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Inhibition of a plant virus infection by analogs of melittin

(antiviral/tobacco mosaic virus)

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ABSTRACT **An approach that enables identification of specific synthetic peptide inhibitors of plant viral infection is reported. Synthetic analogs of melittin that have sequence and structural similarities to an essential domain of tobacco mosaic virus coat protein were found to possess highly specific antiviral activity. This approach involves modification of residues located at positions analogous to those that are critical for virus assembly. The degree of inhibition found correlates well with sequence similarities between the viral capsid protein and the melittin analogs studied as well as with the induced conformational changes that result upon interaction of the peptides and ribonucleic acid.**

Plant viral diseases are responsible for substantial crop losses worldwide (1). In the past 10 years, several transgenic strategies have been developed to control plant viruses (2, 3). Recently, antiviral peptides with activity against herpes simplex virus or human immunodeficiency virus (HIV) have been identified by using rational design approaches (4, 5). These studies showed that small peptides, whose primary sequences were taken from proteins encoded by specific animal viruses, were able to impair viral infectivity (6, 7). We present here the use of peptides that embody features responsible for the function of a viral capsid protein for development of antiviral compounds.

Tobacco mosaic virus (TMV) is a member of the tobamovirus group of plant viruses and the complete nucleotide sequence of its 6.4-kb RNA genome is known (8). cDNAs from which infectious transcript can be derived are available (9), and the structure of the virion has been determined by x-ray diffraction analyses (10). As for other plant viruses, TMV coat protein (CP) serves two roles in virion structure: binding to the viral RNA and interaction with other CP subunits for the assembly of virions (8–10). In particular, amino acids Thr-37, Arg-90, and Arg-92 are located internally in the virion and interact with phosphate groups on three nucleotides of the viral RNA (10). The protein is structured in a four α-helix bundle that comprises a framework for viral capsid assembly (10).

Melittin, a 26-amino acid amphipathic peptide (11), has been widely used for investigating intra- and intermolecular structures. Melittin has also recently been reported to reduce HIV-1 production (4). This was proposed to be due to the affinity of melittin for the gag/pol precursor, therefore preventing processing of gag/pol by the HIV protease. When the sequence of melittin was compared with the sequence of TMV CP, we observed that melittin exhibits sequence and structural similarities to a region of the TMV CP known to be critical for protein–protein and protein–RNA interactions. The amino acids of particular interest—Ala-74, Val-75, and Asp-77—are involved in intersubunit interactions, while Arg-90 and Arg-92 are implicated in RNA binding (Fig. 1; ref. 10). In particular, recent studies using a TMV infectious clone pointed out the importance of Asp-77 as a target to disrupt the normal assembly of CP (14). Since Lys-7 of melittin lies in a homologous position as the critical Asp-77 and is in a region of similar amino acid sequences in melittin and TMV CP (Fig. 1), melittin and analogs of melittin described earlier as having a single substitution at position 7 (15, 16) were tested for their ability to reduce the infectivity of TMV.

MATERIALS AND METHODS

Peptide Synthesis. The peptides were prepared using methylbenzhydrylamine polystyrene resin and standard *t*-Boc chemistry in conjunction with simultaneous multiple peptide synthesis (17). Final cleavage and deprotection were carried out using a "low–high" hydrogen fluoride procedure with a 24-vessel cleavage apparatus (18, 19). The peptides were then purified by preparative reversed-phase (RP) HPLC using a DeltaPrep 3000 RP-HPLC (Millipore) combined with a Foxy fraction collector (ISCO). Analytical RP-HPLC and laser desorption time-of-flight mass spectroscopy (Kompact Maldi-Tof mass spectrometer, Kratos Analytical Instruments), were used to determine the purity and identity of the peptides.

Virus. TMV (U1 strain) was propagated on *Nicotiana tabacum* cv. Xanthi nn and purified as described (20, 21). Purified tomato mosaic tobamovirus (ToMV) was a gift from Yuichiro Watanabe (Teikyo University, Tochigi, Japan). ObNLD3 is a mutant of the tobamovirus Ob that is capable of eliciting necrotic local lesions on *N. tabacum* harboring the *N* gene (20) (H. Padgett, personal communication). It was propagated on *N. tabacum* cv. Xanthi NN. Sunn hemp mosaic tobamovirus (SHMV) was propagated in *N. benthamiana*. ObNLD3 and SHMV were purified as described (20, 21).

