ANTIBODY ENGINEERING STUDIES USING FILAMENTOUS BACTERIOPHAGE DISPLAY TECHNOLOGY

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by

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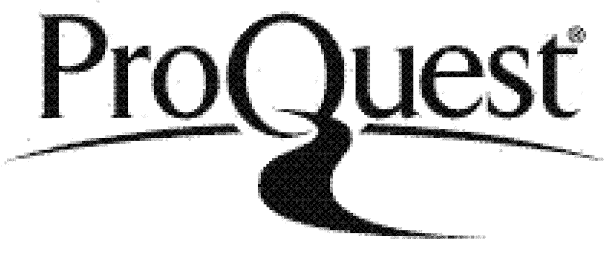

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Antibody engineering studies using bacteriophage display technology

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The work described here uses phage antibodies (PhAbs), combining site-directed mutagensis and molecular modelling to explore a number aspects of phage antibody engineering.

In one study the characteristics of a phage-displayed multi-functional fusion protein were investigated. Bi-functional fusion proteins of staphylococcal IgG binding protein and a single chain Fv antibody (scFv) were displayed on the phage surface. Both moieties functioned although position in the fusion affected function. The protein A moiety of the displayed bi-functional protein provides an affinity handle to facilitate detection and purification of antibody fragments.

The anti-hen egg lysozyme monoclonal antibody, HyHEL-10, has been a focus for studies antibody structure-function relationships. This antibody was used in the production and display on bacteriophage of a hybrid scFv which contained the light chain variable region of HyHEL-10 and the heavy chain variable region of a structurally-related but functionally distinct antibody, AS32. By using a combination of site-directed mutagenesis, complementary determining region (CDR) grafting and molecular modelling, a number of contact and non-contact residues that are important in determining the affinity of HyHEL-10 for lysozyme were identified.

An important application of phage technology is in the creation of natural or synthetic antibody repertoires. A semisynthetic library was created by randomisation of heavy chain CDR3 of AS32 and antibody fragments with new specificities were selected from it. In a separate study using the "single pot" PhAb library (Nissim *et al.*, 1994), to pan against intact germlings of the fungal plant parasite *Phytophthora infestans* and a peptide-BSA conjugate, two populations of antibodies were selected which recognised surface located epitopes of intact *P. infestans* and the peptide conjugate respectively.

The surface location and distribution of the cognate epitopes of the anti-*P. infestans* PhAbs were confirmed by electron microscopy or fluorescence microscopy using rhodamine labelled PhAbs, illustrating the use of fluorescent PhAbs as immunolocalisation reagents.

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Abbreviations

Å	angstrom unit
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pairs
BSA	Bovine serum albumin
cDNA	complementary DNA
CDR	complementarity determining region of immunoglobulin variable domain
cfu	colony forming unit
DMF	dimethyl formamide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediainetetracetic acid
Fab	antigen binding fragment from papain digestion of immunoglobulin
Fc	crystallizable fragment from papain digestion of immunoglobulin
Fd	immunoglobulin heavy chain variable domain and first constain domain
FR	immunoglobulin variable domain framework region
Fv	immunoglobulin variable domain
H	immunoglobulin heavy chain
HEPES	N-2-hydroxyethylpiperazine-N'-2-sulphonic acid
Ig	immunoglobulin
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobases
kDa	kilodaltons
L	immunoclobulin light chain

min	minute(s)
MOPS	3-(N-morpholino) propane-sulphonic acid
NBT	nitro blue tetrazolium
nt	nucleotide
PEG	polyethylene glycol
pfu	plaque forming unit
PhAb	phage antibody
QH_2O	Millipore filtered, double distilled water
scFv	single-chain immunoglobulin variable domain
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N',N',-tetramethyl ethylenediamine
Tris	Tris-(hydroxymethyl)-methylamine
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
X-gal	5-brome-4-chloro3-indolyl-β-D-galactopyranoside

Other abbreviations used are explained in the text or are found in Sambrook et al.(1989).

CHAPTER 1

INTRODUCTION

Antibodies, initially called antitoxins in the late nineteenth century (von Behring & Kitasato, 1890), are crucial components of immune systems. After several decades of intense research it is now possible to engineer these molecules for use in a very wide range of applications in medicine, science and biotechnology. Functional antibodies and their derivatives can now be produced in a numerous heterologous organisms including microbes, plants and animals. In this introduction, an overview is presented of recent developments in antibody engineering and of the many existing and potential applications of this rapidly developing technology.

1.1 General concepts

1.1.1 Antibody properties

Antibodies can bind antigens with a high degree of specificity and can discriminate between two very closely related antigens. These properties are fundamental to the numerous applications of these remarkable molecules.

There are two terms to describe the specificity of antibody, one, major specificity, is used define the species of antigen bound by the antibody, e.g. dinitrophenol vs hen egg lysozyme; the other, fine specificity, is used to define the particular pattern of cross-reactions amongst related antigens, e.g. dinitrophenol vs trinitrophenol or hen egg lysozyme vs Japanese quail egg lysozyme.

The strength or degree of binding between an antibody and antigen can be described thermodynamically by terms such as affinity and avidity. Values for the K_a ($K_{association}$), the affinity constant of antibodies, range from 10^4 to 10^{14} M^{-1} (Pressman and Grossberg, 1968). Whereas affinity describes the interaction between a paratope and its epitope, avidity describes the multivalent binding between an antibody and multiple epitopes on a given antigen or organism. Thus the monovalent binding of Fab fragments to their antigen reflects the antibody's affinity, while the bivalent binding of $F(ab')_2$ fragments or whole immunoglobulin molecules reflects the antibody's avidity.

Another striking characteristic of antibodies is their diversity. There are at least 10^8 (and potentially even more than 10^{11}) antibodies with different specificity in humans, and in mouse more than 10^{10} (Berek and Milstein, 1988). Antibody diversity is so great that virtually any foreign macromolecule can by recognised. The diversity of antibodies, combined with their specificity, makes them powerful bio-molecular tools in science, medicine and biotechnology.

1.1.2 **Molecular genetics of immunoglobulins**

Immunoglobulin genes have drawn the interest of a wide range of biologists. The elucidation of the mechanisms by which DNA rearrangement and somatic mutation contribute to antibody diversity has provided new insights into molecular genetics, and studies on the cell-type specificity of immunoglobulin gene expression has yielded important information on gene expression in mammals. The immunoglobulin gene superfamily itself presents important questions to those interested in evolution and the study of antibody-antigen interactions has been of great value in the understanding of protein/protein interactions. The wealth of information gained in such studies has been of great value in the development of recombinant antibody technology.

The chromosomal localisation of immunoglobulin genes has been known since 1978. There are also some orphan V genes, identified by *in situ* chromosome hybridisation or by the use of somatic cell hybrids, which have been mapped outside the major Ig gene locus (Cox *et al.*, 1994). Chromosomal positions have been assigned by banding and linkage analysis (Medrano and Dutrillaux, 1984).

The Ig loci are composed of multiple genes that give rise to V (variable) and C (constant) regions of the proteins, separated by large stretches of DNA that are never transcribed. Exons have been identified at the 5' end of each Ig locus. The structure of Ig V genes in mouse and humans are very similar apart from the total number of genes in each species. The basic structure of the V genes in both species is as follows. Starting from the 5' of the Ig genes, there are the clusters of V (variable) segments, D (diversity) segments (in the case of the heavy chain), J (joining) segments and the C (constant) region genes. All functional C genes that have been linked are found to have the same transcriptional orientation with respect to each other as well as to their respective J cluster. Not all members of a particular species show the same organisation of the C_H locus. Polymorphic individuals are sometimes found that harbour duplications and deletions of particular genes.

Ig V genes have a simple structure which is highly conserved between heavy and light chains, as well as between species. These genes always consist of two exons which roughly correspond to two functional domains, the leader, or signal peptide, is encoded by the L exon, while the V exon codes for the V region. The signal peptide, which is approximately 20 amino acids in length, provides a hydrophobic amino-terminus necessary for transport of the nascent polypeptide into the endoplasmic reticulum (Milstein *et al.* 1972), where the signal peptide is cleaved off. The two exons are separated by a small intron which varies from about 80 to 300 nucleotides in length. This intron nearly always interrupts codon -5 of the signal peptide, codon 1 being the first amino acid of the mature (cleaved) polypeptide.

V_H genes can be classified in families, based on the DNA sequence homology. There are nine identified mouse V_H gene families which have nucleotide sequence identities of approximately 80% or greater within each family, and seven in human. Analyses of V gene families has relied mainly on Southern blotting, which, due to co-migrating gel-bands, is likely to provide underestimates of the number of V gene families. Recently, PCR has been employed to amplify the V_H segments from a single individual by priming from heptamers (Tomlinson, *et al.*, 1992), and the repertoire from this individual has been used to build filamentous bacteriophage libraries of human antibodies with known components (Hoogenboom and Winter, 1992 , Nissim, *et al.*, 1994, Griffiths, *et al.*, 1994).

Mouse V_κ genes can also be usefully classified into homologous sets of sequences, or gene families, by nucleotide sequence or hybridisation criteria. There are 24 V_κ groups classified as 18 Trp-35 and six Cys-23 (Potter *et al.*, 1982). Human κ light chains appear to be much less diverse than murine chains, and have just been placed into six subgroups (V_κ I-VI) (Kabat, *et al.*, 1991; Cox *et al.*, 1994). In contrast to the mouse κ locus, studies of inbred mice to date have revealed only two germline V_λ genes (V_λ 1 and V_λ2) (Bernard, *et al.*, 1978, Brack, *et al.*, 1978). However, the human λ locus is considerably more complex than the mouse λ locus. Comparison of V_λ protein sequences indicates that there are nine subgroups (V_λI-IX) (Anderson *et al*, 1984; Williams and Winter, 1993). It has been found that the usage of immunoglobulin V genes is limited, and only some of the V genes have been found to be frequently used in both artificial and natural repertoires (Cox *et al.*, 1994; Knight, 1992; Griffiths *et al.*, 1994).

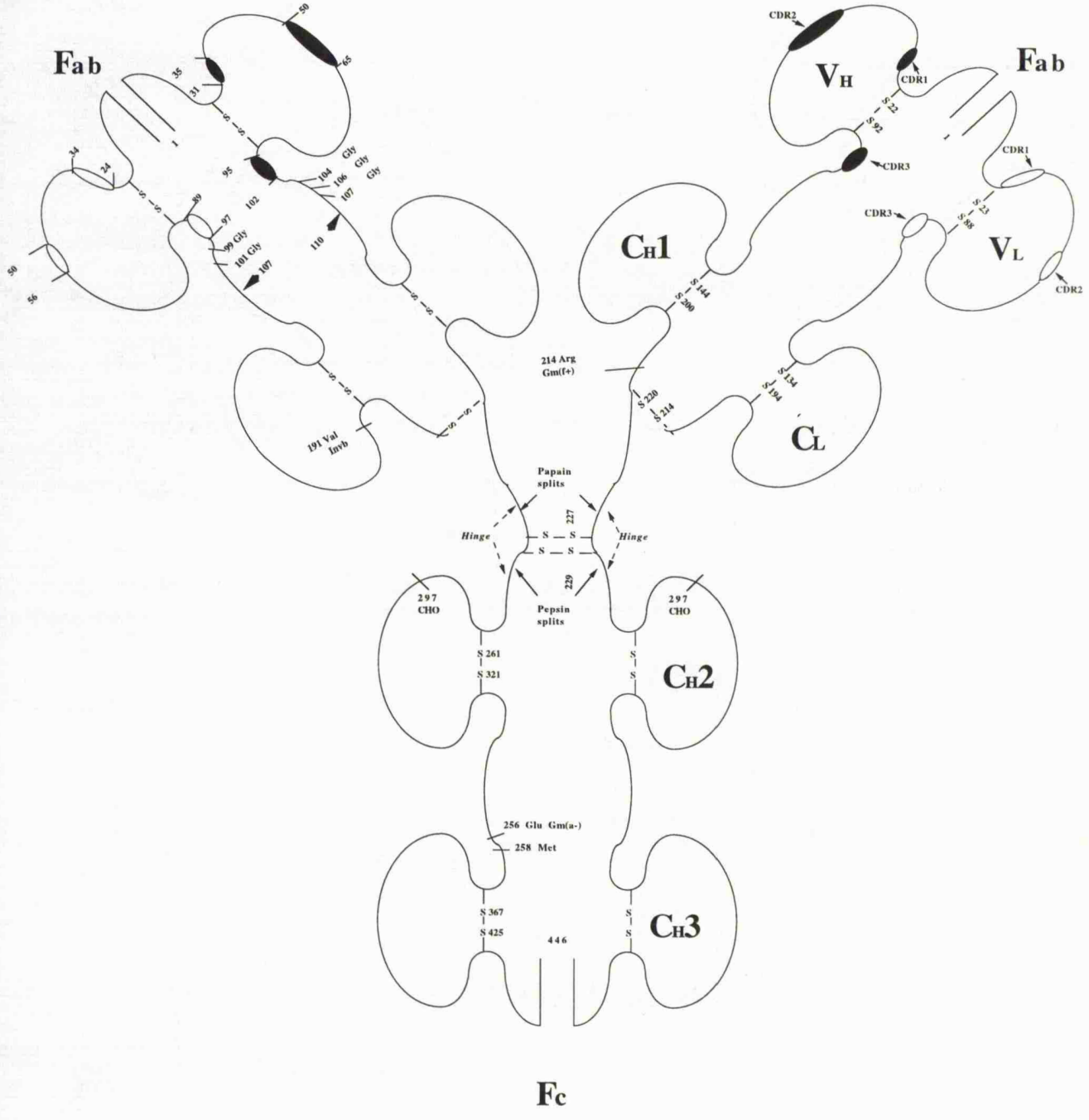
From the multicomponent gene segments, the exons encoding the variable domains are assembled during lymphocyte development by a site-specific recombination reaction known as V(D)J recombination. In immunoglobulin heavy chains, the first two hypervariable loops (CDR-H1 and CDR-H2) are drawn from limited V_H gene segments which are combined with a

few D segments and J_H segments to create the third hypervariable loop (CDR-H3). This loop is exceptionally variable in sequence and length (Wu *et al.*, 1993), because the joining of the segments is imprecise, different reading frames of the D segments may be used, nucleotides can be inserted and deleted at junctions, and the D segments can recombine as D-D fusions (Sanz, 1991). In light chains, the first two hypervariable loops (CDR-L1 and CDR-L2) and much of the third (CDR-L3) are drawn from V_λ and V_κ gene segments. These segments are combined with J_λ and J_κ segments to complete the third hypervariable loop (CDR-L3). This loop has limited variability. Thus, most of sequence diversity is encoded by the heavy chains. The V(D)J recombination generates much of the primary diversity in the binding specificity of Igs. However, antibody repertoires are modified through somatic hypermutation and selection of high affinity variants following each round of antigenic stimulation, so as to continuously refine the pool of memory B cells (Berek, 1993). This somatic hypermutation is a key element in antibody diversification and in the maturation of the immune response, and we now know that the frequency of such mutation is extraordinarily high after antigenic stimulation (Berek and Milstein, 1987).

1.1.3 **Structure of immunoglobulins**

Immunoglobulins are multifunctional glycoproteins constructed by four polypeptide chains. Limited proteolytic digestion and cleavage of disulphide bonds (Porter, 1959) revealed that two of the chains are identical heavy chains (approximately 51-72 kDa) and two are identical light chains (approximately 25 kDa). As the schematic representation shown in Figure 1-1 indicates the monomeric antibody molecule may be described as Y-shaped. Each arm of the Y contains one complete light chain and the amino-terminal end of a heavy chain, while the base is comprised of the carboxyl-terminal ends of the heavy chains. Systematic internal homologies in the amino acid sequences suggested that immunoglobulins are composed of multiple variants of related domains, with the basic folding unit containing approximately 100 amino

Fig. 1-1. Schematic view of four-chain structure of human $IgGl_{\kappa}$ molecule. Numbers on right side: actual residue numbers in protein EU (an immunoglobulin) (Edelman *et al.*, 1969); Numbers on left side of Fab fragment are aligned for maximum homology. Light chain and heavy chain numbers are as in Kabat *et al.* (1991). Heavy chains of EU protein possess residue 52A, three residues 82A, B, C, and lack residues 100A, B, C, D, E, F, G, H, I, J, K, and 35A, B. Thus residue110 (at the end of the variable region) is 114 in actual sequence. Complementarity-determining variable regions (CDRs) (hypervariable regions) are indicated as ovals (open ovals for VL CDRs and close ovals for VH CDRs). V_H and V_L are light and heavy chain variable regions respectively; CH1, C_H2, C_H3 are domains of the heavy chains constant region. C_L is the constant domain of the light chain. The hinge region in which the two heavy chains are linked by disulphide bonds is indicated approximately. Attachment of carbohydrate is at residue 297. Arrows at residues 107 and 110 denote transition from variable to constant regions. Sites of action of papain and pepsin and locations of a number of genetic factors are indicated. Modified from Kabat *et al.* (1991).


Fab
50
65
35
31
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24
95
104
106
107
Gly
Gly
Gly
89
97
102
99 Gly
101 Gly
107
110
50
56
CDR2
VH
CDR1
Fab
S 22
S 92
CDR3
CDR1
CDR3
S 23
S 98
VL
CDR2
CH1
S 144
S 200
214 Arg
Gm(f+)
S 220
S 214
S 134
S 194
CL
191 Val
Inv b
Papain
splits
227
Hinge
Hinge
S — S
S — S
229
Pepsin
splits
297
CHO
297
CHO
S 261
S 321
CH2
256 Glu Gm(a-)
258 Met
S 367
S 425
446
CH3
Fc

acids. Light and heavy chains contain two and four or five domains respectively. Sequence analyses revealed that the first N-terminal domains of both light and heavy chain exhibit relatively high variability, and are termed the variable (V) domains; the remainders of both chains show much lower variability, and are termed constant (C) domains (Kabat *et al.*, 1991).

The immunoglobulin domains have a characteristic tertiary structure consisting of two twisted anti-parallel β-sheets which form a β-sheet "sandwich" structure, and a minor α-helix component (Chothia and Janin, 1981). The two β-sheets of the variable domain are inclined at 30° to one another, and pinned together by a disulphide (S-S) bond between cysteine residues at conserved positions in the sequence. It has been demonstrated the relative orientation of the two β-sheets may vary by up to 18° in variable domains and up to 10° in constant domains (Lesk and Chothia, 1982). This characteristic folding is termed the immunoglobulin fold (Poljak, *et at.*, 1973; Schiffer, *et al.*, 1973; Chothia, *et al.*, 1985).

The variable domains contains three discrete hypervariable regions that are separated by relatively less variable framework regions (FRs) (Wu and Kabat, 1970). By analysis of amino acid sequence variability, Kabat and his co-workers identified the hypervariable regions which are involved in antigen-binding. These hypervariable regions vary in both length and type of amino acids and are also known as complementarity-determining regions (CDRs). Even the hypervariable regions are not completely random in sequence; rather, most hypervariable regions fit into one of a few main chain " canonical structures" (Chothia and Lesk, 1987, Chothia, *et al.* 1989). Detailed analyses of antibody structure have been recently presented by Padlan (1993) and Davies and Chako (1993).

1.2 Structure and modelling of antibody combining sites

The study of IgG or IgG fragments by x-ray crystallography has shown that the antibody combining site (ACS) is formed by the juxtaposition of six hypervariable loops or CDRs, three

from the light chain variable domain and three from the heavy chain variable domain, supported on the β-barrel framework. Further exploration of the antibody combining site has involved physical modelling procedures by which of CDR conformation may be predicted.

1.2.1 **Features of antibody combining sites**

Examination of the occurrence of particular amino acids in different immunoglobulin regions and crystallographic studies indicate that Asp, His and Tyr are more likely to be found in the CDRs than in the framework regions (Kabat *et al.*, 1977; Padlan, 1990; Mian, *et al.*, 1991), while cysteine is more likely to be in the FRs. Apolar aliphatic residues (Ala, Val, Ile, Leu) are represented as frequently in the FRs as in other globular proteins but are much less likely to be found in the CDRs. Aromatic residues (Phe, Tyr, Trp), which are usually buried in globular proteins and in the FRs, are more exposed to solvent in the CDRs. These aromatic residues contribute large areas to the antigen-binding surface and may also reduce the loss of conformational entropy upon antigen binding (Padlan, 1990). The potential mobility of these aromatic side chains have been found to play very important roles in antibody antigen interactions (Mian, *et al.*, 1991). Analysing the antigen-binding sites of three lysozyme–anti-lysozyme complexes revealed that aromatic residues play a large part in antigen binding (Padlan, 1990). No apolar aliphatic residues were found to interact with the lysozyme.

Antibody hypervariable loops have been described as having canonical structures. In such canonical structures, a few key residues are relatively conserved such that the overall secondary structure of the hypervariable regions is maintained. Most changes in the sequences of hypervariable regions maintain the same main-chain structure. Similarly, changes in amino acid side chains do not affect canonical structure unless the amino acid changed is amongst the few that have been shown to be critical (Chothia and Lesk, 1987, Chothia, *et al.* 1989). Implicit in the sequences of V_λ segments are at least three major conformations for CDR-L1 loop and at least two for the CDR-L2 loop. The variability of CDR-L3 is also limited. It

ranges in size from 7 to 11 residues in human λ light chains (Combriato and Klobeck, 1991) and is most commonly 6 residues in κ light chains (Kabat *et al.*, 1991), but can vary between 5 and 8 residues (Timmers *et al.*, 1993). Thus, this loop also conforms to a canonical structure. Implicit in the sequences of V_H segments are three major conformations for the CDR-H1 loop and five for the CDR-H2 loop. In combination they provide seven different folds (Chothia *et al.*, 1992). The third hypervariable region of the heavy chain, however, is the most variable of the hypervariable regions. It varies in length from 2 to 26 (Wu, *et al.* 1993), and does not conform to a canonical structure like the other hypervariable regions. This region is likely to provide a huge range of structures. The combinations of different loops decorated with side chains, create a wealth of binding sites ranging from flat surfaces to pockets (Amit *et al.*, 1986; Alzari *et al.*, 1990; Webster *et al.*, 1994).

1.2.2 **The structural features of antibody antigen complexes**

As a result of x-ray crystallographic and molecular modelling studies of Fab fragments, the structural features of antibody-antigen complexes are well understood. From the analysis of structures of hapten, peptide and protein antibody complexes, three kinds of antibody combining sites have been identified, namely cavity-type for anti-hapten antibodies, groove-type for anti-peptide antibodies and planar-type for anti-protein antibodies (Webster, *et al.*, 1994). Antibody-antigen interactions have been extensively reviewed by several groups very recently (Braden and Poljak, 1995; Wilson and Stanfield, 1994; Webster, *et al.*, 1994; Rees, *et al.*, 1994; Cygler, 1994; Mariuzza and Poljak, 1993).

A general consensus on antibody antigen complexes includes the following points:

1. The CDR residues are involved in contact with the ligands. The ligand-contacting residues are seen to originate mostly from the C-terminal part of CDR-L1, the first and sometimes also the middle position in CDR-L2, from the whole of CDR-L3, CDR-H1 and CDR-H3, and from

the N-terminal part and middle of CDR-H2. The CDR-L3 and CDR-H3 are seen to play a prominent role, not only in ligand binding, but also in the contact with the opposite domain and in the contact with the other CDRs (Padlan, 1993).

2. Surface complementarity between antibody and antigen interface plays a predominant role in antibody-antigen interactions. In some cases, surface complementarity alone can create high specificity and high affinity antibody-antigen interactions (Jeffrey, *et al.*, 1993). The complementarity buried surface area in antibody-hapten complexes varies from 137Å to 291Å, in antibody-peptide complexes from 400Å-503Å and in antibody-protein complexes from 680Å-879Å.

3. The centre of the antibody-antigen contact interface is the primary structure base, and determines the affinity of binding (Padlan, 1993; Hawkins, *et al.*, 1993).

4. Less than 40% of CDR residues normally contact the antigen. The number of van der Waal's interactions can vary from 20 to 68 for antibody-hapten interactions, from 21 to 81 for peptide-antibody interactions and from 75-111 for protein-antibody interactions. In antibody-hapten interfaces, from 1 to 6 hydrogen bonds and salt bridges can be created. About 4 to 16 hydrogen bonds and salt bridges are found in antibody-peptide combinations and about 10 to 24 in antibody-protein interactions.

5. Across all of the antibody-antigen complexes which can be described in three dimensional structure terms, affinities can vary from 1.7×10^5 M^{-1} to 3.4×10^{10} M^{-1}. There is a strong correlation between the number of contacts and the observed affinities in the same types of antibody-antigen interactions (e.g. between different hapten-antibody complexes). There are no strong correlation between affinities and the number of hydrogen bonds or/and salt bridges. However such interactions are sometimes significant. Thus, a single mutation which causes the

loss of one hydrogen bond between lysozyme and the anti-lysozyme antibody HyHEL-5 can reduce the binding affinity by about 1000-fold (Chacko, *et al.*, 1995).

6. The role of water molecules in the interface between the antigen and antibody provides an indication of the extent to which the two surfaces are complementarity. Furthermore, internal water forms hydrogen bond bridges between antibody and antigen, thus stabilising the complex (Bhat, *et al.*, 1994).

7. Some changes in the antibody and/or protein antigen structure often accompany binding.

8. Cross-reactions with closely related antigens are frequently observed.

1.2.3 **Structural considerations in engineering antibody specificity and affinity**

In the last few years, CDR grafting techniques have been employed to 'humanise' murine mAbs. A number of strategies have been employed to work towards this objective including murine antibody variable domain resurfacing (Roguska, *et al.*, 1994). However, the grafting of entire or partial CDRs is particularly significant in the general context of the elucidation of antibody/antigen interactions since specific information can be acquired on the significance to the interaction of particular CDRs (Gulliver, *et al.*, 1994; Riechmann, *et at.*, 1988a; Verhoeyen, *et al.*, 1988). From the protein engineering point of view, the functional transplanting of an active fragment from one protein to another protein framework to produce new activities molecules is attractive.

CDR grafting and structural studies of antibody combining sites have revealed that the function of CDR fragments cannot be completely isolated from the framework which supports them. Indeed some framework residues can be major determinants of CDR function (Chothia, *et al.*, 1989; Tramontano, *et al.*, 1990; Padlan, 1993). The CDR1 loop, for example, is anchored into the framework at one end by residue 24, at the other end by residue 34 and in the middle by residues 27 and 29. Site chains from residues 4, 24, 27, 34, 71, 78, and 94

determine the shape of the platform upon which CDR1 folds. Similarly, the residue occupying position 71 has a major effect upon CDR2 (Tramontano, *et al.*, 1990), and that at position 94 has effects on CDR3 conformation (Chothia and Lesk, 1987; Chothia, *et al.*, 1989).

Several of the platform residues have been identified as a 'Vernier' zone (Foote and Winter, 1992) which fine-tunes CDR structure. In general, sequence differences in the CDRs alter the antigen-binding surface. Small shifts in the loop conformations relative to each other, which may significantly change the binding specificity and affinity may result from sequence differences in both CDRs and FRs (Panka, *et al.*, 1988; Foote and Winter, 1992; Shaw and Margolies, 1992). Furthermore, by analysing five antibody antigen complexes and the available amino acid sequence data on antibody variable regions, the specificity-determining residues in antibodies have been identified as being, in the main, also the most variable residues (Padlan, *et al.*, 1995).

Such studies have identified important framework residues (Chothia, *et al.*, 1987, 1989, Padlan, 1993; Foote and Winter, 1992). Site directed mutagenesis (Tempest, *et al.*, 1991; Foot and Winter, 1992) and modelling of combining site structures (Queen, *et al.*, 1989; Carter *et al.*, 1992) have been used to gain further information on important framework residues.

1.2.4 **Modelling of antibody combining sites**

Many investigators have attempted to model antibody combining sites (ACSs) using two distinct approaches, namely homology-based modelling or *ab initio* modelling. Rees and co-workers have developed an automated procedure that combines both of these approaches, and are able to routinely construct all six loops with a high level of accuracy (Martin, *et al.*, 1989, Pedersen, *et al.*, 1992).

The common feature of the homology approaches has been to examine known antibody crystal structures and select the loops from these on the basis of length and/or sequence. The construction of a physical model of the Fv of MOPC315 was done by Padlan *et al.* (1976) representing the first homology-based modelling of an antibody combining site. The template they used was the corresponding domains of the McPC603 Fab, the structure of which had been previously determined crystallographically (Segal *et al.*, 1974). Subsequently, the same basic procedure was applied to model a number of antibodies, including GLOOP-1-5 (de la Paz, *et al.*, 1986), J539 (Feldmann, *et al.*, 1981), HyHEL-10 (Smith-Gill, *et al.*, 1987) and D1.3 (Chothia, *et al.*, 1986). So far, homology-based methods have had only limited success. When the modelled structure is compared with the corresponding crystal structure, the root-mean-square (RMS) deviations for C_αs of CDRs could be as high as 6.5 Å. The model of D1.3 built by Chothia *et al.* (1986) is more satisfactory, with RMS deviation of 0.5-0.97 Å (backbone-N, C_α, C, C_β) for five of the six loops with CDR-H1 having an RMS of 2.07 Å.

By exploiting the antibody structure database to select conformations on the basis of the conservation of critical residues, Chothia and Lesk (1987) have demonstrated that canonical structures exist for five of the six the hypervariable loops. These critical residues, which affect loop packing (e.g., bulky residues such as Trp, Tyr, or Phe), can form hydrogen bonds or salt bridges (e.g., Ser, Thr, Asn, Gln, Asp, Arg, or Lys), or are able to adopt unusual conformations (e.g., Gly or Pro). This approach is currently completely manual although it is a prime candidate for automation. The major problem, with any such method has been the limited size of the knowledge base since the structure of relatively few antibody loops is known in detail. A significant advance in methods currently in use is that the size of the database is extended by the use of information on general protein loop structure.

Several different *ab initio* methods have been used in antibody modelling. Stanford and Wu (1981) constructed a model of the backbone structure of the ACS of MOPC315 by analysis of

backbone torsion angles for tripeptides observed on other β-sheet proteins (Stanford and Wu, 1981). A related approach is the conformational search algorithm (Bruccoleri *et al.*, 1988), which generates all possible loop conformations by a tree search. Levinthal's group have proposed a random search method which combines molecular dynamics and the generation of random sets of conformations for the CDRs (Fine *et al.*, 1986). The *ab initio* conformational search algorithms saturate the conformational space available to a loop and select the appropriate structure on the basis of its energy, calculated using an empirical energy function. These approaches solve the problem of a limited knowledge base but in doing so fail to make use of the existing knowledge and, in consequence, are extremely expensive in CPU time (total period of time that a Central Processing Unit is used to actually process instructions).

The combined algorithm developed in Rees's group makes use of segments from all known structures (not restricted to antibody structures) as the database from which to choose the most similar segments for the CDR loop of interest. A conformational search program such as CONGEN (Bruccoleri and Karplus, 1987) is then used to obtain the lowest energy conformations. Each conformation is then screened and the one with the smallest hydrophobic exposed area is selected. This combined algorithm has been assembled into an automated immunoglobulin domain modelling program, AbM (Oxford Molecular Ltd.).

1.3 Antibody engineering

Conservation of immunoglobulin structure is very important in connection with the engineering of antibodies and antibody fragments by molecular biotechnology. Recombinant antibodies ranging from intact IgG molecules to single CDR loops have been cloned and expressed (Winter and Milstein, 1991; Wright *et al.*, 1992). Furthermore, variable domain gene repertoires can be cloned or synthesised, and desired binders selected following the display of the gene products of the repertoire on the surface of filamentous bacteriophage (Winter *et al.*, 1994). All of these immunoglobulins and derivatives can be successfully

assembled in both prokaryotic and eukaryotic (non-lymphoid) cells (Oi *et al.*, 1983; Cabilly *et al.*, 1984). It has also been shown that recombinant antibodies can be secreted out from the cell cytoplasm (Cattaneo and Neuberger, 1987; Skerra and Pluckthun, 1988; Better *et al.*, 1988) or retained in it, or targeted to the nucleus (Biocca *et al.*, 1990).

1.3.1 **Cloning strategies**

PCR-based methods have been extensively used for cloning V_H and V_L genes or intact Igs into expression vectors. Primers can be designed for amplification of most families of V genes, as the nucleotide sequences at the 5' and 3' ends of rearranged V genes are relatively conserved (Orlandi, *et al.*, 1989). Two sets of primers for the 3' end of the variable regions can be designed, one located at the J region and the other at the C region. The design of primers for the 5' end is less straightforward, and mixed primers have been based within the signal sequence (Larrick *et al.*, 1989) or at the N-terminal end of the first framework region. Priming in framework one (FR1) takes advantage of the conserved nature of these sequences, making it feasible to use relatively few degenerate primers to amplify most of the different variable regions (Orlandi *et al.*, 1989). The disadvantage of this method is that it may introduce amino acid substitutions into the FR1. Since the residues at the N-terminal of FR1 are normally very close to the antibody combining site (Padlan, 1993) such substitutions could potentially affect antibody-antigen interaction. Priming in the signal sequences is generally the more appropriate method since the leader sequence is removed from the mature antibody molecule, and variation in its sequence will not therefore affect antibody-antigen interaction.

1.3.2 **Expression of recombinant antibodies**

Both eukaryote and prokaryote expression systems have been employed successfully to express recombinant antibody genes: bacteria, yeast, baculovirus/insect cells, plant cells and mammalian cells. Each has its own property, advantages and potential applications.

1.3.2.1 **Bacterial expression systems**

As bacteria have a high growth rate, bacterial expression systems have become the most commonly used tool for producing recombinant antibodies at low cost. There are many factors influencing antibody expression levels in bacteria, including the structure of the expression vector, the primary structure of the gene and amino acids of the recombinant antibodies, the bacterial strains and growth conditions used.

In the construction of expression vectors, promoters from a range of different sources have been used to drive the expression of the recombinant antibody genes. Commonly used promoters are *lac* (Skerra and Pluckthun, 1988), P*ara*B (Better *et al.*, 1988), T7 (Condra *et al.*, 1990), *trp* (Huston *et al.*, 1988). All of these are inducible promoters, *lac* is a moderate promoter, but the rest are all strong promoters. Cytoplasmic expression of antibody fragments in bacteria results in the formation of insoluble and inactive protein aggregates, inclusion bodies (Cabilly *et at.*, 1984). Yields of intracellular-produced antibody fragments may be high, but only a small fraction of the yield can be reconstituted *in vitro* to produce active fragments (Sandhu, 1992). Secretion to the bacterial periplasm, however, results in the production of active antibody fragments. Targeting to the periplasm has been achieved by fusing the antibody genes to one of a range of different signal peptides, including those from OmpA, pelB, PhoA, Bla, OmpF, StII (Skerra, 1993), Sp*ssi* (Ueda *et al.*, 1993) and P43 (Wu *et al.*, 1993). All seem equally successful.

Several different species of bacteria have been used for the expression of antibody fragments, including both Gram-positive and Gram-negative types. Two commonly used Gram-positive bacteria are *Bacillus subtilis* (Wu *et al.*, 1993) and *Streptomyces lividans* (Ueda *et al.*, 1993). Yields are 5 mg/L being obtained from *B. subtilis* and 1 mg/L from *S. lividans*. The Gram-negative bacterium, *Escherichia coli* is the most commonly used organism for the expression of antibody fragments (Skerra 1993). Different *E. coli* strains have been found to exhibit

markedly different expression levels. A comparison of several commonly used strains, including TG1, BMH71-18 (Ward *et al.*, 1989), W3110wt, *E. coli* B (Boss *et al.*, 1984), Y1089, LE392 and HB101, demonstrated that MM294, *E. coli* B and W3110wt gave relatively high expression levels (Duenas *et al.*, 1994, Ayala *et al.*, 1995). However, the expression levels are strongly dependent on the nature of individual recombinant antibody fragments. Expression levels of different antibody fragment in the same expression system, can vary by more than 100 fold (Kelly *et al.*, 1992). The reasons for these differences are largely unknown although such factors as the presence of positive charges within 20-30 amino acids downstream of the signal peptide which can inhibit protein traslocation to the periplasm may sometimes be involved (Li *et at.*, 1988, Ayala *et al.*, 1995). There are several other strategies which have been shown to increase expression levels of functional antibody fragments in *E. coli.* Growth at low temperature and mild induction (Shibui and Nagahari, 1992), fusion with *E. coli* proteins such as the maltose- binding protein (Bregegere *et al.*, 1994) have been used to permit expression of functional Ig fragments in *E. coli.*

Detection and purification of antibody fragments is often facilitated by the addition of a tag. Tags which have been used for this purpose include the first antibody constant domain, immunodetectable peptides such as HSV tag or C-myc tag, and the *Strep tag* which displays intrinsic binding activity to streptavidin and thus enables detection and affinity purification (Schmidt and Skerra, 1993). Calmodulin, which is a highly acidic protein and binds to peptide and organic ligands with high affinity, has also been employed as a versatile antibody fragment tag (Neri *et al.*, 1995). All of the above tags are usually fused to the C-terminal of antibody fragments. In contrast the short FLAG tag (DYKD) offers an attractive N-terminal 'tail' which allows sensitive detection and affinity purification (Knappik and Pluckthum, 1994).

The bacterial expression of antibody fragments has become a familiar task in molecular biology. However, this system has not produced functional, intact antibodies successfully, and

furthermore its failure to glycosylate proteins makes it unsuitable whenever glycosylation is essential for protein function.

1.3.2.2 **Yeast expression systems**

Yeast expression systems offer a way to avoid the problems of inclusion bodies and poor *in vitro* assembly of Ig fragments in *E. coli*. The yeast *Saccharomyces cerevisiae* can recognise mammalian secretion signal sequences (Hitzeman *et al.*, 1983), However when immunoglobulin λ and μ cDNA specific for NP was expressed in yeast *S. cerevisiae* under the control of the yeast 3-phosphoglycerate kinase (PGK) promoter (Wood *et al.*, 1985), only about 25% of total immunoglobulin was secreted from the yeast. There is evidence to suggest that the yeast signal sequences are more effective at secreting mammalian proteins from yeast (Smith *et al.*, 1985). Horwitz *et al.* expressed the chimerical Ig L6 in *S. cerevisiae* by fusing the antibody fragment to the yeast invertase signal sequence and driving expression of the gene by the PGK promoter (Horwitz *et al.*, 1988). Culture supernatants contained about 100 mg/L of light chain and 50 to 80 mg/L of heavy chain. A good proportion (50 to 70%) of the heavy chain was associated with light chain to produce antibody fragments which were functionally indistinguishable from the Fab products of *E. coli* and the Fab prepared by papain digestion of the SP2/0-derived L6 chimerical antibody.

The methylotrophic yeast *Pichia pastoris* has been shown to posses excellent characteristics for the high-level expression of various heterologous proteins, either intracellularly localised or secreted into the culture supernatant. Recently, high expression levels of scFv (100 mg/L) were achieved in small scale shake flasks (Ridder *et al.*, 1995). *P. pastoris* combines the features of a eukaryotic secretion machinery with the fast growth and the requirement for non-complex growth media of bacteria (Cregg *et al.*, 1993). Furthermore, induction can be efficiently regulated by the supplementation of methanol as the carbon source. These features make *P. pastoris* an attractive host for antibody fragments expression. General advantages of

the yeast expression system are that expression is constitutive, and that the culture supernatants contain relatively few proteins other than antibody fragment, thus facilitating purification.

1.3.2.3 **Plant expression systems**

Heterologous gene expression is now well established and, plant agriculture offers the possibility of economical, large-scale production of many heterologous proteins (Whitelam *et al.*, 1993; Whitelam, 1995). To date, several antibodies and antibody fragments have been expressed and functionally assembled in both the cytoplasm and the extracellular space of plants. (Whitelam 1995; Ma, *et al.*, 1995). Routinely, the Ti plasmid of *Agrobacterium tumefaciens*, which mediates exogenous gene transfer into the plant cell nuclear genome, has been used to transform *Nicotiana tabacum* plants with antibody genes (Hiatt *et al.*, 1989; During *et al.*, 1990; Ma *et al.*, 1994), and antibody fragment genes (Owen *et al.*, 1992; Tavladoraki *et al.*, 1993). Antibody and antibody fragment expression levels in the plant system are controlled by several molecular and cellular factors (Whitelam *et al.*, 1993), including promoter activity, transcript processing and protein stability. The promoters used for heterologous gene expression in plant have been extensively reviewed by Whitelam *et al.* (1993), and the most commonly used has been the cauliflower mosaic virus (CaMV) 35S promoter or its derivatives (Whitelam *et al.*, 1993) which acts as a strong, constitutive promoter in most organs of transgenic plants (Jensen *et al.*, 1986).

Although the antibodies biosynthesised in plants have exhibited binding activities equivalent to the parental monoclonal antibody (Hiatt *et al.*, 1989; During *et al.*, 1990; Ma, 1994), and can be used in immunotherapy (Ma *et al.*, 1994; Ma *et al.*, 1995), plant antibodies may, as a result of glycosylation patterns, have different biological properties (Hiatt, 1990). Unusual glycosylation may influence biodistribution, serum half-life, and effector functions.

However, therapeutic applications are by no means the only potential application of antibody expression of plants. It has been shown that the expression of antibody fragments in plants against plant functional antigens such as phytochrome or plant hormones can be used to modulate the plant phenotype (Owen *et al.*, 1992; Artsaenko *et al.*, 1995). Furthermore, the expression of antibody fragments directed against plant viruses can delay symptom development upon infection (Tavladoraki *et al.*, 1993). Similar strategies are possible against a wide range of different plant pathogens including bacteria, fungi as well as viruses. It may also be possible to use plant-expressed antibody fragments which bind pollutants such as pesticides as the basis for an economical strategy for the cleanup of polluted soil or water.

1.3.2.4 **Other expression systems**

Several other eukaryotes expression systems have been developed. The baculovirus/insect cell system provide some advantages over conventional prokaryotic expression systems since it is capable of signal peptide cleavage, N-linked glycosylation, and correct cellular compartmentalisation and extracellular secretion. Mouse immunoglobulins genes (IgG1, κ; IgG2a, κ) have been expressed in the baculovirus expression system under control of the baculovirus polyhedrin protein, which causes abundant expression late in infection. The expressed antibody was correctly processed, glycosylated, and assembled into normal heterodimer H2L2 (Hasemann and Capra, 1990) at a yield of 5 mg/L. Using a different antibody, correct Fc domain function was demonstrated (zu Putlitz *et al.*, 1990). However, large-scale production is difficult in insect cultures because viral infection ultimately causes cell death.

The early attempts at antibody engineering used mammalian cell expression systems (Neubeger, 1983; Sharon *et al.*, 1984). The mammalian cell may indeed be the best system for expressing therapeutic antibodies. Lymphoid cells in particular have the ability to correctly process and posttranslationally modify immunoglobulins. Antibodies produced by lymphoid

cells can be a suitable isotype for the desired effector functions. However, the production levels are normally less than conventional murine myelomas and hybridomas. To overcome problems of low expression in lymphoid cell cells, nonlymphoid cell systems have been developed as a practical and valuable complement to other antibody and antibody fragment expression systems (Wright *et al.*, 1992; Dorain *et al.*, 1994). Several nonlymphoid cells, such as C6 glioma, PC12 pheochromocytoma, Chinese hamster ovary (CHO) cells and HeLa cells, have been tested for the ability to assemble and secrete immunoglobulins (Cattaneo and Neuberger, 1987). Expression levels were very low in HeLa and CHO cells. However the co-amplification of μ heavy chain and λ light chain genes in CHO cells with marker genes such as *dhfr* and *adenosine deaminase* resulted in high level expression of the humanised monoclonal antibody CAMPATH-1H (Wood *et al.*, 1990; Page, *et al.*, 1991). Furthermore, the CHO transfectomas are easily scaled up and can be adapted to growth under serum-free conditions. The CHO produced antibodies retain effector functions such as complement-dependent cytotoxicity (CDC), and antibody dependent cell-mediated cytotoxicity (ADCC).

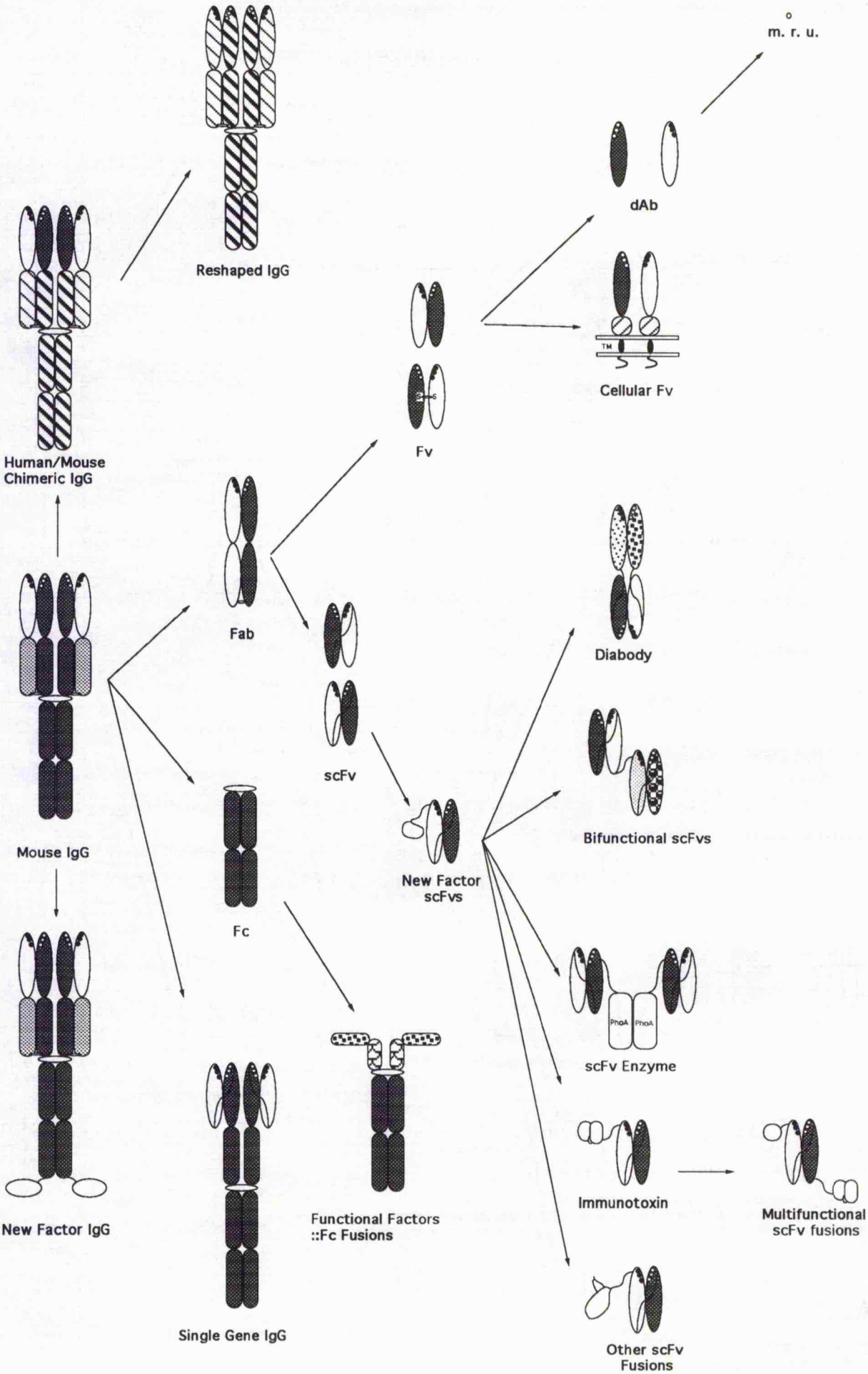
In addition to non-human mammalian cell systems, whole mammals, namely transgenic mice, have been used to produce human antibody light chains (Davies *et al.*, 1993), chimeric mouse/human anti-human interleukin-2 receptor antibodies (Cahill *et al.*, 1993) and human antibody repertoires (Neuberger, 1994).

1.3.3 **Engineered antibody and antibody derivatives**

Recombinant DNA technology can be used to engineer a wide range of intact antibodies and antibody fragments which possess novel structures and multiple functions. Fig. 1-2 illustrates a number of the many possible variants on this theme.

