Characterization of the Reovirus \( \lambda 1 \) Protein RNA 5'-Triphosphatase Activity*

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Characterization of the phosphohydrolytic activities of recombinant reovirus \( \lambda 1 \) protein demonstrates that, in addition to the previously reported nucleoside triphosphate phosphohydrolase and helicase activities, the protein also possesses RNA 5'-triphasphatase activity. This activity was absolutely dependent on the presence of a divalent cation, \( \text{Mg}^{2+} \) or \( \text{Mn}^{2+} \), and specifically removes the 5'-phosphate at the end of triphosphate-terminated RNAs. Kinetic competition analysis showed that nucleoside triphosphate phosphohydrolase and RNA 5'-triphasphatase reactions are carried out at a common active site. These results strongly support the idea that, in addition to its role as an RNA helicase during transcription of the viral genome, \( \lambda 1 \) also participates during formation of the cap structure at the 5' end of newly synthesized reovirus mRNAs. The \( \lambda 1 \) protein represents only the third RNA triphosphatase whose primary structure is known and the first described in a double-stranded RNA virus.

The genome of mammalian reoviruses is made of 10 double-stranded RNA segments and is enclosed in a capsid made of two concentric layers of viral proteins (1). Considering their genome structure and because reoviruses replicate in the cytoplasm of infected cells, they must encode their own transcriptional and replicative enzymes. Reovirus cores contain enzymes that modify the 5' end of newly synthesized mRNAs by adding a cap structure similar to the one found on cellular mRNAs (\( \text{m}^7 \text{GpppGpC} \)). Capping increases the stability and translation efficiency of reovirus mRNAs (4, 5). This cap structure includes the 5'-terminal guanosine present on all reovirus mRNAs (2, 3). The formation of the 5' cap structure on reovirus mRNAs proceeds via a well-characterized mechanism schematized as shown in 1-4 (where \( \text{SAM} \) is \( \text{S-adenosyl-L-methionine} \) and \( \text{SAH} \) is \( \text{S-adenosyl-L-homocysteine} \)).

\[
\begin{align*}
\text{pppG} + \text{pppC} & \rightarrow \text{pppGpC} + \text{ppPi} \ (\text{RNA polymerase, } \lambda 3) \\
\text{pppGpC} & \rightarrow \text{pppGpC} + \text{P}i \ (\text{RNA triphosphatase, } ?) \\
\text{pppG} + \text{pppGpC} & \rightarrow \text{pppGpGpC} + \text{Ppi} \ (\text{guanylyltransferase, } \lambda 2) \\
\text{GpppGpC} + \text{SAM} & \rightarrow \text{m}^7 \text{GpppGpC} + \text{SAH} \ (\text{methyltransferase, } \lambda 2, ?)
\end{align*}
\]

**REACTIONS 1–4**

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Following the start of transcription, an RNA triphosphatase (polynucleotide phosphohydrolase) removes the 5' end \( \gamma \)-phosphate of the nascent RNA molecule, generating a 5'-diphosphate end. Guanylyltransferase then donates a GMP moiety derived from GTP to form a 5'-3'-triphasphate linkage typical of cap structure. The cytidine residue, present on all reovirus mRNAs, can also undergo 2'-O-methylation by a cytoplasmic methyltransferase (6).

Recent studies indicate that the minor core protein \( \lambda 3 \) contains the catalytic site of the reovirus RNA polymerase, whereas the \( \lambda 2 \) protein has been defined as the reovirus guanylyltransferase (7, 8). Biochemical evidences are lacking for the protein acting as methyltransferase to produce the methylated 5' cap structure (\( \text{m}^7 \text{GpppGpC} \)), although a role has been suggested for the reovirus \( \lambda 2 \) protein (9). In contrast, the nature of the core protein involved in the RNA triphosphatase activity has remained unknown. The \( \lambda 2 \) and \( \lambda 3 \) proteins were shown to interact with \( \lambda 1 \) of the reovirus core (7, 10, 11), a protein that exhibits an affinity for nucleic acids (12). This \( \lambda 1 \) protein was recently shown to be responsible for the nucleoside triphosphate phosphohydrolase (NTPase) activity present in reovirus cores (13–16), an activity postulated to be involved in the RNA triphosphatase reaction (17, 18). This idea is still controversial (13), but no direct evidence was available to support or rule out the hypothesis.

In this report, recombinant \( \lambda 1 \) produced in yeast cells and previously used to demonstrate NTPase/helicase activity (14) was examined for RNA triphosphatase activity. Results obtained indicate that the reovirus \( \lambda 1 \) protein does possess the ability to remove exclusively the 5'-'\( \gamma \)-phosphate at the end of a triphosphorylated mRNA molecule. This finding strongly supports the idea that \( \lambda 1 \) participates as an RNA triphosphatase during formation of the cap structure on newly synthesized reovirus mRNAs. Competitive inhibition analysis also demonstrated that the RNA triphosphatase and NTPase activities are carried out at a common active site of the \( \lambda 1 \) protein. The \( \lambda 1 \) protein represents only the third RNA triphosphatase whose primary structure is known and the first RNA triphosphatase, which is described in a double-stranded RNA virus.

