Identification and Analysis of *Plasmodium falciparum* Genes Mediating Cytoadherence

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Summary

Malaria is the most important parasitic disease of man: almost two billion people live in areas in which the most pathogenic human *Plasmodium* species is transmitted. The parasite *P. falciparum* accounts for more than 90% of world-wide malaria morbidity and mortality: every year it is responsible for about 500 million clinical malaria cases and for up to 2.7 million deaths world-wide.

A characteristic feature of infections with *P. falciparum* is the ability of infected red blood cells (iRBC) to adhere to venular endothelial cells. This adhesive behaviour is manifested by the iRBC some 16-20 hours into the intraerythrocytic cycle, when the parasite matures to the early trophozoite stage. At this stage, it expresses and transports molecular adhesins to the red cell surface, leaving ring-infected erythrocytes to be the predominant form of parasites found in the peripheral blood. This phenomenon, called sequestration, is proposed to be the key event in vital organ failure, and by adhesion to the microvasculature of the brain, leads to the major life-threatening manifestation of falciparum malaria: cerebral malaria.

The major parasite-derived adhesin, incorporated in the iRBC membrane, was detected 1984 and termed *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1). PfEMP-1 was originally described as a strain specific, high molecular weight (250-350 kDa), highly polymorphic protein on the surface of *P. falciparum*-infected RBCs and it was thought to be involved in adhesion to the already identified receptors on the surface of the endothelial cells, such as CD36, thrombospondin (TSP), inter-cellular adhesion molecule-1 (ICAM-1), and others. Later on (1991), it was shown, that PfEMP-1 undergoes antigenic variation in cloned isolates and that changes in antigenic type are accompanied by changes in the binding specificity for host receptors. Further progress was made 1995 when the genes encoding PfEMP-1 were identified and sequenced: It was shown that each variant antigen type was encoded by a single gene. These genes, termed *var* genes, were shown to be present at 50-100 copies per haploid genome, to be highly polymorphic, both within a single parasite and between isolates, but to have a similar basic structure. Briefly: this structure includes a cysteine-rich interdomain region (CIDR), 2-5 Duffy binding like domains (DBL) and a acidic terminal sequence (ATS). Nevertheless, the causal proof, that *var* genes are indeed involved in sequestration *in vivo* has to be shown.

It was the aim of the presented thesis, to undertake new and innovative approaches, in order to identify and analyze possible parasite-derived ligands, involved in sequestration: a schizont-specific *P. falciparum* cDNA expression library was constructed and introduced in simian COS 7 cells by transient transfection. In a first approach, these transfected COS 7 cells were used in a newly developed *in vitro* assay. Screening cells, expressing ICAM-1 or CD36 on their surface were used to identify parasite-derived genes, whose gene products

confer adherence to these host cell receptors. In a second approach, the transfected COS 7 cells were screened with antibodies against surface molecules of iRBCs to identify the genes involved. A more direct, third approach was applied by transfecting COS 7 cells with fragments of *var* genes, identified by PCR amplification using primers binding to conserved *var* gene domains, such as DBL1 or ATS, in order to analyze the role of *var* genes in cytoadherence. And finally, in the fourth approach, *var* gene domains, expressed as 6xhis-tagged proteins in *Escherichia coli*, were used to analyze binding of these selected domains (CIDR- and DBL1 domain) to different host receptor molecules, expressed on the surface of Chinese hamster ovary (CHO) cells.

With the fourth approach, we were able to show, that the CIDR domain of *var* genes binds to chondroitin sulphate A (CSA) in a dose dependent manner. This binding could be inhibited by 50 µg of soluble CSA (62.4%) and by chondroitinase ABC treatment (up to 94.4%) of the CHO cells.

Unfortunately, all approaches using transfected COS 7 cells, did not lead to the identification of new or known adhesive molecules. In the course of this thesis it became evident, that the AT-richness of the *P. falciparum* genome (69% in coding and 86% in non coding-regions) might be responsible for this lack of expression of *P. falciparum* genes in COS 7 cells, since the existence of AUUUA-motifs can mediate mRNA decay in mammalian cells, a motif which is abundant in *P. falciparum* mRNA.

Nevertheless, it was possible during this thesis, to establish the techniques of transient and stable transfection of mammalian cells, to improve screening techniques by the use of green fluorescent protein (GFP) as a reporter system and to pick and isolate single cDNA clones from transfected COS 7 cells.

Abbreviations

AA	amino acid
ABI	Applied Biosystems Incorporation
AEC	3-amino-9-ethyl-carbazole
amp	ampicillin
AP	alkaline phosphatase
ATP	adenosine triphosphate
ATS	acidic terminal sequence
BCRR	basic case reproduction rate
BII	Basel Institute of Immunology
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
CAPS	3-cyclohexylaminopropan-1-sulphuric acid
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CHO cells	Chinese hamster ovary cells
CIDR	cysteine-rich interdomain region
clag gene family	cytoadherence-linked asexual gene gene family
CSP	circumsporozoite protein
CSA	chondroitin sulphate A
CSase	chondroitinase (Chondroitin lysase)
DBL domain	Duffy binding like domain
DEAE-dextran	diethylaminoethyl-dextran
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMF	N,N-Dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxy nucleotide triphosphate
DPBS	Dulbecco's phosphate buffered saline
DTT	dithiothreitol
EB	ethidium bromide
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetate
EIR	yearly entomological inoculation rate

ELAM-1	Endothelial Leukocyte Adhesion Molecule-1
EtOH	ethanol
FACScan	fluorescence-activated cell scan
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GAG(s)	glycosaminoglycan(s)
gDNA	genomic deoxyribonucleic acid
GFP	green fluorescent protein
HBD	HEPES-buffered Dulbecco's modified Eagle's medium
HEPES	N-2-Hydroxyethylpiperazine-N-2-ethanesulphoric acid
his	histidine
HPS	heparan sulphate
HRP	horseradish peroxidase
HSVgD	*Herpes simplex* virus glycoprotein D
hu	human
ICAM-1	intercellular cell adhesion molecule-1
IFA	indirect immunofluorescence assay
IgG	immuno globulin type G
IMDM	Iscove's modified Dulbecco's modified Eagle's medium
IPTG	isopropyl-β-D-galactoside
iRBC(s)	infected red blood cell(s)
kan	kanamycin
kb	kilobase
kDa	kilodalton
LB	Luria Bertani
mAb	monoclonal antibody
MeOH	methanol
MHC	major histocompatibility complex
MSP	merozoite surface protein
mRNA	messenger ribonucleic acid
NTA	nitrilotriacetic acid
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	polyethylene
PECAM-1	platelet / endothelial cell adhesion molecule-1
P. falciparum	*Plasmodium falciparum*
PfEMP-1	*Plasmodium falciparum* erythrocyte membrane protein-1

Pfu DNA polymerase	*Pyrococcus furiosus* DNA polymerase
PNG	Papua New Guinea
PP	polypropylene
PS	polystyrene
RBC(s)	red blood cell(s)
rif gene family	repeated-interspersed family gene family
RNA	ribonucleic acid
RNAse	ribonuclease
RPMI	Rosewell Park Memorial Institute
RT	room temperature
SA-AP	streptavidine connected to alkaline phosphatase
SDS	sodium dodecyl sulphate
SOC	solution C
SSC	saline sodium citrate
STET	sodium-Tris-EDTA-triton buffer
STI	Swiss Tropical Institute
Taq DNA polymerase	*Thermus aquaticus* DNA polymerase
TBE	Tris-boric-EDTA
TE	Tris-EDTA
tet	tetracycline
TM	thrombomodulin
TNFα	tumor necrosis factor alpha
TRAP	thrombospondin related adhesive protein
Tris	tris[hydroxymethyl]aminomethane
TSP	thrombospondin
u	unit(s)
UTR	untranslated region
UV	ultraviolet
VCAM-1	vascular cell adhesion molecule-1
WHO	World Health Organisation
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosylpyranoside

1. Introduction

Malaria is the most important parasitic disease of man: about 40% of mankind lives at risk to become infected with one of the four human-pathogenic *Plasmodium* species (WHO, 1997). Whereas three species give rise to considerable malaria morbidity (*P. vivax*, P. *malariae* and *P. ovale*) only *P. falciparum* results in high mortality: every year it is responsible for about 500 million clinical malaria cases and for up to 2.7 million deaths world-wide (WHO, 1997).

One of the major questions in malaria research is why infections with *P. falciparum* malaria are often lethal, if untreated, as compared to the other species. A possible explanation lies in the additional mechanism, that has been developed by this parasite, to evade the human immune response and to avoid clearance by its host: while all four human-pathogenic *Plasmodium* species have an almost completely intracellular life cycle in the human host and show genomic plasticity, only *P. falciparum* uses complex cellular interactions between parasite and human host, which lead to the sequestration of infected red blood cells (iRBCs).

The present study is within this context and focuses on a central event of immune evasion, the sequestration of iRBCs. The opening chapters will provide an introduction to *P. falciparum* malaria and to the immune evasion strategies used by this parasite.

1.1. *Plasmodium falciparum* malaria

1.1.1. Life cycle, transmission and exposure

Life cycle

The life cycle of the protozoan parasite *P. falciparum* involves two hosts: female mosquitoes, exclusively of the genus *Anopheles* and humans. No animal reservoir or free living stages are known. Infection with *Plasmodium falciparum* occurs by injection of few sporozoites, perhaps between five and twenty (Coppel *et al.*, 1998), into the bloodstream of the human host during the blood meal of an infected female mosquito. Within minutes after entering the blood circulation, sporozoites are arrested in the liver and enter hepatocytes, where they multiply and develop into liver schizonts. After 5-14 days of parasite multiplication, the hepatocytes rupture and release thousands of merozoites into the bloodstream, where they exclusively invade red blood cells. The intracellular parasites undergo maturation from ring stages to trophozoites to schizonts within 48 hours. Each schizont gives rise to 8-32 merozoites until the iRBC finally disrupts, thereby releasing the merozoites, which find their way into another erythrocyte within a few seconds, starting a new cycle of intraerythrocytic asexual replication. Often an adhesive behaviour is manifested by the iRBC some 16-20

hours into the intraerythrocytic cycle, when the parasite matures to the early trophozoite stage. At this stage, it expresses and transports molecular adhesins to the red cell surface, which are responsible for the sequestration of iRBCs to the deep vascular bed of organs or for the rosetting with uninfected red blood cells. This phenomena of sequestration will be dealt with in chapter 1.2.2.

While propagation of intraerythrocytic parasites may persist for months, occasional ring stages develop into sexual forms of the parasite, termed micro- and macro gametocytes, which are infective for the female *Anopheles* mosquitoes. In the mosquito midgut, the gametocytes differentiate into gametes (1 macro- or 8 micro gametes, respectively) and, after fertilisation and zygote formation, transform into ookinetes. Each ookinete penetrates the midgut wall, develops into an oocyste and begins with sporogony: upon maturation, one oocyste contains thousands of sporozoites, which are released into the hemolymph. The sporozoites invade the salivary glands of the mosquito from where they are injected into a human host during the next blood meal.

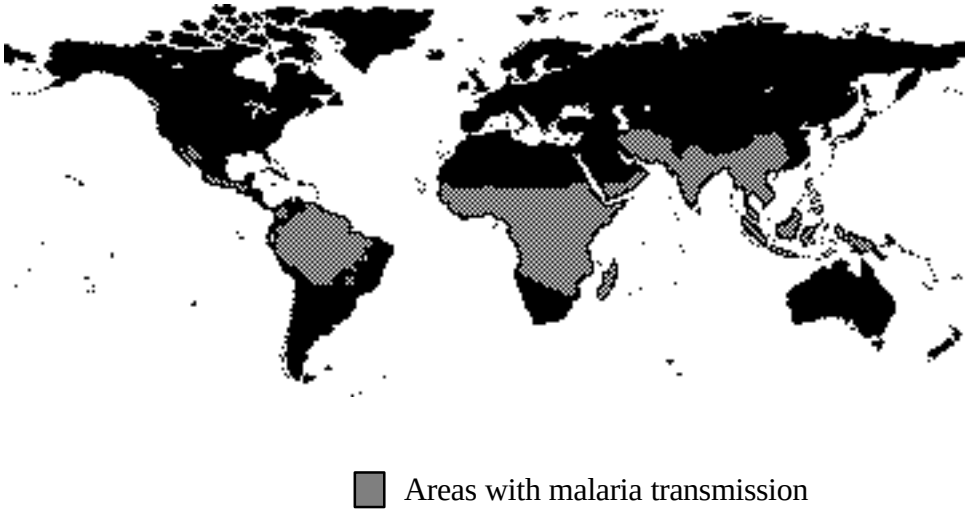
Transmission

The transmission of *P. falciparum* malaria in a certain region depends on many factors, such as the natural environment, vector and parasite population and behavioural and social situation of man.

Natural factors limit the area of transmission of malaria: it is restricted to the habitat of the *Anopheles* species that is located at altitudes below 2200-2500 metres over sea-level and at places where open water pools provide breeding sites. At temperatures below 17°C, the life cycle of *P. falciparum* will not be completed in the mosquito and will therefore terminate the transmission cycle (Macdonald 1952). These factors limit the area of transmission to 91 countries where 2460 million people, or about 40 % of the total world population live (Figure 1.1, WHO 1997).

Man-made factors include environmental, social, economic and demographic factors. Their influence aims in two directions: they influence the density of the vector (e.g. additional, man-made breeding sites) as well as the availability of the host (e.g. migration behaviour, urban settings or the amount of money, which can be spend for preventive measures like bednets).

Figure 1.1 Areas with malaria transmission



Exposure

The level of exposure is regarded as the key factor shaping the presentation of malaria in the human host. The degree of exposure shapes the pattern of host morbidity and host immunity. This pattern can be used to describe a defined area as holoendemic or hypoendemic (see: Table 1-I). Exposure can be characterized by the yearly entomological inoculation rate (EIR) and the basic case reproduction rate (BCRR). Whereas the EIR is determined also by the host's availability, the BCRR is determined by the mosquito only (population density, feeding behaviours, longevity, duration of sporogony). The BCRR gives a good estimate for transmission stability in malaria areas, and subsequently for endemicity, describing the average number of secondary cases to which a single malaria case would give rise after one passage through one mosquito. Unstable malaria occurs in areas where the BCRR lies just above 1, the minimal level for malaria to sustain itself. In rural sub-Saharan Africa, the BCRR may reach values of up to 1000 (Bradley, 1995)

A moderate EIR often results in parasite prevalences and densities which undergo significant changes throughout a year. These changes are often due to the availability of surface water and humidity, resulting in the lowest vector density at the end of the dry season. Under these circumstances, malaria transmission is seasonal but stable. A high EIR is often correlated with a year-round stable malaria transmission. Under such conditions, the prevalence of *falciparum* malaria infection and the respective parasite densities peak during early infancy at the individual level (Bradley, 1991, Table 1-I).

Table 1-I Endemicity levels classified by parasite prevalence*

Level	Prevalence
Holoendemic	Parasite rate in the one-year age group constantly over 75%, spleen rate in adults high (New Guinea type) or low (African type), parasite density declining rapidly between 2-5 years of live and then slowly.
Hyperendemic	Parasite rate in children of 2-9 years constantly over 50%.
Mesoendemic	Parasite rate in children of 2-9 years as a rule 11-50% (may be higher during part of the year).
Hypoendemic	Parasite rate in children of 2-9 years as a rule less than 10% (may be higher during part of the year).

*adapted from (Molineaux, 1988)

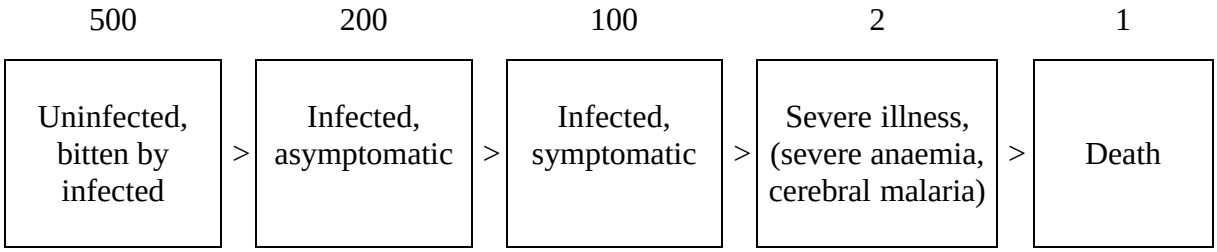
1.1.2. Infection, disease and immunity

Infection

The symptoms of all human malaria infections are manifold and may include distinct attacks of fever, often associated with headache, myalgia, chills, sweating and/or anaemia. If present, the attacks of fever are correlated with the asexual intraerythrocytic life cycle of the parasite: Fever occurs at the time of rupture of iRBCs (approx. every 48 hours in *P. falciparum* malaria) leading to the classical sequence: cold stage, then hot stage, then sweating. Rigors are common and splenomegaly is often a consequence.

In areas where infection with *P. falciparum* constantly occurs (holoendemic), it appears that only a small proportion of all infected people fall ill. Therefore, it will be useful to distinguish between malaria as an infection and malaria as a disease: the proportion of *P. falciparum* infections developing from asymptomatic, to uncomplicated symptomatic, to severe, and finally fatal disease for children under 5 years is illustrated in Figure 1.2 (adapted from: Marsh, 1992). In semi-immune adults, the ratio of asymptomatically : symptomatically infected would rather be 10-20 than 2. WHO (1997) estimates *P. falciparum* to be responsible for about 400-500 million clinical cases each year, resulting in the death of 1.5 to 2.7 million individuals world-wide, with the worst situation in children under 5 years old, living in sub-Saharan Africa (app. 1 million deaths per year).

Figure 1.2 The spectrum of malaria, from infection to mortality



The clinical outcome of an infection (i.e. a first infection, without considering the influence of the history of previous *P. falciparum* infections) in an individual person may depend on a wide range of factors, arising from the parasite, the host and the environment:

Factors arising from the parasite could include virulence factors like growth and multiplication rates, expression of a cytoadherent phenotype, toxin production, immune evasion ability and, more and more important, drug resistance.

Factors arising from the host can be dependent on the 'genotype' which codes for a red cell polymorphism (Stirnadel *et al.*, 1999), glucose-6-phosphate dehydrogenase deficiency (Cappadoro *et al.*, 1998), polymorphism in immune response genes such as T-cell receptors (Murillo *et al.*, 1992), and polymorphism in endothelial receptors like the Kilifi-mutant of ICAM-1 (Fernandez-Reyes *et al.*, 1997), or can be dependent on the actual 'phenotype', as defined by the acute hormonal disposition (pregnancy), by the nutritional status (e.g. iron supplementation, Menendez *et al.*, 1997 and Beck *et al.*, 1999), by concomitant diseases (Ravindran *et al.*, 1998), and finally by the presence of another simultaneous *P. falciparum* infection, since a high multiplicity of *P. falciparum* infections appears to be protective against clinical malaria in young children (Al Yaman *et al.*, 1997).

Factors arising from the environment could be related to the level of knowledge about malaria and its prevention (bednets) or the availability of health care in the community.

All these factors might influence the outcome of malaria but no clear correlation between any of these factors and the severity of disease could be established up to now. Nevertheless, it was proposed, that the overall incidence of uncomplicated or severe malaria morbidity in a population depends largely on its exposure history, since immunity to malaria might develop slowly upon repeated infection and can be short-lived (Molyneux, 1995).

Disease and immunity

The first 3-6 months after birth, infants born from immune mothers are protected from the disease, and after this period, clinical manifestations depend on transmission intensity:
In areas of very high endemicity (holoendemic), the most frequent manifestation of severe malaria is severe anaemia, characteristically occurring in the first year of life (Snow *et al.*, 1994 and Kitua *et al.*, 1996). Disease susceptibility rapidly declines after the first year of life, as antimalarial semi-immunity is acquired.
The relationship between multiple infections and the development of immunity, and its relation to morbidity has only recently been brought under extensive examination (Al Yaman *et al.*, and Beck *et al.*, both 1997) and led to the hypothesis, that in the first months of life, defence against infection relies mainly on fever and related cytokine activities, and individual infections are of short duration. However, older children develop a chronic, low-level parasitaemia with multiple infections of many different genotypes. These chronic infections seem to confer cross-protection against newly inoculated parasites - a phenomenon that has been called 'premunition', in contrast to 'semi-immunity' (Smith *et al.*, 1999).
Therefore adults are mostly asymptomatic but with the exception of primigravidae mothers, which become highly susceptible to malaria infections, probably by providing a CSA-rich placental substrate, which selects for CSA-binding parasites, a phenotype, primigravidae mothers might not have had encountered before, thereby leading to maternal malaria (Fried & Duffy, 1996 and Maubert *et al.*, 1997).

In areas of lower endemicity and seasonal transmission (EIR 10-20), exposure can be insufficient to induce significant immunity. As a result, all age groups are susceptible to severe disease, but complications are most prominent in children below 1 to 4 years of age, with severe anaemia being a problem in children less than 1 year. In contrast, cerebral malaria is typically seen in older children (Greenwood *et al.*, 1991, Snow *et al.*, 1994).
People lacking continuous exposure like during epidemics or travellers and migrating populations therefore have to be considered as non-immune and all age groups are at equal risk to develop severe and fatal disease.

The overall exposure and its temporal distribution in an area determines the immune status of the residing individuals. The observation, that prevalence of *P. falciparum* infections is not always 100% and that parasitic densities remain relatively stable over time (despite the theoretical 8 to 32-fold multiplication every 48 hours), both illustrate the effective, but not complete, immunity in highly exposed individuals. Kwiatkowski (1992) proposed three effector mechanisms to control asexual erythrocytic infection:

(-1.) an immediate monocyte/macrophage-derived tumor necrosis factor alpha (TNFα)-response to parasite toxins, possibly released during iRBC disruption, causing fever and other non-specific mechanisms to constrain parasite growth.

(-2.) an early T cell-derived interferon-γ response, which in combination with TNFα stimulates macrophages and neutrophils to attack parasites.

(-3.) a slowly evolving B-cell response that is eventually capable of eradicating the infection.

The fact, that the acquisition of individual immunity occurs over several years supports the view, that immunity is strain-specific, or variant-specific respectively. The finding, that high multiplicity of *P. falciparum* infections appears to be protective against clinical malaria in young children in Tanzania or in PNG (Al Yaman *et al.* and Beck *et al.*, both 1997 and FŠrnert *et al.* 1999) additionally supports this hypothesis. The enormous diversity of *P. falciparum* antigens allows the parasite to avoid complete clearance by the host's immune system, since the immuno-dominance of repeat regions might lead to poor defence reactions, especially in individuals frequently confronted with new infections ('smoke screen': Day and Marsh, 1991) Antigenic variation supports the immune evasion since a new variant can lead to a weak defence reaction, when a slightly different variant was encountered before by the immune system: In presence of the new, slightly different variant, old memory B cells may be triggered, whose antibodies fail to recognize the new variant ('antigenic sin': Good *et al.*, 1993).
Therefore, the interaction between the parasite and the host's immune system is mostly determined by the parasite's ability to actively evade the immune response. The main strategies involved in immune evasion are given in the next chapter:

1.2. Immune evasion

Two main strategies are involved in immune evasion of *Plasmodium falciparum* parasites: the first strategy involves the phenomenon of adherence, and the second strategy deals with the phenomenon of antigenic variation. Both strategies are coupled with each other: since adhesive determinants on the surface of the parasite or on the surface of the iRBCs are likely to be antigenic, it would be useful for the parasite to be able to switch from one adhesin to another. On the other hand, antigenic variation has to be limited, since any given adhesin should still be able to mediate adherence to a given receptor.

1.2.1. The role of adherence

The need of adherence for *P. falciparum* is two-fold: first, infection of the host cells (to evade the immune system) requires host-parasite recognition events, which are mediated by adhesion- and signalling molecules, and secondly, iRBCs express on their surface parasite-derived adhesins, which mediate adherence to endothelial cells in the deep vascular bed of different organs (sequestration), and allows the parasite to avoid spleen-dependent killing of iRBCs. (Langreth and Peterson, 1985).

In its vertebrate host, the malaria parasite spends most of its life cycle intracellulary, shielded from an attack by antibodies. Only the sporozoites injected by the mosquito and the merozoites emerging from disrupted iRBCs are extracellular stages, and even then for a very short time. For both, sporozoites and merozoites, accurate targeting is facilitated by the abundance of the appropriate host cells (hepatocytes and erythrocytes). Invasion into the host cells requires the specific recognition of the host cell's surface molecules by molecules from the parasite's plasma membrane, often followed by additional adhesion and signalling events mediated by the secretion of proteins by micronemes and rhoptries, specialized organelles in the anterior pole of both extracellular stages, sporozoites and merozoites.

Two sporozoite surface proteins were described to participate in hepatocyte invasion: the circumsporozoite protein (CSP, Nussenzweig & Nussenzweig, 1989) and the thrombospondin related adhesive protein (TRAP, Robson *et al.*, 1988). CSP covers the entire surface of sporozoites uniformly, but is not expressed in blood stages nor in the initial stages of parasite development in mosquitoes. By disrupting the CSP gene, it has been shown, that CSP is essential for sporozoite development in the mosquito (Menard *et al.*, 1997). In the vertebrate host, a conserved region (region 2) of CSP mediates initial attachment to the glycosaminoglycans (GAGs), which are expressed on the surface of the liver cells. About one third of the CSP molecule consists of tandemly repeated motifs, which vary widely in sequence within each plasmodium species, and might therefore be responsible for the 'camouflage' of the protein.

The second protein, TRAP, is found in small clusters on the plasma membrane of sporozoites and in micronemes, and contains a sequence of amino acids homologous to the region 2 of CSP. As excepted, TRAP also binds to liver GAGs (Muller *et al.*, 1993), but its most important role seems to be to provide the mechanical support necessary for gliding and invasion, since the protein is connected, via a transmembrane and cytoplasmic domain, to the cytoskeleton of the parasite (Sultan *et al.*, 1997).

Red cell invasion on the other hand is a complex, poorly understood multistep process, during which different proteins of the merozoite adhere to the red cell. Two proteins fit into the context of this thesis and will be mentioned here: EBA175 (Erythrocyte binding antigen 175), since it was one of the first *P. falciparum* proteins, which was successfully expressed in COS cells (Sim *et al.*, 1994) and p235 (in *P. yoelli*), since it is encoded by a large multigene family (approximately 50 copies per haploid genome) with each nucleus in a single developing schizont transcribing and expressing a different variant (Preiser *et al.*, 1999).

After the penetration of the red cell by the merozoite, the parasite matures and starts to express and transport molecular adhesins to the surface of the iRBC. The functions of these adhesins will be presented in the next chapter:

1.2.2. Sequestration, rosetting and auto-agglutination

A characteristic feature of infections with *P. falciparum* is the ability of iRBCs to adhere to venular endothelial cells (MacPherson *et al.*, 1985). Adhesive behaviour is manifested by the iRBC some 16-20 hours into the intraerythrocytic cycle, when the parasite matures to the early trophozoite stage, at which time it expresses and transports molecular adhesins to the red cell surface, leaving ring-infected erythrocytes to be the predominant form of parasites found in the peripheral blood. These adhesins have been shown to be associated with protrusions on the surface of the iRBC, called 'knobs'. For many years knobs were said to be necessary but insufficient for adhesion, but it is now recognized, that parasite lines exist, which are able to adhere to endothelial cells *in vitro*, despite the lack of knobs (Biggs *et al.*, and Udomsangpetch *et al.*, both 1989), but that knobs might be necessary under physiological shear stress (Crabb *et al.*, 1997). Additionally, knobs have been shown to be the site at which ligands for some host receptors are expressed (Nakamura *et al.*, 1992). These adhesins on the surface of iRBCs mediate adherence to endothelial cells (sequestration), to uninfected RBCs (rosetting, David *et al.*, 1988) and to other iRBCs (auto-agglutination, Roberts *et al.*, 1992).

Sequestration:

The peripheral withdrawal of iRBCs was recognized over one hundred years ago, as was the accumulation of these cells in small blood vessels of vital organs (Bignami & Bastianelli, 1889). For the parasite it may offer a possibility of avoiding passage through the spleen and its non-specific clearance mechanisms. Although this advantage has not been formally proved, the advantage which sequestration confers to *P. falciparum*, is sufficient to

be maintained in natural populations, whereas it is rapidly lost *in vitro* in the absence of a selective pressure or *in vivo* in splenectomized animals (David *et al.,* 1983). For the host, sequestration is a pathological event, tolerated to varying degrees with respect to the site of sequestration and the amount of sequestered iRBCs. Nevertheless, sequestration has been proposed to be the key event of vital organ failure in the pathogenesis of cerebral malaria, and cytoadherence in the brain plays a major role, as indicated by studies which quantified the amount of sequestered iRBCs in the brain vessels from fatal cerebral malaria cases (MacPherson *et al.,* 1985 and Aikawa *et al.,* 1992). However, the discussion, whether these findings indicate post-mortem artefacts and the fact, that cerebral malaria also occurs, although rarely, in *P. vivax* malaria, a species which shows no sequestration, indicates, how many questions are still unanswered (This argumentation is reviewed in P. today (1994), Vol. 10, Nr. 10: Grau and de Kossodo / Clark and Rockett / Berendt *et al.*).

Undisputed is the theory, that in each venule where sequestration occurs, a certain degree of obstruction to flow will occur. This might be sufficient to decrease the efficiency of metabolite exchange in these segments of the microvasculature. Additionally, the lower flow rates may favour further sequestration, since binding to CD36 seems to be more sensitive to shear stress than ICAM-1 (see below, Cooke *et al.,* 1994, and reviewed by: Cooke & Coppel, 1995). Furthermore, sequestered iRBCs themselves are metabolically highly active and release large amounts of lactic acid, which may worsen the biochemical imbalance in the tissue (White *et al.,* 1985). In addition to adherence to endothelial cells, iRBCs can also adhere *in vivo* to platelets, monocytes, lymphocytes (Barnwell *et al.,* 1985), uninfected RBCs (rosetting, David *et al.,* 1988) and other iRBCs (auto-agglutination, Roberts *et al.,* 1992).

Rosetting and auto-agglutination:

An additional complexity has come up with the recognition, that iRBCs can adhere to uninfected RBCs to form 'rosettes' (David *et al.,* 1988) or to auto-agglutinate with other iRBCs (Roberts *et al.,* 1992). Unlike sequestration, rosetting is a property of only some strains, varying quite dramatically in the extent to which they form rosettes (Wahlgren *et al.,* 1994) but was found in all 4 species of human malaria parasites (Lowe *et al.,* 1998). Most (e.g. Carlson *et al.,* 1990 and Rowe *et al.,* 1995) but not all (Al Yaman *et al.,* 1995) studies, which have examined the relationship between rosetting and disease, suggest that parasites of the rosetting phenotype are more likely to occur in patients with severe disease. It has also been shown in some (e.g. Carlson *et al.,* 1990) but not all (Reeder *et al.,* 1997) studies, that antibodies, which disrupt rosettes are less common in patients with disease. Nevertheless, it seems likely that the ability to form rosettes might be a virulence factor, since it had been suggested that the parasite, cocooned within a group of uninfected red blood cells may rapidly reach and invade these cells, thereby diminishing its extracellular, vulnerable stage.

On the other hand, no difference could be found in the growth rates of strains capable to form rosettes, compared to those that cannot (Clough *et al.*, 1998).

The molecular basis for adhesion of *P. falciparum*-infected RBCs was started to be investigated by the advent of electron microscopy (Miller, 1969), and, as it became possible to cultivate intraerythrocytic forms (Trager and Jensen, 1976), by the development of *in vitro* cell adhesion models (Udeiyna *et al.*, 1981), which were subsequently improved by flow chambers (Cooke *et al.*, 1994) or by appropriate *in vivo* models (e.g. Willimann *et al.*, 1995). The host's receptors for iRBCs and the ligands on the surface of iRBCs, responsible for sequestration and/or rosetting, which are characterized up to now, will be introduced in the next two chapters.

1.2.2.1. Host receptors involved in sequestration

Over the past ten years, a number of molecules expressed on the surface of endothelial cells have been shown to bind erythrocytes infected with *P. falciparum*, varying from members of the immunglobulin superfamily to substituted sugars.
In chronological order, these are: thrombospondin (TSP) (Roberts *et al.*, 1985), CD36 (Barnwell *et al.*, 1985), ICAM-1 (CD54) (Berendt *et al.*, 1989), VCAM-1 (CD 106), E-selectin or ELAM-1 (CD62E) (both Ockenhouse *et al.*, 1992a), chondroitin sulfate A (CSA) (Rogerson *et al.*, and Robert *et al.*, both 1995), PECAM-1 (CD31) (Treutiger *et al.*, 1997) and P-selectin (CD62P) (Udomsangpetch *et al.*, 1997). Indirect evidence also suggests a role for the $\alpha_v\beta_3$ integrin (CD51 / CD61) (Siano *et al.*, 1998).
As possible receptors on the surface of uninfected red blood cells, i.e. receptors mediating rosetting, CD36 (Handunnetti *et al.*, 1992), complement receptor 1 (CR1, CD35) (Rowe *et al.*, 1997) and heparan sulfate (HPS) (Chen *et al.*, 1998a), were described. Additionally, the AB0 blood group antigens have been identified as receptors, but they influence the size of the rosettes, rather than the frequency of rosetting (Carlson and Wahlgren, 1992).

The molecular structure of these molecules, their expression and the evidence for their role in malaria will be presented in the following (see also: Table 1-II).

Thrombospondin (TSP)

TSP is a secreted glycoprotein consisting of 3 identical 150 kDa subunits, linked to each other by disulphide bonds. It is stored within platelets and is synthesized as well by endothelial cells, macrophages and melanoma cells like C32 cells. It is a multifunctional, multidomain protein that binds many different ligands such as heparin, fibrinogen or collagen.

Nearly all isolates collected from malaria patients and analyzed after a brief *in vitro* culture bound to TSP in static assays (Sherwood *et al.,* 1987). With electron microscopy, it was shown, that soluble TSP binds the surface of iRBCs specifically at knobs (Nakamura *et al.*, 1992). Lately, the importance of TSP as a receptor *in vivo* was diminished by the finding, that the strength of binding of iRBCs to TSP is possibly too low to allow interaction in a post capillary venule under flow conditions (Cooke *et al.*, 1994). More recently, it was argued, that soluble TSP might be capable of bridging parasite-induced membrane alterations on iRBCs and endothelial CD36 to increase binding (Siano *et al.*, 1997).
PfEMP-1 (Baruch *et al.*, 1996) and Pfalhesin (Lucas and Sherman, 1998) were identified as possible ligands in binding of TSP.

CD36

CD36 (also called glycoprotein (GP) IIIb, GPIV and GP88) is an integral membrane glycoprotein existing as a monomer of about 88 kDa, consisting of two extracellular loops, interspaced by a membrane-associated region, and two membrane-spanning regions at both termini (Greenwalt *et al.*, 1992). CD36 was originally isolated from platelets, but it is also present on the surface of monocytes, microvascular endothelial cells , some epidermal cells, on erythroid precursors and weakly on erythrocytes (Handunnetti *et al.*, 1992), and a wide variety of cultured cell lines. CD36 is a receptor for the extracellular matrix proteins collagen and TSP. The binding site to TSP (which might be important in cytoadherence of iRBCs, see: Siano *et al.*, 1997) is located on AA 87-99, whereas two malaria binding sites were mapped to AA 8-21 and 97-110 (Asch *et al.*, 1993). Furthermore, a site-directed, single mutagenesis of the histidine at position 242 of human CD36 to tyrosine (rodent CD36) showed the importance of that residue in the conformation of the iRBC-binding domain(s) of CD36 (Serghides *et al.*, 1998). Mature iRBCs bound to purified CD36 immobilized to plastic, and purified soluble CD36 inhibited the adherence to HUVEC and C32 melanoma cells (Barnwell *et al.*, 1989). In contrast to huICAM-1-mediated adhesion, iRBCs bound to CD36 under physiological flow conditions by firm, static attachment (Cooke *et al.*, 1994) whereas co-expression of CD36 and huICAM-1 had a synergistic effect on iRBC binding (McCormick *et al.*, 1997), indicating that cytoadherence *in vivo* will most likely result from the sum of several interactions between iRBCs and endothelial receptors (Newbold *et al.*, 1997).
Like TSP, CD36 bound specifically to the knobs of iRBCs (Nakamura *et al.*, 1992). Most wild isolates investigated *in vitro*, bound to CD36 (Hasler *et al.*, 1990), and the most common interactions appeared to be between PfEMP-1 and CD36 (Baruch *et al.*, 1996) but also between Pfalhesin and CD36 (Crandall *et al.*, 1994 and 1996) and between Sequestrin and CD36 (Ockenhouse, 1991).

Intercellular Adhesion Molecule-1 (ICAM-1, CD54)

ICAM-1 belongs to the immunoglobuline (Ig) gene superfamily molecules (Imhof & Dunon, 1995), and is a glycoprotein of approximately 100 kDa. ICAM-1 is expressed on the surface of lymphocytes, monocytes, macrophages, the vascular endothelium, and on transfected cell lines like L-huICAM-1- (Van Kooyk *et al.*, 1993) and CHO-huICAM-1 cells (Hasler *et al.*, 1993). Under physiological conditions, ICAM-1 is the ligand for lymphocyte function-associated antigen-1 (LFA-1, CD11a/CD18, $\alpha_L\beta_2$, Staunton *et al.*, 1990), and for complement receptor 3 (CR3, MAC-1, CD11b/CD18, $\alpha_M\beta_2$, Diamond *et al.*, 1990). In addition to the binding of iRBCs, ICAM-1 had also been found to act as an endothelial receptor for human rhino-virus (Marlin *et al.*, 1990). Two studies (Ockenhouse *et al.*, 1992b and Berendt *et al.*, 1992) have assigned two distinctly different sites in ICAM-1 to be responsible for binding to LFA-1 and iRBCs respectively.

Furthermore, ICAM-1 can be present in a biologically active, soluble form in human serum (Gearing and Newman, 1993) and increased plasma levels of soluble ICAM-1 were detected during acute phase of *P. falciparum* induced malaria. This indicates a high level of surface expression of ICAM-1 at this stage of infection (Wenisch *et al.*, 1994b). In general, the expression of ICAM-1 is upregulated by stimulation with interleukin-1 (IL-1), TNFα or interferon-γ. In an *in vivo* model, it was shown that the endothelium of the mid brain showed the highest responsiveness to such an inflammatory stimulus (Willimann *et al.*, 1995), indicating a correlation between the expression of ICAM-1 and cerebral malaria. This association is supported by results from a large case-control study in Kilifi, Kenya, in which the adhesion of iRBCs to ICAM-1 was highest in the cerebral malaria category, compared to asymptomatic control (Newbold *et al.*, 1997). Also in Kilifi, a coding polymorphism in the N-terminal domain of ICAM-1 (K29/M; i.e. in the mutant, the lysine at position 29 is replaced by a methionine) was found with a high frequency. Carriers of this mutant were shown to have increased susceptibility to cerebral malaria (Fernandez-Reyes *et al.*, 1997).[1]

Under physiological flow conditions, iRBCs tether and roll along ICAM-1, even at high wall shear stress (Cooke *et al.*, 1994). This is in contrast to an immobilizing effect of ICAM-1 on leukocytes.
In contrast to CD36 and TSP, many parasite strains fail to bind to ICAM-1 *in vitro*, but those which do, presumably bind via PfEMP-1 (Baruch *et al.*, 1996).

[1] The binding site of the rhinovirus is in a area that overlaps the K29 residue (Craig *et al.*, 1991) and therefore, protection from rhinovirus is a possibility for the high frequence of the ICAM-Kilifi-mutant.

