Entry from the Cell Surface of Severe Acute Respiratory Syndrome Coronavirus with Cleaved S Protein as Revealed by Pseudotype Virus Bearing Cleaved S Protein

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Severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV) is known to take an endosomal pathway for cell entry; however, it is thought to enter directly from the cell surface when a receptor-bound virion spike (S) protein is affected by trypsin, which induces cleavage of the S protein and activates its fusion potential. This suggests that SARS-CoV bearing a cleaved form of the S protein can enter cells directly from the cell surface without trypsin treatment. To explore this possibility, we introduced a furin-like cleavage sequence in the S protein at amino acids 798 to 801 and found that the mutated S protein was cleaved and induced cell fusion without trypsin treatment when expressed on the cell surface. Furthermore, a pseudotype virus bearing a cleaved S protein was revealed to infect cells in the presence of a lysosomotropic agent as well as a protease inhibitor, both of which are known to block SARS-CoV infection via an endosome, whereas the infection of pseudotypes with an uncleaved, wild-type S protein was blocked by these agents. A heptad repeat peptide, derived from a SARS-CoV S protein that is known to efficiently block infections from the cell surface, blocked the infection by a pseudotype with a cleaved S protein but not that with an uncleaved S protein. Those results indicate that SARS-CoV with a cleaved S protein is able to enter cells directly from the cell surface and agree with the previous observation of the protease-mediated cell surface entry of SARS-CoV.

A causative agent of the severe acute respiratory syndrome (SARS) that spread worldwide in 2002 to 2003 is a newly identified SARS coronavirus (SARS-CoV) (29). SARS-CoV is an enveloped virus with a positive-sense, single-stranded genome RNA of 29 kilobases (25) and is classified into the group 2 CoVs (34).

SARS-CoV spike (S) protein is a class I fusion glycoprotein having a molecular mass of ca. 200 kDa and is responsible for attachment to its receptor and subsequent entry into cells (6, 17, 31). During the maturation of the SARS-CoV S protein, its cleavage by intracellular protease is not observed (6, 35), which is similar to findings with the S protein of group 1 CoVs, such as human CoV 229E and transmissible gastroenteritis virus (4, 17, 19, 31). However, cleaved S1 and S2 (cl-S) subunits are detected on virions and in cells infected with murine CoV mouse hepatitis virus (MHV) and some other group 2 CoVs (36, 39). Although Bergeron et al. reported that certain proprotein convertases facilitate the processing of SARS-CoV S protein (3), others failed to find any subunits corresponding to S1 and S2 in cells infected with SARS-CoV or transfected with an S protein expression plasmid (27, 32, 35, 39). Uncleaved S proteins of SARS-CoV and others consist of regions that correspond to the S1 and S2 subunits of the MHV S protein. The receptor-binding domain (RBD) of SARS-CoV is located at amino acids 270 to 510 of the S1 subunit (2), making it similar to the RBD of group 1 CoVs (4, 10, 39) but different from that of MHV, which has its RBD in an N-terminal, 330-amino-acid region of the S1 subunit (21). Membrane-anchored S2 subunits of CoVs and corresponding regions in SARS-CoV S protein are structurally similar to the class I fusion proteins of other enveloped viruses (6, 7, 12). However, the fusion peptide that plays a crucial role in virus-to-cell fusion is located at the N terminus of the membrane-anchored subunit in influenza virus and human immunodeficiency virus (HIV), which in SARS-CoV and other CoVs is located in an internal region of the S2 subunit (6, 8, 12, 18, 24, 30, 37).

SARS-CoV was reported to enter target cells by two different pathways, one via an endosome and the other from the cell surface (27, 32, 33). In the endosomal pathway, the virus particle that attached to its receptor, angiotensin-converting enzyme 2 (ACE2) (23), is trafficked into the endosome, and then the S protein is cleaved at amino acid position 678 by an endosomal protease, cathepsin L (CPL), which activates the S protein fusion activity (5). This results in a fusion of the viral envelope and endosome membrane and subsequent internalization of the virus genome into cells (5, 33). On the other hand, the virion S protein attached to cell surface ACE2 is activated for fusion by proteases such as trypsin, which induces cleavage at amino acid position 667 of the S protein, 11 amino acids upstream of the CPL cleavage site (5, 22), which results in the fusion of the virus envelope and plasma membrane and subsequent viral entry from the cell surface (27). Entry from the cell surface is therefore possible only in the presence of proteases that activate the S protein. In both pathways, the
cleavage and fusogenic activation of SARS-CoV S protein are a critical step for virus entry into cells.

