Synthesis of Disease-Associated Proteins in Viroid-Infected Tomato Leaves and Binding of Viroid to Host Proteins

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ABSTRACT

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Comparison of protein synthesis from uninfected and potato spindle tuber viroid (PSTV)-infected tomato leaves showed that three proteins of 14, 33, and 90–100 kDa were enhanced in infected tissue. The 33 kDa protein was the most prominent. These proteins were distributed in soluble subcellular fractions. A 33 kDa protein that bound PSTV in vitro was present at detectable levels in the soluble fractions from infected but not uninfected cells. Both the infected and uninfected fractions also contained PSTV-binding proteins of 52–55, 35, 31–32, and 16–20 kDa. With the exception of the 35 and 33 kDa proteins, all the other PSTV-binding proteins also were detected in in vivo complexes of PSTV with subcellular constituents obtained by density-gradient fractionation of 20,000–70,000 *g* pellet fractions from infected cells. The PSTV-binding proteins in the complexes are of host origin. Treatment of the purified PSTV-cellular constituent complexes with pronase or triton-X-100 released PSTV; thus PSTV appears to be noncovalently bound to proteins in these complexes.

Additional keywords: soluble host proteins, viroid cellular constituent complexes

Viroids, the smallest known autonomously replicating pathogens, are linear or covalently closed single-stranded RNA molecules of 246–375 nucleotides that lack mRNA activity (5). Consequently, viroid replication seems to depend on host enzymes and direct interaction between the infecting RNA and host proteins, nucleic acids, and/or other components. Comparison of protein species from uninfected and potato spindle tuber viroid (PSTV)-infected tomato leaves, as judged by their staining after separation by gel electrophoresis, showed that the amounts of a 14 kDa protein and a 140 kDa protein (consisting of two 70 kDa subunits) were enhanced in infected as compared with uninfected leaves (7,12). These proteins accumulated, significantly, within 1–2 wk after symptom appearance. Proteins from tomato leaf nuclei recently have been found to form complexes with PSTV in vivo (22). The nuclear PSTV-binding proteins have been identified as 41 kDa protein, 31 kDa protein, and histones. No information, however, has been given on the possible occurrence of PSTV complexes or PSTV-binding proteins with other cellular components.

This paper reports the in vivo enhancement of certain proteins and the occurrence of PSTV-binding proteins in extracts of viroid-infected tomato leaf tissue. I present evidence that the synthesis of 14, 33, and 90–100 kDa proteins is enhanced in PSTV-infected tomato leaves and that the 33 kDa protein is predominant. In addition, I show that a PSTV-binding protein of 33 kDa occurs at detectable levels in infected tomato leaf cells. Preliminary results of this work have been published (8,9).

MATERIALS AND METHODS

Preparation of subcellular fractions. Leaf tissue from PSTV-infected or uninfected tomato plants (*Lycopersicon esculentum* Miller 'Rutgers') was homogenized or ground in buffer containing 1 M sucrose, 10 mM Tris-Cl (pH 7.5), 5 mM MgCl2, and 10 mM 2-mercaptoethanol (17). The extract was filtered (17), and the filtrate was centrifuged at 1,000 *g* for 10 min. Nuclei were purified from the pellets (17). The supernatant was centrifuged at 20,000 *g* for 20 min. This supernatant was centrifuged at 70,000 *g* for 2 hr. The 70,000 *g* supernatant was saved (hereafter referred to as the soluble fraction). The 70,000 *g* pellets were suspended in a buffer containing 10 mM Tris-Cl (pH 7.5) and 5 mM MgCl2 and layered onto a discontinuous gradient of 20–60% sucrose (w/v) in the same buffer. The gradients were centrifuged for 30 min at 35,000 rpm in a Spinco SW 41 rotor. Ten fractions were collected and their contents were concentrated eightfold by ethanol precipitation. Five-microliter aliquots of each sample were bound to nitrocellulose membranes for analyses by dot hybridization (20) using ^{32}P-labeled, nick-translated, cloned PSTVcDNA (11). PSTV was distributed in most fractions but was concentrated in two fractions that sedimented rapidly through the gradient (hereafter referred to as PSTV-cellular constituent complexes).

