

Contents

Declaration	ii
Abstract	iii
Publications arising from this research	v
Acknowledgements	viii
List of figures	xiii
List of abbreviations	xvi
PREFACE	1
CHAPTER ONE- INTRODUCTION	3
1.1 Life cycle of <i>P. falciparum</i>	3
1.2 Ultrastructure of the merozoite	5
1.3 Erythrocyte invasion	7
1.4 Major merozoite proteins	10
1.4.1 Merozoite surface protein 1	10
1.4.2 Rhoptry proteins	12
1.4.3 Dense granule proteins	15
1.4.4 Microneme proteins and host-receptor interactions	16
1.4.5 Merozoite proteases and invasion	21
1.5 <i>P. falciparum</i> and the erythrocyte membrane	23
1.5.1 Structure of the erythrocyte membrane	23
1.5.2 Erythrocyte protein 4.1	27
1.5.3 Erythrocyte surface modifications	30
1.5.4 Alterations to the erythrocyte skeleton	35
1.5.5 Transport pathways in <i>Plasmodium</i> -infected erythrocytes	40
1.6 Phage display technology	42
1.6.1 Phage display libraries	43
1.6.2 Applications of phage display to <i>P. falciparum</i>	44
1.7 Research objectives	47
CHAPTER TWO- MATERIALS AND METHODS	49
2.1 Purification of human 4.1R	49
2.1.1 Preparation of erythrocyte membranes	49
2.1.2 Method A: salt-based purification of 4.1R	50
2.1.3 Method B: detergent-based purification of 4.1R	51
2.1.4 Protein quantitation and gel electrophoresis	51
2.1.5 Densitometric scanning of Laemmli gels	52
2.1.6 Immunoblot of purified 4.1R	52
2.2 Concentration and biotinylation of purified 4.1R	53
2.2.1 Streptavidin-peroxidase test	53
2.3 Culturing of asexual <i>P. falciparum</i> parasites	54
2.3.1 Preparation of human erythrocytes for parasite cultures	54
2.3.2 Preparation of parasite cultures from frozen stock	55
2.3.3 Continuous culture method	55
2.3.4 Freezing of parasite cultures	56
2.4 Isolation of <i>P. falciparum</i> RNA	56

2.4.1	Extraction of total RNA	57
2.4.2	Assessment of RNA quality and removal of contaminating DNA	58
2.4.3	Isolation of mRNA from total RNA	58
2.5	Construction of <i>P. falciparum</i> phage display libraries	59
2.5.1	Synthesis and end-modification of parasite cDNA libraries	60
2.5.1.1	First and second strand cDNA synthesis	60
2.5.1.2	End-modification and size fractionation of cDNA inserts	61
2.5.1.3	Electrophoresis of end-modified cDNA	62
2.5.2	Construction of phage display libraries	63
2.5.2.1	Cloning of inserts in T7Select vector arms	63
2.5.2.2	<i>In vitro</i> packaging and plaque assay	64
2.5.2.3	Plate lysate amplification	65
2.6	Biopanning of <i>P. falciparum</i> phage display libraries	66
2.6.1	Identification of <i>P. falciparum</i> cDNA inserts	67
2.6.1.1	Insert size determination	67
2.6.1.2	DNA sequencing and bioinformatic analysis	68
2.7	Cloning of <i>P. falciparum</i> gene sequences	68
2.7.1	Isolation of genomic DNA from asexual parasites	68
2.7.2	Preparation of PCR products for subcloning	69
2.7.3	Preparation of pET15b and pGEX-4T-2 protein expression vectors	70
2.7.4	Ligation of vector and parasite insert DNA	71
2.7.5	Transformation of DH5 α competent cells	71
2.7.6	Plasmid DNA purification and parasite insert verification	72
2.7.7	Sequence verification	72
2.8	Expression of recombinant parasite proteins	73
2.8.1	Transformation of BL21-CodonPlus [®] competent cells	73
2.8.2	Induction of target protein expression	74
2.8.3	Purification of glutathione S-Transferase and histidine-tagged parasite proteins	74
2.8.3.1	Extraction of soluble proteins	74
2.8.3.2	Affinity capture of histidine-tagged proteins	75
2.8.4	Verification of 6His parasite protein expression	76
2.9	Cloning of 4.1R domain sequences	76
2.9.1	Reticulocyte total RNA extraction	76
2.9.2	Reverse transcription PCR	77
2.9.3	Purification and restriction enzyme digestion of PCR products	78
2.9.4	Preparation of pGEX-4T-2 vector and ligation to 4.1R DNA	79
2.10	Expression of 4.1R recombinant domains	79
2.10.1	Transformation of Rosetta [™] 2 (DE3) competent cells	79
2.10.2	Induction of target protein expression	80
2.10.3	Purification of GST-tagged 4.1R domains	80
2.10.4	Verification of GST-4.1R protein expression	81
2.11	Interaction studies between recombinant proteins	81
2.11.1	Blot overlay assays	81
2.11.2	Pull-down assays	82

