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The Internal Domain of Hordeivirus Movement Protein TGB1 Forms *in vitro* Filamentous Structures

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Abstract—The 63 kDa hordeivirus movement protein TGB1 of poa semilatent virus (the PSLV TGB1 protein) forms viral ribonucleoprotein for virus transport within a plant. It was found using the dynamic laser light scattering technique that the internal domain of TGB1 protein forms *in vitro* high molecular weight complexes. According to results of atomic force microscopy, a part of these complexes is represented by globules of different sizes, while another part consists of extended filamentous structures. Similar properties are also characteristic of the N-terminal half of the protein and are obviously due to its internal domain moiety. The data support the hypothesis that upon viral ribonucleoprotein complex formation, the N-terminal half of the PSLV TGB1 protein plays a structural role and exhibits the ability to form multimeric filamentous structures (the ability for self-assembly).

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Key words: hordeivirus, TGB1 movement protein, internal domain, filamentous structures

Transport of a number of RNA-containing viruses within an infected plant is carried out in the form of ribonucleoprotein complexes (RNP complexes) [1]. These complexes (the virus transport form), formed with participation of viral RNA and non-structural movement proteins of viruses, are involved in the intracellular translocation of the viral genome from the RNA replication sites to the cell wall, cell-to-cell movement through cytoplasmic channels of plasmodesmata interconnecting adjacent cells (intercellular transport), and along the phloem (long-distance movement). Transport of hordeiviruses requires three movement proteins encoded by the triple transport gene block (TGB), and only one of these proteins, encoded by the first TGB gene (TGB1 protein) is involved in the formation of viral transport form, whereas two other proteins, TGB2 and TGB3, provide for intracellular transport of viral RNP complexes to plasmodesmata. Within a plant the hordeivirus genome is transported in the form of an RNP complex at all stages of the spread of the viral infection [2, 3].

In our laboratory we have been studying for a number of years the properties of the 63 kDa TGB1 hordeivirus protein of poa semilatent virus (PSLV). We recently proposed a model of the domain organization of this protein [4] according to which the PSLV TGB1 consists of three domains: N-terminal (NTD) with disordered structure, internal structured domain (ID), and C-

Abbreviations: AFM, atomic force microscopy; DLS, dynamic laser light scattering; HELD, NTPase/helicase domain; ID, internal domain; N63K, N-terminal half of the protein including NTD and ID; Ni-NTA agarose, agarose-Ni^{2+}-nitrilotriacetate; NTD, N-terminal domain; PSLV, poa semilatent virus; RNP complex, ribonucleoprotein complex; TGB (1-3) proteins, proteins encoded by the triple transport gene (1-3) block.

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terminal domain exhibiting NTPase/helicase activities (HELD). The first two domains comprise the N-terminal half of the protein, which exhibits RNA-binding activity [5]. RNA-binding, NTPase, and RNA-helicase activities have been shown for HELD [5-7].

Studying biochemical and physicochemical properties of the PSLV TGB1 protein and its complexes is of great interest for understanding of the viral transport form structural organization and the mechanism of its function. In our previous work [4] we studied some properties of domains within the N-terminal half of the protein. It was shown, in particular, that the ID domain is able to form high molecular weight complexes and cooperatively interact with RNA.

In this work we further investigate the internal domain of the PSLV TGB1 protein. High molecular weight complexes formed by this domain are visualized as extended filamentous structures. The complexes contain an insignificant amount of RNA and are, evidently, the product of internal domain self-assembly. Similar properties are also characteristic of the whole N-terminal (nonenzymic) protein half and are due to its ID moiety.

