Identification of epitopes in cucumber mosaic virus using a phage-displayed random peptide library

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Antigenic sites in the cucumber mosaic virus (CMV) coat protein (CP) have been identified using a polyclonal antiserum prepared against glutaraldehyde-fixed virions. Antibodies were used to screen a random peptide library of heptamers displayed on the surface of a bacteriophage. Eight of 36 (22%) sequenced phage clones had inserts resembling a putative virion surface domain of the CMV CP. This region has the sequence LETDEL, corresponding to amino acids 194–199 in the Fny-CMV CP. The binding of phage clones to Fny-CMV antiserum was inhibited by a synthetic peptide representing this region. Six of 36 (17%) phage clones contained sequences corresponding to a C-terminal sequence in the Fny-CMV CP, which is thought to be internal in assembled virions. This sequence, EHQRSTSGV, represents amino acids 206–215 and all but the P residue were observed in at least one clone. Four of 36 (11%) sequenced phage clones carried sequences that matched a portion of the sequence RLLLPDSV, corresponding to amino acids 89–96 in the Fny-CMV CP. This region was also identified as the antigenic site recognized by a monoclonal antibody (MAb23C10E4). Eleven percent of the phage (4 of 36) contained sequences matching at least three amino acids of the N-terminal region in the CMV CP. The positions of the antigenic sites seen in this study are consistent with a predicted structure for the CMV CP.

Introduction

A knowledge of virus epitopes can provide a perspective on the surface architecture of virions. This is of particular interest in trying to understand the interactions of viruses with host factors and insect (or other) vectors. Epitopes correspond to the antigenic determinants of proteins, which are recognized by the binding sites (paratopes) of antibodies (Van Regenmortel, 1992). Close, geometrically and chemically specific contacts are defined by amino acid residues in the protein (Smith & Petrenko, 1991). Historically, protein epitopes have been classified as continuous (linear) or discontinuous (conformational) (Barlow et al., 1986). Although most antigenic determinants are discontinuous (Benjamin et al., 1984; Van Regenmortel, 1992), a greater emphasis has been placed on continuous epitopes, due to the readily available amino acid sequence data (Bottger et al., 1995; Stephen et al., 1995).

The antigenic structures of many plant viruses have been investigated through the identification of epitopes recognized by monoclonal and polyclonal antisera. This has been accomplished largely through the use of synthetic peptides, although early work was done with coat protein (CP)-derived peptide fragments and some information has come from sequence and mutational analysis (Al Moudallal et al., 1982, 1985). Most plant viruses whose antigenic structures have been well characterized have rod-shaped virions, including members of the following genera: Tobamovirus (Al Moudallal et al., 1985; Van Regenmortel, 1986; Dore et al., 1987, 1988), Potyvirus (Shukla et al., 1988; Jordan, 1992; Vuento et al., 1993; Andreeva et al., 1994; Desbiez et al., 1997), Potexvirus (Koenig & Torrance, 1986), Furovirus (Torrance et al., 1993; Commandeur et al., 1994; Pereira et al., 1994; Chen et al., 1997), Tobravirus (Legorburu et al., 1996) and Closterovirus (Pappu et al., 1993). Tobacco mosaic virus is perhaps the best antigenically characterized of all plant viruses and epitopes have been localized at the extremities and along the surface of virions (Dore et al., 1988, 1990).