**EXPERIMENTAL PROCEDURES**

Expression and Recovery of Recombinant \( \lambda 1 \)—Expression, recovery, and enrichment by zinc chelate affinity chromatography of recombinant \( \lambda 1 \) protein produced in the *Pichia pastoris* expression system has been previously described (14).

Synthesis of RNA Triphosphatase Substrates—The RNA triphosphatase substrates were transcribed in *vitro* from the XboI-digested pBluescript II SK (+) (Stratagene) with T7 RNA polymerase (Pharmacia Biotech Inc.). The transcripts (89 nucleotides) containing the sequence pppGp...a t h e 5' were synthesized in the presence of \( \gamma ^{32} \text{P} \text{GTP} \) (ICN; 4500 Ci/mmol) or \( \beta ^{32} \text{P} \text{GTP} \) prepared as described by Furuichi and Shatkin using \( \gamma ^{32} \text{P} \text{ATP} \) (ICN; 4500 Ci/mmol) (15).

1The abbreviation used is: NTPase, nucleoside triphosphate phosphohydrolase.
Sequential conversion of \( \gamma^{32}\text{P} \)ATP to \( \beta^{32}\text{P} \)GDP and \( \beta^{32}\text{P} \)GTP was monitored by chromatographic separation on polyethyleneimine cellulose followed by autoradiography (data not shown). The original DNA templates in transcription reactions were removed by DNase treatment (RQ1 DNase, RNase free, Promega), and the transcription products were extracted with phenol/chloroform and precipitated with ethanol. The RNA molecules labeled either at their \( \gamma \) or \( \beta \)-phosphate residue were resolved by electrophoresis on 6% polyacrylamide-urea gel, located following autoradiography, excised from the gel, and purified by elution overnight at 4 °C in a buffer containing 300 mM sodium acetate and 20 mM Tris-HCl, pH 8.0.

For competition assays, an unlabeled triphosphorylated RNA substrate was synthesized as described above and purified on polyacrylamide-urea gel. The amount of unlabeled RNA substrate was evaluated by spectroscopic absorption at 260 nm.

GTPase and RNA Triphosphatase Assays—The reactions were performed in a buffer containing 30 mM Hepes-KOH, 2 mM MgCl\(_2\), 0.2 pmol of \( \gamma^{32}\text{P} \)GTP (ICN; 4500 Ci/mmol) or 0.15 pmol of labeled RNA substrates and 2.5 ng (18.2 fmol) of recombinant \( \lambda \) protein in a total volume of 15 \( \mu \)l. The reactions were incubated at various temperatures and stopped by the addition of 0.1 M EDTA at times indicated, and aliquots (2 \( \mu \)l) were applied onto plastic-backed polyethyleneimine cellulose sheets (Aldrich). The reaction products were separated by ascending chromatography in 0.375 M potassium phosphate buffer (GTPase) or 0.8 M acetic acid, 0.9 M LiCl buffer (RNA triphosphatase); TLC plates were then air-dried and subjected to autoradiography.

The Michaelis-Menten constants (\( K_m \)) were determined by the isotope dilution method with unlabeled GTP or unlabeled triphosphatemodified RNA substrate. For quantitative evaluation, the spots corresponding to the radiolabeled substrates and reaction products were identified following autoradiography and excised from the gel, and purified by elution overnight at 4 °C in a buffer containing 300 mM sodium acetate and 20 mM Tris-HCl, pH 8.0.

The effect of MgCl\(_2\) concentration (A), temperature (B), and RNA concentration (C) on the \( \lambda \)-associated RNA 5'-triphosphatase activity was examined. Reactions were performed using \( \gamma^{32}\text{P} \)-labeled RNA under standard conditions as described under "Experimental Procedures," except that the MgCl\(_2\) (A) or RNA (C) concentrations were varied. Incubations were all performed at 37 °C except for in B, where the enzyme was incubated at various temperatures for up to 1 h. Inhibition experiments were conducted in standard conditions in the presence of varying concentrations of inhibitors (GTP or RNA). Inhibition constants (\( K_i \)) were calculated by plotting 1/\( v \) against the concentration of inhibitor (Dixon plots).
RESULTS

