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Importance of SARS-CoV spike protein Trp-rich region in viral infectivity

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ABSTRACT

SARS-CoV entry is mediated by spike glycoprotein. During the viral and host cellular membrane fusion, HR1 and HR2 form 6-helix bundle, positioning the fusion peptide closely to the C-terminal region of ectodomain to drive apposition and subsequent membrane fusion. Connecting to the HR2 region is a Trp-rich region which is absolutely conserved in members of coronaviruses. To investigate the importance of Trp-rich region in SARS-CoV entry, we produced different mutated S proteins using Alanine scan strategy. SARS-CoV pseudotyped with mutated S protein was used to measure viral infectivity. To restore the aromaticity of Ala-mutants, we performed rescue experiments using phenylalanine substitutions. Our results show that individually substituted Ala-mutants substantially decrease infectivity by >90%, global Ala-mutants totally abrogated infectivity. In contrast, Phe-substituted mutants are able to restore 10–25% infectivity comparing to the wild-type. The results suggest that the Trp-rich region of S protein is essential for SARS-CoV infectivity.

Spike (S) protein is the outermost component of the virion, and is crucial for the entry of coronaviruses into host cells. S protein is type I viral protein and responsible for the attachment of virus to host cells [1–3] and for instigating the fusion of the virus envelope with cell membrane. All coronavirus S2 proteins have a highly conserved ten-residue sequence Y(V/I)KWPW(W/Y)VWL, which is rich in aromatic amino acids with 3 to 4 tryptophan (Trp) residues. The last five residues of this region probably form the beginning of the membrane-spanning domain which is also called transmembrane (TM) domain, [4,5]. This region is so called Trp-rich region, membrane-proximal external region (MPER), proximal-membrane region or pretransmembrane region (preTM) etc; in this study it is referred as Trp-rich region.

The function of this Trp-rich region is intriguing and remains unknown. Similarly Trp-rich regions also exist in the TM of all lentiviruses, although they may differ somewhat with regard to the number of Trp residues contained, the length of the sequence in which these are interspersed, the properties of the other amino acids present, and the distances within the linear sequence of the Trp-residues between themselves and with the putative membrane-spanning domains.

According to current models for HIV entry [6–10], and the high preference of Trp residues for residing at the external face of membranes [11–15], the proximal-membrane Trp-rich region appears to reside on the envelope surface at the membrane–water interface of the lipid bilayer. Following activation, the Trp-rich region is believed to undergo sequential conformational changes, from a reverse turn to an amphipathic helical structure capable of intimately interacting with the viral membrane through certain Trp residues and, concomitantly, with the cell membrane through others [16]. With their relatively bulky indole side chains, the Trp residues, most likely synergizing with the fusion peptide [12], would then destabilize both membranes and drive the energetically unfavorable lipid merging. This interaction permits the formation and expansion of the fusion pore in the late stages of the entry process [17,18]. Here, we examined the putative functions of the Trp-rich region of SARS-CoV S protein.

Materials and methods

Reagents. Calcium chloride ($CaCl_2$), sodium chloride (NaCl), potassium chloride (KCl), disodium hydrogen phosphate anhydrous (Na_2HPO_4), potassium phosphate monobasic (KH_2PO_4) and 4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid (HEPES) were all Ultra grade and purchased from Sigma–Aldrich. Rabbit anti-S antibody was kindly provided by Prof. Ding Xiang Liu (Institute of Molecular and Cellular Biology of Singapore).

Cell cultures. Vero E6 cells (Vero C1008 [ATCC CRL-1586^{TM}]) and 293T cells (293T/17 [ATCC CRL-11268]), were maintained in DMEM with L-glutamine (Gibco), supplemented with 10% new born calf serum and 50 μg/ml penicillin–streptomycin (Gibco).

Plasmids. pNL4-3Luc$^+$Env$^-$Vpr$^-$ and pcDNA3.1-OPT9-S were kindly provided by Prof. Zhang Linqi (Aaron Diamond AIDS Research Center).

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Table 1
Primers used to produce S protein mutants

