RNA helicase: a novel activity associated with a protein encoded by a positive strand RNA virus

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Received August 1, 1990; Revised and Accepted November 8, 1990

ABSTRACT

Most positive strand RNA viruses infecting plants and animals encode proteins containing the so-called nucleotide binding motif (NTBM) (1) in their amino acid sequences (2). As suggested from the high level of sequence similarity of these viral proteins with the recently described superfamilies of helicase-like proteins (3-5), the NTBM-containing cylindrical inclusion (CI) protein from plum pox virus (PPV), which belongs to the potyvirus group of positive strand RNA viruses, is shown to be able to unwind RNA duplexes. This activity was found to be dependent on the hydrolysis of NTP to NDP and Pi, and thus it can be considered as an RNA helicase activity. In the *in vitro* assay used, the PPV CI protein was only able to unwind double strand RNA substrates with 3' single strand overhangs. This result indicates that the helicase activity of the PPV CI protein functions in the 3' to 5' direction (6). To our knowledge, this is the first report on a helicase activity associated with a protein encoded by an RNA virus.

INTRODUCTION

The identification of a nucleotide binding motif (NTBM) (1) in the amino acid sequence of a protein is showing to be a good criterium to predict nucleotide hydrolisis activity or nucleotide binding capacity for the protein. Proteins with helicase activity, currently defined as an activity that unwinds duplex polynucleotides by using a nucleoside triphosphate as an energy source (7), in many cases contain this motif (2). According to amino acid sequence comparisons, three superfamilies of helicases, which include the NTBM containing proteins encoded by most positive strand RNA viruses, have been recently defined (2-5). It is interesting to note that the degree of sequence similarity between NTBM containing proteins encoded by positive strand RNA viruses which have comparable genome structures can be lower than the level of similarity found between the NTBM-containing protein of certain groups of these viruses and the NTBM-containing proteins of apparently distantly related viruses and cellular helicases. This is the case of the potyvirus cylindrical inclusion (CI) proteins, which present nine amino acid sequence motifs in common with the flavi- and pestivirus NTBM-containing proteins and with the eukaryotic translation initiaton factor eIF-4A-related family of helicase-like proteins (2,4), whereas the NTBM-containing protein of picorna-like viruses, whose genomic structure is similar in many aspects to that of potyviruses (8), has been recently considered to be related with SV40 large T-antigen (5), a well characterized DNA and RNA helicase (9,10).

We have recently described the purification of PPV CI protein from infected *Nicotiana clevelandii* leaves and found that it presents a nucleic acid stimulated ATPase activity (11). In this paper we report that the purified PPV CI protein is able to separate the strands of partially hybrid RNA molecules, supporting the hypothesis on the RNA unwinding activity of the NTBM-containing proteins of positive strand RNA viruses.

MATERIALS AND METHODS

Substrates

ssRNAs used to obtain the partial dsRNA substrate A were prepared by run-off transcription of linearized plasmids purified by CsCl gradients. Transcription assays were run as recommended (Promega, Transcription systems, protocol # 1) using α^{32}P-UTP (12 μM, 400 Ci/mmol) for the synthesis of labelled transcript. The unlabelled strand (approximately 230 nt) was transcribed from plasmid pXds2 (a pX1 (12) derivative in which the SmaI-HindIII fragment of pPPV1 (13) had been inserted) digested with NdeI and PvuII. The labelled strand (62 nt) was transcribed from pT4ps2 (a pT7.4 (14) derivative containing the PstI-PvuII fragment of pUC18 (15) between the PstI and SmaI cloning sites) digested with EcoRI and PvuII. The unlabelled strand of substrate RNA B is the same one used to obtain substrate RNA A (Figure 1). The labelled strand (19 nt) was transcribed from pXst15 (a pX1 (12) derivative containing the 204 nt HincII-PvuII fragment of pUC18 (15) cloned into its StuI site) digested with SmaI and PvuII. The unlabelled strand of substrate RNA C was transcribed from pT4M2 (a pT7.4 (14) derivative containing the 156 nt HindIII fragment of phage ϕ29 DNA (16)) digested with SmaI and PvuII. The labelled strand (19 nt) was transcribed from pXds2 digested with HincII and PvuII. After DNaseI treatment and phenol-chloroform extraction

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of the transcription reactions, the aqueous phase was filtered through a 1 ml Sephadex G50−80 column. The amounts of unlabelled and labelled transcripts synthesized were quantitated by EtBr staining of the RNA after PAGE and scintillation counting, respectively. Annealing of the partially complementary transcripts was performed with a twenty-fold excess of the unlabelled over the labelled strand in the conditions previously described (10).

