RNA-dependent RNA polymerase complex of
Brome mosaic virus: analysis of the molecular structure
with monoclonal antibodies

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Viral RNA-dependent RNA polymerase (RdRp) plays crucial roles in the genomic replication and subgenomic transcription of Brome mosaic virus (BMV), a positive-stranded RNA plant virus. BMV RdRp is a complex of virus-encoded 1a and 2a proteins and some cellular factors, and associates with the endoplasmic reticulum at an infection-specific structure in the cytoplasm of host cells. In this study, we investigate the gross structure of the active BMV RdRp complex using monoclonal antibodies raised against the 1a and 2a proteins. Immunoprecipitation experiments showed that the intermediate region between the N-terminal methyltransferase-like domain and the C-terminal helicase-like domain of 1a protein, and the N terminus region of 2a protein are exposed on the surface of the solubilized RdRp complex. Inhibition assays for membrane-bound RdRp suggested that the intermediate region between the methyltransferase-like and the helicase-like domains of 1a protein is located at the border of the region buried within a membrane structure or with membrane-associated material.

Introduction

RNA replicase, which directs RNA-dependent RNA synthesis, plays a crucial role in the replication of RNA viruses. By analogy with phage Qβ replicase, the RNA replicase of eukaryotic positive-stranded RNA viruses consists of both virus-encoded and host-encoded proteins (Buck, 1996). Viral RNA replicase complexes studied to date associate with various intracellular membranes in infected cells (Ahlquist et al., 1994; Buck, 1996). This makes it difficult to purify or crystallize the replicase complexes, and has slowed the biochemical study of the structural and functional properties of viral replicase. Crystal structures have been reported for the recombinant soluble RNA-dependent RNA polymerase (RdRp) subunits of Poliovirus and Hepatitis C virus (Hansen et al., 1997; Ago et al., 1999; Bressanelli et al., 1999; Lesburg et al., 1999), and these structures have provided some insights into the RdRp structures of other RNA viruses (O’Reilly & Kao, 1998). However, the gross structures of the viral replicase complexes, which include several viral factors and possibly some host factors together with intracellular membranes, remain unclear.

Brome mosaic virus (BMV), a well-studied plant RNA virus, is the type member of the genus Bromovirus in the family Bromoviridae in the alphavirus-like superfamily (Kao & Sivakumaran, 2000). The BMV genome is composed of three RNAs designated RNA1, RNA2 and RNA3. Subgenomic RNA4 is transcribed from the complementary strand of RNA3 (Miller et al., 1985). The RNAs are capped with 7-methylguanylate at the 5’ end, and have a tRNA-like structure at the 3’ end (Kao & Sivakumaran, 2000). RNA1 and RNA2 encode the 109 kDa 1a and 94 kDa 2a proteins, respectively, which are required for RNA replication (Kao & Sivakumaran, 2000). The 1a protein has capping-associated activities (Ahola & Ahlquist, 1999). RNA3 encodes the 32 kDa 3a protein and the 20 kDa coat protein, which is translated from RNA4 (Ahlquist, 1992). The 3a protein is required for cell-to-cell movement of the virus (Schmitz & Rao, 1996). The coat protein is required for encapsidation, cell-to-cell movement and systemic spread of the virus (Okinaka et al., 2001; Sacher & Ahlquist, 1989; Schmitz & Rao, 1996).

BMV RdRp is partially purified as a complex of 1a and 2a proteins with some cellular factors, including eukaryotic elongation initiation factor 3 or a related protein (Horikoshi et al., 1988; Quadt & Jaspars, 1990; Quadt et al., 1993). In barley plants infected with BMV, 1a and 2a proteins co-localize at cytoplasmic infection-specific structures at the sites of viral RNA synthesis (Dohi et al., 2001). In barley protoplasts and yeast cells, 1a and 2a proteins co-localize at the sites of viral RNA synthesis, which takes place in the perinuclear en-
doplasmic reticulum (ER) (Restrepo-Hartwig & Ahlquist, 1996, 1999), suggesting that BMV replicase complexes associate with the cytoplasmic ER membrane. Viral RdRp activity was initially fractionated in a membrane-bound state with 1a and 2a proteins during purification (Hardy et al., 1979; Maekawa & Furusawa, 1984; Horikoshi et al., 1988; Quadt & Jaspars, 1990).