Vascular Cell Adhesion Molecule-1 (VCAM-1, CD106), Endothelial Leukocyte Adhesion Molecule-1 (ELAM-1, E-Selectin, CD62E), and P-Selectin (CD62P)

VCAM-1 can be present in two alternatively spliced isoforms of a single gene, but its predominant form is that of a glycoprotein of approximately 100 kDa. Like ICAM-1 it belongs to the Ig gene superfamily and is expressed by vascular cells involved in leukocyte activation and migration, and on bone marrow stromal cells. Again, a soluble form has been detected in the serum of patients suffering from cancer or inflammatory diseases (Wenisch *et al.*, 1994a and Imhof & Dunon, 1995). Under physiological conditions, VCAM-1 is the receptor for the very late antigen 4 (VLA-4, CD49d/CD29, $\alpha_4\beta_1$), expressed on leukocytes, and binds weakly to the $\alpha_4\beta_7$ integrin on the surface of gut homing lymphocytes and activated platelets (Vonderheide & Springer, 1992). It is also upregulated during inflammation in response to TNFα, IL-1 and IL-4 (Masinovsky *et al.*, 1990). Together with ICAM-1, it regulates tight adhesion of leukocytes to endothelium, and recruitment of leukocytes to sites of inflammation (Imhof & Dunon, 1995).

ELAM-1, now designated E-Selectin (Bevilacqua *et al.*, 1989), is a glycoprotein of 110 kDa, exclusively expressed by inflamed endothelial cells. Like P-Selectin (140 kDa, expressed by activated platelets and endothelium), it belongs to the selectin family. Both proteins show a typical mosaic structure, consisting of a sugar binding domain (N-terminus), a epidermal growth factor (EGF)-like domain, several short consensus sequences, a transmembrane domain and an intercellular C-terminus. A variety of glycoproteins have been reported to bind to these selectins. Elevated levels of circulating, soluble E-Selectin, are specific for diseases that provoke marked endothelial activation, including malaria (Gearing and Newman, 1993)

In the context of malaria, both VCAM-1 and ELAM-1 were found to bind weakly to iRBCs from a particular patient's isolate called CY36. CY36-infected RBCs also bound weakly to ICAM-1, but adhered well to CD36. Infected RBCs from this isolate were subsequently selected *in vitro* by sequential panning over purified immobilized CD36, ICAM-1, VCAM-1 and ELAM-1. A parasite clone, that bound strongly to all of these receptor proteins, was isolated, which demonstrates the potential of natural parasite isolates to bind to a variety of endothelial cell surface proteins (Ockenhouse *et al.*, 1992a). However, VCAM-1, as well as ELAM-1 and P-Selectin, seem to be uncommon binding targets for iRBCs and the parasite-derived ligand remained unidentified.

Platelet / Endothelial Cell Adhesion Molecule-1 (PECAM-1, CD31)

PECAM-1, a glycoprotein of 130 kDa, also belongs to the Ig gene superfamily as well, and its expression is restricted to endothelial and intravascular cells including granulocytes, monocytes and platelets (Newman *et al.*, 1990). Its physiological function is to mediate transendothelial migration of leukocytes, its main ligand is $\alpha_v\beta_3$.

In the context of malaria, RBCs infected by a particular strain (FCR3S1.HUV), were selected by panning over PECAM-1. After multiple rounds of selection, iRBCs bound to PECAM-1, expressed on transfected L-cells, or directly to recombinant PECAM-1 absorbed onto plastic (Treutiger *et al.*, 1997). Binding was blocked by both, mAbs to domains 1-4 of PECAM-1, and soluble PECAM-1. The significance of this interaction is hard to gauge, since PECAM-1 appears to be confined to areas of cell-cell contact between endothelial cells and to be absent from the luminal face, to which iRBCs adhere, although interferon-γ may redistribute the receptor expression to the surface of the endothelium (Romer *et al.*, 1995). The ligand on the surface of iRBCs is unknown.

Interestingly enough, in the large case-control study in Kilifi, Kenya (Newbold *et al.*, 1997), CD31 was used as a negative control.

The integrin $\alpha_v\beta_3$ (CD51 / CD61)

The integrins comprise of a large family of molecules that mediate adhesion in immune and inflammatory responses between cells, and between cells and the extracellular matrix. Integrins consist of a large α chain, with multiple cation-binding sites, non-covalently paired with a smaller β chain. The β_3 chain (CD61) associates with the α_{IIb} chain on platelets (CD41/CD61) or the α_v chain (CD51) on a variety of cultured cells, including endothelium. The $\alpha_v\beta_3$ integrin (254 kDa) recognizes multiple matrix proteins such as fibrinogen and thrombospondin and is constitutively expressed on microvascular endothelium. Its expression is rapidly increased at sites of vascular inflammation and damage (Swerlick *et al.*, 1991).

In the context of malaria, it was shown (Siano *et al.*, 1998) that binding of iRBCs to endothelial cells under flow conditions could be inhibited by a mean of 45% by using anti-α_v antibodies. Furthermore, this adherence was independent of the addition of thrombospondin or other adhesion mediators. The ligand(s) on the surface of iRBCs is unknown.

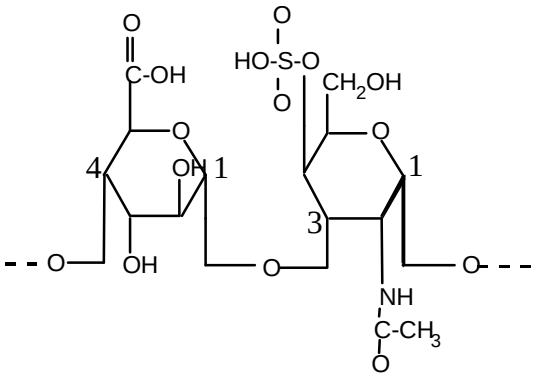
Chondroitin sulfate A (CSA)

The glycosaminoglycan (GAG) CSA was the first receptor for iRBCs showing, that sequestration not only depends on protein-protein interaction, although GAGs were already known to be involved in adherence of sporozoites to hepatocytes (Pancake *et al.*, 1992).

GAGs, the polysaccharide chains in proteoglycans, are made up of disaccharide repeating units, containing a derivative of an amino sugar, either glucosamine or galactosamine.

At least one of the sugars in the disaccharide has a negatively charged carboxylate or sulfate group. Hyaluronate, chondroitin sulfate, keratan sulfate, heparan sulfate (HPS) and heparin are the major GAGs. The classical structure of CSA is one of repeating units of alternating glucuronic acid [1->3] and N-acetylgalactosamine-4-sulfate [1->4] (see: Figure 1.3), although it is now clear, that GAGs are more heterogeneous than previously suspected (Karamanos *et al.*, 1994).

Figure 1.3 Disaccharide unit of CSA



In CSB, glucuronic acid is replaced by iduronic acid and in CSC, the N-acetylgalactosamine is predominately 6-sulphated. However, these GAGs do not mediate adherence (Gysin *et al.*, 1997). In the vascular system, the only abundant CSA containing proteoglycan is thrombomodulin (TM), a transmembrane protein, which often has a CSA chain attached at one of two sites (Gerlitz *et al.*, 1993). CSA contributes to the role of TM, principally binding and inactivating the circulating coagulant thrombin. TM is found widely on vascular endothelium (~100'000 molecules per cell), and interestingly, in the context of malaria, it is expressed at high levels on the syncytiotrophoblasts, lining the maternal blood sinusoids (Maruyama *et al.*, 1985 and Maubert *et al.*, 1997), a site to which iRBCs have recently been shown to bind in the placenta (Fried and Duffy, 1996). CSA is also found on the vascular endothelium of the lung and the brain, indicating a possible role in the development of cerebral malaria (Prudhomme *et al.*, 1996). Binding of iRBCs to CSA was almost completely inhibited *in vitro* by competition with soluble CSA oligosaccharides, with the minimum length being 7 disaccharide repeats (Beeson *et al.*, 1998) or by chondroitinase ABC treatment of the cells. CSA is also present on many cultured cell lines, including C32- and Chinese hamster ovary (CHO) cells. Very recently, it had been shown, that PfEMP-1 mediated binding of iRBCs to CSA (Reeder *et al.*, 1999).

Table 1-II Adhesion molecules implicated in malaria sequestration

CD Number	Other names	Main function	Main distribution	Size kDa	Main ligands	Parasite ligands	Comments
CD31	PECAM-1	Transendothelial migration of leukocytes	EC., M., P.	130	$\alpha_v\beta_3$	?	uncommon binding target.
CD36	GPIIIb	Flag for phagocytosis of cells undergoing apoptosis	EC., M., P.	88	TSP, collagen	PfEMP-1 Pfalhesin Sequestrin	most common binding phenotype of parasites confers tight binding.
CD51 / CD61	$\alpha_v\beta_3$	Distribution of cells via extracellular matrix molecules	EC., M., P.	254	CD31, laminin TSP	?	uncommon binding target.
CD54	ICAM-1	Leukocyte adhesion to endothelium in inflammation	EC., M., DC	90 to 115	LFA-1 ($\alpha_L\beta_2$) MAC-1 ($\alpha_M\beta_2$)	PfEMP-1 Pfalhesin	Rolling receptor for iRBCs, the binding site has been mapped.
CD62E	E-Selectin	Rolling of lymphocytes on activated endothelium	EC.	110	ESL-1	?	uncommon binding target.
CD62P	P-Selectin	Platelet-Adhesion to neutrophils & monocytes	act-EC., P.	140	PSGL-1	?	uncommon binding target.
CD106	VCAM-1	Recruitment of leukocytes to inflammation-sites	EC.	90 to 110	VLA–4 $\alpha_4\beta_7$	?	uncommon binding target.
-	TSP	various	EC, also soluble	450	CD36	PfEMP-1	Physiological role in question due to the weakness of bin-ding under flow conditions.
-	CSA	binding and inactivating sol. thrombin	on EC and syncytiotrophoblasts	-	thrombin	PfEMP-1	Appears to be important in malaria during pregnancy.

Legend to Table 1-II

9 host molecules, described to be involved in iRBC sequestration, are listed and compared relative to their size, function, distribution and role in *P. falciparum* malaria.

Abbreviations used:
DC: dendritic cell, EC: endothelial cell, act-EC: activated EC, ESL-1: E-selectin ligand-1, LFA-1: Lymphocyte function-associated antigen-1, M: Monocytes, P: Platelets, PSGL: P-Selectin glycoprotein ligand-1 and VLA-4: very late antigen-4.

1.2.2.2. Parasite-derived or -modified ligands involved in sequestration

A number of parasite-derived or -modified candidates, involved in sequestration, have been identified, including *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1, Leech *et al.*, 1994), sequestrin (Ockenhouse *et al.*, 1991), modified erythrocyte band 3 anion transporter, also termed Pfalhesin (Sherman *et al.*, 1992 and Crandall *et al.*, 1994). Of all these above mentioned ligands, PfEMP-1 is the only parasite derived protein for which full sequence information is available and it is the only adhesion mediating molecule, which has been shown by several laboratories to be parasite encoded and to be located on the iRBC surface. For two recently described proteins, the *rif* gene family encoded molecules on the surface of iRBCs (rifins, Cheng *et al.*, 1998) and the proteins, encoded by the *clag* gene family (Bowman *et al.*, 1999), no involvement in cytoadherence has been shown. On the other hand, direct (Baruch *et al.*, 1996 and 1997) and indirect (Gardner *et al.*, 1996 and Chaiyaroj *et al.*, 1994) experiments by a number of investigators showed PfEMP-1 to be involved in binding to diverse receptors (TSP, CD36, ICAM-1 and CSA, see above). Furthermore, PfEMP-1 is also involved in rosetting via binding to CD36 (Handunnetti *et al.*, 1992), complement receptor 1 (CD35) (Rowe *et al.*, 1997) and heparan sulfate (HPS) (Chen *et al.*, 1998a).

Role of Sequestrin and Pfalhesin in sequestration

Evidence for an involvement of sequestrin in cytoadherence of iRBCs was attained from indirect experiments: Ockenouse *et al.*, (1991) were able to demonstrate binding of CD36 to an antigen from knobless parasites, today considered to be most likely PfEMP-1 (Deitsch and Wellems, 1996), which they named sequestrin.

On the other hand, the modified erythrocyte band 3 anion transporter (Pfalhesin) is a clearly defined host receptor, but modified by the intraerythrocytic parasite (Crandall and Sherman, 1991). They showed that iRBCs bind via a 65 kDa protein, present in the membrane of iRBCs. This protein was recognized by mAbs, specific for the cytoplasmic domain and the N-terminal side of the membrane-spanning region of human band 3, but was not recognized by an antibody specific for the C-terminal part of the membrane spanning region. Later on, a CD36-like binding domain was found in this protein (Crandall and Sherman, 1996) and finally it was shown, that TSP mediates parasitized erythrocyte band 3-related adhesin binding (Lucas and Sherman, 1998).

Role of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1) in adhesion

PfEMP-1 was originally described as a strain specific, high molecular weight (250-350 kDa), highly polymorphic protein on the surface of *P. falciparum*-infected RBCs (Leech *et al.*, 1994). Its biochemical characteristics of insolubility in non-ionic detergents and extreme sensitivity to proteases, together with its location and degree of antigenic diversity in field isolates suggested, that it was the *P. falciparum*-equivalent of the phenotypically variable antigen (SICA antigen, Brown and Brown, 1965) of *P. knowlesi*. Indeed, in the early nineties, *in vitro* data from two laboratories (Biggs *et al.*, 1991 and Roberts *et al.*, 1992) showed, that PfEMP-1 did undergo antigenic variation in cloned isolates and that changes in antigenic type were accompanied by changes in the receptor binding specificity. More recently, it was suggested that the presence of antibody to specific PfEMP-1 variants protects against subsequent clinical infection with isolates expressing that variant (Bull *et al.*, 1998).
Further progress was made 1995, when the genes encoding PfEMP-1 were identified and sequenced. It was shown that each variant antigen type was encoded by a single gene (Baruch *et al.*, Smith *et al.*, and Su *et al.*, all 1995). These genes, termed *var* genes (Su *et al.*, 1995), were shown to be present at 50-100 copies per haploid genome, to be highly polymorphic, both within and between isolates, but to have a similar basic structure (see also 1.2.3.1.). Briefly: this structure includes a cysteine-rich interdomain region (CIDR), 2-5 Duffy binding like domains (DBL), a transmembrane domain, and an acidic terminal sequence (ATS).

The **ATS** represents the C-terminal, intracellular part of PfEMP-1 and is possibly involved in interactions with other parasite derived proteins and with sub membranous proteins of the RBC cytoskeleton, which might be responsible for the correct presentation and anchoring of PfEMP-1. Furthermore, the ATS domain is the most conserved domain of the *var* genes.

Different **DBL** domains identified as candidates for binding: Very recently it was shown, that antibodies to the DBL3 domain and to the CIDR domain of a CSA-binding PfEMP-1 also inhibited binding of iRBCs to CSA (Reeder *et al.*, 1999). Specific DBL1 domains were shown to mediate rosetting via binding to CR1 (Rowe *et al.* 1997) or to HPS (Chen *et al.*, 1998a).

The **CIDR** domain was the first domain, which has been found to mediate binding: using an expressed *var* gene from the Malayan Camp (MC) parasite, Baruch and co-workers (1997) expressed the CIDR domain as a glutathione-S-transferase fusion protein and showed that this domain mediated binding to CD36.

For huICAM-1, which is also a receptor for PfEMP-1 (Baruch *et al.*, 1996) no binding domain of PfEMP-1 could yet be characterized, but most ICAM-1 binding PfEMP-1 variants, identified so far, were encoded by *var* genes, which encode for a larger than average molecule, containing five DBL domains (Gardner *et al.*, 1996 and Smith *et al.*, 1998).

TSP-binding to PfEMP-1 was shown to be insensitive to immune serum, binding showed no variant-specific characteristics, and it was not pH-dependent, in contrast to the binding of CD36 or ICAM-1 to PfEMP-1. These data suggested that TSP-binding occurred at another molecule, possibly at the modified erythrocyte band 3 anion transporter (Gardner *et al.*, 1996).

Little data exists for the involvement of PfEMP-1 in binding to other receptors, such as VCAM-1, E-selectin, P-Selectin and PECAM-1.

A possible role of PfEMP-1 in rosetting was first proposed, when CD36 was found to be present on iRBCs (Handunnetti *et al.*, 1992). Recent data support the role of PfEMP-1 in rosetting: by growing rosetting and non-rosetting lines from the rosetting clone R29 (Rowe *et al.*, 1997), it was shown that a single dominant *var* gene is expressed in rosetting but not in non-rosetting parasites. This gene was sequenced and fragments were expressed on the surface of COS 7 cells. Only the construct containing the first DBL domain (DBL1), but none of the other constructs permitted binding of RBCs. Similar results were obtained by another group (Chen *et al.*, 1998a) using a different parasite genotype, expressing a distinct rosetting-associated *var* gene. Thus, it seems likely that the DBL1 domain of the expressed PfEMP-1 variant mediates rosetting. The binding of the DBL1 domain to the surface of RBCs was proposed to be mediated either via complement receptor 1 (CR1, CD35, Rowe *et al.*, 1997) or via heparan sulfate (HPS, Chen *et al.*, 1998a).

1.2.3. Antigenic diversity and antigenic variation

Antigenic diversity as a consequence of variation is considered to be a second crucial strategy in immune evasion, coupled with the expression of adhesins on the surface of iRBCs. The difficulty lies in the trade-off between variation (to evade the immune system) and conformity (to bind the receptors) of the parasite-derived ligands involved (Reviewed in Borst *et al.*, 1995).

Antigenic diversity is defined as the expression of antigenically different alleles of a gene at a single genetic locus in different clones of the parasite (Reeder and Brown, 1996). Several plasmodial antigens, such as the merozoite proteins 1 and 2 (MSP-1 and MSP-2) highlight the phenomenon of antigenic diversity, since many different alleles of these genes exist in a *Plasmodium falciparum* population and therefore, an immune response directed

against one antigen encoded by a certain allele may not be effective against an infection with parasites expressing a different allele (Prescott *et al.*, 1994).

Antigenic variation, on the other hand, is defined as the ability of a clonal population to switch its antigenic phenotype. In the context of *P. falciparum*-derived adhesins, antigenic variation is the mechanism of immune evasion, which has to deal mainly with the trade-off mentioned above and was attributed to *P. falciparum* in 1991 (Biggs *et al.*, 1991). The phenomenon was already well known from African trypanosomes (the causative agent of sleeping sickness), which express variant surface glycoproteins (VSGs) on their surface during the course of infection (Reviewed in Borst and Rudenko, 1994).

Biggs *et al.*, (1991) could demonstrate that parasite clones, isolated from a parental clonal culture, were serologically distinct from the parental clone, despite the absence of any selective pressure. Furthermore, it was found in the same experiment, that this antigenic switch was accompanied by changes of the molecular weight of PfEMP-1. Further studies (Roberts *et al.*, 1992) revealed that antigenic variation of PfEMP-1 is due to the differential expression of *var* genes, leading to the exposure of phenotypically different PfEMP-1 variants on the surface of iRBCs. These differences are manifested in distinct antigenic and adhesive properties of these variants (Scherf *et al.*, 1998).

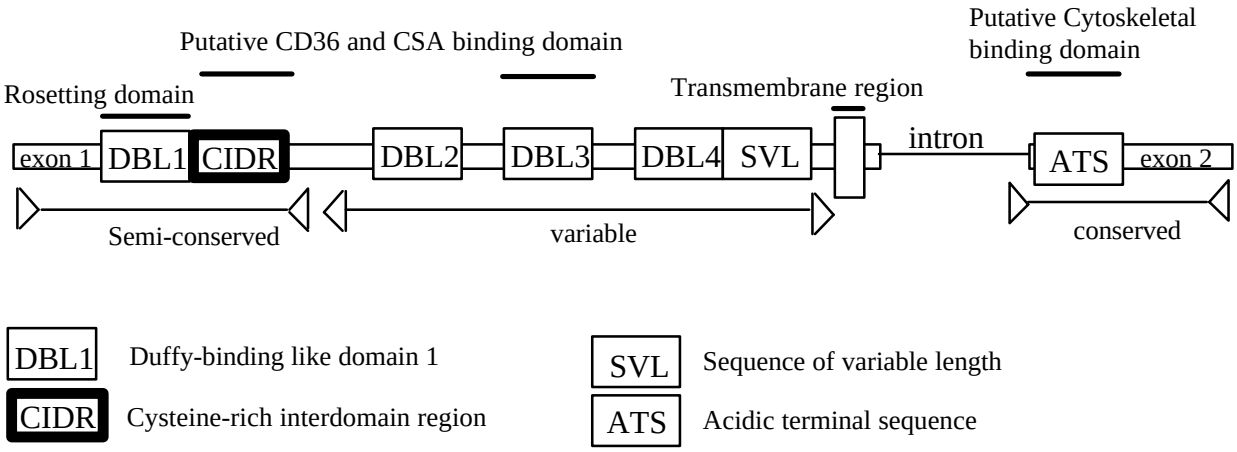
1.2.3.1. Role of *var* genes and expression of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1) in antigenic variation

Structure

All *var* genes have a two-exon structure. The large 5' exon (exon 1) codes for the extracellular part of the protein and is highly diverse, containing 2-5 cysteine-rich Duffy binding like (DBL) domains and a cysteine-rich interdomain region (CIDR) of 300-400 amino acids, located between DBL1- and DBL2 domain. Exon 1 terminates with a sequence of variable length (SVL) and a short hydrophobic region which shows characteristic features of a transmembrane segment (Engelman *et al.*, 1986). The DBL domains, which show broad homologies to domains previously identified in other malarial proteins such as the Duffy binding protein of *P. vivax* and the erythrocyte binding antigen (EBA 175) of *P. falciparum* (Chitnis and Miller, 1994) are highly diverse and can only be identified by a few conserved residues; however, the DBL1- and the subsequent CIDR domain are the most conserved regions of exon 1, and were found in all *var* genes so far, leading to the suggestion, that the DBL1- and CIDR domain together comprise a conserved head structure (Su *et al.*, 1995). The number of DBL domains may be indicative for the binding phenotype of the corresponding PfEMP-1 molecule, since most ICAM-1 binding PfEMP-1 molecules are encoded by *var* genes, which encode for a larger than average molecule, containing five DBL domains

(Gardner *et al.*, 1996 and Smith *et al.*, 1998). Additionally, the CSA binding PfEMP-1, termed *var*^{CSA} (Scherf *et al.*, 1998) contains only two DBL domains.
The smaller 3' exon (exon 2) is well conserved and codes for a segment, that is rich in acidic amino acids, therefore termed acidic terminal sequence (ATS), representing the intracellular portion of PfEMP-1, which is probably involved in anchoring the protein to the cytoskeleton of the iRBC. The two exons are separated by an intron sequence of approximately 1 kb. A schematic diagram of a *var* gene containing 4 DBL domains is given in Figure 1.4

Figure 1.4 Schematic of the general structure of a var gene



Distribution

The *var* gene family shows extensive diversity and consists of 50-150 *var* genes per haploid genome, distributed throughout all 14 chromosomes (Rubio *et al.*, 1996), and located at chromosome-internal sites (chromosome 4, 7, 8, and 12; Thompson *et al.*, and Fischer *et al.*, both 1997), as well as within nearly all subtelomeric regions, next to the non-coding repetitive element Rep20 (Hernandez-Rivas *et al.*, and Fischer *et al.*, both 1997). It has been shown that subtelomeric *var* genes are more closely related to each other than to sequences found in central regions, indicating, that recombination might more frequently occur among subtelomeric *var* genes (Rubio *et al.*, 1996). Furthermore, a sequence termed pf60, which has been shown to correspond also to a large gene family (Carcy *et al.*, 1994), shows strong homology to the conserved ATS domain of *var* genes and hybridized primarily to chromosomes 4, 7, and 12, where they are located next to the chromosome-internal *var* genes (Thompson *et al.*, 1997). On the other hand, the recently described *rif* and *clag* gene families were found next to the subtelomeric *var* genes (Bowman *et al.*, 1999).

Expression

From studies correlating *var* gene expression with antigenic and adhesive properties of iRBCs, it appears that only a limited number, probably only one, *var* gene variant is expressed at any given time (Rowe *et al.*, 1997, Chen *et al.*, 1998b, and Scherf *et al.*, 1998), with *var* gene switching occurring at a rate of ~2% per generation *in vitro* (Roberts *et al.*, 1992 and Smith *et al.*, 1995). Scherf and colleagues (1998) showed, that only one distinct *var* gene was expressed after selection of parasites on endothelial receptors (CD36, CSA, and ICAM-1), termed var^{CSA}, var^{CD36} and var^{ICAM-1} respectively. var^{CSA}, and var^{CD36} were located in subtelomeric regions whereas var^{ICAM-1} was present within a central domain of chromosome 12. Taken together, these data suggest, that expression from individual *var* genes is controlled by a mechanism that allows activation in situ (Scherf *et al.* 1998) , without need for gene conversion or telomeric exchange events, such as these described in trypanosome VSG switching (Borst and Greaves, 1987). While there is thought to be transcription from the entire *var* repertoire during the first few hours after red cell invasion (Chen *et al.* 1998b), only a single var gene appears to be active during the time in parasite development, when PfEMP-1 is actively translated and transported to the surface of iRBCs (Scherf *et al.*, 1998). Very recently Deitsch *et al.* (1999) found evidence, that *var* gene expression is under epigenic control, possibly through changes in the chromatin structure.

1.2.3.2. PfEMP-1 and immune selection

Given that each particular 'brood' or 'strain' of *P. falciparum* has a limited repertoire of distinct PfEMP-1 variants, among which switching can occur, it was proposed (Gupta and Day, 1994) that the selective pressure excerted by human immune responses, directed against PfEMP-1, is sufficient to structure the parasite population, such that an overlap in the repertoire is minimized between 'strains' of *P. falciparum.*

According to that 'distinct strain theory', the specific anti-PfEMP-1 response, elicited by a particular parasite strain after one or a few exposures, will reduce multiplication of that particular type in the human host and render transmission of that strain less likely with each subsequent exposure (Gupta *et al.*, 1996). This model is supported by a field study (Contamin *et al.*, 1996), showing that clinical episodes in Senegalese children were associated with recently inoculated 'new' strains of *P. falcipar*um. More recently, Bull *et al.* (1999) found in 21 children from Kenya an inverse relationship between each child's repertoire of agglutinating antibodies and the agglutination frequency of the isolated parasites, indicating an immune selection of rare surface variants.

On the other hand, a phylogenetic analysis of 103 DBL1 domains could not support the theory of distinctly segregating repertoires of *var* genes (Ward *et al.*, 1999).

2. Goals and Objectives

The goal of the studies presented in this thesis was to identify and clone schizont-specific *Plasmodium falciparum* gene(s) involved in cytoadherence by expression in simian COS 7 cells . A secondary goal was to bring forth a direct proof, that *var* gene-encoded molecules can mediate binding to host cell receptors.

To this end, the project had the following developmental objectives:

A.) Objective 1: from the phenotype to the genotype (Shotgun approaches 1 and 2)

1. Isolation of schizont-specific mRNA of synchronized *Plasmodium falciparum* cultures after confirmation of adhesion of iRBCs to CD36 and huICAM-1 and construction of a mammalian expression cDNA library into the pcDNA3.1-vector.
2. Establish an *in vitro* adhesion assay with CD36 and huICAM-1 expressing screening cells on seeded, library-transfected COS 7 cells.
3. Identification of transfected, adhesion-mediating COS 7 cells, isolation of cDNA clones, and identification of *P. falciparum* genes mediating adherence (Shotgun approach 1).
4. Identification of COS 7 cells expressing iRBC-surface molecules with previously generated monoclonal antibodies (PhD-thesis of K. Willimann, 1996) (Shotgun approach 2).

B.) **<u>Objective 2: from the genotype to the phenotype</u>** (Direct approaches 1 and 2)

1. Design of degenerated primers to amplify semi-conserved domains from *var* genes (DBL1-, CIDR- and ATS domains) from an adherent and a non adherent strain.
2. Screening of the cDNA library to identify full-length *var* genes.
3. Performing *in vitro* assays with *var* gene-transfected COS 7 cells to identify *var* genes (or *var* gene domains) which mediate adhesion to host cell receptors (Direct approach 1).
4. *E. coli*-expression of the conserved DBL1- and CIDR domains as 6xhis-tagged proteins under native conditions.
5. Analysing the binding of these purified domains to host cell receptors, involved in cytoadherence (Direct approach 2)

3. Materials and Methods

3.1. Materials

3.1.1. Cell lines, *Plasmodium falciparum*- and *Escherichia coli*-strains.

Most of the mammalian cell lines used in this work were obtained as a gift from other research groups, three cell lines were purchased from the American Type culture collection (ATCC, Table 3-I). All *P. falciparum* strains were obtained from the laboratory of H. Matile at F. Hoffmann-La Roche Ltd (Table 3-II). The *E. coli* strains used in this work are listed in Table 3-III

Table 3-I Mammalian cell lines

Name	**Phenotype / expression of receptors involved in sequestration**	**Source (Reference)**
Bosc23 cells	Easily transfectable cell line derived from human kidney cell line HEK 293.	B. Imhof, Centre Medical Universitaire, Genf, CH (Pear *et al.*, 1993)
Bosc-huICAM-1-EGFP cells	stably transfected Bosc23 cells, expressing huICAM-1 on the cell surface and cytosolic GFP.	produced
C32 cells	Human amelanotic melanoma cells, expressing CD36 and CSA.	ATCC, Rockville, ML, USA (Udeiyna *et al.*, 1985 and Pouvelle *et al.*, 1998)
CHO cells	Chinese hamster ovary cells, expressing CSA.	H. Matile, Hoffmann-La Roche Ltd, Basel, CH
CHO-huCD36 cells	stably transfected CHO cells, expressing CD36 and CSA.	ATCC, Rockville, ML, USA (Scherf *et al.*, 1998)
CHO-huICAM-1 cells	stably transfected CHO cells, expressing ICAM-1 and CSA.	ATCC, Rockville, ML, USA (Scherf *et al.*, 1998)

Name	Phenotype / expression of receptors involved in sequestration	Source (Reference)
COS 7 cells	Derived from the African green monkey kidney cell line CV-1 by transformation with an origin defective SV40 virus.	B. Imhof, Centre Medical Universitaire, Genf, CH (Gluzman, 1981)
L-cells	Mouse fibroblast like cells.	C. Figdor, the Netherlands Cancer Institute, Amsterdam, NL
L-huICAM-1 cells	stably transfected L-cells, expressing huICAM-1 on the surface.	C. Figdor (Van Kooyk *et al.*, 1993)

Table 3-II *Plasmodium* strains

Name	Phenotype	Source (Reference)
ItG2.F6	knobby and adherent to C32- and L-huICAM-1 cells.	H. Matile, Hoffmann-La Roche Ltd, Basel (Magowan *et al.*, 1988)
K1	knobless and not adherent	H. Matile

Table 3-III *E. coli* strains

Name	application	Source
M15	protein expression (3.2.5.)	Qiagen, Basel, CH
MOSblue competent cells	T/A cloning (3.2.1.9.)	Amersham, Buckinghamshire, UK
Top10F'	cDNA library	Invitrogen, Leek, NL
Top10F' electrocompetent cells	electroporation	Invitrogen, Leek, NL

3.1.2. Vectors

6 different vectors were used during this work. 5 of these vectors are commercially available from Invitrogen (pcDNA3.1 and pcDNA3.1-EGFP), Amersham (pMOSblueT-vector) and Qiagen (pQE30 and pREP4). See suppliers' catalogues for further information. The plasmid pRE4 is a kind gift of Drs. Gary Cohen and Roselyn Eisenberg, University of Pennsylvania, PA, USA (Cohen *et al.*, 1988) and was already used earlier for expression of plasmodial proteins in COS 7 cells (Chitnis and Miller, 1994). It contains a SV40 origin of replication, a Rous sarcoma virus LTR as a promoter and the SV40 early polyadenylation signal. The *Herpes simplex* virus glycoprotein D-gene (HSVgD-gene) was cloned in the HindIII cloning site. The HSVgD-gene has a unique PvuII restriction site 81 bp downstream the signal peptide cleavage site and a unique ApaI restriction site 213 bp upstream of the C-terminal hydrophobic stretch, which we used to generate chimeric proteins, flanked by the HSVgD leader sequence (5') and the HSVgD transmembrane and cytosolic domain (3').
The purposes of these vectors are summarized in Table 3-IV.

Table 3-IV Vectors

Name	**Purpose**	**Source**
pcDNA3.1	cDNA-cloning and expression in mammalian cells	Invitrogen, Leek, NL
pcDNA3.1-EGFP	Expression of GFP in mammalian cells. Transfection- and expression-control	B. Imhof, Centre Medical Universitaire, Genf, CH
pcDNA3.1-huICAM-1-EGFP	Chimeric expression of huICAM-1 with GFP at COOH-end	produced
pMOSblueT-vector	T/A cloning of PCR-products	Amersham, Buckinghamshire, UK
pQE30	*E. coli*-expression with a 6xhis-tag at NH_2-end	Qiagen AG, Basel
pREP4	Kanamycin-resistance and lac-operon repressor	Qiagen AG, Basel
pRE4	Chimeric expression in mammalian cells with HSVgD flanking regions	Drs. Gary Cohen and Roselyn Eisenberg, University of Pennsylvania, PA, USA

3.1.3. Antibodies and secondary reagents

Antibodies and secondary reagents that have been applied in FACScan analysis, western blots and in immunohistochemical assays are listed in Tables 3-V to 3-VII.

Table 3-V Monoclonal Antibodies

Name	Specificity	Source (Reference)
MEM112 (CD54)	human ICAM-1	Anawa AG, Wangen, CH
OKM*5	human CD36	Ortho Diagnostic System, Raritan NJ, USA
Penta His	5xHis-tag	Qiagen AG, Basel
anti-6x Histidine Clone AD 1.1.10	6xHis-tag	R&D Systems, Minneapolis, MN, USA
αGFP (polyclonal)	GFP	Clonetech, Heidelberg, D
ID3	AA 11-19 of the mature HSVgD protein	Drs. Gary Cohen and Roselyn Eisenberg, University of Pennsylvania, PA, USA
DL6	AA 272-279 of the mature HSVgD protein	Drs. G. Cohen and R. Eisenberg
Sup 9.22	GYGLFHKEKMIL motif of the SPf66-peptide	Dr. G. Pluschke, STI, CH
SupI 202/7	iRBC (crossreacts with human Lymphocytes)	All the following mAbs were generated by K. Willimann (1996,
SupII 3c2/9	iRBC (crossreacts with human lymphocytes)	PhD-thesis) and recognized iRBC and intraerythrocytic *P. falciparum*
SupX 128	trophozoites	stage proteins.
SupX 216	iRBC	
SupX 304/3	late schizonts and merozoites	
SupX 323/3	schizonts	
SupX 331	merozoites and rings	
SupX 435/3	surface of intracellular stages	
SupX 509	schizonts	

Table 3-VI Conjugated monoclonal Antibodies

Host	Specificity	Conjugate	Source
goat	mouse IgG F(ab')2	FITC	Cappel, Durham, NC, USA
goat	rabbit IgG (H+L)	AP	Jackson ImmunoResearch, West Grove, PA, USA
goat	mouse IgG	AP	Sigma, Buchs, CH
rabbit	mouse total Ig	HRP	DAKO Corporation, Zug, CH
goat	mouse IgA+G+M (H+L) human serum adsorbed	HRP	Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA
goat	human IgG (γ)	HRP	Kirkegaard & Perry

Table 3-VII Polyclonal sera

Host	Specificity	Source
pooled human sera	undefined, COS 7 cells adsorbed prior to use.	Malaria exposed adults from PNG

3.1.4. Reagents

The majority of chemicals and reagents were purchased from Merck, Darmstadt, D. Reagents supplied by others are listed in Table 3-VIII.

Table 3-VIII List of chemicals and reagents

Name	Supplier
30% Acrylamide/Bis solution (37.5:1)	Gibco BRL Life Technologies, Basel, CH
AEC Tablets	Sigma, Buchs, CH
Agar	Difco Laboratories, Detroit, MG, USA
Agarose (electrophoresis grade)	Gibco
Albumax II (low endotoxin)	Gibco

Name	Supplier
Albumin, Bovine Fraction V (BSA)	Sigma
Bacto tryptone	Difco
Bacto yeast extract	Difco
BSA, nuclease free	New England Biolabs
CAPS, 99% pure	Aldrich chemicals, Buchs, CH
Cellfectin	Gibco
Chloroquine (Diphosphate salt)	Sigma
CSA, sodium salt from bovine trachea	Sigma
CSase ABC from *Proteus vulgaris*	Sigma
DEAE-dextran (chloride form)	Sigma
N,N-Dimethylformamide (DMF)	Sigma
DMEM (high glucose)	Gibco
DMRIE-5	Gibco
DMSO	Fluka, Buchs, CH
dNTPs (100 mM each)	Pharmacia, DŸbendorf, CH
Drymilk (Rapilait)	Migros, Basel, CH
DTT	Calbiochem, San Diego, CA, USA
FCS, ultra low IgG	Gibco
Ethidium bromide 1% solution	Boehringer Mannheim
G418 sulphate (Geneticin)	Gibco
Gas mixture CO_2	Carbagas, Basel, CH
Giemsa solution	Fluka
Glycogen (1mg / ml)	Boehringer Mannheim
HEPES	BDH Laboratory Supplies, Poole, UK
HPS, sodium salt from bovine kidney	Fluka
IMDM	Gibco
IPTG	Appligene Oncor, Basel, CH
L-Glutamine 200 mM	Gibco
Lipofectin	Gibco
LipofectAmine	Gibco
LipoTaxi	Stratagene, Heidelberg, D
Ni-NTA Agarose	Qiagen AG, Basel, CH
RPMI 1640 Medium	Gibco
Saponin (from *Gypsophila*)	Sigma
SDS	Serva, Heidelberg, D

Name	Supplier
SERVA Blue R	Serva
Sodium pyruvate (100 mM)	Gibco
SuperFect	Qiagen, Basel, CH
Tris base (TRIZMA)	Sigma
Triton X-100	Sigma
Trypanblue 0.4% solution in 0.85% saline	ICN Biomedicals Inc., Costa Mesa, CA, USA
Trypsin-EDTA solution (10 x concentrate)	Gibco
Tween 20	Fluka
Urea, enzyme grade	Gibco
Xylene cyanol	Fluka
X-gal	Appligene Oncor

3.1.5. Equipment and consumables

A list of items used in addition to the standard laboratory equipment and consumables is given in Table 3-IX.

Table 3-IX List of equipment and consumables

Name	Supplier
0.22, 0.44 µm syringe membrane filter	Schleicher and Schuell, Dassel, D
0.22 µm bottle top filter (Steritop)	Millipore, ZŸrich, CH
3 well glass slides	Semadeni, Ostermundigen, CH
10 well glass slides	BioMŽrieux, Lausanne, CH
12- 24- 48-well plates	Costar at Integra Biosciences, Wallisellen, CH
ABI Prism 310 Genetic Analyzer	Perkin Elmer, Foster City, CA, USA
Electroporator (EasyjecT)	Equibio, Boughton, Montchetsea Kent, UK
Electroporation cuvette	Equibio
Eppendorf Thermomixer 5436	Dr. Vaudaux AG, Schšnenbuch, CH
FACScan	Becton Dickinson, San Jose, CA, USA
FACScan tubes (PS, Falcon 2052)	Falcon Labware, Oxnard, CA, USA
Freezing vials	Nunc Inc., Naperville, IL, USA
HyBond-C extra nitrocellulose membrane	Amersham, Buckinghamshire, UK
HyBond-N+ Nylon membrane	Amersham

Name	Supplier
Hybridizer 400HY	Bachofer AG, Reutlingen, D
Hybridizer HB-2D	Techne, Cambridge, UK
Inverted fluorescence microscope	Zeiss, Basel, CH
Mini-PROTEAN II Electrophoresis cell	Bio-Rad Laboratories AG, Glattbrugg, CH
Needle scalpel	Moria-Dugast, Paris, F
PAP pen	Anawa AG, Basel, CH
Petri dishes, tissue culture treated	Falcon
Petri dishes, bacterial	Greiner Labortechnik, KremsmŸnster, A
round bottom Tubes (PP, Falcon 2059)	Falcon
Sonicator (Branson Sonifier)	Skan AG, Basel, CH
Whatman 3MM filter paper	Whatman, Maidstone, UK
X-ray films (Kodak X-Omat AR)	Kodak

3.1.6. Media, Buffers, Solutions

Media, buffers and solutions for cell culture work were prepared with Millipore water, sterilized by filtration through 0.22 µm bottle top filters. Uncompleted media was stored up to 3 months at 4°C. If the media was older than 2 weeks, 2 mM L-Glutamine was added when completed with FCS.