2.11.2.1	Densitometric scanning of Laemmli gels	83
CHAPTER THREE- RESULTS		84
3.1	Purification of 4.1R	84
3.1.1	Detergent-based purification of 4.1R	85
3.1.2	Salt-based purification of 4.1R	89
3.1.3	Protein biotinylation	91
3.2	Construction of <i>P. falciparum</i> phage display libraries	91
3.2.1	RNA extraction	91
3.2.2	Preparation of parasite cDNA	92
3.2.3	<i>In vitro</i> packaging and plate lysate amplification	93
3.3	Identification of interacting parasite peptides	95
3.3.1	Size determination of cDNA inserts	95
3.3.2	Sequencing and bioinformatic analyses	96
3.3.3	Annotated <i>P. falciparum</i> proteins	99
3.4	Identification of a common filament repeat motif	105
3.5	Cloning of <i>P. falciparum</i> sequences binding to 4.1R	107
3.6	Expression and purification of recombinant parasite proteins	109
3.7	Cloning of 4.1R domain sequences	113
3.8	Expression and purification of GST-4.1R domains	115
3.9	Binding studies between 6His- <i>PfJ</i> and GST-4.1R domains	117
CHAPTER FOUR- DISCUSSION		122
4.1	Utility of <i>P. falciparum</i> phage display libraries	122
4.2	<i>P. falciparum</i> proteins binding to 4.1R	124
4.2.1	Low complexity domains	124
4.2.2	Coiled-coil domains	125
4.2.3	Hypothetical proteins	126
4.2.4	Annotated enzymes	128
4.2.5	Erythrocyte invasion proteins	129
4.3	Conclusion	134
4.3.1	Closing remarks	135
APPENDIX		137
A-1	Reagents and standard laboratory methods	137
A-1.1	Laemmli SDS-PAGE	137
A-1.2	Fairbanks SDS-PAGE	138
A-1.3	Preparation of samples for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)	139
A-1.4	Silver staining of SDS-PAGE gels	139
A-1.5	Protein transfer and immunoblot	139
A-1.6	Incomplete RPMI culture medium	140
A-1.7	Complete RPMI culture medium	141
A-1.8	Microscopic analysis of stained blood smears	141
A-1.9	Phenol-chloroform extraction and ethanol precipitation of nucleic acids	142
A-1.10	DNA sequencing and preparation of acrylamide gels for electrophoresis	142

