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Letter

Roles of the functional domains and conserved residues of the severe fever with thrombocytopenia syndrome virus L protein provide insights into the viral RNA transcription/replication mechanism

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Dear Editor,

The severe fever with thrombocytopenia syndrome virus (SFTSV) is an emerging tick-borne pathogen belonging to the genus *Bandavirus*, family *Phenuiviridae*, which causes high fatality rates ranging from 12% to 30% (Kim et al., 2013). In the absence of efficacious drugs and vaccines, therapies for SFTS are performed mostly according to the clinical manifestations accompanied by supportive treatment. The SFTSV polymerase protein (SFTSV-L), encoded by its L segment, plays a critical role in genomic replication and transcription. Similar to other single negative-sense RNA viruses (sNSRVs), such as the influenza A virus (FluA) and the La Crosse virus (LASV), the SFTSV-L can be divided into three main functional regions, with the RNA-dependent RNA polymerase (RdRp) region flanked by the endonuclease region at the N-terminus and the cap-binding functional region at the C-terminus (Sanchez and de la Torre, 2005; Pflug et al., 2014; Vogel et al., 2020a; Wang et al., 2020). The RdRp region is the core functional domain, which includes a linker (l), an RdRp core, a PA-CL-like domain (PA-CL), and a PB2-NL-like domain (PB2-NL), while the C-terminal cap-binding region can be further subdivided into a blocker (b), arms, a lariat and a cap-binding domain (CBD), according to their functioning modes (Vogel et al., 2020a; Wang et al., 2020). Basing on the atomic model of SFTSV-L (PDB: 6L42) compared with the analogs of LASV-L and FluA polymerase complex, the entire process of RNA synthesis is speculated which involved close cooperation between RdRp region and the other domains, accompanied by appropriated conformational changed (Vogel et al., 2020; Wang et al., 2020).

To better understand the functional mechanism of SFTSV-L, the roles of functional domains and its conserved motifs/residues in affecting SFTSV RNA transcription/description were evaluated via a reporter-based minigenome system. First, truncated L proteins lacking domains associated with the RdRp region, including endoN (△endoN-L), arm1 (△arm1-L), CBD (△CBD-L), blocker (△b-L), arm2 (△arm2-L) and lariat (△lariat-L) were constructed (Supplementary data). Expression of each construct was confirmed in the plasmid transfected cells via indirect immunofluorescence assays (IFAs) (Supplementary Fig. S1). However, deleting endoN, arm1, CBD, blocker, arm2 or lariat domains of SFTSV-L significantly impaired viral RNA transcription and replication, as green fluorescence was not observed in cells transfected with any of the mentioned truncated L protein expression plasmids by using the eGFP-based minigenome system (Supplementary data, Fig. 1A). It suggested that each of the six domains in association with the RdRp domain had an essential role in SFTSV-L functioning for viral RNA replication/transcription.

Single-site mutation is an effective strategy which has been used before to investigate the role of key site in RdRp activity (Noda et al., 2020), because the amino acid substitution may alter the protein surface charge, disulfide linkage, and pair interactions between residues. Here, the sites of highly conserved amino acids located in the nuclease conserved motif (H80, P111, D112, E126, K145) of the endoN domain, the catalytic active cavity (1125-SDD-1127) of the RdRp core, as well as the sites responsible for conformational stabilization (V1517, Y1797) and in the cap-binding pocket (Y1745, Y1719, F1703, R1843) of the C-terminal domain were investigated via single-point mutation (Fig. 1B) (Wang et al., 2020). To clarify their influence on L protein function, these vital amino acids of the SFTSV-L were mutated to alanine. Of the three mutants within the endoN domain, fluorescence was observed in cells transfected with the P111A plasmid at comparable levels to the wild-type L, while D112A and E126A mutants resulted in the complete inhibition of fluorescence. Previous studies have demonstrated the indispensable role of two aspartates of the SDD motif locating in RdRp core domain in the catalytic active of L protein of influenza virus, poliovirus, and arenavirus (Biswas and Nayak, 1994;

