The p27 Protein Is Present at One End of Citrus Tristeza Virus Particles

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ABSTRACT

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Sequence analysis of the citrus tristeza closterovirus (CTV) genome indicated the presence of an open reading frame upstream of the capsid protein (CP) gene that codes for a protein of 27 kDa (p27). p27 is a diverged CP, showing 41% deduced amino acid sequence similarity with the CP. Previous reports indicated that CTV particles were composed of a single protein, the CP. However, adsorption of extracts of CTV-infected tissue to enzyme-linked immunosorbent assay (ELISA) plates with p27-specific antibodies and their detection with antibodies to the CP suggested that the p27 and CP were associated. The association of p27 with the virion was indicated by positive reverse transcription and polymerase chain reaction results using the p27-adsorbed material and two sets of primer pairs corresponding to the 5′ and 3′ ends of the CTV genome. Immunoelectron microscopy demonstrated that p27 antibodies decorated a 75- to 85-nm-long segment at one end of the virus particle, but not the main portion of the particle, for both a Florida and an Israeli strain of CTV. Conversely, the CP antibodies decorated the majority of the virus particle, but not a terminal 75- to 85-nm-long segment. Both normal length (about 2,000 nm) and shorter particles contained the p27 segment.

Additional keywords: electron microscopy.

Citrus tristeza closterovirus (CTV), the most destructive virus of citrus, is transmitted by several species of aphids in a semipersistent manner (3). Virus particles are approximately 2,000 nm long and 10 to 12 nm in diameter (12). They contain one positive-sense, single-stranded RNA molecule of 19.3 kb (2,11), and biochemical characterization of purified preparations suggested a single capsid protein (CP) with a molecular mass of 25 kDa (4,21). Recent evidence also showed that a range of shorter, defective RNA (D-RNA) molecules may be encapsidated in shorter particles (16,17). Sequencing of the genome of the Florida CTV strain T36 revealed the presence of an open reading frame encoding a protein of 27 kDa (p27) with 41% deduced amino acid sequence similarity with the CP (11,19). This open reading frame, located upstream of the CP gene, is expressed in vivo (7).

The diverged CP (dCP) gene, first reported for beet yellows closterovirus (BYV) (5), is characteristic of closteroviruses (5,13,19). Recently, Agranovsky et al. (1) found that p24, the dCP of BYV, was localized to a 75-nm-long segment at one end of the filamentous virus particles, and suggested that virions of closteroviruses have a polar structure and are composed of two CPs. Here, we present two types of evidence to demonstrate the presence of p27 in CTV particles: (i) indirect, using enzyme-linked immunosorbent assay (ELISA) with antibodies specific for both the CP and p27, and immunocapture-polymerase chain reaction (PCR) with two sets of primer pairs to show the association of p27 with the viral genome, and (ii) direct, using immunoelectron microscopy with the same specific antibodies.

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MATERIALS AND METHODS

Virus source. Florida CTV strains T36 (quick decline) and T30 (mild) were maintained under greenhouse conditions at the University of Florida Citrus Research and Education Center, Lake Alfred. The host plants were *Citrus excelsa* Wester or *C. sinensis* (L.) Osbeck cultivar Madame vinous. The Israeli seedling yellows strain VT was propagated in Alemow (*C. macrophylla* Wester) seedlings as described previously (4).

Indirect ELISA. Four different antibodies were used for reactions with the CP: two polyclonal antibodies, CREC35 and G604, produced in rabbit and goat, respectively, to an *Escherichia coli*-expressed CP (15); and two monoclonal antibodies, 3DF1 and 3CA5 (24). A previously described polyclonal antibody, prepared to an *E. coli*-expressed p27, was used to detect p27 (7).

Double antibody sandwich indirect (DAS-I) ELISA was performed using a modification of the method of Garnsey and Cambra (10). Incubations with coating and secondary antibodies were performed at 4°C overnight. Sample and tertiary antibody incubations were performed at 37°C for 4 and 2 h, respectively. The tertiary antibodies conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis) used were goat anti-rabbit (for the detection of CREC35 and p27 antibodies), goat anti-mouse (for the detection of 3DF1 and 3CA5), and rabbit anti-goat (for the detection of G604). Plate-trapped antigen indirect ELISA (10) was used to determine the reactivity of the *E. coli*-expressed p27. Each experiment was repeated at least three times with duplicate wells containing the same sample.