Name	Sequence 5′–3′ [a]
S1194WA5	GTACGAGCAGTACATCAAG**GCC**CCCTGGTACGTGTGGCTGGGC
S1194WA3	GCCCAGCCACACGTACCAGGG**GGC**CTTGATGTACTGCTCGTAC
S1196WA5	TACATCAAGTGGCCC**GCC**TACGTGTGGCTGGGC
S1196WA3	GCCCAGCCACACGTA**GGC**GGGCCACTTGATGTA
S1197YA5	ATCAAGTGGCCCTGG**GCC**GTGTGGCTGGGCTTC
S1197YA3	GAAGCCCAGCCACA**GGC**CCCAGGGCCACTTGAT
S1199WA5	CAAGTGGCCCTGGTACGTG**GCC**CTGGGCTTCATCGCCGGCCTG
S1199WA3	CAGGCCGGCGATGAAGCCCAG**GGC**CACGTACCAGGGCCACTTG
S1202FA5	GCCCTGGTACGTGTGGCTGGGC**GCC**ATCGCCGGCCTGATCGCC
S1202FA3	GGCGATCAGGCCGGCGAT**GGC**GCCCAGCCACACGTACCAGGGC
S11946WA5	CGAGCAGTACATCAAG**GCC**CCC**GCC**TACGTGTGGCTGGGCTTCAT CG
S11946WA3	CGATGAAGCCCAGCCACACGTA**GGC**GGG**GGC**CTTGATGTACTGCT CG
S11949WA5	AAG**GCC**CCCTGGTACGTG**GCC**CTGGGCTTCATC
S11949WA3	GATGAAGCCCAG**GGC**CACGTACCAGGG**GGC**CTT
S11969WA5	AAGTGGCCC**GCC**TACGTG**GCC**CTGGGCTTCATC
S11969WA3	GATGAAGCCCAG**GGC**ACAGTA**GGC**GGGCCACTT
S119469WA5	AAG**GCC**CCC**GCC**TACGTG**GCC**CTGGGCTTCATC
S119469WA3	GATGAAGCCCAG**GGC**CACGTA**GGC**GGG**GGC**CTT
S119469WA 1197YA5	GTACATCAAG**GCC**CCC**GCCGCC**GTG**GCC**CTGGGCTTCATCGCCGG
S119469WA 1197YA3	CCGGCGATGAAGCCCAG**GGC**CAC**GGCGGC**GGG**GGC**CTTG ATGTAC
S119469WA 1197YA1202FA5	CC**GCCGCC**GTG**GCC**CTGGGC**GCC**ATCGCCGGCCTGATCGCCATCG
S119469WA 1197YA1202FA3	CGATGGCGATCAGGCCGGCGAT**GGC**GCCCAG**GGC**CAC**GGCGGC** GG

[a] Mutated base pairs are bold and underlined.

Primers. Primers designed to encode S protein mutants are listed in Table 1.

Construction of clones containing S protein mutants. Based on plasmid pcDNA3.1-OPT9-S which contains codon-optimized S gene, a series of plasmids containing mutated S gene (Fig. 1 and Table 2) were produced using QuickChange® II XL site-directed mutagenesis kit (Stratagene), following the manufacturer's instructions. The PCR reaction contains 5 μl of 10× reaction buffer, 3 μl (10 ng) of dsDNA template, 3 μl (125 ng) of primers (forward and reverse each, Table 1), 1 μl of dNTP mix and 3 μl of QuickSolution, 1 μl of PfuUltra HF DNA polymerase (2.5 U/μl), and 32 μl ddH_2O. The amplification program was performed at 95 °C, 1 min; 18 cycles of 95 °C, 50 S; 60 °C, 50 S; 68 °C, 12 min; followed by a final elongation step at 68 °C for 7 min. The PCR amplification products were digested by DpnI (1 μl of 10 U/μl DpnI in 50 μl amplification products) at 37 °C for 1 h. The DpnI-treated DNA was transformed into XL10-Gold® Ultracompetent cells, and then the cells were grown on LB-ampicillin agar plates at 37 °C for >16 h to produce clones.

Table 2
Mutations introduced in Trp-rich region of S protein

Name/ abbreviations	Description	Sequence (aa 1190–1204)
Wild-type	Control	Q Y I K ***W*** P ***W Y*** V ***W*** L G ***F*** I I
1. *Global mutations*		
WYF5A	W1194A + W1196A + Y1197A + W1199A + F1202A	Q Y I K **A** P **A A** V **A** L G **A** I I
WY4A	W1194A + W1196A + Y1197A + W1199A	Q Y I K **A** P **A A** V **A** L G ***F*** I I
W3A	W1194A + W1196A + W1199A	Q Y I K **A** P **A** ***Y*** V **A** L G ***F*** I I
W2A-4/6	W1194A + W1196A	Q Y I K **A** P **A** ***Y*** V ***W*** L G ***F*** I I
W2A-4/9	W1194A + W1199A	Q Y I K **A** P ***W Y*** V **A** L G ***F*** I I
W2A-6/9	W1196A + W1199A	Q Y I K ***W*** P **A** ***Y*** V **A** L G ***F*** I I
2. *Single mutations*		
W1194A	W1194A	Q Y I K **A** P ***W Y*** V ***W*** L G ***F*** I I
W1196A	W1196A	Q Y I K ***W*** P **A** ***Y*** V ***W*** L G ***F*** I I
Y1197A	Y1197A	Q Y I K ***W*** P ***W*** **A** V ***W*** L G ***F*** I I
W1199A	W1199A	Q Y I K ***W*** P ***W Y*** V **A** L G ***F*** I I
F1202A	F1202A	Q Y I K ***W*** P ***W Y*** V ***W*** L G **A** I I
W1194F	W1194F	Q Y I K **F** P ***W Y*** V ***W*** L G ***F*** I I
W1196F	W1196F	Q Y I K ***W*** P **F** ***Y*** V ***W*** L G ***F*** I I
Y1197F	Y1197F	Q Y I K ***W*** P ***W*** **F** V ***W*** L G ***F*** I I
W1199F	W1199F	Q Y I K ***W*** P ***W Y*** V **F** L G ***F*** I I

