. Natural mutants of *A. thaliana* that exhibit embryonic and vegetative developmental defects were shown to encode a truncated form of AtFKBP70. Complementation of such mutants with the full-length protein restored normal developmental phenotypes.

Another naturally occurring *A. thaliana* mutant exhibits dramatic growth defects due to deletion of AtFKBP42 (Kamphausen *et al.*, 2002; Geisler *et al.*, 2003). Such mutants exhibit twisted growth and have organs that are dramatically shorter than in wild type plants, giving rise to the designation "twisted dwarf mutants". AtFKBP42, as mentioned previously, is localised to the cytosolic side of the plasma and vacuolar membranes. Geisler and colleagues suggested that AtFKBP42 plays an important role in plant development by regulating auxin transport through its interactions with AtPGP1 and AtPGP19 (Geisler *et al.*, 2003). Auxins are a class of plant hormones that effect several aspects of plant development, with the stimulation of cellular elongation and leaf development being the most important functions. Because plants lacking AtFKBP42 do not respond to brassinolide, a steroid-like hormone essential for cell division and morphogenesis (Ecker, 1997), Kamphausen and colleagues proposed that AtFKBP42 interacts with

the membrane-bound brassinolide receptor and is involved in the propagation of the hormone-induced signalling cascade (Kamphausen *et al.*, 2002).

An analogous role in initiating the cellular response to growth-promoting signals was suggested for a recently identified FKBP from *Drosophila melanogaster* (Munn and Steward, 2000). DmFKBP52 (alternatively named 'shut-down') is expressed in germline stem cells and is an absolute requirement for oogenesis and spermatogenesis, with natural mutations of the gene encoding DmFKBP52 resulting in male and female sterility. Because DmFKBP52 exhibits a PPIase domain fused to a TPR domain, the authors speculate that this protein plays a pivotal role in signalling events that regulate cell division.

Although not studied in great detail, hFKBP36 has been proposed to be involved in the developmental process in some manner. This is suggested by the finding that the gene encoding this protein maps to a region of chromosome 7 that is deleted in patients with the developmental disorder known as William's syndrome (Meng *et al.*, 1998).

1.4.2.6. Miscellaneous functions

A number of assorted functions for various FKBPs have been proposed. A role for a subclass of microbial FKBPs as virulence factors is well described. *Legionnella pneumophila* is an intracellular bacterial pathogen and the etiological agent of Legionnaires' disease, a distinct form of pneumonia. The finding that a strain of *L. pneumophila* devoid of a 24-kDa surface protein was dramatically reduced in its infectivity of macrophages in culture led to this protein being designated the acronym Mip (*M*acrophage *i*nfectivity *p*otentiator; Cianciotto *et al.*, 1989). It wasn't until Fischer and colleagues identified regions of homology with FKBPs, and subsequently showed a recombinant form to have FK506-inhibitable PPIase activity, that Mip was shown to be a member of the FKBP family (Fischer *et al.*, 1992). Interestingly, the structural organisation of Mip - a homodimer comprised of monomers with a C-terminal PPIase domain – is very similar to that of FkpA.

Legionella species can infect various cell types. In their natural aquatic habit they colonise free-living protozoa, while in mammals, they replicate within alveolar macrophages, epithelial cells and blood monocytes. Using a Mip$^-$ strain of *L. pneumonophila* complemented with site-directed or deletion variants of Mip, it

was recently shown that the PPIase activity of Mip appears to play no part in infection of *Acanthamoeba castellanii*, a natural host. However, the same study showed that the PPIase activity seems necessary to establish full virulence in a guinea pig model of human infection (Köhler *et al.*, 2003). The physiological function of the PPIase activity of Mip, therefore, remains unclear.

The mechanism by which Mip acts as a virulence factor remains unknown, but it seems clear that it is involved in the ability of *L. pneumophila* to survive within the host cell rather than in the actual invasion process, as Mip^- *L. pneumophila* strains associate with amoeboid host cells as efficiently as Mip^+ strains (Cianciotto *et al.*, 1990). Whether Mip is involved in the replication process or in some form of resistance mechanism that inhibits phagosome-lysosome fusion inside the host cell remains unanswered.

Mip is not unique to *L. pneumophila*, with at least 35 species of *Legionella* expressing a Mip homologue (Ratcliff *et al.*, 1997). The remarkable degree of conservation in the PPIase domain among all the *Legionella* Mip homologues argues in favour of this enzymatic activity playing a physiological role. Mips have also been identified in other intracellular pathogens. *Chlamydia trachomatis*, the causative agent of lymphogranuloma venereum, one of the most prevalent sexually transmitted diseases, expresses a 27-kDa homologue (Lundemose *et al.*, 1993), and *Coxiella burnetii*, the etiological agent of Q fever, produces a 25.5-kDa homologue (Mo *et al.*, 1995). *Trypanosoma cruzi*, the protozoan responsible for Chagas' disease, or American trypanosomiasis, produces a Mip that, unusually, is secreted (Moro *et al.*, 1995; Pereira *et al.*, 2002). The processed form of this protein is 18.8 kDa in size and is secreted predominantly by the invasive trypomastigote form. Unlike the archetypal *L. pneumophila* Mip, the *T. cruzi* homologue appears to function in the process of host cell penetration. Both FK506 and a non-immunosuppressive derivative, both of which inhibit the PPIase activity of recombinant *T. cruzi* Mip, potently inhibit this invasion process, arguing in favour of a PPIase-mediated invasion process (Moro *et al.*, 1995).

A role for hFKBP65 in the secretion of tropoelastin has been suggested by Davis and colleagues (Davis *et al.*, 1998). Tropoelastin is a soluble protein that, upon secretion, assembles into elastin - insoluble elastic fibrils that constitute a major component of the extracellular matrix. Immunoprecipitation of tropoelastin from crosslinked foetal bovine chondrocyte lysates resulted in the co-

immunoprecipitation of FKBP65. The presence of a putative endoplasmic reticulum (ER) retention sequence at the C-terminus of FKBP65 (Coss *et al.*, 1995), suggests that FKBP65 may play a role in the folding of tropoelastin within the ER. This is reminiscent of the proposed function of CypB in the folding of procollagen, the soluble form of collagen, the other major component of the extracellular matrix (Smith *et al.*, 1995). Although still speculative, the fact that proline residues comprise approximately 12% of tropoelastin leads to the attractive proposition that the PPIase activity of FKBP65 functions in the folding of this protein.

A role for hFKBP38 in preventing apoptosis has recently been suggested (Shirane and Nakayama, 2003). Yeast 2-hybrid studies showed hFKBP38 to interact physiologically with both Bcl-2 and Bcl-x_L, two essential proteins that prevent cells from undergoing untimely apoptosis. The localisation of these proteins in the mitochondrion is a pre-requisite for their anti-apoptotic function, and Shirane and Nakayama provided evidence that hFKBP38 targets and anchors these two proteins to the membrane of this organelle. Interestingly, the possibility that hFKBP38 acts as an endogenous regulator of calcineurin was suggested by the finding that both these proteins co-immunoprecipitated in the absence of FK506. The phosphatase activity of calcineurin is inhibited by this binding event.

The findings that FKBP12 expression levels in the brain are as much as 50 times higher than in the immune system (Steiner *et al.*, 1992) and expression of this protein is elevated during neuronal regeneration (Lyons *et al.*, 1995), suggests that FKBP12 plays a role in either neuroregeneration or neuroprotection processes. Indeed, recent work has shown increased levels of hFKBP12 in surviving neurons of the brain in patients suffering from Parkinson's disease, Alzheimer's disease and dementia (Avramut and Achim, 2002). FKBP12 is not the only member of the FKBP family present in neurons, with FKBP25, FKBP38, FKBP52 and FKBP65 also showing high levels of expression (Gold *et al.*, 1999; Brecht *et al.*, 2003; Klettner *et al.*, 2001). Intriguingly, the distribution of FKBP12 in neurons closely matches that of calcineurin (Lyons *et al.*, 1995; Dawson *et al.*, 1994). The main impetus in understanding the functions of FKBPs in neurons comes from the potent neurotrophic and neuroregenerative effects of FK506 and its analogues. Certain analogues show remarkable neurotrophic effects – the non-immunosuppressive analogue GPI-1046 is effective at low picomolar concentrations (Synder *et al.*, 1998). Unlike most neuroregenerative compounds, uncontrolled outgrowth of

healthy neurons does not occur with FKBP ligands as they appear to be selective only for damaged neurons (Hamilton *et al*, 1997; Steiner *et al.*, 1997). The development of FK506 analogues specifically for this purpose is the focus of intense pharmaceutical interest. The mechanisms by which these drugs exert their effects, and the physiological function of FKBPs in neurons, remain unknown.

1.5. PROJECT OBJECTIVES

P. falciparum contains at least three cyclophilins (Hirtzlin *et al.*, 1995; Reddy, 1995; Berriman and Fairlamb, 1998) and recent work by Bell and colleagues shows that two of these (PfCyp19A and PfCyp19B) appear to be the major CsA-binding proteins of the parasite, suggesting an involvement in the anti-malarial activity of this drug (Gavigan *et al.*, 2003). The likely presence of members of the other class of immunophilins, the FKBPs, in the parasite was suggested by the anti-malarial activities of both FK506 and rapamycin (Bell *et al.*, 1994).

The work documented in this thesis was undertaken to understand the mechanism by which these two drugs exert their anti-malarial activities. This project had four main aims:

1) to identify *P. falciparum* FKBP homologues;
2) to provide evidence that FK506 and rapamycin mediate their anti-malarial activities through one or more *P. falciparum* FKBPs;
3) to ascribe cellular functions to *P. falciparum* FKBP(s);
4) to study the potential of non-immunosuppressive derivatives of FK506 as novel anti-malarial drugs.

Chapter 2

Materials & Methods

2.1. CHEMICALS AND REAGENTS

All reagents were from Sigma Aldrich (Tallaght, Ireland) unless otherwise stated. FK506 was a kind gift of Fujisawa GmbH (Munich, Germany). All derivatives of FK506 were a kind gift of Kosan Biosciences, Inc. (Hayward, California, USA). The RIG plasmid was a kind gift of Wim Hol (University of Washington, USA). The anti-PfPP5 antiserum was kindly provided by Mo-Quen Klinkert (University of Hamburg, Germany). All reagents used during electrophoresis were of electrophoresis grade. All chemicals used for cell culture were cell culture tested. Double distilled, deionised water was used throughout.

2.2. CULTURE OF AND EXPERIMENTS WITH *P. FALCIPARUM*

2.2.1. Routine culture

P. falciparum FCH5.C2, a cloned subline of strain FCH5/Tanzania adapted to grow in horse serum (Bell *et al.*, 1993), was maintained in continuous culture in human erythrocytes (whole blood obtained from the Irish Blood Transfusion Board, St. James Hospital, Dublin, and erythrocytes extracted as described in section 2.2.2) according to the method of Trager and Jensen (1976). Parasites were cultured routinely in culture medium (RPMI 1640 medium supplemented with 25 mM HEPES, 0.01% (v/v) neomycin sulphate, 0.18% (w/v) sodium bicarbonate, 50 μg/ml hypoxanthine, 10% heat-inactivated horse serum) and washed erythrocytes (at a haematocrit of 2.5% or 5% [v/v]). Parasites were maintained in Petri dishes at 37°C in a candle jar. Culture medium was replaced depending on the parasitemia. Parasitemia was monitored by microscopic examination of Giemsa-stained smears.

2.2.2. Treatment of whole human blood

Erythrocytes were prepared from whole human blood as follows. A portion of whole blood was mixed with 1/10 volume of PIGPA solution (50 mM sodium pyruvate, 50 mM inosine, 100 mM glucose, 500 mM disodium hydrogen phosphate, 5 mM adenine, made up in 0.9% NaCl) and incubated at 37°C for 1 h with periodic mixing. The mixture was centrifuged in a Sorvall RT6000D benchtop centrifuge (DuPont, Hertfordshire, UK) at 500 x *g* at 4°C for 10 min and the supernatant and

buffy coat removed by aspiration. The pellet was washed twice with sterile, cold phosphate buffer saline (PBS – Oxoid, Hampshire, UK) and once in incomplete culture medium (culture medium without horse serum or neomycin sulphate) with careful removal of any residual buffy coat between each wash. Packed erythrocytes were resuspended in an equal volume of culture medium to give a final haematocrit of 50%. Erythrocytes prepared in this manner were stored at 4°C and deemed suitable for use for 1 week.

2.2.3. Synchronisation of *P. falciparum* cultures

Synchronisation of *P. falciparum* cultures to a specific developmental stage was achieved by the method of Lambros and Vanderberg (1979). Briefly, cultures were centrifuged at 650 x *g* at room temperature for 10 min. The pellet was resuspended in pre-warmed, filter-sterilised sorbitol (5% w/v) and left for 5 min to allow the sorbitol to accumulate in erythrocytes infected with mature parasites and promote osmotic lysis. Parasites were centrifuged again, washed in pre-warmed incomplete medium and the resulting pellet resuspended in culture medium and cultured as normal. The above treatment resulted in cultures of ring-stage parasites only (0 to ~18 h post invasion). More highly synchronised cultures were obtained by repeating the sorbitol treatment 36 h after the initial treatment (for rings [~0 to 6 h post invasion]), followed by growth for a further 12 h (for late rings/early trophozoites [~12-18 h post invasion]), 24 h (for mature trophozoites [~24-30 h post invasion]) or 36 h (for schizonts and segmenters [~36-48 h post invasion]).

2.2.4. Harvesting of *P. falciparum* cultures

Free parasites were released from infected erythrocytes according to the method of Zuckerman (1967). Briefly, cultures (10-20% parasitemia) were centrifuged at 650 x *g* at 4°C for 10 min and washed twice in cold PBS. The pellet was incubated with ice-cold saponin solution (0.05% [w/v] prepared in saline sodium citrate [SSC] buffer [150 mM NaCl, 15 mM sodium citrate, pH 7.0]) for 20 min on ice with vigorous shaking every 5 min to lyse erythrocyte membranes and release parasites. Freed parasites were pelleted by centrifugation at 975 x *g* at 4°C for 15 min and washed twice with cold SSC buffer. Pellets were either used immediately or resuspended in freezing solution (10% [v/v] glycerol, 2 mM phenyl-

methylsulphonyl fluoride [PMSF], 1 μg/ml pepstatin A, 20 μg/ml leupeptin, prepared in PBS) for storage at –80°C.

2.2.5. Preparation of whole cell extracts of *P. falciparum*.

Extracts of *P. falciparum* were prepared from harvested pellets. When frozen pellets were used, cells were thawed slowly on ice to minimise protease activity. Pellets were incubated with 1% (v/v) Triton X-100 for 30 min on ice, with vigorous mixing every 5-10 min, and centrifuged at 18,000 x *g* for 5 min at 4°C in a microfuge. The supernatant was carefully removed to a fresh microfuge tube, leaving behind the unwanted cellular debris. The supernatant was centrifuged twice more to ensure removal of residual cellular debris.

2.2.6. Isolation of *P. falciparum* genomic DNA

Asynchronous parasites, harvested from human erythrocytes as described in section 2.2.4., were lysed in 10 volumes of lysis buffer [10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 10 mM EDTA, 400 mM NaCl, 5% (v/v) sodium dodecyl sulphate (SDS), 0.2 mg/ml proteinase K (Roche, Basle, Switzerland)] overnight at 37°C. The lysate was incubated with an equal volume of phenol/chloroform/isoamyl alcohol (15:14:1) for 15 min at 65°C, and centrifuged at 18,000 x *g* for 10 min. The extraction was repeated before precipitating the genomic DNA from the aqueous phase by incubating with 2 volumes of ethanol at –70°C for 30 min. The precipitated DNA was pelleted by centrifugation at 18,000 x *g* for 15 min, left to air dry for 10-15 min, and resuspended in an appropriate volume of deionised water (dH_2O).

2.2.7. Inhibition of growth of *P. falciparum*.

To assess the effects of FK506 and other related compounds on cultured *P. falciparum*, asynchronous parasitised human erythrocytes at 0.8% parasitemia and 2% haematocrit were grown in RPMI culture medium supplemented with the appropriate compound in 96-well flat bottom microtitre plates for 72 hours. Compounds were diluted from stock solutions into culture medium, and then serially diluted two-fold in wells of the microtitre plates down to sub-inhibitory concentrations. Following incubation, the effect of the compounds on parasite

growth was determined using the parasite lactate dehydrogenase (LDH) assay of Makler et al. (1993). This assay correlates the activity of *P. falciparum* LDH (PfLDH) to parasite growth, exploiting one of the biochemical characteristics that distinguishes PfLDH from host LDH. The parasite enzyme has the ability to utilise 3-acetyl pyridine adenine dinucleotide (APAD) as a co-factor to generate pyruvate from lactate (Makler *et al.*, 1993). Human erythrocyte LDH can also use APAD, but at a much slower rate. The activity of PfLDH results in the formation of the reduced form of ADAP. Briefly, 10 µl of each culture were transferred to wells in a fresh 96-well plate and mixed with 50 µl of Malstat (Flow Inc., Portland, Oregon, USA), which contains L-lactate and APAD. 10 µl of a mixture of nitroblue tetrazolium (NBT) and phenazine ethosulphate (PES) (1:1 mixture of NBT [2 mg/ml] and PES [0.1 mg/ml]) were added to each well and the plate was incubated at room temperature in the dark for 1 h or longer This allows for the detection of PfLDH as, in the presence of the reduced form of APAD, NBT is itself reduced, forming a blue formazan product than can be detected either visually or spectrophotometrically at 650 nm. Uninfected erythrocytes served as negative controls to determine the background level of LDH in human erythrocytes, while parasites cultured in the absence of inhibitors served as positive controls for the measurement of 100% PfLDH activity. Dose response curves were constructed for each drug. The drug concentrations required to inhibit parasite growth by 50% (IC_{50}) were determined graphically from the respective dose-response curves.

2.2.8. Stage-specific susceptibilities of *P. falciparum* to inhibitors

To assess which developmental stages of the intra-erythrocytic cycle of *P. falciparum* was most susceptible to FK506 and rapamycin, growth inhibition assays were set up exactly as described in section 2.2.7, except cultures were first synchronised to the desired stage (see section 2.2.3) and growth was assessed at various time points during drug exposure.

2.2.9. Assessment of cidal or static inhibitory effect of compounds

To assess whether inhibitory effects of compounds were reversible or irreversible, mature trophozoite-stage cultures were incubated with high concentrations of drug for 0, 3, 6 or 9 h. At relevant time points, cultures were

washed free of drug by transferring samples to microfuge tubes, mixing with pre-warmed incomplete medium, and centrifuging at 500 x *g* at room temperature for 10 min. The pellets were washed once more with pre-warmed incomplete medium, and finally resuspended in pre-warmed complete medium, maintaining the original parasitemia and haematocrit. Cultures were then transferred to microtitre plates and incubated for a further 48 h (one complete erythrocytic growth cycle). Blood smears were made for each culture, stained with Giemsa, and parasites were counted and morphologically characterised.

2.2.10. Metabolism of compounds by cultured parasites.

The ability of parasites to metabolise 18-ene-20-oxa-FK520 and 13-dM(Me)-18-ene-20-oxa-FK520 into other forms was assessed by treating *P. falciparum* cultures with 5 μM of these compounds for 48 hours (corresponds to ~IC_{30}). Cultures were transferred to microfuge tubes and frozen at –70°C in preparation for analysis by mass spectrometry. Cells treated identically, but exposed to FK520 or 13-dM(Me) FK520, served as controls. Mass spectrometry was performed by Maria Fardis and Peter Revill at Kosan Biosciences Inc, Hayward, California, USA.

2.3. CLONING OF *PfFKBP35*

2.3.1. Amplification of *PfFKBP35* gene by polymerase chain reaction (PCR)

The PfFKBP35 sequence was identified in the genome of *P. falciparum* 3D7 by tBLASTn analysis (www.ncbi.nlm.nih.gov/BLAST/) using the consensus FKBP domain amino acid sequence described by Kay (1996). Primers (5'-GCGCGGATC CATGACTACCGAACAAGAATTTG-3' and 5'-GCGCCTGCAGCTTATAAGAA ATATTAATTTGC-3') were used to amplify the coding sequence of the predicted protein with a *Bam*HI site and a *Pst*I site at the 5' and 3' ends, respectively, to facilitate subsequent cloning into the pQE30 expression vector (see section 2.3.4). "Hot-start" PCR was performed using ~2 μg *P. falciparum* genomic DNA (section 2.2.6.), 0.4 μM primers, 2.5 units of *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, California, USA) and 0.2mM each of dATP, dTTP, dGTP and dCTP (Roche)

in a Hybaid thermocycler (94°C for 5 min, 55°C for 1 min, 72°C for 1 min; followed by 29 cycles of 94°C for 1 min, 55°C for 1min, 72°C for 1 min; followed by 1 cycle of 94°C for 1 min, 55°C for 1 min, 72°C for 10 min).

2.3.2. Visualisation of DNA by agarose gel electrophoresis

DNA samples (PCR samples, purified plasmids, restriction enzyme-treated plasmids, etc.) were typically analysed by mixing 5 µl of sample with 1 µl of 6X loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol, 10 mM EDTA) and running through a 0.7 – 1.0% (w/v) agarose gel prepared in tris acetate EDTA (TAE) buffer (40 mM Tris-HCl, 2 mM EDTA, 1 M acetic acid). Gels were typically run at 60-100 V until the desired degree of separation was achieved (assessed visually by migration of loading buffer). Gels were stained by immersion in ethidium bromide (0.5 µg/ml) until the desired degree of staining was achieved (typically ~15 min), washed briefly in dH_2O, and DNA was visualised by exposing the gel to ultraviolet (UV) light using an AlphaImager 2200 gel imaging system (Alpha Innotech Corporation, San Leandro, California, USA).

2.3.3. Purification of PCR products

PCR products were purified by one of two methods, both of which utilised glass fibre membranes to affinity-purify DNA. If only a single product was visible on an ethidium bromide-stained agarose gel, then the PCR product was purified directly from solution (i.e. the PCR sample) using a High Pure PCR purification kit (Roche). If other products resulting from non-specific priming were evident, the product of interest was excised from an agarose gel and purified using a PerfectPrep Gel Cleanup purification kit (Eppendorg, Hamburg, Germany).

2.3.4. Generation of pQE-PfFKBP35

pQE30 (Qiagen, Crawley, UK) was isolated from *E. coli* XL-1 Blue cells (Stratagene) using a Qiagen Mini plasmid purification kit (Qiagen). Purified pQE-30 and the PCR-amplified PfFKBP35 coding sequence (see section 2.3.3) were sequentially digested with *Bam*HI and *Pst*I (Roche). Briefly, 10 µl reactions were set up in microfuge tubes using 0.5-1 µg DNA, 10 units of *Bam*HI, 1 µl of 10X

*Bam*HI buffer and 3 µl of dH_2O. Tubes were incubated for 2 h in a 37°C water bath. DNA was purified using phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation (see section 2.3.7), and ligated together using 1 unit of T4 DNA Ligase (Roche) in a 16°C water bath overnight. The ligase enzyme was subsequently inactivated by incubating the mixture at 65°C for 10 min. Competent *E. coli* XL-1 Blue cells, prepared as described in section 2.3.8, were transformed using the heat-shock method (see section 2.3.9) and plated on to L-agar (10% tryptone, 5% yeast extract, 5% NaCl, 1% agar) supplemented with 100 µg/ml of ampicillin (Amp; Roche) and incubated overnight at 37°C. Resulting colonies were screened for the presence of the desired construct (pQE-PfFKBP35) as described in section 2.3.10.

2.3.5. Generation of pMAL-PfFKBP35

The *PfFKBP35* gene was sub-cloned from pQE-PfFKBP35 into pMAL-c2X (New England Biolabs, Hertfordshire, UK). The *PfFKBP35* insert from pQE-PfFKBP35 was amplified by PCR using the same conditions as described in section 2.3.1, except using pQE-PfFKBP35 as template. This product, along with purified pMAL-c2X, was treated as described in section 2.3.4. In this case, *E. coli* TB1 (New England Biolabs) was used as the recipient strain, to create pMAL-PfFKBP35.

2.3.6. Generation of pMAL-PfFKBP35-His_6, pMAL-FKBP-His_6, pMAL-TPR-His_6 and pMAL-His_8 by inverse PCR

pMAL-PfFKBP35 served as the template in an inverse PCR to create pMAL-PfFKBP35-His_6. Briefly, two primers (5'-GCGCCTCGAGTCATTTCTTAT AAGCTGCAGGCAAGC-3' and 5'-GCGCCTCGAGTTAGTGATGGTGATGGT GATGATTTGCACTATTTTTTTTTTC-3') were used such that priming occurred in opposite directions from the extreme 3'end of the PfFKBP35 coding sequence. This resulted in the replication of pMAL-PfFKBP35 with the addition of sequence encoding a His_6 tag, a stop codon and a *Xho*I site at one end, immediately downstream of the PfFKBP35 sequence, and a *Xho*I site at the other. The PCR conditions and cycle parameters were as described in 2.3.1, except with 10 ng of template per reaction, and an elongation time of 16 min. The resulting product was digested with *Xho*I and subsequently ligated to itself to create pMAL-PfFKBP35-

His_6. This construct in turn served as the template in inverse PCRs to generate pMAL-FKBP-His_6 (directs expression of MBP-FKBP-His_6 [i.e. the FKBP domain of PfFKBP35 only]) using the primers 5'-GCGCACTAGTCATCACCATCACCAT CAC-3' and 5'-GCGCACTAGTTCTAAAGCTTAATAATTCAATTTC-3'; pMAL-TPR-His_6 (directs expression of MBP-TPR-His_6 [i.e. the TPR domain of PfFKBP35 only]) using the primers 5'-GCGCACTAGTGAAGCTAAAAAAAGTA TATAT-3' and 5'-GCGCACTAGTGGATCCGAATTCTGAAATC-3' and pMAL-His_6 (directs expression of MBP-His_8 - note that an extra two His residues resulted from a cloning artifact) using the primers 5'-GCGCACTAGTCATCACCATCACC ATCAC-3' and 5'-GCGCACTAGTGGATCCGAATTCTGAAATC-3'. These primer sets introduced the recognition sequence for *Spe*I immediately following the stop codon in each construct, facilitating circularisation of the respective PCR products by digestion with *Spe*I and ligation with T4 ligase.

2.3.7. Recovery of DNA by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation

DNA was recovered from solution by vigorously mixing samples with an equal volume of phenol/chloroform/isoamyl alcohol (15:14:1) and centrifuging at 18,000 x *g* for 5 min in a microfuge. Supernatants were carefully removed to fresh microfuge tubes and mixed well with an equal volume of chloroform and centrifuged again at 18,000 x *g* for 5 min. Supernatants were carefully removed to fresh microfuge tubes and mixed with 2 volumes of 100% ethanol and 1/10 volume 3M sodium acetate (pH 7.5) and incubated at –20°C for ~30 min. The precipitated DNA was pelleted by centrifugation at 18,000 x *g* for 15 min, left to air dry for 10-15 min, and resuspended in an appropriate volume of dH_2O.

2.3.8. Preparation of competent *E. coli* cells

A single colony of the desired strain of *E. coli* was used to inoculate 50 ml of L-broth (10% tryptone, 5% yeast extract, 5% NaCl) and the culture grown overnight at 37°C with agitation (200 rpm). 4 ml of this culture was used to inoculate 400 ml of fresh L-broth in a 2-l flask, and grown to an optical density (OD) at 595 nm (OD_{595}) of 0.375. The culture was divided equally amongst four pre-chilled sterile polypropylene tubes and left on ice for 5-10 min. Cells were centrifuged at 1600 x *g* for 7 min in a Sorvall RC-50 Plus centrifuge at 4°C and left

to decelerate without applying the brake. Supernatants were discarded and the pellets gently resuspended in 20 ml sterile, ice-cold $CaCl_2$ solution (60 mM $CaCl_2$, 15% [v/v] glycerol, 10 mM PIPES, pH 7.0). Cells were centrifuged at 1100 x *g* for 5 min at 4°C, and resulting pellets were resuspended in 20 ml of ice-cold $CaCl_2$ solution and incubated on ice for 30 min. Cells were centrifuged at 1100 x *g* for 5 min at 4°C, and resulting pellets were resuspended in 4 ml of ice-cold $CaCl_2$ solution, dispensed into pre-chilled sterile microfuge tubes (typically 500-1000 µl volumes), snap frozen in liquid nitrogen, and transferred immediately to a –70°C freezer.

2.3.9. Transformation of *E. coli*

Competent *E. coli* XL-1 Blue cells were transformed using the heat-shock method essentially as described by Maniatis *et al.* (1982). 200 µl amounts of competent cells, thawed on ice for 30 min, were mixed with 6 µl of ligation mixture in sterile microfuge tubes and left on ice for 30 min. The tubes were heat-shocked for 2 min in a 43°C water bath, placed back on ice for a further 2 min prior to addition of 1 ml of pre-warmed L-broth, and incubated in a 37°C water bath for 1 h. 100 µl volumes of each sample were plated in triplicate on L-agar supplemented with 100 µg/ml Amp and incubated overnight at 37°C. Cells transformed with the parental vector served as positive controls, while cells transformed with the DNA sample digested with the relevant enzymes but left unligated served as negative controls.

2.3.10. Screening of transformants

Putative transformants obtained after overnight growth on L-agar (supplemented with appropriate antibiotic, usually Amp) were screened by a number of methods. All methods involved replica-plating colonies of putative transformants onto fresh L-agar (supplemented with appropriate antibiotic) using either sterile cocktail sticks or pipette tips and using the remainder of the colony for the screening procedure. Once clones were identified, DNA sequence analysis (performed by the Advanced Biotechnology Centre, Imperial College, London, UK) was used to confirm that the constructs included the correct sequence.

2.3.10.1. Rapid colony screening

Colonies were mixed with 30 µl of pre-warmed lysis buffer (10% [w/v] sucrose, 100 mM NaOH, 60 mM KCl, 5 mM EDTA, 0.25% [v/v] SDS, 0.05% [w/v] bromophenol blue) in microfuge tubes and incubated in water bath at 37-45°C for 5 min. Samples were placed on ice for 5 min and centrifuged for 5 min at 18,000 x *g* for 5 min in a microfuge to pellet cellular debris. 15 µl of each supernatant were loaded directly onto an agarose gel and treated as described in section 2.3.2. Following ethidium bromide staining, recombinant plasmids were identified by virtue of slower migration through the gel when compared to the parental vector.

2.3.10.2. Small scale induction

The rapid colony screening approach is not applicable when there is an insignificant size difference between the construct of interest and the parental vector. In such cases, colonies were screened by assessing their ability to express the recombinant protein of interest. Putative transformants were used to inoculate 3 ml of L-broth (supplemented with appropriate antibiotic) in a test-tube. Cultures were grown overnight at 37°C with agitation (200 rpm). The following morning, two 1/10 dilutions of each culture were prepared in L-broth (supplemented with appropriate antibiotic) and grown until they reached an OD_{600} of 0.5-0.7 (or whatever OD_{600} was recommended for the particular vector system). To one set of cultures, filter-sterilised isopropylthiogalactopyranoside (IPTG – Melford Laboratories, UK) was added to the recommended concentration (typically 0.35 – 1.0 mM), while the other set of cultures were supplemented with sterile dH_2O (same volume as IPTG) to serve as negative controls. The cultures were grown for a further 3 hr at 37°C. ~1 ml of each culture was transferred to fresh microfuge tubes (the remainder of each culture was discarded) and centrifuged at 18,000 x *g* in a microfuge for 5 min. Pellets were washed in PBS, re-centrifuged, and finally resuspended in 40 µl of PBS. 40 µl of 2X SDS loading buffer (see section 2.5.3) were added, and 5 µl volumes analysed by SDS-PAGE (see section 2.5.3). Production of recombinant protein from each original colony was assessed by comparing protein profiles (following Coomassie blue staining of SDS-PAGE gels [see section 2.5.5]) for samples in the presence/absence of IPTG.

2.3.10.3. Screening by PCR

PCR was performed on plasmids obtained from putative transformants to confirm the presence of the insert. Bacterial colonies were lysed by mixing with a few microlitres of dH_2O and boiling for 10 min. Following a brief spin to pellet cellular debris, 5 μl of the lysate was used as the template in a PCR (conditions as described in 2.3.1) using primers specific for either the insert itself or vector regions flanking the insert. Alternatively, plasmids were obtained by purification using either a Qiagen Mini plasmid purification kit (Qiagen) or a FastPrep Mini plasmid purification kit (Eppendorf).

2.3.10.4. Screening by restriction endonuclease digestion

The presence of the insert of interest was assessed by the ability of restriction endonucleases, for which specific recognition sites had been introduced, to release the insert. Plasmids were obtained by purification using either a Qiagen Mini plasmid purification kit (Qiagen) or a FastPrep Mini plasmid purification kit (Eppendorf) and restriction endonuclease digestion was performed using the appropriate enzymes, as described in 2.3.4. The presence of the released insert in the resulting mixture was assessed by electrophoresis through an agarose gel.

2.4. Analysis of Expression by Reverse Transcriptase PCR (RT-PCR)

Two-stage RT-PCR was performed on total RNA isolated from *P. falciparum* using RNA Isolater. Firstly, cDNA synthesis was performed on ~2μg RNA in the presence of oligo$(dT)_{12-18}$ primer, 0.2 mM of each dNTP, 1/20 RNAguard, 10 mM dithiothreitol and 30 units of avian myeloblastosis virus (AMV) reverse transcriptase (all from Roche). The samples were incubated at 42°C for 45 min, followed by 5 min at 95°C to inactivate the enzyme. Amplification of cDNA was performed as for the PCR described in section 2.3.1, except with the template being cDNA rather than genomic DNA.

2.5. PROTEIN ANALYSIS

2.5.1. Determination of protein concentration by Bradford assay

Protein concentration was determined by the method of Bradford (1976), which is based on the binding of Coomassie Brilliant Blue G to protein. A series of known amounts of bovine serum albumin (BSA, typically ranging from 1-15 μg) were made up to 100 μl with PBS, mixed well with 1 ml of Bradford reagent (0.01% [w/v] Coomassie Brilliant Blue G-250, 0.25% [v/v] ethanol, 10% [v/v] orthosphosphoric acid, filtered and stored at 4°C) and left at room temperature for 20 min. Absorbances of the BSA protein standards were measured at 595 nm, and a standard curve was constructed, from which the concentration of samples of the protein of interest, prepared and analysed in the same manner as the BSA standards, could be calculated.

2.5.2. Determination of concentration of citrate synthase by Beer-Lambert Law

Prior to use in the chaperone assays, the ammonium sulphate suspension of citrate synthase was dialysed into ≥100 x volumes of TE buffer (50mM Tris-HCl, pH 8, 2mM EDTA), concentrated to the original volume in a Microcon-10 concentrator (Millipore, Billeria, Massachusetts, USA), and the concentration was determined according to the Beer-Lambert law, $A = \varepsilon cl$, where A = absorbance at 280 nm, ε = molar extinction coefficient {1.78 $(mg/ml)^{-1}$ cm^{-1} for citrate synthase at 280 nm [Buchner *et al.*, 1998]}, c is protein concentration and l is the pathlength of the cuvette (typically 1 cm).

2.5.3. Analysis of proteins by SDS-polyacrylamide electrophoresis (SDS-PAGE)

Protein samples were electrophoretically separated by SDS-PAGE according to the method of Laemmli (1970). Separating gels typically consisted of 0.375 M Tris-HCl, pH 8.8, 12.5% (v/v) acrylamide/bisacrylamide (Protogel, National Diagnostics, Hessle Hull, UK), 0.1% (v/v) SDS, 0.06% (v/v) ammonium persulphate (APS) and 0.05% (v/v) N, N, N , N -tetramethyl-ethylenediamine (TEMED), and stacking gels consisted of 0.125 M Tris-HCl, pH 6.8, 4% (v/v)

acrylamide/bisacrylamide, 0.1% (v/v) SDS, 0.1% (v/v) APS and 0.1% (v/v) TEMED. Running buffer consisted of 0.025 M Tris-HCl, 1.9 M glycine, 0.1% (w/v) SDS. Protein samples were typically solubilised in an equal volume of 2X SDS loading buffer (0.125 M Tris-HCl, pH 6.8, 4% [v/v SDS, 20% [v/v] glycerol, 10% [v/v] β-mercaptoethanol, 0.002% (w/v) bromophenol blue, filtered to remove particulate material) by boiling for 5-10 min. The desired amount of protein sample (maximum volume of 30 μl) was loaded onto gel, and electrophoresed at 100 V through the stacking gel and 150-250 V through the separating gel. Proteins separated by this technique were visualised either by staining with Coomassie Blue (section 2.5.5), or transferred to a membrane for Western immunoblotting (section 2.5.7).

2.5.4. Separation of proteins by PAGE under non-denaturing or non-reducing conditions

For non-denaturing PAGE, gels were set up and run as described for SDS-PAGE (section 2.5.3) except no SDS was included in the system (i.e. separating/stacking gels, running buffer and loading buffer). For non-reducing PAGE, β-mercaptoethanol was omitted from the loading buffer.

2.5.5. Coomassie Brilliant Blue staining of SDS-PAGE gels

Gels were stained with Coomassie Blue reagent (0.15% [w/v] Coomassie Brilliant Blue R-250, 45% [v/v] methanol, 10% [v/v] acetic acid, filtered to remove particulate material) until the desired degree of staining was achieved (typically 1-4 h). Gels were subsequently destained in a solution of 20% (v/v) methanol/7.5% (v/v) acetic acid. When gels were sufficiently destained, they were exposed to white light using an AlphaImager 2200 gel imaging system and photographed.

2.5.6. Concentration of protein by precipitation with trichloroacetic acid (TCA)

In many cases, samples of low protein concentration were treated with TCA prior to separating by SDS-PAGE in order to avoid the lengthy silver staining procedure afterwards. Samples were mixed with an equal volume of ice-cold 25% (w/v) TCA, kept on ice for 5 min, and centrifuged at 18,000 x *g* for 5 min to pellet

precipitated proteins. The supernatant was discarded and the pellet resuspended in the desired volume (typically 20 µl so as to be able to load the whole sample into a well) of 1X reducing SDS loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% [v/v SDS, 10% [v/v] glycerol, 5% [v/v] β-mercaptoethanol, 0.002% (w/v) bromophenol blue, filtered to remove particulate material). If samples turned yellow upon addition of SDS loading buffer, the pH was rectified by adding a few crystals of Tris. Samples were boiled for 5-10 min prior to loading on gels.

2.5.7. Western immunoblotting

After electrophoresis, unstained polyacrylamide gels were sandwiched with polyvinylidiene difluoride (PVDF, Roche, pre-treated as per manufacturer's instructions) and immersed in a blotting tank with transfer buffer (0.025 M Tris-HCl, 1.9 M glycine, 24% [v/v] methanol). Proteins were transferred at 100 V for 1 h. After transfer, the membrane was blocked by soaking in 5% (w/v) non-fat milk prepared in Towbin's solution (0.1 M Tris-HCl, pH 7.4, 0.9% [w/v] NaCl] for 1 h. The blocked membrane was soaked with a solution of primary antibody diluted to the desired concentration (typically 1:500 – 1:10,000, depending on the particular antibody) with 5% (w/v) non-fat milk for 1-4 h, after which time the membrane was washed well in Towbin's/Tween solution (Towbin's solution supplemented with 0.05% [v/v] Tween-20). These steps were repeated with a solution of the secondary antibody. Bands were detected using a chemiluminscence system (Roche) according to the manufacturer's instructions using a Kodak X-OMAT 1000 automatic developer.

2.5.8. Stripping and re-probing PVDF membranes

Membranes were re-used by heating at 60°C in a solution of 62.5 mM Tris-HCl, pH 6.8, 2% (v/v) SDS, 0.7% (v/v) β-mercaptoethanol for 30 min. The membrane was drained and washed three times in Towbin's/Tween solution for 10 min. After blocking with 5% (v/v) non-fat milk, membranes were probed with desired primary antibody and processed as described in section 2.5.8.

2.6. PURIFICATION OF RECOMBINANT PfFKBP35

2.6.1. Harvesting and lysis of *E. coli* cells

Recombinant proteins were produced by inoculating L-broth, supplemented with 100 μg/ml Amp, with overnight cultures of the strain of *E. coli* harbouring the desired plasmid, and grown at 37°C with agitation at 200rpm to an OD_{600} of 0.5-0.7. Protein expression was induced by the addition of 0.35mM IPTG and the culture incubated for an additional 3 hours. Cells were harvested by centrifugation at 6,000 x *g* for 15 minutes at 4°C *g* in a Sorvall RC50 Plus centrifuge using a GSA rotor. Pellets were either used immediately or frozen at –20°C. Depending on the subsequent application, pellets were prepared for lysis by resuspending in either AAC buffer or MCAC-0 buffer (see section 2.6.2 and 2.6.3, respectively) supplemented with Complete Mini protease inhibitor tablets (Roche). The cells were lysed by passage through a French Press, and clarified by spinning at 35,000 x *g* in a Sorvall RC50 Plus centrifuge using an SS-34 rotor for 1 h at 4°C.

2.6.2. Amylose affinity chromatography

Amylose resin (New England Biolabs) was poured into a 1.5 cm x 5 cm column (Biorad, Hercules, California, USA) and washed with ≥ 8 column volumes of amylose affinity column (AAC) buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA). Clarified lysate (prepared in AAC buffer – see section 2.6.1) was applied to the column at a flow rate of 1-2 ml/min using a peristaltic pump, and resin washed with ≥12 column volumes of AAC buffer. Bound proteins were eluted by washing the resin with 3-5 column volumes of MBP elution buffer (AAC buffer supplemented with 10 mM maltose).

2.6.3. Nickel-chelate affinity chromatography

A nickel-nitrilotriacetic acid-agarose column (HiTrap chelating column, Amersham Pharmacia, Buckinghamshire, UK) was equilibrated with 10 column volumes of metal-chelate affinity chromatography (MCAC) buffer (25 mM sodium phosphate, 500 mM NaCl, pH 7.4) prepared without imidazole (MCAC-0). Clarified lysate (prepared in MCAC-0 buffer – see section 2.6.1) was applied to the column at a flow rate of 2-3 ml/min using a peristaltic pump. Resin was washed

with 30 column volumes of MCAC-95 buffer (MCAC-0 supplemented with 95 mM imidazole), followed by 10 column volumes of MCAC-110 buffer. The MCAC-110 fraction (1 x 50 ml) was collected and concentrated by ultrafiltration through Amicon Ultra 15 concentrators (Millipore)

2.6.4. Ion-exchange chromatography

An anion-exchange column (Mono Q; Amersham Pharmacia) was equilibrated with 5-10 column volumes of start buffer (20mM Tris-HCl, pH 8.0, 25 mM NaCl). The concentrated MCAC-110 eluate (see section 2.6.3), dialysed overnight at 4°C against ≥ 100 x volumes of start buffer, was applied to column at a flow rate of 1-2 ml/min. Resin was washed with 20 column volumes of a 9:1 mixture of start buffer:end buffer (end buffer = 20mM Tris-HCl, pH 8.0, 1 M NaCl), followed by 5 column volumes of a 4:1 mixture of start buffer:end buffer. The latter fraction was concentrated by ultrafiltration through Amicon Ultra 15 concentrators to ~500 µl, and concentrated further (typically to ~100 µl) using a Microcon-10 concentrator (Millipore).

2.6.5. Cleavage of MBP-tag from MBP-fusion proteins

MBP-fusion proteins in MBP elution buffer (fusion proteins purified by nickel-chelate chromatography were dialysed overnight at 4°C against ≥ 100 x volumes of MBP elution buffer) were mixed with factor Xa (Novagen, Nottingham, UK) using 1 unit of protease per 50 µg fusion protein, and incubating at 22°C for 24 h.

2.6.6. N-terminal sequencing

Protein samples were separated by SDS-PAGE and transferred to PVDF membrane. Following transfer, the membrane was rinsed in dH_2O followed by a 5-10 s soak in methanol. The membrane was then stained with amido black solution (0.1% [w/v] amido black, 1% [v/v] acetic acid, 40% [v/v] methanol) for 1 min, and destained by rinsing well with dH_2O. The membrane was air-dried and the stained band(s) of interest were analysed by Alta Bioscience, University of Birmingham, UK.

