CHAPTER TWO- MATERIALS AND METHODS

2.1 Purification of human 4.1R

MilliQ water and reagent grade chemicals were used for the preparation of buffers and solutions. All reagents and samples were kept on ice water throughout the duration of the preparation. To prevent protein degradation, buffers and extraction solutions used in the preparation of erythrocyte membranes and purification of 4.1R were supplemented with PMSF, a reversible protease inhibitor. A 400mM stock solution was prepared in dimethyl sulphoxide (DMSO) and added dropwise to a final concentration of 0.1mM. Centrifugation steps were performed in a Beckman J2-21 centrifuge at 4^{o}C, unless stated otherwise.

2.1.1 Preparation of erythrocyte membranes

Erythrocyte membranes were prepared from whole blood by hypotonic lysis (Dodge et al., 1963). Sixty ml of venous blood was drawn from healthy individuals and collected in acid citrate/dextrose tubes to prevent clotting. Blood samples were centrifuged at 4^{o}C in a Jouan BR3.11 at 1100 *g* for ten minutes, the plasma aspirated and buffy coat removed. The packed cells were washed three times in 0.9% saline and centrifuged as before to remove any remaining buffy layer. This was important as white blood cells contain proteolytic enzymes, capable of degrading membrane proteins. The erythrocytes were transferred to 50ml Beckman tubes, lysed with ice cold lysis buffer (3.0mM $NaPO_4$, 0.1mM EDTA, pH 8.0) and centrifuged for 15 minutes at 17 500 *g*. The supernatant of the lysed cells and tightly packed residual white blood cells were aspirated. The membranes were resuspended in fresh lysis buffer and washed as before until they appeared white.

2.1.2 Method A: salt-based purification of 4.1R

4.1R was purified from erythrocyte membranes according to a modified method of Tyler and colleagues (Tyler et al., 1979; Tyler et al., 1980). Band 6 or glyceraldehyde-3-phosphate dehydrogenase was firstly extracted from the membranes by incubation for 30 minutes on ice in 20 volumes of phosphate buffered saline (PBS: 5mM $NaPO_4$, 1mM EDTA, 155mM NaCl, pH 8.0). The ghosts were centrifuged at 17 500 *g* for 5 minutes, washed once in PBS and pelleted as before. They were then successively resuspended in 20 volumes of 5mM $NaPO_4$, 1mM EDTA, pH 8.0 and 0.3mM $NaPO_4$, 0.1mM EDTA, pH 9.2 solutions, followed by a final rinse in 0.1mM EDTA, pH 9.2. Centrifugation steps were carried out in between washes at 17 500 *g* for 15 minutes. The vesicles were depleted of spectrin and actin by incubation at 37^{o}C for 30 minutes in extraction solution (0.1mM EDTA, pH 9.2, 0.1mM β-mercaptoethanol). The vesicles were subsequently pelleted at 17 500 *g* for 25 minutes, rinsed in fresh solution and centrifuged at 120 000 *g* for 30 minutes at 4^{o}C in a Beckman L8-M ultracentrifuge. They were then resuspended in an equal volume of 4.1R extraction solution (1M KCl, 1mM EDTA, pH 7.6, 1mM Pefablock® *SC) and incubated at 37^{o}C for 30 minutes. Pefablock® *SC is an irreversible serine protease inhibitor and was added to limit the degradation of 4.1R. The vesicles were pelleted in the ultracentrifuge and the supernatant dialysed in snakeskin tubing against two changes of chilled column buffer (5mM $NaPO_4$, 1mM EDTA, 20mM KCl, 0.5mM DTT, pH 7.6) for 2 hrs at room temperature. 4.1R extracts were immediately loaded onto a column of 2ml DEAE-cellulose anion exchange matrix, equilibrated in column buffer at 8^{o}C. After loading, the column was washed with column buffer containing 60mM KCl under gravity feed to remove minor contaminants. Pure 4.1R was eluted with column buffer containing 100mM KCl and 10 times 200μl aliquots of eluate collected. Protein-containing samples were detected by measuring the absorbance at 280nm (A_{280}) UV light in a Beckman DU-65 spectrophotometer calibrated with column buffer. Aromatic amino acids in proteins absorb efficiently at this

wavelength, which enabled the detection of 4.1R. The appropriate samples were prepared for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the 4.1R containing fractions pooled for concentration and biotinylation.

2.1.3 Method B: detergent-based purification of 4.1R

A detergent-based method described by Becker and colleagues (Becker et al., 1983) was also used for purifying 4.1R from erythrocyte membranes. Band 6, spectrin and actin-depleted ghosts were prepared as detailed in 2.1.2. The vesicles were resuspended in an equal volume of 4.1R extraction buffer (0.2M glycine, 1mM EGTA, 2% Tween 20, pH 9.8, 1mM Pefablock® *SC), stirred overnight on ice and then centrifuged at 120 000 *g* for 30 minutes at 4^{o}C in a Beckman L8-M ultracentrifuge. The supernatant was immediately chromatographed at 8^{o}C on a column of 2ml DEAE-cellulose matrix, equilibrated in 0.05M glycine, 0.5mM EGTA, 0.5mM DTT, pH 9.8. After loading the 4.1R crude extract, the column was washed with equilibration buffer under gravity feed. A continuous gradient of 0-350mM sodium chloride in the same buffer was then applied at a flow rate of 0.3ml/minute, using a SJ-1211 peristaltic pump connected to a Hoefer SG series gradient maker. Approximately 20ml of eluate was collected in 500μl aliquots using a Pharmacia LKB Redi fraction collector and protein detected by A_{280} UV light. Samples were prepared for SDS-PAGE and the appropriate fractions pooled for concentration and biotinylation.

2.1.4 Protein quantitation and gel electrophoresis

The protein concentrations of samples from different stages of the purification protocol were determined using the Coomassie Plus® Protein Assay Reagent Kit. A standard curve was drawn up using dilutions of a 2mg/ml BSA stock solution. To solubilise erythrocyte membrane proteins, 5μl aliquots were first added to an equal volume of 5M NaOH, while 10μl purified extracts were added directly to Coomassie reagent. The

absorbance was measured after five minutes with visible light at a wavelength of 595nm on a Beckman DU-65 spectrophotometer.

Membrane proteins and purified 4.1R were analysed by SDS-PAGE on 12% Laemmli gels (Laemmli, 1970) (A-1.1) and 3.5-17% exponential gradient Fairbanks gels (Fairbanks et al., 1971) (A-1.2). The polyacrylamide gels were prepared in a Hoefer Mighty Small Dual Casting Tray or Hoefer Sturdier vertical slab gel unit (SE400). Aliquots of protein samples were solubilised in 1X suspension solution (A-1.3), approximately 5μg loaded onto the gel and electrophoresed as in A-1.1. The gels were stained overnight in Coomassie blue stain (0.5% Coomassie Brilliant Blue R-250, 10% acetic acid, 25% methanol) and destained in 10% acetic acid, 10% methanol, followed by several changes in 10% acetic acid. For protein detection as low as ~1ng (Coomassie dye sensitivity ~100ng), the gels were stained with silver and visualised with sodium carbonate and formaldehyde developing solution (A-1.4).

2.1.5 Densitometric scanning of Laemmli gels

Coomassie stained gels were washed several times in MilliQ water to remove the acetic acid. Gels were scanned using a Hoefer GS300 transmittance/reflectance scanning densitometer. The area under the 4.1R peak was determined using the accompanying software, and the 4.1R yield calculated after the purification.

2.1.6 Immunoblot of purified 4.1R

To confirm the presence and identity of 4.1R, proteins were transferred from a stained 12% Laemmli gel to nitrocellulose paper using a TE Series Transphor Electrophoresis Unit (A-1.5). The blot was primed with rabbit anti-4.1R antibody (1:1000) and incubated with a secondary goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (1:1000). 4.1R

bands were visualised using 1, 3, diaminobenzidine as a substrate and hydrogen peroxide (A-1.5).

