Chapter 2

Materials and methods

2.1 Production of recombinant membrane protein ectodomains

The full-length ectodomains of membrane proteins were produced in soluble, recombinant form using an established system based on transient transfection of the HEK293E human cell line (Durocher *et al.,* 2002). The cells, adapted for suspension culture, were maintained in Freestyle media (*Invitrogen*) supplemented with 1% fetal bovine serum, 50 µg/ml of geneticin (*Sigma*), 100 units/ml penicillin (*Invitrogen*) and 100 µg/ml streptomycin (*Invitrogen*), incubated at 37°C, 5% CO_2 and 70% humidity with orbital shaking at 120 r. p. m.

2.1.1 Design and construction of expression plasmids

All expression plasmids used for the production of membrane protein ectodomains are listed in Table 3. The sequences encoding the full-length ectodomains were codon optimised for expression in mammalian cells, using the *GeneART* gene synthesis service (*Invitrogen).* The optimised sequences were then cloned into pTT3-based expression vectors (Durocher *et al.,* 2002) containing a region coding for the immunoglobulin-like domains 3 and 4 rat Cd4 (Brown and Barclay, 1994) together with either

i. A 17-amino acid peptide substrate for the *Escherichia coli* biotin ligase BirA (Brown *et al.,* 1998; Bushell *et al.,* 2008) or
ii. The pentamerisation domain of the rat cartilaginous oligomeric matrix protein (COMP) (Tomschy *et al.*, 1996) with an ampicillin resistance protein (TEM) β-lactamase (Bushell *et al*., 2008) or
iii. A hexa-His tag as represented in Figure 5.

Endogenous signal peptides were included for erythrocyte membrane proteins, however those of *Plasmodium* proteins were replaced by the leader sequence of the mouse variable κ light chain 7-

33 (Crosnier *et al.*, 2010) during gene synthesis. The potential N-linked glycosylation sites (N-X-S/T) of *Plasmodium* proteins were also systematically removed by substituting alanine for serine/threonine at these sites.

Flanking unique NotI (5') and AscI (3') restriction endonuclease recognition sites were introduced to all sequences encoding the membrane protein ectodomains during gene assembly and were used for cloning into the expression vectors. Briefly, after digestion with NotI (*New England BioLabs, NEB*) and AscI (*NEB*) overnight at 37°C both the vectors and inserts were resolved by agarose gel electrophoresis (Section 2.1.1.2) and purified using the QIAquick Gel Extraction kit (*QIAGEN*) as per manufacturer's instructions. Ligations were performed overnight at 16°C with T4 DNA Ligase (*Roche*) using 20 ng of vector and 50 ng of insert DNA per reaction in ligation buffer (50 mM Tris-HCl, 10 mM MgCl*2*, 1 mM ATP, 10 mM dithiothreitol, pH 7.5). Chemically-competent *E.coli* TOP10 cells (*Sigma*) were transformed with the ligation products and positive clones were selected on LB-agar plates containing 100 µg/ml of ampicillin. The presence of an insert of the correct size was confirmed by colony PCR using vector-specific primers flanking the insert site, A and B (Table 2) as described below (Section 2.1.1.1). Plasmids were then purified using the QIAprep Miniprep Spin kit (*QIAGEN*) according to the manufacturer's instructions, tested again for the correct insert via NotI and AscI digestion and sequenced with primers A and B. Sequence-verified expression plasmids were used to transform TOP10 cells. Single positive clones were then inoculated into 100 ml cultures of LB broth supplemented with 100 µg/ml of ampicillin and grown overnight at 37°C with shaking at 200 r. p. m. Plasmids were purified from these cultures using the PureLink^{TM} HiPure Plasmid Maxiprep kit (*Invitrogen*). The purified plasmid DNA was assessed for quantity and quality by measuring the absorbances at 260 nm (A_{260}) and 280 nm (A_{280}) using a spectrophotometer. In terms of

purity, A_{260}/A_{280} ratios between 1.8-1.95 were considered to be acceptable. All plasmid DNA preparations were diluted to 1 mg/ml in buffer TE for use in transfections.

To express fragments of ectodomains, appropriate segments of coding sequence were PCR-amplified from the expression constructs of the full-length ectodomains, using flanking primers (Table 2) containing NotI (forward primer) and AscI (reverse primer) restriction sites, and cloned into expression vectors as described before.

