Multiple presentation of foreign peptides on the surface of an RNA-free spherical bacteriophage capsid

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We have produced a plasmid expression vector for the coat protein of RNA bacteriophage MS2. The vector has been modified to introduce a unique KpnI restriction site within the coat protein gene at a site corresponding to the most radially distant feature of the bacteriophage capsid, namely the top of the N-terminal β-hairpin (between residues 15 and 16). Insertion of DNA oligonucleotides at this site allows the production of chimeric MS2 coat proteins having foreign peptide sequences expressed as the central part of the hairpin. We have produced chimeras with a number of different peptide sequences (up to 24 amino acids in length) chosen because of their known antigenic properties. The chimeric coat proteins self-assemble into largely RNA-free phage-like capsids in *Escherichia coli* and can be easily disassembled and reassembled in vitro. Such peptide-presenting particles may have a number of biotechnological applications, including use as a cost-effective, synthetic vaccine. We have tested the antigenicity of one such construct in vivo in mice and have shown that these particles are immunogenic and that antibody titres against the inserted peptide epitope can be obtained.

Introduction

Although small linear peptides are capable of eliciting the production of antibodies that react with proteins containing the sequences as continuous epitopes, the peptides themselves are poor antigens (Porter & Whelan, 1986). The classical approach to improving immunogenicity involves chemical coupling of the peptide to large carrier molecules, such as keyhole limpet haemocyanin (KLH) and/or use of an adjuvant on administration (Harlow & Lane, 1988). Several groups have attempted an alternative route of peptide epitope presentation using genetic engineering to introduce foreign peptide sequences into the coat proteins of simple viruses in the hope that the epitope will be presented as part of the assembled viral capsid (Smith, 1985; Clarke et al., 1987; Dedieu et al., 1992). A great deal of effort has been directed towards the use of filamentous bacteriophages for this purpose (Scott & Smith, 1990; Greenwood et al., 1991). Here we present results with an alternative bacteriophage system, namely the simple spherical RNA bacteriophage MS2, which may have a number of advantages over the filamentous phage systems.

MS2 is a male-specific coliphage which has been extensively characterized genetically and biochemically (Fiers, 1979). The genome consists of a positive-sense ssRNA of 3569 bases, which is encapsidated in a T = 3 surface lattice containing 180 copies of a single coat protein subunit (*M*, 13.75K). The mature phage also contains one copy of the maturation protein (the product of the A gene), which functions during the phage life cycle by binding to bacterial pilus and is taken up into the host bacterium with the phage RNA, whilst the remaining coat protein subunits are dissociated. The phage genome encodes just two other polypeptides, a replicase subunit and a lysis protein, which is produced later in infection to allow escape from the host cell. Neither of these proteins is part of the final capsid.

The structure of the phage capsid is known at atomic resolution from X-ray crystallography, although the position of the A protein was not revealed by the electron density map (Valegård et al., 1990). The coat protein subunit is unusual in that it does not have the conserved anti-parallel β-barrel topology seen in virtually every other spherical RNA virus coat protein structure (Rossmann & Johnson, 1988). In the phage capsid the coat proteins are packaged as non-covalent dimers which are secured by interdigitation of C-terminal α-helices from each monomer. Underneath the helices lies an extensive β-sheet comprising five β-strands from each subunit, whereas at the N terminus of the protein the polypeptide is folded into a β-hairpin structure which forms the most radially distant feature of the mature capsid.
capsid (Fig. 1). A feature of the structures of simple viruses which must avoid immune neutralization is the introduction of extended polypeptide loops, which are the immunodominant epitopes, in otherwise conserved three-dimensional folds (Rossmann & Johnson, 1988). Therefore we postulated that potentially MS2 could be used to mimic such a system by insertion of foreign peptide sequences at the top of the β-hairpin, provided that such insertions do not lead to aberrant folding or assembly. A previous attempt to use a member of the MS2 family of phages in this way has been reported, although insertions were made essentially at random and only involved model sequences (Kozlovskaya et al., 1988). A similar rationale has been used successfully to insert foreign sequences into the B-C loop of the VP1 protein of poliovirus (Burke et al., 1988; Dedieu et al., 1992).

