The movement proteins of cowpea mosaic virus and cauliflower mosaic virus induce tubular structures in plant and insect cells


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The movement proteins (MP) of cowpea mosaic virus and cauliflower mosaic virus (CaMV) are associated with tubular structures in vivo which participate in the transmission of virus particles from cell to cell. Both proteins have been expressed in plant protoplasts and insect cells. In all cases, immunofluorescent histochemistry showed that the MPs accumulate intracellularly as tubular extensions projecting from the cell surface. Additionally, electron microscopy revealed intracellular MP aggregates in CaMV MP-expressing cells. The data presented establish common features for the tubule-forming MPs: no other virus gene products are required for tubule formation and unique plant components (e.g. plasmodesmata) are not essential for tubule synthesis.

Introduction

The cell-to-cell movement of plant viruses is associated with the modification of plasmodesmata mediated by the action of virus-encoded proteins called movement proteins (MP). So far, two functional groups of MP have been identified (Maule, 1991; Lucas & Gilbertson, 1994). In one group, exemplified by tobacco mosaic virus MP, virus movement is mediated without overt changes in plasmodesmatal structure but with an increase in the plasmodesmatal size exclusion limit (Wolf et al., 1989). Typically, viruses associated with this group are able to move from cell to cell in the absence of coat protein and therefore virions. In the second group, exemplified by cowpea mosaic virus (CPMV) and cauliflower mosaic virus (CaMV) MPs, electron microscopy suggests that the virions move through plasmodesmata and that the role of the MP is to modify these channels structurally through the formation of tubules that are able to accommodate virus particles (Maule, 1991; Lucas & Gilbertson, 1994). Using immunogold labelling, the MP has been identified as a structural component of the tubules for CPMV (van Lent et al., 1990a), red clover mottle virus (Shanks et al., 1989), tomato spotted wilt virus (TSWV; Storms et al., 1995), grapevine fan leaf (Ritzenhaler et al., 1995) and tobacco ringspot (Wiecorek & Sanfacon, 1993) nepoviruses and CaMV (Linstead et al., 1988). However, the detailed compositions of the tubules and the mechanism for their formation have not yet been determined for any of these viruses.

Although the MP tubules are associated with plasmodesmata in infected tissue, they have also been seen as intracellular projections from the surface of infected protoplasts (van Lent et al., 1990a; Perbal et al., 1993; Storms et al., 1995; Ritzenhaler et al., 1995) lacking both a cell wall and plasmodesmata. In all cases the tubules exhibit a clear polarity extending from the surface of the protoplasts into the culture medium, thus mimicking the assembly of the tubule from the infected plant cell, through the plasmodesmata, into the cytoplasm of the neighbouring cells. This phenomenon has been used to determine whether other virus gene products are involved in tubule formation. Transient expression of the MP gene from CPMV (Wellink et al., 1993) or TSWV (Storms et al., 1995) in protoplasts resulted in tubule formation, thereby excluding a role for other virus gene products. In the case of TSWV, this was also shown after expressing the MP gene in Spodoptera frugiperda cells from a baculovirus vector where, surprisingly, surface tubules were again formed despite the cells being of non-plant origin (Storms et al., 1995).
The MPs of CPMV and CaMV have been subjected to extensive mutational analysis (Wellink et al., 1993; Thomas & Maule, 1995a,b) and there is some evidence (Thomas et al., 1993) that they might have a related structure, despite the absence of significant amino acid sequence similarity. To extend the comparative analysis of these two proteins it was necessary to establish whether the tubules made by the CaMV MP required other virus gene products for their formation, and to develop comparative expression systems. The CaMV MP has already been expressed in Escherichia coli (Citovsky et al., 1991; Thomas & Maule, 1995b) and S. frugiperda cells using a baculovirus vector (Zuidema et al., 1990; Maule et al., 1992). The experiments presented here provide further evidence for a common function for tubule-forming MPs in that the CaMV MP similarly requires no other virus gene product for tubule formation and, like TSWV MP, both CPMV and CaMV MPs form tubules on the surface of insect cells. The latter phenomenon has provided the opportunity for a more detailed electron microscopy analysis of MP tubules.

