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Use of macromolecular assemblies as expression systems for peptides and synthetic vaccines

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The past decade has witnessed the development of numerous systems for the presentation of antigens on the surface of self-assembling macromolecules. Although the sites for insertion were initially chosen empirically, the determination of the three-dimensional structures of a number of carrier macromolecules has enabled structure-based insertional mutagenesis to be used increasingly. Furthermore, it is now feasible to determine the structure of an inserted sequence as presented in a heterologous environment, making it possible to correlate the detailed structure of a peptide with its immunological properties.

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Abbreviations

CLP	core-like particle
CPMV	cowpea mosaic virus
3D	three-dimensional
FMDV	foot and mouth disease virus
HRV14	human rhinovirus 14
HSV1	herpes simplex virus type 1
MHV	mouse hepatitis virus
PVX	potato virus X
TMV	tobacco mosaic virus
VP	virus protein

Introduction

Over the past 15 years, there has been much interest in the development of polypeptides derived from pathogens as novel vaccines. Though free peptides can confer protection, it is generally found that their immunogenicity can be enhanced by presenting them on the surface of a macromolecular assembly [1]. Initial studies involved chemically crosslinking synthetic peptides to a variety of moderately sized carrier proteins [2]. Subsequently, attention has turned to genetically fusing polypeptide sequences to self-assembling macromolecules. To use such a method, it is necessary that the fused sequence does not interfere with the assembly process and that it is exposed on the assembled particle. Suitable sites have been identified either empirically or on the basis of a known three-dimensional (3D) structure. In this review, we examine the use of self-assembling systems as a method of presenting epitopes and the future prospects for such systems.

Bacteriophage

Since the pioneering work which demonstrated that filamentous phage could be used to express foreign antigens on the virion surface [3], the uses to which such 'phage display' technology has been put have increased rapidly. In particular, the use of filamentous phage to display random libraries of amino acid sequences that bind to a variety of ligands has attracted considerable attention. Because this use of phage display has been extensively reviewed recently [4,5], it will not be discussed further here. We will, however, discuss the display of pre-defined polypeptides on the surface of filamentous phage.

The capsids of filamentous phage such as fd, f1 and M13 consist of one major (pVIII) and four minor (pIII, pVI, pVII and pIX) proteins. There are approximately 2700 copies of the 50 amino acid long pVIII protein per virion, which form a helical structure around the genomic single-stranded DNA, and about five copies of each of the minor constituents [6]. To investigate the potential of filamentous phage as an epitope presentation system, de la Cruz *et al.* [7] inserted tetrapeptide repeats from the circumsporozoite protein of the malarial parasite *Plasmodium falciparum* between the functional domains of the minor capsid protein pIII of phage f1. The recombinant phage were immunogenic in rabbits but gave variable responses in mice. To increase the number of copies of the insert on the modified phage, antigenic sequences were inserted near the N terminus of the major capsid protein, pVIII, of fd [8] and M13 [9]. However, it has proved impossible to recover assembled particles when the modified pVIII protein contains inserts longer than about six amino acids. This limit to the size of the insertion may reflect constraints imposed during the initiation of assembly [10]. The insertion of a five amino acid sequence near the N terminus of pVIII does not appear to alter the architecture of the phage particles [9] and fibre diffraction studies [11••] indicate that such inserts are presented in an extended conformation between two α helices on the surface of the virions. To express longer sequences fused to pVIII, hybrid fd phage containing a mixture of modified and wild-type pVIII have been constructed. The resulting particles contained 10–30% of the modified protein. Such hybrids particles expressing epitopes from *Plasmodium falciparum* [8,12] or the V3 loop of HIV-1 [13] have been shown to elicit an appropriate immune response when injected into experimental animals.

