The Fate of the Transport Protein of Tobacco Mosaic Virus in Systemic and Hypersensitive Tobacco Hosts

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SUMMARY

The transport protein of tobacco mosaic virus (TMV) (Mr 30,000, 30K non-structural protein) was detected on Western blots, using an antiserum to a synthetic C-terminal nonapeptide. Accumulation of this protein in subcellular fractions of inoculated leaves was measured during TMV infection of Nicotiana tabacum cv. Samsun and cv. Samsun NN. In cv. Samsun, a systemic host, the 30K protein appeared transiently in a crude membrane fraction but accumulated more stably in cell walls. In cv. Samsun NN, which is a hypersensitive host giving only localized infection, the early accumulation (up to 40 h; before any necrosis was visible) was the same as in cv. Samsun. However, as soon as necrosis was visible, the amount of 30K detected in the cell wall fraction decreased sharply and coat protein synthesis stopped. This drop in the amount of 30K protein is most easily interpreted as a side-effect of the hypersensitive reaction and may explain why TMV infection becomes localized in leaves of cv. Samsun NN.

The outcome of plant virus infections depends on the ability of the infectious agent to spread from the sites of initial infection to the neighbouring healthy tissue and eventually, through the vascular system, to the entire plant. Virus spread is thought to require the help of a viral protein, the 'spreading factor' or 'viral transport factor', which is therefore critically important in pathogenesis (Atabekov & Dorokhov, 1984). For tobacco mosaic virus (TMV), this factor has been identified as the Mr 30,000, 30K non-structural protein. Well-characterized point mutants in the 30K protein coding sequences are temperature-sensitive with respect to transport (Leonard & Zaitlin, 1982; Ohno et al., 1983; Zimmer & Hunter, 1983; Meshi et al., 1987) and additional evidence that the 30K protein functions as a spreading factor comes from work with plants transgenic for the 30K protein gene (Deom et al., 1987).

The mode of action of the transport protein is not understood. It has been suggested that it modifies the wall and/or the plasmodesmata between adjacent infected and uninfected cells to allow movement of the infectious particles (Atabekov & Dorokhov, 1984) and, indeed, the 30K protein of TMV was recently observed in the plasmodesmata of infected leaves (Tomenius et al., 1987). Here we examine the kinetics of accumulation of the 30K protein in different subcellular fractions, as was previously achieved for non-structural protein P3 of alfalfa mosaic virus (AMV), which is also thought to be a transport factor (Godefroy-Colburn et al., 1986).

In addition to its role in virus transport, the 30K protein may be involved in symptomatology because nitrous acid-induced mutations of the common strain of TMV which changed the symptoms induced in Nicotiana tabacum cv. Java (carrying the N' gene) from systemic to necrotic have been mapped to the 30K cistron (Nitschko, 1987). We therefore compared the
subcellular location of the 30K protein in *N. tabacum* cv. Samsun, in which TMV infection is systemic, and in cv. Samsun NN, in which infection is localized and necrotic.

Detection of the 30K protein was performed using an antiserum to a synthetic nonapeptide that corresponded to the theoretical C terminus of the 30K protein (sequence ATVAESDSF; Goelet *et al.*, 1982), coupled to keyhole limpet haemocyanin (KLH) with glutaraldehyde. A 2 kg New Zealand White rabbit was immunized with the peptide–KLH conjugate (purchased from Biochrom GmbH, Berlin, F.R.G.) by an initial injection (1 mg conjugate emulsified in Freund's complete adjuvant, injected intradermally and intramuscularly in 12 different sites) followed by three boosters (0.75 mg conjugate in Freund's incomplete adjuvant) at 4 week intervals. The antiserum was made from blood collected 3 weeks after the last booster and was stored at −20 °C in 50% glycerol.

