PEPTIDES MIMICKING EPITOPES ON MALARIAL ANTIGENS FROM RANDOM PEPTIDE LIBRARIES DISPLAYED ON PHAGE

Submitted by

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Abstract

This study investigates the applications and utility of phage-displayed random peptide libraries in malaria research. Monoclonal antibodies against two asexual stage malaria antigens were used to identify peptide mimics of the antigens for potential use in serology or antimalarial therapy. The ring-infected erythrocyte surface antigen (RESA) is a dense granule protein of *Plasmodium falciparum* which binds to the cytoskeletal structure of the erythrocyte after parasite invasion. It is currently under trial as a vaccine candidate. In an effort to characterize further the antibody responses to this antigen, two independent libraries of random peptides expressed on the surface of filamentous phage were panned on a monoclonal antibody (MAb18/2) against RESA. One library consisted of a potentially constrained 17-mer peptide fused with the gpVIII phage coat protein and the other displayed an unconstrained 15-mer as a fusion with the minor phage coat protein gpIII. Several rounds of biopanning resulted in the enrichment of clones from both libraries that interacted specifically with the MAb18/2 antibody, in protein blotting and ELISA experiments. Nucleotide sequencing of the random oligonucleotide insert revealed a common predominant motif: S/TAVDD. Several other clones had related but degenerate motifs. Importantly, antisera from individuals with prior exposure to malaria recognized these peptides. Immunization of a mouse with phage displaying a mimotope resulted in a serum that recognized the synthetic form of the mimotopes, as well as both recombinant and native forms of RESA. Thus, a monoclonal antibody against a malarial antigen can select common mimotopes from different random peptide libraries and these peptides can be used to reconstitute aspects of the immune response generated by this antigen in the field.

In a similar approach, a monoclonal antibody (MAb4G2) against the vaccine candidate *P. falciparum* apical membrane antigen-1 (PfAMA-1) was used to pan random peptide

libraries in an attempt to isolate mimotopes of PfAMA-1. Two different peptides were isolated that bound specifically to MAb4G2 in ELISA and Western blotting experiments. However, these peptides were found not to be authentic mimotopes of PfAMA-1, as PfAMA-1 could not inhibit the binding of the peptides to MAb4G2 in competition assays. It is envisaged that there are many uses for phage peptide-display technology in malaria research.

Statement of Authorship

Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted whole or in part from a thesis by which I have qualified for or have been awarded another degree or diploma.

No other person's work has been used without due acknowledgment in the main text of the thesis.

This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

31/8/00

(Christopher Adda)

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Contents

List of abbreviations

3D	three dimensional
ABRA	antigen which contains repeats of acidic and basic residues
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AIDS	acquired immune deficiency syndrome
AMA-1	apical membrane antigen 1
AP	alkaline phosphatase
BSA	bovine serum albumin
CMV	cucumber mosaic cucumovirus
cpn60	protein chaperone 60
CSA	chondroitin sulfate A
CSP	circumsporozoite protein
DNA	deoxyribose nucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
dsDNA	double stranded DNA
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ELISA	enzyme-linked immunosorbent assay
ESMS	electrospray ionization mass spectrometry
EPO	erythropoietin
Fab	antibody fragment containing the antigen-binding site
FIRA	falciparum interspersed repeat antigen FC27 S-antigen, the
FITC	fluorescein isothiocyanate
gpIII	gene III protein
gpVIII	gene VIII protein
HBsAg	small hepatitis B virus surface antigen
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase

HSP-70	70kDa heat shock protein
ICAM	intercellular adhesion molecule
IgG and IgM	immunoglobulins G and M
IL	interleukins
IRBC	infected red blood cells
i.p	intraperitoneally
KAHRP	knob-associated histidine-rich protein
LB/Tc	agar plates containing tetracycline
MAb	monoclonal antibody
MESA	mature parasite-infected erythrocyte surface antigen
MHC	major histocompatability complex
MMP	matrix metalloproteinases
mono	monomer
MSP	merozoite surface protein
NCF	nitrocellulose membranes
NMR	nuclear magnetic resonance
OPD	O-phenylenediamine
PAI-1	plasminogen activator inhibitor 1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PECAM-1	platelet/endothelial cell adhesion molecule-1
PEG	polyethylene glycol
Pf11.1	*Plasmodium falciparum* antigen 11.1
Pf332	*Plasmodium falciparum* antigen 332
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein-1
PNG	Papua New Guinea
pNPP	p-nitrophenyl phosphate
poly	polymer
PVDF	polyvinylidene difluoride
PVM	parasitophorous vacuolar membrane
PvMSP1	*Plasmodium vivax* merozoite surface protein-1
RESA	ring infected erythrocyte surface antigen
scFv	single chain variable fragment
SDS	sodium dodecyl sulphate

SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SIP	selectively infective phages
ssDNA	single stranded DNA
TBS	tris buffered saline
TNF	tumour necrosis factor
TPO	thrombopoietin
Tris	tris (hydroxymethly) amino methane
TSP	thrombospondin
URBC	uninfected red blood cells
VCAM	vascular cell adhesion molecule
V_H and V_L	variable heavy and variable light
VR1	tissue-type plasminogen activator

In the labours of scientific endeavour, as in the practices of agriculture, there are those who seek the most promising land and prepare it for seed. Still others sow that ground with hardy stock, and provide the germen with nourishment and protection. And if, by the actions of man and nature the crop bears fruit, there will also be those ready to reap the harvest. From fertile lands there comes enough to feed the entire community. Each and every scientist will set himself one or more of these tasks, and in so doing adds to the collective wealth of the scientific profession.

— Maurizio Toscano.

Chapter 1:

Introduction

1.1 Malaria

Malaria has been a global problem for many centuries, and it continues to be a problem into the 21st Century. The malarial parasite not only infects humans but also other vertebrates of various genera. One third of the global human population is exposed to malaria. Approximately 90% of cases occur in sub-Saharan Africa with the remaining 10% occurring in south and south-east Asia and central and south America (278). The resources and funding required to combat malaria are not available in these countries due to the poor socioeconomic conditions (61). In comparison to the acquired immune deficiency syndrome (AIDS) and cancer, relatively little money is being spent on addressing the problem of malaria as it receives little publicity in the Western countries. Yet, malaria is responsible for up to 3 million deaths and 300-500 million cases each year and this number is growing due to evolutionary and environmental factors such as drug and insecticide resistance and global warming (45, 115, 166). Thus there is an urgent need for an inexpensive means of combating the global malaria problem with vaccination likely to be the most suitable solution.

It was believed for many centuries that malaria was caused by 'bad air', hence the name (45). It has now been over a century since Alphonse Laveran and Sir Ronald Ross, using little more than a microscope, identified the parasite as the cause of malaria and characterized its life cycle (59). As advances in science and technology have occurred over the last century, more information has become available. Yet, no vaccine has been found to rid the world of this life-threatening disease.

1.1.2 Malaria life cycle

Malaria is a protozoan eukaryotic parasite of the genus *Plasmodium*. Of the four species; *P. falciparum, P. malariae, P. ovale* and *P. vivax* (143) which infect humans, *P. falciparum* is the greatest cause of human mortality as its symptoms can include coma and severe anaemia, often resulting in death (271). The life cycle of *P. falciparum* is complex as it involves two hosts: the vector, a female Anopheline mosquito (178) and the human host.

The parasite in the sporozoite stage is released from the salivary glands of the mosquito and injected into the blood stream of the vertebrate host prior to removal of blood by the vector. Within 30 minutes, it travels to the liver where it invades the hepatocytes and develops asexually through the hepatic stage, over the next 7-10 days (156). Upon synchronous hepatocytic rupture, between 10,000 and 30,000 merozoites are released into the circulatory system and within seconds invade erythrocytes to begin the erythrocytic cycle (235). Erythrocytic invasion entails a defined cascade of events: recognition and attachment to the membrane; reorientation of the merozoite; production of a junction between the apical end of the merozoite and erythrocyte via deposition of rhoptry-derived proteins and lipids; invagination of the erythrocyte membrane and evolution of a parasitophorous vacuolar membrane (PVM), a membrane that is initially continuous with that of the erythrocyte; and the resealing of the membrane post-invasion (41, 244). Within the erythrocyte, the parasite undergoes continuous change over 48 hours through the ring, trophozoite and schizont stages within the PVM (92). The schizonts then divide, producing between 4 and 36 new merozoites per red cell (23) that concurrently rupture from the erythrocytes. This effects the destruction of a proportion of the body's erythrocytes and releases parasite debris into the serum, resulting in the symptomatic fevers of the disease (143). The merozoites then proceed to invade other erythrocytes.

During the erythrocytic cycle a small percentage of parasites, responding to unknown signals, differentiate within the erythrocyte into male and female gametocytes (251) and are taken up by the mosquito vector when it takes a bloodmeal. Less than 1% of the parasites consumed in the blood meal survive in the gut of the mosquito to undergo the sporogenic cycle. Due to the drop in temperature within the mosquito's gut and other factors, the gametes are released from the erythrocytes and fertilization occurs in which the gametes form zygotes and rapidly develop into ookinetes. The sexual stage continues in the epithelium of the mosquito's stomach through which the ookinetes penetrate and develop to form oocysts (235). Haploid sporozoites are released from the oocysts and migrate to the salivary glands where the cycle can begin anew. The cycle within the mosquito takes approximately 10-12 days in tropical conditions, however, this varies depending on temperature (162) (Fig. 1.1.1).

1.1.3 The erythrocytic cycle

During the erythrocytic asexual stage of development, malarial parasites are in a homeostatic environment shielded from the immune system by the erythrocyte membrane (104). The erythrocyte membrane consists of a stable but flexible phospholipid bilayer containing various proteins in a fluid environment. Underlying the membrane is a cytoskeletal network of proteins, which provide the erythrocyte with structural stability so that it can withstand the shear forces as it transverses the body at high speeds through narrow capillaries (249). A range of parasite derived proteins have been shown to form structures referred to as knobs on the surface of *P. falciparum*-infected erythrocytes giving them an irregular 'bumpy' morphology. These proteins including the knob-associated histidine-rich protein (KAHRP) (123), erythrocyte membrane protein-1 (PfEMP1), and PfEMP-3 (28, 131), are either inserted through the membrane or anchored just below the membrane in small clusters causing protrusions on the surface of the erythrocyte (69). Due to the formation of knobs, the landscape of the red cell surface is changed and hence, it might be expected that the infected erythrocyte would be recognized as abnormal and be removed by the spleen. However, the parasite has evolved a mechanism of adhering to the

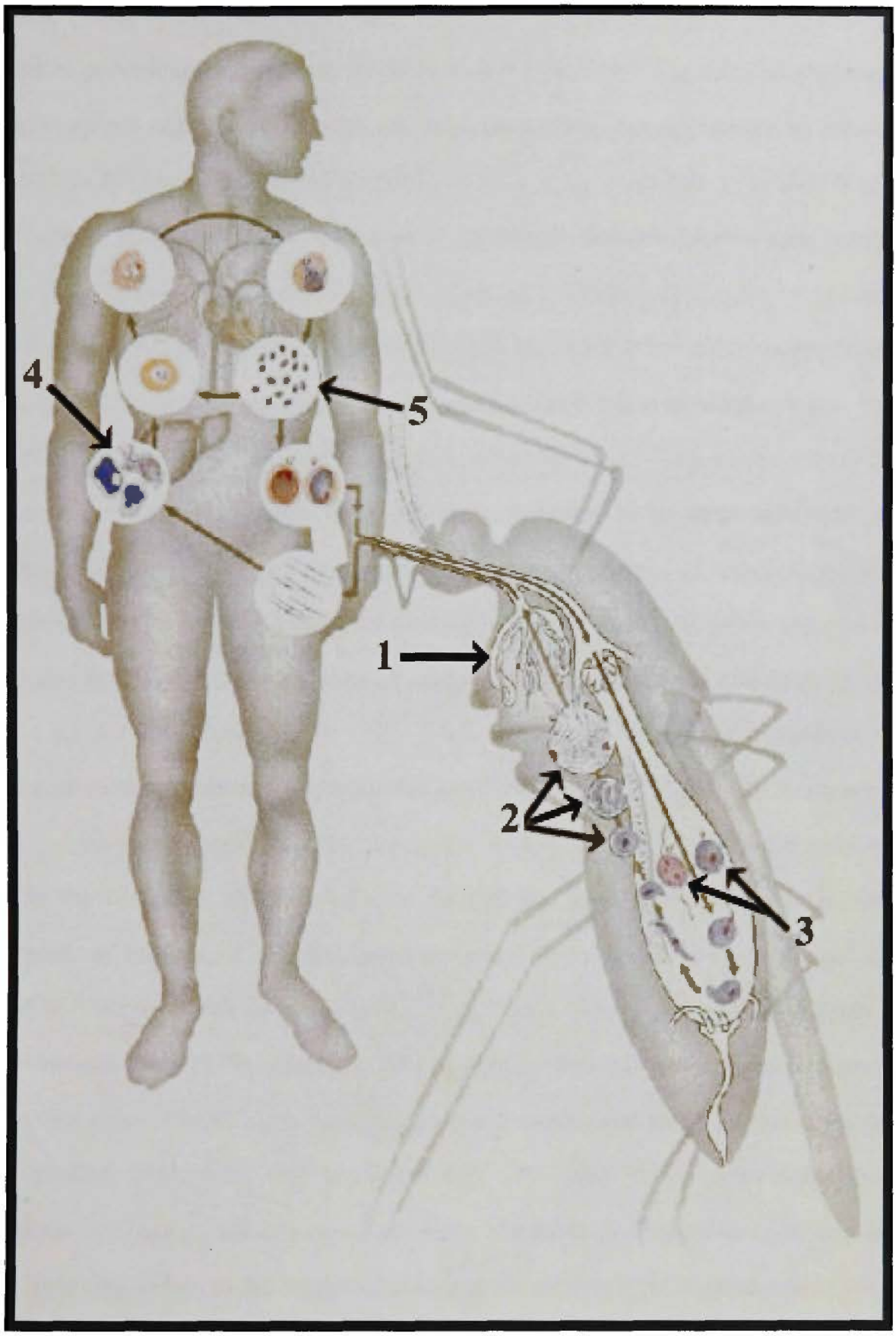


Figure 1.1.1. Life cycle of the malarial parasite. (1) Sporozoites in the salivary glands of the mosquito. (2) Oocysts in the stomach wall. (3) Male and female gametocytes. (4) The hepatic stage. (5) Release of merozoites from the liver which proceed to invade the erythrocytes where both sexual and asexual cycles continue. Adapted from the World Wide Web at http://www.rph.wa.gov.au/labs/haem/malaria/history.html. Presented by Richard Davis and Graham Icke of the Division of Laboratory Medicine at Royal Perth Hospital, Australia.

endothelial lining of the blood capillaries, referred to as cytoadherence, which has been suggested to prevent splenic removal by preventing the passage of the infected erythrocyte through the spleen (222). In some cases, the infected red cells autoagglutinate by adhering to themselves (63) or to uninfected erythrocytes in a process referred to as rosetting. A parasite protein displayed on the surface of *P. falciparum*-infected erythrocytes, referred to as the *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1), has been implicated in mediating this adhesion within rosettes (52, 220). However, other non-parasite derived proteins including immunoglobulins G and M and the blood group A and B antigens have also been suggested to play a role in rosette formation (25, 57, 227). Cytoadherence, when it occurs in the microvasculature of the brain is believed to be associated with the development of an often fatal complication of malaria known as cerebral malaria. Cytoadherence appears to result from the binding of ligands displayed on the infected red cell surface (eg. PfEMP-1), to a variety of receptors on the endothelial cell surfaces (see Section 1.1.6 Immune Evasion) (6, 163). There is evidence to suggest that where the infected erythrocytes predominantly bind the endothelial receptor ICAM, the incidence of cerebral malaria is increased (70, 185). Originally, it was proposed that cytoadherence was caused by the occlusion of the blood flow through the brain by cytoadherent infected erythrocytes, or rosettes of infected and uninfected erythrocytes. This occlusion was proposed to lead to a lack of oxygenation of the brain cells thereby causing death in cerebral malaria cases (179). Although this hypothesis still has some support, there is evidence that other factors such as localized nitric oxide production in the brain and altered cytokine levels, may also contribute (55, 139, 260). People who suffer from thalassaemia and sickle cell anaemia have been shown to be protected from cerebral malaria. This may be due to the decreased ability of the abnormal red cells to rosette (48).

The erythrocyte predominantly contains haemoglobin and lacks the organelles usually present in other mammalian cells. During the erythrocytic cycle, the parasite structurally alters the erythrocyte through the production of various proteins (85) and degrades the haemoglobin producing as a waste product, haemozoin, an insoluble polymer of toxic

haem. The haemoglobin is believed to supply the parasite with most of the amino acids it requires for protein synthesis (196).

1.1.4 Transport pathways

The import of proteins, including haemoglobin, and other macromolecules from the erythrocyte cytosol to the food vacuole within the parasite, occurs via a cytostome, which results from the invagination and budding inwards of the PVM and the parasite plasma membrane (114). However, *Plasmodium* cannot live on haemoglobin alone. In order to survive within the erythrocyte, the parasite also needs to import nutrients from the exoerythrocytic environment. Such nutrients include glucose, ions and other molecules. Increased rates in the uptake of molecules such as glucose, amino acids and nucleosides have been demonstrated in parasitized erythrocytes (91). There is also evidence for the uptake of lipids from the serum (118). Various models have been presented but the mechanisms of transport within the parasitized red blood cell have not been described in great detail (91, 92, 114). Membranous finger-like extensions of the PVM, referred to as the tubo-vesicular network, have been suggested to assist in nutrient uptake but not in the export of proteins (155, 188). It has also been suggested that a parasitophorous duct pathway within the infected erythrocyte is responsible for importing macromolecules from the external medium although, the presence of a parasitophorous duct is still hotly debated (250).

Various *Plasmodium* proteins, including RESA, MESA, Pf332, PfEMP-1 and KAHRP are transported to the erythrocyte membrane, yet the mechanisms of protein export are largely unknown and many exported *Plasmodium* proteins lack the typical N-terminal hydrophobic signal sequences that are found in secreted proteins of other eukaryotes. Although a clearly defined golgi has not been demonstrated, it is now generally accepted that membranous 'whorls' within the parasite cytoplasm function as the parasite golgi, as they have been suggested to resemble various components of the mammalian secretory

pathway and malarial homologues of mammalian proteins involved in protein trafficking have also been identified (8 , 93, 152).

1.1.5 Immune response

During the proliferation of the malarial parasite within the human host, the host immune system responds by developing both humoral and cell-mediated immune responses. The humoral immunity comprises an antibody-mediated response to various parasite proteins. The production of antibodies against parasite antigens takes 10-14 days. Thus, antibodies produced in the first encounter with the parasite can play a protective role in subsequent infections. This is the basis of protection in people who live in areas where malaria is endemic. After the sporozoites are injected into the bloodstream, antibodies produced to the surface proteins on the sporozoite, such as the circumsporozoite protein (CSP), can block hepatocytic invasion (50). Once within the hepatocytes, a cell-mediated immune response can destroy the infected cells after recognition by T cells. When the merozoites are released from the hepatocytes, immune responses to merozoite antigens, such as the merozoite surface proteins 1 and 2 (MSP-1 and MSP-2) and apical membrane antigen 1 (AMA-1), can prevent erythrocyte invasion (46, 167, 183). The parasite proteins expressed on the surface of the red cell are also targets for immune attack as antibodies to these proteins can affect their function. For example, antibodies against PfEMP-1 can potentially prevent the cytoadherence of parasitized red cells since infected erythrocytes recognized by the immune system are cleared from circulation if they pass through the spleen. Parasite derived proteins and toxins have been shown to activate cytokines such as tumour necrosis factor (TNF) and interleukins (IL), as a result of a cell-mediated immune response, through T-cells and macrophages. Cytokine responses have also been associated with the symptomatic fevers of malaria infection (82, 137). For those individuals who live in malaria-infected regions, as do 40% of the global population, there is always the possibility of continuously being reinfected (278).

1.1.6 Immune evasion

Despite the many points of attack by the immune system on the invading pathogen, the parasite has evolved various mechanisms to evade annihilation. Cytoadherence may be one of these mechanisms. The parasite-derived protein, PfEMP-1, has been identified as an important molecule in mediating cytoadherence (241). There is evidence that it is inserted through the red cell membrane and functions as a receptor which binds to a range of host cell-adhesion molecules including the cell surface glycoprotein (CD36) (16, 24), intercellular adhesion molecule (ICAM-1) (30), vascular cell-adhesion molecule (VCAM-1) (193), chondroitin sulfate A (CSA) (211), thrombospondin (TSP) (216), E-selectin (193) and the platelet/endothelial cell adhesion molecule-1 (PECAM-1/CD31) (255). Cytokines such as TNF and IFN-γ have been shown to induce the expression of these host cell-adhesion molecules within the vasculature system (29). The ability of PfEMP-1 to bind to such a diverse array of molecules means that it may sequester the infected red cell in a range of tissues and organs to avoid splenic clearance (256). PfEMP-1 is encoded by a group of about 50 genes called the *var* genes (223), which have a high switching rate of about 2% per generation (215). This means that at any one time, 2 out of every 100 infected red cells derived from a homogeneous population have a high probability of expressing an antigenically different version of PfEMP-1 on its surface. The PfEMP-1 molecules expressed on each infected cell are variant specific and cannot bind all host cell-adhesion molecules (186, 259). Furthermore, PfEMP-1 is also the target of variant-specific agglutinating antibodies (33). This mechanism of antigenic variation allows the parasite to effectively evade the immune response by expressing different phenotypic versions of PfEMP-1, which bind to different host cell-adhesion molecules and produce a different antibody response (32, 33). As it takes around 10-12 days for the host to mount a response to each new antigen, this allows waves of parasitemia to occur, each with a novel PfEMP-1.

Another mechanism of immune evasion is the camouflage offered by the use of host cells as a site of reproduction. For example, while the parasite is developing within the

hepatocyte, the host cell is processing and presenting foreign antigens for recognition by T-cells which would lead to the destruction of the hepatocyte. This cell-mediated response takes 10-12 days to effect, however, the parasite evades the imminent immune attack by completing the hepatocytic cycle within 7-10 days. Within the erythrocyte, the parasite is shielded from immune attack, as antibodies are unable to penetrate the plasma membrane and the erythrocyte is unable to present peptides in a MHC restricted manner. The parasite is not detected until the late stages of erythrocytic development when it presents foreign molecules on the surface of the host cell and also changes the properties of the cell, at which stage it has the ability to cytoadhere.

One characteristic feature of many *Plasmodium* proteins is that they contain regions of repetitive sequence that form immunodominant epitopes, which are recognized by antibodies in the sera of individuals regularly infected with malaria. Examples of such proteins include the ring infected erythrocyte surface antigen (RESA), the falciparum interspersed repeat antigen (FIRA), the S-antigen, the antigen which contains repeats of acidic and basic residues (ABRA), CSP, Pf332, Pf11.1, MSP-1 and 2 (13, 129, 209, 232, 243, 269). It has been found that antibodies to some of these antigens are cross-reactive with the repeat regions of other proteins (3, 49). As many of these antibodies appear to have no role in host protection from malaria, it has been suggested that these repetitive sequences may function as a 'smoke screen', reducing the ability of the immune system to respond effectively to the important functional epitopes on parasite proteins by skewing the immune response toward non-protective epitopes (9, 143, 209).

1.1.7 Vaccine technology

Most of the current antimalarial vaccines are aimed at interrupting parts of the malarial life cycle or essential processes carried out by the parasite, such as the production and processing of various antigens, the invasion of hepatocytes and erythrocytes, the process of cytoadherence by mature stages and the transmission of the parasites to the mosquito. These vaccines are aimed at stimulating both cellular and humoral immune responses. A

cellular response could result in the production of T-cells that recognize and destroy infected hepatocytes as well as the intracellular parasite, whilst the humoral response would ultimately result in the production of antibodies that bind and neutralize malarial proteins. One example is the vaccine candidate antigen, AMA-1. AMA-1 is believed to be instrumental in the invasion of erythrocytes by merozoites. Protective antibodies to AMA-1 have been identified which inhibit erythrocyte invasion and hence provide protective immunity when an immunized animal is challenged with malaria (71). Due to antigenic variation, the complexity of the malarial life cycle and the ability of the parasite to evade the immune response, single vaccine candidate antigens have afforded only partial protection of populations in vaccine trials (127). Hence, the current vaccine formulations target a combination of protective epitopes from many different malarial antigens expressed at various stages of the life cycle (127).

Antibodies play an important role in the protective immune response. Over the years, a range of antibodies and sera has been characterized. Some of these antibodies bind to malarial proteins and inhibit processes essential to the malarial life cycle, such as erythrocyte invasion. Whilst it is known what antigens these antibodies recognize, their exact epitopes in many cases are not known and hence, we lack a detailed understanding of the consequences of the antibody-antigen interaction. One approach that is being used to help identify the epitopes of protective antibodies is the use of random peptide libraries displayed on phage. This approach is analogous to finding which of 10^7 different keys available, opens a 'molecular lock'. The use of rapid screening methods and easy replication of the peptide has made this approach a rapid and effective way of identifying the epitopes and mimotopes (peptides that structurally mimic the epitope) recognized by antibodies.

1.2 Phage display technology

Phage display technology is a powerful molecular tool, which involves the expression of a recombinant viral structural protein, containing a foreign protein or peptide, on the surface

of bacteriophage. Large libraries of diverse molecular structures can be expressed on the surface of phage. The phage library can then be screened for phage with a particular phenotype or a desired binding specificity. An essential feature of phage display technology is that it links phenotype with genotype, such that, the expression of a particular phenotype on phage is physically linked to the genetic information contained within the phage particle. The expression of the desired phenotype can be achieved by incorporating the relevant genetic material into the phage genome. The desired binding molecule can be rapidly replicated and its ability to interact with another molecule, easily characterized.

In 1985 George Smith, considered by many to be the father and pioneer of phage display technology, developed a bacteriophage vector that could display a small foreign peptide of a defined length on the surface of a filamentous phage particle, with the genetic information encoding that peptide contained within the phage particle (236). The phage containing the foreign peptide was able to invade bacteria in the same manner as a wildtype phage particle indicating that the fusion protein was still functional. A library of random peptides, at first only six amino acids in length, was then constructed and displayed on the surface of phage. In this original library each phage particle expressed only one of 10^7 different peptides (197). The entire phage library could be contained in a small volume of less than 1 ml and easily replicated by infecting *E. coli* cells. Using a monoclonal antibody, Smith *et al.* (197) screened the library using a technique referred to as biopanning in order to identify peptides that bound specifically to the antibody. This involved reducing the diversity of the population of peptides at each round in the biopanning process. A number of peptides were isolated, some of which contained a consensus motif that was identical to the epitope of the antibody. Another peptide contained no such motif and showed no similarity to the epitope at the primary sequence level, yet was able to bind specifically to the antibody. Peptides of this type are able to mimic the conformational shape and charge profile presented by the epitope and are

referred to as mimotopes as they have a different primary sequence to the epitope, yet make similar molecular contacts with the antigen-binding region of the antibody.

Since the introduction of phage display, numerous applications of this technology have been described. The epitopes recognized by various antibodies have been defined without needing a precise knowledge of the antigen against which they were raised. Epitopes recognized by protective disease-related antibodies have been mimicked and the specificity of that protection, replicated. Phage-displayed peptides that bind to hormones, proteins, carbohydrates, DNA and other compounds have been identified using rapid screening techniques. Since its discovery, much work has been done to optimize this technology and adapt it for use in different biological situations. Various libraries, not only of peptides but also proteins, including antibody fragments, receptors and polypeptides have been designed along with a suite of affinity selection and screening techniques.

The approach of constructing large combinatorial libraries has not been confined to using phage as a framework. Molecules have been displayed on bacterial components such as pili, flagella and surface proteins as well as yeast (35). Other viral vectors include lambda (246), T4 and P4 bacteriophage (130, 158) and the eukaryotic viruses such as baculovirus (40). However, the most commonly used vector is still M13 filamentous bacteriophage.

1.2.1 Phage biology

Filamentous phage are preferred for use in phage display libraries because of their ease of use and flexibility. They range from 1-2 μm in length with a width of 6-7 nm and infect *Escherichia coli* cells displaying F pili. Filamentous phage contains a circular single-stranded DNA genome that encodes 10 proteins, 5 of which are virion structural proteins. The genome is enclosed in a protein coat encoded predominantly by gene VIII. There are approximately 2700 copies of the gene VIII protein comprising the protein coat. At either end of the virion are a number of minor coat proteins specific to each end. At one end of

the phage particle are 4-5 copies of the gene VII protein and 4-5 copies of the gene IX protein. These are involved in the initiation of phage assembly and maintenance of the stability of the viral particle. At the other end of the phage particle are 3-5 copies of the gene III protein which is required for infection of the host cell and 4-5 copies of the gene VI protein involved in the termination of the viral assembly process. Unlike the lambda-like or T-type phages, filamentous phage are secreted from the host cell after viral replication, thus the host cell remains intact and viable and continuously secretes phage into the medium. The gene III protein (gpIII) and the gene VIII protein (gpVIII) have been predominantly used as the frameworks for expressing foreign peptides and proteins on the surface of phage (180, 237).

1.2.2 M13 life cycle

M13 filamentous bacteriophage can only infect male *E. coli* cells expressing an F pilus. GpIII is essential for phage infectivity. It is composed of three domains, a C-terminal domain (gpIII-D3) which is attached to the viral particle and two N-terminal domains (gpIII -D1 and gpIII -D2) which effect infectivity. The gpIII -D2 domain attaches to the end of the F pilus, which retracts into the cell allowing gpIII to transverse the bacterial outer membrane and the gpIII -D1 domain to interact with host cell TolQRA complex on the inner membrane. It has been suggested that the TolQRA complex in association with gpIII forms a channel through which the viral DNA is extruded into the cytoplasm of the host cell, leaving behind the protein coat (213). The circular single stranded viral DNA (plus strand) is converted to a double stranded replicative form using the host cell apparatus. The replicative form is then used to express all of the viral genes. The viral gene II product nicks the plus strand of the replicative form so that copies of the circular single stranded DNA can be produced from the minus strand using the host cell apparatus in a process referred to as rolling circle replication. In the initial stages of viral infection the newly synthesized plus strands of circular single stranded DNA are converted to the replicative form and used for protein synthesis and DNA replication. In the latter stages the accumulating single stranded DNA is bound by the viral gene V protein (gpV-DNA)

for viral assembly. The five viral structural proteins are synthesized and inserted into the inner membrane of the host cell with the N-terminal domains within the periplasm. GpIII and gpVIII contain signal sequences that are cleaved during membrane insertion. As gpIII is relatively large, it is believed that the bacterial export machinery encoded by the *sec* genes assists in the protein folding and membrane insertion. The phage assembly process involves both the gene I protein and the gene IV protein which form a pore in the membrane of the host cell from which the viral particle is extruded. The gene VII proteins and the gene IX proteins are assembled first. The gene V protein in the gpV-DNA complex is replaced by gpVIII coat as the phage is extruded from the host cell. Finally, the gene VI protein is assembled followed by gpIII, which completes phage assembly (Fig. 1.2.1) (95, 180, 237).

1.2.3 Types of phage libraries

There are many different types of libraries displayed on phage (Fig. 1.2.2). In most cases the recombinant protein is expressed on either gpIII or gpVIII referred to as the type 3 and type 8 libraries respectively (239). In the type 3 libraries, the construct encoding the recombinant protein can be included as a fusion on the phage genome such that all copies of gpIII will contain a foreign protein sequence. If the phage genome contains genes for both the wildtype gpIII as well as the recombinant gpIII then there will be a mixture of recombinant and wildtype gpIII produced: this system is referred to as type 33. Where the foreign protein is large, as in the case of antibody libraries, the recombinant gpIII is encoded by a plasmid within the phage particle, referred to as a phagemid. Helper phage, which contain a defective origin of replication, are used to produce wildtype phage proteins within the same *E. coli* host, such that, the resulting phage particle contains the phagemid and expresses only a single copy of the recombinant gpIII. These are referred to as the type 3+3 libraries.

Random peptide libraries displayed on gene VIII are referred to as type 8 libraries. There is a similar range to the type 3 libraries in that they include types 8, 88 and 8+8. GpVIII is

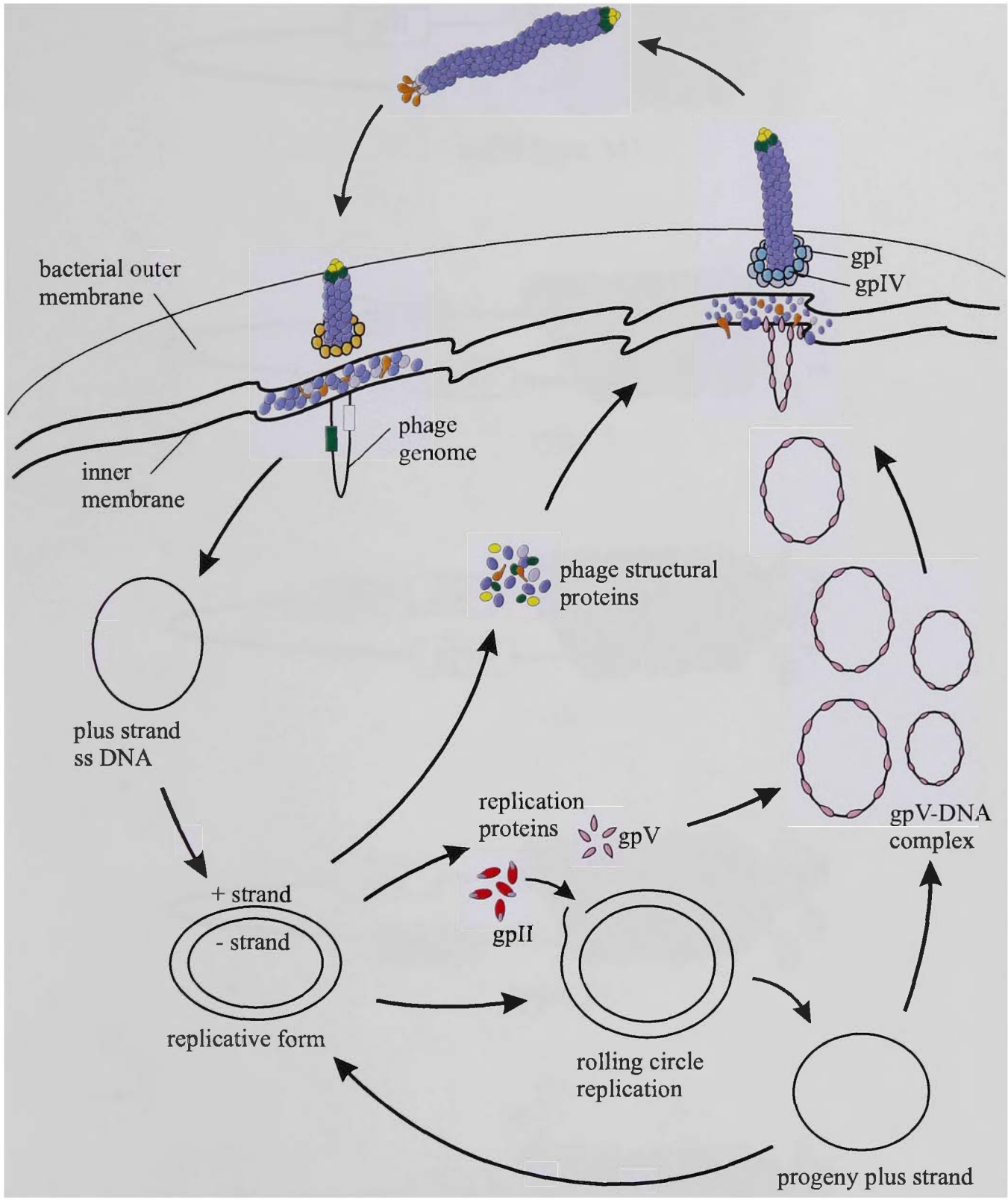


Figure 1.2.1. Replication of phage. During the initial infection step, the phage genome is extruded into the cytoplasm of the bacterial cell leaving behind the protein coat. The single-stranded circular DNA is replicated to produce the double-stranded replicative form which is used to express the viral proteins involved in replication (gpII, gpV, gpX), assembly (gpI , gpIV) and viral structure (gpVIII, gpIII, gpVII, gpVII, gpIX). Rolling circle replication is the process by which phage DNA is produced initially for DNA replication and protein expression and latter for phage assembly. Adapted from Smith, 1988 (237).

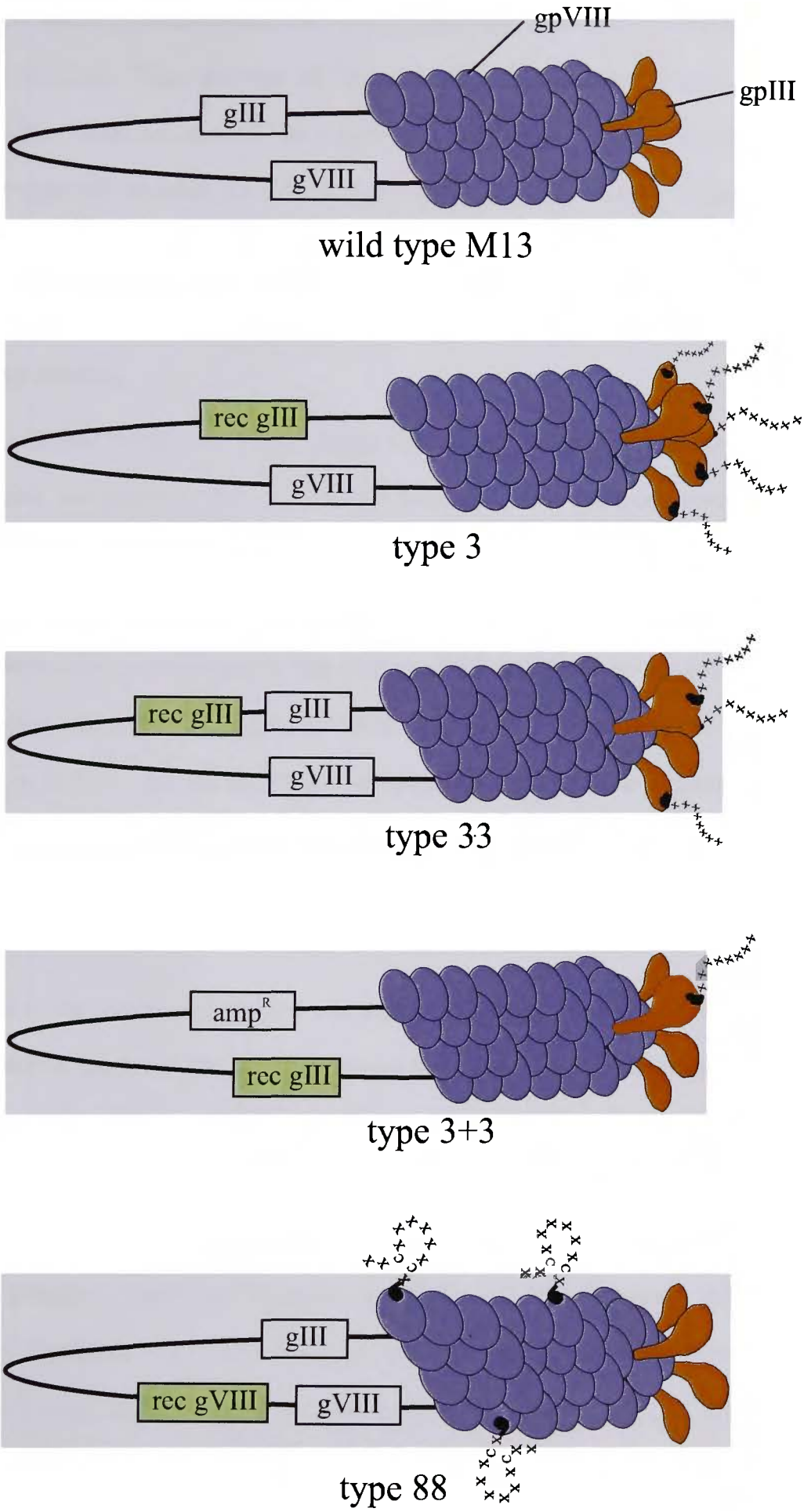


Figure 1.2.2. Some of the different types of phage libraries. Genes encoding recombinant proteins are coloured green. 'X' represents any of the 20 amino acids. Type 3 and type 88 are representative of the 15-mer and 17-mer libraries respectively used in this work. Adapted from Smith, 1993 (239).

about 8 kDa which is smaller than the 42 kDa gpIII and does not tolerate being linked to large proteins (132). Thus the type 88 libraries are generally used for the expression of small peptides. There are about 2700 copies of gpVIII expressed on the surface of phage hence, the type 88 libraries, in which there are about 240 copies of the recombinant protein expressed, are the most practical.

1.2.4 Biopanning

Biopanning is the process by which phage expressing desired specificities and binding characteristics are selected and enriched for from a diverse pool of biomolecules. This process usually involves a sequential reduction in the diversity of the phage library over repeated rounds of panning (67). The diversity relates to the complexity and number of different biomolecules contained in the library. This is limited by factors including the transformation efficiency of cells, the lower expression frequencies of codons rarely expressed in *E. coli* and the repertoire of molecules that can be expressed on phage without having adverse effects on phage production or host cell biology. These and other limitations have been reviewed previously (218).

The success of the selection procedure can depend not only on the type of library used, but also the method used for the selection procedure (75). The panning process generally involves immobilizing a target molecule, probing the molecule with the phage library and eluting phage that bind. The eluted phage are usually amplified and again panned on the immobilized molecule as the phage that bind specifically are enriched for over repeated rounds of panning. There are however, variations to this approach that have been successfully employed (Fig. 1.2.3). In many cases the target molecule is immobilized on a matrix, which may include microtitre plates (111), immunotubes (75), and polystyrene coated beads (86). The molecule can be allowed to bind directly to the matrix or indirectly via a linker, such as streptavidin that is coated on the matrix then incubated with a biotin-labeled molecule (231). The method of immobilization can vary depending on a number of factors, including, the properties of the target molecule. For example, biotinylation may

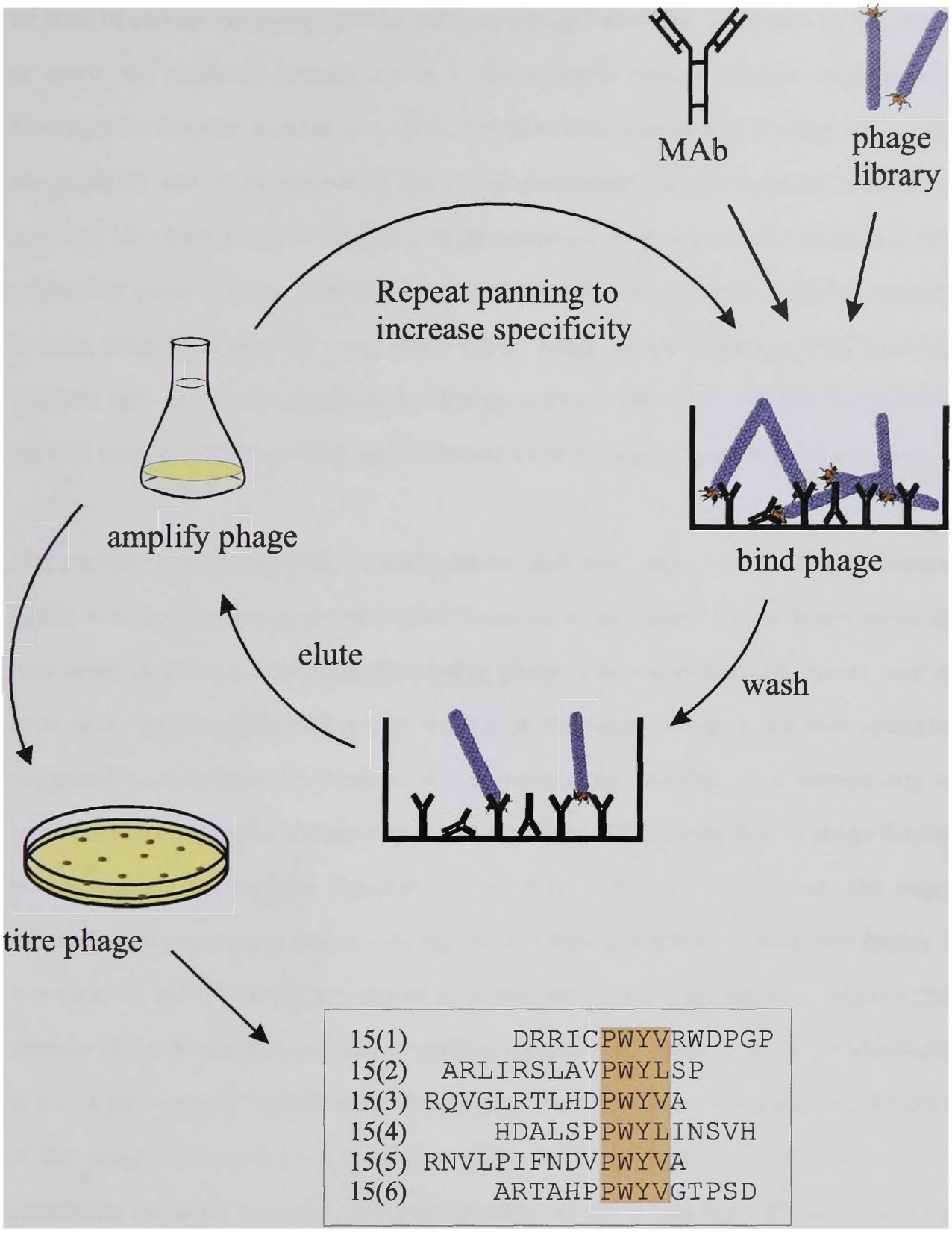


Figure 1.2.3. A method of biopanning used to isolate peptides that bound to a monoclonal antibody (MAb). The MAb was immobilized in the wells of a microtitre plate and probed with the phage peptide library. Unbound phage were removed by washing and then the bound phage were eluted with acid and amplified in liquid culture. The isolated phage were repanned on the MAb for 3-4 rounds. The titre of the phage was determined at each round in the panning procedure and individual clones were picked and sequenced to identify the expressed peptide. Common motifs within the peptide sequences were also identified.

be used to elevate the molecule from the matrix to prevent steric hindrance by the matrix or orient the molecule in such a way as to expose a binding domain. Alternatively, biotinylation may be avoided as it may interfere with a particular binding site on the molecule. In one study comparing the use of polystyrene plates to magnetic beads as matrices on which to immobilize the target molecule, in this case interleukin-8, it was found that panning using magnetic beads was significantly more efficient for isolating specific phage than panning using plates (171). These results relate specifically to IL-8 and may vary depending on the target molecule and the conditions used, however, they do provide some comparison which may be of use when designing biopanning experiments.

The amount of target molecule immobilized has also been suggested to affect the success of the selection procedure, as incomplete covering of the matrix by the target molecule may result in the selection of matrix-binding phage. The use of blocking agents such as skim milk, and immobilizing a high density of the target molecule are two strategies suggested to overcome this problem (2). In some cases however, it is unnecessary to immobilize the molecule. Selectively infective phages (SIP) is a method of phage display which exploits the modular structure of the gpIII (150). In this method, the target molecule is expressed as a fusion with the two N-terminal domains of gpIII. The library is expressed on the C-terminal domain of gpIII attached to the phage particle, which in the absence of the N-terminal domains of gpIII is not infective. When a phage particle binds to the target molecule, infectivity of the phage particle is restored enabling amplification of the phage and the desired specificity (245). In this case it is unnecessary to first immobilize the target molecule. Another advantage of SIP is that high affinity binders are not excluded in the selection procedure as the binding phage do not need to be eluted from a matrix.

Another novel approach used to identify peptides that target specific organs, involved *in vivo* screening of peptide libraries displayed on phage. Phage libraries were injected intravenously into anaesthetized rodents and the circulatory system was used as a vehicle

to transport the phage through the organism. After a few minutes, phage that bound to specific organs were recovered generally by removing the organs, homogenizing them and adding *E. coli* cells to the suspension, so that infection could occur. The phage were amplified and the procedure repeated 2-3 times (207). In this approach, undefined target molecules on the surfaces of organs that are exposed to the circulation were presented in their natural environment and membrane-bound native state. *In vivo* selection has therefore enabled the *in situ* isolation of ligands to membrane-bound molecules and target molecules that may be difficult to isolate or express as recombinant proteins due to their hydrophobic properties. Furthermore, this approach has allowed the rapid identification of peptides that target specific organs which could be used for the *in vivo* targeting of therapeutics.

The first round in the panning process is the step which results in the greatest decrease in the diversity of the library, as phage which do not bind the target molecule are selected against. Conversely, it is also the step after which the phage that bind the target molecule show the greatest diversity. Therefore, it is important to optimize the chances of retaining a diverse pool of binding phage after the first round that could be repanned to select for binders with specific properties, for example phage displaying high affinities for the target molecule, in subsequent rounds of panning. Factors which can affect the diversity of the phage pools at each round in the panning procedure include the stringency of the panning procedure and methods used to elute the bound phage. The stringency of the washing procedures directly affects the range of affinities displayed by the binding phage. If conditions of low stringency are used in the panning process, then a greater diversity of binders are selected for in comparison to high stringency conditions, which tend to select for high affinity binders. Therefore, in order to select for a pool of phage in the initial round of panning which has a high diversity, conditions of low stringency are generally used. Conversely, conditions of high stringency can be used to select of high affinity binders in subsequent rounds of panning. Mutagenesis has also been used to increase the affinities of the selected binders (77, 122, 279). In one example, a cyclic 8 amino acid

peptide was identified that bound and activated the erythropoietin receptor with an affinity of 10 μM. Mutagenesis was then used to create libraries of related cyclic peptides from which peptides were isolated that had affinities 10 to 50 times greater that the original peptide. Interestingly, when the cysteines in these peptides were mutated to serine residues, the affinities of these peptides were reduced by at least 1000 fold illustrating the importance of the disulphide constraints in generating peptide conformations that had high binding affinities (279).

The affinities displayed by the binding phage can be related to the number of copies of the recombinant coat protein expressed on the surface of the phage particle. This is referred to as avidity. Phage displaying several peptides may appear to have a higher relative binding affinity than phage expressing a single copy of the recombinant protein however, the higher avidity may be the result of multiple low affinity interactions (276). Therefore, libraries such as types 8 and 88 may not be as suited for the selection of high affinity binders, in comparison to other libraries displaying fewer copies of the recombinant protein. Library diversity, stringency of the panning process and methods of elution are also addressed later in the context of peptide libraries. In situations where the library does not contain phage that bind the target molecule with sufficient affinity, the isolation of 'non-specific' phage, including phage which bind the blocking agent and/or matrix can present a problem (2).

The amplification of the eluted phage is another factor which can affect the success of the panning procedure. Eluted phage are allowed to infect *E. coli* cells displaying F-pili, with only one phage infecting each cell. The *E. coli* are then grown either in liquid broth or on solid media. Amplification on a solid medium involves plating the infected cells on multiple agar plates and harvesting the grown colonies, by scraping them from the plate and resuspending them in a liquid medium (240). Culturing the infected cells in liquid media is not as labour intensive, however it was initially thought that liquid culturing promoted a bias in the library towards clones that had a growth advantage as the result of

different growth rates among clones. It has since been found that a similar level of competition among clones occurs when cells are cultured on solid media and therefore there is no significant advantage over liquid culture amplification (173).

1.2.5 Applications of phage display

The use of phage display to isolate desired binding molecules from large libraries has been applied to an enormous number of biological problems. These include defining epitopes and mimotopes recognized by different antibodies, identifying peptides that mimic conformation-dependant binding sites of various receptors, identifying peptides that bind and enter specific cell types, identifying a source of protein-binding molecules, identifying peptides that could be used as immunogens and therapeutic agents and identifying antibodies that recognize specific antigens to name a few (20, 38, 68, 79, 192, 198). The huge number of phage display applications can be broken down into three general approaches according to the nature of the binding molecule being displayed: 1) antibody fragments, 2) protein fragments and 3) random peptides.

1.2.6 Display of proteins and enzymes

Phage are able to express a wide range of exogenous proteins as fusions with either gpIII or gpVIII. In many cases, entire proteins or protein domains can be displayed in a correctly folded form. Indeed there are many examples of displayed proteins that retain their function on the surface of phage. Thus phage is a suitable framework to examine such phenomena as enzyme-substrate or receptor-ligand interactions. Random mutagenesis in conjunction with phage display technology has enabled the identification of novel substrates of penicillin G acylases (267). The first enzymically active heterodimer displayed on the surface of phage was the 86 kDa penicillin G acylase which was expressed as a fusion with both gpIII and gpVIII. The unprocessed 133 kDa fusion protein is transported to the periplasm where it is processed into the α and β subunits and correctly folded to form an active enzyme displayed on the surface of phage. It was found that the multivalent display of the enzyme was not attainable due possibly to the limited

size of the phage secretion pore formed by the gene IV protein (267). Thus there is probably a limit to the size and valency of the proteins that can be expressed on phage, highlighting a possible limitation of this technology.

In efforts to construct and identify novel enzymes which catalyse defined reactions, phage display approaches have proved useful. One approach has been to select for enzymatic activity by expressing enzymes, with mutations in their binding sites, as fusion proteins with calmodulin-tagged gpIII. The enzyme library is then panned on a substrate, which is attached to a calmodulin-binding peptide. The calmodulin-binding peptide attaches to the calmodulin-tagged gpIII, such that the enzyme is linked to the substrate. In cases where the substrate is converted to a product, an anti-product affinity reagent is used to isolate the phage which is attached to the product via the calmodulin-binding peptide. Calcium chelators are used to dissociate the calmodulin-peptide complex (80). There are also other examples where various substrates have been selected on libraries of enzymes in order to identify novel binding specificities and enzymes with varying affinities (273).

Phage display has also been used to study protein-DNA interactions. Phage displaying mutated zinc finger proteins were selected on DNA sequences in order to identify zinc finger proteins with new DNA-binding specificities (138, 210). This approach can be applied to identify proteins that recognize specific sites on DNA with high affinity (117).

Phage display is now widely used in the study of protein-protein interactions particularly where it is necessary to explore the structure-function relationships of multifunctional proteins. Using a panning procedure which first involved negative selection, thrombin and a domain of tissue-type plasminogen activator (VR1) were selected on a phage library of plasminogen activator inhibitor 1 (PAI-1) mutants. Amino acid residues that are important in the interaction of PAI-1 with either thrombin or the VR1 domain were identified (262). In some cases, proteins have been used as a scaffold for the display of random peptides on the surface of phage. These random peptides on surface-exposed regions of the protein are

usually constrained by the protein framework and tend to function in a similar manner to a protein receptor. Proteins which bind to *Taq* polymerase, human insulin and a human apolipoprotein A-1 variant have been isolated from a library of phage expressing thirteen randomized amino acid residues, exposed on the surface of the α-helical bacterial receptor domain of staphylococcal protein A (189). Thus phage display enables one to isolate, from complex libraries containing tens of millions of variants, molecules with improved functional properties. The different classes of functional proteins that are able to be expressed on phage continues to expand.

1.2.7 Phage display of antibodies

Antibody fragments have been displayed on the surface of phage in a monovalent format such as Fab or scFv fragments (226). The Fab fragment includes a part of the Fc region as well as the variable region of the antibody whilst the scFv consists only of the variable complementarity determining regions from the heavy and light chains linked by a peptide linker. The phage display of antibody fragments has facilitated the isolation of antibodies with rare binding specificities and high affinities. The display of Fab fragments on phage has been employed to isolate antibodies to a large and ever increasing variety of molecules. Some examples are the botulinum neurotoxin produced by the bacterium *Clostridium botulinum* (94), malarial antigens (108), the protein kinases (149) and even the surface proteins of mammalian cells (204). It has been suggested that phage antibody display and bacterial production of recombinant antibodies could overcome many of the limitations of hybridoma technology by providing more control over the antibody selection process in terms of affinity and specificity, and also in the production of high affinity monoclonal antibodies and antibody reagents (94).

The advantages of phage display technology over traditional hybridoma technology has been widely recognized particularly in terms of speed, simplicity and diversity of available antibodies (264) (56) (176) (14). The affinities of antibodies isolated from phage display libraries depends to a large extent on the source of the antibodies and the panning

strategy used to isolate the desired clones from this library. Antibody libraries tend to be made with DNA from three different sources: 1) hybridomas expressing à desired antibody specificity, 2) peripheral blood cells from organisms immunized with an antigen to which an antibody is desired and 3) naïve donors.