In addition to the filamentous phage, the coat proteins of other types of bacteriophage have also been modified to express foreign sequences. For example, the coat protein

of the small spherical RNA-containing bacteriophage MS2 has been modified to express epitopes up to 24 amino acids long [14]. Particles of MS2 contain 180 copies of a single coat protein arranged in a T=3 lattice (Fig. 1a) and the foreign sequences were inserted in the N-terminal β hairpin [15]. When expressed in *Escherichia coli*, the modified coat proteins can self assemble into empty virus-like particles which are immunogenic. In view of the fact that the structures of a number of MS2 mutants have been solved recently [16], this system may provide a means of determining the 3D structure of inserted sequences. Two systems involving bacteriophage λ have also been developed for displaying foreign protein sequences. These involve making fusions either to the C terminus of V tail protein [17] or to the N terminus of the D capsid protein [18•]. In the former case, only a fraction of the V proteins are modified and the system allows the expression of proteins as large as tetrameric β-galactosidase; in the latter case, all copies of the D protein could be modified to express peptides of significant length.

Non-infectious eukaryotic systems

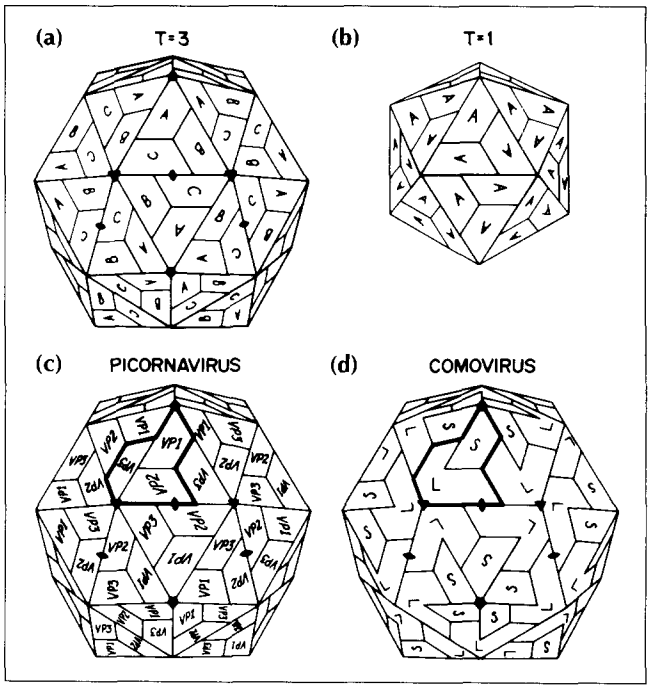
These systems are based on proteins which can self assemble when expressed in either a homologous or heterologous system. Although many of the examples described below are based on macromolecular assemblies produced by viral proteins, the particles themselves are not infectious.

The Ty element from *Saccharomyces cerevisiae* encodes a 50 kDa protein (TYA) which can self assemble into particles which range in size between 15 and 39 nm [19]. Particles of a more homogeneous size (11–16 nm) can be produced when a truncated form of TYA containing amino acids 1–381 is expressed in yeast [19,20]. It has proved possible to fuse substantial lengths of heterologous sequences (>20 kDa) after amino acid 381 without abolishing the ability of the particles to assemble; particles expressing portions of HIV-1 proteins at this site were weakly immunogenic [20]. Immunological analysis of the 1–381 particles indicates that although the N terminus of the TYA protein is on the exterior of the assembled particles, the C terminus lies on the interior [21]. This probably explains the relatively poor immunogenicity of sequences fused to the C terminus. Analysis of regions of the TYA proteins essential for particle formation indicates that there are many non-essential sequences, including the N-terminal 40 amino acids [22•]. This information should greatly assist the development of new TYA-based systems.