Infectivity Assays. Plants of the local lesion host *N. tabacum* cv. Xanthi NN were topped and kept 1 day in the dark before inoculation. Purified virus was diluted in inoculation buffer (20 mM potassium phosphate, pH 7/1 mM EDTA), and then peptides dissolved in 5 mM Mops (pH 7) were added to achieve the desired final concentration. Half leaves of each of two leaves on *N. tabacum* cv. Xanthi NN plants were inoculated either with virus alone or with virus plus the corresponding peptide. Five or six leaves were used in each experiment. The infectivity assays were repeated at least five times. At 4 days postinoculation, the number of necrotic local lesions was counted and the ratio between the number of lesions produced

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Abbreviations: TMV, tobacco mosaic tobamovirus; CP, coat protein; ToMV, tomato mosaic tobamovirus; SHMV, sun hemp mosaic tobamovirus.

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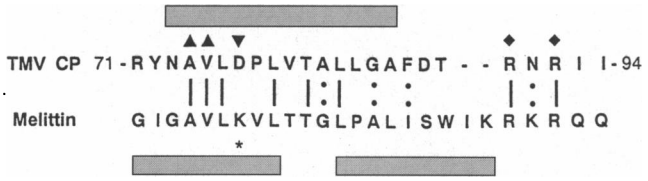

FIG. 1. Sequence and structural homologies between TMV CP (amino acids 71–94) and melittin. Boxes indicate right radial α-helix of TMV CP (10) and the two α-helices of melittin (12). Identical and similar amino acids are indicated by vertical lines and double dots, respectively. ▲, Residues involved in intersubunit CP hydrophobic contacts; ▼, intersubunit pairs of carboxyl groups; ◆, amino acids forming ion pairs with phosphate groups on TMV RNA (13); *, amino acid 7 in melittin substituted in this study.

by virus incubated with peptide and those produced by virus alone was determined.

Band-Shift Assays. TMV RNA (500 ng) extracted from purified virions as described (22) was incubated with peptides and electrophoresed on a 1.2% agarose gel in TAE buffer under nondenaturing conditions (23). After electrophoresis, the gel was stained with ethidium bromide and photographed. Two DNA fragments that migrated faster and slower than TMV RNA, respectively, were included in each sample as internal controls of binding and mobility.

CD Measurements. All measurements were carried out on a J-720 CD spectropolarimeter (Jasco, Easton, MD) in conjunction with a Neslab RTE 110 waterbath and temperature controller (Dublin, CA). The spectra were measured in a 1-mm pathlength cuvette. Data were taken with a 0.2-nm step size, 8-s average time, and 20-nm/min speed, and the results of 10 scans were averaged. CD spectra were taken at 10C, in 10 mM Tris·HCl/1 mM EDTA buffer, pH 8. Concentration of peptides was determined by UV spectroscopy (16). The concentration of TMV RNA was 22.5 μg/ml for all experiments.

RESULTS AND DISCUSSION

TMV and most tobamoviruses elicit necrotic local lesions at the sites of infection when inoculated onto *N. tabacum* cv. Xanthi harboring the resistance gene *N* (13). The *N* gene was originally identified in *Nicotiana glutinosa* (24) and has been recently isolated (13). The number of local lesions produced is indicative of the infectivity of the viral inoculum (1). Because of the similarity in the amino acid sequences of the TMV CP and melittin in a region of the CP known to be essential for protein–protein and protein–RNA interactions (Fig. 1), a study was initiated to examine the interaction of melittin with TMV and TMV RNA and its effect on the infectivity of the virus.

