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 methylvbenzyldiamine 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 (Kompakt 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 tobacco mosaic virus (ToMV) was a gift from Yuichiro Watanabe (Teikyo University, Tochigi, Japan). ObNLD3 is a mutant of the totabivirus 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

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

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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. 2A). The inhibition of infection by subK7I was dose dependent between 0.1 and 5 μM (Fig. 2B). 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 coinduction 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 inhibi-
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 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. 5A–C). TMV RNA was found to undergo substantial conformational changes upon addition of either melittin or subK7I (Fig. 5A 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. 5A). 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. 5B). 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. 5C). The analogy between this spectral change and that produced upon addition of melittin to TMV RNA (Fig. 5A) 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
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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