Two self-assembling proteins from hepatitis B virus have been developed as epitope-presentation systems. The first to be developed was based on the 22 nm particles formed by the surface antigen (HBsAg). These particles contain lipids and approximately 100 molecules of 226 amino acid long S protein. Expression of this protein in a variety of systems leads to production of lipoprotein particles similar to those found during infection. Initial studies revealed that fusion to the herpes simplex virus gD protein or the insertion of an 11 amino acid epitope from poliovirus near a major antigenic region of the S protein still permitted the formation of particles [23,24]. Since then, HBsAg-based particles have been successfully used to express antigens from a number of pathogens including *Plasmodium falciparum* [25,26] and HIV [27]. To help identify potential sites for the insertion of foreign sequences, a prediction of the structure of the S protein on the basis of topological data has recently been made [28].

The 21 kDa core antigen protein of hepatitis B virus (HBcAg) assembles into 27 nm particles when expressed in a number of different systems. To investigate the use of such particles as carriers of foreign sequences, the immunodominant epitope from foot and mouth disease

Figure 1



Examples of the icosahedral capsid types of viruses used for the construction of chimaeras. **(a)** The T=3 quasi-equivalent capsid is formed from 180 subunits. The trapezoids labelled A, B and C correspond to identical gene products located in 3 different structural environments. The central triangle formed from one copy each of subunits A, B and C is similar to the triangle centred on the threefold axis of the T=1 structure. However, instead of having a fivefold axis at each of the three vertices of the triangle, there is a fivefold axis at the top vertex and threefold axes (quasi sixfold) at the bottom two vertices. This structure is an approximate model for the structure of bacteriophage MS2 capsids. **(b)** The T=1 capsid contains 60 identical subunits (represented by trapezoids labelled A) arranged with exact icosahedral symmetry. This is an appropriate model for parvoviruses. **(c)** The pseudo-equivalent (P=3) picornavirus capsid is closely related to the T=3 capsid except that trapezoids VP1, VP2 and VP3 correspond to different gene products. **(d)** The pseudo-equivalent (P=3) comovirus capsid is very similar to the picornavirus capsid except that VP2 and VP3 are a single polypeptide (L) with two structural domains. The S protein is equivalent to VP1.

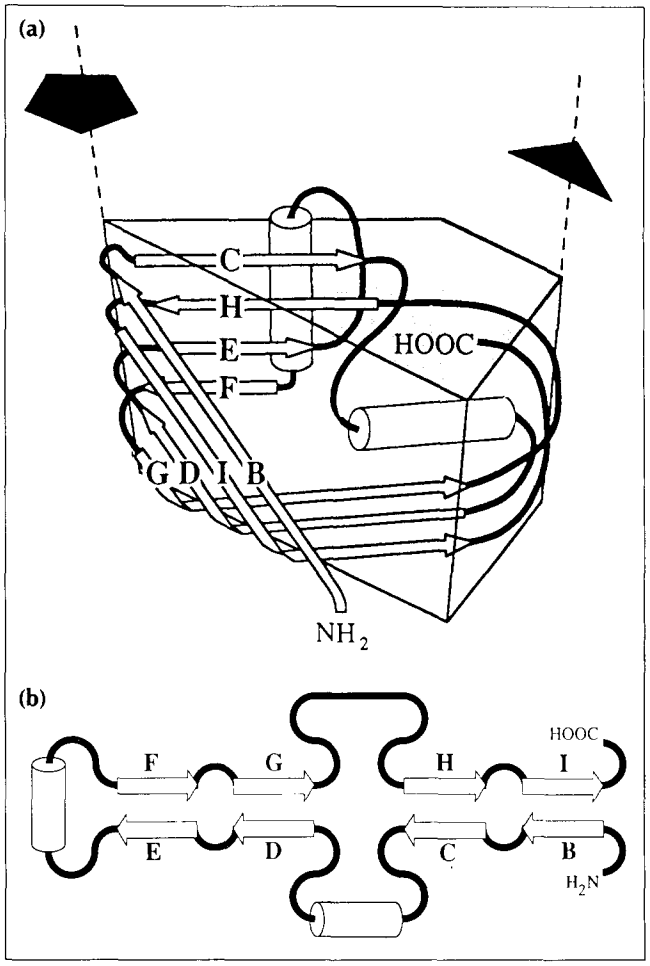
virus (FMDV) was fused to the N terminus of the core antigen and resulting particles were shown to be highly immunogenic [29], though the immunogenicity depends critically on the precise site of insertion [30,31]. Such particles have subsequently been used to express epitopes from pathogens such as SIV [32], HIV [33] and human papillomavirus [34]. HBcAg particles expressing a C-terminal fragment from SPAG-1, a sporozoite surface antigen of *Theileria annulata*, have recently been used in a vaccination trial with partial success [35•].

