RNA helicase activity of Semliki Forest virus replicase protein NSP2

Marta Gomez de Cedrón[a], Neda Ehsani[b], Marja L. Mikkola[b], Juan Antonio García[a], Leevi Kääriäinen[b,*]

[a]*Centro de Biologia Molecular (CSIC-UAM), Universidad Autónoma, Canto Blanco, 28049 Madrid, Spain*
[b]*Institute of Biotechnology, Biocenter Viikki, P.O. Box 56 (Viikinkaari 9), FIN-00014 University of Helsinki, Helsinki, Finland*

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Abstract Semliki Forest virus replicase protein nsP2 shares sequence homology with several putative NTPases and RNA helicases. NsP2 has RNA-dependent NTPase activity. Here we expressed polyhistidine-tagged nsP2 in *Escherichia coli*, purified it by metal-affinity chromatography, and used it in RNA helicase assays. RNA helicase CI of plum pox potyvirus was used as a positive control. Unwinding of α-^{32}P-labelled partially double-stranded RNA required nsP2, Mg^{2+} and NTPs. NsP2 with a mutation, K192N, in the NTP-binding sequence GVPGSGK$_{192}$SA could not unwind dsRNA and had no NTPase activity. This is the first demonstration of RNA helicase activity within the large alphavirus superfamily.

Key words: Alphavirus; RNA replication; Helicase

1. Introduction

Semliki Forest virus (SFV), a member of the alphavirus genus of the Togaviridae family, has a positive-stranded RNA genome of 11.5 kb (42S RNA). The RNA replication takes place in the cytoplasm by an RNA-dependent RNA polymerase consisting of non-structural proteins nsP1–4, which are cleavage products of a 2342 aa polyprotein (for review see [1]). The parental 42S RNA is copied to a complementary 42S RNA minus-strand, which is used as a template for the synthesis of new 42S RNA plus-strands and a subgenomic 26S mRNA. The RNA replication and transcription of the 26S RNA take place in association with cytoplasmic vacuoles (CPVI), which are modified endosomes and lysosomes. The role of nsPs in RNA replication has been studied over the past 20 years [1]. NsP1 is an RNA capping enzyme [2–6] and a specific 'initiation factor' for the synthesis of 42S RNA$^-$ strands [7]. NsP4 is the catalytic subunit of the polymerase [1,8,9], and nsP3 is a phosphoprotein with poorly defined, but essential functions in RNA replication [1,10,11].

On the basis of sequence analysis, biochemical and genetic data, nsP2 (799 aa in SFV) consist of two functional domains. The C-terminal half has a papain-like proteinase domain [12], and a nuclear localization signal responsible for the transport of nsP2 to the cell nucleus [13]. Mutations in the C-terminus affect the level of 26S RNA, suggesting that nsP2 serves as an initiation factor for the synthesis of the subgenomic RNA ([14] and references therein).

The N-terminal half of nsP2 has sequence homology (aa 183–416) with the nucleoside triphosphatase (NTPase) and RNA helicase protein superfamily 1 [15]. However, among the large group of alphavirus-like viruses RNA helicase activity has not been demonstrated for any of the NTP-binding helicase motif-containing proteins. We have previously shown that SFV nsP2 has NTPase activity, which is stimulated by single-stranded RNA suggesting that this protein might be an RNA helicase [16,17]. Here we show directly that nsP2 is indeed an RNA helicase, which is an essential activity for virus replication.

2. Materials and methods

2.1. Expression in Escherichia coli and purification of NSP2 and NSP-GNS

NsP2 and its NTPase-negative variant nsP2-GNS were expressed in *E. coli* using two plasmids, pHAT-ns2 encoding wild type nsP2, and pHAT-ns2/GNS encoding nsP2 with a point mutation K192N in the NTP-binding sequence [18]. Both constructs had N-terminal His$_6$ tags [16]. *E. coli* JM109 (DE3) harboring the plasmids was grown on an LB plate in the presence of 50 μg/ml ampicillin. A single colony was inoculated into 20 ml of 2×YT medium containing 100 μg/ml ampicillin and grown for 16 h at 37°C. A 10 ml inoculum of the overnight culture was added to 1 l of 2×YT medium containing 100 μg/ml of ampicillin, and grown to $OD_{600} = 0.6$ at 37°C, transferred to 24°C, and induced with 50 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 h at 24°C. All further steps were carried out at 4°C. The cells were pelleted by centrifugation at 15 000×*g* for 10 min, and resuspended in 30 ml of 1.25× buffer A (50 mM Tris-HCl pH 8.0, 5 mM imidazole, 1 mM dithiothreitol (DTT), 0.1% Tween 20, and 1 mM phenylmethylsulfonyl fluoride). The cell suspension was passed twice through a French press cell (SLM Instruments, Inc.) at 10 000 lb/in^2. The broken cells were centrifuged at 15 000×*g* for 15 min, and 5 M NaCl was added to the supernatant (S15) to a final concentration of 1 M.