In this latter category, the antibodies are usually of lower affinity as the genes for the antibodies have not undergone somatic mutation, which is an important step in the affinity maturation process that *in vivo*, results in high affinity antibodies (51, 165). However, one report has described the isolation of scFv fragments of sub-nanomolar affinities from libraries constructed from naïve human donors. In this case it was necessary to construct an extremely large and diverse library. Some of the antibodies isolated from this library recognized the cytotoxic molecule doxorubicin, which is also immunosuppressive (264). This example illustrates an advantage of phage display technology over traditional antibody production methods, particularly in the production of antibodies to cytotoxic and immunosuppressive compounds. Another advantage of phage display over hybridoma technology is the ability to rapidly select for specific, high affinity antibodies to antigens which naturally have poor immunogenicity (51, 274). It is generally believed that multiple rounds of panning and reamplification tends to select for clones which bind with high affinity, however, this is quite often not the case due to problems associated with the enrichment for specific high affinity antibodies (75).

Antibody display has also provided a new approach in vaccine design and the search for antibodies with therapeutic potential. In one study a scFv library was designed using the human V_H and V_L genes from immune and non-immune hepatitis C (HCV) patients respectively. By screening synthetic peptides corresponding to viral core and envelope proteins of the hepatitis C virus on the library, scFvs were isolated that bound to epitopes also recognized by polyclonal antibodies in HCV-infected sera (51). This further illustrates how antibody libraries may be employed to rapidly isolate antibodies against antigens of low immunogenicity and to defined epitopes of medical importance.

Antibody display has also been applied to the study of antigen-antibody interactions. Since the DNA sequence of each scFv clone can be determined rapidly, it is possible to ascertain the amino acid residues in the complementarity-determining regions of antibodies and begin to understand the antibody-antigen interaction at a molecular level (108). In cases where there is structural data from X-ray crystallography, this information becomes particularly valuable (77). Antibodies to carbohydrates play an important role in the immune response to various pathogens yet these antibodies tend to be of low affinity and relatively little is known about carbohydrate-antibody interactions. The phage display of Fab fragments has been used to isolate high affinity monoclonal antibodies that bind carbohydrate moieties of medical importance. This has provided some insight into the carbohydrate-antibody interaction at a molecular level (83). Phage display has also been used to isolate antibodies that localize to specific carbohydrates within plant cells (274).

Antibody display has facilitated the isolation of recombinant antibody fragments for intracellular targeting of therapeutics. Through screening a scFv library on breast tumour cells expressing the growth factor receptor ErbB2, phage displaying an anti-ErbB2 scFv were isolated that bound ErbB2 and underwent receptor-mediated endocytosis into mammalian tumour cells resulting in the expression of a reporter gene encoding the green fluorescence protein. Thus it is possible to isolate phage that bind specific cell receptors, leading to endocytosis of the phage particle and delivery of the phage-derived gene, via the appropriate trafficking pathway and to the correct cellular location for expression (204). This approach has potential for defining cellular receptors and intracellular targeting molecules for use in drug delivery systems and gene therapy.

Phage antibody display has been used to rapidly identify structural information on antigens, particularly the tertiary and quaternary structures, in cases where crystallography and nuclear magnetic resonance (NMR) spectroscopy are difficult to employ. Fab fragments which recognize various components of the human immunodeficiency virus

type 1 surface glycoprotein gp120 have been isolated from phage display libraries. These monoclonal antibody fragments can be used to provide information about the topography of gp120. The size and carbohydrate content of this antigen make it difficult to be studied using crystallography and NMR. Whilst the phage display approach does not provide definitive structural information, it can provide insights which would be difficult to obtain by other means (84).

Antibody display has allowed for the production of reagents with potential for applications in diagnostics and therapeutics. Human erythrocytes have been screened on a phage display library of Fab fragments in order to isolate antibodies which recognize cell surface antigens. This approach could be useful in serology, diagnostics and in studying the immune response to antigens present on the human erythrocyte (234). Phage display libraries have also been employed to isolate antibodies involved in the pathogenesis of autoimmune disease (257), the diagnosis of mammalian pathogens (94) and the diagnosis of plant diseases. The cucumber mosaic cucumovirus (CMV) was screened on a phage-displayed scFv library in order to isolate antibody fragments that specifically recognized the CMV in naturally infected plant tissues (287). However, problems have been described relating to the stable production and purification of large quantities of bacterially expressed antibody fragments, as hybridoma cultures produce greater yields of antibody in comparison to bacterial cultures (18, 94). Although, this problem is being addressed by the expression of antibody fragments in other cell types (18).

One limitation of any monoclonal antibody-based therapeutic for use in humans is the anti-mouse antibody response. Since many scFv and Fab fragments are of mouse origin, it is sometimes necessary to humanize these molecules by grafting the complementarity-determining regions onto a human antibody framework. Traditionally humanization of antibodies has been time consuming and generally results in a consequential loss of antibody affinity. Antibody humanization using phage display has provided a new

approach to rapidly screening libraries of mutants for humanized antibodies that bind with increased affinity (19).

1.2.8 Phage display of peptides

Many antibody fragment libraries are biased towards binding molecules with desired specificities, for example by prior immunization of donor animals or by using hybridoma nucleic acid as the starting material. By contrast, peptide libraries are generally unbiased. Generally, random oligonucleotides containing all possible codons are inserted into the gene encoding the coat protein, which results in phage displaying random peptides on their surface (283). Random peptide libraries have been constructed with an insert length of between 6 and 43 residues and with a variety of constraints imposed by the presence of cysteine residues (281). Such libraries serve to increase the diversity of molecular structures that are available for affinity selection. Peptides that interact with a range of molecules, such as monoclonal and polyclonal antibodies and other binding proteins, can be selected without having any prior knowledge about the structure or interactive sites on the molecule. For example, peptides that bind to polyclonal antibodies in the sera of diseased individuals have been identified (64) without having any knowledge about the nature of the authentic epitopes or antigens. Thus phage-displayed peptide libraries could potentially play an important role in elucidating the mechanisms involved in disease pathogenesis as well as in vaccine design and development. The applications of peptide libraries are diverse, ranging from identifying the epitopes of monoclonal antibodies to elucidating the binding motifs for receptors. Interactions involving carbohydrates and nucleic acids are also amenable to study using phage-peptides. Several studies have identified peptide mimics of carbohydrate antigens that when immunized into animals were able to elicit an anti-carbohydrate response analogous to the original antibody (202, 206).

The ability to select peptides with the desired properties depends largely on the diversity of the library and the strategy used for selection. A major limitation to the diversity of any

library is the ability of the host *E. coli* cells to express the recombinant peptides. Peptides which are in some way lethal to the host cell, or inhibit protein expression and other cellular processes will not be represented in the library. The length of the peptide displayed in the library also plays a role in the diversity of the library. Even if it were theoretically possible to express all of the 10^9 peptide variations in a 7-mer library, it is not possible to do so for a 15-mer library as the number of possible peptide variants is 10^{17}, however the maximum library diversity is less than 10^{10} peptides due to the relatively low transformation efficiency of bacterial cells (66). However, long peptides can also be considered as a sliding series of smaller peptides. For example, a 15-mer peptide is really 10 different 6-mer peptides each of which have variable adjacent residues. Libraries of long peptides also have the ability to form a range of more complex molecular shapes which increases the chances of selecting a peptide that binds the target molecule. This may be particularly relevant when isolating mimotopes to discontinuous epitopes.

A further consideration of the success of identifying binders from a random peptide library is stringency. If the stringency in the selection procedure is too high then there is a decreased chance of retaining low affinity peptides that are nevertheless specific. Conversely, if the stringency is too low then non-specific binders that have a growth advantage may dominate.

The elution strategy can also affect the outcome of the selection procedure. One of the exploitable properties of phage is their ability to withstand highly acidic conditions and still remain infective. Acid elution of bound phage is a method commonly used to recover binding phage (98, 170, 268, 277), but this approach used alone, can fail to recover high affinity binders (21). Other methods of specific elution that have been attempted include the use of corresponding antigens in the case of antibodies (284) and other ligands that bind the target molecule (112). The addition of *E. coli* cells directly to the site of the phage-target molecule interaction, such as in the wells of a microtitre plate, has been

shown to result in the infection of the *E. coli* cells by the bound phage although the mechanism of infection is unknown. However, it is not known whether this method is adequate to elute high affinity binders. Another approach utilizes nitrostreptavidin which exhibits a reversible attraction for biotin. In this case the biotinylated target molecule is immobilized to a matrix via nitrostreptavidin. Phage that bind the target molecule can be eluted from the matrix, whilst still attached to the target molecule, by an excess of free biotin. This approach also enables the selection of high affinity binders (21). Alternatively, the SIP method of phage display is another approach that could be employed to overcome problems associated with the selection for high affinity binders (see Section 1.2.4 Biopanning) (245).

Antibodies can recognize continuous linear amino acid sequences in proteins and also discontinuous or conformational epitopes in which the epitope is not a linear sequence but consists of amino acids that are widely spaced in the primary sequence but are brought together in the folded protein. The use of random peptide libraries to identify peptides that bind to various antibodies is, to date, probably the most common application of phage display technology. Generally, when screening peptide libraries on monoclonal antibodies a range of different peptides are isolated that often contain a common or consensus motif which in many cases is similar, if not identical, to the native epitope of the antibody (1, 268). It is also common to select for peptides that mimic the conformation of the epitope (20). These peptides are referred to as mimotopes as they have a different primary amino acid sequence to the native epitope, but possess similar structural characteristics.

Random peptide libraries have been used to characterize antibody-peptide interactions. In one study, monoclonal antibodies that bind human interleukin-2 (IL-2) were screened on a 9-mer peptide library in order to characterize their native epitopes. Peptide consensus motifs were identified that corresponded to regions of the primary sequence of IL-2. One such motif (TTFM) was identical to a region of the primary sequence whilst the other consensus motif (**LXLXDSRNLXFRPR**) was very similar to a region of the primary

sequence (**LNLAQSKNFHLRPR**). When the consensus motif was compared with the 3D model of IL-2, one monoclonal antibody was found to bind to a loop region connecting a helix and a beta strand, whilst the other bound to a partially exposed loop region connecting two helices. This work has therefore provided structural information on the specificities of the monoclonal antibodies and the immunogenic regions of human interleukin-2 (268).

In another study, three monoclonal antibodies against the small hepatitis B virus surface antigen (HBsAg) were used to identify peptides that mimicked the conformational epitopes of the three antibodies. Some of the isolated peptides were found to contain a combination of motifs which showed homology to different regions in the primary sequence of HBsAg. It was suggested that, when in the folded state, these different regions in the HBsAg primary sequence come together to form the epitopes of the relevant antibodies. Based on the data obtained from the mimotopes, a model for the 3D structure of HBsAg was proposed (134). Thus peptide libraries are useful in providing structural information relating to antibody-antigen interactions however, this information is not limited to the characterization of just linear epitopes. Epitope-mapping studies can also allow the characterization of the three dimensional surface of entire antigens if a panel of monoclonal antibodies is available. In the latter example, a library displaying 20-mer peptides was used, illustrating the applicability of different libraries to different situations. If a 9-mer library was used in place of the 20-mer, a different repertoire of peptides may have been isolated which may not have provided that same structural information as the 20-mer mimotopes.

Polyclonal antibodies have also been used for the isolation of specific peptides (89, 90, 110, 164, 280). Panning on polyclonal sera can be more complicated in comparison to identifying peptides that bind to monoclonal antibodies, as the target molecules with the desired specificities are present in a complex mixture of antibodies, are fewer in number and there is usually more than one type of target molecule within the serum. Differential

selection procedures utilizing infected and normal sera have been established to enable the isolation of mimotopes to specific antigens involved in disease pathogenesis (106, 174, 205). In one novel example, a DNA-based selection procedure in conjunction with phage display was used to isolate peptides that bound to hepatitis C virus (HCV) antibodies present in human sera. This essentially involved panning a random peptide library on sera from two HCV-infected patients to generate two pools of phage peptides. dsDNA was produced from one phage pool with the minus strand being retained and biotinylated. ssDNA (+ strand) was isolated from the second phage pool. The DNA from each pool was then combined and the complementary DNA sequences allowed to hybridize. Streptavidin coated beads were used to isolate the hybridized DNA which was then transformed into bacteria to produce a pool of phage with binding characteristics for sera from both the HCV-infected patients (26). This unique approach (which has the advantage of DNA hybridization over protein affinity) has allowed for the rapid isolation of peptides that bind target molecules from within a complex mixture of molecules.

It has been reported that libraries in which the peptides are conformationally constrained tend to provide superior mimotopes, in terms of affinity and specificity, than libraries of unconstrained or linear peptides (126, 148). This point has been further illustrated in studies which showed that a reduced and alkylated peptide showed decreased binding to a monoclonal antibody compared with the cyclized form of the peptide (1). Other studies have also suggested that there may be difficulties associated with mimicking discontinuous epitopes with linear unconstrained peptides (65, 279). This problem has been addressed through the use of libraries expressing constrained peptides. Whilst a random peptide library should theoretically contain a small proportion of disulphide bonded constrained peptides, the engineering of one cysteine into the random peptide sequence dramatically increases the probability of selecting for a constrained or cyclized peptide. When two cysteines are incorporated, one on either side of the random peptide insert, then the majority of the peptides are cyclized. The smaller the random peptide flanked by cysteines, the more constrained the peptide will be. By constraining the peptide

in such a manner, the number of different structural conformations that can be attained are limited and therefore, the library will consist of molecular structures that have a dramatically reduced flexibility and hence will be more defined. Furthermore, in the case of conformational epitopes, the constrained nature of the peptide could potentially mimic the native state of the epitope to a greater degree than the unconstrained form. However, irrespective of which type of library is used, one would be more likely to identify a mimotope of a discontinuous epitope as opposed to the native discontinuous epitope regardless of whether a constrained or unconstrained library is used. This is because the probability of a library containing the exact epitope in the right conformation is much lower than the probability of the same library containing a panel of peptides with similar binding properties to the epitope. It has also been suggested that constrained peptides form better immunogenic and antigenic mimotopes than unconstrained peptides (67).

Several studies have reported the use of libraries having several cysteine residues engineered within the peptide insert (148, 195). In one dramatic study, libraries expressing peptides flanked by cysteine residues at either or both ends were used to identify integrin-binding peptides. These peptides were found to contain a common RGD motif essential for integrin binding, and are able to block integrin function and cell adhesion. Interestingly, most of the isolated peptides contained at least two cysteines indicating a preference for the selection of constrained peptides (148). Furthermore, in this and other studies (146, 147, 195) it was found that conformationally constraining the peptides by incorporating flanking cysteine residues markedly improved the binding affinities of the integrin binding peptides. Moreover, one peptide containing four cysteine residues (ACDC**RGD**CFCG), and hence capable of forming two disulphide bonds, was found to be a 20-fold more potent inhibitor of cell adhesion than similar peptides containing a single disulphide bond and 200-fold more potent than linear RGD peptides (148). This peptide was later used in targeted chemotherapy to deliver the anticancer drug doxorubicin to the tumour vasculature in a mouse model (15). This illustrates the

importance of libraries expressing conformationally constrained peptides for isolating specific peptides with high binding affinities.

As mentioned briefly in Section 1.2.6, constraints on the flexibility of random peptides can be introduced by engineering the peptide insert into the framework of another polypeptide. Frameworks that have been used include immunoglobulins (168), the human pancreatic secretory trypsin inhibitor (219), the α-amylase inhibitor, Tendamistat (172), the α-helical bacterial receptor domain of staphylococcal protein A (189) and the minibody, which contains more than one randomized region (266). It is not surprising that the immunoglobulin framework and related molecules have been employed for the grafting of random peptide libraries, since the V_H-V_L antigen-binding region can be viewed as six random peptide libraries constrained by the β sheet protein framework (168, 191). However, other polypeptides have also been examined for their abilities to display random peptides. McConnell and Hoess (172) expressed random peptides in two regions (amino acids 38-40 and 60-65) on the α-amylase inhibitor Tendamistat. Several features of this protein make it a suitable scaffold for random peptides, including its small size (74 amino acids), the availability of both NMR and X-ray structural information and its considerable stability due, in part, to the two disulphide bonds found in the protein (212). Tendamistat-displayed peptides that bind to the anti-endothelin monoclonal antibody, A8, were isolated and interestingly, were found to differ dramatically from peptides that were isolated from simple libraries of random peptides expressed on gpIII of phage and selected on the same antibody. This study reinforces the view that there are a number of ways in which peptides can bind to the same molecule and illustrates how the context in which they are displayed influences which peptide sequences will be isolated. One advantage proposed by the authors is that structural studies of peptides in the Tendamistat framework are much simpler since advantage can be taken of the known structure of Tendamistat itself.

Random peptide libraries have not only been used in epitope mapping and mimotope identification but also in a range of other applications. Intracellular signalling pathways have been studied by identifying peptides that bind to the receptors involved in signal transduction. Small peptide agonists, which possess activity that is comparable to the native protein, have been isolated from random peptide libraries. Two notable examples relate to the isolation of peptide agonists which mimic the properties of the polypeptide hormones erythropoietin (EPO) and thrombopoietin (TPO). EPO has been shown to interact with the EPO receptor (159), upon which an intracellular signalling cascade results in the stimulation of erythropoiesis. The extracellular domain of the EPO receptor was screened on a cyclic peptide library from which was isolated a cysteine-flanked 8 amino acid peptide (GGCRIGPITWVCGG) with an affinity of about 10 μM. Phagemid display mutagenesis was then used to obtain a group of 14 amino acid cyclic peptides that had 10 to 50-fold higher binding affinities and a common consensus motif (YXCXXGPXTWXCXP). Interestingly, these peptides were found to be full EPO receptor agonists *in vitro*, even though they lacked primary sequence homology to EPO, as they were able to stimulate the same intracellular signalling cascade as EPO and support cell proliferation. Furthermore, the peptides were found to also stimulate erythropoiesis in *in vivo* animal models (279).

In the other example, screening of peptide libraries on the TPO receptor resulted in the isolation of a 14 amino acid peptide (IEGPTLRQWLAARA) which has nanomolar affinity for the TPO receptor and was shown to stimulate the *in vitro* proliferation of a TPO responsive cell line. Remarkably, when this peptide was dimerized, it was found to be 4000-fold more potent than the monomeric form and equipotent to recombinant TPO. Moreover, the dimer was shown to increase megakaryocyte formation from human bone marrow cells *in vitro*, to a similar degree as recombinant TPO, and increase platelet production in mice (73). Thus it is possible to isolate potent mimetics of proteins that also have therapeutic potential. These examples further illustrate how the context in which a peptide is presented can have a dramatic effect on the binding properties of the peptide.

Conversely, peptide antagonists have also been identified using random peptide libraries. Intact human platelets have been screened on a peptide library in order to identify a peptide antagonist of the thrombin receptor (87) and similarly, antagonists to calmodulin have been identified (203). Peptide antagonists to angiogenin have also been isolated that inhibit the interaction of angiogenin with actin which ultimately could result in the inhibition of tumour growth and provide a new anticancer therapy (112).

One area where random peptide libraries displayed on phage have had a large impact is in the identification of peptide ligands of proteins, substrates and enzymes. 15-mer peptides with the consensus motif, Arg-X-Tyr-Trp, have been isolated after the selection of porcine pancreatic α-amylase on a random peptide library. The high affinity peptide ligands were also found to cross-react with barley α-amylase with dissociation constants in the nanomolar range (277). These ligands could potentially be used as antagonists of amylase functions. Peptide substrates for metalloproteinases have also been isolated using a similar approach (242). In a dramatic example, Koivunen *et al.* (145) have identified a 10 residue peptide that inhibits matrix metalloproteinases (MMP) 2 and 9, but not several other MMPs. This disulphide-constrained peptide not only inhibits these enzymes, but it selectively targets angiogenic blood vessels and thus inhibits the migration of tumour cells *in vivo*. This study not only demonstrates that peptides with useful biological functions can be obtained using phage display but that small peptides can have a variety of functions.

Peptide ligands that inhibit conformational changes in the human immunodeficiency virus (HIV) type-1 glycoprotein gp120, necessary for HIV attachment to the cellular receptor, CD4, have been identified from libraries of random peptides. These peptides have been shown to inhibit the binding of CD4 with gp120 from three different HIV strains. It has been suggested that the peptide ligands interact with a conserved domain on gp120 and may prove useful in the development of inhibitors of HIV entry (98).

Phage display has also been used to identify peptides that could be used as lead compounds in the development of peptidomimetic drugs. It has been suggested that the use of the D-enantiomers of peptides in drug development is more appropriate as they are not efficiently processed by the immune system and do not succumb to proteolytic degradation by the body. The D-enantiomers of proteins have been produced and used to select for L-peptides whose D-enantiomers bind the natural L-enantiomeric protein in what has been referred to as mirror-image phage display (228).

Several groups have extended the phage display technology to select for peptides that bind and enter a variety of different cell types using a variety of approaches including intact cultured cells and *in vivo* selection techniques using live organisms. The advantages of selecting peptide libraries on whole intact cells is that the cell surface receptors will be presented in their native conformation, purification of the specific protein receptor is not required nor is any previous knowledge about the targeted receptors (66, 107). However, peptides will be obtained that bind to many different surface molecules and it is difficult to target desired receptors specifically. In addition, extensive characterization is required after peptides have been enriched in order to identify the binding partner(s). Peptides that bind specific organs or cell types could be used for diagnostic imaging, for the targeted delivery of drugs or for the delivery of DNA in gene therapy applications. Cell-specific peptides that bind the membrane surface of human neutrophils have been isolated from libraries displaying linear 9-mer and cyclic 6-mer and 10-mer peptides. The peptides were found to induce receptor-mediated functions in neutrophils resulting in an increase in the intracellular calcium concentration (170). Thus using whole cells, cell-specific diagnostics and modulators of intracellular signal transduction pathways can be identified without prior knowledge of the receptors involved. Currently there is no gene therapy technique available for transfecting large masses of muscle in the body, which in some cases is necessary for the treatment of inherited myopathies such as muscular dystrophy. Using a combined *in vitro* and *in vivo* selection procedure, peptides which bind specifically to

muscle tissue were isolated from a 7-mer peptide library. Three rounds of selection were performed *in vitro* on muscle cells and two rounds were performed *in vivo* by injecting mice with the library, removing the relevant tissues and amplifying the bound phage. A peptide was isolated which the authors suggest could potentially be used to target therapeutic gene constructs to muscle cells (225).

Pasqualini and Ruoslahti dramatically demonstrated the power of phage display in selecting peptides that are able to specifically target a variety of organs in mice. Peptides targeting the vasculature of the brain, kidney, lung, pancreas, skin, intestine, uterus, adrenal gland and prostate have been isolated by injecting peptide libraries into mice (199, 207). It was found that peptides specific for the brain, when coated onto red blood cells, were able to localize these cells to the brain. Clearly, these peptides have considerable promise for targeting drugs and genes to desired tissues (199). Indeed, the same group have isolated peptides using phage display that localize specifically to tumour blood vessels. Interestingly, one of these peptides contained the integrin binding RGD motif. They also report the exciting observation that the efficacy of the anticancer drug doxorubicin against transplanted tumours in mice was enhanced when coupled to these peptides (15). The authors suggest that this strategy of targeting the vasculature of tumours rather than the tumour itself has advantages since the tumour is starved of nutrients and mutations in the tumour cells are unlikely to overcome this effect.

In another notable example involving tissue-specific targeting, peptides that had previously been shown to target the lung microvasculature when injected into mice (207) were found to bind specifically to the 55 kDa lung surface zinc metalloprotease, membrane dipeptidase (MDP). Interestingly, one peptide (CGFECVRQCPERC) was found to inhibit MDP enzyme activity by binding to the active site (208). This illustrates not only how small peptides can be used for tissue-specific targeting of therapeutics, but also highlights the multifunctional properties that many of these peptides possess. In other studies, peptides that bind cell receptors and undergo endocytosis have also been isolated

from mammalian cells (133). These peptides could potentially be used in the development of tissue-specific diagnostics and targeted delivery of therapeutics.

Many peptide libraries referred to previously have been primarily of random peptides displayed on phage. In many cases however, the desired peptide many not be represented in the libraries, which may pose a problem particularly for those trying to identify an epitope of an antibody. In order to assist in the isolation of the important disease-related epitopes on specific proteins, gene fragment libraries can provide a more focused approach compared with random peptide analysis. Genes that encode proteins of interest can be randomly fragmented and inserted into the genes encoding the coat proteins of filamentous phage. The result is a phage library displaying random peptides of various sizes, derived from the open reading frame of the protein of interest. Many of the fragments are likely to assume their native folded structure. In one study, a gene fragment library composed of a malarial protein, AMA-1, was constructed to aid in the fine mapping of epitopes recognized by two monoclonal antibodies (MAb5G8 and MAb1F9). Two protein fragments were obtained that overlapped by 19 amino acids. Thus the epitope for MAb5G8 was mapped to a 19 amino acid sequence within the AMA-1 prosequence. MAb5G8 was also simultaneously screened on a 15 residue random peptide library which produced a single binding peptide. Remarkably, the 15 residue random peptide contained within it a AYP motif which was identical to that found within the 19 amino acid sequence isolated from the gene fragment library. Furthermore, a synthetic 19 amino acid peptide containing the AYP motif was shown to inhibit the binding of AMA-1 to MAb5G8 whereas the same peptide lacking the AYP motif showed no inhibition (60). This work illustrates how gene fragment and random peptide libraries may complement each other in the elucidation and fine mapping of epitopes as well as providing structure-function information on important proteins. This approach also provides a rapid alternative to epitope mapping, which may facilitate vaccine design. Gene fragment libraries may also be useful for identifying discontinuous epitopes that require a specific polypeptide conformation. However, the expression of a fragmented gene may in some

cases result in the absence of a discontinuous epitope in the library, particularly if such an epitope is comprised of amino acids that are derived from extreme ends of the protein.

Peptides that mimic non-proteinaceous molecules have also been identified using random peptide libraries. In one example, peptide mimotopes of the low molecular weight mycotoxin, deoxynivalenol, were isolated from a random 7-mer peptide library by screening a monoclonal antibody that binds deoxynivalenol (284). This approach has also been used to identify peptides that mimic carbohydrates (125, 202, 230). Carbohydrate antigens are important targets for the development of anti-cancer therapeutics as they constitute the most abundant antigens on the surface of the cancer cell and appear to provide the tumour with a selective growth advantage. Vaccine development using traditional methods has been tedious since the carbohydrate antigens are poorly immunogenic, difficult to purify and synthesize, and do not elicit a lasting immune response (206). Using monoclonal antibodies that recognized cancer-related carbohydrate antigens, mimotopes were isolated from a 15-mer library that were able to elicit the production of protective antibodies. These antibodies were found to be cytotoxic to a fibrosarcoma cell line expressing the relevant carbohydrate antigen (206). Thus random peptide libraries can play an important role in the development of therapeutic vaccines against both proteinaceous and non-proteinaceous antigens of pathogens. Mimotopes of carbohydrates have also been identified that bind to a range of carbohydrate-binding antibodies (121, 261, 282) and the protein concanavalin A (194).

Peptides that bind to non-proteinaceous compounds such as streptavidin and DNA have also been identified (53, 113, 140, 270). In one interesting study, a 12-mer library was screened on the anti-cancer drug, paclitaxel otherwise known as Taxol (217). Paclitaxel has been shown to promote microtubule assembly by binding to tubulin (157) and also to induce apoptosis by causing the phosphorylation and hence inactivation of the anti-apoptotic protein, Bcl-2 (119), however, no interaction between Bcl-2 and paclitaxel has previously been observed. The authors initially expected to identify peptides that

mimicked tubulin, however, no such peptides were identified. Interestingly, isolated peptides were found to exhibit significant similarity to regions of Bcl-2. Furthermore, subsequent studies revealed that paclitaxel binds to Bcl-2 with a dissociation constant of about 400 nM and this binding results in a conformational change of Bcl-2. Therefore, peptides isolated using phage display have enabled the identification of a new molecular target for paclitaxel and the delineation of the molecular basis of this anti-cancer drug (217).

1.3 Applications of peptide display in malaria research

The ability of the malarial parasite, *Plasmodium*, to evade its host's immune response has inhibited progress towards the eradication of the disease (see Section 1.1.6 Immune Evasion). Antigenic variation has been the major stumbling block in the design of antimalarial vaccines as the parasite can produce new variants of red cell surface-exposed proteins in each new generation of parasites (215). However, people who live in malaria-endemic regions are able to develop some level of acquired immunity to the disease (44, 58, 214). Antibodies have been shown to be important in the protective immune response, both in humans and in animal models (54, 58, 62) and a range of protective antibodies has been characterized that bind to various malarial proteins and inhibit processes essential to the malarial life cycle (71, 258). For a number of inhibitory antibodies, the corresponding antigens have been identified but the exact epitopes are not known. Phage peptide-display has been applied in a few studies (1, 78, 79, 247) to assist in the development of antimalarial vaccines by trying to reproduce or mimic an immune response. For example, mimotopes to a monoclonal antibody that recognizes a repeat region of the *P. falciparum* circumsporozoite surface protein have been isolated from a 6-mer library. These mimotopes are able to elicit an immune response that recognizes a recombinant form of the protein containing the repeat region (247). Similarly, mimotopes of a fragment of the *P. vivax* merozoite surface protein-1 (PvMSP1) have been identified which produce an immune response that not only recognizes PvMSP1, but also the *P. cynomolgi* MSP-1 (78, 79). Thus, screening of random peptide libraries has allowed the selection of

mimotopes that are similar to the original antigen but are different enough to produce a range of antibodies that cross-react with variants of the original antigen (79). This range of cross-reactive antibodies could assist in vaccine design by producing an immune response that is protective against pathogens, like the malaria parasite, whose antigens are antigenically variable. In this study, the applications of peptide display in malaria research are investigated in relation to two vaccine candidate antigens, RESA and PfAMA-1. Monoclonal antibodies to both these antigens are used to screen peptide libraries with the aim of identifying mimotopes to the original antigens, which could assist in the understanding of the immune response to these antigens.

1.3.1 Mimotopes of RESA

The Ring-infected Erythrocyte Surface Antigen (RESA/Pf155) is a 155 kDa protein produced by the most pathogenic of the human malaria parasites, *Plasmodium falciparum*. Although some culture-adapted parasite strains do not express RESA, it is found in all field isolates of *P. falciparum* that have been tested suggesting that it facilitates survival of the parasite *in vivo* (200). RESA is produced in the final stages of schizont development and stored in dense granules within the developing merozoite (7). Following rupture and merozoite reinvasion, RESA is secreted into the newly formed parasitophorous vacuole, then transported, by an unknown mechanism, to the red cell membrane skeleton (72). RESA has been shown to interact with erythrocyte spectrin and to stabilize the erythrocyte membrane (74, 105). Here it remains for up to 24 hours (43). After associating with spectrin, RESA is phosphorylated, possibly by an exoparasitic kinase, primarily at serine but also at some threonine residues. Dephosphorylation is initiated some 3-4 hours post-invasion with little phosphorylation present after 12 hours. The purpose of phosphorylation is unknown, but has been suggested to cause disruption to interactions between cytoskeletal proteins, or affect the degree to which RESA binds spectrin (103).

The RESA gene consists of 2 exons separated by a short intron located 195 nucleotides downstream of the 5' end of the open reading frame. The first exon encodes a putative

signal sequence, the second exon encodes 1007 amino acids which include two distinct blocks of tandem repeats (Fig. 1.3.1) (97). The spectrin-binding region of the RESA polypeptide has been mapped to the region between amino acids 670 - 770 (102). RESA also contains a region that has homology to the conserved "J region" of the *E. coli* molecular chaperone, DnaJ (39). This region may be responsible for the proposed chaperone-like activity of RESA (74, 104). These functional regions of the RESA molecule are flanked by two regions of repetitive acidic amino acid sequence, the so-called 5' and 3' repeat regions. These acidic repeats represent immunodominant epitopes (97), and are recognized by sera of people who are naturally exposed to malaria (201). Indeed, a number of studies examining malaria endemicity and other seroepidemiological parameters have relied on synthetic peptides corresponding to the linear repeat sequences of RESA (181, 214, 221). The function of the repetitive sequences of RESA is not clear. Many malaria antigens have extensive regions of their amino acid sequence comprised of repetitive sequences, some of which are probably the targets of protective immune response (13). Other repeats, including some that are recognized as dominant epitopes by the host's immune system may function as molecular "smoke screens", decreasing the host's ability to mount an effective immune response (9, 143).

Although RESA is not exposed at the surface of the infected erythrocyte (7) and is not essential for growth *in vitro* (47), evidence from several studies have indicated that antibodies against RESA can inhibit the invasion of merozoites into the host erythrocyte (4, 269). Moreover, immunization of *Aotus* monkeys with recombinant RESA offered some protection from malaria challenge (62). This has led to the idea that antibodies to the RESA molecule might cross-react with another malarial protein that plays an important role in invasion or development of the intraerythrocytic parasite. A di-acidic motif found within both the 5' and 3' repeat regions of RESA is also found within the repeat regions of the falciparum interspersed repeat antigen (FIRA), the FC27 S-antigen, Pf332, Pf11.1 and erythrocyte band 3 (13, 129, 269). A human monoclonal antibody (MAb33G2) has been shown to cross-react with Pf322 and RESA (169). Indeed, anti-Pf322 antibodies that

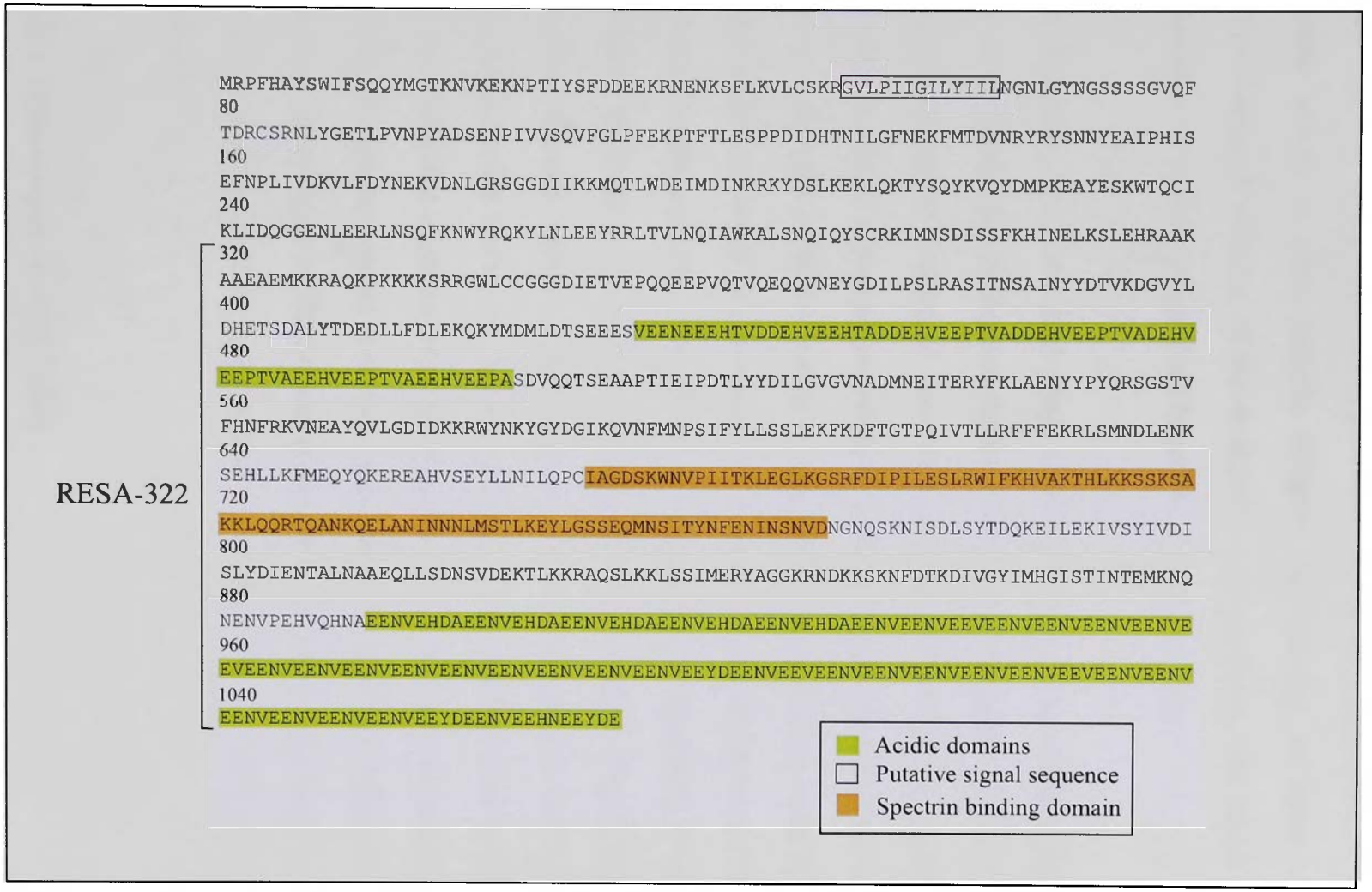


Figure 1.3.1. Amino acid sequence of RESA. The recombinant RESA-322 lacks the N-terminal 322 residues.

cross-react with the acidic repeat regions at the C-terminus of RESA were found to inhibit growth of parasites even when the parasite strain did not express RESA (269). These studies suggest that antibodies recognizing the repeat regions of RESA may be important anti-malarial agents due to their promiscuous binding activity and to the presence of di-acidic motifs in many parasite antigens. Interestingly, in recent clinical trials, the Combination B vaccine, of which RESA is a component, was found to reduce parasite densities by 62% in 5-9 year old Papua New Guineans (10).

In this study, we have used phage peptide technology to obtain information about the binding specificity of an anti-RESA monoclonal antibody, MAb18/2. MAb18/2 was raised against a C-terminal recombinant fragment of RESA (residues 893 - 1073) containing the 3' repeat sequences (11). It has also been shown to cross-react with the 5' repeat sequence of RESA which is also rich in acidic amino acids (11). MAb18/2 has been used extensively as a research tool to reveal aspects of the role of RESA in the newly infected erythrocyte (74, 105). Due to the availability of MAb18/2, it was deemed to be a suitable antibody for use in investigating the applications of phage peptide-display in malaria research. MAb18/2 was used to pan two independent random peptide libraries displayed on the surface of filamentous phage. Peptides that bind to the antigen-binding site of MAb18/2 might be expected to mimic the structure of the acidic repeat regions of RESA. It was anticipated that these peptides would provide information about the fine structure of epitopes on RESA and might prove useful in understanding the nature of the epitopes of cross-reactive antigens that may help parasites evade the protective immune response.

1.3.2 Mimotopes of PfAMA-1

The apical membrane antigen-1 (AMA-1) is a protein identified in multiple species of *Plasmodium* and believed to be a member of a family of cysteine-rich rhoptry proteins which includes MAEBL (187). These proteins are thought to be erythrocyte-binding proteins involved in host cell invasion. AMA-1 is produced by mature schizonts in the

final stages of the erythrocytic cycle and is stored within the parasite in the neck of apical secretory organelles, which are referred to as the rhoptries. After erythrocytic rupture and prior to invasion, a processed version of AMA-1 is released from the rhoptries and redistributed to the surface of the apical region of the merozoite. Therefore, AMA-1 is thought to be instrumental in the erythrocytic invasion process (253).

P. falciparum AMA-1 is initially an 83 kDa protein which is proteolytically processed at the N-terminus to a 66 kDa form (184). It contains 16 conserved cysteines which when disulphide-bonded in the native state, delineate the molecule into three distinct ectodomains (Fig. 1.3.2). The disulphide-bonded ectodomain of AMA-1 has been shown to be conserved between species of *Plasmodium* suggesting that AMA-1 may play a pivotal role in parasite survival. The unprocessed AMA-1 molecule contains a prosequence preceded by a putative signal sequence at the N-terminus. The ectodomain is anchored to the membrane by the transmembrane domain and a short cytoplasmic domain at the C-terminal end extends into the parasite cytosol. Unlike many other asexual stage *Plasmodium* antigens, AMA-1 lacks regions of repetitive sequence which often form immunodominant epitopes (124).

AMA-1 is currently an important vaccine candidate as it has been shown to induce a protective immune response in rodents and primates. Mice immunized with recombinant refolded ectodomain of AMA-1 from the murine parasite *P. chabaudi* (PcAMA-1) have been shown to be partially protected when challenged with malaria, whereas mice immunized with unfolded PcAMA-1 were not protected (12). This suggests that the protective immune response is dependant upon a native conformation stabilized by the disulphide bonds (124). The passive transfer of antibodies has also been shown to be protective (12), further supporting the assertion that AMA-1 has an important functional role in parasite survival.

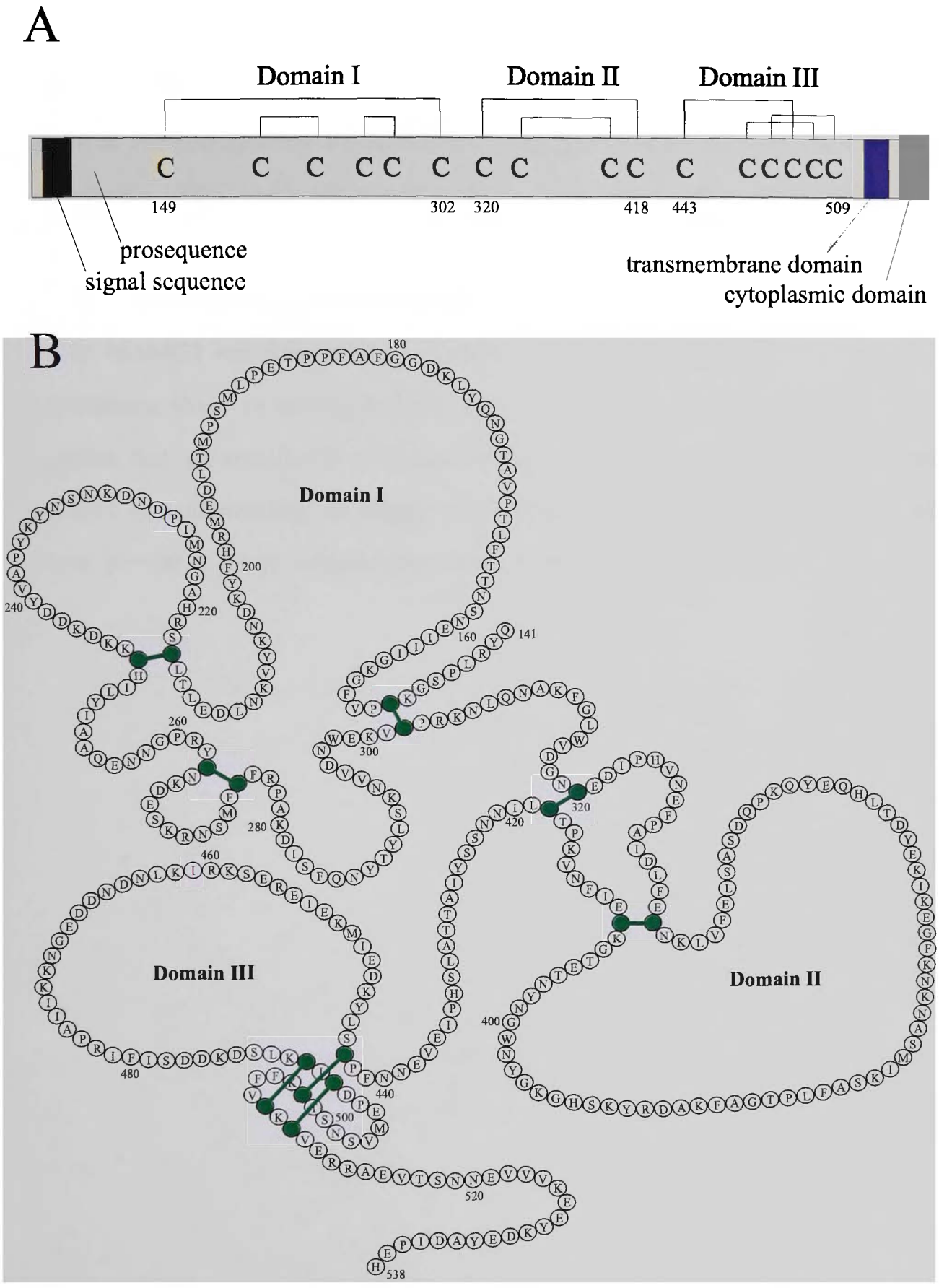


Figure 1.3.2. Structure of the apical membrane antigen-1 (AMA-1). (A) Cartoon illustrating the structure and disulphide bonds of PfAMA-1 (B) Primary sequence of the ectodomain of PfAMA-1 illustrating how disulphide bonds between the conserved cysteines (green circles) delineate the molecule into three domains. Adapted from Hodder *et al.* 1996 (124).

In this study, a protective monoclonal antibody raised against the ectodomain of *P. falciparum* AMA-1 (MAb4G2) was used to identify protective epitopes on AMA-1 which could be included as part of a vaccine formulation. MAb4G2 is a rat monoclonal antibody of subclass IgG2a which reacts with AMA-1, in its native conformational state, from multiple *P. falciparum* strains. Whilst the exact epitope of MAb4G2 is unknown, it is likely that it recognizes a critical conformational epitope on the ectodomain of PfAMA-1 (144). MAb4G2 was screened on a 15 amino acid random peptide library displayed on filamentous phage to identify peptides that bind to the monoclonal antibody. Such peptides that are specifically recognized by MAb4G2 might be expected to represent epitopes or conformational mimotopes of critical functional epitopes on PfAMA-1 and hence, provide an insight into the nature of the protective immune response.

Chapter 2:

Materials and Methods

2.1 Compounds

A MilliQ water filtration system was used to produce fresh sterile deionised water that was used for all buffers and reagents.

2.2 Parasites

The *Plasmodium falciparum* cloned lines FAC-8 and D10 were continuously cultured as described by Trager and Jensen (254). Briefly, *P. falciparum* were cultured in human erythrocytes that were maintained at a 7% hematocrit in RPMI 1640 (CSL, Australia) supplemented with 25 mM HEPES, 21.6 mM $NaHCO_3$, 2.1 mM L-glutamine, 35 μg/ml gentamicin (Sigma), 4.3% inactivated AB+ human serum and 0.22% Albumax (GIBCO BRL, Grand Island, NY), pH 7.3. The parasitemia was maintained below 15% and determined by calculating the number of parasitized erythrocytes in relation to the total number of erythrocytes in stained smears. Smears of parasitized erythrocytes were stained using the Quick Dip (Histo Labs, Riverstone, NSW) staining method in accordance with the manufacturer's instructions.

2.3 Phage library preparation

The 15-mer phage-peptide library was kindly provided by George Smith, University of Missouri, Columbia, MO, USA (231) and the 17-mer library by Jamie Scott, Simon Fraser University, British Columbia, Canada (37). These libraries were constructed by incorporating random oligonucleotide inserts of defined length into gene III or gene VIII. Both libraries carry a tetracycline resistance gene that allows for their selection on media

containing tetracycline. The diversity of the 15-mer library is estimated to be about 3.2×10^9 particles and was provided at a concentration of 2×10^{14} particles/ml (238). The 17-mer library, which has a diversity of 1.2×10^{12} clones, was provided as a primary library stock of 1×10^{12} particles/ml, which had not been amplified since the initial amplification when the library was made (36). Phage were amplified, based on a procedure by Smith and Scott (240). A single colony of *E. coli* K91 grown on minimal media was inoculated into 5 ml of LB media and grown to log-phase at 37°C. 2 ml of this log-phase culture was inoculated with phage (~1×10^{10} particles) and incubated at room temperature for 20-30 min to allow infection of the phage into the *E. coli* K91. The infected *E. coli* K91 were then inoculated into 200 ml of LB media containing 50 µg/ml tetracycline and shaken overnight at 37°C. The supernatant was twice clarified by pelleting the cells at 10,000 × g for 15 min and 0.2 volumes of PEG solution (20% polyethylene glycol 8000, 2.5 M NaCl) was added. The sample was incubated at 4°C for at least 2 h before being centrifuged at 16,000 × g for 50 min to precipitate the phage. The supernatant was discarded and the pellet briefly recentrifuged at 653 × g for 3 min to collect the residual supernatant, which was discarded. The centrifuge bottles were allowed to drain for 20-30 min in order to remove all residual supernatant. The phage pellet was resuspended in 1 ml of TBS (50 mM Tris, 150 mM NaCl, pH 7.5) and stored in the dark at 4°C with 0.02% NaN_3. no NaN_3 was added when phage were used for mouse immunizations. Instead, phage were applied to a Detoxi-Gel Endotoxin Removing Gel (Pierce Chemical Co. Rockford, IL) column in order to remove bacterial lipopolysaccharide which may be toxic to the mice. The column was prepared in accordance with the manufacturer's instructions by regenerating it with 5 column volumes of 1% sodium deoxycholate, followed by washing with 5 column volumes of water and 1 column volume of PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 8). The phage were applied to the column and gently drawn through with the aid of a syringe. The eluted fractions were titred for phage and the phage containing fractions were stored at 4°C.

2.4 *Escherichia coli*

E. coli K91 strain was kindly provided by George Smith, and was maintained on minimal media and stored indefinitely as glycerol stocks. Glycerol stocks were prepared by combining 800 µl of log-phase *E. coli* culture with 150 µl of 100% glycerol and vortexing until a homogenous solution was obtained. The glycerol stocks were then snap frozen in liquid nitrogen and stored at -80°C. *E. coli* K91 containing phage were also stored as glycerol stocks. Phage were recovered by scraping cells from the glycerol stock and growing them overnight at 37°C in 200 ml of LB media containing 50 µg/ml tetracycline. Phage were isolated as described previously.

2.5 Preparation of media

M9 Minimal media and Lauria Bertani (LB) media was prepared as described previously (224).

2.6 Panning the phage libraries

An adaptation of the technique used by Parmley and Smith (197) was used to screen the phage-peptide libraries on both MAb18/2 (11) and MAb4G2 (a kind gift from Alan Thomas, Dept. of Parasitology, Biomedical Primate Research Centre, Rijswijk, The Netherlands). MAb18/2 (5 µg/ml) or MAb4G2 (10 µg/ml) in 100 µl of binding buffer (0.1 M $NaHCO_3$ pH 8.6) was coated onto the wells of a 96 well microtitre plate (F96 Maxisorb Nunc-Immuno plates) overnight at 4°C in a humidified container. The wells were blocked for 2 h at 4°C with 400 µl of blocking solution (0.5% BSA, 0.1 M $NaHCO_3$, pH 8.6). Phage (approx. 10^{11} particles) in 100 µl of probing solution (0.5% BSA in TBS) were added to the wells and gently shaken for 50 min at room temperature. After incubation the wells were washed vigorously 2 times in the first round and 4 times in subsequent rounds of panning with TBS-T (0.5% Tween 20 in TBS) to remove non-binding phage. Phage bound to the antibody were eluted with 100 µl of elution solution (0.1 M glycine, pH 2.2) for 15 min at room temperature and neutralized using 7 µl of 2 M

Tris. In some cases 1 µg of the appropriate antigen (recognized by the MAb), diluted in 100 µl of probing solution, was added to the wells and shaken for 30 min at room temperature in order to elute the bound phage. The titre of eluted phage was estimated and a 80 µl aliquot of the eluted fraction was used to infect *E. coli* K91 cells for amplification.

2.7 Phage titrations

Phage were titred according to the procedure described by Scott and Smith (231). Phage were subjected to serial tenfold dilutions using 100 µl of water in a 96 well (non-protein binding) plate (Nunc International). To each of the phage dilutions, 100 µl of log phase *E. coli* K91 cells were added and incubated at room temperature for 20 min to allow infection of the phage into the *E. coli* cells. A 20 µl aliquot of each dilution was spread onto agar plates containing 100 µg/ml tetracycline (LB/Tc), the plates were inverted and incubated overnight at 37°C. Phage infection of bacteria confers resistance to tetracycline and such colonies were counted and expressed as colony forming units per ml (cfu/ml). The cfu/ml is equivalent to the number of phage per ml.

2.8 Immunoprecipitation of parasite-produced RESA from culture supernatants

Protein-A Sepharose CL-4 beads (Pharmacia Biotech Inc, San Francisco, CA) were reconstituted according to the manufacturer's instructions. 250 µl of packed reconstituted beads were combined with 23 µg of MAb18/2 in 0.5 ml of TBS and incubated with rotation for 1 h at 4°C. 1.5 ml of culture supernatant from ring-stage *P. falciparum* D10 strain laboratory cultures was then added and incubated as before for a further hour. Protein-A Sepharose beads were pelleted by low speed centrifugation and washed 5-6 times in TBS to remove unbound material. Bound antibodies and RESA were removed from the beads by boiling for 5 min in sample buffer (10% glycerol, 63 mM Tris pH 6.8, 2% SDS, 0.0025% bromophenol blue) containing 100 mM dithiothreitol (DTT), the beads

were pelleted and the supernatant analysed by SDS-polyacrylamide gel electrophoresis followed by Western blotting.

2.9 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

10^{10} phage particles from selected clones and other proteins were boiled for 3 min in the presence of 0.5 volume of sodium dodecyl sulphate (SDS) sample buffer plus 10% β-mercaptoethanol or 50 mM DTT and applied to precast 10-20% Tricine gels (Novex, San Diego, CA.). The gels were immersed in tricine buffer (0.1 M Tris, 0.1 M Tricine and 3.5 mM SDS, pH 8.3). The phage proteins were electrophoretically separated in the Novex XCell II SDS-PAGE apparatus overnight at 4°C and 30 volts, until the bromophenol blue dye marker exited from the bottom of the gel. Kaleidoscope Prestained Standards (Biorad) were used to estimate the molecular weight of the electrophoresed proteins. This procedure was carried out when immunoprecipitates were analysed.

2.10 Western blotting

SDS-PAGE separated proteins were transferred to polyvinylidene difluoride (PVDF)-Plus transfer membrane (Millipore. Bedford, MA) using the Western Transfer apparatus model TE22 (Pharmacia Biotech Inc, San Francisco, CA) at 4°C and 100 volts for 2 h according to the manufacturer's instructions. The PVDF-Plus transfer membrane was blocked overnight in 10% blotto (10% skim milk powder in TBS) to quench the remaining protein binding sites and probed with MAb18/2 or MAb4G2 diluted 1:1000 in 5% blotto with agitation for 45 min at room temperature. After washing 3 times for 5 min, horse radish peroxidase (HRP) conjugated anti-mouse IgG (Sigma Chemical Co. St Louis, MO) or HRP conjugated anti-rat IgG for MAb4G2 (Sigma Chemical Co. St Louis, MO) diluted 1:2000 in 5% blotto was used as a secondary antibody and incubated as before for 45 min at room temperature. Binding of the secondary antibody was detected using enhanced chemiluminescence (ECL) (Pierce Chemical Co. Rockford, IL) according to the manufacturer's instructions. To detect the binding of phage, the PVDF-Plus transfer membrane was stripped of antibodies by incubating it in stripping solution (2.5 mM

glycine and 3.75 mM NaCl, pH 2.2) for 15 min at room temperature. The membrane was then washed 5 times for 5 min in TBS, blocked overnight in 5% blotto and probed with an anti-M13-HRP conjugated antibody (Pharmacia Biotech, Quarry Bay, Hong Kong) diluted 1:1000 in 5% blotto for 45 min at room temperature. After a further three washes for 5 min each, antibody binding was detected using ECL.

For experiments involving mouse sera, the membrane was probed with mouse sera diluted 1:100 or MAb18/2 diluted 5 μg/ml in 10% blotto for 50 min at room temperature and then washed 6 times for 20 min in TBS-T. Anti-mouse IgG-HRP diluted 1:2000 in 10% blotto was used as the secondary antibody and incubated for 50 min at room temperature. Binding of the secondary antibody was detected using ECL.

2.11 Dot blotting experiments

10^{10} phage of each clone were spotted in triplicate onto nitrocellulose membranes (NCF) (Millipore. Bedford, MA) with the aid of a dot blotting apparatus (BioRad) and adsorbed onto the NCF with the aid of a vacuum pump. The membrane was incubated twice for 20 min in two changes of 5% blotto and then divided into three strips with each strip containing one of every clone. One NCF strip was probed with MAb18/2, washed 3 times for 5 min with TBS-T and probed with HRP conjugated anti-mouse antibody. Another NCF strip was probed with HRP conjugated anti-M13 antibody and the third strip was probed with HRP conjugated anti-mouse antibody alone. All antibodies were diluted 1:1000 in 5% blotto and the membrane strips probed for 45 min at room temperature with agitation. After washing the NCF strips 3 times for 5 min in TBS-T, antibody binding was detected using ECL in accordance with the manufacturer's instructions. For dot blotting experiments involving MAb4G2, immobilized phage and PfAMA-1 (0.1 μg) were probed with MAb4G2 followed by anti-rat-HRP conjugated antibody.

