Ultrastructural localization and epitope mapping of the methyltransferase-like and helicase-like proteins of Beet yellows virus

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Monoclonal antibodies (MAbs) specific to the methyltransferase (MT) and helicase (HEL) domains of the closterovirus Beet yellows virus (BYV) were used for immunogold labelling of ultrathin sections of virus-infected Tetragonia expansa plants. MAbs 4A2 and 4A5 from the MT panel, and 1C4 from the HEL panel, specifically labelled distinct closterovirus-induced membranous structures, the ‘BYV-type vesicles’, thus suggesting that the closterovirus MT-like and HEL-like proteins co-localize in these structures. Probing of the MT and HEL MAbs with synthetic octapeptides spanning the sequences of the recombinant MT and HEL fragments that had been used as immunogens showed that 4A5 and 4A2 recognized a single epitope, SRLLENET (aa 686–692 in the BYV 1a protein), and 1C4 reacted with the DDPF epitope (aa 2493–2496). These epitopes apparently reside on the exposed parts of the membrane-associated molecules of the closterovirus MT-like and HEL-like proteins. Two other epitopes determined for the MT MAbs that were nonreactive in the immunogold labelling, namely TMVTPGEL (aa 750–757; MAbs 3C5, 4B4 and 4C5) and SREQLVEA (aa 806–813; MAb 2A4), are possibly buried in the MT domain fold or shielded by membranes or other proteins involved in the viral replicative complex.

Introduction

The Closteroviridae is a family of positive-strand RNA viruses of plants whose members are characterized by several peculiarities of their genome structure, distinct particle morphology and the specific cytological effects they cause (reviewed in Bar-Joseph et al., 1979; Lesemann, 1988; Dolja et al., 1994; Agranovsky, 1996). The RNA genomes of closteroviruses contain two main gene modules, namely the 5′-terminal replicative module consisting of ORFs 1a and 1b which code for the conserved domains of papain-like cysteine protease, methyltransferase (MT), helicase (HEL) and RNA-dependent RNA polymerase (Agranovsky et al., 1994; Karasev et al., 1995; Klaassen et al., 1995), and the 3′-terminal module encoding proteins involved in encapsidation and cell-to-cell transport of the virus RNA (Agranovsky et al., 1994, 1995, 1998; Peremyslov et al., 1999; Alzhanova et al., 2000). In recent experiments with infectious cDNAs of Lettuce infectious yellows virus (LIYV) (Klaassen et al., 1996; Yeh et al., 2000), Beet yellows virus (BYV) (Peremyslov et al., 1998), and Citrus tristeza virus (CTV) (Satyanarayana et al., 1999), it was shown that both ORFs 1a and 1b are necessary and sufficient for closterovirus RNA replication. These genes are expressed by an apparently complex strategy including ribosomal frameshifting and proteolytic processing (reviewed in Agranovsky, 1996). As demonstrated for BYV, the 1a product is a polypeptide that may undergo multiple cleavages. It was found that the leader protease releases itself in vitro by cleaving the Gly-588/Gly-589 bond in the 1a product (Agranovsky et al., 1994). In addition, immunoblot analysis of the BYV-infected plants with monoclonal antibodies (MAbs) specific to the BYV
Fig. 1. For legend see facing page.
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Methods

- MAbs and antigens. Production of MAbs to the bacterially expressed BYV MT and HEL immunogens, encompassing aa 665–813 (MTR motifs I–III) and 2450–2630 (HEL motifs V–VI) of the BYV 1a protein, respectively, was described previously (Erokhina et al., 2000).