**Methods**

**Antisera.** A polyclonal serum recognizing the C-terminal 30 residues of the overlapping 48 kDa/58 kDa proteins of CPMV has been described before (Wellink et al., 1987). A further polyclonal antisera specific for the 58 kDa and 48 kDa proteins was prepared by injection of the CPMV 58 kDa protein expressed in E. coli (J.-C. Boyer, unpublished results) into rabbits. Both antisera recognize the 58 kDa and 48 kDa proteins of CPMV. The two CaMV MP (P1) antisera used (Harker et al., 1987; Maule et al., 1992) have also been described before.

**MP expression from recombinant baculoviruses.** For CaMV MP expression in insect cells, an *Autographa californica* nucleopolyhedrovirus (AcMNPV) recombinant expressing the CaMV (Strasbourg isolate) MP under control of the polyhedrin promoter (AcMNP1; Zuidema et al., 1990) was used.

To obtain a recombinant baculovirus expressing the CPMV (Nigerian isolate) MP from the AcMNPV p10 promoter, the sequence for the 48 kDa protein was cloned as a BglII fragment into the BamHI site of the transfer vector pAcAs3 (Vlak et al., 1990) to give pAcAs1. Plasmid pAcAs3 contains p10 flanking sequences and a β-galactosidase marker gene cassette. The BglII fragment was synthesized by PCR from plasmid pTM8S (Kasteel et al., 1993) using the primers 5' GTATATTCT-GCCACAGATCTGGCATGGAAGACATTATCCACC 3' and 5' CGG-GGCAAAGAAGGCTTACGCAATTCTCTGGAAAGCC 3', the newly created BglII sites are underlined. The techniques used were essentially as described by Sambrook et al. (1989).

Recombinant viruses were obtained by co-transfecting S. frugiperda Sf21 cells with AcMNPV AcM021 DNA (linearized with Bsu36I) and pAcAs1 DNA using the Lipofectin method as described by Groebe et al. (1990). The AcM021 is a p10-based expression vector that contains a unique Bsu36I site in the p10 locus (Martens et al., 1994). Recombinant viruses were selected as blue-coloured plaques upon addition of X-Gal, and subsequently plaque-purified (Brown & Faulkner, 1977) to homogeneity.

For baculovirus expression, the *S. frugiperda* cell line IPLB-Sf21 (Vaughn et al., 1977) was maintained as monolayers in Hink's medium (Hink, 1970) supplemented with 10% (v/v) fetal bovine serum (FBS). Sf21 cells were infected with recombinant viruses at a m.o.i. of 5 TCID<sub>50</sub> per cell as described by O'Reilly et al. (1992) and incubated for 48 h or 64 h.

**Transient expression of CaMV MP in Arabidopsis protoplasts.** A vector for the expression of CaMV MP in plant cells was prepared by excising the gag gene from pSLJ4D4 (Jones et al., 1992) using Ncol and BamHI and replacing it, after filling in of the 5' extension of the Ncol site using the Klenow fragment of E. coli polymerase I with a PCR-derived copy of CaMV gene I. The gag gene I DNA fragment was adapted with the PCR primers to give a blunt end and BamHI site at the 5' and 3' ends, respectively. The resulting plasmid (pSLJ4D4gI) contained the complete MP coding sequence between the CaMV 35S promoter and octopine synthase 3' poly(A) signal.

*Arabidopsis thaliana* C24 protoplasts were isolated from 3-4 week-old plants grown on GM medium following the methods of Damm & Willmitzer (1988) and Karesch et al. (1991a). Protoplasts (0.75 × 10<sup>4</sup>) were treated with 10 μg of pSLJ4D4 or pSLJ4D4gI using PEG-mediated DNA uptake as described by Damm et al. (1989) and Karesch et al. (1991b). Treated protoplasts were incubated in darkness for 17 h at 26 °C. CaMV-infected protoplasts were isolated from infected Chinese cabbage plants as described previously (Perbal et al., 1993).