MATERIALS AND METHODS

Expression of recombinant protein genes in *E. coli* cells, purification of $(His)_6$ recombinant proteins by affinity chromatography on Ni-NTA agarose, and protein electrophoresis in SDS-polyacrylamide gel. The previously obtained recombinant plasmids [4, 5] were used to transform M15 *E. coli* cells containing the high-copy repressor plasmid pRep-4. Clones expressing recombinant protein genes corresponding to the NTD and ID domains in the N-terminal half of TGB1 protein (N63K) were grown overnight at 37°C on a standard 2×YT medium in the presence of ampicillin (100 μg/ml) and kanamycin (25 μg/ml). The overnight culture was diluted tenfold and grown at 37°C to absorption 0.8 at 600 nm. Protein expression was induced by addition of isopropyl β-D-thiogalactopyranoside (final concentration 1-2 mM) for 2-4 h. Cells were pelleted at 6000 rpm for 10 min in a Beckman J-21 centrifuge (Beckman, USA). The recombinant proteins, fused at the N-terminus with the sequence containing six histidyl residues, were chromatographed on Ni-NTA agarose in accordance with the Qiagen (USA) isolation protocol under denaturing conditions. Cells were lysed in buffer A (100 mM NaH_2PO_4 and 10 mM Tris-HCl, pH 8.0) containing 6 M guanidine hydrochloride. After centrifugation for 10 min at 6000 rpm, the supernatant was mixed with Ni-NTA agarose and incubated under gentle stirring for 1 h at room temperature in a column. The column was washed in succession by B, C, and D buffers (100 mM NaH_2PO_4 and 10 mM Tris-HCl) with 8 M urea and pH 8.0, 6.3, and 5.9, respectively. Recombinant proteins were eluted from the column by buffer E (100 mM NaH_2PO_4 and 10 mM Tris-HCl) with 8 M urea, pH 4.5. Obtained fractions were analyzed by SDS-PAGE in 15 or 20% polyacrylamide gels according to Laemmli. Gels were stained with Coomassie Brilliant Blue R-250.

Dynamic laser light scattering (DLS). Recombinant protein preparations were dialyzed against 10 mM Tris-HCl, pH 7.5, and analyzed using the DLS technique. Measurements were carried out on a Zetasizer Nano ZS device (Malvern Instruments Ltd., Great Britain) with He-Ne laser (633 nm, 10 mW) as a light source. Temperature of samples was maintained within 0.1°C using the Peltier thermostatting system. Light scattering was measured at an angle of 173°. Detection and processing of autocorrelation functions were carried out using the Dispersion Technology Software (DTS) version 5.10. Usual polystyrene cells with 10 mm optical path were used for the experiments. The sample volume in a cell was 1 ml. Measurements were carried out in 10 mM Tris-HCl, pH 7.5, in the protein concentration interval 0.05-0.15 mg/ml. In some experiments protein preparations were incubated for 30 min at 25°C with the mixture of RNAase A (0.25 μg) and RNase T1 (2 units/μg protein, activity of original preparation was 1000 units/μl).

Atomic force microscopy (AFM). After DLS analysis samples were diluted tenfold and 2.5-5.0 μl of solution was deposited on freshly cleaved mica surface for adsorption. After 10-15 min the samples were rinsed in a flow of distilled water to remove unadsorbed material and dried at room temperature. Topographic images of particles were obtained using a Nanoscope III atomic force microscope operating in tapping mode at the scanning frequency 0.5 Hz. Commercially available silicon cantilevers fpN11S and fpN11 with typical force constant 11.5 N/m were used. The standard tip curvature radius for this type of cantilevers is 10-25 nm. In this geometry one of the AFM artifacts – increased lateral dimensions of the objects under study compared to real ones – is typically observed [8]. This effect is especially pronounced upon visualization of particles having dimensions comparable to those of the cantilever edge. In this case significantly enlarged lateral dimensions are observed. The real particle size may be increased by value of the tip edge radius from every side. The obtained images were processed using the specialized FemtoScan Online software. The presented here particle dimensions were estimated with account for this broadening effect.

RESULTS

Internal domain of hordeivirus TGB1 protein forms multimer complexes. Figure 1 shows the scheme of the PSLV TGB1 protein domain organization according to our previous hypotheses [4]. The first two domains (NTD and ID) form the N-terminal half of the protein. The

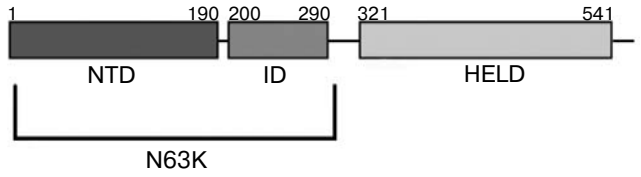

Fig. 1. Scheme of domain organization of TGB1 movement protein of poa semilatent hordeivirus. The bracket points to the N-terminal protein half including the NTD and ID domains. Figures indicate amino acid residues designating domain borders.

recombinant protein, corresponding to the internal domain (ID) of the PSLV hordeivirus movement TGB1 protein, was expressed in *E. coli*, purified by affinity chromatography under denaturing conditions, and then renatured. The polypeptide includes amino acid residues (a.a.) from 201 to 290 of 576 a.a. of the wild-type TGB1 protein. The calculated molecular mass of this recombinant protein is 11.24 kDa, and the theoretical p*I* value is 9.2.

