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The C-terminal domain of the 2b protein of *Cucumber mosaic virus* is stabilized by divalent metal ion coordination

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ABSTRACT

The main function of the 2b protein of *Cucumber mosaic virus* (CMV) is binding permanently the double stranded siRNA molecules in the suppression process of post-transcriptional gene silencing (PTGS). The crystal structure of the homologue *Tomato aspermy virus* (TAV) 2b protein is known, but without the C-terminal domain. The biologically active form is a tetramer: four 2b protein molecules and two siRNA duplexes. Regarding the complete 2b protein structure, we performed a molecular dynamics (MD) simulation of the whole siRNA–2b ribonucleoprotein complex. Unfortunately, the C-terminal domain is proved to be partially unstructured. Multiple sequence alignment showed a well conserved motif between residues 94 and 105. The negatively charged residues of the C-terminal domain are supposed to take part in coordination of a divalent metal ion and stabilize the three-dimensional structure of the C-terminal domain. MD simulations were performed on the detached C-terminal domains (aa 65–110). 0.15 M $MgCl_2$, $CaCl_2$, $FeCl_2$ and $ZnCl_2$ salt concentrations were used in the screening simulations. Among the tested divalent metal ions Mg^{2+} proved to be very successful because Asp95, Asp96 and Asp98 forms a quasi-permanent Mg^{2+} binding site. However the control computations have resulted in any (at least) divalent metal ion remains in the binding site after replacement of the bound Mg^{2+} ion. A quadruple mutation (Rs2DDTD/95–98/AAAA) was introduced into the position of the putative divalent metal ion binding site to analyze the biological relevance of molecular modeling derived hypothesis. The plant inoculation experiments proved that the movement of the mutant virus is slower and the symptoms are milder comparing to the wild type virus. These results demonstrate that the quadruple mutation weakens the stability of the 2b protein tetramer–siRNA ribonucleoprotein complex.

1. Introduction

Cucumber mosaic virus (CMV) and *Tomato aspermy virus* (TAV) belong to the *Cucumovirus* genus in the family *Bromoviridae* [1]. CMV infects over a thousand plant species and cause serious crop losses in agriculture, principally in vegetable and ornamental cultures while the host-plant range of TAV is significantly narrower. The genome of cucumoviruses consists of three single-stranded, positive-sense RNA molecules and codes for five viral proteins. Elements of the viral replication complex, protein 1a and 2a, are encoded by RNA 1 and 2. RNA 2 encodes for another small protein (2b) in a shifted and overlapped open reading frame (ORF), which has a key role in the suppression of RNA silencing and takes part in the regulation/induction of symptom expression [2]. RNA 3 encodes the movement protein (3a, MP) and coat protein (3b, CP). The main function of the CP is to encapsidate the viral RNA while the MP participates in the transport of the pathogen to the adjacent plant cell through plasmodesmata [3–5]. The virus particle of *cucumoviruses* is assembled from 180 CP subunits with a $T = 3$ truncated icosahedral symmetry [2,6,7].

The structure of the smallest cucumovirus coded protein, 2b, is mainly alpha helical and it is composed of 110 amino acids (aa). During viral infection it is localized in the nucleus [8]. The major function of the 2b protein of *cucumoviruses* is to bind permanently the double stranded siRNA molecules to suppress the post-transcriptional gene silencing (PTGS) process [9–11]. The crystal structure of the homologue TAV 2b protein is already known [12], but the structure of the C-terminal domain (aa 69–110) is absent. The C-terminal domain is proved to be toxic when it was expressed in *Escherichia coli* [13]. The known part of the 2b protein

