5'-Dephosphorylated 2',5'-Adenylate Trimer and Its Analogs

INHIBITION OF TOBACCO MOSAIC VIRUS REPLICATION IN TOBACCO MOSAIC VIRUS-INFECTED LEAF DISCS, PROTOPLASTS, AND INTACT TOBACCO PLANTS*

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The effect of the 5'-dephosphorylated 2',5'-adenylate trimer and its 2',5'-trimer core analogs on the inhibition of tobacco mosaic virus (TMV) replication was determined in tobacco leaf discs, protoplasts, and whole tobacco plants, using infectivity tests and enzyme-linked immunosorbent assays. A structure-activity-metabolic stability-toxicity analysis of the 2',5'-adenylate trimer core molecule in TMV-infected Nicotiana glutinosa was determined. Modification at either the 6-amino position of the adenylate residues (i.e. inosinate trimer core) or at the 2' terminus (i.e. A-A-ara-A or A-A-Tu) inhibited replication of TMV. Modification of the 3'-hydroxyl group of the adenylate residues to 3-deoxyribose (i.e. the 2',5'-cordycepin tri-mer core) inhibited TMV replication better than the 2',5'-adenylate trimer core molecule. With enzyme-linked immunosorbent assays, there was complete inhibition of TMV replication by 200 nm 2',5'-adenylate trimer core for 60 h and by 200 nM 2',5'-cordycepin trimer core for 96 h. The amount of 2',5'-oligouronucleotides associated with the leaves was determined using 2',5'-[3H]cordycepin trimer core; 1 x 10^12 mol/cm^2 of plant leaves inhibited TMV replication by 99%. No 2',5'-phosphodiesterase activity was detected in TMV-infected and noninfected leaf extracts. Therefore, the 2',5'-trimer cores were potent inhibitors of TMV replication at nanomolar concentrations, i.e. at 1000-fold lower concentration than that required in mammalian systems.

Protection is conferred to virus-infected mammalian cells by the formation of interferon which induces the synthesis of at least two enzymes. One of these enzymes is the 2',5'-adenylate synthetase which in the presence of certain double-stranded RNAs converts ATP to a series of unique 2',5'-triphosphate 2',5'-linked oligoadenylates, Knight et al. (8) have reported on the intracellular accumulation of the 5'-dephosphorylated 2',5'-adenylate molecules (referred to here as 2',5'-adenylate cores) in mouse L-cells following treatment with interferon. They concluded that because the amount of cores seemed to be independent of the concentration of the 2',5'-oligoadenylate 5'-triphosphate molecule, 2',5'-oligoadenylate cores may play a separate role in the inhibition of DNA and cellular reactions. Because of the rapid hydrolysis of the naturally occurring 2',5'-oligoadenylate molecule in cells, there have been reports on the enzymatic and chemical synthesis of the 5'-triphosphates and corresponding cores in attempts to increase the metabolic stability and retain inhibition of protein synthesis (9–13). In addition, Rapport et al. (14) have reported that the 5'-diphosphate of 3',5'-adenylate molecule activated the 2',5'-A,-dependent endonuclease to hydrolyze mouse L-cell rRNA. This laboratory has recently reported that the 2',5'-cordycepin tetramer 5'-triphosphate analog complexes with and activated the 2',5'-A,-dependent endonuclease to hydrolyze vesicular stomatitis virus mRNA and inhibits protein synthesis (10).

Because of the natural occurrence of the 2',5'-oligoadenylate core in mammalian cells, our laboratory and other laboratories have studied the effect of core compounds on cellular processes in animal cells, tumor growth in whole animals, and inhibition of virus replication in plants (15–31).

For example, we have demonstrated that the 2',5'-oligoadenylate cores and 2',5'-cordycepin trimer core inhibit transformation of Epstein-Barr virus-infected lymphocytes (20) by inhibiting the synthesis of the Epstein-Barr virus-induced nuclear antigen (23), augment natural killer cell activity, inhibit TMV replication in tobacco plants (28–29), and inhibit chordrosarcoma growth in animals (25). It has been demonstrated that the 2',5'-oligoadenylate core analogs do not exert their action by 5'-repophosphorylation or activation of the 2',5'-A,-dependent endonuclease (26, 27). There have been suggestions that cores are taken up by cells (19), may be repophosphorylated (17, 32), or may act by pathways independent of interferon (16, 23–26, 31). Chapekar and Glazer (31) and Eppstein et al. (24) have reported that 2',5'-cores act as prodrugs due to degradation by esterases. However, this does not appear