Immunocapture reverse transcription coupled with PCR (RT-PCR). The CTV particles adsorbed from infected tissue to the ELISA plates were used for RT-PCR, using a modification of the procedure by Nolasco et al. (18). After reading the color reactions, the plates were washed once with sterile, distilled water and stored at –20°C.

The RT reaction was performed in the plate wells by adding 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 10 mM dithiothreitol, 2.5 mM $MgCl_2$, 0.1 mM dNTPs, and 100 pM of each primer in a final volume of 90 µl. The samples were incubated at 70 to 75°C for 5 min and cooled to room temperature. The avian myeloblastosis virus reverse transcriptase (10 U), *Taq* polymerase (2.5 U), and RNasin (20 U) were added in a final volume of 10 µl. The samples were incubated at 42°C for 45 min, and then transferred to 200-µl tubes for 40 cycles of incubation at 94, 50, and 72°C for 1 min each.

The primers used were specific for a region at the 5′ end of the CTV genome between nucleotides 61 and 588 (5′-TAGGTCCAGCCTTTAAGC-3′ and 5′-CACGTCTGTGAAGTCTTG-3′, genome sense and antisense, respectively) and the p23 gene (5′-GCTAGCACCATGGATAATACTAGCGGAC-3′ and 5′-AGATCTTCAGATGAAGTGGTGTTC-3′, genome sense and antisense, respectively), located between nucleotides 18,394 and 19,023 near the 3′ end of the CTV genome (11,19).

Immunogold labeling and direct immunoelectron microscopy. Crude extracts of bark from CTV-infected citrus were diluted with high salt Tween buffer (HST) (50 mM Tris-HCl [pH 7.4], 3% NaCl, and 0.1% Tween-20). The CTV-T36 particles were adsorbed to Formvar-coated grids using a CP-specific antibody (G604 IgG, diluted 1:5,000) and the serologically specific electron microscopy procedure described by Derrick (6). For labeling (14), antibodies specific to the CP (CREC35, diluted 1:10,000) and p27 (diluted 1:500) were reacted with the virus particles on separate grids and detected using 10-nm gold-conjugated goat anti-rabbit IgG (EY Laboratories, Inc., San Mateo, CA). Antibodies were diluted with HST. All incubations were performed for 1 h at room temperature. An antibody raised in rabbit to the CTV p18 protein (8) was diluted 1:100 for use as a negative control.

Immunoelectron microscopy of CTV-VT particles from crude and purified preparations was conducted as described by Agranovsky et al. (1). Samples were incubated on Formvar-coated grids for 3 to 5 min and washed for 1 min. The samples were then reacted for 30 min with antibody (diluted 1:50 for p27 and 1:500 for CP), washed, and stained with uranyl acetate.

RESULTS

Detection of p27 using ELISA. The presence of p27 in preparations containing CTV particles was determined using ELISA. Extracts from CTV-infected tissue were incubated in plates coated with antibodies to p27 and detected using polyclonal and monoclonal antibodies to the CP. Samples infected with either T30 or T36, but not the uninfected samples, reacted positively (Table 1). These results suggest that the CP and p27 were associated in the infected tissue. Because of the similarity between p27 and the CP (7), it was necessary to demonstrate that the antisera were not cross-reactive to the heterologous antigens. For this purpose, the *E. coli*-expressed CP and p27 were included as controls. No positive reactions were observed when these proteins were tested similarly (Table 1). However, the CP, but not p27, was readily detected when CP antibodies were used for both coating and as secondary antibodies (Table 1). Similarly, when p27 was coated to the plate, it was detected with the p27-specific antiserum (optical density at 405 nm [OD_{405}] = 0.166 for p27, OD_{405} = 0.007 for the CP, and OD_{405} = 0.012 for bacterial proteins without the recombinant peptides). These results confirm those previously published; the p27 antiserum reacts specifically with its antigen and does not cross-react with the CP (7). Positive ELISA results also were obtained with CTV by coating with the CP antibodies and detecting with the p27 antibody (Table 1), further indicating the association between the CP and p27. Neither the CP nor the p27 recombinant antigen was detected in this ELISA test (Table 1), further demonstrating the specificity of the antibodies.

To determine if p27 was associated with the virions, we tested the amplification as cDNA of two CTV sequences from the material immunocaptured in the ELISA plates coated with the p27 antiserum, using RT-PCR and specific primers. Both sequences, one located at each of the 5′ and 3′ ends of the CTV genome, were amplified from the immunocaptured material from the CTV-infected tissue (Fig. 1). However, no amplified products were obtained from uninfected samples. Similar results were obtained using the ELISA plates coated with the CP antibody (Fig. 1).

