PROCEEDINGS OF THE BALKAN SCIENTIFIC CONFERENCE OF BIOLOGY IN PLOVDIV (BULGARIA) FROM 19TH TILL 21ST OF MAY 2005 (EDS B. GRUEV, M. NIKOLOVA AND A. DONEV), 2005 (P. 253–262)

STUDY ON THE AFFINITY AND SPECIFICITY OF THE INTERACTION BETWEEN POTATO SPINDLE TUBER VIROID AND TOMATO PROTEIN VIRP1

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ABSTRACT. Potato Spindle Tuber Viroid (PSTVd), the infectious agent causing diseases of many economically important crops (potato, tomato, etc.), is one of the smallest RNA pathogens [1]. Its genome (360nt) consists of single stranded, circular RNA with high degree of complementarities, which folds into a specific rod-like secondary structure [2-4]. PSTVd RNA has no protein coding capacity, but is able to replicate autonomously. To accomplish its replication, transport and pathogenicity PSTVd RNA needs to interact with different host protein factors during its life cycle.

In an attempt to identify host factors that play a role in the viroid life cycle, the potato spindle tuber viroid (PSTVd) RNA-binding protein from tomato, called Virp1, was recently isolated and characterized [5, 6]. Virp1 represents a new class of an RNA binding protein and it seems to interact specifically with viroid (+) RNA. Its cellular function is still unclear, but a possible role in viroid replication and RNA-mediated chromatin remodeling is discussed.

In the present work we report a high affinity and specificity of the interaction between PSTVd RNA and Virp1. Analyzing the binding reaction we confirm the published recently results [5, 6] but also extend the knowledge about establishing the optimal binding conditions, assessing the binding specificity and measuring the dissociation constant of PSTVd RNA-Virp1 interaction.

KEYWORDS. PSTVd, RNA-binding protein, EMSA

INTRODUCTION

Among the broad spectrum of plant pathogens, viroids are the smallest and the simplest. Their presence in the field cause enormous losses in the yield of many economically important plants like potato, tomato, hops, citrus etc. [7, 8].

Naturally, viroids are RNA pathogens. Their genome consists of a circular, covalently closed, single stranded RNA molecule, which is characterized by high degree of self complementarity and specific secondary structure [2-4]. The viroid RNA molecule is not protected by coat protein and does not code for any peptides. Therefore the pathogenic effects of viroids, their ability to replicate and move are encoded in their RNA sequence and the resulting secondary structure. It is postulated that viroids contain binding signals for one or more cellular constituents.

Potato Spindle Tuber Viroid is a type member of *Pospiviroidae* family. All representatives of *Pospiviroidae* are characterized by their specific rod-like secondary structure, nuclear localization and asymmetric mode of replication. Based on comparative sequence analysis a model was proposed that divides the rod-like secondary structure into five domains, namely the central conserved region (CCR), flanked by pathogenic (P) and variable (V) domains and two terminal domains left (TL) and right (TR), respectively [9]. In the beginning it was suggested that each domain is responsible for a particular function. Later on the experiments showed that the situation is more complex and more than one domain can take part in one function.

In an attempt to identify host factors that play a role in the viroid life cycle, the potato spindle tuber viroid (PSTVd) RNA-binding protein from tomato, called Virp1, was isolated and characterized [5]. Virp1 represents a new class of RNA binding proteins and it seems to interact specifically with viroid (+) RNA. Its cellular function is still unclear, but a possible role in viroid replication and RNA-mediated chromatin remodeling is discussed [5]. Using the three-hybrid system, it was found that Virp1 interacts with the terminal right domain (TR) of PSTVd (+) RNA [10]. Based on comparative sequence analysis of *Pospiviroidae* members a potential recognition motif in the terminal right part of PSTVd was proposed and was proved lately as a determinant of specific interaction [10, 11].