A-1.11	Genomic DNA extraction from human white blood cells	144
A-1.12	Plasmid DNA purification: alkaline lysis method	145
A-1.13	SOC medium	146
A-2	Sequences and vector maps	147
A-2.1	Two-base anchored primers	147
A-2.2	Spectrin and band 3 primers for size standards	147
A-2.3	T7Select 10-3b vector map and cloning cassette	147
A-2.4	pET15b vector map and cloning cassette	148
A-2.5	pGEX-4T-2 vector map and cloning cassette	150
A-2.6	PCR primers for amplification of 4.1R domain sequences	151
A-2.7	Spectrin/4.1R♠ and 4.1R♣ specific <i>P. falciparum</i> binding peptides and corresponding nucleotide sequences	152
A-2.8	Secondary structure of <i>P. falciparum</i> filament repeat motifs	154
A-2.9	<i>P. falciparum</i> peptide sequences selected for cloning into protein expression vectors	156
A-3	List of suppliers of chemicals and equipment	158
REFERENCES		162

List of figures

Figure 1- Life cycle of <i>Plasmodium falciparum</i>	4
Figure 2- Ultrastructure of the <i>P. falciparum</i> merozoite	6
Figure 3- Diagrammatic representation of erythrocyte invasion by <i>Plasmodium knowlesi</i>	8
Figure 4- Diagram depicting the gene structure of <i>P. falciparum</i> and <i>P. vivax</i> erythrocyte binding proteins	17
Figure 5- Matrix depicting parasite ligand-host receptor invasion pathways in <i>P. falciparum</i>	21
Figure 6- Schematic model of the erythrocyte membrane	24
Figure 7- Schematic representation of the genetic and proteomic elements of 4.1R	28
Figure 8- Principles of phage display technology	45
Figure 9- Band 6, spectrin and actin extraction from erythrocyte membranes	84
Figure 10- Detergent-based extraction of 4.1R from erythrocyte membranes	85
Figure 11- Purification of 4.1R from crude extracts	87
Figure 12- Densitometric scanning of erythrocyte membrane proteins	88
Figure 13- Elution of 4.1R and contaminating proteins from a DEAE ion exchange column	89
Figure 14- Salt-based extraction of 4.1R from erythrocyte membranes	90
Figure 15- Assay to confirm protein biotinylation	91
Figure 16- Analysis of <i>P. falciparum</i> RNA preparations	92
Figure 17- Gel filtration of <i>P. falciparum</i> end-modified cDNA	93
Figure 18- Plaque assay	94
Figure 19- Analysis of parasite cDNA inserts cloned into the T7Select 10-3b vector	95

Figure 20- Sequence analysis of virions biopanned against 4.1R	98
Figure 21- mRNA expression profiles of EBA-175 and EBA-181	99
Figure 22- mRNA expression profiles of annotated <i>P. falciparum</i> enzymes	102
Figure 23- Coiled-coil domains in 4.1R specific <i>P. falciparum</i> myosin-like motifs	107
Figure 24- PCR amplification of parasite 4.1R binding sequences	108
Figure 25- Verification of the presence of <i>P. falciparum</i> inserts	108
Figure 26- Partial DNA sequence of 6His-EBA-181	109
Figure 27- Expression and purification of 6His- <i>PfJ</i>	111
Figure 28- Expression of a GST-tagged 4.1R binding peptide from EBA-175	112
Figure 29- Analysis of human reticulocyte RNA	113
Figure 30- RT-PCR of 4.1R domains	114
Figure 31- Confirmation of 4.1R domain inserts	114
Figure 32- Exon map of 4.1R cDNA	115
Figure 33- Expression of GST-4.1R domains	116
Figure 34- Purification and immuno-detection of GST-4.1R domains	117
Figure 35- Blot overlay of 6His- <i>PfJ</i> with native 4.1R and GST-tagged 4.1R domains	118
Figure 36- Histidine pull-down of GST-4.1R recombinant proteins	119
Figure 37- GST-10kDa/6His- <i>PfJ</i> solution binding assays	121
Figure 38- Proposed model of EBA-181 function during <i>P. falciparum</i> erythrocyte entry	133