2.7. FUNCTIONAL ANALYSIS OF RECOMBINANT PfFKBP35

2.7.1. PPIase assay

The PPIase activity of recombinant proteins was assessed by the method of Kofron *et al.* (1991) using the tetrapeptide substrate succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide (Bachem, Bubendorf, Switzerland). All reagents were pre-equilibrated at 0°C prior to use. In a 1ml glass cuvette, recombinant protein (50 – 400 nM final concentration diluted from 120 μM stock) was mixed with 100 μl α-chymotrypsin (60 mg/ml in 1 mM HCl), and the volume brought up to 975 μl with assay buffer (50 mM HEPES, 100 mM NaCl, pH 8.0 at 0°C). The reaction was initiated by the addition of 25 μl substrate (4 mM tetrapeptide in 470 mM anhydrous LiCl prepared in trifluoroethanol). Changes in absorbance due to released *p*-nitroanilide were monitored at 390nm at 0°C over a 3 min period in a Shimadzu UV-1601PC UV-vis spectrophotometer with a thermostatted cuvette holder. The first order rate constant (k) was calculated from the slope of the plot of $\ln(A_{max} - A_t)$ against t, where A_{max} is the maximum absorbance and A_t is absorbance at time t. The catalytic efficiency (k_{cat}/K_m) was determined by plotting rate constants against enzyme concentration. In assays in which drugs were included, they were added as 1 μl amounts of 1,000 x the desired concentration, prepared in appropriate solvent. 1 μl of solvent alone served as a control. To monitor non-enzymatic, spontaneous isomerisation, the assay was performed in the absence of recombinant protein.

2.7.2. Calcineurin inhibition

The phosphatase activity of 40 nM bovine brain calcineurin in the presence or absence of inhibitors was measured using the ProFluor serine/threonine phosphatase assay (Promega, Southampton, UK). This assay is based on the fluorescence of a rhodamine-conjugated peptide substrate that, upon dephosphorylation by calcineurin, is digested by a protease. Because only the cleaved product is fluorescent, the level of fluorescence can be correlated with calcineurin activity. To ensure that reduction of rhodamine fluorescence was not due to inhibition of the protease, a control aminomethylcoumarin-conjugated peptide, whose fluorescence is independent of phosphorylated state, was incorporated into each reaction. Each reaction (100 μl volume), supplemented with

calmodulin, was performed according to the manufacturer's instructions. For reactions that included recombinant protein alone or drug alone, 5 µl of 20 µM stock solution were added (final concentration of 1 µM); for reactions that included both recombinant protein and drug, 2.5 µl of 40 µM stock solutions were used (final concentration of 1 µM for both). Fluorescence was monitored using a Perkin Elmer LS 50B fluorescence spectrophotometer.

2.7.3. Citrate synthase aggregation assay

The thermal denaturation of pig heart mitochondrial citrate synthase was achieved essentially as described by Buchner *et al.* (1998). Citrate synthase (1.5 µM monomer, prepared as described in section 2.5.2) was incubated at 43°C in 40 mM HEPES, pH 7.5, for 30 min and aggregation during the denaturation process was measured by monitoring the increase in absorbance at 360 nm in a Shimadzu UV-1601PC UV-vis spectrophotometer with a thermostatted cuvette holder using a quartz microcuvette. The effects of additional components on citrate synthase aggregation were assessed as described in the relevant results chapters.

2.7.4. Rhodanese aggregation assay

The thermal denaturation of bovine liver rhodanese was achieved essentially as described by Pirkl *et al.* (2001). Rhodanese (4.4 µM) was incubated at 44°C in 40 mM sodium phosphate, pH 8.0, for 30 min, and aggregation was monitored as for citrate synthase (section 2.7.3.). The effects of additional components on rhodanese aggregation were assessed as described in the relevant results chapters.

2.7.5. Citrate synthase activity assay

The ability of MBP-PfFKBP35-His_6 to prevent the loss of activity of citrate synthase upon heat treatment was assessed essentially as described by Buchner *et al.* (1998). Briefly, citrate synthase (0.15 µM monomer) was incubated for 10 min at 43°C in 40 mM HEPES buffer, pH 7.5. At time points, samples were removed and stored on ice. The activity of citrate synthase remaining in each sample was assayed by mixing 20 µl of the sample with 980 µl of freshly prepared assay buffer (100 µM oxaloacetic acid, 100 µM 5, 5'-dithio-bis(2-nitrobenzoic acid) [DTNB], 150 µM acetyl CoA, made up in Tris-EDTA (TE) buffer [50 mM Tris-HCl, 2 mM

EDTA, pH 8.0]), and monitoring the increase in absorbance at 412 nm at 25°C for 3 min. The specific activity of each sample was calculated by plotting absorbance against time, obtaining the slope and using the formula: specific activity = slope (V/εdvc), where V is the test volume (1 ml); ε is the molar extinction coefficient for DTNB (13,600 $M^{-1}cm^{-1}$); d is the pathlength of the cuvette (typically 1 cm); v is the volume of the sample added to the cuvette (20 µl); c is the concentration of citrate synthase. *S. cerevisiae* Hsp90 served as a control.

2.8. Investigation of Binding Partners of PfFKBP35

2.8.1. MBP–pull-down assay

50 µl volumes of amylose resin were dispensed into microfuge tubes. Resins were washed three times by adding 1 ml of AAC buffer (see section 2.6.2), mixing on an end-over-end mixer for 2 min, and spinning at 4,000 x *g* for 5 min. Following washing, resins were gently resuspended in 300 µl AAC buffer and 5-10 µg of bait protein (i.e. MBP-PfFKBP35-His_6 or MBP-His_8) were added. Tubes were mixed on an end-over-end mixer for 1 h at 4°C, after which time unbound protein was removed by washing the resins five times with 1 ml of AAC buffer. Following washing, resins were gently resuspended in 300 µl AAC buffer and 5-10 µg of the protein of interest (e.g. yHsp90) or parasite extract were added. Tubes were mixed on an end-over-end mixer for 1 h at either 4°C or room temperature, after which time unbound protein was removed by washing the resins eight times with 1 ml of AAC buffer. Final pellets were resuspended in an equal volume of 2X SDS loading buffer and boiled for 10 min. Resins were pelleted by spinning at 4,000 x *g* for 5 min, and supernatants loaded directly onto SDS-PAGE gel. Gels were subsequently stained by Coomassie Blue, or subjected to Western immunoblotting.

2.8.2. His_6–pull-down assay

50 µl volumes of iminodiacetic acid-sepharose resin were dispensed into microfuge tubes. Resins were washed thrice by adding 1 ml of MCAC-0 buffer (see section 2.6.3), mixing on an end-over-end mixer for 2 min, and spinning at 4,000 x *g* for 5 min. 200 µl of 100 mM $NiCl_2$ (pre-filtered through a 0.4 µm filter,

Millipore) were added and tubes were mixed on an end-over-end mixer for 1 h at 4°C. Resins were subsequently treated exactly as described for MBP–pull-down assays (section 2.8.1.) except MCAC-0 buffer replaced AAC buffer.

2.8.3. "Far-Western" analysis

Proteins, separated by SDS-PAGE, were transferred to PVDF membrane as described in section 2.5.7. Following the blocking procedure, membranes were soaked in a solution of 30-40 μg MBP-PfFKBP35-His_6 (or MBP-His_8 as a negative control) made up in 5% (w/v) non-fat milk for 4 h at 4°C. Membranes were then washed well in Towbin's/Tween solution and exposed to primary and secondary antibodies as described in section 2.5.7.

2.8.4. Glutaraldehyde cross-linking

In a total volume of 30 μl, equimolar amounts of proteins of interest were mixed together in a microfuge tube in cross-linking buffer (10 mM HEPES, pH 7.4, 25 mM KCl, 2 mM DTT, 0.02% [v/v] Nonidet P-40). Tubes were incubated on ice for 1 h, and cross-linking initiated by adding 3.3 μl of 20 mM glutaraldehyde. Tubes were mixed and left on ice for a further 1 h after which time 50 μl of 0.1 M Tris-HCl (pH 8.0) were added to terminate the cross-linking reactions. Following mixing, tubes were incubated at room temperature for 30 min, mixed with 28 μl of 4X SDS-loading buffer (0.25 M Tris-HCl, pH 6.8, 8% [v/v SDS, 40% [v/v] glycerol, 20% [v/v] β-mercaptoethanol, 0.002% (w/v) bromophenol blue, filtered to remove particulate material) and boiled for 5-10 min. 20-30 μl samples were separated by SDS-PAGE, and gels transferred to PVDF membrane and subjected to Western immunoblotting.

Chapter 3

Identification of a *P. falciparum* FKBP

3.1. INTRODUCTION

The first hint of the presence of FKBPs in *P. falciparum* came from the report of the inhibitory actions of FK506 and rapamycin in cell culture experiments (Bell *et al.* 1994). Their anti-malarial effects have only been assessed on asynchronous cultures and no information is available regarding the susceptibility of particular parasite developmental stages within the erythrocyte (rings, trophozoites and schizonts) to these drugs. Exposure of *P. falciparum* to CsA in culture results in inhibition of schizont maturation (Matsumoto *et al.*, 1987). Such information can provide important insights into the mechanism of action of anti-malarial compounds. Work performed in this chapter describes the effects of FK506 and rapamycin on the intra-erythrocytic development of *P. falciparum*.

One possible explanation for the drugs' effects is that an important cellular process mediated by a putative *P. falciparum* FKBP is being affected. Three homologues of cyclophilin have been described in *P. falciparum* (Reddy, 1995; Hirtzlin *et al.*, 1995; Berriman and Fairlamb 1998), and recent evidence supports the idea that the anti-malarial activity of CsA is mediated through at least two of these parasite cyclophilins (Gavigan *et al.*, 2003). No reports of FKBPs in malarial parasites were made prior to this work.

Previous attempts at identifying *P. falciparum* FKBP homologues, based on probing a genomic DNA library with a *P. falciparum* sequence fragment that showed high similarity to mouse FKBP52, proved unsuccessful (Hanley and Bell, unpublished results). At the beginning of this present study, efforts to sequence the genome of *P. falciparum* were well underway. Since FKBP domains exhibit high levels of sequence similarity, we set out to identify *P. falciparum* FKBP homologues using a data-mining approach. The work performed in this chapter describes this analysis.

3.2. RESULTS

3.2.1. Effects of FK506 and rapamycin on intra-erythrocytic development of *P. falciparum*

In order to investigate if a particular stage of parasite development inside the host erythrocyte was more susceptible to FK506 and rapamycin, cultures were

synchronised so that each of the developmental stages (rings, trophozoites and schizonts) could be assessed individually – under normal culture conditions, *P. falciparum* exhibits asynchronous development, resulting in a variety of developmental stages being present simultaneously. For this purpose, the parasite life cycle was divided into four stages by sorbitol treatment (stage 1, early rings [0-6 h post invasion]; stage 2, late rings/early trophozoites [12-18 h post invasion]; stage 3, mature trophozoites [24-30 h post invasion]; stage 4, schizonts [36-42 h post invasion]). Each set of cultures was exposed to a range of FK506 or rapamycin concentrations and the effect on parasite growth was determined for each after 12, 24, 36 and 48 hr of drug exposure by measuring the levels of parasite LDH. The IC_{50} values for stage 1 cultures after 24 hour exposure to drugs (during which time the cells would have been expected to progress to mature trophozoites) were 64 μM and 90 μM for FK506 and rapamycin, respectively (Fig. 3.1). The corresponding values for stage 2 cultures, which would have been expected to progress through to schizonts, were 13 μM and 6 μM. Since the only difference between these sets of cultures was the stages of the parasite exposed to drug, it suggests both FK506 and rapamycin exert their optimal effects on trophozoites. Although difficulties in interpreting such data arise due to factors such as possible differences in rate of drug uptake by the different parasite stages, the general trend of trophozoites appearing more susceptible to FK506 and rapamycin was followed.

In order to ascertain whether FK506 and rapamycin have cidal or static effects on parasite growth, mature trophozoite-stage cultures were incubated with high concentrations of either drug for set periods (0, 3, 6 or 9 h), after which the cultures were washed free of drug and reincubated for a further 48 h (one complete erythrocytic growth cycle). Direct counting of blood smears made from these cultures showed that the growth of parasites exposed to either drug for ≥ 3 hours was significantly inhibited (Fig. 3.2). The effect of rapamycin on parasite growth was even more potent than FK506, with 9 hours of exposure to this drug resulting in complete annihilation of parasites within a single life cycle. Control cultures exposed to drug vehicle exhibited normal progression through the various stages, with the number of rings decreasing over time as they progressed into trophozoites and subsequently into schizonts. In agreement with the data presented in Fig. 3.1, both FK506 and rapamycin were shown to have dramatic effects on trophozoites.

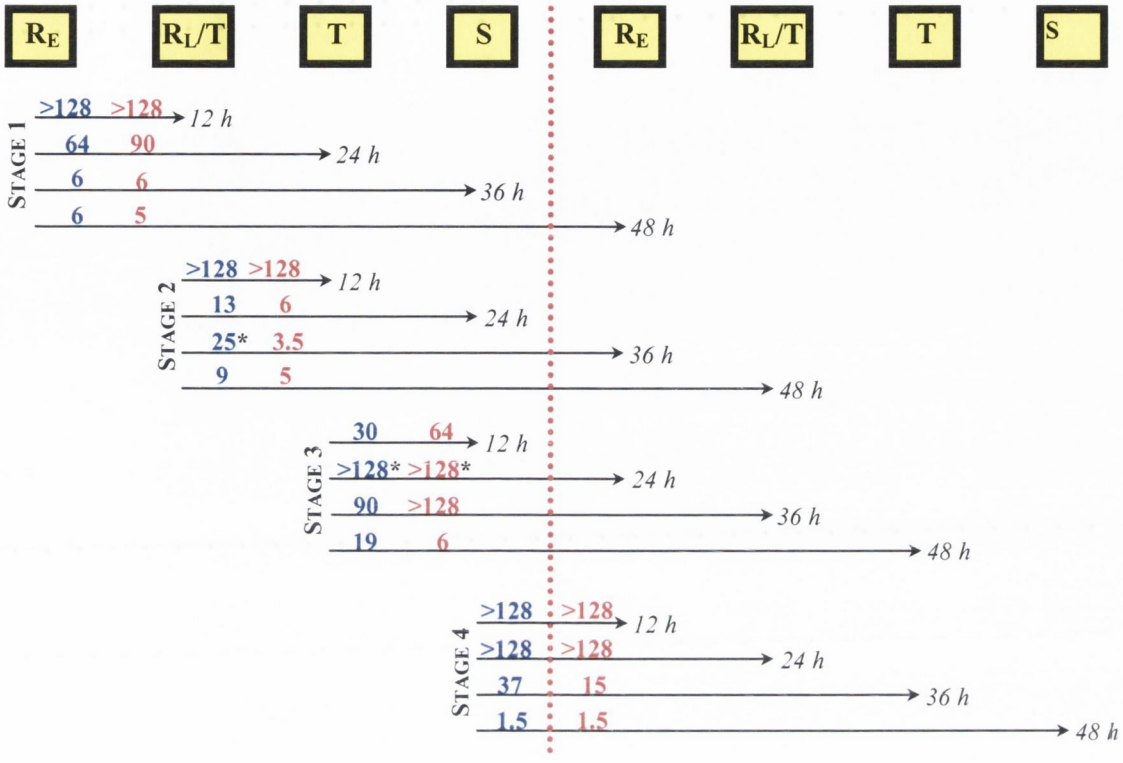


Fig. 3.1. Stage-specific effect of FK506 and rapamycin on growth of *P. falciparum*. Cells were synchronised to early rings (R_E : corresponds to 0-6 h post invasion), late rings/early trophozoites (R_L/T: 12-18 h post invasion), mature trophozoites (T: 24 - 30 h post invasion), or schizonts (S: 36-42 h post invasion). Each synchronous culture was treated with various concentrations of FK506 or rapamycin, and the effect on parasite growth determined for each after 12, 24, 36 and 48 hr of drug exposure. The IC_{50} values for growth inhibition by FK506 (blue) and rapamycin (red) on each set of cultures is given (μM – average values of duplicate determinants). Asterisks indicate IC_{50} values higher than those from preceding time points as a consequence of surviving parasites multiplying upon entering a second intra-erythrocytic life cycle (indicated by dotted red line). Arrows indicate expected progression of parasite development during the course of drug exposure.

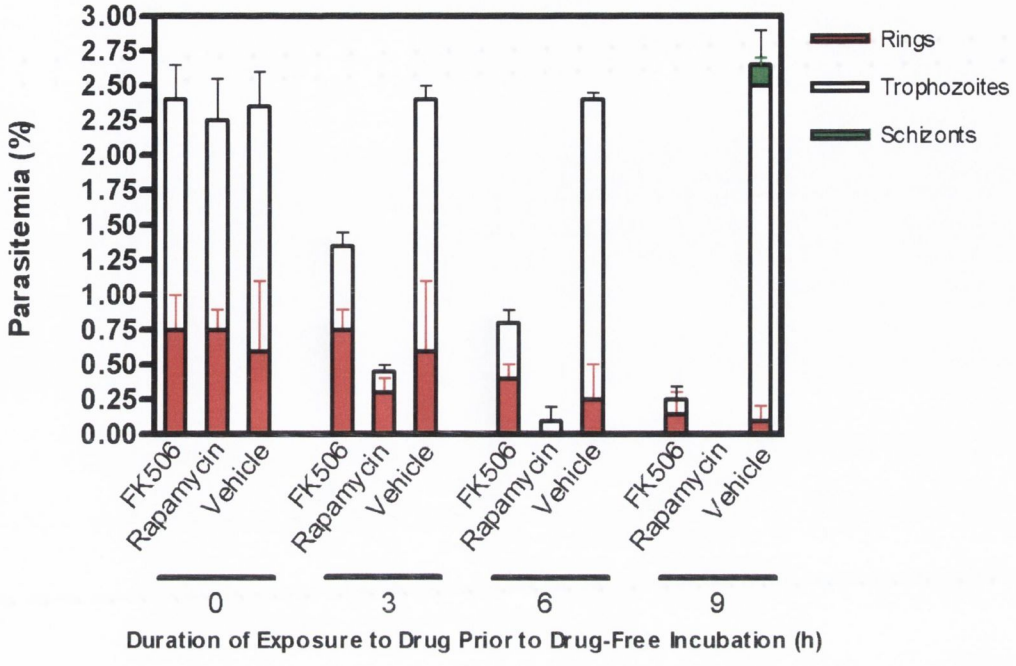


Fig. 3.2. Cidal/static effects of FK506 and rapamycin on trophozoite-stage *P.falciparum* growth *in vitro*. Cultures were synchronised to the trophozoite stage of development (24-30 h post invasion) and exposed to the 30 μM drugs for 0, 3, 6 and 9 h. Following this, the drugs were washed from the culture medium and the cells were reincubated for an additional 48 h, which represents one complete life cycle. Blood smears were stained with Giemsa, and stained parasites were counted and morphologically characterised. Bars show SEM of duplicate samples, with parasitemias determined by counting at least 1000 erythrocytes per slide.

Neither drug caused immediate overt toxicity, as judged by analysis of blood smears prepared immediately after period of drug exposure (data not shown). Rather, the inhibitory effects were only evident after the drug-free re-incubation. It appears, therefore, that exposure to the drugs beyond a certain time period caused irreversible damage to the parasites. Taken together, these inhibition data suggest that the target of FK506 and rapamycin is predominantly present during the trophozoite stage of the intra-erythrocytic life cycle of *P. falciparum*.

3.2.2. FKBPs from *P. falciparum*

The FKBP domains of FKBPs exhibit significant levels of sequence similarity. This characteristic facilitated a bioinformatic approach to identify a *P. falciparum* FKBP homologue, exploiting the rapid dissemination of genomic sequence produced by the *P. falciparum* sequencing consortium prior to the publication of the full genome sequence. Previous work by Kay which analysed the sequences of 30 FKBP-domain proteins derived from a range of mammals as well as eukaryotic and prokaryotic microorganisms, identified a core of highly conserved amino acids stretching for 107 residues (Kay, 1996). This conserved sequence (Fig. 3.3) served as the basis for designing a search string for using in a tBLASTn (www.ncbi.nlm.nih.gov/BLAST/) search against the available *P. falciparum* raw sequence data. This approach identified an open reading-frame (ORF) within a contig of chromosome 12 that putatively encoded a protein with a predicted molecular weight of 34.8 kDa showing significant similarity to the FKBP consensus sequence (Fig. 3.3A).

When the sequence of this putative 34.8-kDa FKBP was used as the search string in a second round of tBLASTn analysis, two regions of interest were identified. An ORF from chromosome 13 encoded a putative 25-kDa protein. However, the similarity to the consensus FKBP domain was very low (22.6% identity - Fig. 3.3B). A contig derived from chromosome 11 DNA harboured a sequence fragment showing significant similarity to a C-terminal region of the putative 34.8-kDa protein used as the search string. Further analysis revealed this ORF from chromosome 11 to contain a sequence identical to an expressed sequence tag (EST) previously generated by researchers in the University of Florida as part of the *P. falciparum* genome sequencing consortium (Dame *et al.*, 1996). This EST, which encoded a polypeptide showing significant similarity to a region of mouse

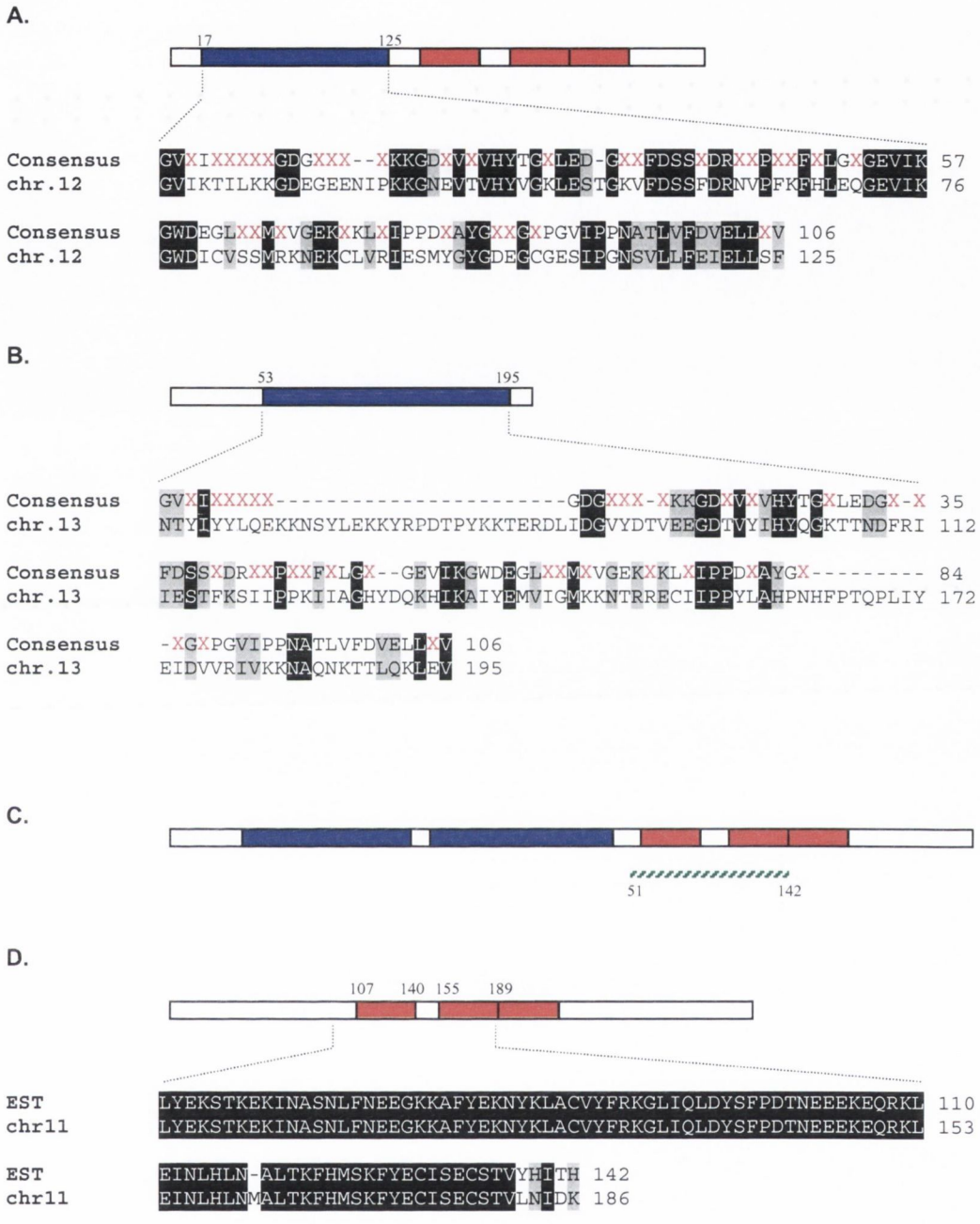


Fig. 3.3. Identification of FKBP-like sequences from the genome of *P. falciparum*. Alignment of FKBP consensus sequence (modified from Kay, 1996) with the deduced amino acid sequence of **A.** an ORF from chromosome 12 (accession # NP_701815), and **B.** an ORF from chromosome 13 (NP_705042). The proposed modular structure of the proteins is shown above the respective sequence alignments (blue = FKBP domain; red = TPR motif) **C.** Schematic representation of region of similarity between an EST from *P. falciparum* (green hatched line), encoding a 142 residue polypeptide, and mouse FKBP52 (modular structure shown). **D.** Alignment of the same EST with a putative protein encoded by a gene from chromosome 11 (NP_700985). Identical residues are highlighted in black, with conservative substitutions highlighted in grey. Red X's within the consensus sequence represent any residue.

FKBP52 (Fig. 3.3C), was used by Hanley and Bell for probing a *P. falciparum* genomic DNA library in a previous search for a *P. falciparum* FKBP homologue (Hanley, S. and Bell, A. unpublished results). As further genomic sequence data became available, however, it became apparent that this FKBP52-like protein was not a member of the FKBP family. The corresponding ORF that was subsequently identified did not encode an FKBP domain (Fig. 3.3D). Rather, its similarity to FKBP52 was based on the C-terminal portion of FKBP52 rather than its N-terminal FKBP domain. Thus, the ORF from chromosome 12 appeared to represent the only FKBP-encoding gene in *P. falciparum*.

The intron-less, 915-bp sequence of the ORF encoding the putative 34.8-kDa FKBP (Fig. 3.4) was used as the basis for designing DNA primers that were designed so as to incorporate *Bam*HI and *Pst*I restriction endonuclease sites at the 5' and 3' ends, respectively, of the amplicon to facilitate subsequent cloning. These primers successfully amplified a DNA fragment of the expected size of ~950bp (Fig. 3.5A). In order to ensure that this ORF was a functional gene, rather than a segment of non-coding DNA, reverse transcriptase PCR was performed using RNA isolated from erythrocytic-stage parasites. A band of the expected size was visualised on an agarose gel, indicating that the ORF was indeed an expressed gene (Fig. 3.5B). As the protein product was predicted to be 34.8 kDa, it was named PfFKBP35.

3.2.3. Sequence analysis of PfFKBP35

Analysis of the derived amino acid sequence of PfFKBP35 shows it to be comprised of a single, N-terminal, FKBP domain and a C-terminal TPR domain (Fig. 3.3A and Fig. 3.4). The FKBP domain exhibits 44% identity and 72% similarity to the archetypal FKBP, hFKBP12 (Fig. 3.6). This degree of identity is consistent with that of FKBP domains from other proteins. Importantly, from the point of view of correlating the anti-malarial activity of FK506 with effects on the activity of this protein, twelve of the fourteen residues that have been shown in hFKBP12 physically to contact FK506 (van Duyne *et al.*, 1993; Kay, 1996) are conserved in the PfFKBP35 sequence. However, the amino acids from hFKBP12 that are known to interact with calcineurin in the hFKBP12-FK506-calcineurin ternary complex (Ke and Huai, 2003) show limited conservation in the primary

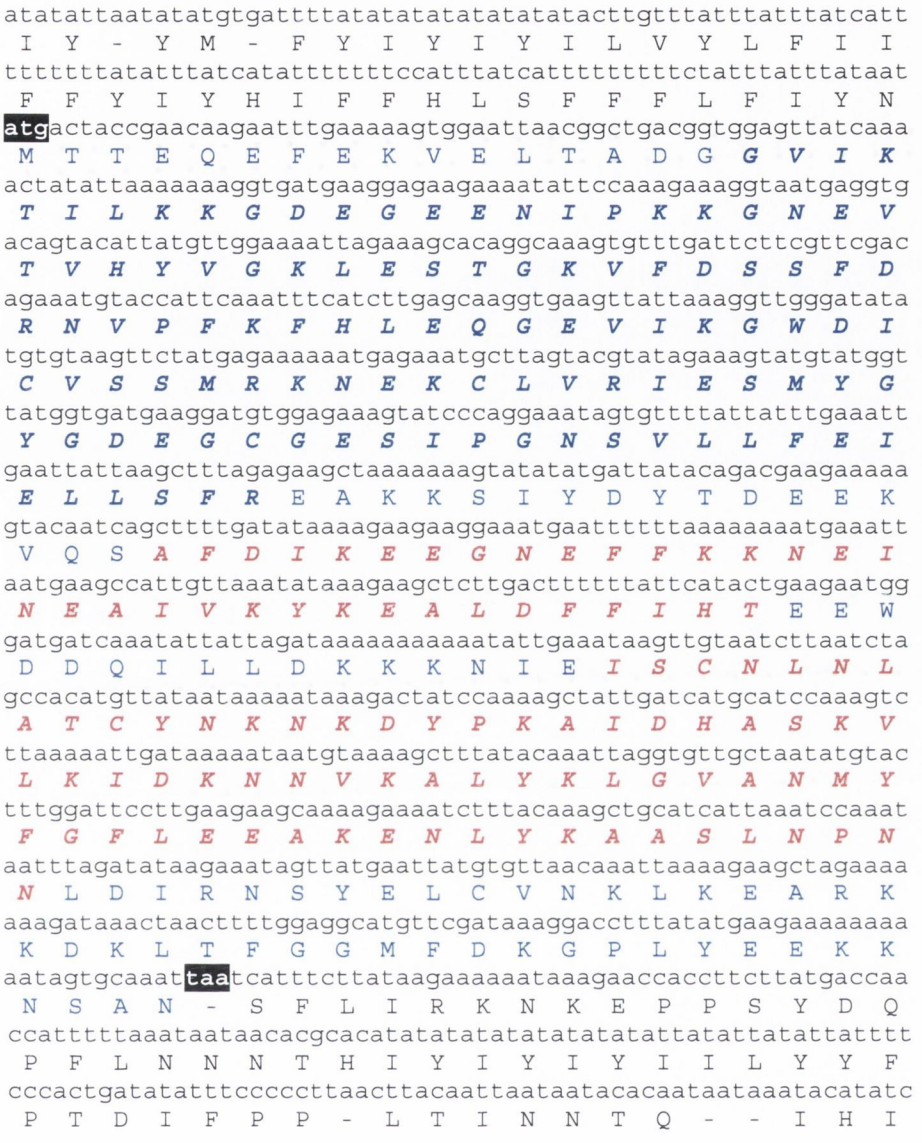


Fig. 3.4. *In silico* translation of a region of a contig from chromosome 12 of *P. falciparum*. An ORF putatively encoding an FKBP (start and stop codons highlighted in black) was identified within the contig. The amino acid sequence of the proposed protein is highlighted in colour beneath the corresponding nucleic acid sequence. The FKBP domain is highlighted in blue italics and the TPR regions are highlighted in red italics.

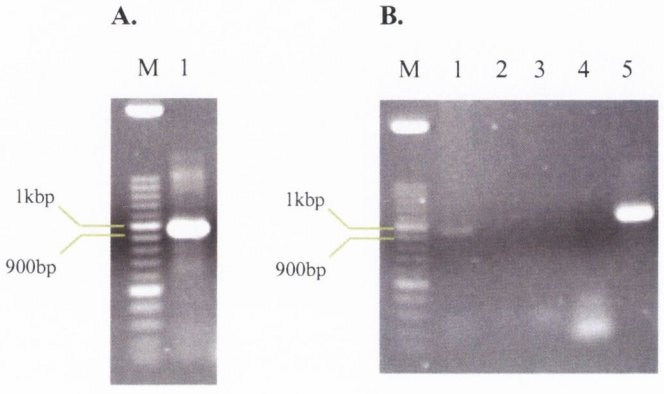


Fig. 3.5. Amplification and analysis of expression of *PfFKBP35*. **A:** PCR-amplification from genomic DNA using primers based on the ORF from chromosome 12 run on a 0.7% agarose gel. A band of the expected size (950 bp) was obtained (lane 1). **B:** RT-PCR amplification from RNA using the same primers, run on a 0.7% agarose gel. Lane 1 shows a faint band corresponding to PCR-amplified cDNA from the ORF. Lanes 2-5 are controls (2=no RNA added: 3=no reverse transcriptase added: 4=no template DNA added: 5= genomic DNA used as template). 5 µl volumes of each reaction were analysed. M= DNA ladder.

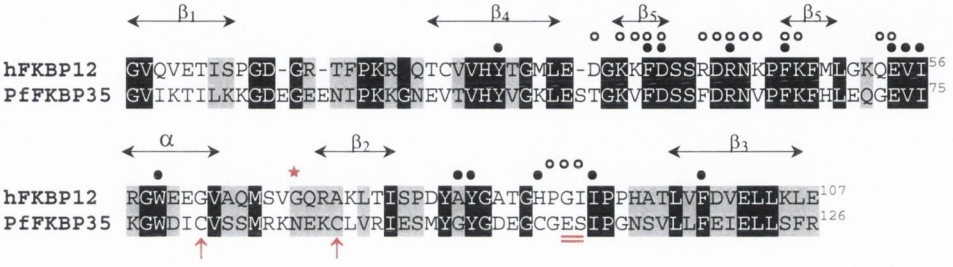


Fig. 3.6. Alignment of hFKBP12 with the FKBP domain of PfFKBP35. Identical residues are highlighted in black, with conservative substitutions highlighted in grey. Black circles above the hFKBP12 sequence indicate the residues that interact with FK506 (Kay, 1996), and white circles indicate those that interact with calcineurin in the hFKBP12-FK506-calcineurin ternary complex (Ke and Huai, 2003). The red double-underline indicates an unusual hydrophilic doublet in PfFKBP35. The red star designates a glycine residue that lies on the rear surface of hFKBP12 and is highly conserved throughout the FKBP family, but is substituted by an asparagine in PfFKBP35. The red arrows indicate two cysteine residues of PfFKBP35 that may be spatially adjacent to each other. The predicted secondary structure of PfFKBP35 corresponds to that of hFKBP12, with the residues forming the five strands of the β-sheet (β_5 comprises two sections) and intervening α-helix indicated by β and α respectively.

sequence of PfFKBP35. The Gly89/Ile90 doublet in hFKBP12 is a highly conserved region of all FKBPs. Only a handful of known FKBPs exhibit substitutions at this position, and those that do generally retain the hydrophobic character of this dipeptide. The hydrophilic nature of this doublet in PfFKBP35 is striking. Gly69 of hFKBP12 is an exceptionally conserved position throughout the FKBP family. The corresponding residue of PfFKBP35, however, is asparagine. The primary sequence of PfFKBP35 shows the presence of three cysteine residues within the FKBP domain, while most FKBP domains only contain a single cysteine. Examination of the atomic structure of hFKBP12 (van Duyne *et al.*, 1993) suggests that two of these cysteine residues of PfFKBP35 (Cys81 and Cys91) may be within close enough proximity to form a disulphide linkage (Fig. 3.7).

CD-BLAST analysis shows the protein to contain three TPR motifs towards the C-terminus (Fig. 3.3A and Fig. 3.4). The presence of these repeats is of interest as these are implicated in a diverse range of protein-protein interactions in a multitude of protein families. TPRs are degenerate 34-amino acid repeat motifs that are most often found as multiple, tandemly repeated units (Lamb *et al.*, 1995; Blatch and Lässle, 1999; D'Andrea and Regan, 2003). Due to amino acid degeneracy, TPR motifs are characterised principally by secondary structure similarity as each 34-residue repeat forms two anti-parallel α-helices. Although these motifs exhibit limited identity at the primary sequence level, there is a loosely conserved pattern of eight amino acids showing similarity in size, hydrophobicity, and charge (Fig. 3.8). This pattern is more or less adhered to in the three TPR motifs of PfFKBP35. This is only the second reported TPR-containing protein in *P. falciparum*, the first being a serine/threonine protein phosphatase 5 (PP5) homologue (Dobson *et al.*, 2001; Lindenthal and Klinkert, 2002).

The overall domain architecture of PfFKBP35 shows remarkable similarity to other known FKBPs, particularly hFKBP36, hFKBP37 and hFKBP38 (Fig. 3.9). Although hFKBP51 and hFKBP52 include a second FKBP domain, their overall modular structure is otherwise very similar to PfFKBP35. Little is known about either hFKBP36 or hFKBP38, but hFKBP37, hFKBP51 and hFKBP52 are known to interact with heat shock protein 90 (Hsp90) as part of hetero-oligomeric receptor complexes (reviewed in Petrulis and Perdew, 2002; Pratt and Toft, 2003). It has been shown that the similarity between functionally different TPR motifs is

Fig. 3.7. X-ray crystal structure of hFKBP12 complexed with FK506 (PDB accession # 1FKF). The bottom panel is rotated 180° relative to the top panel. Bound FK506 is depicted as a blue spacefill model to emphasise the position of the binding pocket of hFKBP12, whose backbone is shown as a ribbon. The FKBP domain of PfFKBP35, as for most FKBP domains, is predicted to form the same generalised structure. The positions of two spatially adjacent cysteines of PfFKBP35 (Cys81 and Cys91) are highlighted in red (corresponding to Gly62 and Ala72 of hFKBP12). Gly69 (yellow) of hFKBP12 represents one of the most conserved positions throughout the FKBP family, but is substituted for Asn in PfFKBP35. The position of the Gly89/Ile90 dipeptide of hFKBP12, substituted for a hydrophilic dipeptide in PfFKBP35, is highlighted in green.

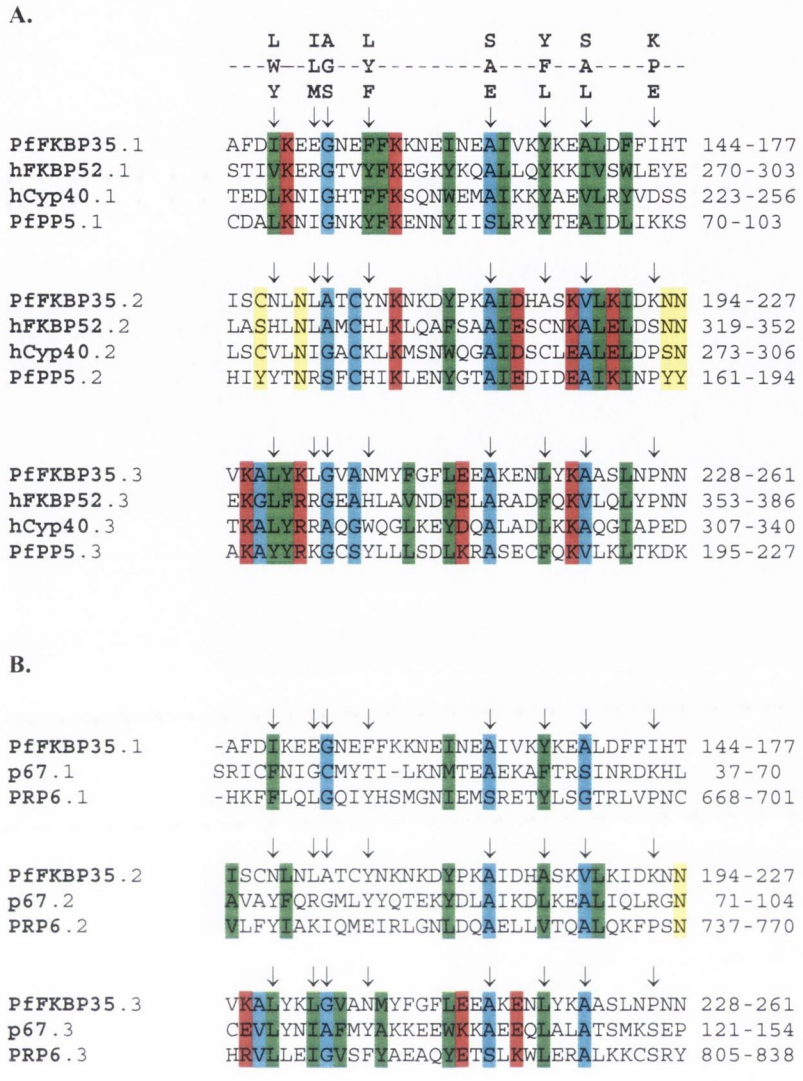


Fig. 3.8. Alignment of TPR motifs of PfFKBP35 with TPR motifs of other proteins exhibiting tripartite TPR domains. **A.** Alignment with TPR motifs from Hsp90-binding proteins. The first TPR motif of each protein is aligned in the top panel, the second TPR motif of each is aligned in the middle panel, and the third TPR motif is aligned in the bottom panel. Shown are: hFKBP52 (accession # Q02790); hCyp40 (A45981); and PfPP5 (AL049185). **B.** Alignment with TPR motifs from proteins that do not interact with Hsp90. Shown are $p67^{phox}$ (P19878), a neutrophil oxidase factor, and PRP6 (P19735), a yeast protein involved in pre-mRNA splicing. Arrows above each panel indicate the positions of the eight loosely conserved residues that comprise the TPR consensus sequence: position 4 (W/L/F), 7 (L/I/M), 8 (G/A/S), 11 (Y/L/F), 20 (A/S/E), 24 (F/Y/L), 27 (F/Y/L), and 32 (P/K/E). Colour coded amino acids correspond to small (blue), large hydrophobic (green), charged (red) and polar (yellow) residues that are conserved amongst the aligned sequences. Numbering corresponds to the positions of the TPR motifs in the respective proteins.