**Protein analysis.** Insect cells or treated protoplasts were harvested, washed in fresh culture medium and the proteins analysed by SDS-PAGE (Laemmli, 1970) in 10% polyacrylamide. After electrophoresis, gels were either stained with Coomassie brilliant blue or used for immunoblotting to nitrocellulose. For immunodetection, blots were blocked overnight with 5% (w/v) fat-depleted milk powder and 0.01% (v/v) NP40 in PBS pH 7.2, incubated with antiserum (anti-CPMV MP diluted 1:3000 or anti-CaMV MP (Maule et al., 1992) diluted 1:1000) for 2 h, washed with PBS and further incubated with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Tago) for 1 h. After washing the immunoblots were developed using NBT and BCIP (BRL Life Technologies) as substrates. All incubations were done at room temperature.

**Immunofluorescence microscopy.** Sf21 cells were grown on sterile glass coverslips in small Petri dishes and infected with the recombinants AcCPMV-MP or AcAM1. As controls Sf21 cells were infected with AcMO21 parental virus, wild-type AcMNPV or buffer. At 48 h and 68 h post-infection (p.i.) the cells were fixed with acetone at −20 °C for 7 min, washed with PBS and blocked for 45 min with 1% (w/v) BSA in PBS. The cells were then incubated for 1 h with anti-CPMV MP or anti-CaMV MP serum (Harker et al., 1987) diluted in 1% (w/v) BSA in PBS, then washed extensively before incubating with FITC-conjugated horse anti-rabbit antibody (Nordic). After three 10 min washes with PBS, the cells were covered with glycerol–PBS containing Citifluor (Agar) and examined using a Leitz Laborlux S UV microscope.

*A. thaliana* and Chinese cabbage protoplasts were fixed and immunostained using anti-CaMV MP serum (Maule et al., 1992) as described by van Lent et al. (1991).

**Electron microscopy and immunogold labelling.** Negative staining and immunogold labelling of AcCPMV-MP or AcAM1-infected insect cells was performed after incubation for 48 or 64 h as described for protoplasts by van Lent et al. (1991). These cells were also fixed with aldehydes, dehydrated and embedded in LR Gold (London Resin Company) and sections were immunogold-labelled with antibodies against CPMV or CaMV MPs and Protein A–gold complexes with 7 nm or 10 nm gold particles, as described by van Lent et al. (1990b).

Additionally, AcAM1-infected insect cells were fixed with aldehydes and infiltrated with 2:3 m-sucrose in distilled water for 16 h. These specimens were then cryo-fixed by immersion in liquid propane using a Reichert KF80 cryofixation unit. Cryo-sections were prepared in a Reichert Ultracut S equipped with a FCS cryo-sectioning chamber. Cryo-sections
were immunogold-labelled as described before and/or stained by applying a thin film of a mixture of 0.5% methyl cellulose and 0.5% uranyl acetate in distilled water. Specimens were examined in a Philips CM12 transmission electron microscope.

Results

Transient expression of CaMV MP in plant protoplasts

Previous work has shown that transient expression of CPMV MP in plant protoplasts results in the formation of tubules similar to those seen following virus infection of protoplasts, except that in the former case the tubules do not contain virus particles (Wellink et al., 1993). To see whether the CaMV MP tubules could similarly form independently of any other virus protein, an expression vector containing CaMV gene I downstream of the CaMV 35S promoter was introduced into *A. thaliana* protoplasts. After treatment of protoplasts with this plasmid vector and incubation for 17 h the cells were harvested and assayed for the MP accumulation by immunoblot analysis and for tubule formation by immunofluorescent staining.

Immunoblot analysis with anti-CaMV MP serum (Fig. 1a) identified a range of polypeptides whether the protoplasts were treated with the control vector (pSU4D4) or the MP expression vector (pSU4D4gI). Most of these represented cross-reaction of the serum with host proteins but, in protoplasts treated with pSU4D4gI, a more intense band co-migrating at 46 kDa with *E. coli*-expressed CaMV MP indicated accumulation of expressed MP (Fig. 1a, lane 3).