The antiserum was tested against the 30K protein synthesized in vitro under the direction of an artificial mRNA preparation made from TMV particles by partial decapsidation with 1% SDS at pH 7.5 (15 min at 37 °C), followed by repeated zonal centrifugation to select particles which were 50% to 60% stripped (A260/A280 1.45 to 1.52), and then RNase treatment and phenol extraction (PSV-RNA, average Mr 900000; Nitschko, 1987). PSV-RNA (30 μg/ml) was translated in a wheatgerm extract containing [35S]methionine as described by Godefroy-Colburn *et al.* (1985). After translation, the reaction mixture (70 μl) was treated with 10 μg/ml RNase in 20 mM-EDTA for 30 min at 37 °C (Berna *et al.*, 1985) and then adjusted to 500 mM in NaCl and centrifuged at 12000 g for 30 min. SDS–PAGE analysis (not presented) showed that the pellet contained about 50% of the 30K protein, but less than 10% of the wheatgerm proteins. The pellet was redissolved in 70 μl of electrophoresis sample buffer (ESB: 4.5% SDS, 9 M-urea and 7.5% 2-mercaptoethanol in 75 mM-Tris–HCl pH 6.8) at 80 °C for 5 min. This procedure eliminated a 30K wheatgerm protein which reacted non-specifically with the antiserum (not shown). A blank translation medium without added mRNA was similarly treated and used as a negative control. The samples were analysed by SDS–PAGE in 9% polyacrylamide (Laemmli, 1970) and blotted onto nitrocellulose (Towbin *et al.*, 1979), for 1·5 h at a constant current of 2 mA/cm². The efficiency of transfer of the 30K protein was 80 to 90% (data not shown). Indirect immunoreactions on blots using peroxidase-conjugated sheep IgG against rabbit IgG as the second antibody, quantification of radioactivity in gels and on blots, and reflection densitometry of the immunoreactions were as described by Berna *et al.* (1986). The molar amount of 30K protein present in the 35S-labelled samples was derived from the radioactivity recovered from the 30K protein bands after SDS–PAGE, and the knowledge that this protein contains 10 methionine residues (Goelet *et al.*, 1982).

The immunoblot of 35S-labelled products (Fig. 1, lanes 2 and 8) showed one major band which corresponded to radioactive 30K (lane A) and which was absent from the blank translation medium (lane 1). The 29K and 23K translation products (thought to originate by initiation at the second and third AUGs of the 30K cistron respectively; Goelet *et al.*, 1982; Nitschko, 1987) were also detected. In addition, the antiserum reacted faintly with a few wheatgerm proteins, present in both lanes 1 and 2. The 30K, 29K and 23K bands were absent from the immunoblots when the antiserum had been preabsorbed with the uncoupled nonapeptide, proving the specificity of the reaction (lane 8'). In contrast, the reaction with wheatgerm proteins was not affected by serum preabsorption. Densitometry showed that, under the conditions used, the intensity of the 30K-specific reaction on immunoblots was proportional to the amount of radioactive 30K transferred; the highest amount tested was 0·15 pmol, i.e. 4.5 ng per lane (data not presented).

