Novel Catalytic Activity Associated with Positive-Strand RNA Virus Infection: Nucleic Acid-Stimulated ATPase Activity of the Plum Pox Potyvirus Helicaselike Protein

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The cylindrical inclusion protein of potyviruses contains the so-called nucleoside triphosphate binding motif, an amino acid sequence motif present in proteins encoded by most positive-strand RNA viruses, some double-strand RNA viruses, apparently all groups of double-strand DNA viruses, and also several single-strand DNA viruses. Further sequence analysis has allowed to include the cylindrical inclusion protein of potyviruses as a member of a superfamily of helicase-like proteins. In this paper we show that the purified cylindrical inclusion protein of plum pox potyvirus interacts with RNA and ATP and copurifies with a nucleic acid-stimulated ATPase activity. To our knowledge, this is the first time that this kind of enzymatic activity has been experimentally associated with a positive-strand RNA virus-encoded protein.

Several sources of information have led to hypotheses about the involvement of different protein activities in the life cycle of RNA viruses. Recent technical advances have made it possible for several of these proposals to be empirically tested. Unfortunately, there are only a few cases in which there is experimental evidence about the particular biochemical process performed by an RNA virus-encoded protein. This is the case of the RNA-dependent RNA polymerase activity of proteins encoded by plus-, minus-, and double-strand RNA viruses (13). Another enzymatic activity unequivocally assigned to virus-encoded proteins is the protease activity that is responsible for polyprotein processing during the expression of several positive-strand RNA viruses (2, 24). Based on sequence similarity studies (5–8, 11, 16), a novel enzymatic activity, a nucleoside triphosphate-dependent RNA unwinding activity, has been proposed to be associated with the nucleotide binding motif (NTBM)-containing proteins encoded by the majority of positive-strand RNA viruses. In accordance with this hypothesis, a nucleic acid-stimulated ATPase activity, which could be related to helix unwinding, is shown in this paper to be expressed in plants infected with plum pox virus (PPV), a member of the potyvirus group of positive-strand RNA viruses. This activity was found to be associated with an NTBM-containing protein encoded by PPV, the cylindrical inclusion (CI) protein.

MATERIALS AND METHODS

Plant material. *Nicotiana clelandii* plants were infected with PPV (Rankovic isolate) by rubbing them with sap from infected plants and carborundum as an abrasive. The inoculated leaves were rinsed with distilled water, and the plants were incubated in a growth chamber at relative humidity of 85% and a daily cycle of 14 h of light (22°C) and 10 h of darkness (20°C). Leaves were collected for CI protein purification 6 to 7 weeks after inoculation.

ATPase activity assays. ATPase activity assays were performed with approximately 0.5 µg of purified CI protein and the indicated amounts of other protein samples in a buffer containing 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH (pH 7.5), 2.5 mM Mg(CH₃CO₂)₂, 1 mM dithiothreitol, and 0.1 mM [γ-³²P]ATP (approximately 150 Ci/mol) in a final volume of 30 µl. When indicated, KCl and/or various nucleic acids were included in the reaction mixture. Poly(A) was purchased from Sigma (average size of approximately 3,000 nucleotides), and poly(dA) was purchased from Boehringer-Mannheim (average size of 255 nucleotides). The reaction was carried out at 25°C because at this temperature a good balance between enzymatic activity and protein stability was observed (data not shown). Samples were taken after different incubation periods; the reaction was stopped in ice by adding EDTA to a concentration of 0.2 M, and samples were analyzed by polyethyleneimine-cellulose thin-layer chromatography with 0.15 M formic acid–0.15 M LiCl (pH 3) as the liquid phase. Autoradiography of the dried chromatographic plates served to locate the positions of ATP and P_i. Equivalent-sized pieces of thin layer surrounding these positions were cut, and the radioactivity in each piece was measured to quantitate the ATP hydrolyzed.

