Virus-specific proteins in cells infected with tomato black ring nepovirus: evidence for proteolytic processing in vivo

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The synthesis of proteins encoded by the RNA of tomato black ring virus (TBRV) in vivo was studied in protoplasts by direct labelling with [35S]methionine, and in protoplasts and plants by immunoblotting experiments with specific antisera. Comparison of the proteins synthesized in infected and mock-inoculated protoplasts suggested that proteins of Mr 120K, 90K, 80K, 57K and 46K were virus-specific. The proteins derived from the RNA-1-encoded polyprotein detected by immunoblotting were a stable 120K protein and, only in protoplasts, small amounts of a 90K protein which contains the C-terminal part of the 120K protein and the polymerase domain. The results suggest that the polymerase and the adjacent protease function in vivo largely or solely when combined in a 120K protein. The proteins derived from the RNA-2-encoded polyprotein detected by immunoblotting were 59K and 57K proteins, which reacted with antiserum to TBRV particles, and a 46K protein. In extracts of infected Nicotiana clevelandii and Chenopodium quinoa made soon after inoculation, the 59K protein was more abundant than the 57K protein; later samples contained similar quantities of each protein. The 57K protein comigrated with protein extracted from virus particles. The results of amino acid sequencing suggested that the 57K protein is derived from the 59K protein by the loss of nine C-terminal amino acids. Antiserum to a peptide adjacent to the 57K protein in the 150K polyprotein detected a 46K protein in protoplasts and plant tissue. The results support the processing scheme for TBRV polyproteins proposed after analysis of the products of in vitro translation.

Introduction

Tomato black ring nepovirus (TBRV) has a bipartite genome: RNA-1 [7359 nucleotides + poly(A)] encodes a polyprotein with an M, of 250K (Greif et al., 1988) and RNA-2 [4662 nucleotides + poly(A)] encodes a polypeptide of 150K (Meyer et al., 1986). When TBRV RNAs are translated in vitro, each polyprotein is cleaved by a proteolytic activity contained in the RNA-1-encoded 250K polyprotein (Demangeat et al., 1990, 1991). All the proteins detected in vitro could be located within the polyproteins and a genetic organization was proposed (Demangeat et al., 1990, 1991) which resembled that predicted from the nucleotide sequences (Greif et al., 1988) (Fig. 1). Of the three proteins produced in vitro by cleavage of the 150K polyprotein, the C-terminal protein corresponds to the viral coat protein. However, this protein migrates slightly more slowly than that extracted from purified virus particles and is called the 59K protein (Demangeat et al., 1991); previous estimates of the Mr of TBRV coat protein (Randles et al., 1977), including the protein detected in earlier experiments on TBRV-infected protoplasts (Fritsch et al., 1978), were 57K. Another unusual feature found by Demangeat et al. (1990) was that although the 120K cleavage product of the 250K polyprotein contains both the region thought to be a protease and the region thought to be the virus polymerase, it is never cleaved into these products in vitro.

These results suggested either that the cleavages observed in vitro are incomplete or that the cleavage of the polyproteins in vivo differs from that observed in vitro. This paper describes experiments designed to determine the occurrence, in TBRV-infected protoplasts and in leaf tissues, of cleavage of the 250K polyprotein to yield the 120K and 90K proteins, and cleavage of the 150K polyprotein to yield the coat and 46K proteins.

Methods

Virus purification. Particles of isolate S of TBRV (Fritsch et al., 1984) were purified from systemically infected leaves from Nicotiana clevelandii plants as described by Fritsch et al. (1978). Some virus
proteolytic maturation. Numbers indicate the positions of the terminal sequences of the synthetic peptides T, P and C. Polyproteins are assessed by the ability to take up and hydrolyse fluorescein diacetate, cv. Xanthi plants and inoculated following the procedure of Barker & Harrison (1977). The protoplasts were kept at 20 °C under constant illumination at 3000 lux. After culture for 48 h, protoplast viability, was >70%; more than 70% of inoculated protoplasts were deemed to
ate-conjugated antibody to TBRV particles.

Analysis of viral proteins by immunoblotting. After separation by SDS-PAGE the proteins were electroblotted to a nitrocellulose sheet (Schleicher & Schuell) for 90 min at 400 mA essentially as described by Towbin et al., (1979). Blots were then reacted with a 1:1500 dilution of antiserum followed by a 1:2000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Niesbach-Klösgen et al., 1990). The specificity of the reactions was assessed by mixing the antiserum with an excess of the specific immunogen prior to the reaction and observing competitive inhibition.