Virp1 was isolated from tomato a cDNA expression library by using longer-than-unit-length PSTVd (+) RNA as a probe. In this work, we used a nearly monomeric PSTVd (+) RNA transcript. Here we present experiments, which have been

performed to prove the specific binding activity of Virp1 towards monomeric or shorter PSTVd.

MATERIAL AND METHODS

1. Recombinant constructs

PSTVd (+) RNA of isolate KF-440-2 *(accession number X58388*) was used in this study. The longer-than-unit-length PSTVd (+) RNA was obtained by *in vitro* transcription with SP6 RNA polymerase as described [12] using *Eco*RI- linearized plasmid pHa106 [13].

R-79-wt was obtained after PCR amplification of pHa106 plasmid DNA with the two DNA oligonucleotides 19GEM (5' CCGGAATTCGAGCTCGCCC) and T3Sau(5'GGTCTAGAAATTAACCCTCACTAAAGGGCCGACAGGAGTAATTC C). The resulting PCR product was cloned in the T-easy vector system (Promega, Madison, USA), yielding plasmid pR79-wt. After digestion with *Ava*II, plasmid pR79-wt was transcribed with T3 RNA polymerase delivering transcript R79-wt.

GEM 7Zf(+) RNA was obtained by *in vitro* transcription of *Pvu*II linearized plasmid pGEM 7Zf(+) using T7 RNA polymerase.

pHisVirp1Δ [5] was a generous gift from Dr. Martinez de Alba

2. Expression and purification of Virp1Δ

The plasmid pHis-Virp1Δ has been described earlier [5]. It encodes Virp1Δ, a His-tagged N-terminally truncated form of Virp1 containing all the domains necessary for the specific interaction with PSTVd (+) RNA. Cultivation of BL21(DE3) *E. coli* carrying the plasmid was done as described previously [14]. The protein was purified on a Ni-NTA agarose column under native conditions according to a protocol provided by the manufacturer (Qiagen, Hilden, Germany). To remove imidazole, the sample was concentrated to about one fifth of the volume in a Centricon Centrifugal Filter Device YM-30 (Millipore, Billerica, USA). After addition of 1 ml of dilution buffer containing Tris-HCl 20 mM pH 7.5, 150 mM NaCl, the sample was again concentrated to about half (further dilution of salt concentration might cause unspecific protein-protein association). The quality of the purified protein was analyzed on a 12% SDS-PAGE and its concentration was determined by Bradford Assay [15].

3. Electrophoresis mobility shift assay (EMSA)

All reactions of complex formation between Virp1Δ and PSTVd (+) RNA were carried out for 30 minutes in a total volume of 10 µl binding buffer with a final concentration of 10 mM HEPES-KOH, 50 mM KCl, 100 µM EDTA pH 7.9, and 5% glycerol. All reactions contained 1 mg/ml of baker's yeast tRNA (Roche Molecular Biochemicals, Switzerland). The concentration of Virp1Δ and PSTVd (+) RNA was varied as indicated under Results. The RNA-protein complexes were separated by electrophoresis on a 5% non-denaturing polyacrylamide gel (acrylamide/ bisacylamide 29:1) using as running buffer 0.5 x TBE (50 mM Tris, 50 mM boric acid and 1 mM EDTA, pH 8.3) at about 10 V/cm at room temperature. The gels were

dried and visualized either by autoradiography or quantified by phosphorimager analysis (Storm 840, Molecular Dynamics) with the aid of Image Quant software.

RESULTS

In vitro and *in vivo* studies have suggested that the interaction of Virp1 with PSTVd RNA is specific [5, 6].

For the present work, it was our objective to characterize the extent of affinity and specificity of this interaction.

We present a detailed study on the binding reaction by examining the complex formation at different protein concentrations, salt concentrations and incubation temperatures.

To study the RNA-binding activity of Virp1Δ, the His-tagged Virp1Δ was cloned in *E.coli* strain BL21 (D3) and purified under non-denaturing conditions (the data are not shown). For simplicity, the His-tagged derivative protein subsequently will be referred to as Virp1Δ.