2.12 ELISAs

Enzyme-linked immunosorbent assays (ELISAs) were performed, using a procedure similar to that described by Harlow and Lane (120). In all ELISAs, the washing steps and incubations were performed as described for phage-ELISAs. In phage-ELISAs, 96 well microtitre plates (F96 Maxisorb Nunc-Immuno plates) were coated with MAb18/2, polyclonal rabbit antisera raised against rat chaperone 60, anti-cpn60 (a kind gift from Prof. N. Hoogenraad, Department of Biochemistry, La Trobe University, Australia), MAb4D6, a mouse monoclonal antibody reactive with apical membrane antigen (AMA-1) from *Plasmodium chabaudi* (kindly donated by R. Anders, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. Unpublished) or MAb4G2 and MAb4F2, the isotype control antibody, (both a kind gift from Alan Thomas, Dept. of Parasitology, Biomedical Primate Research Centre, Rijswijk, The Netherlands) (5 µg/ml) diluted in 100 µl of binding buffer overnight at 4°C in a humidified container. The wells were blocked for 2 h at 4°C with 400 µl of blocking solution. Phage, diluted in 100 µl of probing solution, were added to the wells and incubated for 45 min at room temperature with gentle shaking. The wells were then washed 5 times using TBS-T to remove non-binding phage. Bound phage were detected with an anti-M13-HRP conjugated antibody (Pharmacia Biotech, Quarry Bay, Hong Kong) diluted 1:1000 in 100 µl of probing solution and incubated for 45 min at room temperature with gentle shaking. The wells were then washed 5 times with TBS-T. O-phenylenediamine (OPD) prepared in accordance with the manufacturer's instructions was used as an enzyme substrate and spectrophotometrically analysed at 450 nm or upon the addition of 50 µl of 3 M HCl per well at 492 nm. In some cases 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Sigma Chemical Co. St Louis, MO) prepared in accordance with the manufacturer's instructions was used as the enzyme substrate and spectrophotometrically analysed at 410 nm. For competition experiments, 10^{10} phage particles were added to the MAb18/2-coated or the MAb4G2-coated wells in the presence of increasing concentrations of recombinant RESA (RESA-322, (74)), synthetic peptide or PfAMA-1 (donated by R. Anders).

In ELISAs involving the binding of MAb18/2 or MAb4G2 to immobilized phage peptides in the presence of competing synthetic peptides, 10^{10} phage particles of each clone were coated onto microtitre plates. The phage were then probed with 0.44 μg/ml of MAb18/2 or 0.5 μg/ml of MAb4G2, in 100 μl of probing solution, in the presence of the relevant competing synthetic peptide. Binding was detected using an anti-mouse-HRP conjugated antibody (Amersham Australia Pty Ltd) or anti-rat-HRP conjugated antibody (Sigma Chemical Co. St Louis, MO), diluted 1:1000 in probing solution.

In ELISAs involving the detection of synthetic peptides, RESA or PfAMA-1, the synthetic peptides, RESA-322 or PfAMA-1 were coated onto the wells of microtitre plates. MAb18/2, MAb4G2 or MAb4G2, diluted in probing solution, were added to the wells as primary antibodies and detected using an anti-mouse IgG-HRP conjugated antibody or, for MAb4G2 and MAb4F2, HRP conjugated anti-rat IgG diluted 1:1000 in probing solution as described above. For competition experiments, increasing concentrations of synthetic peptide were added with the primary antibody.

ELISAs involving pooled human sera from Papua New Guinea (PNG) were performed in essentially the same manner as described above. 10 μg/ml of each synthetic peptide and 5 μg/ml of RESA-322 were coated onto microtitre plates and probed with human sera diluted to the relevant concentration in probing solution. An anti-human IgG-HRP conjugated antibody (Amersham Australia Pty Ltd) diluted 1:1000 in probing solution was used to detect the human sera.

In ELISAs involving mouse serum, 20 μg/ml of each synthetic peptide and 5μg/ml of RESA-322 were coated onto microtitre plates and probed with the relevant concentration of mouse serum. In competition experiments, 100 μg/ml of each synthetic peptide or 20 μg/ml of RESA-322 were added with the mouse serum. An alkaline phosphatase (AP) coupled anti-mouse IgG antibody (Sigma Chemical Co. St Louis, MO), diluted 1:1000 in

probing solution, was used to detect the mouse sera. Binding was detected using p-nitrophenyl phosphate (pNPP) alkaline phosphatase substrate (Sigma Chemical. Co. St Louis, MO) prepared according to the manufacturer's instructions and spectrophotometrically analysed at 405 nm.

2.13 Oligonucleotide primers

The oligonucleotide primers used for PCR amplification and sequencing of the oligonucleotide inserts were synthesized and reverse phase HPLC purified (Bresatec Pty Ltd, Australia). The lyophilized primers were resuspended in 1 ml of water and stored at -20°C. The primers and their respective concentrations are as follows. 15-mer library (gpIII): 5' primer, GAT AAA CCG ATA CAA TTA AAG, 8.02 pmol/µl; 3' primer, CAC AGA CAA CCC TCA TAG, 0.112 pmol/µl. 17-mer library (gpVIII): 5' primer, CTG AAG AGA GTC AAA AGC, 5.07 pmol/µl; 3' primer, CAA TTT CTT AAT GGA AAC, 9.4 pmol/µl.

2.14 PCR of individual colonies

Polymerase Chain Reaction (PCR) was employed to amplify the region of the phage genome encoding the peptide sequence. Colonies of *E. coli* K91 were picked from LB/Tc plates and inoculated into 20 µl of water. Phage DNA was released from the cells by boiling for 10 min. The cell debris was pelleted by centrifugation at 14240 × g for 5 min and 2 µl of the supernatant was used in the PCR reaction. The PCR reaction also contained 10 µl of 5 times PCR buffer containing 10 mM $MgCl_2$ (Promega), 5 µl of a 10 mM 2'-Deoxynucleoside 5'-Triphosphate (dNTP) solution comprising equal amounts of all four dNTPs (Pharmacia Biotech), 1 µl of each primer, 0.2 U of Taq DNA polymerase (Promega) and made to a total volume of 50 µl with water. A DNA Thermal Cycler Model 480 (Perkin Elmer) was used to amplify the deoxyribose nucleic acid (DNA) contained in the PCR reaction under the following conditions: An initial step of 95 °C for 10 min; followed by cycles of 95°C, 45°C and 72°C each for 30 sec and repeated

25 times; and finally 72°C for 7 min. Agarose gel electrophoresis and ethidium bromide staining was used to visualize the product of the PCR reaction.

2.15 Agarose gel electrophoresis

Agarose gel electrophoresis was performed essentially as described previously (224) using the submarine DNA electrophoresis apparatus (BioRad). Agarose gels were prepared by boiling 50 ml of 2% agarose in TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8). 1 µl of 10 mg/ml ethidium bromide was added to the molten agarose and poured into an appropriate mould to set at 4°C. 5 µl of the PCR reaction was combined with 1 µl of DNA agarose loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol) and loaded into the agarose gel. The DNA was electrophoretically separated at 100 V for 30 min submerged in TAE buffer and visualized using an ultraviolet transilluminator (Stratagene). Mark VI and Mark VIII (Amersham) DNA molecular weight markers for agarose gels were used to determine the molecular weight of the PCR products.

2.16 Nucleotide sequencing

Prior to sequencing, the PCR products were purified from the primers and other components of the PCR reaction mix using the Qiaquick 8 PCR purification kit (Quiagen) in accordance with the manufacturer's instructions. The purified PCR product was analysed spectrophotometrically at 260 nm and the DNA concentration calculated using a formula previously described (224). Each PCR product was diluted to 5 pmol per 16 µl with water and 1 µl of the relevant primer added. The PCR products were sequenced at least two times using automated dye terminator cycle sequencing (Amersham Australia Pty Ltd) by SUPAMAC, Department of Molecular & Clinical Genetics, Royal Prince Alfred Hospital, Australia. In some cases, the sequencing reaction was done using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) according to the manufacturer's instructions. Briefly, 8 µl of the Terminator Ready Reaction Mix

was combined with 2 µl of the PCR product, 1 µl of the relevant primer and 9 µl of water. A DNA Thermal Cycler Model 480 (Perkin Elmer) was used to amplify the DNA contained in the sequencing reaction under the following conditions: (96°C for 30 sec, 50° C for 15 sec, 60°C for 4 min) repeated 25 times. The sequencing products were purified by combining the sequencing reaction mix with 2 µl of 3 M potassium acetate, pH 5.6 and 50 µl of 95% ethanol, vortexing and incubating at 4°C for 10 min. The samples were centrifuged at 14000 × g to pellet the DNA and the supernatant discarded. 250 µl of 70% ethanol was used to wash the DNA pellet and then aspirated. The DNA pellet was dried using a Savant Speed Vac Concentrator and sent to be electrophoretically analysed at the Monash University Department of Microbiology, Microbial Biotechnology and Diagnostic Unit, Automated DNA Sequencing Facility, Australia. Sequences were analysed using DNASIS V2.1 (Hitachi Software Engineering Co., Ltd) computer software.

2.17 Peptide synthesis

Peptides 15(1), CFDYAPYVSAVDDIC; 17(3), GLKNCTVQPWDATDVCD; 17(3j), GAQLDCTVKTPDVWDCN; and 8-mer, $(EENVEHDA)_4$; cl-2pep, GWLSPSWFEPGLASM; cl-2pepE, GAGWLSPSWFEPGLASMGA and cl-25pep, TFVPILFWEHELNAS were synthesized and purified to 70-95% purity by Auspep Pty Ltd, Australia. Peptides RESA 0, SNDQKYSIEDSLTIK; 11-mer, $(DDEHVEEPTVA)_2$; BSA-11-mer, $(DDEHVEEPTVA)_3$; and 4-mer, $(EENV)_8$ were synthesized by the Joint Protein Structure Laboratory (Walter and Eliza Hall Institute of Medical Research and the Ludwig Institute for Cancer Research, Australia). The heteropolymer peptide, comprising peptides 15(1), 17(3) and SAVDD, and the cl. 2 polymer peptide (cl-2pep poly) were synthesized by Daniella Salvatore in the laboratory of David Jackson, Department of Microbiology, University of Melbourne, Parkville, Victoria, Australia (136). All peptides were solubilized in PBS to a concentration of 2 mg/ml and stored at -20°C.

2.18 Reduction and alkylation of peptides

20 µl (1 mg/ml) of peptides 17(3), 15(1) and 17(3j) were each combined with an equal volume of 40 mM DTT and incubated at 70°C for 5 min to reduce disulphide bonds. 10 µl of 0.2 M iodoacetamide was then added and incubated for 60 min in order to alkylate each reduced peptide. Electrospray ionization mass spectrometry (ESMS) was used to determine the extent of reduction and alkylation of the peptides. Peptides were subsequently coated onto the wells of a microtitre plate (10 µg/ml) and probed with MAb18/2 as described previously (see Section 2.12 ELISAs).

2.19 Electrospray ionization mass spectrometry (ESMS)

ESMS was performed by Dr. G. Neumann Department of Biochemistry, La Trobe University, Australia on a Perkin-Elmer Sciex API-300 triple quadrupole mass spectrometer fitted with a micro-ionspray ion source and calibrated to an accuracy of ± 0.01% using singly-charged poly(propylene glycol) ions. 1 µl samples at 1 mg/ml peptide were desalted using C18 reverse phase microcolumns and analysed in 10 µl of 50% acetonitrile, 0.1% formic acid at a flow rate of 0.2 µl/min. Signal-averaged raw mass spectra (50-100 scans over 5-10 min) were transformed to a true mass scale using P.E.-Sciex BioMultiview software.

2.20 Indirect Immunofluorescence assay

Indirect immunofluorescence assays were carried out essentially as described previously (31). Erythrocytes infected with the D10 strain of *P. falciparum* were smeared on glass slides and allowed to dry at room temperature. The smears were then immersed in cold acetone for 10 min and allowed to dry. The smear on the glass slide was divided into 1 cm^2 squares with nail polish. 0.5 µg of MAb18/2 and various amounts of the synthesized peptides, diluted in 20 µl of probing solution, were added to each square on the glass slide and incubated with gentle swirling of 40 min at room temperature. The slides were then washed 3 times for 5 min in TBS. A fluorescein isothiocyanate (FITC)

labelled anti-mouse antibody (Sigma Chemical Co. St Louis, MO), diluted 1:1000 in 20 µl of probing solution, was then added to each square on the glass slide for 40 min at room temperature. The slides were washed 3 times for 5 min in TBS and allowed to dry. A drop of n-propyl gallate (Sigma Chemical Co. St Louis, MO) prepared according to the manufacturer's instructions was then added to the centre of each square, a glass coverslip mounted on top of the slide was sealed onto the slide using nail polish. Fluorescence was viewed under oil immersion using an Olympus microscope, model BH2 with the BP 485 FT 510 515-565 filter and photos were taken using the Carl Zeiss C 35 Microscope Camera.

2.21 Peptide inhibition of antibody binding to parasite produced RESA

P. falciparum (FAC-8) was grown to approximately 10% ring-stage parasitaemia. The erythrocytes were isolated by centrifugation and incubated with 2 volumes of 0.2% saponin (Sigma Chemical Co. St Louis, MO) at 37°C for 20 min to release the haemoglobin. The lysed cells were washed numerous times in PBS pH 8 until all haemoglobin was removed from the cells. 25 µl of packed cells, diluted in 150 µl of probing solution, were incubated with 0.1 µg MAb18/2 in the presence or absence of peptide for 40 min at room temperature. The cells were washed 4 times with TBS. The amount of bound MAb18/2 was assessed by an AP-coupled anti-mouse IgG (Sigma Chemical Co. St Louis, MO), diluted 1:1000 in 150 µl of probing solution and incubated for 40 min at room temperature. The cells were washed 4 times with TBS followed by the addition of 200 µl of pNPP alkaline phosphatase substrate (Sigma Chemical. Co. St Louis, MO) prepared according to the manufacturer's instructions. Cells were pelleted and the supernatant was analysed spectrophotometrically at 405 nm or 420 nm.

2.22 Mouse immunization experiments

Female BALB/c mice, 10-12 weeks old, were obtained from the La Trobe University Central Animal House. For immunizations using phage, a similar protocol to that described previously was used (109). Briefly, a sample of preimmune sera was obtained at

day 0 and mice were inoculated intraperitoneally (i.p) with 10^{10} phage in 1 ml of PBS. At day 21 a boost i.p injection of 10^{10} phage in PBS was administered and the first eyebleed taken on day 31. A second boost of 10^{10} phage in PBS was administered on day 42 and a second eyebleed taken on day 52. A final boost of 10^{10} phage in PBS was administered on day 63 and a kill bleed taken on day 73. To isolate the sera from blood samples, the blood was incubated at 37°C for 2 h and then stored at 4°C overnight to agglutinate the blood cells. The blood was then centrifuged at $10000 \times g$ to pellet particulate matter and the supernatant decanted and recentrifuged to remove any remaining cells. The sera were then retained and stored at -20°C.

Chapter 3:

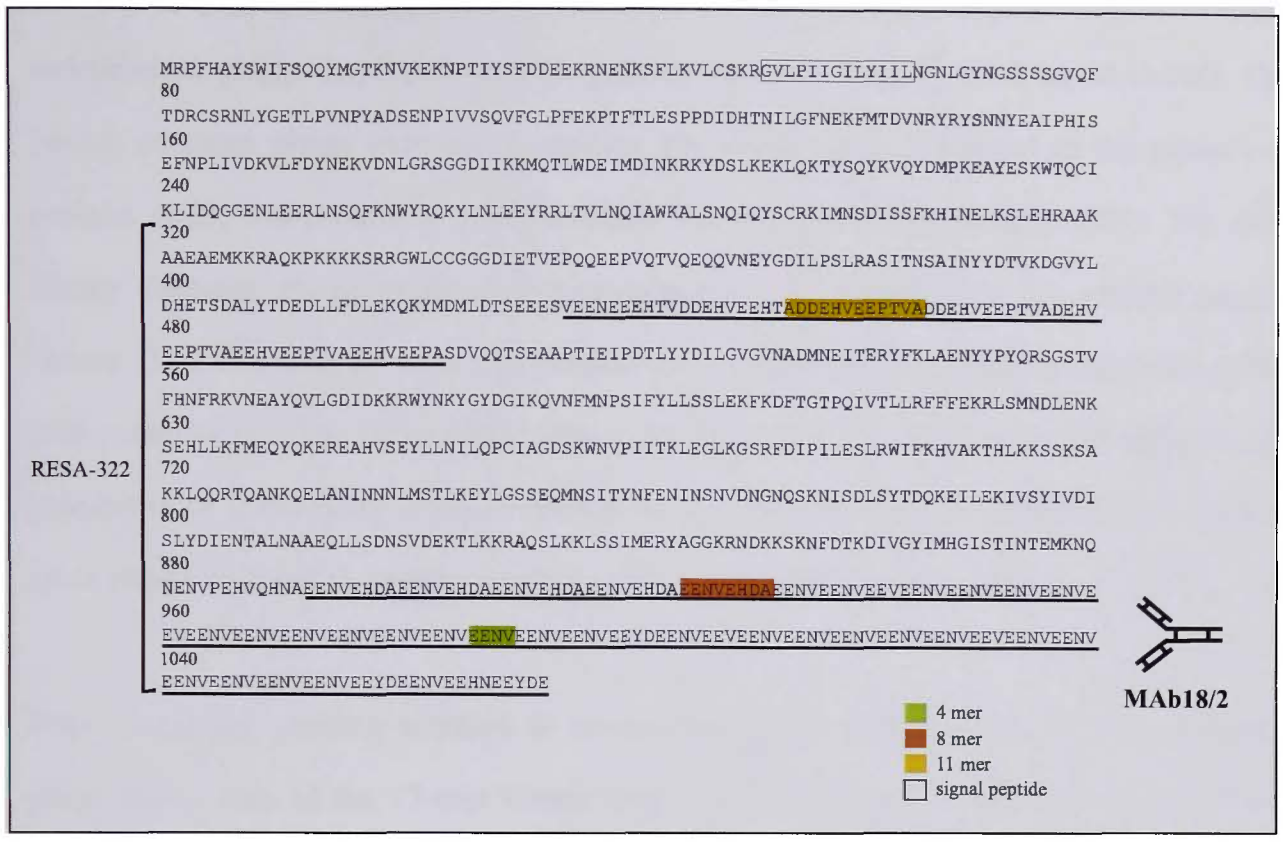
Results

3.1 Isolation of MAb18/2-binding peptides

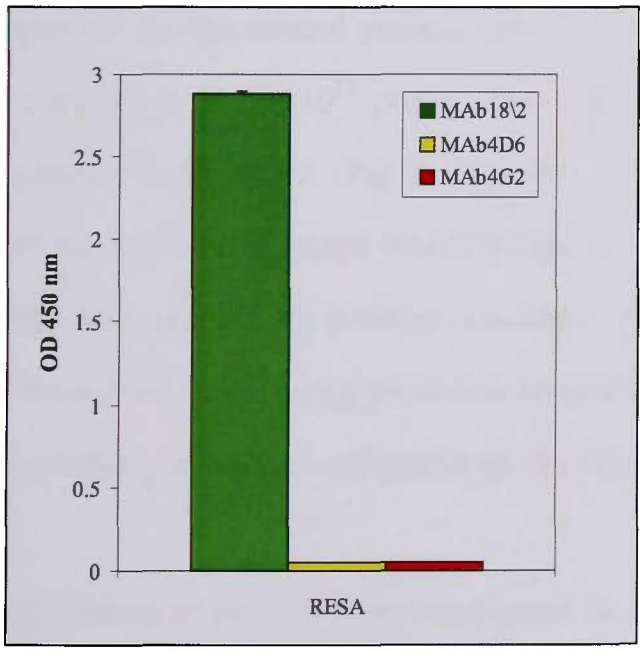
3.1.1 MAb18/2 binds to the acidic repeats of RESA

To confirm that the anti-RESA MAb18/2 recognizes recombinant RESA (amino acids: 322 - 1073), RESA-322 which lacks the N-terminal 322 amino acids (Fig. 3.1.1A) was immobilized onto a microtitre plate and probed with three monoclonal antibodies: anti- *P. chabaudi* AMA-1, MAb4D6; anti-*P. falciparum* AMA-1, MAb4G2; and MAb18/2. Only MAb18/2 bound to the immobilized RESA (Fig. 3.1.1B) illustrating the specificity of the antibody for RESA and that the epitope for the antibody does not rely on the N-terminal 322 amino acids. It was determined that only a very small amount of RESA-322 (~0.1 μg/well) was sufficient for saturation of the binding sites in the wells of a microtitre plate in order to saturate the signal with MAb18/2 in an ELISA (data not shown). In order to confirm that MAb18/2 recognizes sequences in both the 3' and 5' repeat regions of the RESA antigen as has been reported previously (11), synthetic peptides corresponding to regions of the RESA repeats were immobilized onto the wells of a microtitre plate and probed with MAb18/2 (Fig. 3.1.1A). The binding of MAb18/2 to three peptides representing the major repeats of RESA was examined (97). MAb18/2 recognized all 3 peptides: a 4-mer $((EENV)_8)$, a BSA-conjugated 8-mer $((EEHVEHDA)_4)$, and a BSA-conjugated 11-mer $((DDEHVEEPTVA)_2)$, although the 8-mer peptide appeared less reactive than the others (Fig. 3.1.1C). Thus MAb18/2 recognizes sequences both in the 5' and the 3' repeats of RESA, hence the exact epitope is difficult to determine. However, it is reasonable to suggest that acidic residues play an important role in forming the epitope.

A



B



C

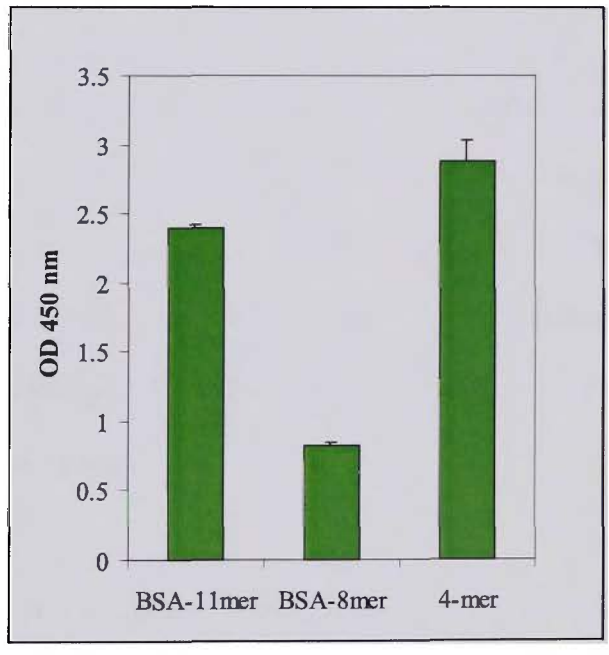


Figure 3.1.1. Binding of MAb18/2 to recombinant RESA-322 and RESA derived peptides. (A) Sequence of RESA. RESA-322 lacks the N-terminal 322 residues. MAb18/2 was raised against residues 893-1073. Acidic repeat regions are underlined. Peptides representing the repeat regions of RESA are coloured. (B) Precoated RESA-322 (5 μg/ml) was probed with MAbs 18/2, 4D6 and 4G2 (5 μg/ml) and binding was detected using HRP-coupled anti-mouse or anti-rat IgG. (C) Immobilized 4-mer and BSA-conjugated peptides, 8-mer and $(11\text{-mer})_3$ (10 μg/ml) were probed with the MAb18/2 (5 μg/ml) and detected using an anti-mouse-HRP antibody.

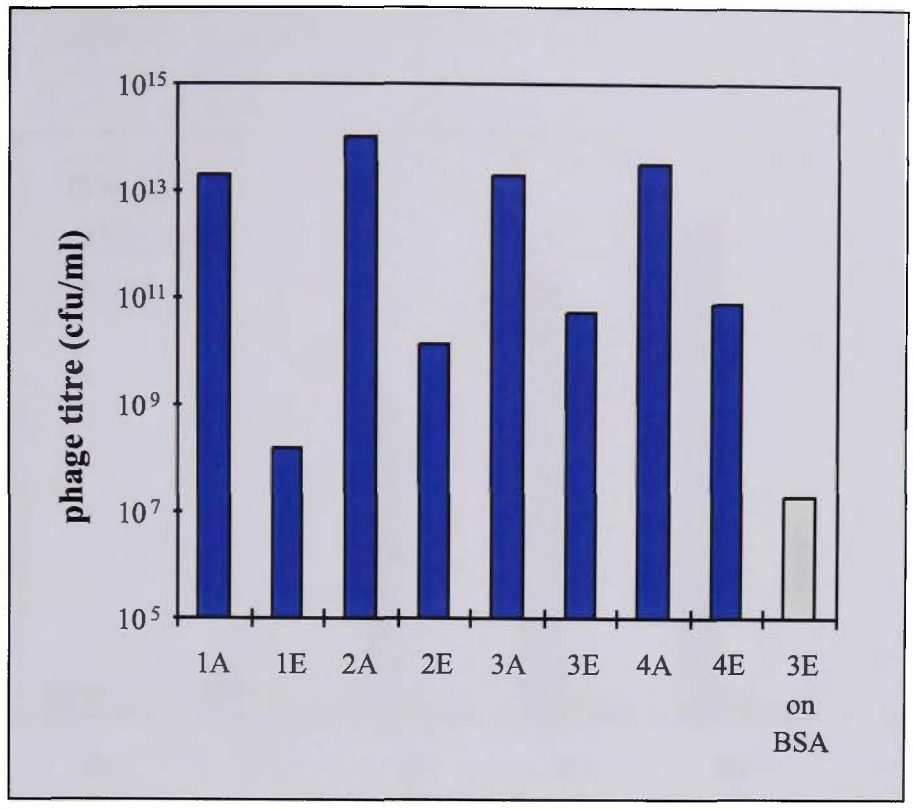
3.1.2 Panning to select pools of phage that bind MAb18/2

To identify peptides that can mimic structural features of the malarial protein, RESA, two independent phage-displayed random peptide libraries were panned on MAb18/2. One library contains phage expressing random 15- residue peptides fused to the minor coat protein, gpIII, and present at approximately 5 copies per phage particle (231). The other library contains phage expressing approximately 250 copies of a 17- residue peptide library ($X_{15}CX$), with an invariant penultimate cysteine residue, as a fusion to the gpVIII coat protein (37). The latter library has a diversity of 1.2×10^8 clones and enhances the possibility of identifying peptides with a second cysteine residue within the 17 residues since phage tend not to express single exposed cysteines (37, 141, 148).

Four rounds of panning resulted in an approximately 1000-fold enrichment of eluted phage in the case of the 17-mer library (Fig. 3.1.2A), whereas in the case of the 15-mer library, approximately only a 10-fold enrichment of eluted phage was observed (Fig. 3.1.2B). When reamplified phage from the third round of panning (Fig. 3.1.2A, 4A) were panned on the control protein, BSA, only 10^7 phage were eluted (Fig. 3.1.2A, 3E) in comparison to the 10^{11} phage which were obtained when the same pool of phage was panned on MAb18/2. (Fig. 3.1.2A, 4E). This suggests that this panning procedure has led to the selection of phage which recognize MAb18/2 and not BSA, which was used as the blocking agent in the panning procedure. However, an increase in the level of enrichment throughout the panning procedure does not necessarily correlate with an increase in the specificity of the isolated clones for the target molecule.

Efficiency of panning is best measured by the ability of the eluted phage to recognize the protein used in the panning, in this case MAb18/2. Thus binding assays on immobilized MAb18/2, using phage from different rounds of panning (phage-ELISAs), were performed. Enhanced binding to MAb18/2 by phage pools from the 15-mer library was exhibited after two rounds of panning (Fig. 3.1.3A) and in the case of the 17-mer library, binding by the phage pools to MAb18/2 was seen after the first round of panning (Fig.

A



B

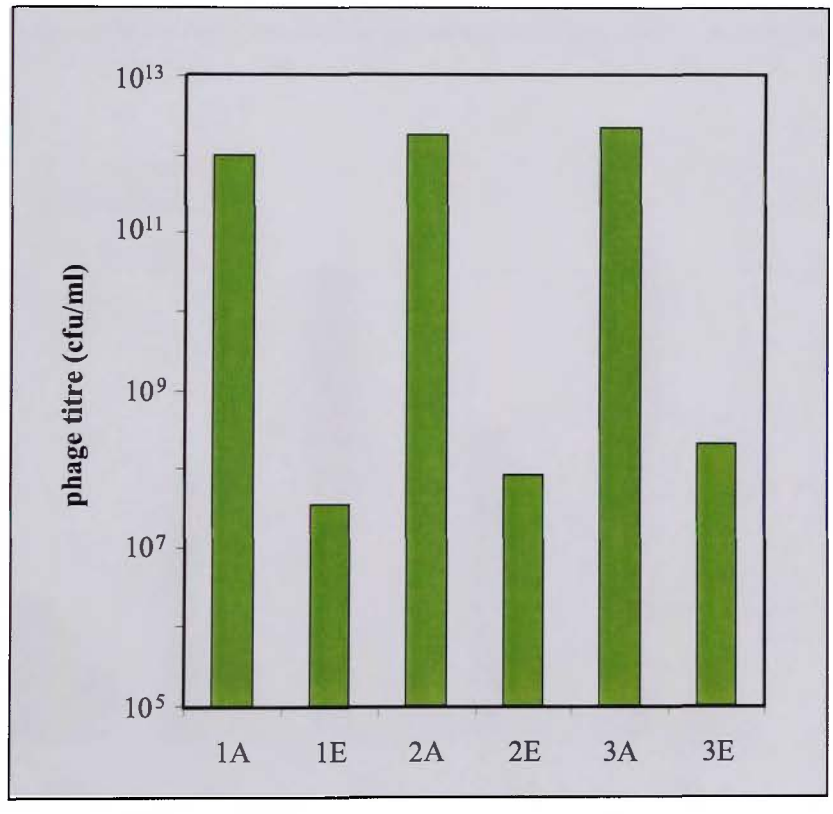
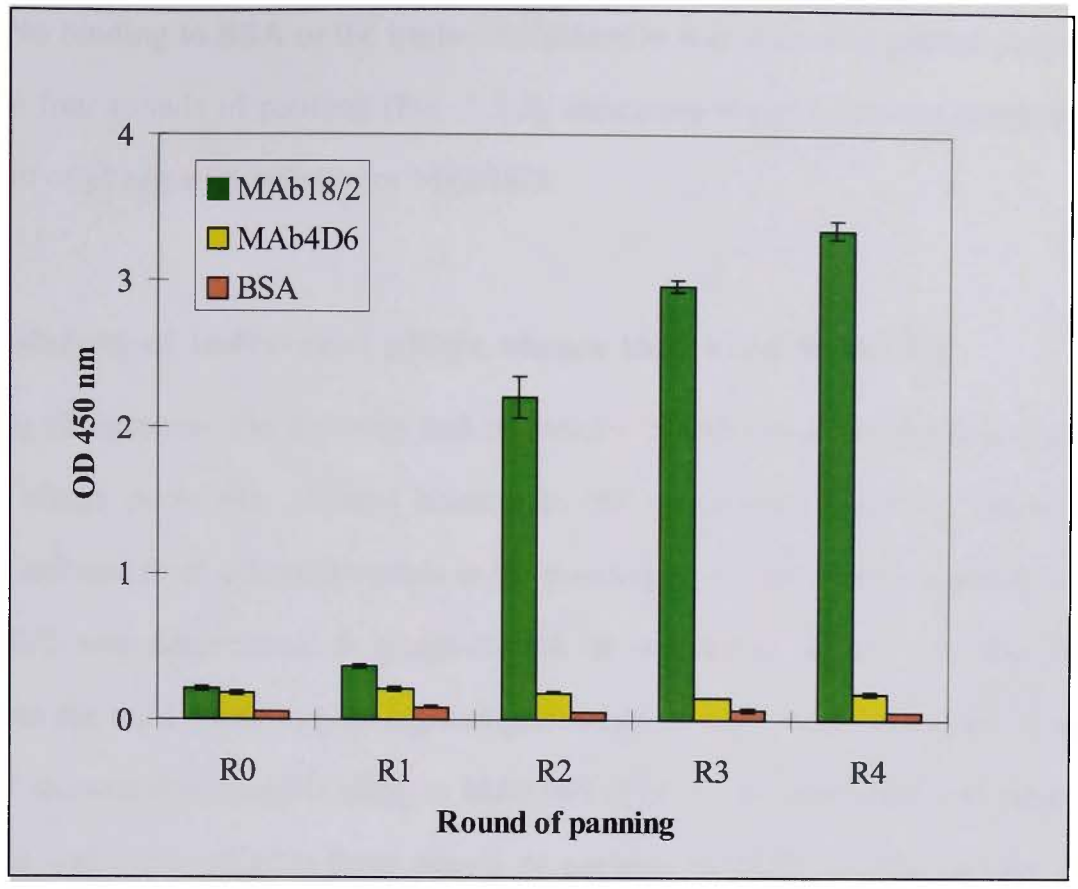


Figure 3.1.2. Enrichment of phage binders during panning on MAb18/2. (A) Titres of 17-mer phage applied to (bars A) and eluted from (bars E) microtitre plate wells containing immobilized MAb18/2 at each round of panning. Phage collected after 3 rounds of panning (3E) were used to pan on a control protein, BSA. (B) Titres of 15-mer phage applied to and eluted from the wells at each round of panning.

A



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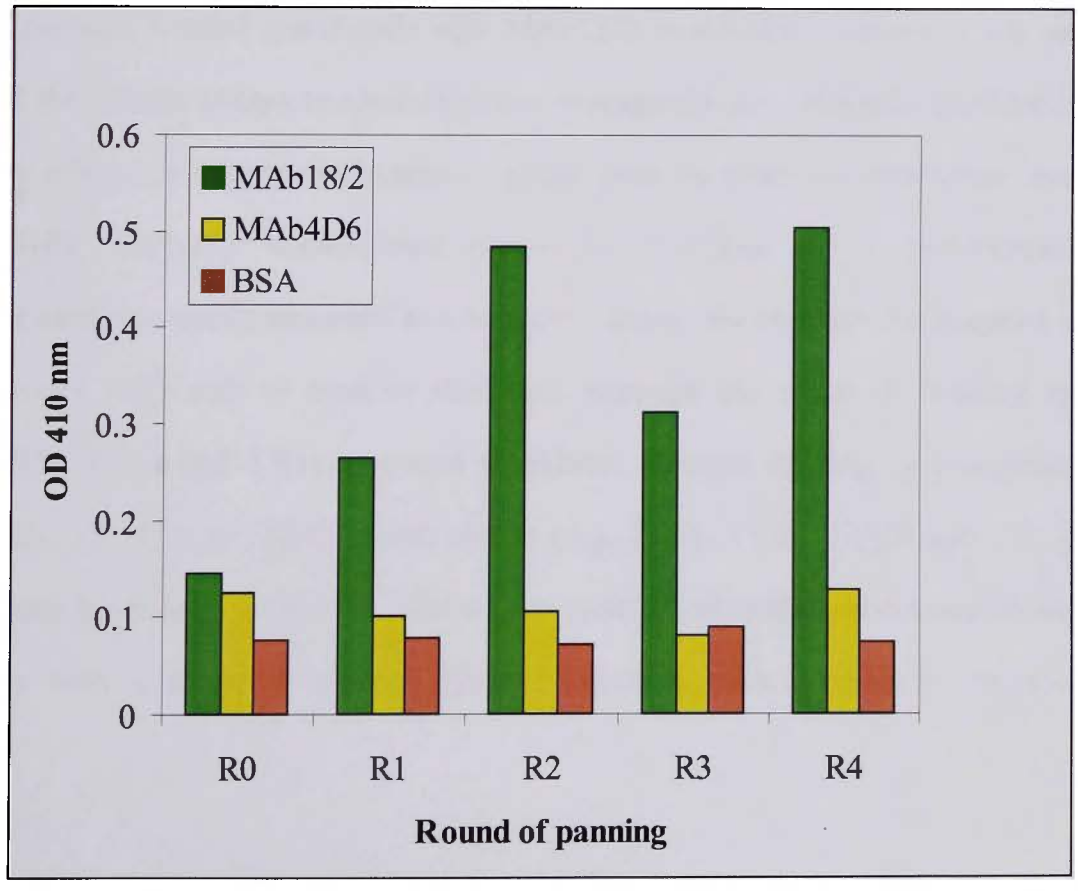


Figure 3.1.3. Enrichment of phage during panning. Phage ELISA of pooled phage after each round of panning on MAb18/2 using the (A) 15-mer library or (B) 17-mer library. 10^{10} pooled phage from each round of panning were added to the wells of a microtitre plate precoated with MAb18/2, anti-AMA-1 (MAb4D6) or BSA. Phage were detected with an anti-M13-HRP antibody.

3.1.3B). No binding to BSA or the irrelevant MAb4D6 was seen with pooled phage from any of the four rounds of panning (Fig. 3.1.3), indicating that the panning resulted in an enrichment of phage with affinity for MAb18/2.

3.1.3 Isolation of individual phage clones that bind MAb18/2

In order to characterize the diversity and specificity of individual phage clones selected from the phage pools that showed binding to the monoclonal antibody, clones were randomly isolated from different rounds in the panning procedure and their ability to bind to MAb18/2 was determined. A phage-ELISA of individual clones from the 15-mer library from the third round of panning was performed. It was found that, of the 10 clones isolated, 7 showed significant binding to MAb18/2 (Fig. 3.1.4), indicating that substantial enrichment was achieved after three rounds of panning on MAb18/2. In the case of the 17-mer library, it was estimated that approximately 50% of the clones isolated after four rounds of panning reacted specifically with MAb18/2. A dot blot experiment was used in the case of the 17-mer library to identify clones that specifically recognize MAb18/2 (Fig. 3.1.5). The efficiency of immobilization of phage onto the filter was confirmed using an anti-M13-HRP antibody. Immobilized phage did not bind to an anti-mouse-HRP conjugated antibody which was used as a negative control. By contrast, the majority of the isolated clones were able to bind to MAb18/2 although the extent of binding varied. Clones 17(3), 17(5) and 17(1) appeared to exhibit stronger binding in comparison to clones 17(2), 17(14) and 17(16). Some clones (eg. 17(6), 17(9), 17(10) and 17(12) did not show any binding to MAb18/2. These data provide preliminary evidence to suggest that clones with a range of relative binding affinities were isolated in the panning procedure.

In order to confirm that the antibody was binding to the peptide displayed on the phage clones, rather than the phage itself, a number of isolated clones were analysed by Western blotting. The proteins of the individual phage were separated under reducing conditions on SDS-PAGE and blotted onto PVDF membranes. MAb18/2 recognized a band that

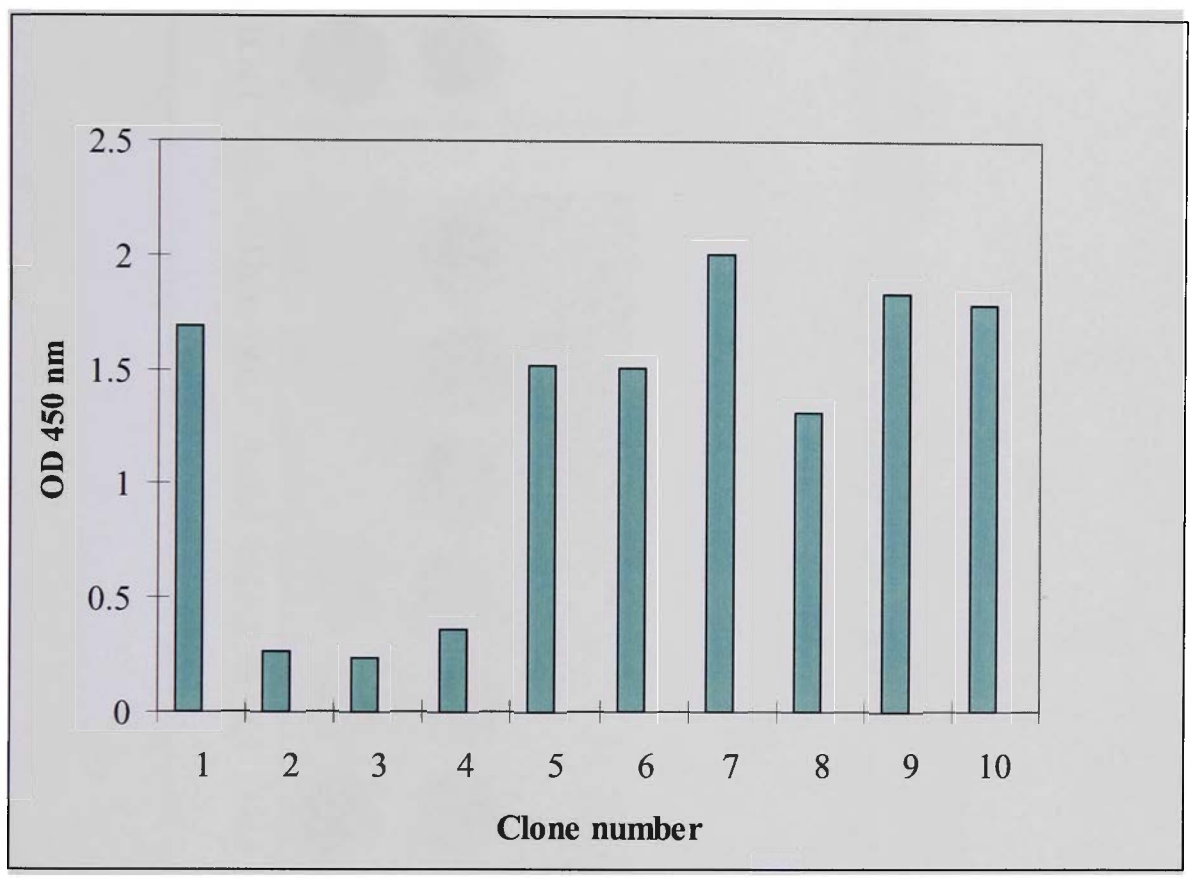


Figure 3.1.4. Phage ELISA of individual 15-mer clones isolated after 3 rounds of panning on MAb18/2. Phage were amplified and 10^{10} phage were added to each well of a microtitre plate precoated with MAb18/2. Bound phage were detected with an anti-M13-HRP antibody.

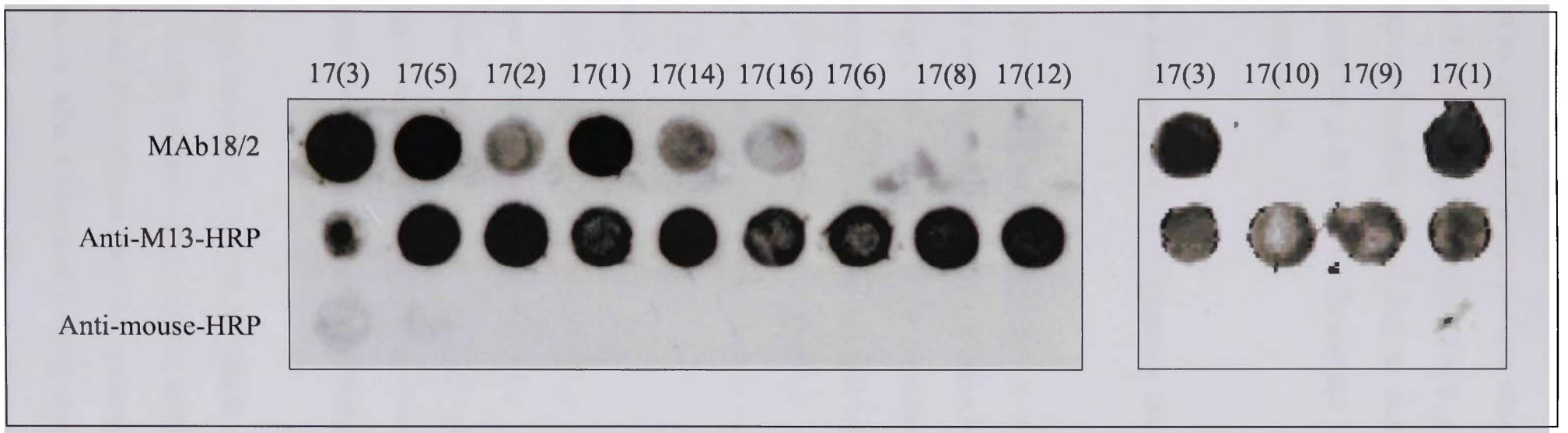


Figure 3.1.5. Dot blot analysis of the binding of selected 17-mer clones to MAb18/2. Phage (10^{10} cfu) were dotted onto NCF in triplicate. Each clone was probed with either: MAb18/2 followed by anti-mouse-HRP, anti-M13-HRP, or anti-mouse-HRP. Binding of the antibodies was visualized using ECL.

corresponds to the expected size of the peptide fused gene product (Fig. 3.1.6). MAb18/2 did not bind to wild-type M13 phage proteins which did not display any foreign peptides (Fig. 3.1.6, M13). This analysis confirms that it is the peptide displayed on the gpIII or gpVIII proteins that confers the binding to MAb18/2. The size difference between peptides expressed on gpIII (15-mer library) and peptides expressed on gpVIII (17-mer library) are clearly visible.

3.1.4 Amino acid sequences of peptide inserts in phage that bind to MAb18/2

Although the precise epitope on RESA that is recognized by MAb18/2 has not been determined, the antibody has been shown to recognize both the 5' and 3' repeat regions of the RESA antigen. The sequences of the peptides that bind to MAb18/2 may help to define the requirements of the epitope recognized by this antibody. In order to elucidate the sequences of the peptides expressed on phage, primers were designed which allowed the amplification of the region of the phage genome that encoded the insert peptide (Fig. 3.1.7A and 3.1.8A). The products of the PCR amplification of the DNA encoding the peptide sequences were expected to be 280 bp for the 15-mer library clones and 192 bp for the 17-mer library clones. It was observed that the PCR products of some clones (eg. Fig. 3.1.7B, lane 2), were slightly smaller in size than others (Fig. 3.1.7B, lane 4). This situation was even more pronounced with clones from the 17-mer library (Fig. 3.1.8B). Individual phage clones after either round 2 or round 4 were propagated and the region of DNA corresponding to the random peptide was sequenced.

Peptide sequences translated from the nucleotide sequences isolated from the 15-mer library after 2 rounds of panning were quite diverse. After 4 rounds of panning, this diversity decreased. The different peptide sequences were compiled and aligned to reveal any common motifs. After 4 rounds of panning, most of the isolated sequences contained a common SAVDD motif within different amino acid sequence contexts (Fig. 3.1.9A). There was some degeneracy in the motif with one peptide (15(5)) having SVEE and

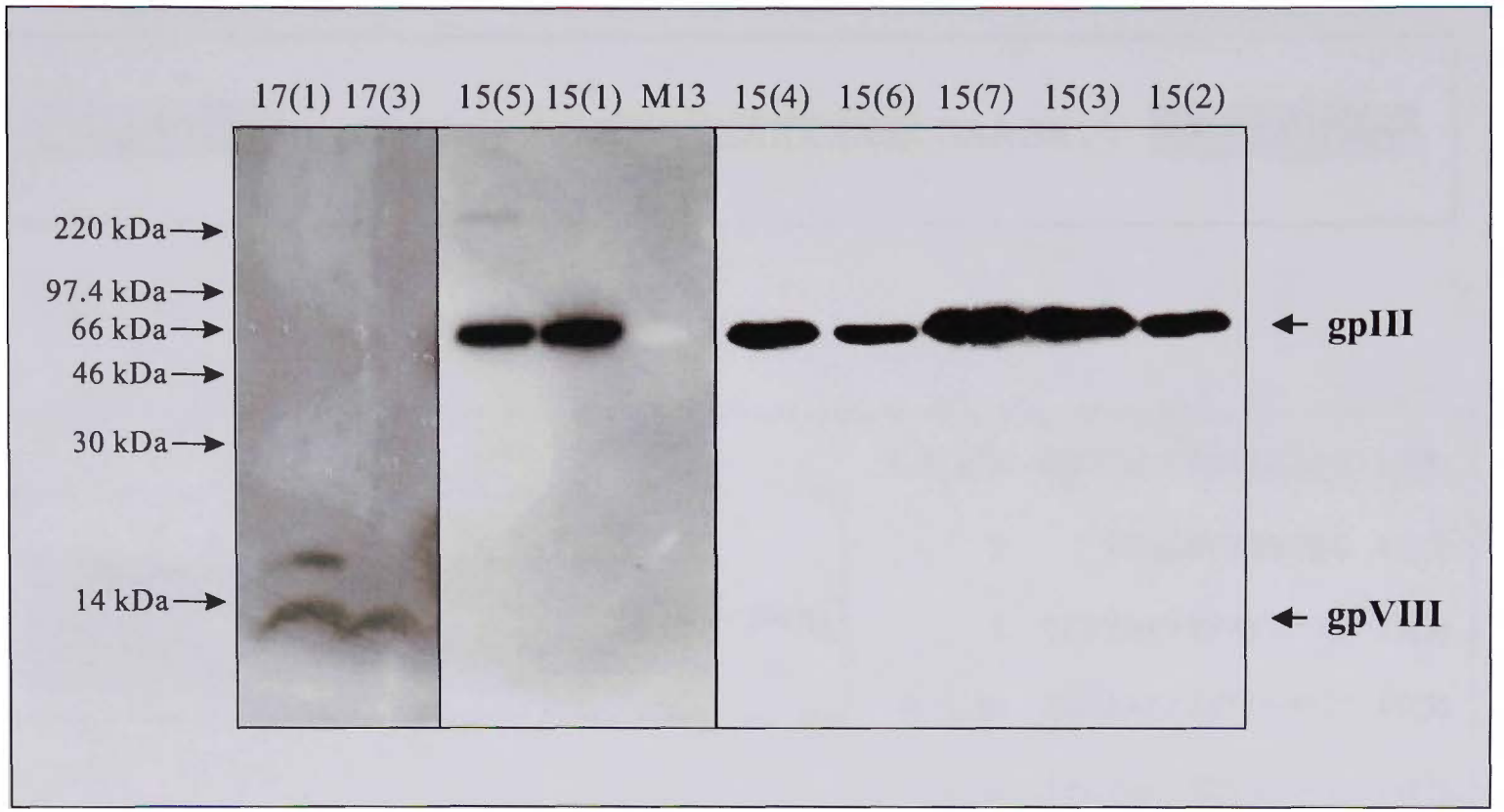


Figure 3.1.6. Interaction of the isolated phage clones with MAb18/2. Western blot analysis of phage clones. Selected phage clones from each of the two libraries and wild-type M13 phage as a control, were separated by SDS-polyacrylamide gel electrophoresis, transferred to PVDF membranes, and probed with MAb18/2. The positions of peptides 15(5), 15(1), 15(4), 15(6), 15(7), 15(3), and 15(2) fused to gpIII and peptides 17(1) and 17(3) fused to gpVIII were detected using MAb18/2 followed by anti-mouse-HRP and visualized by ECL. No binding of MAb18/2 to gpIII on wild-type M13 phage (M13) was observed.

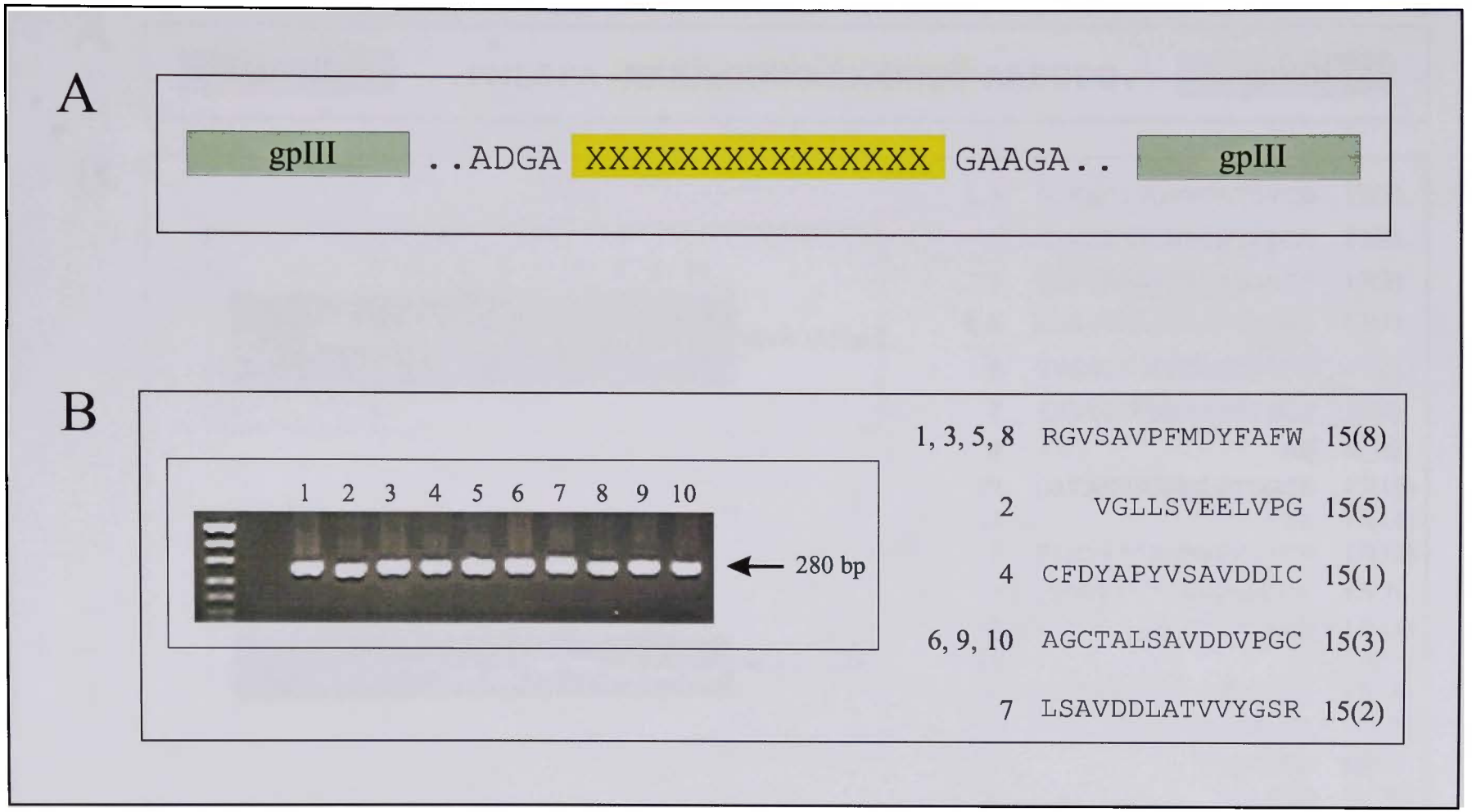


Figure 3.1.7. PCR and sequencing of individual phage clones isolated by panning the 15-mer library on MAb18/2. (A) Schematic showing gpIII containing the random 15 amino acid peptide expressed on phage. (B) 10 individual clones were randomly selected after four rounds of panning, the DNA encoding the peptide was amplified by PCR, visualized by agarose gel electrophoresis (insert) and sequenced. Five different peptides were identified and designated 15(X).