Ni^{2+}-nitrotriacetate resin (NTA; Qiagen, Chatsworth, CA) was used for purification of the polyhistidine-tagged proteins. The resin (2 ml) was washed with distilled water and equilibrated in buffer B (50 mM Tris-HCl pH 8.0, 1 M NaCl, 5 mM imidazole, 0.1% Tween 20). The supernatant was loaded onto the resin and weakly bound proteins were washed with buffer B (10×resin volume). Removal of contaminant proteins was achieved by stepwise addition of imidazole (10–40 mM) to buffer B. NsP2 and nsP2-GNS were eluted with buffer B containing 100 mM imidazole. The eluted proteins were concentrated in an Omega stirred cell (Filtron Technology Corporation, MA) with a 30 kDa Omega membrane at 2 bar. Purified recombinant proteins (0.4 mg/ml in 750 mM NaCl) were stored at −70°C after addition of glycerol to a final concentration of 20%.

2.2. RNA helicase assay

The RNA unwinding assay was performed in 15 μl reaction mixtures containing indicated amounts of nsP2 proteins and buffer (1 mM Tris-HCl pH 7.5, 1.5 mM $MgCl_2$, 1.5 mM DTT, 30 mg/ml bovine serum albumin (BSA), 0.12 U/ml RNasin (Promega)), 2 mM ATP and partially double-stranded RNA substrate. The shorter strand of 62 nt, labelled with [^{32}P]UMP, had 18 nt 3′ and 5′ overhangs, respectively. The longer RNA strand had a 200 nt 3′ overhang, 26 nt complementary to the shorter strand and a 2 nt 5′ overhang [19,20]. After incubation for 20 min at 25°C the reaction was stopped by addition of 1.5 μl of 3% SDS and 150 mM EDTA. Samples were electrophoresed in 8% polyacrylamide gels containing 0.1% SDS and 0.5% TBE buffer. The radioactivity was detected by autoradiography.

*Corresponding author. Fax: (358) (9) 70859560.
E-mail: leevi.kaariainen@helsinki.fi

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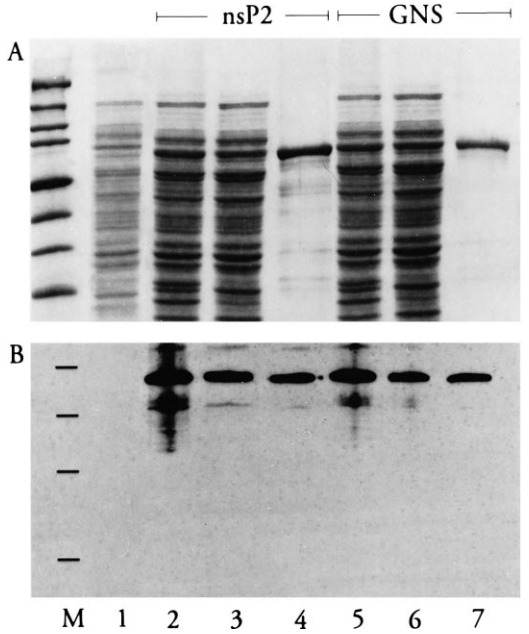

Fig. 1. Purification of recombinant nsP2. SDS-PAGE analysis of SFV nsP2 and nsP2-GNS, which were expressed in *E. coli* and purified. A: Coomassie blue staining. B: Immunoblotting with anti-nsP2 antiserum. Lanes 1: total cell extract before induction; lanes 2: total cell extract with nsP2 5 h after induction with 50 mM IPTG; lanes 3: S15 with nsP2; lanes 4: purified nsP2; lanes 5: total cell extract with nsP2-GNS 5 h after induction with IPTG; lanes 6: S15, nsP2-GNS; lanes 7: purified nsP2-GNS. Lanes M: molecular mass markers; from the bottom in A: 27, 36, 55, 66, 97, 116, 158, 212 kDa, and in B: 30, 43, 67, 97 kDa.