1.3.3.1 **Engineering intact antibodies**

Fig. 1-2. Schematic diagram of a range of engineered antibodies and fragments. Each oval represents a domain. **Human/mouse chimeric IgG**: mouse variable domain fused to human constant domains. **Reshaped human IgG**: human hypervariable loops replaced with those from a mouse antibody. **Single gene IgG**: light chain variable domain covalently linked to IgG heavy chain. **New factor IgG**: Non-antibody sequences, such as growth factors, cytokines, or other effector molecules, are joined to the constant domain of IgG. The fusion may be after C_H1, hinge or C_H2. **Functional factors::Fc fusions**: the variable region of the antibody heavy chain is replaced with a non-antibody sequence, to target Ig effector functions to specific cells. **Fv**: the variable domains of light and heavy chains associated non-covalently or convalently by engineered inter-domain disulfide bonds. **dAb**: single variable domain. **m.r.u.**: single CDR as minimal recognition unit. **Cellular Fv**: heavy chain and light chain variable domains fused to cell surface molecule (such as TcR or MHC molecule). TM stands for the transmenbrane domain. **scFv**: Single chain Fv fragments, in which V_H and V_L domains are linked by a peptide. **Diabody**: small bivalent and bispecific antibody fragment (Holliger *et al.*, 1993). **Bifunctional scFvs**: two scFvs linked by a short linker. **scFv enzyme**: scFv fused to a enzyme (here bacterial alkaline phosphatase as a reporter). **New factor scFvs**: scFv fused to a functional partner such as bacterial thioredoxin or maltose binding protein, to increase solubility and/or function of the scFv in a particular environment (e.g. bacterial cytoplasm). **Immunotoxin**: cellular toxin attached to scFv. **Multifunctional scFv fusions**: the N and C terminus of scFv are attached with different functional domains and demonstrated multifunction.


m. r. u.
dAb
Reshaped IgG
Cellular Fv
TM
Fv
Human/Mouse
Chimeric IgG
Fab
Diabody
scFv
Mouse IgG
Bifunctional scFvs
New Factor
scFvs
Fc
PhoA
PhoA
scFv Enzyme
Immunotoxin
Multifunctional
scFv fusions
New Factor IgG
Functional Factors
::Fc Fusions
Single Gene IgG
Other scFv
Fusions

Catalytic antibodies: For the chemist, the catalytic antibody is likely to be the most intriguing product of the immune system. The ability of antibodies to bind specifically to transition state haptens, can result in catalytic activity in reactions in which the antigen is an intermediate. (Tramontano *et al.*, 1986). To date, about 60 catalytic antibodies have been produced (Stewart and Benkovic, 1993; Lerner *et al.*, 1991). So far, catalytic antibodies do not compare well with natural enzymes. Although several genetic strategies have been used to improve the catalytic activity of catalytic antibodies (Wright *et al.*, 1992) the catalytic activities of antibodies are relatively modest. It seems likely that improvements will result from continued research in this area.

Chimaeric mouse/human antibodies: The human anti-mouse antibody (HAMA) response has limited the clinical applications of mouse monoclonal antibodies. Chimaeric antibodies comprising murine variable domains and human constant domains greatly reduce the HAMA response (Boulianne *et al.*, 1984; Morrison *et al.*, 1984).

Reshaped human antibodies: Even with chimaeric antibodies the mouse V regions may still elicit some immune response in humans thus reducing the effectiveness of the treatment. To counteract this, completely humanised antibodies have been constructed. The first of these, CAMPATH-H1 had been engineered by grafting six CDRs of a rat mAb to human antibody V framework regions (Riechmann *et al.*, 1988a). However the affinity of this humanised antibody was nearly 40 fold lower than the native rat mAb. Subsequent attempts to humanise murine antibodies in which some non-CDR residues were preserved have been more successful in terms of binding affinity and specificity (Gorman *et al.*, 1991; Tempest, *et al.*, 1991; Studnicka *et al.*, 1994). In an alternative strategy, termed variable domain resurfacing, a murine monoclonal antibody has been humanised without loss of antigen binding affinity by the substitution of murine surface amino acids with human equivalents (Roguska *et al.*, 1994).

1.3.3.2 **Engineering antibody fragments**

Recombinant DNA technology has made it possible to construct many types of immunoglobulin related molecules (Winter and Milstein, 1991), which are not produced in nature. Such molecules have the potential to revolutionise some aspects of biomedicine, biotechnology and basic biology. Engineered antibody fragments, can be as small as just a single CDR loops (minimal recognition units or m.r.u) (Winter and Milstein, 1991), or as large as single gene immunoglobulins (Shu *et al.*, 1993), or multi-immunoglobulin fusion proteins.

Due to their small size and relative stability, Fvs and scFvs have been commonly engineered for immunotargeting applications. IgA and IgG antibodies have been found to be refractory to simple proteolytic release of Fv (Huston *et al.*, 1991), which is the variable region fragment consisting of noncovalently associated V_H and V_L domains. In such situations recombinant DNA methods have facilitated the generation of refractory Fv domains. Analysis of Fv fragments shows that they have the same antigen-binding properties as the Fab fragment or whole antibody from which they were derived. Upon dilution however, Fv fragments have a tendency to dissociate into separate V_H and V_L domains (Riechmann *et al.*, 1988b). The presence of the corresponding antigen can stabilise the Fv (Ueda *et al.*, 1993). Several methods also exist for stabilising the Fv by covalently linking V_H and V_L. Firstly the two chains can be cross-linked chemically with glutaraldehyde. With McPC 603, this can be accomplished without any loss of binding affinity. Secondly an intermolecular disulphide bond can be created. It has been shown that the additional disulphide bridge forms spontaneously in the bacterial periplasm and therefore disulphide linked Fvs can be obtained in a fully functional form from *E. coli* (Briggs and Giorasch, 1986). Thirdly, the two domains can be connected by a peptide linker. The primary function of the linker in the scFv molecule is to maintain the close proximity between the two V domains (Tai *et al.*, 1990). The linker is not required to orientate the V domains into a specific structure in order to form the active binding site, this occurs spontaneously (Huston *et al.*, 1991).

The linker peptides have been designed to bridge the 35 to 40 angstroms gap (estimated from monomeric Fv models) between the carboxyl terminus of one variable domain and amino terminus of the other, (Bird *et al.*, 1988; Huston *et al.*, 1988). Either V_L or V_H may be the amino terminal domain, as V_L-linker-V_H or V_H-linker-V_L configurations exhibit identical affinity and specificity (Whitlow and Filpula, 1991). Indeed, a NMR study indicated that this kind of linker did not perturb the folding of linked domains (Freund *et al.*, 1993). Peptide sequences from known protein structures whose lengths and conformations are compatible with bridging the variable domains of an scFv without serious steric interference have been used as linkers (Bird *et al.*, 1988; Takkinen *et al.*, 1991; Mallender and Voss., 1994). In general the linkers consists primarily of stretches of Gly and Ser residues for flexibility, with charged residues such as Glu and Lys interspersed for solubility (Bird *et al.*, 1988; Huston *et al.*, 1988; Whitlow *et al.*, 1993). Evidence has recently been presented which indicates that longer linkers can increase an antibody fragment's affinity and decrease the formation of aggregates (Whitlow *et al.*, 1993). More stable linkers with helical structures have been constructed (Pantoliano *et al.*, 1991) suitable for nonphysiological or industrial applications.

Fab fragments are in widespread use as clinical and research reagents and, as with the other antibody fragments the ability to engineer them provides a means of increasing their potential. Fabs appear to be generally more stable than scFvs, (a factor which might be important for screening purposes), although the yield of functional antibody in bacteria is lower. Yields can sometimes be improved by modifying the secondary mRNA structure (Stemmer *et al.*, 1993) but it seems that the compatibility of the protein sequences with the protein folding and secretary pathway may limit secretion. This proposal is supported by the finding that, for example, a significant increase in the amount of a Fab antibody secreted from *E. coli* was obtained by exchanging the C_κ domain with a C_λ domain (MacKenzie *et al.*, 1994).

1.3.3.3 **Engineering antibody fragment fusions**

Recombinant fusion proteins are widely used for producing artificial multidomain-multifunctional proteins. These fusion proteins have been constructed by using the antigen-binding domain and constant domain (such as CD4, MHC or TCR IgG Fc domain fusion), and demonstrate great potential for use in clinical and basic biological research applications. In this small review, attention is focused on scFv fusion proteins although much of the discussion is also relevant to other recombinant antibody fusion proteins. With a mass equal to one-half that of monovalent Fab, the scFv is well suited to make fusion proteins with effector fusion partners to give bifunctional or multifunctional proteins. Several proteins have been used as fusion partners to immunoglobulin fragments in biotechnology research, to aid in product detection and purification and also to produce novel immunoreagents. The fusion partners used include the Fc binding domain of staphylococcal protein A (Tai *et al.*, 1990; Gandecha *et al.*, 1992), *E. coli.* alkaline phosphatase (Gandecha *et al.*, 1994), β-Lactamase (Kolmar *et al.*, 1992), *E. coli* maltose-binding protein (Bregegere *et al.*, 1994). Bacteriophage coat proteins and bacterial outer membrane proteins have also been used as fusion partners as part of strategies for the selection of genetic variants of immunoglobulin fragments (see chapter 1.4). The *in vivo* properties of scFv fusion proteins may sometimes differ markedly from those of conventional immunoconjugates, as indicated by observations that single-chain Fv immunotoxins exhibit greatly enhanced cytotoxity when compared to conventional cross-linked conjugates (Pastan *et al.*, 1992).

The design of scFv fusion protein constructs has varied according to the purpose for which the fusion protein was intended. Important considerations include the selection of the amino or carboxyl terminal as the site of fusion, a choice which will be influenced by the relationship between the scFv and the fusion partner in the context of the function of the fusion partner. In nature, the C terminus of the V region is attached the N terminus of the constant domain. A construct in which the C terminal of the scFv is attached to the N terminal of the new effector-fusion is most analogous to the original IgG structure. This configuration has been

successfully used to construct immunotoxins by fusing a 40-kDa core of *Pseudomonas* exotoxin (PE40) to the C terminus of several scFvs. Such immunotoxins have been shown to possess enhanced cytotoxity compared to chemically cross-linked IgG-PE40 conjugates (Chaudhary *et al.*, 1989, 1990; Batra *et al*, 1990 a, b; Kreitman *et al.*, 1990). An scFv alkaline phosphatase enzyme fusion, also in this configuration, permitted one step immunodetection and also provided an affinity handle for purification (Gandecha *et al.*, 1994). Since the N termini of antibody variable domains are close to the antigen binding region (Padlan, 1993) it is possible that attachment of a fusion partner to the N terminus might sterically interfere with antigen-binding. However, for bivalent or bispecific scFv fusions such as chelating recombinant antibodies (CRAbs) (Neri *et al.*, 1995), or complex multidomain fusion proteins, it may become necessary to attach fusion partner domains to the N termini of V domains. In such cases, a hinge region may be required between the attached effectors and the N termini of V domains. This hinge region may be topologically and structurally different from the linker used for bridging the V_H-V_L or V_L-V_H domains (Huston *et al.*, 1991). Indeed, immunotoxins with variant hinge regions have shown different folding kinetics, differences in their propensity to aggregate and different yields of correctly folded functional immunotoxin molecules. Immunotoxins incorporating one of the hinge regions investigated exhibited both low aggregation and increases in the rate of formation and the yield of active immunotoxin molecules. A fusion of the native hinge region of the C terminus of the staphylococcal protein A domain B (FB) to an anti-digoxin scFv showed virtually identical digoxin-binding activity to the parental IgG 26-10 (Tai *et al.*, 1990). Furthermore, it has been shown that the two effectors and the antigen-binding site of an α(TGRα)-anti-Tac scFv-PE40 triple fusion protein were all functional. However, the cytotoxicity of this triple fusion protein was about three fold lower than that of the bifuctional scFv-PE40 (Batra *et al.*, 1990 a,b). The activity of scFvs in fusion proteins may be antibody dependent. For example the fusion of two different scFvs, namely an anti-Tac or an anti-TFR, to the C terminus of diphtheria toxin, has different effects

on scFv affinity. The affinity of the anti-Tac scFv is unaltered whereas the affinity of the anti TFR scFv is changed. The cytotoxicity of diphtheria toxin::anti-Tac scFv fusion protein was comparable to that of anti-Tac scFv::PE40 fusion protein (Chaudhary *et al.*, 1990), whereas the diphtheria toxin::anti-TFR scFv fusion protein was less potent than the anti-TFR scFv::PE40 fusion protein against some target cell lines, but more potent against others (Batra, *et al.*, 1991). These investigations have demonstrated the feasibility of complex fusion protein constructs and provide the basis for the design of multifunctional or multispecificity scFv -based immunoreagents.

1.4 **Bacteriophage display technology**

The explosive growth of research on recombinant antibodies is being further boosted by developments in phage display technology. This system was first developed for the display antigenic determinants (peptides) (Smith 1985; Parmley and Smith, 1988) and has been extended to the display of antibody variable domains (McCafferty *et al.*, 1990) and antibody libraries (Clackson *et al.*, 1991). The essential characteristics of the whole immune system may be considered to be mimicked in the bacteriophage display system. Analogous to the B-cell, each phage displays a single antibody species as a fusion of antibody fragment and phage coat protein. Phages carrying antibody fragments with desired binding characteristics (and the genes encoding them) are selected by binding of phage to antigen by virtue of the displayed antibody; and finally soluble antibody fragments are secreted from infected bacteria. The development of phage antibody technology has been recently reviewed (Winter *et al.*, 1994). Here, I attempt to summarise briefly the background and potential of this technology.

1.4.1 **Filamentous bacteriophage biology**

The phages used for the phage display system are currently Ff class of filamentous bacteriophage (*Inovirus andreios*), including f1, M13, fd and their relatives. These phages are

small single stranded DNA viruses which infect male (F^+) strains of *Escherichia coli*. Gene organisation, size sequence and all structure parameters for the viruses in the group are virtually identical.

One of these viruses , M13, has been crystallised and the structure of this virus been solved at 7Å (Glucksman *et al.*, 1992). M13 is about 65Å in diameter with the length dependent on the length of the enclosed genome (Makowski, 1993). The virions of M13, f1 and fd are assembled with the five structural proteins and viral single stranded DNA (ssDNA). The wild type M13 contains 2800 copies of pVIII (product of phage gene *VIII*) making a cylindrical shell about the viral genome. There are five copies each of pVII and pIX at distal end and five copies each of pVI and pIII at proximal end (Makowski, 1993). The mature pIII serves as a host-binding protein, and is a 406-aa polypeptide attached to the virion through interaction with pVI. The structure of pIII is flexible and is an excellent vehicle for the display of foreign peptides. The function of pIII is retained when it is fused to the C-terminus of foreign peptides (Parmley and Smith 1988).

Phage infection is initiated by the binding of gIII proteins to the tip of the F-pilus of *E. coli*, followed by internalisation of the single-stranded viral DNA. This so-called *plus* strand serves as a template for minus-strand synthesis, which starts at a specific origin and results in a double-stranded replicative form (RF). The RF is the template for mRNA transcription, RF replication, and production of progeny ssDNA. The Ff genome encodes ten proteins, only five of them making up final viral particle. Progeny virions are assembled, not in the cytoplasm, but rather by extrusion of ssDNA through the bacterial envelope without killing the cell or preventing cell division. As it emerges from the cell, the ssDNA acquires its extracellular sheath of coat proteins from the membrane. Both pIII and pVIII are synthesised with posttranslationlly cleaved signal peptides, and before incorporation into the virion are anchored in the inner membrane with the N-terminal portion (the bulk of the protein in the

case of pIII) exposed in the periplasm (Smith, 1988; Webster and Lopez, 1985). The pIII appears to have two functional domains, each roughly 200 residues long: an exposed N-terminal domain that binds the F pilus but is not required for virion assembly, and a C-terminal domain that is buried in the particle and is an integral part of the capsid structure (Makowski, 1992). In the virion the C-terminal portion of gVIII protein appears to be inside the virion, close to the DNA, while the N terminus is exposed to the solvent. Once the bacteria has been infected by the phage and is producing pIII, the cell is resistant to f1 phage (Boeke *et al.*, 1982). This property is essential for the generation peptide or antibody libraries, since it ensures that an individual phage will only contain the gene encoding the displayed ligand .

An understanding of bacteriophage biology is very important for the development of the phage display system, especially in connection with the design of improved vectors. Smith (1988) and Webster and Lopez (1985) provide detailed reviews of the biology of filamentous phage.

1.4.2 **Designing phage display vectors**

In order to display peptide or antibody fragments on the surface of filamentous bacteriophage, the surface-exposed domain of the coat protein has to be chosen as the fusion site. Consequently, the N-domain of coat protein III and the N-terminus of coat protein VIII have been used as the insertion or replacing sites (Smith, 1985; Ilychev *et al.*, 1989). In an alternative strategy, a surface exposed domain of the C-terminus of coat protein VI has recently been used for display of a cDNA library derived from *Ancylostoma caninum* (Jespers, *et al.*, 1995).

There are several different vectors for the phage display of foreign peptides. In one of these the fusion gene fragments are encoded entirely within the same phage replicon. This kind of vector has been utilised to display peptides and antibody fragments (Smith, 1985; McCafferty, *et al.*, 1990). Another is the phagemid/helper phage system which has been widely used to

display foreign gene products. The phagemid vectors in this system contain the filamentous phage "intergenic region", which serves as a single strand DNA packaging signal (Breitling *et al.*, 1991; Hoogenboom *et al.*, 1991; Barbas *et al.*, 1991). Phagemids can be packaged into phage particles by superinfection of helper phage such as M13K07 or its mutant VCSM13. The helper phage provides all phage proteins, including pIII, but its genome is poorly packaged in competition with the phagemids, because it contains a defective origin of assembly. On the other hand, phagemid DNA can be incorporated into a λ bacteriophage vector using the filamentous phage replication machinery to circularise its DNA (Hogrefe *et al.*, 1993). The advantage of this lambda vector system is the high efficiency of transformation, reported to be about four times higher than that of the conventional electroporation methods.

In phagemid vectors, the gene of interest/ phage coat protein gene fusion is under control of an engineered promoter/operator. Three kind of promoter have been used to regulate phage coat protein gene/fusion proteins. These are the *lacZ* promoter (Hoogenboom *et al.*, 1991; Barbas *et al.*, 1991), the *phoA* promoter (Garrard *et al.*, 1991) and the coliphage T7 promoter (Breitling *et al.*, 1991). All of these promoters are controlled by the *lac* operator, which is leaky and some authors consider that this will increase the selection pressure for production of deletion mutants. To avoid this potential problem a double *lac* operator has been used to tightly control expression, (Breitling *et al.*, 1991), or the gene encoding the *lac* repressor has been introduced into the phagemids (Orum *et al.*, 1993).

The fusion protein must be directed to periplasm in such a way that the C-terminus of the coat protein is anchored in the inner membrane (when pIII and pVIII are used as vehicle). In the phage vector, the original coat protein signal peptides are used, and in phagemid vectors, *pelB* (Hoogenboom *et al.*, 1991; Barbas *et al.*, 1991) and StII (Garrard *et al.*, 1991) signal peptides have been chosen. In pVI phage display vectors (Jespers *et al.*, 1995), a signal peptide is not

required because pVI is a bacterial inner membrane-associated protein involved in termination of phage assembly (Russel, 1991).

The copy number of fusion protein displayed on a single phage particle is dependent on the coat protein chosen, on vector construction, and on the helper phage and phage host used. Gene III and gene VI coat protein can be used for monovalent to pentavalent display of foreign proteins, whereas the gene VIII coat protein is used solely for multivalent display.

PIII folds as a three-domain protein, and there is a spacer between each domain. An early peptide phage display study demonstrated that a site within the spacer (Smith, 1985), close to the N-terminus (Parmley and Smith, 1988) or at the N-terminus itself (Cwirla *et al.*, 1990) could be used as a fusion site. However for the display of antibody fragments or other proteins on the surface of phage, the two N-terminal domain truncated version of pIII protein was used. Antibody fragments have been fused directly to the C-terminal domain, such as 'pDH188' (Bass *et al.*, 1990; Garrard, *et al.*, 1991) or, as in 'pComb3', through a short flexible linker (GGGGS), which was used to minimise the interaction between antibody fragments and the pIII protein domain (Barbas *et al.*, 1991). Since phages are not infective when assembled solely with the C-terminal domain of the pIII, wild type pIII must be provided by helper phages. The 'pHEN1' vector on the other hand makes antibody fragments fused to the N-terminus of pIII (Hoogenboom *et al.*, 1991), and the phage is infective. The advantage of this kind of vector is that a special phage (M13ΔgIII) can be used as the helper phage. M13ΔgIII helper phage lacks pIII, and is designed to rescue phage particles that incorporate only the pIII fusion from the phagemids (Griffiths *et al.*, 1993). When using helper phages which contain a gIII gene (such as M13K07 or VCSM13) the number of copies of pIII fusion protein assembled on each phage particle is expected to range from zero to five (Winter *et al.*, 1994). In this system, the average displayed foreign protein is further reduced by competition with wild type pIII from helper phage. Further reduction of the expression of the gene III scFv

fusion may be accomplished by the use of *E. coli* strains containing the *lacI*^{q} gene which over expresses *lac* repressor and reduces leakiness of the *lac* operator. When this system is used helper phage superinfection results in the production of fusion phages which have been estimated to display on average less than one fusion protein per particle. These have been termed 'monovalent' phage (Barbas *et al.*, 1991; Garrard *et al.*, 1991; Lowman *et al.*, 1991).

Recently pVI has been employed as the vehicle for the display of foreign gene products. Utilisation of the fusion site at C-terminus of the pVI can be used to display the products of cDNA libraries on the surface of filamentous bacteriophage. This approach facilitates the isolation of biologically important ligands from such libraries (Jespers *et al.*, 1995). The method offers some advantages over undirected display of cDNA libraries through the JungIII fusion/Fos leucine zippers heterodimer approach (Crameri *et al.*, 1994), since the cDNA library product can be directly displayed on phage surface. The pVI fusion system is also suitable for the display of proteins whose C-terminal ends participate in protein conformation and/or biological activity. However, analysis of pIII and pVI fusions with a model protein showed that the pVI fusion had a relatively lower binding efficiency (Jespers *et al.*, 1995).

The major coat protein of filamentous phage, pVIII, can also be used to display peptides (Ilychev *et al.*, 1992; Greenwood *et al.*, 1991; Felici *et al.*, 1991) and antibody fragments (Kang *et al.*, 1991; Huse *et al.*, 1992; Chang *et al.*, 1991). It is surprising that from 900 (Ilychev *et al.*, 1992) to 2700 copies of peptides (up to octapeptides) fused to the N-terminus of pVIII can be incorporated into the phage coat without preventing phage assembly and infection (Iannolo *et al.*, 1995). It was demonstrated that one to twenty-four fd fragments (heavy chain variable domain and first constant domain) fused to pVIII were incorporated along the length of phage particles and that the Fd could associate with coexpressed light chains to produce Fab fragments which were displayed on the phage surface (Kang *et al.*, 1991). Since the pVIII fusion system produces multivalent phage particles it may have

potential for the isolation of low affinity antibodies but in general it less useful than the pIII display system for antibody library screening (Winter *et al.*, 1994).

Any system designed to produce recombinant antibodies from phage display libraries must take account of the importance of downstream characterisation of selected antibody fragments. To facilitate detection and purification of antibody fragments of interest, an antigenic tag has been fused to the antibody fragment C-terminus (Hoogenboom *et al.*, 1991). Production of soluble antibody fragments for characterisation purposes is achieved by three methods: one involves the incorporation of the amber mutation in the phagemid vector and its expression in non-suppressor strains of *E. coli* (Hoogenboom *et al.*, 1991; Huse 1991); a second involves the introduction of a trypsin cleavage site between the antibody fragment and pIII (Breitling *et al.*, 1991; Orum *et al.*, 1993); and finally by designing two restriction enzyme sites at 5' and 3' of gIII fragment which may be utilised to excise the pIII gene when soluble antibody fragments are required (Barbas *et al.*, 1991).

To minimise loss of individual antibody fragments during antibody repertoire cloning into phage display vectors it is necessary to utilise restriction enzymes which occur with low frequency in antibody genes. To this end Chaudhary *et al.*, (1990) investigated the occurrence of several restriction sites in existing antibody gene fragments. Hoogenboom *et al.*, (1991) selected the restriction sites *Sfi* I, *Not* I and *Sal* I for introduction into their multicloning site on the basis of their low frequency in antibody genes.

For the display of Fab fragments, or Fv fragments, phagemids containing two signal sequences and two muticloning sites have been constructed. One cloning site is used to create a fusion gene between phage coat protein and either an fd or a VH fragment. The second multicloning site is used for the light chain. Expression of such genes in *E. coli* results in assembly of Fab or Fv fragments in the bacterial periplasm (Barbas *et al*, 1991).

1.4.3 **Creating phage libraries**

The immune system can produce about 10^8 antibodies with different binding properties at any one time and the creation of libraries as part of a strategy to display these repertoires on the phage surface is a challenging task. To this end native immune repertoires have been copied from unimmunised humans (Marks *et al.*, 1991a), and animals (Gram *et al.*, 1992). Semisynthetic libraries in which variation has been increased by mutation have also been produced and expressed on filamentous phage (Barbas *et al.*, 1992a; Hoogenboom and Winter, 1992).

Native immunoglobulin phage libraries: The native immunoglobulin repertoire is the repertoire present in humans or animals. In principle, this is an IgM rich repertoire. For optimising the efficiency of the library generated from unimmunised human peripheral blood lymphocytes, a set of family-specific PCR primers has been designed (Marks *et al.*, 1991b) to amplify each of human V_H, V_κ and V_λ families. An IgM-specific primer has been used for amplifying first strand cDNA.. This was combined with a light chain cDNA library amplified with V_κ and V_λ constant primers, to create the human native repertoire (Marks *et al.*, 1991a). It is improbable that a native repertoire will contain high affinity antibodies against a particular antigen. This will reduce the likelihood of selection of antibodies with the required specificity by the procedures described in section 1.4.4 below. One resolution of this potential problem is to enhance the avidity of binding by using M13ΔgIII helper phage to generate multivalent phage antibodies (Griffiths *et al.*, 1993). Because the repertoires of V_H and V_L genes are combined at random and original combinations are changed, new specificities, additional to those present *in vivo,* should be generated from this kind of library, From such libraries, several high specificity antibody fragments have been isolated, including those against bovine serum albumin (BSA), turkey egg lysozyme, the hapten phOx (Marks *et al.*, 1991a), human self antigens (Griffiths *et al.*, 1993) and human blood group antigens (Marks *et al.*, 1993). The

affinities of these antibody fragments were in the range 10^5 M^{-1}-10^7 M^{-1}, typical of a primary immune response.

Antibody repertoires have also been generated from genes prepared from unimmunised rodent spleen cells. First strand cDNA was synthesised by random priming from poly(A)$^+$ RNA. The final repertoire was created on an intact pIII fusion vector. It was demonstrated that the specificity of a phage antibody selected from this library was comparable with a mouse monoclonal antibody (Meulemans *et al.*, 1994). It is possible that the library derived from peripheral blood cells or spleen cells could have undergone somatic mutation (Marks *et al.*, 1991a). A potentially 'more native' immunoglobulin library was built by amplifying V genes from bone marrow which was depleted for surface IgG-positive cells from unimmunised rodent. CDNA libraries for μ and κ chains were synthesised by using μ chain and κ chain specific primers. For multivalent display of Fab fragments, a pVIII display vector was used to construct this library. The library was screened only for binding to the hapten progesterone, the binding affinities of Fab fragments selected were low (about 10^4-10^5 M^{-1} measured by competition ELISA), and the fragments cross-reacted with another protein (Gram *et al.*, 1992).

Semisynthetic repertoires: An ideal way of obtaining a totally naive combinatorial library is to chemically synthesise the six CDRs in random ways and present these synthetic CDRs on a varied framework region. Since the heavy chain CDR-H3 generally contributes most in terms of antigen-binding contacts and diversity (Kabat and Wu, 1991), a semisynthetic combinatorial antibody library was generated based on fifteen randomised residues of heavy chain CDR-H3. This library was based on the variable domains of a anti-human tetanus toxoid antibody 7E, and the size of the library was 5 x 10^7. Antibody fragments against the hapten antigen, fluorescence, with affinities in 10^7 M^{-1} range, were selected from this library (Barbas *et al.*, 1992a). The diversity of the library was enlarged by adding three of CDR-H3 loops of lengths

five, ten and sixteen residues and antibody fragments were selected for binding to three hapten conjugates with good specificity and affinity (between 80 to 29 nM) (Barbas *et al.*, 1993). By randomisation of CDR-L1, L3 and CDR-H2, H3 loops of the antibody 4D5 Ab V region, two libraries (about 3 x 10^8 clones) were created, and a single binding specificity was selected against insulin-like growth factor. However screening for binders against two other protein antigens was not successful (Garrard and Henner, 1993).

The V-gene segments are the most a highly appropriate base for building semisynthetic repertoires. A repertoire (2 x 10^7 clones) was first constructed using a short CDR-H3 loop of five or eight random residues with each of 49 segments, and combined with a fixed light chain. Antibodies of high specificity were isolated against two haptens, phOx and NIP (with affinity of up to 10^6 M^{-1}) and human TNF-α, but not against other three protein antigens (Hoogenboom and Winter, 1992). However, by adding a range of CDR-H3 loops of different lengths, from four to twelve residues, a single library was created from which a range of more than 20 binding specificities could be selected (Nissim *et al.*, 1994). The affinities of antibodies selected from this library were moderate (about μM). To optimise the likelihood of obtaining high affinity antibodies, a highly diverse synthetic library is required. A large repertoire (6.5 x 10^{10}) was created using the *lox-cre* site-specific *in vivo* recombination system of bacteriophage P1 (Waterhouse *et al.*, 1993). This library was constructed by using 49 cloned V_H segments (Tomlinson *et al.*, 1992) with CDR-H3 loops of four to twelve residues of random sequences; 26 cloned V_κ segments (Cox *et al.*, 1994) with CDR-L3 loops of eight to ten residues that included one to three residues of random sequences; and 21 cloned V_λ segments (Williams and Winter, 1993), with CDR-L3 loops of eight to thirteen residues that included zero to five residues of random sequences. From this repertoire, Fab fragments were isolated against a range of different antigens and haptens with affinities up to 4 nM.

1.4.4 **Selection of antibodies from phage libraries**

Bacteriophages displaying antibodies (phage antibodies) can be selected by binding to antigen coated solid phase (Barbas *et al.*, 1991; Marks *et al.*, 1991), column matrices (McCafferty *et al.*, 1990), cells (Marks *et al.*, 1993) or biotinylated antigen in solution followed by capture (Hawkins *et al.*, 1992). Non-specific phage binders can be washed away, and specific binders eluted by competition with soluble antigen (Clackson *et al.*, 1991), antibody (Meulemans *et al.*, 1994), alkali (Marks *et al.*, 1991) or acid (Barbas *et al.*, 1991). Enrichment can be achieved by subsequent infection of bacteria with the eluted phage. The resulting amplified population can be used in a further round of selection. Many factors affect the efficiency of selection, including target density, valence of phage-displayed ligand and kinetics of dissociation during washing.

Target density The first round of selection is a key step in the successful isolation of binders with the required characteristics. A large amount of target molecule is usually used in the first round of selection, because the panning procedure used for selection gives only a 1% yield even with strongly binding phage (Scott and Smith, 1990). A high density of antigen should also stabilise binding through multivalent interaction, favouring rebinding of phage that has dissociated. However, a low coating density of target molecule has also been successfully utilised to select an antibody with good binding affinity (Marks *et al.*, 1992). In addition, the effects of coating density may also be influenced by the number of copies of fusion protein assembled on a single phage. It is probable therefore that higher coating densities may be required for the selection of monovalent phage than for multivalent phage (Smith and Scott, 1993).

Multivalent phages versus monovalent phages The binding characteristics of multivalent phage antibodies are influenced by the number of antibody combining sites displayed per phage - the greater the valence, the stronger the binding activity. Monovalent phages on the other hand have similar binding characteristics to the related Fabs or scFvs and are therefore

particularly useful in the selection of antibody fragment clones on the basis of affinity and specificity (Barbas *et al.*, 1991; Lowman *et al.*, 1991). Studies of peptide libraries displayed on multivalent phage have shown that multivalence prevents the separation of moderate affinity peptides (micromolar K_d) from those with high-affinity (nanomolar K_d) after three rounds of panning to a fixed concentration of the ligand (Cwirla *et al.*, 1990). In principle, multiple interactions increase the avidity of phage binding, and slight differences in affinity between antibodies displayed on fusion phages should be amplified by multivalent display - 'avidity boost' (Smith and Scott, 1993). Furthermore, phages with very high affinity and strong avidity will be difficult to elute. Thus strong elution methods will be necessary for high affinity binders. Fortunately the bacteriophage is resistant to extreme pH change, 6M urea for about 10 min. (4M for >20 min.) sulphydryl reagents (e.g. 50 mM dithiothreitol) and trypsin (Smith and Scott, 1993; Lowman *et al.*, 1991; Roberts *et al.*, 1992).

By taking advantage of the extremely high affinity of the non-covalent interaction of biotin and streptavidin, the selection procedure for multivalent phage display system can be further modified. High affinity fusion phage, bound in solution by incubation with excess soluble biotinylated antigen, (but with antigen at lower concentration than the target affinity constant) can be captured by subsequent exposure to streptavidin-coated paramagnetic beads. Using this technique, in which the fusion phages are selected according to their binding affinities, mutant phage antibodies have been selected from a great excess of phages with two- to four-fold lower affinities over many rounds of selection (Hawkins *et al.*, 1992; Hawkins *et al.*, 1993). An alternative procedure for selecting high affinity fusion peptide phages from multivalent phage display could be to reduce the concentration of the target molecules from 1μM for the first round to 0.1nM for following rounds (Scott and Smith, 1990).

Dissociation kinetics High affinity antibodies with slow dissociation kinetics can be selected by the use of long washes with monovalent phage display (Bass *et al.*, 1990). Exploitation of

dissociation kinetics can enhance discrimination between multivalent fusion phages with slightly different affinities. Differences between the dissociation from antigen of two phages with slightly different kinetics should increase with time as a result of the exponential native of the dissociation curve. Such an effect was demonstrated by dissociation of phage antibodies from biotinylated antigen in solution (Hawkins *et al.*, 1992). Using such kinetic selection, even mutant antibodies with a two-fold higher affinity could be selected from a great excess of phages with lower affinity (Hawkins *et al.*, 1993). Washing of phages bound to a solid phase should also allow discrimination on the basis of dissociation kinetics.

1.4.5 **Applications of phage display technology**

The potential applications of phage display and selection technology are immense. For peptide phage display alone, applications include selection of substrate for subtilisin BPN', stromelysin and matrilysin (Matthews and Wells, 1993; Smith, *et al.*, 1995), proteinase inhibitors (Tanaka and Auerswald, 1995), virus assembly inhibitors (Dyson and Murray, 1995), epitope mapping (Lane and Stephen, 1993), whole cell scanning (Fong *et al.*, 1994), DNA binding (Cheng *et al.*, 1995) and new drug discovery (Winter, 1994). No doubt there are other new applications are under development. In a quite distinct group of applications phage display is also playing a very significant role in the development of antibody engineering.

By-passing hybridisation: Making monoclonal antibody fragments without hybridisation was first achieved by building a combined Fab library in the λ phage display vector. This library was derived from total unfractionated cells of an immunised mouse. An anti-hapten Fab fragment with good affinity was isolated (Huse *et al.*, 1989). Because of the limited screening power of this method the original high affinity Fab pairing is not readily selected from the library (Winter and Milstein, 1991). Filamentous phage display provides for much more powerful selection methods. Filamentous phage combinatorial antibody libraries generated from human samples (immunised individuals or patients) have been used to isolate high-affinity

human monoclonal antibody fragments to hepatitis B surface antigen (HBsAg) (Zebedee *et al.*, 1992), and respiratory syncytial virus F glycoprotein (RSV) (Barbas *et al.*, 1992b). The soluble Fab or phage Fab against HBsAg was used to study antigen immunogenicity in humans. This kind of human Fab also has potential for use as a passive immunisation agent for prevention and therapy of viral hepatitis B. The Fab fragment against RSV has been shown to neutralise virus infectivity. The relatively rare autoantibodies against human IgE have also been isolated from a combinatorial library (Vogel *et al.*, 1994). By immunising rabbits with recombinant human leukaemia inhibitory factor (rhLIF), a rabbit monoclonal antibody against rhLIF with high affinity (2.8×10^{-8} M) was isolated from a combinatorial phage display library, which had been created by amplifying rabbit V_H and V_L gene with a few primers (Ridder *et al.*, 1995). Thus, the combination of the use of immunised animals as the source of high affinity antibodies, with the high efficiency selection by filamentous bacteriophage display technology allows the production of monoclonal antibody fragment with high affinity without hybridisation.

By-passing immunisation: V gene repertoires from unimmunised donors have been used to build human phage antibody libraries. The ability to utilise these libraries to generate monoclonal antibodies without immunisation is one of the most significant contributions of phage display technology. To illustrate the utility of such libraries, rare scFv-phages with good affinity and specificity have been selected against turkey egg-lysozyme (TEL), bovine serum albumin (BSA), or the hapten of 2-phenyloxazol-5-one (phOx). ($K_a = 10^7$ M^{-1} for TEL, 2×10^6 M^{-1} for phOx) (Marks *et al.*, 1991). A Fab against human tumour necrosis factor (TNF) with an affinity of 10^7 M^{-1} has also been selected (Griffiths *et al.*, 1993).

Mouse repertoires have also been used to produce phage antibodies against a cytoskeleton antigen (Meulemans *et al.*, 1994), and the hapten progesterone (Gram *et al.*, 1992). As yet

there have been no reports of the isolation of high affinity antibody fragment from native human or animal repertoires.

Semisynthetic library libraries created by randomisation of HCDR3 alone or combined with other randomised CDRs have yielded antibodies to a very wide range of antigens (Hoogenboom and Winter, 1992; Barbas *et al.*, 1991; Garrard and Henner, 1993, Barbas *et al.*, 1993; Nissim *et al.*, 1994), some of them with high affinity (Griffiths *et al.*, 1994).

Affinity maturation: As discussed above the utility of phage antibody selection is not restricted to simply distinguish binders from non-binders. Significantly, the selection of phage scFv mutants that differ in affinity is also possible. This feature allows the use of phage display to mimic affinity maturation in the immune system (Winter *et al.*, 1994). Mutations can be introduced at random *in vitro* into a previously selected low affinity binder by error prone PCR (Leung *et al.*, 1989). Following several subsequent rounds of affinity selection, mutant antibody fragment phages were isolated which displayed improvements in affinity ranging from just two fold (Hawkins *et al.*, 1992) to 13 to 30 fold (Gram *et al.*, 1992). PCR-based site-directed mutagenesis methods have also been employed to introduce random mutations in CDRs. Combined phage display and selection resulted in the isolation of phage antibody fragments with 14-fold improved binding for the hapten phOx 11 (Riechmann and Well, 1993). Another method to improve affinity is based on a combination of splice overlap extension PCR (Ho *et al.*, 1989) and phage selection is termed CDR walking (Barbas *et al.*, 1994). Using this method, an antibody fragment was selected with 56-fold improved affinity compared with the wild type antibody. Affinity maturation has also been achieved by using the synthetic ligase chain reaction combined with phage selection, This method results in the randomisation of all six CDRs and antibody fragments with improved carbohydrate-binding activity have been selected from a library created in this way (Deng *et al.*, 1993; Deng *et al.*, 1995).

Antibody engineering allows the construction of artificial molecules with existing and potential applications in humans, animals and plants. This technology has made the exploitation of human therapeutic antibodies much more realistic, and antibodies or antibody fragments are already being introduced to plants as modulation and protection molecules. The understanding of antibody genetics and antibody structure, especially the structure of antibody combining sites by means of x-ray crystallography, NMR and molecular modelling, is essential for future development of antibody engineering. The ease with which antibodies with desired binding characteristics can be selected through the use of phage display technology provides a boost to the development of such applications. Thus, phage antibody engineering is an exciting field which is interesting in its own right and which is likely to make contributions in many areas of medicine, biology and biotechnology.

1.5 Aims of this thesis

This thesis involves studies on several aspects of antibody engineering, with a particular focus on filamentous bacteriophage antibody display. Phage-display has found applications in many aspects of antibody engineering and has contributed very significantly to the recent remarkable advances in this area.

To investigate further applications of this technology, fourteen α-helices (SpA) were displayed alone, or as fusions with β-sheet (scFv) and the structure and function studied. Some interesting discoveries were made with these artificial phage-displayed hybrid molecules, and these are presented in chapter 3. Further potential applications of phage antibodies (PhAbs) such as surface epitope localisation by means of fluorescent PhAbs or direct visualisation in the electron-microscope were investigated and the results of these studies constitutes chapter 6.

Antibody engineering which has relied heavily upon knowledge of immunoglobulin genetics and structures also provides new methods for studies in these areas. The basic feature of antibodies is their epitope-binding activity, which is related to the structure of the Fv domains. To obtain structure-function information on antibody fragments, a study was designed which employed the techniques of phage display, PCR based site-directed mutagenesis and antibody combining sites modelling (chapter 4). An understanding of the structures of antibody combining sites is a very important foundation for the generation of semisynthetic antibody repertoires from which antibodies with new specificities have been selected. (chapter 5).

Based on information on the structures of V genes, antibody combining sites and the technology of phage display, a "single pot" antibody repertoire was created by Nissim *et al.* (1994). Subsequently, many epitope binding fragments have been isolated from this phage antibody library, and some further applications on this kind of artificial antibody repertoire have been demonstrated in this thesis (chapter 6, 7).

The work presented in this thesis is clearly consistent with the view that phage technology will continue to revolutionise antibody engineering and will provide a general method for the selection of antibody fragments with desired properties.

CHAPTER 2

Material and Methods

2.1 Materials

2.1.1 Reagents

All chemicals were obtained from Sigma, BDH or Pharmacia unless otherwise stated. Oligonucleotides used as primers in PCR reactions were obtained from the DNA synthesising facility in the Protein and Nucleic Acid Chemistry Laboratory of the Biochemistry Department, Leicester University. Deionised distilled water (QH_2O) was prepared using a Milli-Q reagent water system (Millipore). Nucleotides were obtained from Pharmacia. Labelled nucleotides were supplied by Amersham: [^{35}S] α-dATP at a concentration of 370 MBq/ml and with a specific activity greater than 22 TBq/mmol; and [^{32}P] γ-dATP at a concentration of 370 MBq/ml and a specific activity of 110 TBq/mol.

2.1.3 Enzymes

DNA modifying enzymes were purchased from GIBCO Bethesda Research Laboratories (GIBCO BRL), Stratagene or New England BioLabs (NEB). Taq polymerase was supplied by Promega.

2.1.4 *Escherichia coli* culture media

1. *Liquid media*

Recipes for 1 litre of media:

LB: 10g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl

DYT: 16 g bacto-tryptone, 10 g bacto-yeast extract, 5 g NaCl.

SOB: 20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl.

SB: 30 g bacto-tryptone, 20 g bacto-yeast extract, 10 g Mops

After pH adjustment to 7.0-7.2 media were sterilised by autoclaving at 121°C, 15 lb in^{-1}, for 20 min.

SOC: To autoclaved SOB was added, 5 ml 20% glucose, 1 ml 1 M $MgCl_2$ and 1 ml 1 $MgSO_4$, which were all sterilised by autoclaving as above.

2. *Solid media*

To prepare solid media required amount of bacto-agar was added before autoclaving. For agar plates 15 g agar per litre was added. For top agar 6 g agar per litre was added.

2.1.5 Phagemids

pComb 3 (Barbas *et al.*, 1991)

This vector is a pBluescript based vector, which was designed for the cloning of combinatorial Fab libraries. The detailed structure of the vector is given in Fig. 2-1. This phagemid vector was constructed to fuse the antibody Fd chain (VH-CH1 domains) with the C-terminal domain of pIII (C-pIII). A flexible 5-amino-acid tether (GGGGS) which lacks an ordered secondary structure (Huston *et al.*, 1988) was juxtaposed between the expressed Fab and pIII fusion. The light-chain protein (VL-CL domains) were placed under the control of separate *lac* promoter/operator sequences. Both the Fd-C-pIII fusion and the light-chain

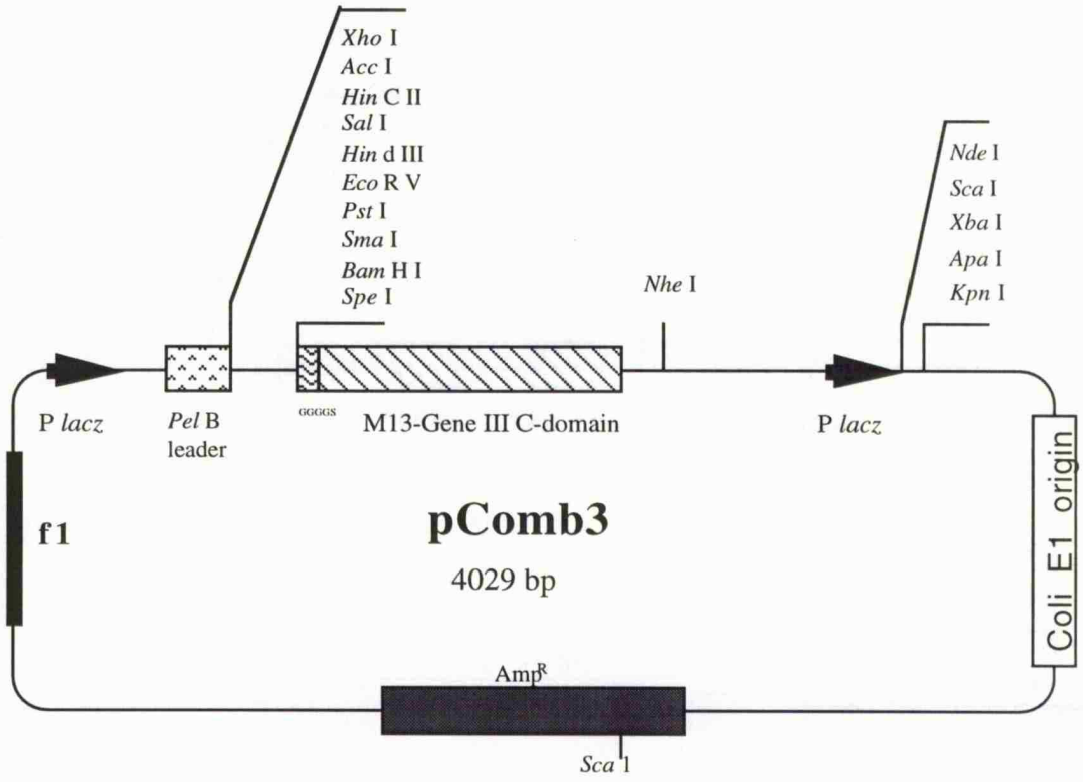


Fig. 2-1 pComb3 map.

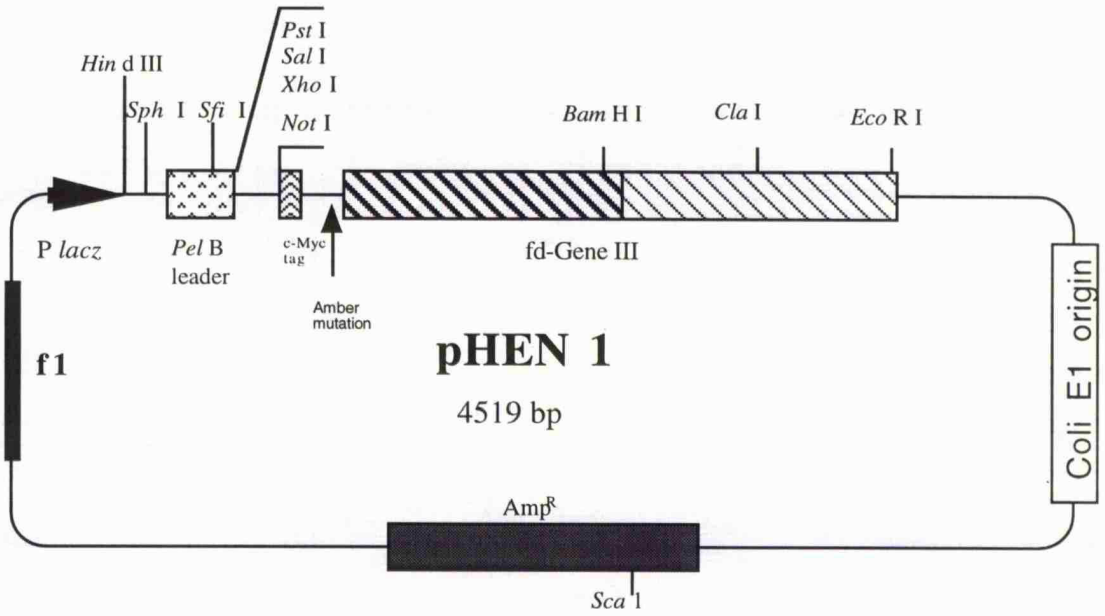


Fig. 2-2 pHEN1 map.

were directed to the periplasmic space by *pelB* leader sequences for functional assembly on bacterial inner membrane. Inclusion of the phage F1 intergenic region in the vector allows for packaging of single-stranded phagemid DNA with the aid of helper phage. The restriction sites *Spe* I and *Nhe* I are located at the 5' and 3' ends of the gIII fragment to facilitate the production of soluble antibody fragments.

pHEN1 (Hoogenboom *et al.*, 1991)

The phagemid pHEN1 (Fig. 2-2) is designed to fuse antibody fragment with N-terminal of full length pIII. The particular differences from pComb3 are an amber codon for producing soluble antibody fragments in non-suppressor strains, such as HB2151 and a c-myc tag for detection and purification of soluble antibody fragments.