A1 Specifically Cleave the β-γ Phosphate Bond at the 5′ Terminus of RNA—In an effort to establish if the reovirus A1 protein can act as an RNA triphosphatase, an RNA substrate labeled at its terminal γ-phosphate was prepared by in vitro transcription in the presence of [γ-32P]GTP (Fig. 1A). This substrate was then exposed to recombinant A1 protein produced in P. pastoris yeast cells, as previously used for the study of A1 NTPase/helicase activity (14). To demonstrate that the RNA 5′-triphosphatase activity is specific to the removal of 5′-γ-phosphate from the RNA molecule, 5′-[β,γ-32P]GTP-terminated RNAs was also tested as a substrate. As shown in Fig. 1B, the A1 protein released a 32P-labeled product at the level of inorganic phosphate from [γ-32P]GTP-terminated RNAs but not from [β,γ-32P]GTP-terminated RNAs (Fig. 1C). The 32P-labeled material from [β,γ-32P]GTP-terminated RNAs was recovered solely at the origin of the chromatogram even after prolonged incubation periods (data not shown), whereas the addition of alkaline phosphatase released the 32P label, thus confirming that the 32P radioactivity was actually present in the free terminal phosphate groups. These results showed that A1 specifically cleaves the β-γ phosphate bond at the 5′ terminus of RNA. Protein extracts prepared from yeast cells transformed with control plasmid vector (pHIL-D2) had no activity in this assay.

Characteristics of the RNA 5′-Triphosphatase Activity—To gain additional insight into the A1-associated RNA 5′-triphosphatase activity, the reaction and its kinetic parameters were further investigated. The activity was absolutely dependent on the presence of a divalent cation, Mg2+ or Mn2+ (data not shown); the activity increased sharply with MgCl2 concentration and was optimal at 0.2 mM, followed by a slightly reduced activity at higher concentrations (Fig. 2A).

The effect of temperature on the RNA 5′-triphosphatase was also analyzed. The temperature optimum of the reaction, as judged by the maximum rate of triphosphate-terminated RNA hydrolysis, was 42°C; however, the activity declined after 20 min at this high temperature (Fig. 2B). A 50-min incubation at 25 and 37°C resulted in similar rates of hydrolysis.

An apparent $K_m$ value of 0.26 μM was determined by a double-reciprocal plot for the RNA substrate after 10 min at 37°C, at which time reaction is still proceeding at its initial rate. The RNA 5′-triphosphatase reaction velocity reached a maximum at $5.7 \times 10^{-14}$ mol/min and a $k_{cat}$ value of 3.1 min$^{-1}$ was estimated (Fig. 2C). Identical results were obtained in three separate experiments.

Common Active Site of the RNA 5′-Triphosphatase and NTPase Activities—Because the A1 protein possesses both RNA 5′-triphosphatase and NTPase activities (14, 15), the possibility that both reactions are carried at a common active site was finally investigated. In the first experiment, the ability of GTP to competitively inhibit the RNA 5′-triphosphatase activity was assessed. As shown in Fig. 3 (A and B), GTP was a linear competitive inhibitor of the RNA 5′-triphosphatase activity. Consistent with a competitive inhibition, the addition of GTP increased the apparent $K_m$ of the RNA 5′-triphosphatase activity, whereas no significant changes were made to the $V_{max}$ value. The $K_i$ for this inhibition was calculated from a Dixon plot to be approximately 2 μM (data not shown), which is comparable with the previously reported $K_m$ value of A1 (14) for GTP (2 μM), as predicted if both RNA and GTP substrates are competing for the same active site.

The reciprocal experiment using unlabeled triphosphorylated RNA substrate to inhibit the GTPase activity was then performed (Fig. 3, C and D). As expected, triphosphatase-terminated RNA also competitively inhibited the GTPase activity in a linear fashion, and an apparent $K_i$ of 0.3 μM was estimated for this inhibition, which is similar to the $K_m$ of A1 for the RNA triphosphatase activity. Other substrates were tested for their capacity to inhibit the GTPase and RNA 5′-triphosphatase reactions. However, no inhibition of either activity could be detected in the presence of large molar excess of GMP or $m^7$GpppG (data not shown). Taken together these data indicate that the RNA 5′-triphosphatase and NTPase activities occur at a common active site that is likely specific for triphosphorylated nucleotides.

DISCUSSION

Previous studies have shown that a NTPase activity is present in reovirus cores, and recent reports have established that the A1 protein is associated with this activity (13, 14, 16, 17). It has been postulated that the NTPase activity can also be responsible for the RNA triphosphatase reaction (18, 19). In this study, we took advantage of our recent analysis of yeast-ex-
pressed A1 NTPase/helicase activity to definitely demonstrate that A1 can hydrolyze the β-γ bond at the 5′ terminus of triphosphorylated RNA molecules. Kinetic analysis of these two phosphohydrolytic activities, NTPase and RNA triphosphatase, showed marked differences. The two activities differ in the MgCl₂ concentration at which maximal catalysis is achieved; the ATPase reaction reached a maximal catalysis at 2.5 mM MgCl₂ (14), whereas the RNA 5′-triposphatase activity is maximal at 0.2 mM MgCl₂. This situation is similar to the vaccinia virus capping enzyme, a protein that hydrolyzes the γ-phosphate of triphosphate-terminated RNAs and can also cleave the γ-phosphate out of free nucleoside triphosphates; with vaccinia enzyme, the NTPase activity also requires a 10-fold higher concentration of MgCl₂ compared with the RNA 5′-triposphatase activity (20 mM versus 2 mM) to reach maximal catalysis (20).