Fractionation of ID preparations in sucrose density gradient has shown that along with low molecular weight protein oligomers, the preparation contains a significant amount of high molecular mass complexes of 440 kDa and more [4]. We further studied conditions of complex formation by dynamic laser light scattering (DLS). This method is used for estimation of both the size distribution of individual protein molecules and protein complexes in solutions [9-12]. Results obtained using DLS are shown as the amount of a certain size particles where the particle size is its hydrodynamic diameter approximated to globular particle parameters and as the volume occupied by particles of this size. Average size of complexes formed by isolated ID domain at 25°C is 50 ± 15 nm ranging from 35 to 70 nm ([4] and this work, Fig. 2a, curve *1*). Large protein complexes of 350 ± 100 nm, occupying an considerable volume in the analyzed preparation (Fig. 2b, curve *1*), are also revealed, although their amount is low, less than 0.1% (Fig. 2a, curve *1*). The sizes of these complexes vary from one

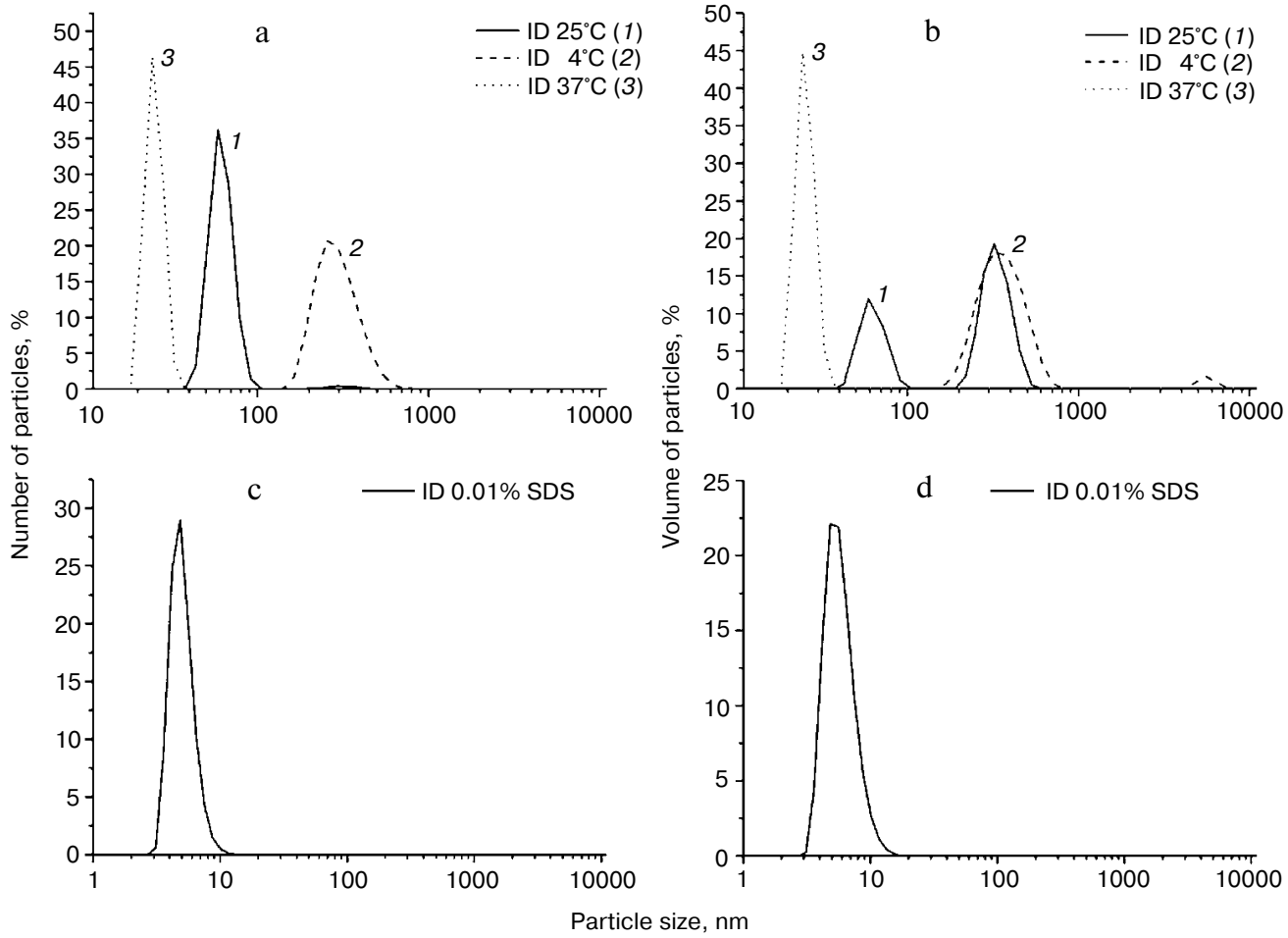

Fig. 2. Characteristics of ID preparation by dynamic laser light scattering (DLS). Size distribution of particles in the ID recombinant protein preparations incubated at different temperatures (a), distribution of particles by volume occupied by them at different temperatures (b); characteristics of ID preparation after treatment with 0.01% SDS: distribution of particles by number (c) and occupied volume (d).

experiment to another and for some preparations they reach about 500 nm. It appeared that the size of complexes and their amount in a preparation depend on incubation temperature. When temperature is decreased to 4°C, large complexes (300 ± 150 nm) become the main component in the preparation (up to 99%) (Fig. 2, a and b, curve *2*). In contrast, upon temperature increase to 37°C, small particles about 15 nm become prevalent in the ID preparation (Fig. 2a, curve *3*). The addition of SDS to 0.01%, not causing formation of micellae, resulted in conversion of complexes into particles of 5.0 ± 2.0 nm (Fig. 2, c and d). These particles might be ID dimers, because stable dimers formed by this domain are revealed upon electrophoresis in denaturing SDS-polyacrylamide gel (data not shown).

