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Biochemical and Biophysical Research Communications 371 (2008) 356–360

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 12 March 2008
Available online 18 April 2008

Keywords:
Trp-rich region
SARS-CoV
Spike protein
Membrane fusion
Alanine scan
Mutant
Infectivity

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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doi:10.1016/j.bbrc.2008.04.044

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-

Table 3
Infectivity of different SARS-CoV pseudotyped with S protein mutants

Mutation on S protein	Name	Infectivity (%)
Wild-type		100[a]
Penta-mutation	WYF5A	0
Tetra-mutation	WY4A	0
Tri-mutation	W3A	0
Double-mutation	W2A-4/6	0.32
	W2A-4/9	0.08
	W2A-6/9	0.12
Single-mutation	W1194A	0.55
	W1196A	0.61
	Y1197A	0.59
	W1199A	0.59
	F1202A	0.02

[a] The infectivity of SARS-CoV pseudotyped with wild-type S protein was set as 100%; the infectivity of each SARS-CoV pseudotyped with S protein mutants was compared with wild-type pseudovirus.

Table 4
Comparison of the infectivity of SARS-CoV pseudotyped with S protein Ala- and Phe-mutants

Position	Infectivity (%)			Infectivity (%) restored by Phe-mutant
	W	A	F	
1194	100	0.55	0.24	0
1196	100	0.61	23.97	23.36
1197	100	0.59	9.13	8.54
1199	100	0.59	11.22	10.73

[*] The infectivity of SARS-CoV pseudotyped with wild-type S protein was set as 100%; the infectivity of each SARS-CoV pseudotyped with S protein mutants was compared with wild-type pseudovirus.

rich region in vaccine and therapeutics development has been demonstrated in HIV research as its epitopes are recognized by broadly neutralizing antibodies from human sera. It may play important roles during the whole fusion process.

Many laboratories have shown that the Trp-rich region plays an important role in HIV infection [11,12,19]. More importantly, this exposed region contains epitopes to which broadly neutralizing antibodies could be raised [11–13]. Indeed, Sainz et al. [4] found that the Trp-rich region of coronavirus type I viral fusion protein induces membrane permeabilization to facilitate the viral entry. Epand et al. [20] claimed that the Trp-rich region of HIV gp41 could promote the formation of cholesterol-rich domains which could facilitate membrane fusion.

Results obtained from our former work [21] show that cholesterol-enriched microdomains, known as lipid rafts, are required for SARS-CoV entry. The cryoEM structures of HIV and SIV reveal that the Trp-rich region forms the "feet" of a tripod-like structure of the spike proteins [22], anchoring the legs (HR1 and HR2 regions) firmly on the lipid rafts of viral surface. In the post-fusion state, the two heptad-repeat regions (HR1 and HR2) form a 6-helix bundle, positioning the fusion peptide closely to the Trp-rich region to drive apposition and subsequent fusion of viral and cell membranes. Because of their similarity in fusion mechanism of SARS-CoV and HIV, we hypothesized that the highly conserved Trp-rich region in SARS-CoV S protein plays a similar role in viral entry as in HIV by anchoring the tripod-like structure of S protein on a lipid rafts-like environment on the viral surface. Such a mechanism of anchoring S protein promotes clustering of S proteins, an effect which facilitates docking of host receptors, and subsequently membrane fusion by merging the cholesterol-enriched microdomains of both virus and host membranes.

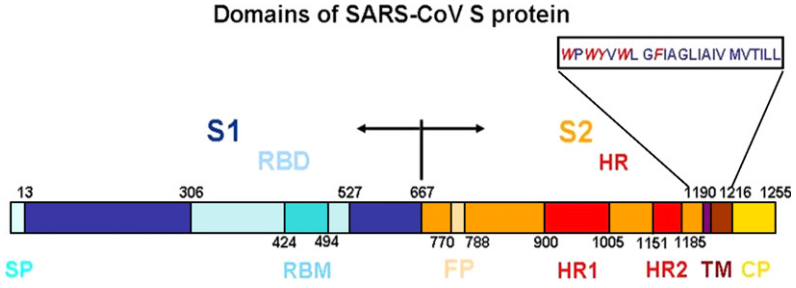

Fig. 1. Schematic representative of SARS-CoV S protein. Modeling and predictive analysis of SARS-CoV S protein. SP, signal peptide; RBD, receptor-binding domain; RBM, receptor-binding motif; FP, fusion peptide; HR, heptad repeat; TM, transmembrane domain; CP, cytoplasmic domain. The number of residues of each region corresponds to their positions in S protein of SARS-CoV. The Trp-rich region is indicated in purple box. The italic red color labeled-residues in Trp-rich region are targeted for mutation in this study.