2.1.1.1 PCR

All PCR reactions were performed with the KOD Hot Start DNA polymerase (*Novagen*) in the thermophilic buffer provided by the manufacturer supplemented with 1 mM of $MgSO_4$ and dNTPs (0.2 mM each). 1 U of the polymerase and 0.3 µM of each primer were used per reaction. When the template was a purified plasmid (at 10 ng per reaction), the cycling parameters used were:

Step	Temperature (°C)	Time (min)
1	94	4
2	94	1
3	58	0.5
4	72	0.33/kbp
5	Go to step 2, repeat for 25 cycles	
6	72	7

When colony PCR was performed for testing bacterial transformants, the Step 1 above was extended to 15 min, but the other cycling parameters were kept the same.

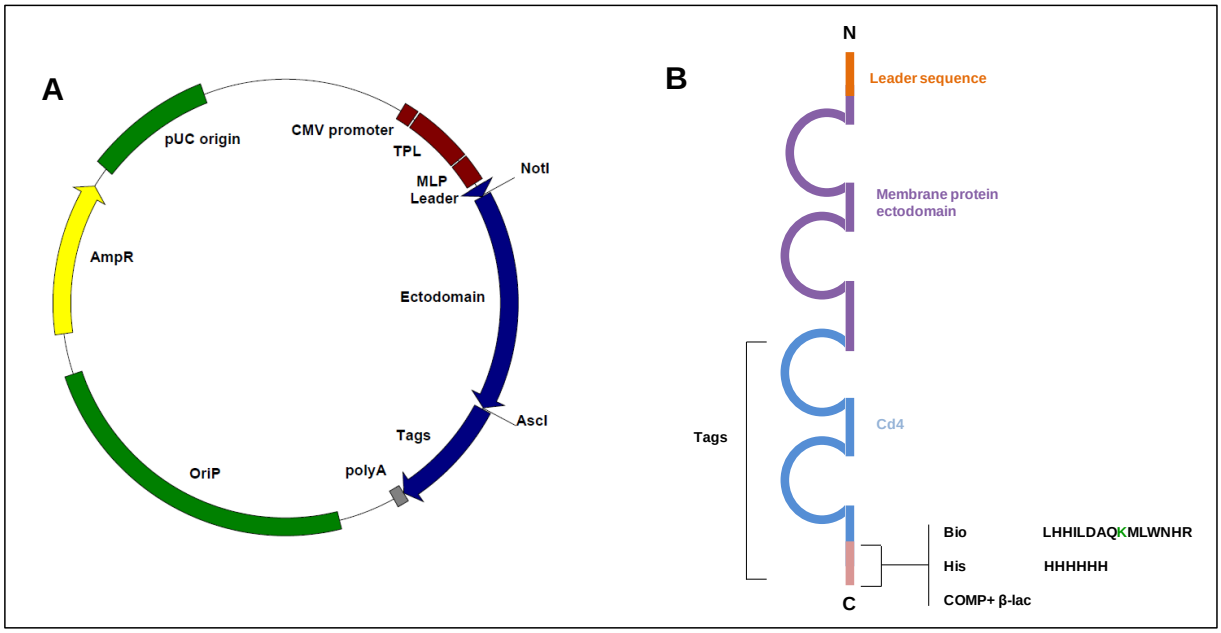

Figure 5. Design of recombinant membrane protein ectodomains. A) Genetic map of expression plasmids used for the production of membrane protein ectodomains (schematic diagram drawn-to-scale). CMV promoter – cytomegalovirus promoter, TPL –tripartite leader sequence, MLP – adenovirus major late promoter enhancer, Leader- leader sequence of the mouse variable κ light chain (included only in expression plasmids of *Plasmodium* proteins), NotI – NotI restriction enzyme recognition site, Ectodomain – sequence encoding the membrane protein extracellular domain, AscI –AscI restriction enzyme recognition site, Tags – sequences coding for domains 3+4 of rat Cd4 and one of the following i) a biotinylatable peptide tag, ii) pentamerisation domain of the rat COMP protein and ampicillin resistance protein β-lactamase or iii) hexa-histidine tag, polyA – SV40 polyadenylation sequence, OriP – Epstein-Barr virus origin of replication, AmpR – β-lactamase gene, pUC origin – bacterial origin of replication. **B)** A simplified schematic of a recombinant *Plasmodium* membrane protein ectodomain with the N-terminal leader sequence derived from a mouse antibody and C-terminal fusion tags. The sequences are given only for the short peptide tags. Cd4: domains 3 and 4 of rat Cd4. Bio: peptide substrate for the biotin ligase BirA. The biotinylatable lysine residue is indicated in green. His: hexa-His. COMP+ β-lac: the pentamerisation domain of the rat COMP protein and the ampicillin resistance protein β-lactamase.

2.1.1.2 Agarose gel electrophoresis

DNA samples diluted in loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF) were loaded onto 1% agarose, TAE-buffered (40 mM tris, 20 mM acetic acid, 1 mM EDTA) gels containing 0.1 µg/ml ethidium bromide. After electrophoresis at a constant voltage of 120V, DNA was visualised using a UV transilluminator (*BIORAD*).