We have chosen to insert six distinct foreign peptide sequences into the MS2 coat protein to illustrate the flexibility and utility of the system. The first epitope is a nonapeptide derived from the haemagglutinin (HA) of influenza virus and is known to be strongly antigenic when presented as a linear fragment on a KLH carrier (Green et al., 1982; Wilson et al., 1984). The second is a decapetide sequence from human IgE responsible for triggering degranulation of mast cells (Stanworth et al., 1990). This sequence is extremely hydrophobic and is believed to function by inserting into the mast cell membrane where it can interact with a G protein. The third is a dodecapetide corresponding to the repeated surface antigen of the malarial parasite Plasmodium falciparum (Greenwood et al., 1991). The fourth and fifth examples are 20-mers derived from the coat proteins L1 and L2 of human papillomavirus type 16 (HPV-16) (Dillner et al., 1990). The final example is a 24-mer derived from the V3 loop of gp120 from human immunodeficiency virus (HIV-1) (Javaherian et al., 1989).

Methods

Construction of the MS2 chimera vectors. The starting plasmid for the production of the chimeric coat protein gene was pTACH-CP (a gift from Dr G. Medina) which contains the MS2 coat protein gene as a 434 bp Xbal–XhoI fragment. This fragment was cloned into the polylinker of M13mpl8, and oligonucleotide-directed mutagenesis to introduce a unique KpnI restriction site was carried out by the method of Sayers et al. (1988) using the Amersham oligonucleotide-directed in vitro mutagenesis system version 2.1 (Fig. 2). The engineering of the KpnI site was such that it did not alter the coding sequence of the MS2 coat protein. The mutant coat protein gene fragment was then reintroduced into the expression vectors pTACH-ACP or a variant, pTACH-ACP. Synthetic DNA oligonucleotides encompassing the foreign peptide sequences to be inserted were annealed and if necessary treated with Klenow polymerase to generate double-stranded substrates for ligation into KpnI-digested pTACH-CP(KpnI) or pTACH-ACP (KpnI), which were then used to transform Escherichia coli TG1 (supE, F[traD36, proAB∗, lacIq, lacZΔM15]) to ampicillin resistance. Transformants were screened for inserts in the correct orientation by subcloning into M13mpl8 as above and dyeoxynucleotide sequencing (Sanger et al., 1977) using the Pharmacia LKB Biotechnology 3′ Sequencing Kit.

Expression and purification of MS2 antigen chimeras. Recombinant MS2 proteins were expressed as follows: 500 ml cultures of E. coli TG1 carrying the appropriate pTACH-ACP or pTACH-CP vectors were grown at 37 °C to an absorbance (at 600 nm: A600) of approximately 0.4 to 0.6 and induced by the addition of isopropyl-β-D-thigalactoside (IPTG) to 1 mM and incubation continued overnight.

Soluble chimeras were purified as follows. Cells were harvested by centrifugation and the pellets resuspended in 50 mM-HEPES, 100 mM-NaCl, 10 mM-DTT, 5 mM-EDTA, pH 7.4, sonicated for 15 min in 1 s bursts followed by 30 s rest periods on ice, and then clarified by centrifugation before the addition of magnesium acetate and DNase I to final concentrations of 6 mM and 10 μg/ml respectively. The mixture was incubated at 37 °C for 30 min to digest contaminating nucleic acids. The slight precipitate that formed was removed by centrifugation, and the MS2 protein in the supernatant was recovered by precipitation with ammonium sulphate. The ammonium sulphate pellet was then dialysed extensively against the resuspension buffer without DTT before being layered onto 15 to 45% (w/v) linear sucrose density gradients formed above a 60% (w/v) sucrose cushion and centrifuged at 28000 r.p.m. at 4 °C for 5 h in a Beckman Ti45 rotor. The peak of absorbance corresponding to assembled material was pooled and further purified over a size exclusion column (Bio-Gel A-1.5M, 5 x 160 cm). Fractions containing pure coat protein were pooled, ammonium sulphate-concentrated and stored at 4 °C until use.