RNA isolation, gel electrophoresis, and transfer. RNA was prepared from the density-gradient fractions or the 70,000 *g* soluble fractions according to the method described by Hadidi, Hashimoto, and Diener (11), separated by gel electrophoresis, and transferred electrophoretically to diazobenzyloxymethyl paper (11).

In vivo labeling of plant proteins. Infected leaves were collected from plants showing systemic PSTV symptoms for 1 or 2 days, and healthy leaves were taken from plants of the same age. At this stage of PSTV symptom appearance, tomato leaf cells are known to synthesize PSTV at a very high rate (10–12). Radioactive labeling of leaf proteins with L-^{35}S-methionine (1106 Ci/mM, NEN) was carried out by uptake of the radioactive material through the petioles (0.5 mCi/4 leaves [2 g] for 5 hr). Proteins were extracted from labeled leaves immediately after the labeling period.

Extractions and gel electrophoresis of proteins. To obtain total proteins, leaf tissue was homogenized and clarified according to the method described by Cremer and Van DeWalle (4), and the proteins were extracted from the clarified homogenate, as described by White and Brakke (21). Proteins were also extracted from labeled or unlabeled subcellular fractions (21). Proteins were separated by electrophoresis on linear 5–20 or 10–20% (w/v) sodium dodecyl sulfate-polyacrylamide gel, as described by

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Laemmli (14). Gels containing labeled proteins were then prepared for fluorography according to the procedure explained by Chamberlain (3).

Protein transfer, binding of PSTV to proteins, and detection of PSTV-bound protein. After electrophoresis, proteins were renatured (2) and blotted, in one direction, to nitrocellulose membranes (19). Purified PSTV was bound to the transferred proteins and then covalently cross-linked (22). PSTV that became covalently bound to proteins was detected by its hybridization under stringent conditions (18) to ^{35}P-labeled, SP6-generated cRNA transcripts of cloned PSTV cDNA (15). After hybridization, nitrocellulose membranes were washed under stringent conditions (18) at 65 C and then treated with RNase A in 0.36 M NaCl (15). Autoradiographic exposures of the washed membranes, using Kodak X-ray films and intensifying screens, were performed at −70 C.

RESULTS

In vivo synthesis of disease-associated proteins. The most prominent alteration previously detected in the protein pattern from PSTV-infected tomato leaf tissue, as judged by the intensity of their staining, is the enhancement in concentration of a 14 kDa protein and a 140 kDa protein that consists of two 70 kDa subunits (7,12). In the present work, synthesis of the 14 kDa protein also was significantly and consistently enhanced in the infected tissue (Fig. 1A, lanes 1 and 2; Fig. 1B, lanes 3 and 4). In addition, Figure 1 shows that, as a result of PSTV infection, the synthesis of disease-associated proteins of 33 kDa (Fig. 1A, lanes 1 and 2; Fig. 1B, lanes 3 and 4) and 92–100 kDa (Fig. 1C) was significantly enhanced in the 70,000 *g* soluble fractions. These or other disease-associated proteins were not present at detectable levels in the density-gradient fractions containing PSTV complexes (see below), or in nuclear fractions from the infected leaves (Fig. 1A, lanes 3 and 4; Fig. 1B, lanes 1 and 2).