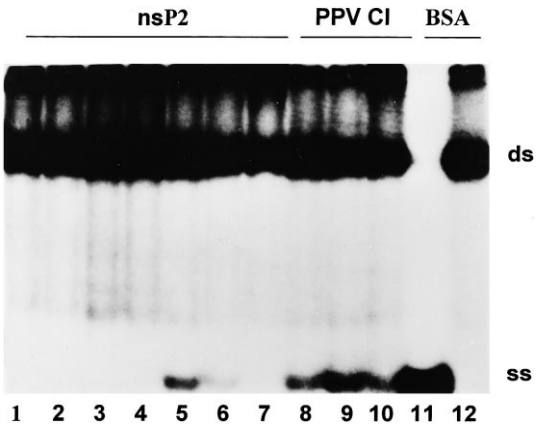

Fig. 2. Detection of RNA helicase activity of nsP2. PAGE analysis of RNA helicase activity of SFV nsP2 and PPV CI. Lane 1: 0.25 μg nsP2 in complete reaction mixture without $MgCl_2$; lane 2: 0.5 μg of nsP2 without $MgCl_2$; lane 3: 0.25 μg nsP2 without ATP; lane 4: 0.5 μg nsP2 without ATP; lane 5: 0.25 μg of nsP2 in complete mixture; lane 6: 0.5 μg nsP2 in complete mixture; lane 7: 1.0 μg nsP2 in complete mixture; lane 8: PPV CI 0.25 μg in complete mixture; lane 9: 0.5 μg PPV CI in complete mixture; lane 10: 1.0 μg PPV CI in complete mixture; lane 11: BSA in complete reaction mixture, heated after incubation to release the ssRNA; lane 12: only BSA in complete mixture without heating after incubation to reveal the position of the partially double-stranded RNA substrate.

3. Results

In order to test the RNA helicase activity of nsP2 the protein was expressed in *E. coli*. As expression of this protein is toxic for *E. coli*, we have developed tightly controlled inducible plasmid constructions. They are based on T7*lac* promoter and $lacI^q$, allowing transcription only in the presence of an inducer such as IPTG. The pHAT plasmids encode a His_6 tag at the N-terminus of the protein to be expressed [21]. Since the expressed nsP2 cannot be kept in solution in physiological salt, 1 M NaCl was used during purification. Significant purification was obtained by metal affinity chromatography carried out as described in Section 2 (Fig. 1, lane 4).

The purified recombinant nsP2 (0.25–1 μg) was used in the RNA helicase assay, and the purified recombinant plum pox potyvirus CI as a maltose-binding fusion protein (PPV CI) served as a positive control [22] (Fig. 2). Strand separation of the dsRNA probe was achieved with PPV CI in a concentration range of 0.25–1.0 μg (Fig. 2, lanes 8–10), respective to NaCl concentrations 33–133 mM. RNA helicase activity was detected with 0.25–0.5 μg of SFV nsP2 (lanes 5, 6) (33–66 mM NaCl), the reaction with 0.5 μg of nsP2 being weak but detectable. In the absence of $MgCl_2$ (lanes 1, 2) or ATP (lanes 3, 4), no RNA strand separation was detectable. Heating of a control sample containing only BSA resulted in complete strand separation revealing the migration of the labelled single-stranded RNA (lane 11), whereas the partially double-stranded substrate is shown in the non-heated control (lane 12). The low salt concentration in the RNA helicase assay was critical for the activity of nsP2 as NaCl concentrations higher than 100 mM inactivated the enzyme (data not shown). Furthermore, the low solubility of nsP2 in higher concentrations even in 1 M NaCl narrowed usable concentrations to the range of 0.1–0.3 μg.

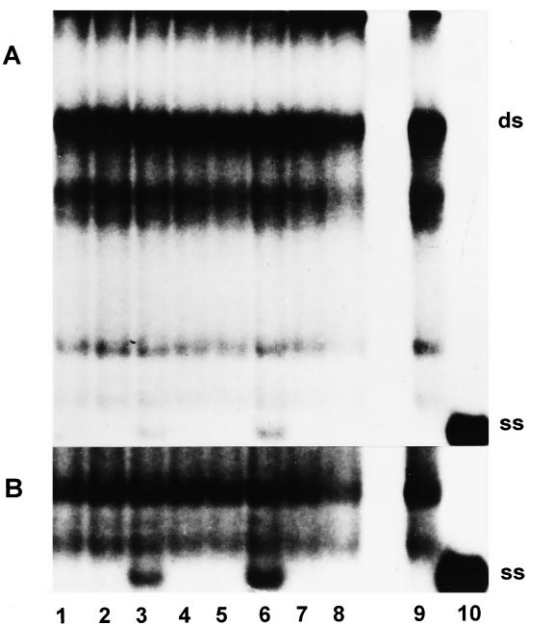

Fig. 3. Effect of protein concentration on RNA unwinding activity of SFV nsP2 and of its NTPase-negative mutant nsP2-GNS (A). Lane 1: 50 ng nsP2-GNS; lane 2: 50 ng nsP2; lane 3: 100 ng nsP2; lane 4: 100 ng nsP2-GNS; lane 5: 200 ng nsP2-GNS; lane 6: 200 ng nsP2; lane 7: 0.5 μg nsP2; lane 8: 0.5 μg nsP2-GNS; lane 9: unheated reaction mixture; lane 10: heated reaction mixture. B: Longer exposure of the region showing the released single-stranded RNA.

