http://dx.doi.org/10.5012/bkcs.2013.34.4.1260

DNA Binding Analysis of Severe Acute Respiratory Syndrome (SARS) Coronavirus Helicase

A-Ram Lee, Soojin Yoon,† Dong-Eun Kim,† and Yong-Joo Jeong*

*Department of Bio and Nanochemistry, Kookmin University, Seoul 136-702, Korea. *E-mail: jeongyj@kookmin.ac.kr*
†Department of Bioscience and Biotechnology, Konkuk University, Seoul 143-701, Korea
Received January 2, 2013, Accepted January 14, 2013

Key Words : Severe acute respiratory syndrome coronavirus, Helicase, DNA binding, Filter binding assay, Fluorimetric titration

Severe Acute Respiratory Syndrome (SARS) is a life threatening disease which has been caused by novel coronavirus, SARS coronavirus (SCV).[1-3] It was claimed infection over 8,000 people, and almost 800 deaths worldwide.[4] Because of the absence of vaccine or effective medicine, SARS is still regarded as a serious concern to human beings. SCV contains about 29.7 kb of genome,[3,5] which is one of the largest among RNA viruses.[6] A single replicase gene (21.2 kb) at the 5'-end of SCV genome is translated into pp1ab (~790 kDa) and pp1a (~490 kDa), which are subsequently processed by viral protease, producing several non-structural proteins (nsPs). The nsPs, especially RNA-dependent RNA polymerase and NTPase/helicase, are reported to be essential for viral replication.

Recent progress in understanding the mechanism of helicases leads to recognizing them as attractive targets for the development of antiviral agent.[7-9] In fact, a lot of attempts have been made to develop inhibitors of SCV helicase.[10-12] Previously, we analyzed ATP hydrolysis activities of SCV helicase in the presence of various types of nucleic acids.[13] We have also shown that cooperative translocation by oligomers of SCV helicase enhances the duplex DNA unwinding.[14] In the present study, DNA binding properties of SCV helicase were investigated by Nitrocellulose (NC) filter binding assay and fluorimetric titration assay.

NC membrane binding assays were performed to investigate the influences of various cofactors on the equilibrium binding of ssDNA to SCV helicase. To determine the optimized condition of SCV helicase binding to ssDNA, we performed a series of ssDNA binding reactions in the presence of various agents as described in Experimental Section. It is well known that nucleotides and divalent cations play an important role in the helicase binding to nucleic acids[15,16] as well as oligomerization of helicase. In the NC membrane binding assay, protein-DNA complexes are bound to NC membrane and unbound DNA passes through the membrane, so that radioactivity quantitation of the membrane provided the fraction of the bound DNA to protein.[15,17] ssDNA binding to SCV helicase in the presence of various agents was performed with dT80 ssDNA and the result is shown in Figure 1. Regardless of the agents included, ssDNA binding to SCV helicase did not show any

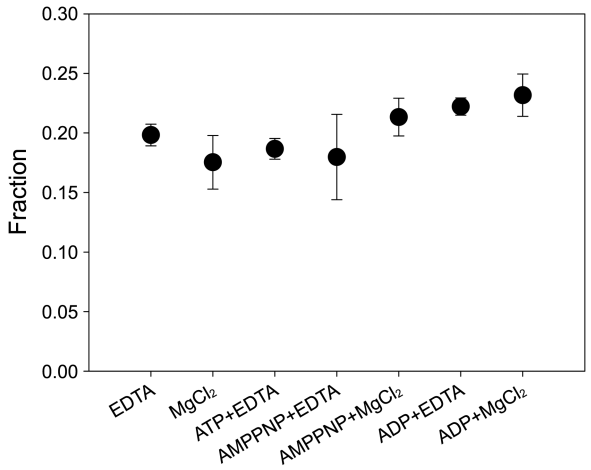

Figure 1. Measurement of ssDNA binding to SCV helicase in the presence of nucleotide cofactors. 500 nM SCV helicase was mixed with 20 nM radiolabeled dT80 ssDNA in the presence of 5 mM EDTA (lane 1), 5 mM $MgCl_2$ (lane 2), 2 mM ATP and 5 mM EDTA (lane 3), 2 mM AMPPNP and 5 mM EDTA (lane 4), 2 mM AMPPNP and 5 mM $MgCl_2$ (lane 5), 2 mM ADP and 5 mM EDTA (lane 6), and 2 mM ADP and 5 mM $MgCl_2$ (lane 7), respectively. After 10 min incubation, the mixture was filtered through NC membrane and the radioactivity was quantitated.