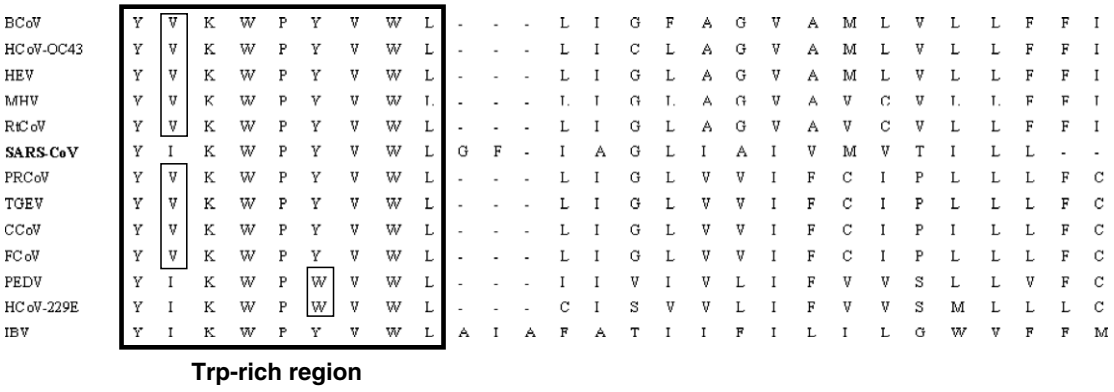

Fig. 2. Conserved motifs in Coronaviruses S protein. Alignment of the C-terminal region of the SARS-CoV and reference coronavirus S proteins. The black box indicates the amino acid sequence Y(V/I)KWPW(Y/W)VWL which is a conserved motif in all three coronavirus groups and SARS-CoV. This region is referred as Trp-rich region in this study.

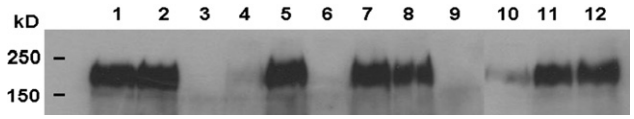

Fig. 3. Expression of wild-type and mutants of S protein in 293T cells. Wild-type S protein and its mutants were expressed in 293T cell. The cells were transfected with plasmids pcDNA3.1-OPT9-S and S mutants using DOTAP transfection reagent. After 48 h of transfection, the cell lysates were resolved on 8% SDS–PAGE, proteins were transferred onto PVDF membrane, and subjected to Western blot using rabbit anti-S antibody as primary probe. The expression of wild-type S protein (lane 1), W1194A (lane 2), W1199A (lane 5), W2A-4/6 (lane 7), W2A-4/9 (lane 8), WY4A (lane 11), and WYF5A (lane 12), were increased, the expression of Y1197A (lane 4), W3A (lane10) were detected at low level; and the expression of W1196A (lane 3), F1202A (lane 6), and W2A-6/9 (lane 9) could not be detected under this condition.

Why is this Trp-rich region so important for the infection of SARS-CoV and other type I viruses? The role of the Trp-rich region in participating in the clustering of gp41 monomer within the HIV-1 envelope has been shown by Saez-Cirion et al. [23]. They reported that the Trp-rich region of EboV glycoprotein predicted to bind the membrane interface, adopted a α-helical structure. This pre-transmembrane sequence might target membranes inherently prone to destabilization of the membrane. This group also reported that sphingomyelin and cholesterol promote HIV-1 gp41 Trp-rich region surface aggregation and membrane restructuring [24]. Furthermore, Guillen et al. [25] identified three membrane-active regions of SARS-CoV S protein using a 16/18-mer peptide scan; one of them is located in the Trp-rich region. Peptides corresponding to this region exert a dramatic effect on leakage for different model membranes, suggesting that this region might be involved in the promotion of the membrane destabilization required for fusion, as well as in fusion pore formation and enlargement.

CryoEM data [22] show that Trp-rich region form the "feet" to interact with the lipid–water interface. New predictive approaches including computation of interfacial affinity and the corresponding hydrophobic moments also suggest that this region is functionally segmented into two consecutive subdomains: one amphipathic at the N-terminal side and one fully interfacial at the C-terminus [26]. The N-terminal subdomain would extend α-helices from the preceding carboxyl-terminal heptad repeat (HR2) and provide, at the same time, a hydrophobic-at-interface surface. Saez-Cirion et al. [23] reported that gp41 Trp-rich peptides have the ability to oligomerizes and insert into the viral membrane interface. In our study, mutation of three or more aromatic residues in this region that functions as "feet" in pre-fusion state would irreversibly change its hydrophobic moments and its ability to anchor stably on the membrane to support the fusion process. Mutation of one or two aromatic amino acid residues in this region partially affects the "feet"-like conformation and virus infectivity.

In the post-fusion state, removing the bulky indole side chains and its weakly charged amine of the Trp residues in the Trp-rich region could disrupt its role in synergizing with the fusion peptide [12] to destabilize both membranes and drive the energetically unfavorable lipid-lipid merging that permits the formation and expansion of the fusion pore in the late stages of the entry process [17,18].

The fusion process is likely aided by the presence of cholesterol-binding motifs found in the S protein sequences. Vincent et al. and Epand et al. [20,27–30] reported that a consensus sequence found in a group of proteins that sequester to cholesterol-rich regions of membranes has the pattern –L/V-(X)(1–5)-Y-(X)(1–5)-R/K-, in which (X)(1–5) represents 1–5 residues of any amino acid. This sequence can induce formation of cholesterol-rich domains. The HIV-1 fusion protein gp41 has a LWYIK motif, consistent with this consensus sequence for this protein sequestering into cholesterol-rich domains and promoting membrane fusion. The SARS-CoV S protein Trp-rich region **Q Y I K W P W Y V W L G F I I** also has a similar motif. Our former data [21] show that lipid rafts are involved in the virus entry, which also supports the notion that the Trp-rich region may interact with lipid rafts in triggering the aggregation of lipid rafts and destabilizing the host cell membrane to promote membrane fusion, furthermore the aromatic amino acid residues in the Trp-rich region play crucial roles in viral infectivity.

Acknowledgment

We thank Prof. Zhang Linqi (*Aaron Diamond AIDS Research Center, Rockefeller University, New York, New York 10016*) for providing plasmids: pNL4-3Luc^{+}Env^{-}Vpr^{-} and pcDNA3.1-OPT9-S.

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