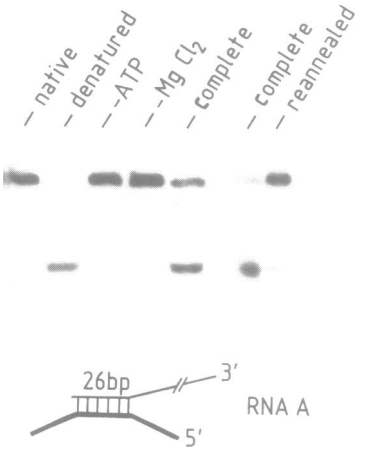

Fig. 1. RNA unwinding activity of purified CI protein. Analysis of the unwinding reaction by SDS-PAGE and autoradiography. The substrate RNA A, schematically drawn below, has a 26 bp long ds region with 3′ and 5′ overhanging ss ends of 18, 18, 2 and ~200 nucleotides. The small RNA strand, drawn with a bold line, was labelled with 32P-UMP. Lane 1, native substrate incubated without protein (incubation did not alter the electrophoretic mobility of the substrate); lane 2, heat denatured substrate; lanes 3−5, native substrate incubated with 0.5 μg of protein from fraction P5 under the RNA helicase reaction conditions; lane 3, without ATP; lane 4, without $MgCl_2$; lane 5, complete reaction mixture; lane 7, the enzymatically separated RNA strands (lane 6) were purified by phenol-chloroform extraction and incubated under annealing conditions.

Helicase assay

The RNA unwinding assay was performed in 30 μl volumes containing approximately 1 ng of radioactively labelled RNA substrate and the indicated amount of purified PPV CI protein in a reaction buffer containing 30 mM Tris-HCl, pH 7.5; 1.5 mM $MgCl_2$; 15 mM dithiothreitol; 30 μg ml^{-1} bovine serum albumin; 0.12 unit μl^{-1} RNasin (Promega); and 2 mM ATP. After incubation for 15 min at 25°C reactions were stopped by adding 0.1 volume of 3% SDS/150 mM EDTA. Samples were loaded in 12% polyacrylamide gels (acrylamide: bisacrylamide, 38:2) containing 0.1% SDS and TBE buffer (17).

RESULTS

As shown in Fig. 1, purified CI protein presented an RNA unwinding activity which absolutely required the addition of ATP and $MgCl_2$ to the reaction mixture. Thin layer chromatography analysis revealed that ATP hydrolysis to ADP and Pi took place during the RNA unwinding reaction. Two nonhydrolyzable analogs of ATP, AMP-PCP and AMP-PNP, failed to substitute ATP for unwinding (data not shown). Given the ATP hydrolysis requirement, the activity responsible for the unwinding of the RNA duplex can be defined as a helicase. The enzymatically separated RNA strands could be reannealed to reconstitute the partially double-stranded substrate (Fig. 1), indicating that RNA

