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Concepts in antibody phage display

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Abstract

This paper introduces the reader to antibody phage display and its use in combinatorial biochemistry. The focus is on overviewing phage display formats, library design and selection technology, which are the prerequisites for the successful isolation of specific antibody fragments against a diverse set of target antigens.

Abbreviations

CDR, complementarity determining region
V_H, variable heavy chain
V_L, variable light chain
scFv, single chain variable fragment
g3p, gene 3 protein
N1, first N-terminal domain of g3p
N2, second N-terminal domain of g3p
CT, C-terminal domain of g3p
IG, intergenic region
RT, reverse transcription

INTRODUCTION TO PHAGE DISPLAY

Animal immunisation followed by hybridoma technology has been used to generate monoclonal antibodies against a variety of antigens. Over the past ten years, advances in molecular biology have allowed for the use of *Escherichia coli* to produce recombinant antibodies. By restricting the size to either a Fab, a Fv or a linker-stabilised single chain Fv (scFv) (Figure 1) such antibody fragments can not only be expressed in bacterial cells but also displayed by fusion to phage coat proteins.[1]

The phage display concept was first introduced for short peptide fragments in 1985.[2] Fragments of the *Eco*RI endonuclease, displayed as a polypeptide fusion (phenotype) to the gene 3 protein (g3p), were encoded on the DNA molecule (genotype) encapsulated within the phage particle. The linkage of genotype to phenotype is the fundamental aspect of phage display. Subsequently, phage display of functional antibody fragments was shown[3] when the V_H and V_L fragments of the anti-lysozyme antibody (D1.3) were introduced, with a linker, into a phage vector at the N-terminus of g3p. Since then, large scFv, Fab and peptide repertoires have been generated using a variety of phage display formats.

One of the major advantages of phage display technology of antibody fragments compared with standard hybridoma technology is that the generation of specific scFv/Fab fragments to a particular antigen can be performed within a couple of weeks. The starting point is usually an antibody library, of either naive or immune origin, comprising a population of, ideally, 10^9–10^{11} clones. After usually two to, maximally, three rounds of selection, the population is enriched for a high percentage of antibody fragments specific for the target antigen. The display of human antibody fragments is of particular interest since any therapeutic derived from these antibody fragments is believed to have a minimal risk of an immune response in patients.

Both scFv and Fab formats have been used successfully in antibody libraries displayed on phage. Fab fragments are usually more stable than Fv fragments and have less potential to dimerise than scFvs.[4] In addition to the V_H and V_L segments, they also possess the constant regions (C_H and C_L) of the heavy and light chains. They are reputed to be displayed at lower

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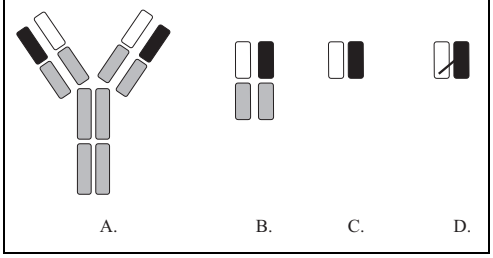

Figure 1: Antibody structure and derived fragments. (A) IgG, 160 kilodaltons (kD); (B) Fab fragment, 50 kD; (C) Fv fragment, 30 kD; (D) scFv with linker, 30 kDa

frequency on the surface of phage, thus alleviating the avidity effects associated with some well-expressed scFvs. Synthetic and naive Fab libraries have been used successfully for the generation of antibody fragments to a variety of antigens.[5–8] Expression of the scFv fragment has a less toxic effect on the cell than the larger Fab molecules.[4] This results in a better yield and diversity in scFv libraries. The remainder of this paper will, therefore, focus primarily on scFv libraries since they are generally the more popular choice.[9]

The oxidising periplasm of *E. coli* favours disulphide bond formation

Expression in *E. coli* ensures that sufficient quantities of scFv, for screening and characterisation, can be produced with relative ease. Many secreted eukaryotic proteins such as antibodies require disulphide bonds for stability, and the oxidising environment of the *E. coli* periplasm, where filamentous phage assembles, provides the appropriate conditions for antibody folding. Human antibodies against human proteins can be isolated from diverse human antibody libraries. Moreover, antibodies to toxic molecules such as doxorubicin[10] can be obtained, a task difficult with immunisation/hybridoma techniques.

