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Amino Acid Substitutions in the S2 Region Enhance Severe Acute Respiratory Syndrome Coronavirus Infectivity in Rat Angiotensin-Converting Enzyme 2-Expressing Cells[▽]

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To clarify the molecular basis of severe acute respiratory syndrome coronavirus (SARS-CoV) adaptation to different host species, we serially passaged SARS-CoV in rat angiotensin-converting enzyme 2 (ACE2)-expressing cells. After 15 passages, the virus (Rat-P15) came to replicate effectively in rat ACE2-expressing cells. Two amino acid substitutions in the S2 region were found on the Rat-P15 S gene. Analyses of the infectivity of the pseudotype-bearing S protein indicated that the two substitutions in the S2 region, especially the S950F substitution, were responsible for efficient infection. Therefore, virus adaptation to different host species can be induced by amino acid substitutions in the S2 region.

The 2002 to 2003 epidemic of severe acute respiratory syndrome (SARS) was caused by SARS coronavirus (SARS-CoV) infection. Initially, SARS-CoV was thought to be transmitted first from marketplace animals to humans and then by human-to-human spread (3, 7, 8). Although marketplace animals may be the immediate source of the SARS-CoV found in humans, SARS-CoV has been detected in other wild animals, e.g., civet cats, raccoon dogs (7), ferrets, and cats (15), suggesting that SARS-CoV may have a broad host range. In addition, rats are suggested to have been an animal vector in the SARS outbreak in the Amoy Gardens in Hong Kong (20). Recently, horseshoe bats have been reported to be a natural reservoir of coronaviruses close to SARS-CoV, and it is suggested that bats are candidate natural reservoirs of SARS-CoV (9, 13). In order to understand how the virus jumped to humans, it is important to elucidate the molecular mechanism of SARS-CoV adaptation to different species.

The SARS-CoV S protein mediates virus entry into cells expressing the receptor molecule angiotensin-converting enzyme 2 (ACE2) (12). The receptor binding domain (RBD), located on the S1 region, is believed to be the critical determinant of virus-receptor interaction (10, 27). It has been shown that amino acid substitutions on the RBD are associated with the SARS-CoV from palm civets adapted to humans (14). Furthermore, a single amino acid substitution on the RBD caused by serial in vivo passage of SARS-CoV in rats was strongly associated with increased infection of rat ACE2-expressing cells (18). Thus, substitution(s) of amino acid residues on the RBD may be one of the critical molecular determinants of SARS-CoV adaptation. On the other hand, in the case of mouse hepatitis virus, one or more amino acid substitutions of the S2 region, in combination with those of the S1 region, are intimately involved in receptor binding and extended host range (2, 16, 19). The presence of a neutralizing epitope within the S2 region of the SARS-CoV suggests that the S protein binding to the host cell surface not only relies on the S1 region but also depends on the global protein structure, including the S2 region (4, 28, 29). These studies suggested that several factors, including amino acid substitutions in the S2 region, in addition to those in the RBD in the S1 region, play roles in determining SARS-CoV infectivity. Thus, analyses of the entire amino acid sequence of the S protein may be necessary to understand the molecular mechanisms of viral adaptation to different species.

Notably, SARS-CoV S protein-mediated entry into cells expressing rat ACE2 has been shown to be extremely low (11, 14). Since the coronavirus serially passaged in vitro acquires amino acid substitutions that might be relevant to virus adaptation to different types of cells (5, 23), we speculated that in vitro passage of SARS-CoV in cells expressing species-specific ACE2 would induce viral adaptation to different animal species. To examine SARS-CoV adaptation to rat ACE2, we compared replication efficiencies for SARS-CoV serially passaged in rat ACE2-expressing cells and for a parental SARS-CoV strain when inoculated to cells expressing rat ACE2.

The SARS-CoV Frankfurt-1 strain was inoculated at a multiplicity of infection (MOI) of 0.01 PFU/cell onto Chinese hamster ovary (CHO) cells transiently transfected with the expression plasmid pcDNArat ACE2, which encodes rat ACE2 (18). The expression of ACE2 proteins was confirmed by Western blotting (Fig. 1A). The plasmids were transfected into the CHO cells at efficiencies of about 80%, as estimated by indirect immunofluorescence methods (data not shown). The virus, which was obtained from the culture supernatants of Frankfurt-1-infected rat ACE2-expressing CHO cells, was reinoculated at an MOI of 0.01 PFU/cell onto fresh CHO cells expressing rat ACE2. The culture supernatants were collected