In contrast to the studies on the antigenic structures of rod-shaped plant viruses, relatively few icosahedral plant viruses have been investigated in detail. Examples include tomato bushy stunt virus (Jaegle et al., 1988), bean pod mottle virus
regions were identified and localized on a three-dimensional peptide library. Two definitive and two putative antigenic epitopes of CMV. A polyclonal antiserum prepared against CCMV CPs, a model for the three-dimensional structure of Bromoviridae demonstrated between the structure of CMV and cowpea et al. resolution by cryoelectron microscopy and image reconstruction (Francki, 1990; Shintaku & Palukaitis, 1990; Perry et al. determinant of aphid transmission (Gera genus are of interest in this regard because the CP is a primary mosaic virus (CMV) and other members of the Cucumovirus et al. 1993; Torrance, 1992) and barley yellow dwarf virus (Rizzo & Gray, 1992). In studies on the surface structures of luteoviruses and a number of other genera, unique antigenicities have been correlated with vector transmission phenotypes (Massalski & Harrison, 1987; Harrison & Robinson, 1988; van den Heuvel et al., 1993; Jolly & Mayo. 1994; Kantrong et al., 1995; Legorburu et al., 1995). Cucumber mosaic virus (CMV) and other members of the Cucumovirus genus are of interest in this regard because the CP is a primary determinant of aphid transmission (Gera et al., 1979; Chen & Francki, 1990; Shintaku & Palukaitis, 1990; Perry et al., 1994, 1998).

CMV, the type member of the genus Cucumovirus (family Bromoviridae), is divided into two main subgroups based on serological and nucleic acid properties (Palukaitis et al., 1992). Serologically, the two subgroups are closely related, as shown by the cross reactions of polyclonal antibodies (Devergne & Cardin, 1973; Wahyuni et al., 1991). Some monoclonal antibodies produced against the CPs of subgroups I and II can differentiate the two, indicating the presence of unique epitopes for each (Porta et al., 1989; Wahyuni et al., 1991). Recently, the molecular structure of CMV has been determined at 23 Å resolution by cryoelectron microscopy and image reconstruction (Wikoff et al., 1997). A remarkable similarity was demonstrated between the structure of CMV and cowpea chlorotic mottle virus (CCMV), another member of the Bromoviridae. Based on the high resolution structure of CCMV and an alignment of amino acid sequences between CMV and CCMV CPs, a model for the three-dimensional structure of CMV is available (Speir et al., 1995; Wikoff et al., 1997).

In the present study, we describe the identification of linear epitopes of CMV. A polyclonal antiserum prepared against glutaraldehyde-fixed Fny-CMV virions was used in ‘biopanning’ experiments to screen a phage-displayed heptapeptide library. Two definitive and two putative antigenic regions were identified and localized on a three-dimensional model of the CMV CP. One of these sequences was also identified as the epitope recognized by a monoclonal antibody prepared against unfixed virions.

Methods

- **Viruses and antibodies.** Fny-CMV, the V and C strains of tobacco aspermy virus (V-TAV, C-TAV) and the J strain of peanut stunt virus (J-PSV) have been described in previous studies (Karawasa et al., 1991; O’Reilly et al., 1991; Perry & Francki, 1992; Perry et al., 1998). Virus was purified from infected Nicotiana tabacum or N. clevelandii by the method of Lot et al. (1972). Polyclonal rabbit antibodies against glutaraldehyde-fixed virions were prepared as described by Francki & Habibi (1972; Francki et al., 1980). The monoclonal antibodies were a gift from H. Hsu. Immuno globulin fractions were initially purified by ammonium sulfate precipitation and ion-exchange column chromatography (Harlow & Lane, 1988). A purified IgG fraction was prepared by protein A chromatography and stored at −20 °C in 50% glycerol. All secondary antibodies were purchased from commercial sources as detailed below.

- **Random peptide library and the selection of phage clones.** The phage-displayed peptide library used in this study was a commercially available kit (Ph.D.-7, New England BioLabs) based on a combinatorial library of random peptide heptamers fused to a minor CP (pIII) of the filamentous coliphage M13. The library consists of 2 × 10^9 bacteriophage particles. random peptide clones and stored at −80 °C.