BMV replicase proteins have been investigated by molecular genetic and biochemical approaches. The 1a protein contains a methyltransferase-like domain in the N-terminal region, and a helicase-like domain in the C-terminal region (Ahlquist, 1992). The methyltransferase-like domain of 1a is involved in the localization of the replicase complex to the ER in yeast cells (Restrepo-Hartwig & Ahlquist, 1999; Chen & Ahlquist, 2000; den Boon et al., 2001). The helicase-like domain of 1a has a globular structure, which resists protease digestion in vitro (O’Reilly et al., 1995). The 2a protein has a central domain conserved in RdRps (Ahlquist, 1992). The polymerase domain of 2a is predicted to have a structure reminiscent of a right hand by analogy with the crystal structure of poliovirus 3D polymerase (Hansen et al., 1992; O’Reilly et al., 1991). Inter- and intra-subunit interactions of the BMV replicase components have been investigated using proteins translated in vitro, and in a yeast two-hybrid system using 1a and 2a derivatives with various site-specific mutations and deletions. The helicase-like domain of 1a interacts directly with the N-terminal region of 2a (Kao & Ahlquist, 1992; O’Reilly et al., 1995). The N-terminal region of 1a interacts with the C-terminal region of 1a and with itself (O’Reilly et al., 1997, 1998). However, the overall structure of the BMV RdRp complex is unclear.

Monoclonal antibodies (mAbs) are powerful tools with which to gain some insight into the structural and functional properties of protein molecules. In this study, we investigated the gross structure of membrane-bound and solubilized BMV RdRp complexes using mAbs raised against 1a and 2a proteins. Immunoprecipitation experiments revealed that the intermediate region between the methyltransferase-like domain and the helicase-like domain of the 1a protein, and the N terminus region of the 2a protein, are exposed on the surface of the solubilized RdRp complex in its active state. Moreover, inhibition assays suggest that the intermediate region between the methyltransferase-like and helicase-like domains of 1a is located just at the boundary of a region buried within the membrane structure.

## Methods

### Virus. BMV strain ATCC 66 (Mise et al., 1994) was used throughout these experiments. Virus particles and viral RNA were isolated as previously described (Okuno & Furusawa, 1979).

### Antibodies. Mouse mAbs specific for 1a and 2a proteins were prepared as described previously (Dohi et al., 2001). mAbs specific to maltose-binding protein (MBP) were obtained as by-products of the production of anti-2a mAbs, in which a fusion protein composed of MBP and 2a protein (MBP–2a) was the immunogen. The immunoglobulin isotype of mAbs was determined using a Mouse Monoclonal Antibody Isotyping Kit (Amersham Biosciences), according to the manufacturer’s recommendations. Alkaline phosphatase-conjugated goat anti-mouse IgG used for ELISA and Western blotting analysis was from Bio-Rad.

### Plasmids. Plasmid pBET1, a vector expressing full-length 1a protein, and pMCB21, a vector expressing MBP–2a, have been described previously (Dohi et al., 2001). Plasmid pMB1amb expressing a fusion protein of MBP and 1a protein (MBP–1a) was constructed as follows: pMALc2 (New England Biolabs) was cut with PstI, blunted with T4 DNA polymerase, and religated to create pMALc2d(Pst). A HindIII linker (5’ AGCTTCTACCTAATAGCGCTAGCTAGTGAAGTAGA 3’), containing a PstI site (shown underlined) and three amber codons in different frames (shown in italics), was inserted into the HindIII site of pMALc2d(Pst) to create pMALc2amb. Plasmid pBB1(Bam), which is a PstI (Mori et al., 1991) derivative with a BamHI site (underlined) upstream from the 1a start codon (shown in italics) (...CGATCCAACT- AAAATG...), was kindly provided by Masashi Mori. The 3 kb BamHI–EcoRI fragment of pBB1(Bam) containing the 1a gene was inserted into the BamHI–SalI site of pMALc2amb to create pMB1amb. Hereafter, all the DNA fragments with incompatible ends (e.g. the EcoRI and SalI-generated ends) were ligated after blunt with T4 DNA polymerase.