Buffers and solutions for mRNA isolation, which were not provided by the Fast Track kit, were prepared with autoclaved and DEPC-treated Millipore water. A list of all prepared media, buffers and solutions is given in Table 3-X to 3-XII.

Table 3-X List of Media

Name	Composition		Usage
DMEM (high glucose) 1 litre	1 box 10 ml 3.70 g ad to	Powder DMEM (high glucose) sodium pyruvate 100 mM-stock $NaHCO_3$ (44 mM) 1 litre with water and adjust to pH 7.0	Stock for Cell culture. Prior use, add 10% FCS (L-Glutamine)
IMDM, 1 litre	1 box 3.02 g ad to	Powder IMDM $NaHCO_3$ (36 mM) 1 litre with water.	Stock for Cell culture. Prior use, add 5% FCS (L-Glutamine)

Name	Composition		Usage
RPMI 1640 for CHO-cell culture, 1 litre	1 box 2.10 g ad to	Powder RPMI 1640 $NaHCO_3$ (25 mM) 1 litre with water and adjust to pH 7.0	Stock for Cell culture. Prior use, add 10% FCS
RPMI 1640 for para-site culture, 1 litre	1 box 2.10 g 2.38 g 50 µg / ml 50 µg / ml 0.5% ad to	Powder RPMI 1640 $NaHCO_3$ (25 mM) HEPES (10 mM) Hypoxanthine Neomycin sulphate Albumax II 1 litre with water	*P. falciparum* culture
RPMI 1640 storage medium	1 box 5.95 g ad to	Powder RPMI 1640 HEPES (25 mM) 1 litre with water and adjust to pH 7.4	Storage of fixed cell-slides (3.2.2.2.)
Binding medium	1 box 5.95 g 5.00 g ad to	Powder RPMI 1640 HEPES (25 mM) Albumax II (0.5%) 1 litre with water and adjust to pH 7.0	Binding assays (3.2.2.2. and 3.2.2.6.)
HBD, 1 litre	1 box 10 ml 5.95 g ad to	Powder DMEM (high glucose) sodium pyruvate 100 mM-stock HEPES (25 mM) 1 litre with water and adjust to pH 7.0	Transfection (3.2.2.5.)
Cell-freezing medium	20% 80%	DMSO FCS	N_2-Stabilates of cells
SOC medium, 1 litre	20 g 5 g 0.5 g 10 ml ad to 20 ml	Bacto tryptone Bacto yeast extract NaCl 250 mM KCl 1 litre with water and adjust to pH 7.0, autoclave, allow to cool down to 60°C before adding 1 M Glucose (0.22 µm filtered)	Culture medium for *E. coli* cultures after electroporation
LB medium	10 g 5 g 10 g ad to	Bacto tryptone Bacto yeast extract NaCl 1 litre with water and autoclave	Culture medium for liquid *E. coli* cultures
LB-Agar	1 litre 15 g	LB medium Agar, then autoclave	Solid culture medium for *E. coli* cultures

Name	Composition		Usage
LB / 50% Glycerol	50% 50%	LB Glycerol, anhydrous, extra pure	Freezing medium for *E. coli* cultures
Super Broth medium	25 g 15 g 5 g ad to	Bacto tryptone Bacto yeast extract NaCl 1 litre with water and autoclave	Culture medium for liquid *E. coli* cultures used for protein expression

Table 3-XI List of Buffers

Name	Composition		Usage
10 x PBS	1.37 M 27 mM 120 mM 15 mM	NaCl KCl Na_2HPO_4 KH_2PO_4 adjust to pH 7.0	Various
1 x DPBS	100 ml 800 ml 200 ml	10 x PBS water 5 x $CaCl_2$ / $MgCl_2$ (add last)	Washing (3.2.2.2.)
1% formaldehyde in 1 x DPBS	540 ml 15 ml	1 x DPBS 37% formaldehyde	Fixation (3.2.2.2.)
1% glutaraldehyde 2% formaldehyde in 1 x PBS	2 ml 2.7 ml 45.3 ml	25% glutaraldehyde 37% formaldehyde 1 x PBS	Fixation (3.2.2.6.)
FACS sheath fluid	0.05% 1x	NaN_3 PBS	FACScan analysis
Phosphate buffer for Giemsa stains	8 mM 18.8 mM	KH_2PO_4 Na_2HPO_4 adjust to pH 7.2	Giemsa stains
Tris-HCl (pH x)	10 mM	Tris base pH adjusted to x with HCl	various
Saponin-lysis buffer	0.15 g 950 ml 50 ml	saponin (0.015%) water 20 x SSC	Saponin lysis (3.2.2.3.)
20 x SSC	3M 0.3 M	NaCl Na_3Citrate adjust to pH 7.0	various

Name	**Composition**		**Usage**
STET buffer	0.1 M 10 mM 1 mM 5%	NaCl Tris-HCl pH 8.0 EDTA pH 8.0 Triton X-100	Rapid boiling protocol (3.2.1.4.)
5 x TBE running buffer	5 M 4 M 10 mM	Tris base boric acid EDTA, pH 8.0	Gel electrophoresis (3.2.1.7.)
Gel loading buffer (blue juice)	30% 10 mM 10 mM a pinch a pinch	glycerine Tris-HCl, pH 8.5 EDTA, pH 8.0 bromphenol blue xylene cyanol	Gel electrophoresis (3.2.1.7.)
TE (storage buffer)	10 mM 1 mM	Tris-HCl, pH 8.5 EDTA	Elution and storage of DNA
10 x Taq PCR buffer	0.5 M 0.1 M 15 mM	KCl Tris-HCl, pH 8.8 $MgCl_2$	PCR (3.2.1.8.)
10 x Pfu reaction buffer	0.1 M 0.2 M 20 mM 60 mM 1% 100µg /ml	KCl Tris-HCl, pH 8.8 $MgCl_2$ $(NH_4)_2SO_4$ Triton X-100 nuclease free BSA	PCR (3.2.1.8.)
1 M Phosphate buffer =Na(P)	1 M 1 M	NaH_2PO_4 (pH 4.1) and Na_2HPO_4 (pH 8.8) were mixed to achieve pH 7.2 (approx. 1:5)	Southern blot (3.2.4.)
Prehybridisation buffer	5.0 parts 3.5 parts 1.5 parts	1 M Na(P) 20% SDS stock in water water	Southern blot (3.2.4.)
Southern buffer	100 mM 150 mM	Tris-HCl, pH 7.5 NaCl	Southern blot (3.2.4.)
Buffer 3	100 mM 100 mM 50 mM	Tris-HCl, pH 9.5 NaCl $MgCl_2$	Southern blot (3.2.4.)
Lysis buffer	50 mM 300 mM	NaH_2PO_4 NaCl adjust to pH 8.0	Protein purification (3.2.5.4.)
Buffer B	8 M 100 mM 10 mM	Urea NaH_2PO_4 Tris-HCl, pH 8.0 adjust to pH 8.0	Protein purification (3.2.5.4.)

Name	Composition		Usage
2 x SDS-Page sample buffer	2.5 ml 2.0 ml 0.4 g 0.31 g 1 mg ad to	0.5 M Tris-HCl, pH 6.8 glycerol SDS DTT bromphenol blue 10 ml with Millipore water	Protein separation (3.2.1.11.)
5 x running buffer	15 g 72 g 5 g ad to	Tris base Glycine SDS 1 litre with Millipore water	Protein separation (3.2.1.11.)
1 x transfer buffer	5 ml 5 ml 40 ml	0.1 M CAPS, pH 11.0 MeOH Millipore water	Western blot (3.2.1.11.)
Buffer A	50 mM 5 mM 140 mM 0.05% 0.25%	Tris base EDTA NaCl adjust pH to 7.4, then add NP 40 gelatine, heat to dissolve	Western blot (3.2.1.11.)
AP staining Buffer	100 mM 0.5 mM	Tris-HCl, pH 9.5 $MgCl_2$	Western blot (3.2.1.11.)
HRP staining buffer	18.8 ml 1.2 ml 10 µl	1 x PBS 0.3% chloronaphtol in MeOH 30% H_2O_2 (Perhydrol)	Western blot (3.2.1.11.)

Table 3-XII List of Solutions

Name	Composition		Usage
5 x $CaCl_2$ / $MgCl_2$	5 mM 5 mM	$CaCl_2$ $MgCl_2$	Preparing of DPBS
Trypsin-EDTA solution	1 part 9 parts	Trypsin/EDTA 10 x concentrate 1 x PBS	Detachment of cells
PBS-EDTA solution	1 part 99 parts	50 mM EDTA, pH 8.0 1 x PBS	Trypsin-free detachment of cells
Chloroquine stock	51.6 mg 10 ml	chloroquine water stock was 0.22 µm-filtered, aliquoted and wrapped in aluminium foil (light sensitive)	Transfection (3.2.2.5.)

Name	Composition		Usage
DEAE-dextran stock	1g 10 ml	DEAE-dextran water stock was 0.22 µm-filtered and aliquoted	Transfection (3.2.2.5.)
Hirt extract solution with proteinase K.	10 mM 0.6% 100 µg/ml	EDTA pH 8.0 SDS Proteinase K	Plasmid isolation (3.2.2.8.)
Amp stock solution Kan stock solution Tet stock solution	50 mg 25 mg 15 mg	ampicillin / ml water kanamycin / ml water tetracycline / ml water	*E. coli* culture (3.2.1.3.)
AEC / DMF Stock solution	20 mg 5 ml	AEC (= 1 tablet) N,N-Dimethylformamide stock was aliquoted and wrapped in aluminium foil (light sensitive).	IFAs (3.2.3.3.)
Staining solution for HRP-labelled mAbs	500 µl 10 ml 1.5 µl	AEC / DMF stock 0.1 M sodium acetate pH 4.8 (pH adjusted with glacial acid) 30% H_2O_2 (Perhydrol)	IFAs (3.2.3.3.)
Serva blue solution	0.5 g 200 ml 1800 ml	Serva blue acetic acid Millipore water Mix 1 hour and filter	Protein staining (3.2.1.11.)

3.1.7. Commercially available kits

During this work, several kits were used. The buffers and solutions provided in these kits were not listed separately in section 3.1.6. (See the supplier's notes and manuals for further information). The kits are listed in Table 3-XIII

Table 3-XIII List of Kit systems

Name	Supplier
Fast Track 2.0 Kit Version D	Invitrogen, Leek, NL
Qiagen mega plasmid purification kit	Qiagen, Basel, CH
Qiaprep Spin Plasmid Kit	Qiagen, Basel, CH
Qiaquick Gel Extraction Kit	Qiagen, Basel, CH

Name	Supplier
pMOSblue T-vector Kit	Amersham, Buckinghamshire, UK
DNA Sequencing Dye Terminator Kit	Perkin Elmer, Rotkreuz, CH
BioPrime DNA labelling System	Gibco BRL Life Technologies, Basel, CH
BLUEGENE Non radioactive Nucleic Acid Detection System	Gibco BRL Life Technologies, Basel, CH
QIAexpress Kit Type IV	Qiagen, Basel, CH

3.1.8. Enzymes

3.1.8.1. Restriction enzymes

Most restriction enzymes and corresponding buffers used in this work were obtained by Gibco BRL Life Technologies, Basel, CH, except ApaI and PvuII, which were obtained by Promega, Wallisellen, CH. A list of all used enzymes is given in Table 3-XIV.

Table 3-XIV List of restriction enzymes

Name	Cutting site	Name	Cutting site
ApaI	5' GGGCC | C 3'	KpnI	5' GGTAC | C 3'
BamHI	5' G | GATCC 3'	NotI	5' GC | GGCCGC 3'
EcoRI	5' G | AATTC 3'	PvuII	5' CAG | CTG 3'
HindIII	5' A | AGCTT 3'	XbaI	5' T | CTAGA 3'

3.1.8.2. DNA polymerases and dNTPs

4 different DNA polymerases were used: Taq DNA polymerases (Gibco BRL Life Technologies AG, Basel, CH and Qiagen, Basel, CH), Native Pfu DNA polymerase (Stratagene, Heidelberg, D), Klenow polymerase (Boehringer Mannheim) and AmpliTaq DNA polymerase (Perkin Elmer).

Nucleotides were often included in Kit systems or were ordered: dNTPs, 100 mM (Pharmacia, DŸbendorf, CH) and dNTPs, 500 mM (Boehringer Mannheim), as well as α-^{32}P labelled dCTP, 250 µCi (Amersham Life Science, Braunschweig, D) and Biotin-14-dCTP, 0.4 mM (Gibco BRL Life Technologies, Basel, CH).

3.1.8.3. Other enzymes

Name	Supplier
Chondroitinase ABC	Sigma, Buchs, CH
Lysozyme	Appligene Oncor, Basel, CH
Proteinase K	Appligene
T4 DNA Ligase	Amersham, Buckinghamshire, UK

3.1.9. Primers

3.1.9.1. Custom-made primers

Primers were manufactured by Gibco BRL Life Technologies, Basel, CH, but *, which were ordered at Mycrosynth, Balgach, CH and **, which were manufactured at the Centre MŽdicale Universitaire (CMU), Geneva, CH. A complete list of the primers used is given in Table 3-XV. Capital letters indicate the coding sequence of a gene and small letters indicate internal restriction sites and/or ribosomal binding sites and/or additional bases, necessary for in-frame ligation. All primers are given in 5' to 3' direction.

Table 3-XV List of primers

Primer name	Sequence	Binding site (Reference)
FW huICAM1**	atataagcttccaccATGGCTCCCAGC AGCCCCCGG	N-terminus of huICAM-1 (3.2.2.11.)
Rev huICAM totGFP**	tataggatccggAGGCGTGGCTTGT GTGTTCGG	C-terminus of huICAM-1 (3.2.2.11.)
FW MSP2 Hind	atataagcttccaccATGAAGGTAATT AAAACATTG	N-terminus of MSP2 (3.2.3.5.)

Primer name	Sequence	Binding site (Reference)
Rev MSP2 Bam	tataggatccatGAATATGGCAAAAGATAAAAC	C-terminus of MSP2 (3.2.3.5.)
FW MSP1 Eco	atatgaattcccaccATGAAGATCATATTCTTTTTA	N-terminus of MSP1 (3.2.3.5.)
Rev MSP1 Bam	tataggatcctcAGGGAGAGGATTTGGTTTACT	AA 342-348 of MSP1 (3.2.3.5.)
FW MSP1 Kpn	gggggtaccATGAAGATCATATTCTTTTTA	N-terminus of MSP1 (3.2.3.5.)
Rev MSP1 Apa	ggggggcccctcAGGGAGAGGATTTGGTTTACT	AA 342-348 of MSP1 (3.2.3.5.)
SEQ HSV FW	CATGGGGTCCGCGGCAAA	AA 21-26 of HSVgD (3.2.3.5.)
SEQ HSV Rev	GGGGGTCTCGGACAGCTC	AA 281-286 of HSVgD (3.2.3.5.)
VARDBL5*	GCACGAAGTTTTGCAGATAT(A/T)GG	ARSFADIG-motif at the 5' end of a DBL1 domain (3.2.4.2.)
VARDBL3*	AA(G/A)TCTTC(G/T)GCCCATTCCTCGAACCA	WFEEWAED-motif at the 3' end of a DBL1 domain
ATS5*	CCTAAATATAAAACATT(G/A)AT(A/T)GAAGT	PKYKTLIE-motif at the 5' end of a ATS domain
ATS3.1*	TCGTTGATTAGGTCGATACCACTATA	YSGIDLIN-motif at the 3' end of a ATS domain
ATS3.2*	TCATTAATTAAATCTATACCACTATA	YSGIDLIN-motif at the 3' end of a ATS domain
DBL1 Fw Bam	cgggatccGCACGAAGTTTTGCAGATATTGG	ARSFADIG-motif with restriction site (3.2.5.2.)
DBL1 Rev Kpn	ggggtaccTTCGGCCCATTCCTCGAACCA	WFEEWAE-motif with restriction site
CIDR Fw Bam	cgggatccAAATGGAAATGTTATTATG	KWKCYY-motif at the 5' end of a CIDR domain
CIDR Rev Kpn	ggggtaccTTGTAGTAATTTATCAATT	IDKLLQ-motif at the 3' end of a CIDR domain

3.1.9.2. Commercially available primers

Name	Purpose
T7 promoter primer (Gibco)	forward sequencing pcDNA3.1 / pMOS
U19mer primer (Amersham)	reverse sequencing pMOSblue T vector
pcDNA3.1/BGH reverse primer (Invitrogen)	reverse sequencing pcDNA3.1
QIAexpress primer type III/IV (Qiagen)	forward sequencing pQE30
QIAexpress primer reverse sequencing (Qiagen)	reverse sequencing pQE30

3.1.10. Molecular weight markers

The following markers were used for DNA gel electrophoresis (3.2.1.7.) and for SDS-Page gel electrophoresis (3.2.1.11.) to estimate the size and the amount of DNA fragments or proteins. All markers were used according the supplier's instructions.

Name	Supplier
1 kb Ladder	Gibco BRL Life Technologies, Basel, CH
Low molecular weight marker	Pharmacia Biotech, DŸbendorf, CH
Rainbow marker 756	Amersham, Buckinghamshire, UK

3.2. Methods

The methods are arranged according to the four approaches used in this work (see: Goals and Objectives). Methods which were not related exclusively to a single approach, are described in the following first chapter of generally used methods:

3.2.1. Generally used methods

3.2.1.1. Cell culture

Maintenance and passaging

All cells were cultivated in a 37°C / 5% CO_2 incubator. L-huICAM-1 cells were cultivated in IMDM containing 5% FCS, supplemented with 1 mg / ml Geneticin as described (Van Kooyk *et al.*, 1993). Non-transfected L-cells were maintained without Geneticin. Bosc23, C32 and COS 7 cells were cultivated without antibiotics in DMEM / 10% FCS. Transfected and non-transfected CHO cells were cultivated in RPMI 1640 cultivation medium / 10% FCS. For passages, all these adherent cells were first washed with PBS, then covered with a minimum amount (800 µl / 75 cm^2 and 400 µl / 25 cm^2) of 1x Trypsin-EDTA solution and placed in the incubator until the cells rounded up (2-5 minutes). Subsequently, the cells were detached from the bottle by tapping or by rinsing harshly with a Pasteur pipette and diluted to the desired cell concentration with fresh medium. Alternatively, all cell types, but the C32 cells, could also be detached by analogous treatment with PBS / 0.5 mM EDTA with a prolonged incubation at 37°C (up to 10 minutes). This treatment was used, if the cells were used for assays, since some cell surface receptors were sensitive to trypsination.

Counting

Detached cells were suspended in complete medium and centrifuged at 300 g for 5 minutes. The supernatant was discarded and the cells were resuspended in the remaining liquid. Depending on the size of the pellet, the suspension of cells was further diluted with 1-3 ml of medium and 10 µl were diluted with an equal volume of Trypanblue solution and transferred to a Neubaur chamber. The concentration of viable cells was calculated by counting the white cells in 16 fields: number of cells x 2 x 10'000 per ml.

Freezing and thawing

Cells were counted as described above. 50'000 cells were mixed with an equal volume of cold, freshly prepared cell-freezing medium in a freezing vial. The vials were placed in a styropor box and stored for 1 to 2 days a -70°C before they were transferred to liquid nitrogen for long term storage. When a new batch of cells was needed, a stabilate was thawed as quickly as possible (at 37°C or by hand) and immediately transferred to cold medium, centrifuged for 5 minutes at 300 g and resuspended in complete medium for cultivation.

3.2.1.2. Parasite culture

Maintenance

Parasite cultures were kept in continuous motion with automated medium exchange twice daily (Matile and Pink, 1990). The *P. falciparum* strains used in this work, were cultured in RPMI 1640 supplemented with 0.5% Albumax II. Infected erythrocytes were replaced by fresh human A+ erythrocytes when culture reached 5-10% parasitaemia.

Counting of *P. falciparum*-infected erythrocytes

Parasitaemia was measured by blood smears: thin films on glass slides were air dried, fixed by covering with MeOH for 2 minutes, air dried, stained for 20 to 30 minutes in 5% Giemsa solution diluted in phosphate buffer, washed under running tap water (chalk enhances the staining) and air dried again. The blood films were analyzed by microscopy using a 1000-fold magnification and oil immersion.

3.2.1.3. *E. coli* culture

E. coli cultures were used for a variety of purposes during this work. The liquid and plate cultures were performed according to standard protocols (Sambrook, Fritsch and Maniatis, 1989), as well as the preparation of glycerol stocks, but if many clones were to be stored: then, the freezing medium was prepared in a 96 well plate, each colony from a LB / agar plate was stripped into one well, the plate was sealed with Parafilm and immediately stored at -70°C.

3.2.1.4. Isolation of plasmids from *E. coli* cultures

The rapid boiling protocol

1.5 ml of an overnight or of an 8-hours culture were transferred to an Eppendorf tube and pelleted with 14'000 g for 1 minute. The supernatant was discarded and the pellet was resuspended in 350 µl of ice cold STET buffer and 25 µl of freshly prepared lysozyme stock solution (10 mg / ml water) was added, mixed by inversion and incubated on ice for 10 minutes. After ten minutes the tube was transferred directly into boiling water for 40 seconds and then briefly cooled down on ice. The tube was centrifuged at 15'000 g for 20 minutes at room temperature. The resulting, sticky pellet was removed with a tooth pick and discarded. The DNA was precipitated from the supernatant with isopropanol as described in 3.2.1.5. The pellet was resuspended in 50 µl TE and stored at -20°C. The rapid boiling method was used if the plasmids were not to be used for sensitive applications, such as ligation or transfection of mammalian cells, or if a lot of colonies had to be screened.

Qiaprep Spin Plasmid Kit

This method was used when highly purified plasmids were needed for further working steps. The kit was used according to the supplier's protocol. It is based on the following principles: *E. coli* cultures are lysed under alkaline conditions (NaOH / SDS) in presence of RNase A. The genomic DNA remains attached to the debris of the cell wall and is pelleted with the SDS-precipitate prior to the application of the supernatant to a spin column containing a silica gel membrane. The plasmid DNA binds to the membrane under the chosen high salt conditions, is washed and is eluted under low salt conditions and a pH around 8.0. The DNA of one culture was eluted in 50 µl TE and stored at -20°C.

3.2.1.5. Precipitation of DNA

DNA was precipitated either with isopropanol at room temperature or with ethanol for 1 hour on ice (precipitation of plasmids) or for 2 hours at -20°C when small particles of DNA were to be precipitated (PCR fragments). Precipitation was performed according to standard protocols (Sambrook *et al.*, 1989). A special application of this method is the Hirt extraction method (3.2.2.8.), where no salt was added for the precipitation, since 5 M NaCl was already added to the DNA solution prior the precipitation of DNA. If small amounts of DNA were to be precipitated, 20 µg of glycogen was added as a carrier.

3.2.1.6. Restriction enzyme digests

If possible, all digests were performed as recommended by the supplier, using the provided 10x reaction buffer. Whenever possible, double digests were performed simultaneously in the appropriate buffer. However, if this was not possible and the enzymes required different buffers, a simultaneous digest was performed, either in the buffer with the lower salt concentration, or a two step digest was performed with a precipitation step in between. In a typical digest, 0.5 to 2 units of enzyme(s) were used for up to 500 ng of plasmid DNA in a reaction volume of 20 µl and the digest was usually performed for 1 hour at 37°C. If the digested DNA was used for salt-sensitive applications such as ligation, the digest was precipitated prior to further use.

3.2.1.7. DNA gel electrophoresis

Agarose gel electrophoresis

Horizontal gel electrophoresis was performed in 1 x TBE as running buffer. A concentration of 0.75% up to 1.5% electrophoresis grade agarose was used to separate the DNA with maximal 50 milliamp•res. The gels were stained for 15 minutes in 1 x TBE containing 1 µg / ml EB. Gels were examined on an UV transilluminator connected to an IBM PC equipped with the Grab-it 2.0 software.
RNase-free gels were poured similarly, with the exception, that all buffers were made with DEPC-treated water and that the EB was already added to the gel before polymerising, in order to keep the time between loading the samples and examination as short as possible.

Estimation of the size and concentration of DNA fragments

The size of DNA fragments was estimated in relation to the fragments of the 1 kb ladder (Gibco). The amount of DNA could be estimated by comparing the intensity of a band with the 1.6 kb band of the 1 kb ladder (100 ng / µl), which represents 10% of the whole marker applied (10 ng per µl marker applied).

Gel extraction

Agarose gel electrophoresis was also used to purify DNA fragments: PCR products or restriction fragments were run on a agarose gel as described above and cut out with a scalpel under UV light (302 nm). The QIAquick Gel Extraction Kit was used to elute DNA from gel fragments.

3.2.1.8. Polymerase chain reaction (PCR)

PCRs were part of several methods. The most commonly used polymerase was Taq polymerase. The Pfu polymerase was used for amplification of templates bigger than 3 kb, AmpliTaq polymerase was used with the sequencing reaction (see 3.2.1.10.), and Klenow polymerase fragment was used for random priming and labelling reactions (3.2.4.3.).

PCR amplification with Taq polymerases

For a set of PCR amplifications, a master mix, containing buffer, dNTPs, primers, and water was usually prepared. A negative control without DNA template and a positive control with appropriate template was always included. The template was added to the mix in a PCR tube and overlaid with mineral oil. Each reaction contained the following components:

DNA template (0.1-1 µg)	x µl
10 x Taq PCR buffer	5.0 µl
10 x dNTPs (2 mM each)	5.0 µl
10 x forward primer (20 ng / µl)	5.0 µl
10 x reverse primer (20 ng / µl)	5.0 µl
Millipore water	29.8 - x µl
Taq DNA polymerase (5 u / ml)	0.2 µl

The Taq DNA polymerase was often added during the initial denaturing step (hot start) and was not added to the mastermix. For most PCR reactions, the conditions were set as follows, but if the reaction conditions differ from this set up it an experiment, the variation is given with the description of that experiment.

5:00 minutes	denaturing at 95°C	
1:00 minute	denaturing at 95°C	
0:45 seconds	primer annealing at [2(A+T)+4(G+C)]-3°C	for 30 cycles
2:00 minutes	extension at 72°C	
10:00 minutes	72°C	

PCR amplification with native Pfu polymerase

The PCR protocol is the same as with Taq polymerase, but the master mix contained a higher concentration of primers:

DNA template (0.1 µg)	x µl
10 x Pfu reaction buffer	5.0 µl
10 x dNTPs (2 mM each)	5.0 µl
"10 x" forward primer (20 ng / µl)	12.5 µl
"10 x" reverse primer (20 ng / µl)	12.5 µl
Millipore water	13.0 - x µl
native Pfu DNA polymerase (2.5 u / ml)	2.0 µl

The native Pfu DNA polymerase was added during the initial denaturing step. The conditions were dependent from AT richness of the primers (annealing temperature) and also from the length of the template (extension time), since native Pfu polymerase synthesizes 1 kb of DNA per 2 minutes. The following profile was used:

5:00 minutes	denaturing at 95°C	
0:45 seconds	denaturing at 95°C	for 25 cycles
0:45 seconds	primer annealing at [2(A+T)+4(G+C)]-3°C	
2:00 minutes	extension at 72°C for each kb of template	
10:00 minutes	72°C	

3.2.1.9. Subcloning

T/A cloning

This method was carried out with the pMOSblue T-vector kit (3.1.7.). The method exploits the template-independent activity of Taq DNA polymerase to add a single adenosine nucleotide to the 3' end of double stranded DNA. Such a PCR product can then be inserted into a thymidine-tailed vector like pMOSblue T-vector and eliminates the requirement for restriction sites being incorporated into PCR primers to produce sticky ends when cleaved. The pMOSblue T-vector allows for blue/white screening.

PCR products were purified by gel extraction and the concentration was estimated by comparing to DNA standards (3.2.1.7.). The vector to insert ratio was adjusted to 1:9 up to 1:14. A master mix was usually prepared containing the following four components, provided by the kit, for each PCR product to be cloned:

10x ligase buffer	1.0 µl
100 mM DTT	0.5 µl
10 mM ATP	0.5 µl
50 ng / ml vector	1.0 µl

For each PCR product to be cloned the following components were added to 3 µl master mix

insert	x µl
water	9.5-x µl
T4 DNA ligase (2 u / reaction)	0.5 µl

3 reactions were prepared for each PCR product to be cloned. One reaction was incubated for 4 hours at room temperature, one reaction was incubated for 2 hours at 16°C before being stored at 4°C overnight. The third reaction was immediately stored at 4°C overnight. The next day the provided MOSblue competent cells were transformed according to the supplier's manual and plated on X-gal / IPTG agar plates and incubated upside down overnight. White clones were picked, DNA was isolated (3.2.1.4.) and the presence of an appropriate insert and its orientation was determined by restriction digest (3.2.1.6) with EcoRI and HindIII and sequencing with the according primers (3.1.9.2.).

Sticky end ligation

This method was the one, predominately used. It allowed cloning of PCR products in-frame into different expression vectors by the use of primers with appropriate internal restriction sites. Vector and insert were digested, whenever possible with two different restriction enzymes to help reducing religation of vector, purified by gel extraction and precipitated. The vector to insert ratio was adjusted to 1:3 up to 1:5. The final ligation mix comprised of the following Amersham products:

2 µl 10 x T4 DNA Ligase buffer
(660 mM Tris-HCl pH 7.6, 66 mM $MgCl_2$, 100 mM DTT, 66 µM ATP)

1 µl T4 DNA Ligase 1:10 diluted with T4 DNA Ligase Dilution Buffer

The ligation mix was kept on ice for up to 1 hour and then transferred to 16°C for 12 to 36 hours. For electroporation (3.2.2.9.), the mix was precipitated and resuspended in 5 µl of water. A negative control without insert was always carried out in parallel to estimate the amount of religated vector.

3.2.1.10. DNA sequence analysis

Sequencing was done using the DNA Sequencing Dye Terminator Kit based on the dideoxy chain termination method (Sanger *et al.*, 1977) with fluorescent dideoxy nucleotides as terminators in a cycle sequencing PCR. This allowed to carry out the reaction in a single tube and to fractionate it in a single 'lane'. Sequence analysis was carried out with an ABI Prism 310 Genetic Analyzer (Perkin Elmer) and ABI Prism software was used to proof read and translate the data into AA sequences.

The following alterations on the recommended protocol were found to improve the quality of the sequence data:

1.) The amount of recommended reaction mix (TRR) per PCR reaction was reduced to one half (4 µl / 20 µl).

2.) The tubes containing the PCR reaction were incubated for 1 minute at 95°C prior to the step cycles of the PCR.

3.) The amount of DNA template was increased from the recommended 100 to 500 ng up to 1 µg per reaction.

4.) After the PCR, the DNA was precipitated in EtOH for 15 minutes on ice, washed and dried and resuspended in 25 µl Template Suppression reagent for each sequence reaction. The sample was mixed thoroughly, denaturated for 2 minutes at 95°C and could then be stored for up to 1 week before being loaded into the sequencer.

3.2.1.11. SDS-Page gel electrophoresis and western blotting

Separation of proteins

The protein aliquots were mixed with 2 x SDS-Page sample buffer and denaturated for 5 minutes at 95°C or, when the aliquots contained imidazole, by incubation at 37°C for 10 minutes. Proteins were separated in a Mini-PROTEAN II Electrophoresis cell (Bio-Rad)

according to the supplier's manual by running a discontinuous polyacrylamide gel (Laemmli, 1970). The acrylamide concentration in the separating (lower) gel was 12.5% and in the stacking (upper) gel 4%. The gels were run in 1 x running buffer with 200 volts. After the run, gels could be stained in Serva blue solution (= Coomassie blue) to visualize the protein bands, or the proteins were transferred to a HyBond-C extra nitrocellulose membrane by a semi-dry western blot. The marker used in Coomassie blue stained gels was the low molecular weight marker (Pharmacia Biotech) according to the supplier's instructions.

The semi-dry western blot protocol

Blotting was performed in a Trans-Blot SD Semidry Transfer Cell (Bio-Rad): the stacking gel was cut off with a scalpel from the separating gel and discarded. The remaining separating gel was measured and ten Whatman 3MM filter paper pieces and one HyBond-C extra nitrocellulose membrane were cut to the same size; five filter paper pieces were prewetted in freshly prepared 1 x transfer buffer and laid onto the Teflon plate of the blotter, onto that stack of filters, the prewetted nitrocellulose, the prewetted gel, and finally the remaining five prewetted filter paper pieces were laid. After air bubbles in this sandwich were removed by rolling a plastic pipette over it, the upper Teflon plate was carefully put on the sandwich and the lid was closed. Blotting was done for 30 minutes at 13 V.

Immunoassay on a western blot

The nitrocellulose was washed 2 x for 5 minutes with 10 ml buffer A in a 50 ml Falcon tube rotating in a Hybridizer HB-2D, as described in 3.2.4.3. at room temperature. The nitrocellulose was then blocked for 1 hour in buffer A / 3% BSA, before the first antibody was applied in an appropriate dilution to the blocking buffer and incubated overnight. The next day, the nitrocellulose was washed 6 x for 5 minutes with 10 ml buffer A, before the second antibody was applied 1:1000 in buffer A / 3% BSA and incubated for 2 hours. Finally the nitrocellulose was washed 4 x for 5 minutes with PBS and was then ready for the visualisation procedure:

If the second antibody was AP-conjugated, visualisation was performed using the BLUEGENE Non radioactive Nucleic Acid Detection System. The nitrocellulose was washed 2 x with AP staining buffer before NBT and BCIP were diluted 1:1000 in AP staining buffer and this solution was applied directly to the nitrocellulose on a glass plate. The reaction was allowed to develop in the dark.

If the second antibody was HRP-conjugated, the nitrocellulose was transferred on a glass plate and the HRP staining solution was poured directly on to the nitrocellulose. Again, the reaction was allowed to develop in the dark.

3.2.2. Shotgun approach (1)

3.2.2.1. Overview

The aim of this approach was to identify *P. falciparum*-derived adhesion molecules by expressing an ItG2.F6-cDNA library in COS 7 cells. The transfected cells were used in a cell-cell adherence assay with screening cells expressing huICAM-1 on their surface, a receptor known to be involved in sequestration of strain ItG2.F6. COS 7 cells, which accumulated bound huICAM-1 expressing cells on their surface, were picked, the cDNA clones were isolated, and used for a re-transfection of COS 7 cells to perform a second screen.

3.2.2.2. Testing for adhesion of *P. falciparum*-infected erythrocytes on monolayers of L-huICAM-1-, L- and C32 cells

Preparation of adhesion assay slides

3-well glass slides were rinsed with 70% EtOH, dried and autoclaved prior to use. The slides were placed in Petri dishes (5 slides per ∅ 145 mm). 200 µl of cell-suspension (500'000 L-cells, L-huICAM-1 cells or C32 cells / ml complete medium) were seeded on each well, the Petri dishes containing the slides were put in a 37°C / 5% CO_2-incubator and the cells were allowed to attach and grow overnight. These slides were used directly the next day (fresh cells) or were fixed and stored for further experiments (fixed cells).

Fixation and storing of adhesion assay slides

Slides with a confluent cell-layer were fixed and stored directly in the surrounding Petri dish: each dish was washed twice with 40 ml DPBS (applying the buffer, swirling the dish moderately 4-5 times and aspirating off the buffer) and then fixed for 1 hour at room temperature in 40 ml of freshly prepared 1% formaldehyde in DPBS. The slides were then transferred to a new dish, washed twice with DPBS and then overlaid with 50 ml RPMI 1640 storage medium. The dishes were then wrapped in Saran wrap and were stored at 4°C for up to 3 months.

iRBC adhesion assay on slides

Fixed or fresh slides were transferred into a new Petri dish, the liquid was aspirated off the black masks of the slides, but not from the wells, and one big circle was drawn around all three wells with a water-repellent PAP-pen. Subsequently, the remaining liquid on the cells was aspirated off and was replaced with 600 µl binding medium. The slides were put

aside until the *P. falciparum* cultures were processed: the iRBC suspensions from parasite cultures (5-10% parasitaemia) were centrifuged and resuspended in binding medium to 1% haematocrit. 900 µl of iRBC suspension were applied on each slide and the cells were allowed to adhere for 1 hour in the incubator (37°C / 5% CO_2), with gentle agitation every 15 minutes. After 1 hour, the slides were washed 4 x with 40 ml of binding medium and then air dried under a ventilator, fixed with MeOH, stained with 5% Giemsa solution and mounted in Eukitt, as described above (3.2.1.2). Adherent iRBCs were counted by light microscopy in 4 mm^2 areas on all three cell mono-layers, fixed and unfixed, with an uninfected RBC suspension as a negative control.

3.2.2.3. mRNA-isolation from a schizont-enriched ItG2.F6 culture

Synchronisation of parasite cultures

Prior to the isolation of mRNA of the strain ItG2.F6, the culture was synchronized for ring stage parasites according to the protocol of Matile and Pink (1990), splitted to 15 10mm culture dishes and then cultivated for an additional 18 hours until most of the stages were late trophozoites or early schizonts. Then, 5 culture dishes were pooled and after centrifugation (four minutes, 350 g, no brake) resuspended to 50% haematocrit in culture medium to perform a final iRBC-adhesion assay, with a small aliquot, as described in 3.2.2.2. After the small aliquot was removed, the remaining parasites from each pool were isolated by saponin lysis.

Saponin lysis (isolation of parasites from an iRBC-culture)

Parasite cultures were sedimented as described above. For the following, all buffers were ice-cold and the centrifuge was cooled to at least 10°C. The pellets were washed twice by resuspending each of them in 10 ml of parasite cultivation medium and centrifugation at 350 g for 5 minutes without break. The supernatant was discarded and the pellets were resuspended in a small volume of medium. These suspensions were brought to the original volume of the parasite cultures (5 cultures = app. 50 ml) with Saponin-lysis buffer and incubated for 20 minutes on ice. Then, the parasites were pelleted with 600 g for 15 minutes and washed twice in 1 x SSC buffer at the same conditions. Finally each pellet was resuspended in 500 µl of PBS.
Such a suspension from 5 cultures was dropped directly into liquid nitrogen. The resulting 13 frozen pearls were stored at -80°C, whereas the suspension from the second and third pool was used to isolate the mRNA.

Isolation of mRNA from *P. falciparum* pellets

The mRNA was isolated from both, fresh and frozen *P. falciparum* pellets, within a week. The fresh suspension on ice (10 synchronized, saponin-lysed cultures of ItG2.F6 in 1000 µl PBS) was processed immediately, the frozen pellets within 6 days. The mRNA isolation was done with the Fast Track 2.0 Kit (Version D) according to the supplier's manual, with the only difference, that the elution buffer was prewarmed to 65°C. After precipitation, the mRNA from both isolations was pooled and after the analysis of the amount and size of the mRNA with a small aliquot on a RNase-free agarose gel (3.2.1.7.), the sample was shipped on dry ice to Invitrogen.