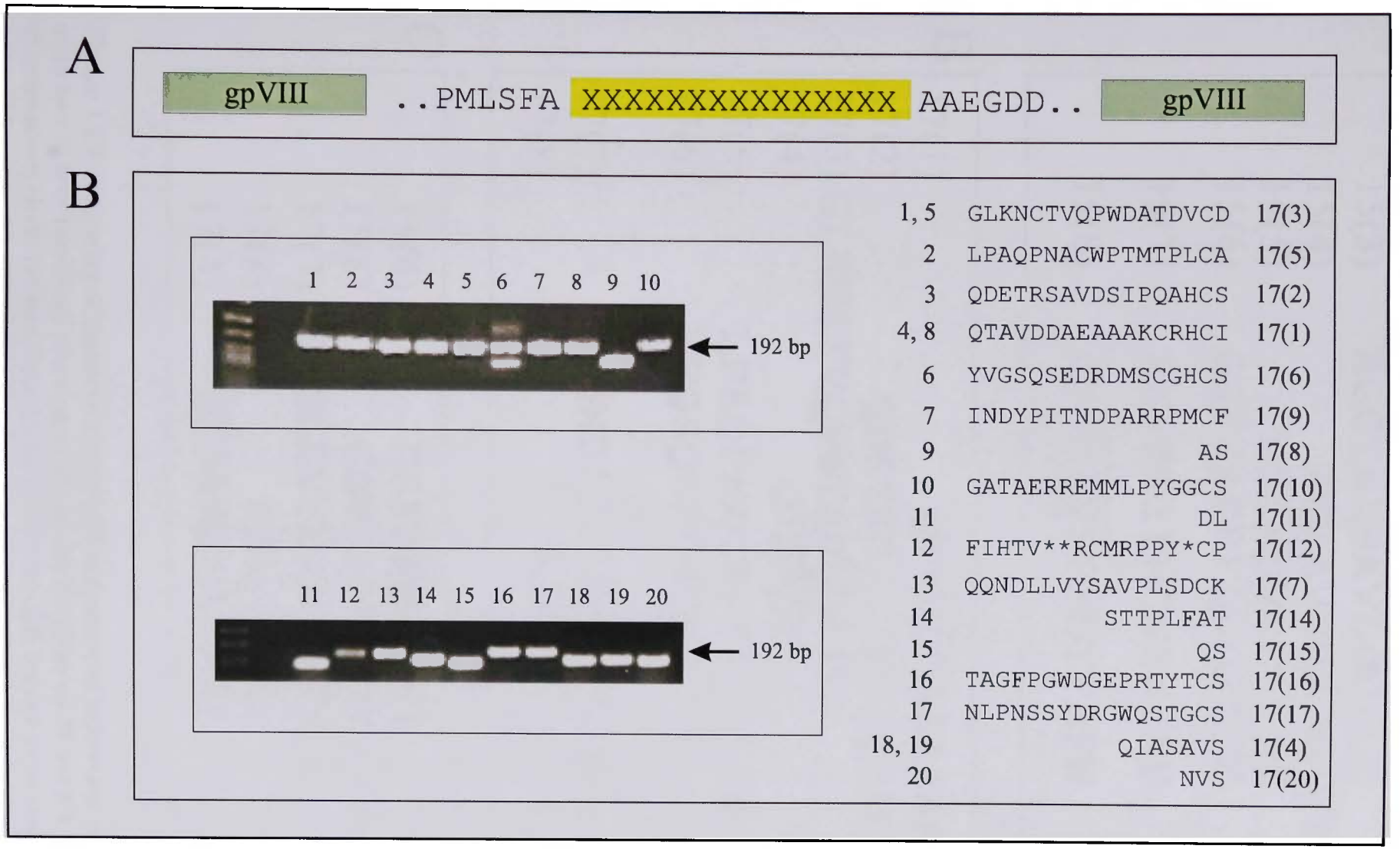


Figure 3.1.8. PCR and sequencing of individual phage clones isolated by panning the 17-mer library on MAb18/2. (A) Schematic showing gpVIII containing the random 17 amino acid peptide expressed on phage. (B) 20 individual clones (1-20) were randomly selected after two rounds of panning, the DNA encoding the peptide was amplified by PCR, visualized by agarose gel electrophoresis (inserts) and sequenced. 17 different peptides were identified and designated 17(X).

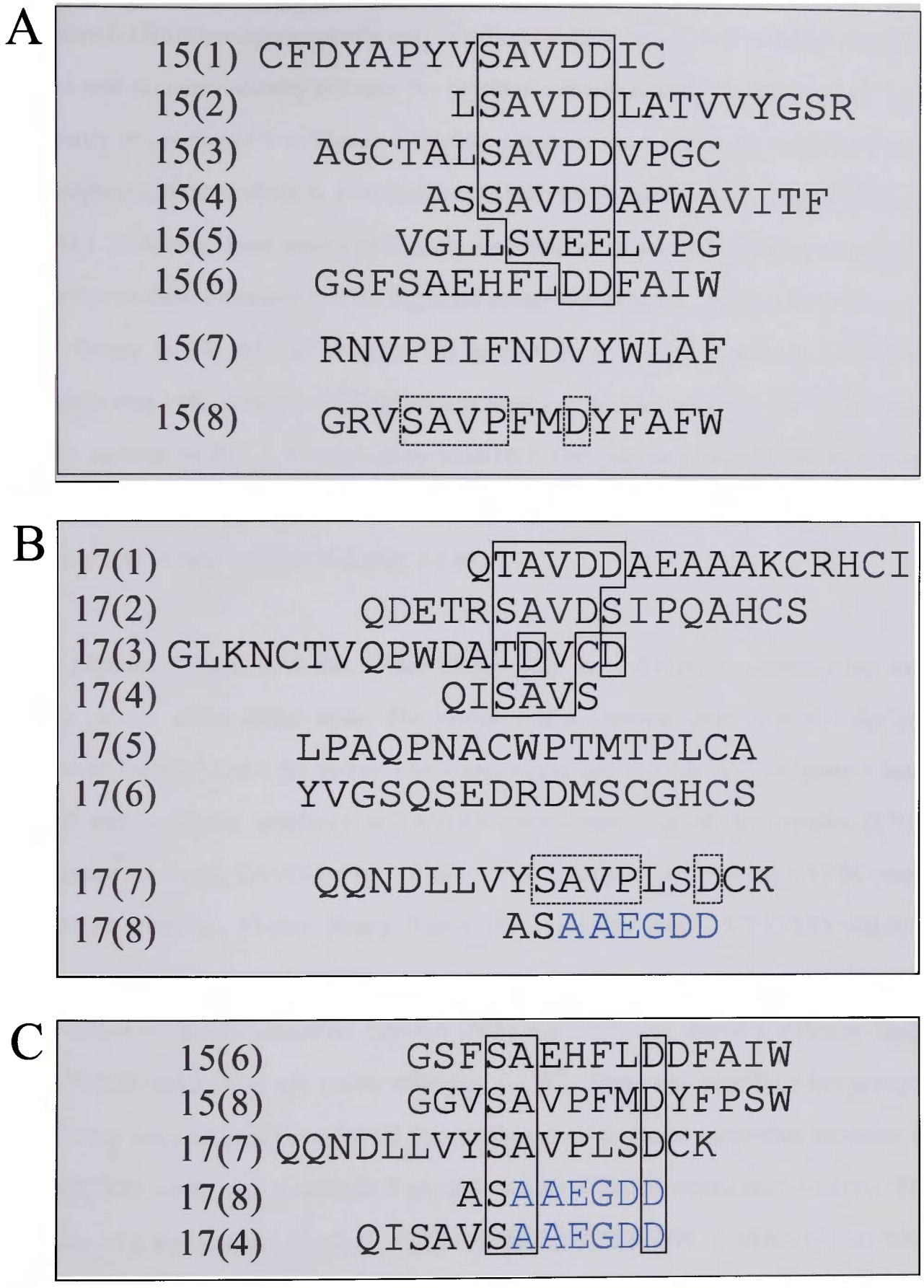


Figure 3.1.9. Summary of deduced amino acid sequences of individual clones obtained after two to four rounds of panning of the phage libraries on MAb18/2. (A) Sequences of inserts from phage isolated from the 15-mer library. (B) Sequences of inserts from phage isolated from the 17-mer library. Regions of similarity within the peptides are boxed. (C) Alignment of selected clones from the two libraries revealing a secondary motif (boxed) within the amino acid sequences. The AAEGDD sequence (blue) in clones 17(4) and 17(8) is derived from the gene VIII protein immediately following the insert.

another ((15(6)) having apparently only the diacidic (DD) residues in common. Although there was no exact identity between the peptides isolated and RESA sequences (97), the majority of the peptides contained a diacidic sequence immediately preceded by a small hydrophobic residue which is a motif found in both the 5' and 3' RESA repeats (VEE and ADD). Acidic residues were significantly enriched in the selected peptides (Fig. 3.1.9). Couet *et al.* have estimated that the expected occurrence of acidic residues in the same 15-mer library is 5% (68). In contrast, the proportion of acidic residues in the isolated peptides was 14%, almost 3-fold higher than expected, perhaps reflecting the acidic nature of the epitope on RESA recognized by MAb18/2. One peptide isolated from the 15-mer library 15(7) did not appear to share any homology to the consensus motif (Fig. 3.1.9A), yet it is able to bind to MAb18/2 (Fig. 3.1.6).

The peptides isolated from the 17-mer library were more diverse in sequence but were again rich in acidic amino acids. The presence of a common motif was less obvious, however the preference for serine, threonine, valine and aspartic acid suggests a loose motif with structural similarity to SAVDD. Interestingly, one of the peptides (17(1)) contained the motif, TAVDD (Fig. 3.1.9B), which is clearly related to the SAVDD motif identified from the 15-mer library. Hence the consensus motif: S/TAVDD which is important for binding to MAb18/2 is proposed. Further comparison of the clones from the two different libraries identified peptides 15(8) and 17(7) that shared a different motif: SAVPXXD, where X is any amino acid (Fig. 3.1.9C). These two peptides when grouped with other selected peptides allowed the assignment of a second consensus sequence of SAXXXXD which was present in 5 peptides within different amino acid contexts. The binding of a representative peptide, 15(6) GSFSAEHFLDDFAIW, to MAb18/2 has been confirmed (Fig. 3.1.6, 3.1.11A and 3.1.12B). Interestingly two of these (peptides 17(8) and 17(4), Fig. 3.1.9C) align with this second consensus motif only if the phage protein backbone of gpVIII to which the peptides are fused is considered to contribute to the binding. This may explain the surprising observation that a phage displaying a peptide insert of only two residues (alanine and serine, peptide 17(8)), was selected from the

library and confirmed for binding to MAb18/2 (Fig. 3.1.12). Peptides were also isolated from these libraries (*ie.* peptides 15(7), 17(5) and 17(6)) that did not conform to any of these consensus patterns. The presence of a number of peptides lacking the full complement of 15 or 17 residues were also observed (eg. peptides 15(5) and 17(4)) (Fig. 3.1.9). These may have resulted from deletions within the DNA insert encoding the peptide, possibly as phage were cultured between rounds of panning. This is supported by the observation that the PCR amplified inserts corresponding to these peptides were smaller in size (Fig. 3.1.7 and Fig. 3.1.8).

3.1.5 Binding specificity of selected phage to MAb18/2

The binding of phage clones to immobilized MAb18/2 was examined using a phage-ELISA (Fig. 3.1.10, 3.1.11 and 3.1.12). Phage clones 15(1), 15(3), 17(1) and 17(3) which showed high affinity binding to MAb18/2 did not bind to the antisera against the irrelevant protein, cpn60 (Fig. 3.1.10), and clones 15(3) and 17(3) also did not bind to an irrelevant monoclonal antibody, MAb4D6 (Fig. 3.1.11). In contrast, phage lacking a peptide (wildtype M13 phage) did not bind to MAb18/2 (Fig. 3.1.10, 3.1.11, and 3.1.12). As can be seen from Figure 3.1.12, phage displaying peptides bind to MAb18/2 in a dose-dependant manner. Increasing numbers of phage of representative clones from the 17-mer and 15-mer libraries were allowed to bind MAb18/2-coated microtitre plates (Fig. 3.1.12). Some clones including 17(1), 17(3) and 15(1) showed high relative binding affinities with measurable signals when only 10^8 phage particles were added (Fig. 3.1.12). Conversely, other clones showed lower relative binding affinities. This was particularly evident in the clones isolated from the 15-mer library, such as clones 15(5), 15(6) and 15(7), which required up to 10^{10} phage particles to give a detectable signal (Fig. 3.1.12B). Phage clones 17(3) and 15(3) which showed high affinity for MAb18/2 did not bind to the antiserum of cpn60. Furthermore, phage lacking a peptide (wildtype M13 phage) did not bind to MAb18/2 even at 10^{11} phage particles (Fig. 3.1.12). One phage clone (17(6)), with the insert sequence, YVGSQSEDRDMSCGHCS, which lacks the consensus motif, was not observed to bind to MAb18/2 (Fig. 3.1.12A). Taken together, these data indicate that the

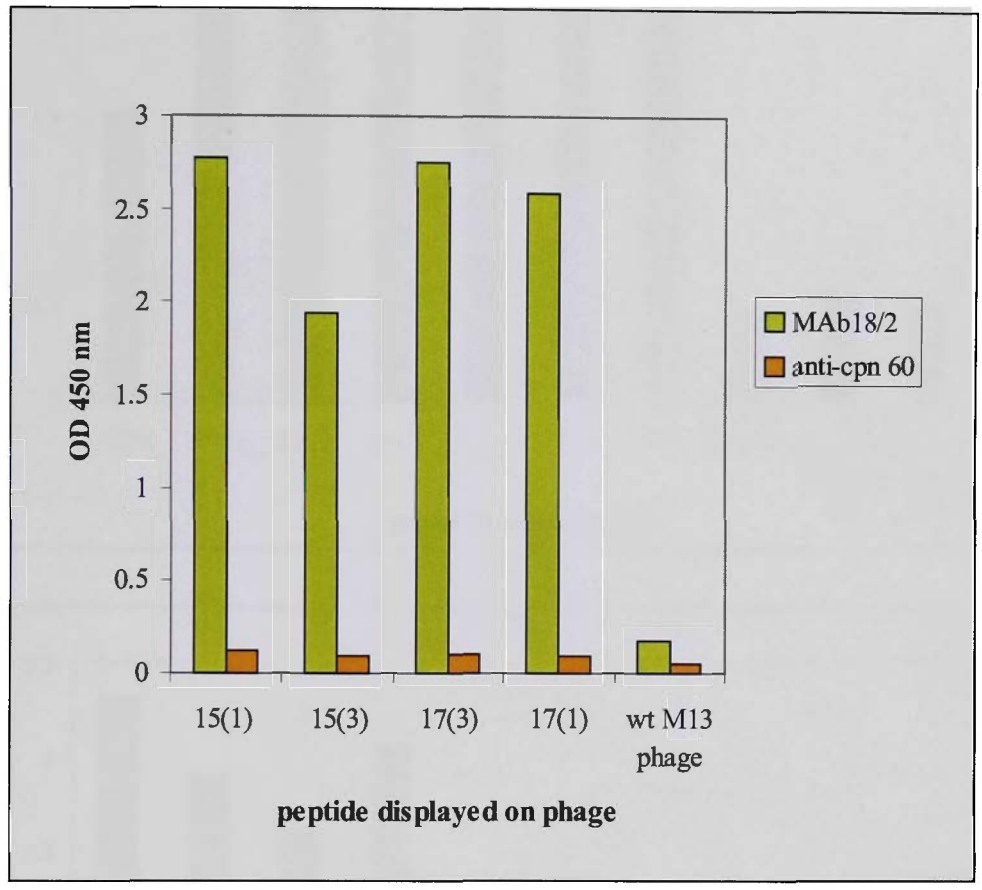
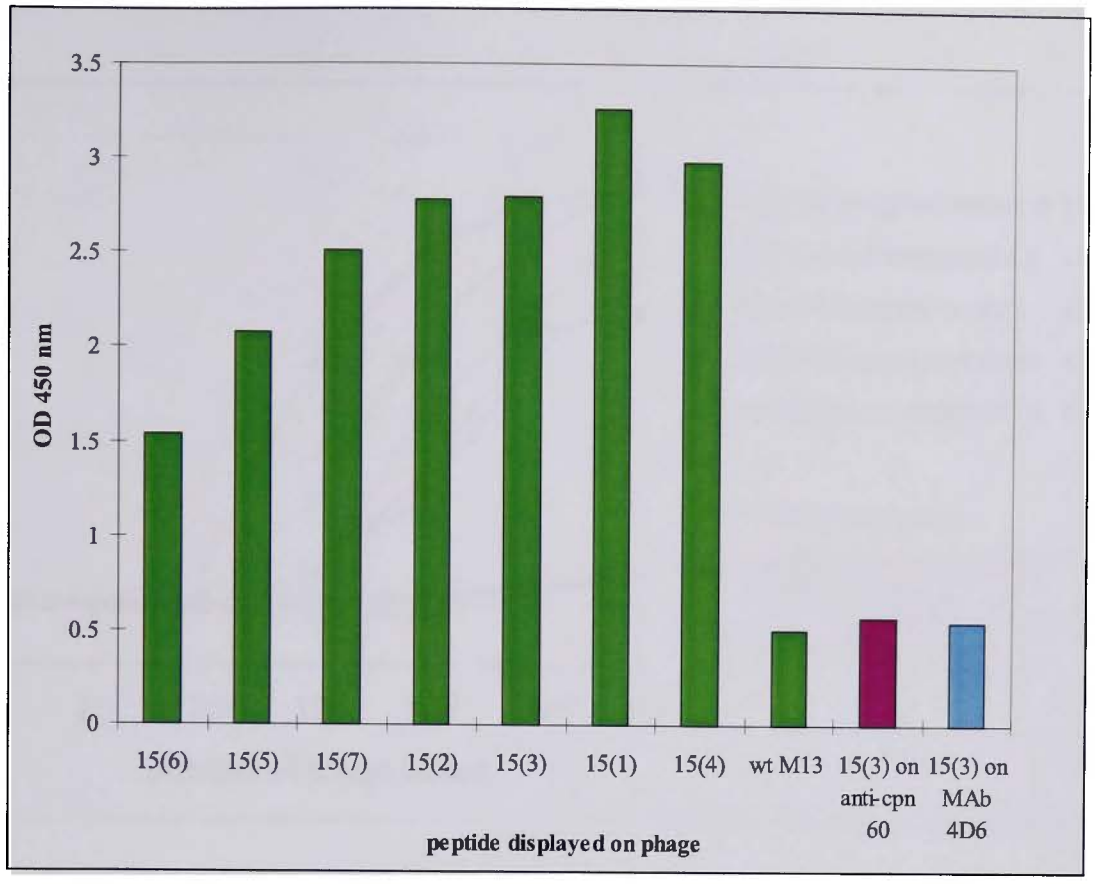


Figure 3.1.10. Study of the specificity of the binding of phage clones isolated by panning on MAb18/2. MAb18/2 and a control antibody, anti-chaperone 60, were immobilized on the wells of a microtitre plate and probed with 10^{10} phage displaying different peptides. Binding was detected using an anti-M13-HRP conjugated antibody. No binding of wild-type M13 phage was detected.

A



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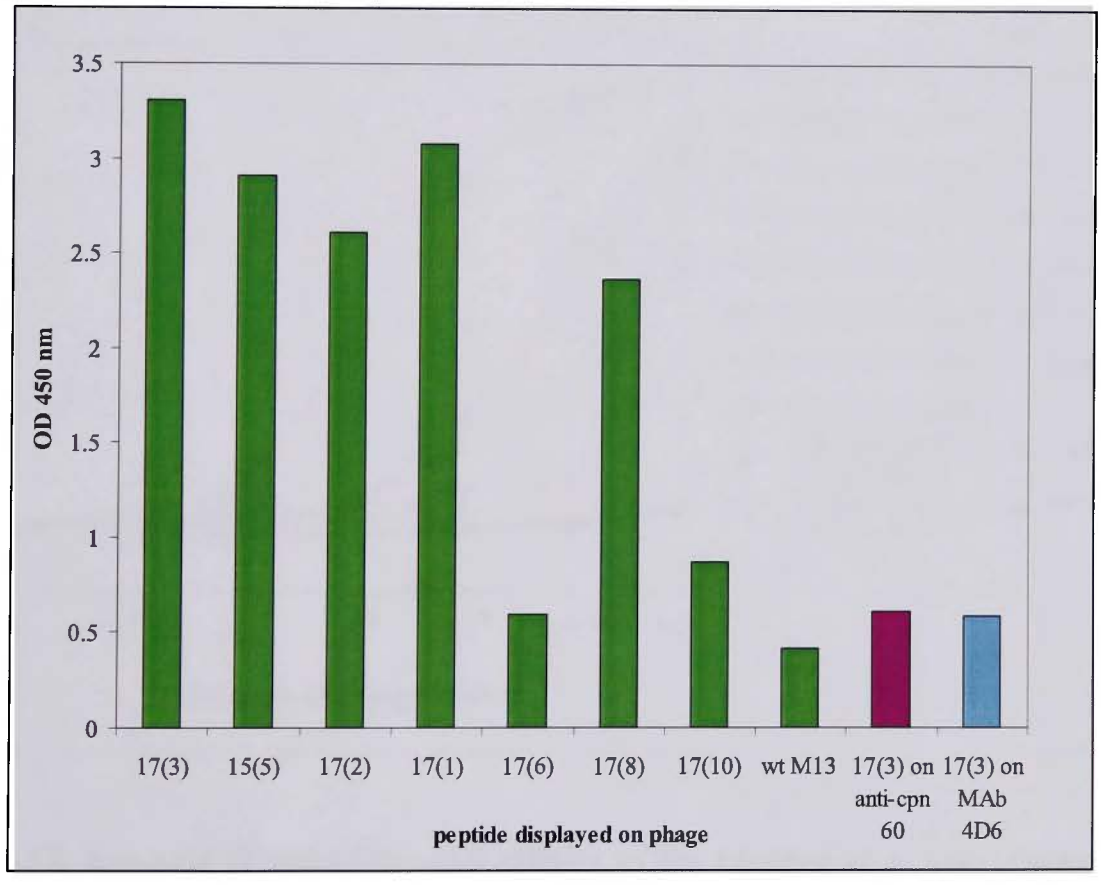
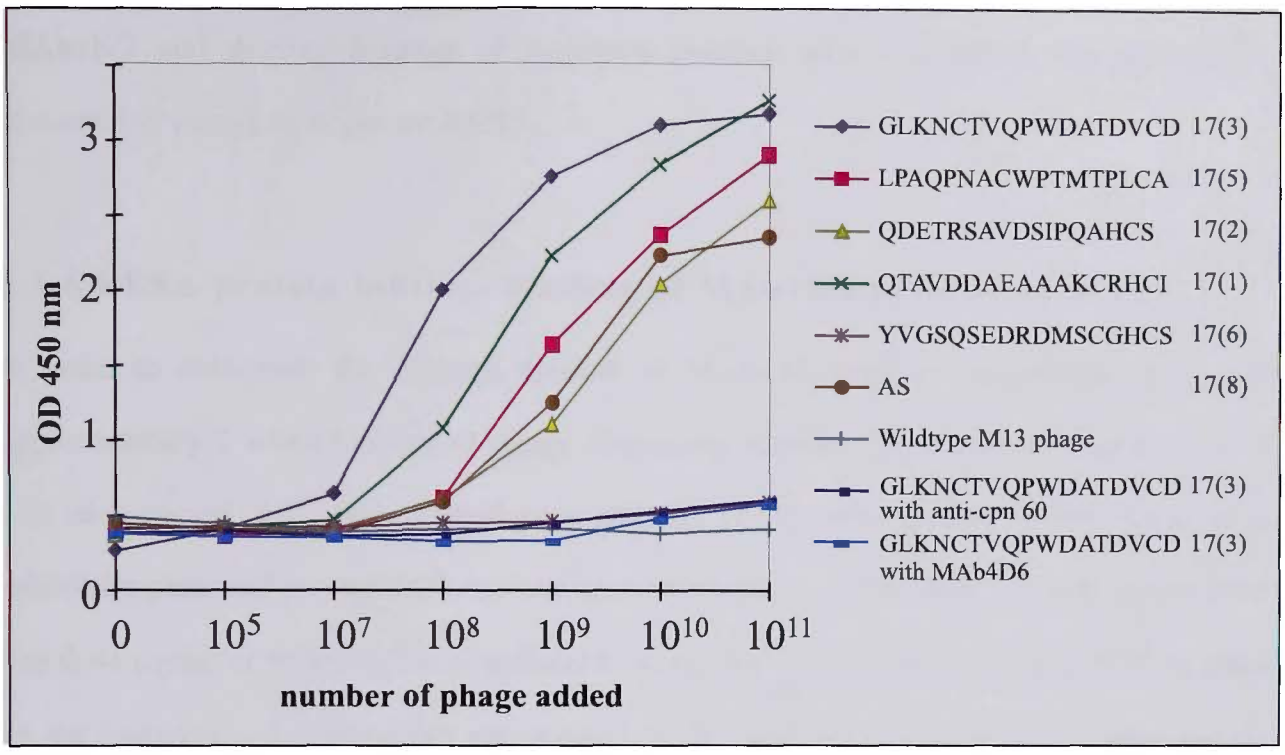


Figure 3.1.11. Analysis of binding of phage clones isolated by panning on MAb18/2. MAb18/2 and control antibodies, MAb4D6 and anti-chaperone 60, were immobilized on the wells of a microtitre plate. MAb18/2 was probed with 10^{10} phage displaying different peptides and wild-type M13 phage, whilst the control antibodies were probed with 10^{10} phage displaying (A) peptide 15(3) and (B) peptide 17(3). Binding was detected using an anti-M13-HRP conjugated antibody. This data is representative of an experiment that was performed on three separate occasions.

A



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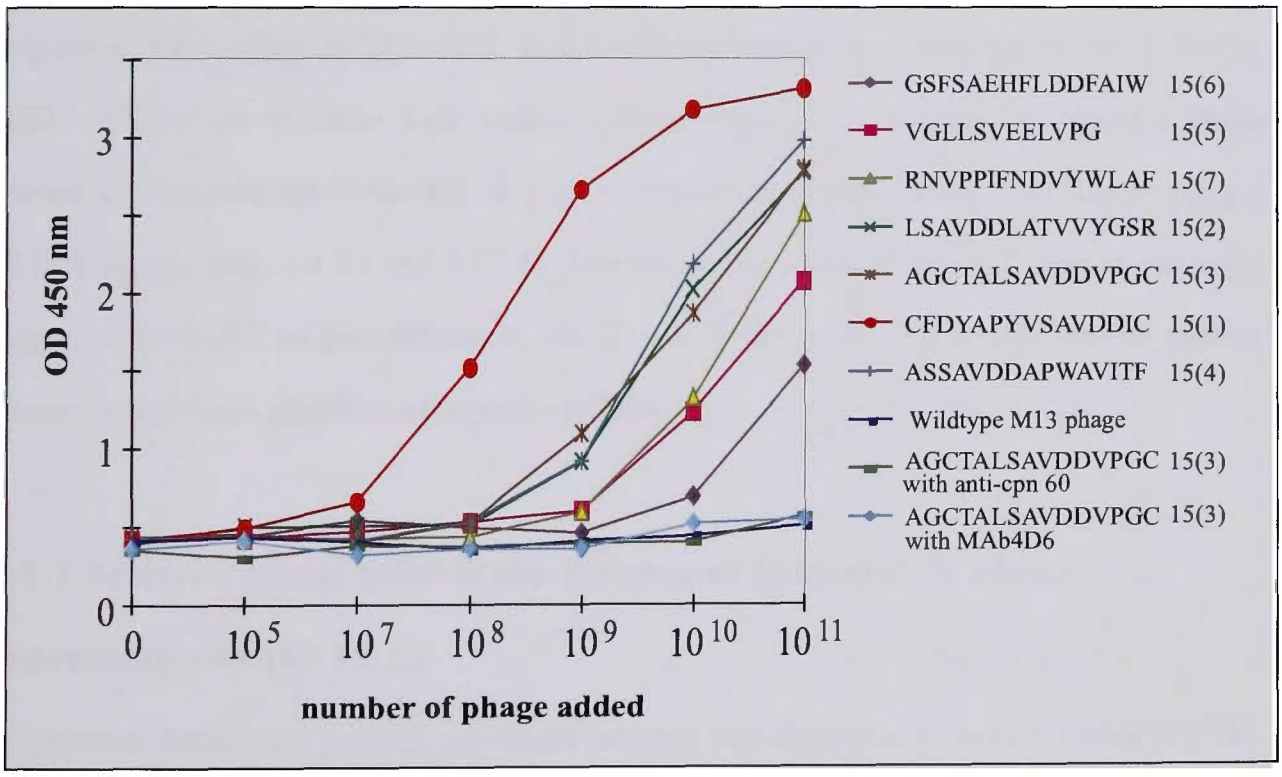


Figure 3.1.12. Analysis of specificity and affinity of the binding of phage-displayed peptides to MAb18/2. Increasing numbers of phage of representative clones from (A) the 17-mer library, (B) the 15-mer library and wildtype M13 phage were applied to wells of a microtitre plate coated with MAb18/2. The binding of peptides 15(3) and 17(3) to two other antibodies, anti-chaperone 60 and anti-AMA-1 (MAb4D6) were also examined. This data is a representative experiment that was performed on three separate occasions.

majority of the isolated peptide sequences bind specifically to the antigen-binding site of MAb18/2 and display a range of apparent binding affinities. Hence the mimotopes presumably mimic epitopes on RESA.

3.1.6 RESA protein inhibits binding of MAb18/2 to selected phage

In order to determine the optimal amount of MAb18/2 required to give an A_{450nm} of approximately 2 when binding to phage displaying peptide 15(1), a dose-response curve was constructed. 10^{10} phage displaying peptide 15(1) were applied to the wells of a microtitre plate and probed with increasing concentrations of MAb18/2. It was determined that 0.44 µg/ml of MAb18/2 was sufficient to occupy 50% of the MAb18/2-binding sites on the immobilized phage (data not shown). If the peptide sequences mimic epitopes on RESA then the RESA protein would be expected to compete with phage displaying these sequences for binding to MAb18/2. A recombinant bacterially-expressed form of RESA, RESA-322 which includes both repeat regions, reduces the binding of selected phage clones to immobilized MAb18/2 in a dose-dependant manner when included in phage-ELISA assays (Fig. 3.1.13 and 3.1.14). Therefore, the isolated peptides bind to the same region of MAb18/2 as does RESA, ie. the antigen-binding site. These data provide further evidence that these peptides are mimics of RESA.

3.1.7 Selected phage inhibit the binding of MAb18/2 to phage expressing peptide 15(1)

Since there were many peptide sequences isolated that all appear to bind to MAb18/2, the question arises as to whether all sequences exhibit the same specificity. In order to examine this, experiments were carried out to determine whether phage displaying a peptide of one particular sequence could block the binding of phage displaying a different peptide to MAb18/2. Immobilized phage expressing peptide 15(1) were probed with MAb18/2 in the presence of another phage clone (Fig. 3.1.15). Reduced binding of MAb18/2 to phage expressing peptide 15(1) was observed in the presence of phage clones 17(3), 17(1) and 15(3). No reduction in binding was observed in the absence of added

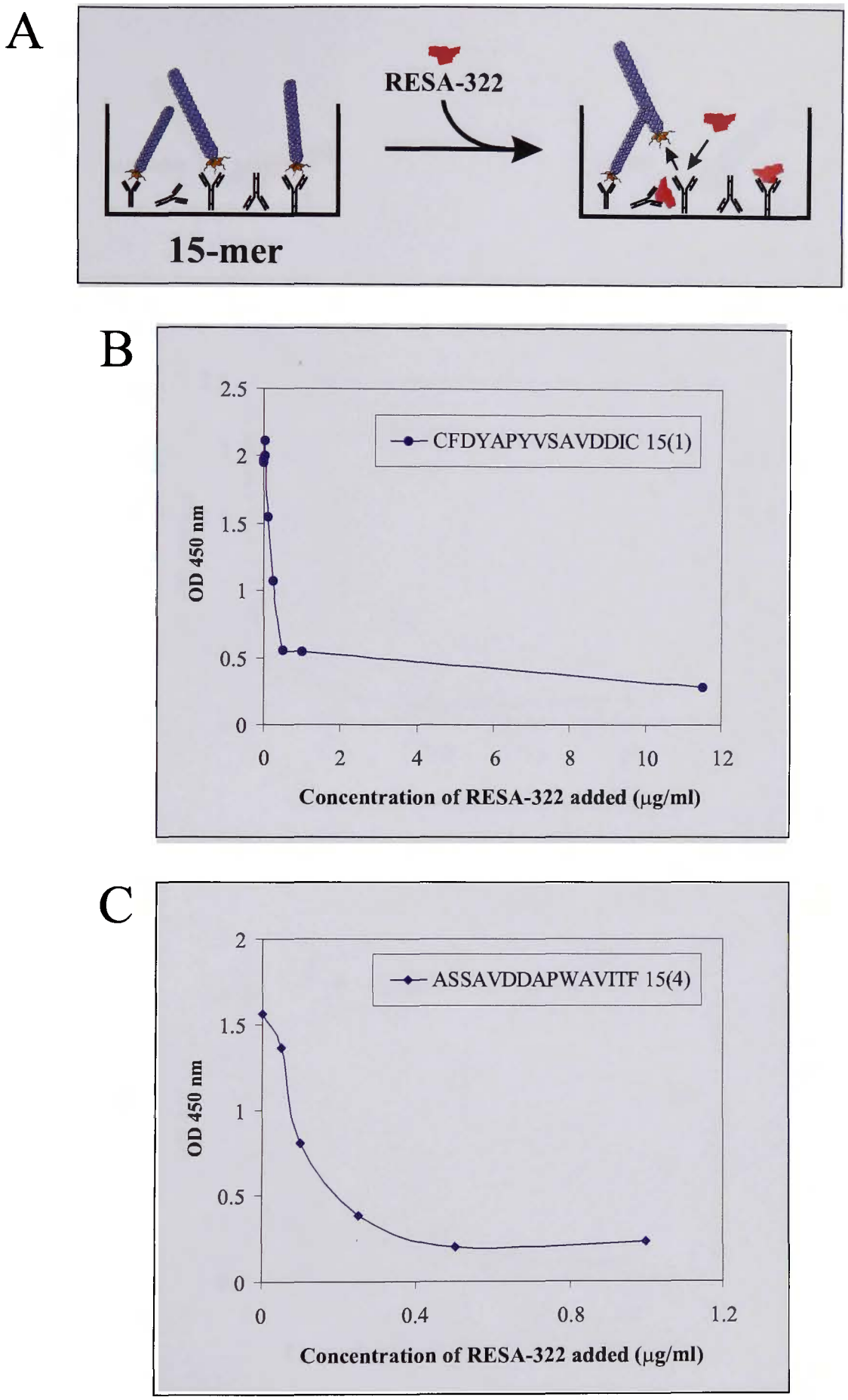


Figure 3.1.13. Inhibition by recombinant RESA-322 of the binding of phage clones to MAb18/2. (A) MAb18/2 (2.5 µg/ml) was used to coat the wells of a microtitre plate and 10^{10} phage from the 15-mer library displaying either (B) peptide 15(1) or (C) peptide 15(4) were added to each well in the presence of increasing concentrations of RESA-322. Binding of phage was assessed using an anti-M13-HRP conjugated antibody.

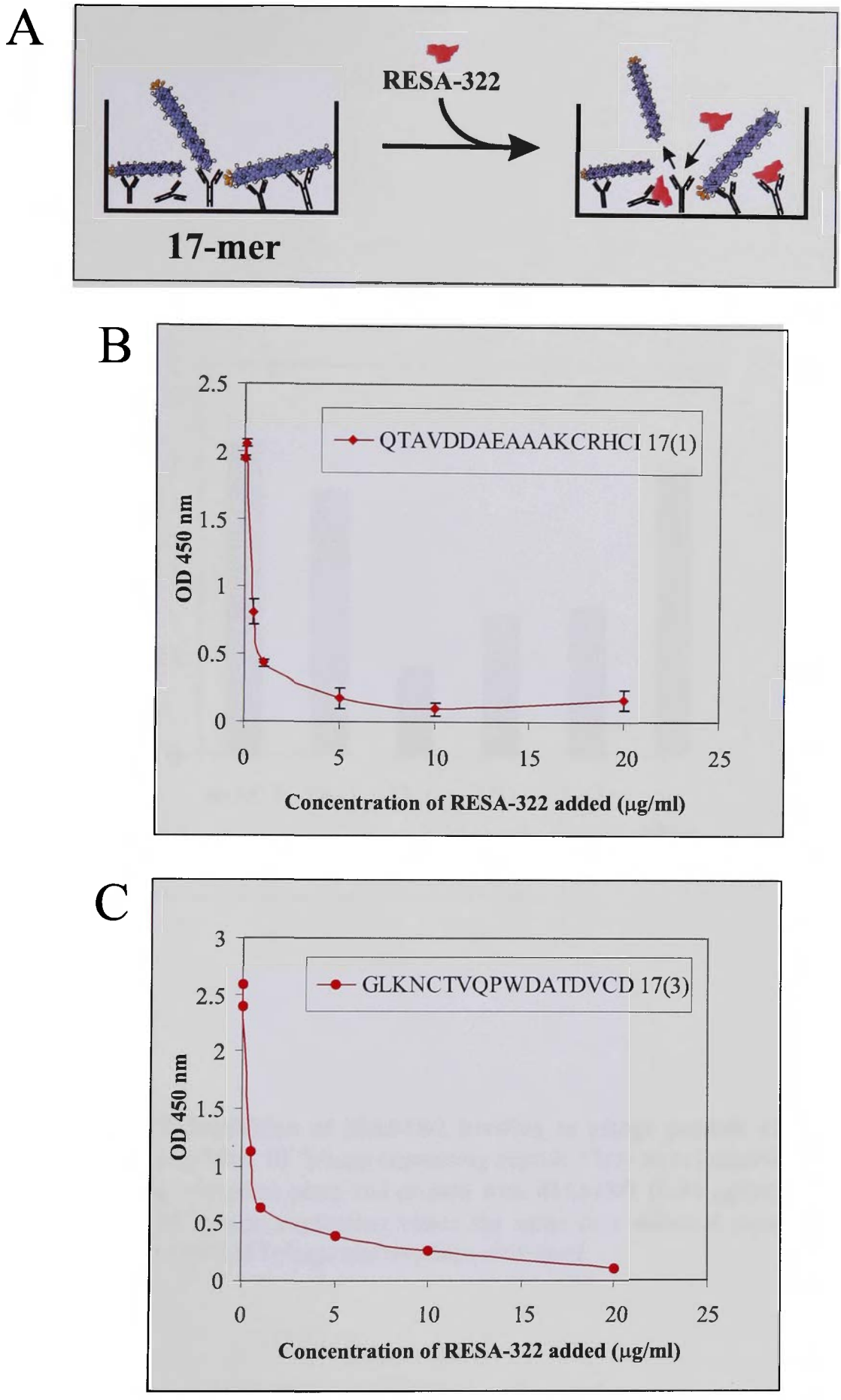


Figure 3.1.14. Inhibition by recombinant RESA-322 of the binding of phage clones to MAb18/2. (A) MAb18/2 (2.5 µg/ml) was used to coat the wells of a microtitre plate and 10^{10} phage from the 17-mer library displaying either (B) peptide 17(1) or (C) peptide 17(3) were added to each well in the presence of increasing concentrations of recombinant RESA-322. Binding of phage was assessed using an anti-M13-HRP conjugated antibody.

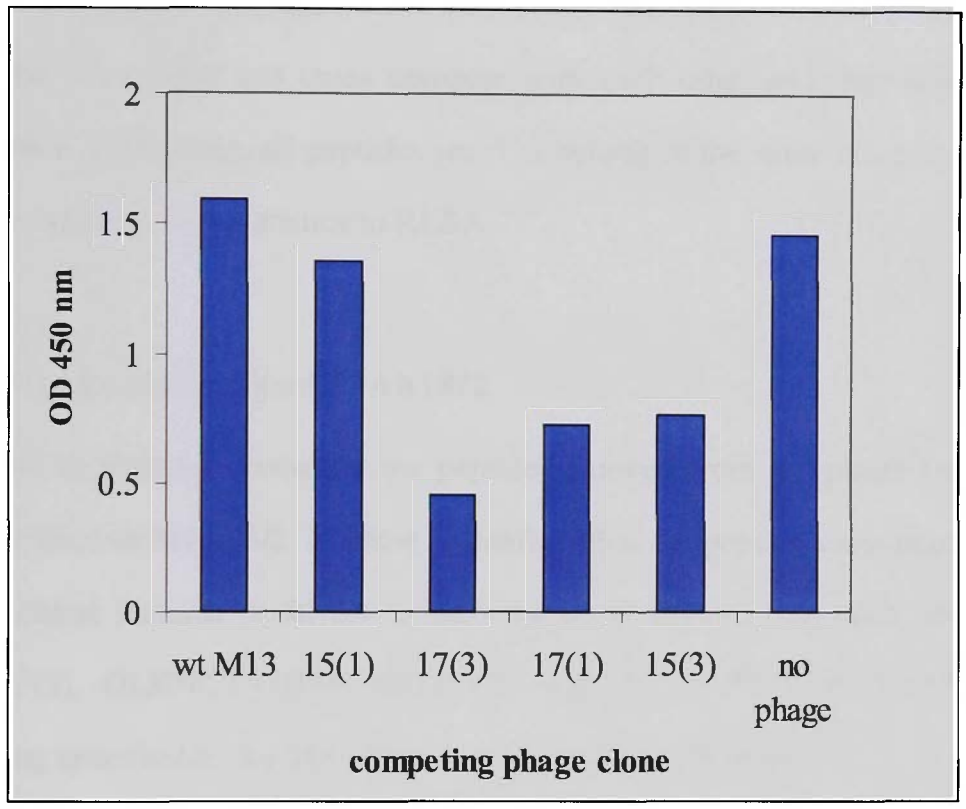


Figure 3.1.15. Inhibition of MAb18/2 binding to phage peptide 15(1) by other phage peptides. 10^{10} phage expressing peptide 15(1) were immobilized in the wells of a microtitre plate and probed with MAb18/2 (0.44 μg/ml) in the presence of 10^{10} phage expressing either the same or a different peptide. As controls, wild-type M13 phage and no phage were used.

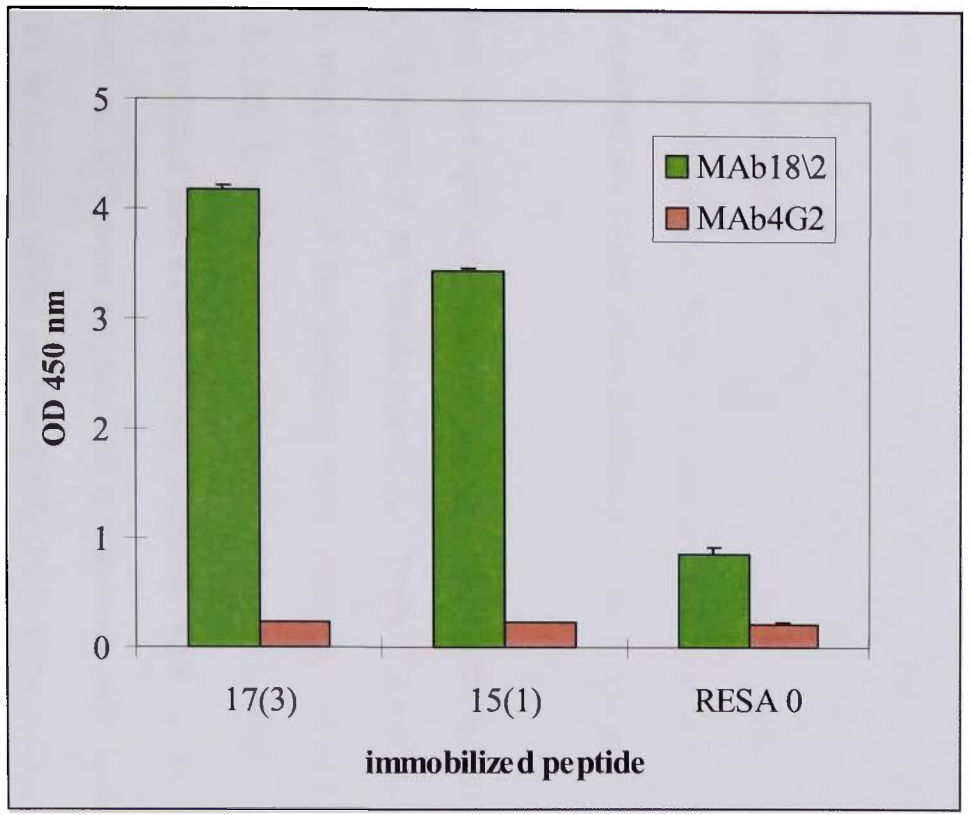
phage nor the addition of phage lacking a peptide (wildtype M13 phage). Surprisingly, no significant reduction in binding was observed in the presence of phage expressing the same peptide (15(1)). The reasons behind this are unknown, however a similar result was obtained when the same experiment was done on an earlier occasion (data not shown). In order to examine this question more thoroughly, it was necessary to perform competition experiments with synthetic peptides rather than phage (see Section 3.1.40). These data indicate that the mimotopes can cross compete with each other and that despite their dramatic sequence differences, all peptides seem to belong to the same class in that they all have similar binding characteristics to RESA.

3.1.8 Synthetic peptides bind MAb18/2

It was important to determine whether the peptide removed from the phage framework was still able to bind to MAb18/2. In order to confirm that the peptide sequences are true mimics of structural features of RESA, a representative peptide from each library was synthesized (17(3), GLKNCTVQPWDATDVCD and 15(1), CFDYAPYVSAVDDIC), and their binding specificities for MAb18/2 were determined. Peptides 17(3), 15(1) when immobilized in the wells of a microtitre plate and probed with MAb18/2 exhibited a high degree of binding in comparison with the same peptides probed with an irrelevant monoclonal antibody, MAb4G2 (Fig. 3.1.16A). No binding by MAb18/2 was observed to a peptide referred to as RESA 0 (SNDQKYSIEDSLTIK), which contains a randomized 15 amino acid sequence from within the RESA protein. A peptide with the same amino acid composition as peptide 17(3) but with a randomized sequence (peptide 17(3j): GAQLDCTVKTPDVWDCN), was also not able to bind MAb18/2 (Fig. 3.1.16B). Hence, the 15 or 17 residue peptide mimotopes can be recognized by MAb18/2 when immobilized in the wells of microtitre plates in the absence of the surrounding framework of the phage, and it is the sequence of amino acids rather than a simple charge or hydrophobicity effect that confers MAb18/2-binding.

A

17(3)	GLKNCTVQPWDATDVCD
15(1)	CFDYAPYVSAVDDIC
RESA 0	SNDQKYSIEDSLTIK



B

17(3)	GLKNCTVQPWDATDVCD
15(1)	CFDYAPYVSAVDDIC
17(3j)	GAQLDCTVKTPDVWDCN

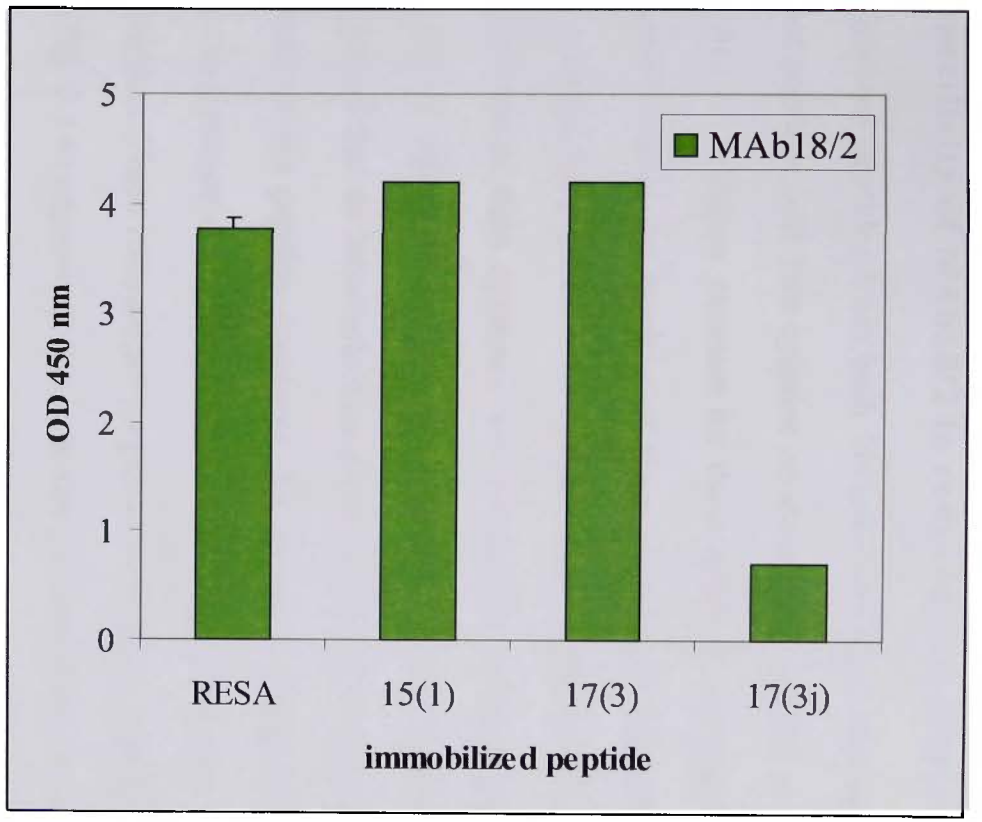


Figure 3.1.16. Specificity of the peptide mimotopes for MAb18/2. (A) Synthetic peptides 17(3), 15(1) and the control peptide RESA 0 (10 μg/ml) were used to coat the wells of a microtitre plate and probed with MAb18/2 (2 μg/ml) and an unrelated antibody, MAb4G2 (2 μg/ml). (B) Recombinant RESA-322 (5 μg/ml) and synthetic peptides 17(3), 15(1) and 17(3j) (10 μg/ml) were used to coat microtitre plates and probed with MAb18/2 (2 μg/ml). Binding was detected using a HRP-conjugated anti-mouse IgG.

3.1.9 Binding specificity of MAb18/2 to reduced and alkylated peptides

A number of the isolated peptides from both libraries contained two cysteine residues. The observation that peptides with two cysteine residues were isolated even from the 15-mer library which had no selection pressure for these residues, suggested that cysteines may play an important role in the binding of these peptides to MAb18/2. Due to the presence of these cysteines, the peptides were cyclized through the cysteine residues when synthesized. The presence of both cysteines was particularly noted in peptides isolated from the 17-mer library where there was an invariant cysteine engineered into the sequence. This suggested that an intramolecular disulphide bond might contribute to the conformation of some of the peptide structures. To further investigate this possibility, phage clones from the cysteine containing 15-mer and 17-mer libraries were separated under reducing conditions. These reduced polypeptides were still recognized by MAb18/2 on a Western blot (Fig. 3.1.6) suggesting that a disulphide bond between the two cysteine residues present in some peptides is not essential for the interaction with the MAb antigen-binding site. MAb18/2 was able to bind to both peptides 15(1) and 17(3) when produced as cyclized peptides (Fig. 3.1.16), however, it was shown that when these peptides are reduced and alkylated, the binding of 15(1) to MAb18/2 is unaffected whereas the binding of 17(3) is significantly reduced. Nevertheless there is still substantial binding of 17(3) to MAb18/2 (Fig. 3.1.17). Clearly the cysteine residues in 17(3) have a greater contribution to the binding of MAb18/2 than those in peptide 15(1), however in both peptides, other residues are clearly more important for the binding.

Electrospray ionization mass spectrometry (ESMS) was used (with the kind assistance of Dr Greg Neumann, Department of Biochemistry, La Trobe University, Australia) to confirm that greater than 95% of the peptides were correctly reduced and alkylated (Fig. 3.1.18, 3.1.19, 3.1.20). Cyclized peptide 15(1) was found to have a molecular weight of 1678.0 Da. When reduced and alkylated approximately 46% of the peptides were alkylated on both cysteines, 48% were alkylated on one cysteine and about 6% were not alkylated (Fig. 3.1.18). In the case of the peptide 17(3) (1861.8 Da, cyclized), up to 50%

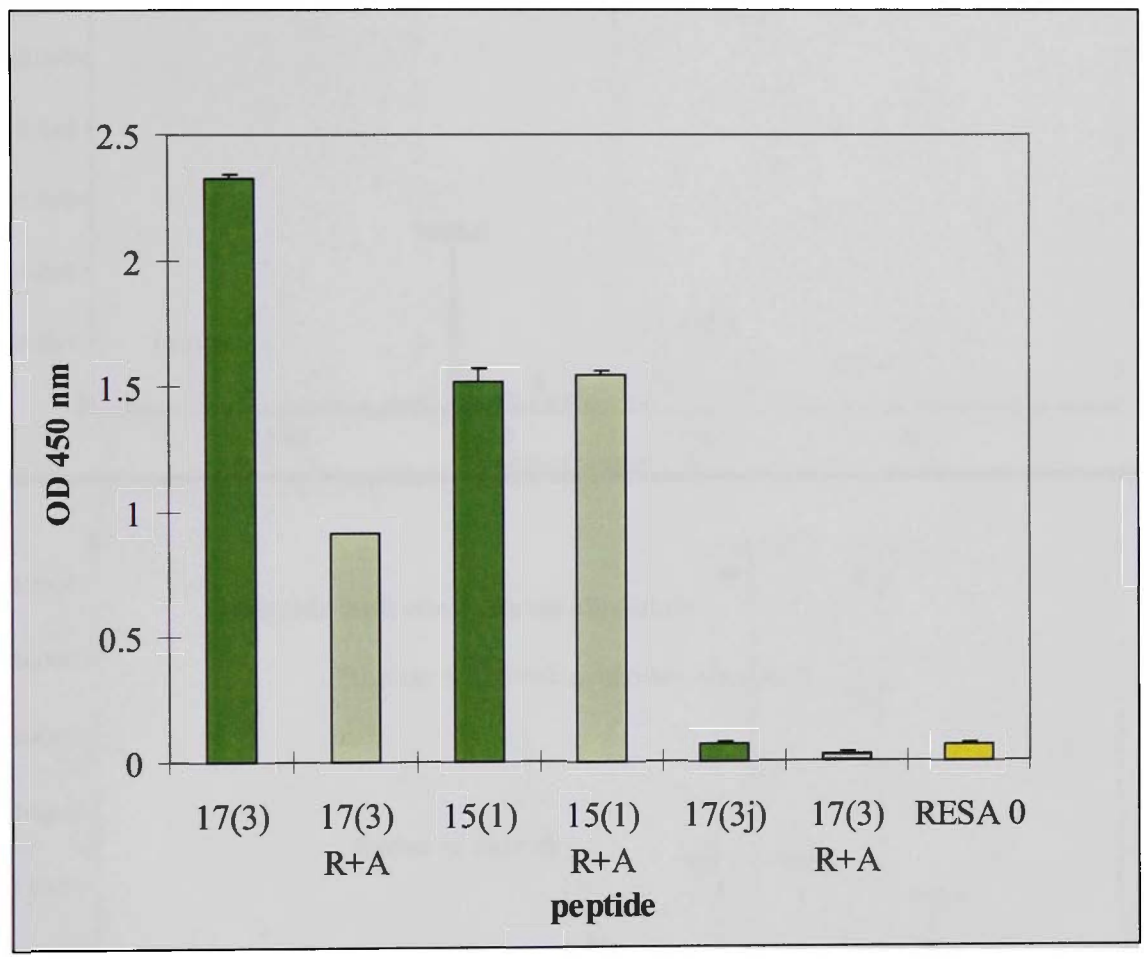


Figure 3.1.17. Effect of reduction and alkylation on binding of mimotopes to MAb18/2. Peptides 15(1), 17(3) and 17(3j) (10 μg/ml), either cyclized or reduced and alkylated (R+A), were used to coat the wells of a microtitre plate and probed with MAb18/2 (2 μg/ml). Binding was detected by HRP-conjugated anti-mouse antibody.

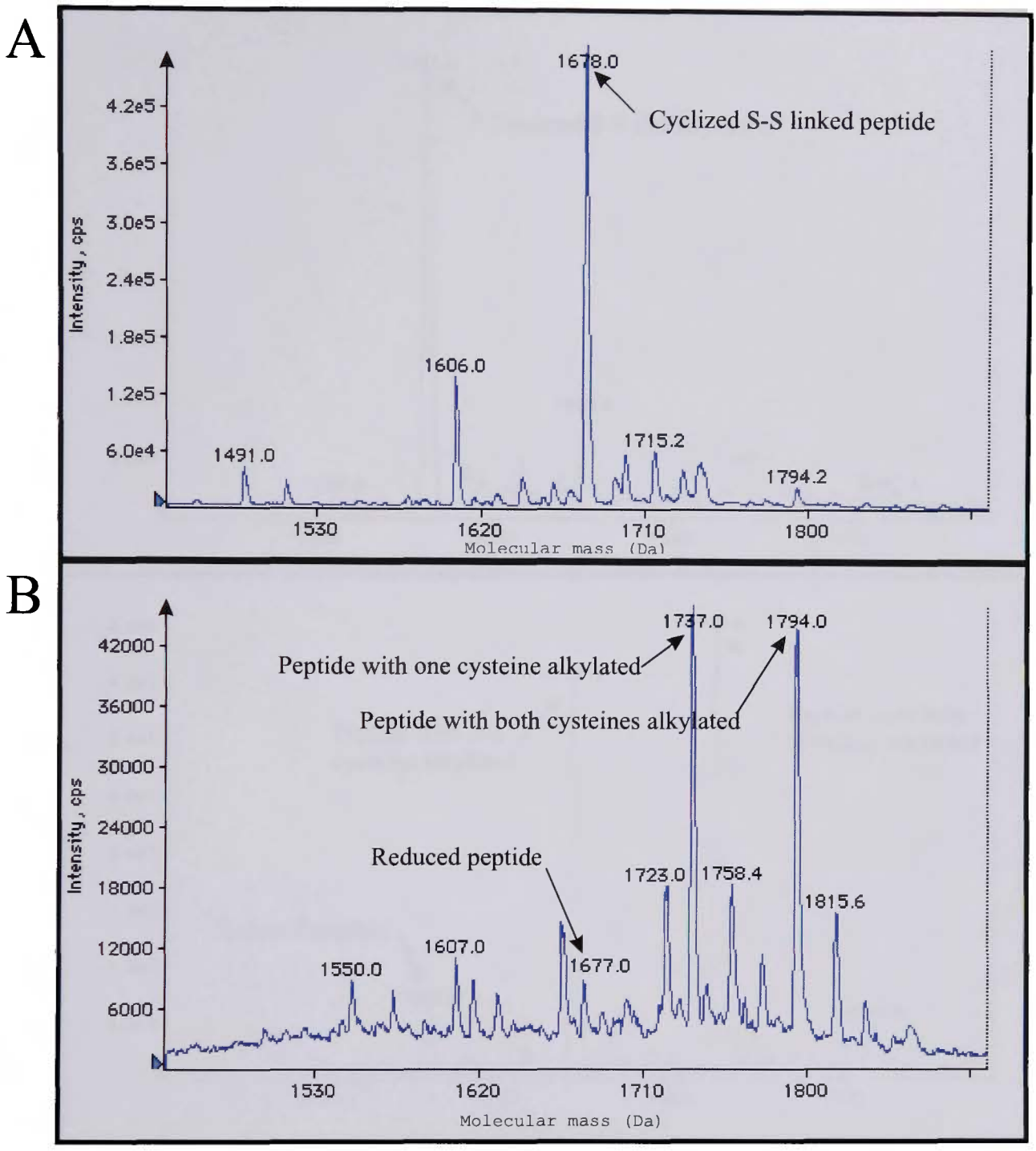


Figure 3.1.18. Analysis of the extent of reduction and alkylation of the peptides by electrospray ionization mass spectrometry (ESMS). (A) Spectrum of desalted peptide 15(1) in a cyclized conformation. (B) Spectrum of peptide 15(1) after reduction and alkylation.