Detection of p27 using electron microscopy. The p27 antiserum was used to determine the location of p27 in the virus particle. Figure 2A shows the localization of the antibodies at one end of the CTV-T36 particles. The labeling was consistently at only one end and was never found in the main portion of the virus particles. When CP antibodies were used (Fig. 2B), they reacted along most of the virus particle, leaving one end undecorated.

TABLE 1. Reactions of citrus tissue extracts and recombinant citrus tristeza closterovirus (CTV) proteins in a double antibody sandwich indirect enzyme-linked immunosorbent assay

	Coating antibodies								
	anti-p27[a]			G604[b,d]	3DF1[b,e]	3CA5[b,f]	G604[c]	3DF1[c]	3CA5[c]
	Secondary antibodies			Secondary antibodies			Secondary antibodies		
Sample	G604	3DF1	3CA5	CREC35[g]			anti-p27		
Uninfected	0.000[h]	0.007	0.005	0.001	0.000	0.000	0.037	0.044	0.009
T30-infected	0.149[i]	0.834	0.746	0.550	0.186	0.166	0.256	0.198	0.071
T36-infected	0.215	0.497	0.530	0.137	0.170	0.115	0.117	0.089	0.024
p27	0.000	0.000	0.000	0.000	0.000	0.000	0.015	0.045	0.013
CP	0.000	0.000	0.000	0.204	0.139	0.152	0.008	0.013	0.013

[a] Using p27 antibodies for coating and capsid protein (CP) antibodies for detection.
[b] Using CP antibodies for coating and detection.
[c] Using CP antibodies for coating and p27 antibodies for detection.
[d] G604 = goat polyclonal anti-CP.
[e] 3DF1 = mouse monoclonal anti-CP.
[f] 3CA5 = mouse monoclonal anti-CP.
[g] CREC35 = rabbit polyclonal anti-CP.
[h] Absorbance values (optical density at 405 nm) were the means of duplicate wells. The results of only one experiment are presented. Two more experiments gave similar results.
[i] Values considered positive (at least double the uninfected control) are indicated as underlined numbers.

Antisera to p18 did not react with any portion of the virus particle (data not shown). Measurements of the CTV-T36 virus particles estimated the modal length of the p27 segment at 75 to 85 nm (Fig. 3A), and the full virus particle at 1,900 to 1,999 nm (Fig. 3B), as previously reported (3). Similar results were observed with CTV-VT, with a range of shorter and full-length particles (2,000 nm), many showing the p27 segment (Fig. 2C and D).

DISCUSSION

The ELISA experiments indicated that the CP and p27 are associated in CTV-infected tissue, since the antibody to either protein "captured" the other protein as well. The possibility of cross-reactivity between the antibodies was precluded, since the use of recombinant antigens gave no positive reaction in the heterologous DAS-I ELISA, yet they were readily detected with their homologous antibodies. In addition, the epitope on the CP recognized by one of the monoclonal antibodies (3DF1) has been identified (20), and that amino acid sequence is not conserved in p27. The specificity of the p27 antibody was previously demonstrated by Western blot analysis (7). Amplification by RT-PCR of the two CTV cDNA sequences from the p27 antiserum immunocaptured material indicated that p27 is associated with the virions.

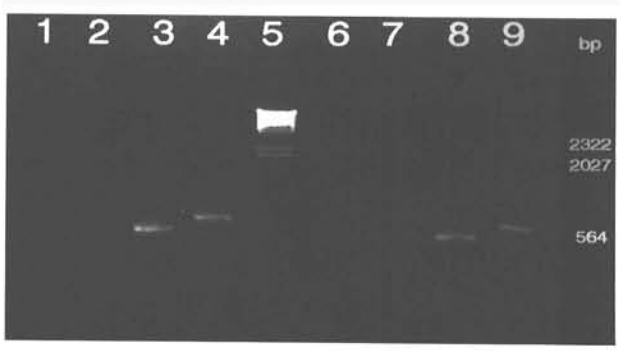

Fig. 1. Agarose gel electrophoresis of reverse transcription-polymerase chain reaction (RT-PCR) products from citrus tissue extracts immunocaptured with anti-p27 antibodies (lanes 1 to 4) or with anti-capsid protein antibodies (lanes 6 to 9) in the same enzyme-linked immunosorbent assay plates providing the data in Table 1. Lanes 1 and 6 are the uninfected controls amplified with a primer pair for the 5′ end region of the citrus tristeza closterovirus (CTV) genome. Lanes 2 and 7 are the uninfected controls amplified with a primer pair for the p23 gene at the 3′ end of the CTV genome. Lanes 3 and 8 are the RT-PCR products from CTV-T36-infected samples using the primer pair for the 5′ end region. Lanes 4 and 9 are the RT-PCR products from CTV-T36-infected samples using the primer pair for the 3′ end. Lane 5 is lambda DNA digested with *Hin*dIII. The size markers in base pairs are indicated to the right.