Largely insoluble chimeras were purified by following the procedure outlined by Cheadle et al. (1992). E. coli cell pellets were obtained as above and then resuspended in 2 x volume of 50 mM-HEPES, 6 M-urea, pH 7.4 by stirring overnight at 4 °C. Next, the sample was clarified by centrifugation (27000 g), 4 °C for 30 min, the supernatant was decanted and diluted at 1 M-urea using 50 mM-HEPES pH 7.4 and 2 ml aliquots were applied to a size exclusion column (Bio-Gel A-1.5M, 5 x 160 cm) which had been previously equilibrated with 50 mM-HEPES, 1 M-urea, pH 7.0 at 4 °C. Fractions (2 ml) were collected at 0.25 ml/min and those containing essentially pure coat protein, as determined by SDS-PAGE, pooled. The coat protein sample was refolded over a rapid desalting column (Pharmacia HR 10/10) in 20 mM-HEPES, 0.1 M-DTT, pH 7.4 and the sample was stored overnight at 4 °C, before being applied to a size exclusion column (Pharmacia Superose-12) equilibrated in the same buffer. The fractions containing the reassembled chimeric capsids were then pooled.

Immunization protocols. TUXCS Number One mice and New Zealand White rabbits were used for the immunization protocols. Five mice were injected subcutaneously at one site with 500 μg of purified MS2–WT (wild-type) or MS2–HA chimeric construct, absorbed on aluminium hydroxide (Alhydrogel) as adjuvant. Two rabbits were injected intradermally at three sites with 1 mg of purified construct in PBS containing no adjuvant. For the mice booster injections were given on days 14, 21 and 26 and blood was collected via the marginal tail vein. For the rabbits boosters were given on week 6, subcutaneously at three sites, and at week 8, intravenously. Blood was collected via the marginal ear vein. Sera were stored at −70 °C until use.

ELISA. The ELISA procedure followed the method originally described by Engvall & Perlmann (1971). Briefly the antigen of interest [50 μl of a 50 μg/ml stock solution in 10 mM-sodium phosphate, 100 mM-sodium chloride pH 7.4 (PBS)] was dispensed into every well of a flat-bottom 96-well polystyrene microtitre plate using a multichannel pipetter and the plate incubated overnight at 4 °C. Next each well was washed four times with a solution of PBS containing 0.1% (v/v) Tween 20 and aspirated using a multichannel
washed/aspirator (Labsystems Autowash II). The wells were then treated with BSA (1% w/v in PBS) for 1 h at 25 °C to block non-specific binding sites, washed to remove excess solution and aspirated as above. The primary antibody was diluted 1:10 with PBS and the titre of this solution determined by successive 1:1 dilutions along a row of wells. A 50 μl aliquot was applied to each well and the plates were incubated at 25 °C for 2 h, washed/aspirated as above and the secondary antibody [e.g. goat anti-mouse IgG conjugated to horseradish peroxidase (HRP)] was added to each well, repeating the above incubation procedure. After the aspiration as above, a 50 μl aliquot of developer solution (100 mM-sodium citrate, 100 mM-sodium acetate, pH 5.0, containing 0.04% w/v 1,2-benzenediamine and 0.004% w/v hydrogen peroxide) was removed to each well and incubated for 10 min at 25 °C. The enzyme reaction was quenched by the addition of 25 μl of 2 M-sulphuric acid. After 5 min the A490 values were measured using an Olivetti PCS286 using FlexiCalc software. Synthetic peptides were covalently coupled to surface-modified polystyrene microtitre plates using established protocols for peptide antigens (Jemmerson, 1987; Larsson et al., 1989).