preference to specific cofactors. Moreover, the fact that only about 20% of ssDNA was bound to SCV helicase indicates ssDNA binding is not so significant in our experimental conditions. The binding efficiency of the present study is less than the expectation based on our previous dsDNA unwinding experiments.[14] We assume that ssDNA binding property of SCV helicase might be different on which kinds of ssDNA the helicase loads, fragmented ssDNA or ssDNA tail of forked DNA. It is likely that SCV helicase has intrinsic DNA binding property, but it is not clear how SCV helicase recognizes and binds ssDNA due to the unavailability of three dimensional structures. Our previous studies showed that ATP hydrolysis activity was enhanced in the presence of single-stranded nucleic acids (ssNA).[13] Although SCV helicase is known to bind only ssNA, exact binding mode is not clear at present. Considering SCV helicase is RNA helicase, therefore, it will be necessary to perform additional binding experiments with RNA in the future

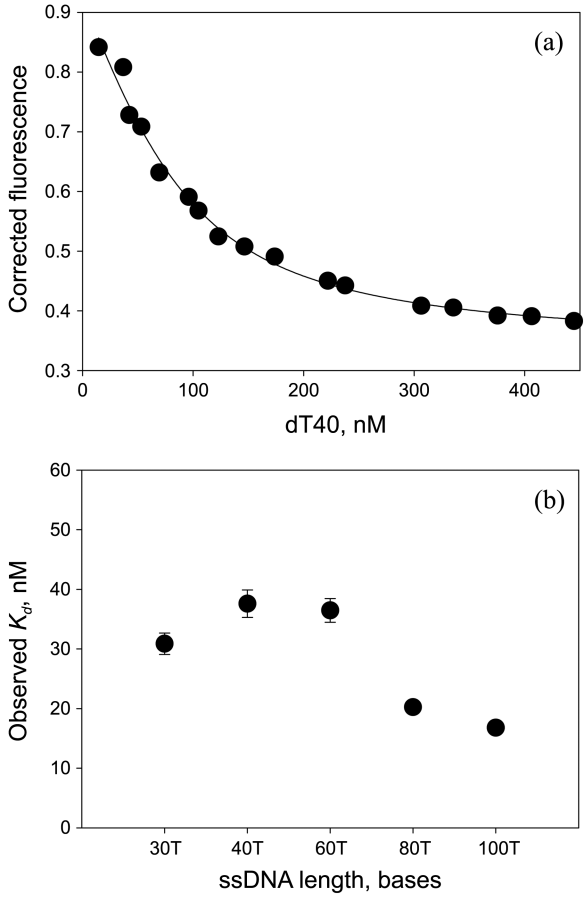

Figure 2. Fluorimetric titration assay of SCV helicase binding to ssDNA. (a) The fluorescence of SCV helicase (100 nM) was measured in the presence of various amounts of dT40 ssDNA. The fluorescence signal was corrected by Eq. (1) and fit into Equation 2-4, by which dissociation constant (K_d) was obtained. (b) Obtained K_d values with standard deviation were plotted as a function of ssDNA length.

study.