1 The abbreviations used are: 2',5'-adenylate trimer core, adenylyl-(2'-5')-adenylyl-(2'-5')-adenosine; 3',5'-adenylate trimer core, adenylyl-(3'-5')-adenylyl-(3'-5')-adenosine; 2',5'-cordycepin trimer core, 3'-deoxyadenyllyl-(2'-5')-deoxyadenylyl-(2'-5')-3'-deoxyadenosine; 2',5'-inosinate trimer core, inosinyl-(2'-5')-inosine; 2',5'-A,-A,-ara-A, adenylyl-(2'-5')-adenylyl-(2'-5')-9-beta-D-arabinofuranosyladenylyl-(2'-5')-9-beta-D-arabinofuranosyladenylyl-(2'-5'); A-Tu, adenylyl-(2'-5')-adenylyl-(2'-5')-tubercidin; HPLC, high-pressure liquid chromatography; TMV, tobacco mosaic virus; AVF, antiviral factor.
2 R. E. Black, E. E. Henderson, W. Pfeiferer, R. Charubala, and R. J. Suhadolnik, manuscript submitted.
to be the case in all mammalian cell systems. For example, we have demonstrated that tritium-labeled 2',5'-cordycepin trimer core is taken up intact by human lymphocytes (27, 28).

Recently, Reichman et al. (29) and Orachinsky et al. (33) reported that human recombinant leukocyte interferon inhibited TMV replication in tobacco leaf discs. Furthermore, the antiviral factor isolated from TMV-infected leaves of Nicotiana glutinosa and human leukocyte interferon induced double-stranded RNA-dependent synthesis of oligoadenylates from ATP in plants, producing nucleotides with antiviral activity (29).

In this study, we report that five 2',5'-adenylate trimer core analogs inhibit TMV replication in TMV-infected tobacco leaf discs, TMV-infected protoplasts, and in whole plants. This study as well as previous studies (28-29) indicate that the antiviral factor (29) or the inhibitor of viral replication (34, 35), released following TMV infection in N. glutinosa plants or protoplasts, may inhibit viral replication via polymerization of ATP to form oligoadenylates.

**MATERIALS AND METHODS AND RESULTS**

**DISCUSSION**

Viral diseases occurring in the animal kingdom and in the plant kingdom present serious problems. Therefore, it is important to understand how the animal and plant cell are protected from virus infection. In the animal cell, interferon is synthesized in response to virus infection which induces the synthesis of 2',5'-linked oligoadenylates. In the plant cell, an antiviral factor has been isolated from N. glutinosa leaves infected with TMV (41). A substance inhibiting virus replication has also been reported to be released from TMV-infected protoplasts of the cultivar in which the infection in the intact plant is localized (34). These inhibitors may be the "protectors" of plants from virus infection. Reichman et al. (29) and Orachinsky et al. (33) demonstrated that some human interferon preparations inhibited TMV replication in tobacco leaf discs. Application of the 2',5'-adenylate trimer core to TMV-infected tobacco leaf discs also inhibited TMV replication (28, 29, 42). TMV, antiviral factor, or interferon induces an enzyme capable of converting ATP to putative oligoadenylates with the ability to inhibit TMV replication (29). These oligoadenylates inhibit protein synthesis when added to either lysates from rabbit reticulocytes or wheat germ extracts via a mechanism different from the 2',5'-A-dependent endonuclease activation. Previous reports indicate that 2',5'-adenylate trimer core and 2',5'-cordycepin core protect plant cells against viral infection (28, 29). This study demonstrates the potency of 2',5'-adenylate trimer core and its 2',5'-analogues and their ability to inhibit TMV replication in infected protoplasts, TMV-infected leaf discs, and the whole plant. In all experiments, the 2',5'-trimer cores were potent inhibitors of TMV replication at nanomolar concentrations. It is not yet clear whether passive equilibrium, uptake, binding, or a combination of uptake and binding is the mechanism by which the inhibition of TMV replication occurs.