2.1.6 Bacterial host strains

Escherichia coli

1. TG1: *supE hsd*Δ5 *thi* Δ[*lac-proAB*] F'[*tra*D36 *pro*AB$^+$ *lacI*^{q} Δ(*lacZ*)*M15*].

   XL-1-Blue: *recA1 endA1 gyrA96 thi-1 hsdr17 supE44 relA1 lac* F'[*pro*AB *lacI*^{q} *ZΔM15 Tn10*(*tetr*)].

These two strains were used as host for the filamentous bacteriophage M13 and also for phagemid and plasmid amplification. These two strains have some general features. Usually they carry a chromosomal deletion called Δ(*lac-pro*AB), which spans the lactose operon and surrounding region. The deletion is partially complemented by an engineered F' plasmid. which carries *pro*AB$^+$ (rescuing proline auxotropy), and *lacZΔM15*, which is the *lac* operon minus the *lacZ'* segment, which is located on the vector DNA molecule. The F' plasmid also carries *lacI*^{q}, a mutation in the *lac* operator that results in over-expression of the operon. The presence of the *pro*AB$^+$ genes on the F' plasmid means that retention of F' can be

selected for by maintaining the bacteria on a proline-deficient minimal medium and also XL-1-Blue can be selected by growth in the presence of tetracycline (10-15μg/ml). This is important because F' bacteria are unstable and can revert to F⁻ if stored for long periods. Loss of the F' plasmid from an M13 host strain would not only result in loss of the *lac* genes but would also render the cells resistant to M13 as the phage infects via the male-specific sex pili. The presence of gene *supE* or *supE44* result in the bacteria translating UAG as glutamic acid (E) rather than 'end'.

2. HB2151: *ara* Δ(*lac-pro*) *thi*/F'*proA*$^{+}$*B*$^{+}$ *lacIqZΔM15*

This strain contained engineered F' plasmid and could be infected by M13, but since the strain lacks the *supE* gene the codon UAG will translated as "end". This strain was used as the host for the production of soluble scFv, when the pHEN1 phage display vector was used.

3. W3110wt: F⁻ *hsd*R⁻ *hsd*M^{+}[p3(kmI)].

TB1: *hsd*R *ara*Δ(*lac pro*AB) *rpsl*(ϕ80 *laZ*ΔM15)

A304: *fhuA*22, *garB*10, *trxB*15::*kan*, *ompF*627, *supD*32, *fadL*701, *relA*1, *pit*-10, *spoT*1, *mcrB*1, *phoM*510

BL21(DE3) *trxB*⁻: F⁻, *ompT*, $r_B^- m_B^-$, (λimm21, *lacI*, *lac*UV5, T7 pol, *int*⁻), *trxB*⁻

These four strains were used for the expression of antibody fragments and fusion proteins. W3110wt, and TB1 served as thioredoxin reductase (TrxB) plus strains for the creation of reduced condition in the cytoplasm of *E. coli*. A304 (Russel and Model, 1986) and BL21(DE3) *trxB*⁻ represented as TrxB minus strains, in which a Tn*kan*^{R} was inserted within TrxB to delete the TrxB gene (Studier and Moffatt, 1986; Holmgren, 1984). In these two

strains, an artificial "oxidising environment" is created to facilitate the formation of scFv disulphide bonds (Proba and Pluckthun, 1995).

The bacterial stocks were preserved frozen in the presence of 15% glycerol at -80°C.

2.1.7 Plant pathogens

The plant pathogens used in this thesis include 16 different species of plant pathogenic fungi. The *Phytophthora infestans* isolates used were 36609, 89/AF1, I137, I53 and I106, which were grown on rough rye agar (60 g rye soaked for 36 hours in that 500 ml of water, boiled for 1 hour and then macerated in a blender to which 15 g agar, 20 g sucrose, water to 1 litre were added. After pH adjustment to 7.0-7.2, media were sterilised as above). After growth in the dark for 12 days at 12°C, a small volume of water (5-10 ml for 9 cm petri dishes) was added and sporangia were induced to release zoospores either by three to four 30 min cycles between 4°C and room temperature, or by incubation at 12°C for four hours. The zoospores were allowed to germinate at room temperature during an additional two hours. Finally the germinating zoospores were collected and washed three times with QH_2O and then re-suspended in QH_2O stored at -20°C.

2.2 Molecular Cloning

The majority of methods used to manipulate DNA fragments such as extraction and quantification of DNA, small-, medium- or large- scale isolation of plasmid or phagemid DNA, restriction enzyme digestion, purification of DNA fragments and vector, dephosphorylation of DNA, DNA agarose gel electrophoresis, preparation of blunt-ended DNA fragments, and ligation of DNA fragments are essentially as described by Sambrook *et al.*, (1989). The following methods were used routinely but differ from those described by Sambrook *et al.*, (1989).

2.2.1 DNA Transformation of *E. coli*

2.2.1.1 DNA transformation of competent *E. coli* cells with PEG/DMSO/Mg^{2+}

The method for routine DNA transformation during manipulation of plasmid DNA, is essentially as described by Chung *et al.*, (1989). Basically, bacteria were grown to the early exponential phase and then treated with a transformation and storage solution (TSS) containing polyethylene glycol (PEG), dimethyl sulfoxide (DMSO), and magnesium. Subsequently, competent cells were mixed with plasmid DNA, kept on ice for 60 min, incubated at 37°C to allow expression of an antibiotic gene, and plated.

2.2.2.2 DNA transformation to *E. coli* by electroporation

1. *Preparation of electroporation-competent cells*

One litre of LB medium containing the appropriate antibiotic was inoculated with 1 : 100 volume of an overnight culture. Cells were grown at 37°C with shaking (225 rpm) to OD_{600} of 0.3-0.4 for about 2 to 2.5 hours. The flask was chilled on ice for 15-30 min. The culture was spun in a pre-chilled rotor at 3,700 rpm for 20 min. The cell pellet was re-suspended and washed twice in ice cold 1 mM Hepes, pH 7.0 (sterile) in volumes of 1,000 ml and 500 ml. The cell was washed again with 20 ml of 10% glycerol in 1 mM Hepes, pH 7.0. The final cell pellet was re-suspended in 2-3 ml of 10% glycerol (to give a cell concentration of 3 x 10^{10}/ml). Cells was pipetted into sterile, pre-chilled micro-centrifuge tubes in 300 µl aliquots and then transferred to -70°C

2. *Electroporation*

A vial of electroporation-competent bacteria was thawed on ice. 50 µl of cell suspension was transferred to a pre-chilled 0.2 cm cuvette (BioRad), 2 µl ligation mix was added, the mixture was shaken to the bottom and the cuvette cooled in ice for 1 min. The Gene Pulser machine (BioRad) was adjusted to 25 µF, 2.5 kV with the pulse controller set to 200 ohms.

The cuvette was dried with tissue and placed in the pre-chilled electroporation chamber. One pulse normally had a time constant of 4.5 to 5 msec. 1 ml of SOC (fresh) was added immediately to the cuvette, the cells re-suspended, transferred to disposable culture tubes, and shaken for 1 hour at 37°C. Fractions were plated on DYT agar plates containing appropriate antibiotics and 1% glucose. Plates were then incubated overnight at 37°C.

2.2.2 Screening of DNA constructs

The cells were screened for insertion of the required DNA fragment as follows.

2.2.2.1 Colony screening by polymerase chain reaction (PCR)

After ligation and transformation with recombinant DNA, colonies were picked from overnight plates with pipette tips. Samples were inoculated onto master plates to allow recovery of the construct, and were also put into 0.5 ml microcetrifuge tubes containing 15 µl PCR reaction mixture [two primers, 1 x PCR buffer (Stratagen), 0.2 mM each of dNTPs 0.5 unit Taq DNA polymerase], mixed well and overlaid with one drop of paraffin oil to prevent evaporation. After a brief centrifugation, the tube was put on a Perkin-Elmer Cetus Thermocycler, the reaction pre-soaked for 5 min at 94°C and then cycled 25 times (94°C for 1 min, 55°C for 15 sec., 72°C for 2 min.). Reaction mixtures were electrophoresed on 1% agarose gel and DNA band were visualised on a UV trans-iluminator. In order to increase specificity and to allow insert orientation to be checked, one of the primers used primed from the insert sequence and the other from the vector sequence.

2.2.2.2 DNA construct screening by DNA hybridisation

Double-stranded DNA probes were used for colony hybridisation. Probe stocks were prepared by suitable restriction digestion and DNA samples, such as insert fragments, were separated on agarose gels. Gel slices containing the required probe DNA were removed

from the gel and the DNA purified by Gene cleaning kit. Probes were labelled with [^{32}P]α-dCTP and used to screen of alkali-lysed colonies on Hybond-N membrane.

2.2.3 DNA Sequencing

Phagemids isolated from the synthetic library were sequenced to enable identification of mutations and CDR aa sequences. Methods modified from Sanger *et al.*, (1977) and also Dye-terminator cycle sequencing methods were used. The dideoxynucleotide sequencing was carried out using the Pharmacia T7 sequencing kit. All solutions required were provided in the kit and sequencing was carried out according to manufacturer's instruction.

Dye-terminator cycle sequencing was carried out using an Applied Biosystems kit according to the manufacturer's instructions . Templates were either phagemid DNA or PCR products from various sources, such as frozen glycerol stocks and rescued monoclonal phagemids. Typically, 1 μg phagemid DNA or 100 ng-500 ng PCR product was purified with Qiagen PCR purification kit and used as template to make up 20 μl reaction mixtures. The PCR cycle reaction involved 25 cycles of 96°C for 30 secs., 50°C for 15 secs. and 60°C for 4 min. The reaction mixture was then either stored at 4°C or immediately purified by phenol extraction according to the manufacturer's instructions. The sequencing reactions were analysed using an Automated DNA Sequencer in the Protein and Nucleic Acid Chemistry Laboratory of the Biochemistry Department, Leicester University. Sequence analysis was performed using SeqEd (Applied Biosystems).

2.3 Polymerase chain reaction-based mutagensis

Splicing Overlap Extension Polymerase Chain Reaction (SOE PCR) provides a powerful method of recombining sequences which does not depend on restriction sites, and also a simple, generally applicable way of using PCR to perform site-directed mutagenesis in vitro (Ho *et al.*, 1989; Horton *et al.*, 1989). This method has also been used to transplant

antibody CDRs by using several overlapping fragments and assembling all 3 CDRs in a single step (Daugherty *et al.*, 1991). The basic principle is that several DNA fragments containing overlapping ends are generated by PCR. These fragments are combined in subsequent 'fusion' reactions in which the overlapping ends anneal, allowing the 3' overlap of each strand to serve as primer for the 3' extension of the complementary strand. The resulting fusion product is amplified further by PCR. Specific alterations in the nucleotide (nt) sequencing can be introduced by incorporating nt changes into the overlapping oligo-nt primers. This technique of site-directed mutagensis was utilised in the antibody combining site study described in chapter 4.

2.3.1 ScFvs construction and display on filamentous phage

To construct the HyHEL-10 scFv, VH and VL genes were amplified by PCR from the plasmid pVHL (Ueda *et al.*, 1993). The 5' primer used to amplify VL contained the linker $(GGGGS)_3$ sequence and the 3' primer used to amplify VH contained 15 bases complementary to the 5' VL primer. The HyHEL-10 scFv was assembled by splice overlap extension (SOE) PCR (Daugherty *et al.*, 1991; Ho *et al.*, 1989). At the same time, two restriction enzyme sites (*Hind* III and *Xba* I) were introduced at the 5' and 3' ends respectively of the HyHEL-10 scFv gene to facilitate cloning into $pC3^{-}$, a modified version of pComb3 (Barbas *et al.*, 1992a) constructed by removal of the light chain cassette.

The scFv AS32 expression plasmids was constructed as previous description by cloning AS32 rearranged VDJ gene from mAb LAS32 (Owen et al., 1992). For constructing scFv AS32 phage display phagemids, $pComb3^{-}$:AS32, the scFv AS32 gene fragment was amplified by PCR with primers VL5RT (5'CG TCT AGA CCC ATG GAC ATC CAA CTG ACC CAG TCT CCA 3') and VH3RTS (5'TC GGT ACC ACT AGT TGA GGA GAC3'), and *Xba* I and *Spe* I sites (outline at primers VL5RT and VH3RTS) wear introduced to 5' and 3' of the AS32 fragment for facilitating cloning, and the scFv AS32 (*Xba* I / *Spe* I)

fragment was cloned into *Spe* I site at pComb3$^-$ vector, which was constructed from pComb3 by eliminating the light chain cassette (Barbas *et al.*, 1992a).

2.3.2 Construction of hybrid, CDR grafted and mutant scFv genes

Six fragments were generated by PCR from the pC3$^-$:AS32 phagemid (Fig. 2-3). These were used to facilitate assembly of a range of scFv genes comprising mixtures of AS32 or HyHEL-10 VH CDRs in the VH framework of AS32. Fragment F_N contains a portion of FR4 of AS32 in which the sequence encoding VH residue Lys94 has been mutated to encode Asn 94. The primers used also introduced *Hind* III and *BstE* II restriction sites at respectively the 5' and 3' ends of the scFv constructs and an *Sst* I site at the 3' end of the VH construct to facilitate subsequent cloning steps. These fragments were used with a combination of PCR, SOE PCR and conventional cloning to assemble phagemids encoding the following mutant or hybrid scFvs:

1. a hybrid scFv comprising the AS32 VH and the HyHEL-10 VL.

2. pC3$^-$:G123, in which HCDRs 1, 2 and 3 of HyHEL-10 have been grafted onto the VH framework of AS32, with the HyHEL-10 VL.

3. pC3$^-$:G123.N, in which HCDRs 1, 2 and 3 of HyHEL-10 have been grafted onto the VH framework of AS32 (Asn residue at VH94), with the HyHEL-10 VL.

4. pC3$^-$:G123.N.D, in which HCDRs 1, 2 and 3 of HyHEL-10 have been grafted onto the VH framework of AS32 (Asp residue at VH27, Asn residue at VH94), with the HyHEL-10 VL.

5. pC3$^-$:G23.N, in which HCDRs 2 and 3 of HyHEL-10 have been grafted onto the VH framework of AS32 (Asn residue at VH94), with the HyHEL-10 VL.

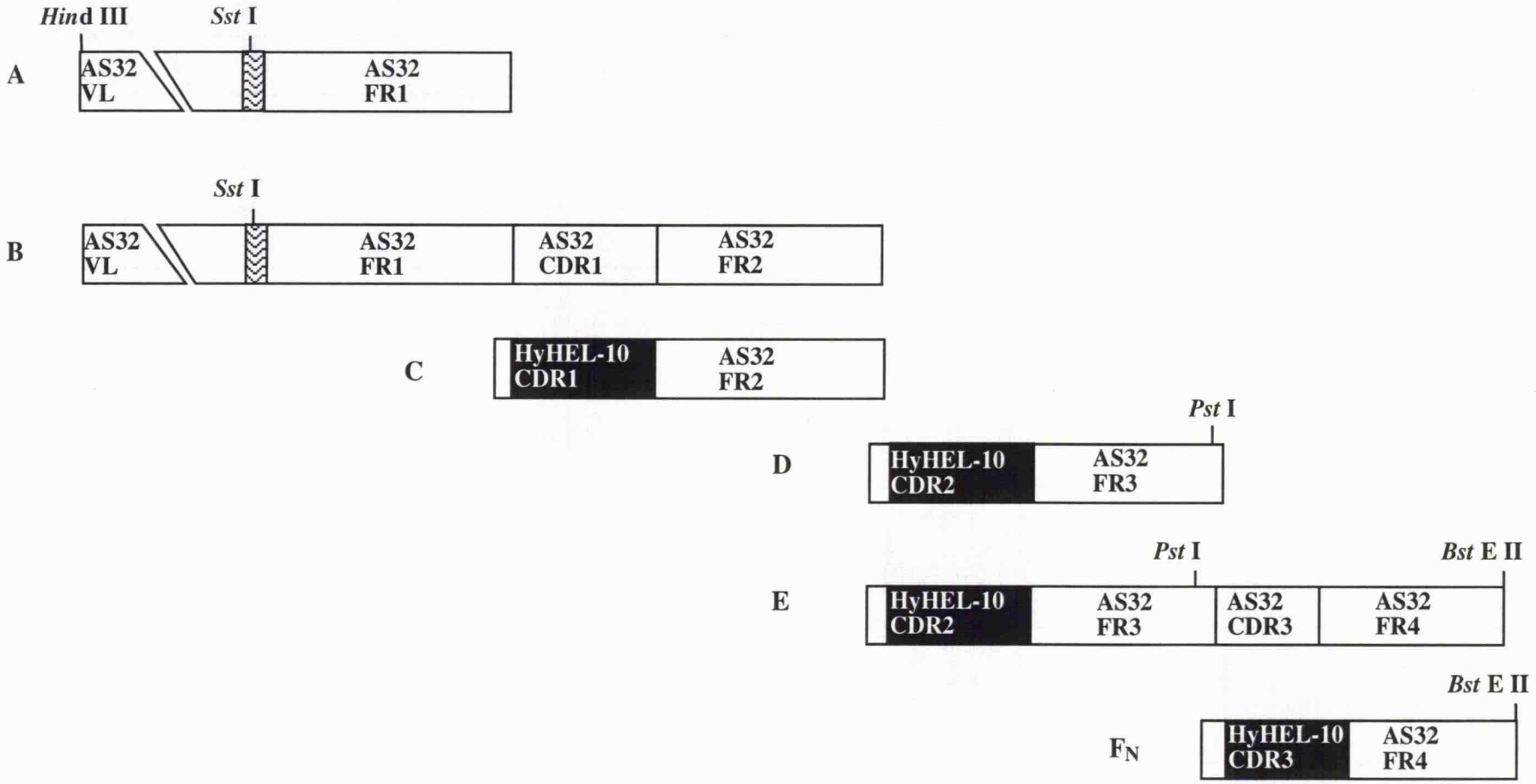


Fig. 2-3. The PCR-generated fragments used for assembly of mutated scFv genes encoding combinations of AS32 or HyHEL-10 VH CDRs in the VH framework of AS32. In fragment F_N the sequence encoding VH residue Lys94 (within framework 3) has been mutated to encode Asn 94.

6. pC3$^-$:G3.N, in which HCDR 3 of HyHEL-10 has been grafted onto the VH framework of AS32 (Asn residue at VH94), with the HyHEL-10 VL.

2.3.3 Construction of a heavy chain Fv gene randomised in CDR3

The phytochrome A binding scFv constructed in the pC3$^-$ vector (2.3.1), pC3$^-$AS32, was used as a template for PCR. Two PCRs were performed. The mixture for reaction 1 consisted of 10 ng of template pC3$^-$AS32, 1 μg of primer 5'_back (5'CTC GAG GTC GAC GGT ATC GAT AAG C 3'), 1 μg of primer HFR3_forward (5'TGC ACA GTA ATA TGT GGC TGT 3'), all four dNTPs (each at 200 μM), and Promega Taq polymerase buffer containing 1.5 mM $MgCl_2$ and 5 units of Taq polymerase in a final volume of 100 μl. Reaction 2 was identical to reaction 1 except that the reaction mixture contained primers HCDR3_random [5'ACA GCC ACA TAT TAC TGT GCA $(NNS)_6$ TGG GGC GCA GGG ACC ACG 3' and C-pIII_forward (5'AAT CAC CGG AAC CAG AGC 3'), in which N is A, C, G, or T (equal-molar) and S is G or C (equal-molar)]. The PCR was performed by 30 cycles of; 1 min denaturation at 94°C, annealing for 1 min at 50°C, and extending at 72°C for 2 min. The resulting PCR products were purified with Gene Clean Kit (BIO 101). Finally, into PCR mixture 3, 100 ng of product 1 from reaction 1 and 100 ng of product 2 from reaction 2 were added as templates for overlap extension with the addition of 1 μg or primer 5'_back and 1 μg of primer C-pIII_forward in a 100-μl reaction mixture as described above. The PCR programme was identical to that for reactions 1 and 2. The resulting fragment was gel-purified by using Gene Clean Kit (BIO 101), digested with the restriction enzyme *Hind* III and *Spe* I and gel purified as above to yield product 3, an scFv fragment with the heavy chain randomised in CDR3.

2.4 Filamentous phage display technology

2.4.1 Phage antibody library construction

This method was used for creating the HCDR3 randomised scFv library.

The final product from SOE PCR was digested with *Hind* III and *Spe* I and purified with Gene Clean II (BIO 101). About 320 ng of purified library product was ligated with 2 mg of the prepared *Hind* III/*Spe* I pC3^{-} (a modified version of pComb3 by eliminating the second multicloning sites) vector in a total volume of 150 ml with 10 U of GIBCO BRL T4 ligase at 15.5°C overnight. Following ligation, DNA was precipitated at -20°C for 2 hours by the addition of 2µl of 20mg/ml glycogen, 15 µl of 3M sodium acetate, pH 5.2, and 330 µl of ethanol. DNA was pelleted at 13, 000 rpm at 4°C for 15 min. The DNA pellet was washed with cold 70% ethanol and dried under vacuum. The pellet was re-suspended in 10 µl of water and transformed by electroporation into 200 µl of *E. coli* XL1-Blue. After transformation, 3 ml of SOC medium was added and the culture was shaken at 200 rpm for 1 hour at 37°C, after which 10 ml of SB containing 20 mg/ml carbenicillin and 10 µg/ml tetracycline was added. (At this stage aliquots of 20, 1 and 0.1 µl were withdrawn for plating to determine the library size). The culture was allowed to grow for an additional hour at 37°C while shaking at 200 rpm. The culture was then added to 100 ml of SB containing 50 µg/ml carbenicillin and 10 µg/ml tetracycline and shaken for 1 hour, after which helper phage VCSM13 (10^{12} pfu) was added and the culture was shaken for an additional 2 hours. After this time, 70 µg/ml kanamycin was added and the culture was incubated at 37°C overnight. The supernatant was cleared by centrifugation (3700 rpm in a GH-3.7 Beckman rotor for 20 min at 4°C). Phage was precipitated by adding 1 : 5 volume of PEG/NaCl solution (20% (w/v) polyethylene glycol 8000 and 2.5 M NaCl), mixed well and incubated on ice for 30 min, followed by centrifugation (9000 rpm for 20 min in a SS34 Sorvall RC-5B rotor at 4°C). Phage pellets were re-suspended in 2 ml of PBS (50 mM phosphate, pH 7.4, 150 mM NaCl) and centrifuged for 3 min to pellet debris, transferred to fresh tubes, and stored at -20°C.

2.4.2 Titration of colony forming units (cfu)

This method is used for all titrations of packaged phagemids.

Phage were diluted in SB (10^{-3}-, 10^{-6}-, 10^{-7}-, and 10^{-8}-fold dilution) and 1 µl was used to infect 50 µl of fresh (OD_{600} = 1) *E. coli* XL1-Blue (selected by additional 10 µl/ml tetracycline) or TG1 grown in SB or 2 x YT medium. Phage and cells were incubated at room temperature for 15 min and then directly plated on LB agar plates containing 150 µg/ml of ampicillin or 70 µg/ml carbenicillin. The plate was incubated overnight at 37°C. The colonies were counted, and plates containing between 20 to 100 were used to calculate phage cfu/ml.

2.4.3 Multiple panning

Method A Micro panning

This method is a modification of that originally described by Parmley and Smith (1988), and was used for selection anti-lysozyme and phytochrome A antibodies from the single random HCDR3 library. Two wells of a microtiter plate (DYNATECH Immunon 4) were coated overnight at 4°C with 25 µl of 10 µg/ml HEL in carbonate-bicarbonate buffer pH 9.6. The wells were washed twice with PBS pH 7.4 and blocked by completely filling the well with 3% (w/v) bovine serum albumin (BSA) in PBS pH 7.4 and incubating the plate at 37°C for 2 hours. Blocking solution was shaken out and the wells were washed twice with PBS by filling and pouring out. 50µl of the phage library (about 10^{11} cfu) was added to each well, and the plate was incubated for 2 hours at 37°C. The phage suspension was removed and the plate was washed twice with water. Each well was washed 10 times with PBS-0.5% Tween 20 by fully filling and incubating at room temperature for 5 min and then pipetting the wash solution up and down five times. The plate was finally washed twice with PBS and twice with Millipore Q water. Bound phage were eluted by the addition of 50 µl of elution

buffer (0.1 M HCl, adjusted to pH 2.2 with solid glycine, containing 1 mg/ml BSA) to each well and incubating at room temperature for 10 min. The elution buffer was pipetted up and down 10 times, removed, and neutralised with 3 µl of 2M Tris base per 50 µl of elution buffer. Eluted phage were used to infect 2 ml of fresh (OD_{600} = 1) *E. coli* XLI-Blue cells for 30 min at 37°C, after which 10 ml of SB containing 100 µg/ml ampicillin and 10 µg/ml tetracycline was added. Aliquots (20, 10, and 0.1 µl) were removed for plating to titrate the eluted phage. The culture was shaken for 1h at 37°C, after which it was added to 100 ml of SB containing 100 µg/ml ampicillin and 10 µg/ml tetracycline and shaken for 1 hour. Then helper phage VCSM13 (10^{12} pfu) were added and the culture was shaken for an additional 2 hours. After this time, 70 µg/ml kanamycin was added and the culture was incubated at 37°C overnight. The phage were prepared by adding 1:5 v/v of 20% PEG and 2.5M NaCl to cleared supernatant, mixing well, incubating on ice for 20 min then pelleted by centrifugation (9000 rpm for 20 min at 4°C). Phage pellets were re-suspended in 2 ml of PBS, and microcentrifugalised for 5 min to pellet debris, transferred to fresh tubes, and stored at 4°C. The phage was then ready for the next panning round.

Method B Immuno- tube panning

This method was used for the selection of phage antibodies against peptide-BSA conjugates or germlings of *P. infestans* zoospores.

Immuno-tubes (75 mm x 12 mm, Nunc; Maxiscorp) were coated with 4 ml of peptide-BSA conjugate (100 µg/ml) or germlings of *P. infestans* zoospores (about 1000 germlings/ml) in 50 mM $NaHCO_3$, pH 9.6 overnight at room temperature. After washing 3 times with PBS, the tubes were blocked by filling the tubes with 2% MPBS (Marvel PBS) and incubating for 2 hours at 37°C. In the meantime, the phage library (10^{13} cfu in 2% MPBS-1%BSA) was incubated at room temperature for 2 hours. After this time, the tubes were washed and the phage particles added, incubated for 30 min at room temperature, systematically inverting

the tube using a rotating turntable, and then left undisturbed for a further 1.5 hours at room temperature. Tubes were washed 20 times with PBS, 0.1% (v/v) Tween 20, 20 times with PBS and three times with QH_2O (each washing step was performed by pouring buffer in and out immediately). Bound phage particles were eluted from the tube by adding 1 ml of elution buffer (0.1 M HCl, adjusted to pH 2.2 with solid glycine, containing 1 mg/ml BSA) and inverting the tube using a rotating turntable for 10 min. The eluted material was immediately neutralised by adding to a tube containing 60 µl of 2 M Tris base. 6 µl of 2 M Tris base was also added to the immuno-tube. The 1 ml eluted phage was used to infect 9 ml of fresh *E. coli* TG1 culture (OD_{600} = 0.5). 4 ml of fresh TG 1 culture was also added in to the immuno-tube to maximise recovery of bound phage. Both cultures were incubated for 30 min at 37°C without shaking. The 10 ml and 4 ml culture were pooled, from which 100 µl of the mixed culture was taken to make serial dilutions of 10^{-1} to 10^{-7}. The dilutions were plated on TYE containing 100 µg/ml ampicillin and 1% glucose and incubated overnight at 37°C. The eluted phage was quantified by counting the number of colonies produced. The remaining 14 ml TG1 culture was centrifuged at 4000 rpm at room temperature for 10 min. The pelleted bacteria was re-suspended in 1 ml of DYT medium and plated on a large Nunc Bio-Assay (150 x 20 style) dish of TYE containing 100 µg/ml ampicillin and 1% glucose. The bacteria were grown overnight at 37°C, and used in the phagemid rescue procedure described below.

2.4.4 Rescuing phagemid after selection

A 5 ml aliquot of DYT was added to the Nunc Bio-Assay dish which contained bacteria from the final step of panning method B and the cells were loosened with a glass spreader. 50 µl of the released bacteria was used to inoculate 50 ml of DYT containing 100 µg/ml ampicillin and 1% glucose. The culture were grown with shaking at 37°C to an OD_{600} of 0.5 (approximately 2.5 x 10^8 cells/ml, using OD_{600} = 1 at 5 x 10^8/ml). This normally required

incubation for about 2 hours. 10 ml of this culture was taken and infected with helper phage VCS M13 at ratio 1 : 20 (phage : cell). This culture was incubated without shaking in a 37°C water bath for 30 min. The infected cells were collected by centrifugation at 3700 rpm for 10 min. The pellet was re-suspended in 50 ml of DYT containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. This cell suspension was added to 250 ml of pre-warmed DYT containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. The cells were grown at 30°C with shaking for 16 hours.

2.4.5 Rescue of phage from individual phagemid clones in ELISA

Single ampicillin-resistant colonies, resulting from infection of *E. coli* TG 1 with eluted phage were inoculated into 150 μl of DYT containing 100 μg/ml of ampicillin and 1% glucose in 96-well plates (Cell wells; Corning) and grown with shaking (200 rpm.) overnight at 37°C. A 48-well plate replicator was used to inoculate approximately 4 μl of the overnight cultures from the master plate into 200 μl fresh DYT containing 100 μg/ml ampicillin and 1% glucose. The plate was incubated with shaking at 37°C for 1 hour. After this time, 25 μl of DYT containing 100 μg/ml ampicillin, 1% glucose and 10^9 pfu of VCS M13 was added to each well, and the plate was incubated at 37°C for 45 min. without agitation. The plate was then shaken at 37°C for 1 hour after which time glucose was removed by centrifugation (Sorvall RT6000B-Sorvall PN11065, 3500 rpm. for 20 min. at room temperature), and aspirating the supernatant. Cells were re-suspended in 200 μl DYT containing 100 μg/ml ampicillin and 25 μg/ml kanamycin and grown for 20 hours with shaking at 37°C. Supernatant containing phage was tested for binding by ELISA.

2.4.6 General method for phagemid rescue

This method was used to study the rescue conditions described in chapter 3.

Single colonies containing phagemids were inoculated and grown in 5 ml of DYT or SB (containing appropriate antibiotics and 1% glucose) at 37°C overnight. 60 µl of the overnight culture was add to 6 ml DYT or SB (containing appropriate antibiotics and 1% glucose) in a 50 ml sterile plastic centrifuge tube and grown at 37°C with fast shaking (250 rpm) for one hour. VCS M13 helper phage was added at a ratio of 1 : 20 (cell : phage). The culture was incubated at 37°C for 30 min without shaking and centrifuged at 3700 rpm and room temperature for 5 min. then the bacteria were re-suspended in 10 ml DYT or SB containing appropriate antibiotics. The culture was incubated with shaking (200 rpm.) at 37°C for 30 min and then kanamycin to a final concentration of 25 µg/ml and IPTG to a final concentration of 0, 0.2 or 1 mM was added. Cells were grown overnight with shaking at 30°C. After which, the cells were pelleted and the supernatant used for detection or purification of antibodies.

2.4.7 Preparing phage particles

This method was used for preparing phage particles for the selection of phage antibodies against *P. infestans* and peptide-BSA conjugates.

The culture containing phage particles was centrifuged at 8000 rpm. for 10 min. (or 3700 rpm. for 30 min.). The supernatant was carefully transferred to a clean centrifuge tube, to which 1/5 volume PEG/NaCl (20% Polyethylene glycol 6000, 2.5 M NaCl) was added, mixed well and incubated for 1 hour at 4°C. The mixture was centrifuged at 8000 rpm. and 4°C for 30 min. The pellet was re-suspended in 40 ml water; 8 ml PEG/NaCl added, mixed well and left for 20 min at 4°C. After this time, the suspension was centrifuged at 3700 rpm and 4°C for 30 min. The supernatant was removed completely by aspirating most of the liquid; and re-centrifuged briefly and aspirating the remaining PEG/NaCl solution. The pellet was re-suspended in 2.5 ml water and the suspension was centrifuged at 7000 rpm. for 10 min in a microcentrifuge to remove most of the remaining cell debris. Phage particle

suspensions were stored at 4°C. (300 ml DYT medium yielded 1 - 5 x 10^{13} cfu phage particles).

2.4.8 Phage antibody ELISA

The analysis of binding of phage to antigens in the work reported in this thesis was performed on bacterial supernatants containing phage essentially as described by Marks *et al.* (1991). Plates were coated with 1 mg/ml HEL, 100 µg/ml peptide-BSA, 10 µg/ml phytochrome A, or 100 µg/ml BSA in 50 mM $NaHCO_3$, pH 9.6 at room temperature overnight. The wells was rinsed three times with PBS and blocked by adding 300 µl per well of 2% MPBS (containing 0.2% sodium azide) and incubating in a sealed container at 37°C for 2 hours. During this time, the supernatant containing phage particles was diluted 1/2 in 4% MPBS (containing 0.2% sodium azide) and incubated for 30 min at room temperature. After blocking, the wells was rinsed three times with PBS, and following the addition of 100 µl of phage particles to each test well, the plate was incubated at 37°C for 1 hour. After which time, the plate was washed six times by immersing the plate in a large container of wash buffer (PBS containing 0.05% Tween-20) and flicking out the buffer. An aliquot of 100 µl of diluted HRP/anti-M13 antibody conjugate (Pharmacia) was added to each test well. The plate was incubated at 37°C for 45 to 60 min. The diluted conjugate was flicked out and the plate washed six times with wash buffer as described above, then rinsed 3 times with QH_2O. 200 µl of substrate solution (ABTS/H_2O_2) was added to each well then the plate was incubated at room temperature for 20 min. Absorbency at a wavelength of 405 nm was read in a microtiter plate reader.

2.4.9 The production of soluble scFv from pHEN1

This method was used for the induction of pHEN1 in *E. coli* HB2151 or other non-suppressor strains to give expression of soluble scFvs.

Individual colonies of non-suppressor strains containing pHEN1:scFv phagemids were inoculated into 150 µl of DYT containing 100 µg/ml ampicillin and 1% glucose in 96-well plates and grown with shaking at 37°C overnight. A 48-well plate replicator was used to inoculate approximately 4 µl of the overnight cultures from the master plate into 200 µl fresh DYT containing 100 µg/ml ampicillin and 0.1% glucose. Following incubation at 37°C until an OD_{600} of 0.9 was reached (after about 3 hours 25 µl of DYT containing 100 µg/ml ampicillin and 9 mM IPTG (final concentration 1mM IPTG) was added into each well. The plate was shake at 30°C for a further 16 to 24 hours then plate centrifuged at 3700 rpm for 10 min. 100 µl of the supernatant solution was used for ELISA analysis. Using similar methods a large volume of soluble scFvs can be produced.

2.4.10 Detection of pIII fusion proteins in colonies

This method is modified from that described by Barbas and Lerner (1991) for the detection pIII :: protein A fusion proteins in bacterial colonies.

The phage particles from affinity column eluates were diluted 1: 10, 000 times, and 1 µl used to infect 100 µl of fresh (OD_{600} = 1) *E. coli* XL1-Blue cells for 15 min. The infected culture was plated on SB agar plates containing 150 µg/ml of ampicillin, 10 µg/ml tetracycline and 1% glucose, then incubated at 37°C for 4 hours. The plate was overlaid with a nitro-cellulose filters soaked in 5 mM IPTG and incubated overnight at 30°C. The filter was removed, incubated in a chloroform chamber for 15 min., transferred to 25 ml of lysozyme buffer containing 50 mM Tris, pH 8, 150 mM NaCl, 5 mM $MgCl_2$, 3% BSA, 0.4 mg/ml lysozyme, and 100 U of DNase, and rocked for 1 hour. Buffer was removed and fresh lysozyme buffer added, and the filter was rocked for an additional hour, after which adhering colonies were removed by gentle rubbing. The filter was finally blocked in 10 ml of PBS containing 4% (w/v) BSA by rocking at room temperature for 1 hour. Filters were then probed by incubating the filter in 10 ml of 1 : 10, 000 diluted rabbit alkaline phosphatase

conjugate for 1 hour at room temperature. The filter was washed three times with PBS/0.5% Tween 20 (10 ml per filter) and then developed with BCIP/NPT substrate solution.

2.4.11 Affinity purification of fusion phage particles

This method used human IgG agarose (Sigma) to affinity purify phage particles displaying four domains of staphylococcal protein A and protein A fusions with scFv AS32.

0.2 ml columns of human IgG agarose were washed extensively with PBS, blocked with 1 ml of 2% MPBS after which 10^{12} cfu of phage (displaying protein A) in 1 ml of 2% MPBS were loaded. Columns were washed with 10 ml of PBS; 2 ml of PBS/0.05% Tween 20; 1 ml of 0.2 M glycine pH 6.0 and 1 ml of 0.2 M glycine pH 5.0. Bound phage particles were eluted by addition of 1 ml of 0.2 M glycine pH2.2 and eluates were neutralised by mixing immediately with 0.2 ml of 1M Tris-HCl pH7.4. Eluted phage was either stored at -20°C or used to infect logarithmic *E. coli* XL1-Blue cultures. A similar method was used to purify phage displaying protein A :: AS32 and AS32 :: protein A fusions from crude medium, in which case, 1 ml of human IgG agarose and 50 ml of phage-containing supernatant was used.

2.4.12 Phage antibody affinity measurement [modified from Friguet *et al.* (1985)]

This method was used to measure dissociation constants of phage antibody scFvs HyHEL-10, G123.N.D, G123.N, G123, G23.N described in chapter 4. The antigen at various concentration was first incubated in solution with the antibody at constant concentration until equilibrium was reached. The concentration of free antibody was then determined by an indirect ELISA.

Each well of the ELISA plate was coated with 100 µl of 10 µg/ml chicken egg lysozyme (HEL) in 50 mM $NaHCO_3$, pH 9.6 at room temperature overnight. HEL at concentrations

ranging from $5x10^{-11}$ M to $1.6x10^{-7}$ M was mixed with a constant amount of PhAbs ($1x10^{10}$ cfu/ml) in the PhAbs ELISA blocking buffer. To each of ten microtubes, 0.25 ml of a range of antigen concentrations were added followed by 0.25 ml of PhAb solution. After incubating for 20h at 20°C, free PhAbs were measured with the procedures described above for PhAb ELISA whilst maintaining the ELISA reaction at 20°C. The final K_D was calculated from the data obtained by using the following equation:

$$A_0 / (A_0 - A_1) = K_D / a_{t1} + 1$$

a_{t1} being the total concentration of antigen; A_o, the absorbency of the antibody in the absence of antigen and A_1, the absorbency of the free antibody in the presence of antigen at a given concentration of a_{t1}.

2.4.13 Phage electron microscopy

This method was used to directly visualise phage antibodies binding to epitopes on the surface of *P. infestans*.

Confirmation that phage from the final round of panning was binding to the surface epitopes of germinated zoospores of *P. infestans* was obtained by ELISA. Collodion (filtered 0.25% solution in amylacetate) coated grids (300-mesh.) were coated with carbon, and then treated with mouse antibodies against *P. infestans* by adding 5 µl mouse serum (1000 dilution in PBS at pH 7.4) and incubating in a sealed container at room temperature for one hour. After which time, the grid was washed three times with PBS at pH 7.4 by adding drops which were absorbed by filter paper underneath the grid. The grids was incubated with 5 µl of germinated zoospores (about 10^5 /ml) at room temperature for one hour in a sealed container. The grids were then washed three times with PBS as described above. Grids were blocked with 5 µl of 1% (w/v) bovine serum albumin (BSA) in PBS at pH 7.4 by incubation in a sealed container at room temperature for 1 hour, and then washed with PBS as

described above. In order to react the PhAbs with germinated zoospores, 5 µl of diluted anti-*P. infestans* phage antibody (about 10^8 cfu/ml in 2% skimmed milk PBS) was added to the grid and incubated in a sealed container at room temperature for one hour. The grid was then washed 3 times with PBS/0.05% Tween 20 at pH 7.4, three times with PBS at pH 7.4 and three times with distilled water as described above. After this time, the grid was fixed in 25% Glutaraldehyde solution for 1 min., and then stained with 2% uranyl acetate in H_2O by dropping the stain onto the grid and drying immediately with filter paper. Finally the fields were viewed with a transmission electron microscope at 10,00-30,000 magnification. Control experiments were performed as described above using VCS-M13 helper phage or phage displaying an antibody fragment with irrelevant specificity.

2.4.14 Fluorescent phage

The coat proteins of intact phage particles can be labelled with fluorochrome using the same methods as are used to label protein molecules in solution. A method for coupling rhodamine isothiocyanate (TRITC) was modified from Goding (1976). A concentration of 10^{12} cfu/ml phage particles in 0.1 M sodium carbonate (pH 9.0) was prepared. TRITC solution was prepared by dissolving the dye in dimethyl sulfoxide (water free) at 1 mg/ml (w/v). This solution was prepared fresh for each labelling reaction. A 50 µl aliquot of the dye solution was added to each 1 ml of phage antibody solution. The dye was added very slowly in 5 ml aliquots, and the phage antibody solution was gently but continuously stirred during the addition. The mixture was incubated in the dark for 8 hours at 4°C. After this time, 1 M NH_4Cl was added to a final concentration of 50 mM, and incubation was continued for a further 2 hours at 4°C. Following dialysis for 24 hours in the dark against three changes of 3000 volumes of PBS the fluorescent labelled phage was stored in 4°C with sodium azide added to 0.02%. The coupling efficiency was measured at wavelength of 495 and 280 nm.

In order to use the fluorescent PhAbs to localise their cognate epitopes on *P. infestans* germlings, aliquots of fresh prepared germlings was washed with QH_2O three times. Fluorescent phage particles (at a concentration of 10^{12}cfu/ml) was added to the germlings, and incubated at room temperature for 3 hour (or 4°C overnight) in darkness. The germlings was subsequently washed three times with PBS and view under the fluorescent microscope at 400 to 1000 magnification, and used green excitation (H546), filters BP 546/12, FT580 and LP590 (Zeiss Fluorescence microscope systems, Germany).

2.5 Protein- and immuno-chemistry

2.5.1 Quantification of protein

Protein concentration were calculated using Coomassie Plus Protein Assay Reagent (Pierce), which is based on the absorbency shift from 465 to 595 nm that occurs when Coomassie blue G-250 binds to proteins in an acidic solution (Bradford, 1976). The 'Plus Assay' is more linear than the original Bradford assay. The experiment was performed in a microtitre plate. Duplicate 10 µl aliquots of serially diluted standard protein solutions (BSA) or test protein solutions were added to microtitre plate wells. After the addition of 300 µl of the reagent to each well the plate was shaken for 10 sec. and analysed on a Dynatec MR5000 microtitre plate reader programmed to calculate the concentration of protein samples directly

2.5.2 Polyacrylamide gel electrophoresis of proteins

A BioRad protein mini-gel kit was used in electrophoresis of proteins for both *in-situ* gel assays and western blotting with the kit assembled according to manufacturer's directions.

The dissociating buffer systems (SDS-PAGE) was used for all gel electrophoresis of proteins. Details of buffer composition and gel mixture preparation for the SDS-discontinuous system, based on the method of Laemmli, (1970), are given in table 2-1.

After addition of TEMED, the resolving gel was poured, overlaid with 0.5 ml of 50% propan-2-ol and allowed to set for 15 to 20 min. During this time, the stacking gel was prepared without TEMED. Once the gel had set the overlay was removed and rinsed with QH_2O. TEMED was added to the mixture of stacking gel which was then poured on the resolving gel. The gel comb was put in place and the gel allowed to set for at least 30 min. Gels were run at 100-150 V for 1.5 to 3 hours in glycine running buffer (14.4 g glycine, 3g Tris base, 1 g SDS, deionised water to 1 litre) until the blue dye present in the sample buffer reached the bottom of the gel.

Protein samples to be run on the discontinuous gels were prepared by adding a volume of 5-30 µl sample to 0.33 volume of 3 x sample buffer (300mM Tris, 2%SDS, 10%glycerol, 5% β-Mercaptoethanol, pH 6.8, 0.003% Bromophenol Blue). Samples were boiled for 1-5 min to denature the protein and immediately placed on ice until loaded onto the gel. Crude bacterial or plant samples were centrifuged for 1 min at 12000 rpm before loading onto the gel.

Table 2-1. Buffer composition and gel mixture preparation (SDS-discontinuous system)

Stock solution	Stacking gel	Final acarylamide concentration in resolving gel mixture (%)[a]					
		6	8	10	12	15	20
Water	2.3	6.55	5.85	5.25	4.55	3.55	1.85
Acrylamide-bisacrylamide (30%:0.8%)	0.66	2.0	2.7	3.3	4.0	5.0	6.7
Stacking gel buffer stock[b]	1.0	-	-	-	-	-	-
Resolving gel buffer stock[c]	-	1.25	1.25	1.25	1.25	1.25	1.25
10% SDS	0.04	0.1	0.1	0.1	0.1	0.1	0.1
10% ammonium persulphate	0.04	0.1	0.1	0.1	0.1	0.1	0.1
TEMED	0.004	0.005	0.005	0.005	0.005	0.005	0.005

[a]The columns represent volumes (ml) of the various solutions required to make 10 ml of gel mixture

[b]Stacking gel buffer stock: 0.5 M Tris-HCl (pH 6.8); 6.0 g Tris is dissolved in 40 ml QH_2O, titrated to pH 6.8 with 1 M HCl (about 48 ml), and brought to 100 ml final volume with QH_2O. The solution is filtered through Whatman No. 1 filter paper and stored at 4°C.

[c]Resolving gel buffer stock: 3.0 M Tris-HCl (pH 8.8); 36.3 g Tris and 48.0 ml 1 M HCl are mixed and brought to 100 ml final volume with water. This buffer is then filtered through Whatman No. 1 filter paper and stored at 4°C.

2.5.3 Coomassie Blue Staining

Proteins separated on SDS-PAGE was stained with Coomassie blue stain prepared by dissolving Coomassie blue R250 (0.1%) in water:methanol:glacial acetic acid (5:5:2 by volume). The solution was filtered through Whatman No. 1 filter paper to remove any insoluble material before use. Each gel was immersed in about 15 ml of stain and agitated at room temperature overnight or at 55°C for one hour. The gel was incubated in 20 ml destaining buffer (30% methanol, 10% acetic acid) overnight with agitation. The destaining buffer was changed at least three times. An alternative to the replacement of destaining solution was to include a few grams of an anion-exchange resin to absorb the stain as it leaches from the gel. Finally, the gel was dried in a dialysis tube.

2.5.4 Western blotting

This method provided a means of transferring proteins to cellulose membranes which could then be probed with antibodies to detect specific proteins. SDS-PAGE mini gels were run as described (2.5.2). The apparatus used was disassembled, the stacker gel removed and the separated proteins from the resolving gel transferred to a piece of nitro-cellulose filter cut to size by a semidry electroblotting system. After blotting, the filter was put in a small container of Ponceau staining (0.2% Ponceau, 3% Trichloroacetic Acid), agitated for 2 min at room temperature and then rinsed with distilled water. Protein bands were stained pink allowing protein transfer to be monitored, and molecular weight markers to be located.

Stain was removed by washing in PBS containing 0.1% Tween-20. The filter was then placed in blocking buffer [PBS pH 7.4 with 0.1% (v/v) Tween-20 and 5% (w/v) Marvel] and incubated at room temperature with rocking for two hours. The filter was then transferred to the primary antibody (an appropriate dilution of the antibody in buffer [PBS pH 7.4 with 0.1% (v/v) Tween-20 and 5% (w/v) Marvel]) and incubated with rocking at room temperature for 1 hour or overnight at 4°C. The filter was washed three times for 15 min each in PBS containing 0.1% (v/v) Tween-20 on a rocking platform. It was then placed in secondary antibody buffer [PBS pH 7.4 with 5% (w/v) Marvel] with the appropriate dilution of secondary antibody, and the filter incubated on a rocking platform for 1 hour. The filter was then washed as described above and finally rinsed in distilled water three times. Treatment of the filter from this point was dependent upon the conjugate attached to the secondary antibody.