To determine the dissociation constant, K_d, fluorimetric titration assay was performed. Based on the fact that the intrinsic fluorescence of protein changes upon binding to nucleic acids,[18] a constant amount of SCV helicase was titrated with increasing amount of dT40 ssDNA. The sample in a cuvette was excited at 280 nm, and the fluorescence emission was measured at 340 nm. Figure 2(a) shows that fluorescence intensity decreases with increasing dT40 concentration. The fluorimetric titration assay was repeated at different lengths of ssDNA (dT30, dT40, dT60, dT80, and dT100), which provided K_d values of 30.9 ± 1.8 nM, 37.6 ± 2.3 nM, 36.5 ± 2.0 nM, 20.3 ± 0.3 nM, and 16.8 ± 1.1 nM, respectively (Fig. 2(b)). While dT30-60 oligomers have similar K_d values, the oligomers longer than dT80 have lower than the shorter ones. Although the minimal binding site size of SCV helicase is not known at present, our previous study showed that length of 15 bases-long 5'-tail of forked DNA was sufficient for binding of a SCV helicase.[14] Therefore, it is assumed that the minimal binding site size of SCV helicase is less than 15 bases at least. The lower K_d values of the oligomers longer than dT80 might be due to extra nucleotides that could provide additional binding position.[19] The binding properties of several helicases from superfamily (SF) 1 and 2 have been reported.[19] For Rep and UvrD helicases, which belong to SF1, similarly with SCV helicase, the reported ssDNA K_d values are 220 and 165 nM, respectively.[20,21] Our studies show that SCV helicase has better ssDNA binding affinity than other SF1 helicases. The fact that SCV helicase is originated from a RNA virus may be the reason of the differences. However, more detailed study is required to reveal the nucleic acid binding properties of SCV helicase.

In the present study, we analyzed ssDNA binding to SCV helicase by NC filter binding assay and fluorimetric titration assay. Interestingly, ssDNA binding to SCV helicase was not affected by cofactors such as $MgCl_2$ and nucleotides. We were also able to determine the K_d values of different lengths of ssDNA. We expect future studies of RNA binding and three dimensional determination of the structure will elucidate the detailed mechanism of nucleic acid binding to SCV helicase.

Experimental Section

Protein, Reagents, and Nucleic Acids. SCV helicase, nsP13, was overexpressed in *E. coli* Rosetta™ competent cells and purified as described previously.[10,11] The protein concentration was determined by 280 nm absorbance and Bio-Rad protein assay system (Bio-Rad).[10] Adenosine 5'-(β,γ-imido) triphosphate tetralithium salt hydrate (AMPPNP) was purchased from Sigma (USA). DNA substrates, which consisting only deoxythymidine (dT), were purchased from Integrated DNA Technologies (Coralville, IA, USA), and purified by 8 M urea polyacrylamide gel electrophoresis (PAGE). The DNA concentration was determined by absorbance measurements at 260 nm and its extinction coefficient. DNA was 5'-end labeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase.

Nitrocellulose Membrane Binding Assay of SCV Helicase. Filter binding was performed using a 96 well Bio-Dot® Microfiltration Apparatus (Bio-Rad) to reduce retention of free single-stranded DNA. NC membrane was presoaked for 10 min in 0.4 M NaOH followed by washing in H_2O until the pH returned to neutral. NC membrane was then equilibrated in binding buffer [20 mM Tris/Cl (pH 7.5) and 50 mM NaCl] for 30 min before use.[22] The binding reaction was initiated by mixing SCV helicase (500 nM) with radiolabeled dT80 ssDNA (20 nM) in a reaction volume of 15 μL. After 10 min incubation, 10 μL aliquot of the mixture was spotted onto 96 well Bio-Dot® Microfiltration Apparatus, filtered, and washed with 100 μL binding buffer. The binding reaction was also performed with various agents (5 mM EDTA, 5 mM $MgCl_2$, 2 mM ATP and 5 mM EDTA, 2 mM AMPPNP and 5 mM EDTA, 2 mM AMPPNP and 5 mM $MgCl_2$, 2 mM ADP and 5 mM EDTA, 2 mM ADP and 5 mM $MgCl_2$) in binding buffer. The radioactivity was quantitated using a Cyclon Phosphor Imager (PerkinElmer) and

the fraction of helicase-DNA complex was calculated.

