The C-terminal region of the movement protein of *Cowpea mosaic virus* is involved in binding to the large but not to the small coat protein

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*Cowpea mosaic virus* (CPMV) moves from cell to cell as virus particles which are translocated through a plasmodesma-penetrating transport tubule made up of viral movement protein (MP) copies. To gain further insight into the roles of the viral MP and capsid proteins (CP) in virus movement, full-length and truncated forms of the MP were expressed in insect cells using the baculovirus expression system. Using ELISA and blot overlay assays, affinity purified MP was shown to bind specifically to intact CPMV virions and to the large CP, but not to the small CP. This binding was not observed with a C-terminal deletion mutant of the MP, although this mutant retained the capacity to bind to other MP molecules and to form tubules. These results suggest that the C-terminal 48 amino acids constitute the virion-binding domain of the MP.

**INTRODUCTION**

*Cowpea mosaic virus* (CPMV) is a plant virus that belongs to the genus *Comovirus* of the family *Comoviridae* (for reviews see Goldbach & Wellink, 1996; Pouwels et al., 2002b). Cell-to-cell movement of CPMV is characterized by transport of mature virions through tubules that are assembled inside the plasmodesmal pore and which contain the RNA-2-coded 48 kDa movement protein (MP) (Wellink & Van Kammen, 1989; Kasteel et al., 1993; Van Lent et al., 1990). Such a tubule-guided cell-to-cell transport system has been described for viruses of different genera including *Caulimovirus*, *Nepovirus*, *Bromovirus* and *Tospovirus* (Perbal et al., 1993; Wieczorek & Sanfacon, 1993; Kasteel et al., 1997; Storms et al., 1995). Similar tubular structures are formed at the surface of infected protoplasts and both in protoplasts and in plant tissue virions appear in a single and continuous row within the tubules (Van Lent et al., 1991). The morphology of the virion-containing tubule suggests that tubule assembly from MP molecules and entrapment of the virions take place simultaneously at or near the plasma membrane. Tubule assembly does not depend on the presence of virions or capsid proteins (CPs), as expression of MP alone in protoplasts also leads to the formation of (empty) tubules (Wellink et al., 1993).

A specific affinity of the viral MP for the CPs or virions is probably essential not only for entrapment of virions during tubule assembly, but also for targeted transport of virions from the cytoplasmic site of assembly to the plasmodesma. Indirect evidence for a specific interaction between the C terminus of the CPMV MP and virions was presented by Lekkerkerker et al. (1996), who showed that a mutant virus encoding an MP lacking the C-terminal 18 amino acids (residues 313–331) was able to form tubules in protoplasts which did not contain virus particles, and the mutant was not able to spread systemically in plant tissue. Furthermore, for *Grapevine fanleaf virus* (GFLV), another member of the family *Comoviridae*, Belin et al. (1999) found that the nine C-terminal amino acids of the MP must be of the same virus origin as the CP for successful systemic spread, and they also proposed a requirement for specific interactions between the movement protein (2BMP) and the coat protein (2CCP).

We describe here further investigations of the affinity of the CPMV MP for virions and for the individual viral CPs by *in vitro* binding assays, i.e. ELISA and blot overlay assays, using purified MP as a probe.

**METHODS**

**Heterologous expression of MP.** The 48 kDa MP and two truncated forms of this protein with six histidine (HIS) molecules linked to their N terminus were expressed in cell line Sf21) insect cells using the Bac-to-Bac system (Gibco BRL).

To isolate the complete MP-coding sequence plasmid pM19GFP2A (Gopinath et al., 2000), containing the full-length cDNA of CPMV RNA-2, was used as starting material. The MP fragment was synthesized by PCR using primers 5’-GGGTAACCATTGAAAAGCATTA

