Isolation and characterization of tubular structures of cowpea mosaic virus

D. T. J. Kasteel,[1] J. Wellink,[2] R. W. Goldbach[1] and J. W. M. van Lent[1]

Departments of Virology[1] and Molecular Biology[2], Agricultural University, Binnenhaven 11, 6709 PD Wageningen, The Netherlands

Tubular structures involved in the cell-to-cell movement of cowpea mosaic virus (CPMV) were partially purified from infected cowpea protoplasts to identify the structural components. A relatively pure fraction could be obtained by differential centrifugation and this was analysed by PAGE and immunoblotting. Besides the movement protein (MP) and capsid proteins (CP) of CPMV, no other major infection-specific proteins could be detected, suggesting that host proteins are not a major structural component of the movement tubule.

Intercellular movement of cowpea mosaic virus (CPMV) is achieved by transport of virions through specialized tubules that are assembled in modified plasmodesmata. It was previously shown *in planta* that the capsid protein (CP) and the 48 kDa movement protein (MP) of CPMV were located in these tubules (van Lent *et al.*, 1990) and mutation or deletion of these proteins resulted in abolition of cell-to-cell movement (Wellink & van Kammen, 1989). In CPMV-infected cowpea protoplasts, the movement tubules occluding virions are extensively formed at the cell surface (van Lent *et al.*, 1991), mimicking the process in plant tissue even in the absence of intact plasmodesmata. This material provides an opportunity for further identification and characterization of components involved in tubule formation. By analysis of deletion and insertion mutants and by transient expression experiments it was shown that the MP of CPMV was the sole viral protein responsible for tubule induction (Kasteel *et al.*, 1993, Wellink *et al.*, 1993).

The typical association between the CPMV movement tubules and the plasma membrane (in protoplasts and in plant tissue) lead to the speculation that one or more host components could be involved, either as a structural component of the movement tubule or in the process of anchoring of the tubule at the plasma membrane. As expression of the MP by a baculovirus expression vector resulted in identical tubule formation at the insect cell surface (Kasteel *et al.*, 1996), it was suggested that host components, if involved at all in the tubule-forming mechanism, should be of a conserved nature (e.g. cytoskeleton proteins).

To identify the major structural components of the movement tubule, tubular structures were purified from CPMV-infected cowpea protoplasts (*Vigna unguiculata* 'California Blackeye') by means of differential centrifugation and analysed for their protein content by gel electrophoresis and immunoblotting. As tubule isolation from intact plant tissue is not feasible, infected protoplasts were used for mass production of tubules. In protoplasts, these tubules protrude from the cell surface and are easy to separate from other cell constituents.

Protoplasts were isolated and inoculated with CPMV RNA or mock-inoculated with water as described by Eggen *et al.* (1989) and screened for infection and tubule formation by immunofluorescence microscopy using polyclonal antisera against CP and MP (Wellink *et al.*, 1987), respectively. Immunofluorescence and negative staining electron microscopy of protoplasts and tubule fractions were carried out essentially as described by van Lent *et al.* (1991).

An important parameter for optimal tubule formation was the viability of the isolated protoplasts, which was estimated by fluorescein diacetate (FDA) staining as described by Power *et al.* (1990). A sample of 100 µl protoplast suspension was mixed with 2 µl of 5 mg/ml FDA in acetone for 2–4 min. Viable protoplasts were identified by fluorescence in a UV-microscope. In general, the number of tubules formed at the cell surface was related to the number of viable protoplasts in the isolated suspension, and only suspensions with 98% or more viable protoplasts upon isolation were used in these experiments. At 48 h after inoculation, suspensions with more then 60% infected cells and numerous tubules were used for isolation of the tubules.

Tubular structures were separated from the infected protoplasts by shaking for 20 min at 80 r.p.m. on a shaker. The protoplasts were then pelleted by centrifugation for 5 min at 60 ***g***. During this centrifugation the tubules remained in the supernatant, as was verified by immunofluorescence microscopy. This supernatant was collected and then tubules were pelleted by centrifugation for 10 min at 15000 ***g*** in an Eppendorf centrifuge. The pellet was resuspended in PBS, pH 7·2, and this partially purified fraction was analysed by

Author for correspondence: Jan van Lent.
Fax +31 317 484820. e-mail jan.vanlent@medew.viro.wau.nl