We have confirmed the binding of PSTVd-Virp1 by applying electrophoresis mobility shift assay (EMSA) (Fig.1a,b). As an RNA component of the binding reaction we have used ^{32}P-labeled Ha106 (+) RNA at a concentration of 10^{-7}M, produced by *in vitro* transcription of recombinant pHa106 plasmid. The protein concentration was varied from $1.7.10^{-6}$ -2.10^{-7}M. The binding reaction included non-specific competitor (yeast tRNA 100ng/ul) to prevent non-specific binding. The reaction mixture was incubated for 30min at room temperature. The formed RNA-protein complexes were separated by electrophoresis on 5% native polyacrylamide gel. At a protein/RNA ratio of 4/1 and 2/1 we could detect two distinct Virp1-Ha106 (+) RNA complexes I and II, which are distinguished by their electrophoretic mobility (Fig.1a). Even at a very low concentration of the protein 2.10^{-7}M (the ratio RNA: protein is 1:2) we were able to detect a formation of RNA:protein complex. New complexes with lower mobility (complex III, IV) have been seen when a high concentration of protein was applied (Fig.1a). This indicates the formation of particles with more protein subunits at this higher protein concentration. By plotting the binding data ([PR/R]:[P]) we were able to calculate Kd=$1.4.10^{-7}$M (Fig.1b), which is an intermediate value for RNA protein interaction.

We studied the influence of temperature for this interaction (Fig.2). The radioactive Ha106 (+) RNA was kept at 4ºC, while the binding mixture was incubated at 0, 4, 16, 28 and 37ºC. We detected RNA-protein complexes at all different temperatures (Fig.2). Moreover the pattern of the RNP complexes remained the same in all temperatures tested.

Next, we investigated the influence of salt on the activity of the protein during its purification and the potential role of salt on the binding reaction (Fig.3). The experiment was performed at 25°C with a part of PSTVd (+) RNA, called R79-wt, which represent 80 nucleotides of the terminal right domain (TR) of PSTVd. As we have already shown R79-wt is fully sufficient for the specific interaction with Virp1 [11]. Two preparations of Virp1, purified with higher (150mM) and lower (75mM) salt concentration, respectively were subjected to EMSA. The protein purified at

75mM salt showed lower activity, since a portion of the RNA was not bound at all. The pattern of binding showed a major complex, and three additional complexes I, II and III. The amount of complexes formed differed in the two protein preparations.

The salt concentration in the binding reaction was additionally varied, ranging from 67 to 667mM. There was no major influence of the salt concentration in this range. The addition of salts until 657mM neither inhibited the RNP complex formation nor the behavior of the free RNA (Fig.3).

As further evidence for the specificity of PSTVd-Virp1 binding, the probability of complex formation between unrelated RNA and Virp1 was investigated. As an unrelated RNA was used GEM3Zf (+) RNA of 323nt length (similar to the length of specific RNA probe), produced by transcription of a part of plasmid pGEM3Zf. No binding was seen with a protein:RNA ratio of 1:1 or 3:1 (Fig.4). In order to be able to see any binding at all, we had to increase the protein/RNA ratio to 28:1 (Fig.4). The signal of this new protein-RNA interaction was not a sharp, most probably due to the instability of the complexes and their subsequent dissociation.

DISCUSSION

In previous experiments, Martinez et al. had shown that Virp1 is interacting specifically with PSTVd, Citrus exocortis viroid (CEVd) and Hop stunt viroid (HSVd) and doesn't interact with RNA of Avocado sunbloch viroid (ASBVd) and Grapevine yellow speckle viroid (GYSVd) (6).

We determined here the Kd of the interaction Virp1/PSTVd, and we studied the influence of different salt concentrations during binding and the specificity of the interaction.

To achieve stringent binding conditions we have studied the interaction between Virp1Δ and PSTVd in the presence of yeast tRNA that prevents a non-specific binding. By titrating the concentration of protein, monovalent salts and incubation temperature we explored the binding reaction in more details.