2.1.2 Transient transfection of expression plasmids

Expression plasmids for recombinant membrane protein ectodomains were transiently transfected into HEK293E primarily using the cationic reagent polyethylenimine (PEI) (Tom *et al.,* 2008a). Cells were split into 50 ml of fresh media at a density of $2.5x10^5$ cells/ml and allowed to recover for 24 hours prior to transfection. Each culture was then inoculated with the transfection mix: 25 µl of the expression plasmid (at 1 mg/ml) and 50 µl of linear PEI (at 1 mg/ml) in 2 ml of non-supplemented Freestyle media that had been incubated together for 10 mins at room temperature. Six days after transfection cells and cell debris were removed by centrifugation at 3220*g* for 5 min, supernatants were then filtered (0.2-µm filter) and stored at 4°C until use. When producing biotinylated proteins, the culture media was supplemented with 100 µM D-biotin and 2.5 µl of a plasmid (at 1 mg/ml) coding for a secreted form of BirA (Bushell *et al.*, 2008) was included in the transfection mix. After harvesting, filtered culture supernatants containing biotinylated proteins were dialysed against 5 L of HBS (0.14 M NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES) over 2 days (7 changes of buffer) in Snakeskin dialysis tubing (10 kDa MWCO, *Thermo Scientific*) to remove excess D-biotin.

2.2 Qualitative and quantitative assessment of recombinant membrane protein ectodomains

2.2.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analysed by SDS-PAGE using Novex NuPage 4-12% Bis-Tris pre-cast gels (*Invitrogen*), as per manufacturer's instructions. Electrophoresis was performed at a constant voltage of 200 V for about 1 hour and proteins were visualized using either the SilverXpress silver staining kit (*Invitrogen*) or a Coomassie brilliant blue (G250) staining solution (*Thermo Scientific*) according to the manufacturers' protocols. The Pierce glycoprotein staining kit (*Thermo Scientific*) was used for specifically detecting glycosylated proteins

2.2.2 Western blotting

Western blotting was used to confirm the size of biotinylated proteins. After separation by SDS-PAGE, proteins were transferred to PVDF membranes using a XCell II blotting module (*Novex*), at a constant voltage of 30 V for 1 hour at room temperature in NuPage transfer buffer (25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA, 0.05 mM Chlorobutanol) (*Invitrogen*) supplemented with 10% methanol. The blots were incubated overnight in HBS and 2% BSA to block non-specific binding sites. They were then probed with horseradish peroxidase (HRP)-conjugated Extravidin (1:1000 dilution; *Sigma*), which was detected by chemiluminescence with Hyperfilm (*GE Healthcare*) exposure. Extravidin is a neutral, deglycosylated form of Avidin with higher specificity.

2.2.3 Enzyme-linked-immunosorbent assays (ELISAs) of biotinylated membrane protein ectodomains

ELISAs were performed as previously described in Bushell *et al.* (2008). Biotinylated proteins were immobilised on streptavidin-coated 96-well plates (*Nunc*) for 1 h before incubation with the primary antibody (diluted to 10 µg/ml in HBS and 1% BSA (HBS-BSA), unless stated otherwise) for another hour. The plates were then washed in HBS and 0.1% Tween-20 (HBST) before incubation with a suitable secondary antibody conjugated to alkaline phosphatase (*Sigma*) for 30-45 mins. Plates were then washed three times in HBST and once in HBS before the addition of *p*-nitrophenyl phosphate (Substrate 104; *Sigma*) at 1 mg/ml (100 µl/well). Absorbance was measured at 405 nm on a PHERAstar plus *(BMG Labtech)* plate reader after 10-15 mins. All the steps in the procedure were carried out at room temperature. In addition to quantifying biotinylated proteins, ELISAs were used for testing the conformational state of ligands by means of specific antibodies that recognise non-linear, heat-labile epitopes. For the latter analysis, proteins were heat-treated for 10 min at 80°C and then incubated on ice for a further 10 min, prior to use in ELISAs with untreated controls.

2.2.4 Normalisation of β-lactamase tagged membrane protein ectodomains

Beta-lactamase tagged pentameric proteins were normalised as described in Bushell *et al.* (2008) by monitoring their enzymatic activity in a nitrocefin (*Calbiochem*) turnover assay. 20 µl of 2-fold serial dilutions of harvested culture supernatants were incubated with 60 µl of nitrocefin (at 125 µg/ml) for a period of 10 mins at room temperature, during which nitrocefin turnover was quantified by monitoring the absorbance at 485 nm on a PHERAstar plus *(BMG Labtech)* plate reader. Normalisation of proteins was achieved either by concentrating with 20 kDa MWCO spin concentrators or diluting as required.