3.2.2.4. Custom-made cDNA library (Invitrogen)

Upon arrival of the *P. falciparum* mRNA, the cDNA library was constructed by Invitrogen with the following specifications:

Starting material:	mRNA of *P. falciparum*
Priming for first strand cDNA synthesis:	Oligo dT (NotI)
Ligation with the following adapters:	BstXI / EcoRI
Separation on an agarose gel and size fractionating at:	500 Bp
Unidirectional ligation into the vector:	pcDNA3.1+
Transformation into the following host vector:	Top10F'

6 tubes containing 2 ml each were shipped on dry ice to the STI, where they were stored at -70°C. The titer of the library was estimated different dilutions of the cDNA library on LB/Agar plates under ampicillin selection. For amplification, 2 ml of cDNA (1 tube) were then diluted in 300 ml LB medium and incubated in a shaker for 3 hours at 37°C. A 12 ml-aliquot of this culture was removed and pelleted, resuspended in 10 ml LB / 50% glycerol, divided in 10 Eppendorf tubes and stored at -70°C (glycerol-stocks of 1 x amplified cDNA). The remaining culture was pelleted and processed according to the Qiagen Mega Plasmid Purification protocol. The DNA was finally eluted in 400 µl TE. The DNA-concentration was measured photospectrometrically and adjusted with the appropriate amount of TE to 1 µg DNA / µl TE. This 1 x amplified cDNA was then aliquoted and stored at -70°C and used for all further experiments.

3.2.2.5. Transient transfection of COS 7 cells

A) Transfections with chloroquine / DEAE-dextran for cell-cell adherence assays

Preparation

Prior the transfection, COS 7 cells were grown to 75-90% confluency in 75 cm^2 tissue culture flasks. 4 x 10^5 COS 7 cells were necessary for one transfection. Aliquot(s) of chloroquine stock solution (5.16 mg / ml) and DEAE-dextran stock solution (100 mg / ml) were thawed on ice, whereas PBS, DMEM / 10% FCS, HBD and Trypsin / EDTA were prewarmed to 37°C. For each transfection, a Falcon 2059-tube was prepared, since PP-tubes are known not to interact with the transfection reagents.

Transfection

To the appropriate amount of HBD (3 ml / transfection) 1/100 volume of chloroquine was added and, after mixing, it was divided into two equal aliquots (A) and (B). To (A) 1/200 volume of DEAE-dextran was added (final concentration of 500 µM) and mixed, whereas (B) was divided into as many 1.5 ml aliquots as transfections were planned. To each aliquot of (B) 3 µg of cDNA was added, then 1.5 ml of (A) was added, the tubes were gently mixed by tapping and put aside until the COS 7 cells were harvested (3.2.1.1.), resuspended in HBD and counted (3.2.1.1.). 4 x 10^5 COS 7 cells, resuspended in HBD (not more than 200 µl) were added to the DNA / DEAE-dextran mixes, then the tubes were vortexed and put into a 37°C water bath for exactly 2 hours. This incubation time is very critical: a shorter incubation would decrease the transfection-rate, but a longer incubation would damage or kill the majority of the COS 7 cells.

Seeding the cells

After 2 hours, the COS 7 cells of each transfection mix were pelleted at 300 g for 5 minutes, the supernatant was carefully aspirated off to 1 ml, the pellet was again resuspended in 10 ml of HBD and the centrifugation step was repeated. Each pellet was finally resuspended in 12 ml DMEM / 10% FCS and seeded in tissue culture-treated Petri dishes (∅ 150 mm). These dishes were placed on a steel plate (to guarantee homogeneous heat conduction) in the incubator for 48-60 hours, with an exchange of medium after the first night, before they were used for a cell-cell adherence assay.

B) Transfections with transfection lipids

Comparative transfection of COS 7 cells with 6 different lipids

4 lipids (Lipofectin, LipofectAmine, Cellfectin and DMRIE-5) were supplied in a transfection reagent sample pack by Gibco BRL, SuperFect by Qiagen and LipoTaxi by Stratagene.

In two 24 well tissue culture plates COS 7 cells were seeded at a density of 3 x 10^4 cells / well in 1 ml of DMEM / 10% FCS one day prior to the transfection. Cells should be 60-80% confluent at the time of transfection. The day of transfection, two fresh 24 well tissue culture plates were taken, 6 rows were labelled from A-F and each column from 1-6. The plates containing the cells were labelled identically. The labelled 36 wells were filled with 300 µl DMEM without serum and 20 µl Lipofectin were added to well A1 as well as 10 µl LipofectAmine (B1), 15 µl Cellfectin (C1), 10 µl DMRIE-5 (D1), 10 µl SuperFect (E1) and 10 µl LipoTaxi to well F1. The plates with the lipids were incubated for 30 minutes at room temperature before 3.2 µg DNA / 300 µl DMEM were added to the wells of column 1 and the plates were incubated again for 15 minutes at room temperature. Then, the lipid-DNA complex was serially diluted by transferring 300 µl from column 1 to 2, mixing, transferring 300 µl from column 2 to 3 and so on, until the final 300 µl removed from well A6 were discarded.

The COS 7 cells were washed once with 1 ml of PBS and the 300 µl lipid-DNA dilutions were transferred from the wells without cells to the corresponding wells with cells. The cells were incubated for 5 hours at 37°C / 5% CO_2, then the lipid-DNA dilutions in each well were replaced with 1 ml of DMEM / 10% FCS and the plates were incubated overnight at 37°C / 5% CO_2. In parallel, a transfection with chloroquine / DEAE-dextran was carried out as described above. These transfected COS 7 cells were seeded in a tissue culture treated Petri dish (∅ 100 mm).

The DNA used in this comparative analysis was the vector pcDNA3.1-EGFP, which, upon expression of GFP, can be easily used as a reporter system: cells expressing GFP will emit bright green light (500 nm) when exposed to UV. Since LipoTaxi yielded the highest transfection-rate (see results), it was further used for the transfection of mammalian cells.

Transfection of COS 7 cells with LipoTaxi for cell-cell adherence assays

The cells were splitted 24 h prior transfection and seeded into tissue culture treated Petri dishes (∅ 100 mm). They had to be 60-80% confluent at the time of transfection. For each transfection 2.7 ml serum-free DMEM was transferred to a Falcon 2052 tube (PS) since PS-tubes yield superior results to PE- or PP-tubes according to the supplier's information.
100 µl of LipoTaxi transfection reagent was added to a tube, the tube was tapped to mix and 5-10 µg of cDNA was added. After incubation for 20 minutes at room temperature, the cultivation medium was removed from the cell culture dishes by aspiration, 5 ml of serum-free DMEM was added to the transfection mix and, with a pipette, the entire mix (7.8 ml) was transferred dropwise to the dish, while swirling the dish. The dish was then incubated for 5 hours at 37°C / 5% CO_2 before adding 8 ml DMEM / 10% FCS, and was further incubated overnight. The next morning, the medium was replaced with 15 ml of fresh, prewarmed DMEM / 10% FCS. The cells were incubated for another 24-72 hours, before they were used for a cell-cell adherence assay.

3.2.2.6. The cell-cell adherence assay

In a preliminary assay, L-huICAM-1 cells were seeded and allowed to grow to an almost confluent cell-layer, before untransfected COS 7 cells were used as screening cells, analogous to the iRBC adhesion assay (3.2.2.2.). Since the COS 7 cells did attach to the dish during the incubation step, the background of screening cells was quite high. Therefore the cell-cell adherence assay was altered as follows: chloroquine / DEAE-dextran transfected COS 7 cells were seeded at a low density (4 x 10^5 COS 7 cells per tissue treated Petri dish, (∅ 150 mm). During the incubation of 24-72 hours after transfection, these mostly singular cells remained so or formed small patches of 4 to 8 cells, which simplified the screening under the microscope.

The cell-cell adherence assay protocol

Prior to the assay, L-huICAM-1 cells were harvested, pelleted and resuspended in binding medium (3.2.1.1.), then, the dishes with the adherent, cDNA-transfected COS 7 cells were taken from the incubator and washed smoothly with 12 ml of binding medium. The screening cells were applied in a 10-fold excess to each dish (4 x 10^6 L-huICAM-1 cells / dish) in 12 ml binding medium. The dishes were then placed on a gently agitated rocker or shaker for 1 hour with a quarter-turn of the dishes by hand every 15 minutes. With a rocker, the screening cells were dispersed more homogeneously than with an orbital shaker, where areas with a high background resulted at the margin of the dishes. After 1 hour, the dishes were washed 4 x with 12 ml of binding medium (applying gently, swirling the dish,

aspirating off) and the cells were overlaid with 10 ml of screening / storing solution (10 mM EDTA pH 8.0 in water). Like that, the assay was ready to be screened under the microscope, but the assay could also be fixed and stored to be screened up to 3 days later.

Fixing and storing the cell-cell adherence assay

The fixing protocol was improved with ongoing numbers of assays. The fixation method used for each specific assay is noted in the corresponding results section.
1st fixation method: after the 4th wash was aspirated off, the dish was washed once with 1 x PBS, then 12 ml of 1% glutaraldehyde / 2% formaldehyde in PBS were applied. The dishes were incubated for 1 hour at 37°C, washed twice with PBS and overlaid with 12 ml of 10 mM EDTA pH 8.0 in water. In that solution, the dishes could be stored at 4°C for up to 3 days or screened directly under the microscope.
The 2nd method was the same as above, but the incubation step was carried out for 1 hour at 4°C.
The best results were obtained using the following fixation method: The cells were fixed for 1 hour at 4°C with 1% glutaraldehyde / 2% formaldehyde in PBS, then the fixation solution was aspirated off, followed by a second fixation step with 50% MeOH / 50% acetone for 15 minutes on ice. The second fixation solution had to be thoroughly mixed before applying it to the dish, since the acetone attacks the plastic. With the 1st fixation step, the cell-cell interaction was fixed, whereas the 2nd fixation step fixed the cells onto the dish. After these two fixation steps, the dishes were treated as above.

Screening the cell-cell adherence assay

The dishes were screened under phase contrast with a microscope using a magnification of 4 to 20-fold. A COS 7 cell was considered positive and marked, if at least 3 independent screening cells (L-huICAM-1 cells) were attached to the surface or at the margin of a single COS 7 cell and only if the background around a positive cell was low, the cell was selected to be picked.
From the second assay on, the screening method was simplified by staining the screening cells with ethidium bromide (EB) prior to the adhesion assay, before the screening cells were harvested. 1/200 volume of 1% EB stock solution was added to the screening cells and incubated for 1 hour at 37°C. The dishes were then screened under dark field by a fluorescent microscope with an UV-filter. If patches of screening cells were found, the light source was switched to normal light, to examine possible adherence of the screening cells to a COS 7 cell.

3.2.2.7. Picking positive COS 7 cells

Positive cells were picked either with a pipette or with a scalpel. A Drummond Pipette with long, reusable, cut off Drummond gel loading tips was used or an Eppendorf Crystal Pipette (1-10 µl), equipped with Milian multiplex gel loading tips: To pick, the screening / storing solution was aspirated off from the cells and the dish was washed once with water, to prevent crystallisation of remaining traces of PBS, and was allowed to dry. 2 µl of water was applied on the area of the cell to be picked, and the cell was observed under the microscope. As soon as the area started to dry out again, the cell could be scrapped of with the tip of the pipette and was sucked in, often together with some neighbouring COS 7 and L-huICAM-1 cells. This small aliquot was immediately transferred into 200 µl of freshly prepared Hirt extract solution containing proteinase K. This pick was either processed further as a "single pick", or additionally picked cells were pooled together in one aliquot of Hirt extract solution and were further processed as a "pooled pick".
In later experiments, positive cells were picked with a needle-scalpel: The dish was also allowed to dry, and before it was completely dry, the cell was scraped off with the scalpel, where the positive cell could actually be seen on the blade of the scalpel.

3.2.2.8. Isolation of cDNA clones using the Hirt extraction method

Immediately after the positive cell(s) were transferred into 200 µl of Hirt extraction solution, the tube was incubated for 30 minutes in a 37°C water bath before adding 50 µl of 5 M NaCl and, after mixing, was stored 4°C overnight. The next day, the SDS / protein-precipitate was spun at 14'000 g for 10 minutes at 4°C. The supernatant was transferred to a fresh Eppendorf tube, 20 µg of glycogen was added as a carrier and 2.5 volumes of absolute EtOH were added to precipitate the DNA; the tube was thoroughly mixed and stored at -20°C for at least two hours. After centrifugation for 30 minutes at 14'000 g, the glycogen-DNA pellet was washed once with 70% EtOH, centrifuged again for 10 minutes, air dried for ten minutes, and redissolved in 2 to 5 µl of dH_2O. The plasmids, isolated in such a manner form a single- or pooled pick were then ready to use for the transformation of *E. coli* cells.

3.2.2.9. Transformation of *E. coli* cells by electroporation

Preparation of electrocompetent cells

Commercially available electrocompetent Top10F' cells were purchased from Invitrogen (Table 3-III). With an aliquot of these cells, further electro-competent cells were prepared: a 10 ml overnight culture of Top10F' electrocompetent cells was diluted with 990

ml LB and was incubated in a shaker at 37°C until an OD 600 of 0.5 was reached. The culture was chilled on ice for 30 minutes and centrifuged at 4000 g, 4°C for 15 minutes. The supernatant was discarded, the pellet resuspended in 1 litre of ice-cold Millipore water and centrifuged again. The resulting pellet was resuspended in 0.5 litre of ice-cold water and incubated 30 minutes on ice, spun and the pellet was resuspended in 20 ml ice-cold water containing 10% glycerol. This suspension was centrifuged again and resuspended in 2 ml of water / 10% glycerol. This suspension was aliquoted (40 µl or 80 µl) in prechilled Eppendorf tubes on dry ice and stored at -70°C.

The electroporation protocol

The *E. coli* cells were transformed with an Equibio electroporator according to the following protocol: electrocompetent cells were thawed on ice, 40 µl transferred to a precooled 2 mm electroporation cuvette and mixed with 2 µl DNA (3.2.2.8.) and incubated for 1 minute on ice. The cuvette was then wiped dry with a Kleenex tissue, tapped to remove air bubbles from the 2 mm gap, and placed into the EasyjecT electroporator to perform a pulse with the following specifications:
voltage: 2.5 kV, capacity: 25 µF, shunt: 201 ½ and pulse time 5 msec.

Further treatment of transformed cells

After the pulse, 800 µl SOC medium without antibiotics were added as fast as possible to the cuvette, mixed and transferred to a cultivation tube. The tube was incubated in a shaker for 1 hour at 37°C. This culture was plated out in 180 µl aliquots on 5 ampicillin plates. The glass spatula was not sterilized between each step, in order to prevent loss of clones. The plates were then incubated upside down over night at 37°C.

3.2.2.10. Analysis of cDNA clones and second screens

Picking of cDNA clones

The colonies were counted and picked with an autoclaved tooth pick. The tooth pick was then used to scratch a corresponding numbered field on an ampicillin plate, equipped with a drawn grid on the back side of the dish. After that, the tooth pick was placed in the corresponding numbered culture tube containing 5 ml LB / ampicillin and was incubated for 8 hours in a shaker at 37°C.

If a pick, from a single cell or from pooled cells, yielded more than 200 colonies, the cultures were pooled after 8 hours in batches of 5-10 prior to the isolation of cDNA clones. Less than 200 colonies per pick were processed individually. The master plate, containing the clones arranged in the grid, was stored at 4°C for up to 1 month and was used as a security back-up for the clones of each pick.

Isolation of cDNA clones from *E. coli* cultures

This was done as described in 3.2.1.4: Isolation of plasmids from *E. coli* cultures and in 3.2.1.5: Precipitation of DNA.

Analysis of the cDNA clones

First, the clones were analyzed for inserts (if any). Each cDNA clone was digested with the restriction enzymes ApaI (Promega) and BamHI (Gibco) in reaction buffer A (Promega) for 1 hour at 30°C and then loaded on a 0.8% agarose gel.

Second screens

Every cDNA clone with an insert was used for a second transfection of COS 7 cells. Therefore, the clones of a single pick, that contained an insert, were pooled in equally amounts. Sometimes, a pool of the clones bearing the biggest inserts, was additionally made. Details are given in the results section. COS 7 cells were transfected with these pools and a cell-cell adherence assay was performed as in the first screen, with one dish per cDNA-pool. The dish was evaluated as the first screen, but with the expectation to see a 10-100 fold increase of positive COS 7 cells.

3.2.2.11. Positive controls of the shotgun approach (1)

A positive control for transfection

The EGFP gene from a commercially available vector (pEGFP-1, Clonetech, Heidelberg, D) was ligated into the pcDNA3.1 vector at the HindIII / NotI sites of the polylinker (see: 3.2.1.9.). This control was used for every further transfection, in order to estimate the transfection-rate in each experiment.

A positive control for picking and Hirt extraction

COS 7 cells were transfected with a cDNA clone containing a DBL1 domain. Four individual cells were picked and the cDNA clone was isolated as described (3.2.2.8.), but instead of a transformation of *E. coli* cells by electroporation (3.2.2.9.), a PCR was performed with the precipitated DNA in order to amplify the DBL1 domain.

Positive controls for expression in COS 7 cells (1)

The construct used to estimate the transfection-rate in each experiment (pcDNA3.1-EGFP) was also an expression control. But EGFP was already optimized for mammalian expression by adjusting the codon usage to a mammalian expression system. For a second construct, the complete gene of huICAM-1 was amplified by PCR from the genomic DNA of L-huICAM-1 cells and cloned in frame into the pcDNA3.1-EGFP vector at the HindIII / BamHI sites of the polylinker. This construct was used as an expression control and as a control for the recognition of leader sequences and the transport of proteins to the surface by COS 7 cells.
Four further constructs, carrying plasmodial genes, will be dealt with in the shotgun approach (2), section (3.2.3.5.).

3.2.3. Shotgun approach (2)

3.2.3.1. Overview

The aim of this approach was to identify *P. falciparum*-derived antigens, expressed intra- or on the surface of COS 7 cells, by the use of mAbs, produced during the PhD-thesis of K. Willimann (Willimann, 1996), which recognized iRBCs and intraerythrocytic *P. falciparum* stage proteins.

3.2.3.2. Transfection of COS 7 cells in 6 well plates

COS 7 cells were seeded in 12 well plates ($9x10^4$ cells / well), allowed to settle down and, after 2 hours, were transfected with the LipoTaxi transfection reagent. The transfection protocol (3.2.2.5b.) was adapted to the size of the wells: for each transfection, 88 µl serum-free DMEM were transferred to a Falcon 2052 tube (PS), then 8 µl of LipoTaxi transfection reagent was added to each tube, the tubes were tapped to mix, and 3-4 µg of cDNA was added to each tube. After 20 minutes, 200 µl of DMEM / 5% FCS was added. All further steps followed the protocol given above. Medium was exchanged after 12 hours, and the

plates were incubated for another 24-48 hours, before they were used for an IFA test. On every plate, one well was transfected with the pcDNA3.1-huICAM-1-EGFP-construct to estimate the transfection- and expression-rate of the transfected cells after 24 or 48 hours and to control the assay conditions by detection of huICAM-1 with an appropriate mAb (see Table 3-V).

3.2.3.3. The indirect immunofluorescence assay (IFA) on adherent cells

Performing the IFA

First, the transfection-rate of the transfected COS 7 cells was checked under the fluorescence microscope, by determining the percentage of green fluorescent cells in the positive control. If the percentage exceeded 50%, the IFA was performed. The cells were washed once with PBS and then either fixed with a freshly prepared and thoroughly mixed 1:1-mixture of Methanol / Acetone for 15 minutes on ice (good mixing is very important since pure acetone attacks the plastic), or not fixed at all, to detect antigens on the surface of the cells only. If the cells were fixed, the fixative was aspirated off, the cells were washed twice with PBS, and then incubated with 200 µl PBS / 5% FCS for 30 minutes at 37°C to saturate and block unspecific binding. After removing the blocking buffer, 150 µl of PBS / 0.5% FCS, containing the first antibody in an appropriate concentration, was applied: αICAM-1 (MEM112) was diluted 1:1000, whilst the supernatants of K. Willimann (Table 3-V) were applied undiluted or diluted 1:20. The plates were wrapped with Saran wrap to prevent evaporation of the antibody-containing solution, and were put on a rocker for 1 hour at room temperature. After this incubation step, the first antibodies were aspirated off, the cells were washed three times with PBS / 0.05% Tween 20, before the second antibody, diluted 1:400 in PBS / 0.5% FCS, was applied. The second antibody was either HRP-labelled rabbit / αmouse total Ig or FITC-labelled goat / αmouse IgG F(ab')2. The incubation step and the further washing steps were the same as with the first antibody. Now the IFA could be screened directly under the fluorescence microscope (FITC) or was ready for the staining procedure (HRP).

The staining procedure for HRP-labelled antibodies

500 µl AEC / DMF stock was mixed in 10 ml of 0.1 M sodium acetate, pH 4.8 (Table 3-XII) and 1.5 µl of 30% H_2O_2 was added. 300 µl of this staining solution was applied to each well, the plate was wrapped in aluminium foil (light sensitive reaction), and put on a rocker for 20 to 40 minutes with a short check of the positive control every ten minutes. When the well with the positive control appeared reddish by eye, the IFA was ready to be screened by light microscopy.

3.2.3.4. Further Steps in the shotgun approach (2)

Transfected COS 7 cells, which reacted with the mAbs of the supernatants, became red (HRP) or were green fluorescent (FITC). These cells were picked as described in 3.2.2.7; if more than one cell reacted per well, these cells were pooled, the cDNA-clones were extracted according to the Hirt extraction protocol (3.2.2.8.), and the DNA was further processed, as described in the shotgun approach (1), and was finally used to perform a second screen. For a second screen, all isolated plasmids, derived from a first screen, were pooled, and this pool was used to transfect COS 7 cells as in the first screen. The second screen was evaluated like the first screen, with the expectation to see a 10- to 100-fold increase of positive cells.

3.2.3.5. Positive controls of the shotgun approach (2)

Positive controls for expression in COS 7 cells (2)

The methods, described in the shotgun approach (2), were also used to establish additional positive controls for the expression of plasmodial proteins in COS 7 cells (see 3.2.2.11.). The MSP2-gene was isolated from cDNA of strain ItG2.F6, using a pair of primers, bearing a HindIII-site in the forward primer and a BamHI-site in the reverse primer (Table 3-XV) and was cloned into the pcDNA3.1- and the pcDNA3.1-EGFP-vector according to the sticky end ligation protocol (3.2.1.9.). A 1050 bp long N-terminal fragment of the MSP1 gene from the strain Ro71, cloned into the pGEM-T vector, was amplified by PCR with a pair of primers, bearing an EcoRI-site in the forward primer and a BamHI-site in the reverse primer to clone it in the pcDNA3.1-vector. Another pair of primers, bearing a KpnI-site in the forward primer and an ApaI-site in the reverse primer was used to clone it into the pRE4 vector for expression of a chimeric protein with the HSVgD flanking regions.
The pRE4 vector has no KpnI site in the polylinker. Therefore the amplified MSP1 fragment was only digested with ApaI, whereas the pRE4 vector was digested with ApaI (sticky end) and PvuII (blunt end) and the insert was then ligated into the sticky/blunt digested vector according to the sticky end protocol, with the only exception of using Pfu polymerase for the PCR amplification to ensure a blunt end at the insert. The KpnI-site was introduced to be able to digest the insert from the vector, since the PvuII-site was lost by ligation.
The 83.1 epitope of MSP1 that was used in the synthetic peptide malaria vaccine SPf66 was present in the MSP1 fragment of strain Ro71 and can be detected with mAb 9.22 (Helg, 1997). The mAb 9.22 was used to detect expression of MSP1, whereas a serum pool of malaria exposed adults from PNG was used to detect expression of MSP2

3.2.4. Direct approach (1) (mammalian expression system)

3.2.4.1. Overview

The aim of this approach was to extract cDNA clones, containing full-length- or parts of *var* genes, from the cDNA library and to use these clones to transfect COS 7 cells to perform cell-cell adherence assays as described in the shotgun approach (1). Two methods were used to identify *var* gene-clones: PCR with appropriate primers and the colony lift protocol with DBL1- or ATS domains as a hybridisation probe.

3.2.4.2. PCR amplification of DBL1- and ATS domains

In a first step, DBL1- and ATS domains were amplified from genomic DNA of the strain K1, in a second step, DBL1 domains were also amplified from the cDNA library. The PCR reaction was performed with Taq polymerase as described in 3.2.1.8., either with degenerated primers, or with a pool of defined primers (Table 3-XV). The reaction conditions of the PCR were gradually optimized by increasing the temperature of the annealing step to diminish the amount of unspecific PCR products. The optimal conditions are given below:

PCR amplification of the DBL1 domains

The primer pair used in this PCR consisted of the two degenerated primers VARDBL5 (forward primer) and VARDBL3 (reverse primer). The conditions were as follows:

5:00 minutes	denaturing at 95°C	
1:00 minute 2:00 minutes	denaturing at 95°C primer annealing and extension at 72°C (2-step PCR)	for 30 cycles
10:00 minutes	72°C	

PCR amplification of the ATS domains

The primers used in this PCR consisted of the degenerated primer ATS5 (forward primer) and a 1:1 pool of ATS3.1 and ATS3.2 (reverse primers). The conditions were as follows:

5:00 minutes	denaturing at 95°C	
1:00 minute 0:45 seconds 2:00 minutes	denaturing at 95°C annealing at 62°C extension at 72°C	for 30 cycles
10:00 minutes	72°C	

Cloning of DBL1- and ATS domains

The resulting PCR products (DBL1 domains of K1 and cDNA, ATS domains of K1) were purified by gel extraction (3.2.1.7.) and ligated by the T/A cloning method into pMOSblue T-vector (3.2.1.9.). The DNA was isolated from positive clones and the remainder of the *E. coli* culture was used to prepare glycerol stocks (3.2.1.3.) Clones were digested with EcoRI and HindIII to analyze the length of the insert. The DBL 1 domains were sequenced in a later step, using the T7 promoter primer and the U19mer primer (3.1.9.2.)

3.2.4.3. Colony lift and southern blot hybridisation (non radioactive)

BioPrime-labelling of the hybridisation probes

All cloned DBL1 domains were amplified by a normal PCR with Taq polymerase as above, but using the 10 x dNTP mixture of the BioPrime DNA labelling Kit. In this mixture, for each dNTP, the concentration is 2 mM, but for dCTP and biotin-14-dCTP, for which the concentration is 1 mM each. In this PCR reaction the annealing temperature was lowered to 65°C (instead of 72°C), since unspecific products were no longer a problem with the cloned templates. The resulting PCR products were purified by gel extraction, eluted in TE and stored at -20°C for further use.

To optimize the following colony lift / southern blot protocol, genomic DNA of strain K1 was used as a template to get random primed, biotinilated hybridisation probes. With these probes, different membranes, buffers and incubation times were tested. The best protocol was then used with the labelled DBL1 domains. The random primed probes were generated with the BioPrime DNA labelling Kit according to the supplier's protocol.

The colony lift protocol

5 agar plates containing ampicillin were used for each colony lift. 100 µl of 1:40'000 diluted 1x amplified cDNA (3.2.2.4.) was plated on each plate, yielding approximately 5000 colonies per plate after overnight incubation. The next day, a positively charged nylon membrane (HyBond-N+) was laid on the colonies of a plate, asymmetrically stabbed 4 times with a needle, carefully lifted from the plate, and placed, with the colonies on the upper side, onto a fresh agar plate. This procedure was repeated with all plates, then all 10 agar plates, the five bearing the nylon membranes and the five with the remainder of the lifted colonies, were incubated upside down at 37°C for 4 to 5 hours. The plates with the regrown colonies were wrapped with parafilm and were stored at 4°C up to 2 weeks. From the other plates the membranes were carefully removed and set, colonies on the upper side, onto Whatman 3MM filter paper; the plates were discarded. Three sets of filter paper were prepared. The first filter (now with the membranes on it) was soaked in 0.5 M NaOH and the membranes were incubated for 5 minutes on it before being transferred by a forceps to the second filter, soaked with 1 M Tris-HCl, pH 7.5. After 5 minutes, the membranes were transferred to the third filter, soaked with 0.5 M Tris-HCl, pH 7.5 / 1.25 M NaCl and were incubated for 5 minutes, before finally being transferred into a dish with 2 x SSC to be washed for 5 minutes. The washed membranes were dried between filters for 2 hours. The DNA on the membranes was then cross linked to the nylon in a UV Strata Linker 1800 (Stratagene) with 'auto link' setting. The nylon membranes could now be stored for up to 3 days between filter papers or were directly processed in a southern blot.

The southern blot protocol for colony lifts

The membranes were applied to glass tubes (Techne), put in a Hybridizer HB-2D (Techne, Cambridge, UK), and were washed for 6 hours at room temperature in 20 ml Prehybridisation buffer, with buffer exchange every 1 hour, and then prehybridized for 2 hours in 20 ml Prehybridisation buffer at 59°C. Hybridisation solution consisted of 20 ml Prehybridisation buffer with 250 µl of the denaturated (10 minutes at 100°C and then snap cooled) BioPrime-labelled pool of DBL1 domains. After 2 hours, the Prehybridisation buffer was replaced by the prewarmed hybridisation solution and the membranes were incubated overnight at 57°C. The next day, the membranes were washed with increasing stringency:
4 washes at 57°C with 50 ml 2 x SSC, 0.1% SDS for 10-15 minutes, followed by 1 wash at 57°C with 50 ml 0.2 x SSC, 0.1% SDS for 10 minutes sharp. Then the membranes were rinsed with 2 x SSC at room temperature and washed once with Southern buffer at room temperature. The membranes were then blocked for 1 hour at 57°C in Southern buffer with 3% BSA to reduce unspecific binding.

Visualisation

Visualisation was done according to the BLUEGENE Non-radioactive Nucleic Acid Detection System: after the membranes were blocked, streptavidine, conjugated to alkaline phosphatase (SA-AP), was applied to the membranes, 1:1000 diluted in Southern buffer for 10 minutes at room temperature. The membranes were then washed 3 x with 100 ml Southern buffer for 10 minutes and 2 x with Buffer 3 for 5 minutes at room temperature. After adding the substrate for the alkaline phosphatase, the reaction was left in the dark to proceed until the colour developed (10-20 minutes). The membranes were then demineralized with Millipore water and were stored between filter papers. A dot blot with decreasing concentration of cloned DBL1 domains in pMOSblue T-vector was used as a sensitivity control for the visualisation step.

3.2.4.4. Colony lift and southern blot hybridisation (radioactive)

Radioactive labelling of the hybridisation probes

Two pools, containing either all cloned DBL1- or all ATS domains respectively, were prepared. Both pools were used as a template to get α-^{32}P labelled hybridisation probes: 25 ng of DNA pool, diluted in 4.2 µl dH2O was denaturated for 10 minutes at 95°C and snap cooled on ice. The following mix was added and incubated at 37°C for 30 minutes:

4.2 µl dH2O containing 25 ng of the denaturated DNA pool (DBL1 or ATS)
2.0 µl 10 x restriction digest buffer React 2 (Gibco BRL)
2.0 µl Fw Primer (200 ng) (VARDBL5 or ATS5)
2.0 µl Rev Primer (200 ng) (VARDBL3 or ATS3.1 + ATS3.2)
3.0 µl dNTPs without dCTP (25 µM each)
0.8 µl nuclease free BSA (10 mg/ml)
5.0 µl α-^{32}P dCTP (10 µCi/µl)
1.0 µl Klenow polymerase fragment (2u/µl)

Radioactive hybridisation and visualisation

The colony lift protocol was exactly the same for radioactive and non-radioactive hybridisation. The southern blot protocol follows the one given above with the following alterations:

- radioactive hybridisation took place in a Bachofer 400HY Hybridizer (Reutlingen, D).
- hybridisation was carried out overnight at 58°C.
- hybridisation solution consisted of 10 ml Prehybridisation buffer mixed with 10 µl of the denaturated (10 minutes at 100°C, snap cooled) α-^{32}P-labelled pool of DBL1- or ATS domains.

Visualisation was performed autoradiographically using x-ray films (Kodak X-Omat AR Scientific Lightening Film) in a Quanta III cassette (Du Pont) containing a Cronex intensifying screen at -70°C for 6 to 96 hours, depending on signal strength. Kodak GB-X Developer and GB-X Fix were used to develop the x-ray films.

3.2.4.5. Isolation of *var* gene clones from the cDNA library

Identification and picking of positive clones

First, every membrane was matched to its plate by the aid of the 4 asymmetrically pinned holes (mirror-oriented). Like that, the colonies, which were stained on the membrane (non radioactive) or were visible on the x-ray films (radioactive), were identified on the plate and a small region, containing the suspected colony, was picked with a pipette tip and applied to 1 ml LB, stirred, and 50 µl of that suspension was taken and diluted 1: 40. 100 µl were plated out on a new plate: such a plate was used for a second screen to verify the first screen. Furthermore, a second screen made it possible to accurately pick a single colony, since the colonies were much less dense than in the first screen.

The second screen was performed exactly as the first one. The remainder of the 1:40 diluted suspension was grown overnight, the DNA was isolated and used as a template in a test PCR with the DBL1 (ATS) primer pair. Single colonies, picked from a second screen, were cultivated overnight, DNA was isolated, the size of the insert was determined by restriction analysis and the insert was tested for a complete DBL1- (ATS) domain by PCR. Some promising inserts were sequenced and used for transfection into COS 7 cells to perform a cell-cell adherence assay, as described in the shotgun approach (1).
After α-^{32}P-labelled clones, carrying a DBL1 domain, were identified, the labelled probes were stripped from the membranes, and the washed membranes were incubated with α-^{32}P-labelled ATS probes to identify possible clones containing both domains and therefore a full length *var* gene.

Stripping radioactive probes from a nylon membrane

250 ml water / 1% SDS were heated to boiling point, removed from the heat source and the membranes were incubated in the hot water for 5 minutes. The procedure was repeated one more time, then the membranes were dried between filter papers and reused.

3.2.5. Direct approach (2) (bacterial expression system)

3.2.5.1. Overview

The aim of this approach was to express parts of *var* genes, namely the CIDR- and the DBL1 domains, as 6 x histidine-tagged (6xhis-tagged) proteins. The domains were amplified from the cDNA (strain ItG2.F6) and from gDNA of the strain K1. The 6xhis-tag was introduced at the N-terminus by cloning the domains into the pQE30 vector. Proteins were expressed in *E. coli* strain M15 and purified using Ni-NTA agarose columns. All these components were part of the QIAexpress Kit Type IV. The binding properties of these domains were evaluated by IFAs and FACScan analysis.

3.2.5.2. Cloning of CIDR- and DBL1 domains into pQE30

In a first step, both domains had to be amplified by a PCR with primers containing convenient internal restriction sites to be able to introduce the insert in-frame into the pQE30 vector. The templates were either cDNA, gDNA or already cloned DBL1 fragments (see 3.2.4.2.). The primer pair used for the amplification of CIDR domains was CIDR Fw Bam and CIDR Rev Kpn, the primer pair for the amplification of DBL1 domains was DBL1 Fw Bam and DBL1 Rev Kpn (see Table 3-XV). PCR and sticky end ligation into pQE30 vector were done as described above (3.2.1.8. and 3.2.1.9.).
All CIDR- and the new DBL1 domains were sequenced, using the QIAexpress sequencing primers (3.1.9.2.).

3.2.5.3. Expression of histidine tagged proteins

Small scale expression

M15 cells, containing a pQE30 plasmid and the pREP4 repressor plasmid, were grown in Super Broth medium supplemented with 100 µg / ml ampicillin and 25 µg / ml

kanamycin. 10 ml LB cultures were grown overnight at 37°C in a shaker. 2.5 ml of this culture was used to inoculate 50 ml of prewarmed Super Broth medium with antibiotics and allowed to grow until an OD_{600} of 0.5 was reached. The culture was left at 37°C or transferred to room temperature, expression was induced by adding IPTG to a final concentration of 1 mM and finished after 2 to 5 hours by harvesting the cells as described above (3.2.2.9.). In order to get a non-induced control, a 1 ml sample was taken immediately prior induction.

Culture growth for preparative purification

The ideal conditions, evaluated by the small scale expression protocol, were the following: 50 ml overnight culture were used to inoculate 1 litre of Super Broth medium, the suspension was allowed to reach an OD_{600} of 0.5 at room temperature before being induced with IPTG and proteins were then expressed for 4 hours at room temperature. Cells were spun down and the pellet was stored at -20°C overnight.

3.2.5.4. Solubilizing of histidine tagged proteins

The protocol is given for preparation under native conditions, whereas alterations for denaturing conditions are given in brackets. For both preparations, the cell pellet was thawed on ice for 15 minutes and then resuspended in lysis buffer (buffer B, containing 8 M Urea) at 5 ml per gram wet weight. For a preparation under native conditions, 1 mg / ml lysozyme was added to the suspension, followed by an incubation on ice for 30 minutes. The suspension was then sonicated on ice with 8 x 20 seconds bursts at 200-300 watt, with a 20 seconds cooling period between each burst. After that, the lysate was drawn 5 times through a narrow gauge syringe needle and distributed among an appropriate number of micro centrifuge tubes. (For a preparation under denaturing conditions, the suspension was distributed among an appropriate number of micro centrifuge tubes and mixed for 1 hour at room temperature in an Eppendorf Thermomixer 5436). In order to pellet the cellular debris, both lysates were centrifuged for 30 minutes at 10'000 g. The pellets were discarded and the proteins were then purified from the cleared lysates according to the protocols given below (3.2.5.5.).

3.2.5.5. Purification of histidine tagged proteins

Principle of the purification using a Ni-NTA resin

The principle of this purification is as follows: Ni-NTA is a tetra dentate chelating adsorbent, which occupies 4 of the 6 ligand binding sites in the co-ordination sphere of the nickel ion, leaving two sites free to interact with the imidazole ring of histidine residues.

Non-specific binding is prevented by adding increasing amounts of imidazole to the washing buffers. Elution of the bound 6xhis-tag protein was achieved by adding 250 mM imidazole to the Ni-NTA agarose columns.

Purification under native conditions

All but two proteins could be isolated and purified under native conditions. An empty 5 ml polypropylene column (Qiagen) was loaded with 1 ml of 50% Ni-NTA slurry per 4 ml of cleared lysate and was equilibrated with 2 x 5 ml lysis buffer. The cleared lysate was applied and resuspended in the resin according the supplier's manual. The resin was then washed as follows: 2 x with 4 ml of lysis buffer (no imidazole), 2 x with 4 ml of lysis buffer / 20 mM imidazole and 1 x with 4 ml of lysis buffer / 35 mM imidazole. After these five washing steps, the protein was eluted in 8 fractions with a total amount of 6.1 ml lysis buffer / 250 mM imidazole, with a first fraction of 500 µl and further fractions of 800 µl.

An aliquot of all eluted fractions, all washes, the flow-through, the cleared lysate, and of the induced and non-induced cell pellets were analyzed by SDS-Page gel electrophoresis.

Purification under denaturing conditions

The two proteins, which could not be isolated and purified under native conditions were purified as above, but instead of using lysis buffer with different concentrations of imidazole, buffer B with decreasing pH was used. The column was washed 4 x with 4 ml of buffer B, pH 6.3 and protein was eluted with 4 x 0.5 ml buffer B, pH 5.9 and later on with 4 x 0.5 ml buffer B, pH 4.5. As above, protein concentration was estimated by SDS-Page gel electro-phoresis.

Dialysis of proteins

The eluted fractions with the highest concentration of purified CIDR- or DBL1 domains were pooled, diluted 1:1 in PBS to prevent precipitation, and then filled into dialysis tubing (cut off 10 kDa, Chemie Brunschwig, CH), that had been boiled in sterile water for 5 minutes and then rinsed with sterile PBS. Dialysis was done overnight against 2 litres of PBS at 4°C. The concentration of protein was determined photometrically. The protein solutions were sterilized by filtration and aliquots were stored at -20°C.