0001-5064

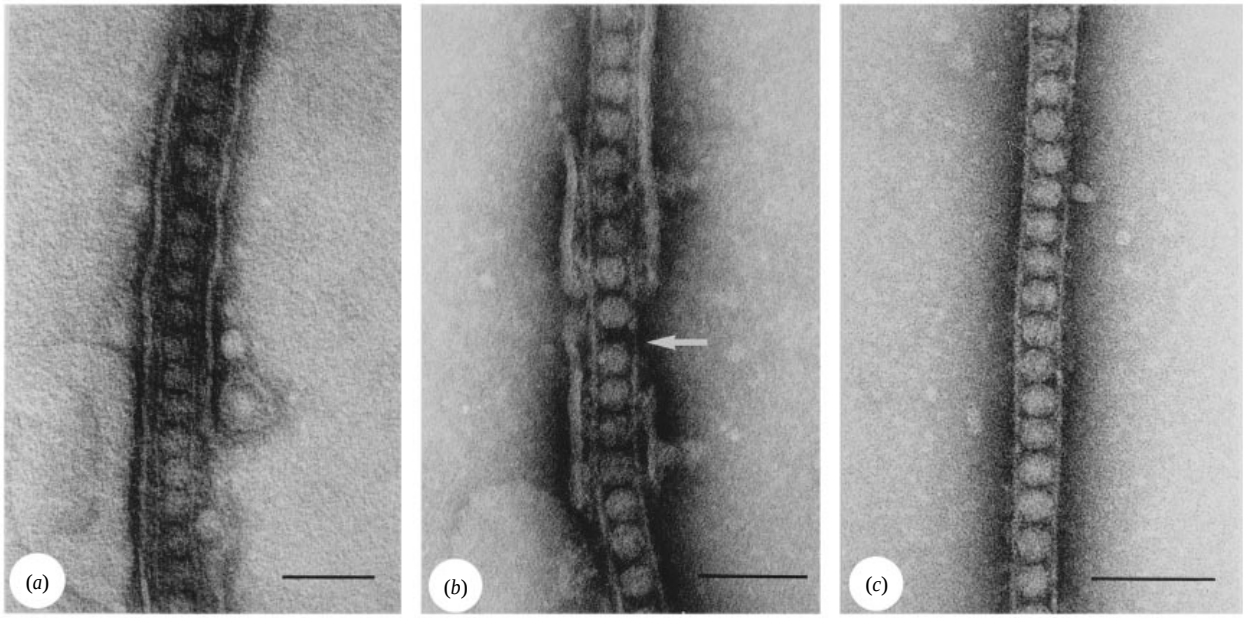

Fig. 1. Electron micrographs of tubules in partially purified fractions. Tubules are encased by the plasma membrane (*a*). After 3 weeks of refrigerated storage in PBS the tubular structure is still intact (*b*), but the plasma membrane has partially disintegrated (arrow). The plasma membrane was then successfully removed by treatment with NP40 (*c*). Bars represent 100 nm.