2.2 Concentration and biotinylation of purified 4.1R

Purified 4.1R was transferred into a 3ml Slide-A-Lyzer dialysis cassette using a sterile syringe. Polyethylene glycol 20 000 was packed around the cassette to draw out excess buffer and the process repeated until the cassette accommodated the entire 4.1R sample. The cassette was attached to a Pierce carousel buoy, dialysed against two changes of chilled PBS (25mM $Na_2HPO_4.2H_2O$, 1.5mM KH_2PO_4, 121mM NaCl, 2.7mM KCl, 0.1mM PMSF, pH 8.0) for 2hrs at room temperature, and then concentrated using the polyethylene glycol to a final volume of approximately 200μl.

Two to 600μg of concentrated 4.1R (1-3μg/μl) was biotinylated with Biotin-NHS ester. The ester is incorporated into proteins under alkaline conditions by forming an amide linkage with amino groups ($R\text{-}NH_2$). Biotin-NHS was dissolved in DMSO and added to the purified 4.1R sample in a 10 fold molar excess and volume ratio of 1:20. The solution was slowly stirred for 4 hrs at room temperature in the dark, and then dialysed overnight against three changes of PBS at 8^{o}C to remove unbound biotin-NHS. The sample was centrifuged at 8 000 *g* for 10 minutes at 15^{o}C, shock frozen in a dry ice/ acetone mixture and stored at -20^{o}C.

2.2.1 Streptavidin-peroxidase test

To verify a successful biotinylation reaction, biotin-labeled 4.1R was detected using streptavidin conjugated to horseradish peroxidase. One μl biotinylated protein was spotted onto a strip of Hybond C nitrocellulose membrane and allowed to dry. Unbound sites were blocked with 3% BSA in TBS for 1 hr at room temperature. The membrane was rinsed in TBS,

incubated with 0.05U/ml streptavidin-peroxidase conjugate in TBS for at least 1 hr and washed three times for five minutes with 0.1% Tween 20 in TBS. Peroxidase signals were detected in a dark room with Supersignal® West Pico Chemiluminescent substrate. The membrane was incubated for five minutes with working solution, prepared by adding equal volumes of the peroxide solution and luminal/enhancer solution supplied in the kit. Excess liquid was drained with absorbent tissue, the strip covered with plastic wrap and then exposed to X-ray film in a cassette for 1 minute. The film was developed with Polycon A working solution diluted 1:5 in water and fixed with Pre-fix working solution.

2.3 Culturing of asexual *P. falciparum* parasites

The FCR3 parasite strain was used for culturing, with all relevant work performed in a laminar flow hood. Sterility was maintained at all times by application of aseptic technique. Equipment and PBS were sterilised by autoclaving at 120^{o}C, 15lb/In^{2}, while media and reagents were sterilised by filtration through 0.22μm filter units. MilliQ water was used for the preparation of all reagents and media.

2.3.1 Preparation of human erythrocytes for parasite cultures

Venous blood was drawn from healthy volunteers and collected in ACD tubes. Samples were centrifuged at 4^{o}C in a Jouan BR3.11 at 1 100 *g* for ten minutes, the plasma aspirated and buffy coat removed. The packed erythrocytes were washed in sterile PBS (10mM $Na_2HPO_4.2H_2O$, 1.5mM KH_2PO_4, 137mM NaCl, 2.7mM KCl, pH 7.4), centrifuged as before and repeated three times to remove any traces of white blood cells. The washed cells were resuspended in an equal volume of incomplete RPMI medium (A-1.6) and this was stored at 4^{o}C for up to two weeks.

2.3.2 Preparation of parasite cultures from frozen stock

Cryotubes containing 1ml frozen parasite cultures were removed from liquid nitrogen storage and thawed in a pre-heated water bath at 37^{o}C. 0.1 volume 12% sodium chloride was added dropwise to the tubes while swirling and then incubated for five minutes at room temperature. Suspensions were transferred to sterile 15ml Falcon tubes and 9 volumes 1.6% sodium chloride added. The tubes were gently inverted and centrifuged in a Jouan BR3.11 at 600 *g* for five minutes at room temperature. The supernatant was aspirated and the parasite pellets resuspended in 9 volumes 0.9% sodium chloride, 0.2% glucose solution. The tubes were centrifuged as before and freshly washed erythrocytes added to a final volume of 750μl. Cells were transferred to sterile Nunc 24cm^2 tissue culture flasks and 5-10ml of 20% complete RPMI, preheated at 37^{o}C, added (A-1.7). Flasks were then gassed for 30 seconds with a 93% N_2, 5% CO_2 and 2% O_2 mix, tightly closed and incubated at 37^{o}C for 48 hrs.

2.3.3 Continuous culture method

P. falciparum parasites were maintained in continuous culture according to a modification of the method described by Trager and Jensen (1976). Cultures containing a 5% haematocrit were incubated at 37^{o}C in 80cm^2 and 175cm^2 sterile Nunc tissue culture flasks. Spent medium was removed from the parasite cultures and supplemented daily with 20ml or 30ml fresh 10% complete media respectively, as well as with 93% N_2, 5% CO_2 and 2% O_2 mix. The parasitaemia was also monitored daily by microscopic analysis of stained blood smears (A-1.8), and this was maintained between 4 and 8%. Cultures were divided between two flasks every second or third day or kept in one flask by aspirating some culture and adding fresh erythrocytes.

2.3.4 Freezing of parasite cultures

Asexual stages were frozen when cultures reached a parasitaemia of around 12% and contained predominantly ring stages. Cultures were transferred to 50ml Nunc tubes, centrifuged at 800 *g* for five minutes and the supernatant removed. The packed cells were resuspended with an equal volume of sterile freezing solution (89mM NaCl, 60mM $Na_2HPO_4.2H_2O$, 23mM KH_2PO_4, 28% glycerol). One ml of this suspension was aliquoted into sterile cryotubes, incubated a room temperature for five minutes and stored in liquid nitrogen.

2.4 Isolation of *P. falciparum* RNA

RNA isolation is complicated by the widespread presence of contaminating ribonucleases (RNases), enzymes responsible for rapidly degrading single stranded RNA. There are a number of sources of RNases including bacteria present in buffers and solutions, skin and clothing as well as airborne particles. To avoid contamination and limit RNase activity, a few basic precautions were exercised:

- A separate area was maintained in the laboratory for RNA work and was wiped with RNaseZap before use.
- Dedicated pipettes, pipette tips, sterile disposable plastic ware and reagents were used at all times.
- Latex gloves were worn and changed at regular intervals.
- RNA was stored in RNase-free Molecular Biology Grade water or diethylpyrocarbonate (DEPC) treated water. Treatment was accomplished by preparing 0.1% DEPC in MilliQ water. The water was incubated overnight and autoclaved to inactive the compound.
- Samples were kept on ice at all times

2.4.1 Extraction of total RNA

The method of (Chomczynski and Sacchi, 1987) was used for extracting RNA from asexual *P. falciparum* parasites. Parasites from four 175cm^2 tissue culture flasks each containing a high parasitaemia (10-15%), were transferred to sterile 50ml Nunc tubes and centrifuged in a Jouan BR 3.11 at 1 100 *g* for ten minutes at 4^{o}C. The supernatant was aspirated and the pellet resuspended in saponin at a final concentration of 0.5% to lyse the erythrocytes. The suspension was incubated at room temperature for five minutes, transferred to 15ml polypropylene tubes, centrifuged for 10 minutes at 1 100 *g* and the parasite pellet washed with PBS until the supernatant was clear. 5ml D* solution (4M guanidine thiocyanate, 25mM sodium citrate pH 7, 0.5% N-laurylsarcosine, 0.72% β-mercaptoethanol) was added to the pellet and resuspended using a 1ml pipette tip. The solution is responsible for releasing RNA from the parasites by disrupting and dissolving cellular components. Guanidine thiocyanate is important for inactivating RNases, thus protecting the RNA from degradation. Half ml of sodium acetate, pH 4.0 was then added and gently mixed, followed by 5ml buffered phenol, pH 4.2 and 1ml chloroform. The solution was centrifuged at 1 100 *g* for 15 minutes at 4^{o}C. RNA was precipitated from the aqueous phase with 1ml isopropanol overnight at -20^{o}C or for 2 hrs at the same temperature (A-1.9). The precipitate was centrifuged at 15 000 g in a Sorvall RMC14 microcentrifuge at 4^{o}C for 30 minutes and then washed with 70% ethanol to remove any residual salt. Air-dried RNA pellets were dissolved in 10-20μl DEPC-treated or nuclease-free water and heated at 65^{o}C to increase solubility. Making use of the fact that an RNA solution of 40μg/ml has an absorbance of 1 at a wavelength of 260nm, the concentration of 1μl RNA in 49μl TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA) was determined with A_{260} UV light in a Beckman DU-65 spectrophotometer.

