Association of the non-structural P3 viral protein with cylindrical inclusions in potyvirus-infected cells

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Potyvirus genomic RNA encodes a large polyprotein that is processed by three virus-encoded proteinases to yield a coat protein and at least six non-structural proteins. A characteristic of potyvirus infections is the aggregation of some of the non-structural proteins to form distinctive, stable structures in infected cells. Cylindrical inclusions (Edwardson, 1966), structures with complex three-dimensional features, are formed by aggregation of the 70K cylindrical inclusion protein (CI) in the cytoplasm of infected cells. Another non-structural protein, the helper component protein, is present in amorphous cytoplasmic inclusions in some potyvirus-infected plants (Martelli & Russo, 1976; Baunoch et al., 1990). Two other non-structural proteins, NIa and NIb, are produced and in the nuclei of cells infected with some potyviruses these proteins aggregate to form nuclear inclusions (Knuhtsen et al., 1974).

We have recently demonstrated the existence, in cells infected with the potyvirus tobacco vein mottling virus (TVMV), of two other non-structural viral proteins (Rodríguez-Cerezo & Shaw, 1991). These proteins were previously identified as the 34K and 42K proteins but, to comply with nomenclatural designations that have recently assumed more common usage, we refer to them here as P1 and P3, respectively. We used anti-P1 and -P3 sera to detect P1 and P3 in immunoblots of extracts of TVMV-infected tobacco leaves and protoplasts. Fractionation of these extracts demonstrated the presence of P1 and P3 in a pellet that sedimented at 30000 g. However, such procedures often generate artefactual binding of proteins to certain fractions and therefore did not provide the type of definitive information on the subcellular location of P1 and P3 that might be useful for the assignment of functions to these proteins. An immunocytological investigation was therefore undertaken to address this issue and we report here the intracellular site of accumulation of the P3 protein of TVMV.

Small pieces of young systemically infected leaves collected from Nicotiana tabacum plants 1 to 3 weeks after mechanical inoculation with TVMV RNA, and from the equivalent leaves of uninoculated plants, were processed for transmission electron microscopy (TEM). The samples were fixed in a mixture of 0.1 to 1% glutaraldehyde and 2% paraformaldehyde in 0.1M-sodium cacodylate buffer pH 7.4, dehydrated in graded ethanol to 70% and embedded in L. R. White resin at 50 to 52 °C (Newman, 1989). Immunocytological labelling was carried out essentially as has been described by Tomenius et al. (1987). Sections were incubated for 1 to 3 h with the primary antiserum [anti-P3 or preimmune serum (Rodríguez-Cerezo & Shaw, 1991)] diluted 10- to 20-fold and for 30 min with colloidal gold-labelled goat anti-rabbit IgG (10 nm diameter particles; Sigma) diluted 25- to 50-fold. At least 20 sections from each of three sets of plants inoculated on different occasions, and from uninoculated control plants, were examined.

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Fig. 1. TEM of thin sections of mesophyll cells from leaves of tobacco plants systemically infected with TVMV. (a, b) Sections incubated with anti-P3 serum; gold label is specifically associated with cylindrical inclusions sectioned longitudinally (ci) or transversely (arrowhead), but not with virus particles (v) or other structures. (c) Section incubated with preimmune serum. ch, Chloroplast; cy, cytoplasm; m, mitochondrion; va, vacuole; w, cell wall. Bars represent 200 nm.
Typical electron micrographs of thin sections of tobacco leaf tissue systemically infected with TVMV that had been incubated with anti-P3 serum and then gold-labelled are shown in Fig. 1 (a and b). Gold particles were observed specifically and almost exclusively associated with the cylindrical inclusions. Fig. 1 (a) contains both longitudinal and transverse views of cylindrical inclusions, revealing the bundle-like and pinwheel-like configurations, respectively, and both were clearly labelled with gold particles. Bundles of virus particles in the same cells were not labelled after treatment of the sections with anti-P3 serum (Fig. 1 a and b) but were labelled when anti-TVMV serum was used (Ammar et al., 1992, and unpublished data).

There was no specific immunogold labelling of nuclei, endoplasmic reticulum, mitochondria, chloroplasts, vacuoles or cell walls after incubation of sections with anti-P3 serum (Fig. 1 a). Sections from infected leaves that had been treated with preimmune instead of anti-P3 serum did not accommodate any specific gold labelling (Fig. 1 c), nor did sections from non-inoculated plants treated in the same manner as those shown in Fig. 1 (a and b).