2.2.5 Purification of His-tagged membrane protein ectodomains

His-tagged proteins were purified from harvested culture supernatants on nickel-charged sepharose columns (HisTrap HP 1 ml; *GE Healthcare*) using the ÄKTAxpress purification system (*GE Healthcare*). In each instance, the column was pre-equilibrated with binding buffer (20 mM sodium phosphate, 40 mM imidazole, 0.5 M NaCl, pH 7.4) at a flow rate of 1 ml/min. The harvested supernatant (> 150 ml) was supplemented with imidazole (10 mM) and NaCl (100 mM), then passed over the column at 1 ml/min. When loading was complete, the column was washed with 15 column volumes (15 CV) of binding buffer to remove non-specific adherents and with 10 CV of elution buffer (20 mM sodium phosphate, 0.4 M imidazole, 0.5 M NaCl, pH 7.4) to recover specifically-bound protein. The eluant was monitored at 280 nm in real-time and collected in 0.5 ml fractions. The two/three fractions with the highest concentration of purified protein (as estimated by measuring the absorbance at 280 nm) were analysed by SDS-PAGE and pooled for downstream applications.

His-tagged proteins recovered from nickel column purification were further purified by gel filtration prior to use in surface plasmon resonance experiments. This step was necessary for removing protein aggregates. The gel filtration column (Superdex Tricorn 200 10/600 GL, *GE Healthcare*) connected to ÄKTAxpress was pre-equilibrated with 2 CV of the running buffer, HBS-EP (10 mM HEPES, 150 mM NaCl, 30 mM EDTA, 0.05% polyoxyethylenesorbitan 20, pH 7.4) before injecting each protein sample (< 1.5 % of column volume). This was followed by further washing with the running buffer at a flow rate of 1 ml/min. 1 ml fractions were collected once 22 ml of running buffer (equivalent to the void volume of the column) had passed through the column. Absorbance at 280 nm and *in silico* predicted extension coefficients were used to estimate the concentrations of peak fractions. The actual sizes of proteins and their

conformational states (i.e. monomer, dimer or oligomer) were deduced from the elution volumes of the peak fractions, by comparison against a standard curve generated using well-defined protein standards from the low molecular weight and high molecular weight gel filtration calibration kits (*GE Healthcare*).

2.3 *In vitro* biotinylation of native Glycophorin A

Native Glycophorin A purified from human erythrocytes (*Sigma*) was re-suspended in HBS and biotinylated *in vitro* using a 20-fold molar excess of EZ-link sulfo-NHS-biotin (*Thermo Scientific*) for 30 mins at room temperature. The biotinylated protein preparations were then dialysed against 5 L HBS overnight (3 changes of buffer) in Slide-A-Lyzer dialysis cassettes (3.5 kDa MWCO, *Thermo Scientific*) to remove free, unconjugated biotin. The biotinylation of Glycophorin A was confirmed by ELISA on streptavidin-coated plates as described in section 2.2.3 for recombinant membrane protein ectodomains.

2.4 Expression and analysis of erythrocyte multi-pass membrane proteins

Erythrocyte multi-pass membrane proteins were transiently expressed with their endogenous signal sequences and C-terminal Myc/DDK tags on the surface of HEK293E cells using TrueORF plasmids commercially available from *OriGene* (Figure 6). To obtain sufficient quantities of DNA for transfection, the TrueORF expression plasmids were propagated in *E. coli* TOP10 cells (*Sigma*) and purified using the PureLink^{TM} HiPure Plasmid Maxiprep kit (*Invitrogen*). All TrueORF clones used were verified by DNA sequencing.