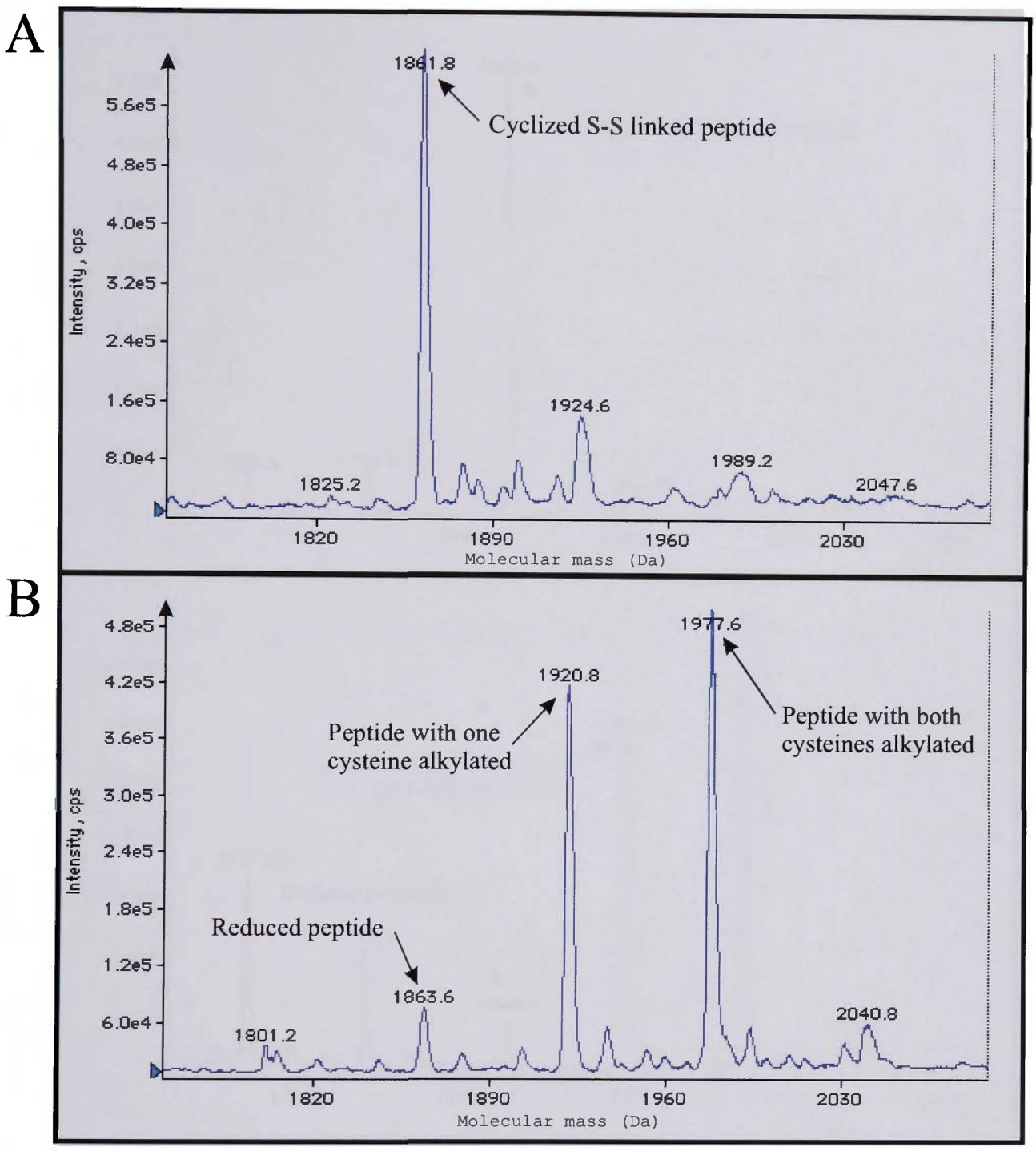


Figure 3.1.19. Analysis of the extent of reduction and alkylation of the peptides by electrospray ionization mass spectrometry (ESMS). (A) Spectrum of desalted peptide 17(3) in a cyclized conformation. (B) Spectrum of peptide 17(3) after reduction and alkylation.

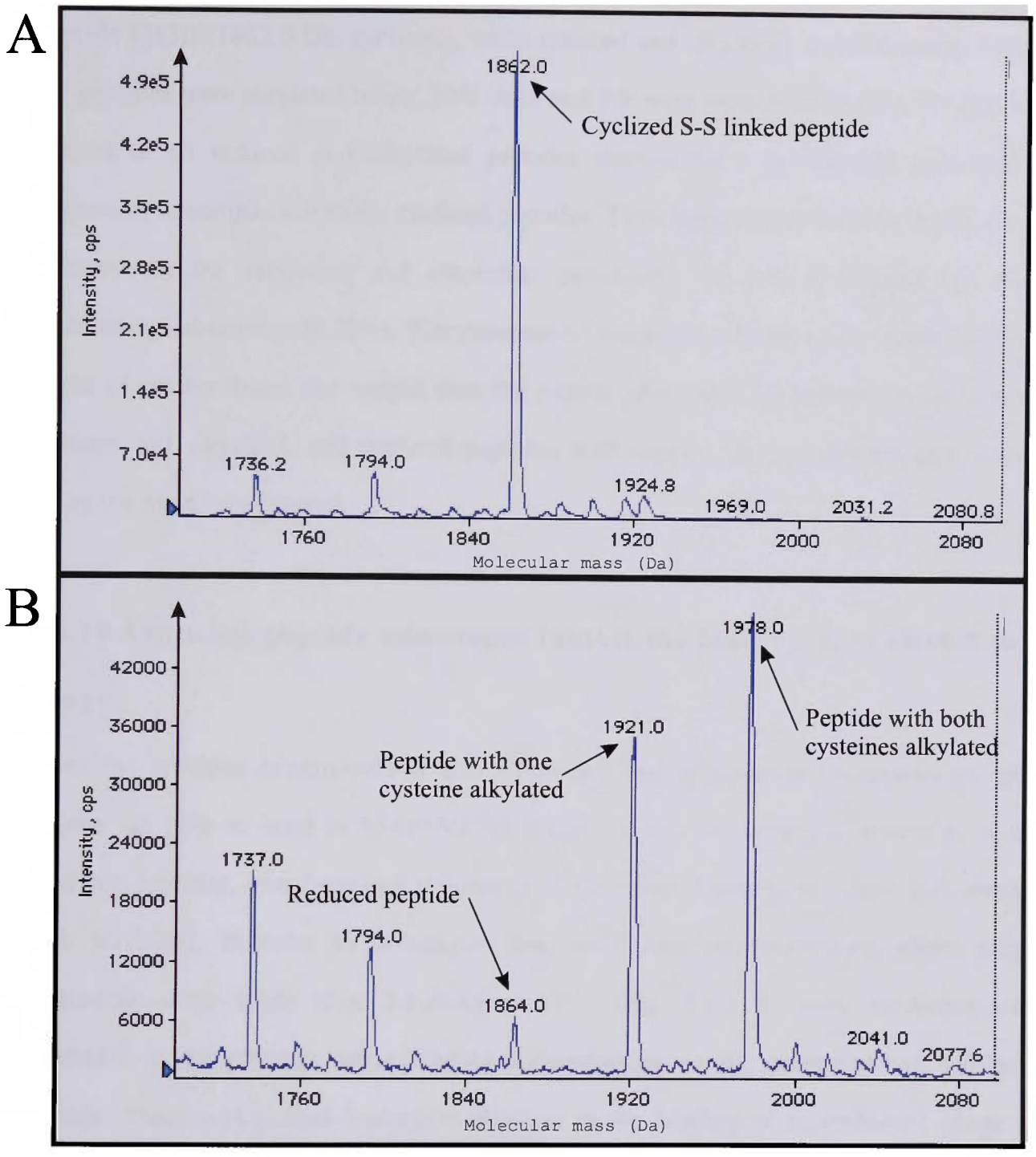


Figure 3.1.20. Analysis of the extent of reduction and alkylation of the peptides by electrospray ionization mass spectrometry (ESMS). (A) Spectrum of desalted peptide 17(3j) in a cyclized conformation. (B) Spectrum of peptide 17(3j) after reduction and alkylation.

of the peptides were alkylated on both cysteines, about 40% on one cysteine, 6% reduced but not alkylated and 4% not reduced (Fig. 3.1.19). For the scrambled version of 17(3), peptide 17(3j) (1862.0 Da, cyclized), when reduced and alkylated, approximately 54% of the peptides were alkylated twice, 39% once and 7% were reduced only (Fig. 3.1.20). The spectra of all reduced and alkylated peptides showed up to a four-fold reduction in intensities in comparison to the cyclized peptides. This decrease was most probably due to dilutions in the reduction and alkylation procedure and was accounted for when conducting subsequent ELISAs. The presence of metal ion adducts was evident (as small peaks of greater molecular weight than the peptide of interest) in the spectra of both the reduced and alkylated, and cyclized peptides with copper, iron, potassium and sodium being the main ions present.

3.1.10 Synthetic peptide mimotopes inhibit the binding of MAb18/2 to phage

From the previous experiments it was concluded that the peptides displayed as gpIII fusions are able to bind to MAb18/2. However, it was important to determine if the synthetic peptides, free from any influences of the phage framework, could still interact with MAb18/2. In order to investigate this, an ELISA was performed where phage displaying either 17(3) (Fig. 3.1.21A) or 15(1) (Fig. 3.1.21B) were incubated with MAb18/2 in the presence of increasing concentrations of the corresponding synthetic peptide. There was a dose-dependant decrease in the binding of recombinant phage to MAb18/2 with both synthetic peptides. This is further evidence that the binding site for MAb18/2 lies within the 17 and 15 residue inserts and that there is little if any influence of the phage framework on binding. Furthermore, a peptide containing the same amino acids as the 17(3) peptide but in a scrambled order (17(3j)) had little effect on the binding of either 17(3) or 15(1) phage to MAb18/2 even at concentrations of 150 μg/ml (Fig. 3.1.21). Thus the primary sequence, and not simply the acidic nature of the peptide, appears to be critical for efficient binding to the antigen-binding site of MAb18/2. In view of the large diversity of sequences that can bind to MAb18/2, an intriguing question was

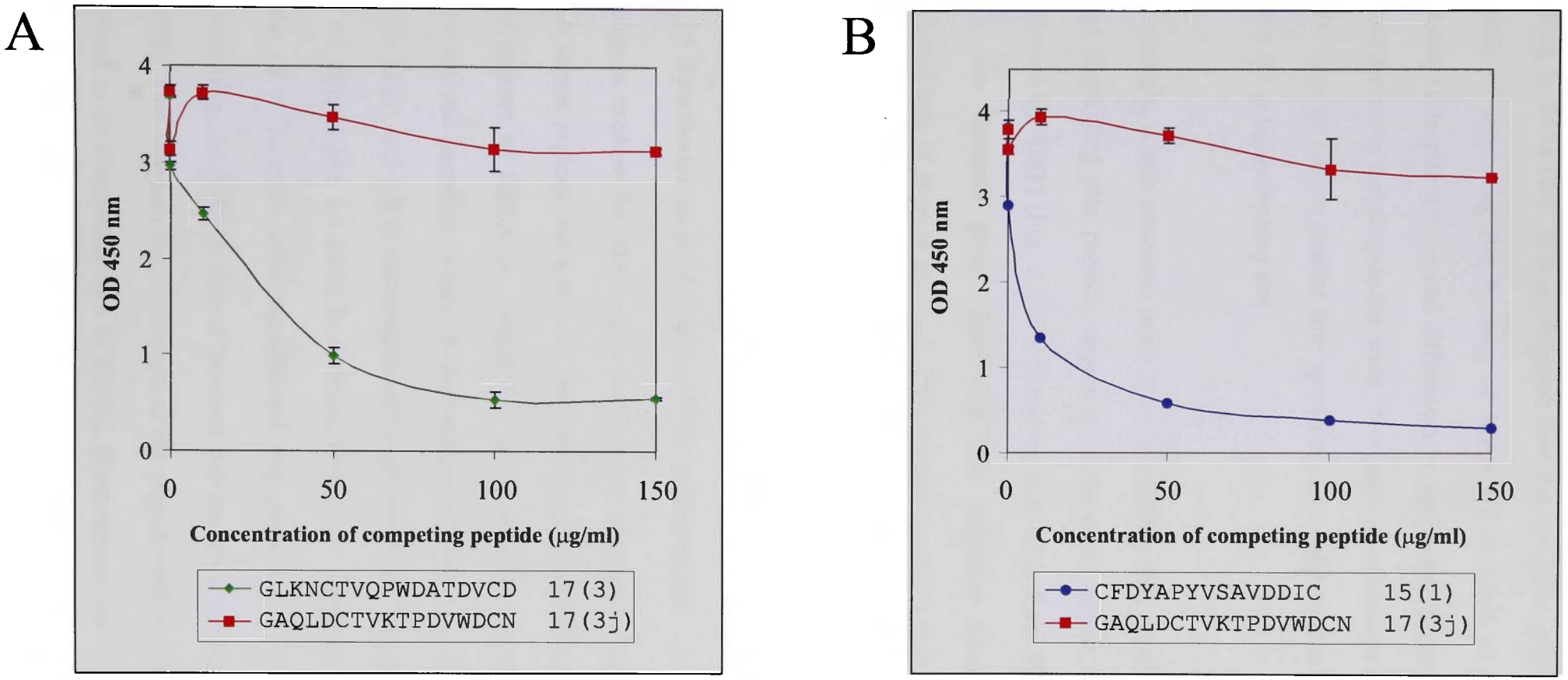


Figure 3.1.21. Inhibition by synthetic peptides of the interaction of phage clones with MAb18/2. 10^{10} phage of (A) phage clone 17(3) or (B) phage clone 15(1) were immobilized on the wells of a microtitre plate and probed with MAb18/2 (0.44 µg/ml) in the presence of increasing concentrations of synthetic peptide 17(3), synthetic peptide 15(1) or the control peptide 17(3j). Binding was detected using an anti-M13-HRP conjugated antibody.

whether a particular peptide was able to compete peptides with different sequences from binding to MAb18/2. It was observed that the synthetic peptides 15(1) and 17(3) were capable of inhibiting the binding of MAb18/2 to most of the other phage-displayed mimotopes despite the marked differences in amino acid sequences (Fig. 3.1.22). It would appear therefore that despite the wide divergence of mimotope sequences identified in this study, they all have a similar fine specificity (*i.e.* they bind to spatially overlapping sites within the antigen-binding site).

Interestingly, it was observed under these conditions that peptide 17(3) does not compete phage displaying this peptide sequence to the same extent as phage displaying other sequences eg. 15(1) (Fig. 3.1.22). However, it is clear from Figure 3.1.21A that peptide 17(3) can compete phage displaying this sequence almost completely if higher concentrations of peptide are used. This apparent paradox may be explained partly by the fact that in the experiment described in Figure 3.1.22, a portion of this peptide may have become aggregated and not have been in a suitable conformation for binding MAb18/2.

3.1.11 Synthetic peptides are RESA mimotopes

In order to explore the relationship between the mimotopes isolated in this study and the RESA repeat regions, we examined the binding of MAb18/2 to peptides representing the major repeats of RESA in comparison to the mimotopes. MAb18/2 recognized all 3 RESA derived peptides: 4-mer, BSA conjugated 8-mer, and BSA conjugated 11-mer as well as 17(3) and 15(1) mimotopes, although the 8-mer peptide appeared less reactive than the others (Fig. 3.1.23A). In contrast, when the 11-mer (not coupled to BSA) and the 22-mer (2 × 11-mer) were immobilized and probed with MAb18/2, no binding was observed (Fig. 3.1.23B). It should be noted that the BSA conjugated 11-mer is 3 × 11-mer in length. This suggests that the length of the peptide and its conformational state may be important in its interaction with MAb18/2. Furthermore, the immobilization on the wells of the microtitre plate may induce a conformation that is not conducive to binding.

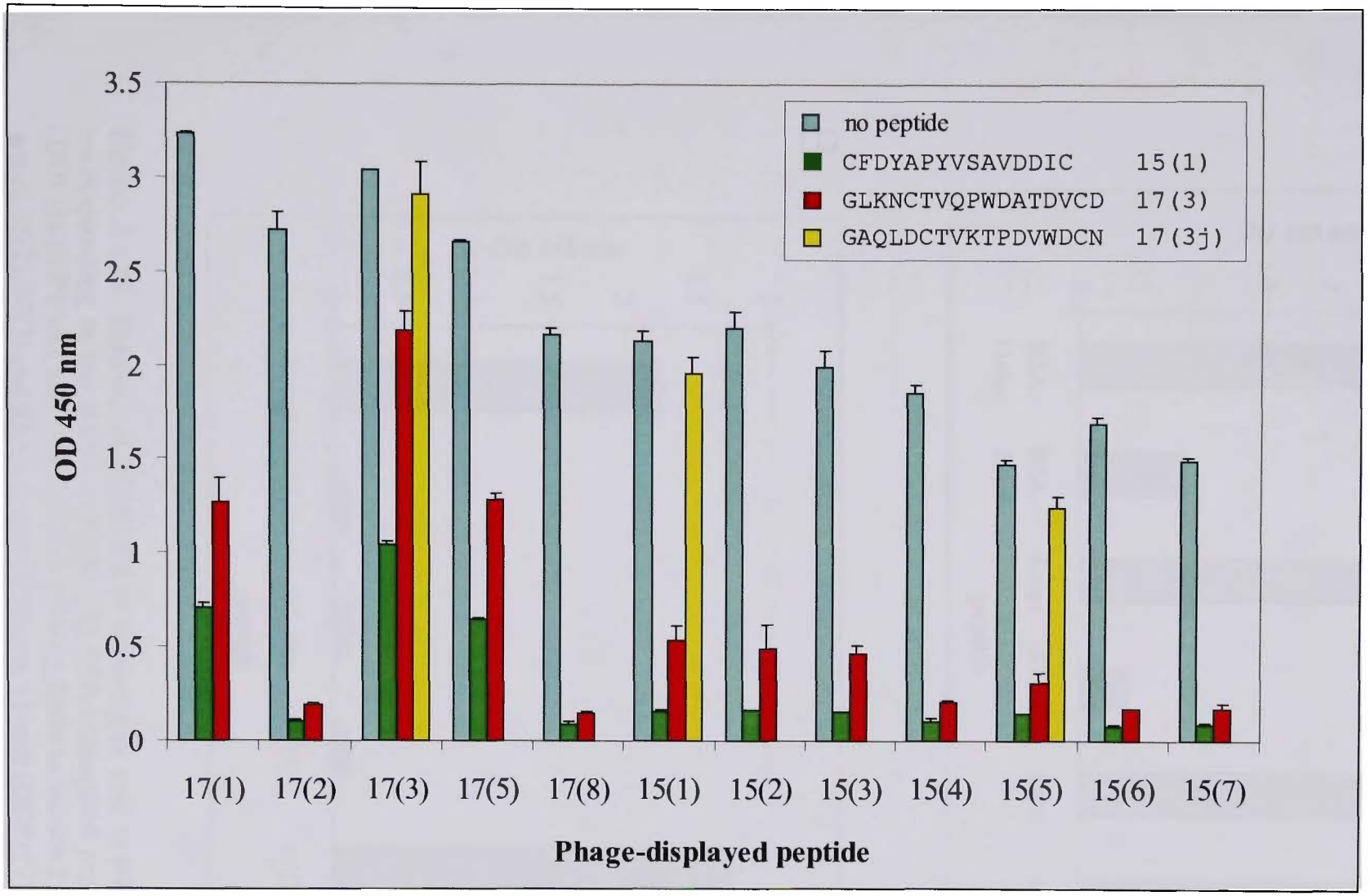


Figure 3.1.22. Inhibition of the binding of phage clones to MAb18/2 by synthetic peptides. Twelve different phage clones (10^{10} phage) were used to coat the wells of a microtitre plate and probed with MAb18/2 (0.44 μg/ml) in the presence of either no peptide (aqua bars), 50 μg/ml of peptide 15(1) (green bars), 50 μg/ml of peptide 17(3) (red bars) or 50 μg/ml of the control peptide 17(3j) (yellow bars). Binding was detected using a HRP-conjugated anti-mouse antibody.

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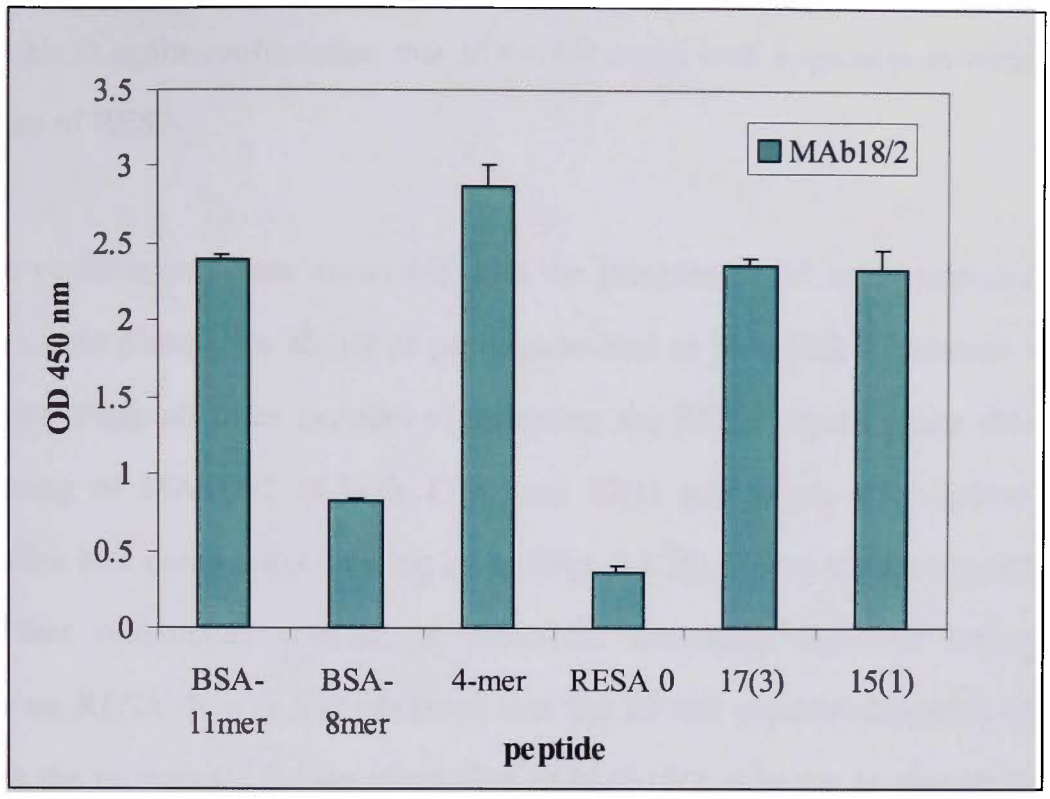

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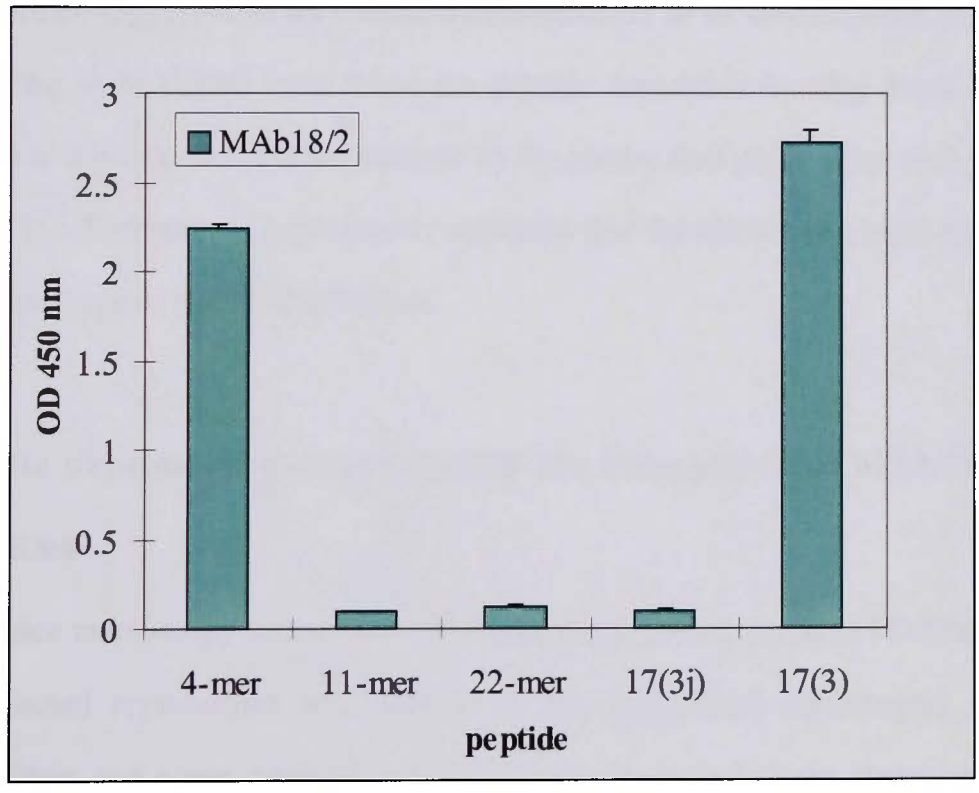

Figure 3.1.23. Binding of MAb18/2 to mimotopes and synthetic peptides corresponding to the RESA repeats. (A) BSA conjugated peptides: 11-mer, $(DDEHVEEPTVA)_3$ and 8-mer, $(EENVEHDA)_4$ (refer to Section 2.17 for details); 4-mer, 15(1), 17(3) and RESA 0; and (B) 4-mer, 11-mer (DDEHVEEPTVA), 22-mer $(DDEHVEEPTVA)_2$, 17(3j) and 17(3) (10 μg/ml) were used to coat the wells of a microtitre plate and probed with MAb18/2 (2 μg/ml). Bound antibody was detected using an anti-mouse-HRP antibody.

Nevertheless, this is again confirmation that MAb18/2 reacts with sequences in both the 5' and 3' repeats of RESA.

In view of the possible problems associated with the presentation of small peptides by adsorbing them onto plastic, the ability of peptides to bind to MAb18/2 in solution was examined. Interestingly all three peptides representing the RESA repeats were able to block the binding of MAb18/2 to both 17(3) and 15(1) mimotopes when added as synthetic peptides in a competitive binding assay (Fig. 3.1.24). These results strengthen the proposal that mimotopes selected on MAb18/2 accurately represent authentic antigenic sites on RESA. It was also observed that the 22-mer peptide competed more effectively with the mimotopes for the interaction of MAb18/2, than the 11-mer peptide, despite the fact that neither peptide recognizes MAb18/2 in an immobilized state (Fig. 3.1.24B). This further suggests that an incorrect conformation of an immobilized peptide can prevent binding to MAb18/2 even when the peptide contains a binding motif. It is possible that critical binding contacts are masked by the plastic thus preventing them from binding to MAb18/2. Both sets of experiments indicated that the mimotopes most closely resembled the 4-mer repeats (EENV) of RESA.

3.1.12 Synthetic peptide mimotopes inhibit the interaction of MAb18/2 with native RESA

Immunofluorescence microscopy studies were performed by probing smears of D10 strain *P. falciparum*-infected erythrocytes with MAb18/2. The parasitized erythrocytes were maintained in culture and a synchronous population was harvested at the ring-stage of infection (Fig. 3.1.25, 3.1.26, 3.1.27). MAb18/2 recognized RESA associated with the membrane skeleton of erythrocytes infected with ring-stage parasites (Fig. 3.1.25). The two synthetic peptides, 15(1), CFDYAPYVSAVDDIC and 17(3), GLKNCTVQPWDATDVCD, inhibited the binding of MAb18/2 to RESA as shown by a decrease in the signal in an immunofluorescence assay (Fig. 3.1.26 and 3.1.27). Five μg of each peptide appeared to be sufficient to almost completely abolish the interaction of

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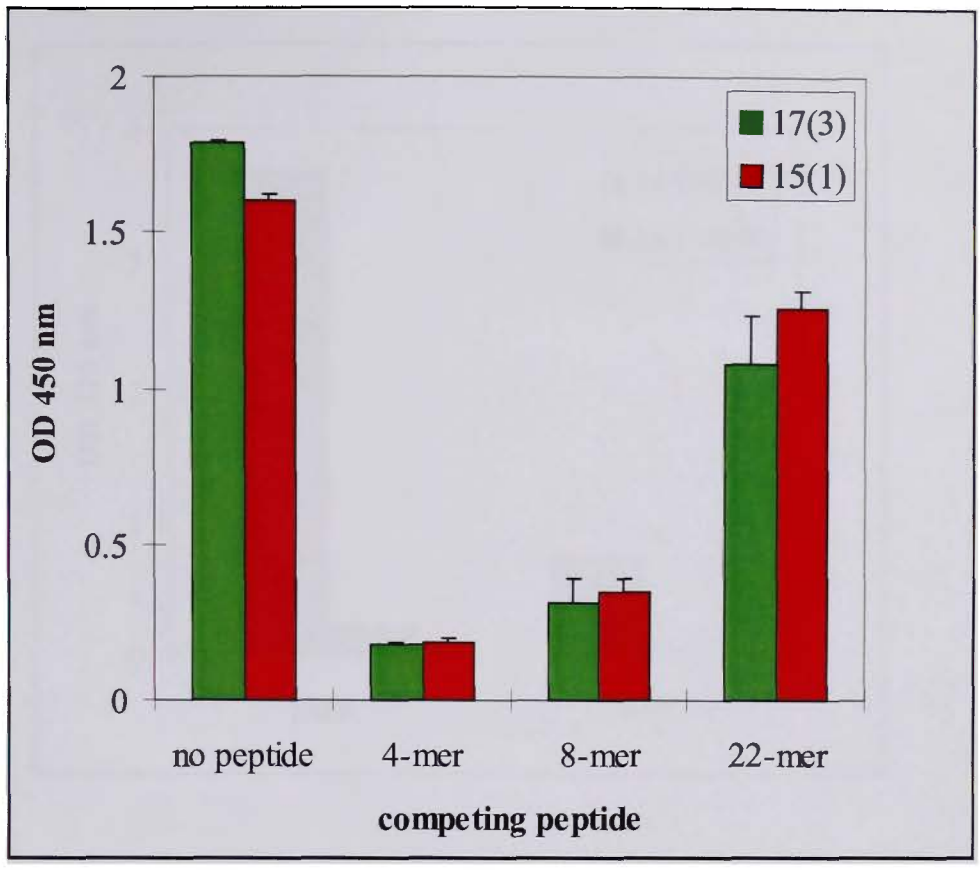

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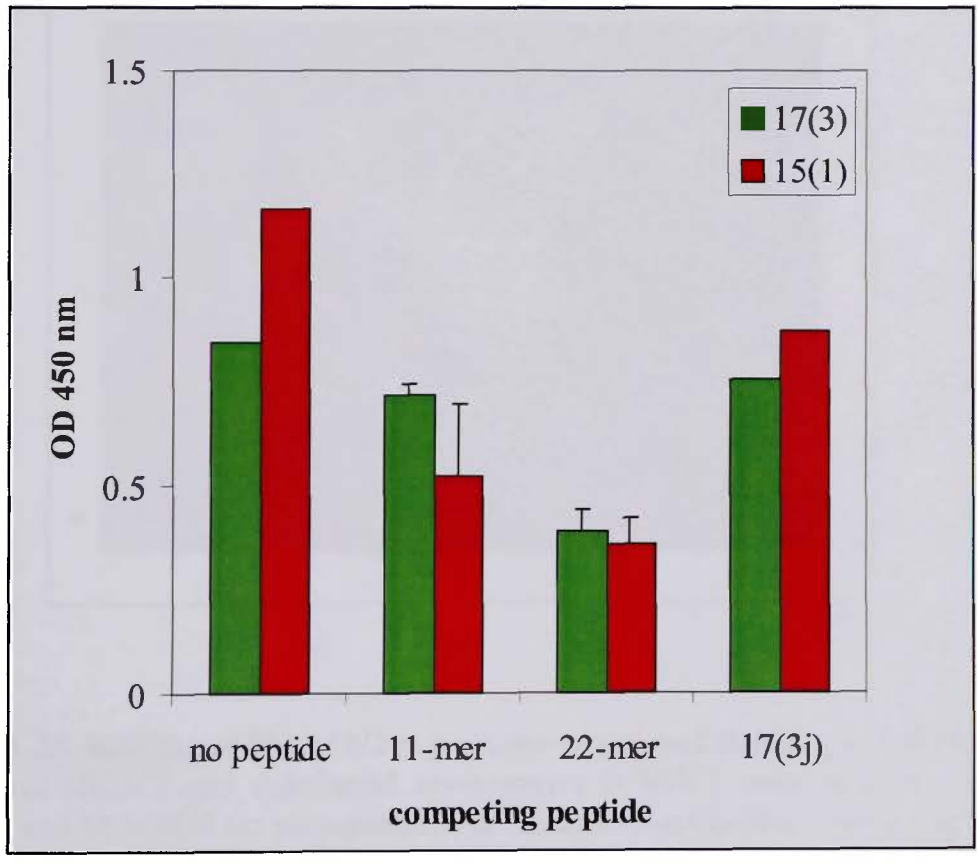

Figure 3.1.24. Peptides derived from the repeat regions of RESA inhibit the interaction of mimotopes 17(3) and 15(1) with MAb18/2. Peptides 17(3) (green bars) and 15(1) (red bars) (10 μg/ml) were used to coat the wells of a microtitre plate and probed with MAb18/2 (0.44 μg/ml) in the presence of 100 μg/ml of (A) the 4-mer, 8-mer and 22-mer $(DDEHVEEPTVA)_2$ or (B) the 11-mer (DDEHVEEPTVA) and 22-mer. Bound antibody was detected using a HRP-coupled anti-mouse IgG. (A) and (B) were performed on different days.

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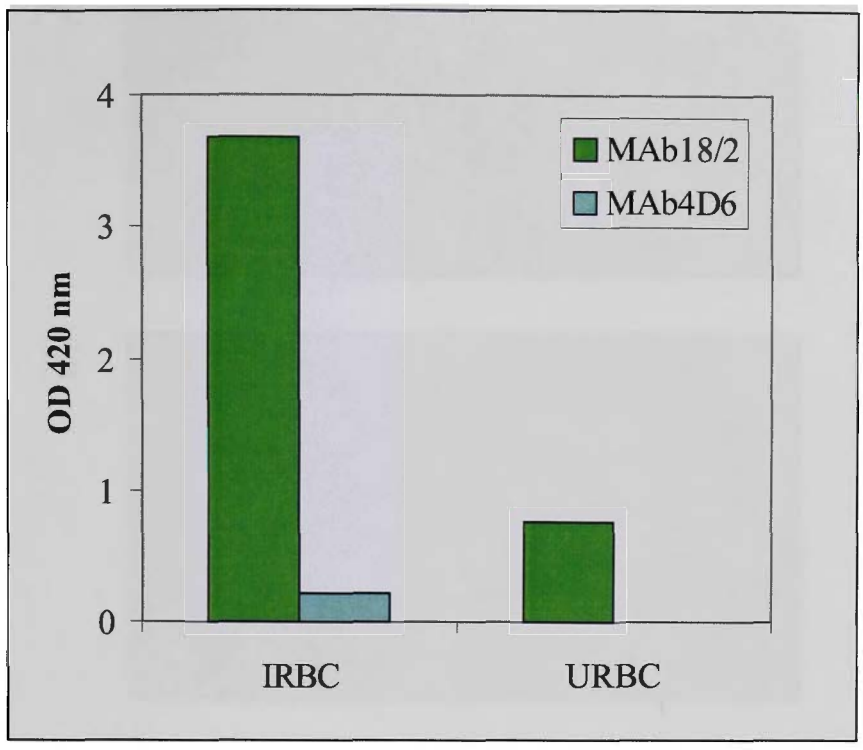

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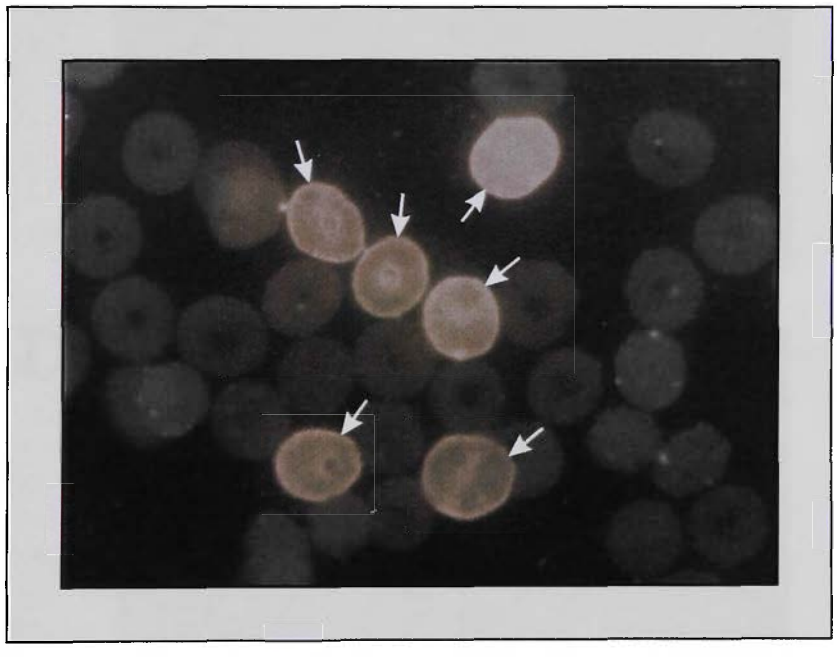

Figure 3.1.25. Binding of MAb18/2 to parasite-expressed RESA. (A) Infected erythrocytes (IRBC) and uninfected erythrocytes (URBC) were probed with MAb18/2 and MAb4D6 (in suspension). An alkaline phosphatase coupled anti-mouse IgG was used as a secondary antibody and binding was detected using the substrate pNPP which was assayed spectrophotometrically at 420 nm. (B) Immunofluorescence experiments were carried out with MAb18/2 to visualize the binding of RESA to the surface of infected erythrocyte membranes. Binding of MAb18/2 was detected using anti-mouse IgG-FITC. A rim-like fluorescence was observed around the circumference of erythrocytes infected with ring-stage parasites (arrows).

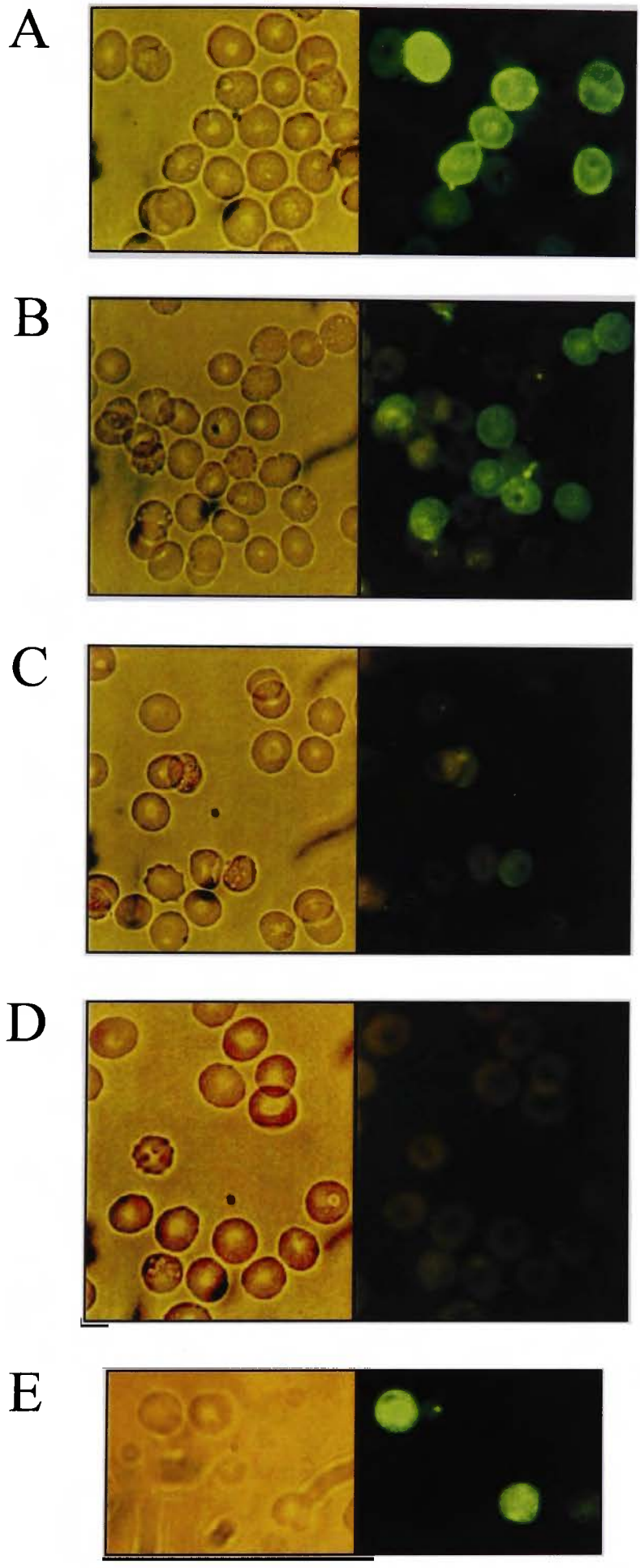

Figure 3.1.26. Inhibition of MAb18/2 binding to parasite-expressed RESA by peptide 15(1). Immunofluorescence experiments were carried out by probing infected erythrocytes with MAb18/2 in the presence of (A) no peptide, (B) 0.5 μg of peptide 15(1), (C)1 μg of peptide 15(1), (D) 5 μg of peptide 15(1) and (E) 5 μg of the irrelevant peptide RESA 0. Binding was detected using anti-mouse IgG-FITC.

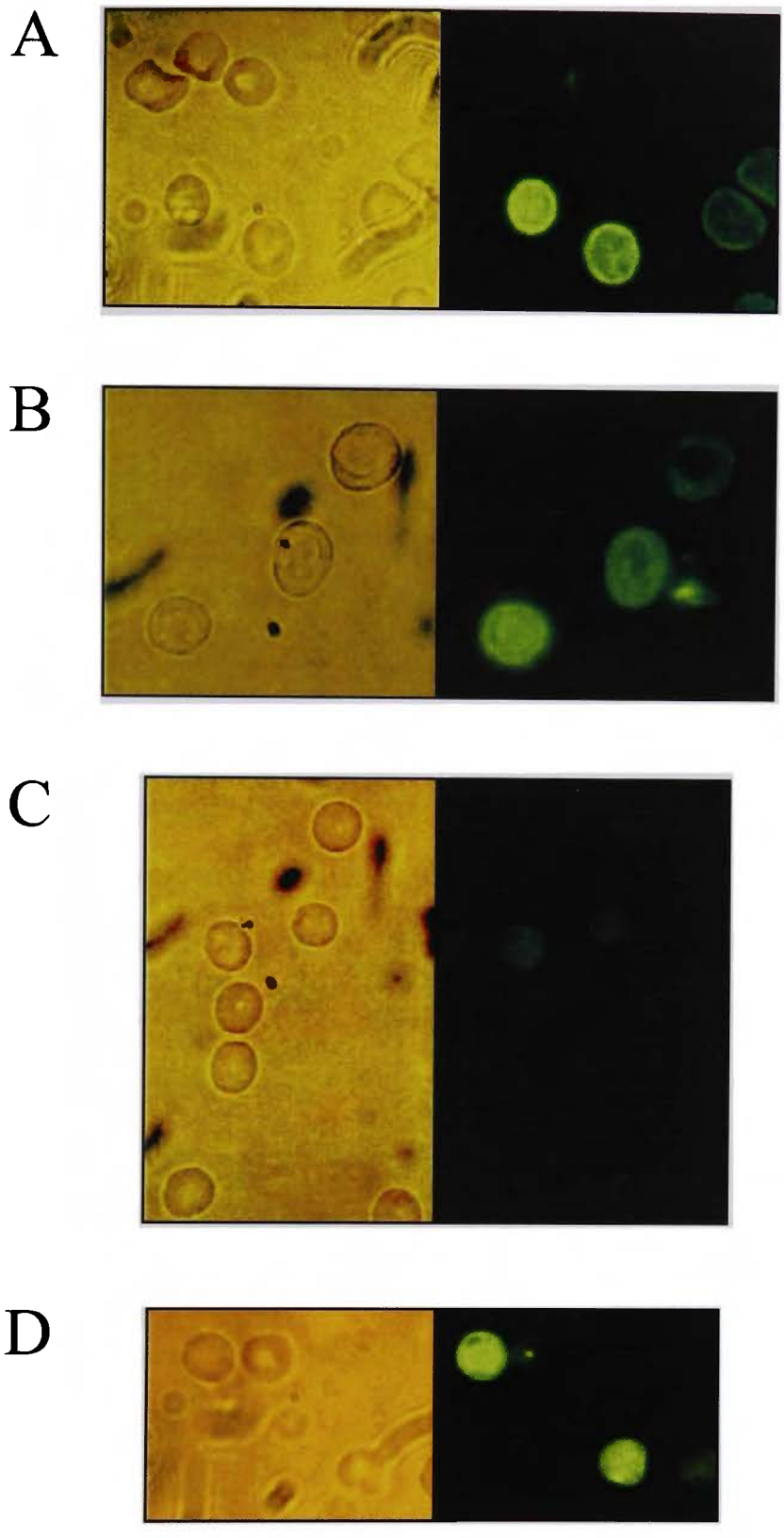

Figure 3.1.27. Inhibition of MAb18/2 binding to parasite-expressed RESA by peptide 17(1). Immunofluorescence experiments were carried out by probing infected erythrocytes with MAb18/2 in the presence of (A) no peptide, (B) 0.5 μg of peptide 17(1), (C) 5 μg of peptide 17(1) and (D) 5 μg of the irrelevant peptide RESA 0. Binding was detected using anti-mouse IgG-FITC.

MAb18/2 with the 'native' RESA in the context of the parasitized erythrocyte (Fig. 3.1.26D and 3.1.27C). An irrelevant peptide, SNDQKYSIEDSLTIK (RESA 0) consisting of a randomized stretch of amino acids from RESA was unable to inhibit fluorescence (Fig. 3.1.26E and 3.1.27D).

Quantitation of the degree of blocking by peptides 15(1) and 17(3) of MAb18/2 binding to RESA *in situ* was obtained by measuring the amount of antibody bound in the presence of increasing mimotope concentrations in a modified ELISA-based format. Briefly, infected erythrocytes containing the ring-stage parasites were lysed to remove haemoglobin and then probed with MAb18/2 which bound to native RESA associated with the erythrocyte membrane. Various concentrations of peptides 17(3) and 15(1) were also incorporated to block this interaction. Following removal of unbound antibody, RESA-bound MAb18/2 was quantitated spectrophotometrically using an alkaline phosphatase-coupled secondary antibody and a suitable substrate (Fig. 3.1.25A) or visualized using immunofluorescence (Fig. 3.1.25B). MAb18/2 recognized infected erythrocytes (IRBC). Uninfected erythrocytes (URBC) only showed autofluorescence. Peptides, 15(1) and 17(3) blocked the binding of the antibody to RESA in a dose-dependant manner whereas an irrelevant peptide had little effect (Fig. 3.1.28). Thus binding of MAb18/2 to authentic RESA (*i.e.* as it is presented by intra-erythrocytic parasites) is inhibited by these phage-derived mimotopes. This emphasizes the structural similarity between these peptides and immunodominant regions of RESA.

3.1.13 Pooled human sera from donors from malarious countries recognize the RESA mimotopes

During an immune response to malaria infection, antibodies to various malarial proteins, including RESA, are produced. Pooled human sera from malaria-infected individuals from a region of Papua New Guinea (PNG) where malaria is endemic has previously been reported to recognize RESA (27). In this study, it was found that the PNG sera bound to recombinant RESA-322 in a dose-dependant manner, whereas Melbourne sera showed

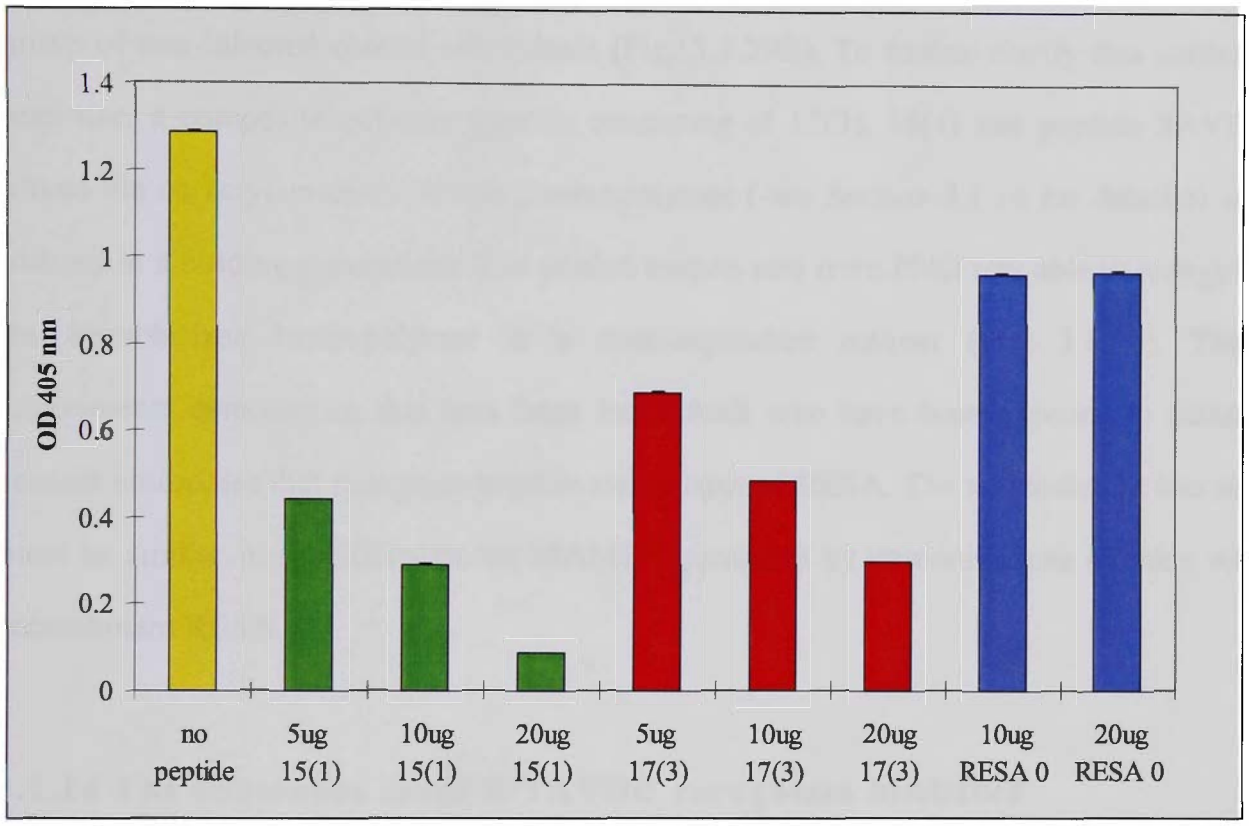

Figure 3.1.28. Quantitation of the inhibition of MAb18/2 binding to parasite expressed RESA by mimotopes. Infected erythrocytes and uninfected erythrocytes (URBC) were probed with MAb18/2 in the presence of no peptide (yellow bars) or increasing amounts of peptides 15(1) (green bars), 17(3) (red bars) and RESA 0 (blue bars). An alkaline phosphatase-coupled anti-mouse IgG was used as a secondary antibody and binding was detected using the substrate pNPP which was assayed spectrophotometrically at 405 nm. Binding of MAb18/2 to URBC (A_{405nm} of 0.45) was subtracted from the other results.

only low binding (Fig. 3.1.29A). To obtain support for the assertion that these mimotopes are relevant to the natural immune response induced by RESA, we examined the ability of the same PNG sera to recognize the mimotopes. In ELISA experiments, the pooled sera from PNG showed a 4-fold higher response to peptide 17(3) and a 3-fold higher response to peptide 15(1) than the background signal observed using pooled Melbourne sera from a group of non-infected control individuals (Fig. 3.1.29B). To further clarify this antibody response, a composite polymer peptide consisting of 17(3), 15(1) and peptide SAVDD linked via an acrylamide backbone (heteropolymer (-see Section 3.1.14 for details)) was utilized in a binding experiment. The pooled human sera from PNG was able to recognize the immobilized heteropolymer in a dose-dependant manner (Fig. 3.1.30). These experiments demonstrate that sera from individuals who have been exposed to malaria contain antibodies that recognize peptide mimotopes of RESA. The antibodies in this sera must be similar in specificity to the MAb18/2 produced by immunizations of mice with recombinant RESA.