Note: Wild-type aromatic residues are shown in bold and italic. Mutated aromatic residues are bold and underlined.

Transient expression of different mutants of S protein in 293T cells. 293T cells were grown to 80% confluence in 75 cm^2 flasks (Nunc), and after trypsinization with Trypsin–EDTA, cells were plated into 6-well plates (5×10^5 cells/well) and cultured at 37 °C, 5% CO_2, overnight. The 293 T cells were transfected with plasmids pcDNA3.1-OPT9-S and S mutants using DOTAP transfection reagent (Roche Applied Science). After 48 h of transfection, cells were collected, cell lysates were resolved by 8% SDS–PAGE, and the expression of S protein and its mutants was investigated by Western blot using rabbit anti-S antibody as primary probe, and HRP-conjugated swine anti-rabbit antibody (DakoCytomation) as secondary probe.

Preparation of pseudotyped SARS-CoV containing different S protein mutants. The pseudoviruses were generated by co-transfection of pNL4-3Luc$^+$Env$^-$Vpr$^-$ and pcDNA3.1-OPT9-S or S mutants into 293T cells using calcium phosphate transfection method. The culture supernatant containing virus was collected on day 2 and 3 after transfection and clarified by filtering it through a 0.45 μm-pore-size filter and concentrated. The virus titer was determined by the reverse transcriptase (RT) activity assay using EnzChek® reverse transcriptase assay kit (Molecular Probes), and the viruses were standardized according the RT assay.

Single-cycle infectivity assay. Vero E6 cells (30,000 cells/well) were seeded in 48-well plates and cultured at 37 °C 5% CO_2 overnight. On the following day, Vero E6 cells were incubated with standardized amounts of pseudoviruses (0.5 U of RT/well) for 1 h and washed. After 48 h of infection, the cells were lysed in 100 μl lysis buffer (Promega). Luciferase activity was determined using luciferase assay kit (Promega). The resultant scintillation was counted for 15 s using a TD-20/20 Luminometer (Tuner Designs).

Results and discussion

To investigate the importance of Trp-rich region in SARS-CoV viral entry, S proteins with mutations in Trp-rich region were prepared. All clones containing different mutated S genes were selected and confirmed by sequencing.

The expression of S protein mutants in 293T cells was detected by rabbit anti-S protein antibody. The results showed that mutants W1194A, W1199A, W2A-4/6, W2A-4/9, WY4A, and WYF5A (see Table 2 for abbreviations) were expressed at high level, whereas others, such as the expression of Y1197A and W3A were detected at low level. No protein expression was detected under our experimental condition for W1196A, F1202A, and W2A-6/9 (Fig. 3).

SARS-CoV pseudotyped with S protein and its mutants was used to determine S protein-mediated infectivity. Pseudotyped retroviruses containing S protein mutants were first generated using a global site-directed mutagenesis and then Ala-scan of aromatic amino acids in the Trp-rich region to determine the positional importance of each Trp, Phe or Tyr. To restore the aromaticity of Ala-mutants, we performed rescue experiments using a Phe-scan. The results show that global substituted mutants, tri-, tetra- and penta-substitution with Ala (WYF5A, WY4A, and W3A) completely abrogate infectivity, while single- and double-substitution with Ala (W2A-4/6, W2A-4/9, W2A-6/9, W1194A, W1196A, Y1197A, W1199A, and F1202A) substantially decrease infectivity by >90% (Table 3). On the other hand, Phe-substituted mutants are able to restore 10–25% infectivity comparing to the wild-type (Table 4). These results suggest that the aromatic residues of the Trp-rich region of S protein are essential for SARS-CoV infectivity.

Coronavirus entry is mediated by type I viral envelope S protein. S protein of SARS-CoV is responsible for receptor-binding and membrane fusion. S protein contains several functional domains (Fig. 1) to support its correct folding and conformation which are crucial for its function. The Trp-rich region is absolutely conserved in members of coronaviruses (Fig. 2) and highly conserved in other RNA viruses such as HIV, FIV, and EboV. The importance of the Trp-