M13 phage biology

Filamentous phage assemblies in the *E. Coli* periplasm

M13 is a filamentous bacteriophage. The native particle is a thin, cylindrical shape, usually 900 nm long and 6–7 nm in diameter.[11,12] It contains a single-stranded DNA genome (6,407 base pairs in length) which encodes 11 genes,[13] five of which are coat proteins. The major coat protein is the gene 8 protein (g8p) which is present in almost 2,700 copies and responsible for encapsulating the phage DNA (Figure 2). The distal end of the phage particle is capped by five copies each of g7p and g9p. At the proximal end, four to five copies each of g6p and g3p are present. M13 bacteriophage infects only male bacteria, ie those *E. coli* cells that bear the F-plasmid which encodes the F-pilus. Infection is mediated by the interaction between g3p of the phage and the F-pilus. Filamentous phage have the characteristic that once they have infected their host cell they do not lyse the cell, but instead are able to replicate and are released from the cell membrane while the host cell continues to grow and divide, in contrast to the lytic phages T4 and T7.

The structure and function of g3p have been well studied. The protein is responsible for phage infection and for release of the phage particle following assembly.[14,15] It has two N-terminal domains (N1 and N2) and a C-terminal domain (CT, Figure 3). N1, which has been shown to be essential for infectivity,[16] interacts with the TolA protein of the TolQRA complex located across the periplasmic space between the inner and outer *E. coli* membranes. The primary interaction of phage with an *E. coli* cell is mediated by N2 binding the F-pilus and it is this association that is thought to bring N1 into close proximity with TolA. It is not yet understood how TolA contributes to phage infection; however, it is known that the F-pilus retracts and that the M13 genome is injected into the cytoplasm. CT is required for the termination of phage assembly in the periplasm and release of the phage from the cell membrane. Once the cells have been infected and phage protein production commences, the cells are no longer able to be infected. The presence of only very small amounts of the g3p of f1 filamentous phage has been shown to be associated with a resistance of the cell to infection from filamentous