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The His-tagged MP and ΔC48MP were purified from AcNPV-MP- and AcNPV-ΔC48MP-infected cells using a Talon Cell Thru column (Clontech). Infected and uninfected insect cells were pelleted in 5 ml lysis buffer (50 mM sodium phosphate, 6 M guanidine.HCl, 300 mM NaCl, pH 8.0) containing 30 μg E64 protease inhibitor ml⁻¹ (Roche). Cells were ruptured by pulse sonication for 6 × 30 s with 30 s intervals and then centrifuged at 1200 r.p.m. in an SS34 Sorvall rotor for 20 min to pellet cell debris. The supernatant was collected and loaded onto a 2 ml bed volume Talon Cell Thru column, previously equilibrated with lysis buffer. The column was first washed with 30 ml lysis buffer followed by 5 ml washing buffer (45 mM sodium phosphate, 5-4 M guanidine.HCl, 270 mM NaCl, 10 mM imidazole, pH 8-0). MP or ΔC48MP was then eluted under denaturing conditions, with 3 ml of washing buffer containing 200 mM imidazole. The eluted proteins were immediately renaturated by a sequence of dialysis steps against renaturing buffer (20 mM Tris/HCl, pH 7-6, 1 mM EDTA, 10 % glycerol, 100 mM NaCl, 7 mM 2-mercaptoethanol) containing decreasing concentrations of guanidine.HCl. Starting with 6 M guanidine.HCl for 6 h, the concentration was halved every 6 h by adding an equal volume of cold renaturing buffer until 750 mM. Except for the first dialysis, which was done at room temperature, all dialysis steps were done at 4 °C. Samples were then dialysed against pure renaturing buffer to remove guanidine.HCl. Protein concentration was determined by Bradford protein assay (Bio-Rad) using goat anti-rabbit conjugated to Alexa 488 as fluorochrome (Molecular Probes). Fluorescence was recorded in a Zeiss LSM 510 laser scanning microscope through excitation with blue laser light at 488 nm and emission through a 505–530 nm bandpass filter.

**Antibodies.** Three antibodies were available to detect the His-tagged CPMV MP. A monoclonal antibody against 6 × His (anti-His) obtained from Clontech could be used to detect denatured forms but not renatured forms of the MP and ΔC48MP. A rabbit polyclonal antibody against a peptide consisting of the 30 C-terminal amino acids of the CPMV MP (denoted anti-48K; Wellink et al., 1987) was most specific to the viral MP, but was not suitable to detect the C-terminally truncated ΔC48MP. A rabbit polyclonal antibody generated against E. coli-expressed 58 kDa protein from CPMV RNA-2, which contains the entire 48 kDa MP sequence (denoted anti-58K; Kasteel, 1999), was able to detect both the MP and ΔC48MP but generally showed some additional bands in Western blots. Insect cell lysates and purified MP and ΔC48MP protein fractions were resolved on 12 % SDS-PAGE gels (Laemmli, 1970), which were stained with Coomassie blue or electroblotted (0-04 mA for 1 h at room temperature) to PVDF Immobilon P membrane (Amersham) in transfer buffer (50 mM Tris base, 192 mM glycine, 20 % methanol, 0-01 % SDS, pH 9-5). On the latter, the His-tagged MP and ΔC48MP were detected with an anti-6 × His monoclonal antibody (Clontech) or anti-MP polyclonal antibody.
ELISA-based binding assay. Purified MP and ΔC48MP were tested for binding to virions in an ELISA assay. Wells of Nunc Axisorp F96 immunoplates were coated with 150 μl of 6·6 μg purified CPMV ml⁻¹ coating buffer (0·05 M sodium carbonate, pH 9·6) overnight at 4 °C. Controls consisted of purified MP and similar amounts of purified virions of the related comoviruses Cowpea severe mosaic virus (CPSMV) and Red clover mottle virus (RCMV) and the unrelated Tobacco mosaic virus (TMV). After rinsing the plates three times with PBS, pH 7·2, containing 0·05% (v/v) Tween 20 (PBS/Tween), the wells were blocked with PBS/Tween containing 5% (w/v) dry non-fat milk for 1 h. The plates were then incubated with 150 μl of 13 μg ml⁻¹ purified MP or ΔC48MP in PBS/Tween containing 1% dry non-fat milk or PBS/Tween as a control for 90 min at room temperature. The plates were rinsed three times with PBS/Tween and incubated with anti-48K or anti-58K antibodies at 37 °C for 1 h. The plates were again rinsed and incubated with secondary antibody conjugated to alkaline phosphatase at 37 °C for 1 h. After rinsing, 150 μl substrate (1 mg p-nitrophenyl phosphate, disodium salt, ml⁻¹ 0·01 M diethanolamine buffer, pH 9·6) was added per well. Reactions were quantified by reading the absorbance at 405 nm using a Bio-Tek Instruments EL 312 ELISA-reader.