If the conjugated enzyme was alkaline phosphatase, the substrate solution contained BCIP and NBT which produce a purple reaction product in the presence of this enzyme. The filter was rinsed as before and then placed in 10 ml of 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$. To this was added 150 µl of BCIP (made up as a stock of 30 mg/ml in DMF) and 150 µl of NBT (made up as a stock of 50 mg/ml in 70% (v/v) DMF). Blots were developed by incubation at room temperature for 5 to 30 min in a dark container. In the case of antibodies conjugated to horseradish peroxidase, the filter was rinsed and developed with the chemiluminscence detection systems supplied by Amersham or Boehringer according to the manufacturer's instructions.

2.5.5 ELISA

This method was used for routine detection of soluble antibody binding activity. The procedure is for a standard enzyme-linked immunosorbence assay and is essentially that

described for phage antibody ELISA. The precise description of each step is given as following protocol.

The antigen was diluted in coating buffer (50 mM $NaHCO_3$, pH 9.6) to a final concentration of between 10 μg/ml to 1 mg/ml. To each well of the ELISA plate, apart from the end well which contained the substrate-only blank, 0.1 ml of the diluted antigen was added. Plates were incubated in a sealed container overnight at 4°C. After coating, plates were rinsed three times with PBS pH 7.4 at room temperature. Between washes plates were emptied by first turning them upside down and shaking over a sink, then hitting them sharply several times on a pile of paper towels. After the third rinse, 0.1 ml of control or antibody solutions were added to each well. All assays were be done in duplicate or triplicate, including non-coated background wells. Plates were incubated at 37°C for one hour, then rinsed three times as described above. After this time, 0.1 ml of immuno-conjugates previously diluted in incubation buffer was added to each well. Depending on the immuno-conjugate supplier, 1000 to 10, 000 fold dilution were used. Plates were incubated as described at 37°C for 45 to 60 min then washed three times as described above. After the final wash, plates were rinsed with distilled water once and emptied of water by hitting them sharply several times on a pile of paper towels. Finally by 0.2 ml of substrate solution was added to all wells, and plates were incubated in a sealed dark container for 20 min after which absorbency was read at a wavelength appropriate to the detection enzyme system used.

2.5.7 Competition/inhibition ELISA

A competition ELISA method was used to distinguish the specificity of two anti-peptide-BSA conjugate PhAbs isolated from the 'single pot' phage antibody repertoire. With the exception of the following two steps the procedure was essentially same as for the standard ELISA. Firstly peptide-BSA conjugate antigen coating was at a concentration of 1 μg/ml. Secondly the phage antibodies (G9, G11) were premixed with a range of dilutions of soluble

G11 scFv (from 1:4 to 1:1024 dilution). Bound phage antibody was detected with anti-M13/HRP conjugated second antibody. In order to test if PhAbs bound specifically to the GDD-BSA related antigen, a similar inhibition assay was performed by adding 10 μg/ml (final) GDD-BSA conjugate to the PhAb solutions and pre-incubating for one hour at room temperature. Subsequent steps were as for the standard PhAb ELISA.

Another competition/inhibition ELISA was performed to compare the IgG binding activity of phage-displayed protein A and native protein A. A range of different concentrations of Human IgG, mouse IgG (3 μg/ml to 500 μg/ml) or native protein A (3 μg/ml to 100 μg/ml) were used as competitors to reduce phage protein A binding to rabbit IgG immobilised on ELISA plates. These competitor solutions (in PBS) were mixed with phage protein A just before being added to ELISA wells coated with rabbit IgG. Otherwise the procedure was as described for the standard ELISA.

2.6 Expression of proteins in *E. coli* and affinity purification

Two bacterial expression systems were used to express potato virus Y (PVY) RNA dependent RNA polymerase (RdRp) and scFvs. By using the T7 promoter based expression system, the RdRp was expressed in an insoluble form in the *E. coli* cytoplasm. For the expression of soluble scFvs in the *E. coli* periplasm and cytoplasm, a maltose binding protein fusion system was used.

2.6.1 Expression of RdRp in *E. coli*

2.6.1.1 Cloning of RdRp gene fragment into the pRSET expression vector

The RdRp gene fragment was amplified from a plant expression vector (pBI220.5:B4, provided by Dr G. Foster) by using PCR. Two primers were synthesised for priming from 5' and 3' regions of full length RdRp. The back primer introduced an *Nco* I restriction site at the 5' end of the fragment, and the forward primer introduced a *Bam*H I restriction site just

downstream of the stop codon (TAA) of the fragment. In addition a $(His)_6$ tag was introduced by the forward primer. The fragment was cloned into a phagemid pRSETB expression vector, in which the RdRp gene was fused to 3' of the gene of a $(His)_6$ short peptide and regulated by the T7 promoter. The construct was transferred into *E coli* strain TG1.

2.6.1.2 Expression of RdRp in *E. coli*

An overnight culture was prepared by inoculating 5 ml of DYT (containing ampicillin at 100 μg/ml) with a single recombinant *E. coli* colony and growing at 37°C with shaking. A aliquot of this overnight culture (0.1 ml) was use to sub-inoculate 100 times DYT (10 ml) containing Ampicillin at 100 μg/ml concentration. The culture was allowed to grow at 37°C with vigorous shaking to an early log phase density (OD_{600}=0.3). After this time IPTG (isopropylthiogalactoside) was added to a final concentration of 1mM and the culture was incubated for a further 1 hour, when the cell density was measured again. An aliquot of 1 ml of this culture was transferred to a sterile tube and incubated for 3 hours. The remaining 9 ml of culture was infected with M13/T7 phage at a ratio of 5 pfu/cell (based on phage titre) and incubated for 3 hours. Cells were precipitated by centrifuging at 3500 rpm for 10 min. The pellet was re-suspended in 1x sample buffer, and boiled for five minutes before electrophoresis on 12% SDS-PAGE gels and western blotting or Coomassie staining.

2.6.2 MBP :: scFv fusion protein expression in *E. coli*

This method was used to express maltose binding protein (MBP) and scFv fusion proteins in the *E. coli* cytoplasm and periplasm using vectors of pMAL_c2 and pMAL_p2. The scFv gene of interest was amplified by PCR with primers of SPL5'(*Xba* I) and SPL3'(*Xba* I). The *Xba* I fragment was cloned into the *Xba* I site of pMAL_c2 and pMAL_p2 and transferred

to *E. coli* strain W3110wt. In some experiments pMAL_c2 constructs were transferred to strain A304 *TrxB*$^-$.

50 ml of DYT (containing 0.2% glucose and 100 μg/ml) was inoculated with 0.5 ml of overnight cultures of cells containing the fusion plasmids. The cultures were allowed to grow at 37°C with good aeration to 2 x 10^8 cells/ml (OD_{600} = 0.5), and then IPTG was added to final concentration of 0.3 mM. The induction was carried out for 2 to 3 hours with continued incubation under the conditions described above. (At this stage, in the pilot experiment, an aliquot of 1 ml culture was taken to act as a negative control and was transferred to a new sterile tube and incubated at same condition without the addition of IPTG). The cells were harvested by centrifugation at 3500 rpm for 10 min. Depending upon whether or not the cells contained pMAL_c2 or recombinants the pellets were treated by one of the following two methods,

Method A. *Fusion protein expression in the cytoplasm of E. coli using pMAL_c2:* The cell pellet was re-suspended in 5 ml of sonication buffer [20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1mM EDTA, 1 μg/ml of pepstatin, 1 μg/ml of leupeptin, 2 μg/ml of apopeptin and 1mM PMSF (phenymethylsulfonyl fluoride)]. The sample was frozen in -20°C overnight and thawed in cold water. After this time, the sample was placed in an ice-water bath and sonicated for three short pulses of 10 seconds with 10 second breaks. The release of protein from *E. coli* was monitored by using Bradford assay. Sonication was repeated several times until the released protein reached a maximum level. The sample was centrifuged at 13, 000 rpm at 4°C for 20 min. The supernatant solution was transferred to a clean tube on ice. This represents the crude, soluble *E. coli* extract. The pellet (insoluble fraction) was re-suspended in 5 ml of sonication buffer described above. Both soluble and insoluble fraction were then analysed by SDS-PAGE gel and Western blotting. The soluble fraction was also applied to an amylose column for affinity purification of the fusion protein.

Method B. *Fusion protein secreted to the E. coli periplasm using* pMAL_p2: The cell pellet was re-suspended in 20 ml of 30 mM Tris-HCl, 1 mM EDTA 20% sucrose, pH8.0, and incubated for 10 min at room temperature with shaking. The sample was centrifuged at 9000 rpm at 4°C for 10 min. The supernatant was saved on ice in a clean tube. The pellet was re-suspended in 20 ml of ice-cold 5 mM $MgSO_4$, and then the sample was incubated in an ice-water bath for 10 min with shaking and then centrifuged as above. The supernatant was saved as above. The pellet was re-suspended in the 20 ml of 30 mM Tris-HCl, 1mM EDTA, pH8.0. The three fractions so produced were all analysed by SDS-PAGE and Western blotting. The antigen binding function of the scFv fusion was tested by the standard ELISA methods.

2.6.3 Affinity purification of synthesised proteins

The methods for affinity purification of synthesised proteins are essentially as described by the manufacturers. Exceptions are that sometimes small (50-100 ml) *E. coli* cultures was used, and all buffers contained 2 mM DTT and a cocktail of four proteinase inhibitors, such as 1 μg/ml of pepstatin, 1 μg/ml of leupeptin, 2 μg/ml of apopeptin and 1mM PMSF. The whole protein purification procedure was carried out at 4°C.

2.7 Molecular modelling of antibody combining sites

The HyHEL-10, G123, G123.N, and G123.N.D Fv models were built by the Immunoglobulin Domain Modelling Program (AbM) version 2.0, (Oxford Molecular Ltd.) (Pederson, *et al.*, 1992), using the AbM standard method and CAMAL method. In the standard method the framework was constructed using the light and heavy chains of 3hfm and CDRs were built as canonical loops except HCDR3 which involved the use of a database search. With the CAMAL method the framework was constructed using the light chain of antibody 1ggi (homologous score 64.22% to both HyHEL-10 and G123) and the heavy chain of antibody

1baf (homologous score 76.79% to HyHEL-10, 83.93% to G123). CDRs built using the combined database search / CONGEN method and database search only method for H3 were as follows:

L1: RAS[Q(SIG)N)NLH

L2: Y[A(SQS)I]S

L3: QQ[S(NSW)P]YT

H1: GDS[I(TSD)Y]WS

H2: YV[S(YSG)S]TY

H3: WDGDY

The residues in brackets were reconstructed using CONGEN and parentheses indicate chain closures. The side chains were built using the *Iterative* algorithm in CONGEN.

The final models were analysed using Insight II (Version 2.3.5, Molecular Modelling Program, Biosym Technologies, Inc, San Diago, CA, 1994) and Quanta (Version 4.0) for molecular similarity analyses.

CHAPTER 3

Filamentous Bacteriophage Surface Display of Functional IgG Binding Domains of Staphylococcal Protein A and Bifunctional Protein A::anti-phytochrome A Single-Chain Fv Fusion Proteins: Functional Interactions between the Domains of the Fusion Protein

3.1 Introduction

Artificial single-chain, multidomain-multifunctional proteins made by means of protein engineering are widely known as new structural and new functional proteins. By making fusion with coat proteins (pIII, pVI or pVIII) of filamentous bacteriophage, many different polypeptides have been displayed on phage surface. One of the many artificial functional single-chain proteins which have been created by protein engineering is based upon the IgG-binding domain(s) of *Staphylococcus aureus* protein A (SpA) (Uhlen *et al.*, 1983; Djojohegoro *et al.*, 1994; Kushwaha *et al.*, 1994). SpA is a cell-wall associated protein of *S. aureus* having an extracellular portion that binds to the Fc of IgG. SpA contains five highly homologous, independent binding domains, identified as fragments A, B, C, D and E (or FA, FB, FC, FD and FE). Each of these domains possesses about 58 amino acids and binds with high affinity to many types of immunoglobulins, including IgG (Moks *et al.*, 1986), human IgM, IgA and IgE (Sasano *et al.*, 1993). The protein A binding sites on human immunoglobulins are located at the Fc and VH regions of VH(III) families (Hillson *et al.*, 1993). The structure of the single FB IgG binding domain of protein A has been resolved by

both X-ray crystallography and 3D NMR (Deisenhofer, 1981; Gouda *et al.*, 1992). Structural analysis has revealed that two α-helices in the N-terminal portion, formed by residues Gln9-Leu17 and Glu25-Asp36, contribute to the binding activity of FB to C_H2 and C_H3 of IgG. Comparison of the structure of free FB in solution and the crystallised FB-Fc complex indicated a significant conformational change in the C-terminal region of FB (Torigoe *et al.*, 1990; Gouda *et al.*, 1992).

FB has been fused to an anti-digoxin scFv and the resulting fusion protein possessed virtually the same scFv and effector binding properties as the parent molecules (Tai *et al.*, 1990). Five IgG binding domains of protein A (Nilsson and Uhlen, 1985) have also been fused to an anti-phytochrome A scFv, and the bifunctional fusion protein was secreted from bacteria (Gandecha *et al.*, 1992). The bacteriophage display system has also been used to display the single IgG binding domain FB, and the four IgG binding domains (FE, FD, FA, and FB) (Djojohegoro *et al.*, 1994; Kushwaha *et al.*, 1994). The resulting protein A phage shows IgG binding activity, and can be enriched from a mixed population of phage by a few rounds of selective panning against IgG. In previous work on phage display of IgG binding proteins, a phage library was created for displaying polypeptides encoded by randomly fragmented chromosomal DNA from *S. aureus* strain 8325-4. A new IgG-binding polypeptide has also been discovered by using the phage library (Jacobsson and Frykberg, 1995). Furthermore, based on SpA structure, a combinatorial alpha-helical bacterial receptor library has been created on filamentous phage surface, and new receptors with multiple substitutions been selected (Nord *et al.*, 1995).

In multidomain-multifunctional proteins, the structural domains show independent folding and independent assembly (Jaenicke, 1987). The folding process of multidomain-multifuntional proteins has been studied in detail by direct measurements of the activity of separate domains following refolding of proteolytically derived fragments (Jaenicke, 1987; Vaucheret *et al.*,

1987). A study of interdomain connections of artificial single-chain multidomain fusion proteins also revealed that the linker between domains could strongly influence the correct folding of the whole single-chain protein (Brinkmann *et al.*, 1992). In an impressive demonstration of the possibility of producing multifunctional single-chain proteins, a transforming growth factor α (TGFα)-anti-Tac scFv-PE40 fusion protein was made in which the two effectors and the antigen-binding site were all shown to be functional (Batra *et al.*, 1990a). Clearly, artificial fusion proteins must involve multidomain folding.

In order to obtain further information on the functional relationships of multidomain and multifunction fusion proteins, a truncated protein A (five IgG binding domains with a truncation at the last IgG binding domain) and its fusion with an anti-phytochrome A scFv were chosen as model proteins for use in the study of effects of interactions between the fusion partners on the function of the fused domains.

3.2 Results

3.2.1 Bacteriophage display of IgG binding domains of protein A

3.2.1.1 Construction of phagemid vectors

For displaying truncated protein A fragments on the surface of M13, the phagemid vector (pComb3PA$^-$) (Fig. 3-1) was constructed to produce a fusion between the protein A fragment and the C-terminal domain of pIII protein (C-pIII). The truncated protein A gene fragment was generated from a protein A fusion protein expression vector pRIT5 (Nilsson and Uhlen, 1985). In the present study, the original protein A signal peptide and the polylinker of pRIT5 were removed. The IgG binding regions remain as in pRIT5 in which the amino acid sequence of the last IgG binding domain (FC) is truncated after Asp36 (Nilsson *et al.*, 1985; Nilsson and Abrahmsen 1990). Two restriction sites were introduced by a back primer of 5'-G TCT AGA CTG CAG ATG GCG CAA CAC GAT GAA GC-3' (*Pst* I), and a forward primer of 5'-GAA

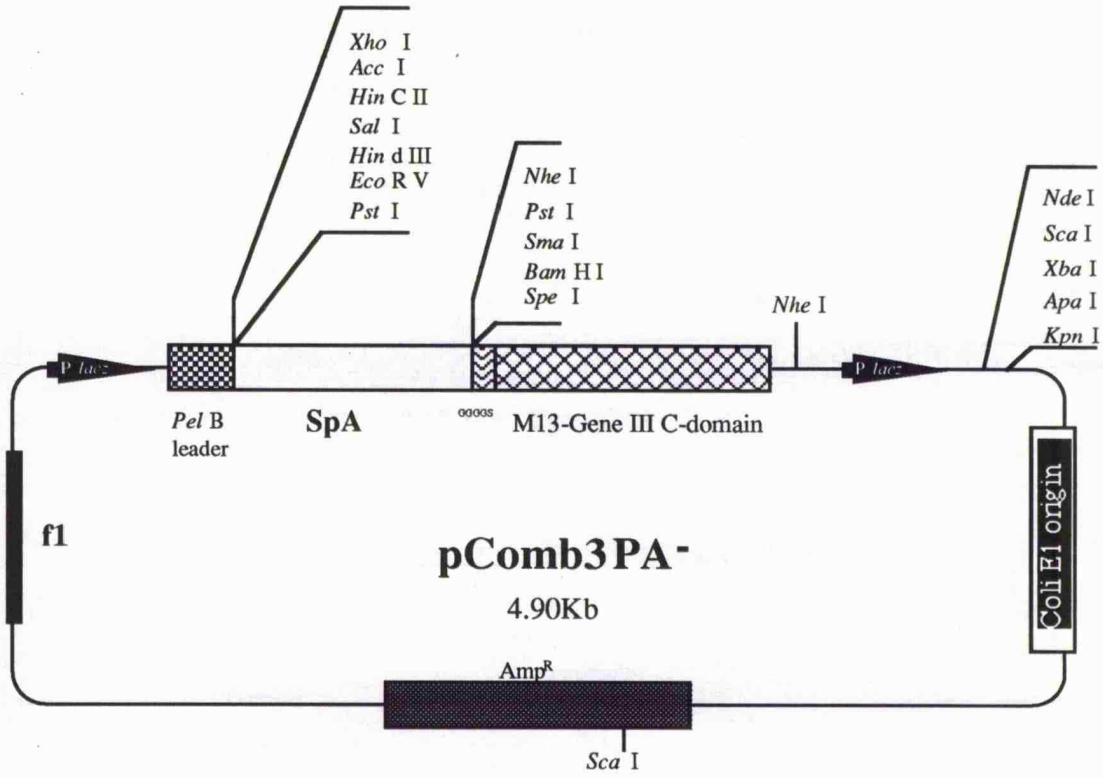


Fig. 3-1. Map of the protein A phage display vector. The SpA fragment was cloned into the pComb3 vector at the *Pst* I site. Apart from this the original pComb3 vector was not modified (see Fig. 2-1).

TCC GCT AGC GTC TTT AAG GC-3' (*Nhe* I). By use of an intermediate plasmid (pSK$^-$, Stratagene, La Jolla, CA), the *Pst* I fragment of the protein A-encoding gene was cloned into the *Pst* I site of phage display vector pComb3 as an in frame fusion with C-pIII-encoding gene. The 3'*Pst* I site was from pSK$^-$, and this strategy introduced 14 residues (ValSerGly SerProGly LeuGlnPro GlyGlySer ThrSer) at the C-terminal of the SpA fragment, and 21 residues (AlaGlnVal LysLeuLeu GluValAsp GlyIleAsp LysLeuAsp IleGluPhe LeuGlnMet) at the N-terminal of the mature SpA protein. In this construct, the protein A::C-pIII fusion gene is under control of the *lacZ* promoter. When used with an *E. coli* strain such as XL-1-blue which contains the *lac*I^q gene and which consequently over-expresses the *lac* repressor (Bass *et al.*, 1990) fusion protein synthesis is relatively tightly regulated. The N-terminal *pelB* signal peptide directs secretion of the fusion protein to the bacterial periplasm, and because of the hydrophobic tail located at the C terminal part of C-pIII, the fusion protein becomes anchored in the inner membrane. In order to minimise interactions between the fusion proteins, there is a flexible spacer (GGGGS) inserted between SpA and C-pIII (Barbas *et al.*, 1991). This construct was used to display four intact IgG binding domains (E, D, A, B) and the first two α-helices of IgG-binding domain C (C$^-$). Thus, the orientation of the primary structure of the protein A::C-pIII fusion is, commencing at the amino terminal, E-D-A-B-C$^-$-(GGGGS)-pIII- .

3.2.1.2 Effects of growth conditions on production of protein A phage

After superinfection with helper phage, VCS M13, and with or without IPTG induction , *E. coli* XL1-blue cells carrying phagemids of pComb3PA$^-$ produced phages assembled with fusion proteins of SpA::C-pIII. The yield of phage particles in terms of colony forming units was 5-10 x 10^{10} cfu per ml of culture supernatant. Yield and rabbit IgG binding activity were not directly correlated, and the proportion of functional fusion protein was influenced by the induction conditions used. When analysed by western blotting, IgG binding activity was increased following induction with increased IPTG concentration (Fig. 3-2). In this experiment

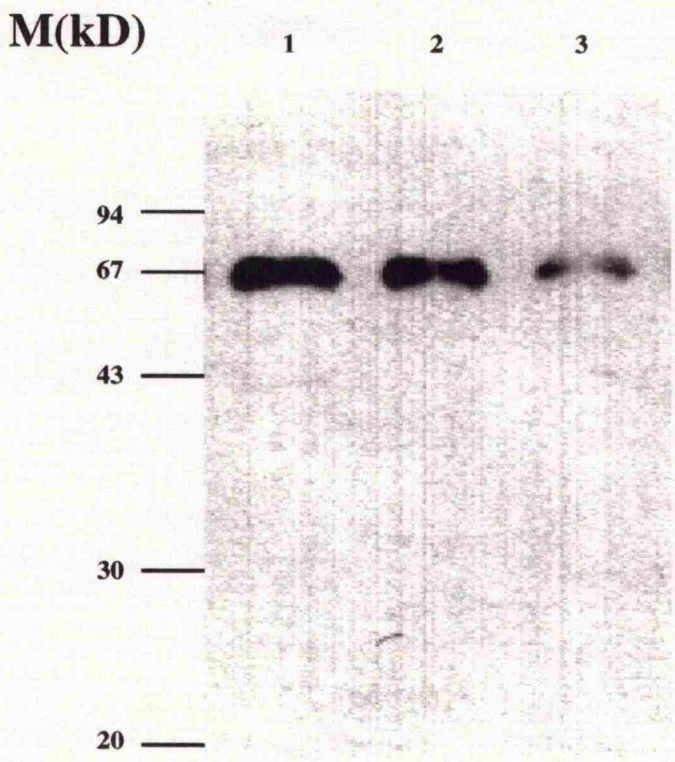


Fig. 3-2. Effects of IPTG induction on the expression of the SpA:C-pIII fusion protein. Protein A phage samples were prepared by infecting recombinant *E. coli* strain XL-1-Blue with helper phage VCSM13, with induction by 1mM IPTG (**lane 1**), 0.2 mM IPTG (**lane 2**) and without IPTG induction (**lane 3**). Equal titres (cfu) of protein A phage particles were loaded onto a 12% SDS-PAGE gel and electrophoresed for about three hours at 100V. The gel was blotted onto nitrocellulose by semidry blotting. Protein A activity was detected with rabbit IgG alkaline phosphotase conjugates followed by incubation with BCIP/NBT at room temperature for 10 min.

each well contained the same number of phage particles and the gel was run under reducing conditions. The protein A::C-pIII fusion protein run at the 67 kDa position. Following transfer to nitro-cellulose membrane and prolonged incubation to optimise refolding, functional SpA::C-pIII fusion protein was detected by direct binding of a rabbit IgG alkaline phosphatase conjugate. After development with substrate (BCIP/NBT), the signals resulting from different induction conditions indicated that the amount of functional fusion protein produced under different induction conditions increased in the following order; zero IPTG induction < 0.2 mM IPTG induction < 1 mM IPTG induction.

3.2.1.3 Purification of protein A phage on human IgG agarose column.

Phage displaying SpA were affinity purified from both crude bacterial culture supernatant and a mixed phage population. The mixed phage was prepared by mixing 10^8 (cfu) SpA phage with non-specific phage particles not displaying SpA (10^{10} cfu), in a total volume of 500 µl. The protein A phage was collected from the mixture by binding to human IgG agarose. About 10^6 (cfu) phage were eluted in a total volume of 500 µl. The purified phage (about 10^2 cfu) was used to infect freshly prepared *E. coli* XL1-Blue, and protein A function was detected directly in lysed bacterial colonies (Fig. 3-3), by the use of rabbit alkaline phosphatase conjugate.

3.2.1.4 Comparison of specificities of protein A phage and native protein A

A competition assay was used to test the specificity of SpA displayed on the phage surface. The results demonstrated that free protein A and human IgG strongly competed with the binding of SpA phage to immobilised rabbit IgG . However, mouse IgG which binds weakly to native protein A failed to compete with the binding of SpA phage to immobilised rabbit IgG (Fig. 3-4).

3.2.2 Bacteriophage display of protein A::scFv fusion proteins

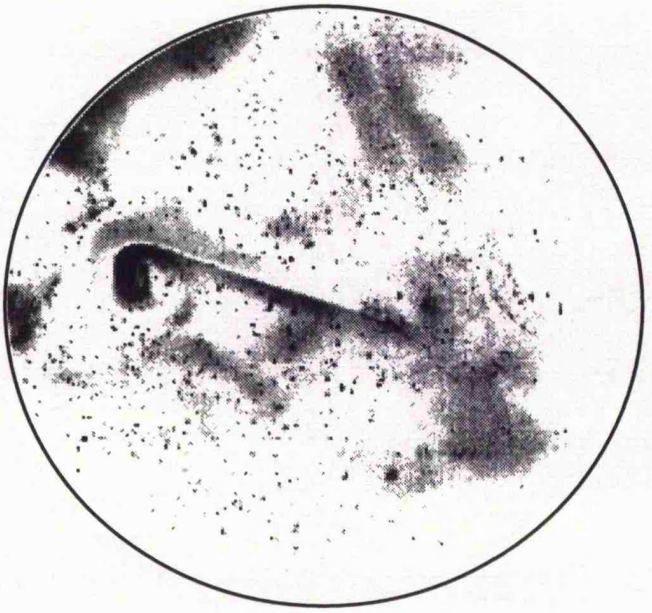
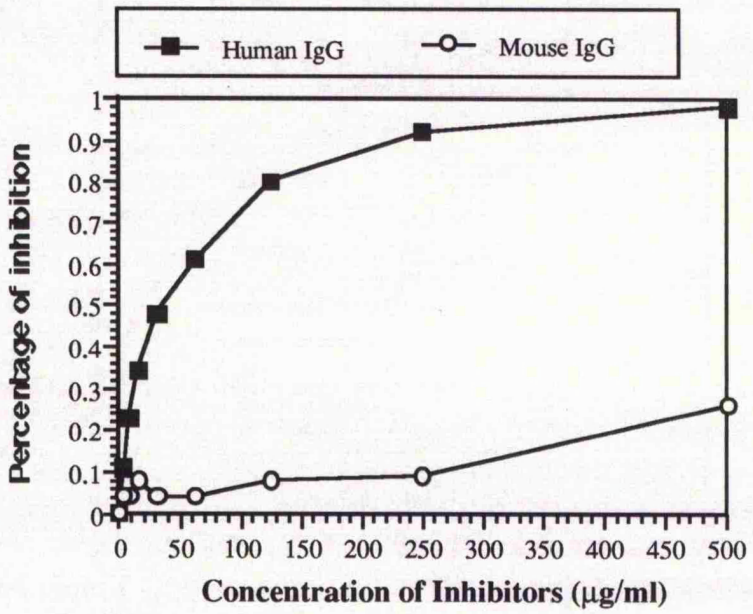


Fig.3-3 Detection of protein A activity directly on protein A phage-infected colonies. After colonies were transferred to nitrocellulose and lysed (see 2.4.10), protein A activity was detected by incubation with rabbit alkaline phosphatase conjugates followed by BCIP/NBT at room temperature. The small dots indicate colonies which produced functional protein A.

A



B

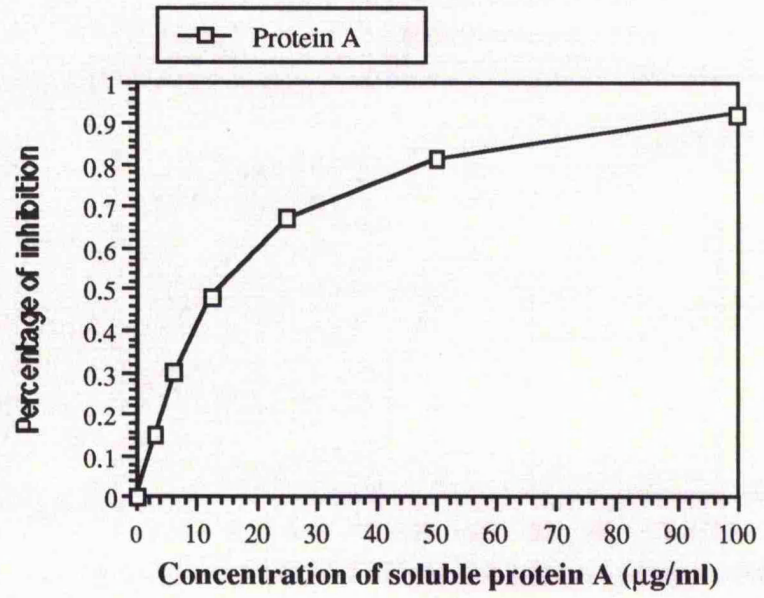


Fig. 3-4. Inhibition assays for protein A phages. A. Inhibition of binding of SpA phage to rabbit IgG by Human and mouse IgGs. Binding of SpA phages to rabbit IgG was reduced over 90% in ELISA signal when human IgG was added to 200 μg/ml. Mouse IgG weakly inhibited SpA phage binding to immobilised rabbit IgG. **B.** Competition between soluble SpA and SpA phage binding to immobilised rabbit IgG. Soluble SpA at 100 μg/ml reduced SpA phage binding by 90%.

3.2.2.1 Construction of fusion protein display vectors

One of the two promoters contained by pComb3 is not required for the present study and therefore a new version of the vector, namely pC3$^-$ was generated by eliminating the second *lacZ* promoter. Two protein A display vectors were constructed, one based on pComb3 (pComb3PA$^-$) and another based on pC3$^-$ (pC3$^-$PA$^-$) (Fig. 3-5). No functional differences were detected between protein A phage particles generated by either of these vectors. Using the pC3$^-$PA$^-$ protein A phage display vector, two protein A scFv fusion constructs were made as shown in Figure 3-7A, B. The pC3$^-$AS32PA$^-$ phage display construct was made by fusing the AS32 gene fragment (*Xba* I/ *BstE* II, *BstE* II 3' overhang was blunted) to the C-terminus of protein A and the N-terminus of the C-domain of pIII at the *Nhe* I/*Sma* I sites. The construct was also made in which two residues (AlaArg) were introduced between the second α-helix of IgG-binding domain C and the N-terminus of the AS32 light chain. As in construct pComb3, there is a flexible linker GGGGS between the scFv AS32 and the C-pIII protein. The construct was screened by using a DNA hybridisation method with radio-labelled AS32 fragment as probe. In the blot shown in Figure 3-6, there are ten positive colonies. One of these contained the AS32 fragment in the correct orientation which could produce protein A::AS32::C-pIII fusion protein and displayed on phage surface.

A second construct was made in which the AS32 scFv gene was located at the amino terminus of the triple fusion protein (Fig. 3-7B). In this case the C terminus of the scFv gene was fused to the N terminus of the protein A gene and the C terminus of protein A gene was fused to the N terminus of C-pIII. No linker sequences were introduced between AS32 and protein A in either construct.

3.2.2.2 Bifunctional display of protein A::AS32

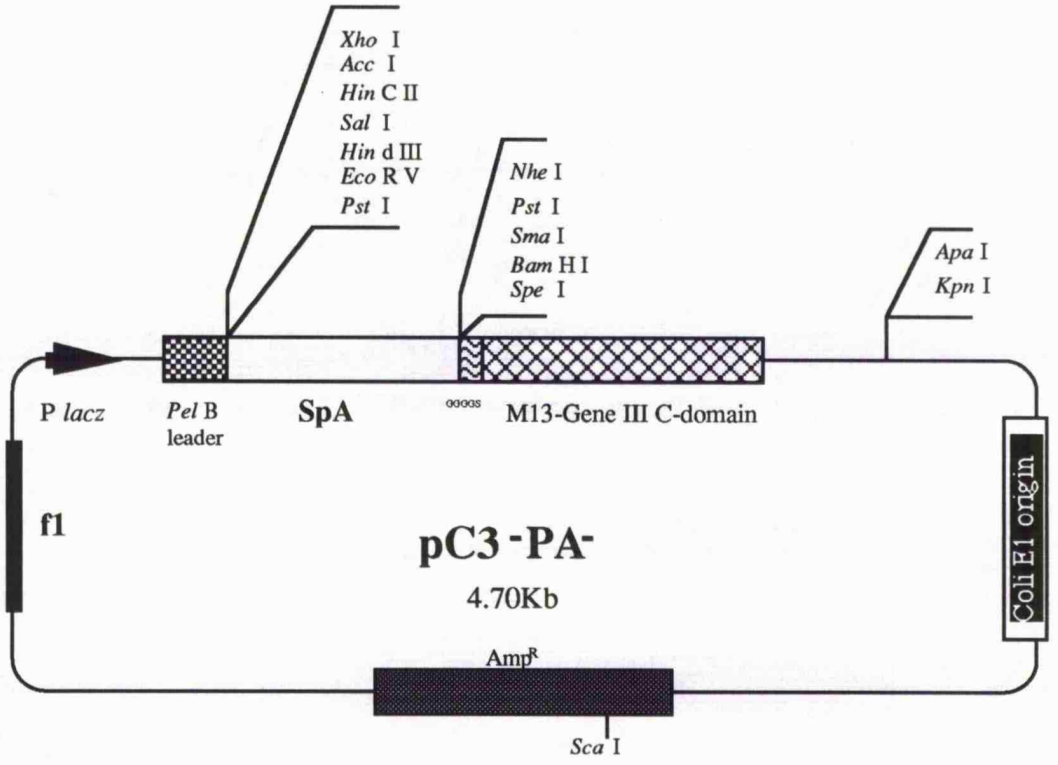


Fig. 3-5. Modified version of protein A phage display vector. One of the lacZ promoter/operators present in pComb3 has been eliminated.

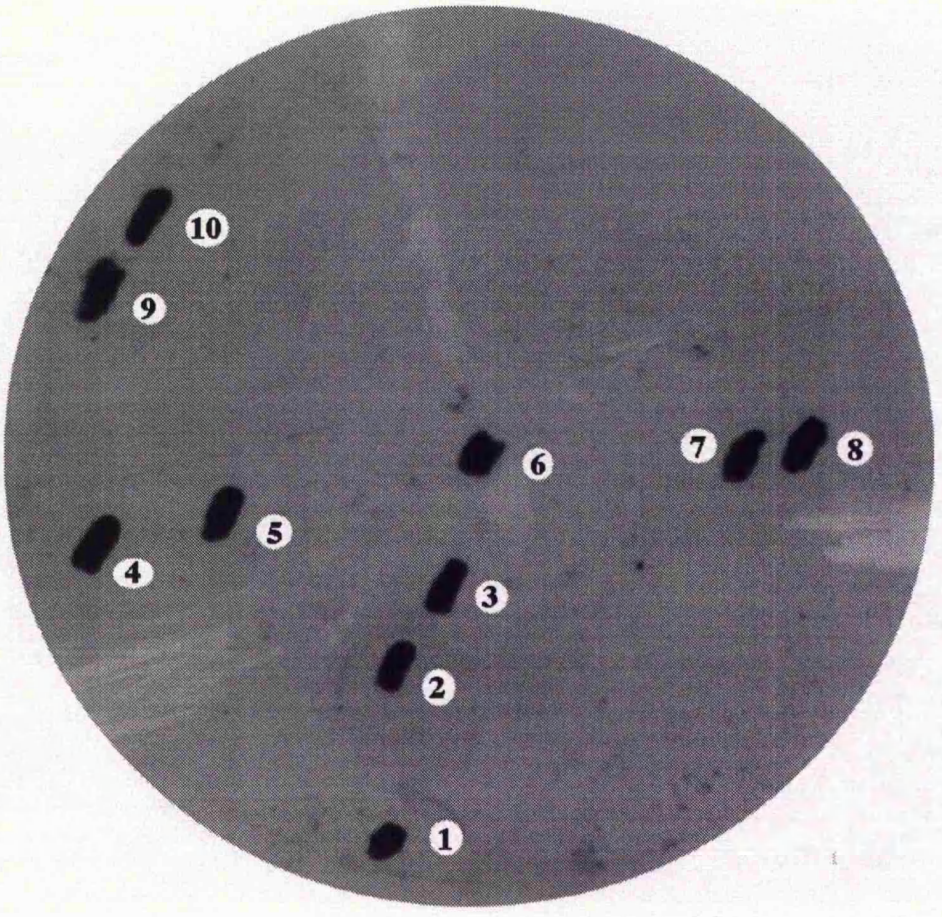


Fig. 3-6. DNA hybridisation screen of pC3-AS32PA- construct. Of 10 positive colonies, one contained pC3-AS32PA- in the correct orientation.

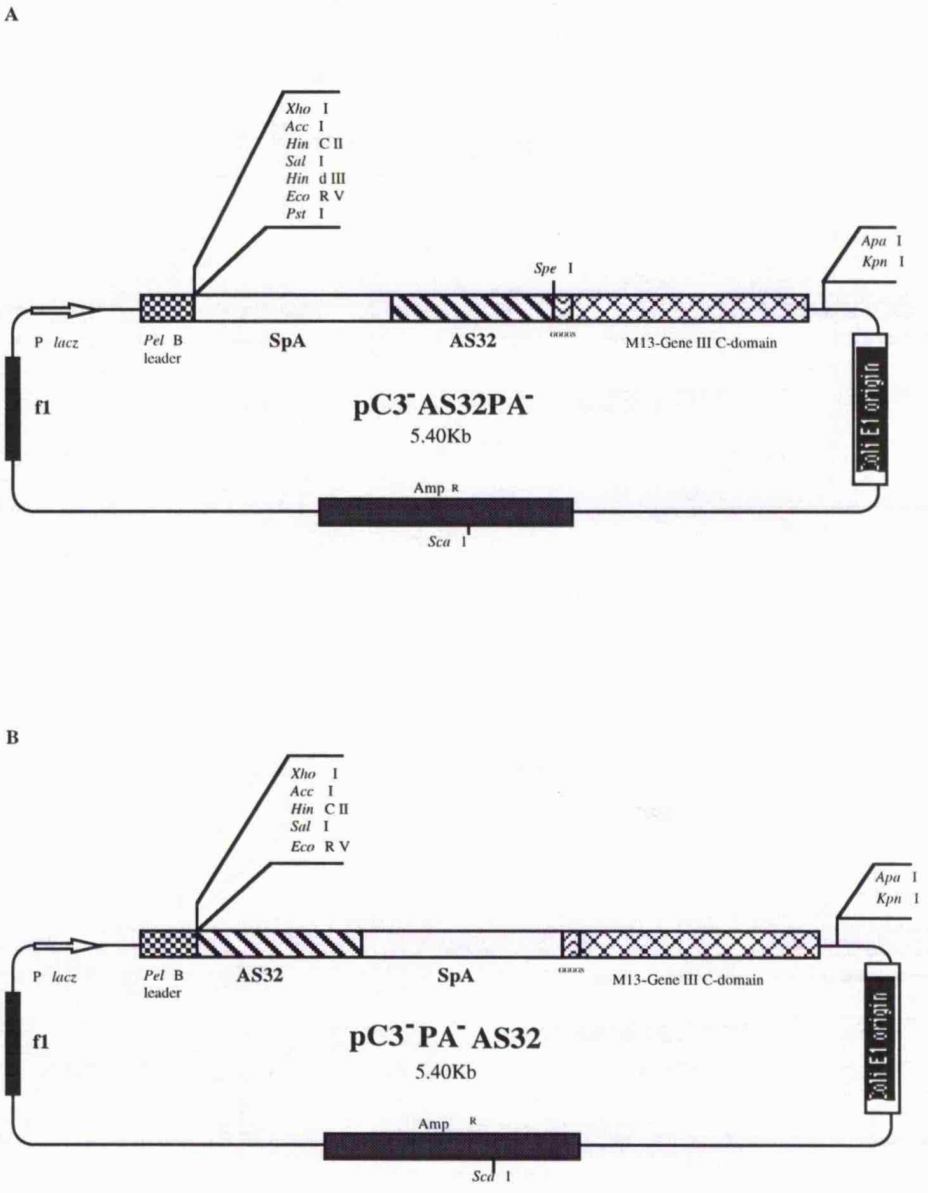


Fig. 3-7. Maps of fusion protein phage display vectors. A. pC3-AS32PA- vector. The scFv AS32 gene was inserted in the middle of the triple fusion gene, resulting in the fusion of the C terminal of scFv AS32 to the N terminal of C-pIII. **B.** pC3-PA-AS32 vector. The scFv AS32 gene was placed at the 5' end of the triple fusion gene, resulting in exposure of the N terminal of scFv AS32.

Both protein A::AS32 and AS32::protein A fusion proteins showed binding activity to phytochrome A and rabbit IgG when displayed on the phage surface. This was demonstrated in experiments which involved affinity purification of fusion protein phage particles on human IgG columns, followed by the detection of binding activity to immobilised phytochrome A by ELISA using sheep anti-phage peroxidase conjugated IgG to detect bound phage. IgG-binding activity was also detected using a similar ELISA with rabbit IgG bound to the microtitre plate. The eluted phage from the human IgG column bound both phytochrome A and rabbit IgG (Fig. 3-8A, B). Although both orientations of fusion proteins bound both IgG and phytochrome A, their binding properties were not the same. Phage particles displaying the fusion protein, scFv AS32::SpA::C-pIII bind to both phytochrome A and rabbit IgG as strongly as AS32 scFv or protein A displayed separately on the phage surface. However, phage particles displaying the fusion protein, SpA::scFv AS32::C-pIII, possess the same IgG-binding activity as protein A phage, but weak phytochrome A binding activity compared with scFv AS32 phage (Fig. 3-9).

3.2.2.3 Interference between protein A and AS32 Domains

To investigate possible interferences between protein A and AS32 in the triple fusion protein, a series of western blotting experiments were carried out to detect epitopes of scFv AS32 and protein A, and also the protein A IgG-binding activity (Fig. 3-10A, B, C, D) An equal titre of PEG concentrated phage particles were separated on denaturing SDS-PAGE and blotted onto nitro-cellulose. Protein A was detected by using a mouse monoclonal anti-SpA antibody (MAb) followed by the goat anti-mouse peroxidase conjugate and developed using the enhanced chemilumincense method. The advantage of using mouse MAb is that protein A binding to mouse IgG is very weak, and therefore interference with the detection of the protein A epitope by non -specific IgG biding is minimal. The protein A::C-pIII fusion was detected as a band with apparent molecular weight of about 67 kDa and the triple fusion of

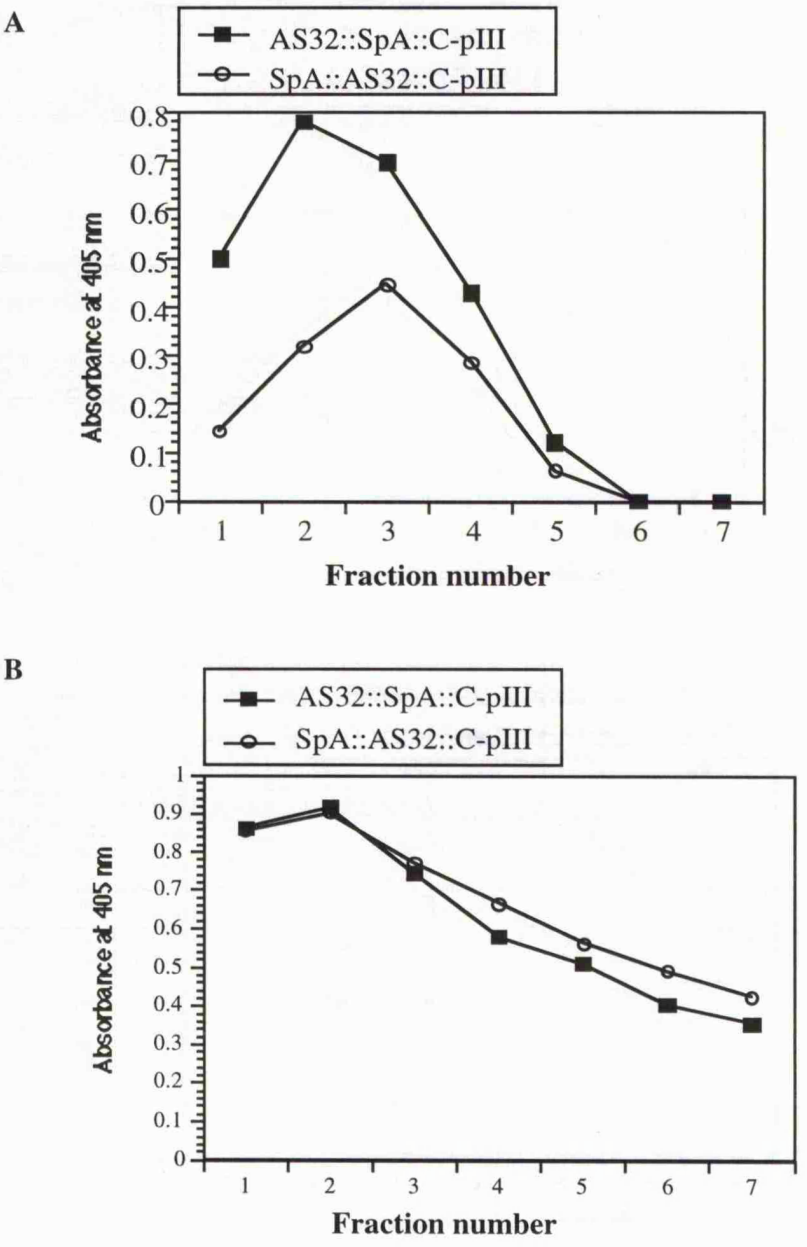


Fig. 3-8. Detection of bifunctionallity of scFv AS32::SpA and SpA::scFv AS32 fusion phages. Equal titres of fusion phage particles were applied to human IgG agarose affinty column, 2ml of eluate was collected in 200 µl aliquots. The eluates were used to test binding activity to Phytochrome A and rabbit IgG immobilised on ELISA plates. **A.** Phytochrome A binding acitvity of eluates. The scFv AS32::SpA::C-pIII fusion phage particles gave a relatively high signal in the phytochrome A coated ELISA wells compared to SpA::scFv AS32::C-pIII fusion phage particles. **B.** Rabbit IgG Binding activity Eluates of scFv As32::SpA::C-pIII or SpA::scFv aS32::C-pIII fusion phages bind to rabbit IgG immobilised on ELISA plates.