When melittin was added to a solution of TMV before inoculation of the leaves of tobacco plants, a small but reproducible reduction (10%) occurred in the number of local lesions induced. Two of the four analogs examined, subK7I and subK7L, substantially reduced TMV infectivity (90% or greater inhibition by subK7I; Fig. 2*A*). The inhibition of infection by subK7I was dose dependent between 0.1 and 5 μM (Fig. 2*B*). Furthermore, pretreatment of tobacco leaves with subK7I from 15 min up to 2 h before inoculation with TMV did not significantly change the number of lesions produced compared with nontreated leaves (data not shown). These results suggest that the melittin analog subK7I interacts with the virus before inoculation, or, alternatively, that the inhibitory activity of the analogs requires cointroduction of the peptide with the virus. These results also indicate that subK7I neither blocks an essential cellular function needed for infection nor triggers a nonspecific host defense mechanism.

To test the hypothesis that the antiviral activity of subK7I is due to its sequence similarity with TMV CP, infectivity inhibition assays were carried out with other tobamoviruses. These viruses were selected based on their sequence homology to the TMV CP at positions 71–94 (Fig. 3*A*). As shown in Fig. 3 *B* and *C*, the degree of sequence homology of a given virus CP with TMV CP correlated with the ability of subK7I to inhibit viral infectivity. No antiviral activity was found for subK7I against SHMV, which is only distantly related to TMV (25), while there was greater inhibition of ToMV, which is closely related to TMV. All these viruses form rigid rods of similar physical dimensions. This result suggests that the overall sequence relations between subK7I and tobamovirus CP are critical to the specificity of the antiviral effect. In contrast, the fact that subK7D, which has the closest similarity to the TMV CP sequence, showed the smallest reduction in infectivity suggests that the similarities at this amino acid level are not the most important component for reducing infectivity. Furthermore, amino acids in the region of the CP that are important for RNA binding (i.e., Arg-90 and Arg-92) are apparently not solely responsible for the specificity of the antiviral activity, since each of the viral CPs studied here contains these amino acids. It should be noted that subK7I and subK7L were found in earlier studies to fold into a β-sheet conformation in the presence of liposomes or negatively charged biopolymers, while subK7D and subK7G have similar ability to fold into amphipathic α-helices to melittin (15, 16). Furthermore, these variations in folding were determining factors for the relative antimicrobial activities of these peptides (16). Thus, while

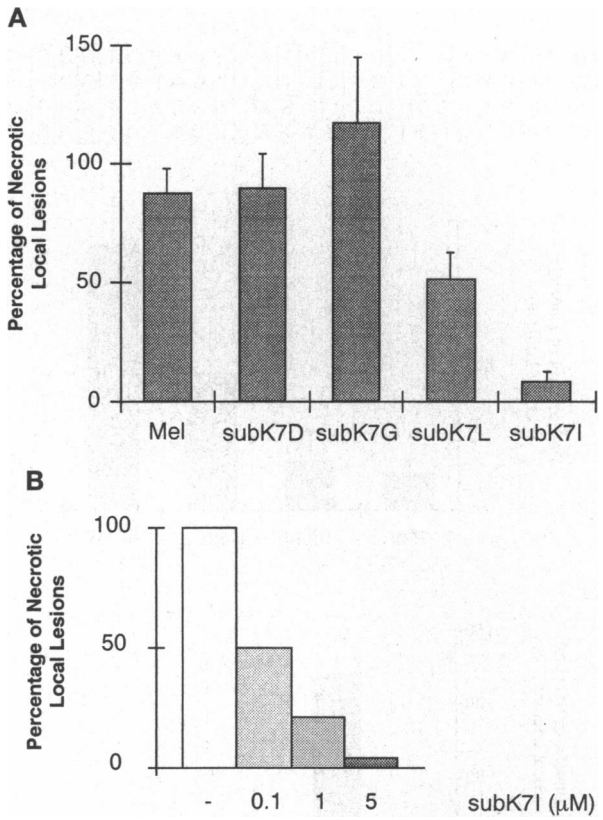

FIG. 2. Effect of melittin and its analogs on infectivity of TMV. (*A*) TMV (50 ng/ml) was mixed with each peptide (5 μM final concentration) and inoculated onto five or six tobacco leaves. Results are presented as percentage of necrotic local lesions elicited by TMV in the absence of added peptides. Error bars indicate standard errors. Analogs are represented as subK7D, subK7G, subK7I, and subK7L, indicating the amino acid replacing Lys-7 in the native melittin sequence. (*B*) Percentage of necrotic local lesions elicited by TMV (50 ng/ml) is shown in the presence of different subK7I concentrations. Open bar represents percentage of necrotic local lesions elicited without peptide added.