RESULTS

Expression and purification of MP

The recombinant baculoviruses AcNPV-MP, AcNPV-ΔAN289MP and AcNPV-ΔC48MP were used to express the MP and truncated forms of this protein in SF21 insect cells. The truncated MP sequences were designed, on the basis of the domain analysis of Lekkerkerker et al. (1996) and Bertens et al. (2003), to generate a mutant MP with a deletion of 48 C-terminal amino acids (ΔC48MP) lacking the putative virion-binding domain and a peptide consisting of the 52 C-terminal amino acids (AN289MP) constituting the putative virion-binding domain. All proteins were expressed with six histidines linked to their N terminus. The MP (AcNPV-MP) and the truncated MP with a deletion of the C-terminal 48 amino acids (AcNPV-ΔC48MP) were highly expressed and readily detected by immunofluorescence (Fig. 1) and on Western blots with cell lysates using the anti-58K antibodies (not shown). In cells infected with AcNPV-MP (Fig. 1A) and AcNPV-ΔC48MP (Fig. 1B) the proteins were present in small and large aggregates mostly located in the periphery of the cell near the plasma membrane. Furthermore, both the MP and the truncated ΔC48MP formed tubules extending from the insect cell surface into the culture medium, indicating that the tubule-forming capacity is not disturbed by the N-terminal tag and the C-terminal deletion. In cells infected with the AcNPV-ΔAN289MP no expression of the truncated peptide could be detected by immunofluorescence or Western blot assays using the anti-48K antibodies, which are specific for the 30 C-terminal amino acids of the CPMV MP (Wellink et al., 1987). The peptide was either not expressed or expressed in amounts that could not be detected.

The His-tagged MP and ΔC48MP were purified from insect cell lysates using Talon column filtration. Initial attempts to purify the proteins from the cell lysates in their native condition failed. Probably the proteins were aggregated into multimeric forms and/or the His-tag was not sufficiently exposed for binding to the column. Both proteins were successfully purified under denaturing conditions after addition of 6 M guanidine.HCl to the lysis buffer prior to loading on the column. Fig. 2 shows a Coomassie blue-stained gel of insect cell lysate (A), purified MP and ΔC48MP (B) and a Western blot of these purified proteins detected with anti-58K antibodies (C). After column purification, the eluted proteins were slowly renatured for further use in binding assays. From 5 × 10⁶ infected insect cells usually 170–210 μg of the MP could be obtained.

Binding between MP molecules

The availability of an antiserum specific for the C terminus of the MP and a C-terminal deletion mutant of the MP allowed us to test binding between MP molecules in vitro using a blot overlay assay. After renaturing, a membrane containing MP and ΔC48MP was incubated with MP. Binding between MP and ΔC48MP was detected using anti-48K antibodies, which only react to the MP but not to the C-terminally truncated ΔC48MP (Fig. 2D). The strong signal in the MP lane (2D, E) is the result of direct binding of the anti-48K antiserum to the blotted MP, while Fig. 2(E) demonstrates that this antibody does not react to ΔC48MP.

Binding of MP to virions

Binding of the MPs to virions of several different viruses was tested in an ELISA assay. The wells were coated with purified virus suspensions or buffer and incubated with MP or ΔC48MP, and bound MP was detected using anti-58K...
antibodies. Average results of three repetitions are illustrated in Fig. 3. The MP showed binding only to homologous CPMV virions, but not to virions of the related comoviruses RCMV and CPSMV or to virions of the unrelated TMV. The truncated ΔC48MP did not show binding even to the homologous CPMV virions.

Binding of the MP to virions could also be shown in a blot overlay assay, where MP and ΔC48MP were blotted onto the membrane and purified CPMV virions were used as the overlay probe. In this assay, virions bound to MP but not to ΔC48MP (Fig. 4D).

**Binding of MP to separate CPMV coat proteins**

As the MP specifically interacted with CPMV virions, it was interesting to investigate whether the protein binds to both L and S CP. Binding of the MP was tested in blot overlay assays resolving both CPs from increasing amounts (5, 10, 20, 40 and 80 μg) of purified CPMV. Bound MP was detected using anti-48K antibodies. Probing of the blotted membrane with MP revealed specific binding to the L protein but not to the S protein of CPMV, even at higher amounts of blotted CPs (Fig. 5B). The truncated ΔC48MP, detected with anti-58K antibodies, showed no
binding to either CP (Fig. 5C; the strong signal in the MP lane is the result of direct binding of the anti-58K antiserum to the blotted MP). As in ELISA, no binding of MP occurred to CPs of the other, related and unrelated, viruses (Fig. 4C).