In order to ‘biopan’ (Parmley & Smith, 1988) for ligands from the peptide library, polystyrene ELISA plates (Costing) were coated with 1:2 ml purified IgG (100 µg/ml in 0.1 M NaHCO3, pH 8.6; 150 µl per well) overnight at 4 °C. Wells were then blocked for 2 h at 4 °C with 300 µl per well of 1 M sodium carbonate containing 5 mg/ml BSA and 0.02% sodium azide. Following blocking, the plates were washed six times with TBST [TBS (50 mM Tris–HCl, pH 7.5, 150 mM NaCl), 0.1% Tween 20 (pH 7.5)]. Ten µl of the original library containing 2 × 10^11 phage was diluted into 800 µl TBST and pipetted into coated and blocked wells (100 µl per well). Panning was carried out for 1 h at room temperature with gentle rocking. The plate was then washed 10 times with TBST and bound phage eluted with 800 µl of elution buffer (0.2 M glycine–HCl, pH 2.2; 1 mg/ml BSA; 100 µl per well) for 10 min at room temperature with rocking. The eluate was transferred to a microfuge tube and neutralized with 120 µl of 1 M Tris (pH 9.1). The entire neutralized elute was added to 20 ml of an early exponential culture of Escherichia coli ER2537 and amplified by incubation at 37 °C with vigorous shaking for 4.5 h. The cells were removed by centrifugation and virus particles purified by PEG precipitation (Smith & Scott, 1993). The phage pellets were resuspended in 200 µl TBS containing 0.02% sodium azide, resulting in titres of between 2 × 10^11 and 1 × 10^12 p.f.u./ml. Second and third rounds of selection were carried out similar to the first except the concentration of Tween 20 was raised to 0.5%. Phage for sequencing were selected following the third round of biopanning. In order to test for a greater diversity of peptide sequences, phage from only two rounds of biopanning were also evaluated.

- **Immunological screening of phage.** The immunological screening procedure was a modification of the method of Folgori et al. (1994). A single colony of E. coli ER2537 was inoculated into 5 ml LB and grown with shaking at 37 °C until mid-exponential phase. Cells were infected with eluted phage from affinity selection at a dilution to yield approximately 500–1000 plaques per 150 mm Petri dish. Infected cells were then transferred to a culture tube containing 3 ml of 0.7% agarose top agar, mixed and immediately layered with 3 ml of LB agar (Sambrook et al., 1989). The plates were incubated for 5 h at 37 °C and inspected for plaque-forming units. Plates with countable plaques were layered with nitrocellulose filters and incubated overnight at 37 °C. Filters were removed and blocked with 5% non-fat milk and neutralized with 120 µl of 1 M Tris (pH 9.1). The entire neutralized elute was added to 20 ml of an early exponential culture of Escherichia coli ER2537 and amplified by incubation at 37 °C with vigorous shaking for 4.5 h. The cells were removed by centrifugation and virus particles purified by PEG precipitation (Smith & Scott, 1993). The phage pellets were resuspended in 200 µl TBS containing 0.02% sodium azide, resulting in titres of between 2 × 10^11 and 1 × 10^12 p.f.u./ml. Second and third rounds of selection were carried out similar to the first except the concentration of Tween 20 was raised to 0.5%. Phage for sequencing were selected following the third round of biopanning. In order to test for a greater diversity of peptide sequences, phage from only two rounds of biopanning were also evaluated.
goat anti-rabbit or anti-mouse IgG antibodies (Sigma; 1/2000 in TBST) was added to the filters and incubated for 2 h at room temperature. Filters were washed and developed using the colorimetric precipitable substrate NBT/BCIP (Sigma; Harlow & Lane, 1988).

Antibody-binding inhibition assay. For a polyclonal antibody-binding inhibition study, multi-well polystyrene ELISA plates were coated overnight with PEG-purified phage particles (3.5 × 10⁹/100 µl) in coating buffer (0.1 M sodium carbonate, pH 9.6). After washing twice with PBST, 300 µl per well of blocking buffer (PBST containing 5% non-fat dry milk and 0.02% sodium azide) was added and plates were incubated at 37 °C for 1 h. Fny-CMV IgG antibodies or preimmune IgG (1 ng/µl) were preincubated with a synthetic peptide with the sequence YSKDDALETDEL (Research Genetics) at 100 ng/µl in blocking buffer containing 10 µl/ml of E. coli lysate and 1 × 10⁻¹¹/ml PEG-purified recognition-negative phage particles at room temperature for 30 min before being added to the phage-coated plates for binding. As a control, the same antibody mixture without added peptide was used. In the monoclonal antibody inhibition assay, plates were coated with 1 µg per well of purified Fny-CMV. MAb23C10E4 was diluted 1/10000 in blocking buffer and preincubated with increasing amounts of PEG-purified phage at room temperature for 30 min before adding to the Fny-CMV-coated plates for binding. For quantification, the plates were washed three times and incubated with alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse IgG antibodies 1/2000 in PBST at 37 °C for 2 h. Plates were washed as described above, and 4-nitrophenylphosphate (Sigma) was added for colour development (Harlow & Lane, 1988). Absorbance was measured at 405 nm. All assays were carried out in duplicate microtiter dishes.