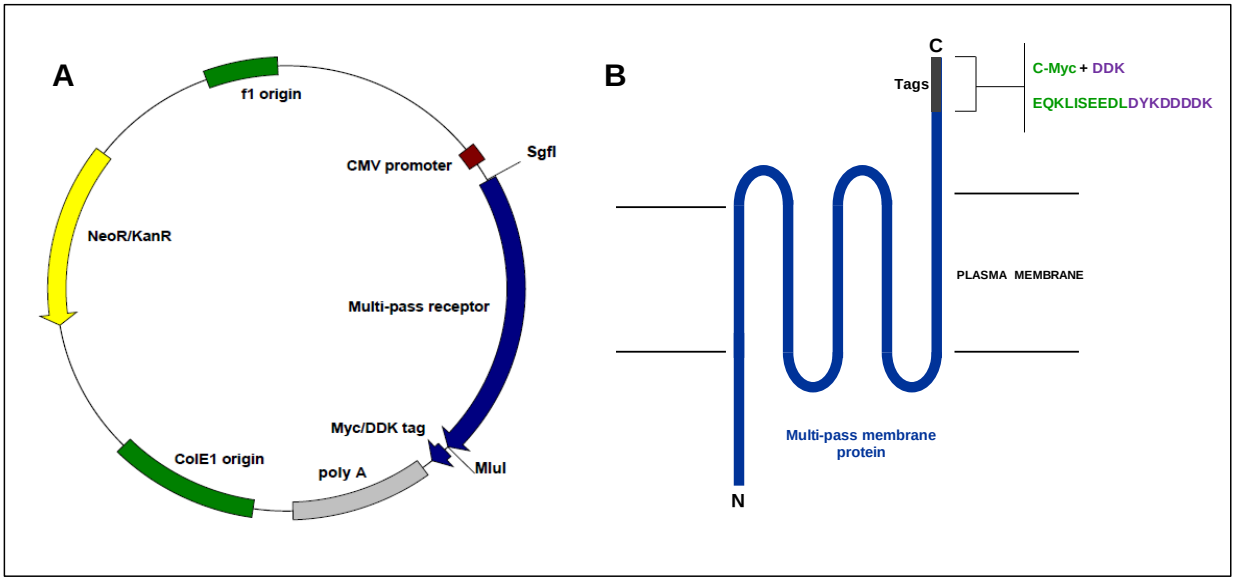

Figure 6. Design of recombinant multi-pass membrane proteins. A) Genetic map of TrueORF plasmids used for the transient expression of erythrocyte multi-pass receptors on the surface of HEK293E cells (schematic diagram drawn-to-scale). CMV promoter – cytomegalovirus promoter, SgfI – SgfI restriction enzyme recognition site, Multi-pass receptor – sequence encoding the multi-pass receptor, MluI –MluI restriction enzyme recognition site, Myc/DDK tag –, polyA – SV40 polyadenylation sequence, ColE1 origin- bacterial origin of replication, NeoR/KanR –neomycin phosphotransferase gene, f1 origin – phage origin of replication. The vector sequence is available at the *OriGene* website http://www.origene.com/destination_vector/PS100001.aspx. **B)** A simplified schematic of a recombinant multi-pass receptor. The peptide sequences of the C-terminal Myc and DDK tags are indicated in green and purple respectively.

2.4.1 Transient transfection of TrueORF plasmids

293Fectin (*Invitrogen*) (Tom *et al.*, 2008b) was primarily used as the reagent for transfecting HEK293E with these plasmids. Each transfection was performed with $2.5x10^7$ cells in 25 ml of media (passaged at half the density a day before), 25 µl of the expression plasmid (at 1 mg/ml), 50 µl of 293Fectin and 2 ml of the OPTI-MEM I media (*Invitrogen*). 293Fectin and DNA were each pre-incubated with 1 ml of OPTI-MEM I for about 5 mins at room temperature prior to being mixed together and then incubated for a further 20 mins at room temperature before addition to cells. The cells were harvested 48 hours after transfection by gentle centrifugation at 199 *g* for 4 mins.

2.4.2 Monitoring expression of multi-pass membrane proteins by flow cytometry

Expression of the recombinant multi-pass membrane proteins was investigated by staining non-permeabilised HEK293E cells, with an appropriate primary antibody (at 0.5 µg/million cells) for 1 h at room temperature with shaking, followed by three washes in HBS-BSA and incubation with a FITC-conjugated secondary antibody for a further 30 mins at room temperature. After being washed thrice in HBS-BSA to remove non-bound antibody, cells were monitored on a BD LSRII cytometer (*BD Biosciences*) using the BD FACS Diva software. Forward scatter (FSC) and side scatter (SSC) voltages of 275 V and 260 V respectively and a threshold of 26,100 on FSC were applied to select the cell population. FITC was excited by a blue laser and detected with a 530/30 filter. The results were analysed using Flow Jo v7.5.3 software (*Tree Star, Inc*).

2.4.3 Confirming expression of multi-pass membrane proteins by confocal microscopy

Expression of the recombinant multi-pass membrane proteins at the surface of HEK293E cells was also confirmed by confocal microscopy. Briefly, cells stained with the appropriate antibodies as described before (section 2.4.2), were counter stained with Slow-Fade Gold-DAPI (*Invitrogen*) on a poly-L-lysine coated microscope slide for a minimum of 10 min at room temperature. Images were captured on a Leica TCS SP5/DM6000 confocal microscope.