Fluorimetric Titration Assay of SCV Helicase. 100 nM SCV helicase in 2.5 mL of the reaction buffer [50 mM Tris/Cl (pH 6.8), 5 mM $MgCl_2$, 50 mM NaCl] was placed in a 10 × 10 × 45-mm quartz cuvette with a magnetic stir bar. Aliquots of the ssDNA solution were added, and the solution was allowed to mix before measuring the absorbance at 280 nm and intrinsic protein fluorescence (E_x = 280 nm and E_m = 340 nm). Fluorescence measurement was performed using a fluoroSENS spectrofluorometer (GILDEN photonics Ltd, UK). Data were corrected for background fluorescence, sample dilution, and inner filter effects, and normalized to 1 to give the corrected fluorescence (Fc) according to Eq. (1),

$$Fc = (F-B)(V-V_0)[1/(F_0-B)] \times 10^{\frac{A_{ex} + A_{em}}{2}} \quad (1)$$

where F is fluorescence intensity, F_0 is initial fluorescence intensity, B is background fluorescence in the absence of SCV helicase, V is final volume, V_0 is initial sample volume, A_{ex} is absorbance at the excitation wavelength, and A_{em} is absorbance at the emission wavelength. Fluorescence were corrected and plotted against [DNA], and the data were fit to Eqs. (2)-(4).[19,23]

$$Fc = fE\cdot(Et - Eb) + fEb\cdot Eb \quad (2)$$

where fE is fluorescence coefficient of free SCV helicase, Et is total SCV helicase concentration, Eb is concentration of SCV helicase bound to DNA, and fEb is fluorescence coefficient of SCV helicase bound to DNA.

$$Eb = \frac{K_d + Et + D - \sqrt{(K_d + Et + D)^2 - 4Et \cdot D}}{2} \quad (3)$$

where K_d is the dissociation constant, D is total concentration of SCV helicase binding sites on the DNA.

$$D = n\cdot Dt \quad (4)$$

where n is the number of SCV helicase that can bind to one DNA molecule, and Dt is total DNA concentration.

Acknowledgments. This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government (NRF-2011-013-C00041).