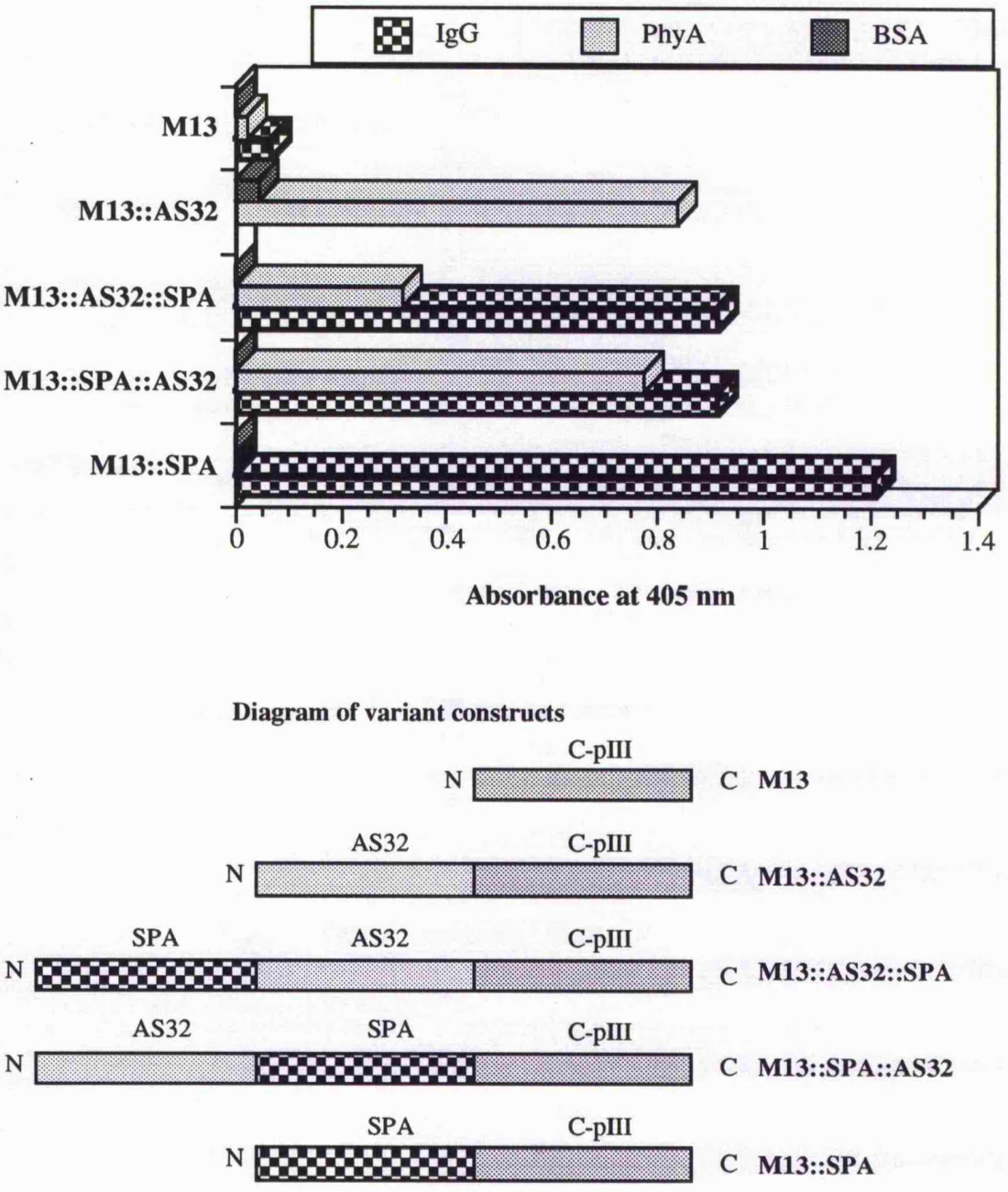
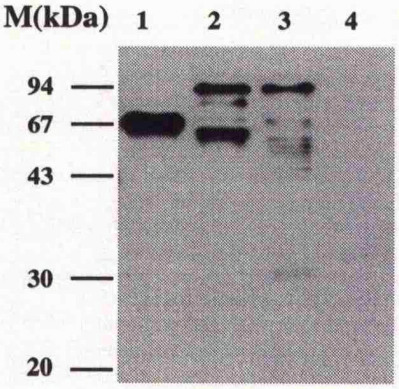


Fig. 3-9 Specificity of fusion phage particles. Binding activity of scFv AS32::SpA::C-pIII (M13::SPA::AS32) to phytochrome A and rabbit IgG. SpA::scFv AS32::C-pIII (M13::AS32::SPA) showed comparable rabbit IgG binding acitvity with SpA phage (M13::SPA) and SpA::AS32 phage, but lower phytochrome A binding activity than AS32 phage and SpA::AS32 phage. A diagram illustrating the fusion phage variants is shown below.

Fig. 3-10. Detection of antigens and Functionality of Fusion Phage Particles with Different Orientations of Fusion Partners. Equal titres of fusion phage particles were cracked in the sample buffer and loaded on 12% SDS-PAGE. Separated proteins were transfered to nitrocellulose. The blots were detected with peroxidase conjugates and followed by a chemiluminscence detection kit. **Lane 1**: Protein A fusion phages. The protein A::C-pIII fusion migrated to the 67KD position. **Lane 2**: ScFv AS32::SpA::C-pIII (M13::SPA::AS32) fusion phages. The scFv AS32::SpA::C-pIII fusion migrated to the 95KD position. **Lane 3**: SpA::scFv AS32::C-pIII (M13::SPA::AS32) fusion phages. The SpA::scFv AS32::C-pIII fusion migrated to the 95KD position **Lane 4**: ScFv AS32 fusion phages. The scFv AS32::C-pIII fusion migrated to the 65KD position. **Blot** A.: A nonspecific rabbit serum was use to detect the protein A binding activity. Proteins from AS32 fusion phage were not bound by nonspecific rabbit serum. Protein A activity was detected from all other sources of fusion proteins. **Blot B**: A mouse monoclonal anti-protein A antibody was used to detect the protein A epitope in proteins from the four different fusion phage particles. Some proteolysis was found in two SpA/scFv fusion phage particles, but very little in SpA phage particles. **Blot C**: AS32 scFv was detected by rabbit anti-scFv AS32 serum. The rabbit Fc binding sites on protein A were blocked by human IgG, as indicated by the absence of significant signal with the SpA-C-pIII fusion. The AS32 conponent of the fusion proteins were not satisfactorily detected on this blot. Many bacterial protein which migrated between the 25 to 40 KD position interacted with the rabbit serum (which had been raised against bacterially produced AS32 protein). **Blot D**: Nonspecific binding of the rabbit serum was eliminated by the inclusion of bacterial lysates in the first antibody dilution buffer. Both the scFv AS32 epitope and SpA Fc binding activity was detected by the rabbit serum. Subsequently, the 95KD band of SpA::scFv AS32::C-pIII fusion protein was detected on this blot.

A


M(kDa)
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2
3
4
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67
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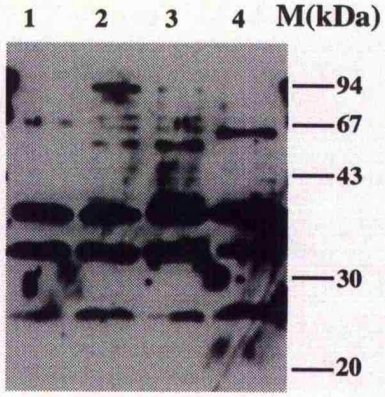
C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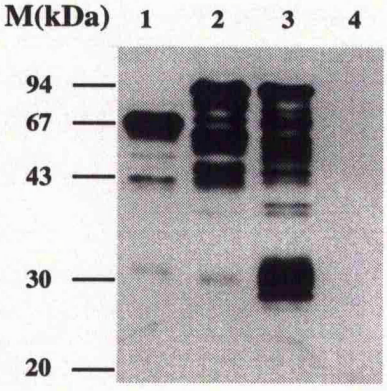
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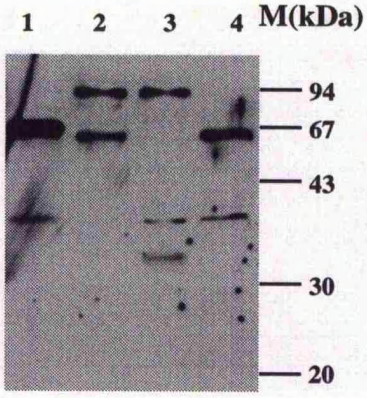
D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4
M(kDa)
94
67
43
30
20

protein A::scFv AS32::C-pIII and scFv AS32::protein A::C-pIII was detected as a band of about 95 kDa. There was no cross reaction between anti-protein A MAb and scFv AS32::C-pIII fusion protein (Fig. 3-10B). The Fc binding activity of protein A was detected by using a non-specific rabbit IgG serum followed by mouse anti-rabbit IgG peroxidase conjugate (Fig. 3-10A). In the lane containing the scFv AS32::protein A::C-pIII fusion phage, a second strong band (about 65kDa) was also detected by the rabbit serum, possibly representing an active proteolytic fragment of protein A (Fig. 3-10 A, D). A comparison of these two blots revealed that some non functional protein A fragments, detected by anti-protein A MAb, were also present. The detection by the anti-protein A MAb of more putative proteolytic fragments in the triple fusion sample than in the protein A::C-pIII sample suggested that the triple fusion was more susceptible to proteolysis (Fig. 3-10B).

For the detection of the scFv AS32, a rabbit anti-scFv AS32 serum was used along with human IgG to block the IgG-binding activity of SpA. The scFv AS32::C-pIII fusion was detected as a 65 kDa band (Fig. 3-10C, D). Since the rabbit anti-scFv AS32 was raised against partially purified bacterial product, many bacterial background bands appeared in the blots used for the detection of the AS32 scFv (Fig. 3-10C). However, this background could be eliminated by including an appropriate amount of bacterial lysate (1mg/ml) in the anti-AS32 scFv antibody solution (Fig. 3-10D). Because the rabbit IgG can bind very tightly to protein A, the blot was first incubated with human IgG in order to block the rabbit IgG binding sites on protein A. Further improvement in background was achieved by including human IgG in the first antibody solution (rabbit anti-scFv AS32 serum). The results show that the IgG binding sites on protein A were completely blocked by human IgG, and only the AS32 scFv was detected by the antiserum (Fig. 3-9 C). However, this procedure did not allow satisfactory detection of the AS32 scFv in the triple fusion protein produced by the construct pC3^{-}AS32PA^{-}. It is possible that the epitopes recognised by the anti-AS32 antibodies might be masked by human IgG binding to the protein A IgG binding sites of the triple fusion.

3.3 Discussion

Phage display of protein A IgG-binding domains: This study was designed to investigate the display of a α-helical multidomain protein on filamentous phage. Protein A consists of five highly homologous IgG-binding domains. As derived from the three dimensional structure of a single protein A IgG-binding domain B (FB), (Deisenhofer, 1981; Gouda *et al.*, 1992), the intact IgG-binding domains of protein A contain fifteen α-helices, with each individual IgG-binding domain being formed by three α-helices (Uhlen *et al.*, 1984; Gouda *et al.*, 1992). Resolution of structure to 2.9 Å by X-ray crystallography (Fig. 3-11) indicates that in each IgG-binding domain, the first two N-terminal α-helices contribute the major binding activity. These two α-helices fold in the anti-parallel form. The remainder of the residues are disordered in the FB-Fc complexes. However, a three dimensional NMR study showed that the free soluble FB fragment folds as three α-helices. In solution, the first N-terminal α-helix is tilted at an angle of 30° with the second α-helix rather than anti-parallel as in the crystal. Furthermore, the last twenty or so residues form the third α-helix in solution which is oriented anti-parallel to the second α-helix (Gouda *et al.*, 1992). In the construct of pComb3PA$^-$ (or pC3$^-$PA$^-$), there were just fourteen α-helices fused to the C terminal domain of pIII. The last α-helix of FC was eliminated when the original construct was made (Nilsson *et al.*, 1985). It was thought that this region of the C domain most likely contributes to flexibility rather than binding (Nilsson and Abrahmsen 1990; Tai *et al.*, 1990). However, by studying a range of different truncated recombinant FB domains, Huston *et al.* (1991) demonstrated that analogues with the third α-helix deleted, bind very weakly to monomeric IgG when compared with full length FB (about 10^4 fold reduction). An amino acid sequence comparison of FC and FB gives a homology of 90% for these two domains and indeed the regions which form the first two α-helices are completely identical (Moks *et al.*, 1986). Based on this information, it was anticipated that the protein A polypeptides displayed on phage would have just four high

A

B

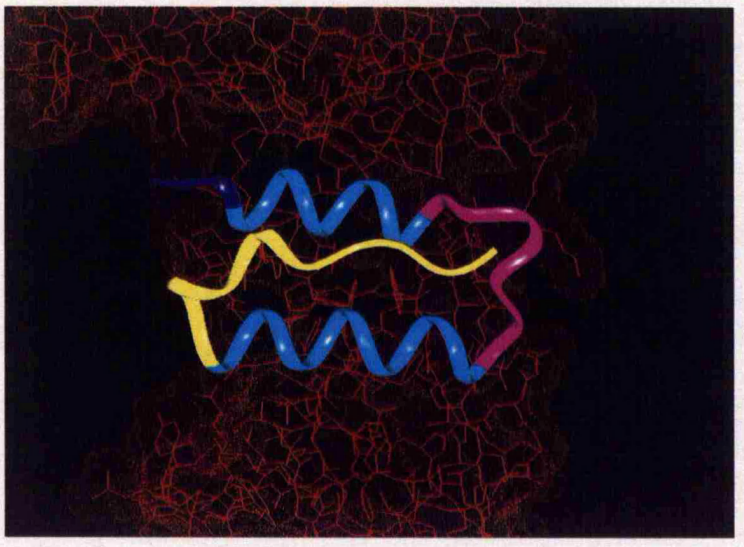


Fig 3-11. **Structure of the FB Fc-binding domain of protein A and its complex with the Fc region.** Molecular modelling utilised a Silicon Graphics workstation and the Insight II Program using the 1fc2 coordinates for the FB-Fc structure (Deisenhofer, 1981) obtained from the Brookhaven Protein Data Bank (Bernstein et al., 1977). A. two α-helices of FB (shown as blue ribbons) bind to Fc at the junction of its CH2 and CH3 domains. The approximate Fc interactions with FB are displayed as a red dot surface. The C terminal portion [forming a third α-helix in solution (Gouda et al., 1992)] is shown in yellow, (and far away from Fc). B. The C terminal portion of FB is disordered in this complex. The two α-helices, which contact Fc, fold as anti-parallel form.

affinity IgG binding sites and that the truncated FC would not contribute significantly to fusion phage's IgG-binding activity.

This study used the pComb3 phage display vector, which results in the production of a fusion protein in which protein A is fused to the C-terminal domain of pIII coat protein. As a result of the deletion of the N-terminal domain of the pIII coat protein this fusion protein can not contribute to the infection of bacteria. Infection requires assembly into the phagemid particle of the full length pIII supplied by the helper phage. Clearly, the protein A phage will be assembled with a mixture of the fusion protein and the full length functional coat protein. The copy number of the fusion protein in the mixture is dependent on growth conditions. In the pComb3 vector, fusion protein synthesis is regulated by the *lac* system. However, even without induction by IPTG, because of the leakiness of the *lacZ* promoter, there is a small amount of fusion protein synthesised. A reduction in amount of protein synthesis can be achieved by using a host strain such as XL-1-Blue which contains the $lacI^q$ gene, and which constitutively expresses the *lac* repressor. In these circumstances the amount of fusion protein is reduced to a level at which, on average, each bacteriophage would contain less than one protein A fusion. In other words, the protein A copy number displayed on phage would expected to be predominantly one (Bass *et al.*, 1990). As shown in figure 3-2, IPTG induction significantly increased the detectable protein A activity and the signal was further increased when increased levels of IPTG were used for induction. Clearly, induction conditions can influence the copy number of displayed protein A.

The binding specificity of the protein A phage appears to be identical to the native soluble protein A. Protein A phage binding to rabbit IgG can be blocked by native protein A and human IgG, but not by mouse IgG.

The protein A phage can be retained on an IgG agarose column, and isolated from a mixture containing non-specific phage. Such protein A display and selection techniques can be used

along with mutational strategies to create a protein A phage library, from which protein A mutants with changed specificity and affinity could be selected. In a protein A site-directed mutagenesis study, it was shown that the pH responsiveness of protein A could be enhanced (Gore *et al.*, 1992), and clearly this kind of study could be boosted by using a protein A phage library.

It is also possible to isolate artificial Fc binding proteins against certain antibody types, to which native protein A cannot bind. Recently, a single Fc-binding domain phage library has been constructed by randomising 13 residues of first two α-helices, and binders were obtained which possessed secondary structures closely similar to the wild-type Fc-binding domain (Nord *et al.*, 1995). In others words, phage display of SpA IgG binding-domains will have great potential for the generation of artificial proteins based on a α-helical structure.

Phage display of protein A::scFv AS32 fusion proteins: The fusion proteins of protein A and AS32scFv were detected as bifunctional proteins displayed on the filamentous phage surface, generated from constructs with either the scFv or protein A at the amino terminal end of the fusion protein (i.e. protein A::scFv AS32::C-pIII or scFv AS32:: protein A::C-pIII). The binding activity of AS32scFv was strongly influenced by the orientation of the fusion protein. When the scFv was located in the middle of the triple fusion protein, phytochrome A binding activity was reduced when compared to that of scFv AS32 displayed alone on the phage surface. In contrast when the scFv occupied the N-terminal position of the triple fusion protein, phytochrome A binding activity was similar to that measured when the AS32 scFv was displayed alone.

Protein A IgG-binding activity on the other hand was not apparently influenced by the orientation of the triple fusion protein. The fact that four IgG-binding domains of protein A were displayed, and that in most cases no more than two IgG molecules can simultaneously

interact with one protein A molecule may explain why the orientation of the fusion had a minimal effect on IgG binding.

The reduction of AS32scFv function when protein A was fused at the N terminus of the scFv may be caused by the presence of a large protein close to the antibody binding site (Padlan, 1993). Furthermore, there was no linker between the fourteen α-helices of protein A and the scFv. It seems likely that the phytochrome A binding site will be masked by this multi-α-helix protein, and that there will be steric hindrance of interactions between antibody binding site and epitope. Evidence supporting this proposal comes from the finding that when the IgG binding sites of the fusion protein were completely blocked by human IgG, the AS32 scFv epitope was not detectable on a western blot (Fig. 3-10). The structural model of the FB-Fc complex (Fig 3-11), indicates that it was likely that the complex of protein A-human IgG would mask the whole epitope of scFv AS32. The N-terminal of antibody variable domain has been shown to be very important to antibody affinity and specificity (Panka *et al.*, 1988). When a large protein is fused to the N-terminal without a linker to limit the interaction of the scFv and fusion partner, it would be expected to sterically influence the binding of the antigen (Padlan, 1993).

Further information on the interactions between SpA and scFv AS32 was obtained in experiments in which the position of the scFv in the triple fusion was changed so that it occupied the N terminal position. Phage displaying this triple fusion were purified by human IgG agarose chromatography and were shown to bind also to phytochrome A (Fig.3-10). Although both the scFv and SpA were functional, it was found that the scFv appeared to bind more strongly to its target epitope than when it did not occupy the N terminus of the fusion protein. Thus if an scFv does not occupy the N terminal position in a fusion protein it is likely that a linker will be required between the scFv and the N terminal fusion partner.

The potential applications of SpA fusion protein are reviewed by Nilsson and Abrahmsen, 1990. ScFv fusions with SpA have been shown to be very useful reagents for detection and purification (Tai *et al.*, 1990; Gandecha *et al.*, 1992). Recently, a pComb3 derived vector (pComb3-SpA) has been constructed for rapid assay of phage-derived recombinant Fabs. These authors have used a SpA fragment to replace the C-pIII fragment and generate a bifunctional Fab-SpA fusion (Sanna *et al.*, 1995). This cloning step, follows the selection of the desired binding activity from combinatorial libraries. In contrast, by using the construct described in this study, scFv::SpA fusion may be directly generated from the phage display vector.

When the scFv is fused to the N-terminal of SpA, there is no detectable reduction in the functionality of the scFv. Thus it would be possible to create an scFv antibody library as scFv::SpA::C-pIII fusions on the phage surface, and selected binders could be directly assayed by generating soluble scFv::SpA fusion proteins. Such scFv::SpA fusion proteins may be particularly valuable in the initial characterisation analysis of scFvs isolated from scFv libraries, since crude *E. coli* medium supernatants commonly contain very low levels of antibody and the SpA moiety of the fusion can be used as the basis for very sensitive downstream immuno-assays.

CHAPTER 4

A study of antibody-antigen interaction through site-directed mutagenesis of the VH region of a hybrid phage-antibody fragment

4.1 Introduction

Somatic mutations of germline V genes, which occur during the course of the immune response, alter the specificity and affinities of antibodies. The detailed analysis of the consequences of these naturally-occurring structural variations in antibody V regions has provided a powerful tool in experimental approaches aimed at relating antibody structure to function (Webster *et al.*, 1994; Davies and Chacko 1993; Berek 1993; Alzari *et al.*, 1990). The monoclonal antibody (mAb) HyHEL-10, directed against hen egg lysozyme, has been a focus for anti-protein antibody studies of the functional effects of somatic mutations (e.g. Lavoie *et al.*, 1992). The crystal structure of the HyHEL-10 Fab/lysozyme complex has been defined to high resolution (Padlan *et al.*, 1989) and it has been shown that all six of the hypervariable complementarity determining regions (CDRs) contribute at least one of a total of 19 antigen-contact residues. The VH region of HyHEL-10 is derived from the germline gene VH36-60 (Smith-Gill *et al.*, 1987).

The development of methods for the cloning and heterologous expression of antibody variable gene sequences, leading to the synthesis of functional antibody fragments, together with the application of methods for site-directed mutagenesis has greatly facilitated structure-function studies of antibody combining sites (e.g. Tsumoto *et al.*, 1995). The value of these methods may be further enhanced by the use of molecular modelling packages designed to predict

antibody combining site structures (Rees *et al.,* 1994). By combining several methods (canonical structure, knowledge-based and *ab initio* modelling), a combined algorithm (AbM) which can predict the structures of all six CDR loops has been developed (Martin *et al.*, 1989; Pedersen *et al.*, 1992; Rees *et al.,* 1994).

The VH and VL region genes from a murine mAb, AS32 have been isolated, which recognises a conserved epitope located in the C-terminal portion of the plant photoreceptor protein phytochrome A (Owen *et al.*, 1992). The VH region gene of AS32 is also derived from VH36-60 and shares 80% identity with the VH region of HyHEL-10. Significantly, 9 of the 11 contact residues of HyHEL-10 VH are also present in the AS32 VH. Here, monovalent phage display technology has been used and combined with chain recombination, site-directed mutagenesis and antibody combining site modelling to identify individual VH residues responsible for the major specificity and high affinity binding of HyHEL-10 to lysozyme.

4.2 Results

4.2.1 Chain recombination experiments

A comparison of derived amino acid sequences reveals that the VH region of the anti-phytochrome A mAb AS32 has high homology with the VH region of the anti-hen egg lysozyme mAb HyHEL-10 (Fig. 4-1). Both VH regions, which are derivatives of the murine VH36-60 germline gene (Near *et al.*, 1984), are characterised by relatively short CDR3 loops; a feature common to many anti-protein antibodies (de la Paz *et al.*, 1986). Significantly, several of the VH region contact residues of HyHEL-10, as identified by high resolution x-ray crystallography of Fab-antigen complexes, are also present in the AS32 VH region at identical positions (Fig. 4-1). All four contact residues of the HyHEL-10 VH CDR1 are found in AS32 VH CDR1. An additional Ala residue is found in VH CDR1 of AS32, which, according to the Kabat numbering system, would be located at position 34. However, analysis of murine

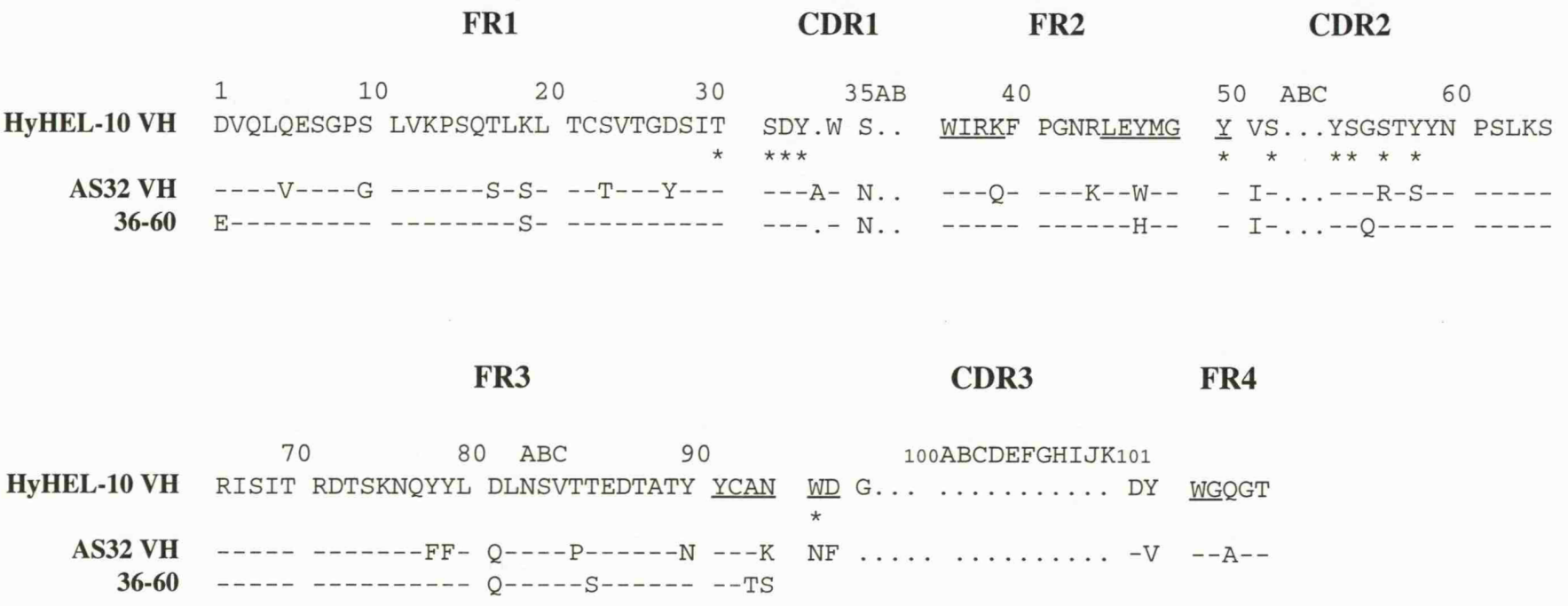


Fig. 4-1 The amino acid sequences of the VH regions of HyHEL-10, AS32 and their progenitor germline gene 36-60. The HyHEL-10 residues that contact HEL are indicated with an asterisk and the HyHEL-10 VH-VL interface residues are underlined. Dashes indicated indentical residues and dots indicate deletions. Residues are numbered according to the Kabat system (Kabat *et al.*, 1991).

immunoglobulin sequences in the Kabat database suggests that insertions between residues 33 and 34 are common and that 50% of the insertions at this position are Ala. By accepting this insertion (i.e. position 33A) residue Trp34 becomes highly conserved among all VH CDR1 sequences (97% as opposed to 53% similarity). Five of the six contact residues from the HyHEL-10 CDR2 are also present in AS32. The single contact reside from the HyHEL-10 CDR3 is not duplicated in AS32. The VL regions of the two mAbs are derived from different germline VL genes and therefore show little homology.

The scFv derived from AS32, when displayed on the surface of filamentous phage, shows strong binding to phytochrome A, and, not surprisingly, very weak binding to hen egg lysozyme (Table 4-1).

Table 4-1. Major specificities of mutant and wild type PhAbs

Phabs/Antigen	Phytochrome A	Lysozyme	BSA
AS32	+++[a]	+/-	-
AS32 VH/HyHL-10	+/-	+/-	-
G3.N	-	+	-
G23.N	-	++	-
G123	-	+++	-
G123.N	-	++++	-
G123.N.D	-	++++	-
HyHEL-10	-	++++	-

[a] Specificities were determined by ELISA. ++++ indicates an ELISA signal > 0.80; +++ indicates 0.51-0.80; ++ indicates 0.21-0.50; + indicates 0.11-0.20; +/- indicates 0.05-0.1; - indicates <0.05.

Similarly, phage-displayed HyHEL-10 scFv shows strong anti-lysozyme activity and no phytochrome A-binding (Table 4-1). A hybrid scFv comprising the AS32VH region with the HyHEL-10 VL region shows no binding to either antigen. Thus, although this hybrid has the HyHEL-10 VL region and a VH region that shows high homology to the HyHEL-10 VH region, and includes nine of its eleven contact residues, this is insufficient to confer appreciable lysozyme-binding activity. This illustrates that among the few residues that differ between the two VH regions, are the determinants of the major specificity and high affinity of HyHEL-10.

4.2.2 CDR grafting, modelling and framework region mutations

Mutation of all three of the VH region CDRs of the AS32 VH-HyHEL-10 VL hybrid to the CDR sequences of the HyHEL-10 VH region (yielding hybrid G123) leads to significant lysozyme-binding activity of the phage-displayed scFv (Fig. 4-2, Table 4-1). However, although appearing to show the same major specificity as the CDR donor, this grafted hybrid scFv, which now possesses all six HyHEL-10 CDRs, displays significantly lower lysozyme-binding activity than the HyHEL-10 scFv (Fig. 4-2).

In order to identify the non-contact residues of the VH region of hybrid G123 that are assumed to be influencing affinity, the G123 and HyHEL-10 scFvs were modelled using AbM (Pedersen *et al.*, 1992) (Fig. 4-3, Fig. 4-4). The model of HyHEL-10 was compared with x-ray crystallography co-ordinates (PDB entry: 3hfm) by using the molecular similarity modelling program Quanta. Using the AbM standard method the accuracy of the model is high and the RMS deviation for the main chains and for all residues of the CDRs are:

	L1	L2	L3	H1	H2	H3
main chain	0.0006Å	0.0007Å	0.0008Å	0.0006Å	0.0008Å	1.373Å
residues	1.694Å	1.157Å	2.035Å	1.469Å	1.456Å	3.118Å

The canonical method was used to model loops L1, L2, L3, H1 and H2 whilst the H3 loop was modelled by a database search method.

Examination of the models revealed that the AS32 (G123) VH framework can associate with the HyHEL-10 VL, even though two very important domain interface residues differ within framework region 2. The Lys residue at position 39 in the HyHEL-10 VH domain, which forms two hydrogen bonds with a Gln residue at position 38 in the HyHEL-10 VL domain, is replaced by a Gln residue in AS32. Also, the Tyr residue at position 47 in framework 2 of the HyHEL-10 VH domain, which forms a van der Waal's interaction with Tyr 96 of the HyHEL-10 VL domain, is replaced by a Trp residue in AS32. The model predicts that both of the

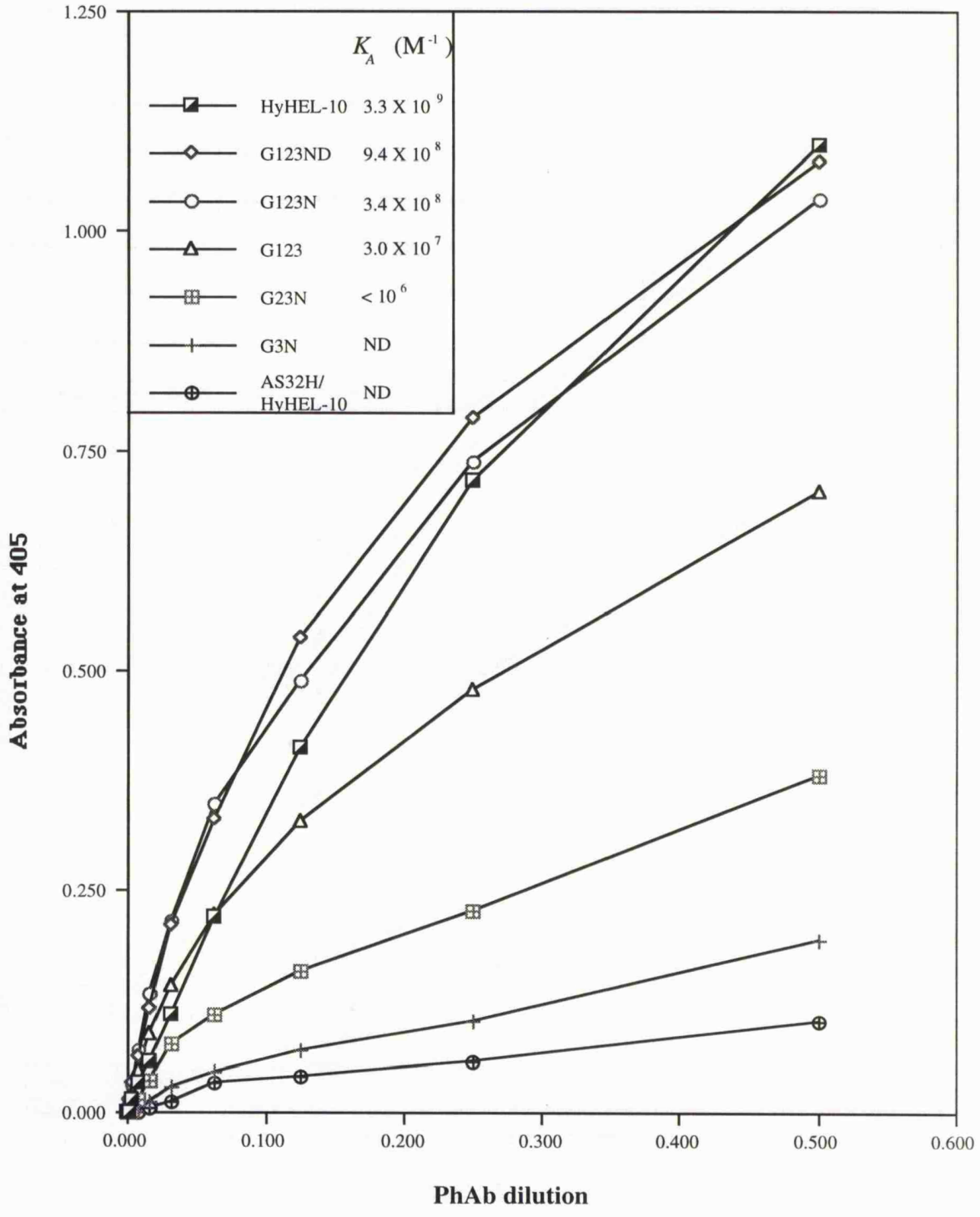


Fig. 4-2. Lysozyme -binding activity and affinity constants of the various phage-displayed scFvs. Crude baterial supernatants, diluted in blocking buffer (1% w/v BSA in PBS) to equal cfu titre, were used the PhAb soures. The affinity constants were calculated from dissociation constants determined by equilibrium competition ELISA.

AS32 framework 2 residues can form similar interactions with the appropriate HyHEL-10 VL residues.

The model also indicates divergence between the structures of the VH CDR3 loops of G123 and HyHEL-10 as a consequence of different residues at position 94 (Fig. 4-3, Fig.4-4). The model predicts that the Lys residue at position 94 in the G123 VH region is very important in determining the structure of the CDR3 loop. An Asn residue occupies this position in the HyHEL-10 VH region. Lys94 in G123 is predicted to alter CDR3 loop structure, particularly the orientation of the side chain of the Trp residue at position 95 (Fig. 4-3). A large shift (6.15Å) is seen in the position of side chain atoms of Trp95 depending on whether Lys or Asn is present in position 94. In addition, the side chain of Lys94 of G123 is predicted to form two hydrogen bonds with main chain oxygens of two other residues within CDR3, Gly97 and Tyr102 (Fig. 4-3).

Because, the model predicts that mutation of residue Lys94 of G123 to Asn94 would restore the native HyHEL-10 VH CDR3 loop structure (Fig. 4-4). Hybrid G123.N, which incorporates this Asn (VH94) mutation, was generated by PCR. This mutant hybrid scFv shows significantly increased lysozyme-binding activity compared with scFv G123, as determined by ELISA (Fig. 4-2). The measured affinity of G123.N for lysozyme is increased by more than 20-fold compared with G123 and is only about 5-fold lower than that of the HyHEL-10 scFv (Fig. 4-2).

The CDR1 loop of the HyHEL-10 VH region belongs to canonical loop class 1 (Chothia and Lesk, 1987), but the CDR1 loop of the G123.N hybrid scFv VH region could not be classified to any canonical loop class because the crucial Asp residue at position 27 in HyHEL-10 is replaced by a Tyr residue in G123.N. When the CDR1 loops of the G123.N and HyHEL-10 VH domains are superimposed, a large shift is seen in the positions of the four contact residues; Thr30, Ser31, Asp32 and Trp33 (Fig. 4-3). The G123.N.D mutant, which

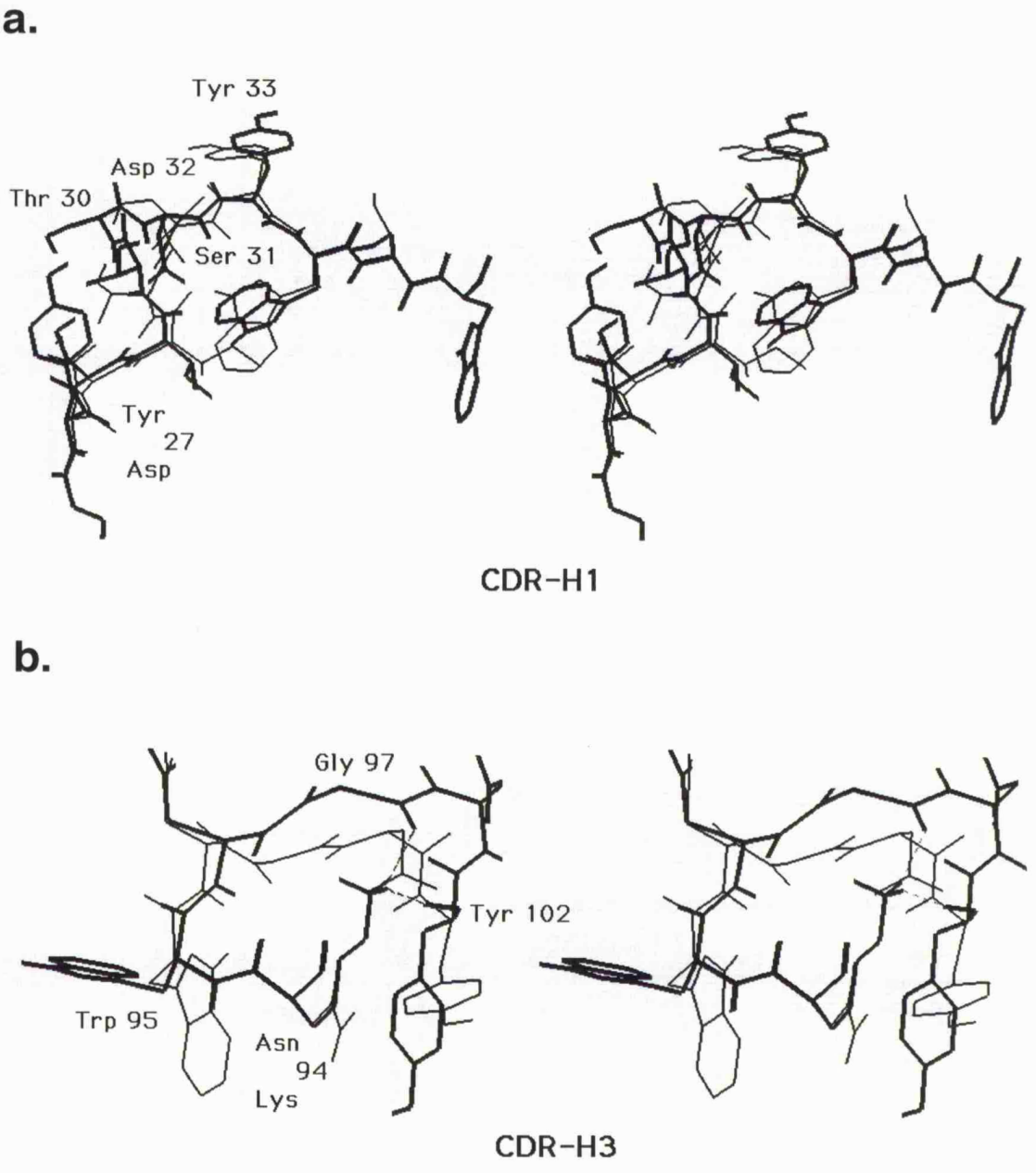


Fig. 4-3. Comparison of the modeled conformations of CDR-H1 and CDR-H3 loops in HyHEL-10 and G123. a: Stereo drawing of CDR-H1 loops. The thick lines represent G123 and the thin lines HyHEL-10. b: Stereo drawing of CDR-H3 loops. The thick lines represent G123 and the thin lines HyHEL-10.

incorporates an Asp at position 27, was generated and was found to have an increased affinity compared with G123.N (Fig. 4-2). The measured affinity of this mutant scFv is only about three fold lower than that of the HyHEL-10 scFv.

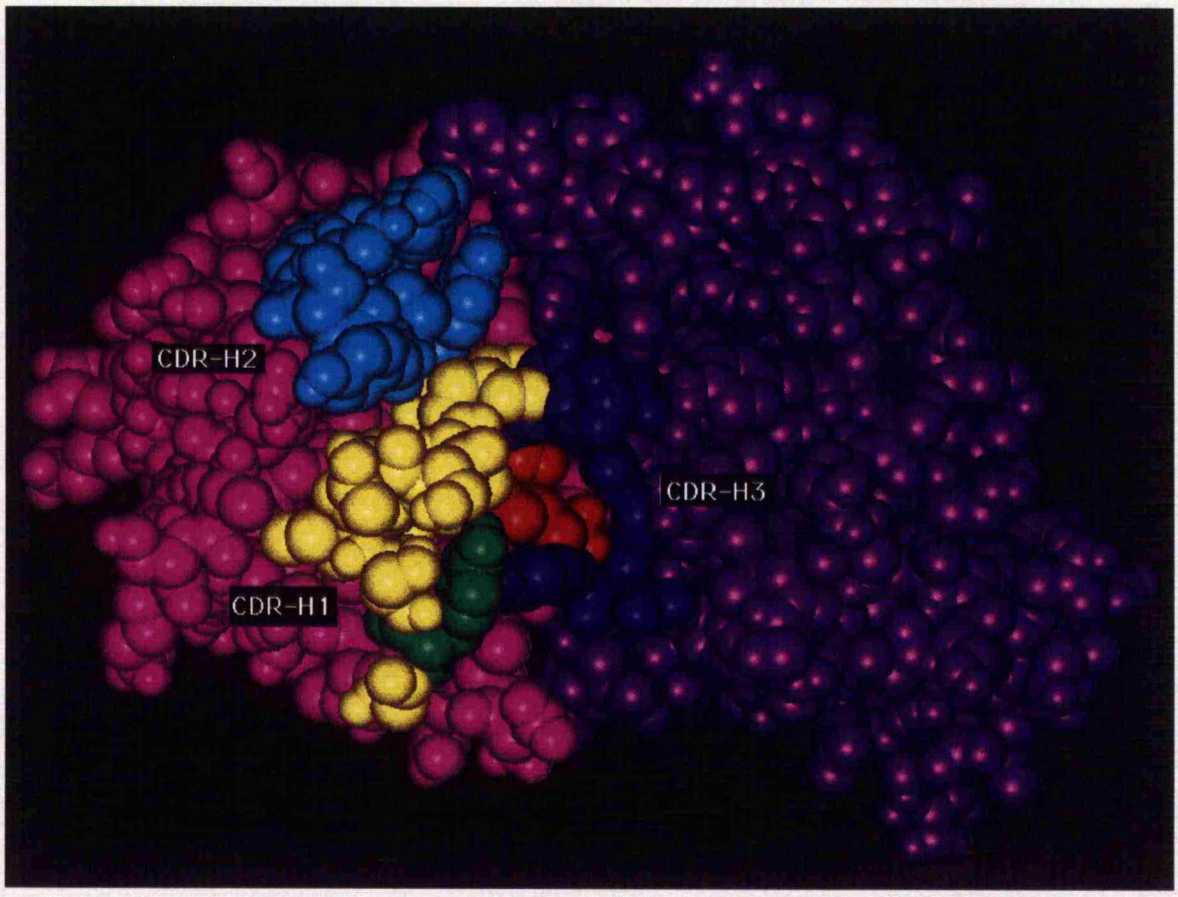
In the hybrid mutant G3.N the Lys94 of AS32 has been mutated to Asn and the CDR3 loop of the AS32 VH region has been replaced by the HyHEL-10 VH CDR3. This mutant which possesses ten of the eleven contact residues of HyHEL-10 displays very weak lysozyme binding activity (Fig. 4-2). When the VH CDR2 loop of G3.N is also mutated to that of HyHEL-10, yielding mutant G23.N, all eleven contact residues of HyHEL-10 are present in the mutant scFv, and an improvement of lysozyme-binding activity is detected. However, the measured affinity of this mutant hybrid for lysozyme is below that which could be detected and is at least 100 fold lower than that of the G123.N mutant (Fig. 4-2). This indicates that the relatively minor differences in the VH CDR1 loops of these two antibodies are playing a significant role in determining affinity. This is consistent with the finding that the C-terminal part of VH CDR1 entirely occupies the central portion of the HyHEL-10 combining site (Fig. 4-4).

4.3 Discussion

Filamentous bacteriophage display systems have been widely used for creating antibody repertoires or peptide libraries from which high affinity ligand can be selected (Winter *et al.*, 1994). Here the phage display system has been used as part of a study of an antibody combining site. For this, the phage antibody behaves as the homogeneous, monovalent antibody fragment, which is important for measuring affinity by equilibrium competition ELISA. Monovalent phage antibody display is based on the fact that the scFv-protein III gene was under the control of the lac promoter/operator system and, in *Escherichia coli* strains which constitutively contain a high level of the lac repressor, the number scFv-protein III fusion molecules is restricted. Infection with helper phage results in the production of a large

Fig. 4-4. **Spacefill models of the combining sites of G123 (a) and HyHEL-10 (b).** The VH framework regions are pink, the CDR-H1 loop is yellow, the CDR-H2 loop is light blue and the CDR-H3 loop is dark blue. Reside H27 (Tyr in G123, Asp in HyHEL-10) is green and residue H94 (Lys in G123, Asn in HyHEL-10) is red. The VL domain is purple.

a



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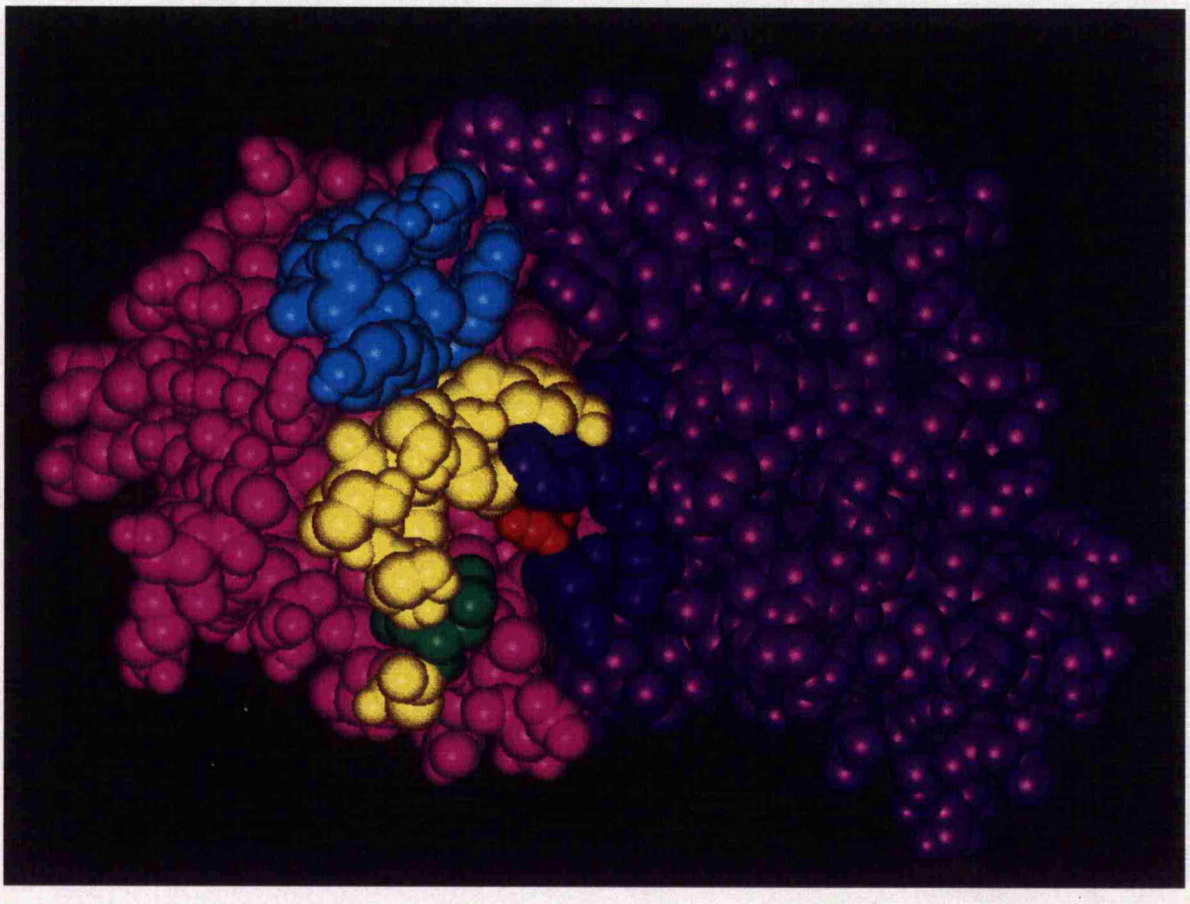


excess of native gene III products, and this ensures that assembled bacteriophages will be essentially monovalent with respect to the number of scFv proteins they carry (Bass *et al.*, 1990). The PhAb immuno-assay is a highly sensitive detection system, in which only about 10^4 phage particles per well can be detected. This kind of sensitivity, combined with the finding that PhAbs in bacterial culture supernatants are relatively pure and do not require extensive purification, facilitates rapid characterisation of many recombinant antibodies. Furthermore, the affinity of monovalent PhAbs can be readily determined using equilibrium ELISA.