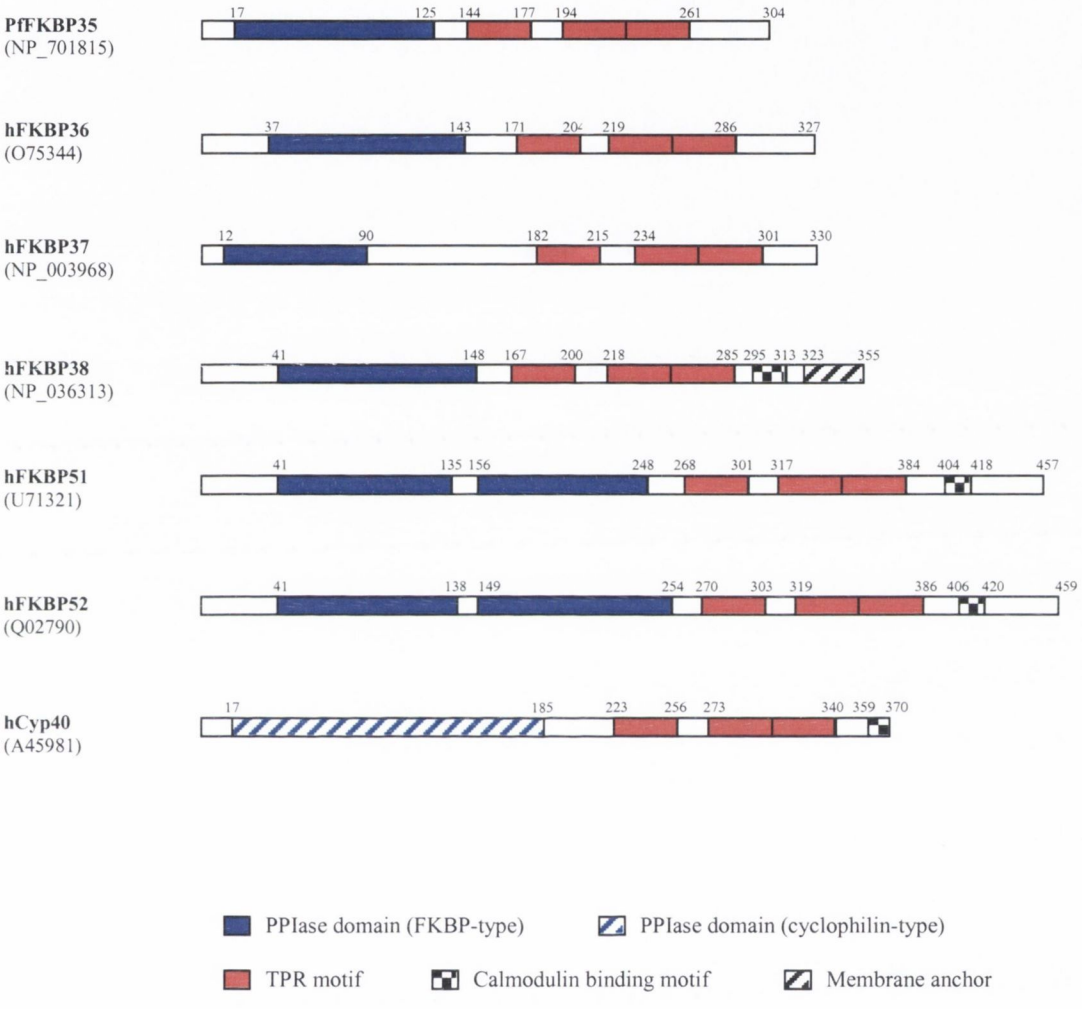


Fig. 3.9. Comparison of the domain architecture of PfFKBP35 with other TPR-containing PPIases. Accession numbers are given in parenthesis.

typically confined to the eight-residue consensus sequence described above, whereas functionally similar TPR motifs exhibit additional similarity outside this core sequence (Blach and Lässle, 1999). Due to the similarity in domain architecture between PfFKBP35 and Hsp90-binding FKBPs, the three TPR motifs of PfFKBP35 were compared to the corresponding motifs of a number of Hsp90-binding proteins (Fig. 3.8A). This analysis revealed a number of similar residues outside the core eight-residue sequence. When the three TPRs of PfFKBP35 were compared with the respective TPR motifs of proteins that do not interact with Hsp90, the level of similarity was far less (Fig. 3.8B).

3.2.4. FKBPs from other *Plasmodium* species

Of the nearly 120 species of *Plasmodium* known, at least 40 infect mammals (Bruce-Chwatt, 1985). Some of these species are exploited by malariologists in animal models of malaria. *P. yoelii, P. berghei,* and *P. chabaudi*, parasites specific for mice, represent the most widespread laboratory infection models due to the ease in handling laboratory mice, but avian (*P. gallinacieum*) and simian (*P. knowlesi and P. reichenowi*) model systems are also used. Aside from *P. falciparum*, the only other malarial parasite whose virtually-complete genome has been published is *P. yoelii* (Carlton *et al.*, 2002). A data-mining approach identified a *P. yoelii* FKBP homologue with remarkable similarity to PfFKBP35. This 35-kDa protein exhibits 79% identity and 96% similarity to PfFKBP35 (Fig. 3.10). This protein exhibits the same substitutions in the FKBP domain as noted in PfFKBP35 with the exception of T51 and F59 (nomenclature based on PfFKBP35 sequence), two positions that, in hFKBP12, are involved in calcineurin binding. The corresponding residues in the *P. yoelii* FKBP homologue are identical to those in hFKBP12.

Efforts at sequencing the genomes of the other important malarial parasites used in animal studies of infection are currently underway (www.sanger.co.uk). Analysis of the data currently available for *P. berghei* and *P. chabaudi* failed to reveal complete FKBP genes, but sequence fragments from both organisms were identified that putatively encode proteins with significant similarity to PfFKBP35 (Fig. 3.10). Intriguingly, both these sequences exhibit the same T51D and F59R substitutions (nomenclature based on PfFKBP35 sequence) as observed in the *P.*

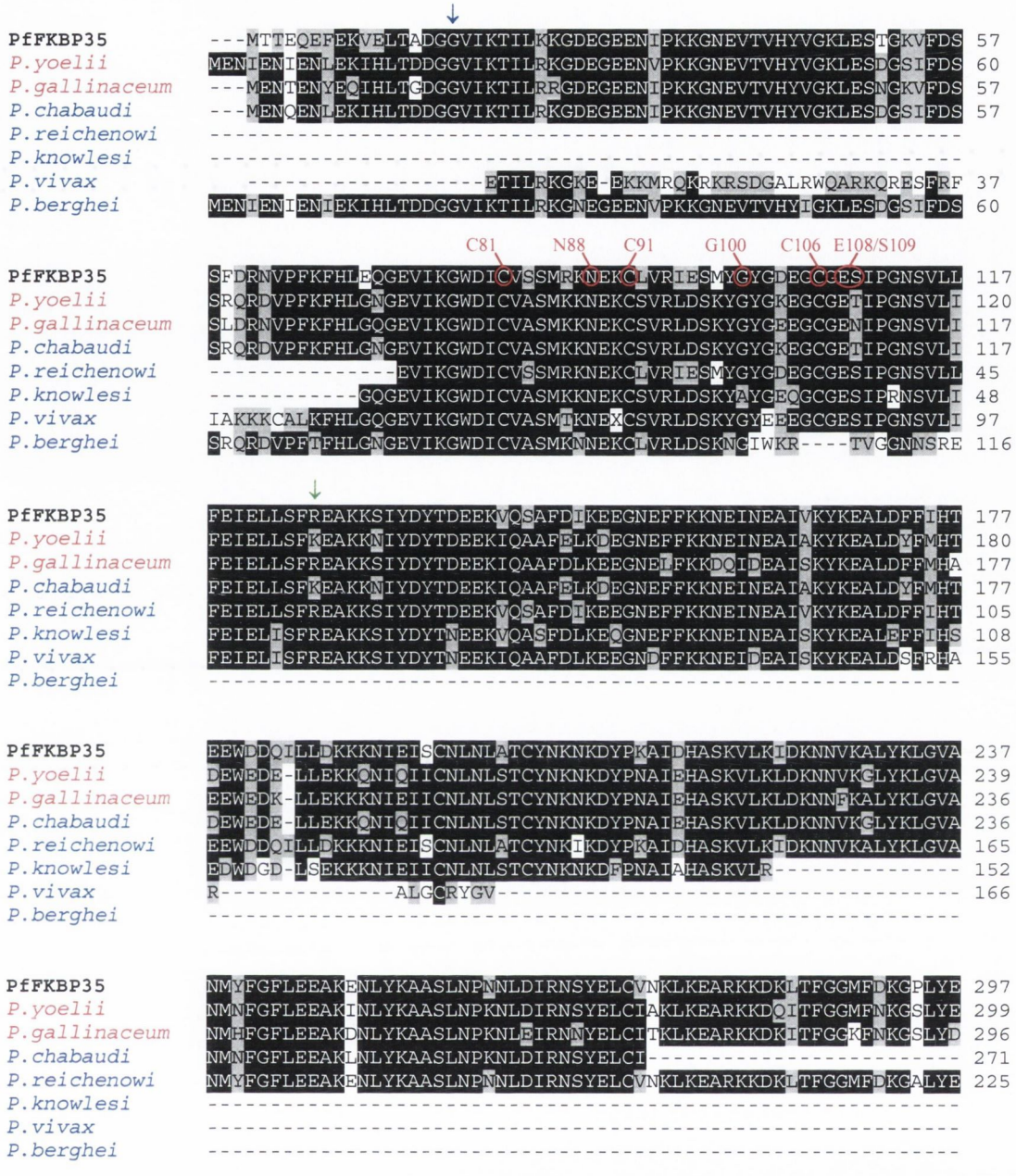


Fig. 3.10. Alignment of PfFKBP35 with putative FKBP proteins from other *Plasmodium* species. Data-mining identified either full-length ORFs (red) or sequence fragments (blue) encoding putative PfFKBP35 homologues. Residues highlighted in black represent amino acids conserved in >50% of aligned sequences. Conservative substitutions are highlighted in grey. Arrows above PfFKBP35 sequence represent the start (blue) and end (green) of the FKBP domain. Circled residues within the FKBP domain of PfFKBP35 correspond to those mentioned in section 3.2.3. Accession numbers: PfFKBP35 (NP_701815); *P. yoelii* (EAA21798); *P. gallinaceum* (331409.c000319831.contig1)*; *P. chabaudi* (PC_RP4866)*; *P. reichenowi* (reich454b08.qlk)*; *P. knowlesi* (pkn264b10.plc)*; *P. vivax* (AZ572610)†; *P. berghei* (BF298302)*. All data accessed on May 18, 2004 at: *www.sanger.co.uk; † www.ncbi.nlm.nih.gov/entrez/query.fcgi.

yoelii FKBP homologue. An ORF encoding a putative 35-kDa FKBP with 81% identity and 96% similarity to PfFKBP35 was identified in the genome of *P. gallinaceum* (Fig. 3.10). Analysis of the *P. knowlesi* and *P. reichenowi* genome data revealed the likely presence of a PfFKBP35 homologue, but as was the case for both *P. chabaudi* and *P. berghei*, only partial ORFs were identified (Fig. 3.10). Of the other three *Plasmodium* species that infect man, only *P. vivax* is currently undergoing full-scale genomic sequencing (http://www.tigr.org/tdb/e2k1/pva1/). Although the available data is far from complete, once again there appears to be an FKBP homologue (Fig. 3.10).

3.3. DISCUSSION

The finding that *P. falciparum* trophozoite stages appear most susceptible to FK506 and rapamycin is not unexpected, as this represents the most metabolically active stage of the parasite's intraerythrocytic life cycle. Rapamycin appears to inhibit parasite growth at a quicker rate than FK506 – for example, a 3 hour exposure of trophozoites to 30 μM rapamycin resulted in ~80% growth inhibition by the end of the following developmental cycle, compared to a ~45% inhibition by FK506. It should be considered, however, that the effects observed in Fig 3.2 after 48 hours of "drug-free" re-incubation may be as a result of drug that had irreversibly bound to intracellular receptors.

FKBPs have been identified in almost every organism in which they have been sought, suggesting that they are involved in important cellular processes. Based on the anti-malarial activities of FK506 and rapamycin, we suspected that *P. falciparum* possessed at least one member of the FKBP family. Analysis of the *P. falciparum* genome database revealed three possible FKBP genes: an ORF from chromosome 12; a second ORF from chromosome 13; and a sequence fragment from chromosome 11. *In silico* translation showed that the ORF from chromosome 12 potentially encoded a 35-kDa FKBP that exhibited significant similarity at the primary sequence level to other FKBPs. Similar analysis of the ORF from chromosome 13 showed the possibility of a 25-kDa FKBP. However, the putative FKBP domain of this protein showed only limited similarity to known FKBPs, exhibiting less than 20% identity to hFKBP12. The sequence fragment from

chromosome 11 showed significant similarity to FKBP52, but this similarity was based on the C-terminal portion of FKBP52 rather than its N-terminal FKBP domain. It was thought that, as further sequence data became available, this sequence fragment would form the latter part of an ORF encoding a *P. falciparum* FKBP52 homologue. However, the ORF that was subsequently identified lacked sufficient upstream sequence to encode an FKBP domain. This FKBP52-like sequence could be the result of recombination events, which suggests that perhaps *P. falciparum* once had an FKBP52 homologue that became redundant. Since the minimum requirement of an FKBP is the presence of an FKBP domain, and the putative gene from chromosome 13 encodes a protein with only limited homology to FKBPs, we concluded that the genome of *P. falciparum* contains only a single FKBP gene on chromosome 12. The presence of only a single FKBP gene is unusual, particularly as the parasite has at least three different cyclophilins, the other major group of PPIase proteins (Hirtzlin *et al.*, 1995; Reddy, 1995; Berriman and Fairlamb, 1998).

RT-PCR analysis confirmed that the ORF from chromosome 12 was transcribed by intra-erythrocytic stage parasites. The deduced amino acid sequence of the protein product (PfFKBP35) revealed a single, N-terminal, FKBP domain and a C-terminal tripartite TPR, a modular structure very similar to certain FKBPs.

Sequence analysis of the FKBP domain of PfFKBP35 showed it to contain many of the important residues typical to FKBPs, as well as some unusual substitutions. Not all FKBPs exhibit PPIase activity *in vitro*, and many don't even bind FK506. Those that do interact with the drug show an exceptionally high degree of conservation within the fourteen residues of hFKBP12 that are known to interact with FK506, according to the atomic structure of the hFKBP12-FK506 complex (Van Duyne *et al.*, 1993). Twelve of these are conserved in PfFKBP35. The other two residues are the least conserved of the fourteen, so substitutions here are not necessarily significant. The Ala81Gly substitution (nomenclature based on hFKBP12) has been observed in a number of other FKBPs, and is a very conservative substitution unlikely to have any major impact on drug binding. However, the His87Cys substitution is a more pronounced, and potentially more disruptive substitution. As the side chain of cysteine is less bulky or rigid than that of histidine, this substitution could possibly have profound effects on drug binding affinity.

Most FKBPs only contain a single cysteine at a variable position, but PfFKBP35 has three. The two cysteines at position 81 and 91 of PfFKBP35 are spatially adjacent and could potentially form a cystine bridge between the α-helix and second β-strand of PfFKBP35 that, if present, may have major implications for the active site. Similar analysis of the FKBP12 homologue from the plant *Vicia faba* (VfFKBP12) showed the possibilty of a disulphide linkage within the active site of the molecule (Xu *et al.*, 1998). Further evidence for this was provided by VfFKBP12 losing its ability to form an FK506-dependent complex with calcineurin in the presence of a reducing agent. The authors also noted the same cysteine residues in the *Arabidopsis thaliana* FKBP12 homologue, leading them to suggest that the redox state in plant cells may modulate the activity of FKBPs. The possibility of a cystine bridge within the active site of PfFKBP35 remains to be investigated.

hFKBP13 exhibits an intramolecular disulphide bond, but this occurs outside the active site, at the rear of the molecule (Schulz *et al.*, 1994). This has been proposed to stabilise the crossing of two loop regions that interconnect β-strands. This crossing of loops is a highly unusual topological feature that, prior to the determination of the structure of FKBP domains, was thought to be prohibited in anti-parallel β-sheets due to difficulties in packing side chains efficiently (Richardson, 1977; Ptitsyn and Finkelstein, 1980). Intriguingly, the Gly69Asn substitution observed in PfFKBP35 occurs within one of these loops (Fig. 3.5). Glycine at this position is one of the most conserved of all residues throughout the FKBP family, even though this residue is not part of the active site. The significance of the Gly69Asn substitution in PfFKBP35 remains unclear.

The other noteworthy change is the substitution of the hydrophobic doublet of Gly89/Ile90 from hFKBP12 to a hydrophilic doublet (Glu108/Ser109) in PfFKBP35. The X-ray crystal structure of the hFKBP12-FK506-calcineurin complex shows that Gly89 and Ile90 form three Van der Waals' contacts with calcineurin (Griffith *et al.*, 1995; Kissinger *et al.*, 1995; reviewed in Ke and Huai, 2003). Futer and colleages showed that substituting Ile90 with other amino acids can severely compromise hFKBP12's affinity for calcineurin (Futer *et al.*, 1995). The involvement of Ile90 in the hFKBP12-rapamycin-TOR complex formation is also evident from analysis of the X-ray crystal structure of this ternary complex

(Choi *et al.*, 1996). Interestingly, the Gly89/Ile90 doublet is a highly conserved region of all FKBPs regardless of whether or not they can sequester calcineurin upon FK506 binding. Although substitutions at this position have been noted in other FKBPs, the hydrophobic character is almost always retained. Due to the positioning of this doublet within a flap region that appears to partially cover FK506 in the hFKBP12-FK506 binary complex (see Fig. 3.7), the substitution observed at this region in PfFKBP35 may have important implications for substrate/drug binding.

Aside from the FKBP domain, PfFKBP35 also includes a C-terminal domain containing three TPR motifs. This is only the second reported TPR-containing protein in *P. falciparum*, the first being a serine/threonine protein phosphatase 5 homologue (Dobson *et al.*, 2001; Lindenthal and Klinkert, 2002). Although the precise physiological function of such motifs remains to be fully elucidated, they are strongly implicated in protein-protein interactions. TPRs have been identified in a wide range of proteins, including FKBPs. The presence of the TPR motifs in PfFKBP35 strongly suggests that this protein has the ability to interact with another protein or proteins. Indeed, comparison of the TPR domains of PfFKBP35 with those of TPR-containing proteins known to interact with Hsp90 suggests that PfFKBP35 may interact with the *P. falciparum* Hsp90 homologue. This notion is supported by the striking similarity of PfFKBP35's modular structure with that of hFKBP37, hFKBP51, hFKBP52 and hCyp40, all of which interact with Hsp90 (reviewed in Petrulis and Perdew, 2002; Pratt and Toft, 2003). The involvement of both hFKBP51 and hFKBP52 in steroid hormone receptor heterocomplexes is the best studied of these. It is the heat shock protein, not the FKBP, that associates directly with the steroid receptor, but the interaction between FKBP and Hsp90 is mediated through the FKBP's TPR motifs, although additional C-terminal regions outside the core TPR domain have been shown to play a role in this interaction (Cheung-Flynn *et al.*, 2003). Indeed, the only other TPR-containing protein of *P. falciparum* to be reported, PfPP5 (Dobson *et al.*, 2001; Lindenthal and Klinkert, 2002), was shown to interact with PfHsp90 (Dobson *et al.*, 2001). Although there is debate as to whether PfPP5 contains three or four TPR motifs, the tripartite arrangement of the human PP5 homologue, which also binds Hsp90, is almost identical to that of PfFKBP35 (Das *et al.*, 1998).

The anti-malarial activity of FK506 and rapamycin suggested that *P. falciparum* expressed an FKBP that played an important cellular role. The finding that other *Plasmodium* species appear to encode an FKBP with remarkable similarity to PfFKBP35 suggests that this protein is evolutionarily conserved, and therefore, likely to be essential. The unusual substitutions observed within the primary sequence of PfFKBP35 suggest that the design of inhibitors specific for this parasite protein over the human ones is feasible. The fact that all of the common organisms used for laboratory models of malaria appear to encode a remarkably similar protein to PfFKBP35 suggests that animal models may serve as useful and valid systems for testing novel PfFKBP35 inhibitors *in vivo* as potential anti-malarials.

Chapter 4

Recombinant Production and Functional Analysis of PfFKBP35

4.1. INTRODUCTION

The previous chapter described the identification of what appears to be the only FKBP gene represented in the genome of *P. falciparum*. The similarity of the FKBP domain of PfFKBP35 with other FKBPs suggested that, like the majority of FKBPs, it would possess PPIase activity. The presence of TPR motifs in PfFKBP35 suggested that this protein might act as a molecular chaperone. In order to test for these activities, a purified protein was required.

Previous work by Bell and colleagues had shown that all the detectable PPIase activity in *P. falciparum* extracts was of the cyclophilin type, suggesting that the physiological concentrations of any FKBP may be low (Bell *et al.*, 1994). This belief was further supported by the failure to purify an FKBP from *P. falciparum* extracts by an affinity chromatography approach using an immobilised FK506 derivative (A. Bell, personal communication), a technique that has been used successfully in other systems (Manning-Krieg *et al.*, 1994). Due to the difficulties in maintaining high numbers of parasites in culture, and the corresponding problems in purifying sufficient quantities of native protein, it was decided to produce PfFKBP35 as a recombinant protein in *Escherichia coli*. The cloning of *PfFKBP35*, its expression in *E. coli* and the purification of its product to homogeneity are described in this chapter. The functional analysis of the purified recombinant protein is also described.

The finding that PfFKBP35 appears to be the only *P. falciparum* FKBP homologue suggests that the anti-malarial actions of both FK506 and rapamycin may be mediated through this protein. This is supported by the conservation of the drug-binding residues within the FKBP domain of the protein. In order to provide evidence for PfFKBP35 being involved in the anti-malarial actions of these drugs, the effects of FK506 and rapamycin on the activities of a recombinant form of the protein were assessed.

4.2. RESULTS

4.2.1. Recombinant Production of PfFKBP35

Numerous obstacles to obtaining a sufficiently pure preparation of recombinant PfFKBP35 for subsequent functional analysis were encountered,

necessitating the need for various cloning strategies. The plasmids constructed during the course of this study are detailed in Table 4.1.

4.2.1.1. Generation of His_6-PfFKBP35

The expression vector pQE-30, which incorporates the sequence for an oligo(His)-tag at the 5′ end of an inserted gene to facilitate subsequent purification of the protein product, was chosen to direct the expression of *PfFKBP35*. The PCR-amplified *PfFKBP35* gene (Fig. 3.5), which included the recognition sites for *Bam*HI and *Pst*I at the 5′ and 3′ ends respectively, was inserted into the *Bam*HI and *Pst*I sites within the multiple cloning site (MCS) of pQE-30, and transformed into *E. coli* XL1-Blue. Transformants were selected on the basis of pQE-30-encoded resistance to ampicillin (Amp). To screen for ampicillin-resistant (Amp^r) colonies harbouring the construct of interest (i.e. pQE-30 with inserted *PfFKBP35* [pQE-PfFKBP35]) rather than the unmodified parental vector, both restriction analysis and PCR were performed on plasmids purified from Amp^r colonies (Fig. 4.1). These techniques facilitated the identification of a clone containing pQE-PfFKBP35. DNA sequencing of purified plasmid from this clone confirmed the presence of the *PfFKBP35* gene.

E. coli were treated with isopropylthiogalactopyranoside (IPTG) to induce expression of His_6-PfFKBP35 from pQE-PfFKBP35. However, no expression of the recombinant protein was detected by SDS-PAGE (Fig. 4.2). Neither growth at lower temperatures (30°C rather than 37°C), nor titration of IPTG (0.5 mM – 3.0 mM, with 4h or overnight incubation periods) induced expression of His_6-PfFKBP35 (data not shown).

Although *P. falciparum* uses the same genetic code as *E. coli*, the frequencies of use of certain codons are widely different between the two (Baca and Hol, 2000) (Table 4.2). This codon bias is proposed to result in translational stalling, whereby *E. coli* effectively runs out of the necessary tRNAs to incorporate the corresponding amino acids into the foreign protein, resulting in diminished or failed expression of recombinant *P. falciparum* proteins. A plasmid encoding four tRNAs recognising codons preferentially used by *P. falciparum* has recently being developed to assist in the overexpression of *P. falciparum* proteins in *E. coli* (Baca

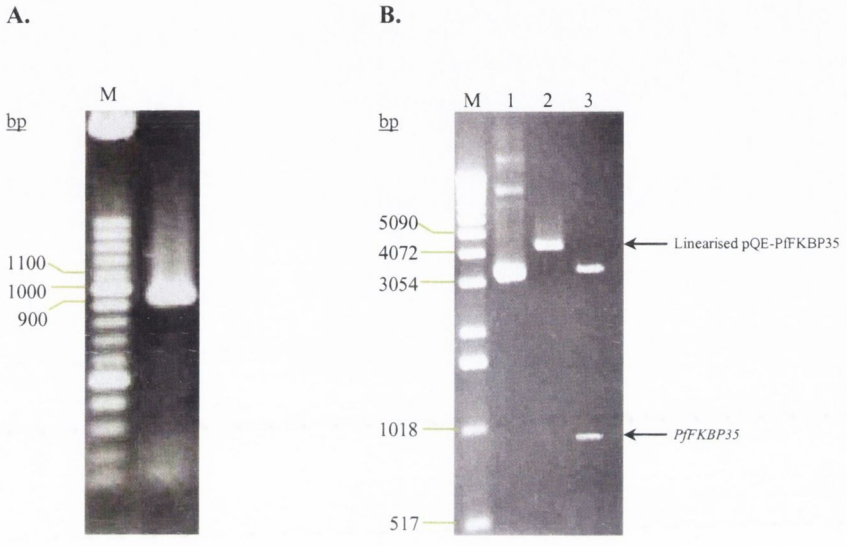


Fig. 4.1. Analysis of pQE-PfFKBP35. Plasmid isolated from Amp^r *E. coli* colonies was subjected to PCR or restriction endonuclease analysis, run through 0.8% agarose gels and stained with ethidium bromide. **A.** PCR amplification of inserted *PfFKBP35*. A band of the expected size (950 bp) was visualised. **B.** Inserted *PfFKBP35* was released from pQE-PfFKBP35 upon treatment with *Bam*HI and *Pst*I (lane 3; ~0.2 µg DNA). Lane 1, ~1 µg untreated pQE-PfFKBP35; lane 2, ~0.2 µg pQE-30 treated with *Bam*HI alone. M = DNA ladder (sizes indicated in bp).

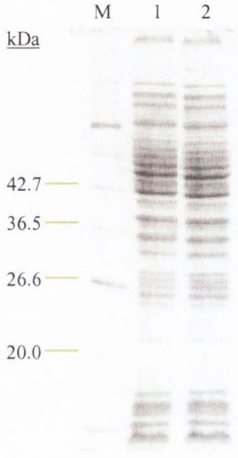


Fig. 4.2. Analysis of production of His_6-PfFKBP35 from *E. coli* transformed with the pQE-PfFKBP35 plasmid. Cells were treated with 1mM IPTG for 4 hours, harvested, lysed by boiling in SDS-PAGE loading buffer and run through an SDS-PAGE gel (12.5% - lane 2). Lane 1 shows an equal volume of lysate from cells treated in exactly the same manner, but exposed to deionised H_2O instead of IPTG. No overproduction of His_6-PfFKBP35 was detected in lane 2. Gel was stained with Coomassie Blue. M = molecular weight markers (sizes indicated in kDa).

Table. 4.2. Comparison of codon usage by *E. coli* and *P. falciparum*.

Amino Acid	Codon	E. coli	P. falciparum
		frequency of codon occurrence (%)	
Ala	GCA	21.4	42.7
	GCC	26.9	10.6
	GCG	35.5	5.0
	GCU	16.2	41.7
Arg	CGA	6.5	9.1
	CGC	39.8	1.5
	CGG	9.7	1.1
	CGU	38.0	11.4
	AGA	3.8	60.5
	AGG	2.2	16.4
Asn	AAC	55.1	13.9
	AAU	44.9	86.1
Asp	GAC	37.1	13.4
	GAU	62.9	86.6
Cys	UGC	55.2	13.1
	UGU	44.8	86.9
Gln	CAA	34.8	86.7
	CAG	65.2	13.3
Glu	GAA	69.1	85.6
	GAG	30.9	14.4
Gly	GGA	10.8	44.2
	GGC	40.2	4.5
	GGG	15.0	9.8
	GGU	34.0	41.5
His	CAC	42.9	14.5
	CAU	57.1	85.5
Ile	AUA	7.2	54.2
	AUC	41.9	6.8
	AUU	50.9	39.0
Leu	CUA	3.7	7.9
	CUC	10.3	2.4
	CUG	49.6	1.9
	CUU	10.4	11.5
	UUA	13.1	62.6
	UUG	12.9	13.7
Lys	AAA	76.7	81.7
	AAG	23.3	18.3
Met	AUG	100	100
Phe	UUC	42.6	16.4
	UUU	57.4	83.6
Pro	CCA	19.2	46.0
	CCC	12.4	10
	CCG	52.4	4.5
	CCU	16.0	39.5
Ser	UCA	12.4	26.0
	UCC	14.8	8.0
	UCG	15.3	4.7
	UCU	14.6	23.1
	AGC	27.7	6.3
	AGU	15.2	31.9
Thr	ACA	13.2	53.1
	ACC	43.4	11.7
	ACG	26.7	9.3
	ACU	16.7	25.9
Trp	UGG	100	100
Tyr	UAC	43.0	10.8
	UAU	57.0	89.2
Val	GUA	15.4	41.1
	GUC	21.5	6.2
	GUG	37.1	12.4
	GUU	26.0	40.3

Red arrows represent codons for which RIG was specifically engineered to encode tRNAs. Green arrows represent codons for which RIG encodes the corresponding tRNAs due to cloning artifacts. Table compiled from data obtained at www.kazusa.or.jp/codon/

and Hol, 2000). These codons encode three different amino acids – AGA/AGC encode arginine (R), ATA encodes isoleucine (I), and GGA codes for glycine (G) – and, accordingly, the construct is termed the RIG-plasmid. Additionally, due to cloning artifacts, the RIG-plasmid carries the genes for tRNAs that recognise TAC (Y), ACA (T) and ACC (T). The latter is particularly fortuitous as ACA represents another codon that is used at a higher frequency by *P. falciparum* than *E. coli*. It should be noted, however, that the codons recognised by the RIG-encoded tRNAs are not the only codons for which *P. falciparum* exhibits preferential use over *E. coli* (Table 4.2).

Examination of the coding sequence of PfFKBP35 showed ~8.5% of the codons to correspond to those for which RIG encodes tRNAs (Fig. 4.3A). In an attempt to boost expression of His_6-PfFKBP35, *E. coli* was co-transformed with pQE-PfFKBP35 and RIG. As RIG provides resistance to chloramphenicol (Chlor), co-transformants were selected on the basis of both Amp^r and $Chlor^r$. To confirm the presence of both plasmids, DNA isolated from colonies exhibiting this phenotype was subjected to PCR analysis, using primer sets specific for RIG and pQE-FKBP35 (Fig. 4.3B). This approach identified clones harbouring both constructs, but upon treatment of these clones with IPTG, no expression of *His_6-PfFKBP35* was detected (Fig. 4.3C). The overexpressed protein visible in Fig. 4.3C corresponds to an unidentified protein often expressed by the RIG plasmid (Hol, W. personal communication). Use of two protease-deficient strains of *E. coli* (strain K12 ER2508, lacking the major cytoplasmic ATP-dependent protease, Lon; and K12 UT5600, deficient in the major periplasmic protease, OmpT - www.neb.com) as hosts failed to express *His_6-PfFKBP35* (data not shown). Due to the failure in directing expression of the recombinant protein from the pQE-30 vector system, it was decided to subclone *PfFKBP35* into an alternative vector.

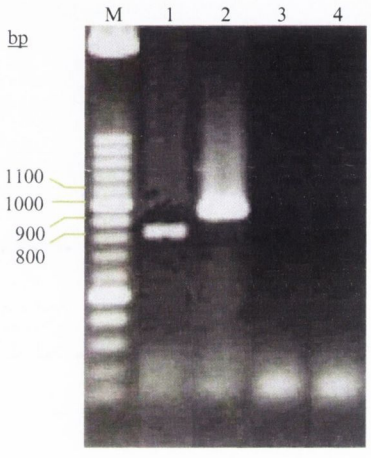
4.2.1.2. Generation of MBP-PfFKBP35

The expression vector chosen to replace pQE-30 was pMAL-c2X (www.neb.com). The MCS of this vector is incorporated into the extreme 3′ end of the *E. coli malE* gene, which is under the control of the strong *trp-lac* hybrid (*tac*) promoter. IPTG induction of P_{tac} results in the expression of maltose-binding

A.

ATG ACT ACC GAA CAA GAA TTT GAA AAA GTG GAA TTA ACG GCT GAC GGT GGA GTT ATC AAA ACT ATA TTA
AAA AAA GGT GAT GAA GGA GAA GAA AAT ATT CCA AAG AAA GGT AAT GAG GTG ACA GTA CAT TAT GTT GGA
AAA TTA GAA AGC ACA GGC AAA GTG TTT GAT TCT TCG TTC GAC AGA AAT GTA CCA TTC AAA TTT CAT CTT
GAG CAA GGT GAA GTT ATT AAA GGT TGG GAT ATA TGT GTA AGT TCT ATG AGA AAA AAT GAG AAA TGC TTA
GTA CGT ATA GAA AGT ATG TAT GGT TAT GGT GAT GAA GGA TGT GGA GAA AGT ATC CCA GGA AAT AGT GTT
TTA TTA TTT GAA ATT GAA TTA TTA AGC TTT AGA GAA GCT AAA AAA AGT ATA TAT GAT TAT ACA GAC GAA
GAA AAA GTA CAA TCA GCT TTT GAT ATA AAA GAA GAA GGA AAT GAA TTT TTT AAA AAA AAT GAA ATT AAT
GAA GCC ATT GTT AAA TAT AAA GAA GCT CTT GAC TTT TTT ATT CAT ACT GAA GAA TGG GAT GAT CAA ATA
TTA TTA GAT AAA AAA AAA AAT ATT GAA ATA AGT TGT AAT CTT AAT CTA GCC ACA TGT TAT AAT AAA AAT
AAA GAC TAT CCA AAA GCT ATT GAT CAT GCA TCC AAA GTC TTA AAA ATT GAT AAA AAT AAT GTA AAA GCT
TTA TAC AAA TTA GGT GTT GCT AAT ATG TAC TTT GGA TTC CTT GAA GAA GCA AAA GAA AAT CTT TAC AAA
GCT GCA TCA TTA AAT CCA AAT AAT TTA GAT ATA AGA AAT AGT TAT GAA TTA TGT GTT AAC AAA TTA AAA
GAA GCT AGA AAA AAA GAT AAA CTA ACT TTT GGA GGC ATG TTC GAT AAA GGA CCT TTA TAT GAA GAA AAA
AAA AAT AGT GCA AAT TAA

B.



C.

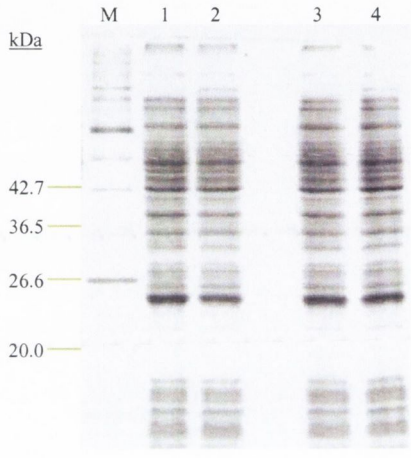


Fig. 4.3. Effect of RIG-plasmid on expression of *PfFKBP35*. **A.** The complete CDS for *PfFKBP35* is shown with the codons corresponding to those recognised by tRNAs encoded by the RIG-plasmid highlighted in colour: red codons represent those for which RIG was specifically engineered to encode tRNAs; green codons representing those for which RIG encodes the corresponding tRNAs due to cloning artifacts (of these 'artifactual' codons, only ACA is depicted in the *PfFKBP35* sequence as the other two codons are not preferentially used by *P. falciparum*). **B.** PCR analysis to confirm co-transformation of *E. coli* with RIG and pQE-PfFKBP35. An Amp^r and Chlor^r *E. coli* colony was lysed by boiling, and the lysate served as template in two separate PCR. Bands of expected sizes were obtained using primers specific for the RIG-plasmid (lane 1, 837 bp) and pQE-PfFKBP35 (lane 2, 950 bp), visualised on a 0.8% agarose gel. Lane 3 and 4 show the lack of PCR products when the lysate from *E.* coli harbouring pQE-30 only served as the template in the respective PCR reactions. 5 µl volumes of each PCR were analysed. M = DNA ladder (sizes indicated in bp). **C.** Lack of effect of RIG-plasmid on expression of *PfFKBP35*. Lysates of *E. coli* transformed with RIG alone (lane 1, uninduced; lane 2, induced with 1mM IPTG), or co-transformed with pQE-PfFKBP35 (lane 3, uninduced; lane 4, induced). Equal volumes of each lysate were resolved by SDS-PAGE (12.5%) and stained with Coomassie Blue. M = molecular weight markers (sizes indicated in kDa).

protein (MBP) and genes inserted within the MCS, therefore, are expressed with MBP fused to the N-terminus of the protein product. Like the oligo(His)-tag, MBP facilitates subsequent purification of recombinant proteins.

Like pQE-30, the MCS of pMAL-c2X includes the recognition sites for *Bam*HI and *Pst*I. Therefore, *PfFKBP35* was directly subcloned from pQE-PfFKBP35 and inserted into the *Bam*HI and *Pst*I sites of pMAL-c2X. The resulting construct, pMAL-PfFKBP35, was used to transform *E. coli* K12 ER2508, a protease-deficient strain that also lacks endogenous MBP. Plasmids isolated from Amp^r transformants were screened for the presence of *PfFKBP35* by both restriction analysis and DNA sequencing. Both of these approaches led to the isolation of a clone harbouring the desired construct (data not shown).

IPTG-induction of the resulting clone resulted in the over-production of two proteins, as judged by SDS-PAGE (Fig. 4.4). Analysis of the relative mobilities of both these bands through SDS-PAGE gels showed the upper band to migrate at approximately 72 kDa, with the lower band migrating at approximately 64 kDa (data not shown). The uppermost of these bands was assumed to represent the protein of interest, MBP-PfFKBP35, because it migrated closer to the expected size of 77.9 kDa. The other band was thought to represent a truncated form of MBP-PfFKBP35, either as a result of proteolytic degradation or translational stalling. Both bands were also observed when the samples were left unheated prior to loading on gels or when β-mercaptoethanol was excluded from the loading buffer (data not shown).

Due to the affinity of MBP for maltose, affinity chromatography using an amylose resin (amylose is an unbranched polymer composed of α(1, 4)-linked maltose units) was performed in order to purify MBP-PfFKBP35 from *E. coli*. SDS-PAGE analysis of fractions collected from this purification procedure showed that both induced proteins were retained on the resin and were eluted off by competing maltose (Fig. 4.5A). However, many host proteins were also retained by the resin, resulting in a very poor degree of purification. Various modifications of the procedure, such as the inclusion of high levels of salt or low levels of maltose in the washing buffer prior to elution, failed to improve the purification (data not shown). To ensure that the contaminants were indeed of *E. coli* origin rather than the result of proteolysis of the MBP-fusion proteins, western immunoblotting, using anti-

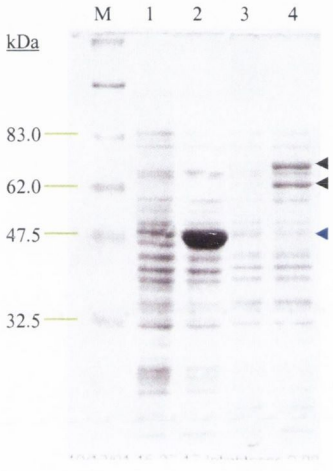


Fig. 4.4. Expression of MBP-PfFKBP35 in *E. coli*. Equal volumes of lysates of *E. coli*, transformed with either pMAL-c2X (lane 1, uninduced; lane 2, induced with 0.35 mM IPTG) or pMAL-PfFKBP35 (lane 3, uninduced; lane 4, induced), were resolved by SDS-PAGE (12.5%) and stained with Coomassie Blue. Black arrow-heads indicate proteins overproduced by pMAL-PfFKBP35 (lane 4). The blue arrow-head indicates overproduction of MBP by parental vector (lane 2). M = molecular weight markers (sizes indicated in kDa).

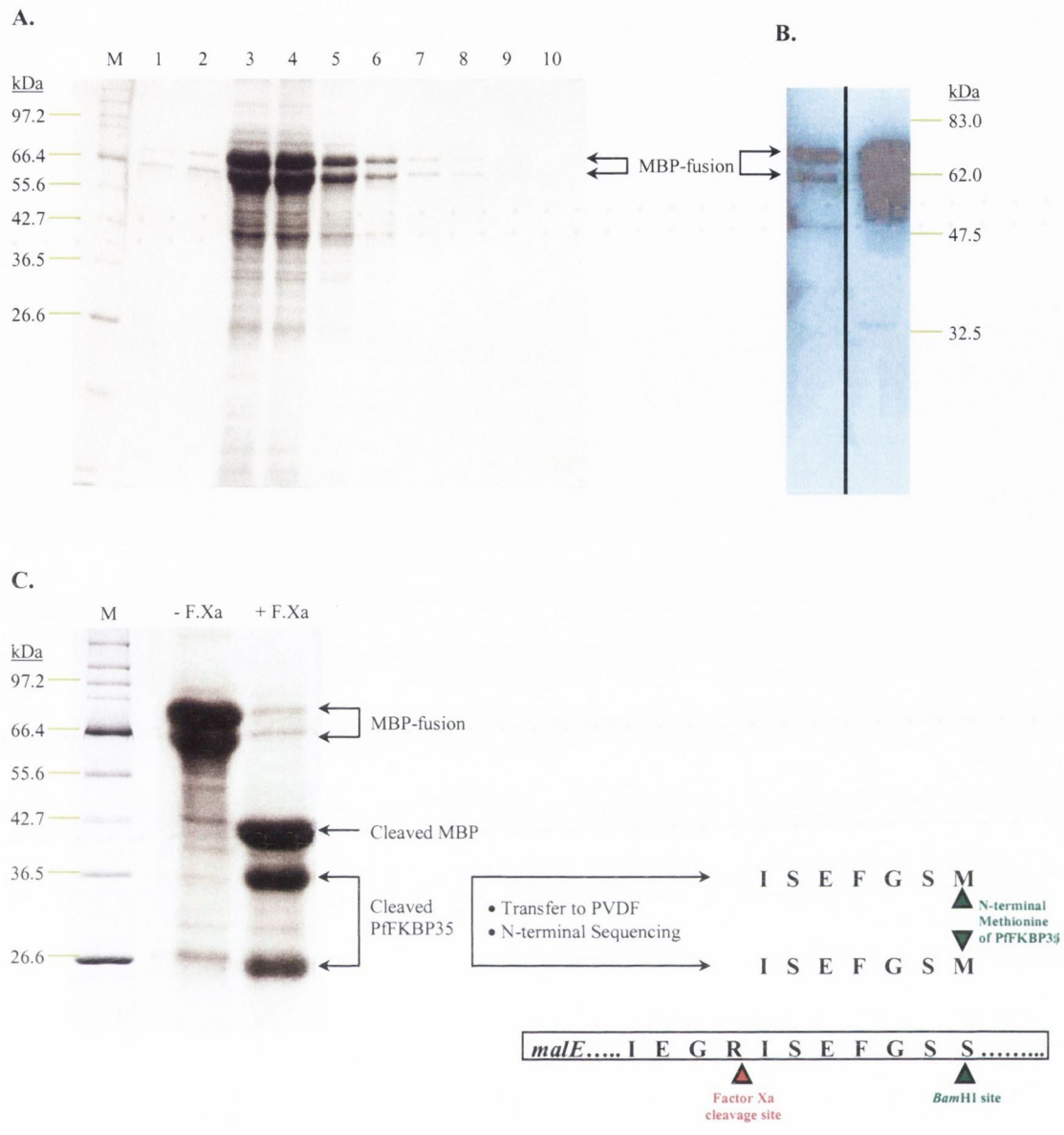


Fig. 4.5. Purification of MBP-PfFKBP35. **A.** Lysate from 3 l of *E. coli* culture was passed through amylose resin and eluates (collected as 10 x 2 ml fractions) resolved by SDS-PAGE (12.5%, lanes 1 – 10, 10 µl of each fraction loaded) and stained with Coomassie Blue. **B.** Eluate #4 from panel A was resolved by SDS-PAGE (0.5 µg total protein), transferred to PVDF membrane, and probed with anti-MBP antibody. The two panels represent the same sample, but with different lengths of exposure. **C.** Cleavage of MBP from MBP-PfFKBP35 by factor Xa. Amylose-purified MBP-PfFKBP35 was left untreated (- F.Xa) or incubated at 22°C for 24 hrs in the presence of factor Xa (+ F.Xa) and analysed by Coomassie-stained SDS-PAGE (~15 µg total protein). A replicate sample of the factor Xa-treated preparation was transferred to PVDF membrane and both the upper and lower factor Xa-cleaved bands were subjected to Edman degradation (seven cycles) to determine the sequence of the N-termini. A portion of the sequence of the protein product from the parental pMAL-c2X vector is shown (boxed) for comparison below the experimentally deduced N-terminal sequences of the two analysed bands. M = molecular weight markers (sizes indicated in kDa).