UV photolabeling. RNA-protein and ATP-protein cross-linking by UV light were performed by incubating approximately 1 ng of a ³²P-labeled 200-nucleotide RNA (3.3 × 10⁸ cpm/µg) or 12.5 µCi of [α-³²P]ATP (200 Ci/mmol) with 2 µg of protein from fraction P5 or 8 µg of bovine serum albumin in 25-µl volumes containing 20 mM HEPES-KOH (pH 7.5)–5 mM Mg(CH₃CO₂)₂ in a multiwell plate on ice and irradiated with a 15-W bulb (254-nm wavelength) from a distance of 2 cm for 1 h. The radiolabeled RNA probe contained sequences derived by *in vitro* transcription of a recombinant pT7.4 plasmid (22) into which the 156-nt *Hind*III fragment of phage Φ29 DNA (25) had been inserted. In the RNA-protein cross-linking experiments, unprotected RNA was digested for 30 min at 37°C with 5 µg of RNase A. The cross-linked RNA-protein and ATP(or ADP)-protein complexes were analyzed by electrophoresis on sodium dodecyl sulfate

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FIG. 1. (a) PPV CI protein purification scheme based on the method of Hiebert et al. (9). S, Supernatant; P, pellet. Buffers: A, 333 mM potassium phosphate buffer (pH 7.5), 0.17% (wt/vol) sodium sulfite, 17% (vol/vol) chloroform, 17% (vol/vol) carbon tetrachloride, and 0.53 M urea; B, 500 mM potassium phosphate buffer (pH 7.5) containing 0.8 M urea; C, 50 mM potassium phosphate buffer (pH 8.2) containing 0.1% 2-mercaptoethanol; D, 20 mM Tris hydrochloride (pH 8.2). Yields of about 14 mg of protein per 100 g of PPV-infected tissue were obtained. (b) Samples of 1/2750 of S1 and S2 fractions and 1/687.5 of the rest of the fractions were subjected to SDS-PAGE (12.5% polyacrylamide) (14) and Coomassie blue staining. The arrow indicates the position of CI protein present in an inclusion protein preparation purified by the method of Martín et al. (17). The molecular weights (in thousands) of protein markers run in the same gel are shown beside the panel. (c) ATPase activity (5-min reaction) in the absence and in the presence of poly(A) (0.21 mM adenosine monophosphate). Samples of 1/9160 of each fraction were used for the enzymatic assays. Poly(A)-stimulated activity could not be detected in the S1 and S2 fractions, probably due to the denaturing properties of the solvent (data not shown).

(SDS)-polyacrylamide (10%) gels (14). The gels were stained with Coomassie blue, dried, and autoradiographed.

Protein retention of poly(A) on nitrocellulose filters. The retention of 5'-end ^{32}P -labeled poly(A) (specific activity, 9,000 cpm/ng) was performed under conditions in which RNA is not retained on nitrocellulose unless it is bound to protein. Samples (50 μl) containing 20 mM HEPES-KOH (pH 7.5), 5 mM $\text{Mg}(\text{CH}_3\text{CO}_2)_2$, 1.5 ng of ^{32}P -labeled poly(A), and the indicated amounts of protein from fraction P5 were incubated at 0°C. In competition assays, the indicated amounts of nonlabeled poly(A) or poly(dA) were present in the reaction mixture. After 30 min of incubation, the samples were diluted with 1 ml of cold washing buffer [20 mM Tris hydrochloride (pH 7.5), 0.1 mM EDTA, 2.5 mM $\text{Mg}(\text{CH}_3\text{CO}_2)_2$] and rinsed twice with

1 ml of this buffer; the radioactivity retained on the nitrocellulose filter was determined by scintillation spectrophotometry.

RESULTS

A poly(A)-stimulated ATPase activity induced by PPV infection associated with CI protein. Extracts from PPV (Rankovic strain)-infected and healthy *N. clevelandii* leaves were fractionated, essentially as described by Hiebert et al. (9), for the purification of CIs from potyvirus-infected plants (Fig. 1a). This method is based on the large size of the cytoplasmic aggregates formed by the potyvirus CI protein and on their stability to shear forces, detergents,

and organic solvents. The major band observed with SDS-polyacrylamide gel electrophoresis (PAGE) (14) of fractions P2 to P5 from infected leaves was absent in extracts from healthy plants (Fig. 1b). The position of this band is the same as that of the recently identified PPV CI protein (17). Also, a poly(A)-stimulated ATPase activity was observed to be PPV infection dependent (Fig. 1c). This activity correlates well with the presence of PPV CI protein throughout the purification procedure (Fig. 1c). Similar results were obtained when fractions from the purification procedure described by Martín et al. (17) were analyzed (data not shown).

The degree of poly(A) stimulation of the ATPase activity varied in different purifications and depended on the amount of protein assayed (data not shown). The level of activity could not be lowered by treatment of the purified fraction with RNases and/or DNase I (data not shown), suggesting either that the basal activity is high or that nucleic acids remain inaccessible to degradation in the protein aggregates. These results contrast with the low basal ATPase activity of the RNA helicases human p68 (12) and mouse translation initiation factor 4A (eIF-4A) (1), which show extensive sequence similarity with the PPV CI protein (8, 16). As with other helicase activities (4), we found by thin-layer chromatography that ATP was hydrolyzed to ADP and P_i (data not shown).