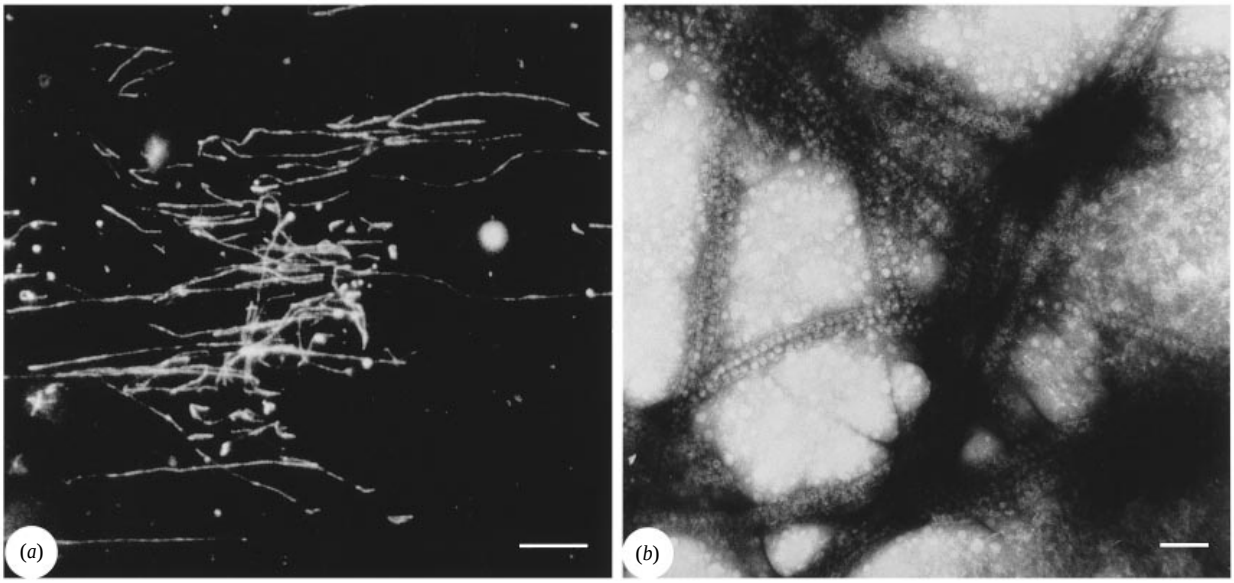

Fig. 2. (*a*) Immunofluorescent staining of the pure tubule fraction, treated with anti-MP serum, showing numerous tubules. (*b*) Electron microscopic image of negatively stained tubules in the same fraction. Tubules are mainly found in large clusters. Bar in (*a*) represents 10 μm, bar in (*b*) represents 100 nm.

electron and immunofluorescence microscopy. Numerous tubular structures of various lengths were observed in the electron microscope and most of them were still surrounded by a membrane (Fig. 1*a*). All tubules contained virus particles. The tubules in this partially purified fraction appeared to be stable for several weeks when stored in PBS at 4 °C, but not at room temperature or frozen. After 3 weeks of storage in PBS only partial breakdown of the surrounding plasma membrane was observed (Fig. 1*b*). To remove these membrane remnants, the fractions were treated with NP40 for 1 h at 4 °C. This treatment had no apparent effect on the tubule structure (Fig. 1*c*).

As this fraction still contained large amounts of cell debris, mainly chloroplasts, further purification was needed. Several

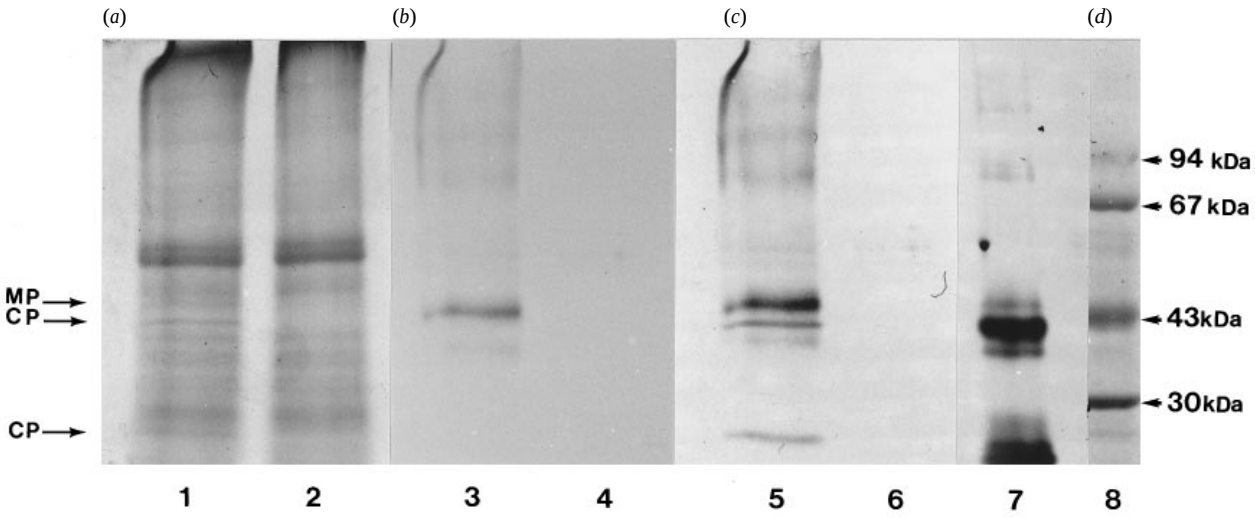

Fig. 3. Silver-stained SDS–polyacrylamide gel (*a*) and immunoblot analysis (*b*, *c*) of purified tubule fractions (lanes 1, 3, 5) and similar fractions of mock-inoculated protoplasts (lanes 2, 4, 6). Blots were treated with anti-MP serum (*b*) and subsequently with anti-CP serum (*c*). The positions of the MP and the CPs VP37 and VP23 are indicated by arrows. (*d*) Lane 7 contains purified CPMV showing VP37 and a truncated form of VP23 (VP20) and lane 8 contains molecular mass markers.

partially purified tubule fractions were pooled and then loaded on a sucrose cushion consisting of 1 ml 40% (w/v) sucrose in PBS with 1% (v/v) NP40 in an Eppendorf tube and centrifuged for 5 min at 15 000 ***g***. The pellet was resuspended in PBS and the purity of the fraction was checked by immunofluorescence and electron microscopy. This final fraction contained numerous clustered tubular structures (Fig. 2*a*, *b*) and very little cell debris when compared to the partially purified fraction.