ELISA assay. Standardized ELISA assays were performed as described (Harlow & Lane, 1988). In a triple antibody sandwich (TAS)-ELISA, plates were coated overnight with anti-Fny-CMV IgG antibodies (1 ng/µl) in coating buffer (0.1 M sodium carbonate, pH 9.6). Purified Fny-CMV was used as an antigen at a 10 µg/ml dilution in 10 mM phosphate buffer, pH 7.0. MAb23C10E4 was used at a 1/4000 dilution in blocking buffer (2% polyvinylpyrrolidone, 0.2% ovalbumin and 0.05% Tween 20 in PBS; Harlow & Lane, 1988). Alkaline phosphatase-conjugated goat anti-mouse IgG antibodies (1/2000 in blocking buffer) were used as secondary antibodies. For antigen-coated plate (ACP)-ELISA, plates were directly coated with purified Fny-CMV (10 µg/ml) in coating buffer. Additional steps were as described for the TAS-ELISA.

Western blot. A variable amount of Fny-CMV (0 to 100 ng) purified from infected plants was denatured by boiling in loading buffer and separated on a 12% SDS-PAGE gel as described by Laemmli (1970). Proteins were electrophoretically transferred to a PVDF membrane (Millipore). The membranes were blocked in TBS containing 5% non-fat milk at room temperature for 2 h, then incubated with MAb23C10E4 (1/4000 in TBS containing 0.05% of Tween 20) at room temperature for 2 h. Following three washings with TBST, the membranes were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG antibodies (Sigma; 1/2000 in TBST) at room temperature for 2 h. The membranes were washed as described above and bound antibodies were visualized using the NBT/BCIP substrate.

Results
Identification of epitopes in the Fny-CMV CP

To characterize clones from the phage-displayed peptide library which bound to the anti-Fny-CMV polyclonal serum, 36 biopanned phage clones were randomly picked and sequenced. The peptide amino acid sequences of these clones are shown in Table 1, wherein the sequences have been grouped according to shared motifs and aligned with sequences of the CMV CP. Six sequence motifs were identified corresponding to six sites in the CMV CP (Table 1). From the second round of biopanning, 12 of 17 clones (70%) were found to contain insert sequences matching with at least three amino acids in the primary sequence of the CMV CP. Seven clones had three amino acid matches with the CP, four had four amino acid matches and one had six amino acid matches. Three contiguous identical amino acids are known to be sufficient to confer antibody recognition (Felici et al., 1991; Trifilieff et al., 1991), although the possibility of mimotopes (Geyson et al., 1986; Stephen et al., 1995) confounds interpretations of significance (discussed below). Nine of 19 clones (47%) from the third round of biopanning carried insert sequences resembling a sequence of the CMV CP. Five of the clones had five amino acid matches with CMV CP motifs 4 and 5. One clone contained the heptapeptide EHQLNRA, which could be aligned at either of two positions in the CMV CP (motifs 2 or 6) to give three amino acid matches. The clone containing the heptapeptide QLVTDEL was found three times. Combining the results obtained from the second and third round panning shown in Table 1, 17% of the sequenced phage clones contained at least three amino acids corresponding to residues 206–215 in the Fny-CMV CP (motif 6). This region has the sequence EHQRIPTSGV and all but the P residue were observed in at least one clone. Twenty-two percent of the phage had sequences resembling the peptide LETDEL (motif 5; amino acids 194–199 in Fny-CMV CP). Eleven percent of the phage contained inserts matching at least three amino acids in the sequence RLLLPSDV (motif 4; amino acids 89–96). The remaining 11% of the sequenced phage clones with CMV sequences had three or four amino acid matches with the N-terminal region of the Fny-CMV CP (motifs 1, 2 and 3).