The NTPase and RNA 5′-triposphatase activities of A1 also exhibit important binding differences for their respective substrates. Apparent $K_m$ values of 1 μM and 2 μM were respectively determined for the NTPase and GTPase reactions (14) compared with 0.26 μM measured for the RNA 5′-triposphatase activity reported in this study. RNA 5′-triposphatases have been isolated from a variety of cellular and viral sources, but few detailed biochemical studies have been performed. However, the reported $K_m$ value of 0.26 μM for the RNA 5′-triposphatase activity of A1 is in the same range as the $K_m$ of other characterized RNA 5′-triposphatases from vaccinia virus (1 μM), Saccharomyces cerevisiae (1.4 μM), and rat liver nuclei (0.15 μM) (20–22).

Gene reassortment analysis and biochemical studies of a recombinant A1 protein have both demonstrated that A1 exhibits a preference for ATP, whereas the 5′-terminal nucleotide in each of the reovirus mRNAs is a guanosine (13, 14). It was therefore intriguing that a putative RNA triphosphatase would exhibit a preference for ATP and not GTP found as reovirus mRNA 5′-terminal residue. However, the present study clearly demonstrated that A1 exhibits an approximately 4-fold lower $K_m$ for GTP-terminated RNAs (0.26 μM) compared with the $K_m$ for ATP (1 μM). Thus, the A1 protein has a greater affinity for GTP-terminated RNAs than for any other free nucleotides. Interaction of A1 with nucleic acids can occur in the absence of a triphosphorylated end (12), and it is thus quite possible that additional interactions with the rest of the nucleic acid molecule stabilize the triphosphorylated RNA–enzyme complex.

The ability of the A1 protein to hydrolyze NTPs and triphosphatase-terminated RNAs raised the question of whether these reactions are carried out at a common or independent phosphohydrolysis active site. Based on kinetic competition analysis, it clearly appeared that the two reactions are carried out at a common active site. Similarly, competitive inhibition and simultaneous mutational inactivation of RNA triphosphatase and NTPase functions of the vaccinia virus capping enzyme have also suggested that both reactions occur at a single active site (20, 23). Recently, an RNA triphosphatase activity has also been assigned to the NS3 protein of the West Nile flavivirus, a protein that possesses NTPase activity (24). In this case, differences in optimal reaction conditions for NTPase and 5′-RNA triphosphatase activities have been presented as an indication that hydrolysis occurs at different sites, although no kinetic competition analyses have been performed to support this idea (24). When additional examples of RNA triphosphatases are identified, it will become possible to determine if the ability to remove the γ-phosphate from both free NTPs and triphosphorylated RNAs can be considered as a common property exhibited by these enzymes.

The A1 protein is a major component of the viral core and appears as a multifunctional protein; in addition to 5′-RNA triphosphatase activity, it possesses NTPase and helicase activities (14). Other viral RNA 5′-triposphatases can also catalyze additional enzymatic reactions. The D1 subunit of the vaccinia virus capping enzyme has both RNA 5′-triposphatase and guanylyltransferase activities (24–26). The West Nile Virus NS3 protein is also a multifunctional protein; it contains a protease activity in its amino-terminal part, a helicase in the central region, and the RNA triphosphatase tentatively assigned to the carboxyl-terminal domain (23, 27–29).

Although very few primary structures of RNA 5′-triposphatases are actually known, it has been noted that the LRRP amino acid sequence found in the reovirus A1 protein (30) is similar to the West Nile Virus NS3 protein (LRPR) (24) and vaccinia virus D1 subunit sequence LKPR (23), the only two other RNA triphosphatases whose primary structure is known. Because no actual structure-function studies have been performed, the importance of this motif remains purely speculative. Furthermore, another somewhat degenerate motif also seems to be present on the reovirus A1 protein (RDETGLM), vaccinia virus capping enzyme D1 subunit (RPNNTSLE), and West Nile virus NS3 (RTNTITLE). These motifs are also found on various putative 5′-RNA triphosphatases of other flaviviruses and DNA viruses. Interestingly, a substitution of the glutamate residue in this latter motif of the vaccinia virus capping enzyme inactivated the triphosphatase but did not affect the guanylyltransferase activity (23). This suggests that these consensuses motifs may have a functional significance, although further studies will be needed to firmly establish their exact nature and importance.

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