**SDS-PAGE and Western blot analysis.** SDS-PAGE for the analysis of proteins followed the procedure of Shägger & von Jagow (1987) without alterations. Western blots followed the procedure of Towbin et al. (1979), with the following alterations for preabsorption blots. To obtain sufficient quantities of antibody for the preabsorption assays rabbit anti-MS2-HA IgG was isolated and affinity-purified according to the methods described by Harlow & Lane (1988). Three replicates of purified MS2-WT and MS2-HA proteins, and a synthetic nonapeptide covalently crosslinked to KLH using the method outlined by Gnann et al. (1989), were separated on a 3 to 20% (w/v) SDS-polyacrylamide linear gradient gel according to Laemmli (1970) and then electro-phoretically blotted onto 0.2 μm nitrocellulose sheets. After blocking of non-specific sites with 1% (w/v) BSA, 0.5% (v/v) Tween 20 in PBS (30 min at 37 °C) the sheet was divided into thirds, and purified rabbit anti-MS2-HA IgG (at a titre of 1:800), undiluted or preabsorbed with MS2-WT at two different molar ratios of carrier molecule to antigen, was used as the primary probe. Visualization of the bands followed binding of HRP-linked anti-rabbit IgG (at a titre of 1:1000) and development of the peroxidase activity using the Amersham ECL (Western blotting detection system).

**Results**

To incorporate the foreign peptide sequences at the appropriate position, we have modified an existing clone of the MS2 coat protein gene (G. Medina & P.G. Stockley, unpublished results) using site-directed mutagenesis to incorporate the following changes in nucleotide sequence, A1380T and T1383C (n.b. sequence numbers are relative to the MS2 wild-type genome;
Fig. 3. SDS-PAGE and Western blot analysis of chimeric MS2 proteins. IPTG-induced cultures of E. coli TG1 carrying the appropriate pTACH-ACP vectors were harvested by centrifugation and the pellets resuspended in a two- to threefold volume of 50 mM-Tris-HCl, 0.5% (w/v) Sarkosyl, pH 6.5 before being disrupted by sonication. Total sonicates were loaded onto SDS-PAGE (16.5% T:6% C) gels according to the procedures of Shagger & von Jagow (1987). (a) Gels stained with Coomassie blue R250 in methanol-acetic acid solution. Lane 1, purified MS2 wild-type phage; lane 2, Mr standards, indicated by arrowheads, from top to bottom 43K, 29K, 18K, 14K, 6.2K and 3.4K respectively; lane 3, MS2 WT; lane 4, MS2-HA; lane 5, MS2-IgE; lane 6, MS2-L1; lane 7, MS2-L2; lane 8, MS2-Mal; lane 9, MS2-gp120. (b) Gels such as those in (a) were blotted onto 0.2 μm nitrocellulose membrane according to the procedures of Towbin et al. (1979), probed by treatment with a rabbit anti-MS2 WT polyclonal antibody followed by HRP-conjugated goat anti-rabbit IgG and the proteins were visualized as described in Methods. Lane 1, MS2-WT; lane 2, MS2-HA; lane 3, MS2-IgE; lane 4, MS2-L1; lane 5, MS2-L2; lane 6, MS2-Mal; and lane 7, MS2-gp120. The doublets in some lanes are due to cross-contamination between lanes during loading of the gel.

Fiers, 1979) which produces a unique KpnI restriction enzyme cleavage site at position 1378 in the gene sequence (Fig. 2). Synthetic DNA oligonucleotides corresponding to the sense and antisense strands of the foreign epitopes and carrying the appropriate flanking nucleotides for insertion at the KpnI site were then annealed and ligated into the expression construct. Positive clones in the correct orientation were then identified by DNA sequencing. This method of insertion results in duplication of the codons for glycine and threonine at positions 14 and 15 in the coat protein thus leading to the inserts being flanked by the sequence Gly-Thr. The size of the inserted non-MS2 sequences is thus increased by two to 11 amino acids for the HA sequence (MS2-HA), 12 amino acids for the human IgE (MS2-IgE), 14 amino acids for the malarial antigen (MS2-Mal), 22 amino acids for the HPV-16 inserts (MS2-L1 and MS2-L2) and 26 amino acids for the HIV sequence (MS2-gp120).