Thus, the internal domain of TGB1 protein is able to form multimer complexes readily aggregating at lower temperatures. At higher temperature and in the presence of ionic detergent at low concentrations, these complexes dissociate to particles, evidently low molecular weight oligomers.

Structure visualization in preparations of internal domain. Atomic force microscopy (AFM) was used to visualize structures formed by the internal domain of TGB1 protein. Figure 3 (a and b) shows typical topographic images of the ID preparation placed onto the surface of mica after DLS analysis. The ID preparation represents a heterogeneous mixture of particles of different types and sizes. The preparation contains a significant quantity of globules of different sizes: globules of 1.5 ± 0.2 nm as well as smaller (from 0.4 nm) and larger (up to 3.8 nm) globular particles. Along with globules, the preparation contains filamentous structures with a height of 1.4 ± 0.4 nm and diameter (measured at the half-height) of about 25.0 ± 7.0 nm and length up to 300 nm. These filamentous structures often form snarls. Figure 3b shows these filamentous structures at higher magnification. A certain number of larger complexes that we determine as snarl aggregates is also detected in preparations.

Thus, complexes formed by the internal domain of TGB1 protein are represented by different size globular homooligomers and extended filamentous structures able to form snarls and their aggregates. It is quite probable that large complexes, revealed by the DLS technique, are snarls aggregates. Evidently, similar aggregates and aggregates of low molecular weight protein oligomers are formed in protein preparations at lower temperatures. At the same time, small complexes, detected by DLS as structures with mean size about 50 nm, are most likely visualized as filamentous structures. This hypothesis is supported by the fact that 550 nm long filamentous virions of potato virus X are estimated by DLS as particles with hydrodynamic diameter 40-50 nm [12].

Role of RNA in formation of high molecular weight complexes in the internal domain preparations. We showed in our previous work that the internal domain of TGB1 protein is capable of unspecific and cooperative interaction with RNA [4]. It is known that in the course of isolation of many recombinant RNA-binding proteins contamination by nucleic acids is often observed. Although ID was isolated under denaturing conditions in the presence of 6 M guanidine hydrochloride and 8 M urea, in our case such possibility also could not be completely excluded.