Phage with a single epitope were preferentially selected

Using the anti-Fny-CMV polyclonal serum as ligate to screen the random peptide phage library, 53% of the phage randomly picked from the third round of biopanning were without matches to the CMV CP. Owing to its polyspecificity, the CMV antiserum could enrich phage which are not virus specific. With the aim of increasing selection efficiency, we tried an additional selection by immunoscreening the enriched phage pool after the third round of biopanning. Phage clones were plated as plaque-forming units, transferred onto nitrocellulose filters and probed with the same polyclonal Fny-CMV antiserum used in the enrichment steps. One negative and seven positive plaques were randomly picked. All seven positive clones had the consensus sequence LXTDEL (where X represents any amino acid), with one of the sequences
Table 1. CMV CP amino acid sequence motifs and the sequences of peptides in phage selected with an anti-CMV polyclonal antiserum

CMV CP amino acids in bold type are those which are represented in at least one phage peptide sequence. The underlined amino acids are those which mimic a CMV coat protein epitope (motif). Numbers in parentheses following a phage peptide sequence indicate the number of phage clones isolated (when greater than one) which contained the identical peptide sequence.

<table>
<thead>
<tr>
<th>CMV CP amino acid sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motif 1</td>
</tr>
<tr>
<td>SAPS</td>
</tr>
<tr>
<td>Others</td>
</tr>
</tbody>
</table>

Second-round biopanning

| HWSPPSL | EQTINQW | LRPSIF | YLTDEF | AIWQHGV | KSFNSPH |
| HFTTRL | LETDELH | LVTDEL | WHQVST | EHORPAL | SGTVHAR |
| QVARTSF | LETDELH | TPHNPAL | EHPWPLL | |
| TQVAR | TPHNPAL | |
| TSFGSFNSPH | |
| HFTT | RLLL | L | |
| LEHQRPA | TPHNPAL | |
| THQRPA | TPHNPAL | |
| TSFGSFNSPH | |
| LEHQRPA | TPHNPAL | |
| TSFGSFNSPH | |
| LEHQRPA | TPHNPAL | |
| TSFGSFNSPH | |

Third-round biopanning

| GHQVSRL | EQHQLNRA† | HQLHPV | SLPTLT | EHQNLRA† |
| EQHQLNRA† | LVTDEL | QLVTDEL | |
| EHQNLRA† | |

* The numbers in brackets indicate the amino acid positions in the CMV CP according to the scheme of Owen et al. (1990).
† The peptide amino acid sequence in this phage clone matched three amino acids at two positions in the CMV CP (motifs 2 and 6).

Table 2. Phage pIII fusion peptide sequences from the third-round biopanning and selected in a plaque-binding assay using the polyclonal anti-CMV serum

<table>
<thead>
<tr>
<th>Phage</th>
<th>Amino acid sequence</th>
<th>Immunoreactivity in plaque assay</th>
<th>Average ELISA value (A405)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 4, 7, 9</td>
<td>QLVTDEL</td>
<td>+</td>
<td>0.18</td>
</tr>
<tr>
<td>3</td>
<td>LVTDEL</td>
<td>+</td>
<td>0.21</td>
</tr>
<tr>
<td>8, 10</td>
<td>SLTTDEL</td>
<td>+</td>
<td>0.20</td>
</tr>
<tr>
<td>11</td>
<td>AIWSTRTR</td>
<td>–</td>
<td>0.06</td>
</tr>
</tbody>
</table>

recovered four times (Table 2). The binding of the positive clones to Fny-CMV polyclonal antibodies was confirmed by ELISA. Table 2 shows that the average ELISA value of the positive clones was at least three times as high as that of a negative control clone. These results suggest that the sequence LXTDEL, corresponding to amino acid residues 194–199, is a dominant epitope in the Fny-CMV CP.