Bluetongue virus, a member of the *Reoviridae*, has capsids consisting of seven proteins, termed virus protein (VP) 1–7. The core consists of two major proteins, VP3 and VP7, which, when co-expressed in insect cells using baculovirus vectors, can form core-like particles (CLPs) [36]. It has proved possible to insert foreign sequences into either protein without abolishing their ability to assemble into CLPs [37,38]. In the case of CLPs containing VP7 modified at its N terminus to express 48 residues from the hepatitis B $preS_2$ protein, antiserum raised against the chimaeric protein could recognize the appropriate proteins from hepatitis B-infected patients [37]. The recently determined 3D structure of VP7 [39] should enable other potential sites for the insertion of foreign sequences to be determined.

The 58 kDa major structural protein (VP2) of human parvovirus B19 can self assemble into T=1 virus-like particles (Fig. 1b) when expressed from baculovirus recombinants in insect cells. Two potential sites of insertion were identified: the extreme N terminus and a loop which was believed to be on the particle surface by comparison with the known 3D structure of canine parvovirus [40]. Two epitopes were inserted at either position: a 13 amino acid sequence from the herpes simplex virus type 1 (HSV 1) glycoprotein gD and an 11 amino sequence from the coronavirus mouse hepatitis virus (MHV) [41•]. All four chimaeric VP2 molecules retained their ability to assemble into virus-like particles. Immunogold labelling studies suggest that epitopes expressed on the loop are more accessible than those fused to the N terminus. Indeed, more recent studies on porcine parvovirus have indicated that the N terminus of VP2, which cannot be visualized by X-ray crystallography, is located internally in the capsids [42]. For all four constructs, mice immunized with the chimaeric particles were partially protected against challenge with a lethal dose of the appropriate pathogen [41•]. In an alternative system, the minor capsid protein, VP1, of parvovirus B19 was modified to express a 147 amino acid sequence from hen egg white lysozyme. When co-expressed with VP2 in insect cells, capsids could still form and the enzymatic activity of the insert could be detected [43].

The self-assembly properties of the coat proteins of two plant viruses have been used to design potential antigen-presentation systems. In the first instance, an eight

Figure 2



Structure of subunits of icosahedral viruses. **(a)** The tertiary structure of the β-sandwich fold seen in the subunits of most RNA and DNA viruses solved to near atomic resolution. (A notable exception, however, is the subunit of bacteriophage MS2 which has a five-stranded β-meander sheet [15].) The modular nature of the subunits with the β-sandwich fold is closely similar in volume and shape to the trapezoids shown in Figure 1. The orientation of the sandwich with respect to the fivefold and threefold axes of a T=1 particle is indicated by the filled pentagon and triangle respectively. The strands of the β sheet are labelled B through I and are connected by loops referred to as B–C, D–E, F–G and H–I. **(b)** The β sandwich shown in an 'unrolled' form, showing the β strands B though I in order within the primary sequence of the subunit. The primary sites used for insertion have been the B–C and the E–F loops. The β strands are shown as arrows, the loops as solid black lines and the α helices as cylinders.