3.2.5.6. Binding assays with histidine tagged proteins on cells expressing known ligands

Two different methods were tested: IFAs on adherent cells similar to the IFAs described above (3.2.3.3.) and FACScan analysis with the cells in suspension.

Cell-protein IFAs on adherent cells

Cells expressing huICAM-1 (L-huICAM-1 cells) and cells expressing CD36 and CSA (C32 cells), as well as cells, expressing no ligand involved in cytoadherence (L-cells), were used to perform these assays. Cells were seeded and grown in tissue culture treated multi well plates (12, 24 or 48 well plates). Cells were either fixed on ice with ice-cold Methanol for 1 minute prior to the application of proteins or thereafter, before the first antibody was applied. The medium was removed from the wells, the cells were washed with PBS and, if desired, fixed with MeOH and air dried (2-5 minutes). The cells were incubated for 1 hour in PBS / 5% FCS, pH 6.8 or in binding medium, pH 7.0 to saturate unspecific binding sites. Proteins were applied in an appropriate dilution in binding medium for 1 hour at room temperature, then the wells were washed 2 x with binding medium and the first antibody (αhis) was applied 1:1000 in PBS / 0.5% FCS. After a 1 hour incubation step at room temperature, the wells were washed twice with PBS / 0.05% Tween 20 and the second antibody (αmouse) was applied 1:1000 in PBS / 0.5% FCS for 1 hour at room temperature. After two washes as above, the cells were ready to be screened (FITC-conjugated second antibody) or were stained according the procedure given in 3.2.3.3. (HRP-conjugated second antibody). Negative (no proteins or PBS instead of the first mAb) and positive controls (mAbs against CD36 and huICAM-1) were always carried out in parallel.

Flow cytometric analysis

Cells were detached by trypsination or with PBS / 0.5 mM EDTA, counted, and a defined number of cells (10^5-10^6) were applied to a Facs tube and incubated in 250 µl PBS / 5% Drymilk for 1 hour on ice, to saturate unspecific binding of the proteins. After 1 hour, the cells were resuspended in 2 ml PBS / 1% BSA, pH defined (6.3 to 7.3, depending on the proteins) and spun again with 300 g (1200 RPM in a cooled Beckman TJ6 centrifuge) for 3 minutes. The supernatant was discarded, the cells were resuspended in the remaining liquid and 100 µl protein diluted in PBS / 1% BSA was added (1-2 µg / tube) and incubated on ice for 1 hour. Cells were washed twice in PBS / 1% BSA as above, but after discarding the supernatant, the tubes were inverted on a Kleenex tissue to drain remaining liquid. First antibody was applied in 50 µl, diluted 1:100 (αhis) up to 1:1000 (αhu-ICAM-1 or αCD36) in PBS / 1% BSA and incubated on ice for 1 hour. Washing steps were repeated, the second

antibody was applied (50 µl αmouse, FITC-conjugated, diluted 1:1000) and incubated as above for 1 hour on ice. After two last washing steps, the cells were resuspended in PBS only and were now ready for FACScan analysis. Negative (no proteins or PBS instead of the first mAb) and positive controls (mAbs against CD36 and huICAM-1) were always carried out in parallel.
FACScan analysis was also used to confirm expression of cell ligands like huICAM-1 or CD36 on mammalian cells. This was done as described here for the protein binding assays, but without proteins and the saturation step.

3.2.5.7. CSase treatment of CHO cells

CHO cells express the polysaccharide CSA on their surface. CSA could be digested with chondroitinase ABC (CSase ABC). Cells, grown as mono-layers in tissue culture flasks, were washed with PBS and incubated with 1 unit CSase ABC per 2 ml PBS for 1 hour at 37°C in the incubator. After this treatment, the cells were of round shape, but not completely detached. PBS / CSase was aspirated off and the cells were detached using either PBS / Trypsin or PBS / EDTA (see 3.2.1.1.).

4. Results

4.1. Results of the shotgun approach (1)

The *P. falciparum* strain ItG2.F6 was tested for cytoadherence on L-ICAM-1- and C32 cells, mRNA was isolated, a cDNA library was constructed by Invitrogen and the library was used to transfect COS 7 cells in order to perform cell-cell adherence assays, as described in 3.2.2.

4.1.1. Analysis of adhesion of iRBCs on L-ICAM-1- and C32 cells

The analysis of adhesion of strain ItG2.F6 was performed twice, as described in 3.2.2.2. The second assay was performed immediately prior to the saponin lysis, the first step of the mRNA-isolation.

In the first assay, cultures of the strains ItG2.F6 and K1 (parasitaemia of 8% and 6% respectively) were used to analyze binding properties on both fresh and fixed C32- and on fixed L cells. A culture of uninfected red blood cells was used as control. In the second assay, binding of strain ItG2.F6 (parasitaemia of 7%) was analyzed on fresh L-, L-ICAM-1- and C32 cells. In the first assay, ItG2.F6 bound to both types of C32 cells, compared to L-cells, strain K1 and uninfected RBCs (Table 4-I). In the second assay binding to C32 cells was confirmed and binding to ICAM-1 was shown, compared to untransfected L-cells (Table 4-II). This assured that the mRNA was isolated from a strain, which bound to huICAM-1 and on C32 cells.

Table 4-I Adherence of iRBCs to C32 cells compared to L cells

Strain	**Type of cells**	**iRBCs / area (3 fields)**	**uninfected RBCs / area**
ItG2.F6	C32 cells fixed	17, 10, 15	00, 01, 00
	C32 cells fresh	09, 11, 08	01, 01, 00
	L-cells fixed	00, 01, 00	00, 00, 01
K1	C32 cells fixed	00, 00, 00	00, 01, 01
	C32 cells fresh	00, 00, 00	00, 01, 01
	L-cells fixed	00, 00, 00	00, 00, 00
none culture of un-infected RBCs	C32 cells fixed	-, -, -	00, 00, 01
	C32 cells fresh	-, -, -	00, 00, 00
	L-cells fixed	-, -, -	00, 01, 00

Table 4-II Adherence of strain ItG2.F6 to L-huICAM-1-cells

Strain	**Type of cells**	**iRBCs/200 cells (4 fields)**	**uninfected RBCs /200 cells**
ItG2.F6	L-cells fresh	00, 00, 00, 01	05, 20, 17, 11
	L-ICAM-1 cells	20, 08, 08, 41	12, 08, 02, 20
	C32 cells fresh	10, 04, 03, 14	02, 04, 04, 05

4.1.2. Analysis of the custom-made cDNA

An aliquot of the isolated mRNA was analyzed on a RNase-free agarose gel. The mRNA appeared as a faint, homogeneous smear, visible from 4000 to 500 bp, with no distinct bands or degradation. The total amount was estimated to consist of 18 µg mRNA. This amount was used by Invitrogen to construct the cDNA library (see: 3.2.2.4.). Invitrogen did a quality control, which we repeated twice with the original and once with the 1 x amplified cDNA library. The results of these controls are summarized in Table 4-III.

Table 4-III Analysis of the cDNA library

	control 1	control 2	control 3	control 4
hours of cultivation prior analysis:	-	17	7	8
Number of recombinants analyzed:	10	10	10	12
Ratio containing an insert:	9 / 10	1 / 10	3 / 10	5 / 12
size range:	0.5 - 3.0 kb	0.7 kb	0.7 - 2.7 kb	0.8 - 4.4 kb
Average insert size:	1.68 kb	0.7 kb	1.33 kb	1.76 kb
ratio of mutated vector:	0 / 10	2 / 10	0 / 10	3 / 12

The original cDNA was used for control 1 (Invitrogen), control 2, and control 3, whereas the 1 x amplified cDNA was used for control 4.

During this thesis, more than 2000 cDNA clones were analyzed, in terms of presence of an insert, insert size or if the insert was part of a *var* gene.
The largest insert found was 4.5 kb, the biggest insert containing a DBL1- or an ATS domain was 3.1 kb and 2.5 kb respectively; however, no insert was found carrying both domains, i.e. no full-length *var* genes were found in the cDNA library.

4.1.3. Analysis of the different transfection methods

In the beginning of this thesis, the COS 7 cells were transfected using the chloroquine / DEAE-dextran technique (3.2.2.5a.), later on, this technique was replaced by using a commercially available transfection lipid. The lipid was chosen by comparing 6 different commercially available transfection lipids (3.2.2.5b.).

4.1.3.1. Transient transfection with chloroquine / DEAE-dextran

Transfection with the pcDNA3.1-EGFP vector (Transfection control, see 3.2.2.11.) never yielded more than 10% of the cells expressing GFP. Furthermore, this method was rather harsh to the cells to be transfected: After a two-hours incubation step, up to 50% of the COS 7 cells died and failed to attach on the Petri dish and were removed by the medium exchange after 12 hours. The remaining cells were visibly damaged and often showed non-typical shapes. Also, they did not multiply as fast as non-transfected COS 7 cells.

4.1.3.2. Transfection with commercially available transfection lipids

The transfection-rates of 6 different transfection lipids were compared to each other and to the transfection-rate, achieved by chloroquine / DEAE-dextran (3.2.2.5b.) with the pcDNA3.1-EGFP construct. The transfection-rate was estimated by counting the percentage of cells expressing GFP. The transfection-rates for each lipid are given in Table 4-IV.

Table 4-IV Comparative transfection with 6 different transfection lipids

		1:1	1:2	1:4	1:8	1:16	1:32
Row A	10 µl Lipofectin	<1%	03%	05%	05%	10%	10%
Row B	10 µl LipofectAmine	25%	25%	10%	10%	05%	05%
Row C	15 µl Cellfectin	02%	05%	10%	05%	05%	02%
Row D	10 µl DMRIE-5 *	30%	30%	30%	30%	10%	10%
Row E	10 µl SuperFect	25%	20%	10%	05%	<1%	<1%
Row F	10 µl LipoTaxi	05%	10%	**30%**	20%	<1%	<1%

* The amount of lipid DMRIE-5 in the sample pack was 10 x less as indicated, therefore, the concentration of lipid could have been 10 x higher than planned.

Two transfections with chloroquine / DEAE-dextran, carried out in parallel, yielded a 10% transfection-rate each, while the LipoTaxi transfection lipid achieved the highest transfection-rates (**30%**) and showed no toxicity to the transfected cells.

In later applications, when LipoTaxi was used according the supplier's manual, the transfection-rates with the pcDNA3.1-EGFP-control plasmid reached up to 70%.

4.1.4. Results of the cell-cell adherence assays

4.1.4.1. Comparison of 8 first-screen cell-cell adherence assays

During this work, 8 first-screen cell-cell adherence assays were performed and continually improved. The first assay could not to be screened due to the unfixed cells, which started to lift off (see: 3.2.2.6.). From the 4th screen on, a transfection with the pcDNA3.1 vector only was always carried out as a negative control for the adherence assay. The 8th assay was the only assay with LipoTaxi-transfected COS 7 cells and included the following improvements: Transfections with the cDNA and with the pcDNA3.1-EGFP (positive control for the transfection and negative control for the binding assay) were done, yielding a transfection-rate of 60% as estimated by the expression of GFP, the screening cells used, were EB-stained L-huICAM-1 cells, the double fixation method was used and positive cells were picked with the scalpel.

Taken together, in all 8 cell-cell adherence assays, 54 COS 7 cells were positive in a first screen and were picked, resulting in the isolation of 1202 cDNA clones, most of them were used to perform a second screen.

All second screens were performed as the corresponding first screen. All clones, isolated from a picked cell of the first screen were pooled to perform a second screen. From the 3rd adherence assay on, a pool of all clones, carrying an insert, was also prepared to transfect COS 7 cells for a second screen.

The cells of two second screens of the 4th adherence assay (inserts > 1 kb of Pool 3.1 and pcDNA3.1-vector only, see: Table 4-V and 4-VI) were washed with PBS, scratched from the dish, lysed and analysed by SDS-Page gel electrophoresis and western blot (3.2.1.11), with the pooled human serum of malaria exposed adults of PNG (Table 3-VII) as first antibody. The pattern of protein bands was compared to each other. A distinct, additional band in the cells, transfected with the pool of inserts > 1 kb was visible in the blot at a size of approximately 66 kDa. This was the first indirect evidence, that a *P. falciparum*-protein, which was encoded by the cDNA library, was expressed by COS 7 cells.

Nevertheless, all second screens were negative. After each failed second screen, the assay was modified, with reference to fixation-, staining- or picking technique (see 3.2.2.6.), or with reference to the used screening cells. A comparison of all eight first-screen cell-cell adherence assays is given in Table 4-V.

Table 4-V Comparison of 8 first-screen cell-cell adherence assays

Nr.	**conditions** (see: 3.2.2.6.)	**positive cells** (COS + screening)	**pick**	**clones** (inserts)	**2nd screens** (composition)
1st	fix:0, stain:0, screen:1	**0**	none	-	-
2nd	fix: 1, stain: 0, pick: 1, screen: 1	**2** (1+18) (1+5)	Pick 1 Pick 2	2 (0) 17 (5)	**1**: 1 x pool of all 19 clones
3rd	fix: 2, stain: 1, pick: 1, screen: 1	**4** (1+4) (1+5) (1+5) (2+12)	Pick 3 Pick 4 Pick 5 Pick 6	0 0 0 45 (8)	**2**: 1x pool of all 45 and 1x pool of 8 clones with insert
4th	fix: 2, stain: 1+2, pick: 2, screen: 1+2	**18** 6x (1+3 or 4) 6x (1+3 or 4) (1+8) & (1+9) 3x(1+4) & (1+8)	Pool 3.1 Pool 3.2 Pool 3.3 Pool 3.4	194 (61) 199 (n.d.) 307 (n.d.) 60 (12)	**3**: 1x pool of 3.1 + 1x pool of 3.4 and 1x with 22 clones with inserts > 1 kb
5th	fix: 2, stain: 1, pick: 2, screen: 1+2	**6** (1+14) (1+6) (1+4) & (1+4) (1+4) & (1+3)	Pick 8.1 Pick 8.2 Pick 8.3 Pick 8.4	1 (0) 282 (232) 0 30 (8)	**4**: 1x with Pick 8.2 and 1x Pick 8.4 and 1x with 17 clones + 1x with clone 84.18
6th	fix:3, stain:1, screen:1	**4** 4x (1+5 to 7)	none*	-	-
7th	fix: 2+3, stain: 1, pick: 3, screen: 1	**6** 2x(1+7)&(1+11) 2x(1+2)&(1+3)	Pick 9 Pick 10	0** 54 (10)	**2**: 1x all 54 clones +1x 10 with insert
8th	fix: 2+3, stain: 1, pick: 3, screen: 1	**14** (1+6) (1+10) (1+4) (1+3) & (1+4) 6x (1+3 or 4) 5x (1+3 or 4)	LTP 1 LTP 2 LTP 3 LTP 4 LTP 5 LTP 6	2 (2) 3 (3) 3 (0) 0 0 3 (3)	**9**: 1 x with a pool of 8 clones with an insert and 1x with each of the eight clones individually

Legend to Table 4-V

For each assay the modifications of the given method, the number of positive cells (bold figures), the number of attached screening cells to 1 or 2 COS 7 cells, the resulting picks, the number of isolated clones, and the composition of the second screen(s) are given. The modifications were defined as follows:

fix: The assay was not fixed (0), fixed with 1% glutaraldehyde / 2% Formaldehyde in PBS for 1 hour at RT (1) or at 4°C (2), fixed with 50% Methanol / 50% acetone for 15 minutes on ice (3), or fixed with (2) followed by (3).

stain: no cells were stained (0), the screening cells were stained with EB (1), the COS 7 cells were counterstained with Evans blue (2).

pick: positive cells were picked with a Drummond pipette (1), with an Eppendorf Crystal pipette (2), or with a scalpel (3).

screen: The screening cells were L-huICAM-1 cells (1) or C32 cells (2).

* the screening cells started to lift off and float before the positive cell was picked.

** The 3 cells of Pick 9 were fibrous and wrinkled, indicating an artefact.

4.1.4.2. Analysis of the isolated cDNA clones of picked COS 7 cells

During this work, 54 COS 7 cells were considered positive (i.e. to mediate adherence to screening cells in a first screen cell-cell adherence assay) and picked, resulting in 22 picks (11 single and 11 pooled picks, see: 3.2.2.7.), yielding in the isolation of 1202 cDNA clones. The clones were pooled and/or used individually to retransfect COS 7 cells for a second screen. The analysis of the clones is summarized in Table 4-VI.

Table 4-VI Analysis of isolated cDNA clones of picked COS 7 cells

Assay	**Pick**	**clones**	**inserts**	**size range**	***var* domains**	**comments**
2	Pick 1 Pick 2	2 17	0 5	 0.6-1.8 kb	 no	
3	Pick 3 Pick 4 Pick 5 Pick 6	0 0 0 45	 8	 0.55-1.7 kb	 no	COS cells of Pick 6 were photographed
4	Pool 3.1 Pool 3.2 Pool 3.3 Pool 3.4	194 199 307 60	61 n.d. n.d. 12	0.2-3.0 kb 0.5-2.0	no no	Transfection with 22 clones with insert > 1 kb (4.1.4.1.) Pool 3.2 and 3.3 stabilated as described (3.2.1.3.)
5	Pick 8.1 Pick 8.2 Pick 8.3 Pick 8.4 Pool 8*	1 282 0 30 645	0 232 8 >400	 0.45-2.5 kb 0.3-3.1 < 3.1 kb	 no yes (DBL1) n.d.	220/232 inserts were identical (1.6 kb). Clone 84.18 (3.1 kb) contained a DBL1 domain. no additional insert found
7	Pick 9 Pick 10	0 54	 10	 0.5-2.1	 no	Picks 9 & 10 were photo-graphed
8	LTP 1 LTP 2 LTP 3 LTP 4 LTP 5 LTP 6	3 3 3 0 0 3	2 3 0 3	2.2 kb 0.8-1.6 kb 2.1 kb	no no no	Two clones (LTP 1.1 and 6.3) carried different fragments of the soluble antigen gene which makes up to 5% of total mRNA in late blood stage parasites (Nicholls *et al.* 1988)

Legend to Table 4-VI

The clones of each pick were analysed for an insert, the size, and if the insert contained a DBL1- or an ATS domain.

* After the precipitation of the cDNA clones of Pick 8.1 to 8.4 only half of the amount was used in the electroporation of *E. coli* cells. When Pick 8.1 and 8.3 yielded no insert, the remainder of Pick 8.1 to 8.4 was pooled (Pool 8) to perform an additional electroporation, yielding in 645 clones. The inserts were not analyzed in detail, but only pool-wise, yielding no insert bigger than 3.0 kb and a 1.6 kb-sized insert in every pool of ten clones, presumable the same insert which was found in 78% of all clones of pick 8.2.

4.1.5. Analysis of the different picking methods

4.1.5.1 Pipette versus needle-scalpel

Picking was either done with a pipette, equipped with a gel loading tip or with a needle scalpel. The essential difference was the accuracy: picking with the pipette was always a 'blind' process, insofar, as the selected cell could not be followed by eye, since it was detached by scratching, often together with surrounding cells, and then sucked in, whereas picking with the scalpel lead to the isolation of the selected cell only, which remained visible on the 'blade' of the scalpel. This fact is reflected by the yield of clones per COS 7 cell: picks with a pipette yielded a mean of 60.5 clones per selected cell, whereas picks with the scalpel yielded a mean of 3.25 clones per selected cell.

4.1.5.2. Results of the positive control for picking and Hirt extraction

In parallel to the fourth cell-cell adherence assay, one transfection with a cDNA clone, containing a DBL1 domain (var 5.4.1) was performed after the chloroquine / DEAE-dextran protocol. After 72 hours, 4 single cells were randomly picked, with a pipette and processed according the shotgun approach (1). A PCR was performed, resulting in an amplified DBL1 domain in 3 out of 4 picks.
Even though a transfection rate of 75% is most probably an overestimation due to the picking method used, it was shown, that it is possible to extract cDNA clones out of relatively few cells by the chosen method.

4.1.6. Analysis of the electroporation protocol

After the fourth cell-cell adherence assay was negative in the second screen, the following hypothesis was tested: plasmids with big inserts, isolated from COS 7 cells, containing attached screening cells in a first screen, were lost during electroporation, due to the small pores generated by electroporation with the given specifications (3.2.2.9.), therefore leading to negative second screens.
In order to test this hypothesis, a cDNA clone (var 5.4.1), was selected to transform *E. coli* cells by using 4 different conditions. The selected plasmid carried an insert of 3.0 kb, resulting in a total size of 8.4 kb. While the capacity, shunt and pulse time was kept constant (25 µF, 201 ½ and 5 msec), the voltage was increased steadily from 2.0 to 3.5 kV. Each transformation was plated, yielding no differences in the number of colonies between the four conditions used.

4.2. Results of the shotgun approach (2)

4 IFAs were performed using the mAbs of K. Willimann, which recognized iRBCs and intraerythrocytic *P. falciparum* stages. The second antibodies used, were either FITC- or HRP-conjugated; the sensitivity and specificity of both second antibodies was the same, yet the latter IFAs were performed with HRP-conjugated antibodies, since picking of cells under visible light was easier than picking under UV-light.

4.2.1. Analysis of the IFAs

The IFAs, performed with the supernatants of K. Willimann (Table 3-V), were performed independently twice. One supernatant (SupI 202/7) could not be used, since it recognized every COS 7 cell, while others never recognized any COS 7 cell. One supernatant (SupX 128/3) repeatedly recognized single COS 7 cells in an assay. All positive cells of a well were picked and pooled and plasmids were propagated in *E. coli*. Plasmid DNA was subsequently isolated and pooled for second screens, but all second screens were negative. The results of the four IFAs are summarized in Table 4-VII.

Table 4-VII Comparison of 4 performed IFAs

mAb	**1. & 2. IFA**	**clones**	**3. & 4. IFA**	**clones**
SupI 202/7	all cells positive	-	all cells positive	-
SupII 3c2/9	negative	-	negative	-
SupX 128/3	4 cells picked	58	3 cells picked	0
SupX 216	negative	-	2 cells picked	12
SupX 304/3	negative	-	1 cell, not picked	-
SupX 323/3	2 cells picked	23	negative	-
SupX 331	negative	-	negative	-
SupX 435/3	negative	-	negative	-
SupX 509	negative	-	negative	-

An additional IFA was performed: As positive control for the shotgun approach (2) a mAb (9.22) recognizing the 83.1 epitope (GYGLFHKEKMIL) of MSP1 (Helg, 1997). By using the same primer pair, which was used to ligate the N-terminus of MSP1 into pRE4, a specific MSP1 product was amplified from the cDNA. COS 7 cells were transfected with the cDNA library and screened with mAb 9.22 in an IFA: Four individual cells were positive and

picked. The pooled pick was used to transform *E. coli* and resulted in 48 clones. Clones were analyzed for the presence and size of inserts:

Pick 9.22

Number of recombinants analyzed:	48
Ratio containing an insert:	17 / 48 (35%)
Size range:	2 x 0.6 / 5 x 0.8 / 1.3 / 2 x 1.6 / 1.7 / 6 x 3.0 kb
Average insert size of the clones analyzed:	1.73 kb
Ratio of mutated vector:	2 / 48 (4%)

All clones were PCR-screened in pools of 6 with primers specific for MSP1. The cDNA and the N-terminus of the MSP1 gene in the pGEM-T vector were used as a positive control for the PCR. No MSP1 product was amplified from the eight pools. Nevertheless, three second screens were performed, one with a pool of all clones carrying an insert, one with a pool of all 48 clones, and one with a pool of the 6 clones, carrying an insert of 3.0 kb. All second screens were negative.
Since it was not possible to detect cDNA clones, which carried an MSP1 gene or -fragment, the feasibility of shotgun approach (2) remained questionable.

4.2.2. Analysis of the expression controls using *P. falciparum* genes

4 expression controls were constructed to proof expression of *P. falciparum* genes in COS 7 cells and to show the correct folding and transport of the corresponding proteins:
1.) MSP2 gene (ItG2.F6) cloned into pcDNA3.1 (HindIII / BamHI)
2.) MSP2 gene (ItG2.F6) cloned into pcDNA3.1-EGFP (HindIII / BamHI)
3.) N-terminus of MSP1 gene (Ro71) cloned into pcDNA3.1 (EcoRI / BamHI)
4.) N-terminus of MSP1 gene (Ro71) cloned into pRE4 (KpnI and PvuII / ApaI)

4.2.2.1. Constructs with the MSP2 gene

The MSP2 gene, which was isolated from the cDNA by PCR, was ligated correctly into the pcDNA3.1- and the pcDNA3.1-EGFP-vector (confirmed by sequencing). Restriction analysis was used to confirm, that the MSP2 gene belonged to the 3D7 family. COS 7 cells were transfected with both constructs and expression of GFP was observed with the pcDNA3.1-EGFP-MSP2-construct. However, in western blots, using a serum pool from malaria exposed adults , no distinct (additional) MSP2 band could be detected. Many other bands indicated, that the serum pool recognized COS 7 cell-specific proteins (see also 4.1.4.1.). A western blot with αGFP revealed a band, of the size of the GFP protein alone, indicating a degradation of MSP2, a mutation or incorrect cloning in the second construct.

4.2.2.2. Constructs with the N-terminus of the MSP1 gene

In order to generate an expression control, for which a mAb was available, the N-terminus of MSP1 (AA 1 - 348), was amplified by PCR. The primers had an internal EcoRI and a BamHI restriction site, respectively. The PCR product was ligated into pcDNA3.1.

A second control plasmid, containing the N-terminus of MSP1, was constructed by amplifying the N-terminal part of MSP1. A pair of primers with internal ApaI and KpnI restriction sites were used. Native Pfu DNA polymerase was used and pRE4 vector was digested with ApaI (sticky end) and PvuII (blunt end), as described in 3.2.3.5. The KpnI site was introduced to cut the insert from the vector, since the PvuII site was destroyed by ligation. The insert replaced the central region of the *Herpes simplex* virus glycoprotein D-gene (HSVgD-gene, AA 33-248), thereby generating a chimeric construct with the leader sequence and the transmembrane and cytosolic domain of the original HSVgD protein.

Both constructs were used to transfect COS 7 cells, but neither in western blots nor in IFAs, the N-terminus was detected by mAb 9.22, although both controls were confirmed by sequencing to be in-frame. Also, neither the mAb ID3 nor DL6, which recognize AA 11-19 and AA 272-279 5' and 3' of the MSP2 insert respectively, recognized the construct, but recognized the original HSVgD protein, indicating no expression of a chimaeric protein, possibly by degradation of the MSP1 gene (see also: 5.1.2.2. and Figure 5.2).

4.2.3. Results of the control using the huICAM-1-EGFP construct

The complete gene of huICAM-1 was amplified by PCR from the genomic DNA of L-huICAM-1 cells and cloned in frame into the pcDNA3.1-EGFP vector at the HindIII / BamHI sites of the polylinker. This construct was used to transfect Bosc23 cell according to the LipoTaxi protocol for stable transfections. The transfected cells were sorted into a 96 well plate by FACScan analysis with an integrated sorter. The plate yielded 5 clones which stably expressed the GFP-huICAM-1 construct on the surface as shown in an IFA, using αICAM-1 mAb MEM112. Furthermore, the green fluorescence was persistent, even after fixation. These cells were supposed to be used as screening cells for further cell-cell adherence assays, since they did not need an additional staining and did not attach as fast to the plastic as L-huICAM-1 cells, yet whether huICAM-1 is correctly presented on the surface of these cells was not tested in an adherence assay.

4.3. Results of the direct approach (1) (mammalian expression system)

The DNA sequences, coding for DBL1- and ATS domains were amplified by PCR from both, the cDNA library and genomic DNA of strain K1, cloned into pMOSblue-T vector, sequenced and used as hybridisation probes in both, radioactive and non-radioactive colony lifts, to identify cDNA clones, carrying a *var* gene insert, to perform cell-cell adherence assays, as described in the shotgun approach (1).

4.3.1. Analysis of 2 *var* gene domains from K1 gDNA

Genomic DNA of strain K1 was used to amplify DBL1 and ATS domains (see: 3.2.4.2.).

4.3.1.1. DBL1 domains of strain K1

With the two-step PCR, as described in 3.2.4.2., three distinctive products of equal intensity were amplified, representing products of 380, 420 and 460 bp. The PCR products were extracted from the gel and ligated by T/A cloning into the pMOSblue-T vector (3.2.1.9.) The ligation, which was transferred immediately to 4°C, resulted in the best ligation efficacy (see: Table 4-VIII).

Table 4-VIII Determination of ligation efficacy with DBL1 inserts

Ligation at	**Insert**	**colonies: blue**	**light blue**	**white**	**Clones picked**
4°C overnight	DBL1	0	1	53	**3**: DBL4.1 to DBL4.3
2 h 16°C / 4°C	DBL1	2	2	42	**3**: DBL16.1 to DBL16.3
4 h RT / 4°C	DBL1	5	2	14	**0**
2 h 16°C / 4°C	none	55	5	08	**0**

Legend to Table 4-VIII

Three clones were picked from the ligation, which was incubated for 2 hours at 16°C before being stored at 4°C over night and from the ligation, which was immediately transferred to 4°C. All six picked clones carried an insert and were named DBL4.1 to DBL4.3 and DBL16.1 to DBL16.3 respectively.

Subsequent sequencing of the clones revealed, that 3 different DBL1 domains had been cloned: clone DBL4.1 was identical to DBL16.1 (399 bp), clone DBL4.2 was the same as DBL16.3 (417 bp) and DBL4.3 was identical to DBL16.2 (387 bp). The sequences of these clones is given in chapter 4.4.1., together with sequences of DBL1 domains, isolated from ItG2.F6 cDNA, and two additional DBL1 domains, lateron isolated from strain K1.

4.3.1.2. ATS domains of strain K1

6 distinctive products of different intensity were visible on a agarose gel after PCR amplification of the ATS domain. The amplified products were: 480, 410, 500, 530, 450, and 590 bp long, sorted according their intensity. Extraction and ligation reactions were the same as above and ligation efficacy was comparable to that above (Table 4-IX).

Table 4-IX Determination of ligation efficacy with ATS inserts

Ligation at	**Insert**	**colonies: blue**	**light blue**	**white**	**Clones picked**
4°C overnight	ATS	4	17	45	ATS4.1 to ATS4.3
2 h 16°C / 4°C	ATS	12	20	60	ATS16.1 to ATS16.3
4 h RT / 4°C	ATS	31	23	28	none
2 h 16°C / 4°C	none	73	21	19	none

Legend to Table 4-IX

Six clones were picked, only four clones carried an insert (ATS4.3 and ATS16.1 to ATS16.3), all of different sizes: ATS4.3: 590 bp, ATS16.1: 480 bp, ATS16.2: 500 bp and ATS16.3: 530 bp.

4.3.2. Analysis of DBL1 domains from ItG2.F6 cDNA

A two-step PCR was performed to amplify DBL1 domains from the cDNA, resulting in a smear of bands, ranging from 480 to 550 bp, indicating that the DBL1 domains of strain ItG2.F6 were generally larger than the DBL1 domains of strain K1. The ligation was incubated overnight at 4°C, yielding 8 blue, 12 light blue and 29 white colonies. 20 white colonies were picked and named DBLs 1 to DBLs 20. 6 clones had an insert, ranging from 500 to 550 bp and were subsequently sequenced, revealing 4 different DBLs sequences: The sequences of clones DBLs 2 and DBLs 14 (414 bp) were identical, as well as the sequences of clones DBLs 16 and DBLs 18 (429 bp). Unique sequences had clones DBLs 4 (471 bp) and DBLs 9 (444 bp). The sequences are given in chapter 4.4.1.

4.3.3. Results of the colony lifts and the corresponding southern blots

All the DBL1 domains were used as hybridisation probes in the following non-radioactive protocol. The isolated, cloned DBL1 domains were biotinilated and hybridized on nylon membranes, carrying colony-lifted cDNA clones (3.2.4.3.). In the radioactive protocol, the ATS domains of strain K1 were additionally used as hybridisation probes (3.2.4.4.).

4.3.3.1. Analysis of the non-radioactive protocol

The cDNA library was diluted 1:40'000 and plated on 4 plates, yielding approximately 5000 colonies per plate. On a fifth plate, the library was diluted 1:400'000, yielding 1000 colonies. After the visualisation procedure, all colonies were visible on the membranes as light purple dots and some deep purple dots.

Four small regions, containing darker colonies, were picked from one plate with approximately 5000 colonies, diluted and plated again. A second screen was performed with these picks, together with a positive and a negative control (cloned DBL1 domain DBL4.2 and cloned ATS domain ATS16.2 respectively). The colonies on these plates were much less dense (approximately 800 colonies) and the darker dots on the membranes of this second screen could accurately be matched to the corresponding colonies. From each plate of the second screen, 2 or 4 darker colonies were individually picked, cultivated and the plasmids were isolated. The positive control resulted in clear, dark dots on the membrane, whereas the negative control showed white dots on a weakly purple background.

Four dark, single colonies were tried to be individually picked from the less dense plate with 1000 colonies, diluted and plated again. No second screen was performed but 3 colonies of each plated pick were randomly picked, cultivated and the plasmids were isolated.

All 24 picks were tested by PCR for the presence of a complete DBL1 domain yielding in 3 cDNA clones, which carried a complete DBL1 domain. Clone var 1.8.3 with an insert of 1700 bp, var 5.3.4 (450 bp), and var 5.4.1 (3000 bp) were used to perform a cell-cell adherence assay (see: 4.3.4.). Clone var 5.4.1. was additionally used in two positive controls (see: 4.1.5.2. and 4.1.6.).

4.3.3.2. Analysis of the radioactive protocol

80 µl of 1:20'000 diluted cDNA library was plated on 5 plates, yielding approximately 800 clones per plate. Membranes of all 5 plates were hybridized in a first step with the P^{32}-labelled DBL1 probes. 30 black dots and no background were visible on the developed x-ray film of membrane 4. From the corresponding plate, 17 small regions were picked and plated for 17 second screens. In these second screens, 8 clones were identified to carry a complete

DBL1 domain and were used to perform a cell-cell adherence assay (see: 4.3.4.). Clone PD4.14.1 (insert 1600 bp) was lateron sequenced (see: 4.3.5).

The P^{32}-labelled DBL1 probes were stripped from the membranes 1, 2, 3 and 5 and the hybridisation was repeated with P^{32}-labelled ATS domains. On the developed X-ray films of membranes 1, 2 and 5, 5 dots were identified, which could represent the same clones on both films. The 5 corresponding small areas were picked and plated for a second screens. The colony lift from this second screen was performed twice, with a 6-hour incubation step in between, to allow re-growth of the colonies. Like that, two membranes with an identical colony-pattern were available; each of which was hybridized independently with P^{32}-labelled DBL1- and ATS probes. The developed x-ray films were then overlaid to identify single colonies carrying both domains, but no such clones were identified.

The film of membrane 3 was used to identify 10 regions, carrying clones with an ATS domain. The corresponding 10 small areas were picked from plate 3 and used for a second screens. Two clones were identified to carry a complete ATS domain and clone PA3.4.1. (insert 2500 bp) was sequenced (see: 4.3.5).

4.3.3.3. Comparison of the two colony lift protocols

The radioactive protocol produced more reliable results than the non-radioactive protocol, since the background was minimal, compared to the very high background of the non-radioactive protocol. Therefore, although the radioactive protocol is much more time consuming, it should be given preference over the non-radioactive protocol.

4.3.4. Results of the cell-cell adherence assays

2 cell-cell adherence assays were performed with the clones, identified by the non-radioactive protocol: In one assay the COS 7 cells were transfected with an equally mixed pool of var 1.8.3, var 5.3.4, and var 5.4.1. In the other one, cells were transfected with clone var 5.4.1 only. Transfection was performed with chloroquine / DEAE dextran (3.2.2.5a.) and the assay conditions were the same as in the 4^{th} cell-cell adherence assay (4.1.4.1.). Both assays were negative, since only a few COS 7 cells were found with one or two attached screening cells on it. The COS 7 cells which were transfected with var 5.4.1 only, were further used in the positive controls for picking and Hirt extraction (see: 4.1.5.2.).

A third cell-cell adherence assay was performed with the 8 clones carrying a DBL1 domain, as identified by the radioactive protocol: The cells were transfected (3.2.2.5a) with an equally mixed pool of the 8 clones. The assay conditions were the same as in the 5^{th} cell-cell adherence assay (4.1.4.1.). The assay was negative.

4.3.5. Sequence analysis of clones PD 4.14.1 and PA 3.4.1

Clone PD 4.14.1 was sequenced to answer the question, why an insert, containing a complete DBL1 domain, is only 1.6 kb long, which could not be a full-length *var* gene. Sequencing revealed a stretch of 6 lysines, encoded by 17 consecutive adenosines (Figure 4.1), which presumably allowed priming of the Oligo dT (NotI) primer, used in first strand cDNA synthesis. Furthermore it revealed a new DBL1 domain, which had not been cloned before from the cDNA library (see: 4.4.1.).

Figure 4.1 Partial sequence of clone PD 4.14.1

```
          1        10        20        30        40        50        60
          |        |         |         |         |         |         |
PD4.14.1  GCACGAAGTTTTGCAGATATAGGTGATATCGTAAGAGGAAAAGATCTATATCTCGGTTATGATAAT
           A  R  S  F  A  D  I  G  D  I  V  R  G  K  D  L  Y  L  G  Y  D  N

   70        80        90       100       110       120       130       140
   |         |         |         |         |         |         |         |
AAAGAAAAAGAACAAAGAAAAAAATTAGAACAGAAATTGTTTAACAAAAAATTTTTCAAGCAAATACATGATGAC
 K  E  K  E  Q  R  K  K  L  E  Q  K  L  F  N  K  K  F  F  K  Q  I  H  D  D

       150       160       170       180       190       200       210
        |         |         |         |         |         |         |
GTGACGTCGACGAGAGGGAGTAATGGGCAGGCTCTAAAAACACGCTACAAAGATGATCGCGAGAAAAATTATTTT
 V  T  S  T  R  G  S  N  G  Q  A  L  K  T  R  Y  K  D  D  R  E  K  N  Y  F

  220       230       240       250       260       270       280       290
   |         |         |         |         |         |         |         |
CAATTACGAGAAGATTGGTGGACGGCGAATCGAGAAACAGTATGGGAAGCCATGACATGCAGCAAGGAGCTTGAT
 Q  L  R  E  D  W  W  T  A  N  R  E  T  V  W  E  A  M  T  C  S  K  E  L  D

       300       310       320       330       340       350       360
        |         |         |         |         |         |         |
AATTCTTCATATTTTCGTGCAACGTGCAATGATACTGGACAAGGTCCATCACAAACCCACAACAAATGCCGGTGT
 N  S  S  Y  F  R  A  T  C  N  D  T  G  Q  G  P  S  Q  T  H  N  K  C  R  C

  370       380       390       400       410       420       430       440
   |         |         |         |         |         |         |         |
GATAAGGACAAGGGCGCAAATGCCGGCAAGCCAAAGGCTGGCGACGGAGATGTAACTATTGTCCCCACATATTTT
 D  K  D  K  G  A  N  A  G  K  P  K  A  G  D  G  D  V  T  I  V  P  T  Y  F

       450       460       470       480       490       500       510
        |         |         |         |         |         |         |
GACTACGTGCCGCAGTATCTTCGCTGGTTCGAAGAATGGGCGGAAGATTTTTGTAGGAAAAAAAAAAAAAAAAG
 D  Y  V  P  Q  Y  L  R  W  F  E  E  W  A  E  D  F  C  R  K  K  K  K  K  K
```

Legend to Figure 4.1

The sequence of clone PD4.14.1 is given from the binding site of the VARDBL5 primer (nucleotides 1-23) to the polyA-stretch, 2 AA downstream the site of the VARDBL3 primer (nucleotides 466-491). The remaining approximate 1000 bp of the insert of clone PD4.14.1 reach into the 5' UTR (sequence not shown).