Detection of N- and C-terminal amino acid of the coat protein. To determine the N terminus, 2 μg of CsCl-purified virus particles in 60 mM-sodium phosphate buffer pH 6.8 was dissociated by heating at 100 °C for 3 min in 80 mM-Tris-HCl pH 8.5, 5% SDS and 12% 2-mercaptoethanol. After SDS-PAGE the coat protein was electroblotted to an Immobilon PVDF membrane as described by the manufacturer (Millipore) using a semi-dry blotter. The piece of membrane corresponding in position to the coat protein was then directly subjected to automated Edman degradation using an Applied Biosystems 470 A protein sequencer equipped with a PTH 120A analyser (Hewick et al., 1981).

To determine the C terminus, the region of the gel containing the coat protein was cut out and the coat protein was electroeluted in a dialysis bag overnight at 180 V in 25 mM-Tris, 192 mM-glycine, 0.1% SDS buffer and concentrated to approximately 500 μg/ml by ultrafiltration in a Centricon 30 microconcentrator. Protein was digested with carboxypeptidase A (Sigma) at an enzyme:protein ratio of 1:30 in 0.1 M-5-methylmorpholine pH 8 for 2 h at 37 °C. The amino acid content was analysed by using a 420 A-130 A derivatization and analysis system (Applied Biosystems).

**Results**

Detection of infection-specific polypeptides by \([^{35}S]methionine labelling\)

In preliminary experiments, protoplasts were inoculated with TBRV, or mock-inoculated, and cultured in the presence of \([^{35}S]methionine\). After electrophoresis some polypeptides were detected in samples of infected but not mock-inoculated protoplasts (Fig. 2a); these had Mr,s of 120K, 90K, 80K, 57K and 46K. In protoplasts sampled 12 h (Fig. 2b), rather than 6 h (Fig. 2a), after adding methionine, the 120K and 57K polypeptides were evident, the 90K polypeptide was obscured by a similarly sized polypeptide in protein from mock-inoculated protoplasts, and the 80K and 46K polypeptides were not detectable. In further experiments, antiseras specific to virus genome-encoded proteins were used to detect viral proteins.

Serological detection of the 120K polypeptide

Protein extracted from infected protoplasts and from infected *C. quinoa* tissue contained a 120K polypeptide
TBRV proteins detected in vivo

Fig. 3. Immunoblot detection of infection-specific polypeptides in protoplasts by using anti-peptide P (a) and anti-peptide C sera (b). Protein was extracted from 2 x 10⁴ mock-inoculated (lanes 1) or TBRV-infected (lanes 2) protoplasts. Lanes 3 and 4 contain samples as for lanes 1 or 2 but were subjected to competitive inhibition with homologous peptide. Numbers to the left indicate the positions and sizes of Mr markers (phosphorylase B, ovalbumin and carbonic anhydrase). Numbers in the centre indicate the 120K and 90K TBRV-specific proteins.

which reacted in immunoblots with anti-peptide P and anti-peptide C sera (Fig. 3). A small amount of a polypeptide of 90K was also detected with anti-peptide P serum, but not with anti-peptide C serum. The polypeptides were absent from protein from mock-inoculated protoplasts and the reaction between the 120K polypeptide and each antiserum was inhibited when peptide C or peptide P was added to the respective antiserum during incubation with the blots (Fig. 3). Therefore the 90K protein corresponds to the C-terminal of the 120K protein obtained by in vitro translation of TBRV RNA (Demangeat et al., 1990). A 120K polypeptide, but not a 90K polypeptide, was also specifically detected in protein from infected C. quinoa tissue. To determine the kinetics of the appearance of the 120K protein and its distribution among subcellular fractions, C. quinoa tissue was taken at intervals after inoculation and fractionated prior to preparing protein samples. The 120K polypeptide was readily detected with anti-peptide P serum in the soluble fraction of samples taken 5 days p.i. (Fig. 4, lane 3). Small amounts were present in this fraction 3 days p.i. (Fig. 4, lane 2), but none was detected in either