PSTV-binding proteins in vitro. Several proteins capable of binding PSTV in vitro were found. Major PSTV-binding proteins of 72–74, 55, 52, and 28 kDa, and several 16–20 and 11–13 kDa were detected in total protein preparations from infected and uninfected tissue (Fig. 2A, lanes 1 and 2). PSTV-binding proteins of 52–55, 35, 31–32, and 16–20 kDa were detected in the soluble fractions from uninfected and infected cells (Fig. 2B, lanes 3 and 4). In addition, a PSTV-binding protein about 33 kDa was detected in the soluble fraction from infected but not uninfected cells (Fig. 2B, lanes 3 and 4, arrow). This protein is very similar in size and in electrophoretic mobility to the 33 kDa disease-associated protein detected in the same fraction from infected but not uninfected tissue (Fig. 1A, lanes 1 and 2; Fig. 1B, lanes 3 and 4). With the exception of the 35 and 33 kDa proteins, all the PSTV-binding proteins found in the soluble fractions were also very similar in size to those found in the complexes (Fig. 2A, lane 3; Fig. 2B, lane 1) or in the density-gradient fractionation of the 70,000 *g* pellet fractions from uninfected cells (Fig. 2B, lane 2).

As reported by Wolff et al (22), no signals of bound viroid were detected when PSTV was absent during the binding step, when the glutaraldehyde fixation was omitted, or when bovine serum albumin was fractionated. In addition, I found that PSTV does not bind to lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, β-galactosidase, or myosin. However, PSTV binds weakly to phosphorylase B and strongly to coat proteins of potato virus y and tobacco etch viruses.

In vivo complexes of PSTV with cellular constituents. Figure 3 (1A) shows the distribution of PSTV in density-gradient fractionated 70,000 *g* pellets from infected cells. PSTV was detected in most fractions; however, it was concentrated in two fractions that sedimented rapidly through the gradient (fractions 6 and 8, referred to as cellular constituent complexes). In contrast, when purified PSTV was added to healthy tissue before extraction

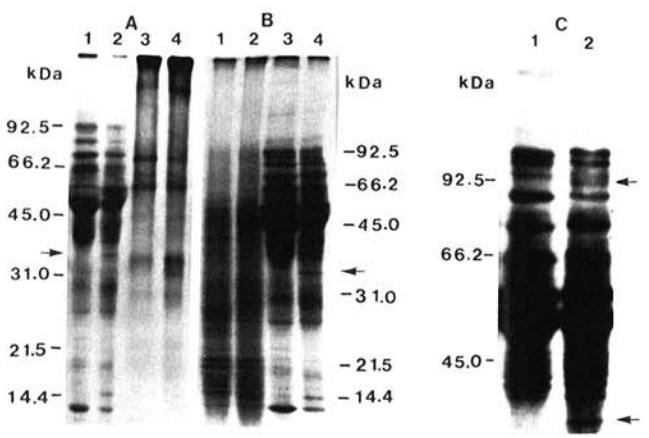

Fig. 1. Fluorographs of gels representing in vivo synthesized ^{35}S-labeled proteins in isolated subcellular fractions from uninfected and infected cells. Analysis was carried out on a 10–20% linear sodium dodecyl sulfate (SDS) polyacrylamide gel (**A** and **B**) or on a 5–20% linear SDS polyacrylamide gel (**C**). **A,** Proteins from the soluble 70,000 *g* fractions from uninfected and infected cells (lanes 1 and 2, respectively). Proteins from density-gradient fractionation of 70,000 *g* pellet from uninfected cells and from the PSTV-cellular constituent complexes (lanes 3 and 4, respectively). Approximately 12 × 10 3 cpm of labeled protein/sample were analyzed. **B,** Proteins from nuclear fractions from uninfected and infected cells (lanes 1 and 2, respectively). Proteins from the soluble 70,000 *g* fractions from uninfected and infected cells (lanes 3 and 4, respectively). Approximately 16 × 103 cpm of labeled protein/sample were analyzed. **C,** Proteins from the soluble 70,000 *g* fractions from uninfected and infected cells (lanes 1 and 2, respectively). Approximately 25 × 103 cpm of labeled proteins/sample were analyzed. The positions of molecular mass markers (kDa) are indicated. Arrows indicate the positions of the 33 and 92–100 kDa disease-associated proteins.