Clone PA 3.4.1 was sequenced, to check whether it contained an ATS domain and reverse sequencing revealed more than 400 bp of a 3' UTR of a *var* gene, which showed 97% homology to the sequence of a *var* gene, found in GenBank[a] (Accession number: L42636 / PFAVAR 7: Su *et al.*, 1995, see Figure 4.2).

Figure 4.2 Alignment of parts of clone PA 3.4.1 and L42636 3' UTR

```
PA 3.4.1     465 TATTTGTGGGTTATATTATAATTTTTATTTATGGGTTAAATATATATTTTTTT  413
                 |||||||||||||||||||||||||||||||||||| ||||||||||||||
L42636      7159 TATTTGTGGGTTATATTATAATTTTTATTTATGGGTTATATATATATTTTTTT 7211

PA 3.4.1     412 TTTTTGTGCATTTGTCTATTTTTTATTTGTGCTTTATATATATATATAGTTT  361
                 |||| ||||||||||||||||||||||||||||||||||||||||||||||
L42636      7212 TTTT-GTGCATTTGTCTATTTTTTATTTGTGCTTTATATATATATATATTTT 7262
```

Finding a complete 3' UTR in clone PA 3.4.1 indicated a good quality of the isolated mRNA, since deadenylation of the polyA-tail is often a first step in mRNA degradation *in vivo*.

Forward sequencing revealed, that the complete insert showed homologies to L42636 and that the insert started more than 1000 bp upstream the ATS domain and extended into exon 1 (Figure 4.3). Therefore this sequence was indeed amplified by RT-PCR from mRNA and not derived from a DNA contamination during mRNA isolation.

Figure 4.3 Alignment of parts of clone PA 3.4.1 and L42636 coding region

```
PA 3.4.1       1 AMLASV-EL--KLGTSSDPLVQCGGIHWVQNFLEDYNKIRHKISHCKENSEQTIC
                   |   |  |    |       |   |||||| || ||  |      |
L42636      4816 KKIKNGQEGDKKYITMKELLKR-----WLEYFLEDYNRIRKKIKLC------T--

PA 3.4.1     KKDCKDKCKCVK----KWISTKKQEWQQIKTRLLEQYKGQDYYNVKTI-LEDLR
             ||   | ||| |    ||   |  ||| |    |||||  |  |  |  ||
L42636       KKE--DGCKCIKGCIEKWVQEKTKEWQKINDTYLEQYKN-DDGNTLTNFLEQFQ

PA 3.4.1     DRPVLNKAIKPCPSLNAFEKSCGLNGTDNSQNGNNNDLVLCMIKTL-KKIDNAK  432
              |     |||||  |  |  ||||| ||||||||||||||    | |||   |
L42636       YRTEFKNAIKPCDGLDQFKTSCGLNSTDNSQNGNNNDLVLCLLNKLQKKISECK 5259
```

4.3.6. Occurrence of full-length *var* genes in the cDNA library

In the direct approach (1), the cDNA library was screened for full-length *var* genes to transfect COS 7 cells. A first step to find full-length *var* genes was the search for clones in the library, which carried both, a DBL1- and an ATS domain. However, no insert with these specifications was found, neither by hybridisation with DBL1- and ATS domains (4.3.3.2.), nor by PCR (3.2.1.8.).

4.4. Results of the direct approach (2) (bacterial expression system)

DBL1- and CIDR domains were amplified by PCR from both, the ItG2.F6 cDNA and genomic DNA of K1, cloned into pQE30 vector, expressed as 6xhis-tagged protein in *E. coli* strain M15, and purified using Ni-NTA agarose columns. The binding properties of these domains were analyzed by IFAs and FACScan analysis (see 3.2.5.6.).

4.4.1. Analysis of all DBL1 domains used for bacterial expression

All DBL1 domains cloned so far into pMOSblue-T vector (4.3.1.1. and 4.3.2) were amplified by PCR with the primer pair DBL1 Fw Bam and DBL1 Rev Kpn, since direct re-cloning into pQE30 was not possible. As a positive control, both, cDNA and gDNA of K1 were used as template in the PCR. Amplified PCR products of all cloned DBL1 domains were gel-eluted, purified and ligated into the pQE30 vector, together with the PCR products of the two control PCRs. All previously cloned DBL1 domains could be recloned in pQE30 as well as three additional new DBL1 domains, two from gDNA of strain K1 and one from the cDNA, termed DBLK3, DBLK4 and DBLs20 respectively.

All 10 DBL1 domains were expressed as 6xhis-tagged protein and were solubilized and purified under native conditions with the exception of DBLs4, which had to be solubilized under denaturing conditions (8 M urea, see 3.2.5.4. and 3.2.5.5.).

The nucleotide and AA sequences of all 10 cloned DBL1 domains is given in Figure 4.4. The AA alignment of these DBL1 domains with 6 DBL1 domains, retrieved from GenBank[a] and the DBL1 domain of cDNA clone PD4.14.1 is shown in Figure 4.5.

Figure 4.4 Sequences of 10 DBL1 domains, expressed as 6xhis-tagged proteins

```
          1        10        20        30        40        50        60        70        80
          |        |         |         |         |         |         |         |         |
DBL16.1   GCACGAAGTTTTGCAGATATAGGTGATATCGTACGCGGCAAAGATCTTTATCTTGGTAATAAGAAAGAACGAGCTAGATTA
           A  R  S  F  A  D  I  G  D  I  V  R  G  K  D  L  Y  L  G  N  K  K  E  R  A  R  L

DBL16.2   GCACGAAGTTTTGCAGATATAGGTGATATCGTACGCGGCAAAGAACTGTATCTTGGTAATAATAAAGAAAAAAAGCAATTA
           A  R  S  F  A  D  I  G  D  I  V  R  G  K  E  L  Y  L  G  N  N  K  E  K  K  Q  L

DBL16.3   GCACGAAGTTTTGCAGATATTGGAGACATTATACGAGGAAAAGATTTGTATCTCGGGTATGATGAAAAAGAAAAAAATCGA
           A  R  S  F  A  D  I  G  D  I  I  R  G  K  D  L  Y  L  G  Y  D  E  K  E  K  N  R

DBLK3     GCACGAAGTTTTGCAGATATTGGTGATATTGTAAGAGGAAAAGATCTTTATTTGGGTGATCAACAAGAAAAAGATAGATTA
           A  R  S  F  A  D  I  G  D  I  V  R  G  K  D  L  Y  L  G  D  Q  Q  E  K  D  R  L

DBLK4     GCACGAAGTTTTGCAGATATTGGAGATATCGTACGCGGCAAAGATCTGTATCTAGGTAATCCACAAGAAAAAGAACAAAGA
           A  R  S  F  A  D  I  G  D  I  V  R  G  K  D  L  Y  L  G  N  P  Q  E  K  E  Q  R

DBLs2     GCACGAAGTTTTGCAGATATTGGAGATATTGTCAGAGGAAAAGATCTATATAGAGGTAATAACAAAGAAAAAGAAAAAAGA
           A  R  S  F  A  D  I  G  D  I  V  R  G  K  D  L  Y  R  G  N  N  K  E  K  E  K  R

DBLs4     GCACGAAGTTTTGCAGATATTGGAGATATTGTCAGAGGAAGAGATTTATATCTTGGAAGGCACGCCAAGAGAGCCACCAAA
           A  R  S  F  A  D  I  G  D  I  V  R  G  R  D  L  Y  L  G  R  H  A  K  R  A  T  K

DBLs9     GCACGAAGTTTTGCAGATATTGGAGATATCGTACGCGCCAAAGATCTATATAGTGGTAATACCCAAGAAAAAAACCGAAGA
           A  R  S  F  A  D  I  G  D  I  V  R  A  K  D  L  Y  S  G  N  T  Q  E  K  N  R  R

DBLs16    GCACGAAGTTTTGCAGATATAGGAGACATTGTCAGAGGAAGAGATCCATTTTATGGTAATACACAAGAAAGTACACAAAGA
           A  R  S  F  A  D  I  G  D  I  V  R  G  R  D  P  F  Y  G  N  T  Q  E  S  T  Q  R

DBLs20    GCACGAAGTTTTGCAGATATTGGAGACATTATACGAGGAAAAGATCTATTTCGTGGTAATAAAAAAAAAAAATCAAACGAA
           A  R  S  F  A  D  I  G  D  I  I  R  G  K  D  L  F  R  G  N  K  K  K  K  S  N  E


                  90       100       110       120       130       140       150       160
                  |        |         |         |         |         |         |         |
DBL16.1   GAAGAAAATTTACAAAAAATTTTTAGGAATATATATGACAATTTGGTGGGTGCAGCAAAAAAACACTACGAAGGTGATAAA
           E  E  N  L  Q  K  I  F  R  N  I  Y  D  N  L  V  G  A  A  K  K  H  Y  E  G  D  K

DBL16.2   GAAGAGAATTTAAAAAAAATTTTCAAGCAAATACATGGTGGATTGAAGAATGGGGAGCTAAAAGCACTCTACCAAAAGGAC
           E  E  N  L  K  K  I  F  K  Q  I  H  G  G  L  K  N  G  E  L  K  A  L  Y  Q  K  D

DBL16.3   AGAAAACAATTAGAAAATAATTTGAAAGAAATTTTCACGCAAATACATGAGGGATTGTCGAAGAACGGCGCACAAAAACAC
           R  K  Q  L  E  N  N  L  K  E  I  F  T  Q  I  H  E  G  L  S  K  N  G  A  Q  K  H

DBLK3     GAAGAGAATTTAAGAAAGATTTTCAAGAAAATATATGAAGGATTGTCGAACAACGGCGCAGAAGAACGCTACAATGGTGAT
           E  E  N  L  R  K  I  F  K  K  I  Y  E  G  L  S  N  N  G  A  E  E  R  Y  N  G  D

DBLK4     GAAAAATTAGAAAATAAGTTGAAAGAGGTTTTTGGGAAAATACATAGTGGATTGACAGATAGTGGTGCAAAAGAACGCTAC
           E  K  L  E  N  K  L  K  E  V  F  G  K  I  H  S  G  L  T  D  S  G  A  K  E  R  Y

DBLs2     GAAAAATTAGAACAGAAATTGAAAGAAATTTTCAAGAAAATACATGGAAATTTGAGGAATGGTGTAAAAGACCACTACAAT
           E  K  L  E  Q  K  L  K  E  I  F  K  K  I  H  G  N  L  R  N  G  V  K  D  H  Y  N

DBLs4     ATAAATCTAAATGGAAAAGAAACAGAAAGAGGAAAATTAGAAAATAATTTGAAAGATATTTTCAAGAATATACATGATGAA
           I  N  L  N  G  K  E  T  E  R  G  K  L  E  N  N  L  K  D  I  F  K  N  I  H  D  E

DBLs9     GATCAATTAGAAAAGAATTTGAAAGAAATTTTCGGGGATATATATAATGACGTGACGTCTAGCGGCAATAATAAGGATGTG
           D  Q  L  E  K  N  L  K  E  I  F  G  D  I  Y  N  D  V  T  S  S  G  N  N  K  D  V

DBLs16    GAAAAATTAGAGAAGAATTTGAAAGAAATTTTCAAGGAAATACATAATGGATTGGATGGGGAAGCACAAGCTCGCTACAAT
           E  K  L  E  K  N  L  K  E  I  F  K  E  I  H  N  G  L  D  G  E  A  Q  A  R  Y  N

DBLs20    AGAGATAAATTAGAAGAGAATTTACCAAAAATTTTCAAGCAAATACATGATGACGTGACGTTCGACGAGAGGAGTAATGGG
           R  D  K  L  E  E  N  L  P  K  I  F  K  Q  I  H  D  D  V  T  F  D  E  R  S  N  G
```

```
                170       180       190       200       210       220       230       240
                 |         |         |         |         |         |         |         |
DBL16.1  GAAAATTTTTATCAATTACGAGAAGATTGGTGGAACGCTAATAGACAACAAGTATGGAACGCGATAACATGCGGCGCTGAG
          E  N  F  Y  Q  L  R  E  D  W  W  N  A  N  R  Q  Q  V  W  N  A  I  T  C  G  A  E

DBL16.2  GGTGATAATTATTTTCAATTACGAGAAGATTGGTGGGCACTTAATAGACAAGATGTATGGAAAGCTATCACATGTGGCGCT
          G  D  N  Y  F  Q  L  R  E  D  W  W  A  L  N  R  Q  D  V  W  K  A  I  T  C  G  A

DBL16.3  TACAATGGTGATACTAAAGATTATTTTCAATTACGAGAAGATTGGTGGGCTCTTAATAGAGACCAAGTATGGAAAGCTATT
          Y  N  G  D  T  K  D  Y  F  Q  L  R  E  D  W  W  A  L  N  R  D  Q  V  W  K  A  I

DBLK3    AAAGATCCAAATTATTATCAATTGCGAGAAGATTGGTGGGCACTTAATAGAGTACAAGTATGGAAAGCTATCACATGTGGT
          K  D  P  N  Y  Y  Q  L  R  E  D  W  W  A  L  N  R  V  Q  V  W  K  A  I  T  C  G

DBLK4    AAAGATGATGCCAAAAAAAAATTTTTATCAATTACGAGAAGATTGGTGGACTGCGAATCGCGCCACAATCTGGGAAGCCATG
          K  D  D  A  K  K  N  F  Y  Q  L  R  E  D  W  W  T  A  N  R  A  T  I  W  E  A  M

DBLs2    GGTGACGGTGATAATTATTATCAATTAAGAGAAGATTGGTGGACTGCAAATCGTGAAACAGTATGGAAAGCTATCACATGC
          G  D  G  D  N  Y  Y  Q  L  R  E  D  W  W  T  A  N  R  E  T  V  W  K  A  I  T  C

DBLs4    GTGACGAAGGGAGGGAAGAATGGGGAGGCTCTAAAAACACGCTACGGAAGTGATAAAGAAAATTATTATCAATTACGAGAA
          V  T  K  G  G  K  N  G  E  A  L  K  T  R  Y  G  S  D  K  E  N  Y  Y  Q  L  R  E

DBLs9    CTAAAAGCACGCTACAATGGTGATACTGATAATTATTATAAACTAAGAGAAGATTGGTGGACTGCTAATAGAGAAACAGTA
          L  K  A  R  Y  N  G  D  T  D  N  Y  Y  K  L  R  E  D  W  W  T  A  N  R  E  T  V

DBLs16   GGTGATACTGATAATTATTATAAATTAAGGGAAGATTGGTGGACAGCGAATAGGCACACCGTGTGGGAAGCTATCACGTGC
          G  D  T  D  N  Y  Y  K  L  R  E  D  W  W  T  A  N  R  H  T  V  W  E  A  I  T  C

DBLs20   CAGGCTCTAAAAACACGCTACAAAGATGATCGCGAGAAAAATTATTTTCAATTACGAGAAGATTGGTGGACGGCGAATCGA
          Q  A  L  K  T  R  Y  K  D  D  R  E  K  N  Y  F  Q  L  R  E  D  W  W  T  A  N  R


                250       260       270       280       290       300       310       320
                 |         |         |         |         |         |         |         |
DBL16.1  AATGATTCTCAATATTTTCGACGAACATGTGGTAGTGATGAAAAAACTGCAACTCGGACTCCTAGTCAATGCCGATGTACC
          N  D  S  Q  Y  F  R  R  T  C  G  S  D  E  K  T  A  T  R  T  P  S  Q  C  R  C  T

DBL16.2  GCGGGTGGTAAATATTTTAGAAATACATGTGTTGGGGGAAGTCCGACTCAAGATGACTGCCGATGCAGGACAAATGATGTA
          A  G  G  K  Y  F  R  N  T  C  V  G  G  S  P  T  Q  D  D  C  R  C  R  T  N  D  V

DBL16.3  ACATGTGATGCGCCACCTGATGCTCAATATTTTCGAGGAACATGTGTAGGTGATGGAAAACATTCAACTCTGGCTAAAGAC
          T  C  D  A  P  P  D  A  Q  Y  F  R  G  T  C  V  G  D  G  K  H  S  T  L  A  K  D

DBLK3    GCTACAATGAAAGATATATATTCAAAAACCACCGATAATGGTAAGCTGCTTCTTTGGAATTATAATTGCGGTCATCATGTG
          A  T  M  K  D  I  Y  S  K  T  T  D  N  G  K  L  L  L  W  N  Y  N  C  G  H  H  V

DBLK4    ACATGCGAAGCGAACGGTACATATTTTCGACCAACGTGCAGTATGAATGGAAGTGGAGCCCAAGCTAATAACAAATGCCGG
          T  C  E  A  N  G  T  Y  F  R  P  T  C  S  M  N  G  S  G  A  Q  A  N  N  K  C  R

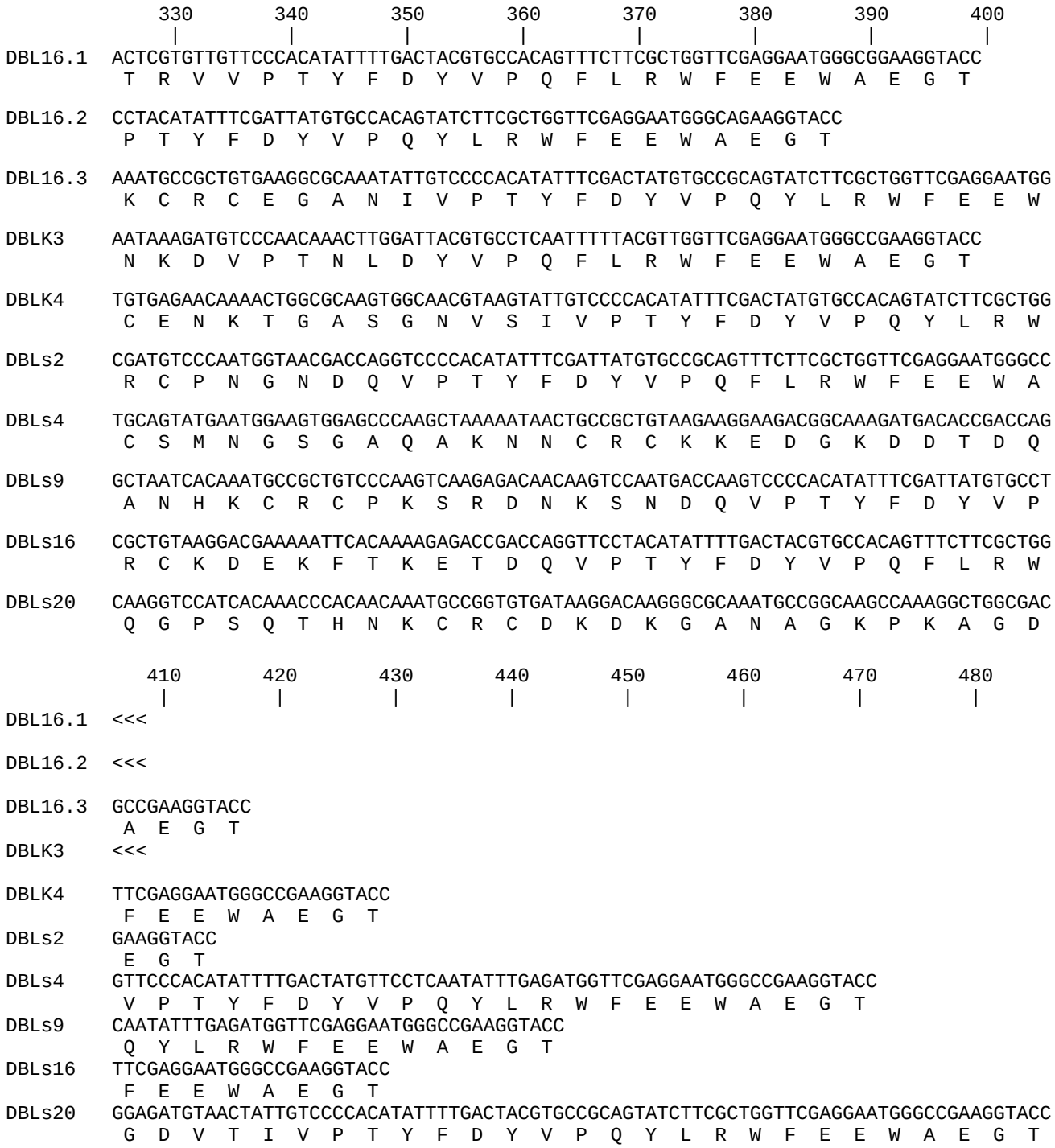
DBLs2    AACGACAGGCTAGGGGGTAATGCATATTTTCGAAAAACTTGTAGTGATACAAAAGGTTCATCTGTAGCTAATCACAAATGC
          N  D  R  L  G  G  N  A  Y  F  R  K  T  C  S  D  T  K  G  S  S  V  A  N  H  K  C

DBLs4    GATTGGTGGACTGCGAATCGCGCCACAGTGTGGGAAGCATTAACATGTGAAGTTGGTAGTGGTACATATTTTCATGCAACG
          D  W  W  T  A  N  R  A  T  V  W  E  A  L  T  C  E  V  G  S  G  T  Y  F  H  A  T

DBLs9    TGGAAAGCCATCACGTGCGGTGCGCCAGAACATGCTTCATATTTTCGTGTAACATGCAGTGATGAACAAGGTGGAGCCGAA
          W  K  A  I  T  C  G  A  P  E  H  A  S  Y  F  R  V  T  C  S  D  E  Q  G  G  A  E

DBLs16   GAAGCTGGGAATGATTCTCAATATTTTCGACCAACATGTGGAGGTAATGAAAAAAATTCAACTCTGGCTAAAGACAAATGC
          E  A  G  N  D  S  Q  Y  F  R  P  T  C  G  G  N  E  K  N  S  T  L  A  K  D  K  C

DBLs20   GAAACAGTATGGGAAGCCATGACATGCAGCAAGGAGCTTGATAATTCTTCATATTTTCGTGCAACGTGCAATGATACTGGA
          E  T  V  W  E  A  M  T  C  S  K  E  L  D  N  S  S  Y  F  R  A  T  C  N  D  T  G
```



Legend to Figure 4.4

The sequence of all ten DBL1 domains (K1, lane 1-5 and ItG2.F6, lane 6-10), expressed as 6xhis-tagged proteins, is given from the site of the DBL1 FW Bam primer (ARSFADIG-motif) to the site of the DBL1 Rev Kpn primer (WFEEWAE-motif). All PCR products showed homologies to DBL1 domains, since all domains contained a common motif ([Q/K]LREDWWX[A/L]NR) typical for DBL1 domains found in almost all published DBL1 domains. This motif is printed in bold.

Figure 4.5 Aligned AA sequences of 17 DBL1 domains

```
          1        10        20        30        40        50        60        70
          |        |         |         |         |         |         |         |
L40608    ARSFADIGDIVRGRDLYLG---------NP-QEIKQRQQLENNL--KTIFGKIY-E------KLNGAEAR--YGNDPE
L40609.3  ARSFADIGDIVRGKDLYLG---------YDDEEKEKRKQLEKNL--KKFFQKIHDDVMKTSGRTNGKKSAEAQKRYND
L40609.2  ARSFADIGDIVRGRDLYRG---------ND-KE-KDR--LEENL--RKIFKKIY-D------NLNDAHVQEHYKDDDK
L42636    ARSFADIGDIVRGKDLYLG---------YDNKEKEQRKKLEQKL--KDIFKKIHKD------VMKTNGAQERYIDDAK
U27338    ARSFADIGDIVRGKDLYLG---------YDDKEKDERKKLENNL--IEIFKKIH-E------NLGTQDAKDHYKKDEE
U27339    ARSFADIGDIVRGKDLYLG---------YDDKEKDERKKLENNL--IEIFKKIH-E------NLGTQDAKDHYKKDEE
DBL16.1   ARSFADIGDIVRGKDLYLG---------NK-KE---RARLEENL--QKIFRNIY-D------NLVGA-AKKHYEGDKE
DBL16.2   ARSFADIGDIVRGKELYLG---------N-NKEK---KQLEENL--KKIFKQIHG-------GLKNGELKALYQKDGD
DBL16.3   ARSFADIGDIIRGKDLYLG---------YDEKEKNRRKQLENNL--KEIFTQIH-E------GLSKNGAQKHYNGDTK
DBLK3     ARSFADIGDIVRGKDLYLG----------DQQEK---DRLEENL--RKIFKKIYE-------GLSNNGAEERYNGDKD
DBLK4     ARSFADIGDIVRGKDLYLG---------NP-QEKEQREKLENKL--KEVFGKIHSGL--TDSGA-----KERYKDDAK
DBLs2     ARSFADIGDIVRGKDLYRG---------NN-KEKEKREKLEQKL--KEIFKKIH--------GNLRNGVKDHYNGDGD
DBLs4     ARSFADIGDIVRGRDLYLGRHAKRATKINLNGKETERGKLENNL--KDIFKNIHDEVTK--GGKNGEALKTRYGSDKE
DBLs9     ARSFADIGDIVRAKDLYSG---------NT-QEKNRRDQLEKNL--KEIFGDIYNDV--TSSGNNKDVLKARYNGDTD
DBLs16    ARSFADIGDIVRGRDPFYG---------NT-QESTQREKLEKNL--KEIFKEIH-N------GLDGE-AQARYNGDTD
DBLs20    ARSFADIGDIIRGKDLFRG---------NKKKKSNERDKLEENLP--KIFKQIHDDVTFDE-RSNGQALKTRYKDDRE
PD4.14.1  ARSFADIGDIVRGKDLYLG---------YDNKEKEQRKKLEQKLFNKKFFKQIHDDVTSTR-GSNGQALKTRYKDDRE
Consensus ARSFADIGDI*RG*DL* G         *   *   *  LE *L    *F  I*                  Y  D

           80        90       100       110       120       130       140       150
           |         |         |         |         |         |         |         |
L40608    ----FFKLREDWWTANRETVWKAITCNAW-GNT-YFHATC-NRG---ERTKGYCRCND--------------D-QVP
L40609.3  ATGNYYKLREDWWNANRDQVWKAITCDAADNDE-YFENSSDGLYVFSNGQ-----CGRNEGK-------------VP
L40609.2  GTKNYYKLRNAWWEANRQTVWKAITCGAA-GGT-YFRQTC-GTG---TWTNEKCRCPI--------------N-DVP
L42636    G-GDFFQLREDWWTSNRETVWKALICHAP-KEANYFIKTACNVG---KGTNGQCHCIG--------------G-DVP
U27338    ---NYYQLREDWWTANRSTVWKAITCHAG-ESDKYFRKTCCSGEWTDDK----CRCKDEEGKN--------ETN-EVP
U27339    ---NYYQLREDWWTANRSTVWKAITCHAG-ESDKYFRKTCCSGEWTD----DKCRCKDEEGKN--------ETN-EVP
DBL16.1   ---NFYQLREDWWNANRQQVWNAITCGAE-NDSQYFRRTCGSDEKTATRTPSQCRCT--------------TR-VVP
DBL16.2   ---NYFQLREDWWALNRQDVWKAITCGAAGG--KYFRNTCVG----GSPTQDDCRCRTN---------------DVP
DBL16.3   ---DYFQLREDWWALNRDQVWKAITCDAP-PDAQYFRGTCVGDGKHSTLAKDKCRCE-------------GAN-IVP
DBLK3     --PNYYQLREDWWALNRVQVWKAITCGATMK--DIYSKTTDN----GKLLLWNYNCGHHV------------NKDVP
DBLK4     --KNFYQLREDWWTANRATIWEAMTCEAN-GT--YFRPTC-SMNGSGAQANNKCRCENKTGAS--------GNVSIVP
DBLs2     ---NYYQLREDWWTANRETVWKAITCNDRLGGNAYFRKTC-SDTKGSSVANHKCRCPNG-------------NDQVP
DBLs4     ---NYYQLREDWWTANRATVWEALTCEVGSGT--YFHATC-SMNGSGAQAKNNCRCKKEDGKD--------DT-DQVP
DBLs9     ---NYYKLREDWWTANRETVWKAITCGAP-EHASYFRVTC-SDEQGGAEANHKCRCP-KSRDN--------KSNDQVP
DBLs16    ---NYYKLREDWWTANRHTVWEAITCEAG-NDSQYFRPTCGGNEKNSTLAKDKCRCKDEKFTK--------ETD-QVP
DBLs20    --KNYFQLREDWWTANRETVWEAMTCSKELDNSSYFRATCNDTGQGPSQTHNKCRCDKDKGANAGKPKAGDGDVTIVP
PD4.14.1  --KNYFQLREDWWTANRETVWEAMTCSKELDNSSYFRATCNDTGQGPSQTHNKCRCDKDKGANAGKPKAGDGDVTIVP
Consensus    ****LREDWW  NR  VW A TC        YF  TC            CRC                   VP

           160       170
           |         |
L40608    TYFDYVPQYLRWFEEWAEDF   1
L40609.3  TNLDYVPQHLRWFDEWAEDF   2
L40609.2  TYFDYVPQYLRWFEEWAEDF   3
L42636    TYFDYVPQYLRWFEEWAEDF   4
U27338    TYFDYVPQYLRWFEEWAEDF   5
U27339    TYFDYVPQYLRWFEEWAEDF   6
DBL16.1   TYFDYVPQFLRWFEEWAEGT   7
DBL16.2   TYFDYVPQYLRWFEEWAEGT   8
DBL16.3   TYFDYVPQYLRWFEEWAEGT   9
DBLK3     TNLDYVPQFLRWFEEWAEGT  10
DBLK4     TYFDYVPQYLRWFEEWAEGT  11
DBLs2     TYFDYVPQFLRWFEEWAEGT  12
DBLs4     TYFDYVPQYLRWFEEWAEGT  13
DBLs9     TYFDYVPQYLRWFEEWAEGT  14
DBLs16    TYFDYVPQFLRWFEEWAEGT  15
DBLs20    TYFDYVPQYLRWFEEWAEGT  16
PD4.14.1  TYFDYVPQYLRWFEEWAEDF  17
Consensus TYFDYVPQ LRWFEEWAE**
```

Legend to Figure 4.5

The AA sequence of 17 DBL1 domains is given:

The published sequences 1 to 4 (Su *et al.*, 1995) were isolated from strain Dd2 (1 + 4) and FCR3 (2 + 3).
The published sequences 5 to 6 (Baruch *et al.*, 1995) were isolated from the Malayan Camp strain.

The consensus sequence is defined as follows:
bold letter: AA is the same in at least 16 of 17 DBL1 domains.
asterix (*): AA is the same in at least 14 of 17 DBL1 domains, or only 2 different AAs were found in the 17 sequences analyzed.

4.4.2. Analysis of all CIDR domains used for bacterial expression

3 distinctive products were amplified by the PCR amplification of the CIDR domain: The PCR with the cDNA as a template resulted in two distinct bands of 520 and 550 bp, whereas the PCR with the gDNA of K1 as template resulted in one band of 530 bp. The PCR products were ligated as described (3.2.1.9.) and resulted in about 260 clones. In total, 42 clones were picked, 10 from K1 and 32 from ItG2 F6 cDNA. All clones were analyzed for presence, size, and sequence of the insert. With small scale expressions, it was checked, if the CIDR domain was expressed as a 6xhis-tagged protein. An overview is given in Table 4-X.

Table 4-X Comparison of 42 clones, tested for expression of a CIDR domain

Clone	**Insert**	**Expression**	**Sequence**	**Clone**	**Insert**	**Expression**	**Sequence**
C1	560 bp	yes	**1 (11xa)**	K22	520 bp	yes	=39
C2	560 bp	yes	=1	K23	520 bp	degr. 100AA	=39 (9xa)
C3	-	-	-	K24	520 bp	yes	=39
C4	500 bp	no	n.d.	C25	560 bp	degr. 100AA	=1 (12xa)
C5	500 bp	no	n.d.	C26	560 bp	yes	=1
C6	500 bp	no	n.d.	C27	520 bp	yes	=10
C7	500 bp	no	n.d.	C28	500 bp	no	n.d.
C8	560 bp	yes	=1	C29	-	-	-
C9	520 bp	yes	**9 (11xa)**	C30	500 bp	no	n.d.
C10	520 bp	yes	**10 (9xa)**	C31	560 bp	yes	=1
C11	500 bp	no	=17	C32	560 bp	yes	**32 (11a)**
C12	-	-	-	C33	500 bp	no	n.d.
C13	560 bp	yes	=1	C34	-	-	-
C14	560 bp	yes	=1	C35	500 bp	no	n.d.
C15	500 bp	no	n.d.	C36	560 bp	yes	=1
C16	500 bp	no	=17	C37	500 bp	no	=17
C17	500 bp	no	**17 (8xa)**	C38	500 bp	no	=17
C18	500 bp	no	n.d	K39	520 bp	yes	**39 (10xa)**
K19	520 bp	yes	=39	K40	520 bp	yes	=39
K20	520 bp	yes	=39	K41	-	-	-
K21	520 bp	yes	=39	K42	520 bp	yes	**42 (9xa)**

Legend to Table 4-X

All 42 cloned CIDR domains were tested for expression and most were subsequently sequenced. Each newly identified sequence was termed according to the clone (bold letters). In all CIDR domains, a polyA-stretch between 9 to 11 adenosines was found (in brackets). Two CIDR domains (CIDRK23 and CIDRC25) had an error in the polyA-stretch, resulting in a frame shift and therefore resulted in degraded proteins (see also Figure 4.6 and 4.7).

Together, 6 different CIDR domains were found and expressed as 6xhis-tagged proteins, termed CIDR C1, CIDR C9, CIDR C10, CIDR C32 (all isolated from the cDNA), CIDR K39 and CIDR K42 (both isolated from gDNA of strain K1).

An additional sequence (termed C17) was found five times in the cDNA, which could never be expressed, since a single mutation at position 122 (see Figure 4.6) led to a stop codon (TAG instead of TGG). All clones, derived from the ligation with the 520 bp PCR product (see above), which were sequenced, carried this mutation, indicating that this error had already been present in the template and was not an artefact of the PCR, since even an error in the first PCR-step, would lead only to 50 % of the PCR product to have this error.

On the other hand, two clones (CIDRK23 and CIDRC25) had a mutation in a polyA-stretch of their insert, which was possibly PCR-derived, since (internal) polyA stretches are known to be possible 'slipping sites' for polymerases (Wagner *et al.*, 1990). The polyA-stretch, common for all isolated CIDR domains, is shown in bold letters in Figure 4.6.

- the clone CIDR K23 had the same sequence as CIDR K39, with the exception of an adenosine missing in a 10xA-stretch, which lead to a frame shift and therefore to a degraded protein of 101 AAs instead of 167 AAs.
- the clone CIDR C25 had the same sequence as CIDR C1, with the exception of an additional adenosine in a 11xA-stretch, which lead to a frame shift and therefore lead to a degraded protein of 100 AAs instead of 189 AAs.

A third clone, CIDR C32, which could be expressed as a complete 6xhis-tagged CIDR domain had a sequence which was identical to the sequence of CIDR C10 for bp 1-211 and then identical to the sequence of CIDR C1 for bp 194-567 (The region of recombination is given in underlined letters in Figure 4.6). Whether this sequence was present in the cDNA or was created during the PCR with the cDNA as a template, was not investigated further.