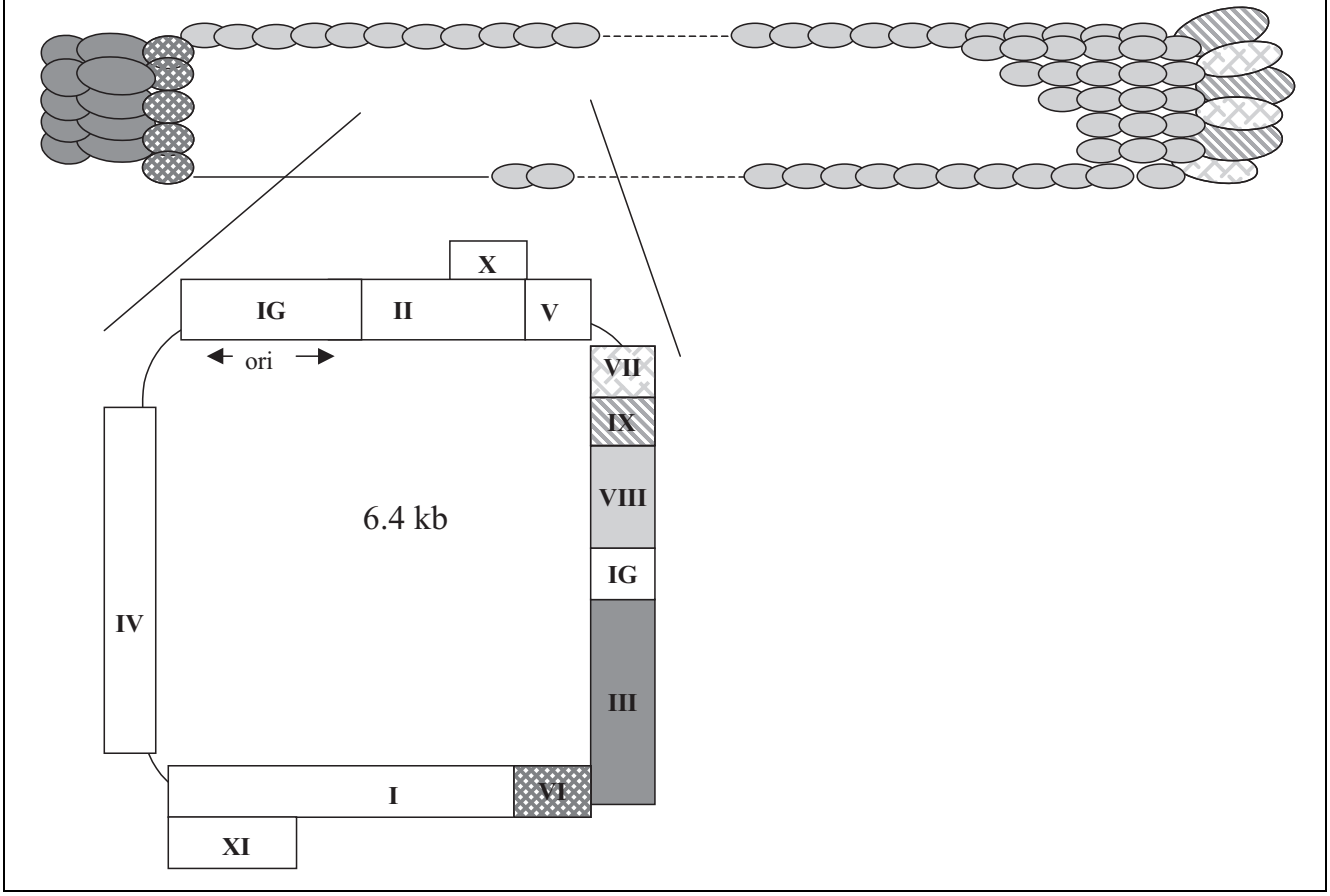

Figure 2: The structure of M13 phage particle. The phage particle is of cylindrical shape (900 nm long) and only 6–7 nm in diameter. Non-structural proteins are unshaded. All coat proteins are shaded according to their corresponding gene in the genome. The intergenic regions are marked by IG. G8p is the major coat protein and is present in about 2,700 copies. The minor coat proteins g3p, g6p, g7p and g9p are present in approximately five copies each

All coat proteins can be used for phage display

phage. Its presence in the *E. coli* membrane disrupts the membrane integrity, causing a number of effects including defective F-pili.[17]

The five coat proteins have all been used as fusion proteins for phage display.[18,19] Genomic or cDNA libraries are favoured as C-terminal fusions to g8p since expression constraints due to frameshifts and stop codons are avoided.[20] C-terminal polypeptide fusions to g3p have also been demonstrated.[21] Since g3p is the most popular fusion protein, this paper will concentrate on display of antibody fragments on g3p in a phagemid system.

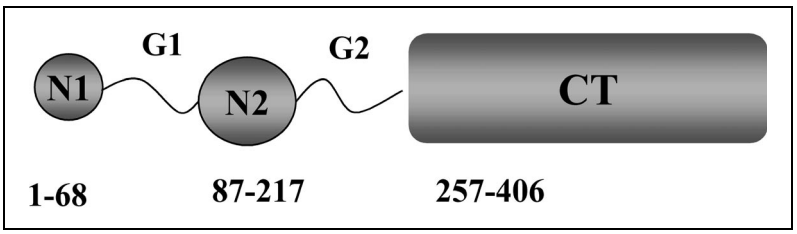

Figure 3: The modular structure of g3p. The N1 domain interacts with the TolA protein in the *E. coli* membrane. The N2 domain binds the F-pilus and the CT is necessary for termination of phage assembly. The glycine-rich linkers, G1 and G2, are thought to confer flexibility to the domains, facilitating the infection process. Antibody fragments can be displayed at the N-terminus of g3p. Alternatively, they can be displayed as an N-terminal fusion to CT

PHAGE DISPLAY FORMATS

Early phage display formats involved the fusion of peptides to the N-terminus of g3p or g8p in the M13 phage vector. The polypeptide or antibody fragment was displayed in a multivalent format, since all copies of the coat protein are translated as fusion proteins,[22,23] although it was not

possible to display larger polypeptides or proteins without affecting the function of g8p. Six residue insertions are usually tolerated, but, when the insert is increased to eight residues, only 40 per cent of phage are infectious and, at 16 residues, this number drops to less than one per cent.[24] Larger fusion proteins to g3p are tolerated, but, in a multivalent display format, such fusions may cause a decrease in infectivity[2] by sterically hindering the interaction of N2 and the F-pilus. These problems are overcome by the use of phagemids.

Phagemid vectors facilitate construction of large libraries

Phagemids are plasmids (~4.6 kilobases) which encode a signal sequence, the phage coat protein and an antibiotic resistance marker. The antibody fragment/polypeptide is cloned upstream of the g3p/g8p coat protein sequence and expression is controlled by the use of a promoter such as *lacZ*. The relatively small size of these vectors means that they have higher transformation efficiencies than phage vectors,[25] hence facilitating the construction of large repertoires or libraries of peptide or antibody fragments. The incorporation of an amber stop codon between the displayed protein and the phage coat protein permits fusion protein expression in suppressor strains of *E. coli* such as TG1.[26] Non-suppressor strains, such as HB2151,[27] will not incorporate a glutamine at the amber codon, thereby resulting in production of only the antibody/polypeptide moiety.