Two expression vectors have been used to produce the chimeric proteins. They are both derived from plasmids carrying the trp-lac ‘tac’ hybrid promoter (Amann et al., 1983). The first (pTACH + CP) is inducible with IPTG as expected but the second (pTACH-ACP) carries a copy of the bacteriophage A gene between the promoter and the coat protein gene. Sequences within this gene confer the property of high level constitutive expression on the coat protein cistron (Kastelein et al., 1983; de Smit & van Duin, 1990). For both vectors SDS–PAGE analysis of cells carrying the appropriate construct revealed production of polypeptides of the expected Mr values, all of which reacted in Western blots with a rabbit anti-MS2 coat protein polyclonal serum (Fig. 3). The chimeras containing the HA, IgE and gp120 inserts also reacted with the appropriate insert-specific monoclonal antibodies (MAbs) (data not shown).

To purify the recombinant proteins and analyse the state of their aggregation, bacterial cell pellets were sonicated and the resultant solutions fractionated by low-speed centrifugation. SDS–PAGE of both pellet and supernatant fractions (not shown) from pTACH-ACP clones revealed that a significant proportion of the MS2-WT and the MS2-HA proteins remained in the supernatant whereas the other chimeras were largely in the pellet fraction. The soluble samples were then analysed on sucrose density gradients (Fig. 4a) in which a rapidly sedimenting fraction containing MS2 coat protein or its HA derivative was readily identified. Electron microscopy of this material showed it to be assembled phage-like particles of diameter approximately 250 Å with a T = 3 morphology. Absorbance measurements at 260 and 280 nm suggested that these particles contain only very small amounts of nucleic acid and are in effect empty capsids.
The insolubility of the other chimeras and portions of the MS2-WT and MS2-HA proteins appears to be the result of constitutive expression in the pTACH-ACP vector system. However, soluble forms of all of the proteins were obtained from cells carrying the pTACH-CP vectors, particularly when the cells were grown at lower temperatures. Examination by electron microscopy of thin sections of the cells expressing chimeric proteins showed that all the chimeras produced assembled phage-like particles (data not shown) which then formed semicrystalline arrays similar to those observed in wild-type phage infections. These insoluble capsid aggregates could be solubilized from cell pellets by treatment with urea according to the procedure of Cheadle et al. (1992) and the disassembled, unfolded chimeric coat proteins purified by gel filtration chromatography. Subsequent dilution to reduce the urea concentration resulted in the formation of soluble reassembled capsid-like particles as judged by electron microscopy. Alternatively the fraction of each chimera remaining soluble after sonications of the cell pellets could be purified by immunoaffinity chromatography on an anti-MS2 coat protein column and yielded essentially homogeneous material (Fig. 4). N-terminal amino acid sequencing of the purified MS2-HA chimera over the first 30 residues confirmed the expected sequence for the insert.

To test the immunogenicity of the chimeric constructs the MS2-HA capsids were used. TUXCS Number One mice were immunized subcutaneously with a series of doses of sucrose gradient-purified empty capsids in a 1:1...
mix (v/v) with the adjuvant Alhydrogel. Booster injections were given at 7 or 14 day intervals. The resultant response was characterized by ELISA using either MS2–HA or MS2–WT as the antigen on the plate (Harlow & Lane, 1988). There was a roughly linear dose–response (data not shown). At the highest dose (500 μg MS2–HA corresponding to 36 μg of insert) the titre (expressed as the half maximum value of the \( A_{490} \)) for the serum at day 42 was 1:7500 ± 300 (Fig. 5). When the carrier alone (i.e. MS2–WT) was used as antigen the titre fell to 1:3200 ± 130, an indication that specific antibody titres against the insert had been produced. The antisera also recognized the HA nonapeptide coupled to KLH, although with a considerably lower titre (1:700 ± 30). Similar anti-MS2–HA sera raised in BALB/c mice also recognized the HA nonapeptide covalently linked directly to the microtitre plate (1:950 ± 40). The specificity of the sera for the inserted peptide was also demonstrated by prior absorption with excess MS2–WT before use in ELISAs or Western blots against MS2–HA, MS2–WT and KLH–HA9, which showed the expected reactivity profile (Fig. 6). KLH–HA was detected only in non-preabsorbed sera (Fig. 6a).