- Preparation of plant tissue for electron microscopy. Healthy or BYV-infected (20–30 days post-infection) tissue of *Tetragonia expansa* was prepared for electron microscopy using two methods: (i) a standard protocol of Epon embedding after fixation with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.0, with or without post-fixation in 0.5% osmium tetroxide (Bendayan & Zollinger, 1983; Riedel et al., 1998), and (ii) low-temperature embedding with Monostep Lowcryl HM20 resin (Polysciences). For the latter, leaf tissue samples were fixed for 2 h at 4°C in a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 (CB). For cryoprotection, the samples were subjected to successive incubations in the 2.3 M sucrose–CB mixtures (1:3, 1:2 and 1:1, v/v; 2 h for each step), and finally in 2.3 M sucrose (overnight at 4°C). The samples were frozen in liquid nitrogen and dehydrated in a Balzers FSU 010 apparatus by freeze substitution (35 h at −45°C and 48 h at 0°C). Ultrathin sections (90–110 nm) were cut with a diamond knife and placed on Formvar-carbon coated grids (75 mesh).

- Immunogold labelling (IGL). Grids were preblocked by incubation for 15 min in phosphate-buffered saline with 1% bovine serum albumin (PBS/BSA) or in CB/BSA, washed (3 × 20 drops) with CB, and incubated overnight at room temperature with primary antibody solutions, BYV antiserum (diluted 1:150 in PBS/BSA or CB/BSA) or individual MAbs (diluted to 2.5–10 μg/ml). After washing, the grids were incubated for 2 h at room temperature with goat anti-rabbit (GAR) or goat anti-mouse (GAM) IgG conjugated to 15 nm gold beads (Biocell), diluted 1:50 in PBS/BSA or CB/BSA, and washed with distilled water. Finally, the grids were stained with 1% uranyl acetate for 30 min and examined in a LEO EM-906 electron microscope.

- Pepscan analysis. Peptide synthesis on a membrane support (Frank, 1992) was carried out on derivatized cellulose sheets using 20 Fmoc-c-amino acid active esters (SPOTs kit, Genosys). Each amino acid coupling step was controlled by spot colour change from blue to green or yellow. After the final cycle, the peptides were N-terminally acetylated with treatment of 0.4 M acetic anhydride (Sigma) in 1-methyl-2-pyrrolidinone, followed by side-chain deprotection in a mixture of dichloromethane, trifluoroacetic acid and triisobutylsilane (Sigma; 2:2:1, v/v), and successive washings with dichloromethane, dimethylformamide and methanol. Prior to the antibody binding assay, the membranes were washed with 0.1 M TBS pH 8.0 and incubated overnight at 4°C with a blocking buffer (Genosys) diluted 1:10 in TBS with 0.05% Tween 20. This and further steps were followed by triple washing of the membranes with TBS/Tween. The membranes were incubated with MAB solution (5 μg/ml) or ascites fluid (1:1000) in blocking buffer for 2 h at room temperature, followed by treatment with anti-mouse goat antibodies conjugated with horseradish peroxidase (Imtek, Russia) for 1 h. The spots were visualized with tetramethylbenzidine substrate solution (Southern Biotechnology Associates, USA).

- Computer analysis of protein sequences. The non-redundant protein sequence database (National Center for Biotechnology Information, NIH, Bethesda) was searched using the BLAST program.
Table 1. Statistics for IGL of different ultrastructures in BYV-infected mesophyll cells probed with antiserum or MAb to BYV CP, individual MAbs to HEL and MT domains of BYV, or heterologous MAbs

(a) Tissue embedded in Epon

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Vesicles</th>
<th>Virion aggregates</th>
<th>Nuclei</th>
<th>Chloroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>As BYV</td>
<td>418 ± 65±1*</td>
<td>8290 ± 44±9</td>
<td>8±8 ± 1</td>
<td>10±7 ± 5±8</td>
</tr>
<tr>
<td>1C4 (HEL)</td>
<td>16±6 ± 3±0</td>
<td>0±6 ± 0±3</td>
<td>2±1 ± 1±5</td>
<td>0±7 ± 0±6</td>
</tr>
<tr>
<td>4A2 (MT)</td>
<td>7±2 ± 2±1</td>
<td>No data</td>
<td>2±7 ± 2±2</td>
<td>1±9 ± 1±3</td>
</tr>
<tr>
<td>4A5 (MT)</td>
<td>5±4 ± 2±3</td>
<td>0±4 ± 0±1</td>
<td>1±1 ± 1±0</td>
<td>0±8 ± 0±8</td>
</tr>
<tr>
<td>3C5 (MT)</td>
<td>7±9 ± 2±5</td>
<td>0±1 ± 0±1</td>
<td>0±3 ± 0±3</td>
<td>0±0 ± 0±5</td>
</tr>
<tr>
<td>3B6, 3H4, 2D5, 2D10, 2C10, 4C5, 4B4, 2A4 (MT)</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>GAM–gold</td>
<td></td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