A phagemid cannot produce infective phage particles alone. A helper phage such as M13KO7 or VCSM13 is required. The helper phage provides the genes which are essential for phage replication and assembly, including a wild-type copy of the coat protein used for display. Cells already containing the phagemid vector are superinfected with the helper phage. Glucose in the growth media represses the *lacZ* promoter, preventing expression of g3p-fusion, which would inhibit superinfection. Once the helper phage genome is incorporated into the cell, the glucose is removed and phage production commences. The M13KO7 genome possesses a modified intergenic region (IG),[28] causing it to be replicated and packaged less efficiently than the phagemid which carries the wild-type M13 IG. This ensures that the genotype (phagemid) and phenotype (g3p–scFv fusion) are linked in a single (phage) molecule, which is the key feature of phage display (Figure 4). The antibody fragment can also be fused to a truncated

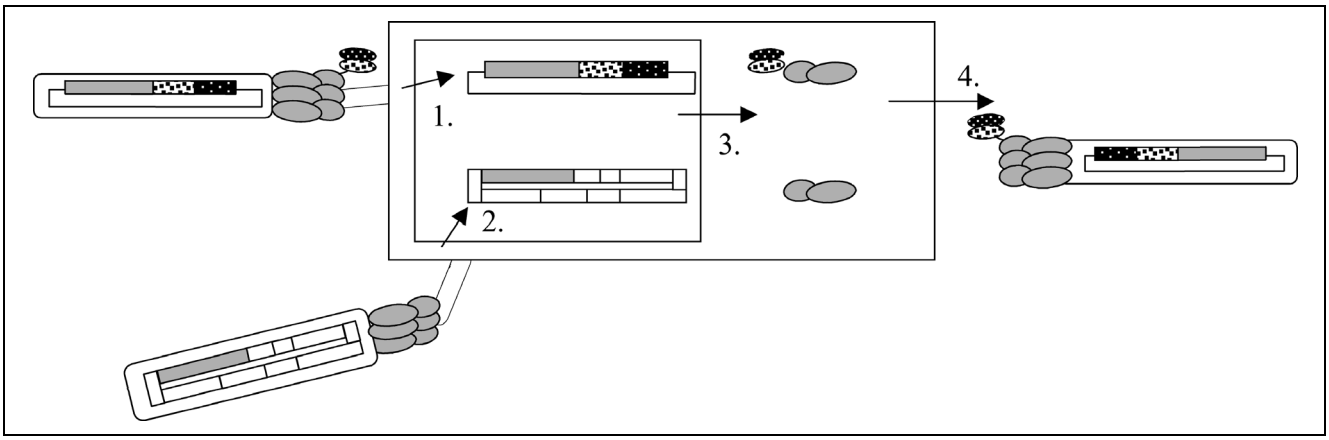

Figure 4: Sequence of events depicting phage infection, assembly and secretion using a phagemid system. (1) Phage displaying scFv infects cell. Single-stranded phagemid DNA is injected into cytoplasm. (2) Helper phage superinfect providing genomic information encoding remaining phage proteins for phage replication and assembly. (3) ScFv–g3p fusion, from the phagemid vector, and all other structural proteins (including wild-type g3p) from the helper phage vector, are directed to the periplasm. (4) Phage assembly occurs in the *E. coli* periplasm. Phage containing DNA encoding scFv sequence (genotype) and displaying scFv (phenotype) are secreted from cell membrane

CT domain in a phagemid system since there will always be copies of wild-type g3p from the helper phage for infection.

Selectively infective phage (SIP) technology (reviewed in Jung *et al.*[29]) exploits the modular nature of g3p and can be used for identifying protein–ligand interactions. The ligand is coupled to N1–N2 either by the use of an expression vector or, if it is sufficiently small (eg an organic molecule), it can be chemically coupled. The receptor/scFv is fused to the CT domain of g3p as part of the phage particle. It is the interaction between the ligand and the receptor which restores the domains of g3p, thereby rendering the phage infectious (Figure 5). The main difference between this technology and standard phage display is that the selection and infection process are coupled. Since interaction leads to infection, there is no need for elution.