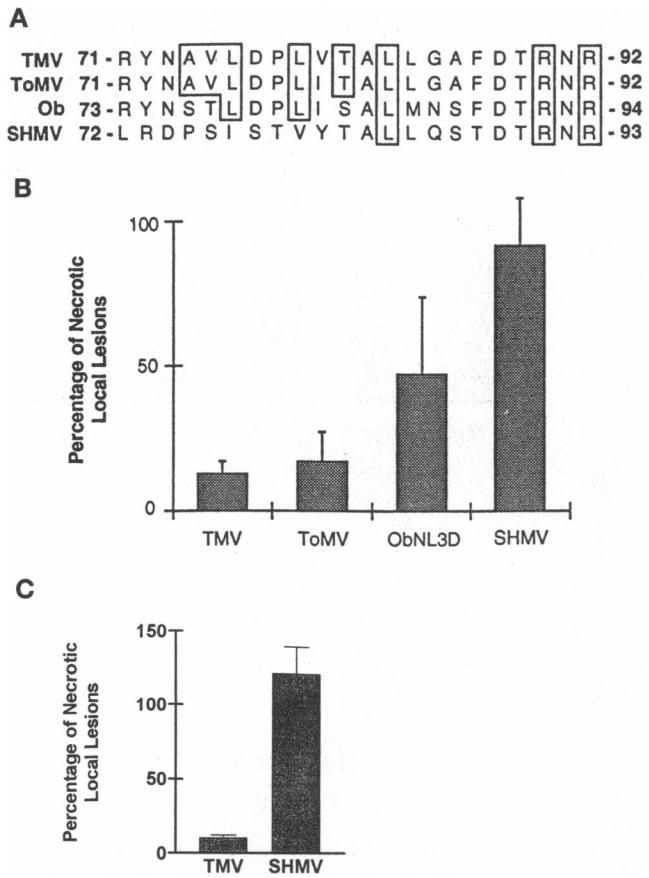

FIG. 3. (*A*) Homologies between TMV CP amino acids 72–93 and those of tobamoviruses ToMV, Ob, and SHMV. Boxes enclose amino acid residues identical to those of melittin as described in Fig. 1. (*B* and *C*) Effect of subK7I on infectivity of different tobamoviruses. Percentage of necrotic local lesions elicited by each virus is shown in the presence of peptide when compared to that elicited by the virus with no peptide added. (*B*) TMV (50 ng/ml), ToMV (50 ng/ml), ObNLD3 (100 ng/ml), and SHMV (500 ng/ml) were mixed with subK7I peptide (5 μM final concentration) and inoculated onto tobacco leaves. (*C*) TMV (250 ng/ml) and SHMV (250 ng/ml) were mixed with subK7I (25 μM).

melittin, subK7D and subK7G exhibited potent antimicrobial activity, no activity was found for subK7I and subK7L. Interestingly, the region of the TMV CP that shows sequence similarity with melittin (i.e., Ala-74 to Ala-86) is known to fold into an α-helix (10) and, in turn, closely resembles the structure of those peptides that did not show significant inhibition of the infectivity of TMV. These results suggest that folding of the peptides is responsible for the inhibitory effect.