(b) Tissue embedded in Lowicryl

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Vesicles</th>
<th>Virion aggregates</th>
<th>Nuclei</th>
<th>Chloroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>As BYV</td>
<td>628 ± 11±9±0</td>
<td>874 ± 24±3</td>
<td>6±3 ± 6±0</td>
<td>4±1±3 ± 8±8</td>
</tr>
<tr>
<td>1470 (BYV CP)</td>
<td>573 ± 16±0±9</td>
<td>&gt; 2500</td>
<td>16±4 ± 1±3±2</td>
<td>6±7 ± 2±4</td>
</tr>
<tr>
<td>1C4 (HEL)</td>
<td>32±9 ± 14±5</td>
<td>1±2 ± 0±9</td>
<td>2±7 ± 1±0 (&lt; 1)</td>
<td>1±5 ± 1±3 (&lt; 1)</td>
</tr>
<tr>
<td>4A2 (MT)</td>
<td>18±0 ± 6±5</td>
<td>1±6 ± 1±5</td>
<td>4±9 ± 2±6</td>
<td>5±5 ± 2±6</td>
</tr>
<tr>
<td>4A5 (MT)</td>
<td>8±2 ± 3±4</td>
<td>2±2 ± 2±0</td>
<td>3±9 ± 3±8 (&lt; 1)</td>
<td>1±1 ± 1±0</td>
</tr>
<tr>
<td>3C5 (MT)</td>
<td>3±2±2 ± 0±2</td>
<td>&lt; 1</td>
<td>0±3 ± 0±2</td>
<td>1±2 ± 1±2</td>
</tr>
<tr>
<td>3B6, 3H4, 2D5, 2D10, 2C10, 4C5, 4B4, 2A4 (MT)</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>2±4 (GLRaV-1 CP)†</td>
<td>2±7 ± 1±9</td>
<td>2±0 ± 1±6</td>
<td>1±7 ± 1±9</td>
<td>1±6 ± 1±3</td>
</tr>
<tr>
<td>52C1 (WSMV CP)‡</td>
<td>1±5 ± 0±8</td>
<td>1±2 ± 1±2</td>
<td>3±0 ± 2±0</td>
<td>0±4 ± 0±7</td>
</tr>
<tr>
<td>4E6 (RGMV)§</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1 (&lt; 1)</td>
<td>&lt; 1 (&lt; 1)</td>
</tr>
<tr>
<td>GAM–gold</td>
<td></td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

* No. of 15 nm gold particles per square unit (± 95 µm²) ± standard deviation, calculated from 20 examined fields. Data in parentheses are counts for the similar ultrastructures of healthy cells. Data in parentheses are counts for the similar ultrastructures of healthy cells.
†, ‡, § Specimens treated respectively with MAb MCA2-4 to GLRaV-1, MAb 52C1 to WSMV or MAb 4E6 to RGMV, as primary antibodies.
|| Specimens treated with goat anti-mouse gold conjugate alone.

(Altschul et al., 1997). Multiple alignments of protein sequences were constructed using the Clustal X program (Thompson et al., 1997) and adjusted manually on the basis of the results of BLASTP searches and previous identification of conserved motifs. Protein secondary structure and solvent accessibility were predicted using the PHD program, with a multiple protein sequence alignment submitted as the input (Rost & Sander, 1994a,b).