MBP antibody, was performed on a representative eluate fraction from the amylose column (Fig. 4.5B). This analysis showed that the most of the contaminants in the eluate were not products of proteolysis, and therefore likely to be of host origin. This also confirmed the presence of the MBP-tag on the two induced proteins. Treatment of eluates from amylose affinity chromatography with factor Xa (a sequence corresponding to the recognition site for this protease is incorporated immediately upstream of the MCS in pMAL-c2X, facilitating the cleavage of the MBP-tag from the fusion protein), successfully cleaved the MBP-tag from MBP-PfFKBP35, as well as the tag from the smaller protein (Fig. 4.5C). N-terminal sequencing of the two MBP-free bands showed both to have identical N-termini (Fig 4.5C) indicating that the lower band was not an N-terminal truncate of the upper band. Taken together, these data seemed to indicate that the smaller of the two bands corresponded to a C-terminal truncate of the larger band, lacking approximately 8-10 kDa of its C-terminus.

As a secondary purification step, ion-exchange chromatography was chosen. It was decided to cleave the MBP-tag from the recombinant protein prior to this secondary purification. As mentioned above, factor Xa successfully cleaved the MBP-tag from amylose-purified MBP-PfFKBP35, as well as the tag from the smaller protein. Although the free-MBP could be removed from the resulting mixture by ion-exchange chromatography, the mixture of the two recombinant proteins could not be separated (Fig. 4.6).

The difficulty in separating MBP-PfFKBP35 from host proteins was unexpected. Due to the success of the pMAL-c2X vector system in expressing the recombinant protein, where pQE-30 had previously failed, there was a reluctance to change expression systems. It was clear, however, that MBP-based purification was not providing the level of purity necessary for subsequent functional analysis of the recombinant protein. Therefore, it was decided to modify the existing pMAL-PfFKBP35 construct to incorporate sequence encoding an oligo(His)-tag at the 3′ end of the *PfFKBP35* insert, with the aim of producing MBP-PfFKBP35-His_6. Not only was this envisaged to provide an additional means of purification, but also to provide a means to eliminate the apparently truncated form of MBP-PfFKBP35.

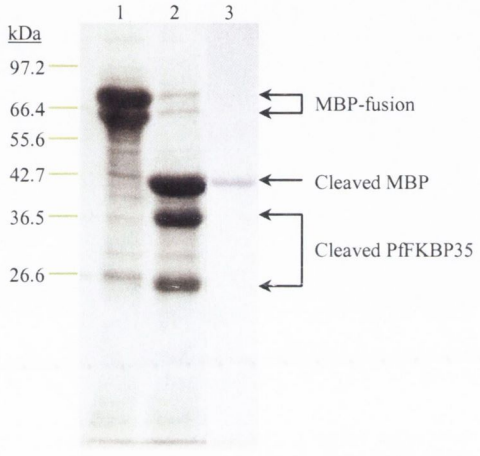


Fig. 4.6. Separation of factor Xa-treated MBP-PfFKBP35 products by ion-exchange chromatography. MBP-PfFKBP35 purified by amylose-affinity chromatography (lane 1; ~10 μg total protein) was incubated with factor Xa at 22°C for 24 h, and the cleaved mixture (lane 2; ~10 μg) subjected to ion-exchange chromatography. The cleaved MBP-tag was eluted by ~100 mM NaCl (lane 3 shows a representative fraction). The remaining two cleaved products co-eluted at approximately 500 mM NaCl (bands barely visible on Coomassie-stained SDS-PAGE gels – not shown). Molecular masses (in kDa) are indicated on the left.

4.2.1.3. Generation of MBP-PfFKBP35-His_6

Modification of the pMAL-FKBP35 plasmid to include sequence encoding an oligo(His)-tag was achieved by an inverse PCR approach (Fig. 4.7). The use of primers that amplified in opposite directions from contiguous priming sites allowed the entire pMAL-PfFKBP35 plasmid to be amplified. Incorporation of *Xho*I recognition sites at the 5′ ends of both primers allowed for subsequent ligation of each end of the PCR product, thereby circularising the DNA molecule. Designing the reverse primer to include the sequence encoding an oligo(His)-tag followed by a stop codon ensured that the resulting product, pMAL-PfFKBP35-His_6, encoded a protein with both an N-terminal MBP-tag and a C-terminal His_6-tag (MBP-PfFKBP35-His_6). The product of this inverse PCR, following restriction with *Xho*I and subsequent treatment with T4 ligase, was used to transform *E. coli* K12 TB1. Plasmids isolated from Amp^r transformants were analysed by restriction analysis with *Xho*I, and PCR with vector-specific primers (Fig 4.8A). The isolation of a clone harbouring the desired construct (pMAL-PfFKBP35-His_6) was confirmed by DNA sequencing. As for cells harbouring pMAL-PfFKBP35, treatment of this clone with IPTG resulted in the expression of two proteins, as judged by SDS-PAGE (Fig. 4.8B).

Nickel-chelate affinity chromatography, exploiting the metal-binding characteristic of oligo(His)-tags, was chosen as the primary means of purification of MBP-PfFKBP35-His_6. This form of chromatography resulted in far superior levels of purity than that achieved with amylose affinity chromatography (Fig. 4.9A). Intriguingly, the protein of higher mobility was also retained by the nickel column, indicating that it included the oligo(His)-tag at its C-terminus. This was confirmed by western immunoblotting using an anti-His_6 monoclonal antibody (Fig. 4.9B). This seemed to rule out the possibility of this band representing a C-terminal truncate of the upper band. However, separation of the two bands was achieved using this approach due to an apparent variation in metal-binding affinity between the two proteins (Fig. 4.9A, compare lane 2 to lane 3). Because the upper of these bands migrated at the predicted molecular weight for MBP-PfFKBP35-His_6 (78.7 kDa – analysis of the relative mobility of this band through SDS-PAGE gels showed it to migrate at approximately 75 kDa (data not shown)), eluates containing this protein were selected, pooled together and concentrated (Fig. 4.9A lane 5).

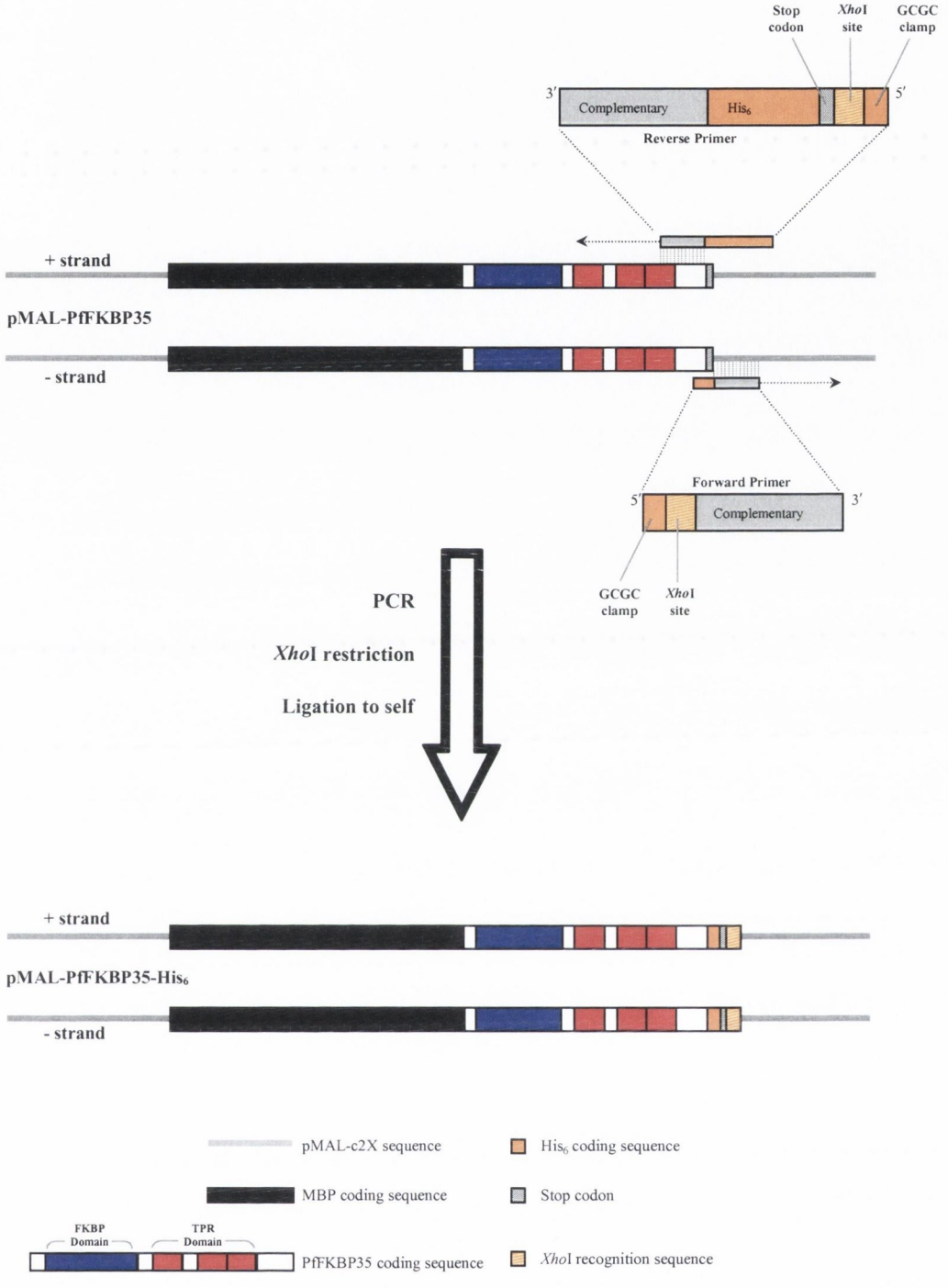


Fig. 4.7. Generation of pMAL-PfFKBP35-His_6 by inverse PCR. Amplification with the forward and reverse primers resulted in the stop codon of the *PfFKBP35* insert being replaced with an oligo(His) tag, followed by a new stop codon and an *Xho*I recognition site. Treatment of the PCR product with *Xho*I, and subsequent treatment with T4 ligase, resulted in the circularisation of the PCR product, yielding pMAL-PfFKBP35-His_6.

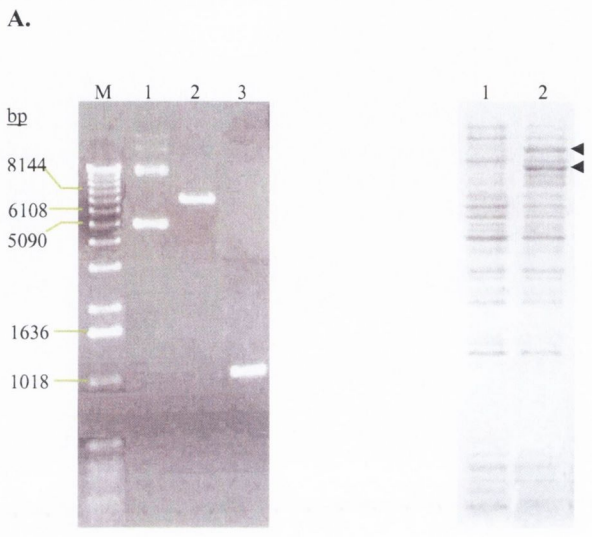


Fig. 4.8. Analysis of pMAL-PfFKBP35-His_6. **A.** Plasmid isolated from Amp^r *E. coli* colonies (lane 1; ~0.5 μg) was subjected to restriction endonuclease treatment with *Xho*I (lane 2; ~0.2 μg) or PCR with primers flanking the insertion site (lane 3; ~0.5 μg), run through 0.8% agarose gels and stained with ethidium bromide. M = DNA ladder (sizes indicated in bp). **B.** *E. coli* clone harbouring pMAL-PfFKBP35-His_6 was treated with IPTG (0.35 mM) as described in Materials and Methods and lysate resolved by SDS-PAGE (12.5%; lane 2) and stained with Coomassie Blue. Lane 1 shows an equal volume of lysate from cells treated in exactly the same manner, but exposed to deionised H_2O instead of IPTG. Arrow-heads indicate proteins overexpressed by the clone.

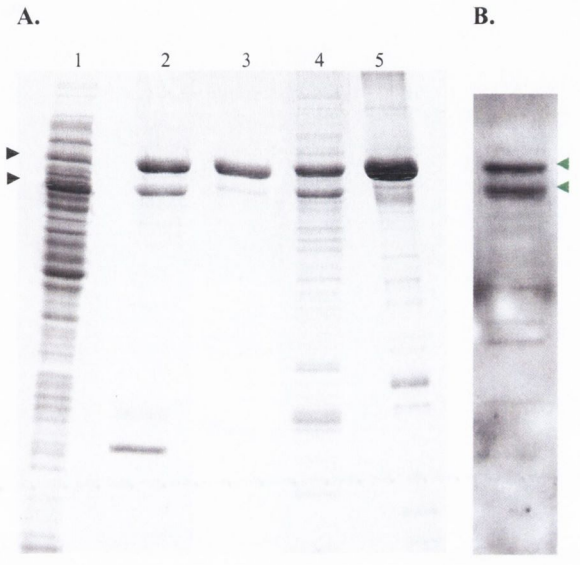


Fig. 4.9. Nickel-chelate affinity purification of MBP-PfFKBP35-His_6. **A.** Lysate from 3 l of *E. coli* cells overexpressing MBP-PfFKBP35-His_6 was loaded onto a column, the resin washed with MCAC buffers and equal volumes of each fraction resolved by SDS-PAGE (12.5% v/v): lane 1, lysate; lane 2, MCAC-85 (85 mM imidazole) wash fraction; lane 3, MCAC-110 wash fraction; lane 4, MCAC-250 wash fraction. The MCAC-110 wash fractions were pooled and concentrated (lane 5; ~6 μg). Gel was stained with Coomassie Blue. **B.** 0.1 μg of the MCAC-85 eluate was resolved by SDS-PAGE, transferred to PVDF membrane, and probed with anti-oligo(His) antibody. Green arrow-heads indicate the bands detected by anti-oligo(His), corresponding to the two overexpressed proteins (black arrow-heads).

As the MBP-tag is quite large, it was preferable to remove it prior to performing functional studies on the recombinant protein. Factor Xa successfully cleaved MBP from MBP-PfFKBP35-His_6, but as was the case for factor Xa-treated MBP-PfFKBP35, problems with purification of the resulting protein mixture arose (Fig. 4.10). The chosen method for purification of factor Xa treated sample was a second round of nickel-chelate affinity chromatography, as the cleaved protein of interest (PfFKBP35-His_6) would still incorporate its C-terminal oligo(His)-tag. Free MBP was separated from the mixture by this method (Fig. 4.10A, lanes 3 and 4), but most of the PfFKBP35-His_6 apparently failed to elute from the column. An alternative strategy was, therefore, devised. It was reasoned that amylose affinity chromatography performed on the factor Xa-treated sample would allow the cleaved PfFKBP35-His_6 protein of interest to flow through the column, while retaining the cleaved MBP fragment. This approach proved somewhat successful, but the PfFKBP35-His_6 sample was not of sufficient purity (Fig. 4.10B, lane 3). It was reasoned that the purity of this sample would be improved by subjecting it to nickel-chelate affinity chromatography. This third round of purification, however, resulted in the loss of PfFKBP35-His_6 (Fig. 4.10B, lane 5). It was thought that the ~66 kDa protein (Fig. 4.10B, lane 5) that was purified from this approach (and also from the approach depicted in Fig. 4.10A, lane 5) could represent a dimer of PfFKBP35-His_6, but performing the purification under denaturing conditions suggested this not to be the case (Fig. 4.10B, lane 6). This was confirmed by subjecting the protein band to N-terminal sequence analysis. This unequivocally showed the protein to correspond to bovine albumin (data not shown). The most likely source of this contaminant was from the commercial preparation of factor Xa used in these experiments, which was isolated from bovine plasma. Although it is supposedly a highly purified preparation, it appears that BSA contaminated the particular batch used in these experiments. Indeed, when pre-cleaved and cleaved samples of MBP-PfFKBP35-His_6 were run side-by-side on SDS-PAGE gels, the contaminating BSA was clear (compare lanes 1 and 2 in both Fig. 4.10A and B). It is strange, however, that the nickel-chelate resin retained the BSA.

Intriguingly, although it proved impossible to purify PfFKBP35-His_6 following cleavage from MBP-PfFKBP35-His_6, the same procedure proved successful in isolating factor Xa-cleaved FKBP-His_6 from MBP-FKBP-His_6, a construct comprising just the FKBP domain of PfFKBP35 fused directly to a His_6

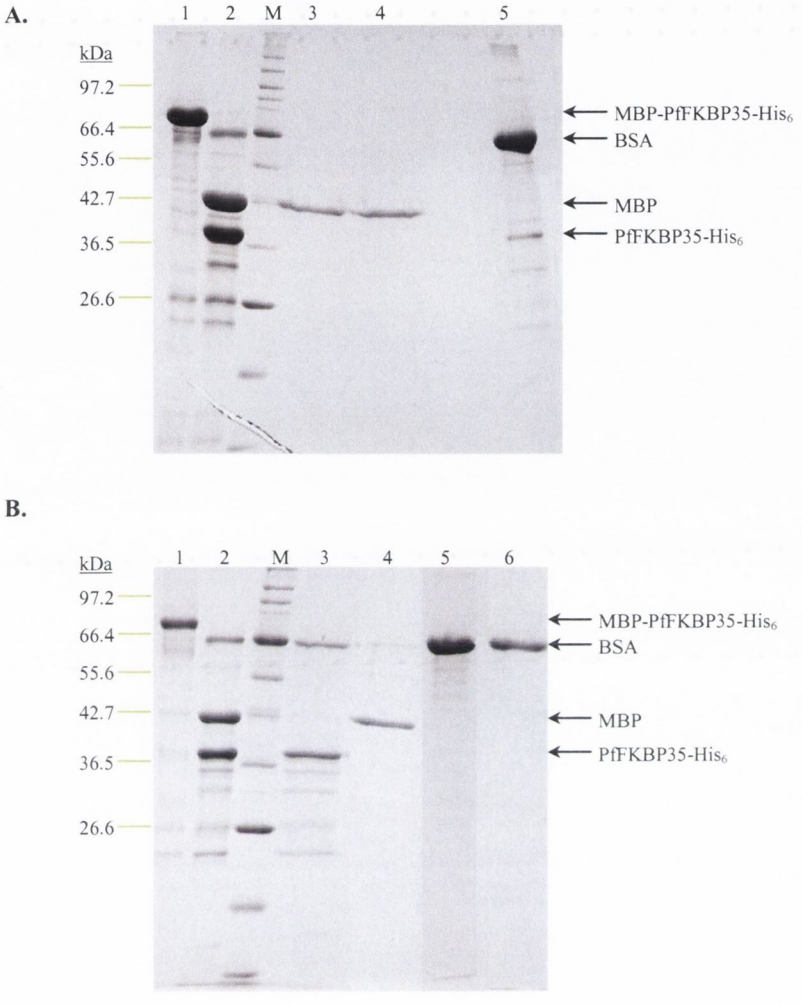


Fig. 4.10. Cleavage of MBP from MBP-PfFKBP35-His_6 by factor Xa. **A.** MBP-PfFKBP35-His_6 purified by nickel-chelate chromatography and concentrated (lane 1), was incubated at 22°C with factor Xa for 24 h (lane 2). This mixture was loaded onto a second nickel-chelate column, washed with 2 x 50 ml MCAC-0 buffer (0 mM imidazole – lane 3 and 4) and eluted with 1 x 50 ml MCAC-500 (500 mM imidazole – lane 5). **B.** As an alternative purification strategy, MBP-PfFKBP35-His_6 treated with factor Xa (lane 2; lane 1 = pre-cleaved MBP-PfFKBP35-His_6) was loaded onto an amylose column, washed with 100 ml AC-0 buffer (0 mM maltose – lane 3) and eluted with 50 ml AC-10 buffer (10 mM maltose – lane 4). The wash fraction (lane 3) was dialysed against MCAC-0 buffer and loaded onto a nickel-chelate column, and washed and eluted as described for panel A. The MCAC-500 eluate (lane 5) was dialysed against MCAC-0 buffer, and subjected to another round of nickel-chelate chromatography, this time under denaturing conditions (lane 6 = MCAC-0/8 mM urea wash fraction). All fractions from columns were concentrated with TCA prior to separation through SDS-PAGE (12.5% v/v) gels. BSA contaminant is visible in lanes 2 and 5 (top panel) and lanes 2, 3, 5 and 6 (bottom panel). Gels stained with Coomassie Blue. M = molecular weight markers (sizes indicated in kDa).

tag – see section 4.2.1.4 (Fig. 4.11 - a different preparation of factor Xa was used here, showing no contaminating BSA). This finding lends support to the theory of peculiarities associated with the C-terminal region of MBP-PfFKBP35-His_6 causing the purification problems.

Due to the failure in obtaining sufficiently pure PfFKBP35-His_6, it was decided to use MBP-PfFKBP35-His_6 for subsequent functional studies. However, further purification of this sample was required. Eluates obtained from nickel-chelate affinity chromatography were pooled together and concentrated (see lane 5 in Fig. 4.9A) and subsequently subjected to ion-exchange chromatography. Eluates from this secondary purification step were pooled together, concentrated and analysed by SDS-PAGE (Fig. 4.12). This sequential use of nickel-chelate affinity and ion-exchange chromatographies resulted in a sufficiently pure sample of MBP-PfFKBP35-His_6 to commence functional studies.

4.2.1.4. Generation of MBP-His_8, MBP-FKBP-His_6 and MBP-TPR-His_6

Prior to commencing functional studies of MBP-PfFKBP35-His_6, it was deemed necessary to produce a control protein in order to rule out any effects of the large MBP-tag. An inverse PCR approach was used to delete *PfFKBP35* from the pMAL-PfFKBP35-His_6 construct thereby creating pMAL-His_8, a construct directing the expression of MBP-His_8 (Fig. 4.13 – an inexplicable cloning artifact resulted in the sequence for an oligo$(His)_8$-tag rather than an oligo$(His)_6$-tag being incorporated into this construct). At this stage it was also decided to generate recombinant forms of the isolated FKBP and TPR domains of PfFKBP35 to study the role of these two domains. As for the full length protein, these were produced with an N-terminal MBP-tag and a C-terminal oligo(His)-tag. The pMAL-PfFKBP35-His_6 construct served as the template in inverse PCRs to generate both pMAL-FKBP-His_6 (directing the expression of MBP-FKBP-His_6 [i.e. the isolated FKBP domain of PfFKBP35]) and pMAL-TPR-His_6 (directing the expression of MBP-TPR-His_6 [i.e. the isolated TPR domain of PfFKBP35]) (Fig. 4.13). All three of these proteins were purified from *E. coli* exactly as described for MBP-PfFKBP35-His_6 (i.e. sequential nickel-chelate affinity and ion-exchange chromatographies - Fig. 4.13B).

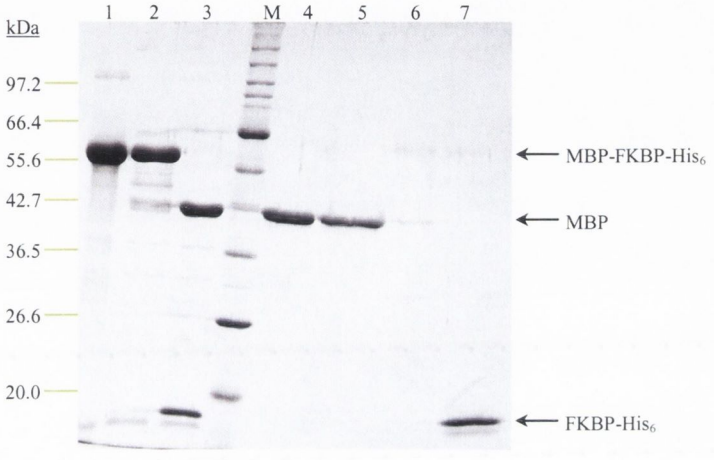


Fig. 4.11. Cleavage of MBP from MBP-FKBP-His_6 by factor Xa. MBP-FKBP-His_6, purified by nickel-chelate chromatography and concentrated (lane 1), was incubated at 22°C with factor Xa for 24 h (lane 2 = sample at 0 h; lane 3 = sample at 24 h). This mixture was loaded onto a second nickel-chelate column (5 ml bed volume; lane 4 = flowthrough fraction), washed with 50 ml MCAC-0 buffer (0 mM imidazole – lane 5) and eluted with 50 ml MCAC-110 (110 mM imidazole – lane 6) and 30 ml MCAC-500 (lane 7). All fractions were concentrated with TCA prior to separation through SDS-PAGE (12.5% v/v) gels. Gel was stained with Coomassie Blue. The cleaved MBP tag was collected in the flowthrough and wash fractions. The cleaved FKBP-His_6 protein was successfully collected in the MCAC-500 eluate. M = molecular weight markers (sizes indicated in kDa).

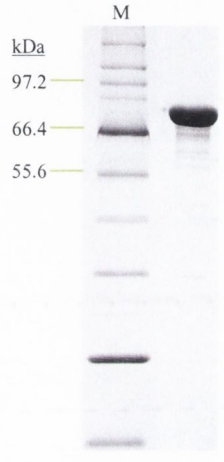
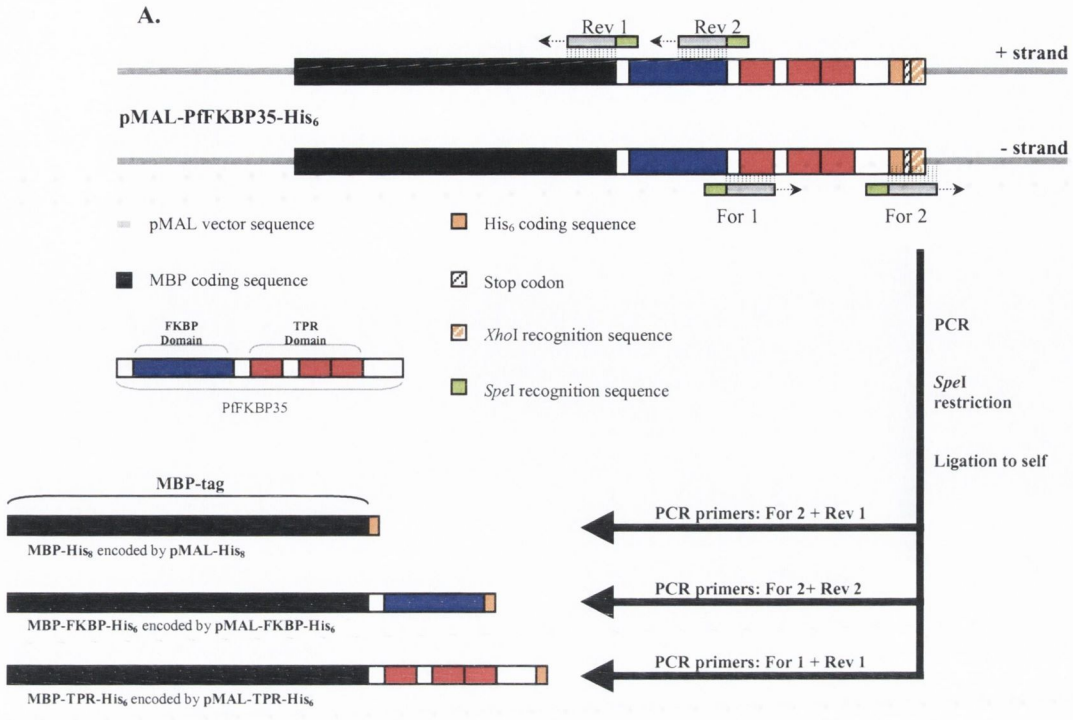


Fig. 4.12. Pure MBP-PfFKBP35-His_6. 3 μg of MBP-PfFKBP35-His_6, purified by sequential nickel-chelate and ion exchange chromatographies, was run through a 12.5% (v/v) SDS-PAGE gel and stained with Coomassie Blue. M = molecular weight markers (sizes indicated in kDa).



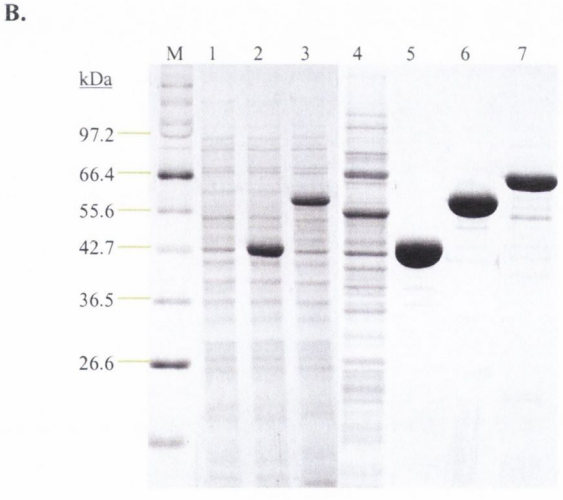


Fig. 4.13. Generation of MBP-His_8, MBP-FKBP-His_6 and MBP-TPR-His_6. **A.** pMAL-PfFKBP35-His_6 served as the template in three separate inverse PCRs to construct pMAL-His_8 (directing expression of MBP-His_8), pMAL-FKBP-His_6 (directing expression of MBP-FKBP-His_6 [i.e. N-terminal region of PfFKBP35]) and pMAL-TPR-His_6 (directing expression of MBP-TPR-His_6 [i.e. C-terminal region of PfFKBP35]). **B.** Overexpression of MBP-His_8 (lane 2), MBP-FKBP-His_6 (lane 3), and MBP-TPR-His_6 (lane 4 – two induced bands visible) following treatment of *E. coli* clones harbouring each individual construct from panel A with 0.35 mM IPTG. Lane 1 = uninduced *E. coli* recipient strain. Lysates resolved by SDS-PAGE (12.5%) and stained with Coomassie Blue. The three proteins were purified as described in section 4.2.1.4.: lane 5 = MBP-His_8; lane 6 = MBP-FKBP-His_6; lane 7 = MBP-TPR-His_6 (all 3 μg). M = molecular weight markers (sizes indicated in kDa).

4.2.2. PPIase activity of MBP-PfFKBP35-His_6

To investigate if MBP-PfFKBP35-His_6 possessed PPIase activity, the chymotrypsin-coupled spectrophotometric assay of Kofron *et al* (1991) was employed. This assay monitors the isomerisation of the peptidyl-prolyl bond in a tetrapeptide (succinyl-Ala-Leu-Pro-Phe) linked at the C-terminus to the chromagen para-nitroanilide (*p*-NA). The anilide bond tethering the chromagen to the tetrapeptide can be hydrolysed by α-chymotrypsin, releasing *p*-NA into solution, but only when the Leu-Pro bond incorporated in the test peptide is in the *trans* conformation. If this peptidyl-prolyl bond is initially in the *cis* conformation, the PPIase activity of an added enzyme can be assessed by monitoring how quickly the α-chymotrypsin-mediated cleavage occurs by measuring the concomitant increase in absorbance of free *p*-NA at 390nm. MBP-PfFKBP35-His_6 exhibited strong PPIase activity (Fig. 4.14A) with a clear concentration-dependent effect (Fig. 4.14B). The MBP-His_8 double tag was shown to confer no PPIase activity to the recombinant molecule. The enzymatic activity of MBP-PfFKBP35-His_6 was significantly less than that exhibited by a recombinant form of human Cyp18 (Fig. 4.14A), a finding that agrees with the general observation that FKBPs are less enzymatically active than cyclophilins, even when substrates optimised for FKBPs are used. The catalytic efficiency (k_{cat}/K_M) of 1.7 x 10^4 $s^{-1}M^{-1}$ for MBP-PfFKBP35-His_6 was within the expected range for FKBPs (Fig. 4.14B).

The PPIase activity of MBP-PfFKBP35-His_6 was abolished by a 20-fold molar excess of FK506 or rapamycin, but not CsA (Fig. 4.15A). The effects of both macrolides on the enzymatic activity of MBP-PfFKBP35-His_6 were shown to be concentration-dependent, with IC_{50} values of 0.32 μM (FK506) and 0.48 μM (rapamycin) against 0.25 μM enzyme (Fig. 4.15B).

From analysis of the PPIase activity of both MBP-FKBP-His_6 and MBP-TPR-His_6, it is evident that the PPIase activity of PfFKBP35 is wholly attributed to its N-terminal portion, which encompasses the FKBP domain, with no such activity being exhibited by the C-terminal, TPR portion (Fig. 4.16A). The finding that the FKBP domain alone has FK506/rapamycin-inhibitable PPIase activity (Fig. 4.16B) provides strong evidence that this is, as expected, the drug-binding domain. As was the case for the full-length protein, CsA at up to 5 μM had no effect on the PPIase activity of the isolated FKBP domain.

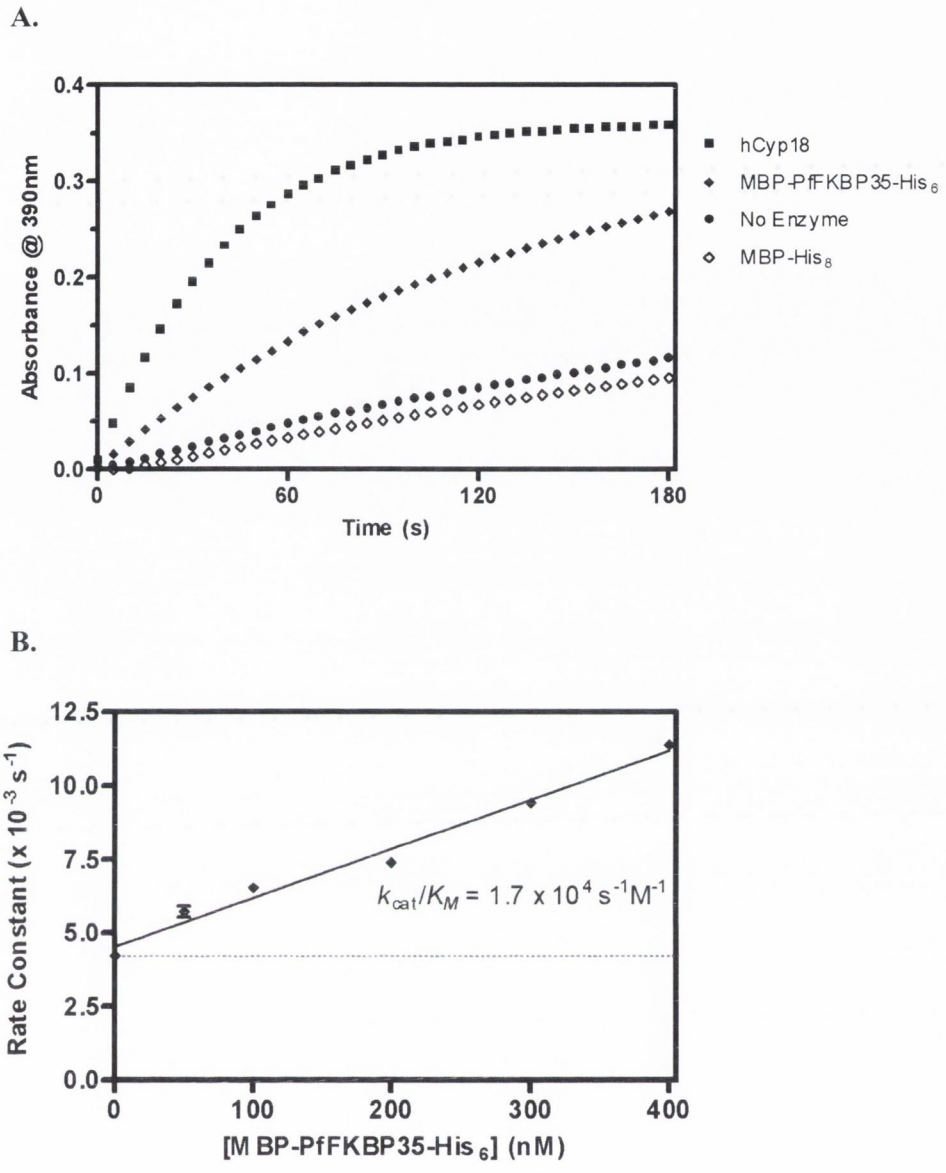
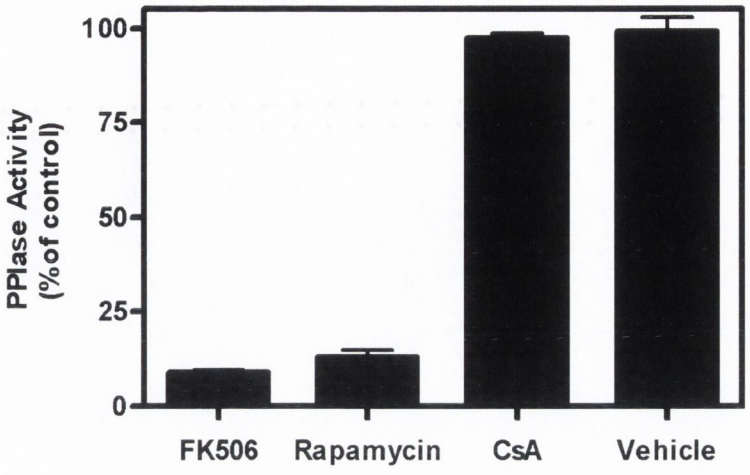


Fig. 4.14. PPIase activity of recombinant PfFKBP35. **A:** Representative curves showing increase in absorbance over time as a consequence of isomerisation of the Leu-Pro bond in a synthetic peptide substrate (MBP-PfFKBP35-His_6 = 250 nM; hCyp18 = 15 nM). **B:** Concentration-dependence of PPIase activity of MBP-PfFKBP35-His_6. The assay was repeated with various concentrations of enzyme, and first order rate constants (k) and the catalytic efficiency (k_{cat}/K_m) were calculated as described in Materials and Methods. The dashed line represents the background isomerisation rate in the absence of enzyme. Bars show SEM of three or more replicates.

A.



B.

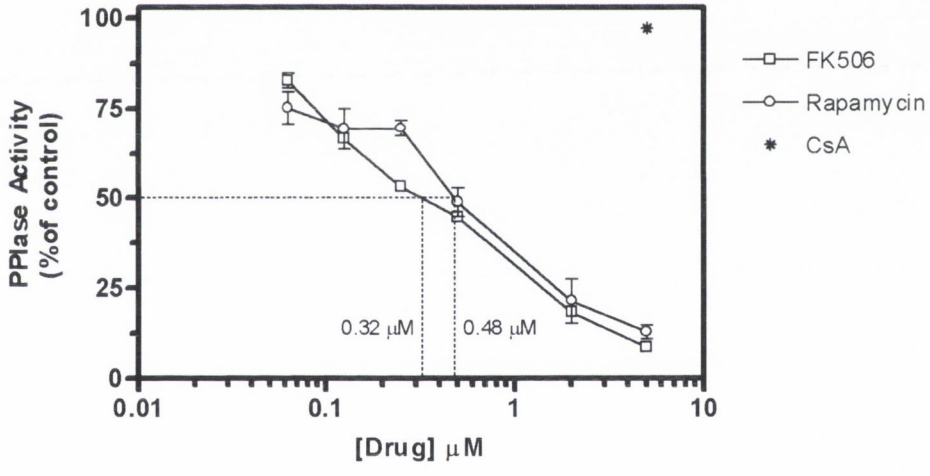


Fig. 4.15. Effects of FK506, rapamycin and CsA on PPIase activity of MBP-PfFKBP35-His_6 (0.25 μM). Activity of 0.25 μM MBP-PfFKBP35-His_6 in the absence of drug was set at 100%. **A.** PPIase assays were performed in the presence of a fixed concentration (5 μM) of drug. The vehicle for FK506 and rapamycin was ethanol. **B.** Dose-dependent inhibition of PPIase activity of MBP-PfFKBP35-His_6. IC_{50} values for FK506 and rapamycin are shown. Bars show SEM of three or more replicates.

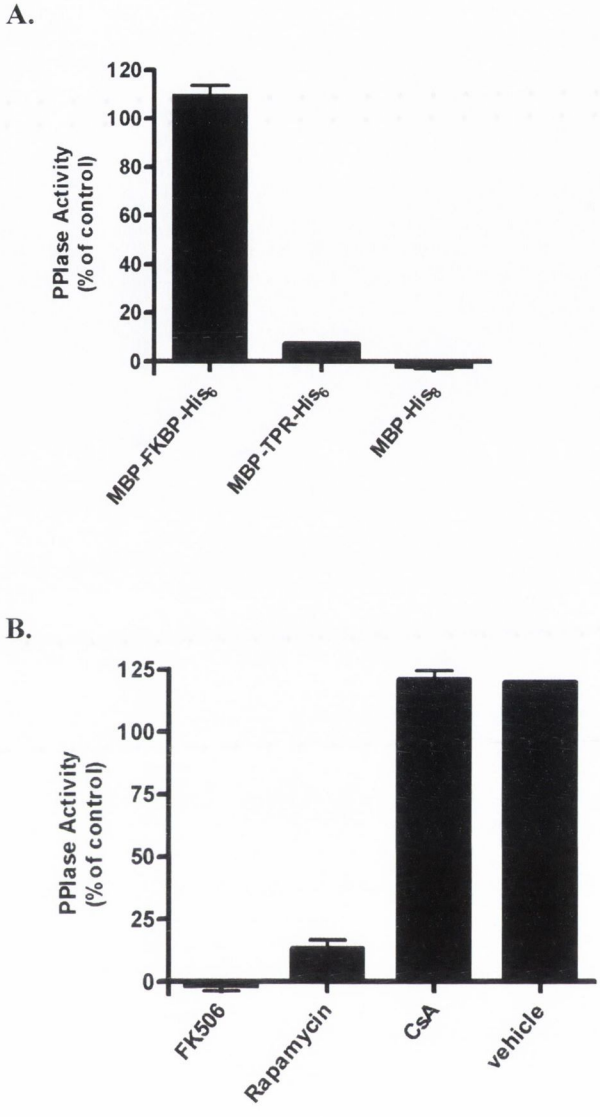


Fig. 4.16. PPIase activity of isolated domains of PfFKBP35. **A.** The PPIase activities of both the FKBP domain (0.25 μM MBP-FKBP-His_6) and TPR domain (0.25 μM MBP-TPR-His_6) of PfFKBP35 were measured and compared with that of the full-length protein (PPIase activity of 0.25 μM MBP-PfFKBP35-His_6 set as 100%). **B.** For the case of MBP-FKBP-His_6, the effects of FK506, rapamycin, CsA (all at 5 μM) and drug vehicle (ethanol) on its PPIase activity were also assessed. PPIase activity of 0.25 μM MBP-FKBP-His_6 set as 100%. Bars show SEM of three or more replicates.

4.2.3. Inhibition of calcineurin by PfFKBP35

To investigate if the complex of PfFKBP35 and FK506 could inhibit calcineurin, the phosphatase activity of bovine brain calcineurin was measured in the presence and absence of PfFKBP35 and/or FK506 using a fluorescence assay (Fig. 4.17). Surprisingly, MBP-PfFKBP35-His_6 inhibited calcineurin in the absence of FK506, albeit weakly, a phenomenon that has only been reported for one other FKBP, hFKBP38 (Shirane and Nakayama, 2003). hFKBP51 has been shown to interact with calcineurin in the absence of FK506 (Li *et al.*, 2002), but the phosphatase activity is only inhibited in the presence of FK506 (Baughman *et al.*, 1997). The interaction of hFKBP51 with calcineurin is mediated by the immunophilin's C-terminal region, not the FKBP domain (Li *et al.*, 2002). The inhibition of calcineurin by PfFKBP35, by contrast, is mediated by the FKBP domain (Fig. 4.17A).

4.2.4. Chaperone activity of MBP-PfFKBP35-His_6

Numerous TPR-containing proteins are known to act as molecular chaperones, including TPR-containing immunophilins, such as hFKBP51, hFKBP52 and hCyp40 (Pirkl and Buchner, 2001). The ability to prevent the thermal aggregation of citrate synthase is the most commonly used model system for assessing chaperone capability of a given protein (Buchner *et al.* 1998). Citrate synthase begins to aggregate within 1-2 minutes of incubation at 43°C, but in the presence of MBP-PfFKBP35-His_6, this aggregation was suppressed (Fig. 4.18A). This effect was also seen for the inhibition of thermal aggregation of another model substrate, rhodanese (Fig. 4.18B). The MBP-His_8 tag had no chaperone effects in either system. FK506 and rapamycin at the concentrations tested were shown to have no inhibitory effect on the chaperone abilities of MBP-PfFKBP35-His_6 in either of the model systems (Fig. 4.18A and B).