References

1. Drosten, C.; Gunther, S.; Preiser, W.; van der Werf, S.; Brodt, H. R.; Becker, S.; Rabenau, H.; Panning, M.; Kolesnikova, L.; Fouchier, R. A.; Berger, A.; Burguiere, A. M.; Cinatl, J.; Eickmann, M.; Escriou, N.; Grywna, K.; Kramme, S.; Manuguerra, J. C.; Muller, S.; Rickerts, V.; Sturmer, M.; Vieth, S.; Klenk, H. D.; Osterhaus, A. D.; Schmitz, H.; Doerr, H. W. *N. Engl. J. Med.* **2003**, *348*, 1967.
2. Drosten, C.; Preiser, W.; Gunther, S.; Schmitz, H.; Doerr, H. W. *Trends Mol. Med.* **2003**, *9*, 325.
3. Rota, P. A.; Oberste, M. S.; Monroe, S. S.; Nix, W. A.; Campagnoli, R.; Icenogle, J. P.; Penaranda, S.; Bankamp, B.; Maher, K.; Chen, M. H.; Tong, S.; Tamin, A.; Lowe, L.; Frace, M.; DeRisi, J. L.; Chen, Q.; Wang, D.; Erdman, D. D.; Peret, T. C.; Burns, C.; Ksiazek, T. G.; Rollin, P. E.; Sanchez, A.; Liffick, S.; Holloway, B.; Limor, J.; McCaustland, K.; Olsen-Rasmussen, M.; Fouchier, R.; Gunther, S.; Osterhaus, A. D.; Drosten, C.; Pallansch, M. A.; Anderson, L. J.; Bellini, W. J. *Science* **2003**, *300*, 1394.
4. Eickmann, M.; Becker, S.; Klenk, H. D.; Doerr, H. W.; Stadler, K.; Censini, S.; Guidotti, S.; Masignani, V.; Scarselli, M.; Mora, M.; Donati, C.; Han, J. H.; Song, H. C.; Abrignani, S.; Covacci, A.; Rappuoli, R. *Science* **2003**, *302*, 1504.
5. Marra, M. A.; Jones, S. J.; Astell, C. R.; Holt, R. A.; Brooks-Wilson, A.; Butterfield, Y. S.; Khattra, J.; Asano, J. K.; Barber, S. A.; Chan, S. Y.; Cloutier, A.; Coughlin, S. M.; Freeman, D.; Girn, N.; Griffith, O. L.; Leach, S. R.; Mayo, M.; McDonald, H.; Montgomery, S. B.; Pandoh, P. K.; Petrescu, A. S.; Robertson, A. G.; Schein, J. E.; Siddiqui, A.; Smailus, D. E.; Stott, J. M.; Yang, G. S.; Plummer, F.; Andonov, A.; Artsob, H.; Bastien, N.; Bernard, K.; Booth, T. F.; Bowness, D.; Czub, M.; Drebot, M.; Fernando, L.; Flick, R.; Garbutt, M.; Gray, M.; Grolla, A.; Jones, S.; Feldmann, H.; Meyers, A.; Kabani, A.; Li, Y.; Normand, S.; Stroher, U.; Tipples, G. A.; Tyler, S.; Vogrig, R.; Ward, D.; Watson, B.; Brunham, R. C.; Krajden, M.; Petric, M.; Skowronski, D. M.; Upton, C.; Roper, R. L. *Science* **2003**, *300*, 1399.
6. Ziebuhr, J. *Curr. Opin. Microbiol.* **2004**, *7*, 412.
7. Crute, J. J.; Grygon, C. A.; Hargrave, K. D.; Simoneau, B.; Faucher, A. M.; Bolger, G.; Kibler, P.; Liuzzi, M.; Cordingley, M. G. *Nat. Med.* **2002**, *8*, 386.
8. Kleymann, G. *Expert Opin. Investig. Drugs* **2003**, *12*, 165.
9. Kleymann, G.; Fischer, R.; Betz, U. A.; Hendrix, M.; Bender, W.; Schneider, U.; Handke, G.; Eckenberg, P.; Hewlett, G.; Pevzner, V.; Baumeister, J.; Weber, O.; Henninger, K.; Keldenich, J.; Jensen, A.; Kolb, J.; Bach, U.; Popp, A.; Maben, J.; Frappa, I.; Haebich, D.; Lockhoff, O.; Rubsamen-Waigmann, H. *Nat. Med.* **2002**, *8*, 392.
10. Jang, K. J.; Lee, N. R.; Yeo, W. S.; Jeong, Y. J.; Kim, D. E. *Biochem. Biophys. Res. Commun.* **2008**, *366*, 738.
11. Lee, C.; Lee, J. M.; Lee, N. R.; Jin, B. S.; Jang, K. J.; Kim, D. E.; Jeong, Y. J.; Chong, Y. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1636.
12. Lee, C.; Lee, J. M.; Lee, N. R.; Kim, D. E.; Jeong, Y. J.; Chong, Y. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 4538.
13. Lee, N. R.; Lee, A. R.; Lee, B.; Kim, D.-E.; Jeong, Y.-J. *Bull. Korean Chem. Soc.* **2009**, *30*(8), 1724
14. Lee, N. R.; Kwon, H. M.; Park, K.; Oh, S.; Jeong, Y. J.; Kim, D. E. *Nucleic Acids Res.* **2010**, *38*, 7626.
15. Hingorani, M. M.; Patel, S. S. *Biochemistry* **1993**, *32*, 12478.
16. Matson, S. W.; Richardson, C. C. *J. Biol. Chem.* **1985**, *260*, 2281.
17. Wong, I.; Lohman, T. M. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 5428.
18. Preugschat, F.; Averett, D. R.; Clarke, B. E.; Porter, D. J. T. *J. Biol. Chem.* **1996**, *271*, 24449.
19. Levin, M. K.; Patel, S. S. *J. Biol. Chem.* **2002**, *277*, 29377.
20. Mechanic, L. E.; Hall, M. C.; Matson, S. W. *J. Biol. Chem.* **1999**, *274*, 12488.
21. Wong, I.; Chao, K. L.; Bujalowski, W.; Lohman, T. M. *J. Biol. Chem.* **1992**, *267*, 7596.
22. Levin, M. K.; Patel, S. S. *J. Biol. Chem.* **1999**, *274*, 31839.
23. Hall, M. C.; Ozsoy, A. Z.; Matson, S. W. *J. Mol. Biol.* **1998**, *277*, 257.