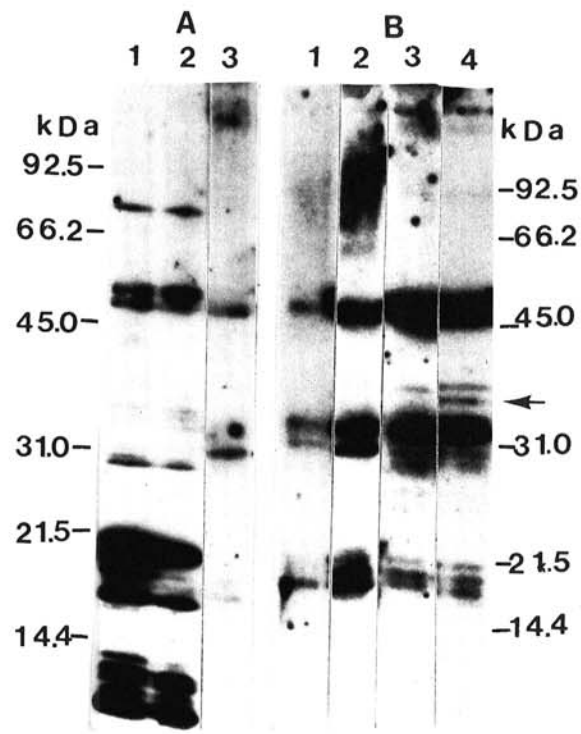

Fig. 2. Autoradiogram of proteins binding to PSTV in vitro. Purified PSTV was bound to electrophoretically separated proteins previously transferred to nitrocellulose membrane and probed with ^{32}P-labeled PSTV cRNA. **A,** Total proteins from uninfected and infected tomato leaf tissue (lanes 1 and 2, respectively). Proteins from PSTV-cellular constituent complexes (lane 3). **B,** Proteins from PSTV-cellular constituent complexes (lane 1). Proteins from density-gradient fractionation of 70,000 *g* pellet from uninfected cells (lane 2); fractions were obtained at the same position in gradient as those of PSTV complexes. Proteins from the 70,000 *g* supernatant fractions of uninfected and infected tissue (lanes 3 and 4, respectively). The positions of molecular mass markers (kDa) are indicated. Arrow indicates the position of the 33 kDa PSTV-binding protein.

and fractionation, the top fractions of the gradient contained most or all the PSTV (Fig. 3, IB).

To determine if there is a possible association of PSTV with proteins in the PSTV-cellular constituent complexes in vivo, the complexes were further purified three times by discontinuous density-gradient centrifugation and then treated with pronase, triton X-100, or RNase A. Each treated sample was then fractionated by density-gradient centrifugation and the resulting fractions were analyzed for the distribution of PSTV. PSTV was released from the purified cellular constituent complexes by pronase or triton X-100 treatment (Fig. 3, IIB and IIC, respectively). The RNase treatment in 0.36M NaCl digested all PSTV (Fig. 3, IID).

RNA extracted from PSTV-cellular constituent complexes and analyzed by Northern blot hybridization contains mainly unit-length ssPSTV (Fig. 4, lanes 1 and 3). On the other hand, RNA extracted from the soluble 70,000 *g* fraction from infected cells contains mainly unit-length dsPSTV (Fig. 4, lane 2). This latter RNA when denatured to ssRNA has the electrophoretic mobility of a unit-length PSTV marker (Fig 4, lane 4). The nondenatured RNA is resistant to RNase A in 0.36M NaCl (not shown).