Figure 4.6 Sequences of 7 CIDR domains, as ligated into the pQE30 vector

```
          1         10         20         30         40         50         60
          |         |          |          |          |          |          |
CIDR C1   GGATCCAGATGGAAATGTTATTATGATGAAAATGAAAAAAATAGTGAAAAAAATAATAATTGTGTA
           G  S  R  W  K  C  Y  Y  D  E  N  E  K  N  S  E  K  N  N  N  C  V
CIDR C32  GGATCCAAATGGAAATGTTATTATGATGAACATAAGCCTAGTAGTAAAAATAATAATAATTGTGTA
           G  S  K  W  K  C  Y  Y  D  E  H  K  P  S  S  K  N  N  N  N  C  V
CIDR C10  GGATCCAAATGGAAATGTTATTATGATGAACATAAGCCTAGTAGTAAAAATAATAATAATTGTGTA
           G  S  K  W  K  C  Y  Y  D  E  H  K  P  S  S  K  N  N  N  N  C  V

CIDR C9   GGATCCAAATGGAAATGTTATTATGATGAAAGTAAGGAAAGTGGACAAAATAATAATTGCATACTA
           G  S  K  W  K  C  Y  Y  D  E  S  K  E  S  G  Q  N  N  N  C  I  L

CIDR C17  GGATCCAAATGGAAATGTTATTATGATGATAGCGATAAAAGTAATAATTGTGTACAAGGAGAATGG
           G  S  K  W  K  C  Y  Y  D  D  S  D  K  S  N  N  C  V  Q  G  E  W

CIDR K39  GGATCCAAATGGAAATGTTATTATGAAAAAACCGATAAAAGTAATAATTGCATACTAGGAAAATGG
           G  S  K  W  K  C  Y  Y  E  K  T  D  K  S  N  N  C  I  L  G  K  W

CIDR K42  GGATCCAAATGGAAATGTTATTATGAAAAAAAAAATGAAAAGGATGGTGGCAAGGACATTAATTTT
           G  S  K  W  K  C  Y  Y  E  K  K  N  E  K  D  G  G  K  D  I  N  F

             70         80         90        100        110        120        130
             |          |          |         |          |          |          |
CIDR C1   GAAGGAACATGGGACACGTTTACACAGGGTAAAAAAGTTAAGCCCTATAATCCATTTTTTTGGGAT
           E  G  T  W  D  T  F  T  Q  G  K  K  V  K  P  Y  N  P  F  F  W  D
CIDR C32  GAAGGAACATGGGACAAGTTTACACAAGGTAAACAAACCGTTAAGTCCTATAATGTTTTTTTTTGG
           E  G  T  W  D  K  F  T  Q  G  K  Q  T  V  K  S  Y  N  V  F  F  W
CIDR C10  GAAGGAACATGGGACAAGTTTACACAAGGTAAACAAACCGTTAAGTCCTATAATGTTTTTTTTTGG
           E  G  T  W  D  K  F  T  Q  G  K  Q  T  V  K  S  Y  N  V  F  F  W

CIDR C9   GGAAAATGGGAGGACTTTACAGGGAACGAGGACGTTACATCTTATAATGTTTTTTTTTATAATTCA
           G  K  W  E  D  F  T  G  N  E  D  V  T  S  Y  N  V  F  F  Y  N  S

CIDR C17  AAAGATTTTCAAAAGGGTCAAAAGGTTACGTCTTATAATGCATTTTTTTGGAAGTAGGTTCATGAT
           K  D  F  Q  K  G  Q  K  V  T  S  Y  N  A  F  F  W  K  *  V  H  D

CIDR K39  GAAAAGTTTCAAAAGGGACAAGAAGTTATGGTCTATCATCCTTTTTTTTGGAGTTGGGTTACTGAA
           E  K  F  Q  K  G  Q  E  V  M  V  Y  H  P  F  F  W  S  W  V  T  E

CIDR K42  TGTGTACTACAAGATGACAAAGCAAGGACATCAGAAGAAAAAAGTATGCACTATAATTCATTTTTT
           C  V  L  Q  D  D  K  A  R  T  S  E  E  K  S  M  H  Y  N  S  F  F

                140        150        160        170        180        190
                 |          |          |          |          |          |
CIDR C1   TGGGTATATCATATGTTACACGATTCTTTAGATTGGAGAAACGAACTTGGGAGTTGTATAGATAAT
           W  V  Y  H  M  L  H  D  S  L  D  W  R  N  E  L  G  S  C  I  D  N
CIDR C32  GATTGGGTTCATGATATGTTACACGATTCTGTAGAGTGGAGAGAAAGACTTAATAGTTGTATAAAT
           D  W  V  H  D  M  L  H  D  S  V  E  W  R  E  R  L  N  S  C  I  N
CIDR C10  GATTGGGTTCATGATATGTTACACGATTCTGTAGAGTGGAGAGAAAGACTTAATAGTTGTATAAAT
           D  W  V  H  D  M  L  H  D  S  V  E  W  R  E  R  L  N  S  C  I  N

CIDR C9   ATTATTGAAATGTTAAATGATTCTATAGAATGGAAAGACAAACTTGATATTTGTATAAATAATAAA
           I  I  E  M  L  N  D  S  I  E  W  K  D  K  L  D  I  C  I  N  N  K

CIDR C17  ATGTTAATCGACTCTATGCAATGGAGAGACGAACATGGTAAGTGTATAAATAAAGATAATGACAAC
           M  L  I  D  S  M  Q  W  R  D  E  H  G  K  C  I  N  K  D  N  D  N

CIDR K39  ATGTTGGACGATTCTATAAAATGGAGGAAAGAACTTGATAATTGCCTAAAAAATGAGAACAAACAA
           M  L  D  D  S  I  K  W  R  K  E  L  D  N  C  L  K  N  E  N  K  Q

CIDR K42  TGGGATTGGGTTTATCATATGTTACATGATTCTTTAAATTGGAGAAAACAACTTGGTAATTGTATA
           W  D  W  V  Y  H  M  L  H  D  S  L  N  W  R  K  Q  L  G  N  C  I
```

```
          200       210       220       230       240       250       260
           |         |         |         |         |         |         |
CIDR C1   GCCAAATCAGGACAATGTGAAAACAAATGTAATAGTAAATGTGAATGTTTTGAACGATGGGTTG**AA**
           A  K  S  G  Q  C  E  N  K  C  N  S  K  C  E  C  F  E  R  W  V  E
CIDR C32  AATGCCAAATCAGGACAATGTGAAAACAAATGTAATAGTAAATGTGAATGTTTTGAACGATGGGTT
           N  A  K  S  G  Q  C  E  N  K  C  N  S  K  C  E  C  F  E  R  W  V
CIDR C10  AATGCCAAATCACAAAATTGTAAAAACAATAAATGTAATAGAGAATGTGGTTGTTTTGCAAAATGG
           N  A  K  S  Q  N  C  K  N  N  K  C  N  R  E  C  G  C  F  A  K  W

CIDR C9   ACGGGAAAATGTAGAAAAGTATGTAAAAATCCATGTGAATGTTATAAACGGTGGATTG**AAAAAAAA**
           T  G  K  C  R  K  V  C  K  N  P  C  E  C  Y  K  R  W  I  E  K  K

CIDR C17  ACATGTATAAGAGGGTGTAAAAGTAAATGTGAATGTTTTCAAAAATGGGTAGATC**AAAAAAAA**GAA
           T  C  I  R  G  C  K  S  K  C  E  C  F  Q  K  W  V  D  Q  K  K  E

CIDR K39  TGTATAAGCAAATGTAATGGTAAATGTGATTGTTTCCAAAGATGGATTGAT**AAAAAAAAAA**CCGAA
           C  I  S  K  C  N  G  K  C  D  C  F  Q  R  W  I  D  K  K  K  T  E

CIDR K42  AATGACGCCAAATCAGGAAAATGTGAAAACAAATGTAATAGCAAATGTGATTGTTTTTTAAAATGG
           N  D  A  K  S  G  K  C  E  N  K  C  N  S  K  C  D  C  F  L  K  W

              270       280       290       300       310       320       330
               |         |         |         |         |         |         |
CIDR C1   **AAAAAAAAA**GAAGAATGGAAGGCAATAAAAGACCATTT**TAA**AAAGCAAAAAGATATTGAACAAGAG
           K  K  K  E  E  W  K  A  I  K  D  H  F  K  K  Q  K  D  I  E  Q  E
CIDR C32  G**AAAAAAAAAAA**GAAGAATGGAAGGCAATAAAAGACCATTTTAAAAAGCAAAAAGATATTGAACAA
           E  K  K  K  E  E  W  K  A  I  K  D  H  F  K  K  Q  K  D  I  E  Q
CIDR C10  GTTGTT**AAAAAAAAA**GACGAATGGGACAAAATAAAAGACCATTTTAACAAGCAAGAAAATATTGGG
           V  V  K  K  K  D  E  W  D  K  I  K  D  H  F  N  K  Q  E  N  I  G

CIDR C9   **AAA**CCGAATTGGAAAAAATTAAAAGACCATTTTCGCAAGCAAAAAGATATTGGAGATGCAGCACAA
           K  P  N  W  K  K  L  K  D  H  F  R  K  Q  K  D  I  G  D  A  A  Q

CIDR C17  GAATGGAAAAATATCAAAATACATTTTCTGAAGCAAGACGATATTGGACAAGAAACACATTGTGAT
           E  W  K  N  I  K  I  H  F  L  K  Q  D  D  I  G  Q  E  T  H  C  D

CIDR K39  TGGGAAAATATCAAAATACATTTTGGCAAGCAAGAAGATA**TGA**GAGAACAAATTGGAGAGGATACA
           W  E  N  I  K  I  H  F  G  K  Q  E  D  M  R  E  Q  I  G  E  D  T

CIDR K42  GTTGTAC**AAAAAAAAA**CCGAATGGAAAAATATCAAAATACATTTTGGGAACCAATATTTTGTCAAG
           V  V  Q  K  K  T  E  W  K  N  I  K  I  H  F  G  N  Q  Y  F  V  K

                    340       350       360       370       380       390
                     |         |         |         |         |         |
CIDR C1   ACACATTGTGATCCTGGCGTAACTCTTGAATTACTTTTTATGAACGACGAACTTTTGAAAAATATT
           T  H  C  D  P  G  V  T  L  E  L  L  F  M  N  D  E  L  L  K  N  I
CIDR C32  GAAACACATTGTGATCCTGGCGTAACTCTTGAATTACTTTTTATGAACGACGAACTTTTGAAAAAT
           E  T  H  C  D  P  G  V  T  L  E  L  L  F  M  N  D  E  L  L  K  N
CIDR C10  CAAAACGTAGGTTTTATCGAATTTAATCACTATGGAGTTCTTGAAGGTGTTTTGGACAAAGATGAA
           Q  N  V  G  F  I  E  F  N  H  Y  G  V  L  E  G  V  L  D  K  D  E

CIDR C9   CGTGAAATGACCCTTAATATTACTTTAAATAATAATTTTTTGAATGATATTAAAGATGCTTATCCA
           R  E  M  T  L  N  I  T  L  N  N  N  F  L  N  D  I  K  D  A  Y  P

CIDR C17  CCTGGCGTAACTCTTGCAGCTGTTTTGGAGGAAGACAAACTTTTGAAAATTATTCAGGATACTTAT
           P  G  V  T  L  A  A  V  L  E  E  D  K  L  L  K  I  I  Q  D  T  Y

CIDR K39  GATCCTGGCATAATTCTTGAAGGTGTTTTGAATTTTGAGAATCTTTTCAAAAATATTAAAGATACT
           D  P  G  I  I  L  E  G  V  L  N  F  E  N  L  F  K  N  I  K  D  T

CIDR K42  CAAGACGGTTTCGACTTAGGAATGAATTCTCCTGATGTTGTTCTTGAATTACTTTTGGAGAAAAAT
           Q  D  G  F  D  L  G  M  N  S  P  D  V  V  L  E  L  L  L  E  K  N
```

```
            400       410       420       430       440       450       460
             |         |         |         |         |         |         |
CIDR C1   AAAGATACTCATGCGAATGCAGATGACATAAAACACATTGAGGCACTGTTGCAAGAAACAGTTTTT
           K  D  T  H  A  N  A  D  D  I  K  H  I  E  A  L  L  Q  E  T  V  F
CIDR C32  ATTAAAGATACTCATGCGAATGCAGATGACATAAAACACATTGAGGCACTGTTGCAAGAAACAGTT
           I  K  D  T  H  A  N  A  D  D  I  K  H  I  E  A  L  L  Q  E  T  V
CIDR C10  CTTTTGAAAAATATTAAAGATACTCATGCGGATGCAAAGGACATAGAAAGAATTGATAAAATGTTG
           L  L  K  N  I  K  D  T  H  A  D  A  K  D  I  E  R  I  D  K  M  L

CIDR C9   GTCAAACAACAGCTGCAGAAAATTGAAGAACGTTTGAAAGATAAAATGCAGGAAGATTTTATTTTT
           V  K  Q  Q  L  Q  K  I  E  E  R  L  K  D  K  M  Q  E  D  F  I  F

CIDR C17  GGGAATACAGAGGAAACAAAACACCTTCGCCAAATGTTGCAACAAGCAGGCGTTGTTGGTGGTGTT
           G  N  T  E  E  T  K  H  L  R  Q  M  L  Q  Q  A  G  V  V  G  G  V

CIDR K39  TATGGGGACGTAAAAGAAATAGATCACATTAAGAAACTGTTGGAAGATGAAGAAAATGTTGGTGGC
           Y  G  D  V  K  E  I  D  H  I  K  K  L  L  E  D  E  E  N  V  G  G

CIDR K42  TTACTTTTGGAAATTATTAAAGATACTCATGCGAATGCAAAGGAAGATGAAATAAAAAACATTGAT
           L  L  L  E  I  I  K  D  T  H  A  N  A  K  E  D  E  I  K  N  I  D

                 470       480       490       500       510       520
                  |         |         |         |         |         |
CIDR C1   GATGGTGTTGCTGCTGGCGGTCCTGGTGGTGCTGGTAGTTATAAGTGTACTGAAGGTGCCAAGGGC
           D  G  V  A  A  G  G  P  G  G  A  G  S  Y  K  C  T  E  G  A  K  G
CIDR C32  TTTGATGGTGTTGCTGCTGGCGGTCCTGGTGGTGCTGGTAGTTATAAGTGTACTGAAGGTGCCAAG
           F  D  G  V  A  A  G  G  P  G  G  A  G  S  Y  K  C  T  E  G  A  K
CIDR C10  CAACAAGCAGGTGTTGTTGGTGGTGGTCGTGGTGGCGAAAATAATACCATAATTGATAAATTACTA
           Q  Q  A  G  V  V  G  G  G  R  G  G  E  N  N  T  I  I  D  K  L  L

CIDR C9   GAGAGAACGAAAACATCAATTGATAAATTACTACAAGGTACC
           E  R  T  K  T  S  I  D  K  L  L  Q  G  T

CIDR C17  GTTGGTAGTGGTGTTGGTGGTACC
           V  G  S  G  V  G  G  T

CIDR K39  GAACATAATACCACAATTGATAAATTACTACAAGGTACC
           E  H  N  T  T  I  D  K  L  L  Q  G  T

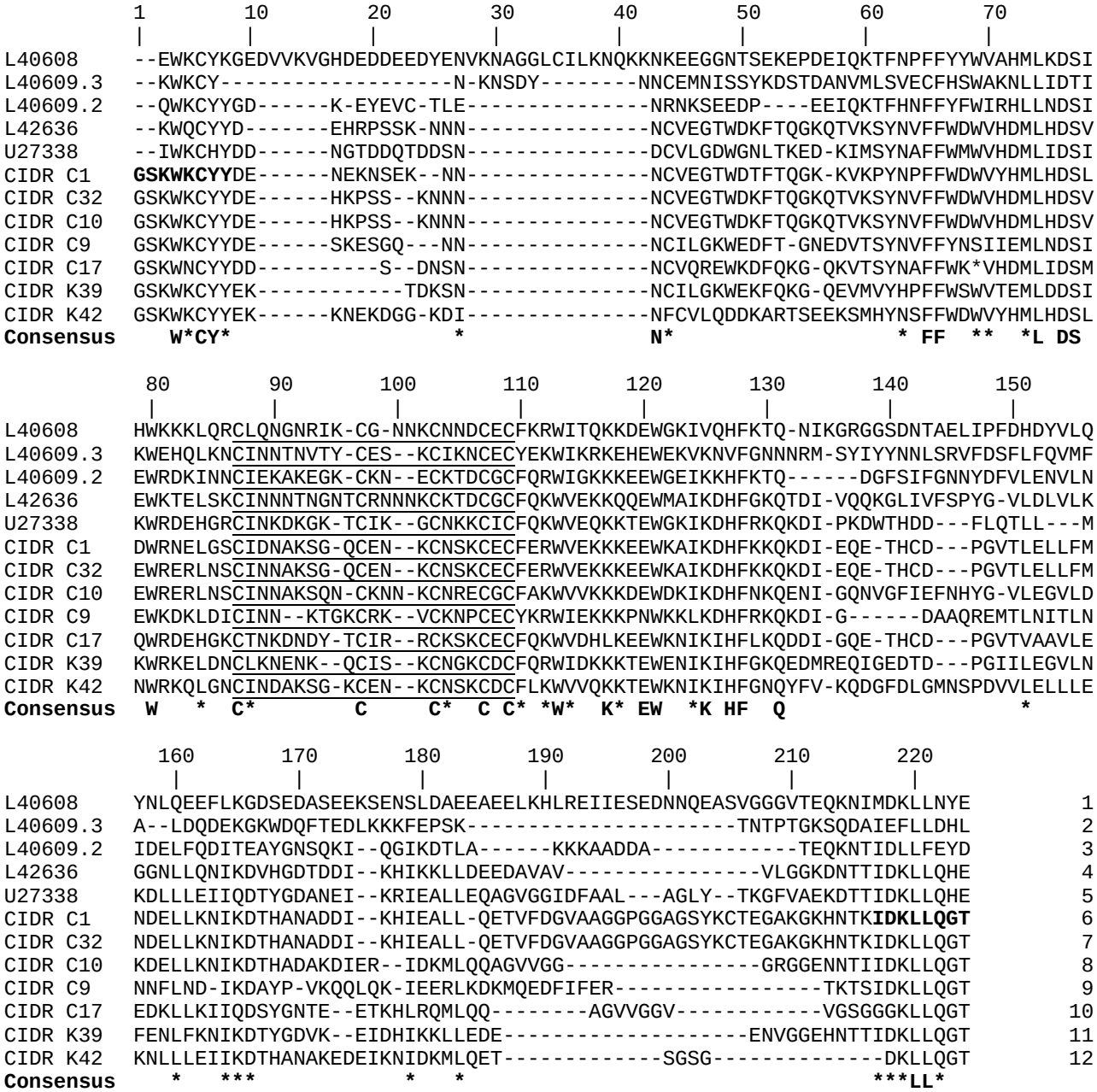
CIDR K42  AAAATGTTGCAAGAAACAAGTGGCAGTGGTGATAAATTACTACAAGGTACC
           K  M  L  Q  E  T  S  G  S  G  D  K  L  L  Q  G  T

          530       540       550       560       570
           |         |         |         |         |
CIDR C1   AAACATAATACTAAAATTGATAAATTACTACAAGGTACC
           K  H  N  T  K  I  D  K  L  L  Q  G  T
CIDR C32  GGCAAACATAATACTAAAATTGATAAATTACTACAAGGTACC
           G  K  H  N  T  K  I  D  K  L  L  Q  G  T
CIDR C10  CAAGGTACC
           Q  G  T
```

Legend to Figure 4.6

The sequences of 7 CIDR domains (5x cDNA, 2x gDNA) are given.
polyA-stretches of all sequences are in bold letters (position 250 to 280), the stop codons at position 305 in CIDR K39 and at position 303 in CIDR C1 are indicated in bold, the mutation (Pos. 122) of CIDR C17, which led to a stop-codon is shown in bold letters, and the putative site of recombination of CIDR C32 (position 197 to 210) is underlined.

Figure 4.7 Aligned AA sequences of 12 CIDR domains



Legend to Figure 4.7

The AA sequence of 12 CIDR domains is shown: The sequences 1 to 5 are the same as in Figure 4.5 (see references noted there). These sequences were aligned with the 6 expressed CIDR domains and with the sequence of the clone CIDR C17, a clone with an internal stop codon. The cysteine-rich motif is underlined. The consensus sequence is defined as follows:

- bold letter: AA were identical in at least 11 of 12 CIDR domains.
- asterix (*): AA were identical in at least 9 of 12 CIDR domains, or only 2 different AAs were found in the 12 sequences analyzed.

4.4.3. Analysis of the expression of 6xhis-tagged *var* gene domains

10 DBL1 domains (see Figure 4.4) and 6 CIDR domains (see Figure 4.6) were expressed as 6xhis-tagged proteins. All but two domains could be isolated and purified under native conditions (3.2.5.4. and 3.2.5.5.). DBLs 4 and CIDR C9 were isolated and purified under denaturing conditions. All 16 domains were subsequently dialysed against PBS.
The size of all 16 purified domains ranged from 18 kDa (DBL16.2) to 28 kDa (CIDR C32). In a preliminary experiment, the domains CIDR C1, CIDR C10, CIDR K39 and CIDR K42 were recognized on a western blot by an αhis-tag mAb (PentaHis, see Table 3-V), as well as by pooled human serum from malaria exposed adults (Table 3-VII).

After storage at -20°C, some dialysed protein fractions precipitated, therefore the protein concentration of all samples was evaluated photometrically and the samples were diluted with PBS to a final concentration of 20 µg / ml and stored again at -20°C. In that concentration no further precipitation was observed. All 16 expressed, purified and diluted *var* gene domains were subsequently used in binding assays as described in 3.2.5.6.

4.4.4. Results of the IFAs, performed with 6xhis-tagged *var* gene domains

The expressed and purified 6xhis-tagged *var* gene domains were tested in 13 IFAs for binding to L-huICAM-1 cells, C32 cells, and L cells as a negative control (see 3.2.5.6.). Two assays were additionally performed with CHO-huCD36 and CHO-huICAM-1 cells. All these assays never gave clear reliable results, but some information, which is summarized below:

- The positive controls (OKM*5 on C32 cells and MEM112 on L-huICAM-1 cells) gave a specific, clear signal, however, OKM*5 did not detect fixed C32 cells.
- The negative control using the αHis mAb without proteins resulted in a light back ground on all cell types used, whereas OKM*5 (on L-huICAM-1 cells and L cells) or MEM112 (on C32- and L cells) showed no background.
- From the 2nd IFA on, cells were always preincubated in PBS / 5% FCS, PBS / 5% drymilk or in Binding medium, which decreased the background.
- No difference was found between applying the proteins in PBS / 0.5% FCS pH 6.3 or pH 7.3 and applying the proteins in Binding Medium pH 7.0.
- In three assays, C32 cells, incubated with CIDR domains and αHis mAb, showed staining, compared to C32 cells with αHis mAb only, to C32 cells, incubated with DBL1 domains and αHis mAb, and to L-cells incubated with CIDR domains and αHis mAb, indicating binding of CIDR domains to C32 cells.

After these assays, it was concluded that:

1) the assays should be analyzed fluorcytometrically, since FACScan analysis uses unfixed cells and can be evaluated quantitatively.

2) unlike L-huICAM-1- and C32 cells, the cells, used in the following protein-binding assays, should only differ in the expression of a single, distinct surface molecule, therefore, only three types of cells (CHO-huCD36, CHO-huICAM-1 and untransfected CHO cells) were used for further assays.

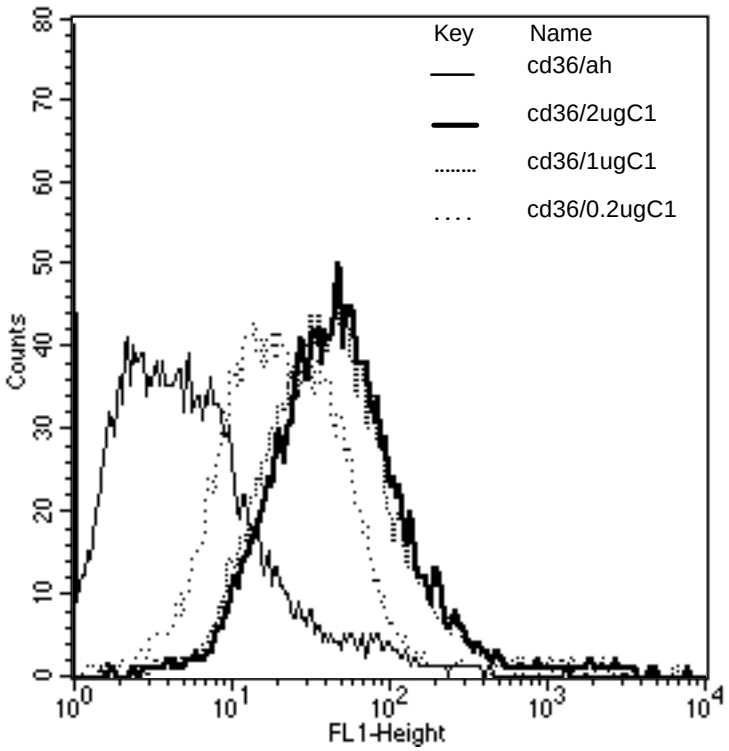
4.4.5. Results of the FACScan analysis, performed with 6xhis-tagged *var* gene domains

All expressed and purified 6xhis-tagged *var* gene domains were tested for binding to cells expressing both, huICAM-1 and CSA (CHO-huICAM-1 cells), and cells expressing both, CD36 and CSA (CHO-huCD36 cells), as well as cells expressing CSA only (CHO cells). In all assays, 10'000 cells were analyzed for each determination, except in one, where 5'000 cells were used (see Figure 4.10).

4.4.5.1. Binding of CIDR domains to CHO-huCD36-, CHO-huICAM-1- and CHO cells

Initially, FACS analysis showed that all 6 recombinant CIDR-domains bound to CHO-huCD36 cells in a dose-dependent manner (Table 4-XI, Figure 4.8). Binding affinity varied, with CIDR C1 binding being the strongest (9x stronger than αhis-antibody without protein) and CIDR K39 the weakest (2.5 x control). Subsequently, it was shown that all CIDR domains also bound to non-transfected CHO cells (Table 4-XI) and CHO-huICAM-1 cells (data not shown), suggesting that binding was mediated through CSA.
At a concentration of 20 µg/ml, all CIDR-domains bound to CHO-huCD36 cells but at a concentration of 2 µg/ml binding was only detectable by FACScan with CIDR C1 and CIDR C32 (Table 4-XI).
In the following figures (Figure 4.8 to 4.11) the analysis of 10'000 (or 5'000) cells per determination is visualized in histogram plots: The number of cells (Counts, Y-axis) is given relative to the intensity of FITC-fluorescence (FL1-Height, X-axis)

Figure 4.8 Binding of CIDR C1 domain to CHO-CD36 cells in a dose-dependent manner



Legend to Figure 4.8

Cytofluorogram of CHO-CD36 cells, incubated with decreasing concentrations of CIDR C1 (20 µg/ml, 10 µg/ml, 2 µg/ml). Bound protein was detected using an αhis-tag mAb followed by detection with FITC labelled αmouse IgG. As negative control, αhis-tag mAb was incubated without prior incubation with protein.

Table 4-XI Binding of all CIDR domains to CHO-CD36 and to CHO cells

amount of protein/ tested cells	αhis-tag neg. control	αCD36 pos. control	CIDR C1	CIDR C9	CIDR C10	CIDR C32	CIDR K39	CIDR K42
20 µg/ml; CHO-CD36			4571	2684	1884	2609	1300	1712
10 µg/ml; CHO-CD36			4259	1482	1813	1838	826	1613
2 µg/ml; CHO-CD36			2128	724	829	1318	616	858
0 µg/ml; CHO-CD36	541	4215						
7 µg/ml; CHO			1026	1563	806	995	842	911
0 µg/ml; CHO	394							

Legend to Table 4-XI

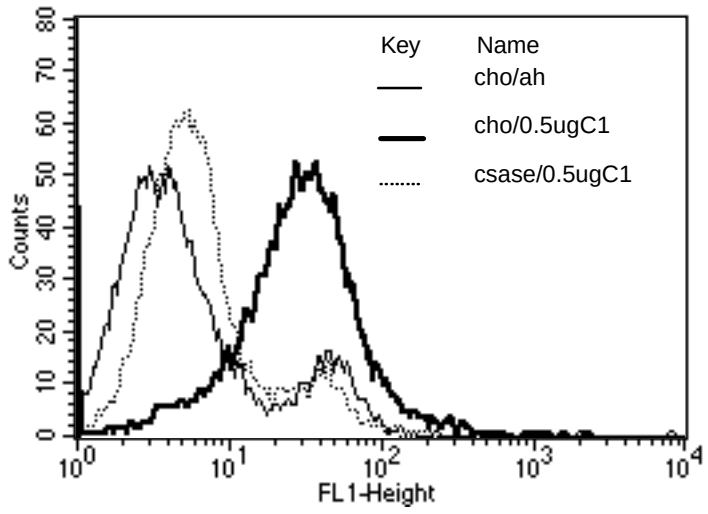
Binding of different recombinant 6xhis-tagged CIDR-domains to CD36-transfected CHO cells and non-transfected CHO cells was measured by cytofluorometry. Depicted is the geometric mean fluorescence after staining with an αhis-tag mAb followed by a FITC labelled αmouse IgG, as measured directly by the FACS analysis. As negative control αhis-tag antibody was incubated in the absence of protein, as positive control CD36-transfected CHO cells were incubated with OKM*5, a monoclonal antibody recognizing CD36. Both controls were visualized using an αmouse-IgG, labelled with FITC.

4.4.5.2. Inhibition of protein binding by CSase ABC treatment of CHO cells

To confirm binding to CSA, CHO and CHO-huCD36 cells were treated with CSase ABC, and binding of 0.2 µg CIDR C1 to treated cells was compared to binding to untreated cells (Figure 4.9). On both cell types binding was decreased after CSase ABC-treatment by at least 90%. Increasing CSase ABC treatment (2 hours) and increasing the concentration of CIDR-domains (0.5 µg CIDR C1, 1µg CIDR C10 or CIDR C32) resulted in a decrease of binding up to 94% when compared to untreated cells (Table 4-XII).

There was no evidence of a difference in binding to CSase ABC treated CHO cells and CSase treated CHO-huCD36 cells, indicating that CD36 was involved in the binding of CIDR C1 (Table 4-XII, Figure 4.9).

Figure 4.9 Binding of the CIDR C1 domain to CSase ABC treated cells



Legend to Figure 4.9

Cytofluorogram of non-transfected CHO cells, untreated and treated with CSase ABC, and incubated with 5 µg/ml CIDR C1.
Bound protein was detected using an αhis-tag mAb followed by detection with FITC labelled αmouse IgG. As negative control, αhis-tag mAb was incubated without prior incubation with protein.

Note:
The negative control in Figure 4.9 (thin line) showed a typical, rather small additional peak, which was often found with all CHO cell types used, but only when the anti 6xHis mAb was involved. The peak was independent of CSA, since both, CSase ABC-treated and untreated cells, showed the same peak. However, this peak was dependent on trypsination: the longer the cells were incubated in presence of Trypsin-EDTA solution, the smaller was the peak. Therefore it was concluded, that the anti 6xHis mAb interacts with a trypsin-sensitive protein, present on the surface of all CHO cell types used.

Table 4-XII Binding of CIDR domains to CSase ABC treated and untreated CHO cells

tested cells	treatment of cells	concentration / protein	mean fluorescence	percentage of binding
CHO-CD36	none	0 µg/ml	661	0 %
CHO-CD36	none	2 µg/ml CIDR C1	983	100 %
CHO-CD36	CSase (1 hour)	2 µg/ml CIDR C1	616	-13 %
CHO	none	0 µg/ml	467	0 %
CHO	none	2 µg/ml CIDR C1	842	100 %
CHO	CSase (1 hour)	2 µg/ml CIDR C1	500	8.8 %
CHO	none	0 µg/ml	563	0 %*
CHO	none	5 µg/ml CIDR C1	2768	100 %*
CHO	CSase (2 hours)	5 µg/ml CIDR C1	687	5.6 %
CHO	none	10 µg/ml CIDR C10	1952	100 %*
CHO	CSase (2 hours)	10 µg/ml CIDR C10	732	12.2 %
CHO	none	10 µg/ml CIDR C32	1691	100 %*
CHO	CSase (2 hours)	10 µg/ml CIDR C32	765	17.9 %

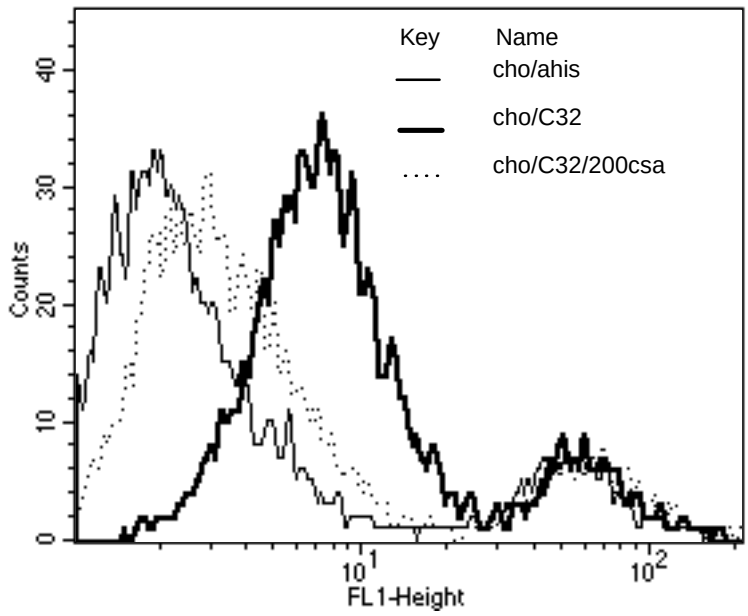
Legend to Table 4-XII

Comparison of binding of three different CIDR-domains to CD36-transfected CHO cells and non-transfected CHO cells, either with or without prior CSase ABC treatment. Binding is measured by cytofluorometry using αhis-tag mAb followed by incubation with FITC-labelled αmouse-IgG. Depicted is the geometric mean fluorescence and remaining percentage of binding after treatment. Percentage of binding was calculated using the value from untreated cells as maximum (100%) and the value of the negative control (αhis-tag mAb only) as minimum (0%). *Note that two independent experiments were carried out and the negative controls were used accordingly.

4.4.5.3. Competition with soluble CSA and -HPS as a control

In parallel, 0.7 µg of each of the 6 CIDR-domains were tested against untreated CHO cells, CSase-treated CHO cells (1 hour treatment), and untreated CHO cells with 200 µg soluble CSA as competitor. Binding was inhibited up to 100% (CIDR C10) by 200 µg soluble CSA, whereas CSase-treatment resulted in 67 to 89 % inhibition of binding. CIDR C32 was also tested against untreated CHO cells and with 50 µg CSA or 200 µg HPS as competitor. Competition with 50 µg CSA resulted in 62.4% inhibition of binding, whereas binding of the same domain was only little affected (18.1 % inhibition) by 100 µg HPS (Table 4-XIII, Figure 4.10).

Figure 4.10 Binding of CIDR C32 domain to CHO cells in presence of free CSA



Legend to Figure 4.10

Cytofluorogram of non-transfected CHO cells, competitively incubated with 7 µg/ml CIDR C32 and 200 µg soluble CSA. Binding was compared against incubation of protein without addition of soluble CSA. Bound protein was detected using an αhis-tag mAb followed by detection with FITC labelled αmouse IgG. As negative control, αhis-tag mAb was incubated without prior incubation with protein. In this assay, 5'000 cells were analyzed for each determination (see 4.4.5.)

Table 4-XIII Competitive binding of CIDR domains and soluble CSA to CHO cells

assay condition	**αhis-tag mAb**	**CIDR C1**	**CIDR C9**	**CIDR C10**	**CIDR C32**	**CIDR K39**	**CIDR K42**
	geometric mean of fluorescence (percentage of remaining binding)						
none	3.94 (0)	10.26 (100)	15.63 (100)	8.06 (100)	9.95 (100)	8.42 (100)	9.11 (100)
CSase	n.d.	5.98 (32.3)	5.22 (10.9)	5.20 (30.6)	4.90 (16.0)	5.10 (25.9)	4.64 (13.5)
+ 200 µg CSA	n.d.	5.26 (20.9)	4.75 (6.8)	3.81 (-3.2)	5.16 (20.3)	5.23 (28.8)	5.29 (26.1)
+ 50 µg CSA					6.20 (37.6)		
+ 100 µg HPS					8.86 (81.9)		

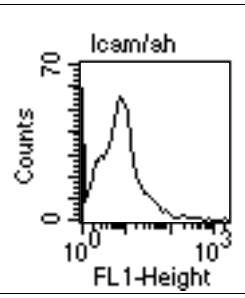
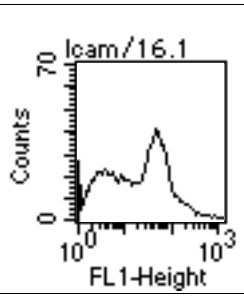
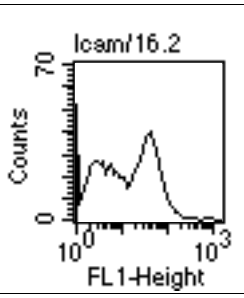
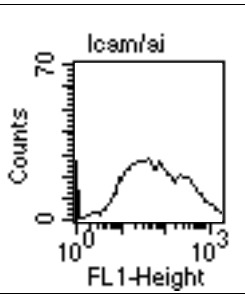
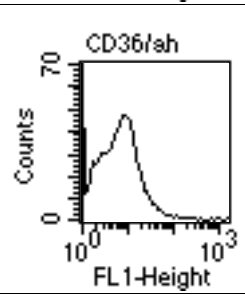
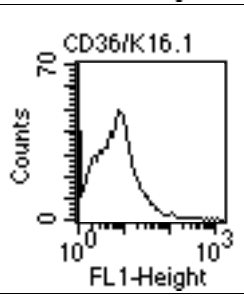
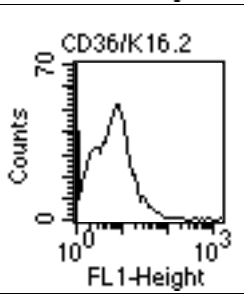
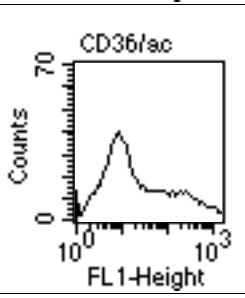
Legend to Table 4-XIII

Competition was measured by cytofluorometry using αhis-tag mAb followed by incubation with FITC-labelled αmouse-IgG. Depicted is the geometric mean of fluorescence and remaining percentage of binding. Percentage of binding was calculated using the value from assays without CSase treatment or addition of soluble CSA as maximum (100%) and the value of the negative control (αhis-tag mAb only) as minimum (0%). In all assays CIDR domains were used at a concentration of 7 µg/ml.

4.4.5.4. Binding of DBL1 domains to CHO-huCD36- and CHO-huICAM-1 cells

In IFAs, using DBL1- and CIDR domains, CIDR domains bound to C32 cells, whereas the DBL1 domains showed no binding. However, the binding of DBL1 domains to CHO-huCD36 and CHO-huICAM-1 cells was also investigated in FACScan analysis assays. In one assay, 1 µg of two DBL1 domains (DBL16.1 and DBL16.2) was found to bind specifically to CHO-huICAM-1 cells, but not to CHO-huCD36 cells (Figure 4.11); 1 µg of all other DBL1 domains did not bind neither cell type (data not shown). But this binding could never be repeated in several subsequent assays, although different pHs in the binding and washing steps were tried (pH 6.3, pH 6.8, pH 7.3 and pH 7.8), and different concentrations of DBL1 domains were tested (1 µg, 2 µg and 5 µg per tube).

Figure 4.11 Binding of 2 DBL1 domains to CHO-huICAM-1 cells

Row 1	Row 2	Row 3	Row 4
			
			

Legend to Figure 4.11

The cytofluorograms in column 1 represent the negative controls (αhis-tag mAb only) on CHO-huICAM-1 cells (upper row) and on CHO-huCD36 cells (lower row), whereas the corresponding positive controls are shown in column 4. The DBL16.1- and DBL16.2 domain bound specifically to CHO-huICAM-1 cells, but not to CHO-huCD36 cells (column 2 and 3).

5. Discussion

The understanding of the molecular mechanisms leading to sequestration and thereby to vital organ failure in human *P. falciparum* infections is fundamental in the comprehension of this disease. Precise knowledge of the parasite-derived ligands and the host cell receptors involved, may firstly point out specific host and parasite factors, that indicate a risk for development of severe disease and secondly may offer novel concepts in therapeutic strategies by developing defined molecules that interfere with or reverse parasite cytoadherence.

Although many receptors expressed on the surface of endothelium cells which line the microvasculature have been identified, the parasite-derived adhesins, expressed on the surface of iRBCs are still much less well studied. Many aspects are hampering the analysis of these adhesins: limited parasite material, (possible) antigenic variation of these adhesins, (possible) interaction of more than one parasite-derived protein in mediating adhesion, and the additional influence of parasite-modified, but not parasite-derived proteins, like the modified erythrocyte band 3 anion transporter, also termed Pfalhesin.

It was the aim of this thesis to identify parasite-derived ligands which are involved in cytoadherence and to provide unequivocal evidence for the causality of their expression and an adherent phenotype.

The following discussion is divided in two parts: the first part discusses the methodological aspects of the approaches pursued during this thesis and discusses the results of the four approaches, which will be compared to recently published results from similar studies. In the second part, it will be discussed, whether and how these results fit into the already existing knowledge of cytoadherence.

5.1. Methodology

The schizont-specific *P. falciparum* cDNA expression library

A good *P. falciparum* cDNA expression library was the fundamental basis of all four approaches pursued during this thesis, especially of the two shotgun approaches. The minimal condition this library had to satisfy, was to carry full-length plasmodial genes, since this is the essential prerequisition for the expression, the correct transport, and introduction of proteins into the COS 7 cell membrane. To guarantee superior quality of the library, it was produced commercially.

But, despite the fact that provided mRNA was of good quality as estimated by gel electrophoresis (4.1.2.), 60% of the clones of the cDNA library did not contain an insert and an additional 12% of the plasmids were recombinant or carried an insert which was not correctly ligated into the polylinker. Since cultivation of cDNA clones never resulted in the loss of a previously identified insert or in mutation of a plasmid, it can be assumed, that cDNA clones, carrying an insert, were stable and that the high ratio of recombined or mutated vector was possibly already present in the original cDNA.

Nevertheless, every gene or domain which was screened for in the cDNA library was present (MSP1, MSP2, DBL1-, CIDR-, and ATS-domain). But no full-length *var* gene was identified in the library by PCR or colony hybridization. No insert larger than 4.5 kb was found in the library which might be explained by mRNA breakage, or premature termination of cDNA synthesis. Since priming of the first strand cDNA synthesis started at the polyA-tail, all inserts carrying a DBL1-domain, should also include the ATS domain, excluding premature termination as sole explanation.