2.4.2 Assessment of RNA quality and removal of contaminating DNA

Purity of the RNA preparation was assessed quantitatively by calculating the A_{260}: A_{280} ratio. A value between 1.8 and 2.0 represents a pure RNA sample, whereas values smaller than 1.8 indicate contamination with proteins and/or phenol. In the event of contamination, RNA was re-purified by phenol-chloroform extraction. The RNA sample was also qualitatively assessed with 1% agarose gel electrophoresis. One μl aliquot was loaded onto a gel containing 10μg/ml ethidium bromide and electrophoresed at 60mA for 30 minutes in a 1XTAE running buffer (50X buffer: 2M Tris-acetate, 50mM EDTA, pH 8.0). RNA was visualised on a UV transilluminator and the quality of the 28S and 18S ribosomal subunits evaluated. Ideally, the intensity of the 28S band should be twice that of the 18S band. The presence of high molecular weight DNA was detected by visualising bands near the wells of the gel. Contaminating DNA was removed by digestion with RNase-free DNaseI. Buffer L (50mM Tris-HCl, pH 7.5, 10mM $MgCl_2$) was diluted 10 times in the crude RNA preparation. One μl DNaseI (10-50U) was added to the sample and incubated at 37^{o}C for 30 minutes, followed by inactivation of the enzyme for fifteen minutes at 70^{o}C. RNA samples were stored at -70^{o}C.

2.4.3 Isolation of mRNA from total RNA

Poly $(A)^{+}$ mRNA was isolated from total RNA using $Oligo(dT)_{25}$ Dynabeads® according to the manufacturers instructions. Use of the mRNA purification kit relies on base pairing between the poly A residues at the 3' end of mRNA and twenty five oligo dT residues covalently coupled to the surface of the Dynabeads. RNA species lacking a poly A tail will not hybridise to the beads and are removed by washing.

In brief, 250μl magnetic beads were transferred to an RNase-free Eppendorf tube and placed in a magnetic particle concentrator (MPC) for

20 seconds. The storage buffer (PBS, 0.02% sodium azide, pH 7.4) was removed and the beads conditioned in binding buffer (100mM Tris-HCl pH 8.0, 500mM lithium chloride, 10mM EDTA, 1% lithium dodecylsulphate, 5mM DTT). An equal volume of binding buffer was mixed with a 1μg/μl RNA sample and the solution added to the conditioned beads. The suspension was mixed by gentle inversion for five minutes at room temperature. After the incubation period, the supernatant was removed from the tube in the MPC for another round of mRNA extraction. The beads were washed twice with 1 ml buffer A (10mM Tris-HCl pH 8.0, 0.15M lithium chloride, 1mM EDTA, 0.1% lithium dodecylsulphate) and once with the same buffer containing no lithium dodecylsulphate. Tubes were placed in the MPC, wash buffer discarded and beads resuspended in 10-20μl 2mM EDTA pH 8.0. The mRNA was eluted by incubating the suspension at 70^{o}C for two minutes. The pure sample was removed using a MPC, transferred into a new tube and stored at -70^{o}C. The 250μl aliquot of Dynal beads was regenerated in 200μl 0.1M NaOH at 65^{o}C for two minutes and used for up to three rounds of extraction.

The concentration of 1μl mRNA in 49μl TE buffer was determined by measuring the absorbance at 260nm UV light. The quality was assessed by calculating the A_{260}:A_{280} ratio and by 1% agarose gel electrophoresis (2.4.2).

2.5 Construction of *P. falciparum* phage display libraries

Construction of phage display libraries relies on the synthesis of high quality parasite cDNA inserts. The strategy was based on the directional synthesis of cDNA using random primers anchored at the poly (A) tail of purified mRNA. Reactions were carried out in the presence of methylated dNTP to protect internal *Eco*R I and *Hind* III restriction sites from subsequent digestion. After cDNA synthesis, the inserts were blunted and *Eco*R I/*Hind* III linkers ligated to their ends. The cDNA was digested with

the respective restriction enzymes and passed through a gel filtration column to remove excess linkers and small cDNA products. Modified inserts were cloned into T7Select 10-3b vector arms and packaged into bacteriophage T7 extracts. Phage particles were propagated in *E. coli* cells which enabled the low-copy number display of large proteins of up to 1200 amino acids on the T7 capsid.

2.5.1 Synthesis and end-modification of parasite cDNA libraries

2.5.1.1 First and second strand cDNA synthesis

P. falciparum cDNA libraries were prepared and end-modified according to the protocol adapted from the Orient Express^{TM} cDNA cloning system. 4μg parasite mRNA was primed with 2μg random two-base anchored primers (A-2.1) and adjusted to a final volume of 20μl with nuclease-free water. The primers were designated oligodT_{12}VN (V= A, C and G; N= any of the four nucleotides) and were designed to selectively anneal to the poly (A) tail of mRNA, thus minimising the occurrence of rRNA and other non-relevant sequences in the library. The mixture was heated to 70^{o}C for 10 minutes to alleviate secondary RNA structure and chilled quickly on ice. The following remaining kit components were added for first strand cDNA synthesis: 10μl 5X first strand buffer (250mM Tris-HCl pH 8.3, 375mM KCl, 15mM $MgCl_2$) 5μl 100mM DTT, 2.5μl 10X methylated dNTP mix and 800U MMLV reverse transcriptase in 50μl final volume. To track the cDNA synthesis, 5μCi [α^{32}P] dATP (800Ci/mmol, 10μCi/μl) was also added, after which the reaction was incubated at 37^{o}C for 60 minutes. The enzyme was subsequently inactivated by heating to 70^{o}C for 10 minutes. The first strand reaction was used in a reaction volume of 250μl, which contained a final concentration of 1X second strand buffer (40mM Tris-HCl pH 7.5, 4.4mM $MgCl_2$, 85mM KCl), 2.4mM DTT, 0.08X methylated dNTP mix, 50U DNA polymerase I and 1.6U RNase H. The reaction was incubated at 15^{o}C for 90 minutes followed by phenol-chloroform extraction and ethanol

precipitation (A1-9). The purified cDNA was stored at -20^{o}C or immediately end modified.

2.5.1.2 End-modification and size fractionation of cDNA inserts

To blunt their overhanging ends, 20μl double stranded cDNA was combined with 3μl 10X flush buffer provided in the kit, 1.5μl 100mM DTT, 3μl 10mM dNTP mix and 1.5U T4 DNA polymerase in a 30μl total volume. The reaction was incubated for 20 minutes at 11^{o}C, after which the cDNA was purified by phenol-chloroform extraction and ethanol precipitation (A1-9). Directional *Eco*R I/ *Hind* III linkers (5'-GCTTGAATTCAAGC-3') (A-2.3) were phosphorylated at their 5' ends with phosphate groups derived from ATP and then ligated to the cDNA inserts. The following components were assembled in a 20μl reaction volume: 10μl blunt-ended cDNA in TE buffer, 2μl 10X ligation buffer (200mM Tris-HCl pH 7.6, 50mM $MgCl_2$), 2μl 1mM ATP, 2μl 100mM DTT, 100pmol directional linkers and 5U T4 polynucleotide kinase. After the phosphorylation reaction at 37^{o}C for five minutes, 2.5μl T4 DNA ligase was added directly to the mixture and incubated overnight at 16^{o}C, followed by inactivation of the enzyme at 70^{o}C for 10 minutes. The cDNA linkers were subsequently digested at 37^{o}C with the relevant restriction enzymes. 10μl 10X *Hind* III buffer and 5μl (100U) *Hind* III enzyme were added to the cDNA mixture and the volume adjusted to 100μl with nuclease-free water. After a 2 hr incubation period, 10μl 10X *Eco*R I adjustment buffer supplied in the kit and 5μl (100U) *Eco*R I were added and digestion continued for a further 4 hrs. The cDNA was purified with phenol-chloroform extraction and ethanol precipitation, resuspended in 100μl TE buffer and stored at -20^{o}C until size fractionation.