Competition of a synthetic peptide with phage for the binding to immune serum IgG

To further support the idea that the sequence LXTDEL is indeed the recognized epitope, we selected a representative clone containing the consensus motif LXTDEL (phage 10 with sequence SLTTDEL) for use in a binding inhibition assay. A synthetic peptide with the sequence YSKDDALETDEL (corresponding to residues 188–199) was used to compete with phage 10 for binding to the immune serum IgG. As a negative control, phage 11, with the sequence AIWSTRTR, was selected. Fig. 1 shows the binding of phage to immune and preimmune IgG in the presence or absence of a fixed concentration of the synthetic peptides. It shows that phage 10 bound specifically to the immune IgG and that the binding was significantly inhibited in the presence of the peptide. The negative control, phage 11, had no significant binding to the immune IgG. This
CMV epitopes

Fig. 1. Inhibition of antibody binding to phage clones by a synthetic peptide. Antibodies were preincubated with (■) or without (□) peptide prior to their addition to polystyrene plates coated with phage. Antibody binding to each of two phage clones was measured in an indirect ELISA assay and is expressed as the average absorbance value of duplicate readings at a wavelength of 405 nm. An IgG fraction of preimmune serum was used as a negative control.

Fig. 2. Inhibition of monoclonal antibody MAb23C10E4 binding to CMV by a selected phage clone. The antibody was preincubated with increasing concentrations of phage prior to addition to polystyrene plates coated with CMV. Antibody binding to CMV was measured in an indirect ELISA assay and is expressed as the average absorbance value of duplicate readings at a wavelength of 405 nm. Phage 21 (♦) was selected using MAb23C10E4 in a filter binding assay. Phages 10 (■) and 11 (▲) were selected using the polyclonal antiserum.

competition inhibition assay confirmed it was the insert sequence of phage 10 which bound to the immune IgG and that the sequence LXTDEL is an epitope in the CMV CP.

Characterization of a monoclonal antibody recognizing disrupted CMV particles

The binding of the monoclonal antibody MAb23C10E4 to virus was characterized by ACP- and TAS-ELISA. In both assays, results were expressed as the ratio of the average ELISA value (absorbance at 405 nm) for wells with purified Fny-CMV to wells without virus (signal to noise ratios). Recognition of Fny-CMV by the monoclonal antibody was much stronger in ACP-ELISA than in TAS-ELISA. In two independent experiments, the ratios obtained with ACP-ELISA were greater than threefold higher than those with TAS-ELISA (e.g. 20.0 versus 6.0 and 7.47 versus 2.0). This suggested that the epitope recognized was internal in the Fny-CMV CP, since virions coated directly onto polystyrene plates in ACP-ELISA are assumed to be disrupted, with unfolding of the CP (McCullough et al., 1985; Smith & Wilson, 1986). The recognition of denatured Fny-CMV by MAb23C10E4 was further confirmed by Western blotting (data not shown). To map the epitope in the CMV CP recognized by MAb23C10E4, we used the phage pool obtained from the first-round biopanning with the CMV polyclonal antiserum to immunoscreen plaques using the monoclonal antibody. One positive plaque was found among a total of 2200. Sequencing of the positive clone (phage 21) revealed that the amino acid sequence of the insert in the phage was LLPDSVI, a sequence which matches six contiguous amino acid residues in the Fny-CMV CP at positions 91–96. The sequence LPDSV was also found in phage isolated from the third-round of biopanning against the Fny-CP and MAb23C10E4. If the peptide displayed by phage 21 mimicked an antigenic site in CMV, it was expected to compete with virions for the same binding site on MAb23C10E4. To test this, we used PEG-purified phage 21 to perform a binding inhibition test. Purified phages 10 and 11 were used as negative controls. Fig. 2 shows that preincubation of MAb23C10E4 with increasing amounts of phage 21 significantly inhibited its binding to the Fny-CMV-coated plate, while phage 10 and phage 11 had no such effect on the binding of the monoclonal antibody to Fny-CMV.