Discussion

We have shown that a number of foreign peptide epitopes, up to 24 amino acids in length, can be inserted into the N-terminal \( \beta \)-hairpin of the MS2 coat protein without significant detrimental effect on the ability of the expressed chimeric proteins to self-assemble into largely RNA-free capsid-like particles in vivo. This is consistent with the location of the hairpin in the phage capsid since it is not involved in any intermolecular contacts (Valegård et al., 1990). The expression construct does not include the MS2 replicase translational operator which is normally located 3′ to the coat protein gene and is believed to function as the assembly initiation sequence (Beckett et al., 1988). The formation of empty capsids upon coat protein expression is therefore accounted for (Rohrmann & Krueger, 1970). Soluble forms of the chimeric capsids are easily purified, but even insoluble forms can be recovered by a simple cycle of disassembly and reassembly in the presence of urea. As expected from the external location of the inserted epitope, chimeric capsids are immunogenic and are able to elicit specific anti-insert responses.

The HA9 peptide sequence, used in the immunogenicity study, was initially identified as a linear fragment capable of generating a strong immune response when presented as part of a synthetic peptide attached to a larger carrier protein (Green et al., 1982). A MAb (Niman et al., 1983) to this peptide has a very high affinity and has been used to purify proteins fused to the nonamer sequence by affinity chromatography (Field et al., 1988). The complex between the nonapeptide and the monoclonal Fab' has been crystallized and the structure
determined by X-ray diffraction techniques (Rini et al., 1992). In the antigen-binding site the peptide interacts with the protein over seven of the nine residues and forms a short extended chain which then forms a type I β-turn. Comparison of the Fab’ and Fab’–peptide complex structures suggests that the antibody undergoes significant conformational change upon antigen binding (‘induced fit’). This implies that the interaction seen in the crystal structure is the lowest free energy form of the complex. It is interesting to speculate whether recognition of the same sequence within a polypeptide loop, i.e. in the context of the MS2 capsid structure, requires the same defined conformation of the peptide. An indication of this comes from the titres of the anti-HA nonamer MAb (Cas-125) for either MS2-HA (1:379 ± 25) or KLH-HA (1:3650 ± 273). The sharply reduced titre for the sequence in the MS2 loop presumably reflects the free energy cost of converting the nonamer into the conformation that is recognized, since the conformation change for the antibody is presumably the same in each case. This result suggests that expression of at least the HA nonamer sequence in the β-hairpin loop constrains its conformation.

These experiments demonstrate the potential utility of the MS2 system for the presentation of foreign peptide sequences on the surface of a spherical bacteriophage capsid. The system has a number of advantages over the filamentous bacteriophage alternatives. The MS2 coat protein is capable of facile self-assembly in the absence of nucleic acid (Rohrmann & Krueger, 1970) unlike the filamentous phages in which assembly is concomitant with encapsidation of nucleic acid (Model & Russel, 1988). Furthermore the filamentous coat protein must undergo post-translational processing and membrane insertion before assembly occurs whereas the MS2 protein is unprocessed. The MS2 system also has the advantage of a detailed molecular model for the coat protein allowing the effects of foreign peptide insertion to be modelled. The apparent ability of MS2 chimeras to produce specific titres against foreign epitopes suggests that they offer a cheap and elegant method for the production of refined vaccines. Indeed, there is no intrinsic reason why the coat protein could not be expressed in a heterologous host such as yeast which might even allow epitopes requiring post-translational modification to be presented.

A big advantage of the filamentous systems over the use of MS2 illustrated here is that expression of foreign peptides in the former system occurs on the surface of live phage containing the DNA encoding the insert. This has led to a number of applications using selection of phage expressing a mixture of inserted random sequences, the selected insert sequences being identified by subsequent phage amplification and DNA sequenc-


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