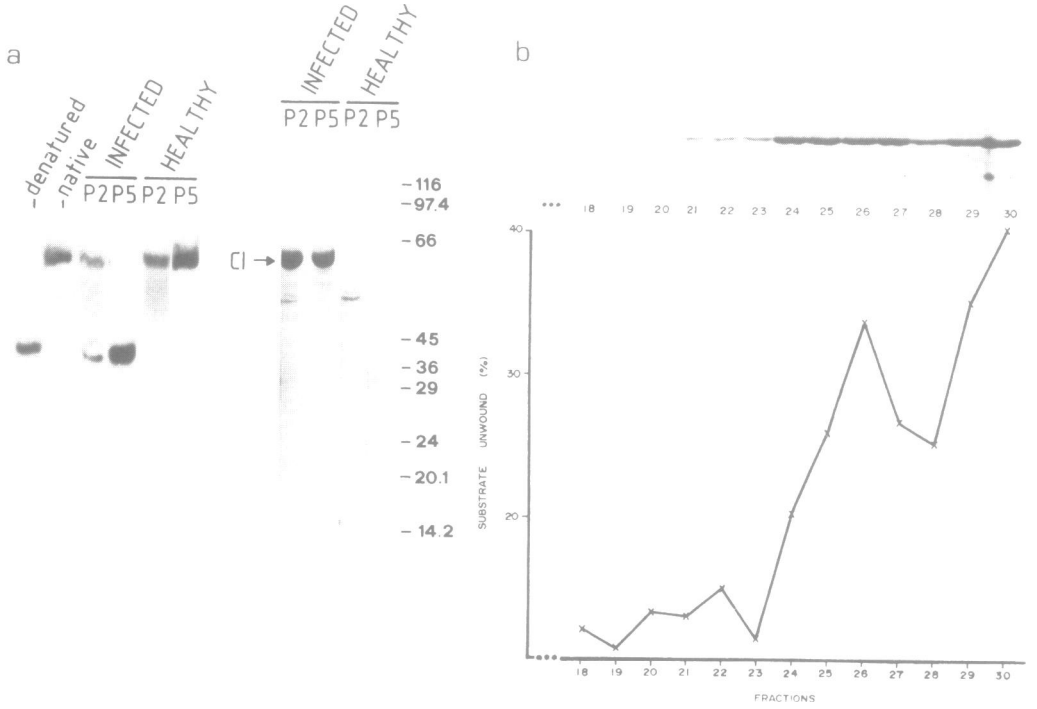

Fig. 2. a, RNA A unwinding assay (left) and SDS-PAGE analysis (right) of a crude fraction (P2) and of purified CI protein (P5) obtained from PPV infected leaves as described (11). Same assay of the corresponding fractions from healthy leaves. b, Cosedimentation of RNA unwinding activity and CI protein. After sedimentation of approximately 1 mg protein from fraction P5 through a 5 ml 50 to 80% sucrose gradient (sedimentation was from left to right), 30 μl and 3 μl aliquots of each 200 μl fraction of the gradient were analysed by SDS-PAGE and Coomassie blue staining (above) and assayed for unwinding (below), respectively.

was not chemically modified during the unwinding reaction in contrast with what has been observed for the RNA unwinding-modifying function isolated from Xenopus eggs (18). A DNA substrate, obtained by hybridization of labelled universal sequencing DNA primer to M13 single strand DNA, failed to be unwound in any of the conditions tested, suggesting that the helicase activity is specific for RNA substrates.

In addition to the clear sequence similarity of the potyvirus CI protein with helicases (2,4), several experimental criteria indicate that the CI protein is responsible for the ATP-dependent RNA unwinding activity: 1) The RNA helicase activity was detected in extracts from PPV infected leaves, but not in extracts from healthy leaves (Fig. 2a). 2) PPV CI protein comigrated with helicase activity throughout its purification procedure (Fig. 2a). 3) The RNA helicase activity cosedimented with PPV cylindrical inclusions in a sucrose gradient (Fig. 2b). 4) CI protein is specifically cross-linked to RNA and ATP (or ADP) (see ref. 11).

The RNA unwinding activity was assayed in the presence of different ribonucleoside triphosphates (Fig. 3), showing a slight preference for purine nucleotides over pyrimidine nucleotides. This is in agreement with the definition of the NTBM as a motif present in purine nucleotide binding proteins (1), although some of them are also able to use pyrimidine nucleotides (19,20). The

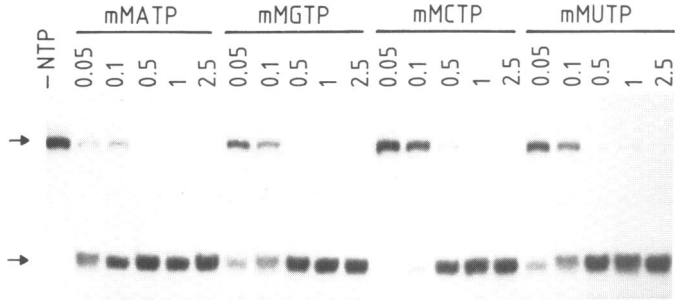

Fig. 3. Nucleotide specificity of the helicase activity. 0.5 mg of purified CI protein were assayed for unwinding of RNA A in the absence (lane 1) and the presence of increasing amounts of each ribonucleotide. Arrows indicate the position of the native (top) and labelled strand of the heat denatured (bottom) substrate RNA A.