Results and Discussion

Clustovirus helicase-like and methyltransferase-like proteins co-localize at the membrane surface of BYV-type vesicles

Electron microscopic examination of the infected T. expansa

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Fig. 2. Distribution of BYV coat protein in Lowicryl-embedded T. expansa tissue as revealed by IGL using antiserum to BYV particles: (a) virion aggregates in the cytoplasm; (b) scattered or aggregated virions in the cytoplasmic matrix surrounding the vesicle clusters. Bars, 1 µm.

tissue revealed characteristic cytoplasmic inclusions of vesicle aggregates, found mostly in the mesophyll and phloem parenchyma cells. In agreement with the earlier observations (Esau & Hoefert, 1971; Lesemann, 1988), these structures appeared either as discrete vesicles (Fig. 1a, small arrows) or as multivesicular clusters (Fig. 1a, large arrows), with the structure of each type surrounded by an additional outer membrane. We consistently observed a higher proportion of single vesicles in
Fig. 3. IGL with MAb 1C4 of BYV-infected T. expansa cell areas containing (a) nucleolus (Nc), nucleus (N), mitochondrion (M), cytoplasm (Cy) and cell wall (W); (b) virus particle aggregate; (c) chloroplast. (d) BYV-infected cell treated with the GAM–gold conjugate alone (with primary antibody incubation step omitted): Vs, vesicle accumulation; C, chloroplast. Embedding in Lowicryl (a, b, d) and Epon (c). Bars, 1 µm.
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Fig. 4. Pepscan analysis of the MAbs specific to the MT and HEL domains of BYV. The 146 aa sequence of MT-19K (a) and the 180 aa sequence of HEL-21K (b) were subdivided into octapeptide units with a two-residue offset, and each was synthesized on a cellulose membrane spot. Horizontal lines mark the first two peptides in a set. (c) Examples of peptide spots revealed by incubation of the membranes with MAbs 3C5, 4A5, 2A4 (MT panel) and 1C4 (HEL panel), followed by treatment with secondary antibody conjugate and tetramethylbenzidine substrate.

The cells of young leaves showing mild vein clearing, whereas in older leaves with symptoms of strong vein clearing, leaf deformation and yellowing, the vesicles were mostly found in aggregates (Fig. 1a and data not shown).

The closterovirus-induced ultrastructures were observed in both the Lowicryl- and the Epon-embedded specimens of the infected cells (Fig. 1a–d). Although the structural details were better discernible after aldehyde–OsO₄ double fixation (Fig. 1a), this treatment did not allow further labelling with the BYV-specific antibodies (data not shown), presumably because of blocking of epitopes after OsO₄ treatment (cf. Bendayan & Zollinger, 1983). In tissues fixed with aldehydes and subjected to IGL, the individual vesicles could not be observed as clearly as in aldehyde–OsO₄-fixed tissues, irrespective of the embedding method. However, the BYV-induced ultrastructures, as well as the cell organelles, were easily recognizable in such specimens (Figs 1b–d, 2 and 3).

The IgG class antibody in the HEL panel, 1C4 (Erokhina et al., 2000), consistently labelled the membranous vesicles in cells of BYV-infected T. expansa embedded in both Epon and Lowicryl (Fig. 1b, c and Table 1). The gold particles were mostly found associated with the outer surface of BYV-type vesicle membranes and the cytoplasmic matrix between them, but not the vesicle lumens (Fig. 1b, c). The IgM class HEL MAbs 1D1, 1A2, 2C6 and 2B5 did not react with any structures specific for the BYV-infected cells (data not shown). Two out of the eleven MAbs in the MT panel (4A5 and 4A2) were also found to label the membranes of the BYV-induced vesicles, although with apparently lower intensity than 1C4 (Fig. 1d and Table 1). The gold labelling with 1C4, 4A5 and
**Table 2. Summary of characteristics of the MAbs in the MT and HEL panels**