The anti-peptide serum was used to assay the 30K protein in subcellular fractions of TMV-inoculated leaves. Six-week-old tobacco plants (*N. tabacum* cv. Samsun or Samsun NN) were inoculated with common strain TMV (purified according to von Wechmar & van Regenmortel, 1970) at 10 μg/ml and were kept at 22 °C with illumination for 16 h each day. Typically, 200 to 300 lesions/leaf were obtained on cv. Samsun NN under these conditions. Subcellular fractions were prepared essentially as described by Godefroy-Colburn *et al.* (1986). The inoculated leaves from three plants (7 g) were chopped in 7 ml of grinding buffer (GB; 10 mM-KCl, 5 mM-MgCl₂, 0.4 M-sucrose, 10% glycerol, 10 mM-2-mercaptoethanol in 100 mM-Tris–HCl pH 8.1 at 4 °C), and
Fig. 1. Immunoassay of the 30K protein of TMV in a wheatgerm translation medium and in subcellular fractions prepared from leaves of *N. tabacum* cv. Samsun. The samples contained the pelletable fraction from 10 μl of wheatgerm translation medium incubated with no added mRNA (lane 1) or with PSV-RNA (lanes 2 and 8/8', containing approximately 4.5 ng of 35S-labelled 30K protein); Pe-30 (lane 3) and cell wall fractions (lane 4) obtained from 150 mg of healthy tobacco leaves; equivalent amounts of Pe-30 (lanes 5 and 5'), Triton extract (lanes 6, 9 and 9'), cell wall fraction (lanes 7 and 7'), Pe-1 (lanes 10 and 10') and S-30 (lanes 11 and 11') of tobacco leaves taken 50 h after inoculation with TMV. Immunoblots were carried out with the anti-nonapeptide serum diluted 1000-fold, either untreated (unprimed lanes) or preabsorbed with the peptide (20 min at room temperature with 6 μg of peptide and 12 μl of phosphate-buffered saline per μl of undiluted serum; primed lane numbers). Reaction with the peroxidase substrate was for 1 h for blots 1 to 8, 5', 7' and 8' and for 5 h for other blots. Lane A is the autoradiogram of lane 2. Arrows point to the major translation products of PSV-RNA. The closed arrowheads adjacent to lanes 9, 10 and 11 indicate the position of the 30K protein in marker Pe-30 samples analysed in the same gels. The open arrowhead in lane 11' points to a non-specific reaction with a host protein.
Fig. 2. Time course of accumulation of the 30K protein in the crude membranes and in the cell wall fraction prepared from TMV-infected leaves of $N.\ tabacum$ cv. Samsun and Samsun NN. Two series of TMV inoculations were made on a batch of cv. Samsun (panels a to c) and on two batches, respectively of cv. Samsun and cv. Samsun NN, treated in parallel (panels d to f). Subcellular fractions of inoculated leaves were prepared at the times after inoculation (h) indicated over the tracks. The coat protein (CP) was assayed in 2 μl of S-30 (corresponding to 0.7 mg of leaf material) by immunoblotting from a 13% gel with an antiserum to the TMV coat protein diluted 20,000-fold (panels a and d). The 30K protein was assayed in the Pe-30 (panels b and e) and cell wall fractions (panels c and f) as in Fig. 1.
filtered by centrifugation through 80-mesh nylon cloth. The filtration residue was washed with 3.5 ml of GB and the combined extracts fractionated into a 1000 g pellet (Pe-1), a 30000 g pellet (Pe-30) and a 30000 g supernatant (S-30). The residue from the second extraction (1.4 g) was extracted twice with 1.4 ml of GB containing 2% Triton X-100, giving the Triton extract, and finally with 2.8 ml of ESB at 80 °C for 5 min, giving the cell wall fraction.

The subcellular fractions prepared from TMV-inoculated Samsun leaves harvested 50 h post-infection (p.i.) were immunoblotted in parallel with similar fractions from healthy leaves. A band with the mobility of the 30K marker was present in Pe-30 (Fig. 1, lane 5) and cell wall fraction (lane 7) of infected leaves, but was absent from the corresponding healthy samples (lanes 3 and 4). It was eliminated by serum preabsorption with the peptide, a further proof of its viral origin (lanes 5' and 7'). Several additional bands in lanes 5 and 7 were not eliminated by serum preabsorption and were therefore non-specific. A band with the mobility of the 30K protein was seen on the immunoblots of the Triton extract, faintly under normal test conditions (lane 6) and more intensely after prolonged incubation with the peroxidase substrate (lane 9, closed arrowhead), but it originated from the host because it was also present in healthy samples (not shown) and resisted serum preabsorption (lane 9'). Faint bands, only detectable under the most sensitive test conditions, were visible at the equivalent positions in the blots of Pe-1 (lane 10) and S-30 samples (lane 11) and could be due in part to the viral protein because their intensity was significantly reduced (lane 10') or abolished (lane 11') after serum preabsorption. These traces of 30K may have originated from contamination by some components of the Pe-30 or cell-wall fractions. In addition, a 29K protein of S-30 reacted with the antiserum (lane 11) but was not virus-related because the reaction resisted serum preabsorption (lane l 1', open arrowhead) and because it was also visible in the healthy samples (not shown).

Thus, after 50 h of systemic infection, the 30K protein was found mainly in the Pe-30 and cell wall fractions of inoculated leaves. This finding agrees with the observations of Tomenius et al. (1987) and of Deom et al. (1987) but differs from those of Watanabe et al. (1984) who found the 30K protein in a nuclear pellet from infected protoplasts. The latter discrepancy may be due to the difference in starting materials or the presence of residual cell wall fragments in the nuclear fraction used by Watanabe et al.