Fig. 1. Transient expression of CaMV MP in protoplasts. (a) Immunoblot of transiently expressed CaMV MP in *A. thaliana* protoplasts. Protoplasts were treated with control plasmid (pSU4D4; lane 2) or CaMV MP expression vector (pSU4D4gI; lane 3) and incubated for 17 h before analysis. Treatment with pSU4D4gI resulted in the accumulation of a 46 kDa polypeptide (arrow) that co-migrated with CaMV MP expressed in *E. coli* (lane 1). (b) Immunofluorescence assay of MP-expressing *A. thaliana* protoplasts showed MP-specific tubules (white arrows) extending from the surface of some protoplasts, similar to those formed after culture of pre-infected protoplasts isolated from CaMV-infected tissue (c). In (a) size markers were bovine albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa) and carbonic anhydrase (29 kDa). Bars in (b) and (c) represent 20 μm.

Fig. 2. SDS–polyacrylamide gel stained with Coomassie brilliant blue (a) and immunoblot analysis (b) of proteins extracted from uninfected (lanes 1, 4), AcMO21- (lanes 2, 5) and AcCPMV-MP- (lanes 3, 6) infected Sf21 cells at 48 h p.i. Immunoblot analysis was performed with the anti-CPMV MP serum prepared against the C-terminal 30 residues of the 58 kDa protein of CPMV.
Immunofluorescent staining of the protoplasts (Fig. 1b) showed that a small proportion of them (3%) displayed threads of fluorescence extending from the cell surface. Supported by electron microscopy evidence, we have previously interpreted these structures as tubules (Perbal et al., 1993). The tubules were variable in length and similar in appearance to those seen in CaMV-infected Chinese cabbage protoplasts (Fig. 1c).

Expression of CPMV MP by recombinant AcCPMV-MP

Sf21 cells were infected with the baculovirus recombinant AcCPMV-MP, baculovirus AcMO21 or treated with buffer. After 48 h incubation, the total cell protein was analysed by gel electrophoresis and immunoblotting. In cells infected with AcCPMV-MP a polypeptide was detected but was absent from cells infected with AcMO21 or buffer-treated cells (Fig. 2a). This protein co-migrated with CPMV MP present in CPMV-infected plant cells (data not shown) and was identified as the CPMV MP from immunoblots using the specific antiserum (Fig. 2b). A smaller polypeptide also reacted with the antiserum and probably represents partial breakdown of the MP.

Characterization of the expression of CaMV MP in AcAM1-infected insect cells has been reported previously (Maule et al., 1992).

Immunolocalization of the CPMV and CaMV MPs

To study the subcellular location of the MPs of CPMV and CaMV in infected insect cells, Sf21 cells were infected with recombinants AcCPMV-MP and AcAM1 and incubated for 48 h and 68 h before preparation for immunofluorescence and electron microscopy. Cells infected with AcMO21, wild-type AcMNPV or treated with buffer were used as controls. In cells infected with recombinant AcCPMV-MP, immunofluorescent labelling with CPMV MP antiserum revealed numerous tubular structures extending from the surface of the cells into the culture medium (Fig. 3a).
Immunogold electron microscopy showed that in the cytoplasm, the CPMV MP was predominantly localized to the plasma membrane (Fig. 3 b). In negative phosphotungstic acid (PTA)-stained whole mounts of AcCPMV-MP-infected cells, in which the cell structure was disrupted by an osmotic shock, many tubular structures were found (Fig. 3 c) and immunogold labelling using the anti-CPMV MP serum identified the CPMV MP as a component of the tubules (Fig. 3 d). These tubules were
the same diameter (35 nm) as those formed following expression of the MP in plant protoplasts and tissue (van Lent et al., 1991) and, as before, they appeared to lack any discernible contents. At higher magnifications no obvious substructure could be noticed. Occasionally the plasma membrane was observed along the tubular structure; it is likely that this membrane was often displaced during disruption of the cell for sample preparation.