3.1.14 The consensus motif S/TAVDD recognizes MAb18/2

Five peptides isolated by panning both peptide libraries on MAb18/2 contained either the sequence SAVDD or TAVDD in widely different contexts. It is tempting to suggest that this motif (S/TAVDD) is necessary and sufficient for binding to the antigen-binding site of MAb18/2. Alternatively, this motif may be important, but residues on either side may also contribute to the binding. In order to investigate the specificity of the consensus motif, S/TAVDD, for MAb18/2 and determine whether this motif alone is sufficient to mediate binding, the peptide SAVDD was produced as a synthetic monomer (SAVDD mono) and as a polymer linked via an acrylamide backbone (SAVDD poly) by Daniella Salvatore in the laboratory of David Jackson, Department of Microbiology, University of Melbourne, Parkville, Victoria, Australia. In addition the three peptides 15(1), 17(3) and SAVDD were assembled in combination on an acrylamide framework (heteropolymer). The assembly of peptides on a polyacrylamide backbone provided an alternative context in which to present the peptide, as it was found in different cases that immobilization of a

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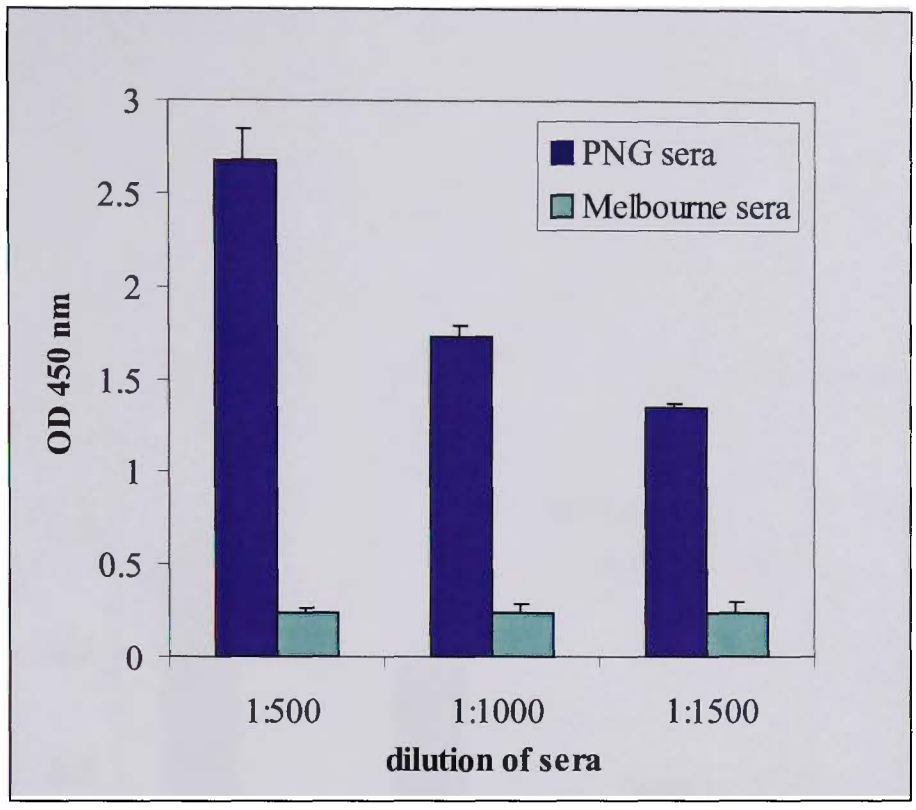

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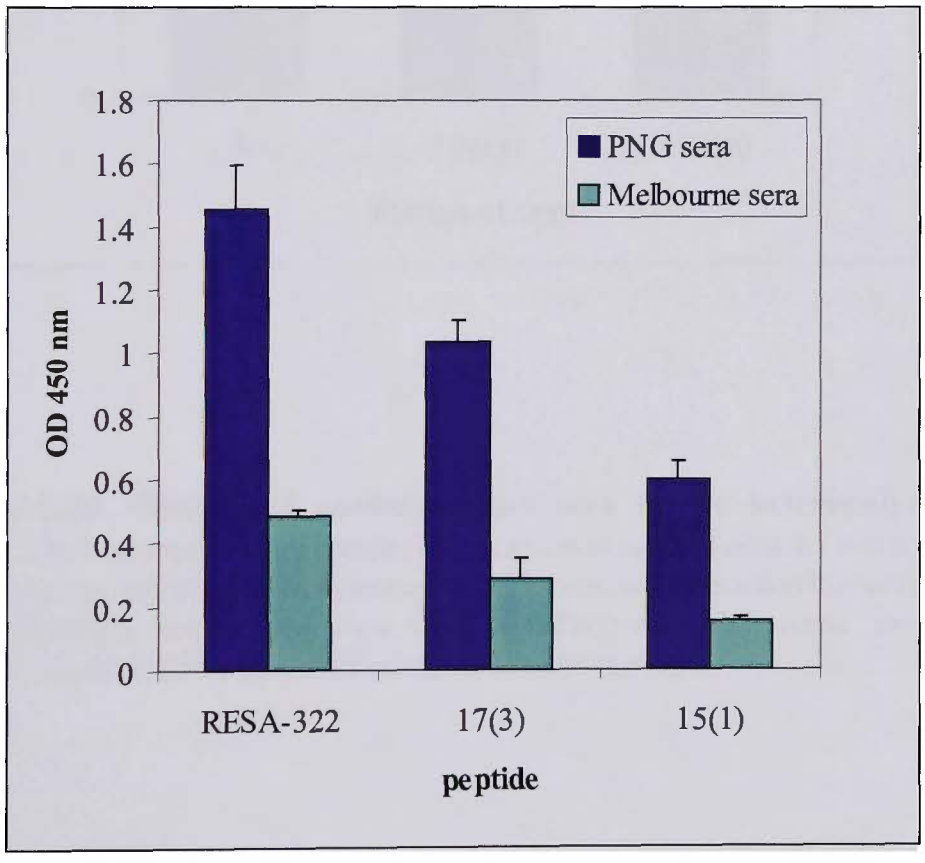


Figure 3.1.29. Binding of pooled human sera to RESA and synthetic mimotopes. (A and B) Recombinant RESA-322 (5 μg/ml) and (B) synthetic peptides 15(1) and 17(3) (10 μg/ml) were used to coat the wells of a microtitre plate and probed with (A) increasing dilutions or (B) a 1:2000 dilution of pooled human sera from individuals from Papua New Guinea (PNG) and Melbourne. Bound antibody was detected with HRP-coupled anti-human IgG.

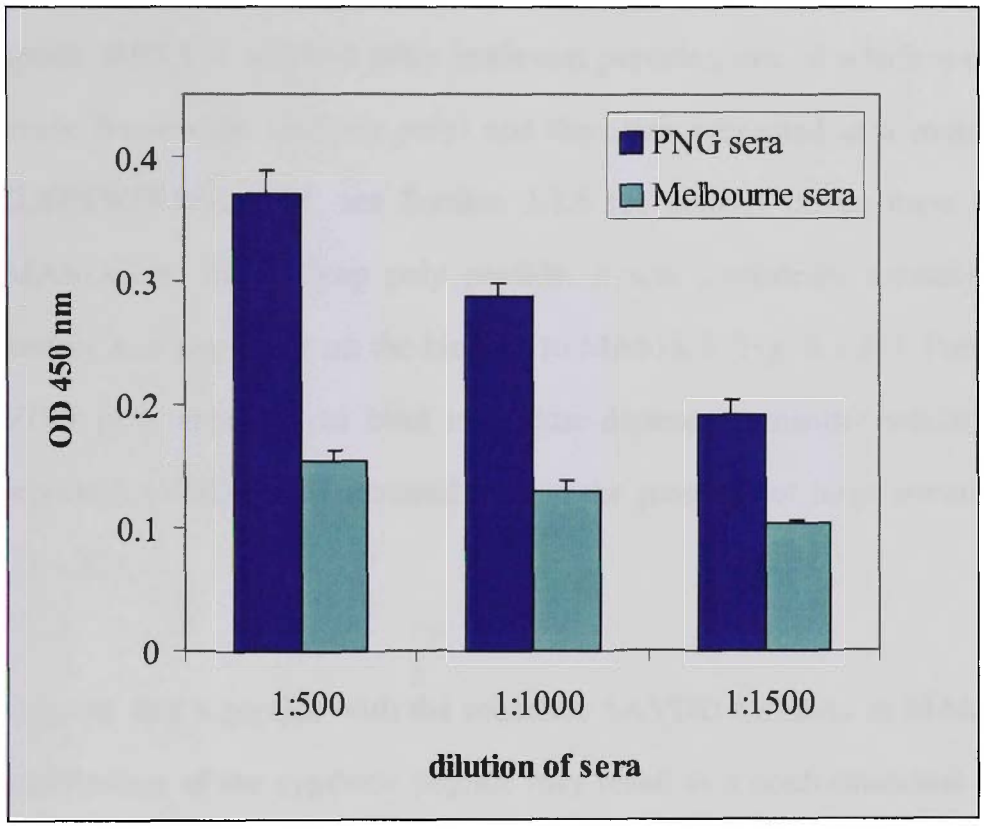


Figure 3.1.30. Binding of pooled human sera to the heteropolymer peptide. The heteropolymer peptide (10 μg/ml) was used to coat the wells of a microtitre plate and probed with increasing dilutions of the pooled human sera from individuals from Papua New Guinea (PNG) and Melbourne. Bound antibody was detected with a HRP-coupled anti-human IgG.

peptide monomer in an ELISA format resulted in a peptide conformation that could no longer recognize the antibody (Fig. 3.1.23 and 3.1.31). In ELISA experiments, MAb18/2 recognized SAVDD poly and the heteropolymer peptides, although the SAVDD poly peptide appeared less reactive than the heteropolymer and peptides 15(1) and 17(3) (Fig. 3.1.31). The peptide, SAVDD mono, did not appear to bind, as was the case for the irrelevant peptide, RESA 0, and two other irrelevant peptides, one of which was coupled to an acrylamide framework (cl-2pep poly) and the other presented as a monomer (cl-2pep. - GWLSPSWFEPGLASM, see Section 3.2.6 for details). Since there was little binding of MAb18/2 to the cl-2pep poly peptide, it was considered unlikely that the acrylamide moiety had any affect on the binding to MAb18/2 (Fig. 3.1.31). Furthermore, peptide SAVDD poly appeared to bind in a dose-dependant manner whilst minimal binding of peptide SAVDD mono occurred only in the presence of large amounts of the peptide (Fig. 3.1.32).

These data suggests that a peptide with the sequence SAVDD can bind to MAb18/2, but that the immobilization of the synthetic peptide may result in a conformational state that renders it incapable of binding MAb18/2. The problem of immobilization rendering peptides incapable of binding to MAb18/2 appears not to be a problem for the mimotopes, 15(1) and 17(3), as the critical residues are available for binding the antibody in the ELISA format. Hence, the length of the peptide as well as its conformational state could be important in the binding interaction in an ELISA format. Further ELISA experiments revealed that the heteropolymer is recognized by MAb18/2 (Fig. 3.1.33A) and binds as well as the combined individual monomers (ie. peptides 15(1), 17(3) and SAVDD) (Fig. 3.1.33B).

Having established that MAb18/2 recognizes the consensus motif, SAVDD, experiments were conducted to identify whether this motif is sufficient to abolish the interaction of MAb18/2 with the mimotopes, 15(1) and 17(3). Competitive binding experiments revealed that the peptide SAVDD mono itself was able to reduce this interaction by

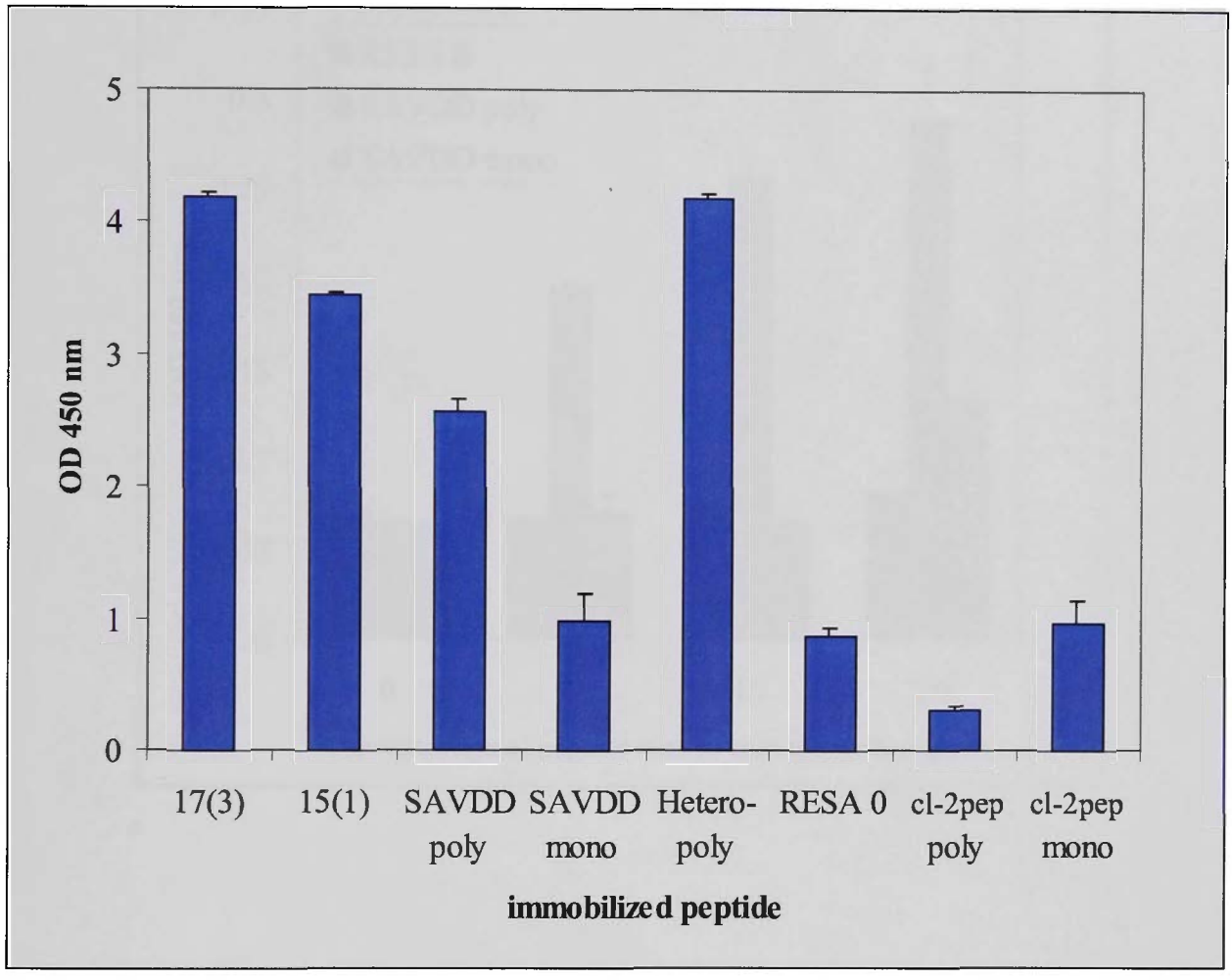


Figure 3.1.31. Binding of MAb18/2 to synthetic monomeric and polymeric peptides. Synthetic peptides presented either as monomers (mono) or coupled to acrylamide to form polymers (poly) (10 μg/ml) were used to coat the wells of a microtitre plate and probed with MAb18/2 (1.5 μg/ml). Binding was detected using an anti-mouse IgG-HRP antibody. RESA 0 and cl-2pep were used as irrelevant peptides.

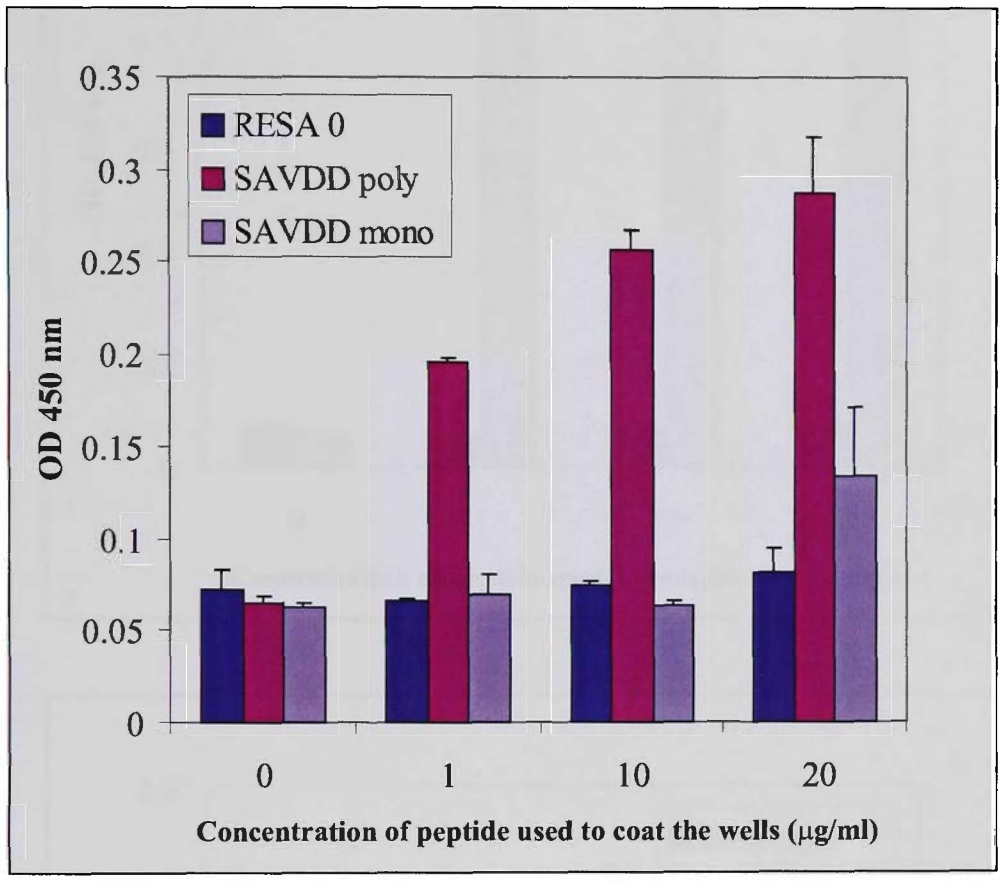
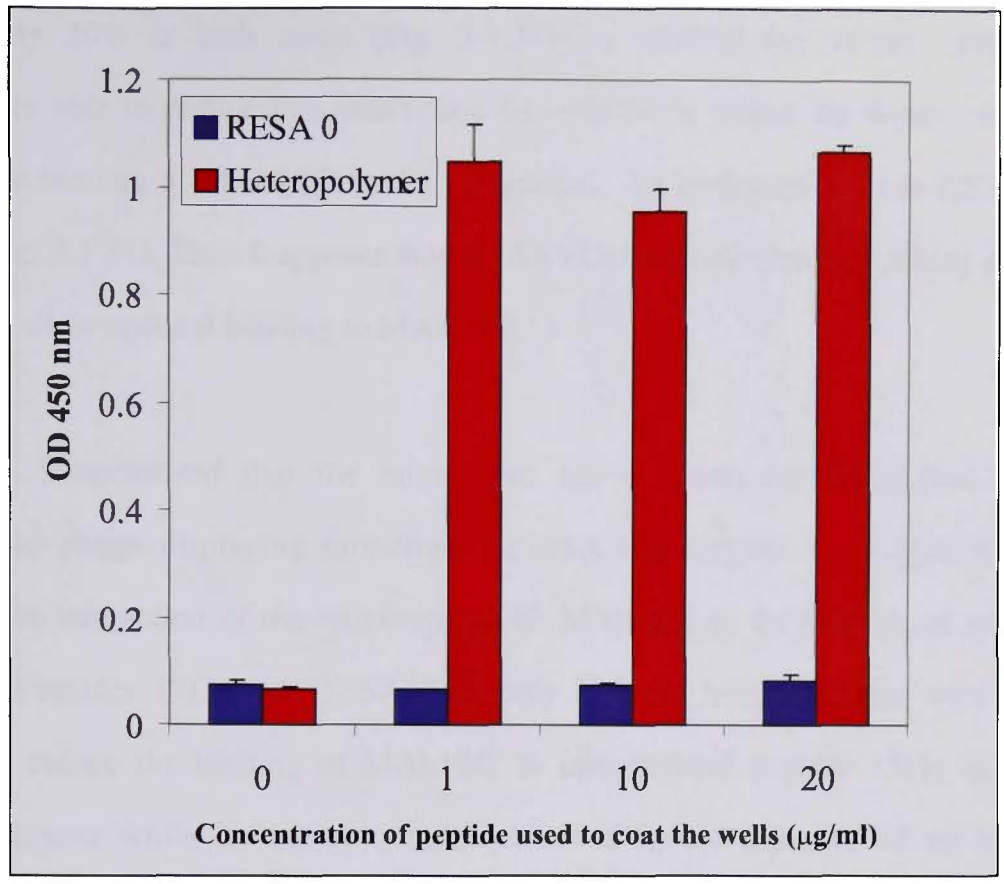


Figure 3.1.32. Binding of MAb18/2 to SAVDD monomer (mono) and polymer (poly) peptides. Increasing concentrations of SAVDD polymer and monomer and RESA 0 peptides were used to coat the wells of a microtitre plate and probed with MAb18/2 (0.7 μg/ml). Binding was detected using anti-mouse-HRP.

A



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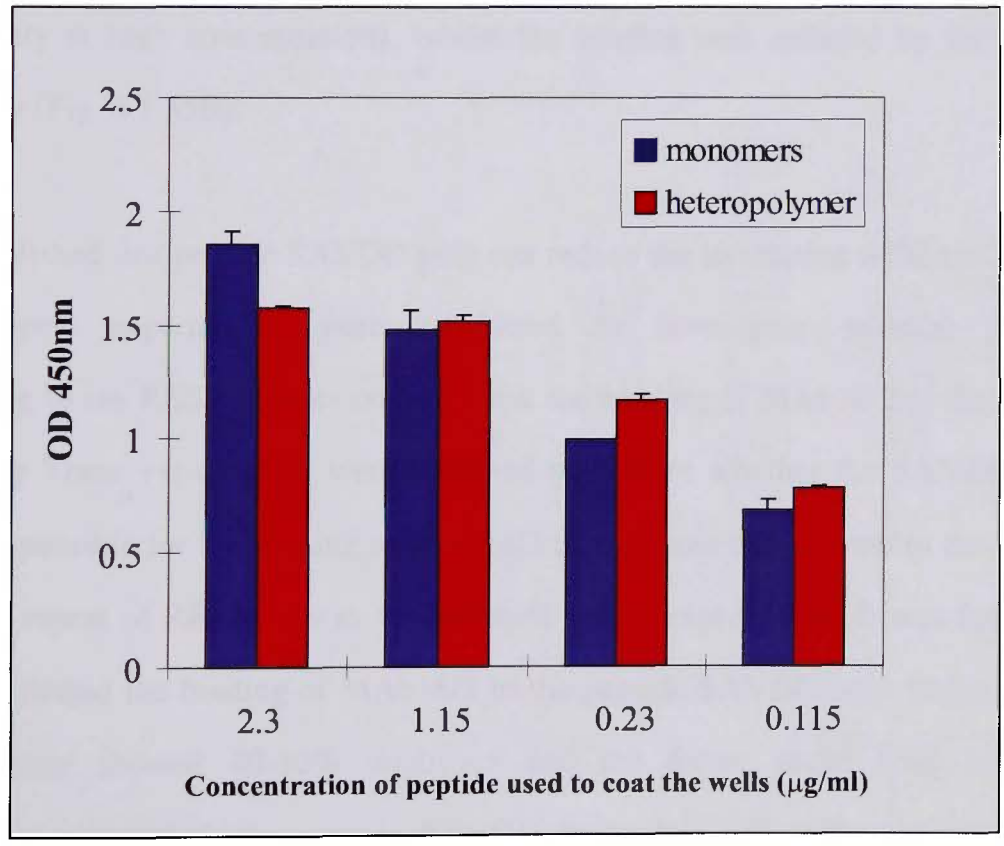

Figure 3.1.33. Binding of MAb18/2 to peptides 15(1), 17(3) and SAVDD either as monomers or coupled to a polyacrylamide backbone as a heteropolymer. (A) Increasing concentrations of the heteropolymer peptide and RESA 0 peptide were immobilized in the wells of a microtitre plate and probed with MAb18/2 (0.7 μg/ml). (B) Decreasing concentrations of the peptides were immobilized either as monomers (in the same well) or as the heteropolymer in the wells of a microtitre plate and probed with MAb18/2 (0.7 μg/ml). Binding was detected using anti-mouse-HRP.

approximately 30% in both cases (Fig. 3.1.34). In contrast the 11-mer and 22-mer peptides were able to reduce this interaction by over 50%, whilst the 4-mer completely abolished the binding of MAb18/2 to the mimotopes. An irrelevant peptide RESA 0 had no effect (Fig. 3.1.34). Thus it appears that the SAVDD peptide alone is lacking structural features that allow optimal binding to MAb18/2.

It has been demonstrated that the mimotopes can compete the interaction between MAb18/2 and phage displaying mimotopes. ELISA experiments were used to further investigate the interaction of the mimotopes with MAb18/2 in the absence of any phage framework. Peptides 17(3), 15(1), SAVDD poly and the heteropolymer were able to significantly reduce the binding of MAb18/2 to immobilized peptide 15(1) in a dose-dependant manner while no inhibition was observed in the presence of an irrelevant peptide, RESA 0 (Fig. 3.1.35A). Interestingly, the peptide SAVDD mono exhibited some inhibition only at high concentrations, whilst the binding was reduced by the peptide SAVDD poly (Fig. 3.1.35B).

Having established that peptide SAVDD poly can reduce the interaction of MAb18/2 with the mimotopes, experiments were conducted to investigate whether peptides corresponding to the RESA repeats could inhibit the binding of MAb18/2 to the peptide SAVDD poly These experiments were designed to explore whether the SAVDD motif itself was responsible for the binding to MAb18/2 in a manner that resembles the binding of the 4-mer repeat of RESA as was suggested in earlier experiments. It was found that the 4-mer abolished the binding of MAb18/2 to the peptide SAVDD poly whilst the 11-mer and 22mer showed 40-50% inhibition and the 8-mer about 75% inhibition (Fig. 3.1.36). As expected, the peptide SAVDD mono had little effect, whilst in the absence of peptide or the presence of RESA 0, no inhibition was seen (Fig. 3.1.36B). These results mirror those obtained when peptides derived from RESA repeats were allowed to compete the interaction of MAb18/2 with the immobilized mimotopes

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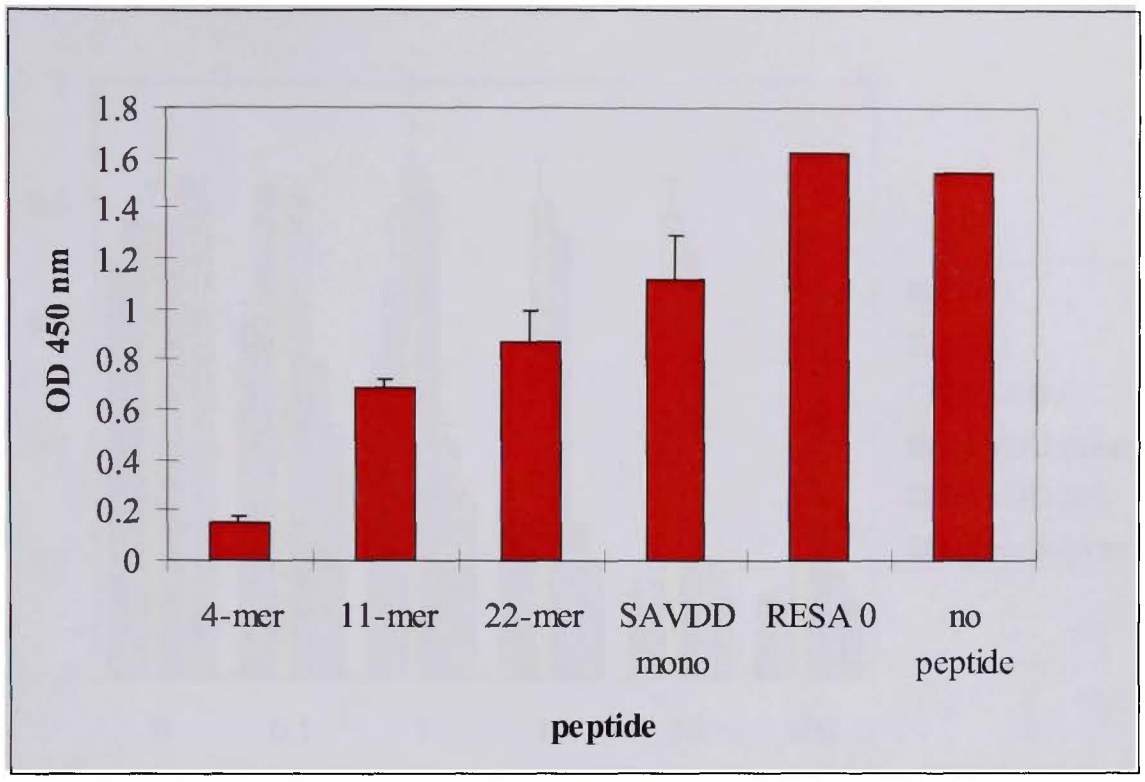

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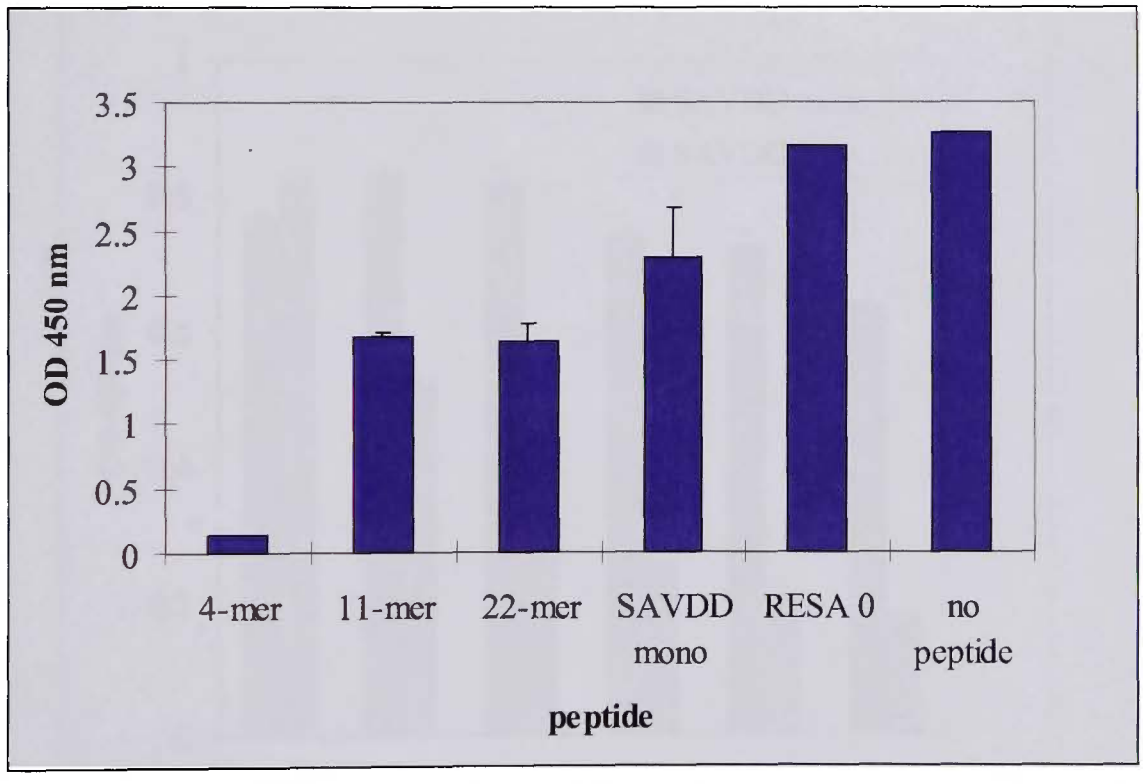

Figure 3.1.34. Inhibition of the interaction of MAb18/2 with RESA mimotopes by synthetic peptides derived from the RESA repeats. Peptides (A) 15(1) and (B) 17(3) (10 μg/ml) were used to coat the wells of a microtitre plate and probed with MAb18/2 (0.44 μg/ml) in the presence of 100 μg/ml of either the 4-mer, 11-mer (DDEHVEEPTVA), 22-mer $(DDEHVEEPTVA)_2$, SAVDD monomer, RESA 0 or no peptide. Binding was detected using an anti-mouse-HRP antibody.

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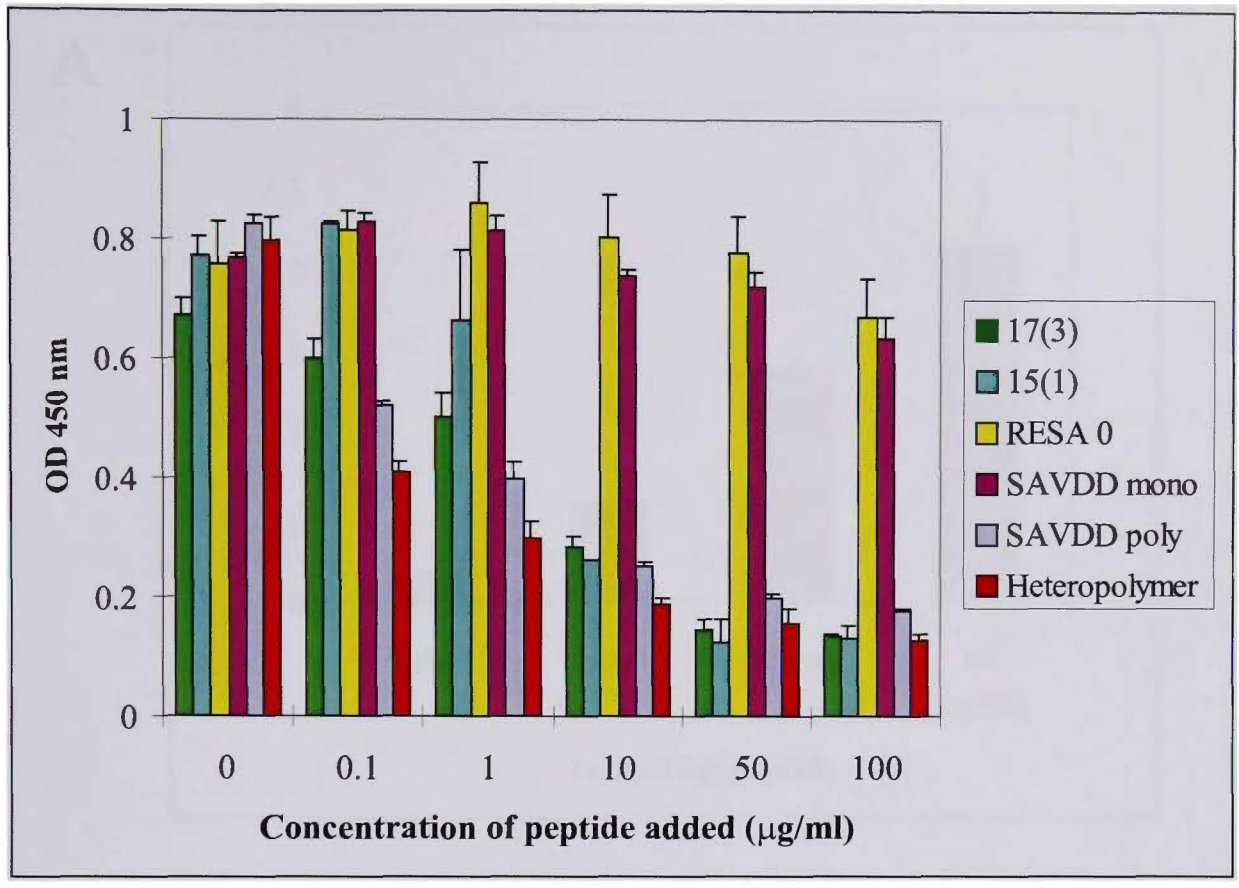

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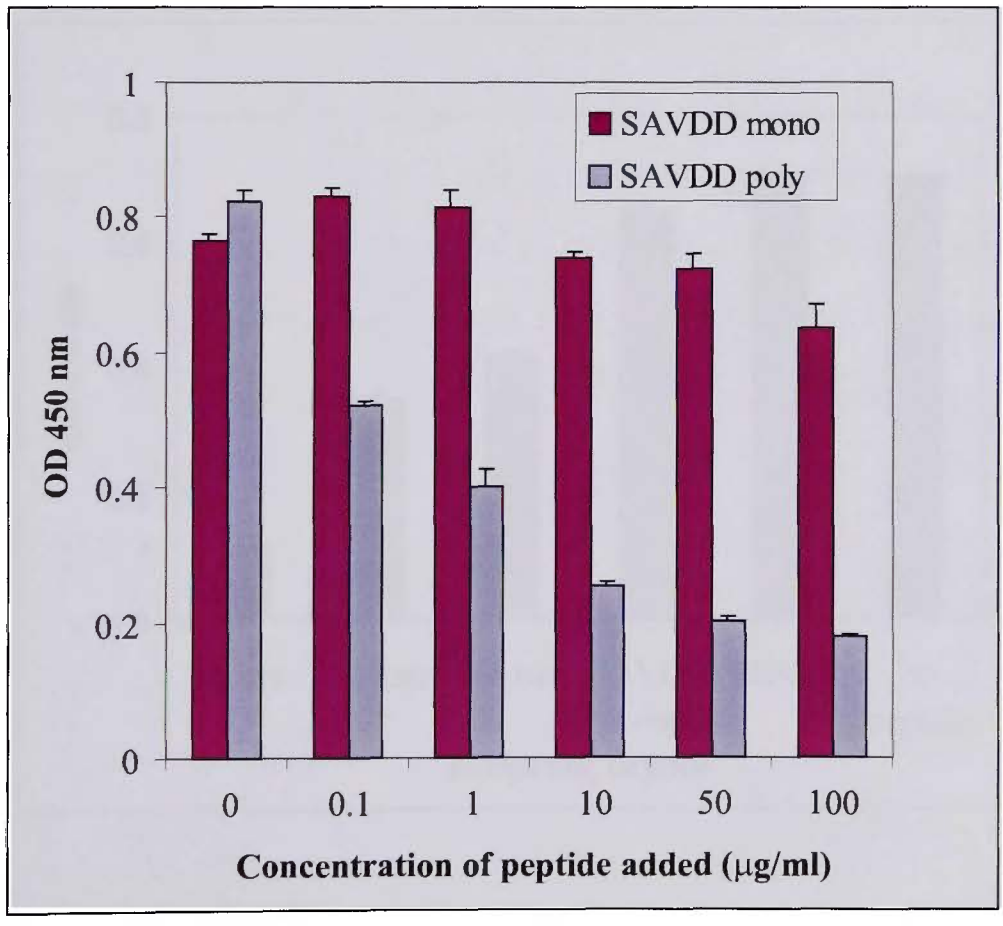

Figure 3.1.35. Inhibition by synthetic monomer and polymer peptides of the interaction of MAb18/2 with peptide 15(1). (A) Peptide 15(1) (10 μg/ml) was used to coat the wells of a microtitre plate and probed with MAb18/2 (0.44 μg/ml) in the presence of increasing concentrations of peptides 17(3), 15(1), RESA 0, SAVDD monomer and polymer, and the heteropolymer peptides. Binding was detected using anti-mouse IgG-HRP. (B) The wells containing the SAVDD monomer and polymer peptides have been highlighted.

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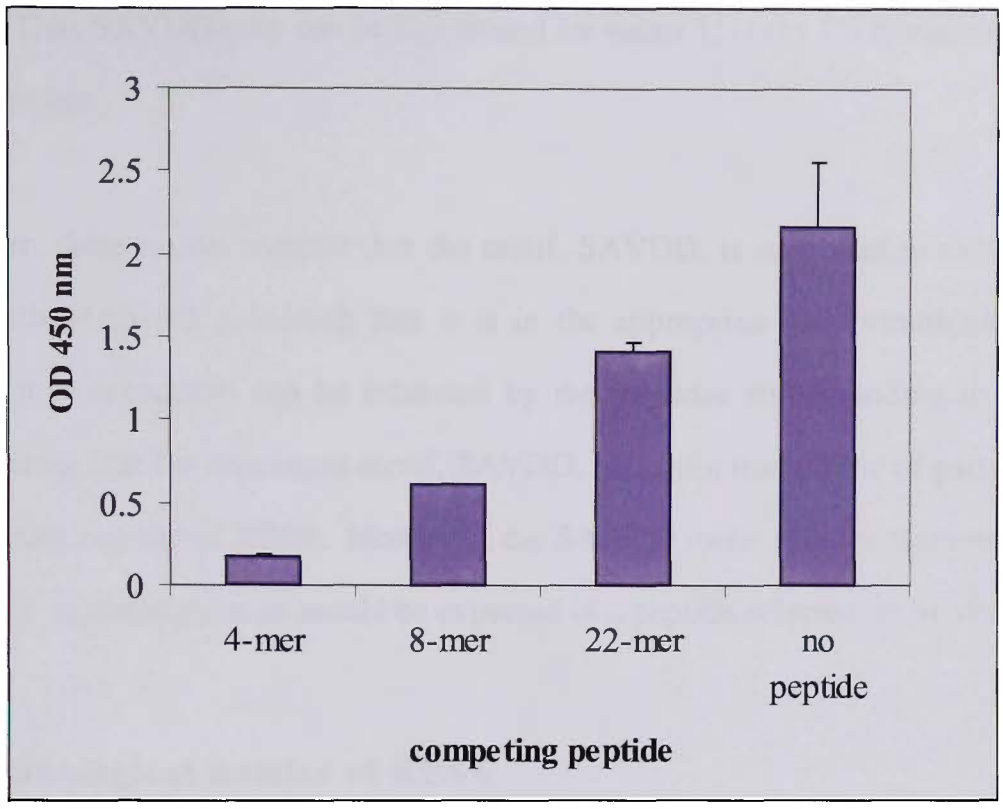

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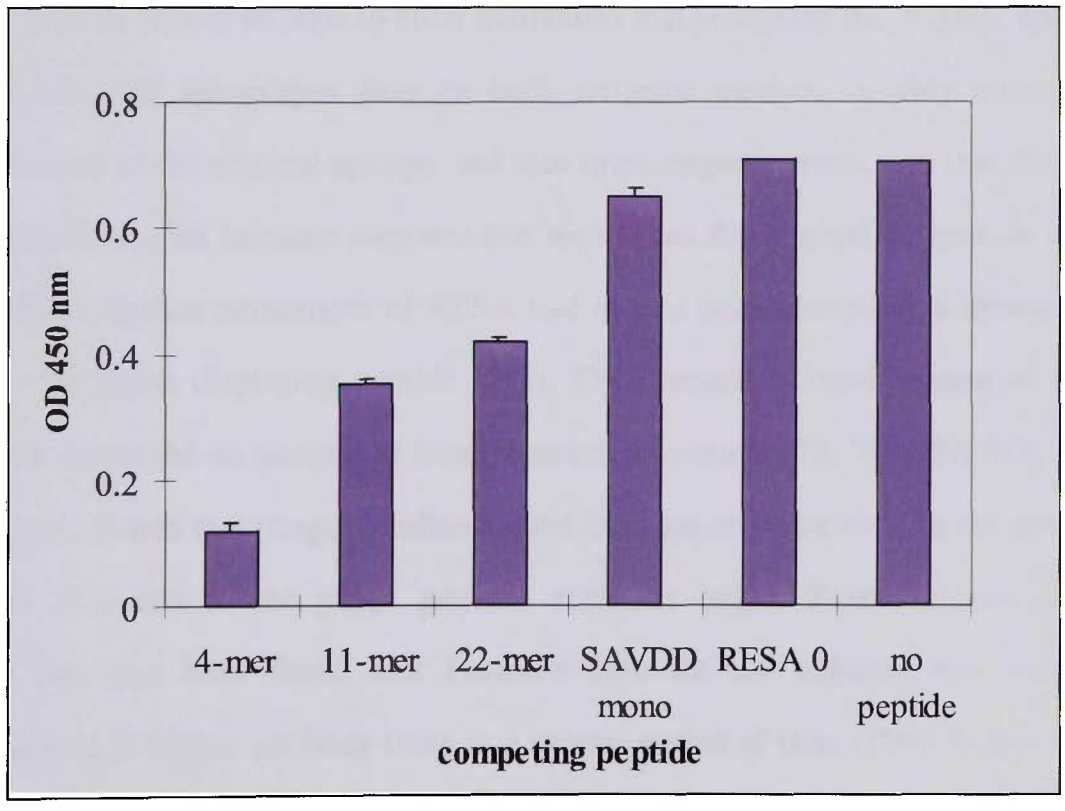

Figure 3.1.36. Inhibition of the interaction of MAb18/2 with SAVDD polymer peptide by synthetic peptides derived from the RESA repeat regions. (A and B) SAVDD polymer peptide (10 μg/ml) was used to coat the wells of a microtitre plate and probed with MAb18/2 (0.44 μg/ml) in the presence of no peptide, or 100 μg/ml of either the 4-mer, 8-mer $(EENVEHDA)_4$, 22-mer $(DDEHVEEPTVA)_2$, 11-mer (DDEHVEEPTVA), RESA 0 or SAVDD monomer peptides. Binding was detected using an anti-mouse-HRP antibody.

(Fig. 3.1.34). Thus SAVDD poly can be substituted for either 15(1) or 17(3) mimotopes in biochemical assays.

Taken together, these results suggest that the motif, SAVDD, is sufficient to mediate an interaction with MAb18/2 providing that it is in the appropriate conformational state. Furthermore, this interaction can be inhibited by the peptides corresponding to RESA repeats illustrating that the consensus motif, SAVDD, is itself a true mimic of parts of the immunodominant regions of RESA. Moreover, the SAVDD motif mimics features of the RESA 5' and 3' repeat regions as would be expected of a peptide selected on MAb18/2.

3.1.15 Immunological mimics of RESA

It has been suggested that in order to confirm that a peptide is an authentic mimic of an epitope, the peptide should be able to elicit antibodies that recognize the original epitope (263). Thus authentic mimotopes must be both antigenic mimics, ie. they mimic the antigenic structure of the original epitope, and also immunogenic mimics, in that they are capable of stimulating an immune response that recognizes the original antigen. In order to confirm that authentic mimotopes of RESA had indeed been identified, a mouse was immunized with phage displaying peptide 17(3). The successful immunization of mice using antigens displayed on phage has been reported previously (78, 79, 109, 175, 202, 275). It has been shown that phage simulate a good immune response even in the absence of additional adjuvants as the phage proteins stimulate helper T-cell activity (275). However, it has also been found that Freund's adjuvant can enhance this immune response resulting in higher antibody titres in a shorter period of time (109). In this case, the mouse was immunized, in the absence of adjuvant, with phage displaying peptide 17(3) and found to elicit a satisfactory immune response. Interestingly, the mouse serum was found to react, in ELISAs, with either peptide 17(3) or 15(1) in a dose-dependant manner despite the fact that the mouse had only been immunized with phage displaying the 17(3) peptide. There was little reactivity of prebleed serum with either peptide 17(3) or 15(1) (Fig. 3.1.37). This is confirmation that there is a structural relationship between

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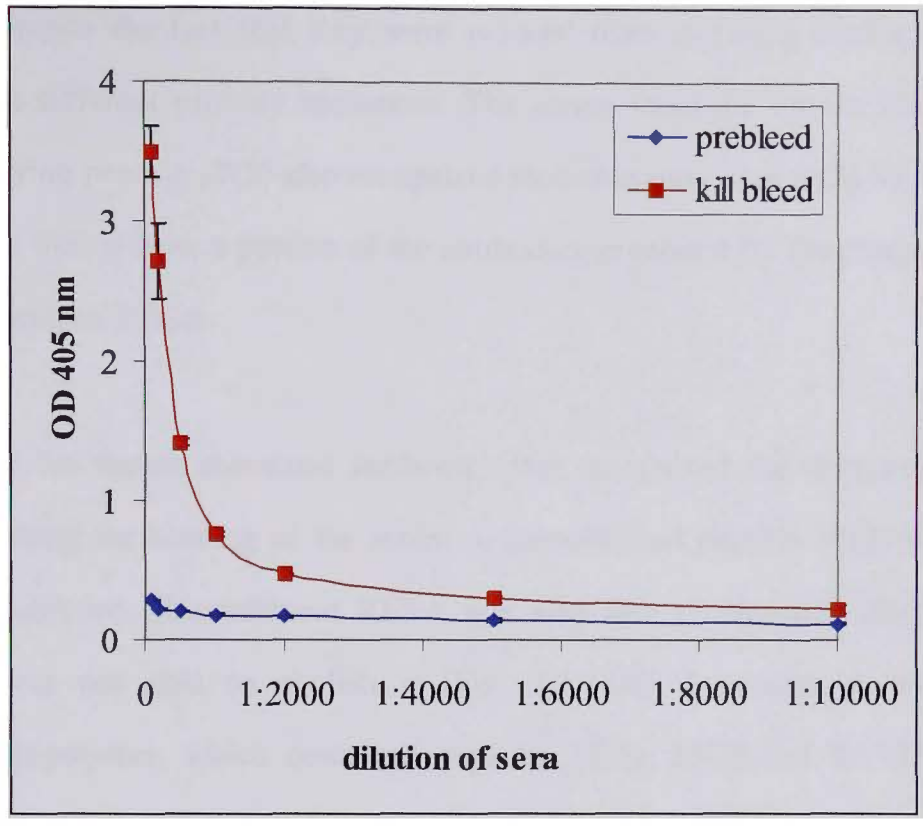

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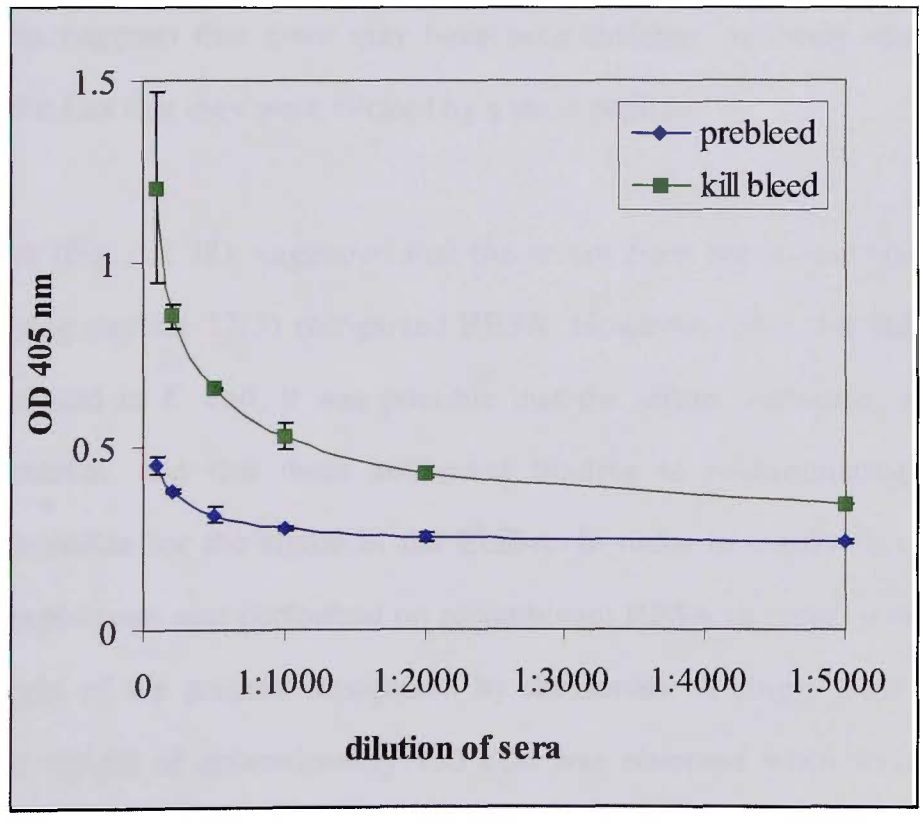

Figure 3.1.37. Titrametric binding of sera from mice immunized with phage displaying peptide 17(3) to synthetic peptides. (A) 17(3) and (B) 15(1) peptides (20 μg/ml) were used to coat the wells of a microtitre plate and probed with dilutions of mouse sera. Binding of the mouse sera was assessed using an alkaline phosphatase-conjugated anti-mouse antibody.

the mimotopes despite the fact that they were isolated from different random peptide libraries and have different primary sequences. The serum from the mouse immunized with phage displaying peptide 17(3) also recognized recombinantly expressed RESA (Fig. 3.1.38), indicating that at least a portion of the antibodies generated by the phage reacted with the original antigen RESA.

Confirmation that the serum contained antibodies that recognized the mimotopes was obtained by inhibiting the binding of the serum to immobilized peptide 17(3) with free peptide 17(3) in solution. Recombinant RESA was also able to diminish this binding somewhat, but was not able to abolish it (Fig. 3.1.39A). Interestingly, when the immobilized heteropolymer, which contained peptides 17(3), 15(1) and SAVDD on a polyacrylamide backbone, was probed with serum, recombinant RESA was not able to inhibit this binding however, peptide 15(1) was able to decrease the binding slightly (Fig. 3.1.39B). This suggests that there may have been different antibody specificities generated despite the fact that they were elicited by a short peptide.

Earlier experiments (Fig. 3.1.38), suggested that the serum from the mouse immunized with phage displaying peptide 17(3) recognized RESA. However, since this RESA was recombinantly produced in *E. coli*, it was possible that the serum contained antibodies against *E. coli* proteins, and that these antibodies binding to contaminating *E. coli* proteins, were responsible for the signal in the ELISA. In order to clarify this issue, a Western blotting experiment was performed on recombinant RESA in order to determine the molecular weight of the product recognized by the serum. A single band with an apparent molecular weight of approximately 130 kDa was observed when recombinant RESA was probed with the mouse serum (Fig. 3.1.40A). When probed with MAb18/2, an identical band representing recombinant RESA was observed and this confirmed that immunization with the RESA mimotope 17(3) results in an immune response that recognizes RESA. This serum appeared to be specific for RESA since it did not react with two other malaria proteins produced recombinantly in *E. coli* (Fig. 3.1.40A). In order to

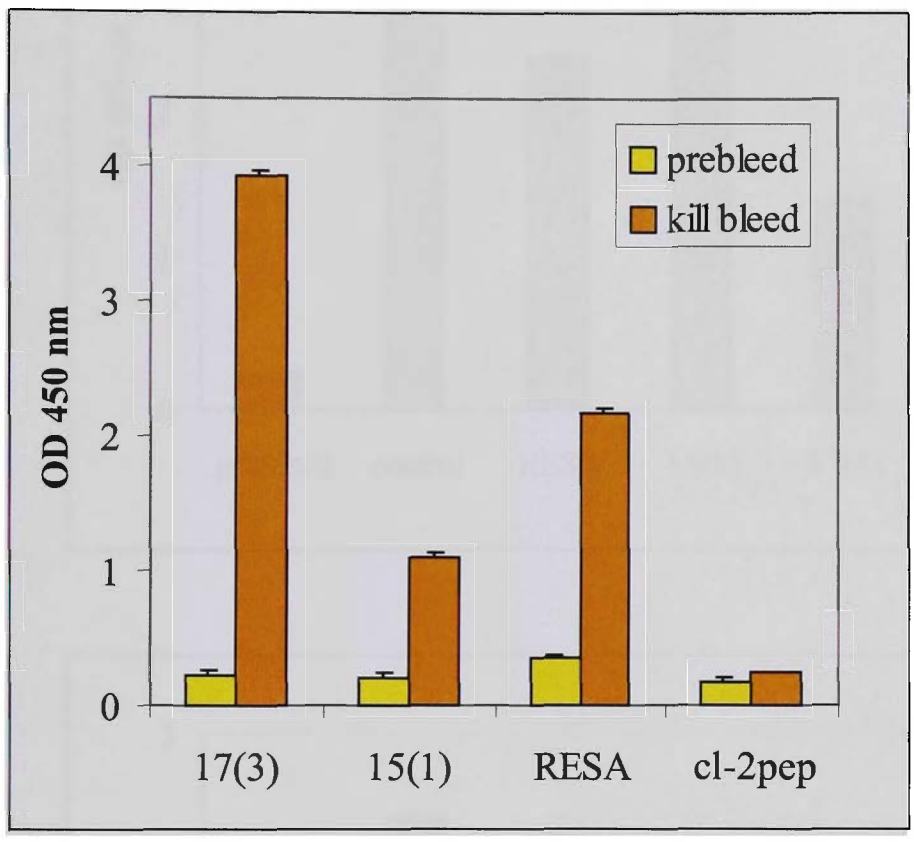

Figure 3.1.38. Binding of sera from mice immunized with phage displaying peptide 17(3) to peptides and RESA. 15(1) and 17(3) peptides (20 μg/ml), recombinant RESA-322 (5 μg/ml) and an irrelevant peptide, cl-2pep (20 μg/ml) were used to coat the wells of a microtitre plate and probed with a 1:150 dilution of mouse sera. Binding of the mouse sera was assessed using an alkaline phosphatase-conjugated anti-mouse antibody.