The chaperone activity of hFKBP52 is localised to its C-terminal TPR domain (Pirkl *et al.*, 2001). To assess whether this was the case for PfFKBP35, the N-terminal (FKBP) and the C-terminal (TPR) domains (MBP-FKBP-His_6 and MBP-TPR-His_6, respectively) were tested for chaperone capabilities with both citrate synthase and rhodanese (Fig. 4.19A). To our surprise, both domains inhibited the thermal aggregation of the model substrates. The effects of FK506 on the chaperone activity of MBP-FKBP-His_6 and MBP-TPR-His_6 were assessed in both

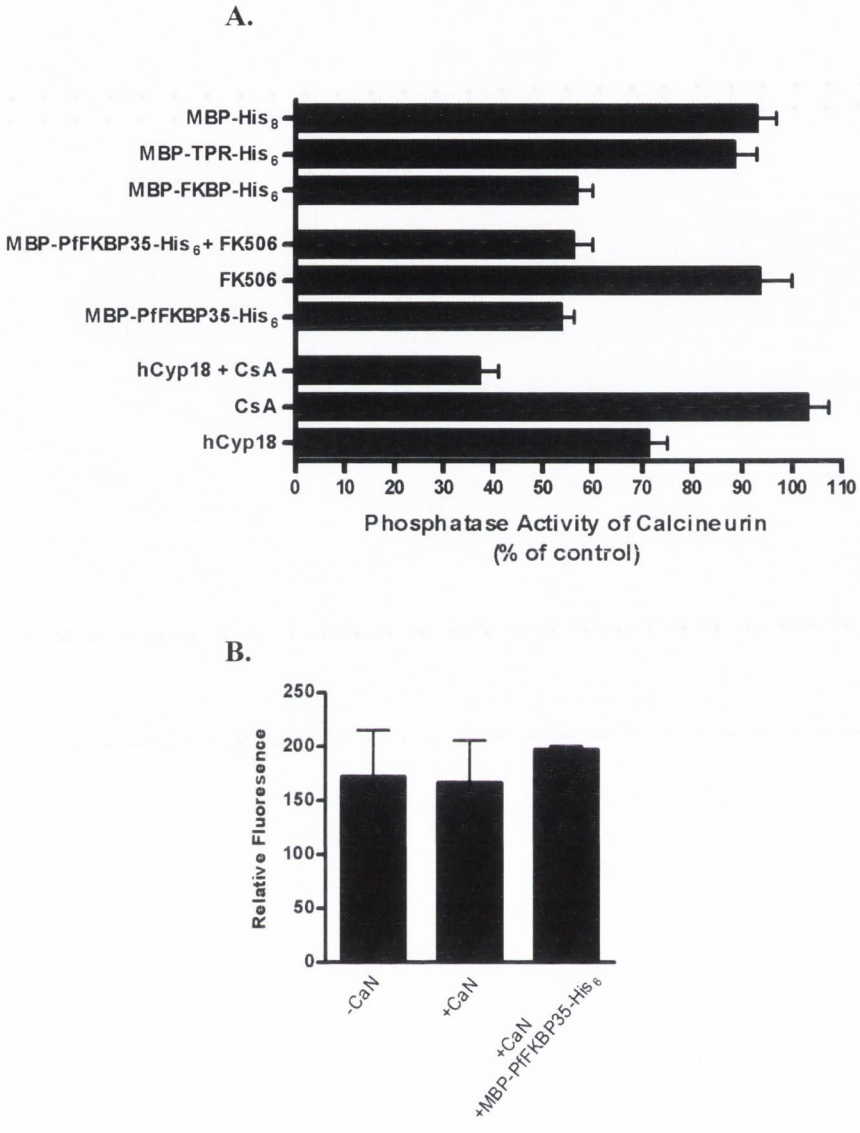


Fig. 4.17. Inhibition of calcineurin by PfFKBP35. **A.** Activity of calcineurin was measured by the fluorescence of a rhodamine-conjugated peptide substrate that, upon dephosphorylation by calcineurin, is digested by a protease. Only the cleaved product is fluorescent. The effects of various proteins and/or drugs on the activity of calcineurin were assessed. The fluorescence attributable to 40 nM calcineurin was set as 100%. All additional components were at 1 μM. **B.** To ensure that reduced fluorescence was not due to inhibition of the protease, a control peptide (conjugated to the fluorophore aminomethylcoumarin), whose cleavage and resulting fluorescence is independent of phosphorylated state, was incorporated into each reaction. No inhibition of protease occurred. Bars show the SEM of five or more replicate experiments. CaN = calcineurin.

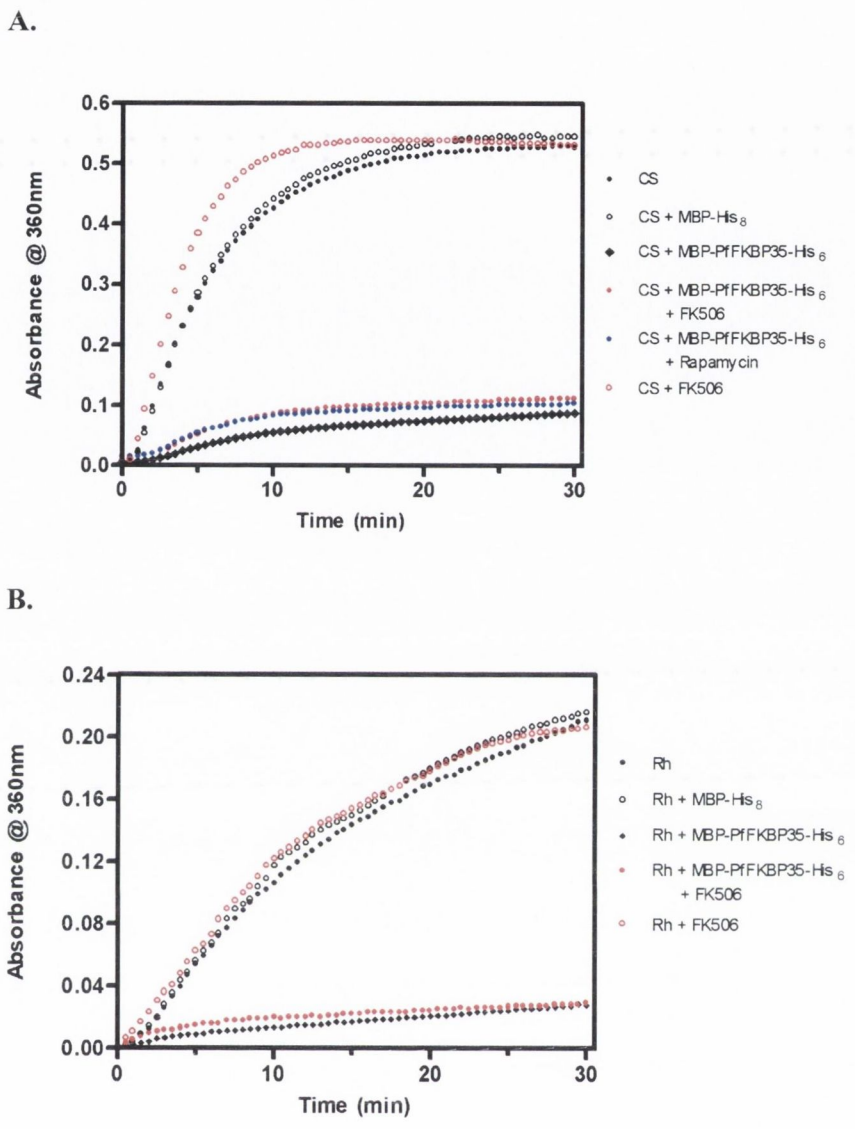


Fig. 4.18. Inhibition of thermal aggregation of citrate synthase and rhodanese by MBP-PfFKBP35-His_6. **A:** Chaperone activity of MBP-PfFKBP35-His_6 (1.5 μM) against citrate synthase (CS - 1.5 μM). FK506 and rapamycin were present, where indicated, at 15 μM. **B:** Chaperone activity of MBP-PfFKBP35-His_6 (4.4 μM) against rhodanese (Rh - 4.4 μM). FK506 present, where indicated, at 22 μM. MBP-His_8 double tag alone had no effect on aggregation of either model substrate.

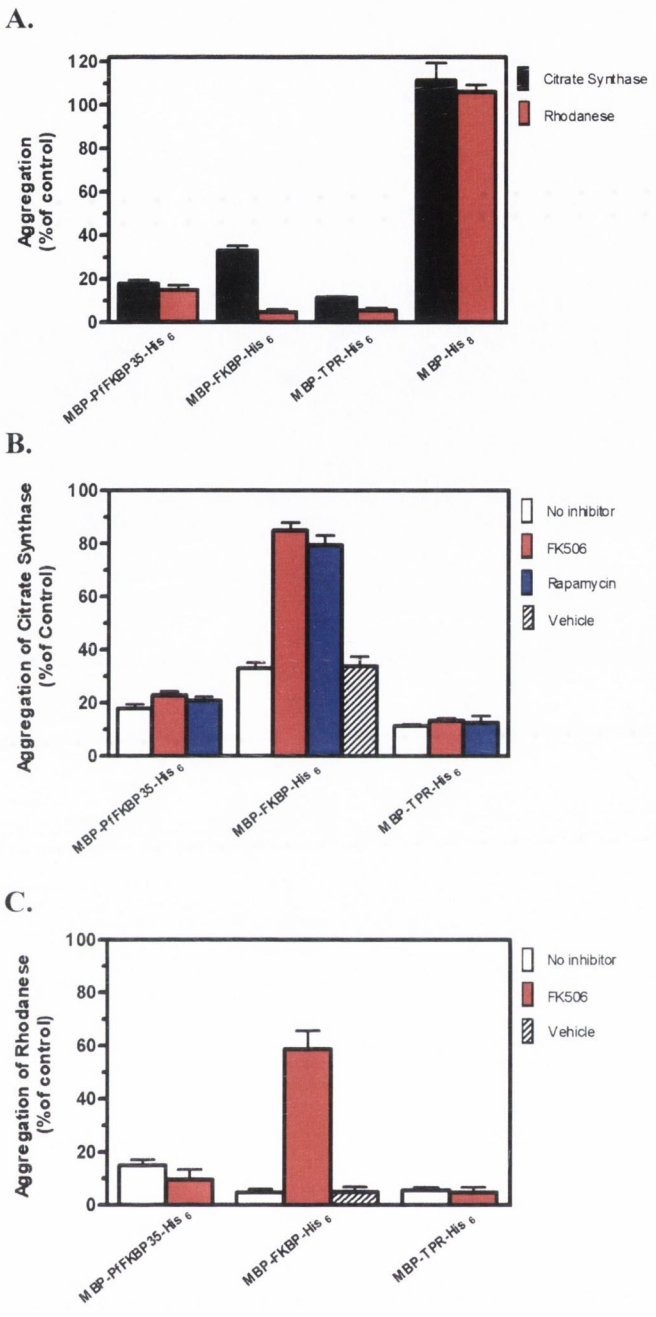


Fig. 4.19. Inhibition of thermal aggregation of citrate synthase and rhodanese by N-terminal and C-terminal domains of PfFKBP35 **A:** Chaperone activity of equimolar amounts of MBP-PfFKBP35-His_6 or MBP-TPR-His_6 on citrate synthase (1.5 μM) and rhodanese (4.4 μM). **B:** Effects of FK506 or rapamycin (15 μM) on chaperone activities of MBP-PfFKBP35-His_6, MBP-PfFKBP35-His_6 and MBP-TPR-His_6 (1.5 μM) on citrate synthase. **C:** Effect of FK506 (22 μM) on chaperone activities of MBP-PfFKBP35-His_6, MBP-PfFKBP35-His_6 and MBP-TPR-His_6 (4.4 μM) on rhodanese. Drug vehicle (ethanol) had no effect on chaperone activity of MBP-FKBP-His_6 against either model substrate. Aggregation was monitored as per Fig. 4.18, and these histograms were plotted using the maximum absorbance values obtained during the heating process. Aggregation (maximum absorbance value) of citrate synthase or rhodanese in the absence of additional components was set as 100%.

model systems (Fig. 4.19B and C). As was the case for the full-length protein, FK506 had no effect on the chaperone activity of MBP-TPR-His_6. However, it did suppress the chaperone activity of MBP-FKBP-His_6. The same inhibitory effect against MBP-FKBP-His_6, but not MBP-TPR-His_6, was also observed with rapamycin in the citrate synthase assay (Fig. 4.19B – rapamycin not tested in rhodanese assay).

While all molecular chaperones can suppress the aggregation of partner proteins, this does not necessarily mean that they prevent the substrate from losing activity. Hsp90, for example, prevents partner proteins from becoming inactivated, while Hsp25 fails to prevent its substrates from losing activity (Buchner *et al.*, 1998). To assess to which group of chaperones PfFKBP35 belongs, the activity of citrate synthase was measured during incubation at 43°C in the presence or absence of MBP-PfFKBP35-His_6. The half-time for the loss of citrate synthase activity in the presence of MBP-PfFKBP35-His_6 (1.6 min; Fig. 4.20) was comparable to that in the absence of additional components (1.1 min). Hsp90 from *S. cerevisicae*, however, significantly slowed down this inactivation process, with citrate synthase exhibiting a half-time of 7.5 min in the presence of yHsp90.

4.2.5. Investigation of binding partners of PfFKBP35

The presence of TPR motifs strongly suggests that PfFKBP35 may play a role in intracellular protein transport or modulation of protein function. The striking similarity in domain architecture between PfFKBP35 and hFKBP37, hFKBP51 and hFKBP52, all of which function in this way, supports this hypothesis. These FKBPs, as discussed in section 1.4.2.2, are found as part of large hetero-oligomeric complexes. Their participation in such complexes appears to be mediated by their interaction with Hsp90. Comparison of the three TPR motifs of PfFKBP35 with the TPR motifs of known Hsp90-binding proteins (Fig. 3.8) suggests Hsp90 to be a possible interacting partner. *P. falciparum* is known to have at least two Hsp90 homologues (Kumar *et al.*, 2003), although a data-mining approach in this study identified two additional putative Hsp90 genes in the *P. falciparum* genome (Table 4.3). To investigate such an interaction, both MBP– and His_6–pull-down procedures were performed by immobilising MBP-PfFKBP35-His_6 to either amylose resin or nickel-chelate resin, mixing with Hsp90 from *S. cerevisiae* (yHsp90) at 37°C, and analysing the wash and elution fractions by SDS-PAGE (Fig. 4.21). For both

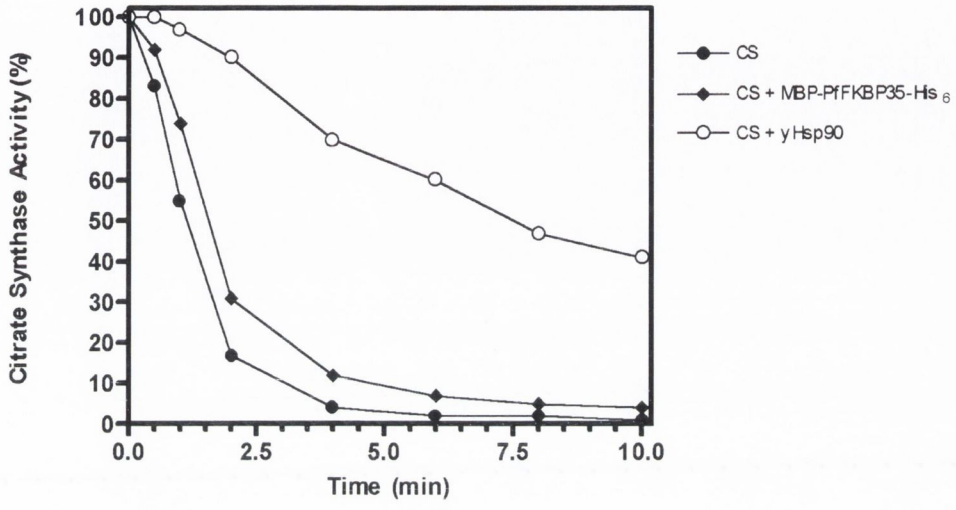


Fig. 4.20. Loss of activity of citrate synthase during incubation at 43°C. 0.15 μM citrate synthase (CS) was incubated at 43°C in the absence of additional components or in the presence of 1.5 μM MBP-PfFKBP35-His_6 or 0.9 μM yHsp90. At indicated time-points, samples were withdrawn and the remaining activity of citrate synthase was measured. The activity of citrate synthase at time = 0 min was set to 100%. All points represent averaged values from at least two replicate experiments.

Table 4.3. *P. falciparum* homologues[a] of major chaperones and co-chaperones

Chaperone	Accession No.	Chromosome	Predicted MW (kDa)	Reference
Hsp40	NP_703333	1	40.3	This study
	NP_473047	2	37.4	This study
	F71623	2	40.3	This study
	NP_472947	2	45.7	This study
	BAA22060	4	70.7	Watanabe, 1997
	NP_703357	5	47.7	This study
	NP_703578	5	61.9	This study
	NP_703949	6	44.7	This study
	NP_704487	8	76.8	This study
	NP_704730	9	43.3	This study
	BAA33726	11	62.3	This study
	NP_701358	11	68.0	This study
	NP_701478	12	28.1	This study
	NP_705208	13	31.2	This study
	NP_705096	13	75.9	This study
	NP_702248	14	48.5	This study
Hsp60	A71610	2	60.8	This study
	NP_703749	6	61.5	This study
	NP_700627	10	62.6	Syin and Goldman, 1996
	NP_701191	11	60.3	This study
	P34940	12	79.4	Holloway *et al.*, 1994
Hsp70	AAC47838	7	97.9	This study
	NP_704366	8	73.9	Bianco *et al.*, 1986; Ardeshir *et al.*, 1987
	CAA48873	9	72.4	Kumar *et al.*, 1988
	NP_701211	11	73.3	This study
Hsp90	CAA82765	7	86.1	Bonnefoy *et al.*, 1994; Su and Wellems, 1994
	NP_701048	11	108.5	This study
	chr12.phat_228 [b]	12	95.0	Kumar *et al.*, 2003
	NP_702306	14	106.9	This study
Co-chaperone				
Hip[c]	NP_703618	5	51.1	This study
Hop[d]	NP_702213	14	66.1	This study
p23	NP_702399	14	30.6	Wiser, 2003

[a] Genes were identified by analysing the *P. falciparum* genome. Except for where a citation appears, expression of genes was not experimental confirmed.

[b] Accession number specific for data stored at www.plasmodb.org

[c] *H*eat shock *i*nteracting *p*rotein

[d] *H*eat shock *o*rganising *p*rotein

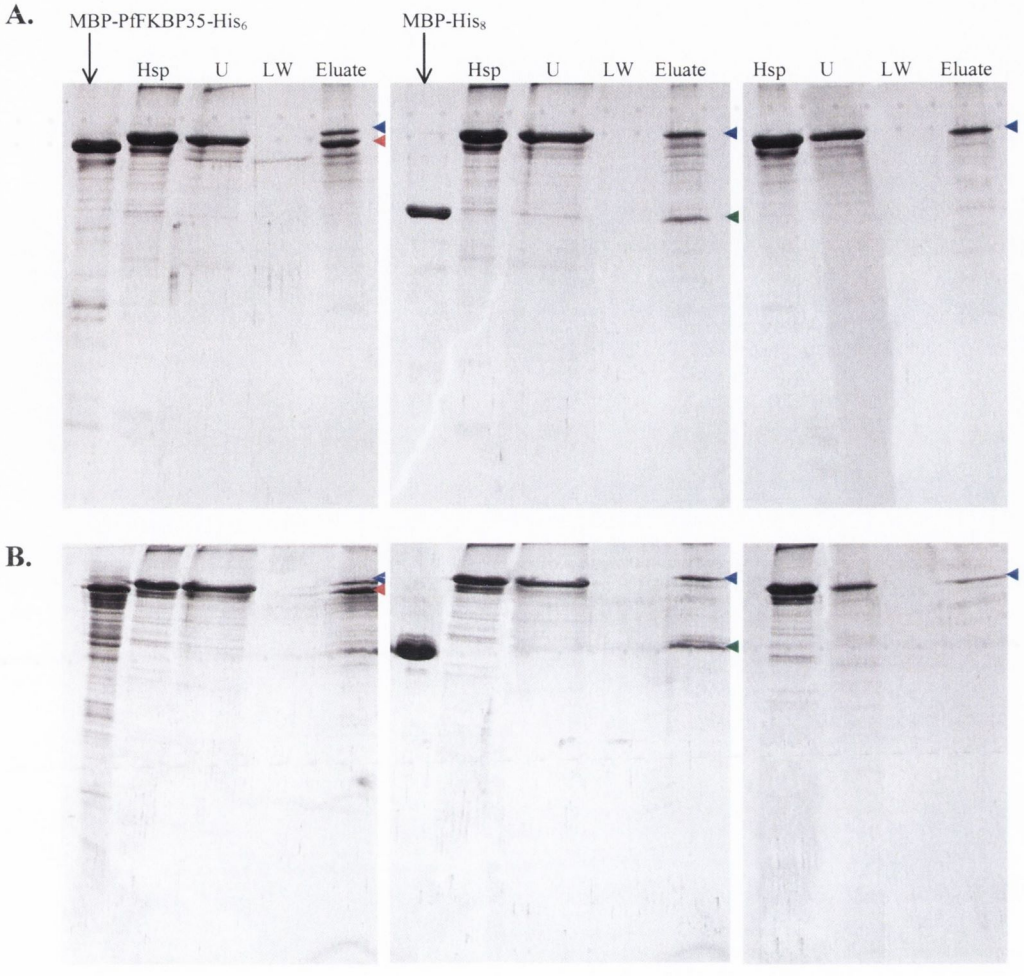


Fig. 4.21. Investigation of possible interaction between PfFKBP35 and Hsp90 by pull-down analysis. **A.** Investigation of interaction by MBP–pull-down technique. Amylose resin was incubated with MBP-PfFKBP35-His_6 (left panel), MBP-His_8 (middle panel) or buffer (right panel) in microfuge tubes, and subsequently mixed with yHsp90 (Hsp) for 1 h at 37^0C. Unbound yHsp90 (U) was removed by centrifugation and the resin washed a number of times with AAC buffer (last wash shown, LW). Bound proteins (including MBP-fusions) were eluted from the resin by mixing with SDS-PAGE loading buffer and boiling for 10 min. Samples were resolved by SDS-PAGE (12.5% v/v) and stained with Coomassie Blue. **B.** Investigation of interaction by His_6–pull-down technique. Nickel-chelate resin was incubated with MBP-PfFKBP35-His_6 (left panel), MBP-His_8 (middle panel) or buffer (right panel) in microfuge tubes, and treated as described for panel A above, with MCAC-O buffer replacing AAC buffer. Arrow-heads indicate eluted proteins: blue, yHsp90; red, MBP-PfFKBP35-His_6; green, MBP-His_8. Approximately 2 μg of each of MBP-PfFKBP35-His_6, yHsp90 and MBP-His_8 were loaded on gels. U, LW and Eluate fractions were precipitated with TCA prior to loading on gel.

procedures, yHsp90 was found in the eluted fractions, but not the pre-elution wash fractions, indicating that yHsp90 was retained by both systems (left panels of Fig. 4.21). However, further analysis showed yHsp90 to be retained by negative control resins (MBP-His_8 immobilised to resin, or resin with no fusion protein immobilised), indicating that yHsp90 was binding to the resin itself. This phenomenon was obtained when yHsp90 was mixed with resins at 4°C and room temperature (data not shown).

The results from the MBP- and His_6–pull-down procedures were inconclusive, so alternative means of assessing any potential interaction between yHsp90 and MBP-PfFKBP35-His_6 were needed. Far-Western analysis, in which a membrane onto which yHsp90 had been transferred was incubated with a solution of MBP-PfFKBP35-His_6 and subsequently probed with anti-MBP antibody, showed no interaction between MBP-PfFKBP35-His_6 and yHsp90 (Fig. 4.22). Extract of an asynchronous *P. falciparum* culture was also used in this analysis in an attempt to identify MBP-PfFKBP35-His_6–interacting proteins directly from the parasite. However, no bands were visible on the autoradiogram (Fig. 4.22). Increasing the duration of exposure of the membrane to MBP-PfFKBP35-His_6 failed to identify any interaction (data not shown). Glutaraldehyde failed to cross-link MBP-PfFKBP35-His_6 to yHsp90 (Fig. 4.23), further suggesting that no interaction occurs between these proteins.

The MBP–pull-down technique described above (results shown in Fig. 4.21A) utilised a commercial preparation of yHsp90. In an attempt to identify an interaction between MBP-PfFKBP35-His_6 and *P. falciparum* Hsp90 (PfHp90), the procedure was repeated using whole cell extract of an asynchronous *P. falciparum* culture in place of yHsp90. The wash and elution fractions were analysed by Western immunoblotting, using monoclonal anti-Hsp90 antibody (previously shown to cross-react with PfHsp90 – data not shown) as the primary antibody (Fig. 4.24A – upper panel). As was the case when yHsp90 was used, however, PfHsp90 appeared to bind to some extent to the resin itself.

PfHsp90 has been shown to interact with PfPP5 (Dobson *et al.*, 2001). In human cells, this phosphatase appears to function in a similar manner to hFKBP52 in steroid receptor complexes, with both proteins linking the receptor complex to dynein (Galigniana *et al.*, 2002). Although hFKBP52 and hPP5 compete with each other for binding to hHsp90, and therefore their participation in hetero-oligomeric

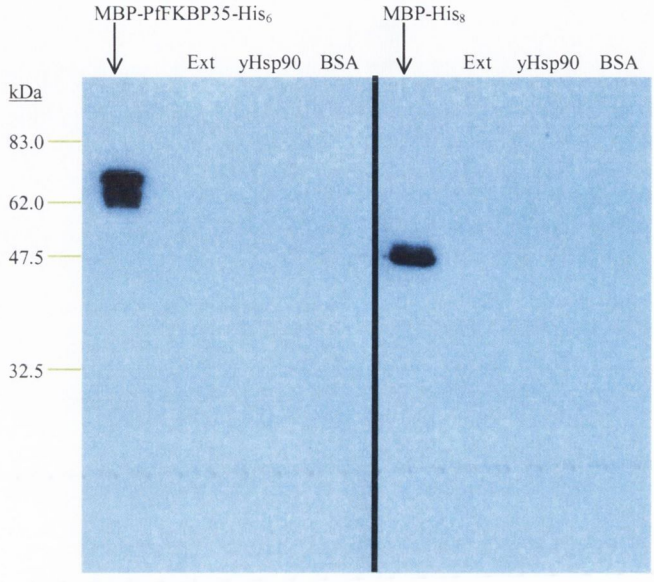


Fig. 4.22. Investigation of possible interaction between PfFKBP35 and Hsp90 by Far-Western analysis. Proteins (indicated above, Ext = *P. falciparum* extract [10 μg]; all others, 2 μg) were resolved by SDS-PAGE (12.5% v/v) and transferred to PVDF membrane. The membrane was divided in two and probed with 40 μg MBP-PfFKBP35-His_6 (left panel) or MBP-His_8 (right panel) for 4 h at 4°C. Both membranes were subsequently washed and subjected to Western immunoblotting using anti-MBP antibody as the primary antibody.

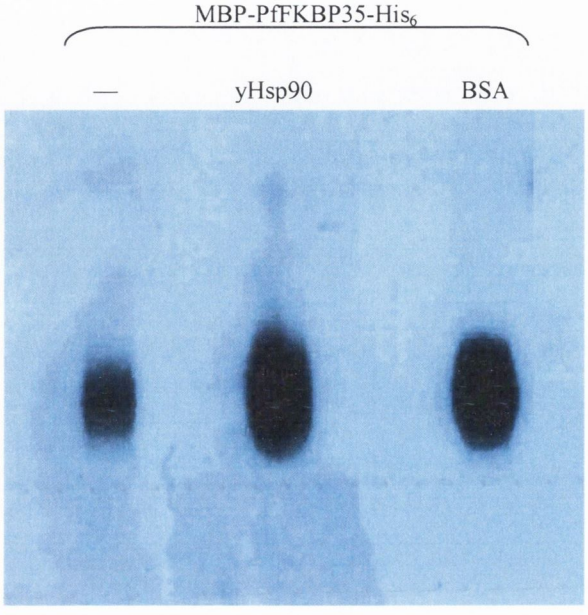


Fig. 4.23. Investigation of possible interaction between PfFKBP35 and Hsp90 by cross-linking analysis. MBP-PfFKBP35-His_6 (4 μg) was mixed with equimolar amounts of yHsp90 or BSA (-ve control) and treated with glutaraldehyde as described in Materials and Methods. Samples were resolved by SDS-PAGE (8% v/v), transferred to PVDF membrane and subjected to Western immunoblotting using anti-MBP antibody as the primary antibody. MBP-PfFKBP35-His_6 treated with glutaraldehyde in the absence of additional components (—) served as the marker.

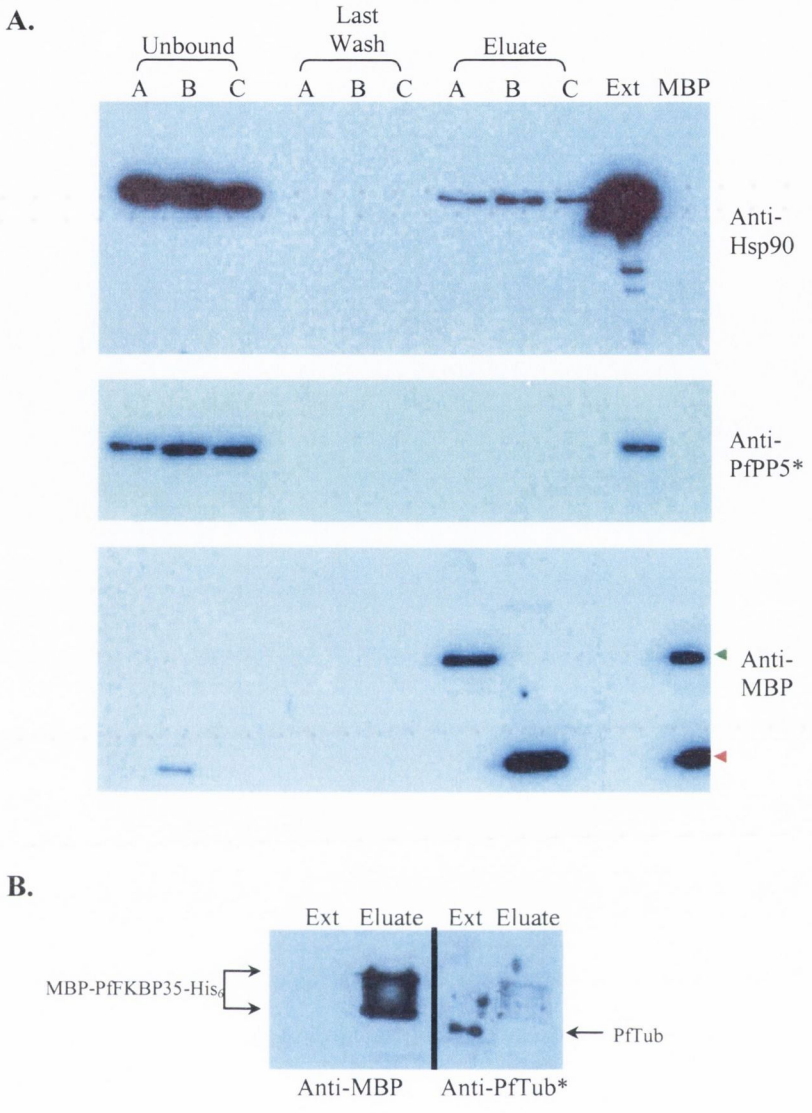


Fig. 4.24. Investigation of possible interacting binding partners of PfFKBP35. **A.** Amylose resin was incubated with MBP-PfFKBP35-His_6 (A), MBP-His_8 (B) or buffer (C) in microfuge tubes, and subsequently mixed with *P. falciparum* extract for 1 h at 37°C. Unbound *P. falciparum* proteins were removed by centrifugation and the resin washed a number of times with AAC buffer (last wash shown). Bound proteins were eluted from the resin by mixing with SDS-PAGE loading buffer and boiling for 10 min. Samples were resolved by SDS-PAGE, transferred to PVDF membrane, and subjected to Western immunoblotting. Each panel represents the same membrane probed with different primary antibodies (indicated to the right – membranes were stripped and prepared for re-probing as described in Materials and Methods). Ext = parasite extract (2 μg); MBP = 1:1 mixture of MBP-PfFKBP35-His_6 (green arrow-head) + MBP-His_8 (red arrow-head - 0.1 μg total protein). **B.** MBP-PfFKBP35-His_6 immobilised on amylose resin was mixed with parasite extract and treated as described in panel A. The left and right panels represents the same membrane probed with different primary antibodies (indicated below panel) PfTub = *P. falciparum* β-tubulin. Asterisk indicates unpurified serum was used as the source of primary antibody.

complexes is mutually exclusive, the possibility of an interaction between PfFKBP35 and PfPP5 was investigated. As judged by MBP–pull-down analysis, no interaction occurred between MBP-PfFKBP35-His_6 and PfPP5 (Fig. 4.24A – middle panel).

The possibility that PfFKBP35 links hetero-oligomeric complexes to the retrograde transport system in *P. falciparum* was investigated by probing the MBP–pull-down fractions with monoclonal anti-dynein antibody, previously shown to cross-react with dynein from *P. falciparum* (Fowler *et al.*, 2001). However, this antibody failed to recognise dynein in asynchronous *P. falciparum* extracts (data not shown). As an alternative strategy, the eluted fraction from the MBP–pull-down procedure was probed with a serum to *P. falciparum* β-tubulin, one of the major components of microtubules. This serum exhibited cross-reactivity to MBP-PfFKBP35-His_6 but no β-tubulin was observed in the eluted fraction (Fig. 4.24B – right panel).

4.3. Discussion

Recombinant production of PfFKBP35 in *E. coli* presented several problems, the most unusual being the appearance of two polypeptides when only one was expected. The co-expression of a second, apparently smaller protein at roughly equal levels to the recombinant protein of interest occurred with both pMAL-PfFKBP35 and pMAL-PfFKBP35-His_6 vectors. Both induced proteins consistently co-purified from amylose resins, once again at equal intensities. Western immunoblotting using an anti-MBP antibody confirmed that this smaller protein included MBP, and therefore its expression was most likely directed from the pMAL plasmid. The difference in size of the two bands was too small for the upper band to correspond to an oligomer of the lower bad. This was further supported by the two bands being visualised on SDS-PAGE gels when samples were unheated prior to loading, and also in the absence of β-mercaptoethanol in the loading buffer. Factor Xa successfully cleaved the MBP tag from both proteins, providing evidence that the two bands seen on SDS-PAGE gels are distinct proteins rather than arising from the same protein running aberrantly as two bands. Proteolytic degradation of the larger protein is unlikely to be the cause of the

observed phenomenon as the two bands were consistently obtained when whole cell lysate was analysed by SDS-PAGE immediately after lysis, and the level of expression of the two proteins was consistently of equal intensity. Lysis of *E. coli* was always performed at 4°C, and protease inhibitors were routinely included in the lysis buffer. It was thought, therefore, that the smaller band corresponded to a C-terminal truncate arising from premature translational termination, possibly due to problems associated with codon bias. However, this proved not to be the case, at least with pMAL-PfFKBP35-His_6, as both proteins encoded by this plasmid were shown to include the C-terminal oligo(His)-tag.

The clone expressing the isolated FKBP domain of PfFKBP35, pMAL-FKBP-His_6, produced only a single band of the expected size, as did pMAL-His_8. Intriguingly, however, the clone expressing the isolated TPR domain, pMAL-TPR-His_6, produced two bands differing in size by ~10 kDa (see Fig 4.13B). This suggests that "two-band" phenomenon might be a result of some peculiarity associated with the C-terminal portion of PfFKBP35 that results in aberrant migration on SDS-PAGE gels. However, the finding that nickel-chelate affinity chromatography successfully separated these proteins from each other argues in favour of the bands corresponding to distinct proteins. The reason for the observed phenomenon remains unclear.

MBP-PfFKBP35-His_6 exhibited PPIase activity that was inhibited by FK506 and rapamycin, but not CsA. This enzymatic activity was shown to be located within the N-terminal FKBP domain of PfFKBP35, as expected. *P. falciparum* proteins with PPIase activity have been described previously (Reddy, 1995; Hirtzlin *et al.*, 1995; Berriman, and Fairlamb 1998), but this is the first FKBP-type PPIase activity identified in the parasite. The fact that FK506 and rapamycin inhibit the enzymatic activity of the recombinant PfFKBP35 makes it reasonable to suggest that the anti-malarial mode-of-action of both these drugs is mediated directly through an inhibitory effect on the enzymatic activity of PfFKBP35 which could affect essential protein folding reactions.

hFKBP12 is the major mediator of FK506-induced immunosuppression, no doubt due to the inability of most other FKBP-FK506 binary complexes to sequester calcineurin. The hFKBP12.6-FK506 complex inhibits calcineurin activity as potently as hFKBP12-FK506 (Lam *et al.*, 1995), while the hFKBP51-FK506 complex is significantly less potent in this regard (Baughman *et al.* 1997). However,

hFKBP51 has been shown to interact with calcineurin independently of FK506 (Li *et al.*, 2002), a finding also reported for the *S cerevisciae* FKBP12 (yFKBP12) homologue (Cardenas *et al.*, 1994), although no reports of an inhibitory effect on calcineurin were made for yFKBP12. The finding that PfFKBP35 inhibits calcineurin in the absence of FK506 is highly unusual. hFKBP38 is the only other FKBP for which this phenomenon has been reported (Shirane and Nakayama, 2003), and hFKBP38 may regulate important cellular processes by modulating the activity of calcineurin. Very little is known about a *P. falciparum* calcineurin homologue, although activity attributable to a calcineurin-like phosphatase has been identified in parasite extracts (Dobson *et al.*, 1999), and preliminary studies of a recombinant form have recently been reported (Kumar *et al.*, 2004). Until the function of this parasite phosphatase is characterised in detail, the biological significance of PfFKBP35's inhibitory effects on calcineurin will remain unclear.

hFKBP51 and hFKBP52, which are similar in their domain architecture to PfFKBP35, are known to act as a molecular chaperones, as are many TPR-containing proteins (Pirkl and Buchner, 2001; Kamphausen *et al.*, 2002; van der Spuy *et al.*, 2000; Pratt *et al.*, 1999; Yano *et al.*, 2004). The recombinant forms of PfFKBP35 used throughout these analyses exhibited chaperone activity against two model substrates, citrate synthase and rhodanese. Equimolar amounts of MBP-PfFKBP35-His_6 resulted in greater than 80% suppression of aggregation of both model substrates, which is similar to the chaperone effects of hFKBP51 and hCyp40 on citrate synthase (Pirkl and Buchner, 2001). hFKBP52 is significantly less efficient in this regard: it took a 14-fold molar excess of hFKBP52:citrate synthase to achieve similar suppression (Pirkl and Buchner, 2001) and even with a 20-fold molar excess of hFKBP52:rhodanese, only around 60% suppression of aggregation was achieved (Pirkl *et al.* 2001). The chaperone activity of MBP-PfFKBP35-His_6 was not affected by either FK506 or rapamycin, suggesting that it is independent of PPIase activity.

In order to localise the chaperone activity to a particular domain of PfFKBP35, the isolated N- and C-terminal domains of the protein were tested independently in the two systems. It was expected that the chaperone activity of PFFKBP35 would be independent of its PPIase activity and be located exclusively in the TPR domain, as is the case for hFKBP52 (Pirkl *et al.* 2001). However, both the FKBP and TPR domains were able to suppress the thermal aggregation of citrate

synthase and rhodanese. Whilst the finding that the FKBP domain can act as a chaperone was unexpected and unusual, it is not unprecedented. A 28.3-kDa FKBP from the archaebacterium *Methanococcus thermoautotrophicum*, which does not include a TPR, prevents the thermal aggregation of rhodanese *in vitro* (Ideno *et al.*, 2000). Its FKBP domain alone displays the same activity, albeit to a lesser extent than the full protein, suggesting that the FKBP domain contributes to the chaperone activity of PfFKBP35. Similarly, the central domain of *E. coli* trigger factor utilises the same binding site for its chaperone and PPIase activities (Patzelt *et al.*, 2001).

However, the observation that the isolated FKBP domain of PfFKBP35 suppresses the aggregation of the two model substrates used in these experiments comes with a caveat – is this really chaperone activity, or is it an enzymatic effect? It is difficult to rule out the possibility that the PPIase activity is having an effect on the substrates by isomerising key peptidyl-prolyl bonds that may prevent them from aggregating. The fact that FK506 and rapamycin inhibit the chaperone activity of the FKBP domain alone, but not that of the full-length protein or TPR domain supports this idea. This leads to an intriguing possibility that PfFKBP35 has the ability to immobilise a substrate with the TPR domain, and subsequently enzymatically alter it with the PPIase activity of its FKBP domain. Such a "hold and fold" functionality, which is similar to the mechanism by which Pin1, the archetypal member of the parvulin family of PPIase, isomerises peptidyl-prolyl bonds, would necessitate the two domains binding the same substrate. Pin1 specifically isomerises Ser-Pro or Thr-Pro peptide bonds, but only when the Ser/Thr residue is phosphorylated (Yaffe *et al.*, 1997). Pin1 interacts with the phosphorylated Ser/Thr residue through its N-terminal WW domain, and induces conformational changes in its substrates through its C-terminal PPIase domain. The fact that both the FKBP and TPR domains of PfFKBP35 independently suppress aggregation of the two model substrates used here shows that both domains bind the substrates. Therefore, we cannot exclude the possibility that the aggregation suppression effect of the full-length protein comes from a combination of true chaperone activity, mediated through the TPR domain, and PPIase activity, mediated through the FKBP domain. However, analyses of the X-ray crystal structures of both citrate synthase and rhodanese shows that, while both are quite rich in proline, none of the peptidyl-prolyl bonds are in the *cis*-conformation. This would suggest that neither of these proteins require the actions of a PPIase to achieve their three-dimensional structure,

thereby suggesting that the observed aggregation suppression effect of the isolated FKBP domain is a result of true chaperone activity. Although low level chaperone-like activity of *E. coli* MalE (i.e. the MBP used in this study) has previously been reported (Richarme and Caldas, 1997), the MBP-His_8 double tag had no chaperone activity in the studies reported here.

The similarity of the domain architectures of PfFKBP35, hFKBP37, hFKBP51 and hFKBP52 suggested that PfFKBP35 might form part of hetero-oligomeric complexes. In mammalian cells these complexes involve the dynamic assembly and disassembly of various chaperones and co-chaperones (Pratt and Toft, 2003). The proteome of *P. falciparum* contains an analogous set of chaperone (Hsp40, Hsp70, Hsp90) and co-chaperone (Hip, Hop, p23) components necessary for such hetero-oligomeric complexes (Table 4.3). hFKBP37, hFKBP51 and hFKBP52 all participate in their respective hetero-oligomeric complexes via an interaction with the Hsp90 component of the complex (Petrulis and Perdew, 2002; Pratt and Toft, 2003), suggesting that PfFKBP35 may interact with a *P. falciparum* Hsp90 homologue, two of which have so far been identified (Kumar *et al.*, 2003). The MBP- and His_6–pull-down techniques used here to investigate such an interaction were inconclusive due to non-specific interactions of both yHsp90 and PfHsp90 with the resins. Far-Western analysis as a means of identifying protein interacting partners is notoriously unreliable due to the possibility of the bait protein (i.e. the protein transferred to the membrane) being in a conformation incompatible for interacting with a partner protein. Therefore, the failure to identify an interaction between MBP-PfFKBP35-His_6 and yHsp90 using this technique was inconclusive, particularly since no positive control was included. The absence of higher molecular weight oligomers following glutaraldehyde treatment of MBP-PfFKBP35-His_6 and yHsp90 suggests that no binding occurred. However, once again, no positive controls were included. Also, although Hsp90 is highly conserved throughout evolution, the possibility of yHsp90 being an inappropriate substitute for PfHsp90 has to be considered. Indeed, the PfHsp90 homologue encoded by the gene from chromosome 7 includes an unusually long acidic domain, and phlyogenetic analysis shows it to be more closely related to plant Hsp90 homologues than to Hsp90 homologues from other organisms (Bonnefoy *et al.*, 1994). While no positive controls were included, the finding that MBP-PfFKBP35-His_6 did not interact with either PP5 or β-tubulin from *P. falciparum* in the MBP–pull-down assay is

unsurprising. In human cells, hPP5 and hFKBP52 compete for binding to hHsp90, so any complex recognised by immobilised MBP-PfFKBP35-His_6 would not be expected to include PfPP5 – their involvement in hetero-oligomeric complexes is mutually exclusive. The manner in which hFKBP52, and indeed hPP5, link their respective hetero-oligomeric complexes to the retrograde transport system is via the motor protein dynein, rather than directly to microtubules (Galigniana *et al.*, 2002). The monoclonal antibody to dynein used here cross-reacts with *P. falciparum* dynein, but only in merozoites and late schizonts (Fowler *et al.*, 2001).