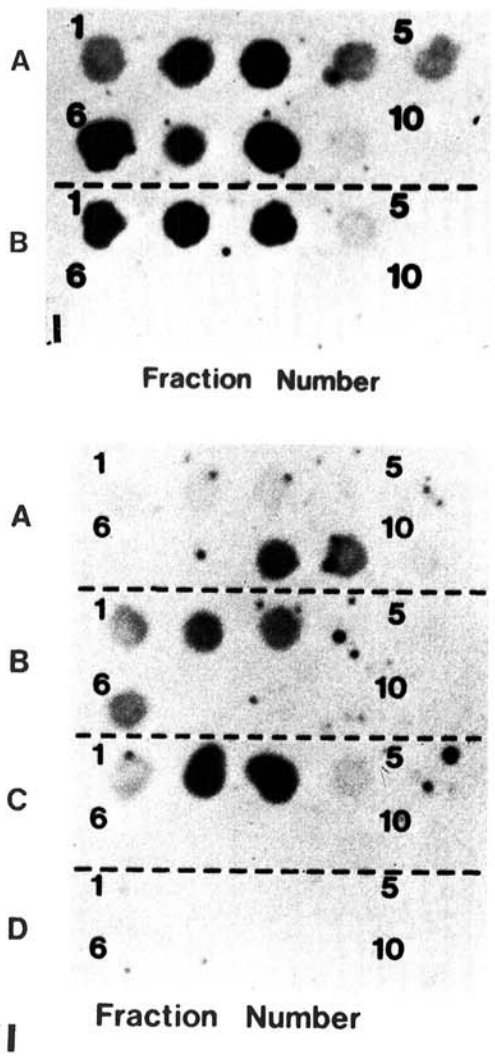

Fig. 3. I, Autoradiogram of dot blot hybridization analysis of PSTV-related RNA from density-gradient fractionated 70,000 *g* pellets. **A,** Fractions from infected tissue. **B,** Fractions from uninfected tissue plus added purified PSTV. The density-gradient fraction numbers 1 and 10 are the top and bottom fractions, respectively. **II,** Autoradiogram of dot blot analysis of the association of PSTV with purified cellular constituent complexes after different treatments. **A,** Untreated; **B,** Pronase treated; **C,** Triton X-100 treated; **D,** RNaseA treated. The density-gradient fraction numbers 1 and 10 are the top and bottom fractions, respectively.

DISCUSSION

The in vivo synthesis of disease-associated protein experiments clearly show that three proteins of 14, 33, and 90–100 kDa are associated with PSTV infection. These proteins are stimulated by PSTV infection and are not encoded by the viroid because PSTV is very small and could not code for proteins of the size observed. The stimulated proteins are distributed in the soluble fraction, but not in the pelleted fractions. This subcellular distribution is similar to that of the 14 kDa and the 140 kDa PSTV-stimulated proteins detected by staining of proteins separated by gel electophoresis (7,12). Thus subcellular distribution of PSTV-stimulated proteins is unlike that of PSTV which accumulates mostly in the nuclei. The in vivo synthesis of the 14 kDa protein but not the 140 kDa protein has been observed when systemic symptoms of PSTV started to appear. These results are in agreement with those of Henriquez and Sänger (12) and Galindo et al (7) who showed by protein staining that the 14 kDa protein was detected at the onset of PSTV symptoms and the 140 kDa protein was detected a few days later. In addition to the 14 kDa protein, the in vivo synthesis of a 33 kDa protein and a 90–100 kDa protein also was observed during the onset of PSTV symptoms. This is the first report of such observation.