<table>
<thead>
<tr>
<th>MAb (panel/isotype)</th>
<th>Reaction with in vivo proteins (IGL)</th>
<th>Reaction with peptides</th>
<th>Predicted structure</th>
<th>Epitope state*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3C5 (MT/IgG1)</td>
<td>−</td>
<td>TMVTPGEL</td>
<td>β-strand – loop</td>
<td>Linear, buried</td>
</tr>
<tr>
<td>4B4 (MT/IgG1)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>4C5 (MT/IgG1)</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>4A5 (MT/IgG1)</td>
<td>+</td>
<td>SRLLENET</td>
<td>α-helix</td>
<td>Linear, exposed</td>
</tr>
<tr>
<td>4A2 (MT/IgG1)</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2A4 (MT/IgG1)</td>
<td>−</td>
<td>SREQLVEA</td>
<td>Loop between β-strands</td>
<td>Linear, buried</td>
</tr>
<tr>
<td>3B6 (MT/IgG1)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>3H4 (MT/IgG1)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2D5 (MT/IgG1)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2C10 (MT/IgG1)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2D10 (MT/IgG1)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>1C4 (HEL/IgG1)</td>
<td>+</td>
<td>KFQEDDPFRS</td>
<td>Hinge between globular subdomains</td>
<td>Linear, exposed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QEDDPFRS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1D1 (HEL/IgM)</td>
<td>−</td>
<td>QEDDPFRS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A2 (HEL/IgM)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2B5 (HEL/IgM)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2C6 (HEL/IgM)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* Apparent state of a given epitope in the membrane-attached protein molecule.
+ Overlapping portions of the immunoreactive peptides (that are likely to represent the epitopes) are shown in bold.

4A2 was stronger on the Lowicryl-embedded than on the Epon-embedded sections (being statistically significant for both types of specimen; Table 1), presumably owing to better preservation of epitopes in the low-temperature procedure. The other MT MAbs showed no specific reactions with the vesicles or other ultrastructures (less than one gold particle per field), except 3C5, which recognized the vesicles in the Epon-embedded but not in the Lowicryl-embedded specimens (Table 1).

Recently, IGL analysis of ultrathin sections of BYV-infected *Nicotiana benthamiana* was done with polyclonal antisera to the virions and to the BYV p65 protein (Medina et al., 1999). In order to produce a positive control, we did IGL of the infected and healthy samples with polyclonal and monoclonal antibodies to BYV particles. Treatment with anti-BYV serum or MAb 1470 of the ultrathin sections of *T. expansa* resulted in high-density gold labelling of filamentous BYV particles and their aggregates, particularly those found among the vesicles (Fig. 2a, b and Table 1). The labelling profile and the counting results obtained for IGL with anti-BYV serum or MAb 1470 were comparable to those of Medina et al. (1999) (Table 1).

In view of the fact that the intensity of labelling of the vesicles with the HEL and MT MAbs was less than that with the BYV CP MAb by one to two orders of magnitude (Table 1), the significance of the IGL data was checked with several negative controls. MAbs 4A5, 4A2 and 1C4 did not react with ultrastructures of healthy cells; in infected cells, these MAbs did not label, or labelled weakly, ultrastructures other than BYV-type vesicles (Fig. 3a, b, c and Table 1). No labelling of infected cells was observed with the gold-conjugated secondary antibody alone (Fig. 3d, Table 1). A low labelling intensity (less than two to three particles per field) was recorded when the infected tissue sections were treated with MAbs to the particles of RGMV, WSMV or GLRaV-1 as primary antibodies (Table 1). Hence, we considered as clearly positive the IGL reactions that were observed both with the Epon- and with the Lowicryl-embedded specimens, and exceeded significantly the background labelling threshold defined in the reactions with the heterologous MAbs. This confidently applies to the labelling of the BYV-type vesicle membranes with MAbs 4A5, 4A2 and 1C4.