Purified tubule fractions and similar fractions obtained from mock-inoculated protoplasts were then analysed by PAGE on a 10% gel (Laemmli, 1970) that was either silver stained (Morrisey *et al.*, 1981) or used for immunoblotting. The silver-stained gel (Fig. 3*a*) showed three protein bands in tubule fractions that were absent in control fractions. These bands correspond to MP and the two CPs of CPMV as was verified on the immunoblot using anti-MP serum (Fig. 3*b*) and, subsequently, with anti-CP serum (Fig. 3*c*). The immunoblot shows an extra MP-specific band, of apparent molecular mass 36 kDa, which probably represents a breakdown product of the MP. Such a species also occurred in insect cells expressing the MP gene of CPMV (Kasteel *et al.*, 1996). Apart from the MP and the CP, no other prominent infection-specific viral or host protein was detected in the partially purified tubule fraction.

These results support the hypothesis that the MP of CPMV is the only major structural component of the tubules. So far, only for cauliflower mosaic virus (CaMV) has an attempt been made to isolate and biochemically characterize tubular structures (Perbal *et al.*, 1993). Upon immunoblot analysis of a fraction obtained in a manner similar to that described here, only the MP of CaMV was detected. Further analysis of this fraction by SDS–PAGE followed by silver staining was not performed, presumably because of the low amount of tubules present in the fraction.

Although they are not a major structural component of the CPMV movement tubule, it remains to be determined if host proteins are in any way functionally involved in the process of tubule formation, e.g. in intracellular protein targeting or in anchoring of the tubule structure to the plasma membrane.

The research was supported by grants from the Foundation of Life Sciences (SLW, D. T. J. Kasteel) of the Netherlands Organization for Scientific Research (NWO) and the Joint Dutch–Israeli Agricultural Research Program (DIARP), grant number 93/29 (J. W. M. van Lent and R. W. Goldbach).

References

Eggen, R., Verver, J., Wellink, J., De Jong, A., Goldbach, R. & van Kammen, A. (1989). Improvements of the infectivity of *in vitro* transcripts from cloned cowpea mosaic virus cDNA: impact of terminal nucleotide sequences. *Virology* **173**, 447–455.

Kasteel, D., Wellink, J., Verver, J., van Lent, J., Goldbach, R. & van Kammen, A. (1993). The involvement of cowpea mosaic virus M RNA-encoded proteins in tubule formation. *Journal of General Virology* **74**, 1721–1724.

Kasteel, D. T. J., Perbal, C.-M., Boyer, J.-C., Wellink, J., Goldbach, R. W., Maule, A. J. & van Lent, J. W. M. (1996). The movement proteins of cowpea mosaic virus and cauliflower mosaic virus induce tubular structures in plant and insect cells. *Journal of General Virology* **77**, 2857–2864.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Morrisey, J. H. (1981). Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Analytical Biochemistry* **117**, 307–310.

Perbal, M.-C., Thomas, C. L. & Maule, A. J. (1993). Cauliflower mosaic virus gene I product (P1) forms tubular structures which extend from the surface of infected protoplasts. *Virology* **195**, 281–285.

Power, J. B., Davey, M. R., McLellan, M. & Wilson, D. (1990). Isolation, culture and fusion of protoplasts. In *Biotechnology Education*, vol. 1, no. 3. pp. 115–124.

van Lent, J., Wellink, J. & Goldbach, R. (1990). Evidence for the involvement of the 58K and 48K proteins in the intracellular movement of cowpea mosaic virus. *Journal of General Virology* **71**, 219–223.

van Lent, J., Storms, M., van der Meer, F., Wellink, J. & Goldbach, R. (1991). Tubular structures involved in movement of cowpea mosaic virus are also formed in infected cowpea protoplasts. *Journal of General Virology* **72**, 2615–2623.

Wellink, J., Jaegle, M., Prinz, H., van Kammen, A. & Goldbach, R. (1987). Expression of the middle component RNA of cowpea mosaic virus in vivo. *Journal of General Virology* **68**, 2577–2585.

Wellink, J. & van Kammen, A. (1989). Cell-to-cell transport of cowpea mosaic virus requires both the 58K/48K proteins and the capsid proteins. *Journal of General Virology* **70**, 2279–2286.

Wellink, J., van Lent, J. W. M., Verver, J., Sijen, T., Goldbach, R. W. & van Kammen, A. (1993). The cowpea mosaic virus M RNA-encoded 48-kilodalton protein is responsible for induction of tubular structures in protoplasts. *Journal of Virology* **67**, 3660–3664.

Received 7 July 1997; Accepted 22 August 1997