The variation of salt concentration was chosen according to the published data describing the stability of complexes between ssRNAs and virus proteins at different salt concentration. It is known that alfalfa mosaic virus movement (AIMV) 3a protein has reduced RNA-binding activity at 0.2M NaCl [16], while the interaction of movement proteins (MPs) of red clover necrotic mosaic virus (RCNMV) and foxtail mosaic potex virus (FoMV) with RNA is stable at 0.4M NaCl [17, 18]. The protein 30K of tobacco mosaic virus (TMV) binds RNA even at 0.6M NaCl, although not sequence-specifically [19]. Our experiment showed that interaction between R79-wt and Virp1Δ is very stabile even at 657mM salts (Fig.3). The concentration of monovalent cations during the purification procedure influences the activity of the protein, possibly by avoiding coagglutination.

The PSTVd-Virp1 complex formation was not influenced by the incubation temperature (0-37ºC) (Fig.2). Moreover the pattern of the formed RNA-protein complexes was unaltered in this temperature range.

The stability of the PSTVd-Virp1 complex was confirmed by the quantitative determination of the dissociation constant Kd= $1.4.10^{-7}$ M (Fig.1b).

The experiment of protein titration shows formation of more than one complex even at very low concentrations of the protein (Fig.1a), which implies possibly a binding at two different sites on the RNA. Indeed, by site directed mutagenesis of an RNA motif, we could prove that this sequence is composed of two motifs [11]. The binding of non-related probe (GEM 7Zf(+)) to Virp1Δ showed formation of weaker complexes at high protein concentration (Fig.4).

In conclusion, we extended previous results on the affinity and specificity of PSTVd-Virp1 interaction.

CONCLUSIONS

PSTVd RNA-Virp1 interaction is highly specific. The quantitative determination of the dissociation constant Kd= 1.4.10-7 M implies a medium binding affinity.

RNA-binding activity of Virp1 is affected by the presence of monovalent ions during its purification.

PSTVd RNA-Virp1 interaction is not influenced by temperature in the range 0÷37°C.

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ACKNOWLEDGMENTS

This work has been supported by a Marie Curie training fellowship to M.G. (contract HPMT-CT-2000-00175).