**Properties of MAbs and their binding sites in the BYV HEL and MT domains**

To probe the epitopes in the MT and HEL domains, we synthesized sets of 71 and 87 octamer peptides spanning (with a two-residue offset) the amino acids 665–813 and 2450–2630 in the BYV 1a product, i.e. the sequences that had been used as respective immunogens to raise the MAbs (Fig. 4a, b; Erokhina...
In the MT MAb panel, 3B6, 3H4, 2D5, 2D10 and 2C10 gave no reaction with any peptide, whereas 3C5, 4C5, 4B4, 4A5, 4A2 and 2A4 reacted strongly with three distinct peptide groups (aa 686–692, 750–757 and 806–813 in the BYV 1a protein; Fig. 4c). MAbs 3C5, 4B4 and 4C5 recognized the peptide TMVTPGEL (with the latter MAb showing a weaker reaction with the neighbour peptide YLTMVTPG), 4A2 and 4A5 reacted with the SRLLENET peptide (the latter also showed weaker binding to LLENETLA) and 2A4 reacted with the SREQLVEA peptide (Fig. 4c). In the HEL MAb panel, 1C4 reacted with three overlapping peptides, KFOEDDPF, QEDDPFRS and DDPFRSEN, whereas 1D1 reacted with only the latter two; the sequences DDPF and DDPFRS (aa 2493–2496 and 2493–2498) are thus the likely candidates for being the respective epitopes (Fig. 4c and Table 2). It should be noted, however, that the actual size of an epitope may be larger than revealed by peptide scanning analysis, and that the amino acids surrounding the key epitope in the native protein globule may influence its reactivity (van Regenmortel, 1992; Commandeur et al., 1994). The MAbs specific to the MT and HEL domains of BYV may thus be classed into two reaction groups: those that did not react with synthetic peptides, presumably because their epitopes are discontinuous or conformation-dependent, and those that apparently recognize linear epitopes (Table 2).

In this study, we sought to reconcile the data from peptide scanning, IGL and computer-assisted predictions of protein secondary structure to assess the state of linear epitopes in the molecules of BYV methyltransferase-like and helicase-like proteins. In spite of the lack of significant sequence similarity, the methyltransferase domain of the viruses of the Sindbis-like supergroup appears to adopt a structural fold similar to that of the classical S-adenosylmethionine-dependent methyltransferases (Ahola et al., 1997; J. Bujnicki & E. Koonin, unpublished observations). The main secondary structure elements of the BYV MT may be predicted from the multiple sequence alignment (Fig. 5). Of the MT MAbs, only 4A2 and 4A5 reacted in IGL of both the Epon- and the Lowicryl-embedded specimens, indicating that the respective epitope SRLLENET (which partially overlaps the MTR I subdomain) is exposed in the membrane-associated protein molecule. This result was somewhat paradoxical in that the SRLLENET sequence is located within a confidently predicted amphipathic α-helix, which appears to be largely, although not completely, buried in the protein globule (Fig. 5). In contrast, the SREQLVEA epitope, which was non-reactive in IGL, appears to be located in a hydrophilic loop between the MTR II and MTR Ila subdomains, and is predicted to be exposed (Fig. 5 and Table 2). It is likely that in the membrane-associated replication complex in the BYV-infected plant cells, the MT domain undergoes conformational changes that affect the properties of these peptides. Alternatively, SREQLVEA might be non-reactive in situ because of its shielding by other proteins in the replication complex. Of the three MAbs specific to TMVTPGEL, none labelled the BYV-type vesicles in the Epon- and in the Lowicryl-embedded specimens. This was not unexpected, because this epitope overlaps with a predicted hydrophobic β-strand and is probably buried in the protein globule (Fig. 5). Furthermore, this epitope is located close to the highly conserved MTR IV motif (Rozanov et al., 1992), which is involved in membrane attachment in the homologous NSP1 protein of Semliki Forest virus (Ahola et al., 1999). Assuming a conserved mechanism of methyltransferase-driven membrane targeting for the Sindbis-like virus replicates (Ahola et al., 1999), the TMVTPGEL sequence may reside close to the membrane-bound surface of the BYV protein and thus be unavailable for interaction with the MAbs. With the lack of structural data for the Sindbis-like virus superfamily methyltransferases, further analysis of MAb reactivity and binding sites may help in envisaging the fold of this essential domain and its contacts in the replicative complex.