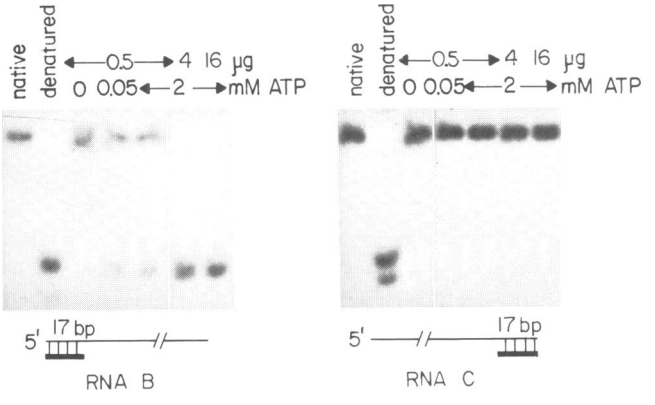

Fig. 4. Substrate requirements for the unwinding reaction. Both substrates possessed a 17 bp ds section and ss overhangs of approximately 200 nt. The labelled strands are drawn with a bold line. The amounts of purified CI protein and ATP used are shown.

same level of activity and the same specificity with respect to the base moiety was observed when dNTPs were used (data not shown).

In most cases, helicases require single stranded regions for initial attachment (7). Substrate RNA A, presented both 5′ and 3′ single strand overhangs (Fig. 1). In order to study whether there was specificity of the RNA helicase activity of PPV CI protein for any of both types of overhangs, unwinding of RNA substrates with long 3′ (RNA B) or 5′ (RNA C) single strands was tested. While RNA B could be unwound with the same nucleotide requirements as RNA A, no unwinding of RNA C could be detected (Fig. 4).

The direction of unwinding has been defined relative to the strand which is initially bound by the helicase (6). Following this assumption, it is expected that RNA unwinding by CI protein, which is apparently specific of RNA substrates with 3′ single strand overhangs, would proceed in the 3′ to 5′ direction. This result correlates with those obtained for the RNA and DNA unwinding activities of SV40 large T antigen (9,10) and for the RNA unwinding activity of human protein p68 (21). Recently, eIF-4A has been shown to present a bidirectional RNA helicase activity. Given that unwinding in both directions by eIF-4A absolutely requires the presence of eIF-4B and that bidirectionality does not seem to be an intrinsic property of helicases (6), the existence of other factors modifying the direction of unwinding of the PPV CI protein cannot be excluded.

DISCUSSION

Although RNA viruses exhibit a great variety of host/vector specificity, morphology and genome structure, the recent analysis of the amino acid sequences of the proteins encoded by these viruses is revealing surprising evolutionary relationships between apparently distantly related RNA viruses infecting plants and animals which may be explained by the mixing of modules of genetic information by recombination events (22,23). Common amino acid motifs in proteins encoded by different RNA viruses have been observed mainly among non structural proteins associated with enzymatic activities. Additionally, these motifs can be also identified in cellular and DNA virus enzymes. The most ubiquitous of these motifs is the G/MDD motif conserved among polymerases apparently encoded by all RNA viruses (24,25). This motif has also been related with the common polymerization domain of DNA polymerases (26). Similarly, most positive strand RNA viruses infecting plants and animals encode helicase like proteins (2). Therefore, it is tempting to speculate that RNA unwinding, shown in this paper to be associated with the PPV NTBM-containing protein, is a common step in the positive strand RNA virus replication cycle. Only positive strand viruses with small genomic RNAs do not encode an NTBM-containing protein (2). Due to the small size of the genomic RNA molecules of these viruses it could be that RNA unwinding is not needed for their replication, but the possibility that a cell encoded helicase is used cannot be ruled out.

Although still scarce, experimental data suggest that the NTBM- containing proteins of positive strand RNA viruses are involved in their genome replication (27–29). In the case of polio and cowpea mosaic virus, one of the particular functions postulated for these proteins in the replication mechanism is to attach the replication complex to membranes (30,31). Furthermore, 2C protein, the NTBM-containing protein of poliovirus, also seems to be involved in the release of newly

synthesized positive sense RNA molecules from the replication complex (27,30), an event which could be nicely explained by the action of an RNA helicase activity. Given the aforementioned ubiquity of helicase-like proteins among positive strand RNA viruses, indicating that RNA unwinding is probably a general requirement for the genomic replication, the study of the enzymatic properties of the potyvirus CI protein will be of interest in the further understanding of RNA replication mechanisms and in the design of strategies to interfere positive strand RNA virus infections.

ACKNOWLEDGEMENTS

We thank Dr. M. Salas, Dr. L. Blanco, Dr. M.T. Martín and M.T. Cervera for critical reading of the manuscript, E. Lozano for experimental collaboration and the Departamento de Química Agrícola (UAM) for greenhouse space. This work was supported by grant BIO88-0257 from CICYT and by grants from Fundación Ramón Areces. S.L. and J.L.R. were recipients of fellowships from Fondo de Investigaciones Sanitarias and Plan de Formaciùn de Personal Investigador, respectively.

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