Isolation and characterization of tubular structures of cowpea mosaic virus

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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 antiserum 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 than 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 15 000 g in an Eppendorf centrifuge. The pellet was resuspended in PBS, pH 7.2, and this partially purified fraction was analysed by

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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. 1a). 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. 1b). 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. 1c).

As this fraction still contained large amounts of cell debris, mainly chloroplasts, further purification was needed. Several
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. 2a, 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. 3a) 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. 3b) and, subsequently, with anti-CP serum (Fig. 3c). 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.

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References


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