In order to perform CDR grafting and variable region framework mutation SOE PCR has been used. The HyHEL-10 VH CDRs were grafted using several mismatch primers which were employed to amplify the corresponding fragments from the AS32 VH gene sequence. There were about 15 nucleotides of complementary overhang between adjacent duplexes. Four of the amplified fragments were successfully assembled in a single PCR reaction in order to completely graft the VH CDRs from HyHEL-10 on to AS32. This method is easier and more rapid than traditional mutagenesis method that have be used for CDR grafting.

By a combination of phage display, site-directed mutagenesis and antibody combining site modelling VH domain amino acid residues has been identified that are crucial for the high affinity binding of HyHEL-10 to lysozyme. The Asn residue at position 94 in HyHEL-10 appears to play an important role in determining affinity, perhaps through affecting the structure of the CDR3 loop. If this Asn residue is replaced by a Lys, as is the case in AS32 and the G123 mutant, there is a predicted alteration in the orientation of the side chain of the Trp residue at position 95 (Fig. 4-3). The Trp residue at position 95 forms the central van der Waal's interactions with the lysozyme residues Arg21, Lys97 and Ser100 (Padlan *et al.*, 1989) and this kind of central contact plays a predominant role in determining the affinity of antibody antigen interactions (Hawkins *et al.*, 1993). In addition, the side chain of Lys94, present in G123, could form hydrogen bonds with other residues within CDR3 and this may reduce the

flexibility of the combining site. The constraint of the CDR3 loop could decrease the range of conformational isomerisms of the antibody combining site. The conformational isomerisms is general phenomenon in antibody combining sites and has been shown to increase affinity in the case of anti-hapten antibodies (Foote and Milstein, 1994; Kranz *et al.*, 1982). There have been several experimental studies that have demonstrated that an Arg residue 94 of the VH region can influence antigen binding activity (Brummell *et al.*, 1993; Chien *et al.*, 1989; Panka, *et al.*, 1988). Specifically, Arg residues at this position are thought to interact with the invariant Asp residues at position 101, forming salt bridges and so stabilising the loops (Chothia and Lesk, 1987). A number of CDR-grafting studies have shown that an Arg residue at position 94 in the VH region of the acceptor, if not also present in the donor, can influence the affinity of the grafted antibodies (Jones *et al.*, 1986; Queen *et al.*, 1989; Tempest *et al.*, 1991). It seems likely that the Lys at position 94 in the AS32 and G123 framework is performing a similar role to Arg in this position by constraining the structure of the CDR3 loop.

Comparing the CDR1 loop sequences of the 36-60 germline gene product and that of HyHEL-10 and AS32, It was found that a hydrophobic residue, Ala, has been inserted into the position between the residue Tyr33 and Trp34 of AS32 during somatic hypermutaion. This Trp34 residue is the canonical structure determining residue for CDR1 (Chothia and Lesk, 1987). The insertion of Ala changes the conformation of the CDR1 loop, especially the packing of the four contact residues (Thr, Ser, Asp and Trp) located at N-terminus of this Ala.

Even when this Ala is removed, as in mutant G123.N, there is still a predicted shift in the conformation of CDR1. This is because the Asp residue found at position 27 in HyHEL-10 is replaced by a Tyr residue in G123.N. Chothia and Lesk (1987) have defined this position as playing a key role to control the main chain conformation of CDR1 and the Tyr residue in this position could disrupt the main chain conformation of the C-terminal part of CDR1 loop, which is located at the centre of combining site (Fig. 4-4). Consequently, the HCDR modelling

analysis suggested that mutation of Tyr27 of G123.N to Asp27 (yielding G123.N.D) would significantly increase antigen-binding activity. However, this mutation had only a modest effect on affinity. This suggests that perhaps residue 27 does not play a crucial structural role in this antibody. Alternatively, it is possible that the effects of a disrupted main chain conformation of CDR1 in G123.N are partly compensated by the presence of the additional Tyr residue. Tsumoto *et al.* (1995) have shown that Tyr residues, which are a common feature of antibody CDRs, play significant roles in the HyHEL-10-lysozyme interaction.

In this chapter I have employed phage display technology and site-directed mutagenesis for the identification of CDR and framework region residues that are crucial for the high affinity binding of HyHEL-10 to lysozyme. Using a hybrid scFv comprising the VL region of HyHEL-10 and a structurally-related VH region from a functionally distinct antibody, in combination with modelling studies, the framework residue at position 94 has been found to play an important role in high affinity binding.

The use of phage display, in combination with SOE PCR for mutagenesis, allows relatively rapid characterisation of the mutant scFv antibodies and eliminates the need for extensive purification of the mutant proteins. Also by using the combined algorithm AbM, a highly accurate antibody combining site prediction can be achieved, providing a rapid approach to the analysis of antibody structure-function relations.

CHAPTER 5

Construction of a semisynthetic single-chain Fv library by randomising heavy chain CDR3

5.1 Introduction

The mammalian immune repertoire is very large and diverse (Berek and Milstein 1987). This diversity results partly from variation generated by V(D)J recombination during immunoglobulin synthesis by B-cells. VJ recombination in the light chain gene is responsible for some variation but VDJ recombination in the heavy chain makes the most significant contribution. Of particular importance is DJ or DDJ joining which results in the generation of diversity in both sequence and length of the heavy chain CDR3.

Filamentous bacteriophage display technology has been used in several strategies to study and engineer antibody repertories. For example, Marks *et al.* (1991a) harvested rearranged V-genes from the mRNA of B-cells of non-immunised human donors and using PCR (Saiki *et al.*, 1985), produced random combinations of heavy and light chains (Huse *et al.*, 1989). The library so produced was displayed on filamentous phage, and antibody fragments with moderate binding affinity (K_d >0.1 μM) were selected against BSA, turkey egg-white lysozyme and the hapten 2-phenyl-oxazol-5-one (phOx). The binding affinity (K_d) of one of the anti-phOx fragments was improved to 1 nM by chain shuffling (Marks *et al.*, 1992). Antibody variable domains have also been amplified from immunised mice (Clackson *et al.*, 1991), rabbits (Ridder *et al.*, 1995) and humans (Barbas *et al.*, 1991), and displayed on the

surface of filamentous phage. Phage with desired binding activities have been selected by panning with antigen, and soluble antibody fragments have been synthesised in, and secreted from, bacteria (Skerra and Plückthun, 1988; Better *et al.*, 1988) and yeast (Horwitz *et al.*, 1988). The advantage of this strategy is that in mRNA from antigen stimulated B-cells compared with resting B-cells, there is an increase in heavy and light V-genes that are predisposed to create antigen-binding combinations (Persson *et al.*, 1991; Clackson *et al.*, 1991; Hawkins *et al.*, 1992). Artificial antigen-binding protein repertories have been created by randomising HCDR3 (Barbas *et al.*, 1992a; Hoogenboom and Winter, 1992) or LCDR1, LCDR3, HCDR2 and HCDR3 (Garrard and Henner, 1993). Relatively long HCDR3s (15 residues) were used to generate the random antibody library by Barbas *et al.*, and hapten binding fragments were isolated with moderate affinity. By using libraries containing relatively short HCDR3 (5 residues), it was possible to isolate antibody fragments against protein antigens, also with moderate affinity (Hoogenboom and Winter, 1992; Garrard and Henner, 1993).

Here, the construction and preliminary study of a semisynthetic HCDR3 library is described. A small library which used a relatively short HCDR3 (6 residues) was constructed on filamentous bacteriophage and PhAbs against phytochrome A or chicken egg-white lysozyme were selected.

5.2 Results

5.2.1 Heavy chain CDR3 randomisation

The anti-phytochrome A scFv AS32 was selected for mutagenesis. An scFv AS32 phage display construct was used as template, SOE PCR was employed to target HCDR3 for mutation. An oligonucleotide was synthesised that is complementary to 19 bases on FR3 and 18 bases on FR4 adjacent to HCDR3 and which contained a randomised sequence in the

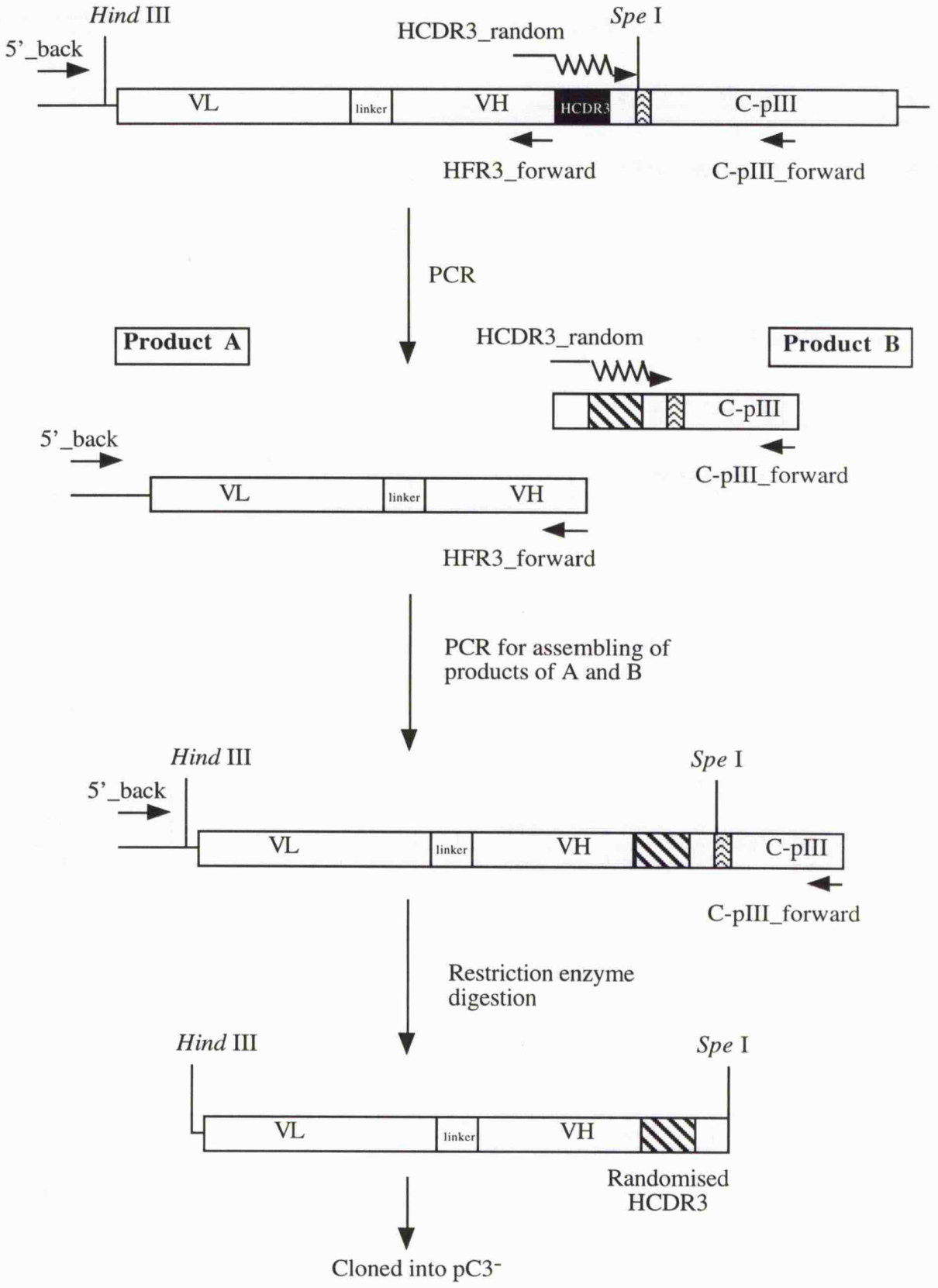


Fig 5-1. Diagram of AS32 HCDR3 randomisation. Primers for PCR mutagenesis are named as 5'_back, HFR3_forward, HCDR3_random and C-pIII forward.

central 18 bases which corresponds to the actual HCDR. By using this oligonucleotide as back PCR primer and another complementary to the downstream sequence in the vector (C-pIII region) as forward PCR primer, a gene fragment that encodes from the middle of FR3 to the middle of C-pIII fragment was generated. The other half of the gene from 5' of scFv AS32 fragment to the 3' end of heavy chain FR3 was also produced by PCR. These two products were then finally combined and fused by PCR (Fig. 5-1) using the back primer complementary to the 5' region of scFv AS32 fragment and the forward primer complementary to the internal sequence of C-pIII. The final PCR product encoded a heavy chain CDR3 of 6 residues in length with a randomised amino acid sequence.

5.2.2 Library construction

The final PCR product, an scFv fragment randomised in sequence only in the CDR3 region, was cloned into the pC3$^-$ vector as an in-frame fusion with C-pIII. This synthetic CDR3 could have $(4x4x2)^6 = 1.0 \times 10^9$ different nucleotide sequences, or $20^6 = 6.4 \times 10^7$ different amino acid sequences. The diversity is not large compared to native antibody repertoires which have been estimated to contain 10^8 -10^{10} members. The diversity of this HCDR3 library was further reduced by the number of transformants that could be studied. In the present case, transformation of *E. coli* with the plasmid DNA resulted in a library of only 3.0×10^4 clones.

5.2.3 Selection

Phage were prepared from the libraries by rescuing with VCS-M13. PhAbs were selected for binding to a microtiter plate wells coated with chicken egg-lysozyme, phytochrome A, digoxin-BSA conjugate, potato virus Y (PVY) particles or ubiquitin. To investigate the selection power of phage antibody display technology, AS32 PhAb was mixed with the synthetic library at ratio of 1: 10^6 cfu (AS32 PhAbs : HCDR3 randomised PhAbs). Specific PhAbs were selected through three rounds of panning, elution, and amplification. The elution

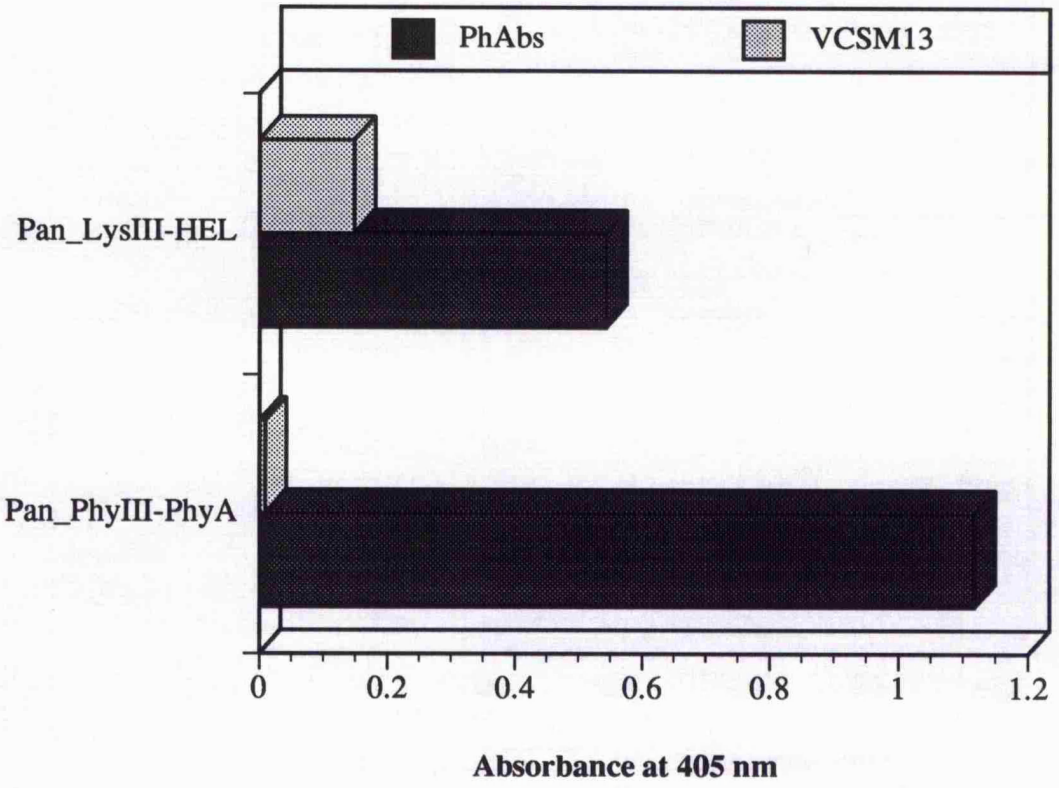


Fig 5-2 Binding activity of polyclonal PhAbs. The binding activity of polyclonal PhAbs from the third round of panning against phytochrome A (PhyA) or chicken egg white lysozyme (HEL) tested against phytochrome A (Pan_PhyIII-PhyA) or lysozyme (Pan_PhyIII-HEL) coated ELISA plates. Helper phage VCSM13 was used as the negative control.

step was performed with acidic-buffer, and resulted in the elution of 1.1 x 10^5, 7.2 x 10^5 and 3.3 x 10^6 phage per well during the course of selection from lysozyme coated plates where the titre of input phage per well were approximately equivalent at 2.0 x 10^9 cfu. Using approximately the same number of input phage and the same elution conditions, 3.0 x 10^4, 2.4 x 10^6 and 7.0 x 10^6 phage per well were eluted from phytochrome A coated plates. Further rounds of panning were also performed, but the eluted phage number was not increased. By using identical methods, selection against three other antigens (Digoxin-BSA, PVY and ubiquitin) was also performed at same conditions, but the enrichment was not observed, indicating that the library might not contain binders to these three antigens.

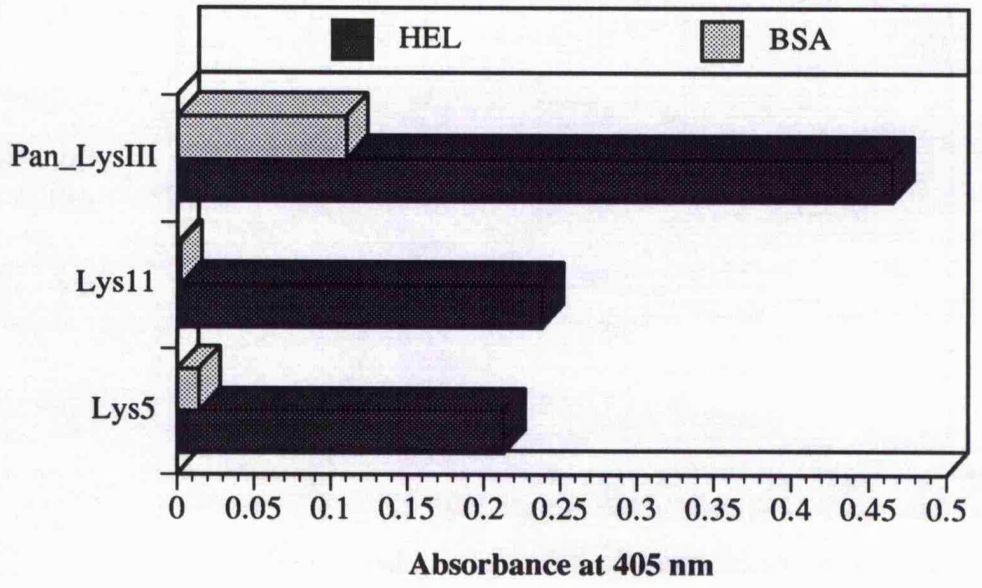
5.2.4 Binding activity of polyclonal PhAbs

The binding activity of polyclonal PhAbs from the third round of panning was analysed (fig. 5-2). The polyclonal PhAbs selected against phytochrome A showed strong binding activity towards phytochrome. Polyclonal PhAbs against HEL showed weak but significant binding activity to HEL in comparison to binding to the negative control antigen BSA.

5.2.5 Binding activity of monoclonal PhAbs

After the third round of panning, several monoclonal PhAbs were produced and tested for binding to corresponding antigens, with BSA as negative control antigen. Two monoclonal PhAbs (Lys5 and Lys11) selected from HEL coated wells showed significant binding activity to HEL, but not to BSA. However the ELISA signals were all lower than those obtained with polyclonal PhAbs from the third round of panning.

Two monoclonal anti-phytochrome A PhAbs (Phy16 and Phy17) selected from the third round of panning showed higher binding activity. Phy17 showed similar binding to phytochrome as the parental AS32 PhAb whereas the binding activity of Phy16 was significantly weaker. Subsequent sequence analysis of Phy 17 showed that it was identical to AS32. The



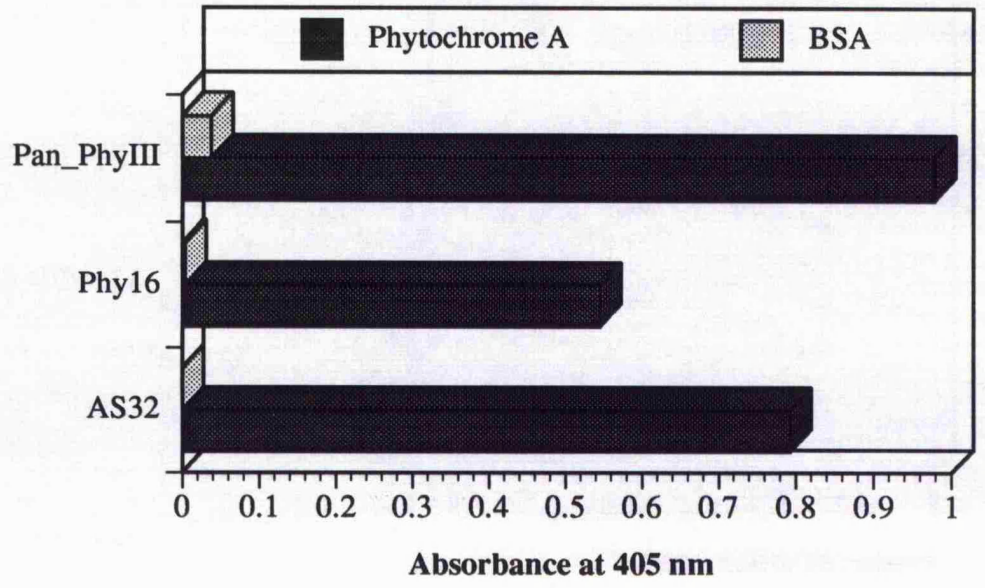


Fig 5-3. Comparison of monoclonal and polyclonal PhAbs binding to Phytochrome A or Lysozyme. Pan_LysIII: polyclonal PhAbs from the third round of panning against lysozyme; Ly5: isolated monoclonal PhAb number 5; Ly11: isolated monoclonal PhAb number 11; Pan_PhyIII: polyclonal PhAb from the third round of panning against phytochrome A; Phy16: isolated monoclonal PhAb number 16; AS32: parental monoclonal PhAb.

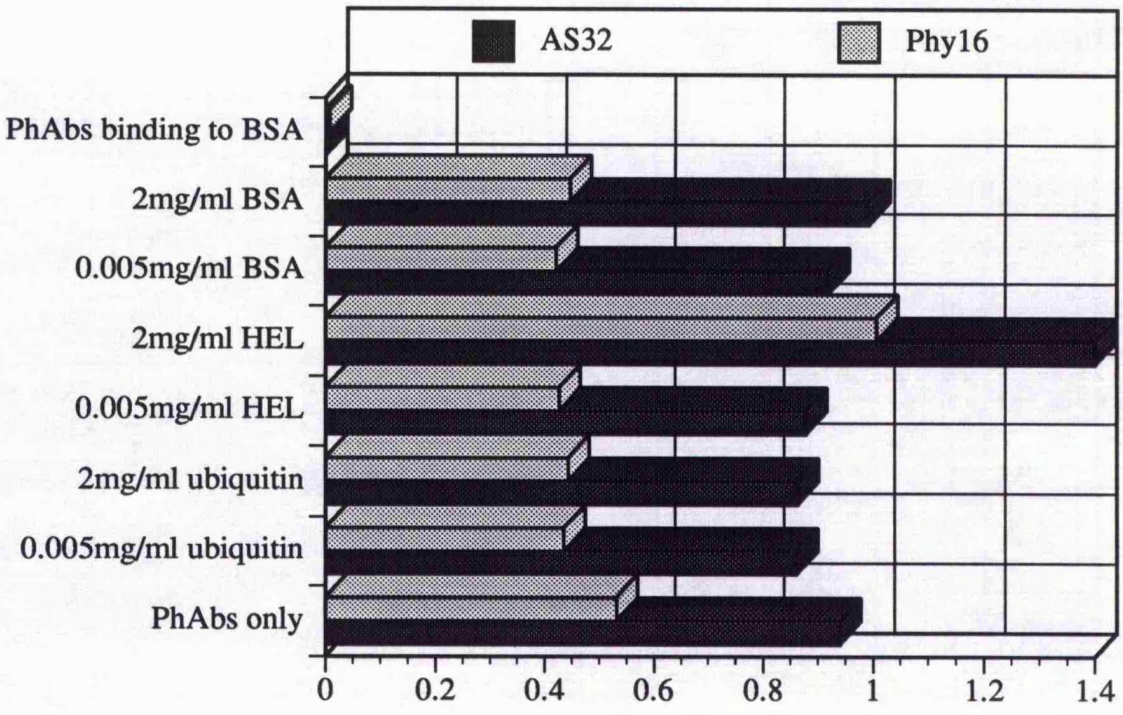


Fig 5-4. Influences of HEL to the antigen binding activity of Phytochrome A binding PhAbs.

monoclonal anti-phytochrome A PhAbs (Phy16) also gave weaker signal than the polyclonal PhAbs (fig. 5-3), as would be anticipated since the strong binder AS32 PhAb was present in the polyclonal PhAb

Another interesting finding was that the binding activities of the monoclonal AS32 PhAb and Phy16 PhAb to phytochrome A was enhanced by adding lysozyme to the solution containing the PhAbs. This enrichment could not be found by adding ubiquitin or BSA (fig. 5-4).

5.2.6 Sequencing

The partial nucleotide sequence of the mutated heavy chain of a representative number of HEL or phytochrome A binding clones was determined. Two anti-HEL monoclonal PhAbs, which show different binding activity, have VH CDR3 as Lys5: SerCysGlySerGlnLeu, and Lys11: GlyProGlyLeuThrGlu. One anti-phytochrome A PhAb (Phy16), which showed weak binding activity compared to scFv AS32 PhAbs, possessed the sequence as SerAlaLeuSerProIle. Another phytochrome A binding PhAb (Phy17) possessed the AS32 sequence, clearly indicating that the panning procedure had succeeded in recovering the AS32 PhAb.

5.3 Discussion

In the past, antibodies had to be generated by immunising animals with antigen. Increasing understanding of the structure of the antibody combining site is allowing manipulation of the binding site to change specificity and affinity and to mimic the immune system and synthesise antigen binding proteins by methods which bypass the animal. Manipulating antibody combining site can be achieved by using a modelling-design approach and also by building artificial antibody repertoires on the phage surface from which binders may be selected.

To obtain experience in the construction and use of random synthetic repertories, a small random HCDR3 library was created and selected against chicken egg white lysozyme (HEL), and phytochrome A. The library size of about 3 x 10^4 members is very small compared with

the natural mouse repertoire which contains about 10^8 members. However, the total mouse repertoire is constructed by using about 600 V_H genes, 250 V_κ genes and 3 V_λ genes with varied lengths for CDR3s. If only one V_H gene and one V_κ gene with fixed CDR3s length (6 residues) were used, the natural repertoire would contain less members. In this respect, the diversity of the synthetic HCDR3 library constructed in this work would be larger than that of the natural repertoire. This library was amplified in bacteria, and the final size of the phage pool was about 10^{13} members. Thus the population for every different PhAb has been amplified approximately 10^8 times by using the monovalent phage display system. Based on this calculation, multiple-rounds of panning (more than three rounds) are not necessary to select a specific monoclonal PhAb from this library, since in the initial (unpanned) phage pool of 10^9 members, there are about 10^4-10^5 individual phage displaying each scFv. Thus three rounds of panning are enough to select particular binders from the semisynthetic antibody library, providing of course that the library contains an scFv with the desired binding specificity

Since the anti-HEL antibody HyHEL-10 is derived from the same V_H gene as AS32, HEL was chosen as one of the target antigens. Two anti-lysozyme monoclonal PhAbs were isolated with good specificity. Comparison of the sequences of HCDR3 of Lys5 and Lys11 with that of

Table 5-1. HCDR3 sequencing comparison of selected PhAbs and AS32 and HyHEL-10

	HCDR3																
	95	96	97	98	99	100	A	B	C	D	E	F	G	H	101	102
Py16	S	A	L	S	-	-	-	-	-	-	-	-	-	-	P	I
AS32	N	F	-	-	-	-	-	-	-	-	-	-	-	-	D	Y
Lys5	G	P	G	L	-	-	-	-	-	-	-	-	-	-	T	E
Lys11	S	C	G	S	-	-	-	-	-	-	-	-	-	-	Q	L
HyHEL-10	W	D	G	-	-	-	-	-	-	-	-	-	-	-	D	Y

HyHEL-10 (table 5-1) shows that all of these sequences have glycine at position H97. The Gly at this position is not present in either of the phytochrome A binders, AS32 and Phy16.

The epitopes of Lys5 and Lys11 are not known. However, since Lys5 and Lys11 are derived from the same germline gene and have identical light chains, it is possible that they bind to the same epitope. It may be that the conserved H97 Gly might play a structural role which contributes to the change in the specificity of AS32 from phytochrome A to lysozyme.

To investigate the role of HCDR3 in AS32 in phytochrome A binding, phytochrome A was chosen as the second target antigen. One of the colonies with good phytochrome A binding activity (Phy16) was sequenced. The sequence of Phy16 HCDR3 is completely different from that of AS32 HCDR3. The affinity of Phy16 appeared to be lower than that of AS32. This may indicate that the original AS32 HCDR3 can tolerate some change without complete loss of binding to phytochrome A. Of course, this is based on the assumption that Phy16 and AS32 (Phy17) bind to the same epitope. Whether or not this is the case could be determined by competition experiments .

For reasons unknown, the phytochrome A binding activity of AS32 PhAbs and Phy16 PhAbs were enhanced by relatively large amounts of lysozyme, but not by BSA and ubiquitin. The molecular weight of BSA is larger than that of HEL, and ubiquitin is smaller but close to that of HEL. Clearly, the enhancement is not due simply to the presence of a large amount of protein, but appears to be caused by a HEL-specific enhancement of phytochrome A binding.

The purpose of this study was to gain experience in the synthesis antibody repertoires and to assess the selection power of panning methods used in the present work. The successful isolation of AS32 from the library indicates that the efficiency of the panning procedures used here is at least 1: 10^6. This work shows that PhAbs with different binding activity can be isolated from quite a small synthetic HCDR3 random library and clearly demonstrates the

potential of the large artificial repertoires which have become available since this work was undertaken.

CHAPTER 6

Selection of phage antibodies to surface epitopes on *Phytophthora infestans*

6.1 Introduction

As a result of their ability to specifically bind to their epitopes, antibodies can be used to detect plant pathogens. Antibody engineering allows the development of recombinant antibodies specifically designed for pathogen-detection strategies with potential advantages in economy of production, detectability and reproducibility. Possibly of greater overall significance in the long term, the specific recognition by engineered antibodies of target molecules produced by the pathogen may provide the basis for novel plant protection strategies. (Owen *et al.*, 1992; Swain, 1991).

Phytophthora infestans is a very important fungal pathogen of potato and tomato. Infections with virulent strains of the fungus are the cause of potato late blight (Rich, 1983). This potato disease is controlled by frequent application of fungicides to the foliage. In addition to the expense of such procedures, there is increasing concern over the liberal use of agrochemicals. Consequently the genetic resistance of the potato plant to blight is becoming increasingly important (Harrison, 1992). *P. infestans* therefore provides an excellent subject for the exploration of the potential of strategies for the recombinant antibody-mediated diagnosis of fungal plant pathogens and protection against them.

Infection by *P. infestans* involves surface contact. Clearly antibodies against surface epitopes of the pathogen could be useful for detection and also, providing that they are important in the pathogenic possess, as a basis for plant protection.

Several conventional immunochemistry techniques have been used for analysis and detection of plant fungi. For example, antisera have been used to estimate amounts of *P. infestans* mycelia in leaf tissues (Harrison *et al.*, 1990); and also monoclonal antibodies have been used to distinguish isolate-, species-, and genera of plant fungi (Hardham *et al.*, 1986). By using synthetic antibody repertoires, the traditional immunisation procedure could be omitted (Winter and Milstein, 1991), and an antibody fragment with the desired specificity could be selected directly from an immune repertoire library expressed on the surface of filamentous bacteriophage (Winter *et al.*, 1994). For a universal phage antibody library, the diversity of the repertories is extremely important.

The ideal universal antibody library would be constructed by chemically synthesising the six complementarity determining regions (CDRs) in a random way and presenting these synthetic CDRs on varied framework regions (FR).

It has been demonstrated that the diversity of a semisynthetic phage antibody library is limited when it is based on a single length HCDR3, and a single V_H gene (Barbas *et al.*, 1992a; Garrard and Henner 1993), or on two different lengths of HCDR3 incorporated into 50 human V_H gene segments (Hoogenboom and Winter 1992). To increase diversity, investigators in Winter's laboratory have cloned 50 human V_H gene segments and 21 V_λ and 26 V_κ segments (Cox *et al.*, 1994; Williams and Winter, 1993) and used them to create a phage antibody library. A diversified "single pot" phage library was created by combining cloned human rearranged V_H genes and a single light chain gene with random nucleotide sequences encoding HCDR3 lengths of 4-12 residues (Nissim *et al.*, 1994). Such phage antibody repertoires have been successfully used to isolate antibody fragments against haptens (Nissim *et al.*, 1994;

Barbas *et al.*, 1992a; Hoogenboom and Winter 1992), proteins (Nissim *et al.*, 1994; Garrard and Henner, 1993) and peptides (chapter 7). Phage antibodies can be treated as detection reagents in the same way as intact antibodies or antibody fragments in ELISA and affinity chromatography (McCafferty *et al.*, 1990), western blotting (Nissim *et al.*, 1994), and also in immunoprecipitation methods (Barbas *et al.*, 1991).

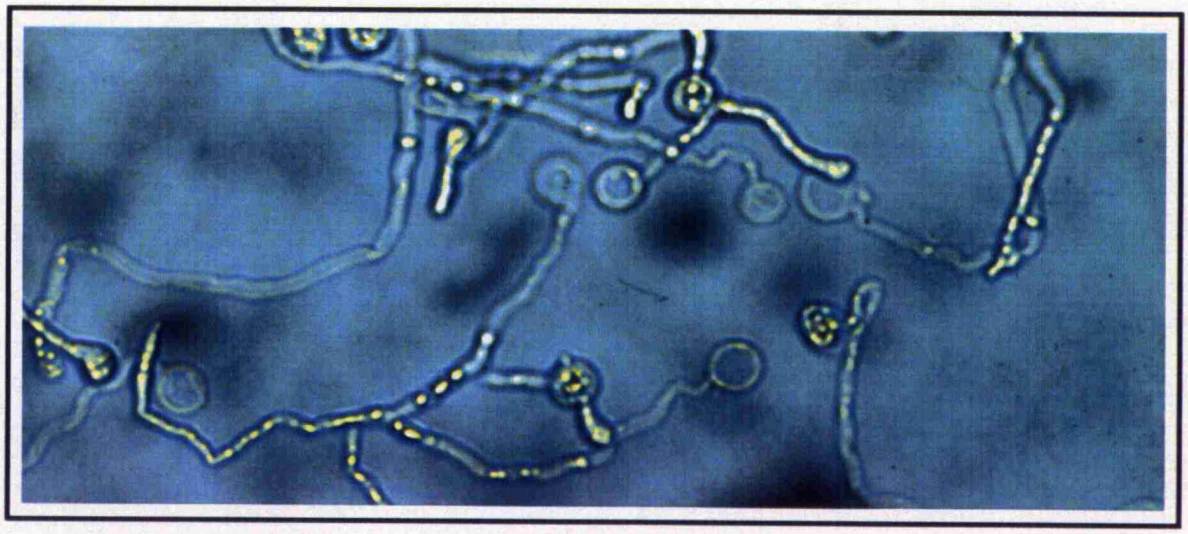
The selection of antibodies from phage-displayed antibody libraries has generally used purified antigens although in one case a virus particle was used (Ziegler *et al.*, 1995). Here, by using the "single pot" antibody library with germinating *P. infestans* zoospores as the target antigen, phage antibodies against surface-exposed epitopes of a complicated intact organism have been selected.

6.2 Results

6.2.1 Isolation of phage antibodies against surface epitopes of germinating zoospores of *Phytophthora infestans*

In preparation for the selection of surface antigens, germinating zoospores of *P. infestans* were washed three times with Q water to remove soluble contaminating antigens from the zoospore culture medium. The intact germinating zoospores were then directly coated onto a solid plastic surface. Germlings adhere readily to the surface of the wells (Fig. 6-1). The "single pot" library (constructed by Nissim *et al.*, 1994) was subjected to five rounds of selection against the immobilised zoospores. In order to promote the selection of antibodies with good affinity and specificity, the coating concentration of the germinating zoospores was reduced ten times after the first two rounds of panning. In addition, the pre-incubation time of the PhAbs with blocking buffer was extended to two hours rather than the half hour normally used for PhAb ELISA.

Fig. 6-1. *Phytophthora infestans* germlings immobilised on microtitre plate well surface used for panning.



Polyclonal PhAbs from each round of selection were analysed for binding activity to germinating zoospores of *P. infestans*. Significant binding to the antigen was detected and the ELISA signal increased with the number of rounds of panning. The specificity of the polyclonal PhAbs was good, with no detectable cross reaction with two unrelated control antigens (Fig 6-2).

Monoclonal PhAbs from the last two rounds of panning were screened for binding activity to germinating zoospores immobilised on ELISA plates. 51% of monoclonal PhAbs showed positive binding to the antigen (Fig. 6-3). Sequence analysis of seven of the PhAb genes revealed that they were all identical. They were derived from the human germline gene DP46 and encoded an HCDR3 10 residues in length (Fig 6-4).

6.2.2 Localising surface epitope on germinating zoospores

Two approaches were employed to use the PhAbs to locate their epitope on the surface of germinating *P.* zoospores. One of these methods involved the use of rhodamine-labelled PhAb prepared using modified conventional fluorochrome-protein labelling methods (see 2.4.14). The fluorescent PhAbs so produced were incubated with germinating zoospores in the dark for three hours at room temperature. Fluorescence staining of the germinating zoospores was viewed in light microscope under actinic irradiation. Controls were made with rhodamine-labelled, antibody-free helper phage particles. The positive fluorescent PhAb-stained germinating zoospores showed strong red fluorescence (Fig. 6-5a). Fluorescence was not evenly distributed on the zoospore surface. The strongest signal was at the tip of the hyphae of the germinating zoospores. The tips of both emergent and elongated hyphae were strongly fluorescently labelled (Fig. 6-5a). The zoospore body was generally weakly fluorescent with a somewhat stronger signal in the region of the spore from which the hypha emerged. In contrast, control germlings showed virtually no detectable fluorescence (Fig. 6-5b).

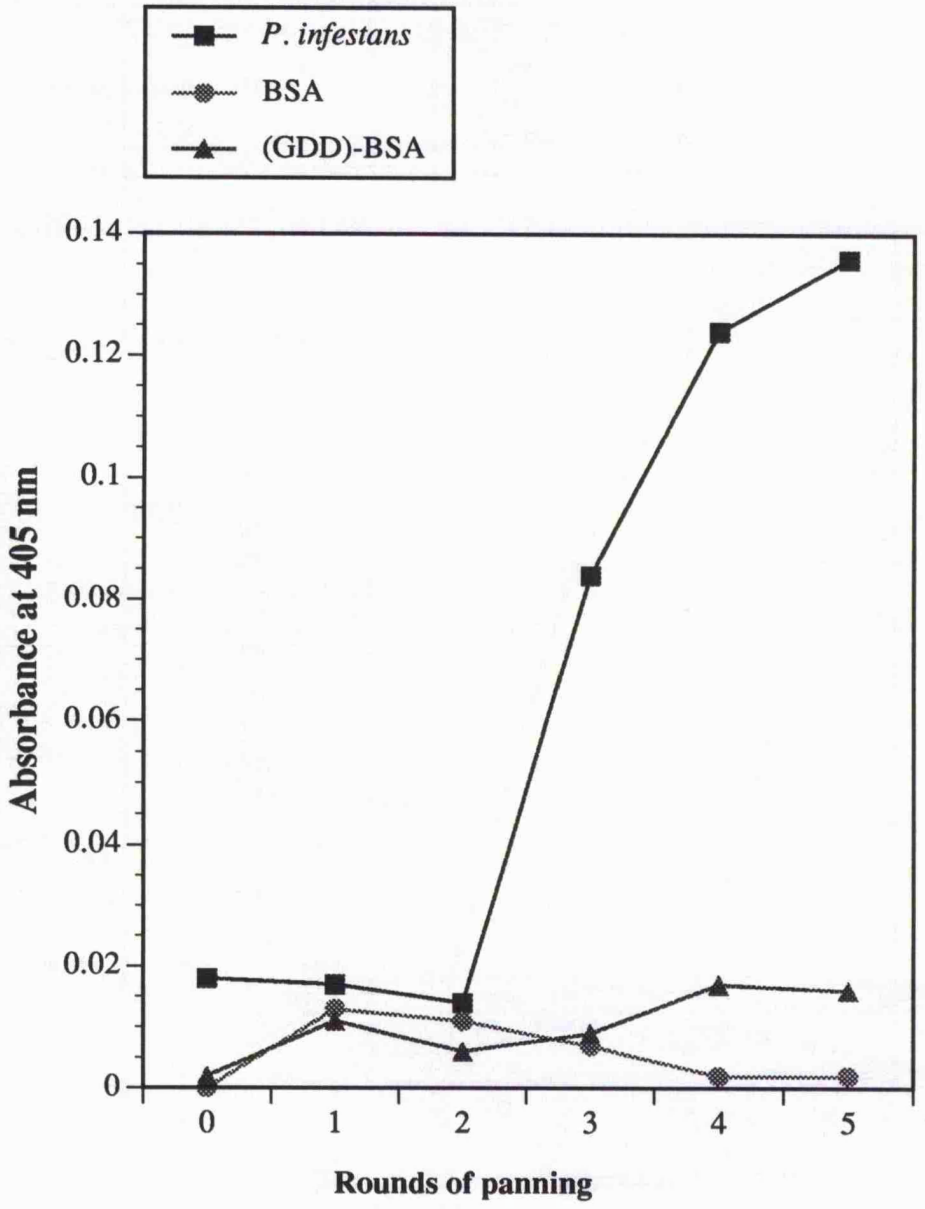


Fig. 6-2. Enrichment of polyclonal PhAbs. PhAbs used here were prepared from following six sources; one was from original library stock and named zero rounds of panned product, others were those eluted PhAbs from each round of panning (from the first to the fifth rounds). The experiment was performed by panning against two targets; germinating zoospores, and a peptide-BSA conjugate (GDD-BSA). Subsequently, GDD-BSA was used as one of the negative control antigens.

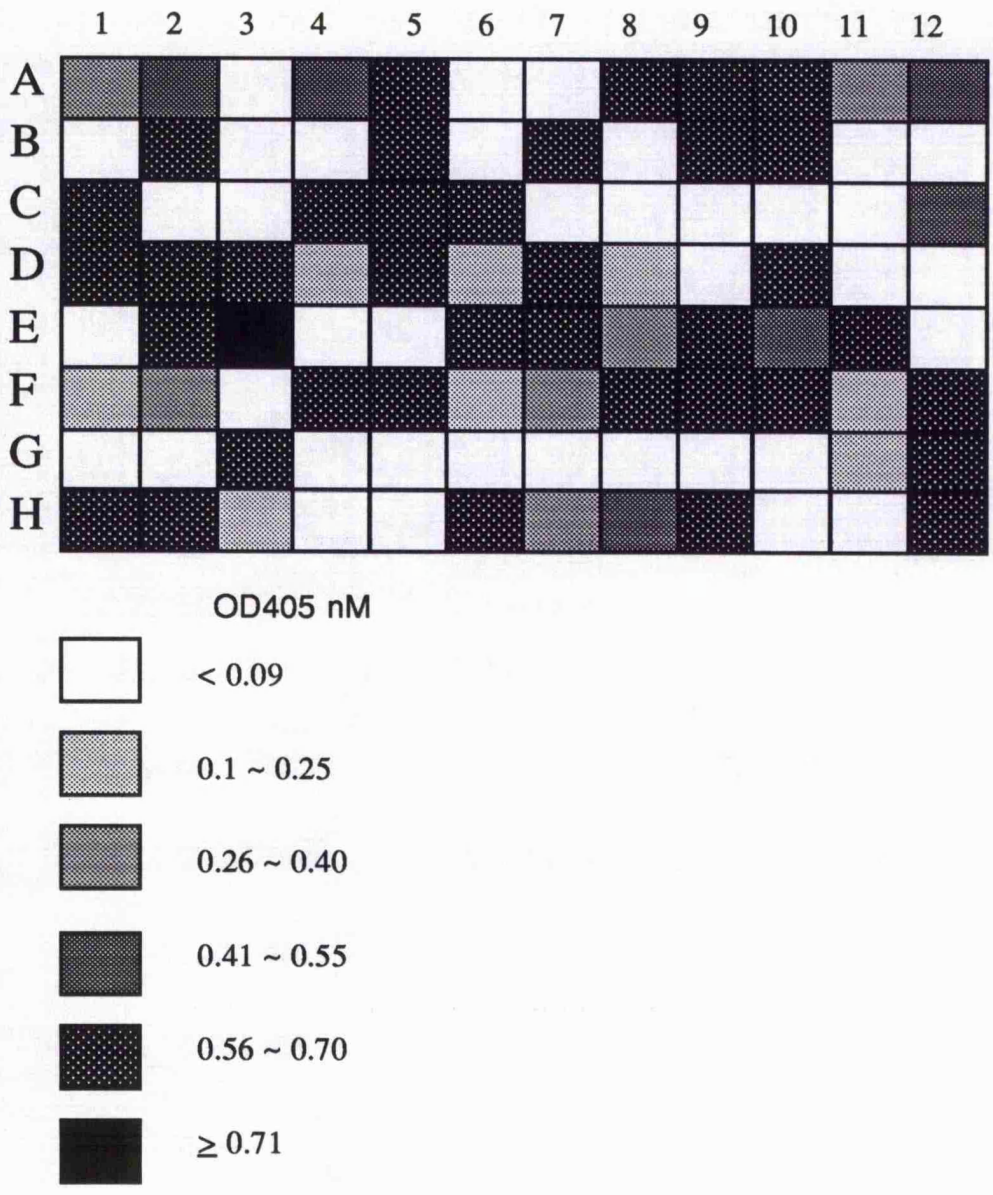


Fig. 6-3. Monoclonal PhAb screening. 96 *E. coli* colonies from the last two rounds of panning were screened for binding activity against germinating zoospores of *P. infestans*.