Therefore we analyzed the possible presence of RNA in ID preparations. First, we analyzed the absorp-

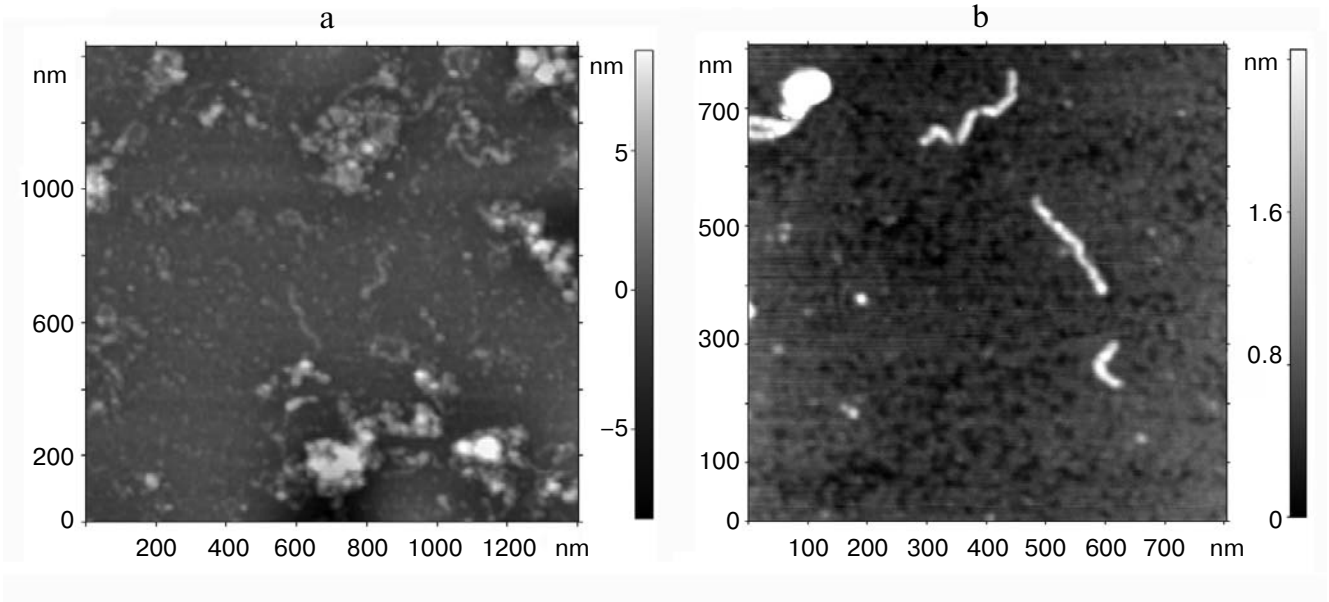

Fig. 3. Topographic image of particles present in ID preparation obtained using a Nanoscope III atomic force microscope operating in tapping mode. The image size is 1.3 × 1.3 μm^2 (a) and 0.8 × 0.8 μm^2 (b).

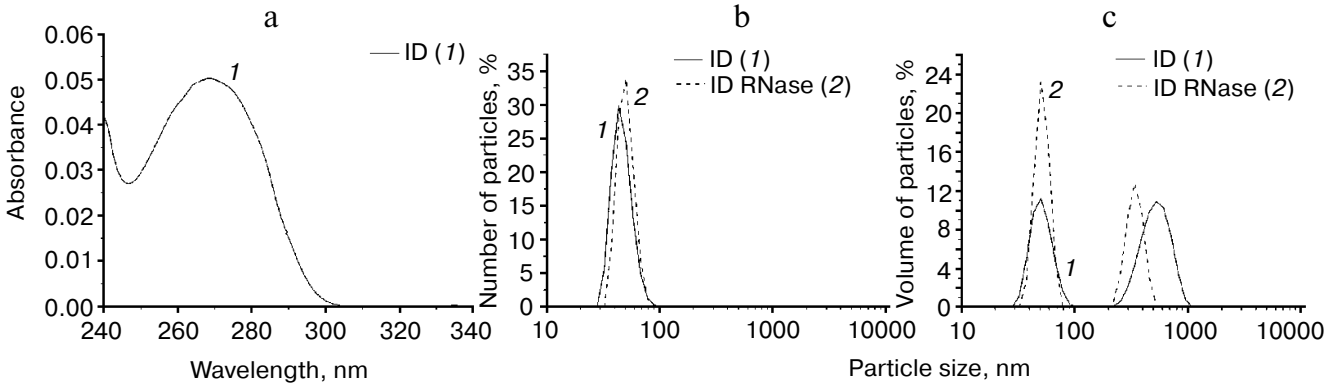

Fig. 4. Detection of RNA in ID preparations. A typical optical adsorption curve of an ID preparation (a); DLS analysis of ID preparation before (curve *1*) and after (curve *2*) RNase treatment (incubation at 25°C for 30 min with a mixture of RNase A (0.25 μg) and RNase T1 (2 units per μg protein): number (b) and volume (c) of particles.