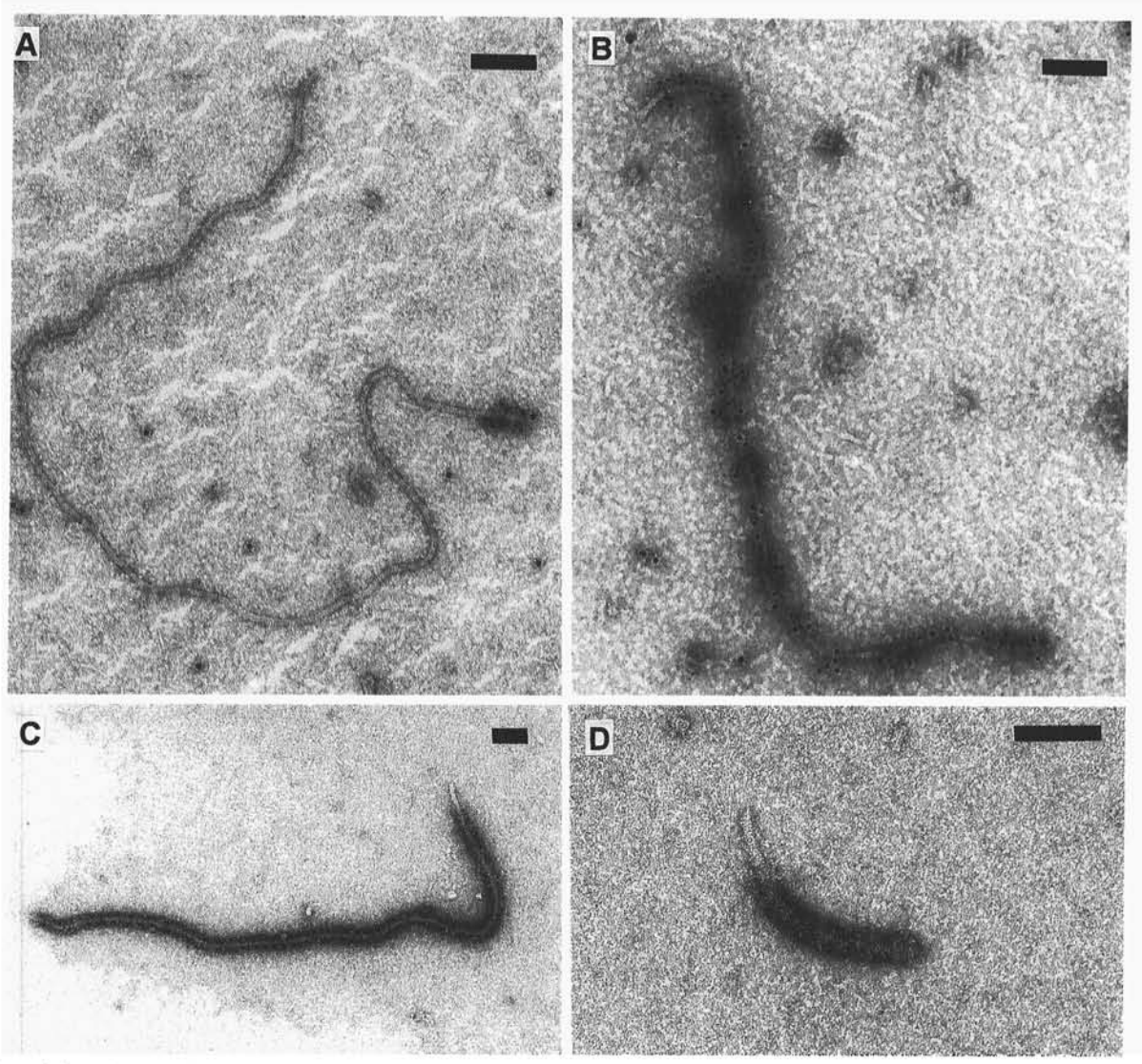

Fig. 2. Transmission electron micrograph of citrus tristeza closterovirus (CTV) particles from tissue extracts reacted with p27- or capsid protein (CP)-specific antibodies. **A,** Gold particles locating the p27 antibodies at one end of a CTV-T36 particle. **B,** Gold particles locating the CP antibodies along the CTV-T36 particle. Note the undecorated p27 segment at the upper end. **C,** Decoration of a CTV-VT particle with CP antibodies with the undecorated p27 segment. **D,** As in C, except a short CTV-VT particle. Note an apparent transient decoration at the junction site of p27- and CP-coated segment. Bar = 100 nm.

Immunolabeling of the CTV particles using the p27 antiserum demonstrated that p27 forms a "tail" at one end of the virus particle, similar to the structure first reported for BYV (1). The segment decorated by antibodies to p27 was approximately 75 to 85 nm long, similar to the corresponding segment (75 nm) of BYV. Our observations indicate that full-length and shorter particles also contain the p27 segment.