2.5 Identifying and characterising low-affinity protein-protein interactions

The highly transient nature ($t_{1/2}$ < 0.1 sec) of low-affinity protein-protein interactions is a major impediment to their identification and characterisation. In the first two methods described below, multimeric reagents were used to increase the avidity of the interactions, thereby prolonging them and enabling their detection. The third method allows molecular interactions to be monitored as they happen in real-time.

2.5.1 Cell-based binding assays with 'prey' protein arrays immobilised on beads

The protocol from Brown (2002) was adapted for detecting binding of recombinant *Plasmodium* membrane protein ectodomains (the 'preys') to receptors on the surface of erythrocytes and HEK293E cells. The mono-biotinylated *Plasmodium* proteins were multimerised by immobilisation on streptavidin-coated Nile red fluorescent 0.4-0.6 µm microbeads (*Spherotech Inc*) by incubation for 1 h at 4°C, followed by sonication for 20 min at a frequency of 35-45 kHz to disrupt aggregates. The multimeric arrays were then presented to cells that had been aliquoted on flat-bottomed 96-well microtitre plates at a density of approximately 3 x 10^5 cells/well (the ratio of cells: beads used was 1:200). After incubation for 1 h at 4°C with the protein arrays, the

cells were washed twice in HBS-BSA and analysed by flow cytometry. The data was acquired on a BD LSRII cytometer (*BD Biosciences*) using the BD FACS Diva software. Nile Red was excited by a blue laser and detected with a 575/26 filter. FSC and SSC voltages of 430 V and SSC 300 V respectively and a threshold of 26,100 on FSC were applied when analysing erythrocytes. HEK293E cells were detected with FSC and SSC voltages of 275 V and 260 V respectively and a FSC threshold of 26,100. The flow cytometry results were analysed using Flow Jo v7.5.3 software (*Tree Star, Inc*). Specificity of detected interactions was confirmed by pre-treatment of cells with the enzymes trypsin, chymotrypsin and neuraminidase and by pre-incubation with monoclonal antibodies.

2.5.1.1 Pre-treatment of cells

All pre-treatments of HEK293E cells and erythrocytes were carried out in non-supplemented Freestyle (*Invitrogen*) and RPMI 1640 (*Invitrogen*) respectively.

A. Trypsin/chymotrypsin treatment of cells

5 x 10^7 cells were incubated with 50 µl of 0.25, 0.5 or 1 mg/ml of tosyl-sulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin from bovine pancreas (*Sigma*) or with 50 µl of 0.25, 0.5 or 1 mg/ml of tosyl lysyl chloromethyl ketone (TLCK)-treated chymotrypsin from bovine pancreas (*Sigma*), for 1 h at 37°C with periodic shaking. The cells were then washed once and treated with 0.5 mg/ml of Soybean trypsin-chymotrypsin inhibitor (*Sigma*) for 10 min on a rotating wheel at room temperature. The cells were finally washed twice before use in binding assays.

B. Neuraminidase treatment

5 x 10^7 cells were incubated with 50 µl of 100 mU/ml *of Vibrio cholera* neuraminidase (*Sigma*) for 1 h at 37°C with periodic shaking, after which they were washed twice prior to binding assays.

C. Pre-incubation with monoclonal antibodies

5 x 10^7 cells were incubated with 25 µg of the monoclonal antibodies for 1 h at room temperature with shaking. The cells were then washed twice before performing binding assays.

2.5.2 Avidity-based extracellular interactions screen (AVEXIS)

The protocol was adapted from Bushell *et al.* (2008). Biotinylated proteins (the baits) were immobilised on streptavidin-coated 96-well plates (*NUNC*) at concentrations sufficient for complete saturation of the available binding surface/well (as determined by ELISA). The plates were then washed twice in HBST and blocked with HBS-BSA (100 µl per well) for 30 mins at room temperature before addition of normalised β-lactamase tagged pentameric proteins (the preys) and incubation for 2 h. The plates were then washed three times in HBST and once in HBS. Nitrocefin (125 µg/ml; 60 µl per well) was added and developed for the stated lengths of time, after which absorbance was measured at 485 nm on a PHERAstar plus *(BMG Labtech)* plate reader. All steps were performed at room temperature.

2.5.3 Surface plasmon resonance (SPR)

Surface plasmon resonance was used to determine kinetic parameters for protein-protein interactions. All SPR experiments were performed on a Biacore T100 instrument using HBS-EP as the running buffer at 37°C.