	FR1	CDR1	FR2	CDR2
A11	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	**SYAMH**	WVRQAPGKGLEWVA	**VISYDGSNKYYADSVKG**

	FR3	CDR3	FR4
A11	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR	**NKLTDRGTQV**	WGQGTLVTVSR

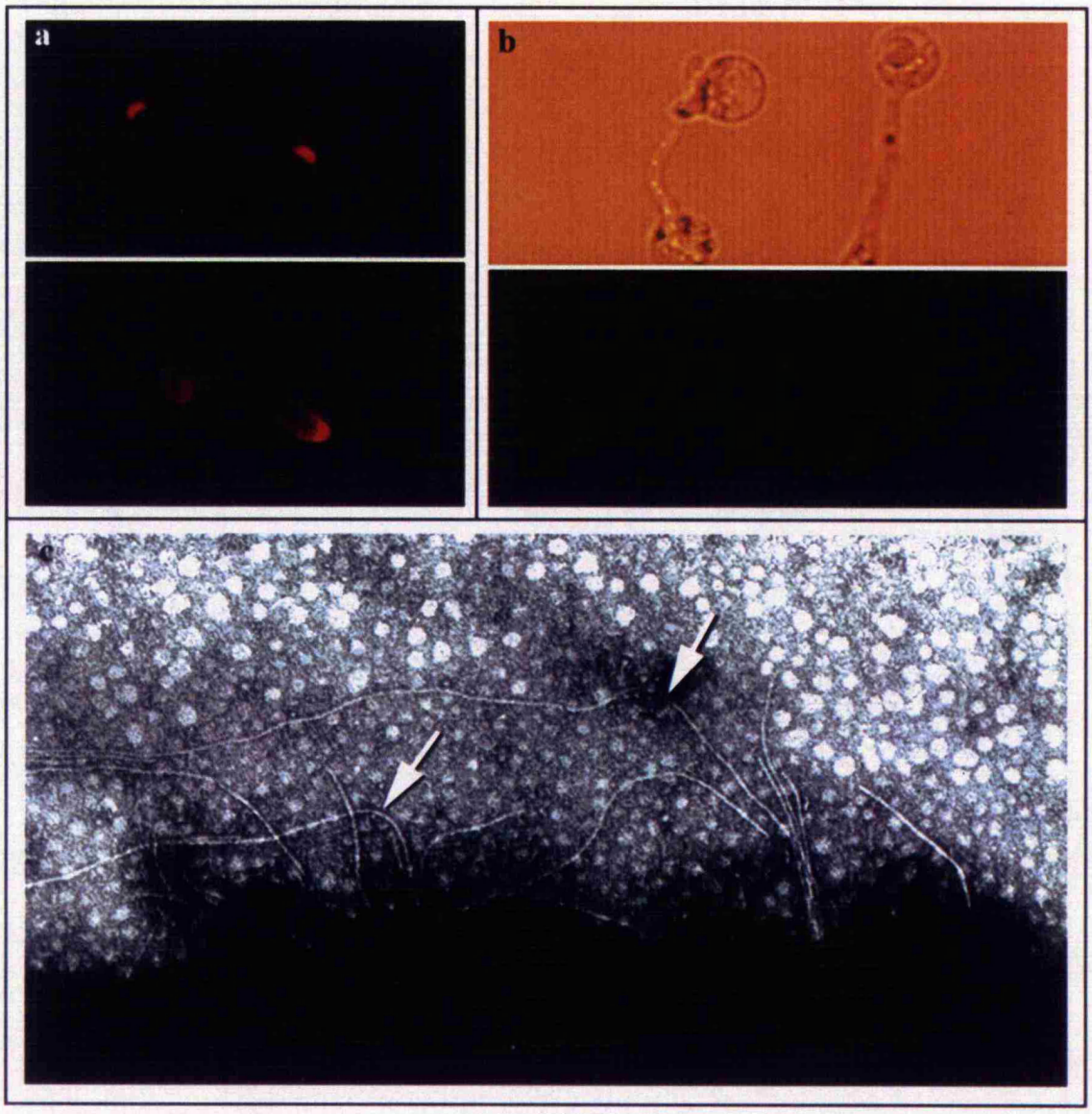
Fig. 6-4. Deduced amino acid sequence of the heavy chain of A11. Frameworks (FRs) and complementarity-determining regions (CDRs) are as defined according to Kabat *et al.* (1991), and shown in boldface.

The second method used to locate the PhAbs epitopes on germinating zoospores involved observation of the PhAb/zoospore complex under the transmission electron microscope. Freshly prepared phage either from the fifth round of panning or unpanned (as a control) were incubated with germinating *P. infestans* zoospores which had been secured to microscope specimen grids by a polyclonal mouse anti-*P. infestans* serum. Electron micrographs of uranyl acetate stained specimens from the fifth round PhAb/zoospore complex showed phage particles attached by one end to the surface of the germinating hyphae (Fig. 6-5c). Since the displayed antibodies are fused to a minor coat protein located at one end of the phage particle, end-on association is consistent with antibody-dependent binding. Thus the PhAbs "pointed" directly to the location of their epitope on the hyphal surface. Both single and aggregated phage particles associated with the surface of germinating zoospores. The aggregation of the PhAbs could be reduced by dilution of the applied phage particles followed by extensive washing with PBS after the primary interaction. By using identical methods, the control phage particle was not found to associate with hyphae. It is interesting to note that bound PhAbs were not spread evenly over the hyphal surface but appeared to be restricted to particular areas. This may indicate that the epitopes recognised by the PhAbs are not evenly distributed on the hyphal surface (Fig. 6-5c).

6.2.3 Binding activity of anti-*Phytophthora* pan_V PhAbs and mouse serum to a range of fungi

In order to compare the different properties of antigen-binding proteins generated by conventional immunisation and selected from artificial phage antibody repertoires, pan_V PhAbs and mouse serum were incubated with antigens from fractions of mycelium homogenates of different plant pathogenic fungi. Pan_V PhAbs were prepared from the fifth round of panning against intact *P. infestans* germinating zoospores immobilised on immuno-

Fig. 6-5. Direct visualisation of filamentous phage binding to surface epitopes of Phytophthora germlings. (a) fluorescence detection of binding of fifth round panned phage to whole germlings (Mag. 400x). (b) germlings treated with control fluorescent phage viewed under bright field and fluorescence conditions (Mag. 400x) (methods see 2.4.14 pp64). (c) electron micrograph of filamentous phage particles (arrowed) associated with the surface of a germ tube tip(Mag. 21,000x) (methods see 2.4.13 pp63).


a
b
c

tubes. Mouse anti-*P. infestans* serum was prepared by standard immunisation procedures with a *P. infestans* mycelium homogenate (serum provided by Dr. J. G. Harrison, SCRI).

The specificities of the mouse serum and pan_V PhAbs were characterised by ELISA using 16 different species of fungal plant pathogens as antigens (provided by Dr. J. G. Harrisson). The pathogens used were members of nine different genera and included five separate species of *Phytophthora* (Fig. 6-6). The polyclonal mouse serum showed approximately equal binding activity to the different species of *Phytophthora.* In contrast the pan_V PhAbs showed strong binding to *P. infestans* and *P. cactorum,* weak binding to *P. drechsleri, P. cryptogea* and *P. erythroseptica* and no significant binding activity towards *P. megasperma.*

Neither PhAbs nor mouse serum showed significant binding activity to *Fusarium oxysporum, Fusarium avenaceum, Trichoderma harzianum, Mortierella hyalina, Polyscytalum pustulans* and *Verticillium tricorpus.* Mouse serum did not bind to *Rhizoctonia solani* or *Chaetonium elatum* whereas PhAbs exhibited weak binding to these species. Strong binding of PhAbs but not mouse serum was also detected against *Pythium sylvaticum* and *Pythium violae.* PhAbs and mouse serum did not show detectable binding to the control negative antigen, BSA (Fig. 6-6).

In order to investigate the interactions of pan_V PhAbs and mouse serum with different isolates of *P. infestans*, soluble fractions of mycelium homogenates were prepared from five different isolates, namely 89/AF1, I137, I53, I106, 36609. Isolate of 36609 was the one used for both the generation of mouse serum and the selection of PhAbs. Mouse serum reacted equally with all of the five isolates of *P. infestans* (Fig. 6-7). Pan_V PhAbs however showed different binding activities towards the different isolates.

6.2.4 Binding activity of monoclonal PhAb A11

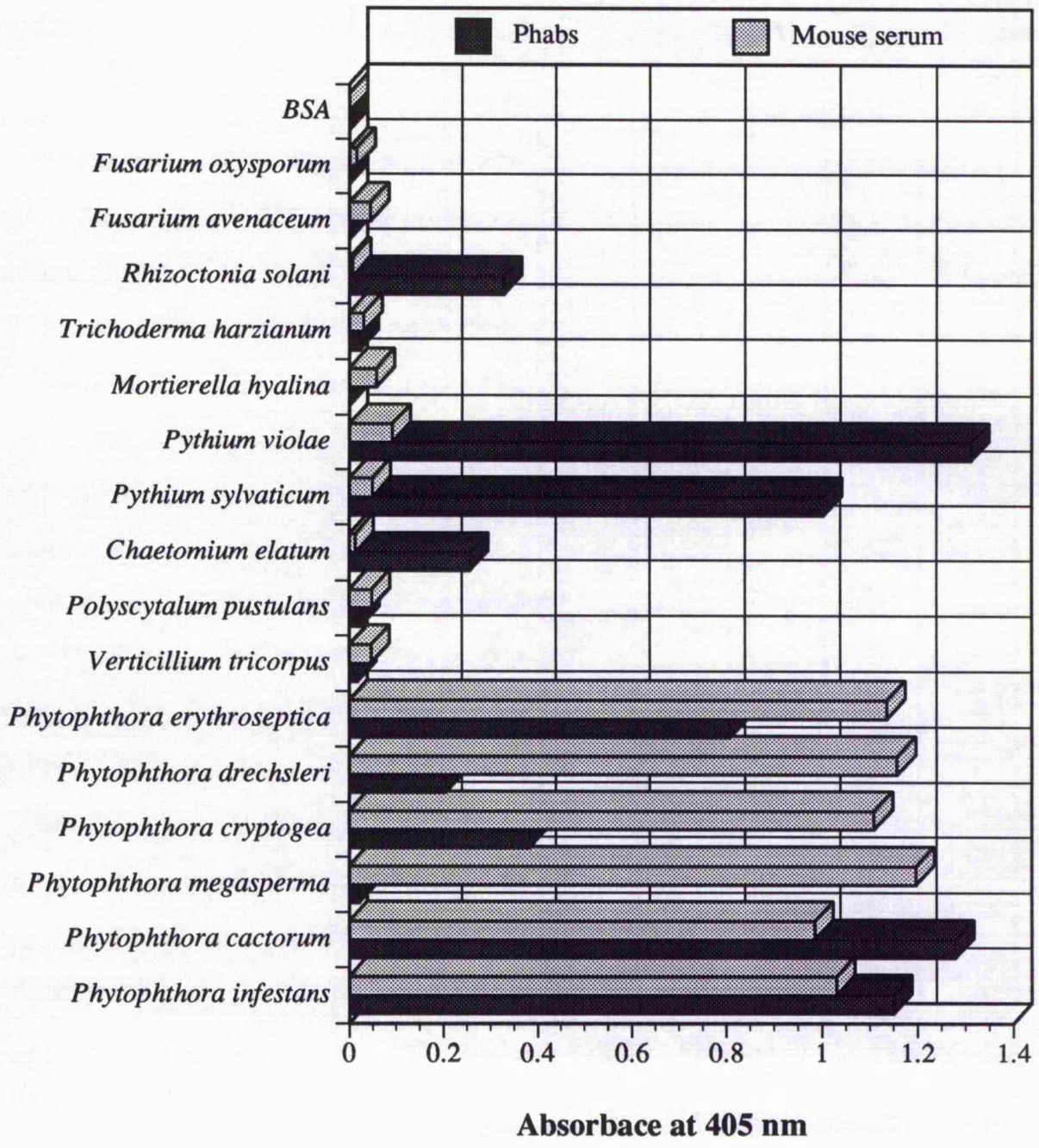


Fig. 6-6. Comparison of binding activity of mouse polyserum and pan_V PhaAbs. The ELISA plate was coated with homogenised fraction of mycelium from 16 different plant pathogenic fungi at a concentration of 20 µg/ml in carbonate-bicarbonate buffer pH9.6. The PhAbs were probed with peroxidase conjugated anti-M13 antibody. The signal was read after incubation with the substrate (ABTS) for 20 min.

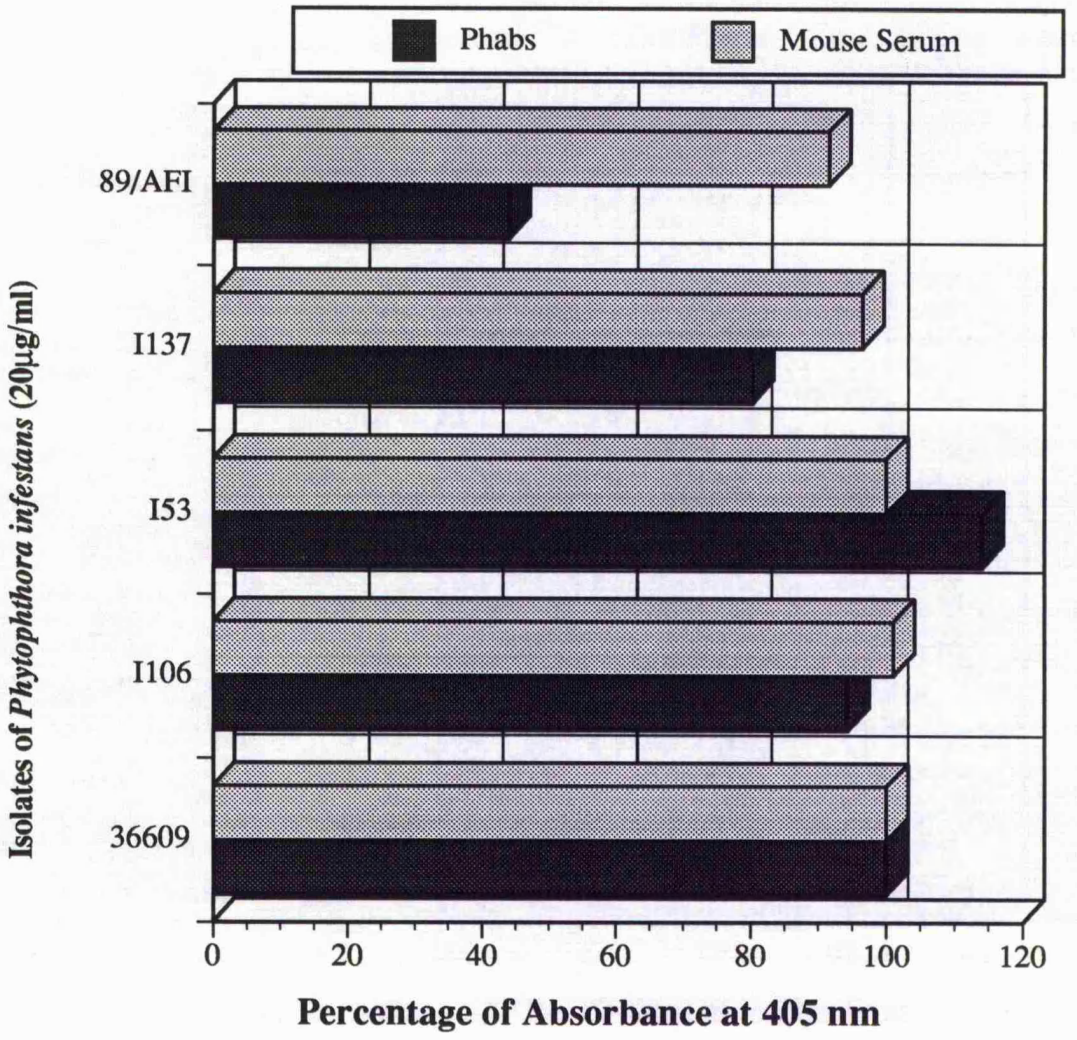


Fig. 6-7. Binding activities of pan_V PhAbs and mouse serum to different isolates of *P. infestans*. The percentage of absorbance was calculated using the signal given by isolates 36609 as 100%.

One monoclonal PhAb, designated A11, was selected for further characterisation and was tested by ELISA for binding activity against 9 different fungi. Overall, the data obtained were broadly similar to those obtained with pan_V PhAbs with the following exceptions. Binding of PhAb A11 to *Chaetomium elatum* was relatively stronger than with pan_V PhAbs. A11 showed relatively weak binding to *Pythium sylvaticum*, but had similar binding activity to *Pythium violae* as pan_V PhAbs. Furthermore, although there was no significant reaction between A11 and *Rhizoctonia solani*, the pan_V PhAbs showed weak but significant reaction towards this species (Fig. 6-8).

6.3 Discussion

In conventional immunisation methods any whole organism or structure is likely to be destroyed within the animal with the consequent release or exposure of any internal contents. Thus all components, whether originally external or internal, have the potential to act as antigens. In the case of selection of antibodies from phage-displayed libraries however, it should be possible by panning against whole organisms, provided they survive the panning process undamaged, to select antibodies which are specifically directed against surface exposed epitopes.

In order to investigate this possibility a "single pot" antibody library was panned against an intact organism, namely, germinating zoospores of *P. infestans*. Usually, panning for rare binders has involved the use of purified antigen at high concentration. In these experiments an unpurified complicated intact organism was used for the selection of binders to surface-exposed epitopes. The concentration of germinating zoospores was not very high (about 1000 per tube in the first two rounds of selection and 100 per tube for the last three rounds). Nevertheless, the ELISA signal obtained at each round of selection of pan_V PhAbs indicated that specific binders were being isolated. The whole surface of germinating zoospores must contain many different antigenic determining regions. It might be expected therefore that with

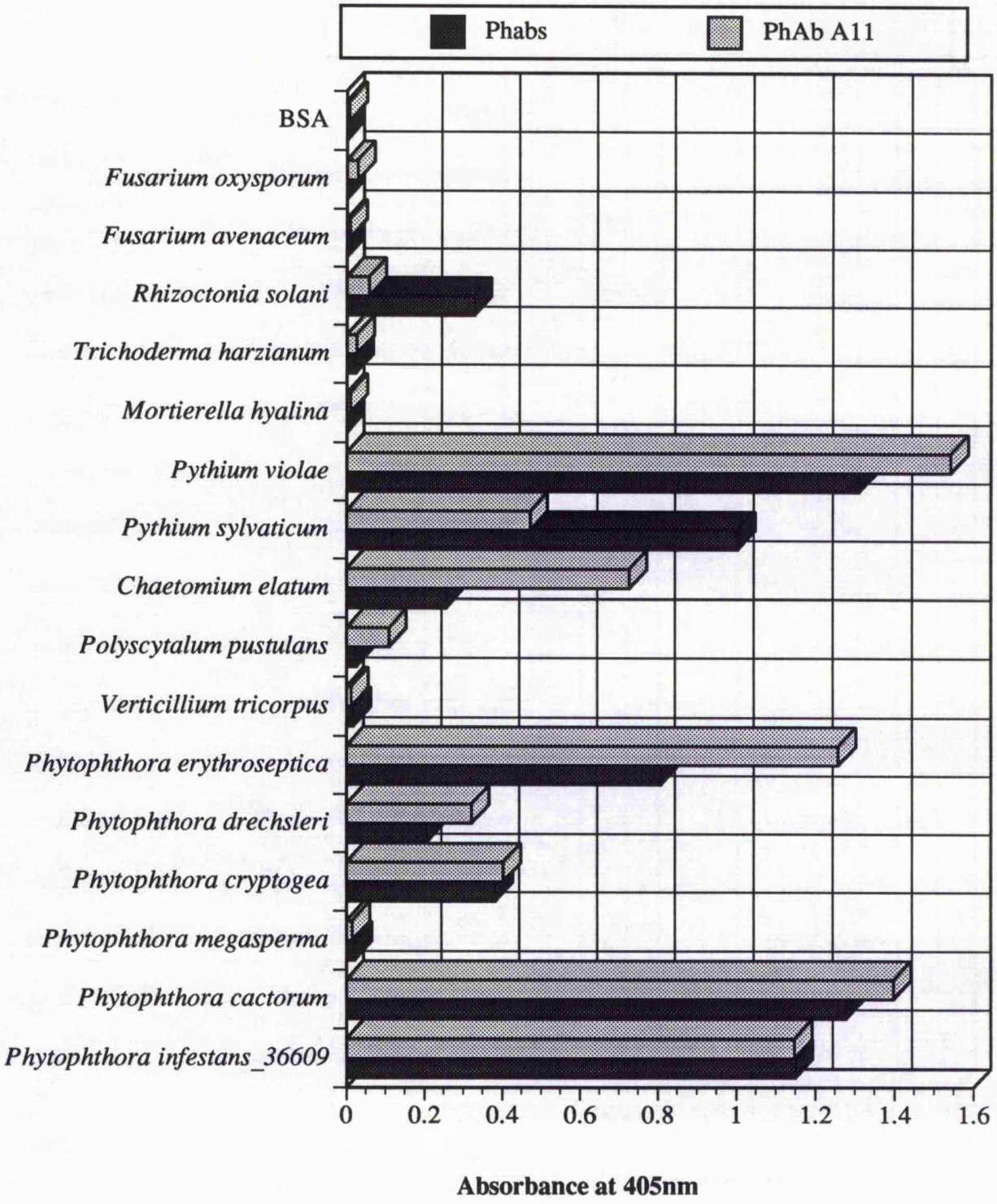


Fig.6-8. Comparison of binding activity of pan_V PhAbs and mono-clonal PhAb A11. The ELISA plates were coated with homogenised fraction of mycelium from 16 different plant pathogenic fungi at a concentration of 20 µg/ml in carbonate-bicarbonate buffer pH9.6. The PhAbs were probed with peroxidase conjugated anti-M13 antibody. The signal was read after incubation with the substrate (ABTS) for 20 min.

the selection process used here, a pool containing a range of different antibody fragments would be generated. However, from seven different randomly chosen colonies, a single identical amino acid sequence was obtained. These preliminary results may indicate that the epitope recognised by A11 was abundant on the entire surface of the germinating zoospores. The possibility cannot be excluded that the "single pot" library was not sufficiently diverse to provide antibodies to a range of the surface-exposed epitopes of *P. infestans*. However in other applications using this "single pot" repertoire with purified antigen, there have been no reports of failures to isolate binders. One possibility is that A11 recognised the most abundant epitope on the surface of germinating *P. infestans* zoospores, because the binding property of A11 is very similar to that of the pan_V PhAbs. Since the nucleotide sequence of only about 13% of selected colonies of the pool of pan_V PhAbs was analysed, the possibility remains that some of them may be different from A11. In order to isolate PhAbs recognising epitopes of low abundance, a high concentration of antigen should be used for selection, or panning methods could be modified by, for example, reducing the washing stringency or using less rounds of panning.

From the point of view of structure, PhAbs differ very significantly from single antibody molecules. The protein domains of PhAbs involved in antigen binding represent a very small proportion of the whole phage particle. The binding characteristics of PhAbs are therefore likely to be similar to but not necessarily identical with an equivalent antibody or antibody fragment. It has been shown that PhAbs can be used as the primary antibody in ELISA and western blotting, and could also be used in affinity chromatography. However, it was reported that in contrast to scFv fragments, anti-p53 PhAbs could not be used in a nucleus staining method. This was attributed to the relatively large volume of the phage particles compared with scFv fragments (Nissim *et al.*, 1994).

The present work demonstrates new methods for the use of PhAbs as detection reagents. In one of these methods the fluorescence labelling of the phage particle allows the direct visualisation of the location of target epitopes. The phage particle has a protein shell, with the amino termini of major coat proteins exposed to the solvent. Thus for the purposes of fluorescence labelling, the phage particle can be treated as a single large protein molecule. The preliminary results reported here demonstrate that it is possible to use fluorescent PhAbs to image germinating *P. infestans* zoospores. Fluorescence labelling of the PhAbs is very similar to the conventional protein labelling method, and is simple and reliable. It might be also possible to use fluorescent PhAbs as fluorescent intact antibodies or antibody fragments in other conventional immunochemical techniques.

Filamentous phage particles can readily be viewed at 20,000 to 35,000 magnification and PhAb /hepatitis B virus complexes have been detected using the electron microscope (Zebedee *et al.*, 1992). The present work demonstrates that PhAbs can also point out the position of their cognate epitope on the surface of the germination tube of *P. infestans* zoospores. This provides a new and precise immunochemical tool for epitope localisation not only on fungal pathogens as demonstrated here but essentially any PhAb epitope. For example, by display and isolation of some identified surface target, the target position could be located directly under the electron microscope without any further modification such as gold labelling.

In order to generate anti-fungal antibodies by conventional immunisation it is necessary to homogenise the fungal material. Thus antibodies will be raised against epitopes which were both inside the intact organism or exposed on its surface. By using the intact organism in conjunction with PhAb repertoires, it is possible to isolate PhAbs which recognise only surface-exposed epitopes. Comparisons of antibodies to *P. infestans* produced by these two methods shows clear differences in binding characteristics. In experiments with 5 different isolates of *P. infestans* the mouse serum interacted equally with all of them. In contrast the

PhAbs might bind less strongly to isolate 89/AF1, indicating that the epitopes recognised by the PhAbs might be less abundant in this isolate than in the other four.

Furthermore, there were differences in interactions with a range of *Phytophthora* species and also with representatives of other fungal genera. The mouse serum recognised all of the *Phytophtora* species investigated but none of the other genera. The PhAbs did not react equally with all of the *Phytophthora* species tested and did not interact at all with *P. megasperma*. This may indicate that the epitopes recognised by the PhAbs are not present in *P megasperma* or are only present at very low abundance. Unlike the mouse serum the PhAbs strongly recognised epitopes of *Pythium* species. Thus it appears that there are epitopes of *P. infestans* isolate 36609 (the isolate used to raise both the PhAbs and the mouse serum) which did not result in the generation of antibodies in the mouse but which did bind PhAbs during the panning process. The localisation of the PhAbs by both light and electron microscope studies shows that their epitopes are surface exposed in the intact organism. In this instance the selection of antibodies from PhAb repertoires has resulted in the isolation of antibodies against epitopes which were not represented (or represented in very low abundance) in serum generated by conventional immunisation.

This work indicates that PhAb repertoires may be of particular significance in the isolation of antibodies against epitopes on the surface of whole complex organisms. Such PhAbs can be used directly (or as a source of soluble fragments) to localise the cognate epitope in light or electron microscopy. Other uses of such antibodies may be in the characterisation and isolation of the cognate epitope by affinity purification, as a probe in cDNA expression libraries and, if the antibody causes phenotypic effects, in the study of the epitope's function.

Chapter 7

Selection of antibodies against a peptide-BSA conjugate from the "Single Pot" library and its expression as functional maltose binding protein fusions in the cytoplasm of *Escherichia coli*

7.1 Introduction

Phage antibody repertoires (such as the 'single pot' library, described in detail in chapter 6.1.) have been used to generate many different binding specificities, including those that are difficult to generate by conventional animal immunisation. Antibodies from such libraries appear to be highly specific and have been used for western blotting and epitope mapping (Nissim *et al.*, 1994; Griffiths *et al.*, 1994).

In conventional procedures for the generation of monoclonal antibodies against haptens or short peptides it is necessary to immunise animals with conjugates of the haptens or peptides to a carrier protein. Complicated screening procedures are required to eliminate anti-carrier antibodies. *In vitro* selection of antibody fragments from artificial immune systems offers a more rapid approach to the selection of monoclonal antibody fragments against haptens or short peptides. In this chapter, a decamer peptide conjugate was used as the target epitope. The peptide was chemically synthesised and coupled to BSA.

The target peptide contains a conserved amino acid sequence motif "GlyAspAsp" found in the 'replicases' or RNA-dependent RNA polymerases (RdRp) (Kamer and Argos, 1984; Bruenn, 1991) of positive-stand RNA viruses of the 'alpha-like' super group (Goldbach *et al* 1991). It

has been demonstrated that the motif plays an important role in virus RNA replication (Mi and Stollar, 1990; Longstaff *et al.*, 1994), and binding of antibodies to this motif results in inhibition of replication *in vitro* (Hayes *et al.*, 1994). Thus, the GDD motif is a possible candidate target in antibody-mediated plant protection strategies involving intracellular expression of anti-viral antibodies. In a successful application of this kind of approach, Maciejewski *et al.*, (1995) reported that intracellular expression of antibodies against HIV reverse transcriptase resulted a high degree of protection against multiple HIV1 strains *in vitro*.

The use of such approaches *in vivo* will require functioning antibodies in the cytoplasm. Most experiments involving high level expression of antibodies in the cytosol of *E. coli*, have resulted in the formation of insoluble non-functional products (Skerra, 1993) and the development of methods to produce functional cytosolic antibodies is proving to be a challenging task. Plückthun and co-workers showed that a low level of soluble, functional antibody can be produced in the cytoplasm of *E. coli* strains lacking the enzyme thioredoxin reductase (TrxB) (Proba *et al.*, 1995). Another approach is to fuse the antibody to a protein which is highly soluble in the cytoplasm. The *E. coli* maltose binding protein (MBP), is an example of a soluble protein which can be expressed to high levels. MBP has previously been successfully used for the production of bifunctional antibody fragment fusion proteins in the bacterial periplasm (Bregegere *et al.*, 1994), and it is investigated in the present work as a suitable fusion partner for the production of functional cytosolic antibodies .

This chapter presents some work on the use of the "Single Pot" library to select antibodies against GDD motif peptide-BSA conjugate, and also some preliminary results on its expression as a functional MBP::scFv fusion protein in the *E. coli* cytoplasm.

7.2 Results

7.2.1 Selection of PhAbs against peptide-BSA conjugate

In order to select PhAbs which bind specifically to the peptide-BSA conjugate, 1% BSA was added to the PhAbs dilution buffer to 'compete out' BSA-specific PhAbs. This kind of competition selection was applied to every round of panning. For the first two rounds of selection, a relatively high concentration of antigen (100 μg/ml) was used to coat the immuno-tube. In order to promote the selection of PhAbs with higher binding affinities, the coating concentration of antigen was reduced 100 times to 1 μg/ml for the last three rounds of panning. An ELISA experiment showed that pan_V PhAbs from the third to the fifth round of panning bound specifically to the peptide-BSA conjugate, but did not bind to the BSA carrier protein alone. The ELISA signal increased with number of rounds of panning (Fig. 7-1).

Monoclonal PhAbs from panning rounds 4 and 5 were selected from a 96 well culture plate. The peptide-BSA conjugate-binders were detected by ELISA. After 4 rounds of selections, 25% of colonies tested were positive; and after 5 rounds of selections, 50% of tested colonies were positive (Fig. 7-2). Partial VH sequences of 10 different colonies were determined. Of the 10 sequences determined 9 were found to be identical. Antibodies with this sequence, derived from the human DP7 germline segment, are referred to here as G11. The heavy chain gene of G11 encoded an HCDR3 length of 6 residues (Fig. 7-3). The remaining sequence which differed from the rest (G9) was derived from the human DP46 segment and encoded an HCDR3 length of 11 residues.

PhAbs from colonies G9 and G11 were used to infect *E. coli* strain HB2151 in an attempt to produce soluble scFv antibody fragments. Soluble G11 scFv was successfully produced, but G9 was not. To investigate possible reasons for the failure to produce soluble G9 two other non-suppressor strains, TB1 and W3110, were also transformed with phagemid DNA. No soluble G9 scFv was detected in the bacterial medium.

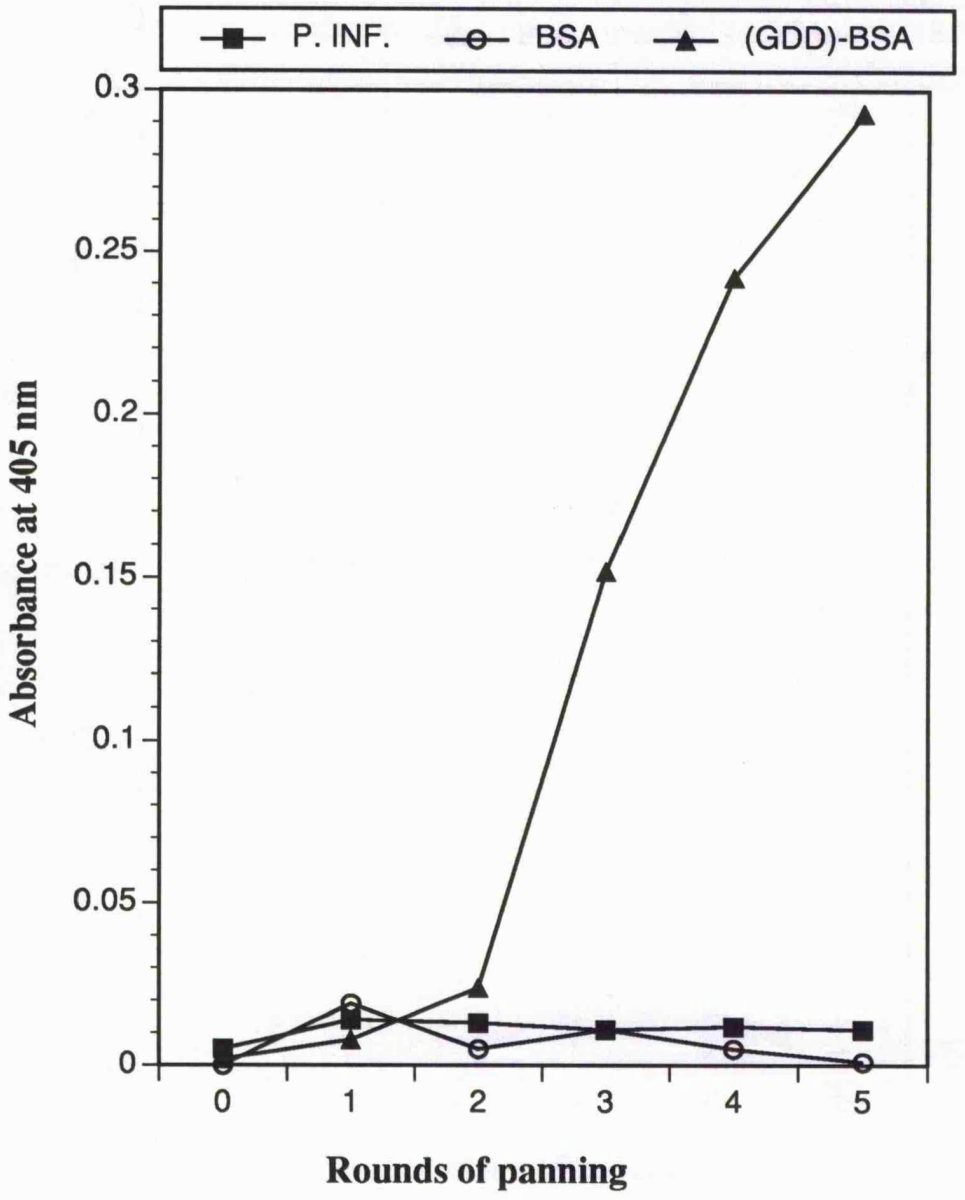


Fig. 7-1 Enrichment of polyclonal PhAbs. The PhAbs used here were prepared from the following six sources; one was from the original library stock and is termed zero rounds of panning and the alters frome 1-5 rounds of panning. The experiment was performed by panning against two targets; the GDD motif peptide-BSA conjugate and germinating zoospores of *P. infestans* (indicated as P. INF.). Subsequently, the germinating zoospores was used as one of the negative control antigens.

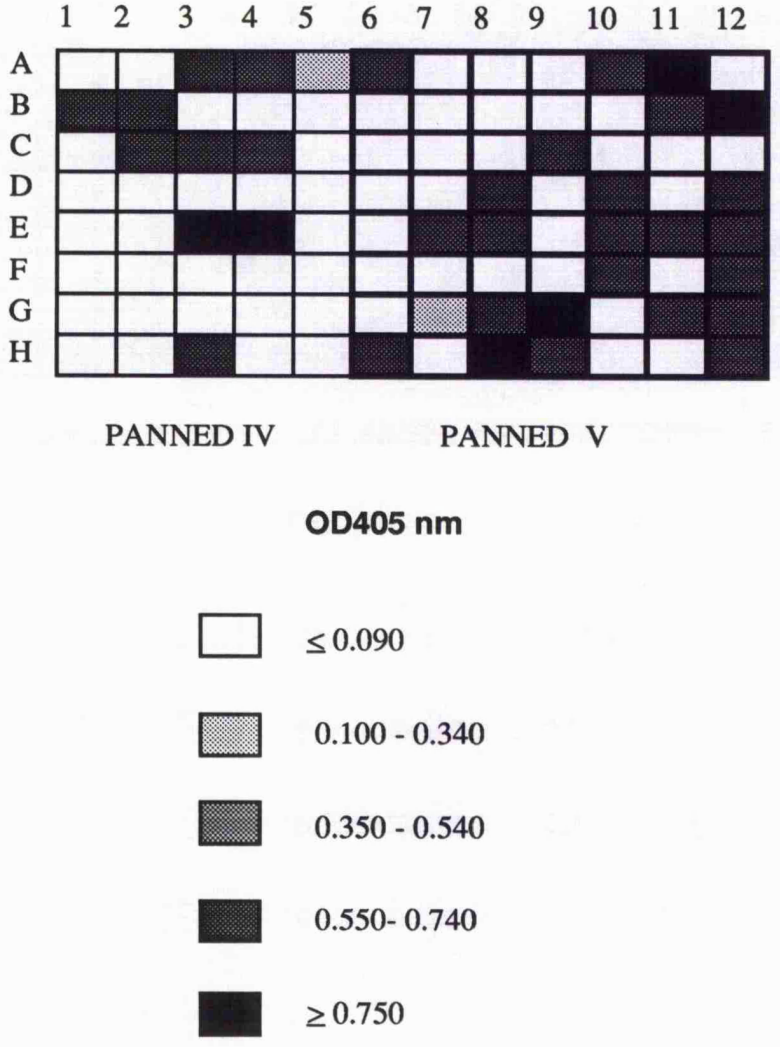


Fig. 7-2 Monoclonal PhAbs screening. Colonies from the fourth and fifth panning rounds were screened for binding to GDD-BSA conjugate.

	FR1	CDR1	FR2	CDR2
G11	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	**SYYMH**	WVRQAPGQGLEWMG	**IINPSGGSTSYAQKFQG**
G9	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	**SYAMH**	WVRQAPGKGLEWVA	**VISYDGSNKYYADSVKG**

	FR3	CDR3	FR4
G11	RVTMTRDTSTSTVYMELSSLRSEDTAVYYCAR	**NPYLIQ**	WGQGTLVTVSR
G9	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR	**GVSFRHKVVNL**	WGQGTLVTVSR

Fig. 7-3. Heavy chain amino acid sequences of anti-GDD motif peptide BSA conjugate scFvs, G9 and G11. Frameworks (FR) and complementarity-determining regions (CDR) are as defined according to Kabat et al. (1991), and shown in boldface.

7.2.2 Characterising G11 and G9

In order to investigate the specificity of G11 and G9, a competition ELISA was performed. Soluble G11 scFv, used as competitor, inhibited the antigen-binding activity of both PhAbs G9 and G11. Soluble G11 strongly reduced the signals detected from PhAb G11, whereas binding of PhAb G9 to peptide-BSA conjugate was less inhibited (Fig. 7-4). At 1/64 (0.0156) dilution of soluble scFv G11, the signal from G11 PhAb ELISA was reduced by 72%, compared with 44% for PhAb G9.

The peptide FVNGDDLLIA was coupled to BSA through its amino terminal. The carboxyl group of the peptide will therefore be accessible to solvent. Usually, in X-ray studies of the structure of Fab-peptide complexes, 7 to 10 residues are visible in the antibody binding pocket (Stanfield and Wilson, 1995), so the "GDD" peptide itself may provide a single epitope. The peptide conjugate probably provides three kinds of epitope: one which is located at the peptide-BSA junction; a second which comprises the entire peptide and a third, which includes the negatively charged C-terminus of the peptide. The observed competition between G9 and G11 may indicate that they may bind to the same epitope. However it is also possible that they bind to overlapping epitopes or that their affinities are different.

7.2.3 Reaction with "parental" protein

A series of experiments were designed to test whether PhAbs selected against the GDD peptide could recognise RdRps which contain this motif. RdRps from two sources were chosen : native RdRp prepared from a crude tissue extract of PVY-infected tobacco plants, and full-length recombinant RdRp synthesised by using the pRSET bacterial expression system.

PVY-infected plant tissue extracts were prepared by homogenisation of tissue frozen in liquid nitrogen, and then extracted in PBS (containing 2 mM DTT). Equal amounts of total soluble

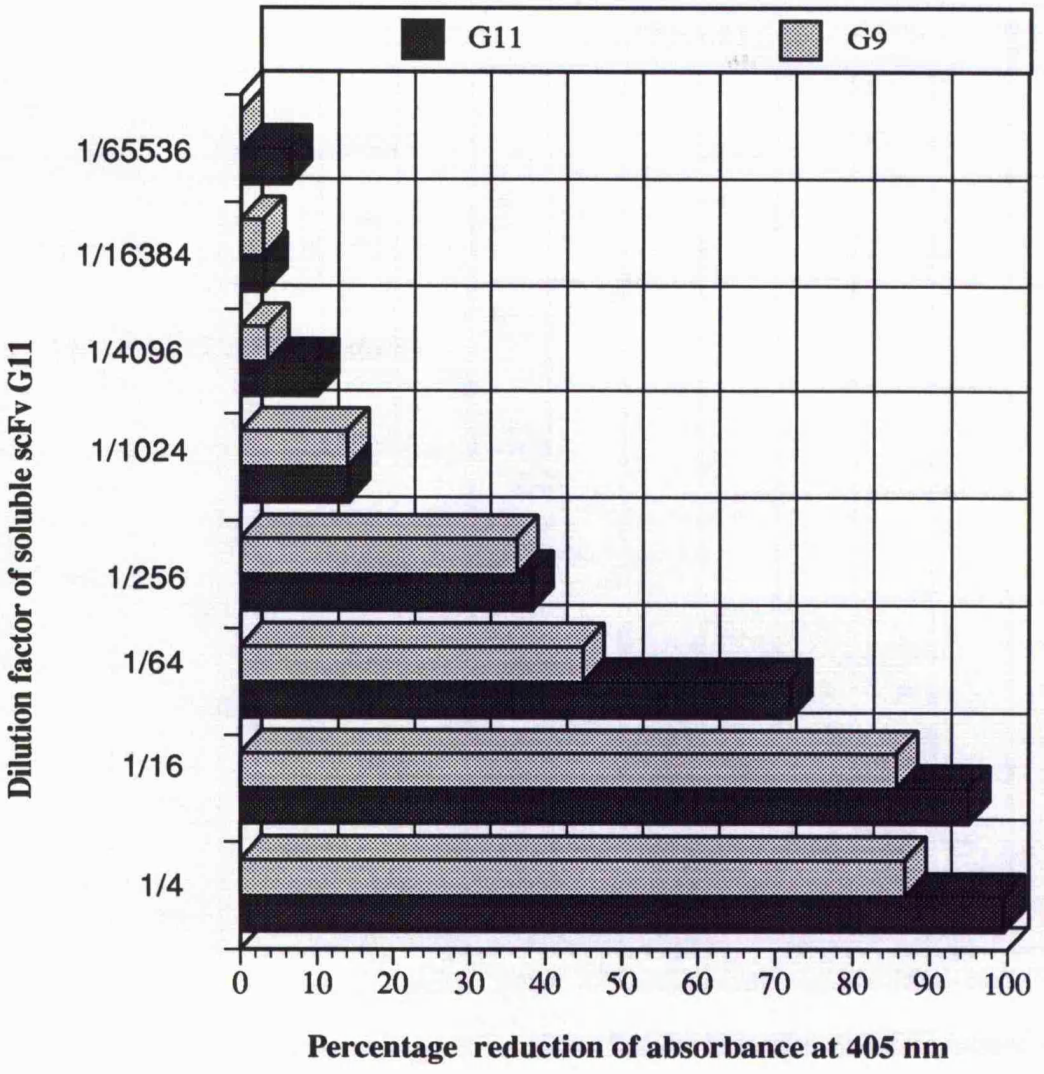


Fig. 7-4. Inhibition of binding activity of PhAbs G9 and G11 against GDD-BSA by soluble scFv G11.

protein from infected and uninfected plants were used to coat ELISA plates. When PhAbs G9 and G11 were incubated with immobilised antigens from PVY-infected plant tissue extracts both gave signals above background (i.e. wells coated with uninfected plant extract) (Fig. 7-5). G9 gave a higher signal than G11. The signal obtained using control (helper) phage was less than 10% of the signals obtained with the selected PhAbs. A further study showed that it was necessary to use freshly prepared infected plant extracts. No signal was detected with frozen and thawed extracts.

Competition ELISA provided evidence that the PhAbs reacted specifically with some component of PVY infected plants. GDD-BSA conjugates competed significantly with the specific interactions between the PhAbs and the "antigen" in infected plant extracts. Furthermore, this kind of competition could not be duplicated with control "antigens" from healthy plant tissue (Fig. 7-6).

A gene encoding the full length of PVY RdRp protein was cloned into pRSET expression vector. The use of this vector also results in the fusion of a short peptide (His)6 to the N terminal of the recombinant RdRp. In order to allow high efficiency purification, a (His)6 tail was also introduced into the C terminal of the recombinant RdRp by the mutation of two residues to His. Since the original RdRp contained 4 His residues at its C termini this resulted in the production of a protein with six histidine residues at both the C and N termini. The RdRp protein was expressed in *E. coli* strain TG1.

PhAb G9 and soluble scFv G11 showed no detectable binding to the bacterial synthesised full length RdRp in western blots. However, using rabbit polyclonal antibodies raised against MBP-RdRp (N-terminal truncated) fusion protein the bacterial synthesised full length RdRp could be detected in western blots as a band which migrated at about the 45kDa position (Fig. 7-7). No signal was detected when G9 and G11 antibody fragments were tested in western blots for binding to the MBP-RdRp (truncated) fusion protein.

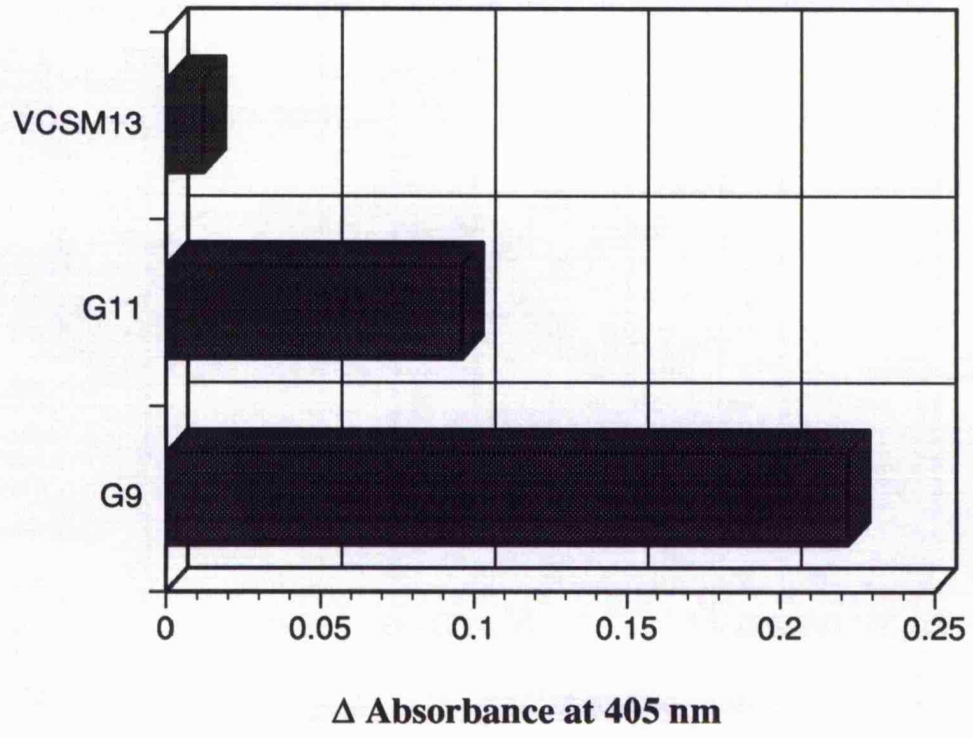


Fig. 7-5 Interaction of phage particles (VCSM13 helper phage, G9 and G11 PhAbs) with antigens from extracts of *Nicotiana* plants infected with PVY (N strain) for two weeks. The figure showed here are signals above those obtained from the wells coated with the extract from a uninfected plant.