A series of plasmids encoding the MBP–1a fusion protein with various lengths of C-terminal truncation was constructed as follows: pMB1amb was cut with KpnI and PstI, blunted, and re-ligated to create pMB1D1. Similarly, pMB1D2, pMB1D3, pMB1D4, pMB1D5, pMB1D6, pMB1D7, pMB1D33 and pMB1D34 were constructed using SmaI, Ndel, Bsu36I, AextI, NruI, AflI, SplI, and EcoT22I, respectively, instead of KpnI as used for pMB1D1. Plasmids pMB1D31 and pMB1D32 were created by inserting the EcoRI–Psp400I fragment of pMB1amb and the EcoRI–DraI fragment of pMB1amb into the EcoRI–PstI site of pMB1amb, respectively. Plasmids pMB1D8 and pMB1D9 were created as follows: pMB1amb was treated with AflI and PstI, digested with exonuclease III, blunted, and re-ligated. Plasmids pMB1D21, pMB1D22, pMB1D23, pMB1D24 and pMB1D25 were constructed as follows: a DNA fragment was amplified by PCR from pMB1amb using a sense primer (5’ GAAGTGCAGACAGCCAA 3’) together with antisense primers (5’ AACTGCAGACACCTCTCCAGTCCGGA 3’), (5’ AACTGCAGAGTACAAGTTCGCAGTCTGC 3’), (5’ AACTGCAGAGTACCTTACTCCTGCTG 3’), or (5’ AACTGCA-GCTCCATCAACCGGGAA 3’), respectively. The amplified fragment was treated with Bsu36I and PstI, and inserted into the BscI–PstI site of pMB1amb, to create pMB1D21, pMB1D22, pMB1D23, pMB1D24 and pMB1D25, respectively.

A series of plasmids encoding MBP–2a fusion proteins with various lengths of C-terminal truncation was constructed as follows: pMCB21 was digested with HindIII, blunted, and ligated with a PstI linker to create pMCB21(Pst). To introduce three amber codons downstream from the 2a ORF in different frames, the PstI–SalI fragment of pMALc2amb was ligated into the PstI–SalI site of pMCB21(Pst) to create pMB2amb. pMB2amb was treated with SfiI and PstI, digested with exonuclease III, blunted, and re-ligated to obtain pMB2D1, pMB2D3, pMB2D4 and pMB2D7. pMB2D11 was created by self-ligation of the fragment from pMB2amb digested with SfiI and PstI. Similarly, pMB2D12, pMB2D13, pMB2D14, pMB2D15, pMB2D16 and pMB2D31 were created using SfuI, Bsu36I, CIIa, SplI, KpnI and AflI, respectively, to digest pMB2amb in place of SfiI in the creation of pMB2D11. pMB2D32 and pMB2D33 were created by ligating the 0.13 kb Afl–IIcII fragment and the 0.35 kb Afl–IcII–HindII fragment, respectively, into the Afl–IcII–PstI site of pMB2amb.
The resulting plasmids were verified by nucleotide sequencing analysis. Regions of the 1a and 2a proteins and the extra C-terminal residues encoded by each plasmid are described in Table 1.

### Protein expression and epitope mapping

The 1a and 2a derivatives were expressed in *Escherichia coli* strain DH5α, and isolated as insoluble inclusion bodies as described previously (Dohi et al., 2001). Interactions between the expressed proteins and the mAbs were investigated by ELISA and Western blotting analysis. For the ELISA, inclusion bodies obtained from 3 ml of a bacterial culture were solubilized with 600 µl of 8 M guanidine hydrochloride, and diluted to 15 ml with 50 mM sodium bicarbonate, pH 9.6. Each well of 96-well microplates (MS-8490F, Sumitomo Co.) was coated with 50 µl of the solution overnight at 4 °C. Each well was washed with TTBS (20 mM Tris–HCl, pH 7.5, 0.5 M NaCl, 0.05% Tween 20) and blocked with 3% (v/v) skim milk in TTBS for 1 h at room temperature. After washing with TTBS, mAbs were incubated at a concentration of 1 µg/ml for 2 h at 37 °C, and positive reactivity was identified after successive incubations with phosphate-agarose conjugated secondary antibody and substrates. For Western blotting analysis, inclusion bodies isolated from 150 µl of bacterial culture were denatured in 10 µl Laemmli sample buffer and separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE; Laemmli, 1970). Western blotting analysis was carried out as described (Nagano et al., 1999) using anti-1a and anti-2a mAbs at a concentration of 2 µg/ml each as primary antibodies.

The SPOTs system (Sigma Genosys) was used to determine the fine epitopes for mAbs. In accordance with the manufacturer’s instructions, a series of peptides with one amino acid overlapping to the neighbouring peptide was synthesized on activated cellulose membrane, and the interactions between each peptide and the mAbs were investigated.