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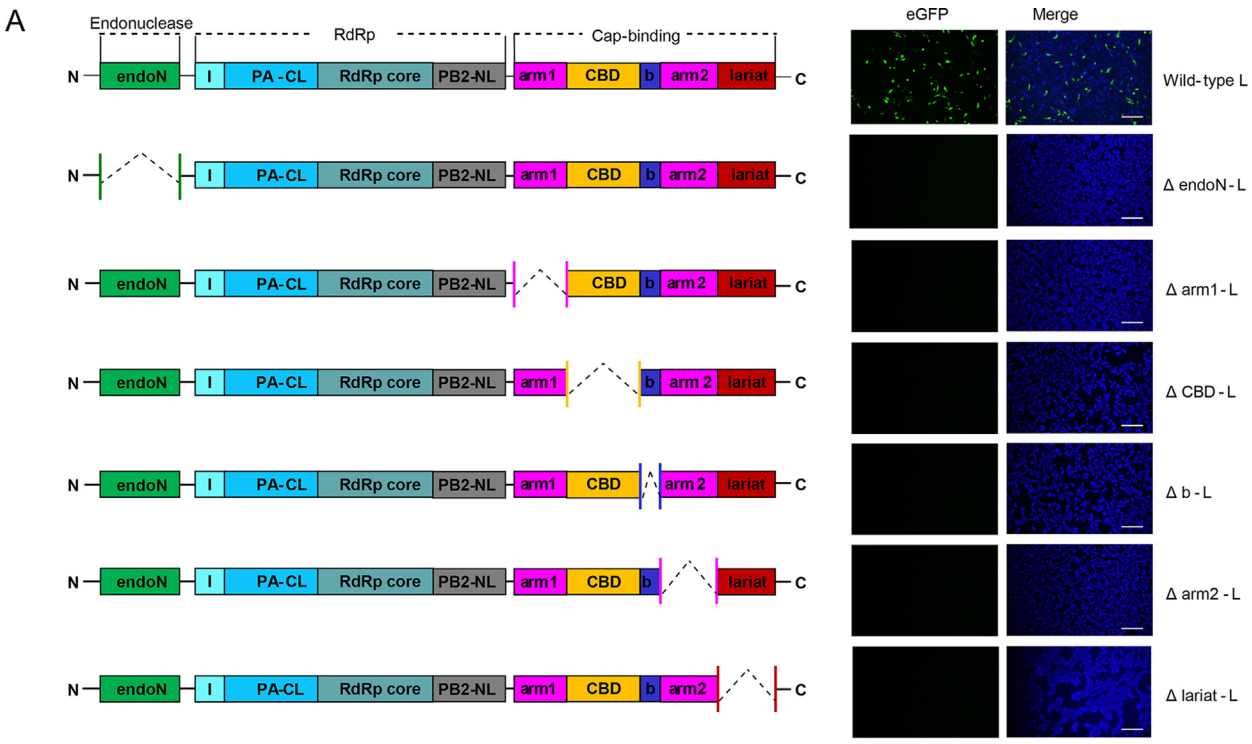