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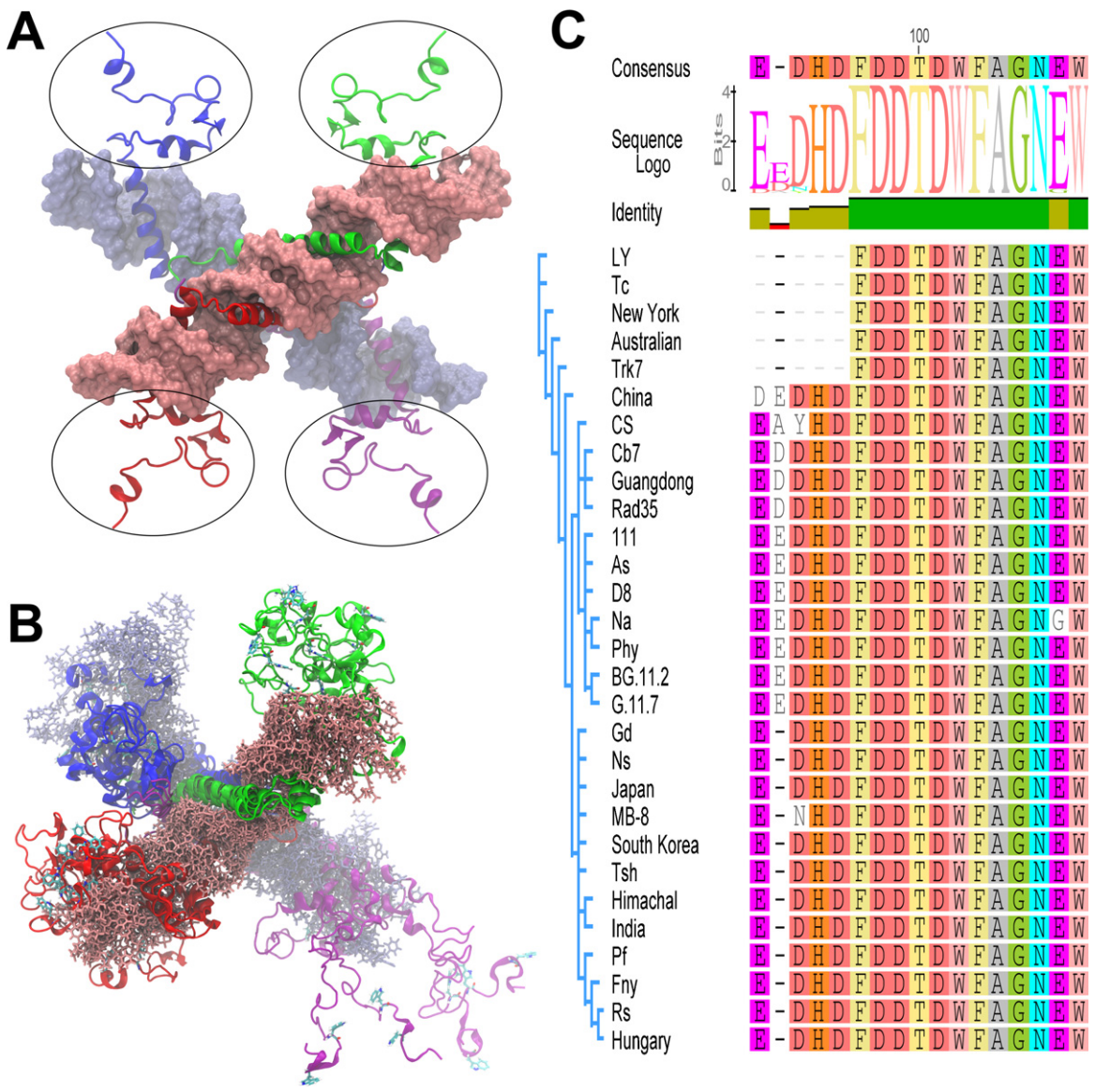

Fig. 1. Structure of the siRNA–2b tetramer ribonucleoprotein complex and the conserved sequence motif between position 94 and 105. The modeled C-terminal domains are encircled. The protein chains of the complex are in cartoon while siRNA duplexes are in molecular surface representation (A). Snapshots of the simulated siRNA–2b tetramer, every 8 ns were superimposed. It can be seen that the C-terminal domain and the siRNA ends become partially unfolded, furthermore the Trp99 and Trp105 residues do not to coordinate to siRNA endings (B). Multiple sequence alignment of 29 different CMV 2b protein sequences (C).

contains two long α-helices. The helical axes are rotated by 120° around each other. The 2b protein forms a pair of hook-like dimers to bind the siRNA duplex. The α-helices fit into the major groove of the siRNA in a sequence-independent and length-preference manner. The biologically active form is a tetramer (Fig. 1A), which is formed by four 2b protein molecules and two siRNA duplexes.

Mainly electrostatic interactions take place between the positively charged protein side chains and the RNA sugar-phosphate backbone at the RNA–protein interface. There is a π–π stacking effect between the conserved Trp50 and the 5′-terminal base, as observed in the TAV 2b X-ray structure [12].

Multiple sequence alignment showed that leucine is conserved at position 50 in numerous CMV strains. Another plant virus protein the *Carnation Italian ringspot virus* (CIRV) p19 binds 21 nt siRNA in a size selective manner where Trp39 and Trp42 act as end-capping residues with the base pairs on each end of the siRNA fragment [14]. The first defective interfering RNA molecule in *Cymbidium ringspot virus* infections was reported by Burgyán et al. [15].

There are also two conserved tryptophans in CMV 2b sequences at position 99 and 105 and these residues may have similar RNA stabilizing function as in CIRV p19 (Fig. 1C). Therefore, in order to gain further insight into the functional and structural features of the siRNA bound 2b tetramer, we performed molecular dynamics (MD) simulations to check the role of the conserved Trp99 and Trp105. Unfortunately the CMV 2b C-terminal domain have become partially unstructured during simulations and we could not detect π–π stacking formation between tryptophan residues and terminal siRNA bases (Fig. 1B). Then, we identified a well-conserved DDTD motif in the sequence alignment at position 95–98 which probably form a divalent metal ion binding site in the 2b C-terminal domain. In order to identify which type of metal ions can bind permanently to this putative site we tested four divalent metal ions. Individually 32–32 ns long MD simulations were performed on the separate C-terminal domain model (aa 65–110) and the system was filled up with 0.15 M $MgCl_2$, $CaCl_2$, $FeCl_2$, $ZnCl_2$, respectively. These MD simulations showed that the 2b C-terminal domain is stabilized by a bound Mg^{2+} ion while the other studied metal ions did not show