### Preparation and assay of BMV RdRp

BMV RdRp fractions were prepared from BMV-infected barley (*Hordeum vulgare* L. cv. Gosheshikoku) seedlings by the method of Horikoshi et al. (1987), with some modifications. The following procedure was carried out at 4 °C. At 7 days after inoculation, about 100 g of inoculated primary leaves and systemically infected secondary leaves was homogenized in a mortar with 300 ml of 50 mM Tris–HCl, pH 7.4, 10 mM KCl, 10 mM magnesium acetate, 1 mM EDTA, 10 mM dithiothreitol (DTT), 0.5% Nonidet P-40 (NP40) to a final concentration of 2% (v/v) NP40, and subjected to centrifugation at 50000 g for 30 min. The supernatant was centrifuged at 30000 g for 30 min and the resulting pellets were suspended in 200 ml of buffer A (50 mM Tris–HCl, pH 8.2, 50 mM KCl, 15 mM MgCl₂, 10 mM DTT, 1 mM EDTA, 20% (v/v) glycerol) to obtain the membrane-bound RdRp fraction. After addition of Nonidet P-40 (NP40) to a final concentration of 2% (v/v), the mixture was stirred for 1 h, and then centrifuged at 30000 g for 30 min to obtain the solubilized RdRp fraction. The supernatant was applied to a DEAE-Biogel (Bio-Rad) column (1.5 × 5 cm). Unbound materials were washed with 150 ml buffer A containing 2% NP40, and then the RdRp activity was eluted with buffer A containing 250 mM KCl and 2% (v/v) NP40 at a flow rate of 0.2 ml/min to obtain the DEAE-purified RdRp fraction.

RdRp activity was assayed by mixing 25 µl of each fraction with 38 µl of an assay mixture containing 50 mM Tris–HCl, pH 8.2, 15 mM MgCl₂, 10 mM KCl, 10 mM DTT, 1.3 mM each of ATP, CTP, and GTP, 1 mM EDTA, 2.5 µM UTP, 2 µCi [3H]UTP (30–60 Ci/nmol, Amersham Biosciences), 1 µg actinomycin D and 20 U RNasin (Promega). Template BMV RNA (25 µg) was also added to assay DEAE-purified RdRp activity, which is template-dependent. After incubation for 90 min at 30 °C, 50 µl was taken for each reaction, and trichloroacetic acid-insoluble radioactivity was measured by the paper-disc method (Horikoshi et al., 1987). In an inhibition assay, 5 µl of mAb solution was added to 25 µl of the RdRp fraction, and incubated at 25 °C for 30 min with occasional shaking, before the RdRp activity was measured. Synthetic peptides used for a competition assay were obtained from Sawady Technology Inc.

### Immunoprecipitation from the DEAE-purified RdRp fraction

DEAE-purified RdRp fraction (200 µl) was incubated with 5 µg of mAb and 50 µl of 20% (v/v) protein A–Sepharose CL-4B (Amersham Biosciences) in buffer A containing 2% (v/v) NP40 overnight at 4 °C with gentle rotation. The resins were washed five times with buffer A containing 2% (v/v) NP40. The resins were suspended in 25 µl of buffer A containing 2% (v/v) NP40, and mixed with 38 µl of the assay mixture to measure RdRp activity as described above.

Alternatively, materials bound to the gel were eluted from the beads by boiling in 60 µl of Laemmli sample buffer (Laemmli, 1970), and 15 µl of each sample was subjected to Western blotting analysis as described (Nagano et al., 1999) using anti-1a and anti-2a mAbs at a concentration of 2 µg/ml each as primary antibodies.

### Results

#### Preparation of mAbs interacting with BMV 1a or 2a protein

Mouse mAbs against 1a and 2a proteins were produced using recombinant full-length 1a and MBP–2a fusion proteins expressed in *E. coli* as described, respectively (Dohi et al., 2001). Eleven mAbs specifically interacting with 1a protein were obtained from two immunized mice, and designated B109, B110, B118, B120, B123, B131, B136, B137, B152, B155 and B157. Thirteen mAbs specifically interacting with 2a protein were obtained from three immunized mice, and designated B201, B202, B212, B213, B215, B217, B218, B219, B221, B222, B223, B224 and B226. All of the anti-1a and the anti-2a mAbs specifically and strongly interacted with recombinant MBP–1a and MBP–2a fusion proteins, respectively, in ELISA (Table 1) and in Western blotting analysis (data not shown), as well as with the 1a and 2a proteins that were contained in the DEAE-purified BMV RdRp fraction during Western blotting analysis (data not shown). The strong reactivity observed with Western blotting suggests that all of the anti-1a and anti-2a mAbs are able to interact with the denatured, unfolded antigens by recognizing continuous linear epitopes. mAb M211 specifically interacted with a recombinant MBP in ELISA (Table 1) and in Western blotting analysis (data not shown), and was used as a control in subsequent experiments. The isotype for all the mAbs was IgG1, except for two mAbs, B152 and B157, which were isotype IgG2b (data not shown).