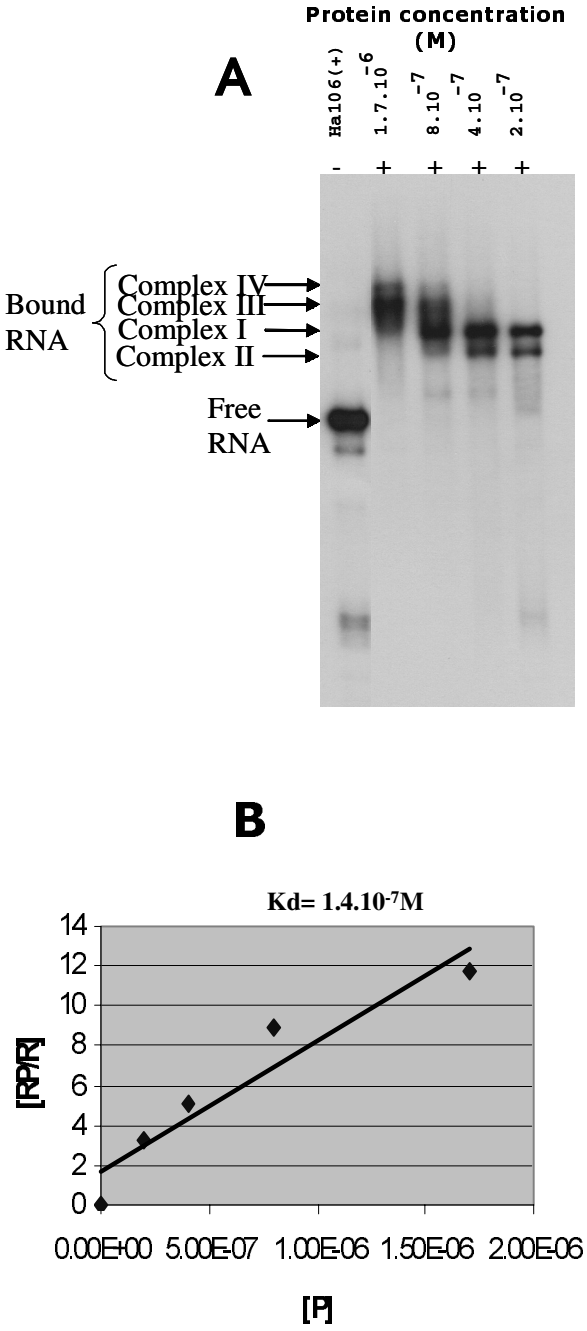

Fig.1 A. *EMSA analysis of binding between Virp1Δ and Ha106 (+) RNA. The RNP complexes were separated on 5% native gel. The RNA is shown in the absence and presence of protein. The positions of free and bound RNA are indicated. The concentration of Ha106 (+) RNA is 100nM, while the concentrations of Virp1 are indicated on the figure.* ***B.*** *Quantitative analysis of data from (A), plotted as bound RNA over free RNA (Virp1-R/R) versus protein concentration. The slope allows determination of the dissociation constant Kd.*

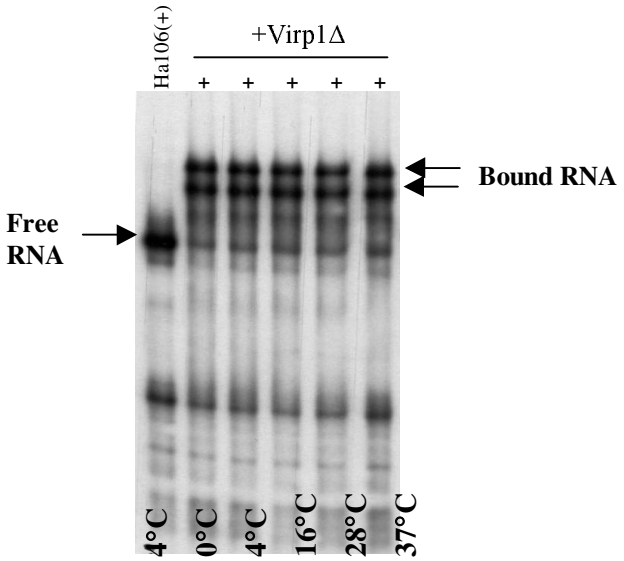

Fig. 2. *EMSA analysis of binding between Ha106 (+) RNA (0.1 μM) and Virp1Δ (0.33 μM). The binding reaction is done at different incubation temperatures (0-37º C). The positions of free and bound RNA are indicated. The RNP complexes are separated on 5% PAGE.*