Additional polyclonal and monoclonal antibodies did not select phage with identifiable virus-specific epitopes

We attempted to identify epitopes in CMV and two other cucumoviruses (TAV and PSV) using five additional anti-CMV monoclonal antibodies (10F10F9, 9D11F11, 15E6F2, 62B7A3, 6G6B4) and three polyclonal antibodies raised against J-PSV, C-TAV and V-TAV. Each serum was used to screen the phage-displayed peptide library and approximately ten phage clones from the third round of biopanning were randomly picked and sequenced. Some consensus sequences could be observed (Table 3), but no identifiable linear epitopes in the respective CPs could be identified.

Discussion

Using a single polyclonal antiserum and a library of random peptides, we have identified two definitive and two putative antigenic regions in the CMV CP. Caution must be employed in interpreting the significance of identified sequences, since some peptides may represent mimotopes. Mimotopes are peptides capable of mimicking assembled, discontinuous epitopes (Geyson et al., 1986); they have also been defined as immunogen-non-homologous sequences representing distinct, alternative ligand structures (Stephen et al., 1995). The region represented by motif 5 is strongly antigenic and half of the sequenced phage heptamers matched five or six amino acids in the CMV CP. Motif 4 was identified by both a polyclonal and
Table 3. Phage pIII fusion peptide sequences from the third-round biopanning selected with polyclonal antibodies to PSV strain J and TAV strains V and C

<table>
<thead>
<tr>
<th>Polyclonal antibody used in biopanning</th>
<th>Amino acid sequence of peptides</th>
<th>Frequency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-PSV-J</td>
<td>SPSAPTH</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>SSSFST</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>ALSIIGK</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>SASIRPQ</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>SLGMHPL</td>
<td>1</td>
</tr>
<tr>
<td>Anti-TAV-V</td>
<td>SHLSTMV</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>SHLSTMML</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>LERTPGK</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>LPYTMWV</td>
<td>1</td>
</tr>
<tr>
<td>Anti-TAV-C</td>
<td>TASFHRN</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>AHRPTLL</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>AHPWLL</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>AKSTLKR</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>LTMTSPI</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>AQGNSVK</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>APWELPL</td>
<td>1</td>
</tr>
</tbody>
</table>

* The number of phage clones picked, sequenced and shown to have the same inserted peptide sequence.

An atomic structure for CMV is not available, but a likely fold for the CMV CP and the relative positions of the antigenic sites can be predicted (Fig. 3). This is based on the work of Wikoff et al. (1997), who have aligned the primary amino acid sequences of the cucumoviruses and bromoviruses. They provide compelling evidence for the overall fold of the CMV CP based on the atomic structure for the related CCMV (Speir et al., 1995). The positions of the antigenic sites seen in this study are consistent with the predicted structure of the CMV CP. Epitope motifs 1, 2, 3 and 6 are found on the N and C termini of the polypeptide chain (Fig. 3). The N and C termini of the CP in many viruses are flexible and surface-located (Westhof et al., 1984), and in a number of cases the termini are known to be antigenic determinants. In CMV and a number of other icosahedral plant viruses, the N and C termini are likely to be on the interior of virions, interacting with RNA or adjoining CP subunits, respectively (Van Regenmortel, 1986; Pereira et al., 1994; Desbiez et al., 1997). In CMV and a number of other icosaedral plant viruses, the N and C termini are likely to be on the interior of virions, interacting with RNA or adjoining CP subunits, respectively (Wikoff et al., 1997). Thus, it is quite possible that although the virus was fixed with glutaraldehyde before being used to immunize rabbits, antibodies were developed against dissociated CP subunits. Even though the region corresponding to epitope motif 5 is predicted to be on the surface of virions, this epitope is also prominent on dissociated CP subunits (unpublished results).