tion spectrum of the protein preparation. There are only two residues of aromatic amino acids within ID, namely these are tyrosine residues in positions 268 and 285 of the full-size PSLV TGB1 protein. Such proteins usually have an adsorption maximum at 275 nm corresponding to that of tyrosine and the 260/280 nm ratio close to 1. Therefore, the presence of even insignificant amounts of nucleic acids in such protein preparations results in adsorption maximum shift towards 260 nm characteristic of nucleic acids. Analysis of several ID preparations showed that their adsorption maximum is in fact shifted towards 260 nm (Fig. 4a), and the 260/280 ratio varies from 1.1 to 1.35. Calibration curve showing the relationship between RNA amount and 260/280 ratio demonstrated that the mean RNA content in ID preparations is about 0.2-0.3% of protein mass. Thus, the ID preparations contain a minor amount of RNA trapped by the protein from *E. coli* lysates and preserved in complexes with this basic protein during its isolation and purification.

The ID preparations were also incubated with RNase A and RNase T1 mixture for 30 min at 25°C and analyzed by DLS and AFM. As shown in Fig. 4 (b and c) the RNase treatment of ID preparations results in decrease in the mean size of large complexes from 500 to 250 nm (Fig. 4c), whereas the mean size of small complexes in the preparations is not changed. Another effect of RNase treatment is slight redistribution of the protein between the populations of large and small complexes: a larger amount of ID is revealed in small complexes (Fig. 4c). These data agree with the supposition that ID preparations contain a certain amount of RNA. On the other hand, since complexes of both types survive RNase treatment, it seems that RNA molecules are not their structural basis. The data of DLS analysis are confirmed by AFM: preparation treated by RNases practically does not differ from untreated protein preparation (data not shown).

The study of exogenous RNA effect on formed ID structures was used as an additional approach. The ID preparations were incubated in the presence of total tRNA preparation (molar RNA/protein ratio 1 : 500). It was shown by DLS that in this case heterogeneous complexes from 90 to 200 nm were formed (mean size about 150 nm) (Fig. 5, curve *2*). AFM showed that the number of protein globules in the preparation noticeably decreased whilst the amount of filamentous structures noticeably increased (data not shown). Formation of extended filamentous structures in the presence of short RNA suggests that although RNA is not the structural basis for complex formation, it might play the role of primer in protein polymerization. It is interesting that similar results were obtained with 20-nucleotide-long

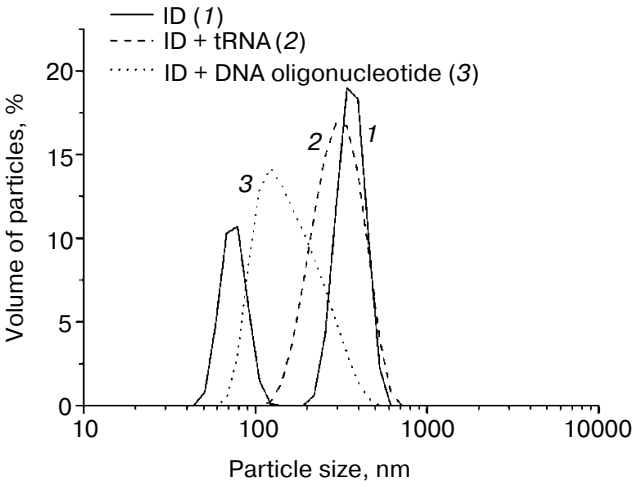

Fig. 5. Complex formation upon addition of low molecular weight RNA and oligodeoxynucleotide to ID preparations; DLS analysis of ID preparation before exogenous RNA introduction (curve *1*) and after addition of tRNA (curve *2*) and oligodeoxynucleotide (curve *3*).