In other studies, it was determined that premixing TMV with melittin or subK7I did not destabilize TMV particles or render it sensitive to RNase T1 (data not shown). Using electrophoretic band-shift assays to detect protein–RNA interactions, it was shown that incubation of TMV RNA with melittin resulted in a significant increase in the electrophoretic mobility of TMV RNA (Fig. 4). These results may indicate that binding caused a conformational change in the structure of RNA. When subK7I was used in similar assays, there was a minor broadening of the RNA band. The change in mobility of the RNA was greatest when both peptides were simultaneously added to the RNA (Fig. 4). In these experiments, the molar ratio of peptide/RNA was 200:1. The absence of free RNA in these assays indicates that the peptides have a strong affinity for TMV RNA. Both melittin and subK7I inhibited infectivity of TMV RNA to the same extent. TMV RNA treated with either 5 μM melittin or 5 μM subK7I elicited 43% ± 28% or

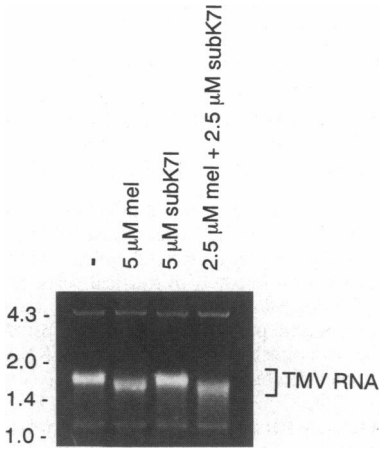

FIG. 4. Electrophoretic mobility-shift assay of TMV RNA in the presence of melittin and subK7I. Concentrations of peptides are indicated above each lane. Lane 1, TMV RNA without peptide added. Range in mobilities of TMV RNA is indicated on the right, and DNA markers together with their sizes (kbp) are indicated on the left.

32% ± 18% of the number of local lesions elicited by untreated TMV RNA, respectively. These results indicate that the effect of subK7I on infectivity of TMV cannot be attributed exclusively to its ability to bind to the viral RNA, although such binding may be one element of inhibition.

To better determine the effect of melittin and subK7I on the structure of TMV RNA, CD studies were carried out. The CD spectrum of free TMV RNA was similar to the spectra of other RNAs (26), with a relative maximum at 265.6 nm and a crosspoint at 247.4 nm (Fig. 5 *A–C*). TMV RNA was found to undergo substantial conformational changes upon addition of either melittin or subK7I (Fig. 5 *A* and *B*). The presence of melittin resulted in a shift to a higher wavelength of the RNA CD spectrum maximum (266.8 nm) and crosspoint (249.8 nm) (Fig. 5*A*). In contrast, the presence of subK7I did not significantly affect the position of either the maximum or the crosspoint but lowered the intensity at the maximum by 4% (Fig. 5*B*). These results support the conclusions of the gel-shift assays that melittin and subK7I bind to and induce different conformational changes in TMV RNA. Melittin and subK7I were found in earlier studies to similarly bind to negatively charged biopolymers (16, 27). However, such interactions resulted in different induced conformations for melittin (α-helix) and subK7I (β-sheet). These results suggest that the binding affinities of melittin and subK7I may not be specific to TMV RNA. This is supported by other preliminary CD studies with viral RNAs from unrelated plant viruses (data not shown).

To compare the conformational changes to RNA caused by melittin or subK7I to the conformation induced by the viral CP on TMV RNA, the CD spectrum was determined for TMV. The maximum of the CD spectrum of TMV RNA in virions was 273.4 nm as compared to the maximum of free viral RNA (265.6 nm; Fig. 5*C*). The analogy between this spectral change and that produced upon addition of melittin to TMV RNA (Fig. 5*A*) suggests that melittin, but not subK7I, can induce a conformation in TMV RNA similar to that induced by the TMV CP. It is therefore suggested that melittin, but not subK7I, is recognized by the RNA as a CP mimic. In contrast, subK7I could therefore be considered a dominant-negative functional analog of the viral capsid in terms of the function of amino acids 71–94 of the CP.