#### Epitope mapping of 1a and 2a proteins

Epitopes for the anti-1a and the anti-2a mAbs were mapped by ELISA using a series of recombinant MBP–1a and MBP–2a fusion proteins with various truncations at their C termini.
Table 1. Epitope mapping of anti-1a and anti-2a mAbs

Interactions of anti-1a and anti-2a mAbs with a series of C-terminal-truncated MBP–1a and MBP-2a proteins were investigated by ELISA. Bacterial inclusion bodies containing recombinant proteins were denatured with guanidine hydrochloride, and applied to microplates as antigens. (−), Same signal level as the negative control; (+), significantly higher signal than the negative control; NT, not tested.

(a) Interaction between anti-1a mAbs and 1a derivatives

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<th>B123</th>
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(b) Interaction between anti-2a mAbs and 2a derivatives

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* pBET1 encodes authentic full-length 1a protein. pMB1amb and pMB1Dn encode full-length and C-terminal-truncated MBP–1a fusion proteins, respectively. pMB2amb and pMB2Dn encode full-length and C-terminal-truncated MBP–2a fusion proteins, respectively. pMALc2 encodes MBP only.
† Non-viral extra residues at the C terminus.
‡ Anti-MBP mAb.
Fig. 1. (A) Schematic map of epitopes for anti-1a and anti-2a mAbs based on the results of ELISA, as shown in Table 1. Anti-1a and anti-2a mAbs are indicated below 1a and 2a proteins, respectively, with arrows pointing to the regions containing their epitopes. The numbers above proteins indicate the positions of the C-terminal or N-terminal (*) amino acid residues of truncated proteins used for epitope mapping. The methyltransferase-like and the helicase-like domains in 1a protein are shown as dotted and cross-hatched areas, respectively. The RNA polymerase domain in 2a protein is shown as a left-hatched area. Regions involved in 1a–1a and 1a–2a interactions and in membrane association (den Boon et al., 2001; O'Reilly et al., 1995, 1998) are indicated above the proteins. (B) Epitopes for anti-1a mAbs B110 and B152, and anti-2a mAbs B212, B215, B217 and B223. Bold type indicates the epitope for each mAb determined using a series of synthetic peptides.

E. coli inclusion bodies containing the recombinant proteins were solubilized with guanidine hydrochloride, and applied to 96-well plates for the ELISA. Anti-MBP mAb M211 interacted with all the MBP–1a and MBP–2a derivatives, but did not interact with the unfused full-length 1a protein, indicating that the experiments were well controlled (Table 1).

Anti-1a mAb B109 interacted with the unfused full-length 1a protein, the MBP–1a fusion protein, and the C-terminal-truncated MBP–1a containing residues 1–919 of 1a, but did not interact with MBP–1a containing residues 1–851 of 1a, or shorter MBP–1a forms (Table 1). This suggests that residues 852–919 of 1a protein contain the epitope for mAb B109. Similarly, interactions between, respectively, the anti-1a and anti-2a mAbs with the MBP–1a and the MBP–2a derivatives were investigated, and the epitopes were mapped (Table 1; Fig. 1A).

Co-immunoprecipitation of 1a and 2a proteins by anti-1a or anti-2a mAbs from the DEAE-purified RdRp fraction

Interactions of 1a and 2a proteins in the DEAE-purified RdRp fraction were investigated by immunoprecipitation experiments with anti-1a and anti-2a mAbs. Anti-1a and anti-
Fig. 2. Specific co-immunoprecipitation of 1a and 2a proteins with RdRp activity from the DEAE-purified RdRp fraction. The fraction was incubated with anti-1a or anti-2a mAbs, and precipitated with protein A-conjugated resin. mAbs used for the immunoprecipitations are grouped with brackets and arranged by the positions of their epitopes. (A, B) Western blots of immunoprecipitated materials. The precipitated proteins were separated on a 10% (A) or 7.5% (B) gel by SDS-PAGE, and Western blotting analysis was used to detect 1a (A) and 2a (B) proteins. An arrow indicates the position of IgG heavy chains, detected by anti-mouse IgG secondary antibody. The positions of 1a and 2a proteins are shown on the right. (C, D) Immunoprecipitation of recombinant MBP–1a (C) and MBP-2a (D) fusion proteins denatured by SDS and a reducing agent. Precipitated proteins were separated on a 10% gel, and analysed by Western blotting using anti-1a (C) and anti-2a (D) mAbs as primary antibodies. The positions of MBP–1a and MBP-2a proteins are shown on the right. (E) RdRp activity of the immunoprecipitated materials. Precipitates were suspended in a reaction mixture containing NTPs, [3H]UTP, actinomycin D and template BMV RNA, and incubated at 30 °C for 90 min. Radioactivity in the acid-insoluble fraction was measured by the paper-disc method. Ratios of the activity in immunoprecipitated fractions to the activity of the original fraction are shown. The values indicate the means ± SE of three independent experiments.