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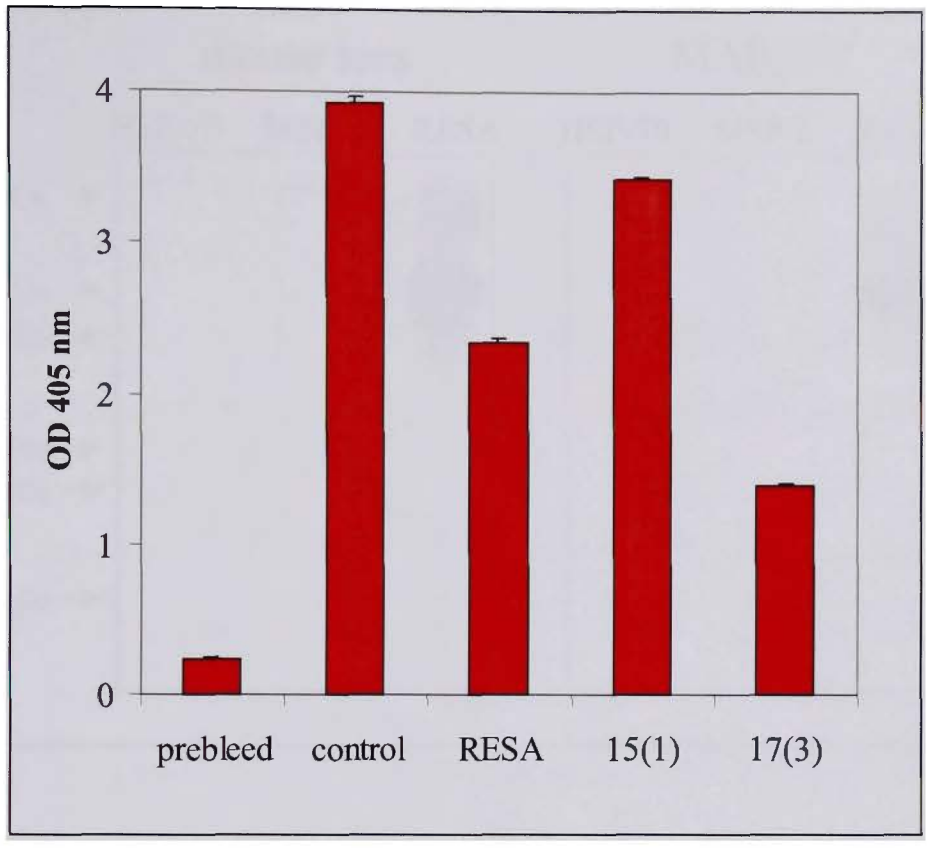

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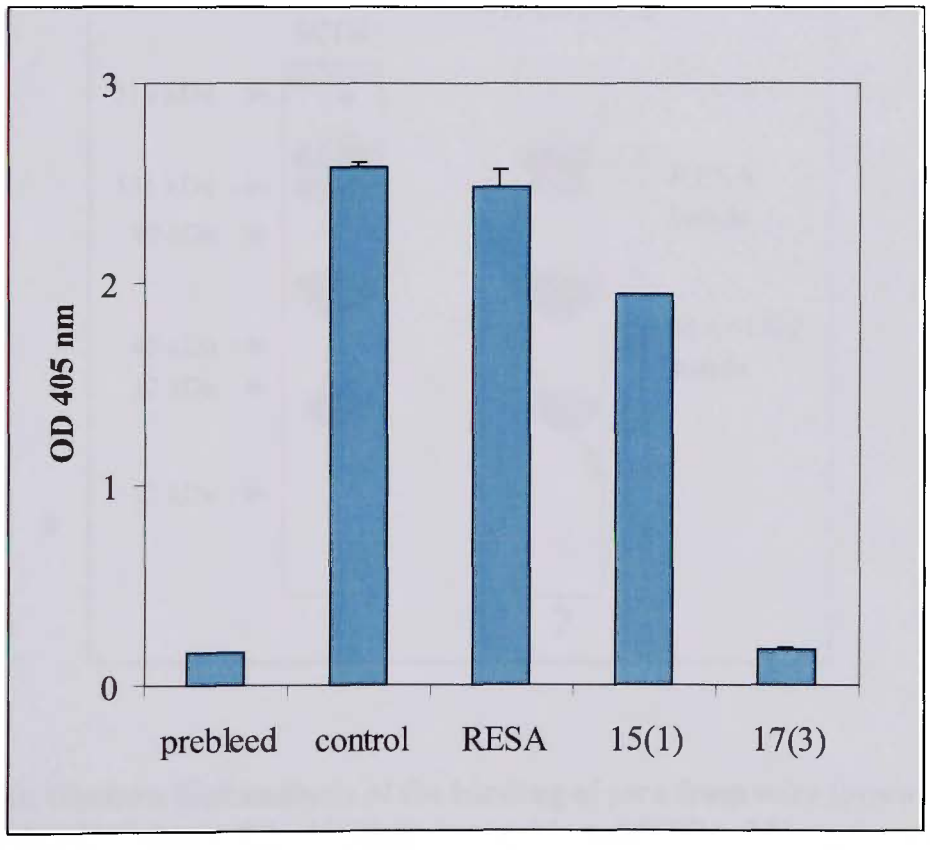

Figure 3.1.39. Inhibition by peptides and recombinant RESA-322 of the binding of sera from mice immunized with phage displaying peptide 17(3) to synthetic mimotopes. 20 μg/ml of (A) peptide 17(3) and (B) heteropolymer peptide were used to coat the wells of a microtitre plate and were probed with a 1:150 dilution of mouse sera in the presence of no peptide (control), peptides 17(3) and 15(1) (100 μg/ml) and recombinant RESA-322 (20 μg/ml). Binding of the mouse sera was determined using an alkaline phosphatase-conjugated anti-mouse antibody.

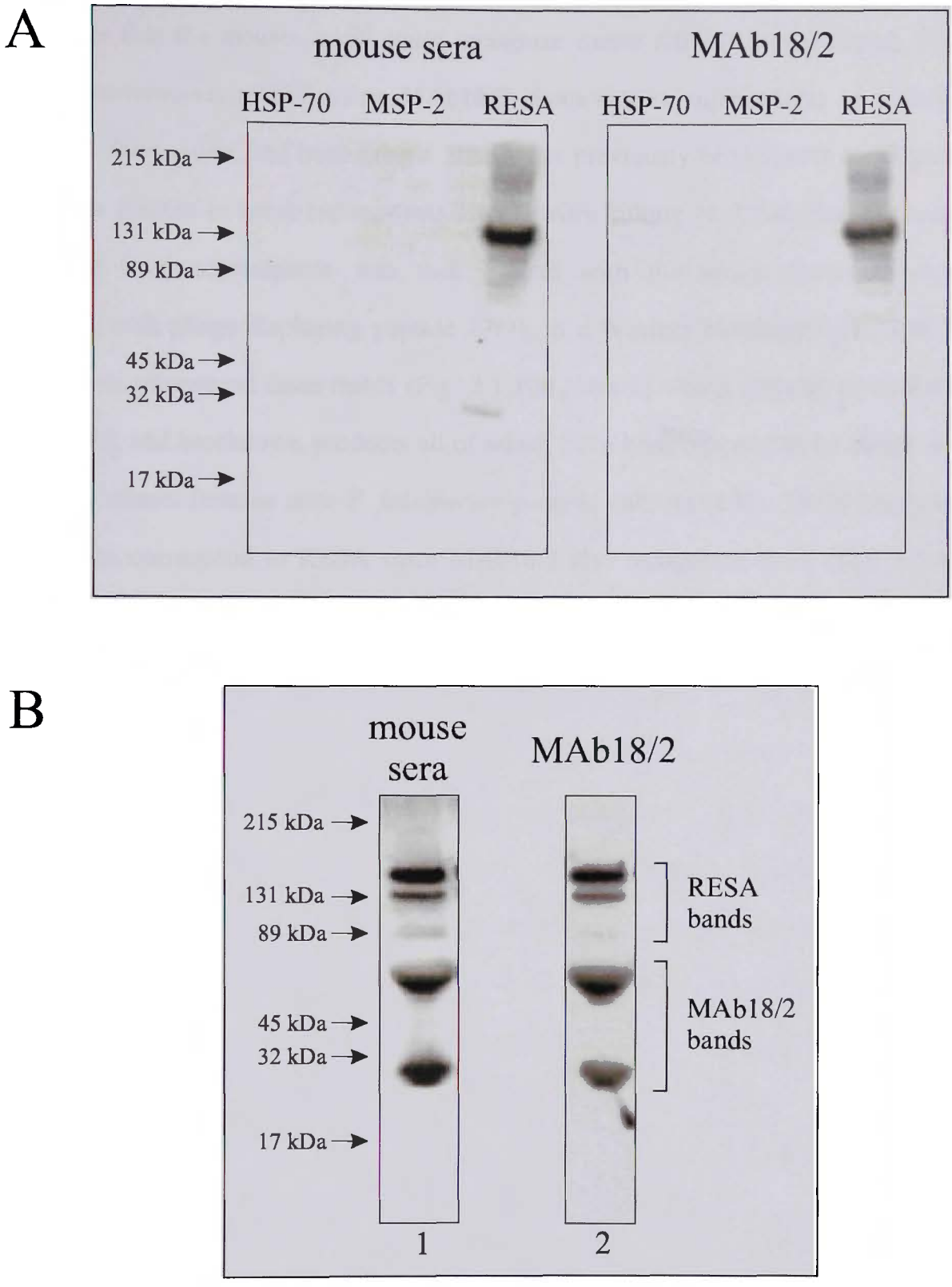

Figure 3.1.40. Western blot analysis of the binding of sera from mice immunized with phage displaying peptide 17(3) to recombinant RESA-322 and parasite produced RESA. (A) Proteins HSP-70, MSP-2 and recombinant RESA-322 (0.3 μg) or (B) RESA immunoprecipitated from the culture supernatants of *P. falciparum* D10 strain laboratory cultures were electrophoresed on SDS-polyacrylamide gels transferred to PVDF membranes and probed with mouse sera or MAb18/2. Antibodies were detected using an anti-mouse-HRP antibody and visualized by ECL.

demonstrate that the mouse serum could recognize native RESA from parasites, RESA was first immunoprecipitated using MAb18/2, from culture supernatants in which the parasite, *P. falciparum*, had been grown. RESA has previously been shown to be present as a soluble protein in spent supernatants after *in vitro* culture of *P. falciparum* parasites (103). The immunoprecipitate was then probed with the serum from the mouse immunized with phage displaying peptide 17(3), in a Western blotting experiment. The mouse serum recognized three bands (Fig. 3.1.40B, lane 1) which correspond to the full length RESA and breakdown products all of which have been reported to be found in the spent supernatants from *in vitro P. falciparum* parasite cultures (103). These bands were confirmed to correspond to RESA since MAb18/2 also recognized them (Fig. 3.1.40B, lane 2). Therefore, it is reasonable to suggest that the antibodies in the mouse serum that recognize RESA have a similar specificity as MAb18/2.

3.2 Isolation of MAb4G2-binding peptides

3.2.1 MAb4G2 binds to recombinant PfAMA-1

Monoclonal antibody 4G2 (MAb4G2) is a protective antibody that recognizes PfAMA-1 and prevents invasion of erythrocytes by the merozoites of *P. falciparum* (144). In order to confirm that MAb4G2 recognizes PfAMA-1, a recombinant form of PfAMA-1, which lacks the signal sequence, the cytoplasmic domain and transmembrane domain, was immobilized and probed with increasing amounts of MAb4G2. MAb4G2 bound to PfAMA-1 in a dose-dependant manner (Fig. 3.2.1A).

3.2.2 Panning and ELISA screening

In an effort to identify peptides that bind to the antigen-binding region of MAb4G2 and hence, mimic the structural features of the protective epitope on PfAMA-1, a library that contains phage expressing random 15- residue peptides fused to the minor coat protein, gpIII, and present at 5 copies per phage particle (231) was used. Enhanced binding of phage pools from the 15-mer library was exhibited after three rounds of panning (Fig. 3.2.1B), suggesting that an enrichment of phage with specific binding characteristics for MAb4G2 resulted from the panning procedure. However, the titres of the bound and eluted phage at each round in the panning process did not reveal an increase in the enrichment of binding phage. This finding supports the notion that the level of enrichment does not necessarily correlate with binding affinity. A second library that contained phage expressing a 17- residue peptide library ($X_{15}CX$), with an invariant penultimate cysteine residue as a fusion to the gpVIII coat protein (37), was also used to pan on MAb4G2. However, an increase in the enrichment of phage during panning was not observed and none of the isolated clones showed specificity for MAb4G2 in subsequent phage-ELISAs (data not shown).

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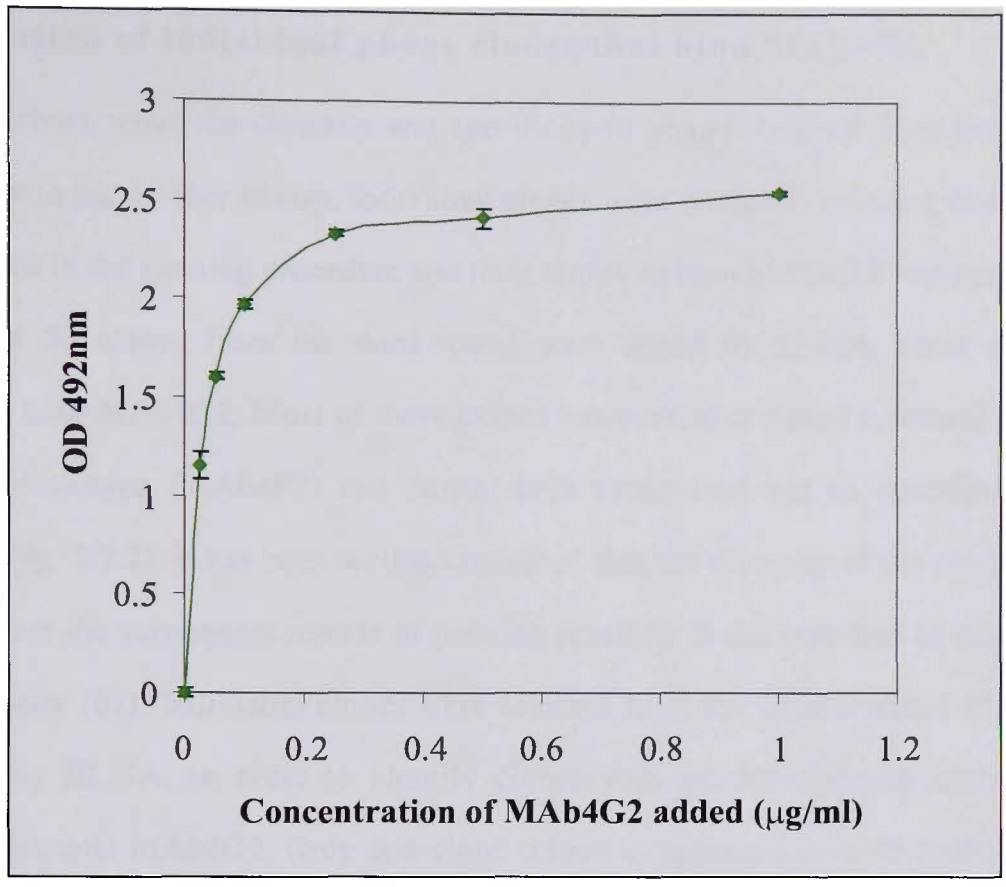

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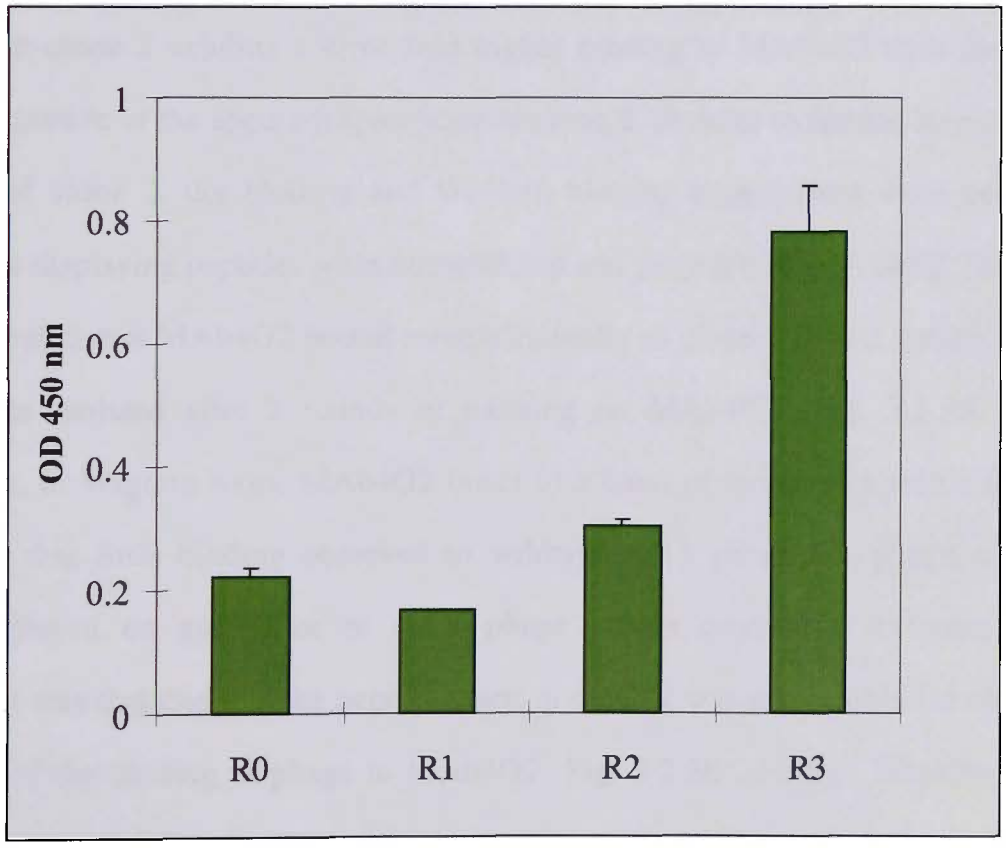


Figure 3.2.1. (A) Binding of MAb4G2 to PfAMA-1. PfAMA-1 (2 μg/ml) was used to coat the wells of a microtitre plate and probed with increasing concentrations of MAb4G2. Binding was detected using anti-rat IgG-HRP. **(B) Enrichment of phage during panning.** Pooled phage after 3 rounds of panning on MAb4G2 were amplified, and 10^{10} phage were added to the wells of a microtitre plate precoated with MAb4G2. Phage were detected with an anti-M13-HRP antibody.

3.2.3 Isolation of individual phage clones that bind MAb4G2

In order to characterize the diversity and specificity of phage obtained from the panning procedure with the 15-mer library, individual clones were randomly selected from rounds two and three in the panning procedure and their ability to bind MAb4G2 was determined. Upwards of 50 clones from the third round were tested by ELISA, most of which appeared to bind MAb4G2. Most of these clones however, also bound a control antibody of the same isotype (MAb4F2) and hence were determined not to specifically bind MAb4G2 (Fig. 3.2.2). It has been well documented that the diversity of the phage clones decreases over the subsequent rounds of panning resulting in the selection of clones with high specificity (67). Individual clones were selected from the second round of panning and tested by ELISA, in order to identify clones with greater diversity and possibly specificity towards MAb4G2. Only one clone (clone 2) appeared to bind specifically to MAb4G2. Figure 3.2.2 is a representative of an ELISA experiment that was performed showing that clone 2 exhibits a three fold higher binding to MAb4G2 than the isotype control, suggestive of the apparent specificity of clone 2. In order to further investigate the specificity of clone 2, dot blotting and Western blotting experiments were performed, where phage displaying peptides were immobilized and probed with MAb4G2. Both assay formats revealed that MAb4G2 bound more efficiently to clone 2 than a variety of other phage clones isolated after 2 rounds of panning on MAb4G2 (Fig. 3.2.3A and B). Furthermore, in Western blots, MAb4G2 binds to a band of the size expected for gpIII. Since there was little binding observed to wildtype M13 phage (ie. phage without a peptide displayed on gpIII) nor to other phage clones containing different peptide sequences, it was deduced that the peptide insert of clone 2 was responsible for conferring the affinity of the binding of phage to MAb4G2 (Fig. 3.2.3B). Further Western blotting experiments of individual phage clones revealed a number of clones that also appeared to bind MAb4G2 specifically. MAb4G2 appeared to react predominantly with clone 25 and to a lesser extent, clones 24, 42 and 44 (Fig. 3.2.3C). The differences in signal intensity between the clones could be due to variations in absolute phage numbers.

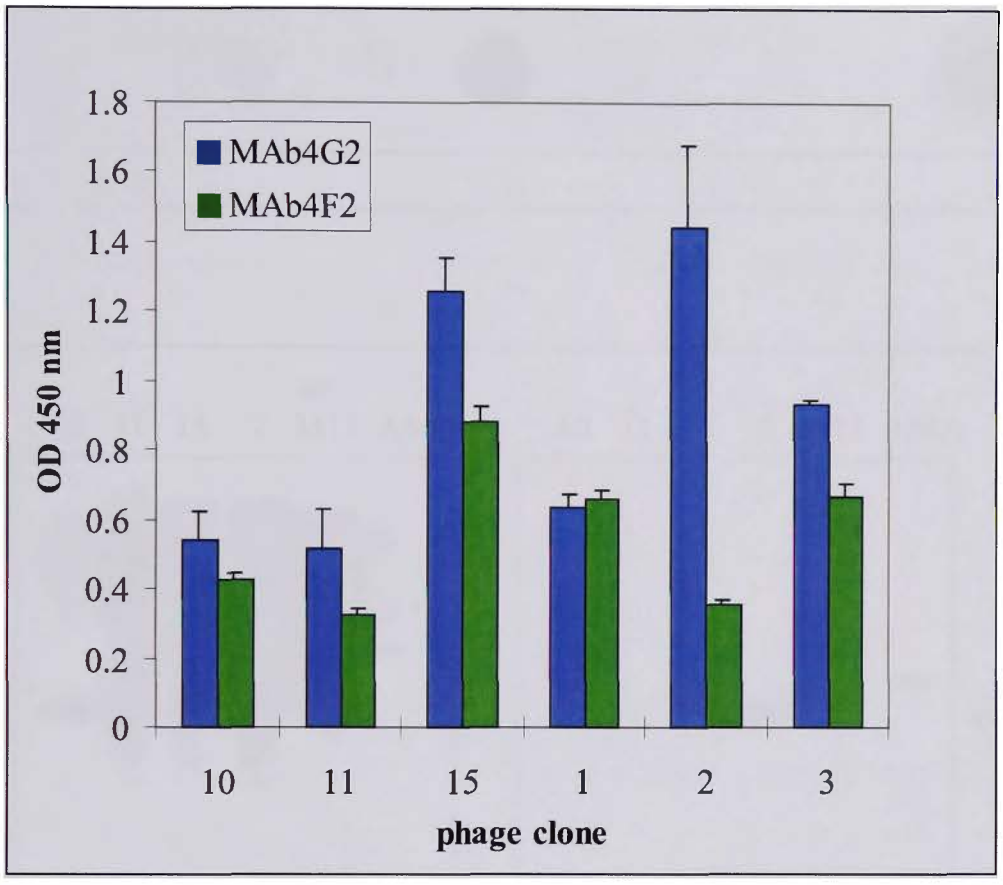


Figure 3.2.2. Phage ELISA of individual 15-mer clones isolated after panning four rounds on MAb4G2. Phage were amplified and 10^{10} phage were added to the wells of a microtitre plate precoated with MAb4G2 or the isotype control antibody, MAb4F2. Phage were detected with an anti-M13-HRP antibody.

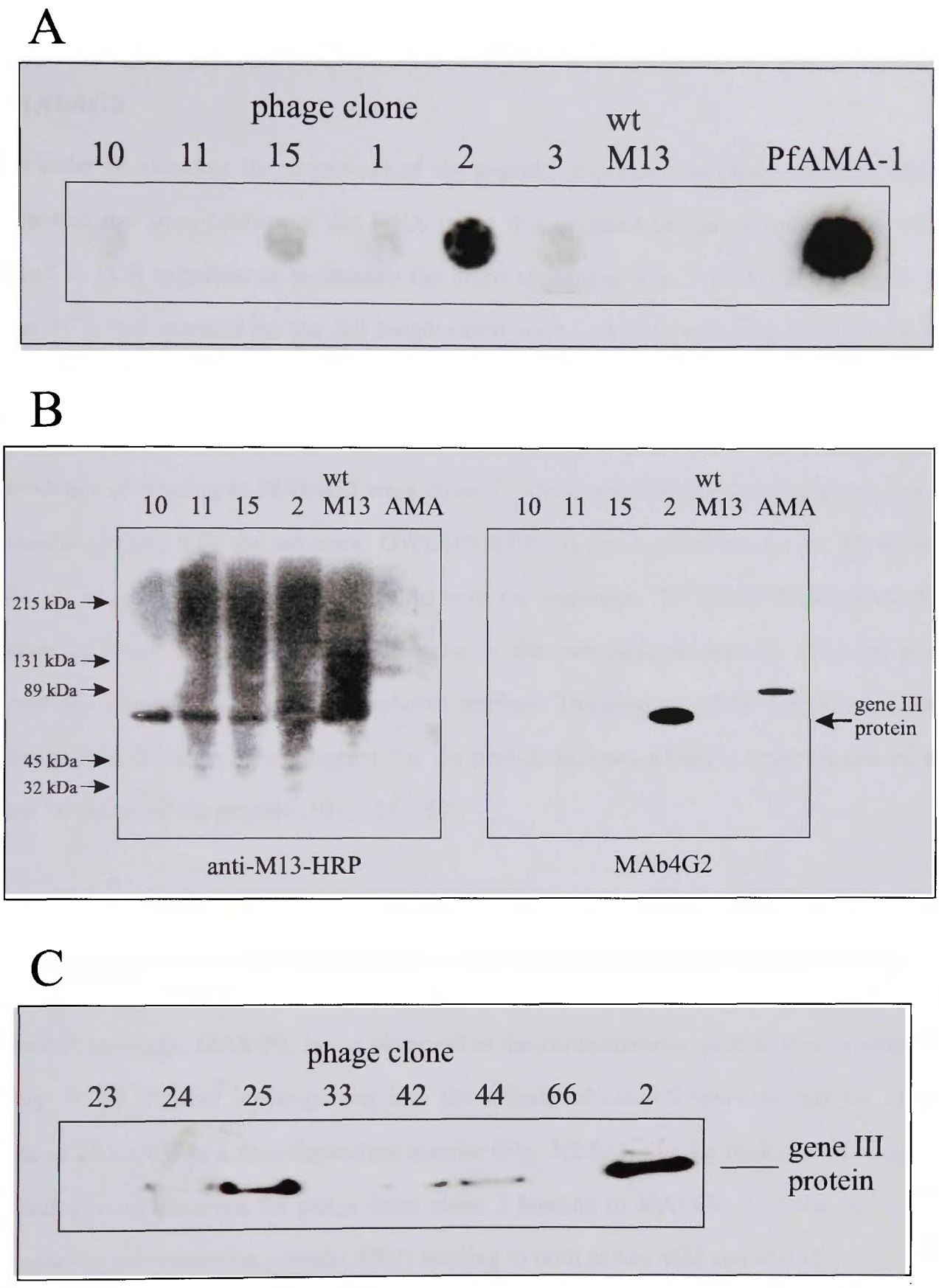


Figure 3.2.3. Interaction of isolated 15-mer phage clones with MAb4G2. (A) Dot blotting experiment of phage clones binding to MAb4G2. 10^{10} phage of clones 10, 11, 15, 1, 2, 3 and as controls, wild-type M13 phage (wt M13) and PfAMA-1 (0.1 μg) were dotted onto NCF and probed with MAb4G2, followed by anti-rat IgG-HRP. Binding of the antibodies was visualized using ECL. No binding was observed to wild-type M13 phage. (B and C) Analysis by Western blotting. 10^{10} of each phage clone were separated by SDS-polyacrylamide gel electrophoresis (10-20% acrylamide), transferred to PVDF membranes, and probed with (B and C) MAb4G2 or (B) anti-M13-HRP. (B) wild-type M13 phage and PfAMA-1 (AMA) were used as controls. Binding was visualized by ECL.

3.2.4 Amino acid sequences of peptide inserts in phage that bind to MAb4G2

In order to elucidate the sequences of the peptides expressed on phage, primers which allowed the amplification of the DNA insert that encoded for the phage peptide, were used in PCR experiments to amplify the insert sequences (Fig. 3.2.4A). The products of the PCR that encoded for the full length insert were 280 bp in size (Fig. 3.2.4B). Of the ten clones that were sequenced there were seven unique sequences, with the sequence TFVPILFWEHELNAS found four times (Fig. 3.2.4B). The two sequences which showed evidence of binding to MAb4G2 were clone 2, which was identified as expressing a 15-residue peptide with the sequence: GWLSPSWFEPGLASM, whilst clones 24, 25, 42 and 44 all expressed a 15- residue peptide with the sequence: TFVPILFWEHELNAS. No obvious consensus motif appears to exist in the two isolated peptides although both peptides contain a number of hydrophobic residues. The presence of the dipeptide, proline and glycine in clone 2 may suggest that the peptide assumes a hairpin structure caused by the 'kinking' of the peptide (101, 128, 182).

3.2.5 Binding specificity of selected phage to MAb4G2

Clones 2 and 25 both bound immobilized MAb4G2 with little binding to an isotype control antibody, MAb4F2, being observed at the concentrations used in this experiment (Fig. 3.2.5). Further investigations into the affinity of clone 2 revealed that the phage bound MAb4G2 in a dose-dependant manner (Fig. 3.2.5A). Similar background levels of binding were observed for phage from clone 2 binding to MAb4F2, and also for phage displaying the irrelevant peptide, 15(1) binding to both MAbs 4G2 and 4F2 (Fig. 3.2.5A). This suggests that it is the peptide expressed on clone 2 phage that binds specifically to MAb4G2 as opposed to a component of the phage framework. Phage clone 25 also bound MAb4G2 in a similar manner to clone 2 with clone 25 showing a slightly higher apparent relative binding affinity (Fig. 3.2.5B).

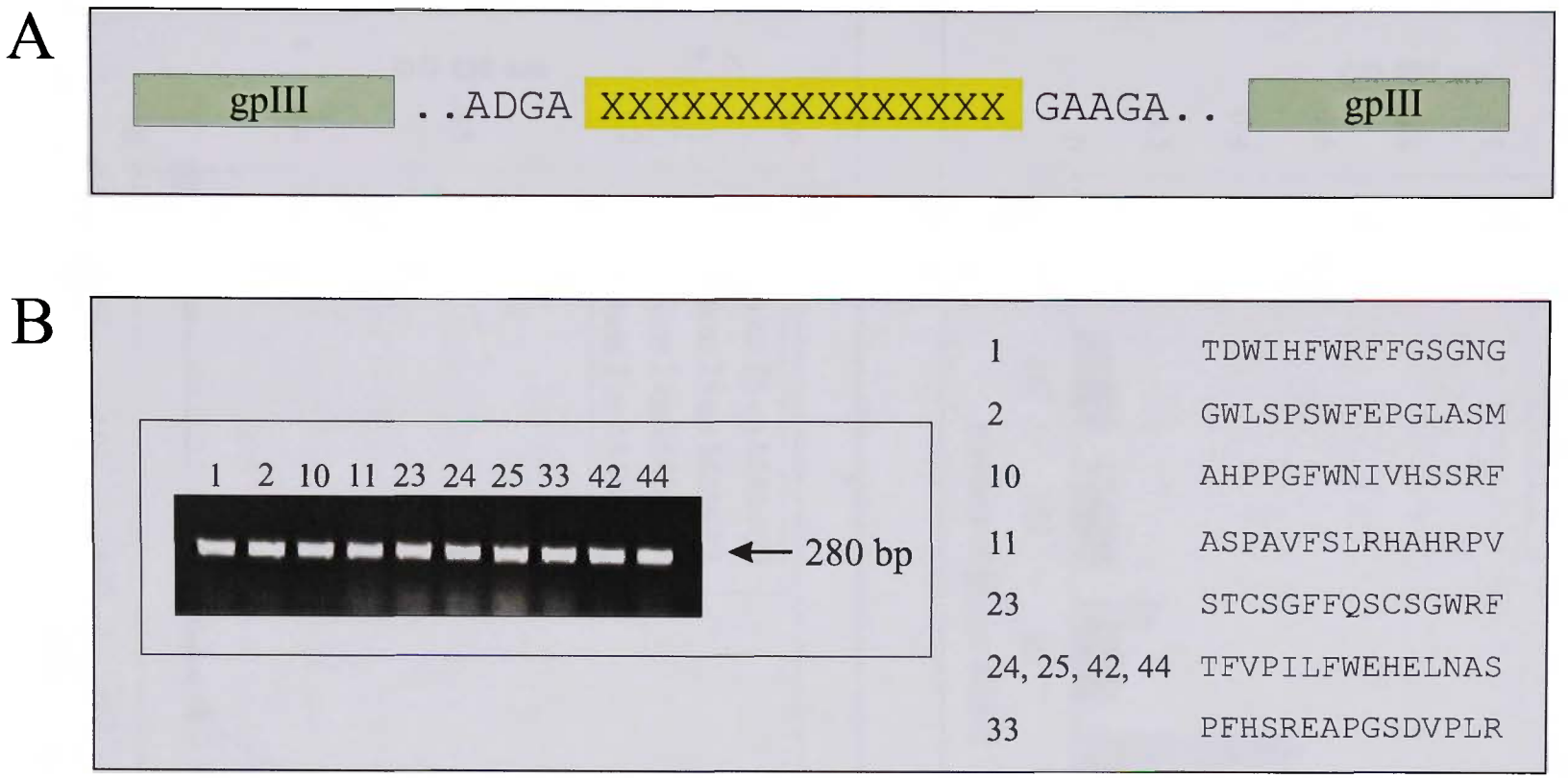
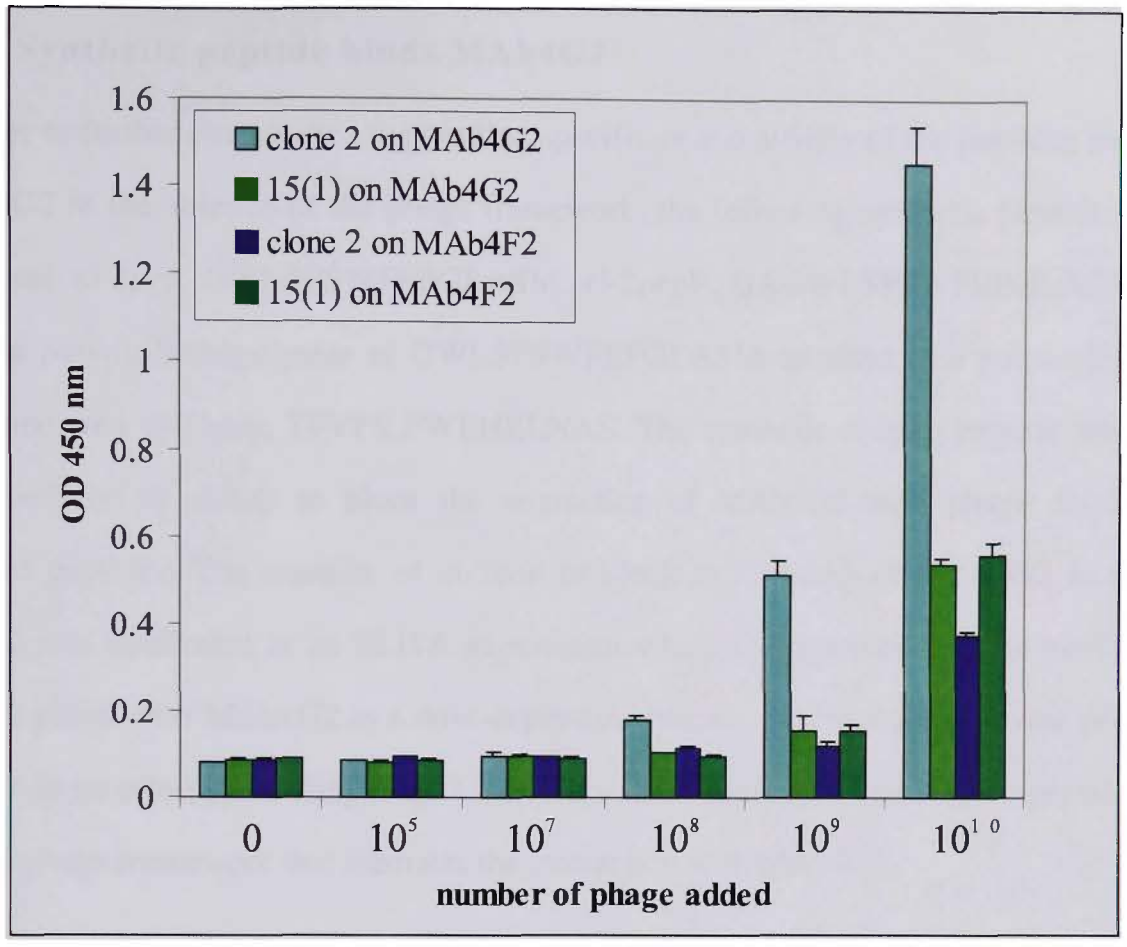


Figure 3.2.4. PCR and sequencing of individual phage clones isolated from panning the 15-mer library on MAb4G2. (A) Schematic showing gpIII containing the random 15 amino acid peptide expressed on phage. (B) 10 individual clones were isolated after four rounds of panning, the DNA encoding the peptide was amplified by PCR, visualized by agarose gel electrophoresis (insert) and sequenced. Representation of the different peptides identified.

A



B

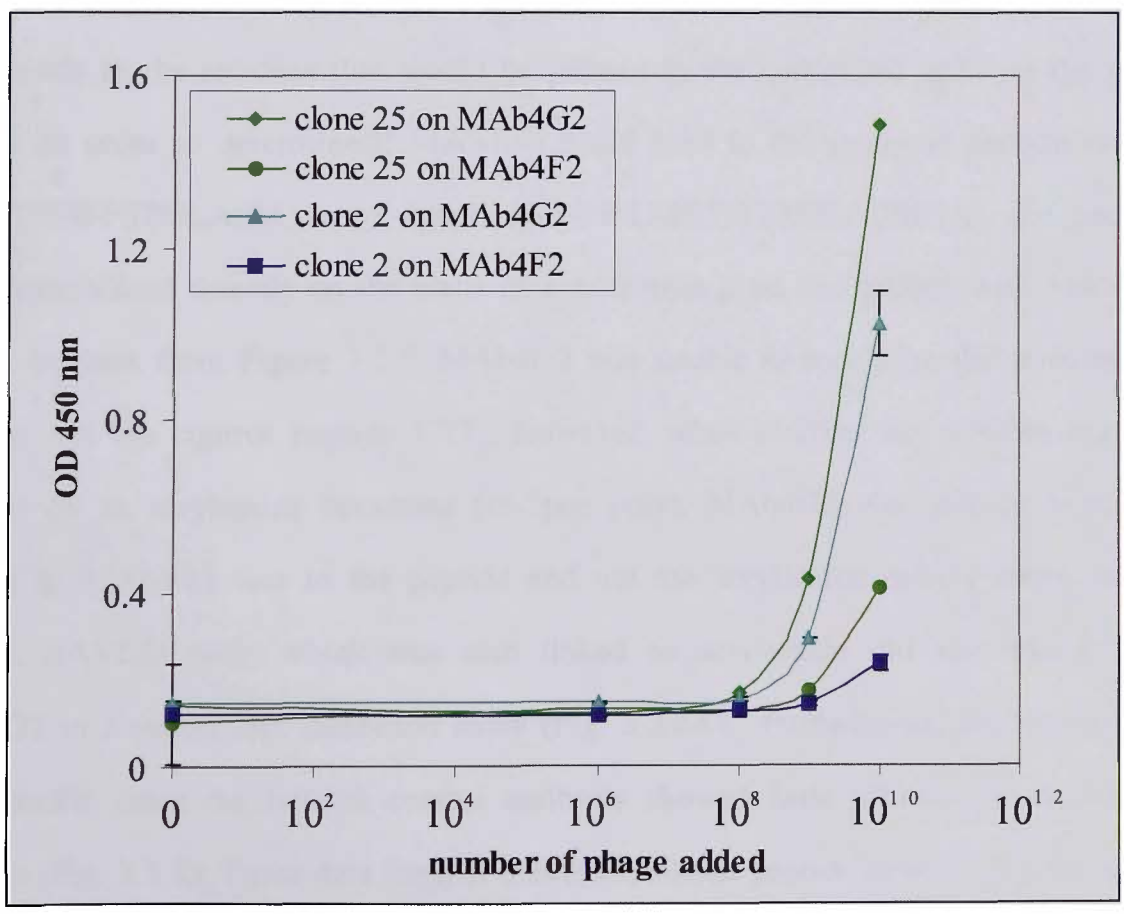


Figure 3.2.5. Analysis of specificity and affinity in the binding of phage-displayed peptides to MAb4G2. (A) Increasing numbers phage of clones 2 (blue bars) and 15(1) (green bars), or (B) clones 2 (blue) and 25 (green) were applied to the wells of a microtitre plate precoated with MAb4G2 and MAb4F2. Binding was detected using anti-M13-HRP.

3.2.6 Synthetic peptide binds MAb4G2

In order to further characterize the binding specificity and affinity of the peptides towards MAb4G2 in the absence of the phage framework, the following synthetic peptides were produced: cl-2pep, GWLSPSWFEPGLASM; cl-2pepE, GAGWLSPSWFEPGLASMGA; cl-2pep poly, a homopolymer of GWLSPSWFEPGLASM attached to a polyacrylamide backbone; and cl-25pep, TFVPILFWEHELNAS. The synthetic cl-2pep peptide was first examined for its ability to block the interaction of MAb4G2 with phage displaying selected peptides. The capacity of cl-2pep to block the binding of MAb4G2 to phage clone 2 was confirmed in an ELISA experiment where cl-2pep inhibited the binding of clone 2 phage with MAb4G2 in a dose-dependant manner, whereas an irrelevant peptide, 15(1), had no effect on binding (Fig. 3.2.6). This again confirmed that it is the peptide and not the phage framework that mediates the interaction with MAb4G2.

Peptide cl-2pepE is identical to cl-2pep but it contains two extra residues at either end that corresponds to the residues that would be present in the context of gpIII on the phage surface. In order to determine if MAb4G2 could bind to the synthetic peptide cl-2pep (GWLSPSWFEPGLASM) or cl-2pepE (GAGWLSPSWFEPGLASMGA), the peptides were immobilized directly on the wells of a microtitre plate and probed with MAb4G2. As can be seen from Figure 3.2.7, MAb4G2 was unable to recognize the immobilized peptides nor the control peptide 17(3). However, when cl-2pep was synthesized as a polymer on an acrylamide backbone (cl-2pep poly), MAb4G2 was able to bind. The binding of MAb4G2 was to the peptide and not the acrylamide moiety since another peptide, SAVDD poly, which was also linked to acrylamide did not interact with MAb4G2 in a subsequent inhibition assay (Fig. 3.2.8A). Furthermore, the binding was also specific since the isotype control antibody showed little affinity for any of the peptides (Fig. 3.2.7). These data suggest that the synthetic peptide alone is able to bind to MAb4G2, however, when it is immobilized to plastic, elements of the interaction site are hidden or unavailable for interaction with the antibody.

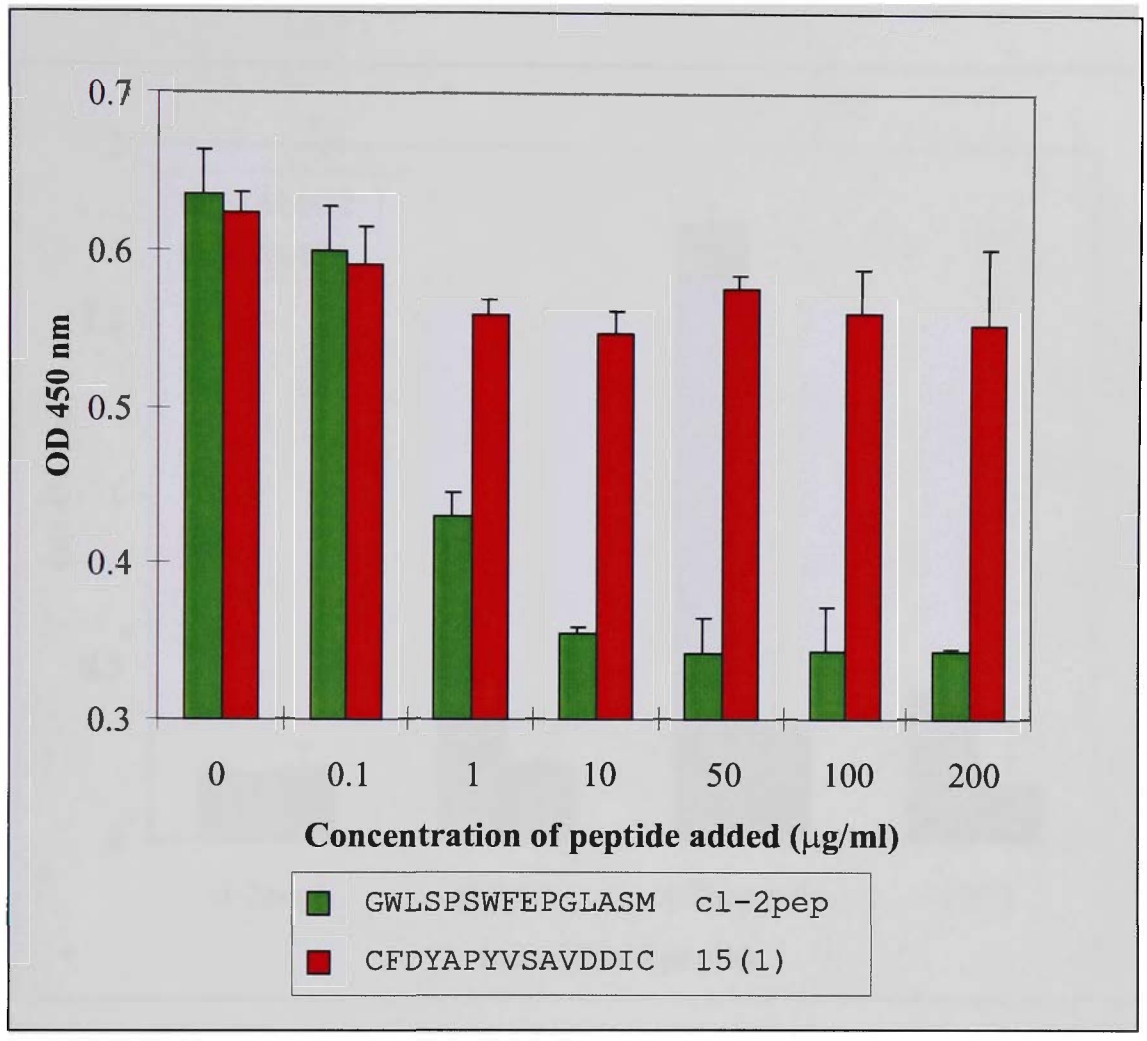


Figure 3.2.6. Inhibition of the interaction of phage clone 2 with MAb4G2 by synthetic peptides. 10^{10} phage of clone 2 were used to coat the wells of a microtitre plate and probed with MAb4G2 (0.5 μg/ml) in the presence of increasing concentrations of the synthetic peptide, cl-2pep or the control peptide, 15(1). Binding was detected using an anti-rat-HRP antibody.

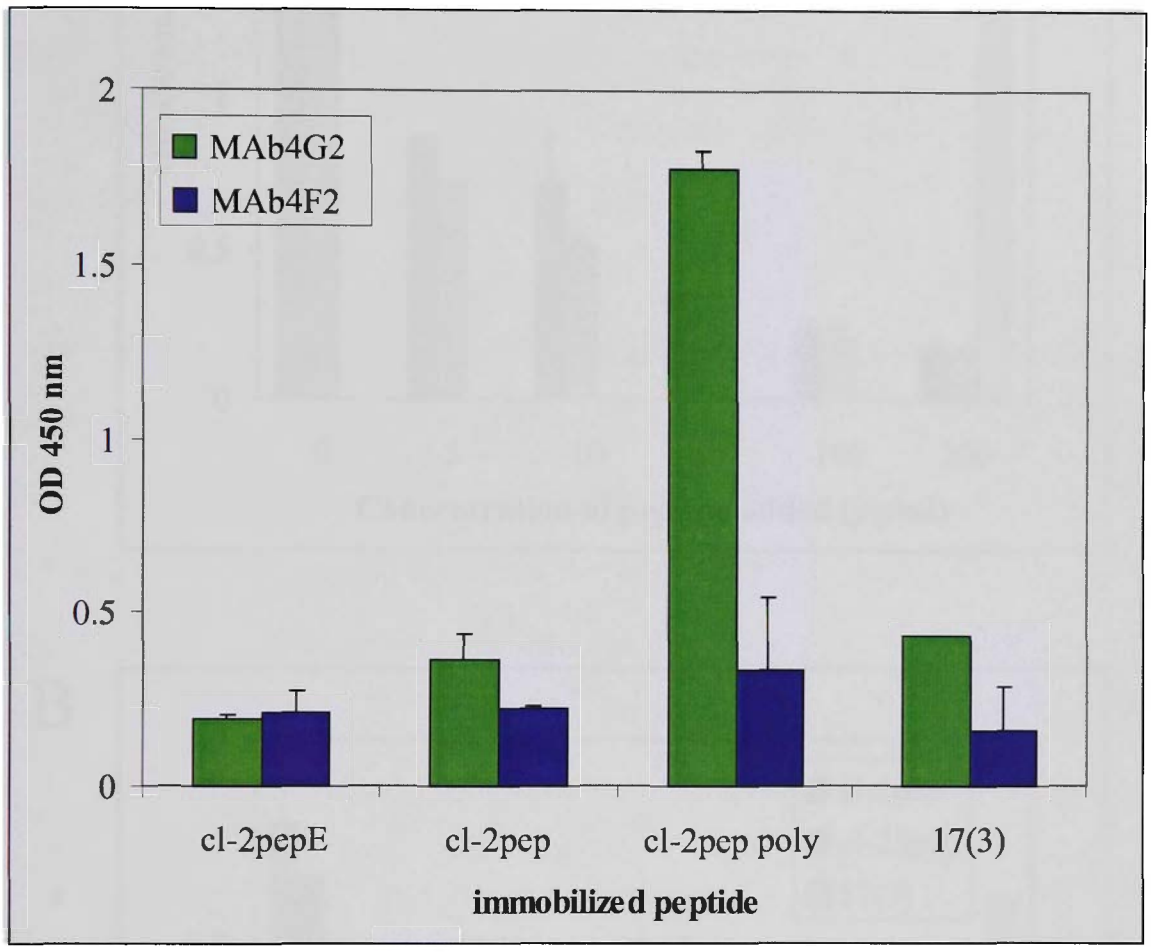
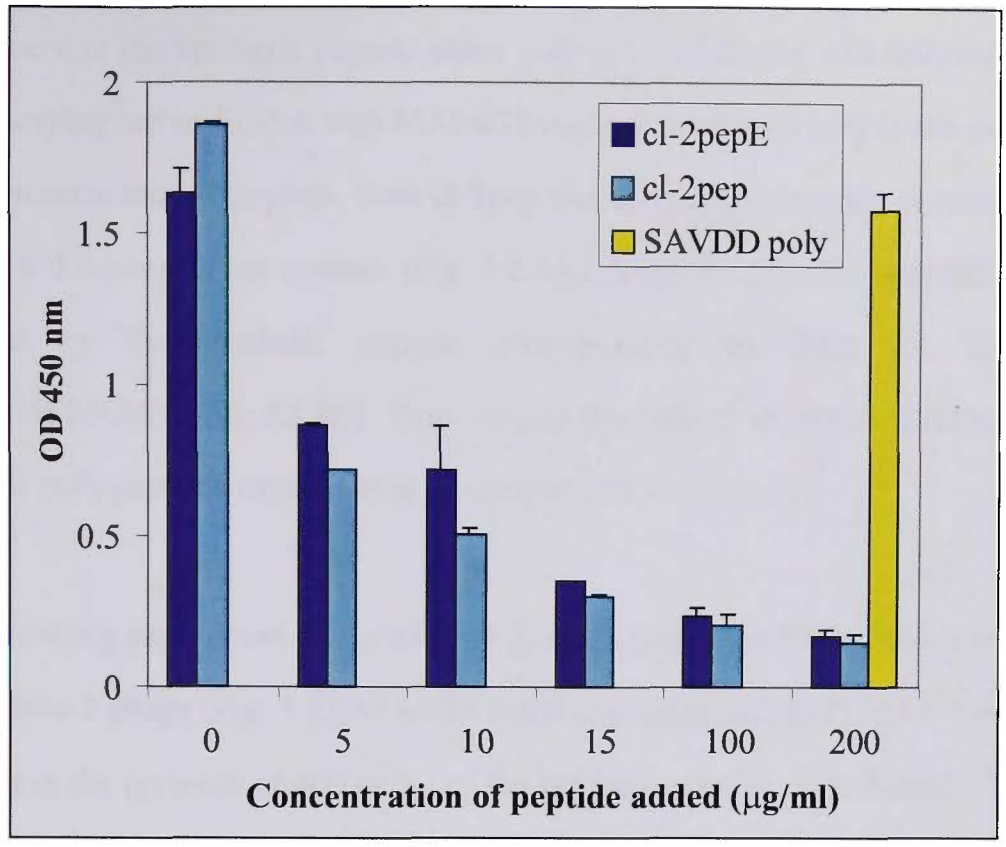


Figure 3.2.7. Binding of MAb4G2 to synthetic monomer and polymer peptides. 20 μg/ml of cl-2pepE (**GA**GWLSPSWFEPGLASM**GA**), cl-2pep, cl-2pep poly (cl-2pep coupled to a polyacrylamide backbone to form a polymer) and the control peptide, 17(3), were used to coat the wells of a microtitre plate and were probed with MAb4G2 or MAb4F2 (10 μg/ml). Binding was detected using an anti-rat-HRP antibody.

A



B

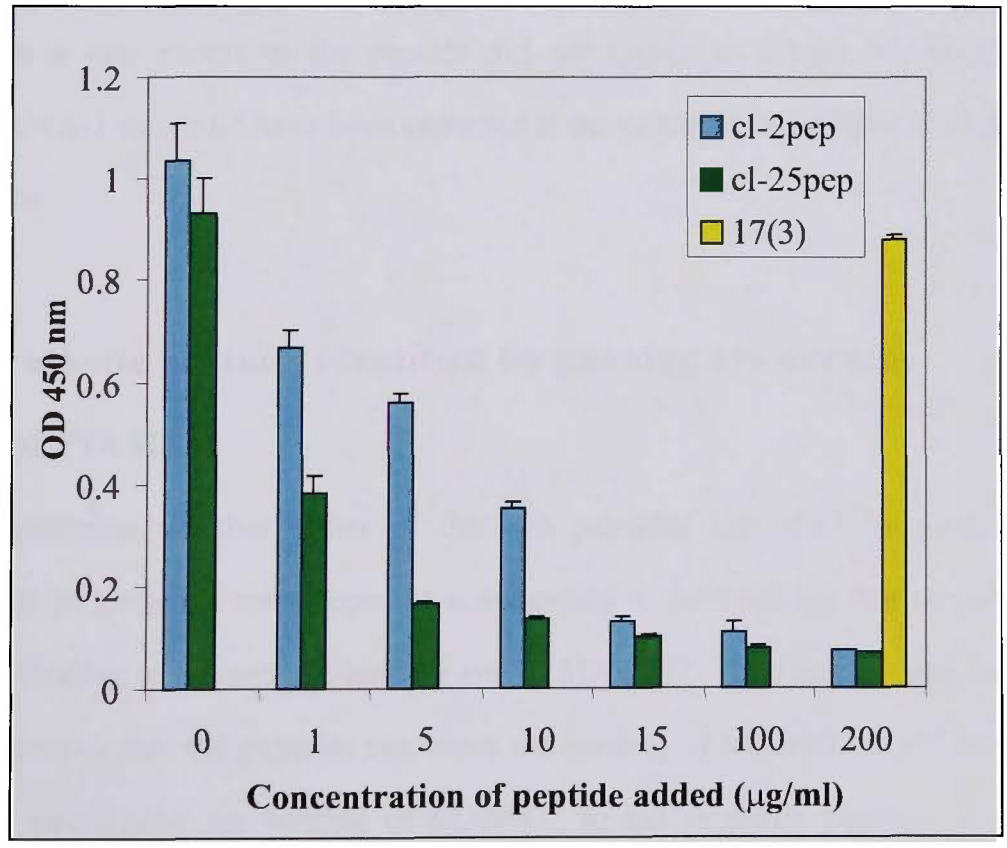


Figure 3.2.8. Inhibition of the interaction of MAb4G2 with cl. 2pep poly by synthetic peptide monomers. cl-2pep poly (2 μg/ml) was used to coat the wells of a microtitre plate and probed with MAb4G2 (10 μg/ml) in the presence of increasing concentrations of synthetic peptides (A) cl-2pepE, cl-2pep or the control peptide, SAVDD poly and (B) cl-2pep, cl-25pep or the control peptide 17(3). Binding was detected using an anti-rat-HRP antibody.

Direct evidence that the synthetic peptide alone was able to interact with MAb4G2 was obtained by carrying out an ELISA with MAb4G2 binding to cl-2pep poly in the presence of varying concentrations of peptide. Both cl-2pep and cl-2pepE were able to inhibit this interaction in a dose-dependant manner (Fig. 3.2.8A). Interestingly, this interaction was also inhibited by the synthetic peptide corresponding to clone 25, cl-25pep (TFVPILFWEHELNAS) (Fig. 3.2.8B). Thus despite the lack of sequence similarity, the binding site for both peptides must overlap to some extent.

In a Western blotting experiment 20 μg/ml of cl-2pep completely inhibited the binding of MAb4G2 to clone 2 phage (Fig. 3.2.9A) whilst binding of MAb4G2 to PfAMA-1 was not inhibited, even in the presence of 400 μg/ml of the synthetic peptide (Fig. 3.2.9B). Thus it appears that the synthetic peptide resembles the structure of the peptide on phage since it can compete with phage displaying the equivalent peptide from binding to MAb4G2. However, even a vast excess of the peptide did not appear to inhibit MAb4G2 from binding to PfAMA-1 as would have been expected if the peptide GWLSPSWFEPGLASM was a mimotope.