Non-biotinylated bait proteins were immobilised on CM5 sensor chips (*GE Healthcare*) via amine groups using an amine coupling kit (*GE Healthcare*). In order to determine suitable coupling conditions, electrostatic pre-concentration of the baits on the sensor surfaces was tested at different pHs by injecting the baits (at 50 µg/ml) in 10 mM sodium acetate (pH 6.0-4.0) at a flow rate of 10 µl/min. Each 2 min pulse of ligand was followed by a 1 min injection of 1 M ethanolamine-HCl (pH 8.5) to remove electrostatically-bound ligand. When immobilising the baits covalently, the sensor surfaces were first activated with a 7 min injection of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 0.05 M N-hydroxysuccinimide (NHS) at a flow rate of 10 µl/min prior to injecting the baits.

When biotinylated proteins were used as baits they were directly immobilised on streptavidin-coated sensor chips (Series S Sensor Chip SA; *GE Healthcare*). Prior to bait immobilisation, the sensor surfaces were activated with three 1 min injections of 1 M NaCl and 50 mM NaOH at a flow rate of 30 µl/min. Biotinylated rat Cd4, the negative control bait, was immobilised in the reference flow cell (flow cell 1) of each chip and each 'query' bait protein was immobilised at a molar equivalent amount to Cd4 in a different flow cell. Flow rates of 30 µl/min and 10 µl/min were used respectively for immobilising recombinant membrane protein ectodomains and native Glycophorin A. To monitor protein-protein interactions, increasing concentrations of purified analyte proteins were injected across the immobilised baits on sensor surfaces at the indicated flow rates. At the end of each injection cycle the surface was 'regenerated' with a pulse of 5 M NaCl. Duplicate injections of the same analyte concentration were performed in each experiment and were superimposable indicating no loss of ligand activity after surface regeneration. Both kinetic and equilibrium binding parameters were derived from the data using the Biacore T100

evaluation software (*GE Healthcare*). Each SPR experiment was performed at least twice using independent preparations of both ligand and analyte proteins.

2.6 Studying protein-glycan interactions

Two ELISA-based assays were used for studying interactions between glycan-binding proteins and sugar moieties.

2.6.1 Lectin binding assay

Biotinylated lectins (*Vector Labs*) were immobilised on streptavidin-coated 96-well plates (*Nunc*) at 10 µg/ml (100 µl/well) for 1 h. The plates were then washed twice in HBST and blocked with HBS-BSA (100 µl per well) for 30 mins. The immobilised lectins were then incubated either with putatively glycosylated β-lactamase-tagged pentamers of recombinant membrane protein ectodomains (100 µl/well) or with purified native Glycophorin (0.02 mg/ml, 100 µl/well) for 2 h. The plates were then washed three times in HBST and once in HBS. The pentameric 'preys' were then detected with nitrocefin as described for the AVEXIS assay in section 2.5.2. To detect native Glycophorin A, the plates were incubated with an anti-Glycophorin A mouse monoclonal, BRIC 256 (diluted to 10 µg/ml in HBS-BSA, 100 µl/well) (*Abcam*) for 1 h and an alkaline-phosphatase conjugated anti-mouse secondary (diluted 1:5000 in HBS-BSA, 100 µl/well) (*Sigma*) for 30 mins before the addition of the alkaline phosphatase substrate, *p*-nitrophenyl phosphate (Substrate 104; *Sigma*) at 1 mg/ml (100 µl/well) and measuring the absorbance at 405 nm on a PHERAstar plus *(BMG Labtech)* plate reader. All steps were performed at room temperature.

2.6.2 High-throughput screen for identifying glycan binding specificities of *P. falciparum* membrane proteins

Mono-biotinylated synthetic carbohydrate probes (*GlycoTech*) were immobilised at 50 µg/ml (100 µl/well) or at the stated concentrations on streptavidin-coated 96-well plates (*Nunc*) for 1 h at room temperature. The plates were then washed twice in HBST and blocked with HBS-BSA (100 µl per well) for 30 mins before addition of normalised β-lactamase tagged pentameric proteins (the preys) and incubation for 2 h at room temperature. The plates were then washed three times in HBST and once in HBS before detection of the prey proteins using nitrocefin as described before in section 2.5.2.

2.7 TABLES

Table 2. Primers used in PCR reactions. The NotI and AscI restriction sites are indicated in purple and red respectively.

Primer name	Sequence (5'⟶ 3')
A	CCACTTTGCCTTTCTCTCCA
B	ATGTCCTTCCGAGTGAGAGA
EBA175 RII-F	GTAGAAAAAAGCGGCCGCCATCAACAACGGCCGGAACACCGCCA
***Pf*EBA175 RII_R**	GTAGAAAAAAGGCGCGCCGACGGCTTCCTGGCTGGTCTGCTCG
***Pr*EBA175RII_R**	GTAGAAAAAAGGCGCGCCCTTCTGTTCCTCGGTGTCGGCCTCG

Table 3. Expression plasmids used for the work described in this thesis.