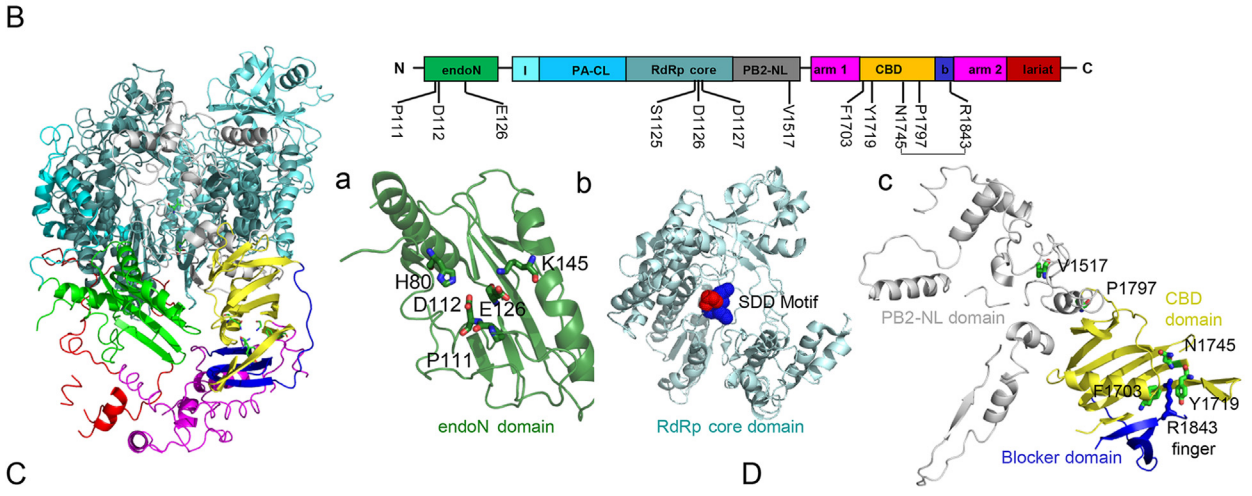

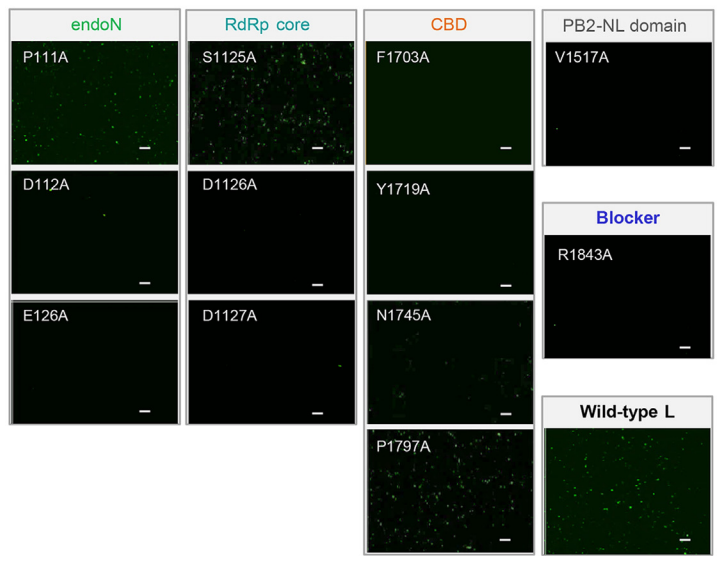

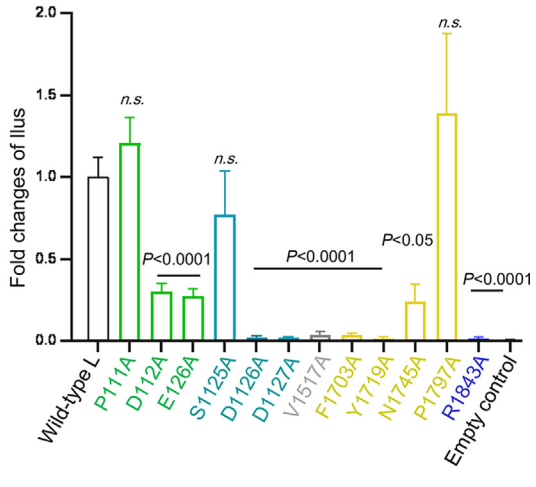

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Fig. 1. Functional evaluation of the domains and conserved sites of the SFTSV L protein. **A** The left panel shows the domain organizations of the complete or domain-truncated SFTSV-L. The endoN domain, linker region (l), PA-C-like domain (PA-CL), RdRp core, PB2–N-likedomain (PB2-NL), arm domains, blocker motif (b), CBD and C-terminal lariat domain are shown in forest green, teal, bright cyan, pale cyan, gray, violet, blue, pale yellow and red, respectively. The images on the right show the evaluation of the domain-truncated L protein via a fluorescence-based minigenome system. Scale bar = 200 μm, 10× magnification. **B** The cartoon on the left shows the 3D structure of SFTSV-L (PDB accession: 6L42). The domains colors are consistent with those shown in (**A**). Three enlarged schematics with the key residues investigated in this study labeled are shown on the right. Their corresponding locations are highlighted in the diagram for SFTSV-L domain organization. **C** The corresponding eGFP expressions via a fluorescence-based minigenome system is presented. Scale bar = 150 μm, 10× magnification. **D** The luciferase assay for evaluating the functional effect of each site mutation via a luciferase-based minigenome system. The evaluating indicator was measured via luciferase light units (llus). The fold change represents the ratio of each mutated group against the wild L protein group. Each mutant labels and bars were colored according to their locations as shown in (**A**). The error bars are standard deviations of the mean of three replicates. The Dunnett's multiple comparisons test was used for statistical analysis. n. s., not signifificant.

Jablonski and Morrow, 1995; Sanchez and de la Torre, 2005). As expected, mutations at the two aspartates of SDD motif (D1126A and D1127A) significantly impaired SFTSV RNA transcription/replication, as fluorescence was not observed in cells transfected with each of the two constructs (Fig. 1C). In contrast, fluorescence was observed when the S1125 was mutated (S1125A). Therefore, it confirmed the critical role of two aspartates of the SDD motif in SFTSV-L functioning, and suggested that the reporter-based minigenome system is an effective method to evaluate the effects of each mutant on SFTSV L protein function. Moreover, no fluorescent signal was observed with the mutant V1517A of the PB2-NL domain and with two of the four mutants, F1703A and Y1719A, in the CBD domain. For the other two mutants in the CBD domain, while a few cells presented fluorescence upon transfection with mutant N1745A, the mutant P1797A showed a very limited impact on the reporter system, as comparable amounts of fluorescent signals were observed in the transfected cells. Mutant R1843A of the blocker domain resulted in severe loss of L protein functions. Taken together, the mutants D112A, E126A, D1126A, D1127A, V1517A, F1703A, Y1719A, and R1843A severely impaired SFTSV-L functions, although these mutations did not show a significant influence on L protein expression in cells (Supplementary Fig. S2). However, this impairment of SFTSV-L functions was not found for the mutants P111A, S1125A, and P1797A.