In the helicase panel, MAB 1C4 was reactive in IGL, thus indicating that the DDPF epitope (residing between the conserved subdomains HEL V and VI; Gorbalenya et al., 1988; Koonin & Dolja, 1993) is exposed on the BYV helicase-like protein molecule (Table 2). This agrees with the fact that a hinge between the corresponding subdomains is exposed in the Superfamily 1 DNA helicase PcrA from Bacillus stearothermophilus, a distant homologue of the viral HEL proteins, whose crystal structure has been determined (Subramanya et al., 1996).

Some peptide sequences established as MAB binding sites in this work are conserved in the 1a proteins of CTV (Karasev et al., 1995) and Grapevine leafroll-associated virus 2 (GRLaV-2; Zhu et al., 1998), the closterovirus most closely related to BYV. Thus, the sequence SRLLENET is completely conserved in the GRLaV-2 1a protein, but contains three substitutions in that of CTV (SRMLENHL). It would be interesting to find out whether this stretch is a common immunoreactive epitope in the MT domains of these viruses. The epitope DDPF is only partially conserved in the proteins of CTV, GRLaV-2 and Beet yellow stunt virus (for the latter, a partial sequence of ORF 1a is available; Karasev et al., 1996). Failure of MAB 1C4 to detect on immunoblots any proteins specific to the citrus tissue infected with CTV (Erokhina et al., 2000) may be due to the change of DDPF to DTPF, or to additional mutations in the amino acid sequences surrounding this key epitope, in the CTV HEL domain.

BYV-type vesicles as possible sites for closterovirus replication in cells

In many, if not all, positive-stranded RNA virus systems, replication is associated with cell membranes (reviewed in Buck, 1996). For assembly of the replicative complexes, some viruses employ the pre-existing membrane organelles, whereas others induce their drastic modification leading to formation of cytopathic ultrastructures (De Graaf & Jaspars, 1994; Buck, 1996). Cytopathological studies revealed that many positive-
Fig. 5. Multiple alignment of the MT domains of a selected set of closteroviruses and other positive-strand RNA viruses [TRV, Tobacco rattle virus (tobravirus); BSMV, Barley stripe mosaic virus (hordeivirus); TMV, Tobacco mosaic virus (tobamovirus); CMV, Cucumber mosaic virus (cucumovirus); SFV, Semliki Forest virus (alphavirus)]. The predictions of secondary structure [H, α-helix; E, extended conformation (β-strand)] and solvent accessibility (b, buried; e, exposed) produced using the PHD program are shown above the alignment. The epitopes recognized by MAbs are shown in bold italics.
strand RNA viruses of plants and animals induce similar modifications of host cell membranes, leading to formation of vesicles 50–100 nm in diameter (Lesemann, 1991). The vesicles originate by budding from membranes of various host organelles, such as endosomes and lysosomes (alphaviruses), tonoplast (tobamoviruses and cucumoviruses), endoplasmic reticulum (bromoviruses), chloroplasts (tymoviruses), perinuclear membranes (luteoviruses) or peroxisomes and mitochondria (tobamoviruses and cucumoviruses), endoplasmic vesicles 50–100 nm in diameter (Lesemann, 1991). The vesicles modifications of host cell membranes, leading to formation of strand RNA viruses of plants and animals induce similar modifications from host membranes, along with their transformation into the vesicle inclusions undergo kinetic changes. It is possible that continuous production of the single vesicles by budding from host membranes, along with their transformation into the multivesicular clusters by invaginations of the outer membrane, serve to increase the number of replication sites during the progression of the infection.

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References


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