Poly(A)-stimulated ATPase activity is associated with PPV CI protein. To identify the protein responsible for the poly(A)-stimulated ATPase activity induced by PPV infection, fraction P5 obtained from infected leaves (Fig. 1a) was sedimented through a sucrose gradient. The PPV-CI protein (detected by SDS-PAGE) comigrated with the ATPase activity assayed in the presence of poly(A) (Fig. 2); a maximum at positions 24 through 27 is clear for both the PPV CI protein and the ATPase. However, a large amount of CI protein and ATPase activity sedimented at the bottom of the gradient, probably because of aggregation of PPV CI protein with other components of the sample. Some activity was also detected in fractions from the top of the gradient. This activity could be due to small amounts of nonaggregated CI protein and/or to another protein component in the sample.

A radiolabeled single-strand RNA probe of 200 nucleotides, obtained by *in vitro* transcription, or [α - 32 P]ATP was incubated with purified CI protein (fraction P5) or bovine serum albumin and irradiated with UV light. This treatment covalently cross-links protein-RNA and protein-ATP complexes (3, 19). The unbound RNA in the RNA-protein cross-linking experiments was digested with RNase A. After incubation, the samples were analyzed by SDS-PAGE (14) and autoradiography. The PPV CI protein was able to bind RNA as well as ATP (or ADP; we have not determined whether it is ATP and/or ADP that becomes cross-linked to CI protein) (Fig. 3). A faint band with an electrophoretic mobility corresponding to approximately 120 kDa, which immunoreacts with sera against CI protein purified from SDS-polyacrylamide gels (data not shown), also interacts with RNA and ATP. This band probably corresponds to a CI protein precursor form produced during the polyprotein processing expression strategy of PPV (15). No protein from fraction P5 from noninfected leaves was photolabeled with 32 P-RNA or [α - 32 P]ATP (data not shown).

Nucleic acid stimulation of the CI-associated ATPase activity. Nucleic acid stimulation of NTPase activity is a general feature for helicase-related proteins (1, 10, 20, 21; reviewed in reference 4). The NTPase activity of each kind of heli-

FIG. 2. Analysis by sucrose gradient centrifugation of purified PPV CI protein. Purified PPV CI protein (fraction P5, approximately 1 mg of protein) to which Triton X-100 was added to a final concentration of 1% was sedimented through a 5-ml 50 to 80% sucrose gradient in 20 mM phosphate buffer (pH 8.2) and 0.1% 2-mercaptoethanol for 1 h at $70,000 \times g$ (maximum). Fractions of approximately 200 μ l were collected. (a) Samples of 30 μ l of the indicated fractions were subjected to SDS-PAGE and Coomassie blue staining; (b) 3- μ l samples were assayed for ATPase activity in the presence of poly(A) (0.21 mM adenosine monophosphate). Sedimentation was from left to right.

case is preferentially stimulated by the type of nucleic acid to which it is functionally related. The ATPase activity associated with the CI protein was stimulated by poly(A) (Fig. 4a); poly(dA) also stimulated ATPase activity, although to a lesser extent. Furthermore, whereas the basal reaction was not affected by the presence of KCl, the stimulations by poly(A) and poly(dA) were sensitive to salt concentration (Fig. 4b). Even though the amount of added poly(dA) was 10-fold the amount of poly(A) (giving rise to similar stimulation efficiencies in the absence of salt), the stimulation of the ATPase activity by poly(dA) was more sensitive than stimulation by poly(A) to salt concentration.

The specificity of CI protein-nucleic acid interactions was studied by retention in nitrocellulose membranes of poly(A) incubated in the presence of purified CI protein (Fig. 5a). The efficiency of the binding was clearly lowered by the addition of salt (Fig. 5a), in accordance with the salt sensitivity of the stimulation of ATPase by poly(A). Higher affinity of the purified protein to poly(A) than to poly(dA), mainly in the presence of salt, could be observed in competition experiments (Fig. 5b and c).