These results were further confirmed by using luciferase-based minigenome assays, which determined the effect of L protein mutants by measuring the *Ranilla* luciferase (Rluc) activity (Supplementary data). As shown in Fig. 1D, the luciferase activities of V1517A, F1703A, Y1719A, and R1843A were significantly impaired (<5% compared to that of the wild-type L), resulting in an undetectable level as similar as the mutants D1126A and D1127A in the SDD motif, which suggested that these conserved sites in CBD and blocker domains played as a critical role as those in SDD motif of RdRp in SFTSV RNA transcription and replication. The luciferase activities for S1125A and N1745A mutants were reduced by 23.5% and 76.5%, respectively, while activities comparable to wild-type L were found for the P111A and P1797A mutants. The mutants D112A and E126A also caused considerably reduced luciferase activities by 70.3% and 73.1%, respectively, compared with the wild-type, differing from the results obtained by using the eGFP-based system. This may be attributed to the different detection sensitivities between the luciferase-based and eGFP-based systems.

Overall, amino acid mutations at nine of the twelve highly conserved sites impaired the activity of SFTSV L protein to varying degrees, which demonstrated their functional importance on SFTSV RNA replication/transcription and suggested their potential as antiviral targets. H80, P111, D112, E126 and K145 are the constituent residues of the motif (H … PD … D/E … K), which is speculated to carry out the endonuclease catalytic activity in the endoN domain function by chelating two cations as cofactors with the negatively charged residues D112 and E126 (Fig. 1B). Our results showed that mutants D112A and E126A, in which the original negatively charged residues were replaced with nonpolar alanine, generated considerably more adverse impacts than P111A. This suggested the importance of negatively charged residues D and E in the cap-snatching process. There used to be a controversy on the role of residue pair V1517–P1797 between the PB2-NL domain and the CBD domain in maintaining compact architecture of cap-binding pocket (Cusack and Rosenthal, 2021; Wang et al., 2021). Later on, the agreement was achieved that it is not likely to form directly interaction among this pairs regarding the 13 Å spatial distance between the two sites based on the L protein structure (Wang et al., 2021). Here, we found that V1517A impaired SFTSV-L protein function rather than P1791A, suggesting that these two sites had different roles and may not pose functional connection. The hydrophobic residues F1703 and Y1719 in CBD participated in forming a cap-binding pocket, which was occupied by R1843 in the blocker domain in the preinitiation stage of the SFTSV-L (Fig. 1B). R1843 and N1745 participate in additional hydrogen bond formation with their surrounding residues to stabilize this conformation, which would otherwise undergo a conformational shift for cap recognition. Our results suggested that a single mutation introduced in any of the four sites can significantly impair L protein functions, suggesting that the occupied conformation of the preinitiation stage of SFTSV-L is critical for cap-recognizing system.

In recent years, novel viruses closely related to the SFTSV were identified, including the Malsoor virus isolated from bats in India which can infect several human cell lines (Mourya et al., 2014), Hunter Island virus isolated from ticks of *Ixodes eudyptidis* which may related to albatross disease (Gauci et al., 2015), Heartland virus isolated from serum sample of tick-bite patients in United States (Brault et al., 2018), and Guertu virus isolated from ticks in China which causes pathological lesions in mice (Shen et al., 2018). These novel emerging SFTSV-related viruses pose serious threat to public health. Therefore, it raises the urgent need to develop effective drugs against these viruses. Alignment of RdRp protein sequences of SFTSV and the related phleboviruses showed that the nine sites of critical role in SFTSV L protein function were highly conserved in the L proteins of SFTSV-related phleboviruses (Supplementary Fig. S3). This suggests that these sites may be of as similar functional importance to SFTSV as to related phleboviruses in viral RNA transcription/replication. Therefore, our findings promoted better understanding of SFTSV-L protein structure in association with its functioning in viral RNA transcription and replication mechanism, which would also contribute to drug development by targeting SFTSV-L as well as L proteins of SFTSV-related viruses.

Footnotes

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