The techniques used here to investigate possible binding partners of PfFKBP35 have numerous drawbacks. No positive controls were available, so conditions for optimal binding were never fully explored. Although the primary antibodies employed for Western immunoblotting strongly recognised their respective target proteins, the possibility exists that they fail to interact with their targets when present in a complex. The presence of tags at either end of the bait protein (i.e. MBP-PfFKBP35-His_6) may also have adversely affected interactions.

The sequence analysis performed in chapter 3 had predicted that PfFKBP35 would have FK506- and rapamycin-inhibitable PPIase activity. The similarity of PfFKBP35's modular structure to certain other FKBPs also suggested it might act as a molecular chaperone. The work described in this chapter shows that FK506 and rapamycin inhibit the PPIase activity, but not the chaperone activity, of PfFKBP35, thereby suggesting that the anti-malarial mode of action of both these drugs might be mediated through an inhibitory effect on the enzymatic activity of PfFKBP35.

Chapter 5

Anti-Malarial Properties of Non-Immunosuppressive Derivatives of FK506

5.1. INTRODUCTION

The work in the previous chapter described the inhibitory effects of FK506 and rapamycin on the enzymatic activity of recombinant PfFKBP35. Although the chaperone activity of the isolated FKBP domain of PfFKBP35 was inhibited by both of these drugs, that of the full-length protein was unaffected. It is reasonable, therefore, to suggest that the anti-malarial mode of action of FK506 and rapamycin may be mediated directly through an inhibitory effect on the PPIase activity of PfFKBP35, and in turn the execution of essential protein folding reactions.

A crucial consideration for the development of therapeutically useful anti-malarials based on the effects of these drugs is that they should lack immunosuppressive capabilities. FK520 (ascomycin) is a natural derivative of FK506 produced by *S. hygroscopicus* subsp. *ascomyceticus* (Hatanaka *et al.*, 1988; Arndt *et al.*, 1999). FK520 differs from FK506 only by an allyl-to-ethyl change at position C-21 (Fig. 5.1), but this modification has little effect on the immunosuppressive nature of the compound as it retains the ability to bind hFKBP12, and the resulting hFKBP12-FK520 complex inhibits calcineurin. Analysis of the three-dimensional structures of both the hFKBP12-FK506 (see Fig. 1.6A and Fig. 1.7A) and hFKBP12-FK520 binary complexes shows that approximately half of each drug molecule is in contact with hFKBP12 and half is solvent exposed (van Duyne *et al.*, 1991; Meadows *et al.*, 1993). The solvent-exposed portions of each drug, together with regions of the hFKBP12 molecule, comprise composite binding surfaces for calcineurin. Modifications of the solvent-exposed regions, through both chemical (Dumont *et al.*, 1992) and genetic (Reeves *et al.*, 2002; Revill *et al.*, 2002) methods have facilitated the design of non-immunosuppressive analogues of FK520 that retain the ability to bind hFKBP12, but are incapable of subsequently inhibiting calcineurin. This chapter describes the anti-malarial nature of FK520 and a number of its synthetic, non-immunosuppressive analogues.

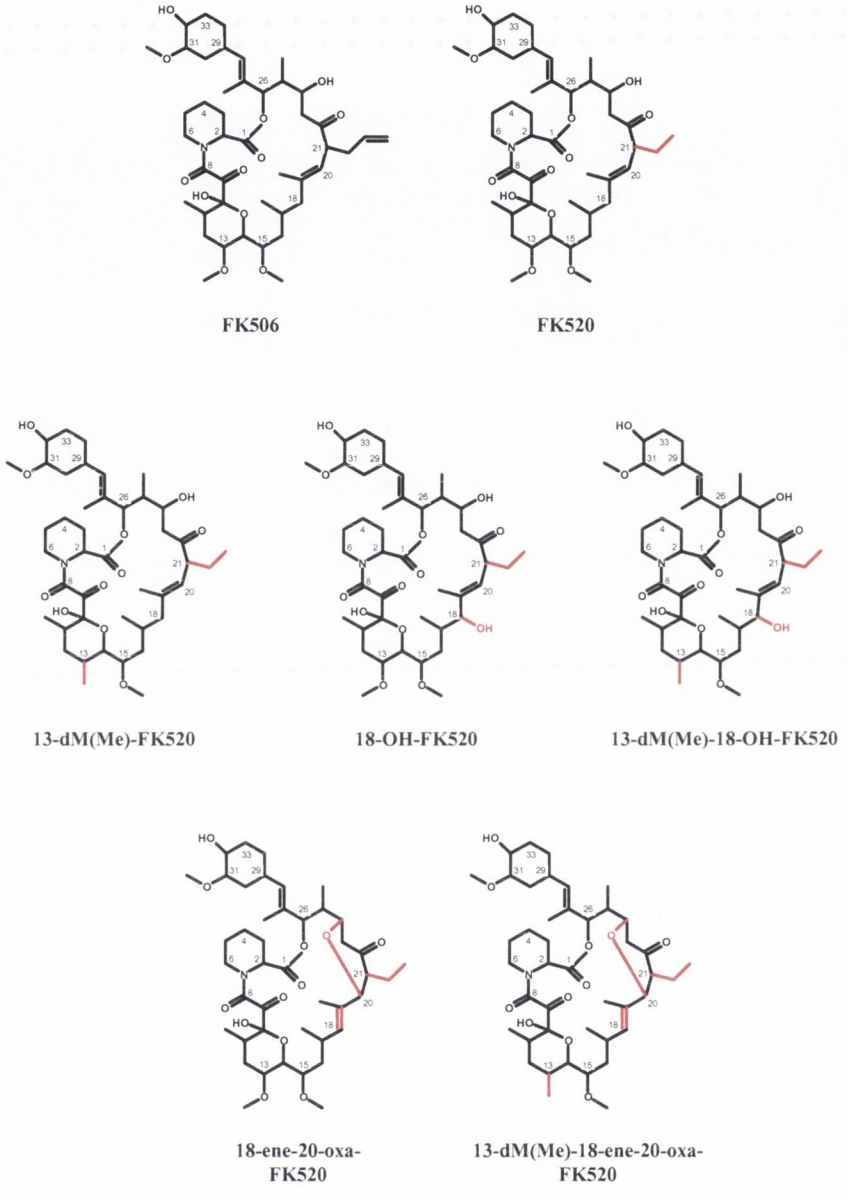


Fig. 5.1. Structures of FK506 derivatives used in this study. FK520 is a natural analogue that exhibits similar immunosuppressive properties to FK506. All other compounds are synthetic, non-immunosuppressive derivatives of FK520. Regions of molecules that differ from FK506 are depicted in red. dM(Me) = desmethoxy-methyl (OCH_3 to CH_3 substitution); OH = hydroxyl group.

5.2. RESULTS

5.2.1. Inhibition of *P. falciparum* growth by FK520 analogues

Five non-immunosuppressive analogues of FK520 (Fig. 5.1) were tested here for their anti-malarial properties. Three of these (13-dM(Me)-FK520, 18-OH-FK520 and 13-dM(Me)-18-OH FK520) can bind hFKBP12, but the resulting binary complexes have low affinity for calcineurin (Table 5.1; Dumont *et al.*, 1992; Revill *et al.*, 2002). In contrast, neither 18-ene-20-oxa-FK520 nor 13-dM(Me)-18-ene-20-oxa-FK520 had measurable affinity for hFKBP12 (Table 5.1). All these compounds exhibited significant dose-dependent inhibitory effects against *P. falciparum* growth in culture (Fig. 5.2). Compounds with the 18-OH or 18-ene-20-oxa substitutions were slightly less active than FK520, by ~3-fold and ~2-fold, respectively.

5.2.2. Effects of FK520 analogues on PPIase activity of PfFKBP35

To investigate if this growth inhibition was mediated by an effect on the enzymatic activity of PfFKBP35, the effects of these compounds on the PPIase activity of MBP-PfFKBP35-His_6 were measured. All the compounds known to interact with hFKBP12 were shown to inhibit the PPIase activity of MBP-PfFKBP35-His_6 (Fig. 5.3). However, the two compounds that do not interact with hFKBP12 caused only minor reductions in enzymatic activity of MBP-PfFKBP35-His_6.

The lack of PPIase inhibitory action by the two 18-ene-20-oxa compounds did not correlate with their anti-malarial activity. We postulated that these compounds might be incapable of interfering with PfFKBP35 function until they are metabolized into 'active', FKBP-binding forms (e.g. by cleavage of the ether bridge between C-20 and C-24) within the parasite. Therefore, lysed parasites and culture media from cultures that had been treated with sub-lethal concentrations of 18-ene-20-oxa-FK520 or 13-dM(Me)-18-ene-20-oxa-FK520 were analysed by mass spectrometry. The starting compounds were detected at concentrations comparable to those of the *P. falciparum*-free control, and no metabolites were found down to the lower limit of detection (W.P. Revill, personal communication).

Table 5.1. Binding of FK520 and analogues to hFKBP12 and calcineurin.

Compound	hFKBP12 binding (K_d)[a], nM	Calcineurin inhibition (K_i)[b], nM
FK520	0.4	49
13-dM(Me)-FK520	1.6	940
18-OH-FK520	0.2	3630
13-dM(Me)-18-OH-FK520	1 ± 0.5	> 14000
18-ene-20-oxa-FK520	> 25	N/A[c]
13-dM(Me)-18-ene-20-oxa FK520	> 25	N/A[c]

[a]K_d = dissociation constant; [b]K_i = inhibition constant (in the presence of hFKBP12); [c]N/A = not applicable. All data from Revill, W.P. (personal communication). Compounds highlighted in blue are capable of binding hFKBP12, those in red have no measurable affinity for hFKBP12.

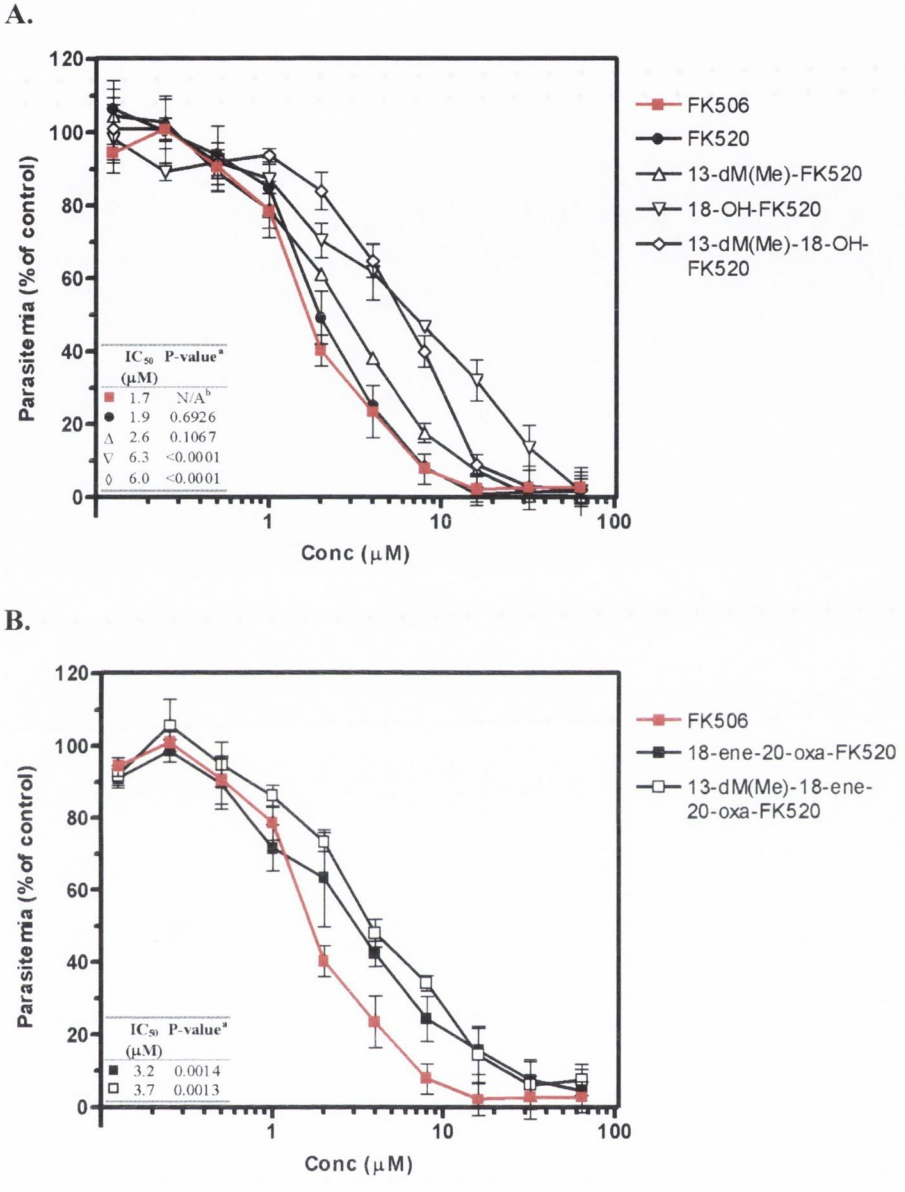


Fig. 5.2. Inhibition of growth of *P. falciparum* in culture by FK506 derivatives. Effects on parasite growth of compounds with high affinity (A) and low affinity (B) for hFKBP12 (FK506 is included in panel B for comparative purposes). Insets show IC_{50} values for cells in culture for each of the compounds. [a] Data for each of the compounds were compared with those for FK506 using an unpaired Student's t-test. [b] Not Applicable. Bars show SEM for four or more replicates.

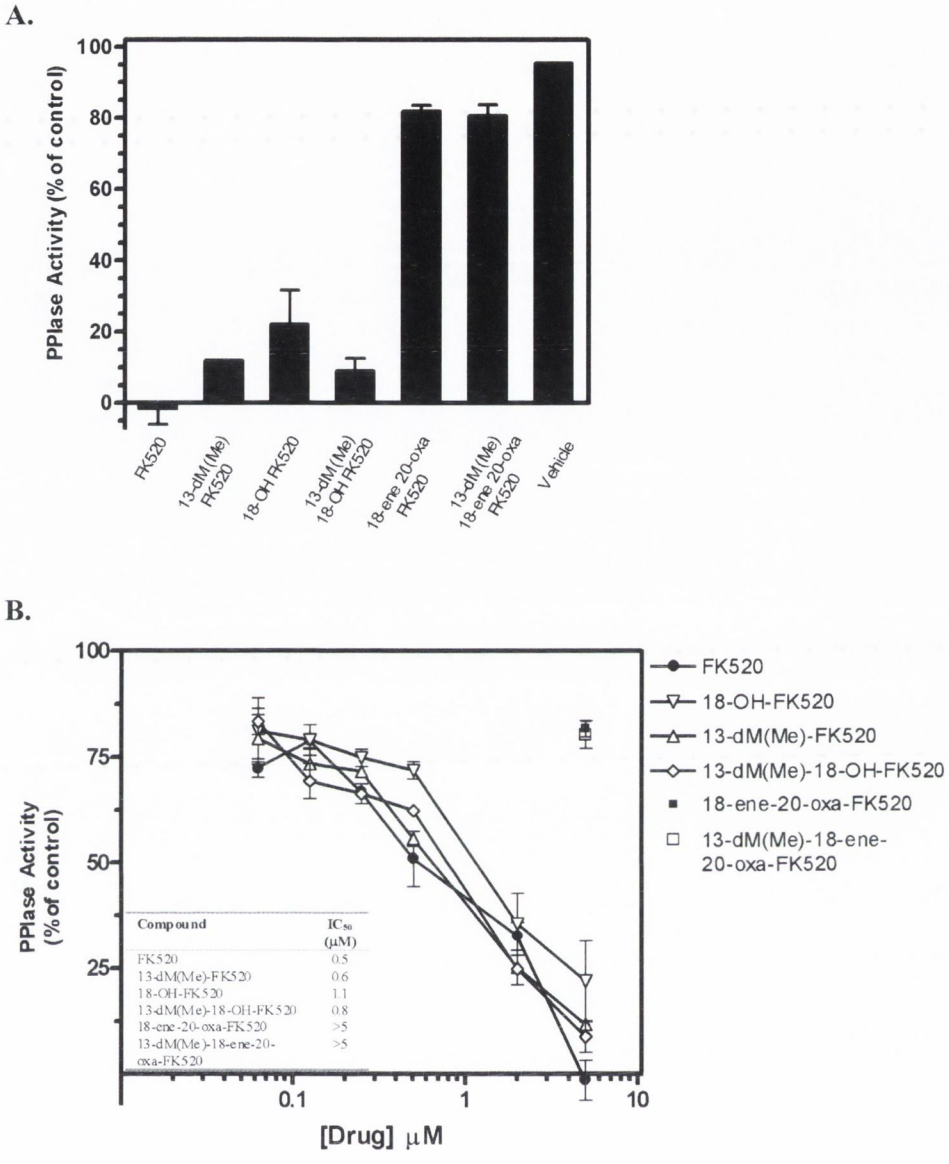


Fig. 5.3. Inhibition of PPIase activity of MBP-PfFKBP35-His_6 by FK520 and analogues. **A.** The activity of 0.25 μM MBP-PfFKBP35-His_6 was assayed at a fixed concentration of inhibitors (5 μM). Vehicle for each compound was DMSO. **B.** Dose-dependent effect of FK520 analogues on PPIase activity of 0.25 μM MBP-PfFKBP35-His_6. The activity of 0.25 μM MBP-PfFKBP35-His_6 in the absence of drug was set at 100%. Inset shows IC_{50} values for PPIase activity for each of the compounds. Bars show SEM of three replicates.

5.2.3. Effects of FK520 analogues on chaperone activity of PfFKBP35

The effects of the FK520 analogues on the chaperone activity of both full-length PfFKBP35 (MBP-PfFKBP35-His_6) and the isolated FKBP domain (MBP-FKBP-His_6) were assessed in both model systems described previously in chapter 4. None of the FK520 analogues had any affect on the ability of MBP-PfFKBP35-His_6 to prevent the thermal aggregation of either citrate synthase or rhodanese (Fig. 5.4). However, as was the case with FK506, all compounds reduced the chaperone activity of the isolated FKBP domain of PfFKBP35. In the absence of inhibitors, MBP-FKBP-His_6 reduced the aggregation of citrate synthase by ~70%, while only a ~20% reduction in aggregation was achieved in the presence of ten-fold molar excesses of any of the inhibitors (Fig. 5.4A). For rhodanese, five-fold molar excesses of FK520, 13-dM(Me)-FK520, 18-OH-FK520 or 13-dM(Me)-18-OH-FK520 allowed MBP-FKBP-His_6 to reduce aggregation by only ~40%, compared to >90% in the absence of inhibitors (Fig. 5.4B). The effects of 18-ene-20-oxa-FK520 and 13-dM(Me)-18-ene-20-oxa-FK520 were intermediate between these extremes.

5.3. Discussion

FK520 is a natural analogue of FK506 with similar immunosuppressive properties. Modifications of the chemical structure of FK520 have facilitated the design of non-immunosuppressive derivatives. The FK520 analogues tested here fall into two groups. The first group, comprising 13-dM(Me)-FK520, 18-OH-FK520 and 13-dM(Me)-18-OH-FK520, are non-immunosuppressive because the complex formed between the macrolide and hFKBP12 is incapable of binding to calcineurin. The non-immunosuppressive character of the second group, comprising 18-ene-20-oxa-FK520 and 13-dM(Me)-18-ene-20-oxa-FK520, results from these compounds' low affinity for hFKBP12 itself. All these compounds, however, exhibited potent inhibitory effects against *P. falciparum* in culture, suggesting that their anti-malarial mode of action is distinct from the immunosuppressive mechanism of FK506/FK520.

Based on the hypothesis that PfFKBP35 is the target for FK506 and its non-immunosuppressive analogues, there are at least two possible models by which these drugs could exert their anti-malarial effects. One such model, by analogy with

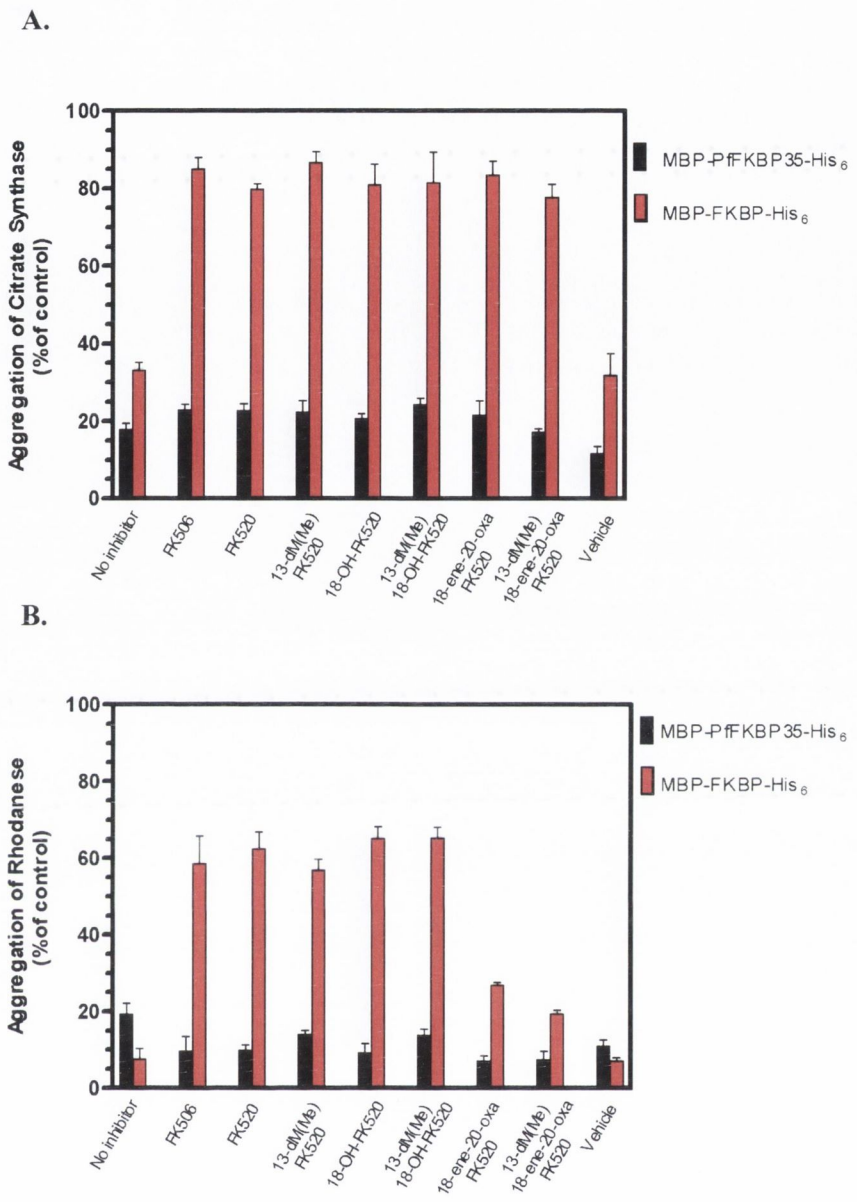


Fig. 5.4. Inhibition of chaperone activity of PfFKBP35 by FK520 analogues. Inhibition of thermal aggregation of 1.5 μM (monomer) citrate synthase (A) and 4.4 μM rhodanese (B) in the presence of equimolar amounts of either full-length PfFKBP35 (MBP-PfFKBP35-His_6) or isolated FKBP domain (MBP-FKBP-His_6). Inhibitors, where included, were present as 10-fold (A) or 5-fold (B) molar excesses. The maximum A_{360} values in the absence of additional components was set to represent 100% aggregation. Bars show SEM of three replicates.

the current models of immunosuppressive and anti-fungal actions of FK506/FK520 and rapamycin, is that the compounds bind first to PfFKBP35, and then the FKBP–ligand binary complex inhibits an essential parasite target. For FK506/FK520-induced immunosuppression, the target of this pathway in T-lymphocytes is calcineurin (Fruman et al., 1994), while the hFKBP12/rapamycin complex inhibits the kinase TOR (Lorberg and Hall, 2004). The anti-fungal mechanisms of FK506 and rapamycin are likewise mediated via FKBP–ligand–calcineurin or FKBP–ligand–TOR ternary complexes, respectively (Foor et al., 1992; Breuder et al., 1994; Odom et al., 1997; Cruz et al., 2001). However, recombinant PfFKBP35 inhibits the phosphatase activity of bovine calcineurin *in vitro* in the absence of FK506. Furthermore, it seems unlikely that the *P. falciparum* calcineurin is involved in mediating the anti-malarial effects of these drugs because each analogue has a different structural modification in the effector domain, yet all retain anti-malarial potency of about the same order. Until more is known about this *Plasmodium* phosphatase, however, it is difficult to rule out its involvement. Due to the absence of a gene encoding any obvious TOR homologue in the *P. falciparum* genome (data not shown), it seems unlikely that a *P. falciparum* TOR homologue is involved in the anti-malarial effects of rapamycin.

A second possible model by which these inhibitors could exert their anti-malarial effect through PfFKBP35 is that the effects on parasite growth are a direct result of inhibition of the protein's cellular activity. The obvious mechanism for such a model is that the macrolides inhibit the protein's PPIase activity. However, two of the compounds tested showed very little inhibition of the PPIase activity of recombinant PfFKBP35. These two compounds, 18-ene-20-oxa-FK520 and 13-dM(Me)-18-ene-20-oxa-FK520, were the only ones tested that are unable to bind hFKBP12. This leads us to conclude that the anti-malarial action of all the analogues is not mediated through inhibition of PfFKBP35's enzymatic activity. A similar conclusion was drawn for the anti-malarial action of CsA – the finding that certain non-immunosuppressive derivatives of CsA are potent anti-malarials, while lacking anti-PPIase action, showed that the anti-malarial mechanism of this class of drugs is probably not a direct result of PPIase inhibition (Bell *et al.*, 1996). This was also shown for the anti-schistosomal effects of cyclosporins (Khattab *et al.*, 1998).

None of the FK520 analogues had any significant effects on the chaperone activity of the full-length PfFKBP35. However, when the isolated FKBP domain

was used in place of the full-length protein, all compounds showed significant inhibitory effects. The finding that the compounds had no effect on the chaperone activity of the full-length protein is most probably due, as in the case of FK506, to the chaperone activity of the C-terminal portion of PfFKBP35 being unaffected. This indicates that these compounds interact with PfFKBP35 at the FKBP domain as expected.

The inhibitory effects of 18-ene-20-oxa-FK520 and 13-dM(Me)-18-ene-20-oxa-FK520 on cell growth and chaperone activity suggests that both these compounds bind PfFKBP35. Perhaps structural limitations on the macrolactone imposed by the 18-ene and/or 20-oxa modifications prevents such compounds binding hFKBP12 without affecting their binding to PfFKBP35. However, as the catalytic active site and drug-binding site of FKBPs are in the same region, the finding that these two compounds fail to inhibit the PPIase activity of PfFKBP35 suggested that no binding occurred. Not all FKBPs bind FK506/FK520. Those that fail to bind these drugs have substitutions at key residues implicated in drug binding. While the primary sequence of PfFKBP35 shows significant differences to that of hFKBP12 at certain residues, most of the drug-binding residues are conserved. These conflicting data lead us to consider that these two drugs interact with PfFKBP35 in a manner which does not result in a loss of enzymatic activity, but does affect the protein's chaperone function.

Although these compounds only inhibited the chaperone activity of the isolated FKBP domain, and not that of the intact protein, it is tempting to suggest that the anti-malarial mode of action of this class of compounds is mediated through inhibition of PfFKBP35's chaperone activity rather than its PPIase activity. Indeed, this may in fact represent the primary intracellular function of PfFKBP35. The mechanism by which trigger factor functions in the folding of newly synthesized polypeptides in *E. coli* is not well understood, but Kramer and colleagues (2004) have recently shown that it is independent of its intrinsic PPIase activity. Indeed, as discussed in section 1.4, the biological significance of the PPIase activity of certain other FKBPs remains controversial, as their functions appear to be independent of enzymatic activity.

The above models of anti-malarial action of FK506 and its analogues are based on the assumption that PfFKBP35 is the target of these compounds. It is possible, however, that these compounds exert their effects through another protein.

As PfFKBP35 is the only FKBP in the *P. falciparum* proteome, and there are no reports of FK506 mediating effects independent of an FKBP, it would appear unlikely that these compounds are targeting another parasite protein. However, the possibility exists that these compounds are affecting erythrocytic FKBPs, resulting in damage to the host cell, thereby indirectly killing the parasite. Indeed, human erythrocytes contain FKBP12 that strongly binds FK506 (Kay *et al.*, 1991). However, the anti-malarial activities of 18-ene-20-oxa-FK520 and 13-dM(Me)-18-ene-20-oxa-FK520, neither of which have appreciable affinity for hFKBP12, suggests that erythrocytic FKBP12 is not involved in the anti-malarial actions of these compounds. hFKBP13 is also found in erythrocytes where it is suggested to regulate the assembly/disassembly of the membrane cytoskeleton (Walensky *et al.*, 1998). Impaired maintenance of this cytoskeleton by inhibiting hFKBP13 could feasibly have an effect on parasites residing within the erythrocyte. However, the affinity of hFKBP13 for FK506 is greatly reduced compared to hFKBP12. Rather, hFKBP13 appears much more sensitive to rapamycin than FK506. As both these drugs have comparable anti-malarial effects, it would appear unlikely that inhibition of erythrocytic FKBP13 explains tha anti-malarial effects of the compounds used in this study.

Further dissection of the mode of action of this class of drugs (and 18-ene-20-oxa-FK520 and 13-dM(Me)-18-ene-20-oxa-FK520 in particular) could potentially lead to the design or selection of more potent derivatives that retain their specificity for the parasite protein. This would be aided by structure-activity data additional to the limited amount presented here (e.g. modifications at the C-18 position of the macrolactone ring structure appear to have a slight negative effect on the anti-malarial properties of these compounds).

Chapter 6

General Discussion

6.1. FKBPs of *P. falciparum*

Prior to the commencement of this work, no FKBP had been reported in *P. falciparum*. Our interest in finding such a protein stemmed from the need to identify and characterise new drug targets and, based on the anti-malarial activities of FK506 and rapamycin, we hypothesised that the parasite contained at least one FKBP through which these drugs mediated their actions.

Although the sequencing of the genome of *P. falciparum* was uncompleted at the outset of this work, the continual release of raw sequence data by the genome sequencing consortium facilitated a data-mining approach for the identification of *P. falciparum* FKBP homologues. Although most organisms contain more than one FKBP isoform, analysis of the *P. falciparum* genome (now complete, with the exception of a few remaining gaps – Gardner *et al.*, 2002) suggested that this organism contains only a single FKBP gene that encodes a 35-kDa protein. Comparison of the predicted amino acid sequence of this protein, PfFKBP35, suggests that it has the capacity to bind FK506 and rapamycin, thereby implicating it in the anti-malarial activities of these two drugs. While BLAST analysis identified a possible 25-kDa FKBP, the poor degree of sequence conservation within its putative FKBP domain (<20% identity to hFKBP12) suggests that it does not belong to the FKBP family. In particular, only three out of the fourteen drug-binding residues of hFKBP12 were conserved in this putative protein, so, even if it does turn out to be an FKBP, it is unlikely to be implicated in the anti-malarial activity of FK506 and rapamycin.

During the course of this study, Braun *et al.* (2003) independently identified PfFKBP35. They showed it to be located in the parasite cytosol, and estimated an intracellular concentration of 50-100 nM. While our attempts at generating recombinant His_6-PfFKBP35 failed, Braun and colleagues successfully produced PfFKBP35 with a C-terminal oligo$(His)_6$-tag by synthesising overlapping oligonucleotides to create an artificial *PfFKBP35* gene optimised for overexpression in *E. coli* by altering the codons bias.

6.2. FUNCTIONS OF PfFKBP35

The finding that the recombinant form of PfFKBP35 used in this study has both PPIase and chaperone activities *in vitro* provides strong evidence that this

protein functions in protein folding events in *P. falciparum*, either as a folding catalyst, a chaperone, or both. This combination of PPIase and chaperone activity is by no means unique to PfFKBP35, as this bifunctionality has been shown for numerous other FKBPs from various organisms (Scholz *et al.*, 1997; Ideno *et al.*, 2000; Iida *et al.*, 2000; Arié *et al.*, 2001; Ideno *et al.*, 2001; Pirkl and Buchner, 2001; Ideno *et al.*, 2002; Kamphausen *et al.*, 2002; Suzuki *et al.*, 2003; Kuzuhara and Horikoshi, 2004). For the majority of FKBPs that exhibit such bifunctionality, the PPIase and chaperone activities are located in different domains. However, while the TPR domain of PfFKBP35 possesses chaperone activity, so too does the FKBP domain. The only other reports of FKBPs whose FKBP domains exhibit both PPIase and chaperone activities are trigger factor of *E. coli* (Patzelt *et al.*, 2001), a 28.3-kDa FKBP from *Methanobacterium thermoautotrophicum* (Ideno *et al.*, 2000) and a 17-kDa FKBP from *Methanococcus thermolithiotrophicus* (Suzuki *et al.*, 2003).

The similarity of the domain architecture of PfFKBP35 with that of certain other FKBPs, such as hFKBP37, hFKBP51 and hFKBP52, suggests that its primary function may be in protein trafficking as part of large hetero-oligomeric complexes (Fig. 6.1). hFKBP51 and hFKBP52 are known to form part of large hetero-oligomeric complexes in human cells comprising the co-operative functions of various chaperones and co-chaperones (Pratt and Toft, 2003). The proteome of *P. falciparum* contains the complete set of chaperone (Hsp40, Hsp70, Hsp90) and co-chaperone (Hop, p23) components necessary for such hetero-oligomeric complexes. Indeed, PfHsp90 and PfHsp70 were recently shown to be present as part of large complexes of up to 300 kDa (Banumathy *et al.*, 2003). Although not studied in as great a detail, experimental evidence suggests that the complex of hFKBP37 and aryl hydrocarbon receptor also includes a number of chaperones and co-chaperones (Petrulis and Perdew, 2002).

A number of approaches were used in this study in an attempt to identify *P. falciparum* proteins that interact with PfFKBP35. Due to the impracticalities involved in obtaining sufficient amounts of *P. falciparum* extract, it is difficult to identify unknown proteins based on *in vitro* pull-down assays like those performed in this study. Therefore, the interaction of PfFKBP35 with a number of 'candidate' binding partners was assessed. One such candidate was Hsp90. hFKBP51 and hFKBP52 are known to participate in these hetero-oligomeric complexes through

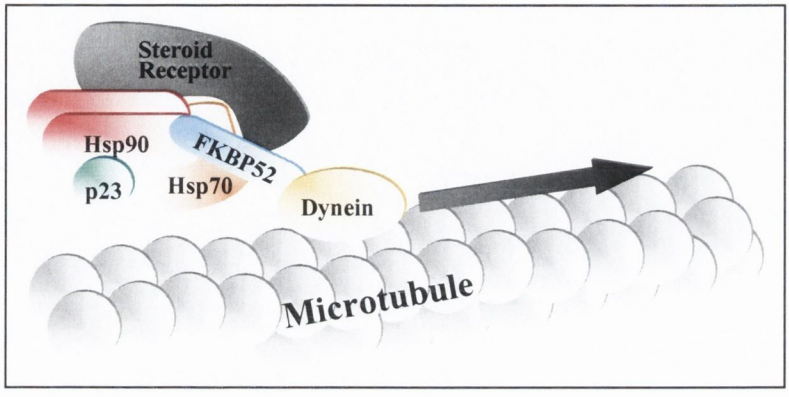


Fig. 6.1. Model of hFKBP52-mediated protein trafficking of steroid receptors. Receptors for glucocorticoid steroid hormones are found in the cytosol as part of large hetero-oligomeric complexes, the assembly of which involves the co-operative actions of chaperones (Hsp40, Hsp70, Hop90) and co-chaperones (Hop, p23). Such hetero-oligomeric complexes are dynamic in nature, constantly assembling and disassembling. Upon binding of steroid by the steroid receptor, hFKBP52 is recruited by the heterocomplex, attaching to the Hsp90 dimer through its C-terminal TPR domain. The interaction of the N-terminal FKBP domain with the dynein motor facilitates the movement of the steroid-bound steroid receptor to its site of action in the nucleus via the microtubule-based retrograde transport system. A similar hFKBP52-mediated nucleocytoplasmic shuttling process has recently been reported for p53 (Galigniana *et al.*, 2004). The arrangement of PfFKBP35's N-terminal FKBP domain followed by a C-terminal tripartite TPR domain is similar to that of hFKBP51, hFKBP52 and hFKBP37.

their TPR-mediated interactions with Hsp90. Two genes encoding Hsp90 homologues have been reported in the genome of *P. falciparum* (Kumar *et al.*, 2003), although the work reported here identified two additional putative Hsp90 genes. The gene from chromosome 7 encoding PfHsp90 has been characterised (Bonnefoy *et al.*, 1994; Su and Wellems, 1994), and recent work has begun to shed light on the functions of its protein product. While the mRNA from this locus is expressed predominantly in the ring and trophozoite stages of the intra-erythrocytic cycle, the protein product was shown to be present in all stages, although at lower amounts in schizonts (Kumar *et al.*, 2003). Sequence analysis of the TPR motifs of PfFKBP35 suggested that PfHsp90 could represent a binding partner. However, the procedures used here to investigate the possible interaction between PfFKBP35 and PfHsp90, and other candidate proteins, were inconclusive and further studies are necessary in this regard.

PfHsp90 encoded by the gene from chromosome 7 exhibits an ATP-binding activity that is specifically inhibited by geldanamycin, a known inhibitor of Hsp90 (Kumar *et al.*, 2003). Geldanamycin was also shown to strongly inhibit the growth of *P. falciparum* in culture (Banumathy *et al.*, 2003; Kumar *et al.*, 2003), with the transition from ring to trophozoite being blocked (Banumathy *et al.*, 2003). It will be interesting to assess the combination of geldanamycin and FK506 (or any of the FK506 analogues) on the growth of *P. falciparum*, in particular to investigate if the combination is syngeristic. The use of drugs in combination for malaria chemotherapy is considered advantageous as it is thought that the probability of parasites developing resistance simultaneously to two drugs that target distinct proteins is extremely low (Boland *et al.*, 2000).

The finding that the recombinant form of PfFKBP35 used in these studies inhibited the activity of bovine calcineurin suggests that PfFKBP35 may regulate cellular processes by modulating the enzymatic activity of calcineurin. Very little is known about *P. falciparum* calcineurin, although preliminary studies of a recombinant form have recently been reported (Kumar *et al.*, 2004). The biological significance, if any, of PfFKBP35's inhibitory effects on calcineurin remains to be addressed. Pull-down experiments using immobilised MBP-PFfKBP35-His_6 could be used to further investigate this interaction. Although highly similar to calcineurin from other organisms, the presence of a 130-residue insert in the *P. falciparum* calcineurin homologue suggests that bovine calcineurin may not represent a suitable

surrogate for binding studies. Co-immunoprecipitation and/or immunofluorescence studies will be useful for studying an interaction between PfFKBP35 and calcineurin *in vivo*.

6.3. Anti-Malarial Mode of Action of FK506, Rapamycin and FK506 Derivatives

One of the primary objectives of this work was to elucidate the mechanism by which these drugs exert their anti-malarial effects. The finding that both these drugs inhibited the PPIase activity of a recombinant form of PfFKBP35 suggested that their anti-malarial effects may be mediated through this protein. There are at least two possible models by which these drugs could exert their anti-malarial effect through PfFKBP35. The first model, by analogy with the current models of immunosuppressive action of FK506 and rapamycin, is that the compounds combine with PfFKBP35, and together these inhibit an essential parasite target. For FK506-induced immunosuppression, the target of this pathway in T-lymphocytes is calcineurin (Fruman *et al.*, 1994), while the hFKBP12-rapamycin complex inhibits the protein serine/threonine kinase TOR (Lorberg and Hall, 2004). The antifungal mechanisms of FK506 and rapamycin are likewise mediated via FKBP-ligand-calcineurin or FKBP-ligand-TOR ternary complexes, respectively (Foor *et al.*, 1992; Breuder et al., 1994; Odom et al., 1997; Cruz et al., 2001). The effects of non-immunosuppressive analogues of FK506 on the growth of *P. falciparum* in culture, however, suggest that this mechanism does not account for the anti-malarial actions of this class of drugs. It seems unlikely that a *P. falciparum* calcineurin homologue is involved in mediating the anti-malarial effects of the FK506 analogues because each had a different structural modification in the effector domain, yet they retain equipotent anti-malarial activity. This is supported by the finding that PfFKBP35 inhibited the phosphatase activity of bovine calcineurin in the absence of FK506. Likewise, the lack of any obvious *P. falciparum* TOR homologue suggests that the anti-malarial actions of rapamycin are distinct from its immunosuppressive actions.

The second possible model by which these inhibitors could exert their anti-malarial effects is through direct inhibition of PfFKBP35's cellular activity. The results presented in this work favour this model, with an inhibitory effect on the chaperone activity of PfFKBP35 possibly being responsible for parasite death.

6.4. PfFKBP35 AS A CHEMOTHERAPEUTIC TARGET: FUTURE DIRECTIONS

Due to the continual emergence of parasites resistant to current anti-malarial drugs, there is a growing need to develop new drugs. Drugs that exert their actions via novel mechanisms are especially desired to counteract the problem of cross-resistance with currently used drugs. This study has led to the identification of the first, and seemingly only, FKBP of *P. falciparum* and the work described in this thesis supports the idea that this protein, PfFKBP35, is worthy for consideration as a novel anti-malarial drug target. Much needs to be addressed, however, before PfFKBP35 can truly be considered a novel drug target. The function of PfFKBP35 remains unknown and a more comprehensive understanding of its cellular function is required before inhibitors can be developed for chemotherapeutic purposes.

The finding that other *Plasmodium* species encode a 35-kDa FKBP with remarkable similarity to PfFKBP35 suggests this protein is evolutionarily conserved and, therefore, likely to play an important role in the development of these parasites in their respective hosts. However, the anti-malarial effects of FK506 and rapamycin have so far only been tested against cultures of *P. falciparum*. CsA, on the other hand, has been shown to exhibit anti-malarial activity with a number of *Plasmodium* species in both culture and animal models of malaria (Thommen-Scott, 1981; Nickell *et al.*, 1982; Cole *et al.*, 1983). More comprehensive experiments, utilising a range of *Plasmodium* species as well as different strains of *P. falciparum*, are required to address the anti-malarial potential of the compounds used in this study. In particular, assessing the potency of these compounds against parasite strains that are resistant to chloroquine and/or other currently used anti-malarials will be important for the further development of this class of compounds. Due to the high degree of similarity between these various *Plasmodium* FKBPs, animal models may serve as useful and valid systems for testing novel FKBP inhibitors *in vivo*.