A structure-activity-metabolic-stability-toxicity relationship of the 2',5'-adenylate trimer core in TMV-infected N. glutinosa was determined. Replacement of the 6-amino group of the adenylate residues in 2',5'-adenylate trimer core with a 6-hydroxyl group (i.e. inosinate trimer core) resulted in a derivative that inhibited TMV replication. When the 2'-terminal adenylate was replaced by ether ara-A or tubercidin (i.e. A-A-ara-A or A-A-Tu), the resulting trimer cores also inhibited TMV replication. When the 3'-hydroxyl of 2',5'-adenylate trimer core was replaced by a hydrogen atom (i.e. the cordycepin trimer core), TMV replication was inhibited better than that observed with the naturally occurring 2',5'-adenylate trimer core. None of these oligoadenylate analogs were toxic to N. glutinosa. Structural modification of the ribose on the 2'-terminal nucleotide in the 2',5'-oligoadenylate molecule results in analogs that are considerably more stable to hydrolysis by the 2',5'-phosphodiesterase in the mammalian system (16, 20, 22); however, 2',5'-phosphodiesterase activity was not detected in N. glutinosa. The fact that there is no increase in hydrolysis of 2',5'-[H]adenylate trimer core in TMV-infected or uninfected N. glutinosa when the amount of cell-free extract was doubled suggests that there is no 2',5'-phosphodiesterase activity as determined under these assay conditions (Table VI). In contrast to the leaf discs and intact plants, where the degradation products of 2',5'-adenylate trimer core and analogs did not inhibit TMV replication (Table III and Fig. 1), in protoplasts a different inhibitory mechanism appears to exist because 3',5'-adenylate trimer core, AMP, and adenosine showed some inhibition of TMV replication (Tables I and II). The increased inhibition of TMV replication by the 2',5'-cordycepin trimer core may be attributed to increased stability to other 2',5'-adenylate degradative enzymes in plants or the inhibition of RNA, DNA, or protein synthesis. The concentrations of 2',5'-adenylate trimer core and trimer analogs required for the inhibition of TMV replication in plants are 1000-fold lower than the concentrations required for the inhibition of transformation of Epstein-Barr virus-infected lymphocytes, the inhibition of tumor growth, the antimitogenic effect, and the antiproliferative effects reported in mammalian systems (16, 17, 19-21, 24-27). The 1000-fold difference could be explained by the metabolic stability of 2',5'-adenylate trimer core in plants due to lack of 2',5'-phosphodiesterase activity (Table VI). These observations are in contrast to reports that 2',5'-cores act as produgs in some mammalian systems due to degradation by esterases (24, 31). This does not appear to be the case in all mammalian cell systems. For example, we have reported that 2',5'-cordycepin core trimer is taken up intact by human lymphocytes (27).

We have previously reported the induction of a plant oligoadenylate synthetase which converted ATP into oligoadenylates when immobilized on poly(rI)-poly(rC) (28, 29, 39). The plant oligonucleotides inhibited TMV replication. More recently, we have shown that these same oligoadenylates, following purification by high pressure liquid chromatography, inhibit protein synthesis in lysates from rabbit reticulocytes; however, we could not detect any 2',5'-A-dependent endonuclease or 2',5'-phosphodiesterase activity in uninfected or TMV-infected plants. Similarly, Cayley et al. (43) reported that the 5'-triphosphate of the 2',5'-oligoadenylate or its binding proteins were not present in tobacco plants.

Our study has demonstrated that the inhibition of TMV replication in intact N. glutinosa occurs with the 2',5'-adenylate trimer core, 2',5'-inosinate trimer core, 2',5'-cordy-
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capin trimer core, 2',5'-A-A-Tu, and 2',5'-A-ara-A. Earlier studies also showed that the 5'-triphosphate of the 2',5'-adenylate trimer molecule, when applied to TMV-infected leaf discs, also inhibited TMV replication (29). There is the possibility that 2',5'-trimer cores inhibit TMV replication via the activation of plant discharging factor (30) to discharge aminoacylated TMV RNA, which inhibits viral protein synthesis. Further studies on the effect of 2',5'-adenylate molecule on plant protein, RNA and DNA synthesis, activation of discharging factor, viral coat protein synthesis, TMV RNA synthesis, and virus assembly are in progress. Abrasion of the plant leaf epidermis with a TMV/carborundum solution containing very dilute solutions of the 2',5'-adenylate core or 2',5'-analogs results in inhibition of TMV replication without any toxicity to the plants. This novel approach to the inhibition of viral replication may have a great potential in the control of plant virus infections.