Fig. 4. Immunoblot detection of infection-specific polypeptides in the soluble fraction extracted from 2 mg (fresh weight) C. quinoa tissue by using anti-peptide P serum. Lane 1, healthy tissue; lane 2, infected leaves 3 days p.i.; lane 3, infected leaves 5 days p.i.; lane 4, infected leaves 11 days p.i.; lane 5, sample as in lane 3 but after competitive inhibition with peptide P. Labelling as in Fig. 3, plus myosin (200K).
Fig. 6. Immunoblot detection of infection-specific polypeptides with anti-peptide T serum. Samples were extracted from protoplasts 48 h p.i. or from the 30000 g pelleted fraction of 2 mg (fresh weight) of *C. quinoa* leaves. Lane 1, mock-inoculated protoplasts; lane 2, TBRV-infected protoplasts; lane 3, as in lane 2 but after competitive inhibition with peptide T; lane 4, infected leaves 3 days p.i.; lane 5, infected leaves 5 days p.i.; lane 6, infected leaves 11 days p.i.; lane 7, leaves from healthy plants; lane 8, as in lane 5 but after competitive inhibition with peptide T. Numbers to the left indicate markers as in Fig. 3. Number to the right indicates the infection-specific 46K protein.

Fig. 5. Immunoblot detection of TBRV coat protein in infected *N. clevelandii* leaves. Protein was extracted from the soluble fraction of 2 mg (fresh weight) of *N. clevelandii* leaves sampled at various times after inoculation. Lane 1, healthy leaf tissue; lane 2, protein from purified virus particles; lane 3, infected leaves 3 days p.i.; lane 4, infected leaves 5 days p.i.; lane 5, infected leaves 11 days p.i. Numbers to the left indicate marker proteins as in Fig. 3. Numbers to the right indicate the immunoreactive 59K and 57K proteins.

荏 sedimentable fraction (data not shown) at any time after inoculation. Essentially similar results were obtained when using anti-peptide C serum.

Serological detection of coat protein

Coat protein is readily detected in infected protoplasts or infected leaves by a variety of serological methods. When protein was extracted from *N. clevelandii* leaves at various times and immunoblotted with antiserum to virus particles, several polypeptides were detected (Fig. 5). Those of 59K and 57K were not present in healthy tissue (Fig. 5, lane 1); the 57K polypeptide comigrated with protein extracted from purified virus particles (Fig. 5, lane 2). In samples extracted from leaves 3 days or 5 days p.i. (Fig. 5, lanes 3 and 4), the 59K polypeptide was predominant, but later samples contained the 57K and 59K polypeptides in approximately equal amounts (Fig. 5, lane 5). Similar results were obtained with leaves from TBRV-infected *C. quinoa* plants.

Serological detection of 46K protein

Immunoblots of protein from TBRV-infected protoplasts reacted with anti-peptide T serum detected a single 46K polypeptide (Fig. 6, lane 2); none was detected in mock-inoculated protoplasts (Fig. 6, lane 1) and the reaction was abolished by the addition of peptide T during the incubation with the antiserum (Fig. 6, lane 3). Similar results were obtained with protein from inoculated leaves from *C. quinoa* plants. Fractionation of the tissue followed by immunoblotting showed that all the reactive 46K polypeptide sedimented at 30000 g or less. Approximately equal amounts were detected in the crude material pelleted at 1000 g and in the fraction pelleted at 30000 g. The maximum amount of 46K was detected 5 days p.i. (Fig. 6, compare lane 5 with lanes 4 and 6). In parallel samples analysed by immunoblotting with antiserum to virus particles, the amount of 57K coat protein detected was much greater 11 days p.i. than 5 days p.i.
Determination of the N and C termini of the 57K coat protein

The sequence deduced for the 10 N-terminal residues of the particle protein was (NH₂)A-G-G-S-Y-A-F-G-E-T. The unambiguous C-terminal sequence of the particle protein was A-T-V(COOH). Therefore the sequence of this protein is between residues 838 and 1348 of the 150K polyprotein sequence (of 1357 amino acids) described by Meyer et al. (1986) and its calculated Mr is 55888. However, for consistency with previously published work we will continue to designate this protein as 57K.

Discussion

The results show that the proteolytic cleavages of the polyproteins encoded by TBRV RNAs observed in vitro (Demangeat et al., 1990, 1991) are the same as, or very similar to, those which occur in vivo. The RNA-1-encoded 250K polypeptide yields a 120K protein, which contains polymerase and protease domains (Greif et al., 1988), and the RNA-2-encoded 150K polypeptide yields coat protein and a 46K protein. However, we were unable to detect a protein corresponding to the N-terminal portion of the 150K polyprotein in immunoblotting experiments with an antiserum to a synthetic peptide from this region of the polyprotein, but this antiserum did not detect 150K polypeptide in in vitro translation products and therefore may not have been very active.