Fractionation was required for removing excess linkers and cDNA synthesis products of less than 300bp. 2ml gel filtration Sepharose® 4B resin was transferred to a mini column and the bed volume packed under gravity flow. The column was equilibrated with 5ml of 1X column buffer

(0.3M sodium acetate) and the cDNA carefully loaded on top of the bed surface. After settling into the resin, the column was washed with 450μl column buffer. A void fraction of 250μl was collected and contained the largest cDNA molecules. The fraction was supplemented with 1μl 10mg/ml glycogen, precipitated with 100% ethanol and the washed pellet dissolved in 20μl TE buffer (A-1.9). The cDNA was stored at -20^{o}C and was ready for cloning into T7Select 10-3b vector arms.

2.5.1.3 Electrophoresis of end-modified cDNA

To confirm the presence of end-modified cDNA and to evaluate the average insert size, a 3μl aliquot was mixed with 2μl stop solution (25mM EDTA pH 7.5, 95% deionised formamide, 0.1% bromophenol blue, 0.1% xylene cyanol FF), denatured at 80^{o}C for at least two minutes and then loaded onto an 8% preheated denaturing acrylamide gel (A-1.10). The average size was determined by comparison to two standard markers, which were synthesised from human genomic DNA sequences using polymerase chain reaction (PCR) and [α^{32}P] dATP (A-2.2).

Human genomic DNA was isolated from whole blood using sodium hydroxide extraction (A-1.11). 25μl PCR included 0.25μg human DNA, 1X PCR buffer (10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$), 0.068mM dNTP, 0.5μM primers, 0.6U TaKaRa *Taq* polymerase and 2.5μCi [α^{32}P] dATP (800Ci/mmol, 10μCi/μl). Reactions were assembled on ice water and placed into an Eppendorf Gradient thermal cycler preheated to 94^{o}C. PCR conditions for the forward 1 (F1) and reverse 1 (R1) primers comprised 30 cycles of denaturation at 94^{o}C for 1 minute, annealing at 55^{o}C for 1 minute and extension at 72^{o}C for 1 minute. Annealing temperature for the forward 2 (F2) and reverse 2 (F2) primer pair was increased to 63^{o}C. 3μl PCR products were mixed with 2μl stop solution (25mM EDTA pH 7.5, 95% deionised formamide, 0.1% bromophenol blue, 0.1% xylene cyanol FF), denatured and electrophoresed alongside the

cDNA sample. Electrophoresis was carried out at 60W for approximately 2.5hrs and the gel exposed to X-ray film overnight at -70^{o}C. Films were developed in a Kodak X-Omat processor (Johannesburg General Hospital, South Africa) and DNA visualised due to the incorporation of α^{32}P dATP into the PCR and cDNA products. The gel was also subsequently exposed to X-ray film in a cassette containing intensifying screens to obtain a darker image.

2.5.2 Construction of phage display libraries

The BLT5403 *E. coli* strain was used for propagating T7 phage particles. Sterility was maintained by autoclaving equipment and specific solutions at 120^{o}C, 15lb/in^{2}. Smaller volumes were sterilised by filtration through 0.22μm filter units. MilliQ water was used for the preparation of all reagents.

2.5.2.1 Cloning of inserts in T7Select vector arms

P. falciparum cDNA fragments were ligated to *Eco*R I/*Hind* III T7Select 10-3b vector arms (A-2.3) according to Novagen's Phage Display System Manual. Parasite cDNA sequences were cloned downstream and in frame with the 10B coat protein gene (after amino acid position 348) such that recombinant fusion proteins were expressed. The reading frame required an AAT (asparagine) codon provided by the *Eco*R I restricted end (amino-terminal side) of cDNA inserts. The 10-3b vector was designed to display polypeptides of up to 1200 amino acids with a copy number of 5-15 fusion proteins per phage particle.

To obtain maximal cloning efficiency with cDNA inserts of average size 1.5 kilobase pairs (kbp), the T7Select System manual recommended an insert: vector ratio of 3:1. For this reason, a 3:1 molar ratio was chosen for cloning the *P. falciparum* cDNA fragments into the T7Select 10-3b vector.

Ligation reactions were prepared in sterile Eppendorf tubes by combining the following components: 1μl (0.06pmol) cDNA, 1μl (0.5μg) T7Select 10-3b vector, 0.5μl 10X ligase buffer provided in the kit, 0.5μl 10mM ATP, 0.5μl 10mM DTT and 1μl (0.4-0.6U) T4 DNA ligase in a 5μl total volume. The reaction was incubated overnight at 16^{o}C and stored at 4^{o}C until use. 1μl control insert DNA was set up as a positive control reaction in parallel with the cDNA fragments to test the efficiency of ligation, packaging and amplification.

2.5.2.2 *In vitro* packaging and plaque assay

Vector constructs were packaged into phage particles by using bacteriophage T7 extracts. Twenty five μl packaging extracts supplied in the kit were thawed on ice water and 5μl ligation reactions added. The samples were gently stirred with a sterile pipette tip and incubated at room temperature for 2hrs. The packaging reaction was stopped with 270μl LB medium (1% w/v tryptone, 0.5% w/v yeast extract, 170mM NaCl, 10mM Tris-HCl pH 7.5) and 20μl chloroform added if the reaction was stored for more than 24hrs prior to amplification.

A plaque assay was performed next to determine the number of recombinants generated. Host *E. coli* cells were streaked on a 90mm LB/ 1.5% (w/v) agar plate containing 50μg/ml ampicillin and incubated overnight at 37^{o}C. The next day, 3ml LB medium was inoculated with a single colony and incubated for 16hrs with vigorous rotation at 37^{o}C in a 50ml sterile Erlenmeyer flask. To 2.6ml of the overnight culture, the following components were added: 7ml LB medium, 300μl M9 salt (375mM NH_4Cl, 440mM KH_2PO_4, 450mM Na_2HPO_4), 140μl 20% glucose, 7μl 1M $MgSO_4$ and 50μg/ml ampicillin. The cells were incubated with shaking for 2 hrs at 37^{o}C in a 50ml Erlenmeyer flask, after which 250μl aliquots were transferred to six sterile 14ml round bottom Falcon tubes.

Serial dilutions of the control and cDNA packaging reactions were prepared in 1ml LB media, and these ranged from one hundred to one million times. 100μl of the phage dilutions and LB medium, which represented a negative control, were added to the respective tubes containing fresh *E. coli* cells. 3ml molten top agarose (10g/l tryptone, 5g/l yeast extract, 5g/l NaCl, 6g/l agarose) at 45-50^{o}C was added to each tube and the contents immediately poured onto LB/agar plates pre-warmed at 37^{o}C and containing 50μg/ml ampicillin. The agarose was spread evenly over the surface, allowed to solidify and the plates inverted and incubated at 37^{o}C for 4 hrs. The size of the library in plaque forming units (pfu) per ml was determined by counting the number of plaques appearing with each plated dilution. The packaging efficiency was expressed as the total number of phage in the reaction per μg vector used. For primary recombinants less that 5×10^{6} pfu, amplification by plate lysate was the preferred method.