Since the cells described above were in the later stages of infection, they did not allow the opportunity to determine whether P3 is associated with cylindrical inclusions at the early stages of infection. In previous work, CI and cylindrical inclusions were detected adjacent to plasma membranes in thin sections of TVMV-infected tobacco leaf mesophyll protoplasts as early as 10 h post-inoculation (p.i.) (Murphy et al., 1991) and, at the same stage, P3 could be detected by immunoblotting of protoplast extracts (Rodriguez-Cerezo & Shaw, 1991). To examine the intracellular site(s) of accumulation of P3 at early stages of infection we therefore inoculated protoplasts by electroporation.

Fig. 2. TEM of thin sections of tobacco protoplasts inoculated with TVMV RNA 10 h (a), 21 h (b) or 45 h (c) before fixation. All sections were incubated with anti-P3 serum; gold label is specifically associated with cylindrical inclusions (ci). Note the stronger labelling of inclusions in (c) than in (b) and the lower part of (a), and the absence of labelling of inclusions in the upper part of (a). Arrow in (b) indicates virus particles. ch, chloroplast; cy, cytoplasm; p, plasma membrane. Bars represent 200 nm.
with TVMV RNA (Luciano et al., 1987; Murphy et al., 1991), collected samples of $2 \times 10^5$ protoplasts 10, 21 and 45 h p.i. and processed them for TEM and immunolabelling as described above.

In thin sections of protoplasts incubated with anti-P3 serum, gold label was associated with only the cylindrical inclusions (Fig. 2). The frequency and intensity of labelling in protoplasts was apparently dependent on the length of time the protoplasts had been infected prior to fixation (Table 1). Thus, in 45 h p.i. samples, 69% of the cylindrical inclusions detected were moderately labelled, 25% were weakly labelled and only 6% were unlabelled. However, at 21 h p.i. 74% of the cylindrical inclusions observed were weakly labelled, and at 10 h p.i. all but two of the 26 cylindrical inclusions detected were unlabelled. Specific gold labelling was not observed in sections of infected protoplasts incubated with preimmune serum or in sections of non-inoculated protoplasts that had been incubated with anti-P3 serum.

In some sections of TVMV-infected protoplasts, virus particles were apparently associated with cylindrical inclusions (Fig. 2b). These did not react with anti-P3 serum but were labelled after treatment with anti-TVMV serum. The subcellular location and aggregation of virus particles and their association with cylindrical inclusions in potyvirus-infected protoplasts and leaf tissues will be reported elsewhere.

The occurrence of immunogold-labelled cylindrical inclusions in sections of TVMV-infected protoplasts treated with anti-P3 serum

<table>
<thead>
<tr>
<th>Intensity of labelling*</th>
<th>10 h p.i.†</th>
<th>21 h p.i.‡</th>
<th>45 h p.i.‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moderate</td>
<td>1 (4%)</td>
<td>8 (21%)</td>
<td>111 (69%)</td>
</tr>
<tr>
<td>Weak</td>
<td>1 (4%)</td>
<td>29 (74%)</td>
<td>40 (25%)</td>
</tr>
<tr>
<td>None</td>
<td>24 (92%)</td>
<td>2 (5%)</td>
<td>10 (6%)</td>
</tr>
<tr>
<td>Total observed</td>
<td>26</td>
<td>39</td>
<td>161</td>
</tr>
</tbody>
</table>

* Number of gold particles/0.09 μm²: moderately labelled, > 4; weakly labelled, 1 to 3.
† Over 2000 protoplast sections examined.
‡ Five-hundred to 1000 protoplast sections examined.

The evidence presented here indicates that the P3 protein of TVMV, or its putative cleavage product of 37K (Rodriguez-Cerezo & Shaw, 1991), is intimately associated with cylindrical inclusions in infected cells. Though there have been many descriptions of potyvirus particles associated with cylindrical inclusions in vivo (Lesemann, 1988), we are not aware of previous reports of an association with these structures of non-structural potyvirus proteins other than CI. Because most of the inclusions observed in protoplasts at 10 h p.i. were not labelled after treatment with anti-P3 serum, P3 may not be essential for the formation of cylindrical inclusions. However, these structures are just beginning to be detectable at this stage of infection (Murphy et al., 1991) and their association with P3 appears to occur shortly thereafter.

The functions of the P3 proteins of potyviruses are not presently known. However, our inability to detect the synthesis of progeny viral RNA in protoplasts or plants inoculated with mutants of TVMV RNA with altered P3 coding regions (P. Klein & E. Rodriguez-Cerezo, unpublished data) may be the result of the involvement of this protein in the replication of potyviral RNA. The association of P3 with cylindrical inclusions at the early stages of infection also suggests such a role since investigations of amino acid sequence homology (Lain et al., 1989) and biochemical activity (Lain et al., 1990) indicate that the potyviral CI is probably a helicase involved in the replication of viral RNA.

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