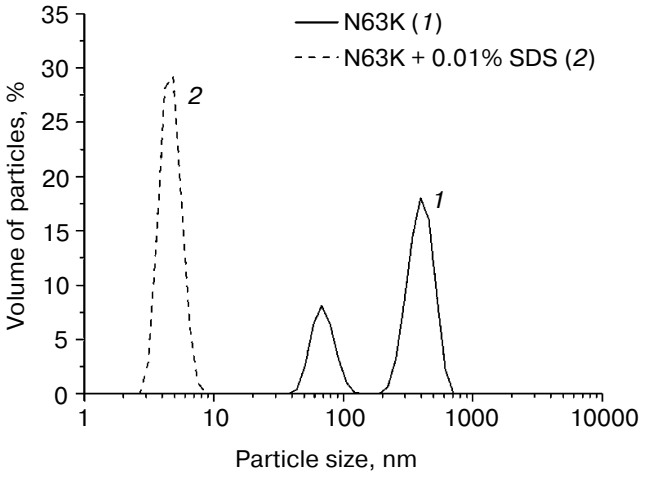

Fig. 6. Analysis of N63K preparation by DLS. Distribution of particles by occupied volume before (curve *1*) and after (curve *2*) addition of 0.01% SDS.

single-stranded oligodeoxynucleotide. The mean size of complexes formed in this case was about 100 nm (Fig. 5, curve *3*).

Thus, filamentous structures found in ID preparations are probably formed due to protein–protein interactions. Minor RNA (or DNA) contaminations in the protein preparations might play the role of a primer initiating formation of multimers.

N-Terminal half of TGB1 protein forms filamentous structures. Properties of the recombinant protein corresponding to the N-terminal half of TGB1 protein (N63K), which includes 1 to 290 a.a. of the wild-type protein (Fig. 1), are generally similar to properties of the internal domain.

Fractionation of N63K preparations in sucrose concentration gradients also shows the presence of a significant amount of high molecular weight complexes [4]. The DLS analysis reveals in N63K preparations two types of particles of 60 ± 20 nm (over 99%) and (250-300) ± 100 nm (less than 0.1%) ([4], Fig. 6, curve *1*). Addition of 0.01% SDS converts complexes to particles of 4.0 ± 1.5 nm (Fig. 6, curve *2*), while RNase treatment has practically no effect on complex sizes (data not shown). Decreasing temperature to 4°C is also accompanied by protein aggregation, and in this case complexes of 350 ± 150 nm become prevalent. In contrast, at 37°C, unlike internal domain, particles of 60 ± 20 nm are preserved while large complexes disappear completely (data not shown). Evidently particles formed by extended N-terminal half of TGB1 protein are more stable compared to structures formed by ID.

AFM of N63K preparations has shown that they contain globules of 1.2 ± 0.2 to 3.0 ± 0.6 nm in height and filamentous often branched structures up to 500 nm in length, 1.2 ± 0.5 nm in height, and apparent diameter at half-height of about 35.0 ± 10.0 nm (Fig. 7a). AFM image of the NTD preparation is shown in Fig. 7b. This domain together with ID is incorporated in the N-terminal half of the TGB1 protein (Fig. 1). It is seen that only small globules 1.5 ± 0.2 nm in height and apparent diameter, measured at height half about 15.0 ± 2.0 nm are found in the NTD preparation. Thus, the N-terminal half of PSLV TGB1 protein is also able to form filamentous structures, and this property is defined by the internal domain rather than by the NTD.