2.5.2.3 Plate lysate amplification

Prior to biopanning, a single round of amplification was necessary for the expression of cloned sequences and their display on the surface of phage capsids. 10ml *E. coli* log phase cells were prepared as described in 2.5.2.2 and mixed with 1×10^{6} phage particles. 1 ml aliquots were transferred to 15ml sterile Nunc tubes and 10ml molten top agarose added. The contents were poured onto a 150mm LB/agar plate containing 50μg/ml ampicillin and then inverted at 37^{o}C for 4 hrs. Phage were eluted by covering the plate with 10ml extraction buffer (20mM Tris-HCl pH 8.0, 100mM NaCl, 6mM $MgSO_4$) and incubating overnight at 4^{o}C.The particles were harvested by transferring the supernatant to a sterile 15ml polypropylene tube and then mixing with 0.5ml chloroform. Tubes were centrifuged at 1 500 *g* in a Jouan BR 3.11 centrifuge for 10 minutes at 4^{o}C to clarify the lysate. The supernatant was collected and the titre of the amplified library determined by plaque assay (2.5.2.2). Libraries were

stored at 4^{o}C for several months and indefinitely at -70^{o}C with 0.1 volume sterile 80% glycerol.

2.6 Biopanning of *P. falciparum* phage display libraries

Phage display libraries were screened in sterile 1.5ml Eppendorf tubes by biopanning against purified 4.1R. Fifty to 150μg biotinylated protein (1-3μg/μl) was immobilised on 50μl streptavidin coated magnetic beads (equilibrated in TBS) at room temperature for at least 1 hr with slow rotation. Unbound protein was removed by collecting the beads with a MPC and washing three times with TBS. Libraries were diluted to 1×10^{6} pfu/ml in 300μl TBS final volume, and incubated with 100μl uncoated beads for 30 minutes with agitation to pre-select background binding. The remaining phage were removed with the aid of a MPC and incubated with 4.1R coated magnetic beads for 1 hour at room temperature. The beads were washed three times for five minutes in 10ml 0.1% Tween 20 in TBS to remove unbound phage and non-specific interactions. Bound particles were eluted for 15 minutes with 200μl TBS, 1% SDS and then amplified at 37^{o}C in 50ml log phase *E. coli* cultures. SDS allowed for efficient disruption of peptide-ligand interactions. It was important to dilute the eluted phage sample at least 200 fold in *E. coli* culture to prevent inhibition of cell growth.

Cultures were prepared by combining 12.5ml of 16hr *E. coli* grows with 35ml LB medium, 1.75ml M9 salt, 0.7ml 20% glucose, 35μl 1M $MgSO_4$ and 50μg/ml ampicillin. The eluted phage were added to the culture and amplified overnight at 37^{o}C with vigorous shaking. Following amplification, sodium chloride was added to the lysed cultures to a final concentration of 0.5M, and cellular debris cleared by centrifugation at 8 000 *g* for ten minutes at 4^{o}C in a Beckman J2-21 centrifuge. The supernatant was transferred to a sterile 50ml Nunc tube and the titre determined by performing a plaque assay. To enrich the phage that bound 4.1R, the first

round library was diluted to 1×10^6 pfu/ml in 300μl TBS final volume and subjected to another three rounds of biopanning. 1ml aliquots of the libraries from the different rounds were stored at -70°C with 0.1 volume 80% sterile glycerol.

2.6.1 Identification of *P. falciparum* cDNA inserts

2.6.1.1 Insert size determination

After fourth round biopanning, the library was titred and individual plaques removed from low density LB/agar plates using sterile pipette tips. The agar plugs were placed in 500μl phage extraction buffer (20mM Tris-HCl, 100mM NaCl, 6mM $MgSO_4$, pH 8.0) overnight at 4°C. DNA was released from the T7 bacteriophage by incubating 10μl phage samples in a final volume of 60μl 10mM EDTA pH 8.0 for 10 minutes at 65°C. The DNA samples were cooled to room temperature and centrifuged at 14 000 *g* for 3 minutes to clarify. *P. falciparum* cDNA insert sizes were determined by PCR and agarose gel electrophoresis. The following PCR was set up in nuclease-free tubes: 2μl phage lysate in a 25μl volume containing 1.25pmol T7SelectUP and DOWN primer (A-2.3) and 1X Promega Master Mix final concentration. The reaction was cycled 30 times in an Eppendorf thermal cycler with denaturation at 94°C for 50 seconds, primer annealing at 50°C for 1 minute and extension at 72°C for 1 minute. To determine the size of the cloned cDNA fragments, PCR products were electrophoresed alongside a 100bp DNA ladder on 1% agarose gels supplemented with 10μg/ml ethidium bromide. This was performed at 60mA for 30 minutes in 1X TAE running buffer. The UP and DOWN primers annealed to the T7Select 10-3b vector at positions flanking the *Eco*R I/*Hind* III cloning site, therefore all PCR products contained 107bp vector sequence. Insert sizes were determined and the largest PCR fragments selected for DNA sequencing.

2.6.1.2 DNA sequencing and bioinformatic analysis

The DNA sequence and correct reading frames of insert sequences were determined using the PCR product pre-sequencing and Sequenase version 2.0 DNA sequencing kits (A-1.10). *P. falciparum* sequences were identified by performing nucleotide-nucleotide (BLASTN) and nucleotide-protein (BLASTX) searches in the PlasmoDB database (http://www.plasmodb.org). Annotated genes were selected based on the best match between the two BLAST searches. Analysis of the corresponding protein/peptide sequences and secondary structure was carried out using algorithms available form the EXPASY proteomics server (http://us.expasy.org) and European Bioinformatics Institute (http://www.ebi.ac.uk/tools/). Solubility profiles of recombinant proteins were obtained using a statistical model for proteins expressed in *E. coli* (http://www.biotech.ou.edu/).

2.7 Cloning of *P. falciparum* gene sequences

Gene sequences encoding proteins binding specifically to 4.1R were amplified from purified *P. falciparum* DNA using PCR. The reaction was primed with oligonucleotides that contained specific restriction endonuclease recognition sites. PCR products were digested with the appropriate restriction enzymes and cloned into protein expression vectors that harbored compatible ends. Successful cloning was confirmed by PCR and the fidelity of the PCR amplification verified by DNA sequencing.

2.7.1 Isolation of genomic DNA from asexual parasites

DNA was extracted from *P. falciparum* parasites which were harvested from a 175cm^2 tissue culture flask (2.3). Once the parasitaemia reached 10-15%, the 30ml culture was transferred to a 50ml Nunc tube and centrifuged at 1 100 *g* for ten minutes at 4^{o}C. The pellet was washed twice with PBS, pH 7.4, centrifuged as before and the packed erythrocytes lysed

with a final concentration of 0.5% saponin at room temperature for approximately 5 minutes. The parasites were pelleted and resuspended in 500μl lysis buffer (40mM Tris-HCl pH 8.0, 80mM EDTA, 2% SDS) supplemented with 0.025mg/ml Proteinase K to degrade contaminating proteins. The suspension was incubated at 37^{o}C for 3 hrs with occasional agitation, after which 1ml MilliQ water was added. Genomic DNA was purified by phenol-chloroform extraction and ethanol precipitation (A-1.9).

2.7.2 Preparation of PCR products for subcloning

Parasite sequences containing 4.1R-specific peptides were selected for amplification and cloning into expression vectors encoding a histidine tag (pET15b) and glutathione S-Transferase (GST) tag (pGEX-4T-2). 5' *Nde* I or *Bam*H I and 3' *Bam*H I or *Xho* I restriction sites were added to the ends of the PCR primers respectively. Four to eight additional nucleotides were also appended at the 5' ends to enable efficient digestion with the respective restriction endonucleases. One μg purified *P. falciparum* DNA was combined in a nuclease-free PCR tube with 0.4μM forward and reverse primers as well as 1X Roche High Fidelity PCR Master Mix in a reaction volume of 50μl. The master mix is composed of two DNA polymerases, namely *Taq* and *Tgo*. The latter enzyme contains inherent 3'-5' exonuclease proofreading activity and therefore increases the fidelity of DNA synthesis by approximately 3-fold when compared to *Taq* DNA polymerase. PCR was performed in an Eppendorf Gradient thermal cycler which was pre-heated to 94^{o}C. To facilitate annealing of the gene-specific region of the primer, five cycles of denaturation at 94^{o}C for 1 minute, annealing at 50^{o}C for 50 seconds and extension at 72^{o}C for 1 minute were carried out. This was followed by 25 cycles to promote amplification of the entire sequence including the restriction sites. Similar cycling conditions were used, except for the annealing step which was increased to 65^{o}C for 45 seconds. 100μl PCR products were purified by phenol-chloroform

extraction and ethanol precipitation (A-1.9). Amplification was confirmed by 1% agarose gel electrophoresis at 60mA for 30 minutes.