The preceding observations led us to postulate that SARS-CoV with cl-S protein that induces cell-to-cell fusion in the absence of proteases is able to enter cells directly from the cell surface, being similar to the cell entry of MHV with cl-S protein (16, 28). To explore this possibility, we have made a cleavage mutant of the SARS-CoV S protein by introducing the recognition sequence of furin-like protease, produced pseudotyped vesicular stomatitis virus (VSV) bearing this S protein, and analyzed whether cl-S protein facilitates direct viral entry from the cell surface.

We have chosen two different sites of S protein to introduce the furin recognition sequence (FRS) (RXR/KR; X indicates any amino acid residue [11]), one at positions 663 to 667 and the other at 798 to 801, since both contain a few endogenous basic amino acids and need not be drastically mutated to introduce FRS. The former corresponds to the site cleaved by trypsin (22) and also to the site of the MHV S protein which is cleaved during biosynthesis (36); however, insertion of FRS in this region failed to produce cleaved and fusogenic S protein in our system, being different from a previous report by Follis et al. (14). Thus, we introduced the FRS into a second region at positions 798 to 801. The mutated S protein was revealed to be fusogenic and was also cleaved by endogenous proteases, most probably by furin. In this study, we have analyzed the relationship of cleavability and fusogenicity of S protein as well as the cell entry pathway of the pseudotype virus (VSV) bearing the cl-S protein (VSV/cl-S) with a mutation at amino acid positions 798 to 801.

FRS was introduced at amino acid positions 798 to 801 in SARS-CoV S protein by an overlapping PCR method (14), and the resultant S protein was designated cl-S. Nineteen C-terminal amino acids were deleted for the efficient pseudotype formation of VSV, as described by Fukushima et al. (15). The mutated cDNA, as well as the cDNA for wild-type S (wt-S) protein lacking the 19 C-terminal amino acid residues, was cloned into a pCAG vector, and the nucleotide sequence was confirmed by DNA sequencing using BigDye Terminator version 3.1 and a 3130xl genetic analyzer (Applied Biosystems, Foster, CA). Figure 1 shows a schematic illustration of these S proteins.