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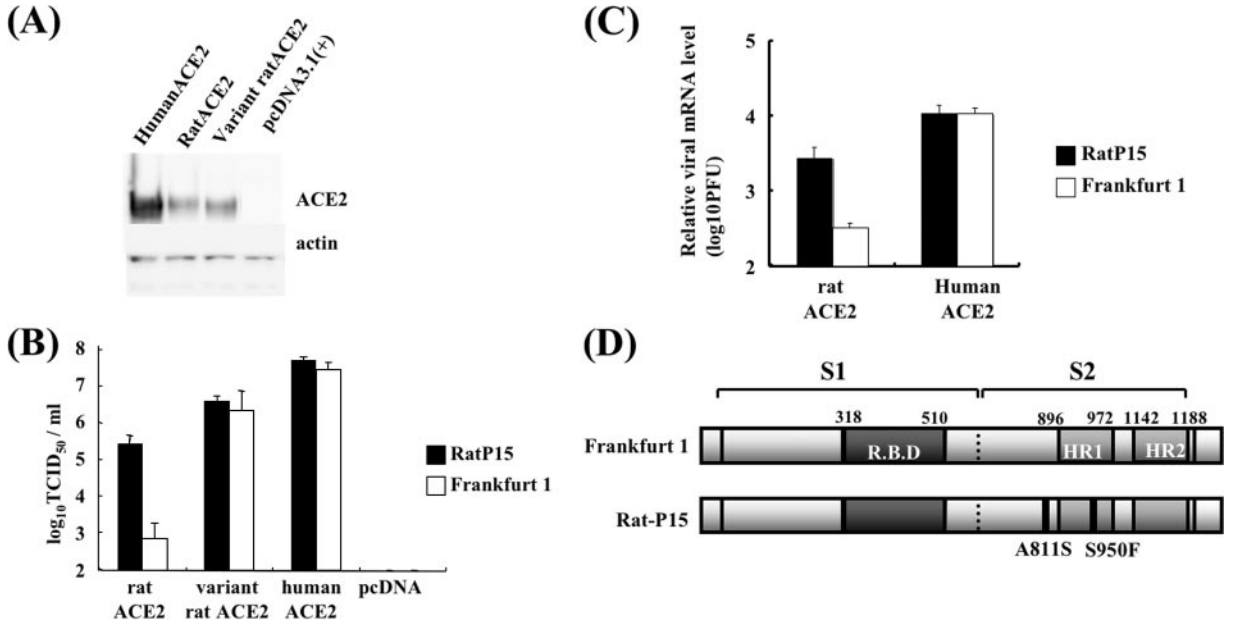

FIG. 1. Comparison of replication efficiencies of the SARS-CoV Rat-P15 strain serially passaged in rat ACE2-expressing cells and that of a parental SARS-CoV strain (Frankfurt-1). (A) Expression of ACE2 proteins. CHO cells were transfected with plasmid pTargeT-hACE2 (6), pcDNArat ACE2, and pcDNArat ACE2MUT2 (18), which encode human ACE2, rat ACE2, and variant rat ACE2 with amino acid residues 82 to 84 (NYS) corresponding to human ACE2 (MYP), respectively. Equal volumes of cell lysates were analyzed by Western blotting using a goat antibody specific for human ACE2 (6) or β-actin. The low signal intensity on rat ACE2 is due to the lower reactivity of the antiserum to rat ACE2. (B) Replication of viruses in CHO cells expressing rat ACE2, variant rat ACE2, or human ACE2. Rat-P15 or a parent Frankfurt-1 strain was inoculated to CHO cells transfected with expression plasmids pcDNArat ACE2, pcDNArat ACE2MUT2, pTargeT-hACE2, or the pcDNA3.1(+) vector. After 72 h, the replications of the virus in the cells were determined as 50% tissue culture infectious doses ($TCID_{50}$)/ml on Vero E6 cells. (C) Assessment of virus entry of Rat-P15 on rat ACE2-expressing cells. CHO cells were transfected with expression plasmids encoding rat ACE2 or human ACE2. After 48 h, 10,000 PFU of Rat-P15 or a parent Frankfurt-1 strain was inoculated. After 5 h, the culture medium was removed, and viral RNAs were isolated from the infected cells. Virus entry efficiency was estimated by quantification of the mRNA9 level with a real-time PCR assay (17). (D) Schematic representation of S proteins of Rat-P15 and Frankfurt 1. Amino acid substitutions at residues 811 and 950 in the S2 region of Rat-P15 are shown. The locations of the RBD, heptad repeat 1 (HR1), and HR2 are shown as filled boxes.