PCR products were initially digested with 10U *Nde* I or *Bam*H I in 50μl volumes containing 40μl PCR amplicons, 1X Promega Core® Buffer D or E, 0.1mg/ml acetylated BSA and 3.5μl nuclease-free water. The reaction was incubated overnight or for two hours at 37°C respectively, and the DNA purified with phenol-chloroform extraction and ethanol precipitation (A-1.9). *Xho* I digestion was carried out in Promega Buffer D at 37°C for 2hrs. The digested products were purified as before and dissolved in 10μl 1X DNA dilution buffer from the Rapid DNA Ligation Kit. Samples were stored at -20°C until required.

2.7.3 Preparation of pET15b and pGEX-4T-2 protein expression vectors

One μg pET15b (A-2.4) and pGEX-4T-2 vector (A-2.5) were prepared for subcloning by linearising with 10U of appropriate restriction enzymes. This was necessary to create compatible ends for ligation with parasite PCR products. Digests were set up in 50μl volumes as described in 2.7.2 and incubated at 37°C for 2hrs. To prevent self-ligation, 5' vector ends were dephosphorylated at 37°C for 30 minutes by adding 1U Calf Intestinal Phosphatase (CIP) to the samples after *Nde* I or *Bam*H I digestion. The vector was purified by extraction with phenol-chloroform and precipitated with ethanol. Linearised plasmids were resuspended in 40μl nuclease-free water, digested with *Bam*H I or *Xho* I and treated with 1U CIP. The vector was purified, dissolved in 10μl 1X DNA dilution buffer from the Rapid DNA Ligation kit and stored at -20°C until use.

2.7.4 Ligation of vector and parasite insert DNA

1-2μl insert DNA was electrophoresed on a 1% agarose gel alongside 10μl of DNA MassRuler™ to estimate the concentrations for ligation. An insert: vector molar ratio of 3:1 was used for ligation, such that the total amount of DNA in the reaction did not exceed 200ng. The reaction was set up in a sterile microcentrifuge tube according to the Rapid DNA Ligation Kit. 9μl DNA (vector and insert) was combined with 1μl 1X DNA dilution buffer, 10μl 2X T4 DNA ligation buffer and 1μl T4 DNA ligase, and incubated at 16°C for 30 minutes. A mock ligation reaction containing all the reagents except insert DNA was prepared to check for self-ligation of the vector. Twenty one μl ligation samples, 3μl 10X Promega Core® Buffer D or MultiCore® Buffer, 1μl 3mg/ml acetylated BSA and 1U *Xho* I or *Sma* I were then combined with water in a 30μl digest volume. The reaction was incubated for 2hrs at 37°C. *Xho* I and *Sma* I linearised re-ligated pET15b and pGEX-4T-2 vector respectively, and therefore acted as an extra control measure for minimising false-positives during transformation of DH5α competent cells.

2.7.5 Transformation of DH5α competent cells

Fifty μl subcloning efficiency DH5α cells, stored at -70°C, were thawed on ice water according to the manufacturers instructions. 5μl of the ligation mixture was added directly to the cells, mixed gently and left on ice for 30 minutes. The samples were heat-shocked at 37°C for 20 seconds and placed on ice for a further two minutes. 450μl LB medium was then added and the cells placed at 37°C in an environmental shaker at 220 rpm for 1hr. Aliquots of 50μl and 450μl transformed DH5α cells were spread onto LB/agar plates containing 50μg/ml ampicillin. The plates were incubated overnight at 37°C. The following day, single colonies were picked from the plates and inoculated in 2ml LB medium supplemented with 50μg/ml ampicillin. Colonies were grown overnight with shaking at 37°C in 15ml

sterile round bottom growth tubes. Glycerol stocks were prepared by diluting the stationary phase cells 1:1 with 60% sterile glycerol and stored at -70^{o}C. The remaining cells were used for plasmid purification and confirmation of successful ligation.

2.7.6 Plasmid DNA purification and parasite insert verification

Two methods were used for isolating plasmid DNA from DH5α cells. Vector constructs were liberated by conventional SDS/alkaline lysis and DNA purified with phenol-chloroform extraction followed by ethanol precipitation (A-1.12). For rapid isolation and purification, the Eppendorf FastPlasmid^{TM} Mini Kit was used. According to the manufacturer's instructions, pelleted *E. coli* cells were lysed in 400μl ice-cold Complete Lysis Solution. The lysate was transferred to a spin column and captured on a solid phase support. The DNA was washed with an isopropanol-containing buffer, eluted in a low-salt buffer and stored at 4^{o}C.

The presence of plasmid insert and its correct size was verified from individual transformed DH5α colonies with PCR. 5μl purified DNA was combined with 0.4μM gene specific primers and 1X PCR Master Mix in a 50μl PCR reaction volume. The same cycling parameters were used as described in 2.7.2. PCR products and a 100bp DNA ladder were resolved by 1% agarose gel electrophoresis.

2.7.7 Sequence verification

Plasmid inserts were verified for reading frame and checked for sequence errors. An LB/agar plate was streaked with a single clone of transformed DH5α cells and submitted for automated DNA sequencing (Inqaba Biotech, South Africa). Sequencing reactions were primed with the pET15b T7 promoter and/or terminator primer or pGEX-4T-2 forward and/or reverse

sequencing primers. Sequencing data were evaluated with ChromasPro (version 1.2) software.

2.8 Expression of recombinant parasite proteins

2.8.1 Transformation of BL21-CodonPlus® competent cells

Production of recombinant proteins in *E. coli* is frequently limited by different codon usage in the bacteria. The expression of heterologous proteins can deplete the pool of rare tRNAs that are normally abundant in the organisms from which the proteins were derived, thus stalling translation. For this reason, BL21-CodonPlus® competent cells were chosen as the host cells for efficient high-level expression of recombinant parasite proteins. These cells contain additional copies of genes that encode the tRNAs for arginine (R), isoleucine (I) and leucine (L). The genes are located on a RIL plasmid and promote translation of heterologous proteins from organisms, like *P. falciparum*, that have AT-rich genomes. An additional co-transfected plasmid, denoted pACYC, confers a chloramphenicol resistant phenotype on the cells.

A 100μl aliquot of competent cells was thawed on ice-water and transferred into a sterile 15ml pre-chilled growth tube. To increase transformation efficiency, 2μl 1:10 XL10-Gold β-mercaptoethanol was added to the cells and incubated on ice for 10 minutes with occasional swirling. Fifty ng pET15b or pGEX-4T-2 vector construct was added and left on ice for a further 30 minutes. The cells were heat-shocked at 42^{o}C for 20 seconds and placed back on ice for two minutes. 900μl SOC medium (A-1.13) was added and the samples placed in a shaker at 250rpm for 60 minutes at 37^{o}C. The cells were spread on LB agar plates containing 50μg/ml ampicillin and 50μg/ml chloramphenicol and incubated overnight at 37^{o}C (2.7.5). A mock plate was also prepared by transforming BL21 cells with vector containing no parasite insert.

2.8.2 Induction of target protein expression

Expression of the target protein required optimisation of numerous factors, which presented potential obstacles for successful production and purification of recombinant protein in *E. coli*. The growth temperature, length of induction with isopropyl-1-thio-β-D-galactopyranoside (IPTG), amount of IPTG and cell density at time of induction were variables that had to be considered.

One ml aliquots of LB medium supplemented with selection antibiotics (50μg/ml ampicillin and 50μg/ml chloramphenicol) were transferred to sterile growth tubes and inoculated with single transformed BL21-RIL colonies. These were incubated overnight with shaking at 37^{o}C, followed the next day by a 1:10 dilution into 10ml fresh LB medium containing no antibiotic. The cells were grown until the absorbance at 600nm was approximately 0.8, and then induced with IPTG at a final concentration of 1mM for a further 3hrs. One hundred μl aliquots of induced and uninduced cultures were solubilised in 1X SDS buffer for electrophoresis on 12% Laemmli gels (2.1.4).