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2',5'-Adenylates Inhibit TMV Replication

Table 1: The inhibition of TMV replication in tobacco protoplasts by 2',5'-adenylate trimer core and analogs

| Nucleotide Core | Concentration (mM) | Inhibition (%) |
|----------------|-------------------|---------------|
| 2',5'-Adenylate trimer core | 2 x 10^-5 | 95 (P < 0.01) |
| 2',5'-Adenylate trimer core | 1 x 10^-5 | 90 (P < 0.01) |
| 2',5'-Adenylate trimer core | 5 x 10^-6 | 85 (P < 0.01) |
| N | 0 | 0 |

Table 2: The inhibition of TMV replication in tobacco protoplasts treated with 2',5'-adenylate trimer core and analogs at various times after infection

| Compound | Time (h) | Inhibition (%) |
|----------|----------|---------------|
| No addition | 0 | 0 |
| 2',5'-Adenylate trimer core | 0 | 95 (P < 0.01) |
| 2',5'-Adenylate trimer core | 12 | 90 (P < 0.01) |

Effect of 2',5'-adenylate trimer core on TMV replication in tobacco plants

The 2',5'-adenylate trimer core was more effective in inhibiting TMV replication in tobacco plants than the 2',5'-adenylate trimer core analogs. The inhibitory effect of the 2',5'-adenylate trimer core was observed 24 hr after inoculation, whereas the 2',5'-adenylate trimer core analogs showed no inhibitory activity. The 2',5'-adenylate trimer core inhibited TMV replication in tobacco plants with an IC50 value of 2.5 x 10^-5 M, whereas the 2',5'-adenylate trimer core analogs showed no inhibitory activity at concentrations up to 2 x 10^-3 M.
2',5'-Adenylates Inhibit TMV Replication

In order to determine the amount of 2',5'-trimer core associated with the plant leaves, 2 x 10^7 M (2.5 x 10^5 dpm) 2',5'-adenylate trimer core (in infecting solution) was applied to N. glutinosa as described in Materials and Methods. Only 0.12% of the 2',5'-adenylate trimer core present in 1 ml of the infecting solution was associated with the leaves (Table 5). Therefore, when 2',5'-adenylate trimer core in the infecting solution is at a concentration of 2 x 10^-6 M, only 2 x 10^-12 moles are associated with each 1 cm² of leaf. About 90% of the 2 x 10^-7 M of 2',5'-adenylate trimer core remained associated with the leaves after extensive washing. No titration was detected in the untreated half-leaves (Table 3), indicating little or no systemic spread of the nucleotides.

Metabolic stability of 2',5'-adenylate trimer core in plant extracts.

2',5'-Adenylate trimer core has been shown to affect mammalian cells at a micromolar concentration (19-22,26,27), whereas it inhibits TMV replication at nanomolar concentration (28-29) (Table 1A, Fig. 1A). Hence, we examined the metabolic stability of 2',5'-adenylate trimer core in lysates of two mammalian cell lines and plant leaves. The assays were done as described in Materials and Methods. 2',5'-Adenylate trimer core was hydrolyzed to adenine and AMP by the 2',5'-adenylate phosphodiesterase present in EAT or Raji cells (Table 5). However, little hydrolysis of 2',5'-adenylate trimer core occurred in N. glutinosa infected and non-infected plant leaf extracts (Table 5).

Table 5. Association of 2',5'-[3H]adenylate trimer core with N. glutinosa leaves

| Time after infection (hr) | 0 | 3 | 6 | 24 |
|---------------------------|---|---|---|----|
| Infection level           | Trained leaf | Treated leaf | Trained leaf | Treated leaf |
|                            | (dpm) | (dpm) | (dpm) | (dpm) |
|                           | 720 | 457 | 14 | 15 |
|                           | 782 | 502 | 21 | 19 |
|                           | 728 | 385 | 20 | 18 |
|                           | 705 | 427 | 17 | 19 |

Assays were done as described in Materials and Methods.

Hydrolysis conditions

| [H]adenine | [H]AMP | [H]adenylate trimer core |
|------------|--------|--------------------------|
| 3          | 4      | 95                       |
| 5          | 7      | 92                       |

Table 4. Association of 2',5'-[3H]adenylate trimer core by electron microscopy

| Concentration (dpm/ml) | Electron microscopy |
|------------------------|----------------------|
| 1 x 10^6               | N. glutinosa leaf    |
| 2 x 10^7               | N. glutinosa leaf    |

Assays were done as described in Materials and Methods.

Percent hydrolysis (average of three experiments) was calculated as:

Percent hydrolysis = 100 x (1 - (dpm of product / dpm of product + dpm of intermediate))

Tables and figures were prepared as described in Materials and Methods.

References

10. Dose for phosphodiesterase (PDE) hydrolyses were as described (10).

Assays were done as described in Materials and Methods.
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