In cells infected with recombinant AcAM1, immunofluorescent labelling with anti-CaMV MP serum similarly revealed the presence of numerous tubular structures emerging from the surface of the infected cell (Fig. 4a). The MP protein was also localized to large aggregates present in the cytoplasm of infected cells (Fig. 4b). Previously, these aggregates were reported to have a hollow fibre-like substructure (Zuidema et al., 1990). Cryo-sections of AcAM1-infected cells revealed more detailed information on the substructure of these aggregates, showing them to have a 'rolled sheet' appearance (Fig. 4c). Negative PTA-stained whole mounts of the AcAM1-infected cells showed numerous tubule-like structures (Fig. 4d). At higher magnification these tubules revealed a detailed structure apparently consisting of an assembly of filaments (Fig. 4e) in a shallow twisted arrangement. The tubules had a mean (n = 17) diameter of 52 nm with an average (n = 7) of 14-7 filaments on the visible surface of each tubule. Immunogold labelling using the anti-CaMV MP serum identified the CaMV MP as a component of the tubules (Fig. 4f).

Discussion

The results of this study confirm that, like the tospovirus MP (Storms et al., 1995), the MPs from the comovirus (CPMV) and caulimovirus (CaMV) groups are able to form tubules in the absence of other virus proteins and can achieve this in cells from outside the plant kingdom. This is despite these viruses having a different genomic organization and expression and different virus particle size.

The observation that these MPs could form tubules extending from the surface of plant cells lacking a cell wall (protoplasts) could be accommodated in a model where the MP's recognized remnants of the plasmodesmata on the inner face of the plasma membrane as foci for the initiation of MP aggregation into tubules. However, the formation of similar structures at the surface of insect cells shows that either homologous target structures/proteins are present in the insect and plant kingdoms or that tubule formation is a spontaneous process when sufficient MP is present. The latter argument is less likely as MP tubules have not usually been seen free within the cytoplasm of plant or insect cells or extending into the plant cell vacuole.

The tubules formed in insect cells by the CPMV MP are morphologically identical to the tubules formed by this protein in plant protoplasts (van Lent et al., 1991; Wellink et al., 1993), exhibiting no pronounced substructure. In contrast, the tubules induced in insect cells by the CaMV MP contain a clear filamentous substructure and, in this respect, resemble the tubules formed by the TSWV MP protein. The tubules formed by the CaMV and TSWV MPs are also of similar dimensions (approximately 50 nm), a size capable of accommodating the respective nucleocapsids. The difference in surface structure between CaMV and CPMV tubules could be related to differences in the N termini of the proteins. Epitope tagging showed that the N terminus of CaMV MP may be exposed on the outer surface of the tubule, and structural alignments between the CaMV and CPMV MPs suggested that the latter could have an N-terminal extension with an α-helical structure (Thomas & Maule, 1995a, b).

Differences between the MPs were seen in their subcellular distribution in insect cells. The CPMV and TSWV (Storms et al., 1995) MPs accumulated adjacent to the plasma membrane whereas the CaMV MP accumulated as large intracellular aggregates. The 'rolled sheet' appearance of the latter supports the view that tubules do not form spontaneously and accords with the observation from epitope tagging experiments in plant cells (Thomas & Maule, 1995a, b) that cytoplasmic CaMV MP has a different conformation from tubule-associated MP.

Although the MPs of CaMV, CPMV and TSWV show no sequence homology, an overall functional similarity (tubule formation) is apparent in that tubules are formed to transport virus particles from cell to cell. This concept is supported by computer analysis of the CaMV and CPMV MP sequences, which has revealed a similarity in the distribution of conserved and variable regions, suggesting a possible structural similarity between the two proteins (Thomas & Maule, 1995a, b). However, the difference in subcellular distribution may point to refined differences in the movement mechanism of these viruses.

In their natural hosts the MPs assemble in what can be considered as a unique plant intercellular organelle, the plasmodesma (for review see Lucas & Gilbertson, 1994). In view of the targeting of the MPs to and assembly of the tubules in these organelles, we must assume that specific intracellular cell trafficking mechanisms are involved. Despite the absence of plasmodesmal components in insect cells, the high level of protein expression and abundant tubule formation for a diverse range of plant virus MPs make the baculovirus expression system an ideal way to make further progress in the analysis of the mechanisms of virus movement.

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