2.8.3 Purification of glutathione S-Transferase and histidine-tagged parasite proteins

The target protein was purified by liberating soluble proteins from BL21 *E. coli* cells and applying the cell lysate to appropriate magnetic agarose beads.

2.8.3.1 Extraction of soluble proteins

After induction with IPTG, the cells were centrifuged at 15 000 *g* for 1 minute. The pellet was frozen at -70^{o}C for five minutes and then placed at room temperature to slowly thaw. Cells from 1ml culture were resuspended and lysed in 200μl BugBuster® protein extraction reagent at

room temperature for 15 minutes with gentle rotation. Prior to lysis, the BugBuster reagent was supplemented with 2μl Protease Inhibitor Cocktail Set III per 1 ml culture to prevent protein degradation. The cocktail displays broad specificity for the inhibition of aspartic, cysteine and serine proteases as well as aminopeptidases. Insoluble cell debris and protein complexes were removed by centrifugation at 17 500 *g* for 10 minutes at 4^{o}C. The pellet was resuspended in 100μl TE buffer and stored with the supernatant at -20^{o}C. Twenty μl of the soluble and insoluble fractions were solubilised in 5X suspension solution (A-1.3) for SDS-PAGE analysis.

2.8.3.2 Affinity capture of histidine-tagged proteins

Histidine-tagged proteins were purified from soluble *E. coli* extracts by affinity capture on His-Select^{TM} magnetic agarose beads. The beads contain an affinity resin charged with nickel, and are designed to selectively bind proteins containing six histidine residues (6His). The pET15b vector (A-2.4) encodes a 5' 6His tag that allows purification of the resultant recombinant protein.

Thirty μl resuspended beads were equilibrated in fresh wash/ binding buffer (20mM Tris-HCl, 0.5M NaCl, 5mM imidazole, pH 7.9) and added to soluble extracts from 1ml induced cells for 1hr at room temperature with gentle rotation. The samples were placed in a MPC for 20 seconds, the supernatant removed and the beads washed three times for 1 minute in 30 volumes wash buffer. 6His parasite proteins were eluted from the beads in 60-100μl elution buffer (20mM Tris-HCl, 0.5M NaCl, 0.5M imidazole, pH 7.9) for 15 minutes. Supernatant containing purified protein was removed and immediately dialysed in a Slide-A-Lyzer MINI dialysis unit against two changes of chilled PBS, pH 7.4 for 2 hrs at room temperature. Pefablock® *SC was added to purified samples to a final concentration of 1mM and stored at -20^{o}C.

2.8.4 Verification of 6His parasite protein expression

Purified protein samples were electrophoresed by SDS-PAGE on 12% Laemmli gels and transferred to nitrocellulose membrane at 50V for 2hrs at 4^{o}C (A-1.5). To confirm the presence of protein, the membrane was stained with 1% Ponceau S diluted in 7% acetic acid and then destained with water. 6His tagged protein was detected by probing the membrane with mouse anti-His antibody (1:2000), followed by detection with goat anti-mouse peroxidase conjugated IgG (1:2000) (A-1.5) using Supersignal® West Pico Chemiluminescent Substrate (2.2.1).

2.9 Cloning of 4.1R domain sequences

2.9.1 Reticulocyte total RNA extraction

A modified method of Chomczynski and Sacchi (1987) was used for extracting total RNA from human reticulocytes. RNA preparations were carried out on a dedicated work area, which was wiped with RNaseZap before use. A number of other strict precautions were taken to prevent contamination with RNases (2.4).

Whole venous blood was collected from volunteers in ACD tubes to prevent clotting. The tubes were centrifuged in a Jouan BR 3.11 at 650 *g* for five minutes at 4^{o}C. This low-speed centrifugation was important for enriching reticulocytes near the buffy coat/plasma interface. The plasma was discarded and the top 2ml of the packed erythrocytes (including white blood cells) were transferred into a 15ml polypropylene tube. The cells were washed with five volumes ice-cold 0.9% saline, centrifuged at 1 100 *g* for 10 minutes and the process repeated three times to remove any visible trace of buffy coat. Tubes were placed at -70^{o}C for at least 2hrs and then slowly thawed in a 10^{o}C water bath. This step was important for lysis of reticulocytes and mature erythrocytes. Intact white blood cells were immediately pelleted at 1 100 *g* for 15 minutes and the RNA-containing

supernatant collected. In this order, 5ml D* solution (2.4.1), 0.5ml 2M sodium acetate (pH 4.0), 5ml buffered phenol (pH 8.0) and 1ml chloroform were added to the supernatant, centrifuged at 1 100 *g* for 15 minutes and the aqueous phase aspirated into a clean 15ml tube. The RNA was re-extracted with phenol-chloroform and precipitated from 1ml aliquots of aqueous phase with an equal volume of isopropanol overnight at -20^{o}C. Precipitates were centrifuged at 15 000 *g* in a Sorvall RMC14 microcentrifuge for 30 minutes at 4^{o}C, pellets washed with 70% ethanol and the RNA resuspended in 20μl nuclease-free water for storage at -70^{o}C. 1μl of the RNA sample was electrophoresed on a 1% agarose gel alongside a MassRuler^{TM} to estimate the concentration and check the quality (2.4.2).

2.9.2 Reverse transcription PCR

The four structural domain sequences of 4.1R (30kDa, 16kDa, 10kDa and 22/24kDa) were reverse transcribed from purified reticulocyte RNA using AMV reverse transcriptase. Primers flanking the relevant domains (A-2.6) were designed and restriction sites appended for ligation with the pGEX-4T-2 protein expression vector (Conboy et al., 1986; Conboy et al., 1988; Conboy et al., 1991; Waller et al., 2003). Approximately 1μg RNA was combined with 0.5μg reverse primers in a 10μl final volume, and incubated at 70^{o}C for five minutes. The samples were snap-cooled on ice-water for five minutes and the solution collected at the bottom of the reaction tube by centrifugation. For first-strand synthesis, the volume was increased to 25μl with the addition of 5μl 5X AMV reaction buffer (250mM Tris-HCl pH 8.3, 250mM KCl, 50mM $MgCl_2$, 50mM DTT), 4μl 2.5mM dNTP mix, 40U RNasin® Ribonuclease inhibitor and 30U AMV reverse transcriptase. Reaction tubes were incubated at 42^{o}C for 1hr and the enzyme inactivated at 70^{o}C for 15 minutes. cDNA products were used for second strand synthesis and amplification in a PCR.

A 50μl reaction including 5μl first strand cDNA, 0.2mM primers and 1X Roche High Fidelity PCR Master Mix was assembled on ice and transferred to a 94^{o}C pre-heated Eppendorf Gradient thermal cycler. The PCR program for the 10kDa domain consisted of 30 cycles of denaturation at 94^{o}C for 1 minute, annealing at 65^{o}C for 30 seconds and extension at 72^{o}C for 1 minute. The annealing temperatures for the 30kDa, 22kDa and 16kDa domains were increased to 67^{o}C, 70^{o}C and 71^{o}C respectively. The presence and size of the amplified 4.1R domain sequences were verified by electrophoresis of 1μl aliquots and 5μl 100bp ladder on a 1% agarose gel.

2.9.3 Purification and restriction enzyme digestion of PCR products

The MiniElute™ Gel Extraction Kit was used for isolating the correct PCR fragments from standard 1% agarose gels as per manufacturer's instructions. 50μl PCR products were resolved by electrophoresis at 60mA for 45 minutes and the DNA bands excised. The gel slices were minimised by removing extra agarose and melted at 50^{o}C for ten minutes in buffer containing guanidine thiocyanate. One gel volume isopropanol was added and the DNA adsorbed on MiniElute membranes by centrifugation. The DNA was washed with a guanidine buffer and eluted with a low salt solution. DNA fragments not requiring gel extraction were purified from PCR using the QIAquick® PCR purification Kit. The DNA was similarly captured on a solid phase support and eluted for storage at 4^{o}C until restriction enzyme digestion.