The function of p27 is still not known. We have previously suggested that p27 could be a movement protein (7). This possibility is not ruled out by the finding that p27 is part of the virus particle. The CPs of several RNA viruses also have a role in cell-to-cell and long distance movement (9,22,23). Alternatively, as suggested for p24 of BYV, p27 of CTV might also be involved in particle assembly or aphid transmission (1). An assembly function could be mediated through the interaction with other viral proteins, including the CP and the CTV HSP70 homolog (p65) or unknown host components. The observation of shorter particles, probably consisting of encapsidated subgenomic or D-RNA (16,17), may indicate that the addition of the p27 segment is required for encapsidation, regardless of the size of the final particle.

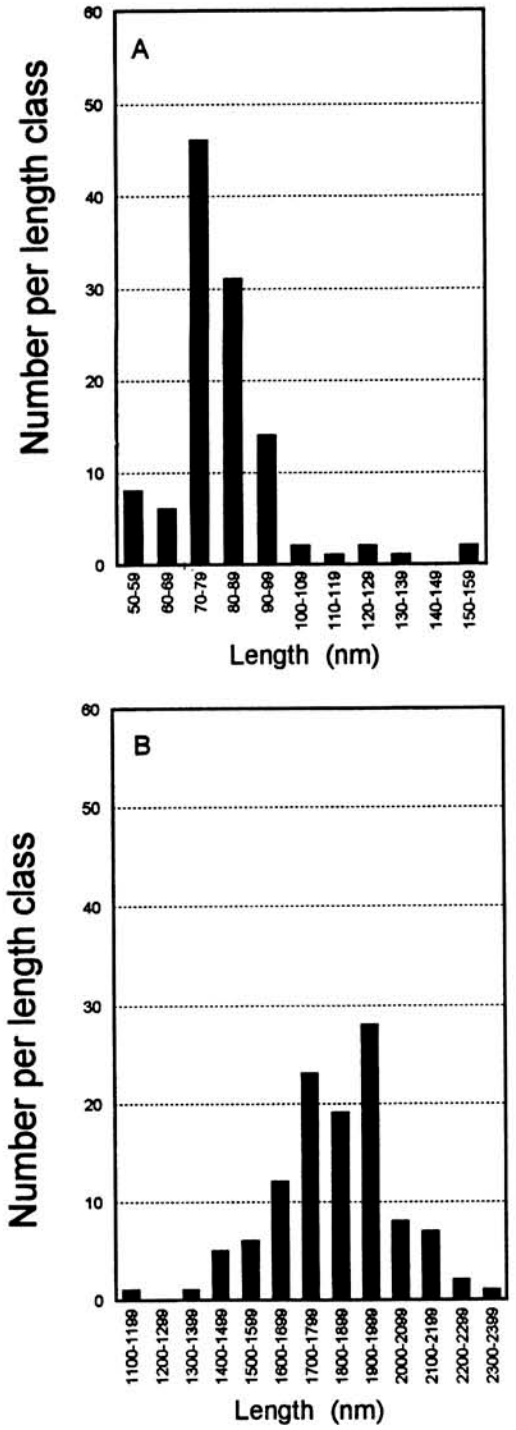

Fig. 3. Length distributions of **A,** p27 segments and **B,** full-length citrus tristeza closterovirus particles of strain T36 detected with the p27 antibody.

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