This study is unusual in that we have used a polyclonal antiserum as the ligate and there are limited reports on the use of polyclonal antisera in combination with phage-displayed peptide libraries to identify epitopes (Kay et al., 1993; Yao et al., 1995; Germaschewski & Murray, 1996). A related and promising application is the use of random peptide libraries to identify disease-related epitopes in the sera of patients (Folgori et al., 1994; Tafi et al., 1997; Yao et al., 1998). In most studies where epitopes have been mapped using random peptide libraries, monoclonal antibodies have been used as ligates. One theoretical advantage in the approach of using polyclonal antibodies is that instead of a single epitope, a set of epitopes might be identified (Germaschewski & Murray, 1996; Yao et al., 1996).

For the identification of the epitope recognized by MAb23C10E4, we used a modified two-step selection strategy described by Folgori et al. (1994). This is characterized by (i) affinity selection of phage from the random peptide library with Fny-CMV polyclonal antibodies and (ii) screening of the enriched phage plaques on filters for recognition by the monoclonal antibody. In theory, this should be a very efficient method so long as the epitope recognized by the monoclonal antibody is also recognized by the polyclonal serum. The enriched phage pool obtained in one round of biopanning with polyclonal antibodies could be repeatedly used for epitope

a monoclonal serum, with up to six matching amino acids. Thus, motifs 4 and 5 represent definitive epitopes. Phage with sequence similarity to motif 6 had at most three contiguous amino acids, although one phage exhibited a fourth, non-contiguous amino acid. Since there were six different phage with sequences resembling a portion of motif 6, it seems likely that this putative domain is indeed an antigenic region. The other three motifs correspond to a putative antigenic domain(s) at the N terminus. These were identified on the basis of only one or two phage clones, with sequence similarities limited to three contiguous amino acids.
screening with different monoclonal antibodies. The use of two different immune sera should also decrease the chance of selecting non-specific phage clones. Using this method we selected a phage clone that carried sequences matching amino acid residues 91–96 (LLPDSV) of the Fny-CMV CP. The antigenic property of the insert sequence was confirmed by a competitive binding inhibition test (Fig. 2). Characterization of MAb23C10E4 by multiple ELISAs and Western blotting has shown that it reacts with disrupted Fny-CMV much stronger than with intact virions, suggesting that the epitope recognized by this monoclonal antibody is not exposed on the surface of the virions. In contrast to the success with MAb23C10E4, when we employed the same method for epitope mapping using eight other CMV monoclonal antibodies, no linear epitopes could be identified. Four of these monoclonals were also used to screen the original phage-displayed peptide library without an initial selection by the polyclonal serum. No sequences with similarities to the CMV CP could be identified. Similar results have been reported by other researchers (Lane & Stephen, 1997; Yao et al., 1995b). Westhof et al. (1984) have suggested that as monoclonal antibodies recognize only a small sample of all antigenic sites, they are more likely to be directed against the numerous patches of protein composed of residues distant in the primary amino acid sequence, rather than against continuous determinants.

We compared phage from the second and third round of biopanning with the polyclonal antisera and found that the percentage of phage carrying CMV CP-related sequences were 71% and 47%, respectively. This indicates that under our experimental conditions a third biopanning step did not favour enrichment of phage clones carrying antigen-specific sequences. As would be expected, repeated rounds of selection resulted in an apparently lower diversity in the phage pool. For example, the percentage of phage clones carrying consensus sequence LxTDE was 18% in the second round and 26% in the third round (Table 1); a fourth round of biopanning led to 71% of the clones with this sequence (data not presented). It appears that multiple selection steps may result in the loss of phage clones that have a lower growth rate or affinity for the ligates.

The approach of using polyclonal antibodies to probe a phage-displayed peptide library and identify epitopes is remarkably informative when successful, but does not appear to be generally applicable. The latter is emphasized by the anomalous results wherein peptides with similarities to linear sequences in the TAV and PSV CPs were not identified. Consensus sequences identified in phage clones selected with the anti-PSV or anti-TAV sera are either mimotopes or represent discontinuous epitopes. The identification and characterization of discontinuous epitopes will be facilitated by the development of an atomic structure for one of the cucumoviruses.

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