Dependence of the RNA helicase activity on NTPs was tested using 0.2 μg of nsP2. 2 mM ATP was optimal as determined by measuring the relative amount of labelled single-stranded RNA band by densitometry. UTP, CTP, GTP, dTTP, dCTP, dATP and dGTP also catalyzed the reaction at 2 mM concentration, varying between 20 and 30% of the value obtained with 2 mM ATP (data not shown).

In order to confirm the specificity of the RNA helicase activity of nsP2 we used lysate from *E. coli* cells, which had been transformed with pHAT plasmid lacking the insert. The lysate was passed over the Ni^{2+} affinity column and treated like the nsP2 preparations. No RNA helicase activity was observed with these preparations (data not shown). As a further control, we used nsP2 variant protein in which the crucial lysine residue in the NTP binding consensus sequence **GVPGS**$\mathbf{GK}_{192}$**SA** [18] had been mutated to asparagine. The nsP2-GNS mutant protein has been shown to lack NTPase activity [16,17]. This protein was purified as a histidine-tagged fusion protein (Fig. 1, lanes 5–7). In RNA helicase assays, the mutant and wild type proteins were used at the same concentrations (Fig. 3A). No RNA helicase activity could be detected for nsP2-GNS. Thus, we conclude that the RNA helicase activity is the property of wild type nsP2.

4. Discussion

Helicases are enzymes which unwind nucleic acid duplexes using an NTP as energy source [23]. Based on sequence comparisons, three superfamilies of helicases or putative helicases have been defined. The alphavirus-like (nsP2-like) proteins together with numerous bacterial DNA helicases have been assigned to superfamily 1. Superfamily 2 includes polypeptides, NS3-like proteins, encoded by poty-, flavi-, and pestiviruses together with several established eukaryotic RNA helicases. Superfamily 3 includes the picornavirus-like (2C-like) proteins together with some viral DNA helicases [18,24]. The best progress in viral RNA helicase research has been achieved with hepatitis C virus NS3 RNA helicase from superfamily 2, the 3D structure of which has been determined [25–27]. On the basis of these results, a molecular mechanism for the RNA unwinding has been proposed [25,27]. Two other helicases, pestivirus NS3 (p80) [28] and plum pox potyvirus CI [19,20,29], belonging to superfamily 2 have been identified by direct demonstration of RNA helicase activity. Of RNA virus members of superfamily 3, NTPase activity has been demonstrated for poliovirus 2C protein, suggesting that all picornaviruses may have an RNA helicase [30–32].

Numerous positive-stranded plant and animal RNA viruses, among them the alphavirus-like viruses, belong to the helicase superfamily 1. Of these, NTPase activity has been demonstrated for nsP2 of SFV [26,27], for the 206 kDa protein of turnip yellow mosaic virus [32], and for a fragment of rubella virus non-structural polyprotein [33]. Here we have shown that SFV nsP2 has RNA unwinding activity, which is dependent on Mg^{2+} and NTP or dNTP, of which ATP was superior in stimulating the helicase activity. Lysate from mock-transfected *E. coli*, purified like nsP2, as well as an NTPase-negative mutant protein (nsP2-GNS) with an amino acid change (K192N) in the NTP-binding sequence were used as negative controls. As a positive control we used plum pox CI protein with previously established RNA helicase activity [22]. Thus, we are confident that the observed RNA helicase activity was due to nsP2 rather than contaminating *E. coli* proteins.

When the cDNA encoding mutated nsP2 was introduced into the infectious cDNA to replace the wild type sequence, no infectious RNA was transcribed. Instead revertants appeared slowly, in which a back mutation had taken place correcting N192 back to K192, the wild type nsP2 sequence [17], indicating that NTPase and RNA helicase activities are essential for the viral RNA replication. By studying the temperature-sensitive mutant ts4 of SFV, we have recently shown that nsP2 is directly associated with nsP1, nsP3 and nsP4 in the RNA polymerase complex [14]. Thus, we assume that the RNA helicase activity of nsP2 is an essential function in the RNA replication of alphaviruses.

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