The kinetics of accumulation of the 30K protein in the Pe-30 and cell wall fractions were followed for 8 days, in parallel with accumulation of the coat protein in S-30. The coat protein (Fig. 2a) was first detected after 30 h and accumulated at a maximum rate between 48 and 71 h p.i., to reach a final level of 1 mg/g of leaves (as judged from densitometry of Fig. 2a, not shown). In Pe-30 (Fig. 2b), the 30K protein was first detected 30 h p.i., its amount increased to a maximum (48 to 71 h p.i.) and then gradually decreased until it was almost undetectable at 119 h p.i. In the cell wall fraction (Fig. 2c), the amount of 30K protein present followed a similar time course for the first 95 h of infection and then remained unchanged until the end of the experiment (191 h p.i.). The behaviour of the 30K protein in cv. Samsun was thus qualitatively similar to that of ALMV P3 in cv. Xanthi nc (Godefroy-Colburn et al., 1986). The similarity was also quantitative: by comparing the intensity of the 30K band in immunoblots of cell wall fractions prepared from three batches of plants 48 to 72 h p.i. with that in marker lanes containing known amounts of translation products, the maximum amount of 30K in cell walls was estimated at 10 to 20 ng/g of leaves and was thus of the same order of magnitude as the amount of P3 in cell walls of ALMV-infected leaves (40 ng/g at 25 °C; Godefroy-Colburn et al., 1986). The maximum amount of 30K protein in intracellular membranes occurred much later in TMV-infected leaves (48 to 71 h p.i.) than in TMV-infected protoplasts (2 to 9 h p.i., Watanabe et al., 1984; 8 to 16 h p.i., Kiberstis et al., 1983). This discrepancy is certainly because virus infection in whole plants (as opposed to that of protoplasts) is not synchronous, the level of infection measured in leaves being a steady-state value which depends on the rate of virus spread under the conditions of the infection. Spreading rate differences may also explain the discrepancy between our results and those of Joshi et al. (1983), which were obtained in leaf discs treated with antibiotics.

In a comparison of N. tabacum cv. Samsun and cv. Samsun NN, plants were inoculated together, grown together and sampled for 3 days. For the first 40 h of infection, i.e. before any
lesion appeared on Samsun NN leaves, viral coat protein was synthesized at the same rate in the two cultivars (Fig. 2d) confirming the observations of Konate et al. (1982). During this period, accumulation of the 30K protein in Pe-30 (Fig. 2e) and in the cell wall fraction (Fig. 2f) was similar in the two hosts and the electrophoretic mobilities of the immunoreactive material were identical.

Differences in the accumulation of the 30K protein became manifest 48 h after infection, when necrotic spots were approximately 0.5 mm in diameter and coat protein synthesis had stopped in cv. Samsun NN (Fig. 2d). As expected from the arrest of virus multiplication, the 30K protein stopped accumulating in Pe-30 and gradually disappeared (Fig. 2e). It also stopped accumulating in the cell wall fraction but, instead of remaining for several days as it did in the systemic host (Fig. 2c), the 30K protein had completely disappeared from this fraction 65 h after infection (Fig. 2f). Essentially similar results were obtained in three replicates.

The disappearance of cell wall-bound 30K protein in Samsun NN has a number of possible causes. (i) Transport of the 30K protein through the normal excretion route may be impaired during necrogenesis. (ii) The necrotic response may prevent binding of the 30K protein by modifying a receptor, for example by lignification (van Loon, 1983). (iii) The necrotic response may induce proteases (van Loon, 1983) which could degrade the 30K protein. (iv) The 30K protein may become sequestered by cross-linking to a wall constituent, e.g. through isodityrosine bridges (shown to exist in extensin; Fry, 1982; Cooper & Varner, 1983), which would render the 30K protein resistant to extraction by SDS and urea. (v) Chemical modification(s) of the 30K protein by enzymes induced during necrogenesis may also destroy the antigenic sites, making immunodetection impossible.

Whatever the mechanism, actual or functional loss of wall-bound 30K protein would impair its function as a viral spreading factor, and this might explain why TMV becomes localized in cv. Samsun NN. Localization of the virus would thus be a side-effect of the hypersensitive response, but would not necessarily occur with the same efficiency in all the hypersensitive hosts. That this is actually the case is shown by a comparison of the hypersensitive reactions to TMV of N gene- and N' gene-carrying tobacco cultivars (van Loon, 1983). It is conceivable that the degree of localization of TMV would depend on the balance between the rate of synthesis or cell wall incorporation of the spreading factor (whichever is limiting) and its rate of loss. This hypothesis agrees with the recent finding of Watanabe et al. (1987) that the virulence of several strains of TMV is related to the rate of synthesis of the 30K protein in infected cells.

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