DISCUSSION

The mechanism of tubule-guided virion movement, employed by CPMV to achieve cell-to-cell movement, implies a specific interaction between the transport tubule, which is built-up from the MP within the plasmodesmal pore, and mature virions. The data reported here indicate that purified and solubilized CPMV-MP molecules bind to mature virus particles in in vitro ELISA-based binding assays and, conversely, virions bind to the MP in Western blot overlay assays. It was further shown that upon removal of 48 amino acids from the C terminus of the MP, virion binding did not occur in either assay. As in both MP and ΔC48MP the tubule-forming domain appears to be functional, as indicated by the formation of tubules at the insect cell surface (Fig. 1), these results suggest that the C-terminal 48 amino acids are essential for the interaction between MP and virions and therefore probably constitute the virion-binding domain of the MP or an essential part of this domain. As it has been shown previously that C-terminal deletions in the MP sequence result in virus mutants that produce empty MP tubules and cannot move from cell to cell (Lekkerkerker et al., 1996; Bertens et al., 2003), the in vitro binding studies reported here reveal a functional binding between MP and virions, in particular the L protein.

Heterologous expression of viral MPs in insect cells and subsequent column purification appears to be a suitable method to obtain functional probes for in vitro binding.
studies. Earlier studies have shown that upon expression of CPMV MP, but also MPs of other viruses employing a similar mechanism of tubule-guided virion movement, the MP retains its tubule-forming capacity in insect cells (Kasteel et al., 1996; Storms et al., 1995). Here it was also shown that a truncated form of the MP, containing the tubule-forming domain but devoid of the virion-binding domain, retained its biological function. The MPs are located at the plasma membrane and assemble into tubules protruding from the cell surface into the culture medium, similar to MP in virus-infected plant cells (Van Lent et al., 1991; Bertens et al., 2003). Attempts to express a peptide of 52 C-terminal amino acids, constituting the putative virion-binding domain, were not successful. The peptide was either not expressed or synthesized in amounts below the detection limit.

Purification of the expressed MPs was only feasible after unfolding the proteins in the insect cell lysate by a denaturing procedure prior to column filtration. Denaturing apparently exposed the His-tagged N terminus sufficiently for efficient binding to the Talon column. Prior to their use as probes in binding assays, the MPs were renatured again. The process of denaturing and renaturing apparently did not affect the intermolecular binding of the MPs (essential for tubule assembly), as in blot overlay assays the MP probe showed binding to the blotted truncated ΔAC48MP (Fig. 2D, E).

A remarkable outcome of the binding assays is the specificity of the MP for its homologous virion. No binding occurred even to virions of the related comoviruses RCMV and CPSMV (for similarities between comoviruses see Haudenshield & Palukaitis, 1998). These results suggest that in vivo only homologous virions can be moved from cell to cell by the viral transport tubule and that the MP is not able to assist the movement of virions even of related viruses. Little, but supportive, evidence is available for this hypothesis from a study performed by Belin et al. (1999) on the nepovirus GFLV, showing that nine C-terminal residues of the 2B movement protein must be of the same virus origin as the 2C coat protein for successful systemic spread.

In Western blot overlay assays, binding of the MP occurs only to the CPMV L protein but not to the S protein (Fig. 5). The significance of this binding for the mechanism of virion movement remains to be elucidated, but the capacity of the MP to bind in vitro to L protein molecules may also indicate a possible early interaction between the MP and this coat protein, i.e. this may occur in the membranous cytopathic structure where CPMV replication, viral protein synthesis and virion assembly take place (De Zoeten et al., 1974; Carette et al., 2000, 2002). MP may thus also be involved in the targeting of virions to the plasmodesma. It is still uncertain how the virion-containing transport tubules are assembled, i.e. how MP and virions are targeted to the plasma membrane/plasmodesma. Recently, Pouwels et al. (2002a) showed that neither the cytoskeleton nor the secretory pathway plays an essential role in this process.

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REFERENCES