Purified PCR fragments were digested for cloning into pGEX-4T-2 vector. Forty μl PCR product, 5μl 10X appropriate Promega Core® Buffer, 0.5μl 10mg/ml acetylated BSA and 10U relevant restriction enzymes were combined with water in 50μl reaction volumes. *Bam*H I and *Xho* I digests were carried out in buffers E and D respectively, while digestion with *Eco*R I was performed in buffer H. All the reactions were incubated at 37^{o}C for

2hrs. DNA was purified after the first and second digests using the QIAquick Purification Kit, and eluted with a final volume of 10μl 1X DNA dilution buffer from the Rapid DNA Ligation Kit. Samples were stored at -20^{o}C until required.

2.9.4 Preparation of pGEX-4T-2 vector and ligation to 4.1R DNA

The pGEX-4T-2 vector (A-2.5) was prepared for subcloning by linearising it with 10U of appropriate restriction enzymes, as described in 2.7.3. Purified samples were resuspended in 10μl 1X DNA dilution buffer from the Rapid DNA ligation Kit and stored at -20^{o}C.

DNA concentrations were determined and vector ligation reactions set up according to the Rapid DNA ligation kit (2.7.4). DH5α competent cells were transformed with the vector constructs (2.7.5). Plasmid DNA was purified from overnight *E. coli* grows for insert verification (2.7.6) and automated DNA sequencing (2.7.7).

2.10 Expression of 4.1R recombinant domains

2.10.1 Transformation of Rosetta^{TM} 2 (DE3) competent cells

The Rosetta 2 (DE3) host strain is a BL21 derivative designed to enhance expression of eukaryotic proteins that contain codons rarely used in *E. coli*. The cells supply transfer RNA for the codons AUA (isoleucine), AGG (arginine), AGA (arginine), CUA (leucine), CCC (proline), GGA (glycine) and CGG (arginine), which are found on a chloramphenicol-resistant plasmid, denoted pRARE.

Twenty μl aliquots of Rosetta competent cells were thawed on ice water and transferred to sterile 15ml growth tubes containing approximately 200ng vector constructs. The contents were gently mixed and left on ice for 5 minutes, followed by heat-shock at 42^{o}C for 30 seconds. Samples

were placed on ice for 2 minutes and then 80μl SOC medium (A-1.13), pre-heated to room temperature was added. The cells were incubated at 37^{o}C for 1hr in an environmental shaker at 220rpm, after which 30μl and 70μl aliquots were spread on LB/agar plates containing 50μg/ml ampicillin and 50μg/ml chloramphenicol. The plates were inverted and incubated overnight at 37^{o}C. Control plates were also prepared by transforming host *E. coli* cells with pGEX-4T-2 vector containing no insert.

2.10.2 Induction of target protein expression

Conditions defining the optimal expression of GST fusion proteins were determined. Overnight *E. coli* grows were induced with 1mM IPTG for 2-3hrs (2.8.2). In an attempt to increase the solubility of recombinant protein, the Overnight Express^{TM} Autoinduction System was used. The system is designed for high-level protein expression and uses lactose to induce synthesis of recombinant proteins. One ml Overnight Express^{TM} medium containing 50μg/ml ampicillin and 50μg/ml chloramphenicol was inoculated with single colonies of transformed Rosetta 2 cells. The cells were grown for 16hrs at 37^{o}C with shaking at 220rpm and aliquots solubilised in 1X SDS buffer for electrophoresis.

2.10.3 Purification of GST-tagged 4.1R domains

Soluble proteins were extracted from induced cultures as described (2.8.3.1). GST-tagged proteins were purified from the extracts by use of MagneGST^{TM} agarose beads. The system consists of reduced glutathione molecules covalently attached to the surface of paramagnetic particles. GST uses glutathione as a substrate and this interaction can be used to isolate GST-fusion proteins from crude cellular lysates.

Thirty μl MagneGST^{TM} beads were equilibrated in wash/binding buffer (4.2mM Na_2HPO_4, 2mM K_2HPO_4, 0.5M NaCl, 10mM KCl). The beads were

transferred to soluble Rosetta 2 extracts in 1.5ml Eppendorf tubes and incubated at room temperature for 1hr with gentle rotation. Tubes were placed in a MPC for 20 seconds, the supernatant removed and the beads washed three times in 30 volumes wash buffer. The first wash was carried out for 5 minutes, followed by two more washes for 1 minute each. The beads were captured by the magnet, supernatant removed and fusion protein eluted in 100μl elution buffer (50mM Tris-HCl pH 8.0, 100mM glutathione-SH) for 15 minutes at room temperature. Purified protein was dialysed against two changes of chilled PBS, pH 7.4 in a Slide-A-Lyzer MINI dialysis unit for 2hrs at room temperature. Pefablock® *SC was added to a final concentration of 1mM and samples stored at -20^{o}C until required.

2.10.4 Verification of GST-4.1R protein expression

Purified recombinant 4.1R domains were electrophoresed on 12% Laemmli gels and transferred to nitrocellulose membranes at 50V for 2 hrs (A-1.5). The transfer was confirmed by staining the membrane with 1% Ponceau in 7% acetic acid. GST-tagged domains were identified by immunoblotting with goat anti-GST antibody (1:2000). This was followed by visualisation with rabbit anti-goat IgG peroxidase conjugated antibody using 4-chloro-1-naphtol as a substrate (A-1.5).

2.11 Interaction studies between recombinant proteins

2.11.1 Blot overlay assays

Blot overlay assays were used for studying protein-protein interactions between recombinant proteins. The objective was to map the interaction between 6His-parasite protein and GST-4.1R domains.

Approximately 5μg of GST, GST-4.1R structural domains and 6His-parasite protein were spotted onto nitrocellulose membrane strips.

Additional binding sites were blocked in 5% BSA in TBS for 1hr at room temperature. The membranes were rinsed in TBS and overlaid with 0.5-1μg (50μl) of either native unbiotinylated 4.1R or appropriate recombinant domain for 2hrs. GST and the 4.1R domains served as negative and positive controls respectively. The strips were washed twice for 1 minute each in TBS and the bound protein fixed to the membrane with 0.5% (v/v) formaldehyde in TBS for 20 minutes. Membranes were washed for 15 minutes in TBS, rinsed and then probed with rabbit anti-4.1R antibody (1:1000) for at least 1hr (A-1.5). Interactions were detected with goat anti-rabbit IgG peroxidase conjugated antibody (1:1000) using Supersignal® West Pico Chemiluminescent Substrate (2.2.1).

2.11.2 Pull-down assays

Pull-down assays were used to verify the interaction between parasite protein and specific 4.1R domain. Furthermore, the dependence of the interaction on concentration was determined by densitometric scanning of 12% Laemmli gels.

6His-parasite protein (~700ng) was coupled to 30μl aliquots of His-Select™ magnetic beads as described (2.8.3.2). Increasing amounts of specific GST-4.1R domain, ranging from 200ng to 2μg, as well as 500ng and 10μg heat denatured protein were added to the beads and incubated for 1hr at room temperature. The denatured samples served as a control to correct for non-specific binding. The beads were rinsed three times with TBS and the protein complexes eluted using 1% SDS in TBS for 10 minutes. Samples were solubilised in 1X SDS buffer and electrophoresed on a 12% Laemmli gel at 30mA for 2hrs with cooling at 4^{o}C. The gel was stained in Coomassie Brilliant Blue overnight, destained and then rinsed thoroughly in MilliQ water.

2.11.2.1 Densitometric scanning of Laemmli gels

The gels were scanned using a Hoefer GS300 transmittance/ reflectance scanning densitometer. Areas under the peaks were calculated using the accompanying software, compared to standard GST-10kDa and 6His-*Pf*J curves and the ratio of bound protein (μg) to parasite protein (μg) determined. A curve was plotted against the amount of 4.1R domain to determine whether the interaction was saturable. To estimate the dissociation constant (Kd) of the interaction, 1/ bound (bound: μg 10kDa bound/ μg *Pf*J) was plotted against 1/ [10kDa]. The Kd value was obtained from the negative inverse of the x-intercept.