Cleavage of the 120K protein to give a 90K product was observed only in protoplasts and then at a relatively low level. Although we cannot exclude the possibility that the 90K protein is unstable and/or barely detectable, the simplest interpretation of this result is that this cleavage is uncommon and not related to an important viral function. It is thought that the protease domain in the 120K protein is responsible for cleaving the polyproteins and, assuming that the proteins observed are significant functional species and not inactive by-products, the results suggest that the protease can function while joined to a functional polymerase. During the processing of the polypeptide of poliovirus it is known that the 3CD protease–polymerase efficiently cleaves the P1 precursor to release capsid protein (Jore et al., 1988; Ypma-Wong et al., 1988).

The results with TBRV-infected cells resemble those obtained with cells infected with cowpea mosaic virus (CPMV). The polymerase and protease domains in the polypeptides encoded by CPMV B RNA (analogous to TBRV RNA-1) are also found in vivo in a single protein of 110K (Dorsers et al., 1984). However, in vitro translation products of CPMV RNA include the separate constituent 87K and 24K proteins as well as the intact 110K species (Goldbach & Rezelman, 1983). Although the 120K TBRV protein resembles the CPMV 110K protein functionally, it is not found in association with membranes (as is the 110K protein) but remains in the soluble fraction when centrifuged at 30000 g. This may reflect differences in the mode of action of the two proteins or it may indicate that structures associated with TBRV replication are unstable during the fractionation procedures used.

In vitro translation of TBRV RNA yields a 59K product which contains the coat protein but migrates slightly more slowly than protein extracted from virus particles (Demangeat et al., 1991). The results presented in this paper show that a similar 59K product is synthesized in infected leaves soon after inoculation, but that a 57K product which comigrates with particle protein appears at later times after inoculation. When virus preparations were examined during purification, the particle protein fraction from partially purified virus contained both a 57K and a 59K protein, whereas that from highly purified virus contained only the 57K protein. Previously reported estimates of the Mr of TBRV coat protein were probably made using such highly purified material.

An explanation for the appearance of two species of coat protein was obtained from the determination of the terminal amino acid sequences of the 57K coat protein. The N-terminal sequence corresponds to a protein commencing at amino acid 838 (as described by Demangeat et al., 1990) and the C-terminal sequence corresponds to a sequence that ends at amino acid 1348, nine amino acids upstream of the end of the 150K polyprotein. Thus the 59K protein may contain the sequence between amino acid 838 and the end of the 150K polyprotein, and the 57K protein may arise from it by the loss of the C-terminal amino acids. It is known that proteases can remove amino acids from the C termini of the coat proteins of tobacco mosaic virus (Harris & Knight, 1952), potato virus X (Koenig et al., 1978) and potyviruses (Shukla et al., 1988). Presumably, as with these viruses, the C-terminal amino acids of TBRV coat protein protrude from the virus particle surface and can be removed without disrupting the virion. This exposed detachable fragment may play a significant role in TBRV biology as, for example, does the protruding N-terminal fragment of potyvirus coat proteins in their transmission by aphids (Atreya et al., 1990). The C-terminal sequence of the 57K protein (PLTIPATV) is very similar to that of the 150K polyprotein of grapevine chrome mosaic nepovirus (GCMV) (PLTIPS; Brault et al., 1989). Perhaps GCMV, which is related serologically to TBRV, differs from it in a property mediated by the protruding C terminus,
perhaps in vector specificity; however, the vector of GCMV is not known.

Some sequence homologies were detected between part of the TBRV 46K protein and putative movement proteins of other viruses (Meyer et al., 1986); the analogous 48K protein in the polyprotein encoded by CPMV RNA plays a role in virus movement (Wellink & van Kammen, 1989). The TBRV 46K protein resembles the CPMV 48K protein in that both are found in association with membranous material. However, the 48K protein is found in the culture medium whereas the 46K protein is not. The 46K protein decreases in abundance later than 5 days after inoculation and is presumably either degraded or sequestered in an unextractable form.

In summary, the production of TBRV-encoded proteins in infected protoplasts and plants has confirmed the model of genome expression derived from the products of in vitro translation. Moreover, the similarity of the expression of the TBRV genome to that of the CPMV genome reinforces the similarities between nepoviruses and comoviruses deduced from the results of nucleotide sequencing studies (Wellink & van Kammen, 1988). Functions have been assigned to the TBRV proteins by analogy, sometimes at third hand, with proteins of other viruses, but none has yet been assigned experimentally except for that of the coat protein. Moreover, even with this well understood protein the results shown in this paper suggest that there may be unsuspected subtleties in the way in which it functions.

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References


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