after 48 hours postinfection (hpi). All the following passages were performed by inoculation of the virus at an MOI of 0.002 PFU/cell and by collection of the culture supernatants at 72 hpi. Passages were performed 15 times, and the virus (Rat-P15) was obtained from culture supernatants. In order to examine whether serial passages of SARS-CoV makes the virus replicate efficiently in rat ACE2-expressing cells, Rat-P15 or a parent Frankfurt-1 strain was inoculated onto CHO cells that express rat ACE2. After 72 h, the virus titers of the culture supernatants were determined. The replication of Rat-P15 was higher than that of the parent Frankfurt-1 strain in rat ACE2-expressing CHO cells (Fig. 1B). In contrast, the replication of Rat-P15 was similar to that of parent Frankfurt-1 strain in CHO cells expressing human ACE2 or variant rat ACE2 with amino acid residues 82 to 84 (NYS) corresponding to human ACE2 (MYP) (18) (Fig. 1B). These results indicate that Rat-P15 came to replicate more efficiently in rat ACE2-expressing cells.

To examine whether the Rat-P15 strain acquired the ability to infect rat ACE2-expressing cells more efficiently than the parent Frankfurt-1 strain, viral infection was determined by quantitative real-time PCR assay after a shorter incubation period (17). Viruses were inoculated onto CHO cells expressing rat ACE2 or human ACE2. After 5 h, virus entry was estimated by measuring the amounts of newly synthesized mRNA9 of SARS-CoV. As shown in Fig. 1C, Rat-P15 propagated more efficiently than Frankfurt-1 strain in rat ACE2-expressing CHO cells by more than 10-fold. This result indicates that serial passages of SARS-CoV in rat ACE2-expressing cells efficiently increased its ability to infect rat ACE2-expressing cells.

To examine whether the Rat-P15 strain acquired amino acid substitutions within the S protein during serial passage, the nucleotide sequence of the S gene was determined as described previously (18). Interestingly, the amino acid sequence of the RBD of Rat-P15 was identical to that of the parent Frankfurt-1 strain. In contrast, two amino acid substitutions on the S2 region were found: serine for alanine at amino acid position 811 (A811S) and phenylalanine for serine at position 950 (S950F) (Fig. 1D). Nucleotide sequencing of this region at passages 1, 3, 5, 7, 9, 11, and 13 revealed that A811S substitution occurred after the 11th passage on rat ACE2-expressing cells, whereas the S950F substitution occurred after the 3rd passage (data not shown). This suggests that the two amino acid substitutions had distinct roles in enhancing viral infection in rat ACE2-expressing cells. On the other hand, virus which was passaged 11 times in cells expressing variant rat ACE2 did not have any amino acid substitutions in the S protein. These results suggest that A811S and S950F substitutions were not solely dependent on infection of CHO cells but were triggered by serial passage in rat ACE2-expressing CHO cells.

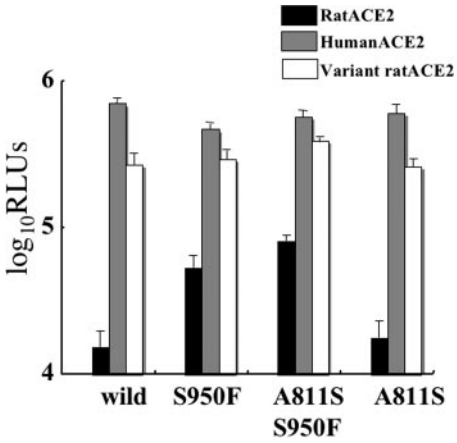

FIG. 2. Analysis of the significance of amino acid substitutions in the S2 region for the efficient entry of the virus into rat ACE2-expressing cells; analysis was done using VSV pseudotypes. VSVΔG*/SEAP-G, in which the VSV glycoprotein gene was replaced with the SEAP (*se*creted-type *a*lkaline *p*hosphatase) gene, was used for generating VSV pseudotypes bearing the C-terminally truncated S protein of SARS-CoV as described previously (6). BHK cells expressing rat, variant rat, or human ACE2 were inoculated with approximately 10^3 infectious units of VSV-St19 (wild), VSV-St19-A811S (A811S), VSV-St19-S950F (S950F), or VSV-St19-A811S-S950F (A811S S950F). At 18 hpi, SEAP activities of culture supernatants were measured by intensities of chemiluminescence reactions of alkaline phosphatase and are represented as relative luminescence units (RLUs). The VSV pseudotype bearing the S950F substitution rather than the A811S substitution efficiently infected rat ACE2-expressing cells.