2a mAbs were added to the DEAE-purified RdRp fraction, and incubated with protein A-conjugated resin. The immunocomplexes were recovered by centrifugation, and analysed by Western blotting. Strong signals corresponding to IgG chains were detected in all precipitations by phosphatase-conjugated anti-mouse IgG antibody used as a secondary antibody (Fig. 2A), indicating that all the mAbs used here effectively bound to the protein A-conjugated resin. A significant amount of 1a protein was co-precipitated with 2a protein when using anti-1a mAbs B110 and B152, the epitopes of which are in the intermediate region between the methyltransferase-like domain and the helicase-like domain of 1a (Fig. 2A, B). Similarly, significant amounts of 2a protein were co-precipitated with 1a protein using anti-2a mAbs B212, B215, B217 and B223, the epitopes of which are in the N-terminal region of 2a (Fig. 2A, B). Lesser amounts of 1a and 2a proteins were detected in precipitations with mAbs B213, B218, B219, B221, B222, B224 and B226, which recognize sequences included in residues 270–310 of 2a protein (Fig. 2A, B). These results indicate that the above mAbs specifically interact with the complex containing both 1a and 2a proteins. The other anti-1a and anti-2a mAbs and an anti-MBP mAb did not detectably precipitate.
either 1a or 2a protein from the RdRp fraction (Fig. 2A, B). Recombinant MBP–1a and MBP–2a fusion proteins that were denatured with SDS and a reducing agent (Laemmli, 1970) were specifically precipitated by all the anti-1a and anti-2a mAbs, respectively (Fig. 2C, D). No bands corresponding to 1a or 2a protein were detected in the immunoprecipitates of similar fractions obtained from mock-inoculated plants (data not shown).

**RdRp activity in the immunoprecipitated fractions**

To investigate whether the immunoprecipitated 1a and 2a proteins constituted a functional RdRp complex, RdRp activity in the immunoprecipitated fractions was measured. The relative RdRp activities are shown in Fig. 2(E). RdRp activity (8–16% of the original activity) was detected in the fractions precipitated with either anti-1a mAbs B110 and B152, or anti-2a mAbs B212, B215, B217 and B223. Low RdRp activities (0–6–14% of the original activity) were detected in the fractions precipitated with anti-2a mAbs B213, B218, B219, B221, B222, B224 and B226. No RdRp activity was detected in the fractions precipitated with other anti-1a or anti-2a mAbs. These results correlate well with the amount of 1a and 2a proteins in the corresponding precipitations described above (Fig. 2A, B), and suggest that the precipitated 1a and 2a proteins are components of an active RdRp complex. These results further suggest that the epitopes for the mAbs that precipitate RdRp activity are located on the surface of the functional RdRp complex.

**Fine mapping of the epitopes located on the surface of the active RdRp complex**

Epitopes for the mAbs (B110, B152, B212, B215, B217 and B223) that interacted strongly with the DEAE-purified RdRp complex were mapped finely using synthetic peptides. A series of peptides consisting of 10 amino acid residues included in a region of 1a between amino acids 514–586 was synthesized with one amino acid overlapping the neighbouring peptides, using the SPOTs system (Sigma Genosys), and their abilities to interact with mAbs B110 and B152 were examined by enzyme-linked assay. mAb B110 specifically interacted with decapeptides containing TDVVP, corresponding to amino acids 544–548 of 1a, and mAb B152 specifically interacted with decapeptides containing SVEVP, corresponding to amino acids 553–557 of 1a (data not shown). These results indicate that B110 and B152 recognize amino acid sequences TDVVP and SVEVP, respectively (Fig. 1B).

Similarly, the epitopes for anti-2a mAbs B212, B215, B217 and B223 were mapped using a series of decapeptides that correspond to amino acids 1–33 in the N-terminal region of 2a. mAbs B212 and B215 interacted with decapeptides containing QWIIDQ, corresponding to amino acids 18–23 of 2a protein, and mAbs B217 and B223 interacted with decapeptides containing DDFVR, corresponding to amino acids 8–12 of 2a (data not shown). These results indicate that B212 and B215 recognize the same amino acid sequence, QWIIDQ, and B217 and B223 recognize the same amino acid sequence, DDFVR (Fig. 1B).