In in vitro reconstitution experiments, several PSTV-binding proteins have been detected in the 70,000 *g* soluble fractions or in the density-gradient fractionation of the 20,000–70,000 *g* pellet fractions from infected or uninfected cells. Some of the PSTV-binding proteins in these fractions are similar in size to those located in the nuclei (22). In the soluble fractions from infected, but not uninfected cells, a protein of 33 kDa bound PSTV. This observation as well as the observations that a 33 kDa protein is stimulated upon infection and that a unit-length dsPSTV is

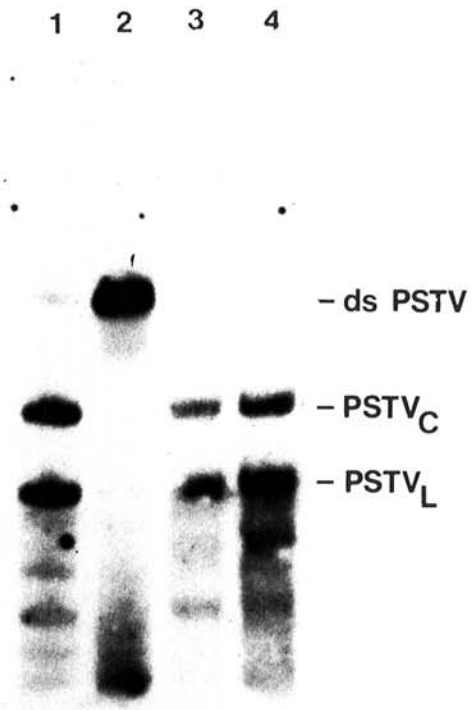

Fig. 4. Autoradiogram of Northern blot hybridization analysis of PSTV-related RNA from the PSTV-cellular constituent complexes and the 70,000 *g* supernatant fraction from infected cells. RNA samples from 1-g tissue each were subjected directly to electrophoresis or after heating for 1 min at 100 C in 50% (v/v) deionized formamide and quenching in ice. Nondenatured RNA (lanes 1 and 2); denatured RNA (lanes 3 and 4). PSTV-related RNA from PSTV-cellular constituent complexes (lanes 1 and 3); and from 70,000 *g* supernatant fraction (lanes 2 and 4). The positions in the gel of unit-length dsPSTV (11) and of unit-length circular and linear PSTV (PSTVC and PSTVL) are indicated.

detected in the same soluble fractions suggest that a 33 kDa protein may play a role in PSTV and/or cPSTV synthesis, processing, transportation, and/or protection. The observations that RNA-directed RNA polymerase in plants is increased in amount by viroid infection (13), and that purified RNA-directed RNA polymerase from tomato leaves, which is capable of copying PSTV in vitro, contains proteins of 33–34 and 67 kDa (1) suggest that the disease-associated 33 kDa protein may be a subunit of the polymerase enzyme. Double-stranded PSTV synthesized by the enzyme may be responsible for viroid movement from cell to cell and systemic movement rather than viroid replication in infected nuclei. Nuclear PSTV is replicated by DNA-directed RNA polymerase II (16).

In addition to host proteins, PSTV also binds to plant viral coat proteins. Recently, it has been shown that velvet tobacco mottle mosaic virus can encapsidate PSTV in vivo (6), thus suggesting binding of PSTV to the viral coat protein. Binding of PSTV to plant viral coat protein in vitro may suggest that this could occur in infected cells and thus be of importance in the field-spread and/or evolution of viroids (6).

The detection of PSTV, which seems to be naturally attached to cellular constituent complexes in the 20,000–70,000 *g* pellet fractions, after fractionation of these pellets by discontinuous sucrose density-gradient centrifugation is surprising. The fact that in reconstruction experiments (Fig. 3, IB) purified PSTV did not bind to host cellular constituents from healthy tissue makes unlikely the possibility that the association of PSTV with the cellular constituent complexes from infected tissue can be generated as a result of adventitious PSTV-cellular constituent complexes interaction. The association of PSTV with these complexes after three successive density-gradient purifications (Fig. 3, IIA) suggests that this association reflects in vivo conditions. The involvement of proteins in this association was demonstrated by release of the PSTV from the cellular constituent complexes by pronase treatment (Fig. 3, IIB). The release of PSTV from these complexes upon treatment with triton X-100 (Fig. 3, IIC) suggests that the PSTV-protein association is not via a covalent linkage.

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