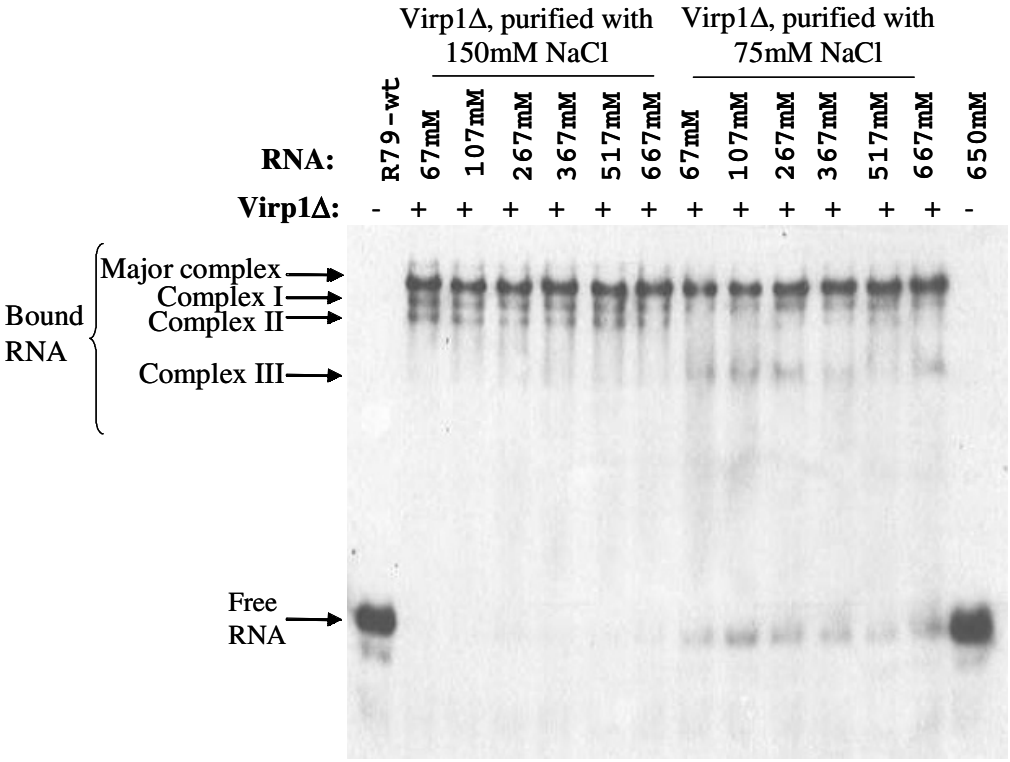

Fig.3. *Binding analysis of R79-wt RNA (1nM) to Virp1Δ (0.1μM) at varying concentrations of salts into the reaction mixture. Two preparations of Virp1, purified with higher (150mM) and lower (75mM) salt concentration, respectively were subjected to EMSA. The RNA is shown in the absence and presence of protein. In line 14 is shown a free RNA, incubated with 650mM NaCl. The RNP complexes were separated on 6% native gel.*

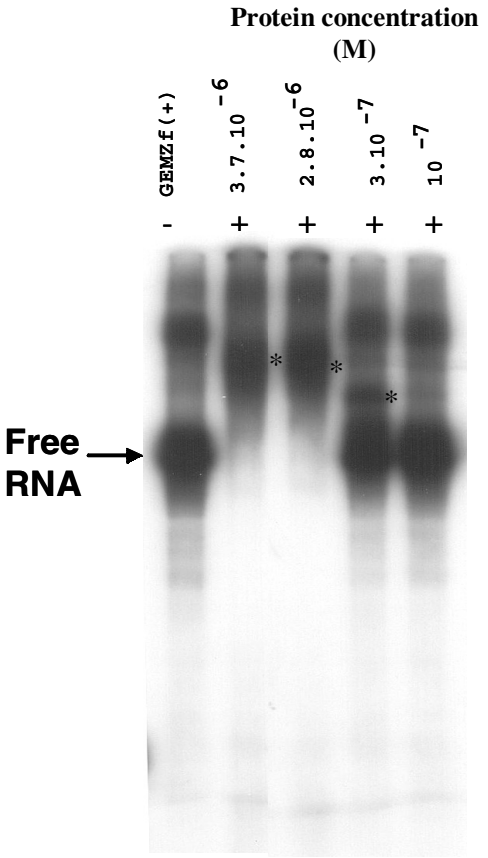

Fig.4. *Titration experiment. EMSA of binding between Virp1Δ and GEMZf (+) RNA. The reaction was carried out at 25ºC. The RNP complexes were separated on 5% native gel. The RNA is shown in the absence and presence of protein. The positions of unstable RNP complexes are indicated with asterix (*). The concentration of GEMZf (+) RNA is 0.1μM, while the concentrations of Virp1 are indicated on the figure.*