**Effects of anti-1a and anti-2a mAbs on the DEAE-purified RdRp activity**

Anti-1a and anti-2a mAbs were tested for their ability to influence RdRp activity in the DEAE-purified RdRp fraction. Of the anti-1a mAbs, B110 and B152 reduced the RdRp activity to approximately 60% and 30% of the control, respectively (Fig. 3). Other anti-1a mAbs did not significantly affect the RdRp activity. Increasing the amount of B110 and B152 from 5 µg to 25 µg did not produce additional effects on
RdRp activity (Fig. 4A). The inhibition of RdRp activity by B110 and B152 was observed throughout the incubation, for at least 120 min (Fig. 4B). Anti-2a mAbs B212, B215, B217 and B223, which recognize the N-terminal region of 2a protein and interact with the DEAE-purified RdRp complex, did not inhibit but rather slightly stimulated RdRp activity (Fig. 3). The other mAbs did not affect RdRp activity (Fig. 3).

To investigate whether the inhibition of RdRp activity by B110 and B152 is epitope-specific, a competition assay was performed. Synthetic nonapeptides P110 (542-PVTDVVPDA-550) and P152 (551-EVSVEVPTD-559), which contain the epitope sequence for mAbs B110 and B152, respectively, were added to the assays with the mAbs. In the presence of peptide P110, B110 did not inhibit RdRp activity, whereas B152 did. Similarly, P152 specifically abolished the inhibition of RdRp activity by B152 (Fig. 5). These results indicate that the inhibition of RdRp activity by B110 and B152 was due to the specific interaction between the mAbs and their epitopes.

mAbs B110 and B152 also reduced RdRp activity in a fraction precipitated with anti-2a mAbs B212 and B221 from the DEAE-purified RdRp fraction, to approximately 50–70% of the control (Fig. 6). This indicates that the epitopes for mAbs B110 and B152 are exposed on the surface of the RdRp complex precipitated by these anti-2a mAbs.

Effects of mAbs B110 and B152 on RdRp activity in the membrane-bound RdRp complex

To investigate the location of the epitopes for mAbs B110 and B152 in the more complex state of RdRp associated with the membrane and with endogenous template RNAs, RdRp activity in the membrane-bound fraction was measured in the presence or absence of the mAbs. The addition of mAb B152 reduced RdRp activity to approximately 60% of the control, whereas mAb B110 had no effect (Fig. 7). This indicates that the epitope for B152 is still accessible on the membrane-bound RdRp complex, but the epitope for B110 is not. To determine the factor(s) that renders B110 inaccessible to its epitope, the effects of B110 and B152 on RdRp activity were tested using the solubilized fraction, which is the supernatant obtained by centrifugation of the membrane-bound RdRp fraction treated with a detergent, NP40. Both B110 and B152 significantly inhibited solubilized RdRp as well as template-dependent...
DEAE-purified RdRp (Figs 4 and 7). Similar results were obtained using the membrane-bound fraction simply treated with NP40. This indicates that some of the factors that were released from the RdRp complex by the NP40 treatment are involved in blocking the access of B110 to the epitope.

Discussion

In this study, we investigated the molecular structure of a BMV replicase complex using anti-1a and anti-2a mAbs.

Locations of the epitopes for anti-1a mAbs in the BMV RdRp complex

mAbs B110 and B152, the epitopes of which are located at amino acid residues 544-TDVVP-548 and 553-SVEVP-557 of 1a protein, respectively, specifically precipitated the DEAE-purified RdRp complex in an active state (Fig. 2), and partially inhibited the enzymatic activity of RdRp (Figs 3 and 4). This indicates that amino acid residues 544-TDVVP-548 and 553-SVEVP-557 of 1a protein are exposed on the surface of the DEAE-purified RdRp complex. Analysis of the 1a protein amino acid sequence with the PHD method using the PredictProtein server program (Rost, 1996) predicts that the epitopes for B110 and B152 are both located in a loop region of the protein structure (O’Reilly et al., 1998). The loop regions of proteins are often highly exposed on the surfaces of proteins (Kuntz, 1972; Rose et al., 1985). Previous studies have shown that the predicted loop region that includes the epitopes for B110 and B152 is located at the hinge between the methyltransferase-like domain and the helicase-like domain (Ahlquist, 1992; Fig. 1), adjacent to the C-terminal protease-resistant domain (O’Reilly et al., 1995). BMV mutants with a two-amino-acid insertion just on or close to the epitopes for B110 and B152 have the ability to replicate in barley protoplasts (Kroner et al., 1990), suggesting that these mutations do not affect the structure of BMV replicase, and that these regions are not included in the catalytic sites of the replicase. mAbs B110 and B152, however, significantly inhibited RdRp activity in the
DEAE-purified fraction (Figs 3 and 4), implying that the binding of the mAbs to the RdRp complex causes a structural change that affects its enzymatic activity. As well as causing a structural change, antibody binding might lower activity by inhibiting a structural change, steric hindrance, or other mechanisms.