List of tables

Table 1- Surface proteins of <i>P. falciparum</i> merozoites	11
Table 2- Rhoptry proteins identified in <i>P. falciparum</i> merozoites	13
Table 3- Characteristics of <i>P. falciparum</i> erythrocyte binding proteins	19
Table 4- Proteins of the junctional complex	26
Table 5- Receptor-ligand interactions between PfEMP1 and human host	33
Table 6- Major <i>P. falciparum</i> proteins that associate with the erythrocyte skeleton	36
Table 7- <i>P. falciparum</i> annotated proteins containing binding sequences specific for 4.1R and spectrin	100
Table 8- Hypothetical <i>P. falciparum</i> proteins binding to 4.1R	104
Table 9- Common 4.1R binding motifs	106
Table 10- Solubility profiles of recombinant <i>P. falciparum</i> proteins	110

List of abbreviations

A	adenine/alanine
ABRA	acidic basic repeat antigen
ACD	acid citrate/dextrose
AMA 1	apical membrane antigen 1
AMV RT	avian myeloblastosis virus reverse transcriptase
APS	ammonium persulphate
Biotin-NHS	D-biotin-N-hydroxysuccinimide ester
BLASTN	basic local alignment search tool nucleotide
BLASTX	basic local alignment search tool protein
bp	base pairs
BSA	bovine serum albumin
C	cytosine
cDNA	complementary deoxyribonucleic acid
Ci	Curie
cm	centimeter
cys	cysteine
°C	degrees Celsius
D	aspartic acid
dATP	deoxyadenosine triphosphate
DBL	Duffy binding like
DBP	Duffy binding protein
DEAE	diethylaminoethyl
DED	death effector domain
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dT	deoxythymidine
DTT	dithiothreitol
E	glutamic acid
EBA	erythrocyte binding antigen
EBP	erythrocyte binding protein
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol bis N, N'-tetraacetic acid
F	phenylalanine
<u>G</u>	glycine/guanine
g	gram

<i>g</i>	relative centrifugal force
GPI	glycosylphosphatidylinositol
GST	glutathione S-Transferase
His	histidine
hrs	hours
I	isoleucine
IgG	immunoglobulin G
IPTG	isopropyl-1-thio- β -D-galactopyranoside
JC	junctional complex
K	lysine
KAHRP	knob-associated histidine-rich protein
Kb	kilobases
kDa	kilodalton
L	leucine
l	liter
LB	Luria Broth
LC	low complexity
M	molar/methionine
mA	milliamps
mg	milligram
ml	milliliter
mm	millimeter
mM	millimolar
MMLV RT	Moloney murine leukemia virus reverse transcriptase
mmol	millimole
mRNA	messenger ribonucleic acid
μ g	microgram
μ l	microliter
μ M	micromolar
MESA	mature parasite-infected erythrocyte surface antigen
MPC	magnetic particle concentrator
MSP 1	merozoite surface protein 1
N	asparagine
ng	nanogram
nm	nanometer
NPP	new permeation pathway
P	proline
PBS	phosphate buffered saline
PCR	polymerase chain reaction

<i>P. falciparum</i>	<i>Plasmodium falciparum</i>
PfEMP1	<i>P. falciparum</i> erythrocyte membrane protein 1
PfRH	<i>P. falciparum</i> rhoptry protein homologue
PfSUB	<i>P. falciparum</i> subtilisin-like proteases
pmol	picomole
PM	plasma membrane
PMSF	phenylmethylsulfonylfluoride
PV	parasitophorous vacuole
Q	glutamine
R	arginine
RAP	rhoptry-associated proteins
RESA	ring infected erythrocyte surface antigen
RhopH	rhoptry high molecular weight proteins
RIMA	ring membrane antigen
RNA	ribonucleic acid
RNases	ribonucleases
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
4.1R	erythrocyte protein 4.1
S	serine
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SERA	serine repeat antigen
T	thymine/threonine
TAE	Tris/acetate/ EDTA
TBE	Tris/borate/EDTA
TBS	Tris buffered saline
TE	Tris-EDTA
TEMED	N,N,N,N-tetramethylethylenediamine
Tris	hydroxymethyl methylamine
U	units
UV	ultraviolet
V	valine/volts
W	watts