In order to analyze the significance of the two amino acid substitutions for the efficient entry of the virus to rat ACE2-expressing cells, a vesicular stomatitis virus (VSV) pseudotyping system (kindly provided by M. A. Whitt) (6) was employed. VSV pseudotypes bearing S protein with a single amino acid substitution (VSV-St19-A811S and VSV-St19-S950F), bearing that with A811S and S950F double amino acid substitutions (VSV-St19-A811S-S950F), and bearing that having the amino acids of the wild type (VSV-St19) were generated. After the expression plasmids encoding rat ACE2, variant rat ACE2, and human ACE2 were transfected to Syrian baby hamster kidney (BHK) cells, each VSV pseudotype was inoculated. All the VSV pseudotypes infected human ACE2- or variant rat ACE2-expressing cells at similar levels (Fig. 2). On the other hand, VSV-St19-A811S-S950F infected rat ACE2-expressing cells more efficiently than VSV-St19. Interestingly, VSV-St19-S950F, which carries the S protein with the S950F substitution, infected rat ACE2-expressing cells more efficiently than did VSV-St19-A811S with the A811S substitution or VSV-St19. This indicates that the S950F substitution has a significant role in the efficient entry mediated by rat ACE2.

It has been shown that a single amino acid substitution in the S2 region affects the maturation of the glycosylation process of SARS-CoV S protein (1). Several lines of evidence indicate that glycosylation of viral envelope proteins is a molecular determinant for virus replication and infectivity (22, 24, 25, 26). Therefore, the wild-type and mutant S proteins were subjected to Western blotting to investigate the effect of the amino acid substitutions on the glycosylation of the S protein. The mutant S protein with a single A811S substitution (S-A811S), as well as the wild-type S protein (S-wt), migrated somewhat more slowly than those with a single S950F substitution (S-S950F) or with A811S and S950F substitutions (S-A811S-S950F) (Fig. 3). When S proteins were digested with endo-H, all mutant proteins migrated with molecular masses comparable to those of undigested controls. In contrast, when the expressed S proteins were digested with PNGase-F, all the mutant proteins showed migration patterns similar to that of the wild-type S protein. This indicates that differences in migration patterns of S-S950F and S-A811S-S950F were due to altered attachment of complex oligosaccharides during maturation. Our results indicate that S950F substitution affected N-linked glycosylation of the S protein and suggest that these differences are well correlated with the increased efficiency of SARS-CoV infection of rat ACE2-expressing cells.

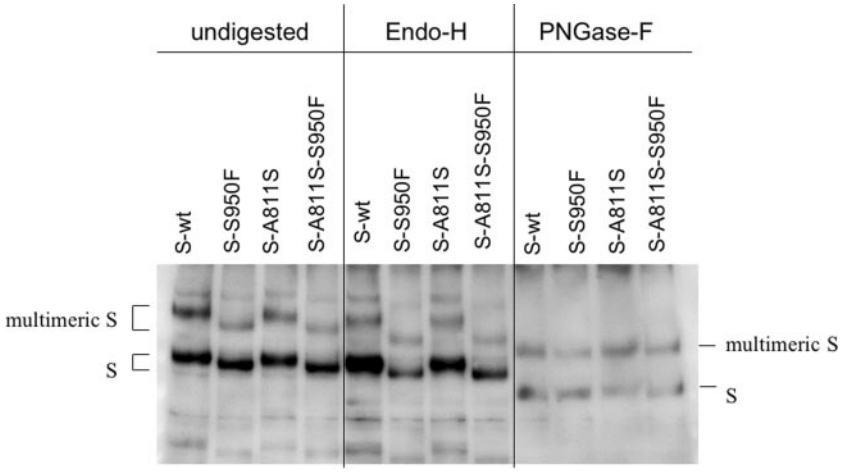

FIG. 3. Analysis of N-linked glycosylation of S proteins. SARS-CoV S proteins with the wild-type sequence (S-wt), an S950F substitution (S-S950F), an A811S substitution (S-A811S), and A811S and S950F substitutions (S-A811S-S950F) that were expressed in 293T cells were treated with endo-H or PNGase-F and then subjected along with undigested samples to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The S protein was detected by Western blotting using a rabbit antibody specific for the S2 region (6).

In contrast to our findings that amino acid substitutions were found in the S2 region, a substitution of tyrosine by serine located in the RBD was detected in the in vivo-adapted SARS-CoV (18). The difference between in vivo and in vitro passage may be attributed to replication sites; the substitution on the RBD seems to be responsible for the efficient replication of the virus on the alveolar area, where ACE2 is expressed at a low level (18), whereas virus replicated in CHO cells where ACE2 was abundantly expressed by transfection of expression plasmids. Alternatively, it seems likely that innate immune responses in rats could select a particular SARS-CoV strain adapted to rats. In summary, SARS-CoV adaptation to a particular animal species can be induced by amino acid substitutions in the RBD within the S1 region but also by those in the S2 region.

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