Species	Protein name (accession number)	Fusion tags	Source of expression plasmid
Rat	Domains 3 and 4 of Cd4 (Cd4 tag) (NP_036837.1)	Biotin	Gavin Wright
		COMP+ β-lactamase	
	Full-length ectodomains of Cd200 and Cd200R	Biotin	
		Transmembrane domain of Cd200R + EGFP	
	α-2,6-sialyltransferase 1 (NP_001106815.1)		Cecile Crosnier
***P. falciparum* (3D7)**	Full-length ectodomains of merozoite surface proteins (Table 1)	Cd4+ Biotin	*GeneART*
		Cd4+ COMP+ β-lactamase	Cecile Crosnier
	Full-length ectodomain of RH5 (PFD1145c)	Cd4+His	
	Full-length ectodomain of EBA175 (MAL7P1.176)		Madushi Wanaguru
	RII of the ectodomain of EBA175 (MAL7P1.176)	Cd4+ Biotin	
		Cd4+ COMP+ β-lactamase	
		Cd4+His	
P. reichenowi	Full-length ectodomain of EBA175	Cd4+ Biotin	*GeneART*
	RII of the ectodomain of EBA175	Cd4+ Biotin	Madushi Wanaguru

		Cd4+ COMP+ β-lactamase	
		Cd4+His	
	Full-length ectodomain of RH5	Cd4+Biotin	*GeneART*
		Cd4+ COMP+ β-lactamase	Madushi Wanaguru
		Cd4+His	
P. billcollinsi	RII of the ectodomain of EBA175	Cd4+ Biotin	*GeneART*
		Cd4+ COMP+ β-lactamase	Madushi Wanaguru
		Cd4+His	
Human	Full-length ectodomains of Glycophorin A (NP_002090.4) and Glycophorin B (NP_002091.3)	Cd4+ COMP+ β-lactamase	*GeneART*
		Cd4+ Biotin	Cecile Crosnier
		Cd4+His	
	Full-length ectodomain of Basigin (NP_940991.1)	Cd4+ COMP+ β-lactamase	
		Cd4+ Biotin	
		Cd4+His	Josefin Bartholdson
	Domain 1 of Basigin (NP_940991.1)	Cd4+ Biotin	
	Domain 2 of Basigin (NP_940991.1)		
	Site-directed mutants of Basigin (Table 6)	Cd4+ Biotin	*GeneART*
	α-2,3-sialyltransferase 1 (NP_003024.1)		*OriGene* (TrueClones)
	CMP-sialic acid transporter (NP_006407.1)		

	Multi-pass membrane proteins (Table 7)	c-Myc+ DDK	*OriGene* (TrueORF clones)
Chimpanzee	Full-length ectodomain of Basigin	Cd4+ Biotin	*GeneART*
		Cd4+ COMP+ β-lactamase	Madushi Wanaguru
		Cd4+His	
	Site-directed mutants of Basigin (Table 6)	Cd4+ Biotin	*GeneART*
Gorilla	Full-length ectodomain of Basigin	Cd4+ Biotin	*GeneART*
		Cd4+ COMP+ β-lactamase	Madushi Wanaguru
		Cd4+His	

Table 4. Antibodies used for the work described in this thesis.

Antibody	Source/reference
Anti-Glycophorin A mouse monoclonals (BRIC 256 and 11E4B7)	*Abcam*
Anti-human BSG mouse monoclonals (MEM-M6/1, MEM-M6/2 and MEM-M6/6)	
Anti-Duffy goat polyclonal	
FITC-conjugated donkey anti-goat secondary	
Anti-c-Myc mouse monoclonal	

Anti- *P. falciparum* Rh5 rabbit polyclonals	Cecile Crosnier
Anti- *P. falciparum* EBA175 RII mouse monoclonals (R217 and R218)	David Narum (Sim *et al*., 2011)
Alkaline phosphatase conjugated goat anti-rabbit secondary	*Jackson ImmunoResearch Laboratories*
Anti- *P. falciparum* EBA175 RVI mouse monoclonal	Mike Blackman (O'Donnell *et al*., 2006)
Anti- *P. falciparum* Rh5 mouse monoclonals	Sandy Douglas
Anti-Cd4 mouse monoclonal (OX68)	*Serotec*
W6/32 mouse monoclonal	
Alkaline phosphatase conjugated goat anti-mouse secondary	*Sigma*
FITC-conjugated goat anti-mouse secondary	
Anti-CD200R mouse monoclonal (OX102)	(Wright *et al*., 2000)
Anti-human BSG mouse monoclonals (MEM-M6/4, MEM-M6/8, MEM-M6/10 and MEM-M6/11)	Zenon Zenonos (Koch *et a*l., 1999)

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