To determine the effect of TMV RNA on the conformation of melittin and subK7I, the spectral properties of each peptide with and without added RNA were determined by using far UV CD. The addition of RNA induces conformational

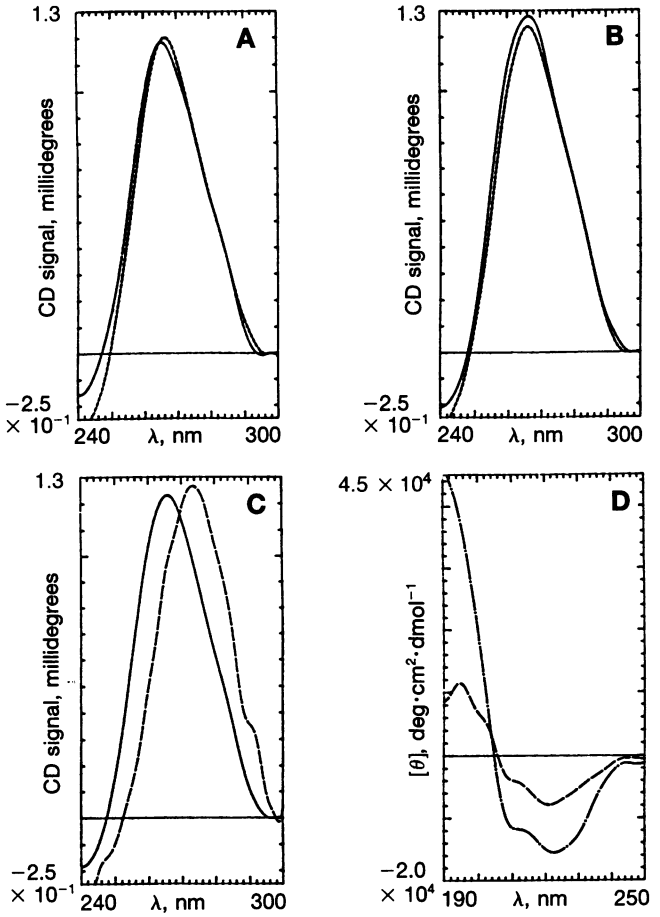

FIG. 5. CD spectra of TMV RNA, TMV, melittin, and subK7I. CD intensities (millidegrees) and mean residue ellipticities ([θ]) are plotted. Spectra in the near UV of TMV RNA in the absence (solid line) or presence (dotted line) of 2.4 μM melittin (*A*) or 2.4 μM subK7I (*B*). (*C*) Spectra in the near UV of free TMV RNA (solid line) and of TMV (virions) at 220 μg/ml (dashed line). (*D*) CD spectra in the far UV showing RNA-induced conformation of melittin (dashed-dotted line) and subK7I (dashed line). CD spectra were obtained after subtracting the spectra of free TMV RNA from the spectra of TMV RNA in the presence of melittin or subK7I.

changes in both melittin and subK7I (Fig. 5*D*). At the micromolar concentrations of peptides used in these studies, both melittin and subK7I showed a random coil conformation in the absence of TMV RNA (15). As shown in Fig. 5*D*, in the presence of TMV RNA melittin folded into an α-helical conformation, while subK7I adopted a distorted β-sheet. As mentioned above, such variations in folding may be related to the differences observed between the antiviral activities of melittin and subK7I, as seen in earlier structure–activity relationship studies with melittin derivatives (15, 16).

In summary, we have identified synthetic peptides with sequence similarities to a specific region of the TMV CP that effectively inhibits infection by TMV but not by distantly related members of the tobamovirus group. Previous studies have shown that peptides whose sequences either correspond to parts of viral proteins or were completely unrelated have activity against mammalian viruses (4–7). This study reports a nonplant peptide that inhibits a plant virus. Our data suggest that the peptide sequences, as a consequence of the relatedness to the CP sequence and the folding induced by RNA, are implicated in the mechanism underlying antiviral activity. The effects of subK7I on the infectivity of TMV may be due to antagonism of the binding of TMV CP to viral RNA as well as interactions between the peptide and virus. The use of well characterized model peptides, combined with a repetitive stepwise sequence alteration of the most effective initial analog, allows one to design peptides with optimized antiviral activities. This approach can also be extended to include the use of combinatorial libraries (reviewed in ref. 28), composed either of sequences homologous to viral proteins or of unrelated small molecules. Such approaches can also be applied to other viruses and/or to a variety of proteins encoded by a given virus.

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