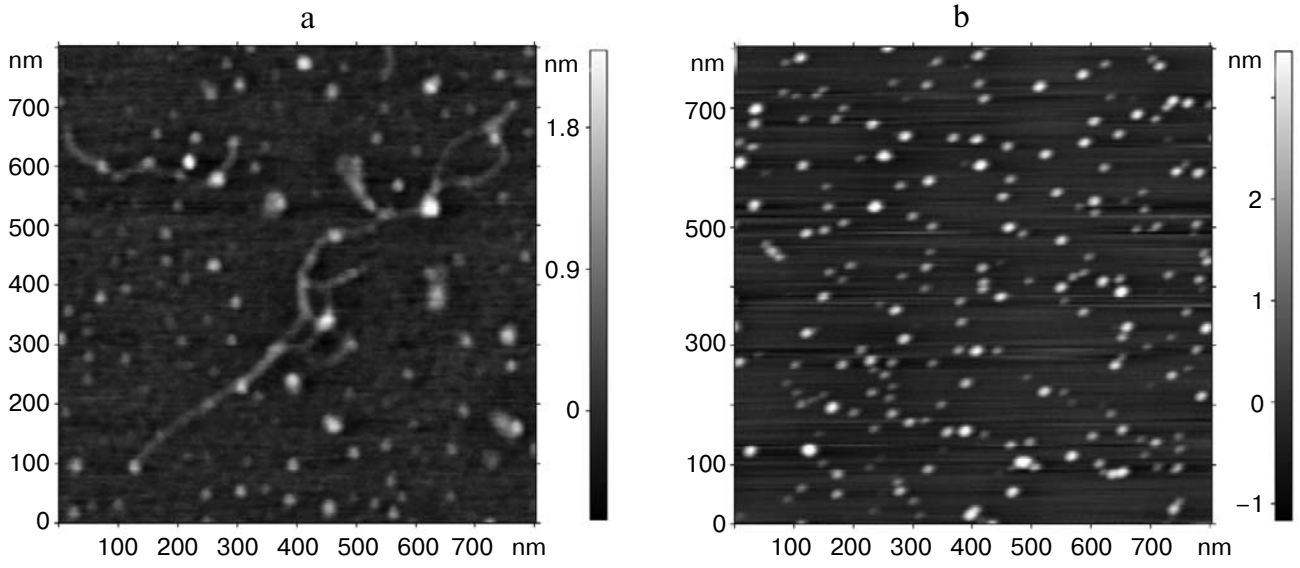

Fig. 7. Topographic image of particles in isolated N63K (a) and NTD (b) preparations obtained using a Nanoscope III atomic force microscope operating in tapping mode. Image size is 0.8 × 0.8 μm^2.

DISCUSSION

Many plant viruses, including those encoding the triple block of transport genes (TGB), need the capsid protein (CP) for cell-to-cell and long-distance virus transport in infected plant. However, in the case of hordeiviruses these processes do not require the CP. As assumed, the hordeivirus genome is transported as an RNP complex formed by TGB1 protein and viral genomic RNA [3, 13-15]. Hordeiviruses differ from most TGB-containing plant viruses by the presence in their movement proteins, encoded by the first TGB gene, of the extended 250-300-a.a.-long N-terminal region (preceding the NTPase/helicase domain) [2]. We supposed earlier [4] that the N-terminal region of hordeivirus TGB1 protein might play the role of CP in viral genome transport in plants.

In this work it is shown using DLS and AFM that internal domain, earlier revealed by us within movement TGB1 protein, as well as the N-terminal half of PSLV TGB1 protein containing this domain, form complexes visualized as filamentous structures up to 500 nm in length. The morphology of complexes is similar to that of ribonucleoprotein complexes obtained *in vitro* in the presence of viral movement proteins and RNA [16, 17]. It should be noted that conditions of complex formation also correspond to those for formation of viral RNP complexes (buffer with low ionic strength or water) [16, 17].

Nucleoproteins forming internal nucleocapsid in a number of animal viruses with negative-stranded RNA genome, including nucleoproteins of influenza and rabies viruses, represent a well-studied class of viral proteins able to form extended filamentous structures [18, 19]. *In vitro* formation of extended structures, morphologically identical to viral ribonucleoprotein complexes (nucleocapsids), was observed both in protein preparations isolated from nucleocapsid and in preparations of recombinant nucleoproteins [19]. The authors believe that formation of RNP-like complexes is determined by the ability of these viral nucleoproteins for self-polymerization/self-assembly. Along with RNP-like structures, preparations of viral nucleoproteins contain protein oligomers of different order [18, 19]. It is interesting that properties of the PSLV TGB1 protein internal domain and N-terminal half are very similar to properties of viral nucleoproteins. Proteins aggregate upon decrease in incubation temperature, whereas dissociation of complexes is observed on temperature increase. RNase treatment has practically no effect on complex sizes. The protein preparations contain minor amounts of RNA. It is shown for the rabies virus nucleoprotein that this can be cellular tRNA [19]. Our preliminary data demonstrate that the similar size of minor RNA (less than 100 nucleotides) can be isolated from ID preparation. Similarly, as it was supposed for influenza virus nucleoprotein [18], we believe that low molecular weight RNA can play the role of a primer initiating the ID multimerization/self-assembly. This supposition is confirmed by experiments with tRNA or oligodeoxynucleotide, because addition of these low molecular weight nucleic acids to ID preparation results in a certain increase in complex sizes (data of DLS) and in increase in the amount of filamentous structures (according to AFM data).

Thus, the results presented in this paper support our hypothesis that the N-terminal half of PSLV TGB1 protein plays a structural role upon formation of viral RNP complex, thus exhibiting the ability for multimerization/self-assembly to filamentous RNP-like structures due to the properties of the ID. The study of the fine structure of formed complexes is the subject of our further investigations.

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