The most conclusive evidence to support the further development of this class of compounds as anti-malarials would be to show that PfFKBP35 plays an indispensable role in parasite development and disease progression through the use of gene knockout experiments. However, although genetic manipulation of *Plasmodium* species is possible (de Konig-Ward *et al.*, 2000), the genetic tools available for studying *Plasmodium* parasites are limited and technically difficult. While interference RNA (iRNA) techniques have been successfully used to ablate protein expression in a growing number of biological systems (Dillin, 2003), the

development of similar procedures for *P. falciparum* have been slow. Three separate groups have reported success in this regard (Kumar *et al.*, 2002; Malhotra *et al.*, 2002; McRobert and McConkey, 2002), but we and others have been unable to reproduce the effects.

Although it is proposed that the compounds used in this study mediate their anti-malarial actions through an inhibition of the chaperone activity of PfFKBP35, the precise mechanisms of their actions will remain unclear until PfFKBP35 can be studied *in vivo*. The ability of the recombinant form of PfFKBP35 used in this study to act as both a folding catalyst and a chaperone *in vitro* is not in doubt. Whether either of these activities represents true intracellular activities of the protein, however, remains to be addressed. In a recent study, Kramer and colleagues showed that, although TF of *E. coli* is one of the most efficient PPIase enzymes *in vitro*, this activity appears to be dispensable for the function of TF *in vivo* (Kramer *et al.*, 2004). Complementation of a *P. falciparum PfFKBP35* null strain with an episomally-expressed PfFKBP35 devoid of PPIase activity could be used to address the significance of the PPIase activity of PfFKBP35. Similarly, the physiological significance of the chaperone activity of PfFKBP35 could be investigated using a form of PfFKBP35 devoid of chaperone activity.

If the PPIase and chaperone activities of PfFKBP35 represent physiological functions of PfFKBP35, the precise nature of these activities needs to be addressed. For example, it will be important to investigate if these two activities are distinct, independent functions of the protein, or if their actions are interdependent. A "hold and fold" function, whereby PfFKBP35 utilises its chaperone activity to immobilise misfolded proteins, and subsequently enzymatically alters the bound substrate with its PPIase activity, is an intriguing possibility. Ascertaining if these activities of PfFKBP35 are directed towards general or specific substrates is also important. For example, does PfFKBP35 function as a general catalyst of peptidyl-prolyl bonds in order to facilitate proteins achieving their correct three-dimensional structures, or does it function to modulate the activity of specific proteins whose activity is dependent upon the isomerisation of key peptidyl-prolyl bonds? The PPIase activity of Pin1, the archetypal member of the parvulin family of PPIases, functions to modulate the activity of a number of proteins (Shen *et al.*, 1998; Zhou *et al.*, 2000; Zacchi *et al.*, 2002; Zheng *et al.*, 2002). Work by two groups has recently shown a key role of Pin1 in modulating the activity of p53, the principal tumour suppressor

in mammals that has been the subject of intense study as it is inactivated in more than 50% of sporadic human tumours. This protein integrates cellular responses to genomic damage, mitotic spindle misassembly and hypoxia by mediating cell cycle arrest through transcriptional regulation of a large array of genes (Yang and McKeon, 2000). The ability of p53 to activate transcription of these genes is dependent upon it binding to specific promoter regions. This DNA-binding capability of p53 is dependent upon the isomerisation of key peptidyl-prolyl bonds within its DNA-binding domain (Zacchi *et al.*, 2002; Zheng *et al.*, 2002). Identifying the precise physiological function of PfFKBP35 will be important in validating it as a novel drug target.

The identification of interacting proteins will be key to elucidating the cellular function of PfFKBP35. The *in vitro* techniques used in this study to identify protein interactions were far from ideal. The difficulties in maintaining very high parasitemias of *P. falciparum* in continuous culture hinder the purification of parasite proteins in large quantities, thereby minimising the possibility of detecting interactions with the bait protein (i.e. PfFKBP35). In the absence of antibodies specific for candidate proteins, mass spectrometric analysis may facilitate the identification of parasite proteins specifically bound to immobilised PfFKBP35. Alternatively, an intact-cell approach, such as the yeast-2-hybrid system, could be used. However, as yet no reliable, representative *P. falciparum* genomic/cDNA library exists for use with this system, due in no small part to the difficulty in expressing *P. falciparum* proteins in heterologous systems. The recently developed technique of *in vitro* expression cloning (Kanai *et al.*, 2002), which involves the *in vitro* translation of proteins from a cDNA library and the subsequent screening for interaction with an immobilised bait protein, could potentially overcome the difficulty of expressing *P. falciparum* proteins in heterologous systems. Also, the application of phage display technology for the identification of interacting proteins from *P. falciparum* has recently been reported (Lauterbach *et al.*, 2003).

Technical difficulties and time constraints during this study hindered the production of antibodies specific for PfFKBP35, but it is envisaged that such antibodies will be invaluable for the further characterisation of this protein. The experiments depicted in Fig. 3.1 and Fig. 3.2 suggest that FK506 and rapamycin exert their anti-malarial effects predominantly on the trophozoite stage. The expression profile of *PfFKBP35* could be addressed by monitoring PfFKBP35

levels in the various developmental stages. An anti-PfFKBP35 antibody would also be invaluable for co-immunoprecipitation and/or co-immunofluroescence experiments for studying interacting partners. Indeed, a combination of anti-FK506 and anti-PfFKBP35 antibodies would allow us to address if PfFKBP35 really is the target of FK506.

Aside from further functional characterisation of PfFKBP35, detailed structural analysis of the protein will have to be undertaken to further strengthen PfFKBP35's potential as a novel drug target. As FKBPs are abundant in human cells, the development of anti-malarial drugs based on FK506 depends upon such drugs specifically inhibiting the parasite protein. Sequence analysis of the FKBP domain of PfFKBP35 suggests that there may be structural differences between PfFKBP35 and hFKBP12 that may allow the parasite protein to be specifically targeted by an inhibitor. This is supported by the finding that both 18-ene-20-oxa-FK520 and 13-dM(Me)-18-ene-20-oxa-FK520 inhibited parasite growth and the chaperone activity of the FKBP domain of PfFKBP35, while having no measurable affinity for hFKBP12. An X-ray crystal structure of PfFKBP35 should provide a greater insight into structural differences between PfFKBP35 and the two predominant human FKBPs, hFKBP12 and hFKBP51, both of whose three-dimensional structures have been determined (Michnick *et al.*, 1991; Sinars *et al.*, 2003).

The compounds used in this study inhibit parasite growth in the low micromolar range. However, for clinical development, inhibitors effective in the low nanomolar range are required. Also, although FK506 and rapamycin are used clinically, they are both large molecules with relative molecular masses (M_r) of 822 and 914, respectively. Due to more desirable pharmacokinetics, smaller molecules ($M_r < 500$) are typically preferred for drug development (Schwarze and Dowdy, 2000). A three-dimensional structure of PfFKBP35 will facilitate a molecular-modelling approach to aid in the design or selection of potential inhibitors specific for PfFKBP35. Unlike FK506 and rapamycin, such compounds may have the potential to serve as novel chemotherapeutic agents against malaria.

References

Allison, A.C. (2000) Immunosuppressive drugs: the first 50 years and a glance forward. *Immunopharmacol* **47**: 63-83.

Anders, R.F., and Saul, A. (2000) Malaria Vaccines. *Parasitol Today* **16**: 444-447.

Arceci, R., Stieglitz, K., and Bierer, B. (1992) Immunosuppressants FK506 and rapamycin function as reversal agents of the multidrug resistance phenotype. *Blood* **80**: 1528-1536.

Ardeshir, F., Flint, J.E., Richman, S.J., and Reese, R.T. (1987) A 75 kd merozoite surface protein of *Plasmodium falciparum* which is related to the 70 kd heat-shock proteins. *EMBO J* **6**: 493-499.

Arié, J.P., Sassoon, N., and Betton, J.M. (2001) Chaperone function of FkpA, a heat shock prolyl isomerase, in the periplasm of *Escherichia coli*. *Mol Microbiol* **39**: 199-210.

Arndt, C., Cruz, M.C., Cardenas, M.E., and Heitman, J. (1999) Secretion of FK506/FK520 and rapamycin by *Streptomyces* inhibits the growth of competing *Saccharomyces cerevisiae* and *Cryptococcus neoformans*. *Microbiol* **145**: 1989-2000.

Avramut, M., and Achim, C.L. (2002) Immunophilins and their ligands: insights into survival and growth of human neurons. *Physiol Behav* **77**: 463-468.

Baca, A.M., and Hol, W.G.J. (2000) Overcoming codon bias: a method for high-level overexpression of *Plasmodium falciparum* and other AT-rich parasite genes in *Escherichia coli*. *Int J Parasitol* **30**: 113-118.

Banerjee, R., and Goldberg, D.E. (2001) The *Plasmodium* food vacuole. In: *Antimalarial Chemotherapy. Mechanisms of Action, Resistance, and New Directions in Drug Discovery.* Rosenthal, P.J. (Ed), Humana Press, Totowa, New Jersey. pp 43-63.

Bang, H., Pecht, A., Raddatz, G., Scior, T., Solbach, W., Brune, K., and Pahl, A. (2000) Prolyl isomerases in a minimal cell: catalysis of protein folding by trigger factor from *Mycoplasma genitalium*. *Eur J Biochem* **267**: 3270-3280.

Banumathy, G., Singh, V., Raghavan, P., and Tatu, U. (2003) Heat shock protein 90 is essential for *Plasmodium falciparum* growth in human erythrocytes. *J Biol Chem* **278**: 18336-18345.

Baughman, G., Wiederrecht, G.L., Chang, F., Martin, M.M., and Bourgeois, S. (1997) Tissue distribution and abundance of human FKBP51, an FK506-binding protein that can mediate calcineurin inhibition. *Biochem Biophys Res Commun* **232**; 437-443.

Becker, J.W., Rotonda, J., McKeever, B.M., Chan, H.K., Marcy, A.I., Wiederrecht, G., Hermes, J.D., and Springer, J.P. (1993) FK-506-binding protein: three-

dimensional structure of the complex with the antagonist L-685-818. *J Biol Chem* **268**: 11339-11339.

Bell, A., Wernli, B., and Franklin, R.M. (1993) Effects of microtubule inhibitors on protein synthesis in *Plasmodium falciparum*. *Parasitol Res* **79**: 146-152.

Bell, A., Wernli, B., and Franklin, R.M. (1994) Roles of peptidyl-prolyl *cis-trans* isomerase and calcineurin in the mechanisms of antimalarial action of cyclosporin A, FK506, and rapamycin. *Biochem Pharmacol* **48**: 495-503.

Bell, A., Roberts, H.C., and Chappell, L.H. (1996) The antiparasite effects of cyclosporin A: possible drug targets and clinical applications. *Gen Pharmac* **27**: 963-971.

Berriman, M., and Fairlamb, A.H. (1998) Detailed characterisation of a cyclophilin from the human malaria parasite *Plasmodium falciparum*. *Biochem J* **334**: 437-445.

Bianco, A.E., Favaloro, J.M., Burkot, T.R., Culvenor, J.G., Crewther, P.E., Brown, G.V., Anders, R.F., Coppel, R.L., and Kemp, D.J. (1986) A repetitive antigen of *Plasmodium falciparum* that is homologous to heat shock protein 70 of *Drosophila melanogaster*. *Proc Natl Acad Sci USA* **83**: 8713-8717.

Bierer, B.E., Mattila, P.S., Standaert, R.F., Herzenberg, L.A., Burakoff, S.J., Crabtree, G., and Schreiber, S.L. (1990) Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin. *Proc Natl Acad Sci USA* **87**: 9231-9235.

Blatch, G.L. and Lässle, M. (1999) The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. *Bioessays* **21**: 932-939.

Boland, P.B., Ettling, M., and Meek, S. (2000) Combination therapy for malaria in Africa: hype or hope? *Bull World Health Org* **78**: 1378-1388.

Bonnefoy, S., Attal, G., Langsley, G., Tekaia, F., and Mercereau-Puijalon, O. (1994) Molecular characterization of the heat shock protein 90 gene of the human malaria parasite *Plasmodium falciparum*. *Mol Biochem Parasitol* **67**: 157-170.

Borel, J.F., Feurer, C., Gubler, H.U., and Stähelin, H. (1976) Biological effects of cyclosporin A: a new antilymphocytic agent. *Agents Actions* **6**: 468-475.

Botham, H., and Plückthun A. (2000) The periplasmic *Escherichia coli* peptidylprolyl *cis,trans*-isomerase FkpA. I. Increased functional expression of antibody fragments with and without *cis*-prolines. *J Biol Chem* **275**: 17100-17105.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. *Anal Biochem* **72**: 248-254.

Braun, P.D., Barglow, K.T., Lin, Y., Akompong, T., Briesewitz, R., Ray, G.T., Haldar, K., and Wandless, T.J. (2003) A bifunctional molecule that displays context-dependent cellular activity. *J Am Chem Soc* **125**: 7575-7580.

Brecht, S., Schwarze, K., Waetzig, V., Christner, C., Heiland, S., Fischer, G., Sartor, K., and Herdegen, T. (2003) Changes in peptidyl-prolyl *cis/trans* isomerase activity and FK506 binding protein expression following neuroprotection by FK506 in the ischemic rat brain. *Neuroscience* **120**: 1037-1048.

Bremen, J.G. (2001) The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. *Am J Trop Med Hyg* **64**(S): 1-11.

Bremen, J.G., Egan, A., and Keusch, G. (2001) The intolerable burden of malaria: a new look at the numbers. *Am J Trop Med Hyg* **64**(S): iv-vii.

Breuder, T., Hemenway, C.S., Movva, N.R., Cardenas, M.E., and Heitman, J. (1994) Calcineurin is essential in cyclosporin A- and FK506-sensitive yeast strains. *Proc Natl Acad Sci USA* **91**: 5372-5376.

Brilliantes, A.M.B., Ondraiš, K., Scott, A., Kobrinsky, E., Ondriašová, E., Moschella, M.C., Jayaraman, T., Landers, M., Ehrlich, B.E., and Marks, A.R. (1994) Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. *Cell* **77**: 513-523.

Brown, D.M., Netting, A.G., Chun, B.K., Choi, Y., Chu., C.K., and Gero, A.M. (1999) L-nucleoside analogues as potential anti-malarials that selectively target *Plasmodium falciparum* adenosine deaminase. *Nucleosides Nucleotides* **18**: 2521-2532.

Bruce-Chwatt, L.J. (1985) In: Essential Malariology, 2nd Edition, William Heiniman Medical Books, London.

Buchner, J., Grallert, H., and Jakob, U. (1998) Analysis of chaperone function using citrate synthase as nonnative substrate protein. *Meth Enzymol* **290**: 323-338.

Budiansky, S. (2002) Creatures of our own making. *Science* **298**: 80-86.

Callebaut, I., Renoir, J.M., Lebeau, M.C., Massol, N., Burny, A., Baulieu, E.E., and Mornon, J.P. (1992) An immunophilin that binds M_r 90,000 heat shock protein: main structural features of a mammalian p59 protein. *Proc Natl Acad Sci USA* **89**: 6270-6274.

Callebaut, I., and Mornon, J.P. (1995) Trigger factor, one of the *Escherichia coli* chaperone proteins, is an original member of the FKBP family. *FEBS Lett* **374**: 211-215.

Cameron, A.M., Steiner, J.P., Sabatini, D.M., Kaplin, A.I., Walensky, L.D., and Snyder, S.H. (1995a) Immunophilin FK506 binding protein associated with inositol 1,4,5-trisphosphate receptor modulates calcium flux. *Proc Natl Acad Sci USA* **92**: 1784-1788.

Cameron, A.M., Steiner, J.P., Roskams, A.J., Ali, S.M., Ronnett, G.V., and Snyder, S.H. (1995b) Calcineurin associated with the inositol 1,4,5-trisphosphate receptor-FKBP12 complex modulates Ca^{2+} flux. *Cell* **83**: 463-472.

Cameron, A.M., Nucifora, F.C., Fung, E.T., Livingston, D.J., Aldape, R.A., Ross, C.A., and Snyder, S.H. (1997) FKBP12 binds the inositol 1,4,5-trisphosphate receptor at leucine-proline (1400-1401) and anchors calcineurin to this FK506-like domain. *J Biol Chem* **272**: 27582-27588.

Cardenas, M.E., Hemenway, C., Muir, R.S., Ye, R., Fiorentino, D., and Heitman J. (1994) Immunophilins interact with calcineurin in the absence of exogenous ligands. *EMBO J* **13**: 5944-5957.

Carlton, J.M., Angiuoli, S.V., Suh, B.B., Kooij, T.W., Pertea, M., Silva, J.C., Ermolaeva, M.D., Allen, J.E., Selengut, J.D., Koo, H.L., Peterson, J.D., Pop, M., Kosack, D.S., Shumway, M.F., Bidwell, S.L., Shallom, S.J., van Aken, S.E., Riedmuller, S.B., Feldblyum, T.V., Cho, J.K., Quackenbush, J., Sedegah, M., Shoaiba, A., Cummings, L.M., Florens, L., Yates, J.R., Raine, J.D., Sinden, R.E., Harris, M.A., Cunningham, D.A., Preiser, P.R., Bergman, L.W., Vaidya, A.B., van Lin, L.H., Janse, C.J., Waters, A.P., Smith, H.O., White, O.R., Salzberg, S.L., Venter, C.J., Fraser, C.M., Hoffman, S.L., Gardner, M.J., and Carucci, D.J. (2002) Genome sequence and comparative analysis of the model rodent malaria parasite *Plasmodium yoelii yoelii*. *Nature* **419**: 512-519.

Carver, L.A., and Bradfield, C.A. (1997) Ligand-dependent interaction of the aryl hydrocarbon receptor with a novel immunophilin homolog *in vivo*. *J Biol Chem* **272**: 11452-11456.

Carver, L.A., LaPres, J.J., Jain, S., Dunham, E.E., and Bradfield, C.A. (1998) Characterization of the Ah Receptor-associated Protein, ARA9. *J Biol Chem* **273**: 33580-33587.

Charng, M.J., Kinnunen, P., Hawker, J., Brand, T., and Schneider, M.D. (1996) FKBP-12 recognition is dispensable for signal generation by type I transforming growth factor-β receptors. *J Biol Chem* **271**: 22941-22944.

Chen, Y.G., Liu, F., and Massagué, J. (1997) Mechanism of TGFβ receptor inhibition by FKBP12. *EMBO J* **16**: 3866-3876.

Cheung-Flynn, J., Roberts, P.J., Riggs, D.L., and Smith, D.F. (2003) C-terminal sequences outside the tetratricopeptide repeat domain of FKBP51 and FKBP52 cause differential binding to Hsp90. *J Biol Chem* **278**: 17388-17394.

Choi, J., Chen, J., Schreiber, S.L. and Clardy, J. (1996) Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. *Science* **273**: 239-242.

Cianciotto, N.P., Eisenstein, B.I., Mody, C.H., Toews, G.B., and Engleberg, N.C. (1989) A *Legionella pneumophila* gene encoding a species-specific surface protein potentiates initiation of intracellular infection. *Infect Immun* **57**: 1255-1262.

Cianciotto, N.P., Bangsborg, J.M., Eisenstein, B.I., and Engleberg, N.C. (1990) Identification of *mip*-like genes in the genus *Legionella*. *Infect Immun* **58**: 2912-2918.

Cianciotto, N.P., and Fields, B.S. (1992) *Legionella pneumophila mip* gene potentiates intracellular infection of protozoa and human macrophages. *Proc Natl Acad Sci USA* **89**: 5188-5191.

Clark, I.A., and Schofield, L. (2000) Pathogenesis of malaria. *Parasitol Today* **16**: 451-454.

Clipstone, N.A., and Crabtree, G.R. (1992) Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature* **357**: 695-697.

Cole, G.A., Nickell, S.P., Mokhtarian, F., and Scheibel, L.W. (1983) Effects of cyclosporine on experimental infections. *Transplant Proc* **15**(S): 2271-2277.

Coss, M.C., Winterstein, D., Sowder, R.C., and Simek, S.L. (1995) Molecular cloning, DNA sequence analysis, and biochemical characterisation of a novel 65-kDa FK506-binding protein (FKBP65). *J Biol Chem* **270**: 29336-29341.

Crabtree, G.R. (1999) Generic signals and specific outcomes: signaling through Ca^{2+}, calcineurin and NF-AT. *Cell* **96**: 611-614.

Craig, A., and Scherf, A. (2001) Molecules on the surface of the *Plasmodium falciparum* infected erythrocyte and their role in malarial pathogenesis and immune evasion. *Mol Biochem Parasitol* **115**: 129-143.

Cruz, M.C., Goldstein, A.L., Blankenship, J., Del Poeta, M., Perfect, J.R., McCusker, J.H., Bennani, Y.L., Cardenas, M.E., and Heitman, J. (2001) Rapamycin and less immunosuppressive analogs are toxic to *Candida albicans* and *Cryptococcus neoformans* via FKBP12-dependent inhibition of TOR. *Antimicrob Agents Chemother* **45**: 3162-3170.

Czar, M.J., Owens-Grillo, J.K., Yem, A., Leach, K.L., Deibel, M.R., Welsh, M.J., and Pratt, W.B. (1994) The hsp56 immunophilin component of untransformed steroid receptor complexes is localised to both microtubules in the cytoplasm and to the same nonrandom regions within the nucleus as the steroid receptor. *Mol Endocrinol* **8**: 1731-1741.

Czar, M.J., Lyons, R.H., Welsh, M.J., Renoir, J.M., and Pratt, W.B. (1995) Evidence that the FK506-binding immunophilin heat shock protein 56 is required for trafficking of the glucocorticoid receptor from the cytoplasm to the nucleus. *Mol Endocrinol* **9**: 1549-1560.

Dame, J.B., Arnot, D.E., Bourke, P.F., Chakrabarti, D., Christodoulou, Z., Coppel, R.L., Cowman, A.F., Craig, A.G., Fischer, K., Foster, J., Goodman, N., Hinterberg, K., Holder, A.A., Holt, D.C., Kemp, D.J., Lanzer, M., Lim, A., Newbold, C.I., Ravetch, J.V., Reddy, G.R., Rubio, J., Schuster, S.M., Su, X., Thompson, J.K.,

Vital, F., Wellems, T.E., and Werner, E.B. (1996) Current status of the *Plasmodium falciparum* genome project. *Mol Biochem Parasitol* **79**: 1-12.

D'Andrea, L.D. and Regan, L. (2003) TPR proteins: the versatile helix. *Trends in Biochem Sci* **28**: 655-662.

Dargan, S.L., Lea, E.J.A., and Dawson, A.P. (2002) Modulation of type-1 $Ins(1,4,5)P_3$ receptor channels by the FK506-binding protein, FKBP12. *Biochem J* **361**: 401-407.

Das, A., Elmendorf, H.G., Li, W., and Haldar, K. (1994) Biosynthesis, export and processing of a 45 kDa protein detected in membrane clefts of erythrocytes infected with *Plasmodium falciparum*. *Biochem J* **302**: 487-496.

Das, A.K., Cohen, P.T.W., and Barford, D. (1998) The structure of the tetratricopeptide repeats of protein phosphate 5: implications for TPR-mediated protein-protein interactions. *EMBO J* **17**: 1192-1199.

David-Pfeuty, T., Chakrani, F., Ory, K., and Nouviandooghe, Y. (1996) Cell cycle-dependent regulation of nuclear p53 traffic occurs in one subclass of human tumor cells and in untransformed cells. *Cell Growth Differ* **7**: 1211-1225.

Davies, T.H., Ning, Y.M., and Sánchez, E.R. (2002) A new first step in activation of steroid receptors. *J Biol Chem* **277**: 44597-44600.

Davis, E.C., Broekelmann, T.J., Ozawa, Y., and Mecham, R.P. (1998) Identification of tropoelastin as a ligand for the 65-kD FK506-binding protein, FKBP65, in the secretory pathway. *J Cell Biol* **140**: 295-303.

Dawson, T.M., Steiner, J.P., Lyons, W.E., Fotuhi, M., Blue, M., and Synder, S.H. (1994) The immunophilins, FK506 binding protein and cyclophilin, are discretely localised in the brain: relationship to calcineurin. *Neuroscience* **62**: 569-580.

de Koning-Ward, F., Janse, C.J., and Waters, A.P. (2000) The development of genetic tools for dissecting the biology of malaria parasites. *Annu Rev Microbiol* **54**: 157-185.

Denny, W.B., Valentine, D.L., Reynolds, P.D., Smith, D.F., and Scammell, J.G. (2000) Squirrel monkey immunophilin FKBP51 is a potent inhibitor of glucocortocoid receptor binding. *Endocrinol* **141**: 4107-4113.

Dillin, A. (2003) The specifics of small interfering RNA specificities. *Proc Natl Acad Sci USA* **100**: 6289-6291.

Dobson, S., May, T., Berriman, M., Del Vecchio, C., Fairlamb, A.H., Chakrabrti, D., and Barik, S. (1999) Characterization of protein ser/thr phosphatases of the malaria parasite, *Plasmodium falciparum*: inhibition of the parasite calcineurin by cyclophilin-cyclosporin complex. *Mol Biochem Parasitol* **99**: 167-181.

Dobson, S., Kar, B., Kumar, R., Adams, B., and Barik, S. (2001) A novel tetratricopeptide repeat (TPR) containing PP5 serine/threonine protein phosphatase in the malaria parasite, *Plasmodium falciparum*. *BMC Microbiol* **1**: 31.

Duffy, P.E., Craig, A.G., and Baruch, D.I. (2001) Variant proteins on the surface of malaria-infected erythrocytes – developing vaccines. *Trends Parasitol* **17**: 354-356.

Dumont, F.J. and Su, Q. (1996) Mechanism of action of the immunosuppressant rapamycin. *Life Sci* **58**: 373-395.

Dumont, F.J., Staruch, M.J., Koprak, S.L., Siekierka, J.J., Lin, C.S., Harrison, R., Sewell, T., Kindt, V.M., Beattie, T.R., Wyvratt, M., and Sigal, N.H. (1992) The immunosuppressive and toxic effects of FK-506 are mechanistically related: pharmacology of a novel antagonist of FK-506 and rapamycin. *J Exp Med* **176**: 751-760.

Dunn, C.R., Banfield, M.J., Barker, J.J., Higham, C.W., Moreton, K.M., Turgut-Balik, D., Brady, R.L., and Holbrook, J.J. (1996) The structure of lactate dehydrogenase from *Plasmodium falciparum* reveals a new target for anti-malarial drug design. *Nature Struct Biol* **3**: 912-915.

Ecker, J.R. (1997) BRI-ghtening the pathway to steroid hormone signaling events in plants. *Cell* **90**: 825-827.

Epand, R.F., and Epand, R.M. (1991) The new potent immunosuppressant FK-506 reverses multidrug resistance in Chinese hamster ovary cells. *Anti-Cancer Drug Des* **6**: 189-193.

Fischer, G., Bang, H., and Mech, C. (1984) Detection of enzyme catalysis for *cis-trans* isomerisation of peptide bonds using proline containing peptides as substrates. *Biomed Biochem Acta* **43**: 1101-1111.

Fischer, G., and Bang, H. (1985) The refolding of urea-denatured ribonuclease A is catalyzed by peptidyl-prolyl cis-trans isomerase. *Biomed Biophys Acta* **828**: 39-42.

Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T., and Schmid, F.X. (1989) Cyclophilin and peptidyl-prolyl *cis-trans* are probably identical proteins. *Nature* **337**: 476-478.

Fischer, G., Bang, H., Ludwig, B., Mann, K., and Hacker, J. (1992) Mip protein of *Legionella pneumophila* exhibits peptidyl-prolyl-*cis/trans* isomerase (PPIase) activity. *Mol Microbiol* **6**: 1375-1383.

Fischer, G., and Aumüller, T. (2003) Regulation of peptide bond *cis/trans* isomerization by enzyme catalysis and its implication in physiological processes. *Rev Physiol Biochem Pharmacol* **148**: 105-150.

Foley, M., and Tilley, L. (1998) Protein trafficking in malaria-infected erythrocytes. *Int J Parasitol* **28**: 1671-1680.

Foor, F., Parent, S.A., Morin, N., Dahl, A.M., Ramadan, N., Chrebet, G., Bostian, K.A., and Nielson, J.B. (1992) Calcineurin mediates inhibition by FK506 and cyclosporin of recovery from α-factor arrest in yeast. *Nature* **360**: 682-684.

Fowler, R.E., Smith, A.M.C., Whitehorn, J., Williams, I.T., Bannister, L.H., and Mitchell, G.H. (2001) Microtubule associated motor proteins of *Plasmodium falciparum* merozoites. *Mol Biochem Parasitol* **117**: 187-200.

Freskgard, P.O., Bergenhem, N., Jonsson, B.H., Svensson, M., and Carlsson, U. (1992) Isomerase and chaperone activity of prolyl isomerase in the folding of carbonic anhydrase. *Science* **258**: 466-468.

Fruman, D.A., Klee, C.B., Bierer, B.E., and Burakoff, S.J. (1992) Calcineurin phosphatase activity is T-lymphocytes in inhibited by FK506 and cyclosporin A. *Proc Natl Acad Sci USA* **89**: 3686-3690.

Fukuda, K., Tanigawa, Y., Fujii, G., Yasugi, S., and Hirohashi, S. (1998) cFKBP/SMAP; a novel molecule involved in the regulation of smooth muscle differentiation. *Development* **125**: 3535-3542.

Futer, O., DeCenzo, M.T., Aldape, R.A. and Livingston, D.J. (1995) FK506 binding protein mutational analysis. *J Biol Chem* **270**: 318935-18940.

Galat, A. (2000) Sequence diversification of the FK506-binding proteins in several different genomes. *Eur J Biochem* **267**: 4945-4959.

Galat, A. (2003) Peptidylprolyl cis/trans isomerases (immunophilins): biological diversity – targets – functions. *Curr Top Med Chem* **3**: 1315-1347.

Galat, A. (2004) A note on clustering the functionally-related paralogues and orthologues of proteins: a case of the FK506-binding proteins (FKBPs). *Comp Biol Chem* **28**: 129-140.

Galat, A., and Metcalfe, S.M. (1995) Peptidylproline *cis/trans* isomerases. *Prog Biophys Molec Biol* **63**: 67-118.

Galat, A., and Revière, S. (1998) In: Peptidyl-prolyl *cis-trans* isomerases. Oxford University Press, Inc., New York.

Galigniana, M.D., Scruggs, J.L., Herrington, J.L., Welsh, M.J., Carter-Su, C., Housley, P.R., and Pratt, W.B. (1998) Heat shock protein 90-dependent (geldanamycin-inhibited) movement of the glucocorticoid receptor through the cytoplasm to the nucleus requires intact cytoskeleton. *Mol Endocrinol* **12**: 1903-1913.

Galigniana, M.D., Radanyi, C., Renoir, J.M., Housley, P.R., and Pratt, W.B. (2001) Evidence that the peptidylprolyl isomerase domain of the hsp90-binding immunophilin FKBP52 is involved in both dynein interaction and glucocorticoid receptor movement to the nucleus. *J Biol Chem* **276**: 14884-14889.

Galigniana, M.D., Harrell, J.M., Murphy, P.J., Chimkers, M., Radanyi, C., Renoir, J.M., Zhang, M., and Pratt, W.B. (2002) Binding of hsp90-associated immunophilins to cytoplasmic dynein: direct binding and *in vivo* evidence that the peptidylprolyl isomerase domain is a dynein interaction domain. *Biochem* **41**: 13602-13610.

Galigniana, M.D., Harrell, J.M., O'Hagen, H.M., Ljungman, M., and Pratt, W.B. (2004) Hsp90-binding immunophilins link p53 to dynein during p53 transport to the nucleus. *J Biol Chem* **279**: 22483-22489.

Gallup, J.L., and Sachs, J.D (2002) The economic burden of malaria. *Am J Trop Med Hyg* **64**(S): 85-96.

Gardner, M.J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., Carlton, J.M., Pain, A., Nelson, K.E., Bowman, S., Paulsen, I.T., James, K., Eisen, J.A., Rutherford, K., Salzberg, S.L., Craig, A., Kyes. S., Chan, M.S., Nene, V., Shallo, S.J., Suh, B., Peterson, J., Angiuoli, S., Pertea, M., Allen, J., Selengut, J., Haft, D., Mather, M.W., Vaidya, A.B., Martin, D.M.A., Fairlamb, A.H., Fraunholz, M.J., Roos, D.S., Ralph, S.A., McFadden, G.I., Cummings, L.M., Subramanian, G.M., Mungall, C., Venter, J.C., Carucci, D.J., Hoffman, S.J., Newbold, C., Davis, R.W., Fraser, C.M., and Barrell, B. (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* **419**: 498-511.

Gavigan, C.S., Kiely, S.P., Hirtzlin, J., and Bell, A. (2003) Cyclosporin-binding proteins of *Plasmodium falciparum*. *Int J Parasitol* **33**: 987-996.

Geisler, M., Kolukisaoglu, Ü.H, Bouchard, R., Billion, K., Berger, J., Saal, B., Frangne, N., Koncz-Kálmán, Koncz, C., Dudler, R., Blakeslee, J.J., Murphy, A.S., Martinoia, E., and Schulz, B. (2003) Twisted dwarf 1, a unique plasma membrabe-anchored immunophilin-like protein, interacts with *Arabidopsis* multidrug resistance-like transporters AtPGP1 and AtPGP19. *Mol Biol Cell* **14**: 4238-4249.

Geisler, M., Girin, M., Brandt, S., Vincenzetti, V., Plaza, S., Paris, N., Kobae, Y., Maeshima, M., Billion, K., Kolukisaoglu, Ü.H., Schulz, B., and Martinoia, E. (2004) *Arabidopsis* immunophilin-like TWD1 functionally interacts with vaculoar ABC transporters. *Mol Biol Cell* **15**: 3393-3405.

Gold, B.G., Densmore, V., Shou, W., Matzuk, M.M., and Gordon, H.S. (1998) Immunophilin FK506-binding protein 52 (not FK506-binding protein 12) mediates the neurotrophic action of FK506. *J Pharmacol Exp Ther* **289**: 1202-1210.

Gormley, J.A., Howard, R.J., and Taraschi, T.F. (1992) Trafficking of malarial proteins to the host cell cytoplasm and erythrocyte surface membrane involves multiple pathways. *J Cell Biol* **119**: 1481-1495.

Goto, T., Kino, T., Hatanaka, H., Okuhara, M., Kohsaka, N., Aoki, H., and Imanaka, H. (1991) FK506: Historical perspectives. *Transplant Proceed* **23**: 2713-2717.

Greenwood, B., and Mutabingwa, T. (2002) Malaria in 2002. *Nature* **415**(S): 670-672.

Griffith, J.P., Kim, J.L., Kim, E.E., Sintchak, M.D., Thomson, J.A., Fitzgibbon, M.J., Fleming, M.A., Caron, P.R., Hsiao, K., and Navia, M.A. (1995) X-ray structure of calcineurin inhibited by the immunophilin-drug complexes. *Cell* **82**: 507-522.

Gu, Y.Z., Hogenesch, J.B., and Bradfield. C.A. (2000) The PAS superfamily: sensors of environmental and developmental signals. *Annu Rev Pharmacol Toxicol* **40**: 519-561.

Hagan, P., Bjorvatn, B., and Jepsen, S. (1999) European Malaria Vaccine Initiative. *Parasitol Today* **15**: 47-48.

Haldar, K., and Akompong, T. (2001) Transport and trafficking in *Plasmodium*-infected red cells. In: *Antimalarial Chemotherapy. Mechanisms of Action, Resistance, and New Directions in Drug Discovery.* Rosenthal, P.J. (Ed), Humana Press, Totowa, New Jersey. pp 27-41.

Haldar, K., Mohandas, N., Samuel, B.U., Harrison, T., Hiller, N.L., Akompong, T., and Cheresh, P. (2002) Protein and lipid trafficking induced in erythrocytes infected by malaria parasites. *Cell Microbiol* **4**: 383-395.

Hamilton, G.S., Huang, W., Connolly, M.A., Ross, D.T., Guo, H., Valentine, H.L., Suzdak, P.D., and Steiner, J.P. (1997) FKBP12-binding domain analogues of FK506 are potent, nonimmunosuppressive neurotrophic agents *in vitro* and promote recovery in a mouse model of Parkinson's disease. *Bioorg Med Chem Lett* **7**: 1785-1790.

Handschumacher, R.E., Harding, G.W., Rice., J., Drugge, R.J., and Speicher, D.W. (1984) Cyclophilin: a specific cytosolic binding protein for cyclosporin A. *Science* **226**: 544-547.

Harding, M.W., Handschumacher, R.E., and Speicher, D.W. (1986) Isolation and amino acid sequence of cyclophilin. *J Biol Chem* **261**: 8547-8555.

Harding, M.W., Galat, A., Uehling, D.E., and Schreiber, S.L. (1989) A receptor for the immunosuppressant FK506 is a *cis-trans* peptidyl-prolyl isomerase. *Nature* **341**: 758-760.

Harrell, J.M., Kurek, I., Breiman, A., Radanyi, C., Renoir, J.M., Pratt, W.B., and Galigniana, M.D. (2002) All of the protein interactions that link steroid receptor•Hsp90•immunophilin heterocomplexex to cytoplasmic dynein are common to plant and animal cells. *Biochem* **41**: 5581-5587.

Harrison, R.K., and Stein, R.L. (1990) Substrate specificities of the peptidyl prolyl *cis-trans* isomerase activities of cyclophilin and FK-506 binding protein: evidence for the existence of a family of distinct enzymes. *Biochem* **29**: 3813-3816.

Hatanaka, H., Kino, T., Miyata, S., Inamura, N., Kuroda, A., Goto, T., Tanaka, H., and Okuhara, M. (1988) FR-900520 and FR-900523, novel immunosuppressants isolated from *Streptomyces*. II. Fermentation, isolation and physiochemical and biochemical characteristics. *J Antibiot (Tokyo)* **41**: 1592-1601.

Hemenway, C.S., and Heitman, J. (1996) Immunosuppressant target protein FKBP12 is required for P-glycoprotein function in yeast. *J Biol Chem* **271**: 18527-18534.

Hesterkamp, T., and Bukau, B. (1996) The *Escherichia coli* trigger factor. *FEBS Letts* **389**: 32-34.

Hesterkamp, T. Hauser, S., Lütcke, H., and Bukau, B. (1996) Escherichia coli trigger factor is a prolyl isomerase that associates with nascent polypeptide chains. *Proc Natl Acad Sci USA* **93**: 4437-4441.

High, K.P. (1994) The antimicrobial activities of cyclosporine, FK506, and rapamycin. *Transplantation* **57**: 1689-1700.

Hinterberg, K., Scherf, A., Gysin, G., Toyoshima, T., Aikawa, M., Mazie, J.C., da Silva, L.P., and Mattei, D. (1994) *Plasmodium falciparum*: the pf332 antigen is secreted by a brefeldin A-dependent pathway and is translocated to the erythrocyte membrane via Maurer's clefts. *Exp Parasitol* **79**: 279-291.

Hirtzlin, J., Farber, P.M., Franklin, R.M., and Bell, A. (1995) Molecular and biochemical characterisation of a *Plasmodium falciparum* cyclophilin containing a cleavable signal sequence. *Eur J Biochem* **232**: 765-772.

Hleb, M., Murphy, S., Wagner, E.F., Hanna, N.N., Sharma, N., Park., J., Li, X.C., Strom, T.B., Padbury, J.F., and Tseng, Y.T. (2004) Evidence for cyclin D3 as a novel target of rapamycin in human T lymphocytes. *J Biol Chem* **279**: 31948-31955.

Holder, A.A. (1999) Malaria Vaccines. *Proc Natl Acad Sci USA* **96**: 1167-1169.

Holloway, S.P., Min, W., and Inselburg, J.W. (1994) Isolation and characterization of a chaperonin-60 gene of the human malaria parasite *Plasmodium falciparum*. *Mol Biochem Parasitol* **64**: 25-32.

Horne, S.M., and Young, K.D. (1995) *Escherichia coli* and other species of the Enterobacteriaceae encode a protein similar to the family of Mip-like FK506-binding proteins. *Arch Microbiol* **163**: 357-365.

Hyde, J. (2002) Mechanisms of resistance of *Plasmodium falciparum* to antimalarial drugs. *Microbes Infect* **4**: 165-174.

Ideno, A., Yoshida, T., Furutani, M. and Maruyama, T. (2000) The 28.3 kDa FK506 binding protein from a thermophilic archaeum, *Methanobacterium thermoautotrophicum*, protects the denaturation of proteins *in vitro*. *Eur J Biochem* **267**: 3139-3148.

Ideno, A., Yoshida, T., Iida, T., Furutani, M., and Maruyama, T. (2001) FK506-binding protein of the hyperthermophilic archaeum, *Thermococcus* sp. KS-1, a cold-shock-inducible peptidyl prolyl *cis-trans* isomerase with activities to trap and refold denatured proteins. *Biochem J* **357**: 465-471.

Ideno, A., Furutani, M., Iba, Y., Kurosawa, Y., and Maruyama, T. (2002) FK506 binding protein from the hyperthermophilic archaeon *Pyrococcus horikoshii* suppresses the aggregation of proteins in *Escherichia coli*. *App Environ Microbiol* **68**: 464-469.

Ideno, A., and Maruyama, T. (2002) Expression of long- and short-type FK506 binding proteins in hyperthermophilic archaea. *Gene* **292**: 57-63.

Iida, T., Furutani, M., Nishida, F., and Maruyama, T. (1998) FKBP-type peptidyl prolyl *cis-trans* isomerase from a sulfur-dependent hyperthermophilic archaeon, *Thermococcus* sp KS-1. *Gene* **222**: 249-255.

Iida, T., Iwabuchi, T., Ideno, A., Suzuki, S., and Maruyama, T. (2000) FK506-binding protein-type peptidyl-prolyl *cis-trans* isomerase from a halophilic archaeum, *Halobacterium cutirubrum*. *Gene* **256**: 319-326.

Jachez, B., Boesch, D., Grassberger, M.A., and Loor, F. (1993) Reversion of the P-glycoprotein-mediated multidrug resistance of cancer cells by FK-506 derivatives. *Anti-Cancer Drugs* **4**: 223-229.

Jayaraman, T., Brilliantes, A.M., Timerman, A.P., Fleischer, S., Erdjument-Bromage, H., Tempst, P., and Marks, A.R. (1992) FK506 binding protein associated with the calcium release channel (ryanodine channel). *J Biol Chem* **267**: 9474-9477.

Kamphausen, T., Fanghänel, J., Neumann, D., Schulz, B., and Rahfeld, J.U. (2002) Characterization of *Arabidopsis thaliana At*FKBP42 that is membrane-bound and interacts with Hsp90. *Plant J* **32**: 263-276.

Kanai, F., Yaffe, M.B., and Stukenberg, P.T. (2002) Using in vitro expression cloning to identify interacting proteins. In: *Protein-protein interactions: a molecular cloning manual.* Golemis, E. (Ed), Cold Spring Harbour Laboratory Press, New York. pp 345-353.

Kay, J.E., Sampare-Kwateng, E., Geraghty, F. and Morgan, G.Y. (1991) Uptake of FK506 by lymphocytes and erythrocytes. *Transplant Proceed* **23**: 2790-2762.

Kay, J.E. (1996) Structure-function relationships in the FK506-binding protein (FKBP) family of peptidylprolyl *cis-trans* isomerases. *Biochem J* 314: 361-385.

Kazlauskas, A., Poellinger, L., and Pongratz, I. (2002) Two distinct regions of the immunophilin-like protein XAP2 regulate dioxin receptor function and interaction with hsp90. *J Biol Chem* **277**: 11795-11801.

Ke, H., and Huai, Q. (2003) Structures of calcineurin and its complexes with immunophilins-immunosuppressants. *Biochem Biophys Res Commun* **311**: 1095-1102.

Khattab, A., Pica-Mattoccia, L., Klinkert, M.Q., Wenger, R., and Cioli, D. (1998) Cyclosporins: lack of correlation between antischistosomal properties and inhibition of cyclophilin isomerase activity. *Exp Parasitol* **90**: 103-109.