3.2.7 The synthetic peptides identified by panning are not true mimotopes of PfAMA-1

In order to determine whether either of the two peptides identified by panning on MAb4G2 might be authentic mimotopes, it is necessary to demonstrate that the peptides are capable of binding to the antigen-binding site of MAb4G2. The simplest way to show this is to demonstrate that the peptides can block the binding of MAb4G2 to PfAMA-1 or that PfAMA-1 can inhibit the binding of MAb4G2 to the synthetic peptides or phage displaying peptides. These points were addressed by experiments conducted using two slightly different formats. In the first format, the synthetic peptides were used to block the binding of MAb4G2 to immobilized PfAMA-1. However, it was found that the clone 2 peptide either alone (cl-2pep) or as an acrylamide polymer (cl-2pep poly) was unable to inhibit the binding of MAb4G2 to immobilized PfAMA-1 (Fig. 3.2.10A and B) despite

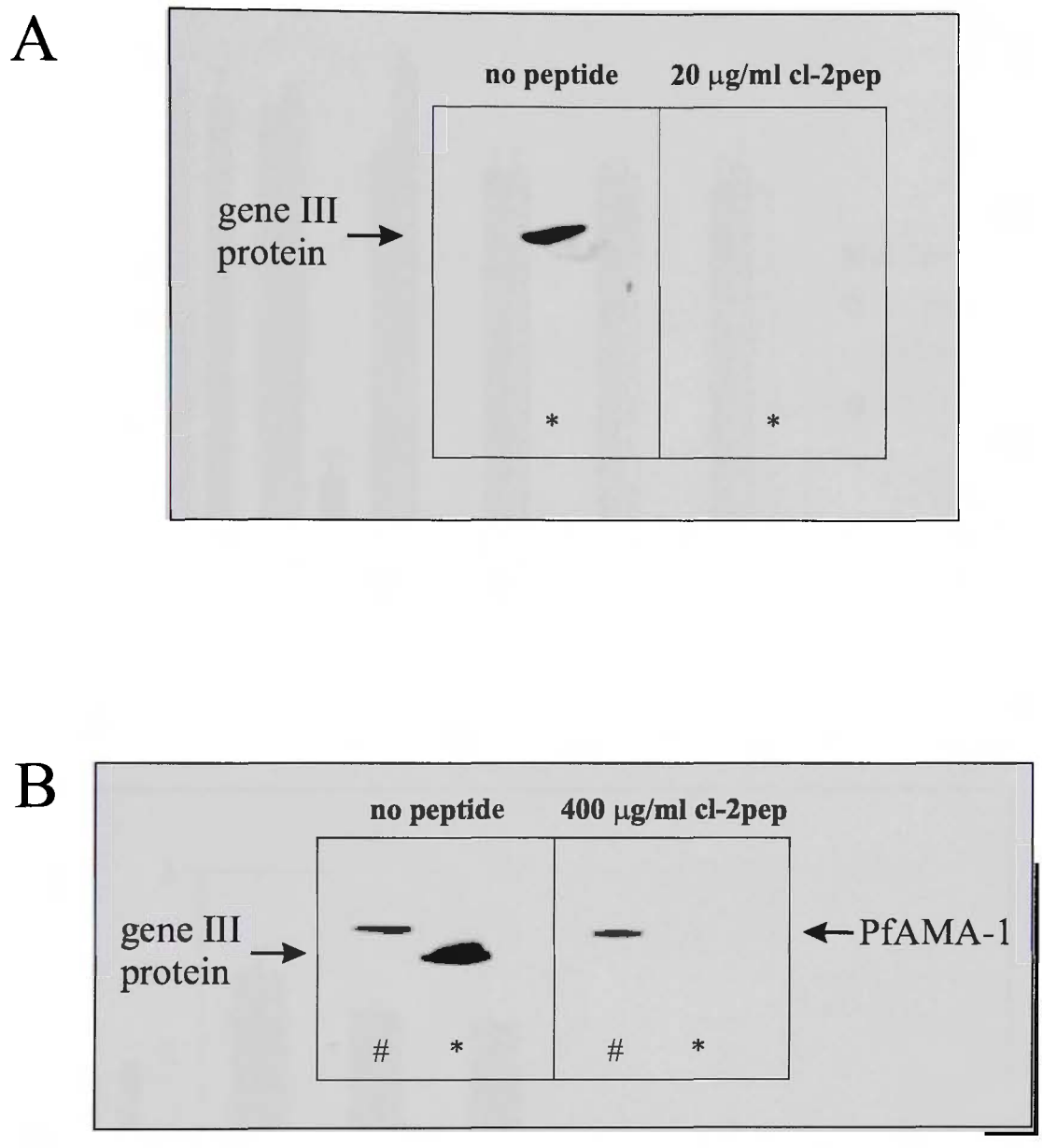
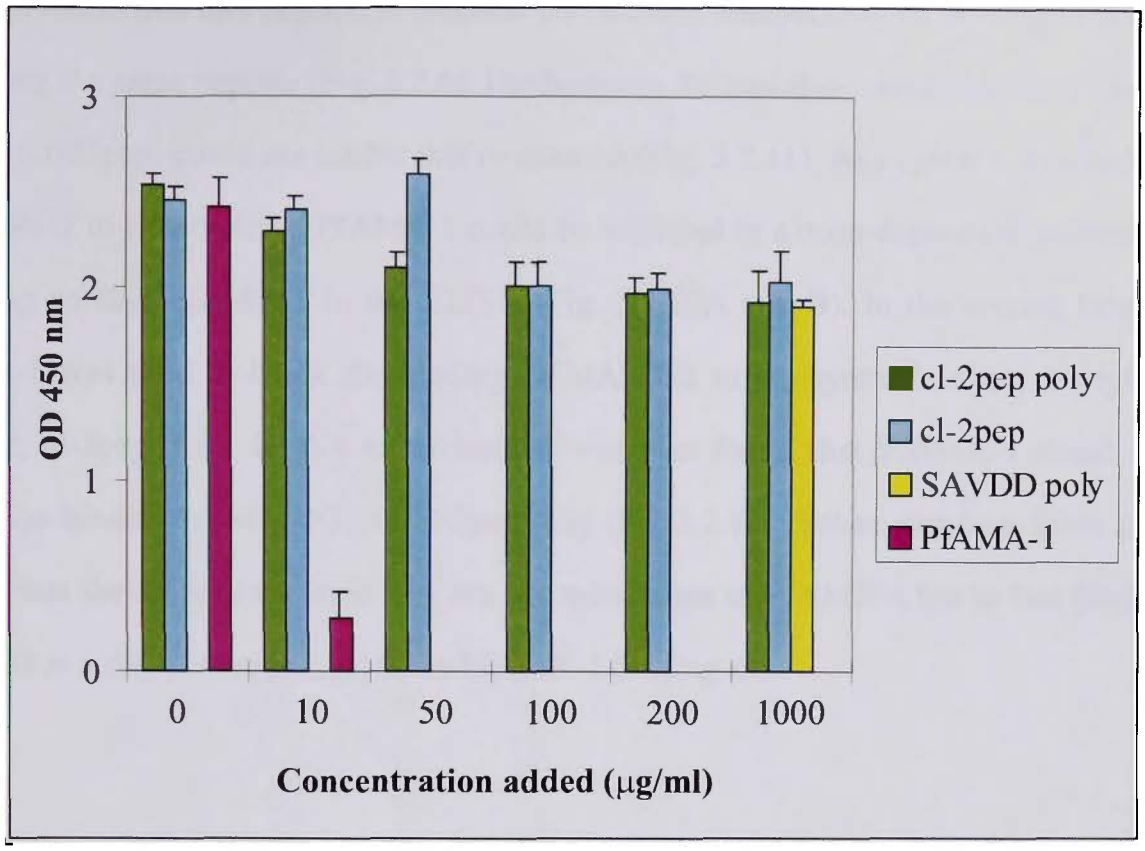


Figure 3.2.9. Inhibition of the interaction of clone 2 phage and PfAMA-1 with MAb4G2 by synthetic peptide, cl-2pep. Analysis by Western blot. (A and B) 10^{10} phage of clone 2 (*) and (B) PfAMA-1 (#) (0.1 μg) were separated by SDS-polyacrylamide gel electrophoresis, transferred to PVDF membranes and probed with MAb4G2 (0.14 μg/ml) in the presence of (A) 20 μg/ml or (B) 400 μg/ml of cl-2pep. Binding was visualized by ECL.

A



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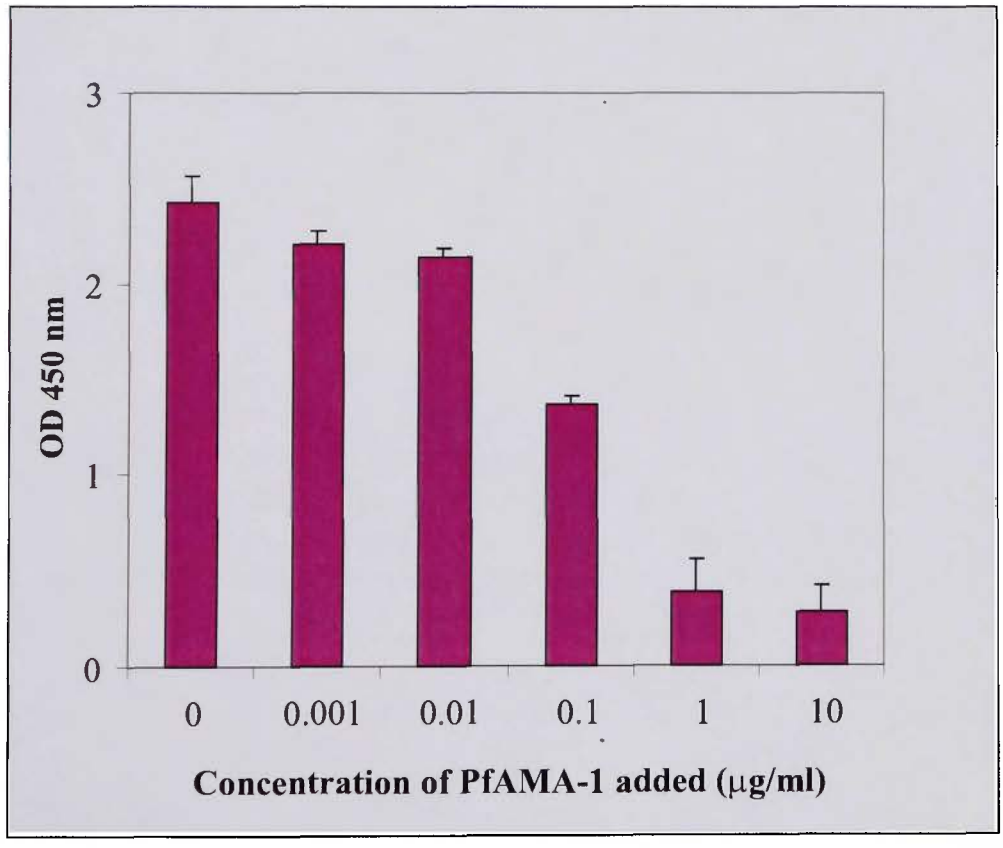

Figure 3.2.10. Inhibition of the interaction of MAb4G2 with PfAMA-1 by synthetic peptides and PfAMA-1. PfAMA-1 (2 μg/ml) was used to coat the wells of a microtitre plate and probed with MAb4G2 (0.05μg/ml) in the presence of increasing concentrations of (A) peptides cl-2pep poly, cl-2pep, SAVDD polymer or (A and B) PfAMA-1. Binding was detected using an anti-rat-HRP antibody.

the observation that this peptide in solution could block MAb4G2 from binding to phage displaying the same peptide (Fig. 3.2.6). Furthermore, its was also found that the clone 25 peptide (cl-25pep) could not inhibit this interaction (Fig. 3.2.11). As expected, the binding of MAb4G2 to immobilized PfAMA-1 could be inhibited in a dose-dependant manner by including soluble PfAMA-1 in the ELISA (Fig. 3.2.10A and B). In the second format, PfAMA-1 was used to block the binding of MAb4G2 to the synthetic clone 2 peptide polymer, cl-2pep poly. In this experiment it was also found that PfAMA-1 could not inhibit the binding of MAb4G2 to cl-2pep poly (Fig 3.2.12). Taken together, these data suggest that the clones identified here are not mimotopes of PfAMA-1 but in fact bind to MAb4G2 at a different region from the PfAMA-1 binding site.

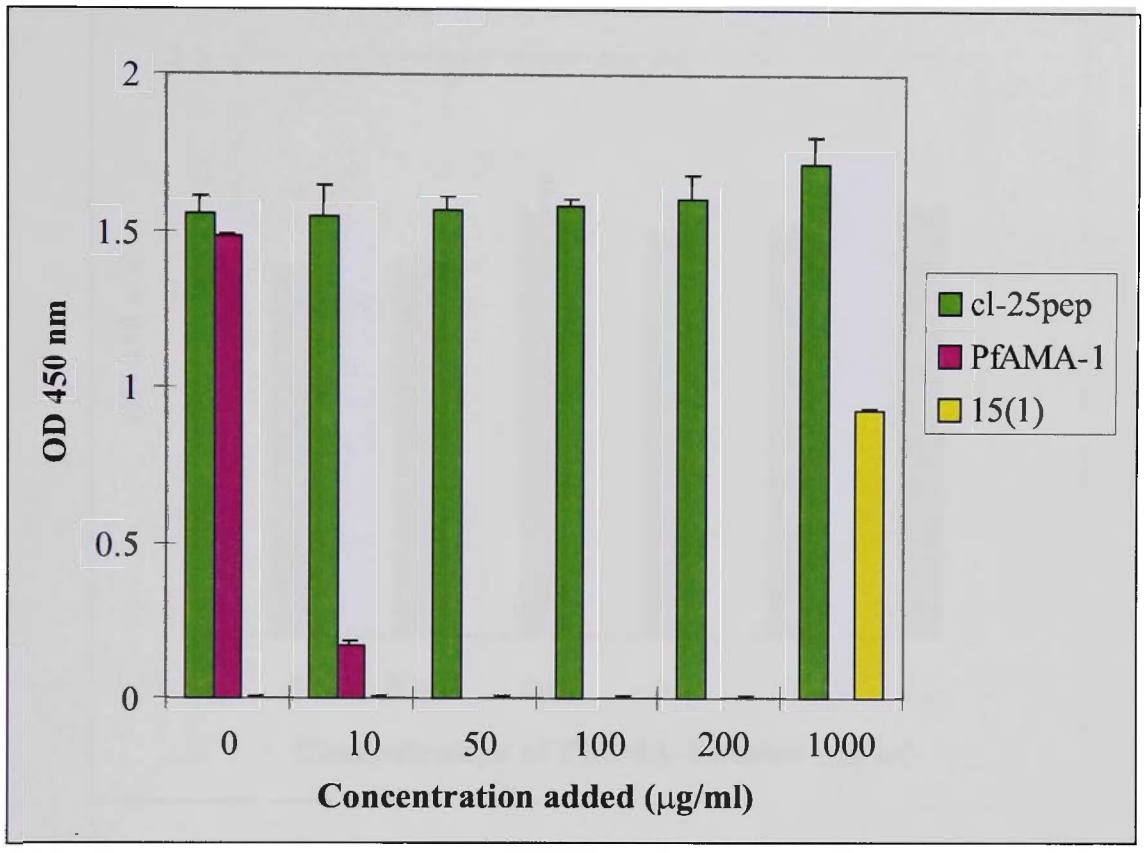

Figure 3.2.11. Inhibition of the interaction of MAb4G2 with PfAMA-1 by synthetic peptides. PfAMA-1 (2 μg/ml) was used to coat the wells of a microtitre plate and probed with MAb4G2 (0.05μg/ml) in the presence of increasing concentrations of peptide cl-25pep (green bars), PfAMA-1 (pink bars) or the control peptide 15(1) (yellow bars). Binding was detected using an anti-rat-HRP antibody.

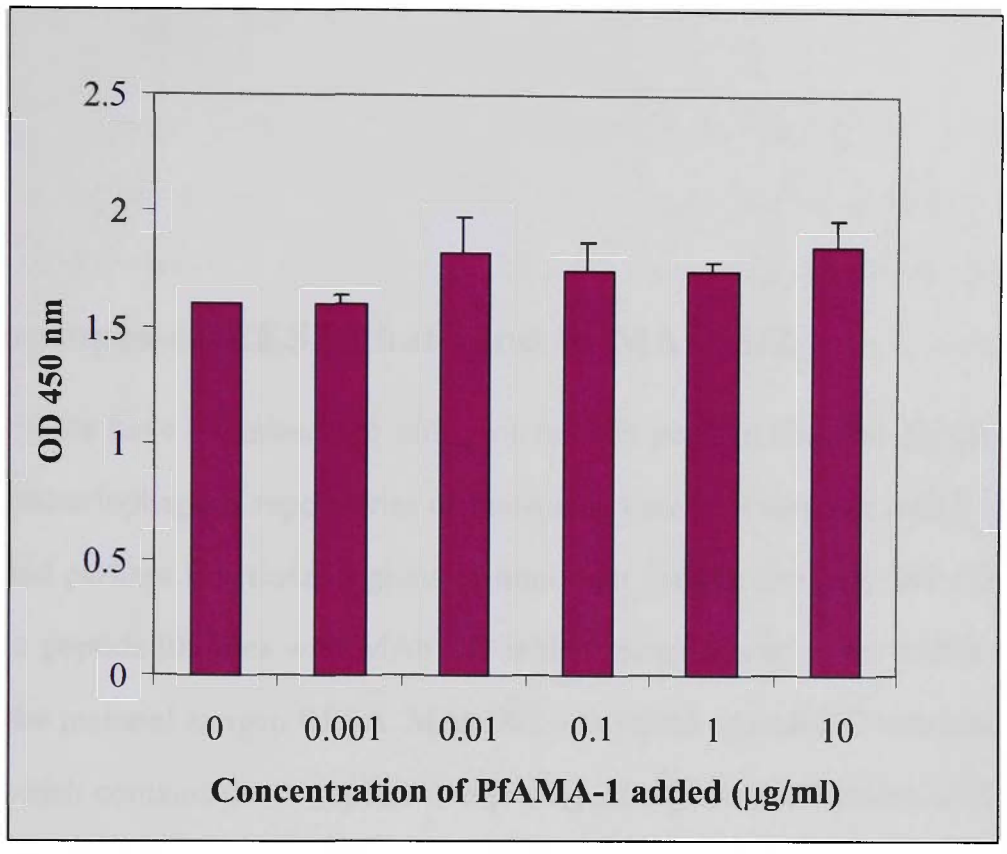

Figure 3.2.12. Inhibition of the interaction of MAb4G2 with cl-2pep poly by PfAMA-1. cl-2pep poly (2 μg/ml) was used to coat the wells of a microtitre plate and probed with MAb4G2 (10 μg/ml) in the presence of increasing concentrations of PfAMA-1. Binding was detected using an anti-rat-HRP antibody.

Chapter 4:

Discussion

4.1 Mimotopes of RESA that bind to MAb18/2

In this study we have examined the utility of random peptide libraries displayed on the surface of bacteriophage as repositories of molecular structures some of which will mimic structural and perhaps functional regions of important malaria antigens. We chose to pan two random peptide libraries with MAb18/2 which recognizes epitopes within the acidic repeats of the malarial antigen RESA. MAb18/2 was raised against a C-terminal fragment of RESA which contains the 3' repeats comprising 29 copies of a 4-amino acid sequence (EENV) plus 5 copies of an extended (8-amino acid) variant of this repeat (EENVEHDA). The exact epitope recognized by MAb18/2 has not been mapped, however it is likely to include the acidic residues present in the 5'and 3' repeats of RESA (97) recognized by MAb18/2.

Panning of two independent random peptide libraries with MAb18/2 identified a set of peptides that bound to the antigen-binding site of this antibody. Although the selected peptides were not identical to sequences within RESA, they shared characteristics that might be expected of RESA mimotopes. It is not clear why there was no selection of peptides that closely resembled the 4-mer sequence, EENV or 8-mer sequence, EENVEHDA. One reason may be that the EENV sequence does not actually represent the best form of the epitope, in the context of the phage. It is also possible that these sequences are not represented in the two libraries used in this study. Alternatively, they may not be tolerated by the phage or the bacteria in which the phage are propagated due to adverse effects, or the production of unfavourable conformations of the phage fusion

protein and hence, would not be accessible for selection during the panning procedure. Alternatively, these peptides may represent very high affinity sites and so not have been eluted by the acid elution method used here.

Isolation of mimotopes (peptides that are not identical to the original epitope but are still able to bind to the antigen-binding site of an antibody due to sufficient structural similarities) from random peptide libraries has been reported by others (78, 106). In one example, a constrained hexapeptide library was screened on a monoclonal antibody raised against the malarial antigen *P. vivax* merozoite surface protein-1 (PvMSP-1). Two groups of peptides that bound specifically to the antibody were isolated, one of which showed no sequence homology to PvMSP-1. Remarkably, these peptides were able to elicit an immune response in mice that recognized recombinant PvMSP-1, indicating that these peptides were indeed mimics of the malarial protein. In the same study, the second group of peptides were found to contain the consensus motif (YSP) that is contained within the primary sequence of PvMSP-1. Whilst these peptides did contain at least three consecutive residues that were identical to PvMSP-1, no peptides containing all 6 amino acids that had previously been identified as the epitope (YSPSGE) were isolated from the library (78). Indeed, it appears to be uncommon to isolate an exact match for an epitope from a random peptide library.

It is interesting to note that the isolation of a number of different peptides, which contain a consensus motif, from random peptide libraries, is not uncommon (1, 34, 78, 177, 285). This may be expected in some cases, particularly where the consensus motif represents the actual epitope of the antibody (78). In this study alone, four different peptides were identified that contained the same SAVDD motif, whilst many others contained a degenerate form of this motif. In some cases however, a consensus motif is not necessarily represented by a sequence of consecutive residues, but may be a sequence containing common amino acids interspersed with random amino acids, for example, SxxDxxK (34). In other cases the random amino acids at certain positions may consist of residues with

specific properties, for example, a positively charged residue or a hydrophobic residue. The isolation of mimotopes that contain consensus motifs may be indicative of limitations in the diversity of peptide libraries or may represent sequences that are favoured by the phage or provide a growth advantage to phage.

In this study, the overall negative charge of the isolated peptides was high, suggesting that the peptides mimic the high glutamate and aspartate content of RESA repeats. The consensus motif identified from the 15-mer library, SAVDD had a closely related counterpart, TAVDD, in the set of peptides enriched from the 17-mer library. The consensus motif **S/TAVDD** most closely resembles the sequences, TVADD, TVADE and TVAEE, which are found within the 5' repeat region of RESA, perhaps suggesting that MAb18/2 may have a higher affinity for epitopes within the 11-mer repeats. This result is slightly surprising since, although MAb18/2 reacts with both 3' and 5' repeats, the antibody was raised against a recombinant protein containing the 4-mer and 8-mer repeats only.

A deeper understanding of this interaction was obtained by competition experiments between the mimotopes and synthetic peptides representing the three dominant repeat motifs in RESA; 4-mer $(EENV)_8$, 8-mer $(EENVEHDA)_4$, and 11-mer (DDEHVEEPTVA). We have shown that MAb18/2 recognized all three synthetic peptides representing the 5' and 3' repeats of RESA, and that all three RESA peptides were able to inhibit the binding of MAb18/2 to the mimotopes (Fig. 3.1.23A and 3.1.24), demonstrating the shared properties of the mimotopes and the RESA-derived sequences. It was interesting to note that the synthetic 8-mer gave the lowest binding to MAb18/2 whereas, it was the 11-mer that was the least effective at blocking the interaction between MAb18/2 and the mimotopes, perhaps reflecting the local conformational differences between peptides attached to a solid substrate and those same peptides in solution. This is further supported by the observation that MAb18/2 when immobilized on the wells of a microtitre plate did not recognize both the 11-mer and 22-mer in the absence of a carrier

protein. However, the 22-mer was more effective than the 11-mer at blocking the interaction between MAb18/2 and the mimotopes. It has been demonstrated in another malarial antigen, the circumsporozoite surface protein (CSP), which like RESA contains an immunodominant repeat region, that numerous linked copies of the repeated motif are required for recognition by a particular monoclonal antibody (190). This finding is consistent with the observation in this study that the 22-mer competes the interaction between MAb18/2 and the mimotopes better than the 11-mer suggesting that higher order structures exist when two or more repeating units are linked. Alternatively, it is possible that the interaction between MAb18/2 and the 11-mer is of low affinity but when the sequence is repeated, there is a dramatic avidity gain.

Can the mimotopes provide an insight into the fine specificity of MAb18/2? The observation that a denatured form of RESA is recognized by MAb18/2 leads to the suggestion that MAb18/2 binds to a continuous epitope on RESA. MAb18/2 was raised against a recombinant protein fragment representing the 3' repeat region (residues 893 - 1073) which includes both the 4-mer and 8-mer repeats. It has also been shown to cross-react with 5' repeat region, which is represented by the 11-mer (11). Therefore, it could be suggested that the epitope of MAb18/2 includes part or all of either the 4-mer or the 8-mer. If the primary sequence of the 4-mer is considered in an extended form $(EENVEENVEENVEENV)_2$ then the rapid successive VEE motif becomes apparent and the only charge on the peptide is associated with the glutamate residues. From this perspective the consensus **S/TAVDD** motif appears to resemble more the 4-mer peptide than the 8-mer or the 11-mer. This is consistent with the finding that the 4-mer and to a lesser extent the 8-mer, compete the interaction of MAb18/2 with the mimotopes more efficiently than either the 11-mer or the 22-mer (Fig. 3.1.24). It is possible that the mimotopes closely resemble the conformational state of the 4-mer more so than the 11-mer despite the earlier observation that the consensus **S/TAVDD** motif closely resembles motifs found in the 5' repeat region of RESA.

Two of the peptide inserts selected from the 17-mer library by panning on MAb18/2 contained large deletions. In the most extreme case the insert from one clone consisted of only 2 amino acids and the other clone insert contained 6 residues instead of the expected 17. Although deviant sequences have previously been observed in this 17-mer library (37), it was nevertheless surprising that a clone containing a peptide insert of only 2 amino acids bound to MAb18/2 while phage containing no insert exhibited no binding activity and phage containing an insert with the full complement of 17 residues, selected at random, did not bind. The most likely explanation for this is that adjacent sequences of the phage protein, gpVIII, combines with the AS insert to constitute a mimotope of MAb18/2. The involvement of the phage environment in binding of phage-displayed peptides to an antibody is a phenomenon that has been previously noted in a study where it was found that synthetic peptides derived from a hexapeptide library could no longer recognize the antibody in the absence of the phage gpIII (78). Interestingly, both the deleted peptides identified in this study end in a serine residue which, when considered along with the next 6 adjacent gpVIII residues (AAEGDD), conforms to a second consensus motif **SAXXXXD** found in examples of MAb18/2 binding clones isolated from both libraries (Fig. 3.1.9C). In order to confirm this, an 8 amino acid synthetic peptide consisting of the two residues AS plus the AAEGDD motif from the phage backbone could be synthesized and tested for binding to MAb18/2.

Of the two libraries we have used, the first consists of 15 completely random amino acids expressed at the N-terminus of the phage protein, gpIII, and the other has 15 random amino acids followed by a fixed cysteine residue which is immediately followed by another random amino acid. This latter library can be considered to be 'semi-constrained' since single cysteine residues are generally not favoured in the phage system and panning tends to select for a second cysteine elsewhere within the peptide (37, 286). Since the position of this second cysteine is not fixed it can, in theory, occupy any position among the other 16 residues thus allowing flexibility in the constraints imparted on the insert by the disulphide bond. We did observe a number of second cysteine residues within the

peptides isolated from the 17-mer library (Fig. 3.1.9B). It was also noted that two peptides containing two cysteine residues were isolated from the 15-mer library, although this library had no particular selection pressure in favour of disulphide bonds. Although disulphide-bond formation between the two cysteine residues probably does occur for at least some of the isolated peptide inserts (286), the disulphide bridges in the selected peptides did not appear to be essential for the binding to MAb18/2 since they were still able to bind to MAb18/2 after elimination of the disulphide bond by reduction and alkylation. The disulphide bond in mimotope 17(3) does appear to play a role in maintaining the integrity of the peptide in order that it can bind efficiently to MAb18/2, since reduction and alkylation leads to a decrease in MAb18/2 binding but does not abolish binding completely (Fig. 3.1.17).

A number of reseachers have advocated that constrained peptides have a more defined conformation, are more stable and that mimotopes isolated from constrained libraries generally exhibit higher affinities than those isolated from unconstrained libraries (126, 154, 203). Thus disulphide bonding within the mimotopes could provide a degree of stability and limit the range of different conformations that linear peptides may attain to those that have the highest affinity and specificity for MAb18/2. Conversely, the insertion of disulphide constraints into some linear peptides that have affinity for the desired target may 'lock' them into conformations that reduce their affinity for the target molecule and will thus not be selected for during panning (281). Unconstrained mimotopes may be able to attain a larger range of conformations, however a proportion of these will probably not be conducive for binding MAb18/2 or may have a lower affinity for the target molecule. Whilst it has been suggested that disulphide constraints placed on peptides reduce the range of targets to which they may bind, when such peptides do bind, they have a greater probability of being more specific and of higher affinity (153). Thus careful consideration should be given when introducing cysteines into a sequence with the aim of stabilizing the conformation and improving the binding properties of a peptide.

Disulphide-bond formation may also play a role in stabilisation of the peptides during their route through the bacterial periplasm or possibly in allowing the peptide to be efficiently packaged on the phage surface and extruded as completed phage particles. It has been suggested that peptides that contain single unpaired cysteines are selected against in the replication of a library as they may pair with one of the cysteines present in the phage framework, hence disrupting the production of that particular phage clone (141). Alternatively, phage expressing random peptides containing a single unpaired cysteine may disulphide bond with another phage expressing a similar peptide, hence excluding those peptides from either being selected for in panning or reamplified. This suggestion was based on the observation that there is a bias against the isolation of phage that contain a single cysteine within the random peptide insert (141). However, if a lone cysteine were to be buried in a hydrophobic microenvironment in the peptide, it may be able to be presented efficiently on the phage surface.

It was observed that 17-mer clones generally exhibited higher relative binding affinities for MAb18/2 compared to the 15-mer clones. Apart from clone 15(1), when 10^9 phage were added to immobilized MAb18/2, the 15-mer clones showed readings in the binding assays of less than 1 OD unit (450 nm), in comparison to the 17-mer clones which showed readings of greater than 1 (Fig. 3.1.12). Clones 17(3), 15(1) and 17(1) appeared to exhibit the highest relative binding affinities. This marked difference in relative binding affinities between the two libraries may possibly be related to the number of copies of the peptide displayed on the surface of the phage particle. The 17-mer clones, displaying about 270 copies of the peptide (37), are likely to have a higher avidity compared to the 15-mer clones, which display only 5 copies of the peptide (231). In other words, the high relative binding affinities displayed by the 17-mer clones may be derived from multiple low affinity interactions with the resulting phage having a high avidity for the antibody. Such a phenomenon has been described previously and has been suggested to impede the selection of high affinity binders (160). Therefore, since the avidity of the phage affects its relative binding affinity, the true affinity of the peptide for MAb18/2 cannot be

determined based solely on these data. Moreover, these data can only provide a qualitative comparison of the binding affinities within each library, which is sufficient for the scope of this study. For a quantitative analysis of the binding affinities of each clone, the individual peptides need to be synthesized and analysed in the absence of the phage framework using, for example, a Pharmacia Biacore Instrument.

In order to overcome the effects of avidity, the monovalent display of molecules has been used. This is particularly suited to the expression of large molecules on the surface of phage that may either disrupt the production of phage or be toxic to the host cell in large numbers (160). In a different approach, using a cysteine flanked 8-mer library, the ability of the polyvalent display of peptides on gpVIII to select low affinity peptides was exploited, in order to select for peptides that bind weakly to the EPO receptor. Wrighton *et al.* (279) identified a consensus peptide (CRIGPITWVC) with a weak dissociation constant of about 10 μM. This peptide sequence was then used as a template to construct a mutagenesis library in a gene III phagemid display system in order to select for higher affinity mutants. In this panning, increasing concentrations of EPO were used to elute the binding peptides. This ultimately resulted in the isolation of a 20 amino acid peptide with an affinity of about 200 nM, which remarkably, can form a dimer and activate the EPO receptor in much the same way as EPO itself (279). Thus polyvalent display systems can also be a means to isolating high affinity peptides.

It is interesting to note that the selection procedure with the 17-mer library resulted in a 1000-fold increase in the signal for the binding of phage to MAb18/2, in comparison to the 15-mer library which showed only a 10-fold increase (Fig. 3.1.2). This difference is consistent with the observed relative binding affinities exhibited by the isolated clones from each library (Fig.3.1.12). The level of enrichment can sometimes provide an indication of the specificity of the eluted phage, as one could expect to find an increase in the specificity of the phage for the target molecule over successive rounds of panning and a subsequent reduction in the diversity of the library (67).

There are a number of factors associated with the panning procedure that affect the affinity and specificity of the eluted phage. Those that have been addressed previously include the amount of target molecule present and the method of immobilization, the stringency of the panning procedure and the method of eluting the bound phage. In this study, MAb18/2 was immobilized directly in the wells of microtitre plates. The use of linkers or coupling reagents such as biotin/streptavidin was deemed not to be necessary as the antigen-binding domain of MAb18/2 was found to be sufficiently exposed. The amount of immobilized antibody remained constant throughout the panning procedure. However, as has been illustrated previously, a subsequent reduction in the amount of the immobilized antibody in the later stages of panning could have been used to encourage the selection of high affinity binders (265).

The stringency of the selection procedure in this study was governed by the number of times the bound phage were washed prior to elution. In the first round of panning, the non-binding phage were removed in two washes and in subsequent rounds, 4 washes were used. The objective in the first round of panning was to retain a large diversity of binding phage regardless of their affinity. The rationale behind the subsequent rounds of panning was to reduce the diversity of the round one phage pool, by removing low affinity and non-specific binders with increased washing strength, while retaining the higher affinity binders.

Acid elution is a method commonly used to elute binding phage (1, 98, 170, 265, 268, 277). Other methods of elution that have been described previously include enzymatic cleavage of the immobilized target molecule and specific elution using a known ligand to the target molecule (as in the above mentioned EPO example (279)). In this study, acid elution was found to be sufficient to elute binding phage, however, it is not known whether all high affinity binders were eluted. It is possible that phage peptides with sequences homologous to the epitope of MAb18/2 were not eluted due to having very

high affinities and that lower affinity mimotopes were obtained instead. The specific elution of binding phage using increasing concentrations of RESA could have been used to ensure that high affinity binders were selected. Therefore, it would be interesting to repeat the panning, but elute the binding phage with an excess of RESA. It has also been suggested that the length of the elution time can also be critical in the type of phage that are eluted (75). The method of acid elution could have been made to be more effective by increasing the elution time from 20 min to 30 min. However, as the objective of the panning procedure was achieved, further optimization of the procedure was not undertaken.

It has been shown that at 50 μg/ml, the synthetic form of peptide 17(3) competes with MAb18/2 for interaction with the phage displaying peptide 17(3) by about 85% (Fig. 3.1.21). Interestingly, when this competition experiment was repeated on a later occasion (Fig. 3.1.22), only a 30% reduction in binding was observed. The reasons behind this difference in results remain unclear, however, we observed that when synthetic peptides were stored in solution for long periods of time, they tended to exhibit a reduced affinity for MAb18/2, in comparison to when the peptide was freshly reconstituted. This reduced affinity could be due to degradation of the peptide, or precipitation of the peptide out of solution. Alternatively, its has been suggested previously, that when in solution, a chemically synthesized peptide may form a different conformation to the same peptide displayed on phage, as it is not restricted in the conformations that it may form by the phage framework (21).

Many of the peptides isolated in the panning of MAb18/2 contained the consensus motif SAVDD (Fig. 3.1.9), and it was important to know if this motif represented the mimotope recognized by MAb18/2. It was established that MAb18/2 could not recognize a synthetic peptide with the sequence SAVDD when immobilized on plastic (Fig. 3.1.31). As previously noted however, the context in which a peptide is displayed can affect the binding properties of that peptide. This was particularly noted in Figure 3.1.23A where

the 11-mer peptide was recognized by MAb18/2 when conjugated to a carrier protein, whereas the same peptide immobilized in the absence of the carrier protein was not recognized (Fig. 3.1.23B). It was therefore decided that a polymeric form of the peptide would be constructed. It is generally recognized that peptide polymers elicit a better immune response, in terms of antibody production, than monomeric peptides (135, 136). It is possible to synthesize the SAVDD peptide in different polymeric forms, eg. as a multimer presenting the peptide in a tandem linear sequence (SAVDDSAVDD...) as in the case of the 4-mer peptide, or as a branched polymer such as the multiple antigenic peptides (MAPs) (248) or as acryloylated peptides (136). The production of synthetic peptide-based immunogens, as a peptide epitope in a tandem linear sequence, has overcome many of the problems traditionally inherent in the design of peptide vaccines and the use of carrier proteins (42, 81, 136, 229). However, there are limitations in this approach, namely the introduction of novel immunogenic sequences at the junction of each epitope and limits in the length of the immunogen that can be synthesized (136). Furthermore, it has been found that peptide polymers in a branched conformation are superior immunogens to linear polymers, in which copies of the linear peptide are presented in tandem (100). It was therefore decided to synthesize a branched polymer. Initially, branched polymers were constructed by incorporating multiple peptides onto a branched oligolysine support referred to as the multiple antigenic peptide (MAP) system (248). In a novel approach, acrylamide has been adapted to produce branched peptide polymeric immunogens. Essentially, peptides are synthesized on a solid-phase support and then acryloylated at the amino terminus with acryloyl chloride. After the peptides are cleaved and purified, exposure to free radicals enables polymerization of the peptides such that the synthetic peptides are attached to a polyacrylamide backbone. Such polymers have been shown to be highly immunogenic (136). Using this novel approach, a homopolymer of the SAVDD peptide was produced in the laboratory of David Jackson (Department of Microbiology, University of Melbourne, Parkville, Victoria, Australia) and ELISA experiments conducted to investigate the ability of MAb18/2 to recognize the SAVDD polymer.

It was observed that MAb18/2 recognizes the polymeric form of the SAVDD peptide to a far greater extent than the monomeric form (Fig. 3.1.32). Furthermore, the monomeric form is only able to partially compete the interaction of MAb18/2 with the mimotopes at high concentrations. This is in contrast to the polymer which blocks this interaction as well, if not better, than the peptides 17(3) and 15(1) (Fig. 3.1.34 and 3.1.35). Thus, MAb18/2 can bind to the SAVDD sequence and hence probably recognizes the consensus S/TAVDD motif in the differing contexts of larger peptides.

One advantage of this approach is that a range of different acryloylated peptides can be combined and polymerized to form a heteropolymer which could be very useful in the production of a multi-antigenic vaccine (136). A heteropolymer peptide was created by polymerizing peptides 17(3), 15(1) and SAVDD together on the same matrix. MAb18/2 was found to recognize the heteropolymer in an ELISA experiment to a similar extent to that shown by the combined individual monomeric forms of the peptides (Fig. 3.1.33B). This data illustrates that polymerization of the mimotopes into a larger molecule has not had an adverse affect on their antigenicity. Indeed, the heteropolymer was able to compete the interaction of MAb18/2 with peptide 15(1) to a greater extent than the individual monomers or the SAVDD poly peptide (Fig. 3.1.35A). Furthermore, the heteropolymer was also found to recognize antibodies in human sera from individuals who reside in a malaria endemic area and had previous infections with *P. falciparum* (Fig. 3.1.30). Thus human antibodies generated during the course of a malaria infection are able to recognize peptides on an acrylamide matrix, illustrating the potential applications of the heteropolymer in seroepidemiological studies.

The production of a humoral immune response to malarial antigens using mimotopes isolated from peptide libraries displayed on phage has been reported previously (78, 247). Indeed mimotopes have been used to mimic the immune response to a number of pathological agents (106, 142, 161). Several studies suggest that there are advantages to

using phage displaying peptides directly as immunogens (76). A major advantage is that the conformation of the peptide displayed on the phage particle would be identical to that selected by the antibody in the panning procedure. This is in contrast to peptides that have been chemically synthesized and coupled to a carrier protein, which may not only be different to the authentic antigen but also to the same peptide displayed on phage. Thus a peptide that is a good antigenic mimic (ie. recognized by antibodies raised against the parent antigen) may not always be a good immunological mimic (ie. generate an immune response to the parent antigen when used to immunize mice). Moreover, the coupling of the peptide to the carrier protein may further chemically alter the peptide, or its conformation, resulting in the reduced affinity of the peptide for the antibody, hence changing the immunological properties of the peptide.

In the context of this study, it seemed possible that the presentation of the peptide in a format other than the surface of the phage could result in the production of a new repertoire of antibodies, which recognize the peptide but do not recognize RESA. Other advantages of using phage as immunogens, as detailed by de la Cruz *et al.* (76), include the minimal cost involved in peptide production and the ease with which phage-displayed peptides can be produced and purified. Phage can also be used to immunize animals without the need for adjuvants whilst still eliciting a good immune response. This has been illustrated previously where a comparative analysis was made of mice immunized with phage in the presence and absence of Freund's adjuvant. It was found that the presence of the adjuvant had no effect in further stimulating helper T-cell activity. Furthermore, it was illustrated that the immune response to phage is T-cell dependent suggesting that the phage can act as its own intrinsic adjuvant (275).

As expected, the immunization of a Balb/c mouse with phage displaying peptide 17(3) resulted in the production of antibodies that primarily recognized phage structural proteins. It was also found that a good immune response to the mimotope displayed on the phage was induced, since mouse sera recognized peptide 17(3) in ELISA experiments.

This induction of peptide-specific antibodies was achieved without the use of adjuvant. Gene VIII protein expression has been considered to be a method of presenting the peptide in a more immunogenic form, since it results in the display of about 270 copies of the peptide on each phage particle. By comparison, expression of peptides on the gene III protein would result in the display of only 3-5 copies of the peptide per phage particle (116). It would generally be expected that a repertoire of antibodies, possibly recognizing different epitopes within the peptide 17(3), would be present in the mouse serum. It is hoped that a portion of these antibodies would probably recognize the region of the peptide 17(3) that mimics RESA. Indeed it was found that the mouse serum did contain antibodies that bound to recombinant RESA, in ELISA and Western blotting experiments. Moreover, Western blotting experiments revealed that serum from the mouse immunized with phage displaying peptide 17(3) also recognized RESA produced by malaria parasites during growth *in vitro* (Fig. 3.1.40). Other malarial proteins including the merozoite surface protein-2, and the malarial homologue of the 70 kDa heat shock protein were not recognized by the mouse serum indicating that the immunization was specific for RESA. Interestingly, the serum was also found to recognize peptide 15(1) although at a lower level than 17(3) (Fig. 3.1.38). This is consistent with earlier results that indicated that RESA was able to compete with phage expressing peptides 17(3) and 15(1) for binding to MAb18/2. Thus although the peptides have different sequences they appear to be antigenically and immunologically very similar. From these immunization data it could be suggested that an immune response has been produced that, at least partly, mimics the immune response of the mice to the recombinant RESA fragment, which allowed the production of MAb18/2. Therefore, not only have true mimics of a malarial antigen been identified but also the immune response to that antigen has been replicated using mimotopes displayed on the surface of phage.

Mimotopes with properties such as those described above, clearly have a role to play in vaccine design. Traditionally, subunit vaccines have been a compilation of antigenic components derived from one or more different proteins, which are used in the hope of

producing the desired immune response. One problem in vaccine design is that the three-dimensional structure of many of these proteins is unknown and this problem is compounded by the fact that immunologically-important conformational epitopes are sometimes lost in the production of recombinant proteins. Unlike subunit vaccines, synthetic peptide immunogens can target the immune response to the important epitopes, even conformational epitopes, through the use of peptides that mimic the epitope (mimotopes). Although RESA is still included in clinical vaccine trials, it is not known whether the repeat regions of this molecule are the important immunological regions for induction of a protective immune response. The results obtained in this study, together with those of others (78), illustrates the potential of phage display technology in the development of peptide vaccines that can be used to target the immune response without the need to first identify the important structural epitopes or even to characterize the aetiological target.

Pf332, a megadalton protein consisting primarily of 11 amino acid repeat sequences (5, 169) contains three sequences (SVTEE, SVTDE and SVTED) that resemble the consensus **S/TAVDD** motif identified in this study. The human monoclonal antibody (MAb33G2) is an efficient inhibitor of parasite invasion *in vitro* (269), and has been shown to recognize Pf332 and cross-react with RESA. Although it is not known whether the antisera generated against the mimotopes identified here are inhibitory, one study has revealed a correlation between antibodies against the RESA 4-mer repeated epitope and protection against malaria infection (17), while another study has demonstrated that antibodies to RESA repeats inhibit malaria invasion (49). Ahlborg and co-workers have also shown that antibodies reactive with RESA repeats inhibit the growth of malaria parasites (4). Interpretation of these results is unclear because of the network of cross reactions that exist between *P. falciparum* antigens containing hydrophobic-diacidic motifs. Peptides isolated by panning phage display libraries may help define more clearly the functional cross-reactivities between antibodies to different malarial antigens. In the context of this study, it would be interesting to determine whether the mouse sera raised

against peptide 17(3) contain protective antibodies. This is unlikely as MAb18/2 is itself not protective and an antibody to Pf332, which has been shown to cross-react with RESA, does not recognize the mimotopes described here (data not shown).

Our results demonstrate that small peptides can mimic features of repeat regions of large *P. falciparum* proteins. The peptide mimotopes described in this study inhibit the binding of MAb18/2 to a recombinant fragment of RESA and also to authentic RESA within the malaria parasite. They also induce a humoral immune response that recognizes both the recombinant and authentic forms of RESA. Such peptide mimotopes and those corresponding to other malarial antigens may prove useful markers for monitoring the sero-epidemiology in communities where malaria is endemic. Numerous studies examining malaria endemicity and other sero-epidemiological parameters have relied on synthetic peptides corresponding to the linear repeat sequences of RESA, (181, 214, 221). It may be that mimotopes isolated using anti-RESA antibodies would more closely mimic the presentation of RESA within the parasite, since any effects of conformation within the repeat regions may not be faithfully represented in short synthetic peptides based solely on primary sequence of the antigen. The observation that sera from individuals from a malaria endemic area recognize a synthetic version of the mimotopes isolated in this study (Fig. 3.1.30), supports this proposal as do the results of the mouse immunization experiment (Fig. 3.1.38). Furthermore, small peptides have obvious advantages over parasite extracts or recombinant antigens in terms of stability and cost effectiveness and it may be possible to identify a small set of peptides that could represent many variants of a highly variable antigen. In addition, these studies could be extended to other antigens of diagnostic or immunological significance. A viable approach would be to pan several libraries on a variety of monoclonal antibodies or affinity purified serum IgG from malaria-infected individuals. The selected mimotopes could then be characterized for their ability to recognize sera from infected individuals.

4.2 MAb4G2-binding peptides

We have demonstrated that random peptide libraries displayed on phage can be used to identify mimotopes that mimic structural features of the malarial antigen, RESA. Our next objective was to identify mimotopes of the malarial antigen, PfAMA-1, using a monoclonal antibody (MAb4G2) that has been demonstrated to bind PfAMA-1 and inhibit asexual *P. falciparum* replication *in vitro*. PfAMA-1 is currently a leading candidate for inclusion in a vaccine against *P. falciparum* (96). Mice immunized with recombinant refolded PcAMA-1 have been partially protected when challenged with malaria (12). Mimotopes of MAb4G2 could be tested for the ability to elicit an immune response that would be equivalent to the specificity and protective properties of MAb4G2. The epitope of MAb4G2 is unknown, however, it is likely to recognize a conformational epitope as it has been shown to react with the ectodomain of PfAMA-1 in its native conformational state, but not in a reduced form (144). Hence, the alternative approach of identifying the epitope and using a synthetic peptide containing this epitope to elicit a protective immune response will most likely be an extremely difficult task.

Despite trying various panning strategies, no mimotopes of PfAMA-1 were obtained from either the 17-mer or the 15-mer libraries. The data obtained clearly illustrates that the isolated peptides specifically bind MAb4G2 in ELISA and Western blotting experiments (Fig. 3.2.3 and 3.2.5). However, it was also shown that these peptides could not compete the binding of PfAMA-1 to MAb4G2, and PfAMA-1 could not inhibit the interaction of MAb4G2 with the synthetic peptide, cl-2pep poly. Taken together, these results indicate that the peptides identified in this study bind to MAb4G2, but at a site distinct from the antigen-binding site. To establish exactly where the peptides bind requires further experiments, but they could be binding close to the antigen-binding site or in the Fc portion of the antibody.

According to published data, most screenings of antibodies on phage-peptide libraries result in the isolation of peptides that recognize the antigen-binding site of the antibody (1, 88, 99,

202, 233, 284). However, peptides with affinity for the Fc region of immunoglobulins (IgG) have also been enriched from peptide libraries. In one example, a decapeptide library was screened on the Fc portion of human IgG resulting in the isolation of a number of peptides. In this study, protein A was used to elute the binding peptides in order to increase the specificity of the isolated peptides. In subsequent analysis, the binding of the peptides to the IgG Fc region was found to be competitively reduced in the presence of increasing concentrations of protein A, suggesting that the peptides may in fact be analogues of protein A (151). Recently however, in a more dramatic example, De Lano *et al.*, using a 20-mer library on gpVIII with a diversity of 4×10^9, isolated two different peptides that bound to the Fc region of human IgG. Further mutagenesis and rescreening of the peptides using a gene III monovalent display system, eventually led to the identification of a 13 amino acid core Fc-binding peptide with the sequence DCAWHLGELVWCT, which adopts a β-hairpin conformation. Remarkably, this peptide could bind IgG with nanomolar affinity and had the same fine specificity as protein A, protein G, rheumatoid factor and the neonatal Fc-receptor. Thus, this 13 amino acid peptide can mimic the IgG binding properties of four different proteins (77). It is relevant to note however, that the Fc-binding peptides were isolated in the absence of the antigen-binding site of the antibody. Thus clearly, it is possible to isolate peptides that bind regions of an antibody not involved directly in antigen-binding, however, a large amount of effort was needed to improve the affinity of the peptides in this example. Generally, one might expect that Fc-binding peptides would have to compete with other peptides in a library that have a higher affinity for the antigen-binding site.

It is possible, therefore, that MAb4G2-binding peptides bind to the Fc-region of MAb4G2. If one compares the amino acid sequences of the MAb4G2 binding peptides, cl-2pep (GWLSPSWFEPGLASM) and cl-25pep (TFVPILFWEHELNAS), with the Fc-binding peptide (DCAWHLGELVWCT) described by De Lano *et al.*, there is no obvious consensus motif, however, there appears to be some similarity in the amino acid composition. All three peptides, apart from cl-2pep, which lacks the histidine residue, contain tryptophan, histidine, glutamate and leucine successively within the space of six residues. If however, the peptides

do in fact bind to a common protein-binding domain then one would expect the peptides to also recognize the isotype control antibody (MAb4F2) to a similar extent, which is not the case in this study. However, MAb4G2 may contain subtle differences in structure to MAb4F2 that allow the peptides to bind with higher affinity. Only further studies will clarify the true binding domain of the isolated peptides. Interestingly, after the submission of this thesis, it was found that MAb4G2 contained an aberrant light chain (252). This leads to the suggestion that the isolated peptides may in fact recognise the MAb4G2 light chain and hence explain why the same peptides did not recognise MAb4F2. Nevertheless, it is clear that the peptides isolated in this study are not mimics of PfAMA-1 and therefore are of little use in the design of an antimalarial vaccine candidate.

MAb4G2 was initially selected on two different random peptide libraries, the 15-mer and 17-mer, however, peptides that were specifically recognized by MAb4G2 were isolated only from the 15-mer library. It is unclear why MAb4G2-binding peptides were not isolated from the 17-mer library, however, this once again suggests that in order to increase the chances of enriching for specific peptides, several different libraries have to be screened. It seems likely that the 17-mer library did not contain peptides with sufficiently high affinity towards MAb4G2 to be isolated in four rounds of panning.

The observation that no true mimotopes were obtained from two different libraries may indicate that a peptide that binds to the antigen-binding site of MAb4G2 may require a complex structure that would be rare in any library of ~ 10^9 clones. Naturally occurring antibodies against AMA-1 in humans and mice have been found to predominantly recognize disulphide-bond dependant conformational epitopes. Furthermore, in the mouse model, refolded domain fragments of PcAMA-1 did not provide protection against malaria challenge, whereas the whole refolded ectodomain did protect (10). Taken together, this suggests that the fine specificity of conformational epitopes in PfAMA-1 is critical for antibody recognition. Perhaps a library of disulphide-constrained peptides should have been panned, as it has been found that constrained libraries are more suited to

identifying high affinity mimics of conformational epitopes in comparison to linear libraries (22, 65). Furthermore, there are many cases where peptides have been isolated from constrained libraries but not from libraries of linear peptides (126, 272). In one such example, two different hexapeptide libraries were selected on a monoclonal antibody raised against the plasminogen activator inhibitor type-1 (PAI-1). One library contained flanking cysteine residues resulting in constrained peptides whilst the other library was unconstrained. Interestingly, mimotopes of PAI-1 were only isolated from the constrained library suggesting that the constrained conformation of the peptide is important when binding to the MAb. This was confirmed by replacing the cysteines with serines, resulting in a significant reduction in the binding of the peptide to the MAb. Based on these observations, it was suggested that unconstrained peptides exist in many different conformations and that the conformation necessary for binding to the antibody may not be well populated, resulting in the lower binding affinities (126). Therefore, it is quite clear that different libraries are suited to different applications. In retrospect, the best approach for each new application would be to use a number of different libraries encompassing short and long peptides, and linear and constrained peptides. In this manner, one is more likely to achieve success with at least one, if not more of the libraries.

In order to increase the probability of selecting for peptides that bound MAb4G2 specifically and with high affinity, PfAMA-1 was used to elute the bound phage from the immobilized antibody at each round in the panning procedure. It was anticipated that PfAMA-1 would compete with those phage that bound to the antigen-binding site on MAb4G2 and therefore, only these phage would be eluted. This method of elution is different from the acid elution used in the panning of MAb18/2, which lacks specificity and elutes phage bound at any site on the antibody. However, this approach did not appear to eliminate the isolation of non-specific binders. In a further modification of the panning procedure, the stringency of the washing was also adjusted to increase the affinity of the eluted phage. Once again, this also did not significantly improve the results of the panning procedure. It appears therefore, that neither of the two libraries examined in this study contained peptides with high enough

affinity for the antigen-binding site of MAb4G2 to be isolated using the procedures adopted in this study.

An unforeseen outcome of this work was the finding that peptides attached to a polyacrylamide framework appeared to bind to the wells of a microtitre plate and to be displayed in a conformation that was conducive to binding the MAbs. The display appeared to be superior to that obtained with the monomeric forms of the peptides. The cl-2pep, when immobilized in the wells of a microtitre plate, was poorly detected by MAb4G2. Interestingly, when cl-2pep was synthesized as a homopolymer (cl-2pep poly), it could be detected by MAb4G2 in the ELISA format suggesting perhaps, that residues critical for binding to MAb4G2 were also binding to the plastic surface and 'masking' them so that they could not bind to MAb4G2. If this was the case, then the production of the acrylamide homopolymer was able to reduce this effect and allow the peptide to bind to the microtitre plate in a conformation that was conducive to binding MAb4G2 at the same time. The chemical interactions occurring between the polyacrylamide framework of the polymer and the plastic surface of the microtitre plate are not known, but they clearly allow the peptide to be displayed in a manner that is different to that for the monomeric form of the synthetic peptide. In the earlier part of this study, the monomeric pentapeptide SAVDD, representing the core motif of the RESA mimotopes, was poorly recognized by MAb18/2 both in solution (Fig. 3.1.35) and immobilized on microtitre plates (Fig. 3.1.32). However, MAb18/2 was able to recognize an acrylamide homopolymer of SAVDD in both free and immobilized orientations. Thus by using the acryloylation technology to produce the peptide polymers, evidence was obtained to suggest that SAVDD was the core sequence recognized by MAb18/2, a result that could not have been reached using the free unmodified pentapeptide. This lends support to the view that this technology has applications beyond the vaccine strategies for which it was developed.

This study has demonstrated that random peptide libraries displayed on phage can be employed in the search for peptides with specific binding to desired antibodies. Such

peptides may be of diagnostic and therapeutic potential as well as of use in probing the molecular interactions between the malaria parasite and its host.

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