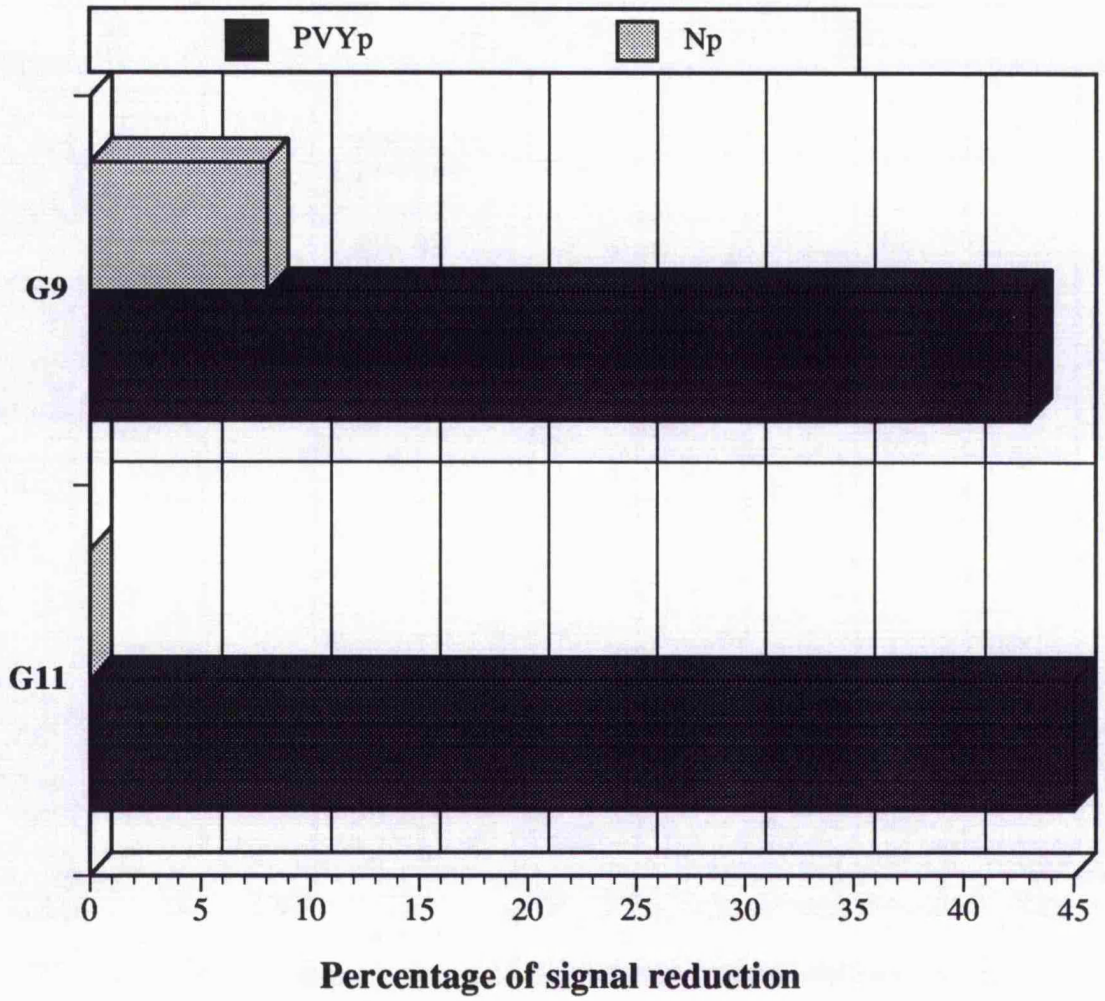


Fig. 7-6 The inhibition of PhAbs (G9, G11) binding to immobilised antigens from plant tissue by GDD-BSA conjugates. Np: Normal *Nicotiana* plants; PVYp: *Nicotiana* plants were infected by PVY (N strain) for two weeks.

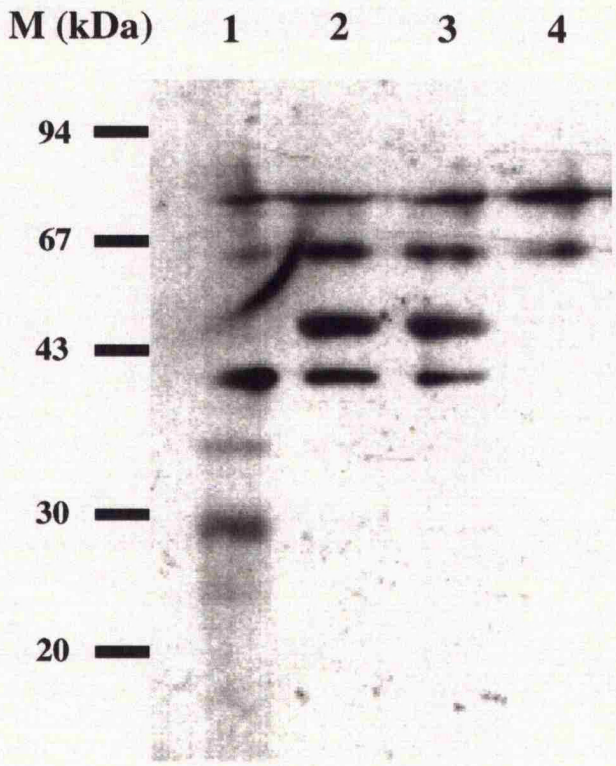


Fig. 7-7. Western blot of *E. coli* synthesised RdRp and probed with rabbit antiserum against truncated RdRp-MBP fusion protein. Total bacterial proteins were prepared from wild type *E. coli* and recombinant *E. coli* strain TG1 (pRSET_B::RdRp) and separated on 12% SDS-PAGE. Lane 1: wild type *E. coli*. Lane 2: recombinant colony 1 infected by M13T7 phage (to introduce T7 polymerase). Lane 3: recombinant colony 2 infected by M13T7 phage. Lane 4: mixture of recombinant colonies 1 and 2 without infection by M13T7.

7.2.4 Expression of MBP::G9 fusion protein

It did not prove possible to produce soluble scFv G9 by transforming non-suppressor strains such as HB2151, W3110 and TB1 with the G9 phagemid DNA. Analysis of the nucleotide sequence confirmed that this was not the result of the presence of an additional amber codon in the reading frame of the G9 HCDR3. It remains unclear why soluble scFv of G9 was not produced at a detectable level.

In order to produce a soluble G9 scFv use was made of the pMAL_c2 and pMAL_p2 maltose binding protein fusion vectors to allow the expression and purification of MBP::G9 fusion proteins. The G9 gene fragment was inserted downstream of the *malE* gene of *E. coli*, which encodes maltose-binding protein, resulting the expression of an MBP fusion protein (Guan *et al.*, 1987; Maina *et al.*, 1988). Here the transcription of MBP-G9 fusion is driven by the strong "tac" promoter and the *malE* translation initiation signals to give high-level expression of the gene of interest (Amann and Brosius, 1985; Duplay *et al.*, 1984). The products may be easily purified in a one-step procedure taking advantage of the affinity of MBP for maltose (Kellerman and Ferenci, 1982). The vectors also carry the *lac*I^q gene, which codes for the lac repressor and keeps expression from P_{tac} low in the absence of IPTG induction.

The use of the pMAL_c2 vector, which encodes MBP without its signal sequence, results in the production of the MBP fusion protein the *E. coli* cytoplasm and a large quantity of both soluble and insoluble MBP-G9 fusion was detected in this location. The amount of soluble protein represented about half of the total fusion protein accumulated (Fig. 7-8). The soluble cytoplasmic fusion protein showed weak binding activity to the peptide conjugate. This binding activity could be detected both before and after purification of the fusion protein. The crude extract containing soluble cytoplasmic MBP-G9 fusion protein gave signals in peptide conjugate coated ELISA wells. Non-specific signals were also obtained with wells coated with

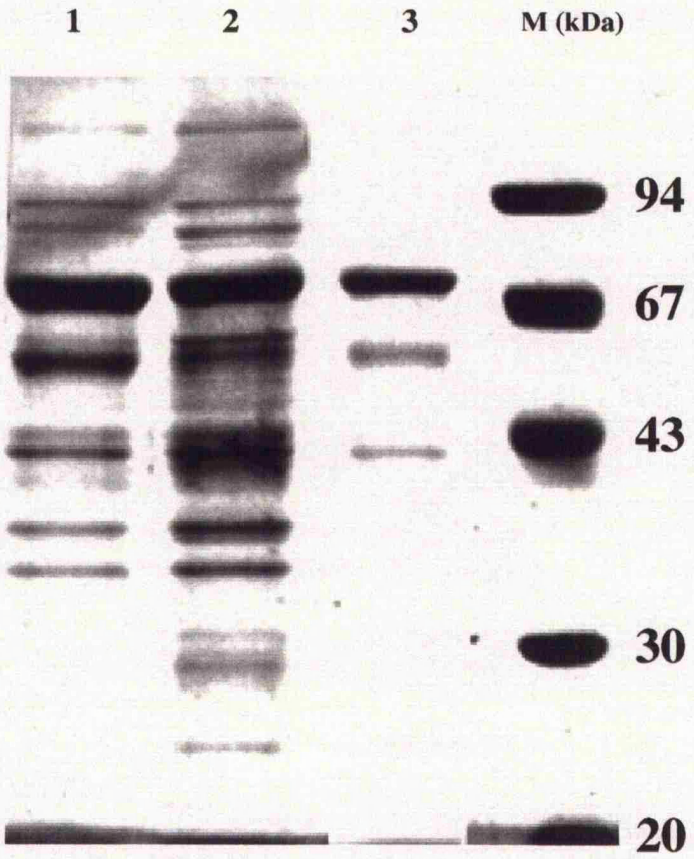


Fig. 7-8. SDS-polyacrylamide gel stained with Coomassie blue of amylose column fractions containing cytoplasmic MBP::G9 fusion synthesised in *E. coli* strain W3110. Lane 1: insoluble fraction from recombinant W3110 (pMAL_c2::G9). Lane 2: soluble fraction prepared by sonication. Lane 3: purified soluble MBP::G9 fusion protein.

negative antigen (BSA), to which G9 PhAbs did not bind. This non specific binding was not detected after affinity purification of the cytoplasmic MBP-G9 (Fig. 7-9).

The pMAL_p2 vector is designed to express MBP fusion proteins complete with the MBP signal sequence which directs the fusion protein to the bacterial periplasm. When this vector was used to direct an MBP-G9 fusion to the periplasm of *E. coli* following IPTG induction, cell growth rate was significantly reduced. This was not observed when the MBP-G9 fusion accumulated in the cytoplasm. Thus the fusion protein appears to be toxic when secreted to the periplasm but not when it is retained in the cytosol. By estimating with coomassie blue staining, the expression level of MBP-G9 when directed to the periplasm was very low compared with that in the cytoplasm. Usually, fusion proteins expressed in the cytoplasm accumulate to about 8 to 15 fold higher levels than that in periplasm. However although much lower levels of secreted MBP-G9 fusion proteins accumulated, the secreted form showed strong binding activity to the peptide-conjugate. This may result from enhanced disulphide bond formation and folding of G9 in the periplasm of *E. coli*. A very weak binding activity to the peptide conjugate was also detected using crude bacterial medium of *E. coli* cultures containing pMAL_p2:G9. This may represent leakage resulting from permeabilisation of the outer membrane of *E. coli* at the elevated expression temperature (Plückthun and Skerra, 1989) (Fig 7-10).

In order to test the possibility of producing a greater proportion of active MBP-G9 in the *E. coli* cytoplasm, the pMAL_c2::G9 plasmid was transferred to an *E. coli* strain BL21(DE3) *TrxB*$^-$ which is deficient in thioredoxin reductase activity. The environment in the cytosol of such strains should be more favourable then the wild type for the formation of disulphide bonds.

A preliminary functional assay indicated that the *TrxB*$^-$ strain did produce more active MBP-G9. The assay also gave a relatively high background signal in BSA coated wells, but this

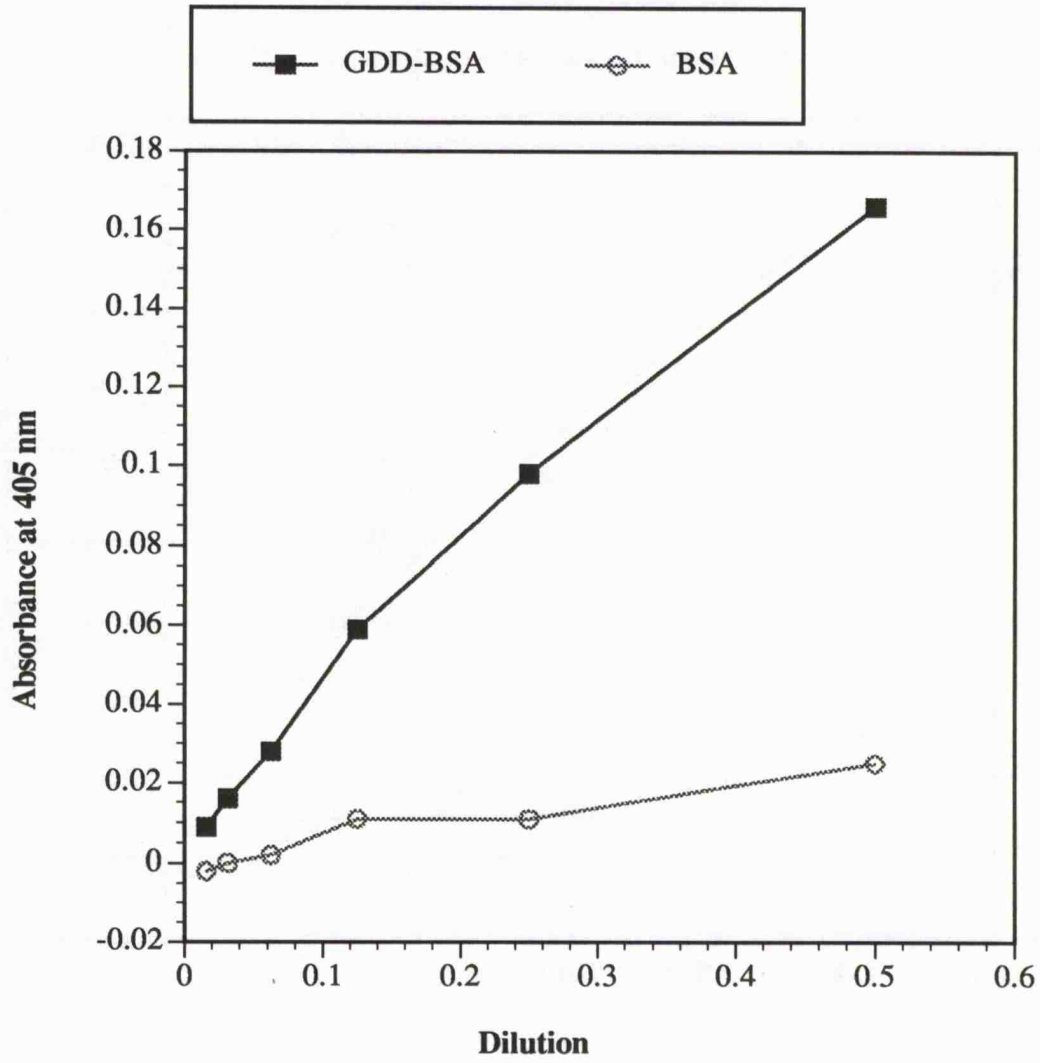


Fig. 7-9 Binding activity of purified cytoplasmic MBP::G9 fusion against GDD-BSA. MBP::G9 fusion was purified from crude soluble cytoplasmic bacterial extract.

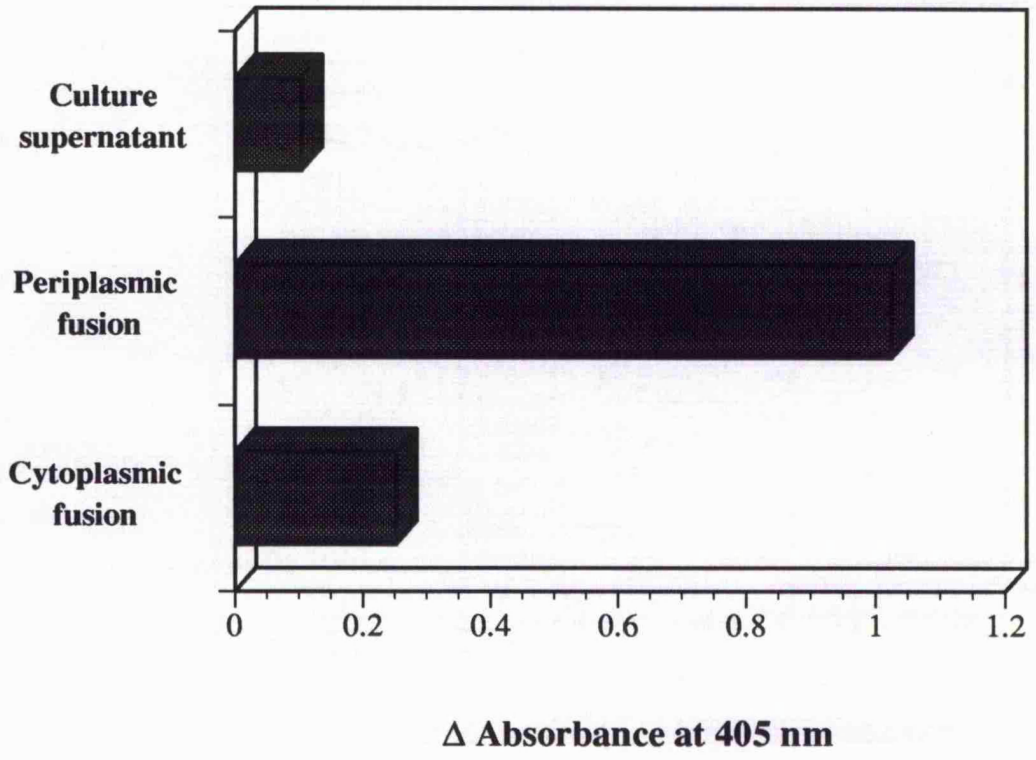


Fig. 7-10 Comparison of GDD-BSA binding activity by MBP-G9 fusion from different sources. The figure showed here are signals above those obtained from the wells coated with BSA. The culture supernatant is the one from *E. coli* expressing periplasmic fusion.

could be reduced by diluting the cytoplasmic extract used. A higher signal to background ratio was obtained for the cytoplasmic extract from the *TrxB*⁻ strain at a dilution of 1:32 than at a dilution of 1:2. At a dilution of 1/32 MBP-G9 from the *TrxB*⁻ strain gave the highest signal with cytoplasmic extract. The scFv binding activity of MBP-G9 was also readily detectable in the absence of IPTG induction. The background signal using uninduced cells and BSA coated wells was lower than that given by IPTG induced samples (Fig. 7-11). This may be caused by leakiness of the *lac* operator used to regulate the expression system (Fig. 7-11). Thus it appears that low level expression of the MBP-scFv fusion in a *TrxB*⁻ strain allows the to scFv fusion protein to fold into a functional form in the *E. coli* cytoplasm. Increasing the expression level of the fusion in the cytoplasm of *TrxB*⁻ strain might not lead to more functional MBP-scFv fusion being accumulated in the cytoplasm of the *E. coli*. This result is very similar to that of Proba *et al.*, (1995) in which an unfused scFv was expressed in the *E. coli* cytoplasm.

7.3 Discussion

7.3.1 Selection of PhAbs against GDD motif peptide

This chapter describes the selection of PhAbs against a peptide conjugate from an artificial immune repertoire. When conventional animal immunisation procedures are used to generate anti-peptide conjugate antibodies, a large population of antibodies against the carrier protein itself are produced in addition to those against the target peptide. These carrier-specific antibodies have to be eliminated by further screening. The elimination of carrier-specific antibodies can be readily achieved during panning procedures for the selection of anti-peptide conjugate PhAbs from artificial immune repertoires by the inclusion of an excess of free carrier protein in the panning solutions. After several rounds of such competing selection, carrier-specific PhAbs could be completely eliminated. As shown in Fig. 7-1, the polyclonal PhAb ELISA signals were increased only against the peptide conjugate during the five rounds of panning. No BSA binding activity was detectable, even though the light chain used in this

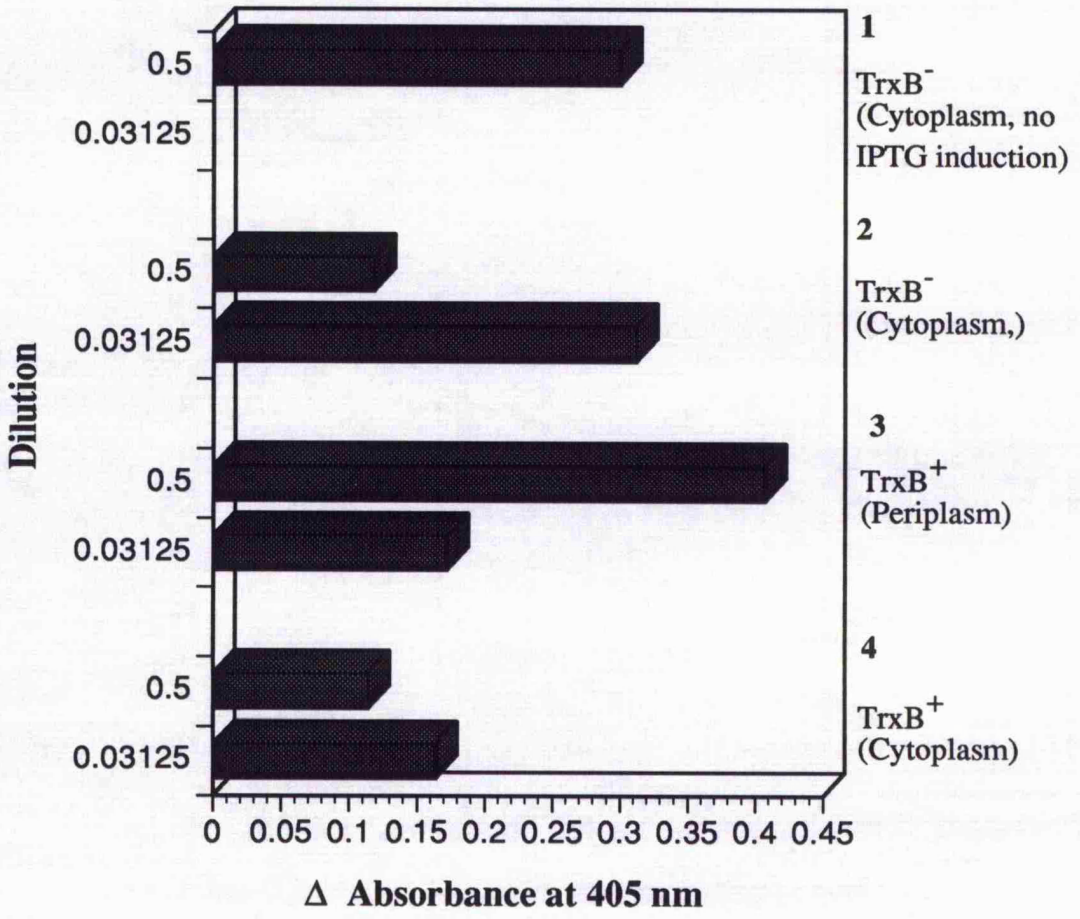


Fig. 7-11 Effects of redox conditions on binding activity of MBP::G9 fusion against GDD-BSA conjugate. $TrxB^-$ indicates the *E. coli* strain BL21(DE3)*TrxB*$^-$; $TrxB^+$ indicates *E. coli* strain W3110. Group 1: Cytoplasmic extract from non-induced recombinant BL21 (DE3)*TrxB*$^-$ containing pMAL_c2:G9; Group 2: Cytoplasmic extract from IPTG induced recombinant BL21(DE3)*TrxB*$^-$ containing pMAL_c2:G9; Group 3: Periplasmic extract from recombinant W3110 containing pMAL_p2:G9: Group 4: Cytoplasmic extract from recombinant W3110 containing pMAL_c2:G9.

artificial repertoire was assembled from a BSA-specific antibody. Furthermore, none of the monoclonal PhAbs selected from ELISA plates bound to BSA at detectable level. Clearly this competing selection, which is only applicable to phage antibody technology , is useful for the direct selection of antibody fragments specific to peptide or hapten conjugates.

Two discrete kinds of scFvs were identified by sequencing about 23% of the PhAbs genes from colonies which gave positive signals in ELISA screening. These two were derived from germline segments VH DP7 and DP46. The sequence first determined for PhAb G11 was found in nine out of ten colonies sequenced. This indicates that the majority of the individual PhAbs in the final selected population were identical. The heavy chain of G11 is encoded by VH DP7 segment and the HCDR3 sequence encodes a short loop of 6 residues in length. The gene segment DP7 was found to be the commonest VH segment in antibodies selected from synthetic repertoires (Griffiths *et al.*, 1994). Groove-type antibody combining sites can be formed in antibodies which contain short HCDR3 and LCDR1 (de la Paz et al., 1986). This kind of antibody combining site architecture favours binding to a loop epitope (Webster et al., 1994), to which the GDD motif was predicted to conform (Kamer and Argos, 1984) (Fig. 7-12). It is possible that G11 binding to the GDD conjugate is based mainly on surface complementarity between the antibody and the antigen interface.

The VH sequence of G9 was quite different from that of G11, being derived from VH segment DP46 and encoding an HCDR3 of 11 residues in length. Detailed analysis of the sequence of HCDR3, indicated that that three positively charged residues (ArgHisLys) were present in the centre of the loop. It is highly possible that these three positively charged side chains participate in charge interactions with the two negatively charged side chains contained by the antigen peptide GDD motif . A model of the GDD peptide showed that the side chains of Asp are surface exposed (Fig 7-12), and highly accessible for recognition by the positively charged residues in the antigen combining sites of antibody G9. Commonly, in Fab-peptide complex

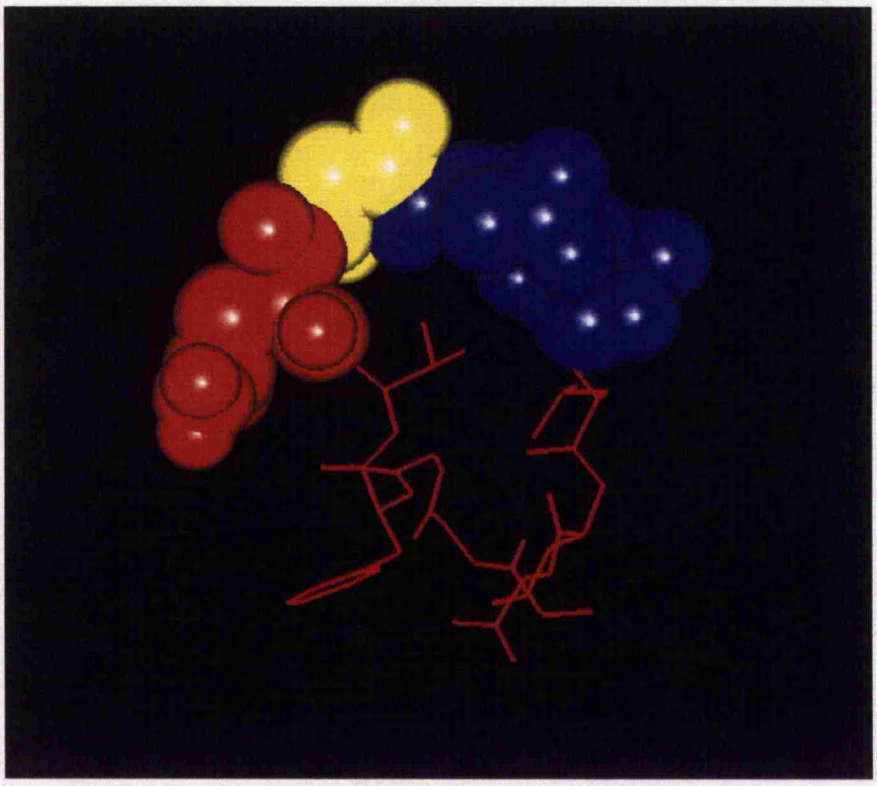


Fig 7-12 **Computer model of loop structure of GDD motif**. Residue Asn shown in red. Residue Gly shown in yellow. Two Asp shown in blue.

structures, side chain recognition is highly specific (Stanfield and Wilson, 1995). The exquisitely specific recognition of antigen by antibody stems from the high complementarity in size and shape between the antigen and antibody binding site, and by the formation of specific hydrogen bonds and charged interactions with the antigen (Padlan, 1993; Davies and Chacko, 1993; Wilson and Stanfield, 1993). The sequence variability of the six complementarity determining region loops allows this high degree of specificity. Ideally, a docking experiment should be performed for the model of GDD peptide and G9 Fv. A competition experiment showed that the fine specificities of G9 and G11 may be different. This could provide further understanding the interaction between G9 and GDD peptide.

Ideally, an antibody raised against a peptide conjugate will recognise the same peptide motif in the native protein. There was inconsistency between the results of analysis by ELISA and western blotting of interactions between the two selected scFvs and full length or truncated synthetic RdRp.

The precise epitopes of G9 and G11 remain unclear. They could be identified by three approaches. Firstly free original synthetic GDD peptide could be used in competition ELISA to investigate the fine specificity; secondly another carrier could be used to make a new conjugate to be used to test the interactions of poly- and mono- clonal PhAbs and thirdly a different conjugate made with a non-BSA carrier could be used in further panning (4 or 5 rounds) of the selected sub-libraries.

Ambiguous results were also obtained from the ELISA of G11 and G9 PhAbs using antigens from extracts of PVY-infected plant tissues. Significantly, G9 and G11 reacted specifically with infected plant tissue, and furthermore the interaction was inhibited by the GDD-BSA conjugate. These results did not agree with the interactions of PhAbs G9 and G11 with bacterial synthesised RdRp. A desirable experiment would involve investigation of interactions

between functional purified PVY RdRp and G9 or G11. Unfortunately, purified native PVY RdRp was not available during this study.

7.3.2 Cytoplasmic expression of a functional MBP::scFv fusion protein

The characteristics of the production of antibody fragments in *E. coli* are different with different antibodies. Some antibodies can be readily synthesised at high yields in *E. coli* expression systems and some can not. An example of such variation in the present work is that soluble G11 scFv can be readily produced from G11 PhAb in the non-suppressor strain HB2151 but G9 could not. Similar results were obtained with two other non-suppressor strains, W3110 and TB1. Other workers have also failed to produce soluble scFv from some selected PhAbs from this synthetic repertoire using HB2151 as host (Nissim *et al.*, 1994).

There are several possible mechanisms for the failure to produce soluble scFvs in non-suppressor *E. coli* strains. One is that, by chance, there is an amber codon present in the synthetic HCDR3 [the codon 'NNS' (N=A, G, T, C; S=G, C) was used to form codons for each residue in the randomised HCDR3 sequence. This codon must occasionally introduce an amber codon (TAG) into the sequence]. The second possibility is that sequences of some antibodies are not well-synthesised in some *E. coli* strains for a variety of reasons such as, for example, codon usage or amino acid usage. It is also possible that the soluble scFv is toxic. In the case of G9, since the sequence was determined and it did not contain a stop codon, one of the other reasons must account for the difficulty in producing it in soluble form.

To confirm the absence of an amber codon, the G9 scFv fragment was cloned into the maltose binding protein fusion vector for expression of MBP-G9 in both the cytoplasm and periplasm of the non-suppressor strain of *E. coli,* W3110. Fusion proteins accumulated in both cellular compartments and showed binding activity towards the peptide conjugate. Successful expression of the maltose binding protein fusion protein in the non-suppressor strain

demonstrates that an amber codon was not present in HCDR3 and the reason for the observed difficulty in producing functional soluble G9 by using the phagemid expression vector pHEN1 remains unclear.

The results of the experiments in which the MBP-G9 fusion was produced in the cytoplasm and periplasm generated an interesting side study. Production of scFvs in the cytoplasm of *E. coli* normally results in the formation of insoluble inclusion bodies. In these experiments however about 50% of the MBP-G9 scFv fusion protein accumulated in the cytosol was soluble. The solubility of the MBP-G9 fusion protein may result from the high solubility of MBP itself.

The MBP fusion expression vector has been used to express an MBP fusion with bovine enterokinase catalytic sub-unit in the bacterial cytoplasm. The MBP-enterokinase produced was non functional including some soluble fusion protein detected in the *E. coli* cytoplasm (Collins-Racie *et al.*, 1995). However, in the case of the MBP-G9 fusion, a small proportion of the MBP-G9 fusion protein was shown to be functional in terms of binding to the GDD peptide-conjugate.

Most scFv fragments probably misfold in the cytoplasm of *E coli* since the reducing conditions in this environment will not favour the formation of the intra-domain disulphide bonds which are essential in the production of the fuctional form of most antibodies. It is noteworthy however that there are some antibodies which do not possess intradomain disulphide bonds (Rudikoff and Pumphrey, 1986;).

In the medium used for the preparation of crude extracts of soluble MBP-G9 fusion protein from *E. coli* lysates 2mM DTT was included to prevent disulphide bound formation after cell lysis when conditions would, in the absence of this reducing agent, tend to become more oxidising. Existing intrachain disulphide bonds are buried within the scFv molecule and would

therefore not be reduced by the DTT. This DTT treatment was applied during all downstream procedures including purification and ELISA. Thus the MBP-G9 fusion should be in the same state as it was in the intact *E. coli*, rather than partially modified after cell lysis. Under these conditions the level of activity observed in cytosolically accumulated MBP-G9 was very low.

In contrast to the low activity of the MBP-G9 fusion accumulated in the cytoplasm, a high level of binding activity to the peptide conjugate was detected with MBP-G9 fusion protein which had been secreted to the periplasm. Although the yield of secreted MBP-G9 was very low, the high level of binding activity observed indicated that a higher proportion of the fusion protein was correctly folded and functional than was the case with cytoplasmic accumulation. These findings are consistent with the fact that secretion to the periplasm has frequently allowed the production of active antibody fragments in *E. coli* (Plückthun, 1991).

Deletion of the thioredoxin reductase gene *TrxB* in *E. coli* results in a phenotype in which the cytoplasm is likely to be a less reducing environment than the wild type. Experiments were conducted to determine whether or not the expression of the MBP-G9 scFv fusion protein in the thioredoxin reductase lacking strain would result in the accumulation of a higher level of functional protein than the wild type. A very preliminary result indicates that relatively high binding activity of the MBP-G9 fusion was indeed produced in the cytoplasm of BL21 (DE3) *TrxB*$^-$ strain. Despite the fact that there was a much higher level of fusion protein accumulated in the cytoplasm than in the periplasm (determined by Coomassie blue staining) the periplasmic fraction showed the highest binding activity. It appears that even in the *TrxB*$^-$ strain a large proportion of the soluble cytoplasmic fusion protein was incorrectly folded, possibly because the redox conditions, although improved compared to the wild type, were insufficiently oxidising to be optimal for antibody folding

This chapter describes the isolation of two anti-GDD peptide conjugate antibodies from a 'single pot' repertoire. However, neither of them recognised bacterial-synthesised RdRp which

incorporates the GDD motif. Furthermore the observed interaction of isolated fragments of one of them, G9, with native RdRp from PVY-infected plant tissue needs further confirmation using purified native RdRp. Although the ultimate objective is to use antibodies recognising the GDD motif in plant protection strategies, there is not yet sufficient information available on antibodies G9 or G11 to determine if they are suitable for this application. A side line of this study revealed that soluble functional scFv fusions can be produced as maltose binding protein fusions in the cytoplasm of *E. coli*, although the binding activity observed was much lower that that observed when the fusion protein was targeted to the periplasm.

Chapter 8

General Discussion

8.1 The study of antibody combining sites

The heavy chain variable domain of HyHEL-10 has been a focus for the study of the architecture of epitope-binding sites. Antibody-antigen interactions involve van der Waal's interactions, hydrogen bonds and salt bridges. Disruption of any of these interaction will reduce the affinity binding of antibody to antigen. Disruptions can be direct or indirect. In the study of this thesis, direct disruptions were shown to be involved in the interactions of G3.N-HEL and AS32H/HyHEL-10-HEL.

The original HyHEL-10-HEL complex involves van der Waal's interactions of heavy chain of HyHEL-10 Ser 56 with HEL Asp101/Gly 102 and HyHEL-10 Tyr58 with HEL Arg21/Ser100/Gly102. Since heavy chain residues 56 and 58 are different in AS32 these van der Waal's interactions with lysozyme may not occur with the mutant antibodies G3.N and AS32H/HyHEL-10. In addition a hydrogen bond between HyHEL-10 Tyr58 and HEL Gly 102 had been identified by x-ray crystallography (Padlan *et al.*, 1989). Since the serine side chain of residue 58 in the heavy chain of the mutant antibodies is much shorter than that of tyrosine which occupies this position in HyHEL10, this hydrogen bond may not form in the interactions between HEL and G3.N and AS32H/HyHEL-10. These differences may account for the lower affinity of G3.N-HEL and AS32H/HyHEL-10-HEL interactions.

Indirect disruption can be identified in the interactions of G23.N, G123 and G123.N with HEL. These mutants contained all of the antigen contact residues in the combining site at exactly the same positions as in HyHEL-10. The 1000-fold difference of affinity found between G23.N and G123.N and HyHEL-10 must therefore have resulted from the differences between the non-contact residues in these mutants at HCDR1 and HFR3 and those of HyHEL-10. Clearly, indirect interruption by such non-contact residues can significantly interfere with the functioning of the entire antibodies combining sites.

This study would be best continued with soluble fragments of the variant mutants. These could be utilised in the following of experiments. Firstly, the precise on- or off- rates of the various mutants could be measured by the techniques of surface plasmon resonance (Chaiken *et al.*, 1992) [This approach was attempted with the mutants as PhAb's. Unfortunately the technique proved unsatisfactory in this application as a result of high background values being obtained with control phage (VCSM13), possibly resulting from the general 'stickiness' of PEG precipitated phage]. Secondly, the antibody-HEL interactions could be studied by using isothermal titration calorimetry (Tsumoto *et al.*, 1995, Sturtevant, 1994); and thirdly, structure-function relationships of HyHEL-10 could be further analysed by NMR or x-ray crystallography.

The role of HCDR3 in antibody combining site studies is of particular importance and has been extensively studied (Sanz, 1991; Wu et al 1993; Kabat and Wu 1991; Brummell *et al.*, 1993). The study of the HyHEL-10 combining site has also shown HCDR3 to play a predominant role in controlling antibody specificity and affinity. The replacement of HCDR3, such as in the mutant G3.N, can alter the specificity of AS32H/HyHEL-10 to HEL. Although HCDR1 and HCDR3 are far apart in the primary sequence, contact between them in the folded antibody may affect the entire antibody combining site. HCDR1 is in fact very close to HCDR3 in the combining site, and the C-terminal part of HCDR1 certainly occupies the centre

of the combining site. It is clear therefore that interactions between HCDR3 and HCDR1 are possible, and that such interactions may change the conformation of HCDR1 and result in the formation of new binding sites. Similarly, interactions between HCDR3 and LCDR3 will be influenced by the structure of HCDR3.

In the natural immune system, HCDR3 is the most diverse of the six CDRs. It is also clear that randomisation of HCDR3 alone and retention of the other five CDRs can generate new specificities. This indicates that the combination of different HCDR3s with otherwise identical CDRs can fold to produce a certain range of different conformations and produce different combining sites. Results consistent with this point of this view, were obtained from the semisynthetic antibody fragment library built by randomising the scFv AS32 HCDR3 of six residues in length. Antibody fragments with new specificity have been isolated. Similarly two antibodies (G9, A11) have been isolated against completely different epitopes from 'single pot' library, (based on no detectable cross interactions of two antibody fragments, such as, G9 against GDD-BSA, A11 against *P. infestans*), although they are derived from the same VH germline gene and share an identical light chain. The only differences between these two antibodies are the sequences and length of HCDR3 (11 residues for G9, or 10 for A11).

8.2 The study of phage display technology

A very important feature of bacteriophage display is that the phage particles carry the encoding gene for the ligand displayed. By affinity selection, both the desired ligands, and the genes encoding them are simultaneously isolated and can be used to regenerate and amplify the corresponding ligand. Before the advent of phage display technology, although it was possible to produce antibodies with desired properties, the isolation of genes encoding them was much more difficult. However once antibody gene fragments are isolated, there is very great scope for modification by molecular technology. One important advantage of PhAb display technology is that it allows very rapid and economical antibody fragment production.

In order to optimise the system, many different phage display vector have been designed and tested (Hoogenboom *et al.*, 1991; Barbas *et al.*, 1991; Kang *et al.*, 1991; Breitling *et al.*, 1991; Garrard *et al.*, 1991; Söderlind *et al.*, 1993; Orum *et al.*, 1993; Hogrefe *et al.*, 1993; Waterhouse *et al.*, 1993; Geoffroy *et al.*, 1994; Jespers *et al.*, 1995; Krebber *et al.*, 1995). Vectors used in studies described in this thesis are pComb3 (Barbas *et al.*, 1991) and pHEN1 (Hoogenboom *et al.*, 1991). A common feature of these two vectors is that the ligand of interest is displayed on the proximal end of the phage particle. There are however several significant differences between them. Firstly, pComb3 has more restriction enzyme cleavage sites in the multi-cloning region compared with those of pHEN1 and it is therefore suitable for the display of a wider range of ligands. Secondly pComb3 has two cloning sites, which is useful for the display of hetero-dimeric proteins, such as Fabs

Thirdly, the greatest advantage of vector pHEN1 is that it contains a c-Myc tag and the amber codon, which are very useful for subsequently producing soluble scFvs for use in binding activity detection. To produce soluble scFvs when pComb3 is the vector, a sub-cloning procedure has to be performed, and furthermore no tag is introduced, to assist in detection of the scFv. However, when pHEN1 is used, soluble scFv can be produced directly by infection of a non-suppressor strain. Fourthly, a very significant difference between the two vectors is that use of pHEN1 displays the ligand as a fusion to the N-terminal of intact phage coat protein III whereas with pComb3 the ligand is fused to the last C-terminal domain of the coat protein. Thus with pComb3 the N-terminal domain of the coat protein has been replaced with the ligand. In contrast, with pHEN1, the ligand is an addition to the intact coat protein and represents an extra protein on the surface of the phage particle. This kind of structure may affect the stability of the ligand displayed. Indeed, scFvs displayed by pHEN1 are found to be easily proteolysed (Winter *et al.*, 1994), and an scFv-gIII fusion protein was barely detectable by western blotting (Jackson *et al.*, 1992). However, in the present work when pComb3 was used, scFv-gIII fusion protein was easily detected by western blotting, and proteolysis was not

significant. However when a large protein (larger than the N-terminal domain of coat protein III) was displayed using pComb3, such as the SpA-scFv AS32 fusion protein, many degraded protein were detected by western blotting.

In conclusion, an improved phage display vector would combine the ease of soluble antibody production, detection and purification provided by pHEN1 with the protection of displayed antibodies from proteolysis provided by pComb3.

Mono-valent phage display is a very important property of some phage display systems. In principle, the mono-valent PhAb relies on the use of the *lac*I^q gene, which reduces 'leakiness' of the *lac* operator. However, some experiments with toxic scFvs indicate that even in such strains, leakage can still occur .

Furthermore, despite the use of the pComb3 vector in an *E. coli* host containing the *lac*I^q gene, bivalent scFv PhAbs were observed by electron-microscopy (Zebedee *et al.*, 1992). This may have resulted from insufficient production of repressor protein of *lac* operon from the *lac*I^q gene in the *E. coli* genome. Over-expression of the repressor can be achieved by cloning the recombinant *lac*I^q gene into the phage display vector, in which the *lac* operon may be more tightly controlled. In the pMAL_2 vector, a recombinant *lac*I^q gene is incorporated to tightly control the *lac* operator. Thus an ideal mono-valent phage display vector should contain the *lac*I^q gene, so that when used with a *lac*I^q containing strain, mono-valent phage display will be favoured.

The gIII protein of filamentous phage is widely used as a basis to display polypeptides, and folds as three domains. The first two domains serve as the infection "finger" and third domain is important for morphogenesis. When the two N-terminal domains are absent. non-infective phages are produced. However, it has previously been reported that phage infectivity can be restored by the addition of the N-terminal domain *in vitro* (Duenas and Borrebaeck, 1994) or

in vivo (Gramatikoff *et al.*, 1994). Based on this discovery, many new phage display systems can be developed. For example, a new 'two hybrid' system has been described which is analogous to the yeast two hybrid system. Indeed, it has been already reported that cognate antibody-antigen pairs can be co-selected by using the phage system (Krebber *et al.*, 1995). Furthermore, purified N-terminal domains can be chemically cross-linked with non-peptide antigens, and, for example, affinity selection based on on-rate and off-rate can be readily performed by changing the incubation time of phage and the N-terminal domain linked target before infection.

A particularly significant feature of phage antibody display is the ability to select higher affinity antibody fragments from a large population of phages carrying lower affinity fragments. The routine methods used in this thesis are not likely to be optimal for distinguishing small differences in affinity. For example, when the small library which was created by randomising HCDR3 of AS32 was mixed with AS32 PhAbs and panned against phytochrome A, it was anticipated that as well as the AS32 PhAbs and some higher affinity mutants might be selected. However amongst 20 randomly selected colonies, no higher affinity binders were selected, and the selected population of parental AS32 at approximately 5% of the total was not high. With panning methods using target antigens immobilised on ELISA plates or immuno- tubes, it is difficult to separate higher affinity binders from moderate binders. As indicated by the work of Hawkins *et al.* (1992,1993) the use of biotinylated Phytochrome A for panning in solution might have allowed more efficient discrimination between AS32 and the weaker binders.

The molecular mass of a phage particle is about 1.6×10^4 kDa (Berkowitz and Day, 1976), and a scFv is about 29 kDa. Clearly, although the mass difference of a phage and a PhAb can be ignored, the mass difference of a scFv and a PhAb is likely to be much more significant, and may affect the binding of displayed scFv. This may result in PhAbs being less suitable for some applications than soluble antibody fragment, such as in some immunohistochemical methods

(Nissim *et al.*, 1994). However, it is certainly possible to use PhAbs as a novel immuno-reagent to directly localise epitopes by electron-microscopy, or light microscopy with fluorescent labelled PhAbs. Optimisation of the use of PhAbs in immunolocation studies will require further investigation of the effects of such factors as PhAb concentration.

To optimise the usefulness of these techniques the following points must be taken into consideration. Firstly for epitope localisation by electron-microscopy, aggregation of PEG precipitated PhAbs must be avoided. Although aggregation can be eliminated by dilution, excessive dilution is clearly undesirable since it will tend to reduce the number of particles available to bind to the epitope. "Bald" phage particles are also undesirable in this application. The proportion of bald phage may be reduced by growing the *E. coli* in inducing conditions since more scFv-pIII fusion will be produced. Of course in this case phagemid DNA might be less well packaged, because the presence of IPTG interferes with phagemid packaging (Breitling *et al.*, 1991). It is possible that $CsCl_2$ precipitated PhAb might aggregate less, in which case $CsCl_2$ phage precipitation may be a good method for preparing PhAb for epitope localisation by electron-microscopy. Bald phage will also be disadvantageous to localisation with fluorescence labelled PhAbs since the bald fluorescent phage will increase the background signal but will not contribute to the localised bound-phage signal. Secondly methods for the fluorescence-labelling of PhAbs require optimisation. In particular the effects of fluorochrome : protein ratio and the suitability of fluorochromes rhodamine require investigation.

Although the present work was used to localise epitopes on the surface of an intact organism it is also possible that PhAbs could be used in immunohistochemistry with sections, with both the light and electron microscopes. This has not yet been tested.

8.3 Heterologous expression of antibody fragments

Heterologous expression of antibody fragment will become a major antibody source in the future. Large quantity, high purity, antibody fragment production is a major goal of antibody engineering. There have been numerous reports of the production of functional antibody fragments in plants in some cases with consequent modification of the phenotype. Functional antibody fragments have also been expressed in the cytoplasm of yeast (Carlson, 1988), which resulted the modified phenotype of the yeast. Since a functional antibody fragment has also been expressed in the cytoplasm of *E. coli* (Proba *et al.*, 1995), it is possible the phenotype of *E. coli* can be modified similarly. The expression of the MBP-G9 fusion in cytoplasm of *E. coli* reported here may indicate that antibody-mediated modification of the cytosol of *E. coli* may be possible. A large quantity soluble MBP-G9 fusion was found to have been produced in the cytoplasm of *E. coli*, but the antigen binding activity was very low. A significant improvement was achieved by expressing the fusion in the *TrxB*$^-$ strain. However, a comparison of the fusion produced in the cytoplasm of the *TrxB*$^-$ strain compared to that produced in the periplasm of the equivalent *TrxB*$^+$ showed that although the amount of soluble fusion protein produced in the periplasm was lower than in the cytoplasm it nevertheless had the highest level of functional antibody. This phenomenon might result from the effects of some unknown protein with *TrxB* like properties which was produced in the absence of the TrxB gene which was necessary to maintain *E. coli* viability. Further improvement might be achieved by over-expression of thioredoxin (TrxA) in *E. coli*. However in some cases the presence of a small amount of functional antibody fragment in the cytoplasm may be enough for the purposes of modification of the microbial phenotype.

8.4 Final conclusions

Phage antibody technology is very useful for many applications, such as antibody combining site studies, antibody antigen interactions, the generation antibody repertoires and the selection of antibody fragments for many different purposes including studies on microbes, plants,

animal and man. Phage antibodies can be directly used as immuno-reagents to perform many immnochemical assays, such as ELISA, affinity chromatography, western blotting, fluorescent straining and immuno-electron-microscopy. This technology will certainly undergo further development in the near future and will undoubtedly be utilised to make contributions to the understanding of the mechanisms and structural basis of macromolecule interactions.

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