amino acid epitope from VP1 of poliovirus type 3 was fused to the C terminus of the tobacco mosaic virus (TMV) coat protein and the fusion protein expressed in *E. coli* [44]. As with wild-type coat protein, the modified protein could assemble into helical virus-like rods when extracts were acidified, greatly aiding purification. The purified rods were shown to be able to elicit the production of anti-poliovirus neutralizing antibodies in rats. More recently, it has been shown that co-expression in *E. coli* of both the TMV coat protein and single-stranded RNAs

containing the TMV origin of assembly can give virus-like particles without the need to acidify the medium [45]. Because RNA-containing particles are more stable than protein-only helices, especially at higher pHs, the latter system could be adapted as an improved way of expressing foreign sequences on TMV virus-like particles. The second plant virus coat protein to be used was from the potyvirus Johnsongrass mosaic virus. Epitopes can be fused to either the N or C terminus of the protein without abolishing the ability of the protein to polymerize into virus-like particles when expressed in *E. coli* [46]. In one case, the assembled particles were shown to be capable of raising an appropriate immune response in mice.

Infectious eukaryotic systems

The group of animal viruses most extensively investigated as potential presentation systems are the *Picornaviridae*. The capsids of these viruses comprise three major structural proteins: VP1, VP2 and VP3, arranged in a pseudo T=3 (P=3) lattice (Fig. 1c). Atomic resolution structures of a number of members of the group have been available since the 1980s and one member of the group, poliovirus, has been used extensively as a live attenuated vaccine for many years. These features have made picornaviruses particularly attractive candidates for structure-based insertion of foreign epitopes. In the case of poliovirus, foreign inserts have been expressed as replacements in the βB–βC loop (Fig. 2) of VP1 and in a number of cases, the particles have been shown to be immunogenic [47,48]. A high resolution structure has been obtained of a poliovirus type 1 chimaera expressing an antigenic site from poliovirus type 2 [49]. Examination of the structure revealed that there were changes not only in the conformation of the inserted loop but also in the neighbouring loops that were required to accommodate the new sequence. Thus, even when chimaeras are made between two closely related viruses, structural changes can occur when a sequence is placed in a heterologous context.

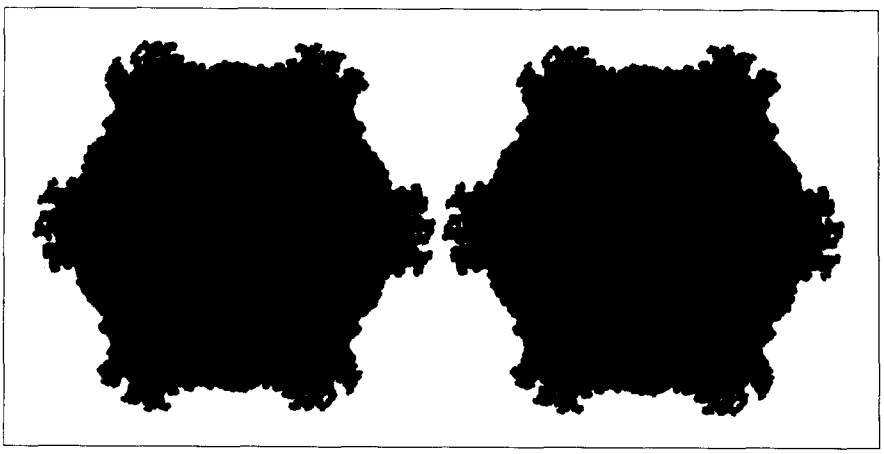
When human rhinovirus 14 (HRV14) was investigated, it was found that the βB–βC loop of VP1 was not a suitable site of insertion; however, it was possible to isolate some viable chimaeras with inserts in the βE–βF loop (Fig. 2) of VP2 [50]. To generate viable chimaeras displaying sequences from the V3 loop of HIV-1, a system of random systematic mutagenesis of the sequences flanking the conserved hexapeptide was employed [51••]. Fifteen out of 25 chimaeras produced in this way were neutralized by one or more of a panel of four anti HIV-1 V3 loop antibodies. The differing responses observed indicate that the flanking residues have a major effect on the antigenicity of the presented sequence. Using a technique related to that used in phage display technology, it has proved possible to immunoselect particularly interesting rhinovirus-based chimaeras from a library [52,53].