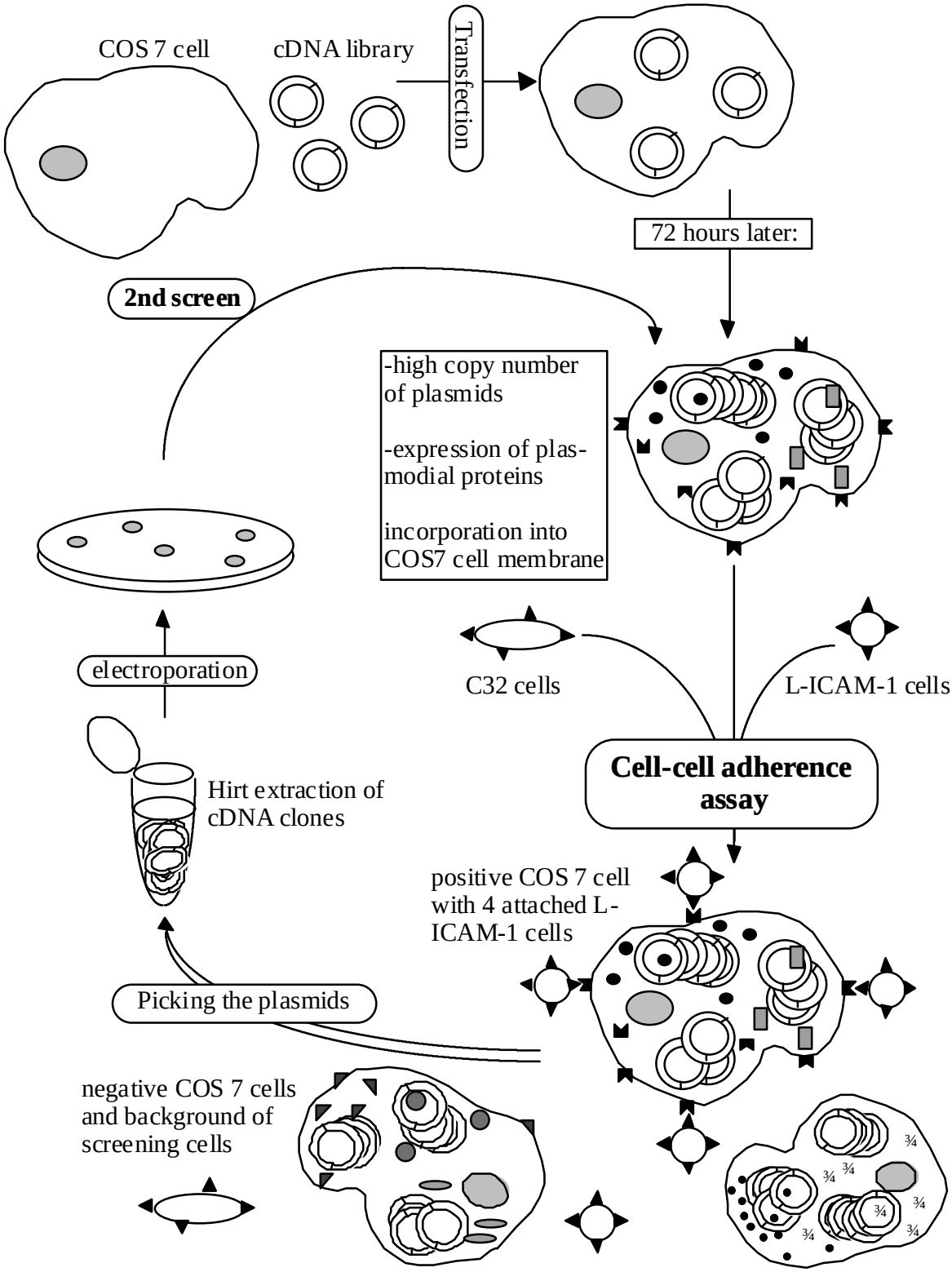
P. falciparum DNA exhibits an exceptionally high AT content of 69% in coding and 86% in non-coding regions (Weber, 1987). This high AT content could lead to false or internal priming of the oligo dT primer (18 T residues and a Not I restriction site). This was actually confirmed by sequencing of clone PD 4.14.1 (see: 4.3.5, Figure 4.1), which contained a DBL1 domain with a stretch of 6 Lysines, encoded by 17 consecutive adenosines. The size of this insert was 1.6 kb, which indicated that the mRNA was intact into the 5' UTR of this *var* gene.

At the beginning of this thesis and after the first description of the *var* gene family, it was calculated, that the large repertoire of *var* genes accounts for 2-6% of the haploid genome, and it was proposed, that the *var* gene family encodes for PfEMP-1 or a similar variant surface molecule (Su *et al.* 1995). Since adhesion of iRBCs is manifested some 16-20 hours into the intraerythrocytic cycle, it was assumed that expression of *var* genes would reach its maximum in the early trophozoite stage. Hence, for the preparation of cDNA synchronized cultures at this stage were used. But recent studies (Chen *et al.* 1998b and Scherf *et al.*, 1998) indicate, that *var* gene mRNA is transcribed from many *var* gene loci during ring stages, whilst probably only one *var* gene seems to be expressed in the late trophozoite stage. In cultures, which were selected by panning for binding to a certain receptor, mostly a single *var* gene transcript is found (Scherf *et al.*, 1998). As a consequence, a cDNA library of unsynchronized *P. falciparum* cultures might contain more different *var* genes, than a library of synchronyzed cultures, but since the synchronized culture bound to huICAM-1 and C32 cells prior the mRNA isolation (see 4.1.1.) adhesion mediating *var* genes were present in the mRNA. 6 different DBL1 and 4 different CIDR domains were found in the library, indicating the presence of at least 6 different *var* gene mRNAs in the synchronized but not selected cultures.

Methodology of the shotgun approaches)

A overview of a simplified shotgun approach (1) is given in Figure 5.1:

Figure 5.1 Schematic overview of the shotgun approach (1)



Since none of the positive first screens could be confirmed in a second screen in this approach, each step of it was extensively tested and improved:

Successful transfection was shown by the presence of plasmids in picked cells of first-screen adherence assays and by the presence of plasmids in cells randomly picked as controls later on (4.1.5.2.) and the GFP reporter system was introduced to estimate transfection efficacy. Using this system, it was shown that LipoTaxi (Stratagene) was superior in both, transient and stable transfection. Compared to the initially used chloroquine / DEAE dextran method, efficacy was up to 100-fold higher and cells were healthier due to a limited toxicity. Although transfection rates were lower than expected, its highly unlikely that failure in the second screens was due to the transfection efficacy.

Picking and plasmid isolation methods were also improved by using a needle scalpel blade and with the addition of glycogen as carrier in precipitation steps (Tracy, 1981). Furthermore, plasmid size did not occur to be limited by electroporation since clone var 5.4.1 (8.4 kb) and huICAM-1-EGFP (7.6 kb) were frequently and successfully used to transform *E. coli* cells.

Although COS 7 cells have been used to express plasmodial genes (Elliott *et al.*, 1990; Chitnis and Miller, 1994; Alano *et al.*, 1995; Smith *et al.*, and Kappe *et al.*, both 1998) and also generally is believed to be an easy and efficacious expression system, there are doubts, whether it would fully allow such an approach as conducted.

Therefore, four control constructs were made to test expression:

-An MSP2 gene, amplified by PCR from the cDNA, was ligated correctly into the pcDNA3.1 and the pcDNA3.1-EGFP vector. Western blots with αGFP revealed a band, the size of the GFP protein alone, indicating either degradation of the MSP2 gene or mutation of the second construct.

- The N-terminus of MSP1 (strain Ro71) was ligated correctly into pcDNA3.1 and into pRE4. Both constructs were used to transfect COS 7 cells, but neither in western blots nor in IFAs, was the N-terminus of MSP1 detected by the supernatant 9.22. Similarly, both the mAb ID3 and DL6, failed to recognize the native HSVgD protein. This also implied, that the transfected plasmids were not correctly transcribed and expressed.

- COS 7 cells were transfected with a pool of 22 different clones from the cDNA, which carried an insert bigger than 1 kb. On a western blot, an additional band of 66 kDa was detected using the serum pool from malaria exposed adults, giving the first evidence of an expressed *P. falciparum*-protein in COS 7 cells.

Previous reports on expressed plasmodial proteins in COS 7 cells, in three cases also used pRE4 (Chitnis and Miller, 1994; Smith *et al.*, and Kappe *et al.*, both 1998). Hence, expression of plasmodial protein in COS 7 cells occurs to be possible and the question remains, why all the four expression controls failed.

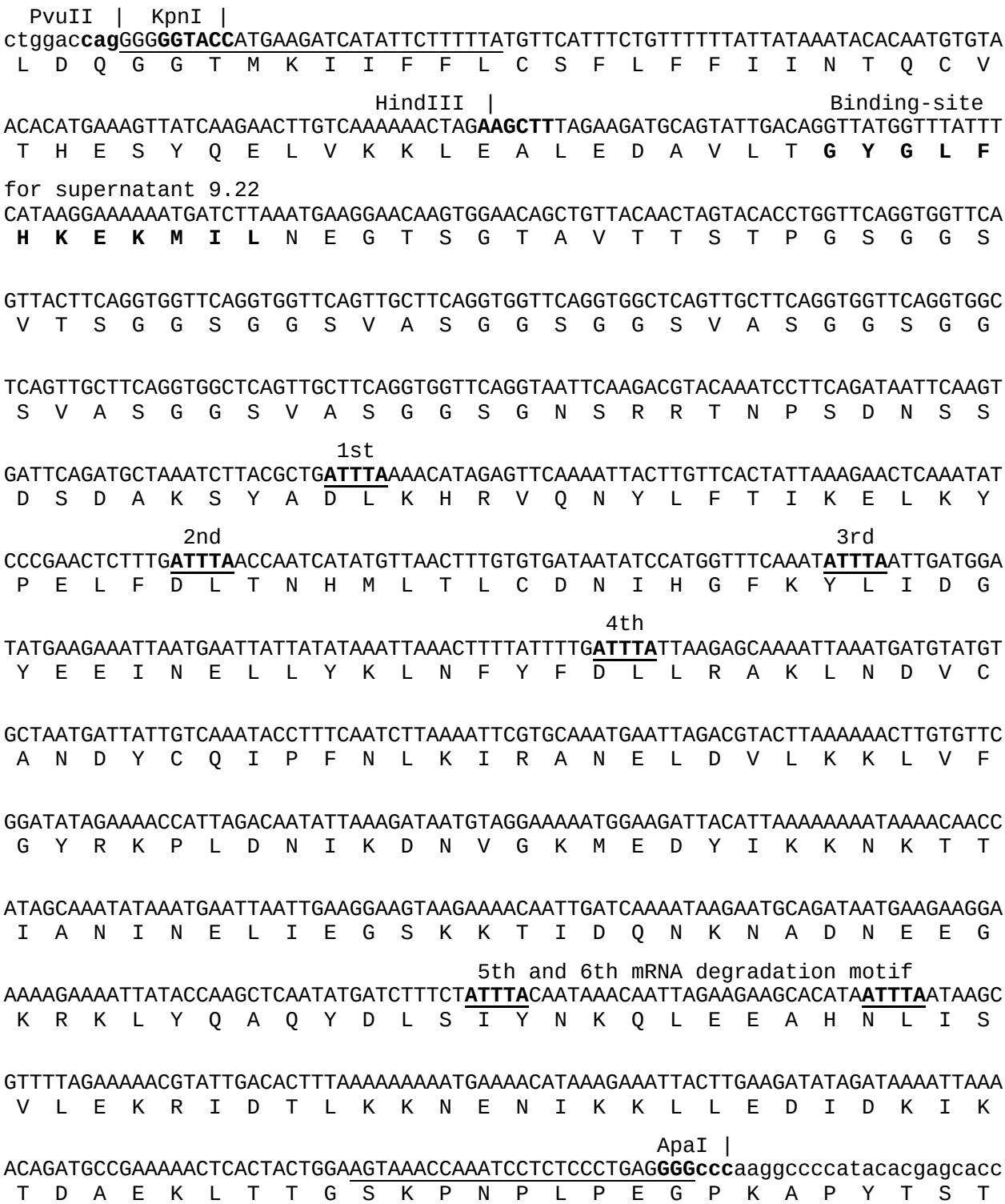
There is evidence that the AT-richness of *P. falciparum* may disturb gene expression in other eukaryotic systems: Pan and colleagues (1999) synthesized a gene encoding MSP1 from the FCB-1 strain adjusted for human codon preferences with a AT content of 55% AT instead of 74% AT, since the high AT content has prevented stable cloning of the parasite derived MSP1 gene. It has been speculated that mRNA-instability motifs (AUUUA) might be responsible for the failure of expression.

Shyu and colleagues (1991) identified an AU-rich element (ARE) in the 3' UTR of the c-fos mRNA. They showed by mutation of the ARE that this sequence controls two steps in the process of mRNA degradation: removal of the polyA tail and subsequent degradation of the transcribed portion of the message, which appears to be dependent on AUUUA pentanucleotides, which are obviously abundant in *Plasmodium* DNA.
AU-rich sequences are found in the 3' UTR of most immediate early mRNAs and are present in labile mRNAs which encode cytokines. A large body of data links the AUUUA pentanucleotide to the selective degradation of these mRNAs (Alberta *et al.*, 1994 and Peng *et al.*, 1996).

However, other studies claim that AUUUA pentanucleotide are not sufficient to promote mRNA decay, but showed, that UAUUUAU, when present in three copies, is sufficient to destabilize mRNA (Lagnado *et al.*, 1994), others identified the nonamer UUAUUUAUU as the key AU-rich sequence motif that mediates mRNA degradation (Zubiaga *et al.*, 1995). Recently, the mammalian ELAV proteins (HuR and HuA) were identified to be implicated in AUUUA-mediated mRNA decay (Myer *et al.*, 1997 and Atasoy *et al.*, 1998).

Of the previously expressed plasmodial genes in COS 7 cells, the coding region of the mRNA of the expressed Pf7 protein contained no AUUUA motif, whereas the coding region of the expressed Pf12 protein contained two AUUUA motifs (Elliott *et al.*, 1990), whereas the six domains of the P. vivax protein (Chitnis and Miller, 1994) and the A4VAR CIDR domain (Smith *et al.*, 1998), which were ligated into pRE4 and expressed in COS 7 cells, contained again no AUUUA motif. On the other hand, the mRNA of the N-terminus of MSP1 (strain Ro71), which was used here as an expression control contained 6 AUUUA motifs (see: Figure 5. 2). Since the mRNA decay occurs to be dependent on the number of AUUUA pentanucleotides in the mRNA (Lagnado *et al.*, 1994), it might be possible, that Pf12 could be expressed in COS 7 cells, whereas the N-terminus of MSP1, containing 6 degradation motifs, could not be expressed in this system.

Figure 5.2 ATTTA motifs in the N-terminus of MSP1



Legend to Figure 5.2:

The sequence of the N-terminus of MSP1 of RO71 (capital letters) and flanking regions of the HSV gD gene (small letters) are given. The primer sites for FW MSP1 Kpn and REV MSP1 Apa are underlined, restriction sites and AA 42-53, recognized by mAb 9.22, are in bold, and the 6 ATTTA pentanucleotides are bold and underlined.

The failure to detect COS 7 cells expressing plasmodial genes and to confirm adhesion in all second screens may indeed reflect that positive cells in a first screen might have been artefacts. The discussed AT-richness also adds to these problems and the assays can only be validated by constructing positive controls, either for the cell-cell adherence assay (shotgun approach 1) or the detection by a mAb (shotgun approach 2).

A recent publication, using almost the same cell-cell adherence assay may shed light on that matter: Smith and colleagues (1998) transfected COS 7 cells with A4VAR constructs, ligated into the pRE4 vector, and grew COS 7 cells on coverslips. During the assay, the cells were overlaid with 2 x 10^6 CHO-huCD36 or CHO-huICAM-1 screening cells. Binding was allowed for 5 to 15 minutes, then the coverslips were flipped onto a stand and centrifuged with 8 g to remove unbound screening cells. Although this wash step was probably harsher than swirling the dish, Smith and colleagues still found 1% of untransfected COS 7 cells having bound five or more CHO-huCD36 cells. This background would have been considered as positive in our assays.

Despite the problems faced in this study, the cell-cell adherence assay can be useful and might be used in the identification of plasmodial adhesins. A mammalian expression system has distinct advantages compared to a bacterial expression system, since proteins are correctly folded and structural epitopes can be detected. However, this expression system proved to be difficult in shotgun approaches, using *P. falciparum* cDNA, but could be used to investigate protein interactions of defined genes or parts of genes.

Methodology of the direct approaches

Since the assays of the direct approaches do not rely on expression of genes mediating cytoadherence, the direct approaches can lead more successfully to identification of defined genes. These defined genes can then be further analyzed using an eukaryotic transfection system as has been shown recently (Smith *et al.,* 1998). However, such a system requires an already known sequence or sequence fragment for screening and thus will not identify different and yet unknown genes like the shotgun screening in COS 7 cells which uses functional properties for the identification.

5.2. Discussion of the results

The main result of this thesis was the finding, that certain CIDR domains of *var* genes can bind to chondroitin sulphate A (CSA) in a dose dependent manner. Under the same conditions, binding of these CIDR domains to CD36, as previously described (Baruch *et al.*, 1997), and binding of DBL1 domains to CD36, huICAM-1 or CSA could not be shown.

All CIDR- and DBL1 domains, which were analyzed for their binding to CSA, CD36 and huICAM-1 were amplified by PCR and expressed in *E. coli*. But whether the sequences of CIDR K23 and CIDR C25 had a PCR-derived mistake in their nucleotide sequence (see 4.4.2.) or were translated pseudo genes, or whether the sequence of the CIDR C32 domain (identical to CIDR C10 for bp 1-211 and then identical to CIDR C1 for bp 194-567, see: Figure 4.6) was a PCR-artefact or an example for diversification by recombination in *Plasmodium falciparum*, the proteins encoded by these sequences bound to CSA. Two additional facts concerning the nucleotide sequence of the *var* gene domains were interesting:

1.) theoretically, the polyA-stretch, in which the mistakes of domains CIDR K23 and CIDR C25 were found (see: Figure 4.6) could represent a motif for the slipping of DNA polymerase to induce a frame-shift in the subsequent translation, analogue to the 'ribosomal slipping site' (Wagner *et al.*, 1990). Such a stretch could lead to a frame-shift into another ORF and thereby altering the adhesive properties from CD36-binding to CSA-binding. Although tempting, this theory could not be applied to the polyA-stretch of the CIDR domains, since in all 6 expressed CIDR domains, both 'alternative ORFs' led to the termination of the protein within 6 to 31 AAs.

2.) Of all CIDR domains, only CIDR C10 contained a ATTTA motif (position 351 to 355, Figure 4.6). Five DBL1 domains contained no ATTTA motif, but DBL16.1, DBL16.2, DBLK3, DBLs4, and DBLs20 each contained one ATTTA motif (position 89 to 93, 90 to 94, 90 to 94, 44 to 48, and 101 to 105). These CIDR- and DBL1 domains could be ligated into pRE4 and expressed as chimeric proteins on the surface of COS 7 cells, to analyze the binding properties of correctly folded domains to any given receptor.

The *E. coli* expressed 6x his-tagged *var* gene domains might not be folded correctly and were surely not glycosilated. However, when Baruch and colleagues (1997) identified the CIDR domain to mediate binding to CD36, they expressed the *var* gene domains as glutathione-S-transferase (GST)-fusion proteins in *E. coli* with purification on glutathione-sepharose. The CIDR domain, denoted rC1-2, was immobilized on plastic and promoted binding of CHO-huCD36 cells but not to untransfected CHO cells or CHO-huICAM-1 cells. Thus, in their assays, rC1-2 and additional CIDR domains bound CD36, but not CSA, whereas all CIDR domains, tested in this project bound CSA, but not CD36.

To date, *E. coli* expressed *var* gene domains have been shown to bind to CD36 via CIDR domains (Baruch *et al.*, 1997), to CSA via CIDR domains (this thesis), and it had been shown that antibodies to the CIDR domain and to the DBL3 domain of a CSA-binding PfEMP-1 variant inhibited iRBC binding to CSA (Reeder *et al.*, 1999). This leads to an array of questions:

> Why did the CIDR domains described by Baruch and colleagues (1997) bind to CD36 but failed to bind to CSA. Similarly, why did the CIDR domains, presented here bind to CSA but not to CD36?
> Is the limited sequence diversity of the CIDR domain sufficient to generate ligands for two different host receptors?
> Are there CIDR domains, which can bind to CSA and to CD36 simultaneously, or is this binding exclusively determined for each CIDR domain, hence are there two subgroups of CIDR domains?
> Can a CIDR domain switch its binding preference? And if so, how?

One possible answer to these questions lies in sequence comparison of different CIDR domains: The CIDR domain, which was first described to bind to CD36, denoted rC1-2 (Baruch *et al.*, 1997) corresponded to AA 576 to 755 of the MC PfEMP 1 gene (Baruch *et al.*, 1995). The CIDR-domains introduced in this work show homologies to AA 527 to 755 of the MC PfEMP 1 gene, therefore include the CD36-binding domain sequence of rC1-2. However, the domains described here were expressed as 6xhis-tagged proteins and showed no binding to CD36 on transfected CHO cells. For this, it is important to note that our forward primer used for amplification recognized a sequence up-stream of the binding site described by Baruch *et al.* (1997), and coded for the amino acid sequence KWKCYY (AA 1-6 in Figure 5.3). Baruch and colleagues used a forward primer coding for KEDKIMSY (AA 33-40 in Figure 5.3), a sequence which they found to be important for the correct folding of the protein in *E. coli*. Due to the observed sequence diversity, amino acids 31 to 39 in our CIDR-domains ($XGX_{(2-3)}V(K/T/M)SY$) were not identical to the described ones in rC1-2 (KEDKIMSY).

In their work, Baruch and colleagues (1997) studied 13 further GST-fusion proteins corresponding to rC1-2 from 8 different parasite isolates, which they tested for binding to CD36. They found an additional 3 proteins (all from the Malayan Camp isolate, but differing in rosetting behaviour or the expression of knobs: MCR^+, MCR^-, and MCK^-) which bound strongly to CD36, 7 proteins which bound weakly to CD36, and 3 CIDR domains which did not bind at all to CD36 (from the Palo Alto strain and from Dd2). None of the CIDR-domains, which weakly bound or failed to bind to CD36 were tested for binding to CSA. These additional 13 sequences were amplified with the degenerated forward primer UNI

179-5', which bound 3' adjacent to 179-5' to the sequence coding for FFWXWVXXML (representing AA 43-52 in Figure 5.3). Therefore, the KEDKIMSY motif was not 'imprinted' on these domains, which might be responsible for the lack of binding to CD36 in some of these domains. Apart from the cysteine-rich motif, Baruch and colleagues (1997) identified 5 additional regions of homology, which they suspected to be involved in CD36 binding (see Figure 5.3). Homology in region five again is probably due to its use as a primer binding site. All other regions of homology also show some degree of homology to the CIDR domains described here, except region 4 which was only found in CIDR K39.

By using primers corresponding to KEDKIMSY, Baruch *et al.* (1997) might have selected for a certain group of var genes containing this motif and thus with CD36-binding properties. Or it might have profound effects on the folding, which again might lead to CD36-binding. In turn, lack of this motif, despite complete presence of cysteins, might allow alternative folding leading to CSA-binding. The question, whether two subgroups of CIDR domains exist, or whether two alternative foldings of one PfEMP-1 variant can lead to both phenotypes could be answered by evaluation of the active domain of different CIDR domains. Strong evidence for the existence of two different genotypes being involved in the binding phenotypes of CIDR domains was found recently: Two *var*-genes have been identified conferring binding to CSA (Scherf *et al.* 1998, Reeder *et al.* 1999). In both cases, expressed *var*-genes were isolated after selection on CHO cells, and a single *var*-gene-transcript was identified. The *var*csa-gene (strain FCR3) was located on chromosome 10 and had an unusual small transcript of 4.4 kb (Scherf *et al.* 1998), whereas the *var*-CS2 transcript (strain ItG2) was 8.1 kb long (Reeder *et al.* 1999). In both cases the change to a new *var*-gene variant was accompanied by the loss of the original binding phenotype (CD36 or CD36 and ICAM1, respectively). Since the change of adhesive phenotype was reflected in a switch of the *var* gene expressed, it excludes a refolding of the expressed PfEMP-1 variant to switch the binding phenotype.

If the CIDR-domain is involved in both CD36 and CSA binding, then co-existence of binding motifs is expected. The location of binding motifs might overlap and might be completely exclusive, thereby leading to subgroups of *var*-genes binding either to CSA or to CD36. Using recombinant gene-fragments, as in this study, might provide evidence for the involvement of various regions in binding. However, such approach will not allow the analysis of more complex molecular interactions, i.e. the concerted action of two or more domains. Reeder and colleagues (1999) showed that the major inhibition of binding was observed with antibodies against DBL3, however, they also showed inhibition of CSA-binding with antibodies against the CIDR-domain. The published sequence from *var*-CS2 (GenBank[a] accession number AF 134154) also contains homologies identified in this study (Figure 5.3) and might be responsible for the binding of the CIDR-domains to CSA.

Figure 5.3 Comparison of rC1-2 and *var*CS2 to the CIDR domains of this thesis

```
           1        10        20        30        40        50
           |        |         |         |         |         |
CIDR C1    KWKCYYDE-NEKNSEKNNN-CVEGTWDTFTQGK--KVKPYNPFFWDWVYHMLHDSLDWRN
CIDR C32   KWKCYYDEH--KPSSKNNNNCVEGTWDKFTQGK-QTVKSYNVFFWDWVHDMLHDSVEWRE
CIDR C10   KWKCYYDEH--KPSSKNNNNCVEGTWDKFTQGK-QTVKSYNVFFWDWVHDMLHDSVEWRE
CIDR C9    KWKCYYDES--KESGQNNN-CILGKWEDFT-G-NEDVTSYNVFFYNSIIEMLNDSIEWKD
CIDR K39   KWKCYY------EKTDKSNNCILGKWEKFQKG--QEVMVYHPFFWSWVTEMLDDSIKWRK
CIDR K42   KWKCYYEKKNEKDGGKDINFCVLQD-DK-ARTSEEKSMHYNSFFWDWVYHMLHDSLNWRK
rC1-2                                        KEDKIMSYNAFFWMWVHDMLIDSIKWRD
varCS2                                             NDFFNFWVAHMLKDSIYWKK
Consensus  KWKCYYDE   K S KNNN CV G WDKFT G    V  YN FFW WV  ML DS  WR
Regions                                           <    Region 1   >

60        70        80        90       100       110       120
|         |         |         |         |         |         |
ELGSCIDNAKSGQCEN-KCNSKCECFERWVEKKK-EEWKAIKDHFKKQKDI-EQETHC----DPGVTLELL
RLNSCINNAKSGQCEN-KCNSKCECFERWVEKKK-EEWKAIKDHFKKQKDI-EQETHC----DPGVTLELL
RLNSCINNAKSQNCKNNKCNRECGCFAKWVVKKKD-EWDKIKDHFNKQENI-GQNVGFIEFNHYGV-LEGV
KLDICINN-KTGKCRK-VCKNPCECYKRWIEKKKP-NWKKLKDHFRKQKDI-G-DAAQREM-----TLNIT
ELDNCLKNENK-QCIS-KCNGKCDCFQRWIDKKKT-EWENIKIHFGKQEDMREQIGEDT---DPGIILEGV
QLGNCINDAKSGKCEN-KCNSKCDCFLKWVVQKKT-EWKNIKIHFGNQYFV-KQDGFDLGMNSPDVVLELL
EHGRCINKDKGKTCIK-GCNKKCICFQKWVEQKKT-EWGKIKDHFRKQKDIPKDWTH-------DDFLQTL
KLERCLENGTK-KCGKN-CNNDCGCFEKWVKQKKEEEWDKIKEHFRKQTDL
 L  CI N K   C    CN  C CF  WV  KK  EW  IK HF KQ DI  Q         PG  LE
                  <           Region 2           >               <

130      140       150       160       170       180       190
|         |         |         |         |         |         |
FMNDELLKNIKDTHANADDI--KHIEALLQETVFDGVAAGGPGGAGSYKC-TEGAKGKHNTKIDKLLQGT
FMNDELLKNIKDTHANADDI--KHIEALLQETVFDGVAAGGPGGAGSYKC-TEGAKGKHNTKIDKLLQGT
LDKDELLKNIKDTHADAKDIER--IDKMLQQA---GVVGGGRGGE------------NNTIIDKLLQGT
LNNNFLND-IKDAYP------VKQQLQKIEERLKDKMQEDFIFER------------TKTSIDKLLQGT
LNFENLFKNIKDTYGDVK--EIDHIKKLLEDE-------------------ENVGGEHNTTIDKLLQGT
LEKNLLLEIIKDTHANAKEDEIKNIDKMLQET------------SGSG---------------DKLLQGT

LMKDLLLEIIQDTYGDANEI--KRIEALLEQAG---V--GGIDFAALAGLYTKGFVAEKDTTIDKLLQHE
L    LL  IKDTH  A     K I   L
Reg. 3>       <    Region 4    >                              <Region 5>
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Legend to Figure 5.3

Sequence alignment of 6 cloned and sequenced CIDR-domains, the rC1-2 CIDR-domain described by Baruch *et al.* (1997), and the partial *var*CS2 CIDR-domain (amino acids 661-729 in the original sequence) described by Reeder *et al.* (1999). Primer binding sites are given in bold letters in rC1-2 and the tested sequences. The consensus sequence is given when 2/3 of the sequences were identical for the respective amino acid.

Additionally, the first four regions of homology are given in italic letters in the rC1-2 sequence, the fifth region of homology is identical to the reverse primer binding site of rC1-2, given in bold letters.

The question remains open, why in our approach only CSA-binding and no CD36-binding CIDR domain was identified. CD36-binding was found in one study with almost all field isolates (Hasler *et al.*, 1990), whereas prevalence of CSA-binding phenotypes have been shown to be low in studies in Papua New Guinea (Rogerson *et al.*, 1995) and Thailand (Chaiyaroj *et al.*, 1996). There are several possible answers to that question:

The prevalence of the CSA-binding phenotype is underestimated. A study on cytoadhesion in human placentas (Fried and Duffy, 1996) revealed that iRBCs from all placentas studied, cytoadhered to CSA, whereas no cytoadhesion was detected in iRBCs from the peripheral blood of these pregnant women. This would imply that underestimation of prevalence of CSA-binding phenotypes is likely.

Additionally, it is possible, that the primer design and identification of CIDR-domains in this study selected a restricted *var*-gene repertoire with a CSA-binding phenotype and a non-CD36-binding phenotype.

Furthermore, it can be speculated that the CSA-binding motif is rather short and might be present in most, if not all PfEMP1 molecules, since Robert *et al.* (1995) showed that mAb 1G11 and 4B2 against CSA could block adhesion of several different *P. falciparum* strains to Saimiri brain epithelium. But iRBCs expressing this CSA-binding motif could also bind to other receptors (e.g. CD36) with a higher avidity. Therefore, CSA-binding might be observed *in-vitro* only, and might be less frequently observed *in-vivo* due to stronger binding forces to other receptors.

5.3. Conclusions and perspectives

Due to the AT-rich genome of *Plasmodium falciparum*, mammalian expression systems might not be useful for shotgun approaches. Nevertheless, they may help to further elucidate products of defined genes or gene fragments. Domains of interest can be expressed on the surface of COS 7 cells as chimeric proteins with *Herpes simplex* glycoprotein D flanking regions by the use of the pRE4 vector. Furthermore, the binding properties of proteins expressed in a mammalian system could be compared to binding properties of *E. coli* expressed proteins.

Genes or gene fragments of interest can be identifed from a cDNA library, either by PCR or by a colony lift. Proteins could then be expressed either as 6xhis-tagged proteins in *E. coli* or on the surface of COS 7 cells using the pRE4 vector. As a control for expression and transport to the surface, the mAbs ID3 or DL6 could be used. Using these approaches, the following interesting questions could be investigated:

- Will our CIDR domains, expressed on the surface of COS 7 cells (in pRE4), still mediate binding to CSA?

- If the KEDKIMSY motif was imprinted on our CIDR domains (by PCR) and if these altered CIDR domains were expressed as 6xhis-tagged proteins in *E. coli*, would these altered CIDR proteins still bind to CSA in a FACScan analysis? Or would they even bind to CD36?

- If a common motif of the CSA-binding phenotype was identified, would it define a distinct subspecies of *var* genes, which can not bind CD36? And will this common motif be similar to the GAG-binding motif (WSPCSVTCG) of TRAP and the region 2 of CSP?

An answer to these questions would increase our knowledge of the adhesion of iRBCs to the endothelium. The understanding of cell adhesion is rapidly gaining importance in the clinical context, since cell adhesion molecules are becoming more and more legitimate targets for therapeutic interventions. But to develop anti-adhesin therapies in malaria, it would be necessary to know the exact binding sites of both, ligands and receptors, and their interaction in iRBC sequestration. Therefore it would be necessary, to correlate the genotype of a given PfEMP-1 variant to its receptor-binding phenotype and further, to correlate the expression of a distinct PfEMP-1 variant with severity of the disease.
With this knowledge, anti-adhesive peptide therapy in cerebral malaria becomes feasible.

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(Hint Hint, ich wünsch' mir ein Photoalbum von und mit euch zum Apéro)

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- Igor hab ich **immer bestaunt** (oft fassungslos und bestürzt), denn er kann brillante Experimente in 2 und ein gutes Montagsseminar in knapp 10 Minuten durchführen und kein Stoss im Billard dauert länger als 2 Sekunden (inkl. anlegen und zielen). Ausserdem kann er geniale Pläne schmieden, wenn es um Sushi geht (gäll Laura) und ist das treueste Fahrradtaxi von Basel (gäll Moni).

- Miri hab ich **immer beschämt** und es tut mir auch nicht leid, denn nie ist sie sooo schön, nie fallen ihr die Locken süsser in die Augen denn dann. Miri, du bist für mich die einzige Rechtfertigung, für die Erfindung der Faaaabföddeli (oder zumindest rot/weiss) und ich würde dich sooo gerne sehen, wenn du das liest!! Allerdings tut es mir leid, dass du jedesmal wenn du mich siehst, panik bekommst, es könnte Montag und der Stickstofftank schon wieder leer sein....

- Tatsuo hab ich **immer bedauert**, weil er A) keinen Platz zum Arbeiten hatte, obwohl er zwei leere Plätze vor Augen hatte, die jeweils nur 5 Minuten am Tag benutzt wurden, B) Zuerst die Micro-codierung lernen musste, bevor er auf RNA Jagd gehen durfte und C) weil er das Grinsen vom blöden ROL (der vom Highscore) dann auch noch im Labor sehen musste. Deshalb kann ich gut verstehen, dass sein Arbeitstag erst dann anfing, wenn meiner schon wieder zu Ende ging.

- Denise hab ich **immer begehrt** als die beste Rauchpausenpartnerin (da doppelte Länge!!), denn nie waren Rauchpausen so nötig (!) und so schön (!!). Ausserdem chattet niemand so NETT (jawoll, nett) im NET (und das noch einhändig) und wer Denise schon mal gesehen hat wie sie Elisa-Platten klopft oder nach Altpapier lechzt, wird verstehen, warum ich gewisse Bilder trotz meines Alters nie werde vergessen können.

- Sylvia hat mich **immer be(ent)geistert** weil sie selbst in der hektischsten Panik und im chaotischsten Tohuwabohu den inneren Frieden fand, um Bomben zu entschärfen, selbst wenn die Stimme des Herrn und Meisters sekündlich nach Lob verlangte und unser aller Nerven blank lagen. Ausserdem bleibt sie wohl für immer die einzige, die es je geschafft hat, im grossen Kühlraum steril zu arbeiten!!

Auf jeden Fall seid ihr alle was **Besonderes**. Das gilt auch für den ganzen Rest des Hauses, besonders für Bea1 (vielen vielen Dank nochmals für den Kuli, er bedeutet mir sehr viel) BeaII (wenn ich nur etwa 25 Jahre jünger wäre....), Moni (für die beste Party meines Lebens, wo ich mich beinahe 25 Jahre jünger gefühlt habe), Claudia (Nie war eine Grünabfuhr so liebevoll...) Christian und Amelie (Dir, Näääääää und dir, Amelie, alles Gute und es gibt noch ein Tomatenfondue...) undundund.

So, das wollt ich nur mal noch gesagt haben bevor ich’s vergesse, ansonsten Billard (Andrea und BeaII!!!) oder auch hotmail (Inkognito68) oder Teflon 4212665 oderoderoder....
P.S. versucht gar nicht erst, das rückwärts zu lesen, es kann nur einen (**immer begnadeten**) Pascali geben!!!

Curriculum vitae

Roland Degen
Born on the 29th of May 1965 in Basel, and citizen of Oberwil (BL), Switzerland

General Education

1972-1976	Primary School Dreirosen in Basel
1976-1984	Gymnasium DeWette (MNG) in Basel, Matura Typus C

Scientific Education

1984-1986	4 semesters basic studies in Mathematics und Physics at the ETH ZŸrich, Switzerland.
1986-1991	10 semesters of studies in Biology (1) at the University of Basel, with Medical Parasitology and Population Biology as main blocks.
1992-1993	MSc-thesis carried out at the Swiss Tropical Institute (STI), supervised by Professor Dr. Leo Jenni: "Analysis of the sexual Compatibility among Ugandan *Trypanosoma brucei*-Isolates".
1996-1999	PhD studies and thesis at the Swiss Tropical Institute, supervised by Dr. B.A. Imhof (Basel Institute of Immunology and Centre MŽdicale Universitaire, Geneva) and Drs H-P. Beck and N.A. Weiss (Swiss Tropical Institute): "Identification and Analysis of *Plasmodium falciparum* Genes Mediating Cytoadherence".

During my studies I attended lectures and courses of the following lecturers:

W. Arber, C. Baroni-Urbani, B. Baur, H.-P. Beck, B. Betschart, T.A. Bickle, K.A. Bienz, C. Boesch T. Boller, B. Bruderer, R. Brun, P. Duelli, S. Fallab, I. Felger, T.A. Freyvogel, W.J. Gehring, U. Gisi, M. Hall, H. Hecker, J. Hofsteenge, L. Jenni, R. Kaminsky, J. Koella, C. Kšrner, C. Lengeler, E. LŸdin, J. Maynard-Smith, J. Meier, S. Mutter, G. Parker, L. Partridge, G.-R. Plattner, G. Pluschke, U. Rahm, H. Riezman, H.F. Rowell, W. Rudin, G. Schatz, B. Schmid, P. Schmid-Hempel, V. Schmid, D.G. Senn, H. Sigel, T. Smith, M. Spiess, S.C. Stearns, J. Stšcklin, D. Subrahmanyam, M. Tanner, A. Van Noordwijk, F. Vollrath, D. Walz, N. Weiss, A. Wiemken, H. Zoller, A. Zumstein.

Professional Experience

1987-1993	Official expert at the bureau of social contributions (WSD) Austrasse 67, Basel. (25%-part time position)
1993-1994	Technical assistance at the mosquito breeding facility of the Swiss Tropical Institute (STI).
1994	Research within the CHEMOTRYP project at the STI.
1995	Practical course and training in Molecular Biology at the Friedrich Miescher Institute (FMI) in Basel
1995	Substitute teacher in Biology and Geography for one semester at the Gymnasium BŠumlihof in Basel

Publications and Meetings

1993 Degen R., Pospichal H., Enyaru J., Brun R. and Jenni L. (1993) Analysis of sexual compatibility among Ugandan *T. brucei* isolates. *10th Annual Swiss Trypanosomatid Meeting*, Charmey. (Oral presentation and Poster)

Degen R., Pospichal H., Enyaru J., Brun R. and Jenni L. (1993) Analysis of sexual compatibility among Ugandan *Trypanosoma (T.) brucei* isolates. *IX. International Congress of Protozoology*, Berlin. (Abstract and Poster)

Degen R. (1993) Analyse der sexuellen KompatibilitŠt bei Ugandischen *Trypanosoma (T.) brucei*-isolaten. MSc-Thesis D161, Library of the Swiss Tropical Institute, Basel.

1995 Degen R., Pospichal H., Enyaru J. and Jenni L. (1995) Sexual compatibility among Trypanosoma brucei isolates from the endemic area in south-east Uganda. *Parasitology Research 81: 253-257*

1998 Degen R., Imhof B., Weiss N. and Beck H-P. (1998) COS7-Cell Expression of *Plasmodium falciparum* Genes mediating Cytoadherence. *10th Malaria Meeting of the British Society for Parasitology*, Edinburgh. (Abstract and Poster)

1999 Degen R., Weiss N. and Beck H-P. (1999) Plasmodium falciparum: cloned and expressed CIDR-domains of PfEMP1 bind to Chondroitin-Sulfate A (CSA). Submitted to Experimental Parasitology.