In contrast to this result, only B152 but not B110 affected RdRp activity in the membrane-bound enzyme fraction (Fig. 7). This suggests that the epitope for B110 was not accessible and was possibly buried in the membrane-bound RdRp complex. Moreover, treatment of the membrane-bound fraction with NP40 rendered the B110 epitope on the RdRp complex accessible to B110 (Fig. 7), suggesting that some factor that otherwise blocks an interaction with B110 was released by the detergent treatment. The blocking factor is unlikely to be a template RNA because B110 inhibited RdRp activity on endogenous templates in the solubilized fraction. Because the epitopes for B110 and B152 are located close to the C-terminal region of the methyltransferase-like domain, which mediates the association of 1a to the ER membrane (den Boon et al., 2001), the region that includes the epitopes for B110 and B152 may be located just on the border of the region buried within the membrane structure or with a membrane-associated material.

The mAbs with epitopes located at residues 1–143, 586–619 or 852–919 of 1a did not interact with the DEAE-purified RdRp complex (Fig. 2), suggesting that the regions that include these epitopes are highly structured and buried inside the 1a protein. Alternatively, these regions may be buried in the highly ordered quaternary structure of the replicase complex and be involved in the formation of the complex. N-terminal residues 1–515 of 1a protein are required for the 1a–1a interaction in a yeast two-hybrid analysis (O’Reilly et al., 1998). The helicase-like domain of 1a protein includes regions essential for its interaction with 2a protein as well as with N-terminal residues 1–515 of 1a protein, and forms a putative globular domain, which is resistant to protease treatment (Kao & Ahlquist, 1992; O’Reilly et al., 1995, 1998). Insertion of two amino acids into the regions that include the epitopes of these mAbs abolishes the ability of the BMV mutants to replicate in barley protoplasts or in yeasts, and disrupts the predicted secondary structure of 1a protein (Kroner et al., 1990; O’Reilly et al., 1998). These data together imply that the regions that include residues 1–143, 586–619 and 852–919 of 1a protein may play an important role(s) in the formation of functional BMV replicase.

Locations of the epitopes for anti-2a mAbs in the BMV RdRp complex

Anti-2a mAbs with epitopes located at N-terminal residues 8–12 or 18–23 of 2a precipitated the RdRp complex in an active state without interrupting RdRp activity (Figs 2 and 3). This indicates that these regions are located on the surface of the DEAE-purified RdRp complex and are not involved in viral RNA synthesis. A BMV mutant with a deletion of residues 3–24 of 2a protein replicates similarly to wild-type BMV in barley protoplasts (Traynor et al., 1991), and 2a fused to GFP at the N terminus is functional in BMV RNA replication in yeast cells (Chen & Ahlquist, 2000). These results strongly suggest that the region near the N terminus of 2a protein is not involved in the formation of the functional BMV replicase.

The mAbs with epitopes located in the region including residues 270–310 of 2a precipitated a small amount of RdRp complex that displayed RdRp activity (Fig. 2). This indicates that the epitope region is not directly involved in RdRp activity. Moreover, most of the RdRp complex in the DEAE-purified RdRp fraction did not interact with these mAbs, suggesting that the region that includes residues 270–310 of 2a protein is buried inside the RdRp complex and is involved in an interaction with some components that constitute the functional replicase complex. These epitopes are included in ‘the RdRp unique region’ implicated in polymerase–polymerase interactions (O’Reilly & Kao, 1998).

mAbs B202 and B201, the epitopes of which are located in the regions that include residues 50–95 and 341–376 of 2a, respectively, did not interact with the DEAE-purified RdRp complex (Fig. 2). This suggests that the epitopes for these mAbs are buried inside the RdRp complex. This is supported by the previous finding that the epitope for B202 occurs in the region required for the interaction of 2a with 1a to form the replicase complex (Kao & Ahlquist, 1992; O’Reilly et al., 1995). Sequence alignment of BMV 2a protein with poliovirus 3D polymerase, the crystal structure of which has been determined, shows that the region that includes the B201 epitope corresponds to the ‘fingers’ subdomain of the poliovirus polymerase, which is thought to be important for polymerase activity (O’Reilly & Kao, 1998).

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References


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