An enveloped RNA animal virus, Sindbis virus, has been developed as a potential source of live attenuated vaccine. Random insertional mutagenesis has been used to construct infectious Sindbis virus chimaeras expressing epitopes from Rift Valley fever virus [54]. Suitable sites for insertion were found in the E2 and E3 glycoproteins. In the case of insertions into E2, the antigens were expressed on the virion surface and could stimulate a partially protective immune response against Rift Valley fever virus.

Plant viruses have a number of potential advantages as polypeptide presentations systems: they are often easy to grow in large (gram) quantities, purification is usually straightforward and many are extremely stable. The *Comoviridae* are bipartite positive-strand icosahedral RNA viruses which are related to picornaviruses [55] (Fig. 1). Comparison of the structures of three members of the group showed that the βB–βC loop (Fig. 2) of the S protein was particularly variable and, like the corresponding loop in VP1 of picornaviruses, was exposed on the virion surface [56•]. In the case of cowpea mosaic virus (CPMV) this

Figure 3

Stereo pair showing the presentation of a 14 amino acid polypeptide from human rhinovirus 14 (HRV14) on the surface of cowpea mosaic virus (CPMV). The L subunits (see Figure 1d) are shown in green and the S subunits in blue. The HRV14-specific sequence is shown in red. The model is based on the 2.8 Å crystal structure of the CPMV-based chimaera.



loop has been shown to tolerate the insertion of foreign sequences [57,58••]. Modified CPMV particles expressing a sequence from gp41 of HIV-1 have been shown to be able to elicit the production of anti-HIV neutralizing antibodies in three strains of mice [59,60], raising the prospect that such particles could form the basis of novel vaccines. In addition it has proved possible to obtain crystals of a chimaera expressing a 14 amino acid insert derived from VP1 of HRV14 which diffract to near atomic resolution [61]. Analysis of the diffraction data has allowed the structure of the insert to be determined (Fig. 3). Comparison of the structure of the loop expressed on CPMV with that which it adopts in its native environment confirms that presentation of sequence on the surface of a heterologous particle can have a profound effect on its structure.

Initial attempts to modify the coat protein of TMV concentrated on making extensions to the exposed C terminus. The results indicated that particle formation is inhibited if every copy of the protein is modified [62,63]. By incorporating a 'leaky' stop codon at the end of the coat protein gene, it has proved possible to isolate particles in which approximately 5% of the coat protein subunits carry the insert [63,64•]. Such particles expressing an epitope from *Plasmodium yoelii* were shown to be antigenic [64•]. By altering the site of insertion, it has proved possible to produce particles in which all the subunits are modified. By inserting a 13 amino acid sequence after residue 154 of the coat protein rather than at the extreme C terminus (amino acid 158), Fitchen *et al.* [65] produced viable particles which could raise antibodies against the insert when injected into mice. Likewise, it has proved possible to express sequences on a surface loop of the viral coat [64•]. In the case of the flexuous rod-shaped virus potato virus X (PVX), it is possible to express whole proteins fused to the N terminus of the coat protein without abolishing particle formation (S Santa-Cruz, personal communication).

Conclusions

Recent work has highlighted the importance of structural constraints on the immunogenicity of peptides expressed on heterologous proteins [66••]. Clearly, the optimization of the sites of insertion to achieve the highest possible levels of antigenicity/immunogenicity of an expressed peptides is an important stage in the development of novel vaccines. The increasing availability of data regarding the structures of self-assembling proteins should assist the identification of such sites. Of possibly even greater significance is the prospect of being able to determine the structure of peptide when expressed on the surface of a heterologous particle and to correlate changes in immunogenicity with variations in the 3D structure of the insert. This could lead to 'fine tuning' of expression systems so that they fully realize their potential.

Acknowledgements

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