Kieffer, L.J., Seng, T.W., Li, W., Osterman, D.G., Handschmucher, R.E., and Bayney, R.M. (1993) Cyclophilin-40, a protein with homology to the P59 component of the steroid receptor complex. Cloning of the cDNA and further characterization. *J Biol Chem* **268**: 12303-12310.

Kissinger, C.R., Parge, H.E., Knighton, D.R., Lewis, C.T., Pelletier, L.A., Tempcyzk, A., Kalish, V.J., Tucker, K.D., Showalter, R.E., Moomaw, E.W., Gastinel, L.N., Habuka, N., Chen, X., Maldonado, F., Barker, J.E., Bacquet, R., and Villafranca, J.E. (1995) Crystal structures of human calcineurin and the human FKBP12-FK506-calcineurin complex. *Nature* **378**: 641-644.

Kino, T., Hatanaka, H., Hashimoto, M., Nishiyama, M., Miyata, S., Goto, T., Okuhara, M., Aoki, M., and Imanaka, H. (1987) FK-506, a novel immunosuppressant isolated from *Streptomyces*. I. Fermentation, isolation and physiochemical and biochemical characteristics. *J Antibiot (Tokyo)* **40**: 1249-1258.

Klettner, A., Baumgrass, R., Zhang, Y., Fischer, G., Bürger, E., Herdegan, T., and Mielke, K. (2001) The neuroprotective actions of FK506 binding protein ligands: neuronal survival is triggered by de novo RNA synthesis, but is independent of inhibition of JNK and calcineurin. *Mol Brain Res* **97**: 21-31.

Kofron, J.L., Kuzmic, P., Kishore, V., Colon-Bonilla, E., and Rich, D.H. (1991) Determination of kinetic constants for peptidyl prolyl *cis-trans* isomerases by an improved spectrophotometric assay. *Biochem* **30**: 6127-6134.

Köhler, R., Fanghänel, J., König, B., Lüneberg, E., Frosch, M., Rahfeld, J.U., Hilgenfeld, R., Fischer, G., Hacker, J., and Steinert, M. (2003) Biochemical and functional analyses of the Mip protein: influence of the N-terminal half and of peptidylprolyl isomerase activity on the virulence of *Legionella pneumophila*. *Infect Immun* **71**: 4389-4397.

Komarova, E.A., Zelnick, C.R., Chin, D., Zeremeski, M., Gleiberman, A.S., Bacus, S.S., and Gudkor, A.V. (1997) Intracellular localization of p53 tumor suppressor protein in gamma-irradiated cells is cell cycle regulated and determined by the nucleus. *Cancer Res* **57**: 5217-5220.

Kramer, G., Rauch, T., Rist, W., Vorderwülbecke, S., Patzelt, H., Schulze-Specking, A., Ban, A., Deuerling, E., and Deuerling, E. (2002) L23 protein functions as a chaperone docking site on the ribosome. *Nature* **419**: 171-174.

Kramer, G., Patzelt, H., Rauch, T., Kurz, T.A., Vorderwülbecke, S., Bukau, B., and Deuerling, E. (2004) Trigger factor's peptidyl-prolyl *cis/trans* isomerase activity is not essential for the folding of cytosolic proteins in *Escherichia coli*. *J Biol Chem* **279**: 14165-14170.

Krishna, S., Eckstein-Ludwig, U., Joët, T., Uhlemann, A,C., Morin, C., Webb, R., Woodrow, C., Kun, J.F.J., and Kremsner, P.G. (2002) Transport processes in *Plasmodium falciparum*-infected erythrocytes: potential as new drug targets. *Int J Parasitol* **32**: 1567-1573.

Krugliak, M., Zhang, J., and Ginsburg, H. (2002) Intraerythrocytic *Plasmodium falciparum* utilises only a small fraction of the amino acids derived from the digestion of host cell cytosol for the biosynthesis of its proteins. *Mol Biochem Parasitol* **119**: 249-256.

Kumar, N., Syin, C., Carter, R., Quakyi, I., and Miller, L.H. (1988) *Plasmodium falciparum* gene encoding a protein similar to the 78-kDa rat glucose-related stress protein. *Proc Natl Acad Sci USA* **85**: 6277-6281.

Kumar, R., Adams, B., Oldenburg, A., Musiyenko, A., and Barik, S. (2002) Characterisation and expression of a PP1 serine/threonine protein phosphatase (PfPP1) from the malaria parasite, *Plasmodium falciparum*: demonstration of its essential role using RNA interference. *Malaria J* **1**: 5.

Kumar, R., Musiyenko, A., and Barik, S. (2003) The heat shock protein 90 of *Plasmodium falciparum* and antimalarial activity of its inhibitor, geldanamycin. *Malaria J* **2**: 30.

Kumar, R., Musiyenko, A., Oldenburg, A., Adams, B., and Barik S. (2004) Post-translational generation of constitutively active cores from larger phosphatases in the malarial parasite, *Plasmodium falciparum*: implications for proteomics. *BMC Mol Biol* **5**:6.

Kumar, S., Epstein, J.E., Richie, T.L., Nkrumah, F.K., Soisson, L, Carucci, D.J., and Hoffman, S.L. (2002) A multilateral effort to develop DNA vaccines against falciparum malaria. *Trends Parasitol* **18**: 129-135.

Kunz, J., Loeschmann, A., Deuter-Reinhard, M., and Hall, M.N. (2000) FAP1, a homologue of human transcription factor NF-X1, competes with rapamycin for binding to FKBP12 in yeast. *Mol Microbiol* **37**: 1480-1493.

Kuzuhara, T., and Horikoshi, M. (2004) A nuclear FK506-binding protein is a histone chaperone regulating rDNA silencing. *Nature Struct Mol Biol* **11**: 275-283.

Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Lam, E., Martin, M., Timerman, A.P., Sabers, C., Fleischer, S., Lukas, T., Abraham, R.T., O'Keefe, S.J., O' Neill, E.A., and Wiederrecht, G.J. (1995) A novel FK506

binding protein can mediate the immunosuppressive effects of FK506 and is associated with the cardiac ryanodine receptor. *J Biol Chem* **270**: 26511-26522.

Lam, E., Martin, M., and Wiederrecht, G.J. (1995) Isolation of a cDNA encoding a novel human FK506-binding protein homolog containing leucine zipper and tetratricopeptide repeat motifs. *Gene* **160**: 297-302.

Lamb, J.R., Tugendreich, S., and Hieter, P. (1995) Tetratrico peptide repeat interactions: to TPR or not to TPR? *Trends Biochem Sci* **20**: 257-259.

Lambros, C., and Venderberg, J.P. (1979) Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J Parasitol* **65**: 418-420.

Laurer, S.A., Rathod, P.K., Ghori, N., and Haldar, K. (1997) A membrane network for nutrient import in red cells infected with the malaria parasite. *Science* **276**: 1122-1125.

Lauterbach, S.B., Lanzillotti, R., and Coetzer, T.L. (2003) Construction and use of *Plasmodium falciparum* phage display libraries to identify host parasite interactions. *Malaria J* **2**: 47.

Lebeau, M.C., Massol, N., Herrick, J., Faber, L.E., Renoir, J.M., Radanyi, C., and Baulieu, E.E. (1992) P59, an hsp90-binding protein. *J Biol Chem* **267**: 4281-4284.

Lees, M.J., Peet, D.J., and Whitelaw, M. (2003) Defining the role for XAP2 in stabilization of the dioxin receptor. *J Biol Chem* **278**: 35878-35888.

Lepre, C.A., Thomson, J.A., and Moore, J.M. (1992) Solution structure of FK506 bound to FKBP-12 R. *FEBS* **302**: 89-96.

Leroux, M.R., and Hartl, F.U. (2001) Cellular functions of molecular chaperones. In: Mechanisms of Protein Folding. 2nd Edit. Pain, R (Ed.) Oxford University Press Inc., New York. pp 212-249.

Li, T.K., Baksh, S., Cristillo, A.D., and Bierer, B.E. (2002) Calcium-and FK506-independent interaction between immunophilin FKBP51 and calcineurin. *J Cell Biochem* **84**: 460-471.

Lindenthal, C., and Klinkert, M.Q. (2002) Identification and biochemical characterisation of a protein phosphatase 5 homologue from *Plasmodium falciparum*. *Mol Biochem Parasitol* **120**: 257-268

Liu, J., Farmer, J.D., Lane, W.S., Friedman, J., Weissman, I., and Schreiber, S.L. (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66**: 807-815.

López-Antuñano, F.J., and Schmunis, G.A (1993) Plasmodia in humans. In: Parasitic Protozoa. Kreier, J.P. and Baker, J.R (Eds), Academic Press, San Diego. **5**: 135-266.

Lopez-Ilasaca, M., Schiene, C., Küllertz, G., Tradler, T., Fischer, G., and Wetzker, R. (1998) Effects of FK506-binding protein 12 and FK506 on autophosphorylation of epidermal growth factor receptor. *J Biol Chem* **273**: 9430-9434.

Lorberg, A., and Hall, M.N. (2004) TOR: the first 10 years. In: TOR-Target of Rapamycin. Thomas, G., Sabatini, D.M., and Hall M.N. (Eds), Springer-Verlag, Berlin. **279**: 1-18.

Lundemose, A.K., Kay, J.E., and Pearce, J.H. (1993) *Chlamydia trachomatis* Mip-like protein has peptidyl-prolyl *cis/trans* isomerase activity that is inhibited by FK506 and rapamycin and is implicated in initiation of chlamydial infection. *Mol Microbiol* **75**: 777-783.

Lyons, W.E., Steiner, J.P., Snyder, S.H., and Dawson, T.M. (1995) Neuronal regeneration enhances the expression of the immunophilin FKBP-12. *J Neurosci* **15**: 2985-2994.

Ma, Q., and Whitlock, J.P. (1997) A novel cytoplasmic protein that interacts with the Ah receptor, contains tetratricopeptide repeat motifs, and augments the transcriptional response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J Biol Chem* **272**: 8878-8884.

Mackrill, J.J., O'Driscoll, S., Lai, A., and McCarthy, T.V. (2001) Analysis of type 1 ryanodine receptor-12 kDa FK506-binding protein interaction. *Biochem Biophys Res Commun* **285**: 52-57.

Makler, M.T., Ries, J.M., Williams, J.A., Bancroft, J.E., Piper, R.C., Gibbins, B.L., and Hinrichs, D.J. (1993) Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. *Am J Trop Med Hyg* **48**: 739-741.

Malhotra, P., Dasaradhi, P.V.N., Kumar, A., Mohmmed, A., Agrawal, N., Bhatnagar, R.K., and Chauhan, V.S. (2002) Double-stranded RNA-mediated gene silencing of cysteine proteases (falcipain-1 and –2) of *Plasmodium falciparum*. *Mol Microbiol* **45**: 1245-1254.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbour, New York.

Manning-Krieg, U.C., Henríquez, R., Cammas, F., Graff, P., Gavériaux, S., and Movva, N.R. (1994) Purification of FKBP-70, a novel immunophilin from *Saccharomyces cerevisiae*, and cloning of its structural gene, FPR3. *FEBS Lett* **352**: 98-103.

Marks, A.R. (2002) Ryanodine receptors, FKBP12, and heart failure. *Fronts Bioscience* **7**: 970-977.

Marx, S.O., Reeked, S., Hisamatsu, Y., Jayaraman, T., Burkhoff, D., Rosemblit, N., and Marks, A.R. (2000) PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell* **101**: 365-376.

Matsuda. S., Shibasaki, F., Takehana, K., Mori, H., Nishida, E., and Koyasu, S. (2000) Two distinct action mechanisms of immunophilin-ligand complexes for the blockade of T-cell activation. *EMBO Rep* **1**: 428-434.

Matsumoto, Y., Perry, G., Scheibel, L.W., and Aikawa, M. (1987) Role of calmodulin in *Plasmodium falciparum*: implications for erythrocyte invasion by the merozoite. *Eur J Cell Biol* **45**: 36-43.

Mattila, P.S., Ullman, K.S., Fiering, S., Emmel, E.A., McCutcheon, M., Crabtree, G.R., and Herzenberg, L.A. (1990) The actions of cyclosporin A and FK506 suggest a novel step in the activation of T-lymphocytes. *EMBO J* **9**: 4425-4433.

McRobert, L., and McConkey, G.A. (2002) RNA interference (RNAi) inhibits growth of *Plasmodium falciparum*. *Mol Biochem Parasitol* **119**: 273-278.

Meadows, R.P., Nettesheim, D.G., Xu, R.X., Olejniczak, E.T., Petros, A.M., Holzman, T.F., Severin, J., Gubbins, E., Smith H., and Fesik, S.W. (1993) Three-dimensional structure of the FK506 binding protein/ascomycin complex in solution by heteronuclear three- and four-dimensional NMR. *Biochemistry* **32**: 754-765.

Mendis, K., Sina, B.J., Merchensini, P. and Carter, R. (2001) The neglected burden of *Plasmodium vivax* malaria. *Am J Trop Med Hyg* **64**(S): 97-106.

Meng. X., Lu, X., Morris, C.A., and Keating, M.T. (1998) A novel human gene FKBP6 is deleted in Williams syndrome. *Genomics* **52**: 130-137.

Meshnick, S.R., and Dobson, M.J. (2001) The history of antimalarial drugs. In: *Antimalarial Chemotherapy. Mechanisms of Action, Resistance, and New Directions in Drug Discovery.* Rosenthal, P.J. (Ed), Humana Press, Totowa, New Jersey. pp 15-26

Meyer, B.K., Pray-Grant, M.G., Vanden Heuvel, J.P., and Perdew, G.H. (1998) Hepatitus B virus X-associated protein 2 is a subunit of the unliganded aryl hydrocarbon receptor core complex and exhibits transcriptional enhancer activity. *Mol Cell Biol* **18**: 978-988.

Michnick, S.W., Rosen, M.K., Wandless, T.J., Karplus, M., and Schreiber, S.L. (1991) Solution structure of FKBP, a rotamase enzyme and receptor for FK506 and rapamycin. *Science* **252**: 836-839.

Miller, L.H., Baruch, D.I., Marsh, K., and Doumbo, O.K. (2002) The pathogenic basis of malaria. *Nature* **415**(S): 673-679.

Missiakas, D., Betton, J.M., and Raina, S. (1996) New components of protein folding in extracytoplasmic compartments of *Escherichia coli* SurA, FkpA and Skp/OmpH. *Mol Microbiol* **21**: 871-884.

Moro, A., Ruiz-Cabello, F., Fernandez-Cano, A., Stock, S.P., and Gonzalez, A. (1995) Secretion by *Trypanosoma cruzi* of a peptidyl-prolyl *cis-trans* isomerase involved in cell infection. *EMBO J* **14**: 2483-2490.

Munn, K., and Steward, R. (2000) The *shut-down* gene of *Drosophila melanogaster* encodes a novel FK506-binding protein essential for formation of germline cysts during oogenesis. *Genetics* **156**: 245-256.

Murphy, S.C., and Bremen, J.G. (2001) Gaps in the childhood malaria burden in Africa: cerebral malaria, neurological sequelae, anaemia, respiratory distress, hypoglycaemia, and complications of pregnancy. *Am J Trop Med Hyg* **64**(S): 57-67.

Nabarro, D., and Taylor, E. (1998) "The Roll Back Malaria" campaign. *Science* **280**: 2067-2068.

Naito, M., Oh-hara, T., Yamazaki, A., Danki, T., and Tsuruo, T. (1992) Reversal of multidrug resistance by an immunosuppressive agent FK-506. *Cancer Chemother Pharmacol* **29**: 195-200

Nickell, S.P., Scheibel, L.W., and Cole, G.A. (1982) Inhibition by cyclosporin A of rodent malaria *in vivo* and human malaria *in vitro*. *Infect Immun* **37**: 1093-1110.

Odom, A., Del Poeta, M., Perfect, J., and Heitman, J. (1997) The immunosuppressant FK506 and its nonimmunosuppressive analogue L-685-818 are toxic to *Cryptococcus neoformans* by inhibition of a common target protein. *Antimicrob Agents Chemother* **41**: 156-161.

O'Keefe, S.J., Tamura, J., Kincaid, R.L., Tocci, M.J., and O'Neill, E.A. (1992) FK-506- and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. *Nature* **357**: 692-694.

Ono, K., Yano, M., Ohkusa, T., Kohno, M., Hisaoka, T., Tanigawa, T., Kobayashi, S., Kohno, M., and Matsuzaki, M. (2000) Altered interaction of FKBP12.6 with ryanodine receptor as a cause of abnormal Ca^{2+} release in heart failure. *Cardiovasc Res* **48**: 323-331.

Pahl, A., and Keller, U. (1992) FK-506-binding proteins from Streptomycetes producing immunosuppressive macrolides of the FK-506 type. *J Bacteriol* **174**: 5888-5894.

Pal, D., and Chakrabarti, P. (1999) *Cis* peptide bonds in proteins: residues involved, their conformations, interactions and locations. *J Mol Biol* **294**: 271-288/

Patzelt, H., Rüdiger, S., Brehmer, D., Kramer, G., Vorderwülbecke, S., Schaffitzel, E., Waitz, A., Hesterkamp, T. Dong, L., Schneider-Mergener, J, Bukau, B., and Deuerling, E. (2001) Binding specificity of *Eschericia coli* trigger factor. *Proc Natl Acad Sci USA* **98**: 14244-14249.

Peattie, D.A., Harding, M.W., Fleming, M.A., DeCenzo, M.T., Lippke, J.A., Livingston, D.J., and Benasutti, M. (1992) Expression and characterisation of

human FKBP52, an immunophilin that associates with the 90-kDa heat shock protein and is a component of steroid receptor complexes. *Proc Natl Acad Sci USA* **89**: 10974-10978.

Pereira, P.J.B., Vega, M.C., González-Rey, E., Fernández-Carazo, R., Macedo-Ribeiro, S., Gomis-Rüth, F.X., González, A., and Coll, M. (2002) *Trypanosoma cruzi* macrophage infectivity potentiator has a rotamase core and a highly exposed α-helix. *EMBO Rep* **3**: 88-94.

Perrot-Applanat, M., Cibert, C., Géraud, G., Renoir, J.M., and Baulieu, E.E. (1995) The 59 kDa FK506-binding protein, a 90 kDa heat shock protein binding immunophilin (FKBP59-HBI), is associated with the nucleus, the cytoskeleton and mitotic apparatus. *J Cell Sci* **108**: 2037-2051.

Petrulis, J.R., and Perdew, G.H. (2002) The role of chaperone proteins in the aryl hydrocarbon receptor core complex. *Chem Biol Interact* **141**: 25-40.

Pirkl, F., and Buchner, J. (2001) Functional analysis of the Hsp90-assocaited human peptidyl prolyl *cis/trans* isomerases FKBP51, FKBP52 and Cyp40. *J Mol Biol* **308**: 795-806.

Pirkl, F., Fischer, E., Modrow, S. and Buchner, J. (2001) Localisation of the chaperone domain of FKBP52. *J Biol Chem* **276**: 37034-37041.

Pourtier-Manzanedo, A., Boesch, D. and Loor, F. (1991) FK-506 (fujimycin) reverses the multidrug resistance of tumor cells in vitro. *Anti-Cancer Drug* **2**: 279-283.

Pratt, W.B., Silverstein, A.M., and Galigniana, M.D. (1999) A model for the cytoplasmic trafficking of signalling proteins involving the hsp90-binding immunophilins. *Cell Signal* **11**: 839-851.

Pratt, W.B., and Toft, D.O. (2003) Regulation of signalling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Exp Biol Med* **228**: 111-133.

Ptitsyn, D.B., and Finkelstein, A.V. (1980) Directed mechanism of the self-organization of proteins: generalized model. *Quart Rev Biophys* **13**: 339-386.

Radanyi, C., Chambraud, B., and Baulieu, E.E. (1994) The ability of the immunophilin FKBP59-HBI to interact with the 90-kDa heat shock protein is encoded by its tetratricopeptide repeat domain. *Proc Natl Acad Sci USA* **91**: 11197-11201.

Ramm, K., and Plückthun A. (2000) The periplasmic *Escherichia coli* peptidylprolyl *cis,trans*-isomerase FkpA. II. Isomerase-independent chaperone activity *in vitro*. *J Biol Chem* **275**(22): 17106-17113.

Ramm, K., and Plückthun A. (2001) High enzymatic activity and chaperone function are mechanistically related features of dimeric *E. coli* peptidyl-prolyl isomerase FkpA. *J Mol Biol* **310**: 485-498.

Ratajczak, T., Carrello, A., Mark, P.J., Warner, B.J., Simpson, R.J., Moritz, R.L., and House, A.K. (1993) The cyclophilin component of the unactivated estrogen receptor contains a tetratricopeptide repeat domain and shares identity with p59 (FKBP59). *J Biol Chem* **268**: 13187-13192.

Ratajczak, T., and Carrello, A. (1995) Cyclophilin 40 (CyP-40), mapping of its hsp90 binding domain and evidence that FKBP52 competes with CyP-40 for hsp90 binding. *J Biol Chem* **271**: 2961-2965.

Ratcliff, R.M. Donnellan, S.C., Lanser, J.A., Manning, P.A., and Heuzenroeder, M.W. (1997) Interspecies sequence differences in the Mip protein from the genus *Legionella*: implications for function and evolutionary relatedness. *Mol Microbiol* **25**: 1149-1158.

Reddy, G.R. (1995) Cloning and characterisation of a *Plasmodium falciparum* cyclophilin gene that is stage-specifically expressed. *Mol Biochem Parasitol* **73**: 111-121.

Reeves, C.D., Chung, L.M., Liu, Y., Xue, Q., Carney, J.R., Revill, W.P., and Katz, L. (2002) A new substrate specificity for acyl transferase domains of the ascomycin polyketide synthase in *Streptomyces hygroscopicus*. *J Biol Chem* **277**: 9155-9159.

Renoir, J.M., Radanyi, C., Faber, L.E., and Baulieu, E.E. (1990) The non-DNA-binding heterooligomeric form of mammalian steroid hormone receptors contains a hsp90-bound 59-kilodalton protein. *J Biol Chem* **265**: 10740-10745.

Revill, W.P., Voda, J., Reeves, C.R., Chung, L., Schirmer, A., Ashley, G., Carney, J.R., Fardis, M., Carreras, C.W., Zhou, Y., Feng, L., Tucker, E., Robinson, D., and Gold, B.G. (2002) Genetically engineered analogs of ascomycin for nerve regeneration. *J Pharmacol Exp Ther* **302**:1278-1285.

Rexin, M., Busch, W., and Gehring, U. (1991) Protein components of the nonactivated glucocorticoid receptor. *J Biol Chem* **266**: 24601-24605.

Reynolds, P.D., Ruan, Y., Smith, D.F., and Scammell, J.G. (1999) Glucocorticoid resistance in the squirrel monkey is associated with overexpression of the immunophilin FKBP51. *J Clin Endocrinol Metab* **84**: 663-669.

Richardson, J.S. (1977) β-sheet topology and the relatedness of proteins. *Nature* **268**: 495-500.

Richarme, G., and Caldas, T.D. (1997) Chaperone properties of the bacterial periplasmic substrate-binding proteins. *J Biol Chem* **272**: 15607-15612.

Richie, T.L., and Saul, A. (2001) Progress and challenges for malaria vaccines. *Nature* **415**(S): 694-701.

Riegel, J.S., Corthesy, B., Flanagan, W.M., and Crabtree, G.R. (1992) Regulation of the interleukin-2 gene. In: Interleukins: Molecular Biology and Immunology. Kishimoto, T. (Ed), Karger, Basel. **51**: 266-298.

Riggs, D.L., Roberts, P.J., Chirillo, S.C., Cheung-Flynn, J., Prapapanich, V., Ratajczak, T., Gaber, R., Picard, D., and Smith, D.F. (2003) The Hsp90-binding peptidylprolyl isomerase FKBP52 potentiates glucocorticoid signaling *in vivo*. *EMBO J* **22**: 1158-1167.

Roberts, C.W., Roberts, F., Lyons, R.E., Kirisits, M.J., Mui, E.J., Finnerty, J., Sohnson, J.J., Ferguson, D.J.P., Coggins, J.R., Krell, T., Coombs, G.H., Milhous, W.K., Kyle, D.E., Tzipori, S., Barnwell, J., Dame, J.B., Carlton, J., and McLeod, R. (2002) The shikimate pathway and its branches in apicomplexan parasites. *J Infect Dis* **185**(S): 25-36.

Rosen, M.K., Michnick, S.W., Karplus, M., and Schreiber, S.L. (1991) Proton and nitrogen sequential assignments and secondary structure determination of the human FK506 and rapamycin FK506 and rapamycin binding protein. *Biochem* **30**: 4774-4789.

Rosenthal, P.J. (2001) Protease inhibitors. In: *Antimalarial Chemotherapy. Mechanisms of Action, Resistance, and New Directions in Drug Discovery.* Rosenthal, P.J. (Ed), Humana Press, Totowa, New Jersey. pp 325-345.

Rosenthal, P.J., and Miller, L.H. (2001) The need for new approaches to antimalarial chemotherapy. In: *Antimalarial Chemotherapy. Mechanisms of Action, Resistance, and New Directions in Drug Discovery.* Rosenthal, P.J. (Ed), Humana Press, Totowa, New Jersey. pp 3-13.

Rotonda, J., Burbaum, J.J., Chan, H.K., Marcy, A.I., and Becker, J.W. (1993) Improved calcineurin inhibition by yeast FKBP12-drug complexes. *J Biol Chem* **268**: 7607-7609.

Sachs, J., and Malaney, P. (2002) The economic and social burden of malaria. *Nature* **415**(S): 680-685.

Salmon, B.L., Oksman, A., and Goldberg, D.E. (2000) Malaria parasite exit from the host erythrocyte: a two-step process requiring extraerythrocytic proteolysis. *Proc Natl Acad Sci USA* **98**: 271-276.

Saul, A. (1999) The role of variant surface antigens on malaria-infected red blood cells. *Parasitol Today* **15**: 455-457.

Scheufler, R.S., Brinker, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F.U., and Moarefi, I. (2000) Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *Cell* **101**: 199-210.

Scholz, C., Stoller, G., Zarnt, T., Fischer, G., and Schmid, F.X. (1997) Cooperation of enzymatic and chaperone functions of trigger factor in the catalysis of protein folding. *EMBO J* **16**: 54-58.

Scholz, C., Mücke, M., Rape, M., Pecht, A., Pahl, A., Bang, H., and Schmid, F.X. (1998) Recognition of protein substrates by the prolyl isomerase trigger factor is independent of proline residues. *J Mol Biol* **277**: 723-732.

Schreiber, S.L. (1991) Chemistry and biology of the immunophilins and their immunosuppressive ligands. *Science* **251**: 283-287.

Schreiber, S.L. and Crabtree, G.R. (1992) The mechanism of action of cyclosporin A and FK506. *Immunol Today* **13**: 136-142.

Schreiber, S.L., and Bernstein, S.L. (2002) Signaling network model of chromatin. *Cell* **111**: 771-778.

Schultz, L.W., Martin, P.K., Liang, S.L., Schreiber, S.L., and Clardy, J. (1994) Atomic structure of the immunophilin FKBP13-FK506 complex: insights into the composite binding surface for calcineurin. *J Am Chem Soc* **116**: 3129-3130.

Schwarze, S.R., and Dowdy, S.F. (2000) *In vivo* protein transduction: intracellular delivery of biologically active proteins, compound and DNA. *Trends Pharmacol Sci* **21**: 45-48.

Seghal, S.N., Baker, H., and Vezina, C. (1975) Rapamycin (AY-22,989), a new antifungal antibiotic. II. Fermentation, isolation and characteristion. *J Antibiot* **28**: 727-742

Shen, M., Stukenberg, P.T., Kirschner, M.W., and Lu, K.P. (1998) The essential mitotic peptidyl-prolyl isomerase Pin1 binds and regulates mitosis-specific phosphoproteins. *Genes Dev* **12**: 706-720.

Shirane, M., and Nakayama, K.I. (2003) Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis. *Nature Cell Biol* **5**: 1-10.

Shou, W., Aghdasi, B., Armstrong, D.L., Guo, Q., Bao, S., Charng, M.J., Mathews, L.M., Schneider, M.D., Hamilton, S.L., and Matzuk, M.M. (1998) Cardiac defects and altered ryanodine receptor function in mice lacking FKBP12. *Nature* **391**: 489-492.

Siekierka, J.J., Hung, S.H.Y., Poe, M., Lin, C.S., and Sigal, N.H. (1989) A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. *Nature* **341**: 755-757.

Siekierka, J.J., Wiederrecht, G., Greulich, H., Boulton, D., Hung, S.H.Y., Cryan, J.H., Hodges, P.J., and Sigal, N.H. (1990) The cytosolic-binding protein for the immunosuppressant FK506 is both a ubiquitous and highly conserved peptidyl-prolyl *cis-trans* isomerase. *J Biol Chem* **265**: 21011-21015.

Silverstein, A.M., Galigniana, M.D., Chens, M.S., Owens-Grillo, J.K., Chinkers, M., and Pratt, W.B. (1997) Protein Phosphatase 5 is a major component of glucocorticoid receptor hsp90 complexes with properties of an FK506-binding immunophilin. *J Biol Chem* **272**: 16224-16230.

Silverstein, A.M., Galigniana, M.D., Kanelakis, K.C., Radanyi, C., Renoir, J.M., and Pratt, W.B. (1999) Different regions of the immunophilin FKBP52 determine its association with the glucocorticoid receptor, hsp90, and cytoplasmic dynein. *J Biol Chem* **274**: 36980-36986.

Sinars, C.R., Cheung-Flynn, J., Rimerman, R.A., Scammell, J.G., Smith, D.F., and Clardy, J. (2003) Structure of the large FK506-binding protein FKBP51, an Hsp90-binding protein and a component of steroid receptor complexes. *Proc Natl Acad Sci USA* **100**: 868-873.

Smith, D.F., Bagenstoss, B.A., Marion, T.N., and Rimmerman, R.A. (1993a) Two FKBP-related proteins are associated with progesterone receptor complexes. *J Biol Chem* **268**: 18365-18371.

Smith, D.F., Albers, M.W., Schreiber, S.L., Leach, K.L., and Deibel, M.R. (1993b) FKBP54, a novel FK506-binding protein in avian progesterone receptor complexes and HeLa extracts. *J Biol Chem* **268**: 24270-24273.

Smith, T., Ferreira, L.R., Herbert, C., Norris, K., and Sauk, J.J. (1995) Hsp47 and cyclophiln B traverse the endoplasmic reticulum with procollagen into pre-Golgi intermediate vesicles. *J Biol Chem* **270**: 18323-18328.

Snyder, S.H., Sabatini, D.M., Lai, M.M., Steiner, J.P., Hamilton, G.S., and Suzdak, P.D. (1998) Neural actions of immunophilin ligands. *Trends in Pharmacol Sci* **19**: 21-26.

Song, Q., Alnemri, E.S., Litwack, G., and Gilbert, L.I. (1997) An immunophilin is a component of the insect ecdysone receptor (EcR) complex. *Insect Biochem Molec Biol* **27**: 973-982.

Spielman, A., and D'Antonio, M. (2001) In: Mosquito. Faber and Faber Ltd., London.

Steiner, J.P., Dawson, T.M., Fotuhi, M., Glatt, C.E., Snowman, A.M., Cohen, N., and Snyder, S.H. (1992) High brain densities of the immunophilin FKBP colocalised with calcineurin. *Nature* **358**: 584-587.

Steiner, J.P., Hamilton, G.S., Ross, D.T., Valentine, H.L., Guo., H., Connolly, M.A., Liang, S., Ramsey, C., Li, J.H.J., Huang, W., Howorth, P., Soni, R., Fuller, M., Sauer, H., Nowotnik, A.C., and Suzdak, P.D. (1997) Neurotrophic immunophilin ligands stimulate structural and functional recovery in neurodegenerative animal models. *Proc Natl Acad Sci USA* **94**: 2019-2024.

Stoller, G., Rücknagel, K.P., Nierhaus, K., Schmid, F.X., Fischer, G., and Rahfield, J.U. (1995) Identification of the peptidyl-prolyl *cis/trans* isomerase bound to the *Escherichia coli* ribosome as the trigger factor. *EMBO J* **14**: 4939-4948.

Stoller, G., Tradler, T., Rücknagel, K.P., Rahfield, J.U., and Fischer, G. (1996) An 11.8 kDa proteolytic fragment of the *E. coli* trigger factor represents the domain carrying the peptidyl-prolyl cis/trans isomerase activity. *FEBS Lett* **384**: 117-122.

Su, B., and Karin, M. (1996) Mitogen-activated protein kinase cascades and regulation of gene expression. *Curr Opin Immunol* **8**: 402-411.

Su, X-Z., and Wellems, T.E. (1994) Sequence, transcript characterization and polymorphisms of a *Plasmodium falciparum* gene belonging to the heat-shock protein (HSP) 90 family. *Gene* **151**: 225-230.

Suzuki, R., Nagata, K., Yumoto, F., Kawakami, M., Nemoto, N., Furutani, M., Adachi, K., Maruyama, T., and Tanokura, M. (2003) Three-dimensional solution structure of an archael FKBP with a dual function of peptidyl prolyl *cis-trans* isomerase and chaperone-like activities. *J Mol Biol* **328**: 1149-1160.

Syin, C., and Goldman, N.D. (1996) Cloning of a *Plasmodium falciparum* gene related to the human 60 kDa heat shock protein. *Mol Biochem Parasitol* **64**: 25-32.

Takahashi, N., Hayano, T., and Suzuki, M. (1989) Peptidyl-prolyl *cis-trans* isomerase is the cyclophilin A-binding cyclophilin. *Nature* **337**: 473-475.

Taraschi, T.F., Trelka, D., Martinez, S., Schneider, T., and O'Donnell, M.E. (2001) Vesicle-mediated trafficking of parasite proteins to the host cell cytosol and erythrocyte surface membrane in *Plasmodium falciparum* infected erythrocytes. *Int J Parasitol* **31**: 1381-1391.

Thommen-Scott, K. (1981) Antimalarial activity of cyclosporin A. *Agents Actions* **11**: 770-773.

Tilley, L., Loria, P., and Foley, M. (2001) Chloroquine and other quinoline antimalarials. In: *Antimalarial Chemotherapy. Mechanisms of Action, Resistance, and New Directions in Drug Discovery.* Rosenthal, P.J. (Ed), Humana Press, Totowa, New Jersey. pp 87-121.

Timerman, A.P., Wiederrecht, G., Marcy, A., and Fleischer, S. (1995) Characterization of an exchange reaction between soluble FKBP12 and the FKBP-ryanodine receptor complex. *J Biol Chem* **270**: 2451-2459.

Trager, W., and Jensen, J.D. (1976) Human malaria parasites in continuous culture. *Science* **193**: 673-675.

Trombetta, E.S., and Parodi, A.J. (2003) Quality control and protein folding in the secretory pathway. *Annu Rev Cell Dev Biol* **19**: 649-676.

Troullier, P. and Olliaro, P.L. (1998) Drug development output from 1975 to 1996. *Int J Infect Dis* **3**: 61-63.

Valent, Q.A., Kendall, D.A., High, S., Kusters, R., Oudega, B., and Luirink, J. (1995) Early events in preprotein recognition in *E. coli* interaction of SRP and trigger factor with nascent polypeptides. *EMBO J* **14**: 5494-5505.

van der Spuy, J., Kana, B.D., Dirr, H.W., and Blatch, G.L. (2000) Heat shock cognate protein 70 chaperone-binding site in the co-chaperone murine stress-inducible protein 1 maps to within three consecutive tetrapeptide repeat motifs. *Biochem J* **345**: 645-651.

Van Duyne, G.D., Standaert, R.F., Karplus, P.A., Schreiber, S.L., and Clardy, J. (1991) Atomic Structure of FKBP-FK506, an immunophilin-immunosuppressant complex. *Science* **252**: 839-842.

Van Duyne, G.D., Standaert, R.F., Karplus, P.A., Schreiber, S.L., and Clardy, J. (1993) Atomic structures of the human FKBP-12 complexes with FK506 and rapamycin. *J Mol Biol* **229**: 105-124.

Venkataramanan, R., Jain, A., Warty, V.S., Abu-Elmagd, K., Alessiani, M., Lever, J., Krajak, A., Flowers, J., Mehta, S., Zuckerman, S., Fung, J., Todo, S., and Starzi, T.E. (1991) Pharmocokinetics of FK506 in transplant patients. *Transplant Proceed* **23**: 2736-2740.

Vermeer, H., Hendriks-Stegeman, B.I., van Suylekom, D., Rijkers, G.T., van Buul-Offers, S.C., and Jansen, M. (2004) An in vitro biassay to determine individual sensitivity to glucocorticoids: induction of FKBP51 mRNA in peripheral blood mononuclear cells. *Mol Cell Endocrinol* **218**: 49-55.

Vial, H.J., Eldin, P., Tielens, A.G.M., and van Hellemond, J.J. (2003) Phospholipids in parasitic protozoa. *Mol Biochem Parasitol* **126**: 143-154.

Vittorioso, P., Cowling, R., Faure, J.D., Caboche, M., and Bellini, C. (1998) Mutation in the *Arabidopsis thaliana PASTICCINO 1* gene, which encodes a new FK506-binding protein, has a dramatic effect on plant development. *Mol Cell Biol* **18**: 3034-3043.

Vogel, G. (2002) An elegant but imperfect tool. *Science* **298**: 94-95.

Walensky, L.D., Gascard, P., Fields, M.E., Blackshaw, S., Conboy, J.G., Mohandas, N. and Snyder, S.H. (1998) The 13-kD FK506 binding protein, FKBP13, interacts with a novel homologue of the erythrocyte cytoskeleton protein 4.1. *J Cell Biol* **141**: 143-153.

Waller, R.F., Keeling, P.J., Donald, R.G.K., Striepen, B., Handman, E., Lang-Unnasch, N., Cowman, A.F., Besra, G.S., and Roos, D.S. (1998) Nuclear-encoded proteins target to the plastid in *Toxoplasma gondii* and *Plasmodium falciparum*. *Proc Natl Acad Sci USA* **95**: 12352-12357.

Waller, R.F., Reed, M.B., Cowman, A.F., and McFadden, G.I. (2000) Protein trafficking to the plastid of *Plasmodium falciparum* is via the secretory pathway. *EMBO J* **19**: 1794-1802.

Wang, T., Donahoe, P.K., and Zervos, A.S. (1994) Specific interaction of type I receptors of the TGF-β family with the immunophilin FKBP-12. *Science* **265**: 674-676.

Wang, T., Li, B.Y., Danielson, P.D., Shah, P.C., Rockwell, S., Lechleider, R.J., Martin, J., Manganaro, T., and Donahoe, P.K. (1996) The immunophilin FKBP12 functions as a common inhibitor of the TGFβ family type I receptors. *Cell* **86**: 435-444.

Watanabe, J. (1997) Cloning and characterization of heat shock protein DnaJ homologues from *Plasmodium falciparum* and comparison with ring infected erythocyte surface antigen. *Mol Biochem Parasitol* **88**: 253-258.

Whitty, C.J.M., and Sanderson, F. (1999) New therapies and changing patterns of treatment of malaria. *Curr Opin Infect Dis* **12**: 579-584.

Whitty, C.J.M., Rowland, M., Sanderson, F., and Mutaningwa, T.K. (2002) Malaria. *BMJ* **325**: 1221-1224.

Wiesner, J., Ortmann, R., Jomaa, H., and Schitzler, M. (2003) New antimalarial drugs. *Angew Chem Int Ed* **42**: 5274-5293.

Wilson, I.R.J.M., Denny, P.W., Preiser, P.R., Rangachari, K., Roberts, K., Roy, A., Whyte, A., Strath, M., Moore, D.J., Moore, P.W., and Williamson, D.H. (1996) Complete gene map of the plastid-like DNA of the malaria parasite *Plasmodium falciparum*. *J Mol Biol* **261**: 155-172.

Wilson, R.J., and Williamson, D.H. (1997) Extrachromosomal DNA in the apicocomplexa. *Microbiol Mol Biol Rev* **61**: 1-16.

Wiser, M.F. (2003) A *Plasmodium* homologue of cochaperone p23 and its differential expression during the replicative cycle of the malaria parasite. *Parasitol Res* **90**: 199-170.

Wiser, M.F., Lanners, H.N., and Bafford, R.A. (1999) Export of *Plasmodium* proteins via a novel secretory pathway. *Parasitol Today* **15**: 194-198.

Xu, Q., Liang, S., Kudla, J., and Luan, S. (1998) Molecular characterization of a plant FKBP12 that does not mediate action of FK506 and rapamycin. *Plant J* **15**: 511-519.

Xu, X., Su, B., Barndt, R.J., Chen, H., Xin, H., Yan, G., Chen, L., Cheng, D., Heitman, J., Zhuang, Y., Fleischer, S., and Shou, W. (2002) FKBP12 is the only FK506 binding protein mediating T-cell inhibition by the immunosuppressant FK506. *Transplant* **73**: 1835-1848.

Yaffe, M.B., Schutkowski, M., Shen, M., Zhou, X. Z., Stukenberg, P.T., Rahfelf, J-U., Xu, J., Kuang, J., Kirschner, M.W., Fischer, G., Cantley, L.C., and Lu, K.P. (1997) Sequence-specific and phosphorylation-dependent isomerization: a potential mitotic regulatory mechanism. *Science* **278**: 1957-1960.

Yang, A., and McKeon, F. (2000) p63 and p73: p53 mimics, menaces and more. *Nature Rev Mol Cell Biol* **1**: 199-207.

Yang, W.M., Inouye, C.J., and Seto, E. (1995) Cyclophilin A and FKBP12 interact with YY1 and alter its transcriptional activity. *J Biol Chem* **270**: 15187-15193.

Yang, W.M., Yao, Y.L., and Seto, E. (2001) The FK506-binding protein 25 functionally associates with histone deacetylases and with transcription factor YY1. *EMBO J* **20**: 4814-4825.

Yano, M., Ono, K., Ohkusa, T., Suetsugu, M., Kohno, M., Hisaoka, T., Kobayashi, S., Hisamatsu, Y., Yamamato, T., Kohno, M., Noguchi, N., Takasawa, S., Okamoto, H., and Matsuzuaki, M. (2000) Altered stoichiometry of FKBP12.6 versus ryanodine receptor as a cause of abnormal Ca^{2+} leak through ryanodine receptor in heart failure . *Circulation* **102**: 2131-2136.

Yano, M., Terada, K., and Mori, M. (2004) Mitochondrial import receptors Tom20 and Tom22 have chaperone-like activity. *J Biol Chem* **279**: 10808-10813.

Yem, A.W., Tomasselli, A.G., Heinrikson, R.L. Zurcher-Neely, H., Ruff, V.A., Johnson, R.A., and Diebel, M.R. (1992) The Hsp56 component of steroid receptor complexes binds to immobilised FK506 and shows homology to FKBP-12 and FKBP-13. *J Biol Chem* **267**: 2868-2871.

Zacchi, P., Gostissa, M., Uchida, T., Salvagno, C., Avolio, F., Volinia, S., Ronai, Z., Blandino, G., Schneider, C., and del Sal, G. (2002) The prolyl isomerase Pin1 reveals a mechanism to control p53 functions after genotoxic insults. *Nature* **419**: 853-857.

Zheng, H., You, H., Zhou, X.Z., Murray, S.A., Uchida, T., Wulf, G., Gu, L., Tang, X., Lu, K.P., and Xiao, Z.X. (2002) The prolyl isomerase Pin1 is a regulator of p53 in genotoxic response. *Nature* **419**: 849-853.

Zhou, X.Z., Kops, O., Werner, A., Lu, P.J., Shen, M., Stoller, G., Küllertz, G., Stark, M., Fischer, G., and Lu, K.P. (2000) Pin1-dependent prolyl isomerization regulates dephosphorylation of cdc25c and Tau proteins. *Mol Cell* **6**: 873-883.

Zubay, G. (1993) In: Biochemistry. 3rd Edition. Wm. C. Brown, Dubuque.

Zuckerman, A. (1967) Harvesting of *Plasmodium falciparum* with saponin. *Bull WHO* **37**: 431-436.