A variation of this technology — termed Cys display — has recently been presented.[30] Here, the g3p and the antibody fragment to be displayed are both expressed separately in the cell, although both with a terminal Cys. In the oxidising periplasm, g3p and scFv can interact, allowing for the formation of a g3p–S–S–scFv fusion which can later be eluted by reducing agents such as DTT. No selection results have so far been reported with this technology, but there is the possibility of unpaired cysteine residues interfering with disulphide bond formation in the scFv, resulting in misfolding and reduced yields.[31]

Competition from g3p of the helper

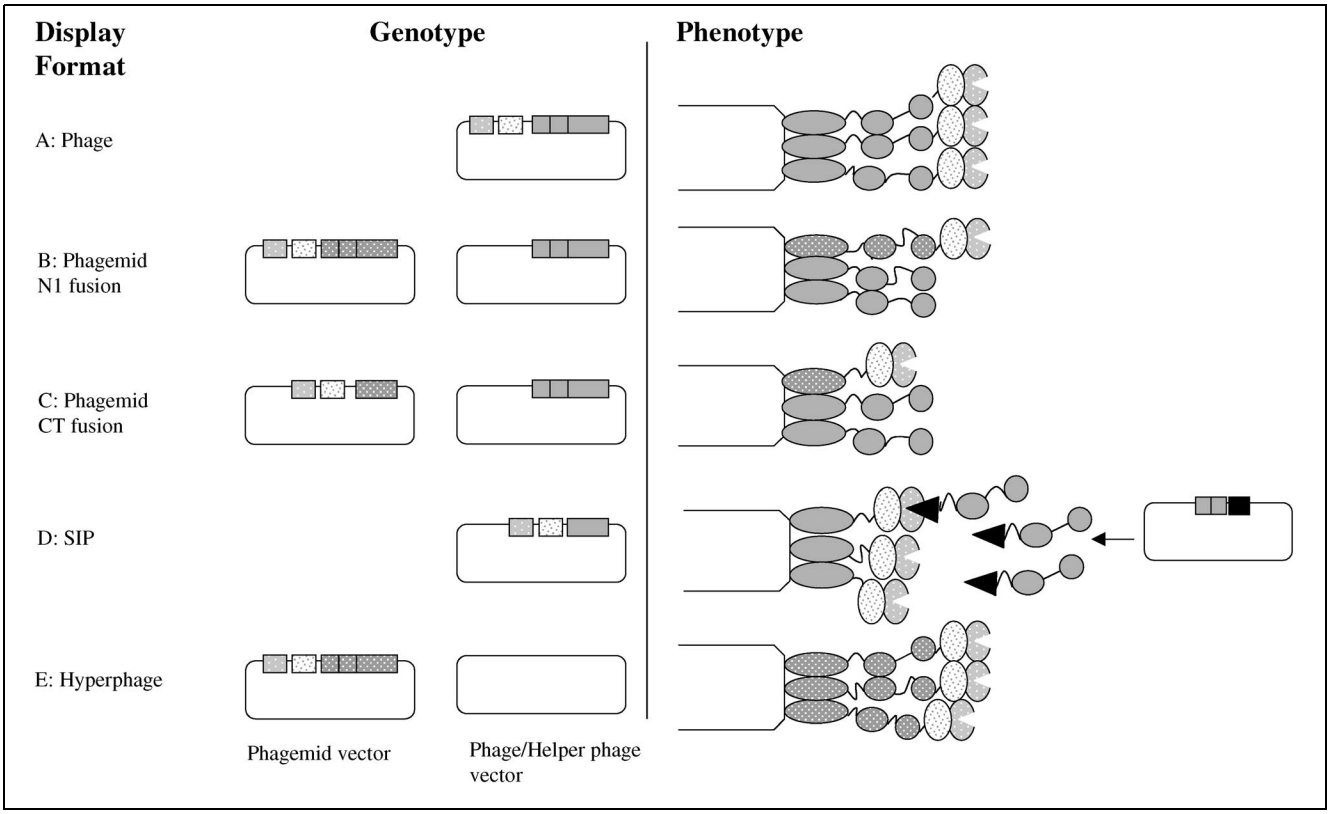

Figure 5: Formats for scFv display and vectors used. (A) Wild-type phage displaying multiple scFvs at the N-terminus of the N1 domain. (B) and (C) scFv displayed at the N-terminus of N1 or CT, respectively. Both these formats incorporate copies of wild-type g3p. (D) SIP technology. scFv is displayed at the end of CT domain of wild-type g3p in the phage vector. Infectivity is dependent on the scFv interacting with a ligand attached to the N-terminal domains. (E) Hyperphage. The helper phage genome lacks a g3p so all copies of g3p are derived from the phagemid vector system, resulting in multivalent phage

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