Different sorts of nucleic acids stimulated the P5 ATPase activity to variable extents. The highest stimulation was obtained by RNAs lacking secondary structure (data not

FIG. 3. Analysis of protein-RNA (a) and protein-ATP (or ADP) (b) complexes by SDS-PAGE (10% polyacrylamide) (14). Coomassie blue staining and autoradiography of UV-irradiated and nonirradiated samples containing ^{32}P -labeled RNA (a) or $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ (b) and 2 μg of protein from fraction P5 or 8 μg of bovine serum albumin (BSA). The molecular weights (in thousands) of protein markers run in the same gel are shown between the panels.

shown). In summary, the CI-associated ATPase activity may be described as preferentially stimulated by single-strand RNA among other nucleic acids. This assumption is in agreement with a possible functional role of the potyviral CI protein as a helicase involved in some step of viral replication.

DISCUSSION

The binding (and hydrolysis) of nucleoside triphosphates is an essential step for the function of proteins and protein complexes involved in diverse biochemical processes. Amino acid sequence similarity searches have resulted in the definition of a cassette of amino acids common to many proteins for which nucleoside triphosphate binding has been demonstrated or postulated. The A/GXXXXGKS/T motif was first observed by Walker et al. (23). Recently, the same cassette of residues was found in proteins encoded by most positive-strand RNA viruses, double-strand DNA viruses, and several single-strand DNA viruses. Although few complete genomic sequences of double-strand RNA viruses have been determined, it seems that the codification of an NTBM-containing protein is also frequent among these viruses (6). In contrast, this motif has not been found in proteins encoded by negative-strand RNA viruses and retroviruses (6; S. de la Luna, personal communication). The search for further sequence similarity between the NTBM-containing proteins of positive-strand RNA viruses has led to their classification in three major groups: the NTBM-containing proteins of viruses like Sindbis virus; viruses like picornavi-

FIG. 4. (a) Stimulation by poly(A) and poly(dA) of ATPase activity associated with PPV CI protein. The ATPase activity of purified PPV CI protein (fraction P5) was assayed under standard reaction conditions (7 min) (see Materials and Methods) with increasing concentrations of poly(A) and poly(dA). (b) Sensitivity to salt concentration of the PPV CI-associated ATPase activity. The ATPase activity was assayed (6-min reaction) in the absence and in the presence of poly(A) (0.021 mM adenosine monophosphate) or poly(dA) (0.21 mM adenosine monophosphate) with increasing concentrations of KCl.

rus; and potyviruses, flaviviruses, and pestiviruses (6). These three groups of NTBM-containing proteins have been included in three distinct superfamilies of NTBM-containing proteins from very diverse origins and are involved in different biochemical processes with the common feature of being related with the unwinding of nucleic acid secondary structures with nucleoside triphosphate hydrolysis as the source of energy (6, 11, 16). However, no experimental evidence for the enzymatic activity of the NTBM-containing

FIG. 5. (a) Retention of ^{32}P -labeled poly(A) to nitrocellulose filters by increasing amounts of purified PPV CI protein (fraction P5) in the absence or presence of 120 mM KCl. (b and c) Retention of ^{32}P -labeled poly(A) to nitrocellulose filters by purified CI protein (fraction P5) in the presence of increasing amounts of competing poly(A) or poly(dA): (b) no salt, 50 ng of protein; (c) 120 mM KCl, 2 µg of protein. The poly(A) retained in the absence of competitor corresponds to a value of 100%.

protein encoded by positive-strand RNA viruses has been reported.

In this paper, we describe a nucleic acid-stimulated ATPase activity present in extracts from leaves infected with PPV. This activity copurifies with the cytoplasmic inclusions formed by the NTBM-containing protein (PPV CI protein) of the virus. In vitro stimulation of this activity showed that it is preferably enhanced by the interaction of the protein with single-strand RNA. However, the specificity seems to be lower than that described for helicases of the superfamily to which the potyviral CI proteins are related by sequence similarity (i.e., eIF-4A [1, 18] and p68 [10]). This low specificity and the high basal activity in the absence of added nucleic acids could be related to the aggregation of the protein in the sample. Alternatively, this result could be due to intrinsic characteristics that may differentiate the potyviral and cellular activities; indeed, although clear similarity was observed among all proteins of the superfamily, closer relationships could be defined among either cellular or viral proteins than between any of the viral proteins and cellular helicases such as p68 or eIF-4A (16).

In summary, the evidence shown in this report suggests that the NTBM-containing proteins encoded by positive-strand RNA viruses present a nucleic acid-stimulated nucleoside triphosphatase activity which, according to the sequence similarity data, is probably involved in RNA unwinding.

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