The expression plasmids pCAG/cl-S and pCAG/wt-S, encoding SARS-CoV cl-S and SARS-CoV wt-S proteins, respectively, were transfected into HeLa cells and HeLa-ACE2 cells by using FuGENE6 (Roche Diagnostics, Mannheim, Germany), and those cells were incubated for 48 h after transfection. HeLa-ACE2 cells that constitutively express ACE2 were established from HeLa cells, following the transfection of a plasmid to express ACE2. The cells cultured for 48 h were also treated with 20 μg/ml of trypsin at room temperature for 5 min and further cultured at 37°C for 2 h to check cell fusion. As shown in Fig. 2A, large syncytia were visible on the HeLa-ACE2 cells transfected with pCAG/cl-S without trypsin treatment, whereas there was no apparent syncytium formation on the cells transfected with pCAG/wt-S. Treatment with trypsin induced fusion of the cells expressing wt-S protein. On the other hand, neither of those S proteins could induce cell-to-cell fusion on HeLa cells, even when they were treated by trypsin. These results strongly indicated that the cleavage of the S protein at amino acid position 798 converted its protease-dependent fusion phenotype to a protease-independent one and that ACE2 is crucial for the induction of cell-to-cell fusion. We then confirmed the cleavage of the S protein in those cells by Western blot analysis using anti-S2 rabbit serum no. 557 (Imgenex, San Diego, CA). Cells treated or untreated with trypsin were lysed with lysis buffer (10% glycerol, 1% Triton X-100, 135 mM NaCl) 5 min after treatment, and cell lysates were subjected to Western blot analysis. As shown in Fig. 2B, an S2 fragment with a molecular mass of 70 kDa was detected in the cell lysates prepared from pCAG/cl-S-transfected HeLa-ACE2 cells but not in the lysate of cells transfected with pCAG/wt-S (Fig. 2B). The 70-kDa protein found in cells transfected with pCAG/cl-S seems likely to correspond to the S2 fragment cleaved at the site where mutations recognized by furin-like protease are inserted, since its putative size was calculated to be approximately 68 kDa. It was also found that a proportion of cl-S protein expressed on HeLa-ACE2 cells was cleaved into a 100-kDa protein from a 240-kDa uncleaved form of S protein by trypsin treatment. The appearance of this 100-kDa protein in the lysates of trypsin-treated cells could result from the cleavage at amino acid position 667 (22), consistent with previous reports, and this subunit has been suggested to be involved in an S protein-mediated fusion (27, 32). Accordingly, cell fusion induction by trypsin of wt-S protein-expressing cells shown in the present study may be due to the
presence of 100 kDa of S2 protein (Fig. 2B). Also, the slightly elevated syncytium formation by trypsin treatment of the cells expressing cl-S protein 48 h after transfection (Fig. 2A, compare HeLa-ACE2 cells expressing cl-S protein and treated with trypsin to similar cells without trypsin treatment) could be attributed to a 100-kDa protein that arose after trypsin treatment. When cl-S protein was expressed in HeLa cells deleting ACE2, cleavage products were not detected, resulting in the absence of cell-to-cell fusion. Treatment of HeLa cells expressing wt-S or cl-S proteins with trypsin facilitated the cleavage of 240 kDa into 100 kDa, which was similar to the type of cleavage observed in HeLa-ACE2 cells; however, no fusion was detected, which indicated to us that ACE2 is crucial for the induction of cell-to-cell fusion. These results suggest that cl-S protein with a 70-kDa S2 subunit can induce fusion in a protease-independent manner.

Recently, Bosch et al. reported that SARS-CoV S proteins were cleaved by CPL at amino acid 678, resulting in a ca. 70-kDa S2 subunit (5), while the cl-S protein cleaved at position 798 shown in this study also resulted in a 60- to 70-kDa S2 subunit, even if the cleavage site was about 120 amino acids apart. This coincidence of the molecular mass of two different S2 subunits could come from the difference of S protein structure used in each experiment. We used full-length S, deleting 19 C-terminal amino acids, while S protein utilized by Bosch et al. was the soluble form of S protein, deleting transmembrane, and cytoplasmic domains consisted of ca. 60 amino acids (5). The full-length S2 subunit cleaved by CPL in SARS-CoV-infected cells would be ca. 100 kDa, similar to the S2 subunit cleaved by trypsin, because cleavage sites of those two proteases are closely located and only 11 amino acids apart (5, 22).

To evaluate the significance of the cleavage of the S protein to the entry pathway of SARS-CoV, pseudotyped VSV/cl-S or VSV/wt-S protein was produced as described previously (15, 26). In brief, expression plasmids for cl-S or wt-S protein were transfected to 293T cells, and those cells were infected with VSV bearing the glycoprotein (G), VSVΔG*G/SEAP, or VSVΔG*G/GFP, at 36 h after transfection. VSVΔG*G/SEAP and VSVΔG*G/GFP have a secretary alkaline phosphatase (SEAP) gene and the green fluorescence protein (GFP) gene in place of the VSV viral G gene, respectively. To confirm the S protein on the pseudotype viruses released in the culture supernatants, the supernatant was collected at 24 h after infection, and pseudotyped VSV was concentrated by ultracentrifugation at 45,000 rpm for 2 hours at 4°C using an SW50.1 rotor (Beckman, Fullerton, CA). The resultant pellets were dissolved in phosphate-buffered saline, pH 7.2, and analyzed by Western blotting after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. To detect S1 and S2 subunits, we used anti-S1 no. 20 (epitope, 20 to 35) and anti-S2 no. 1125 (epitope, 1125 to 1140) produced by the immunization of a chicken with synthetic peptides. As shown in Fig. 3A, a ca. 150-kDa protein and a 240-kDa protein was detected on pseudotyped VSV/cl-S by anti-S1 antibodies, while a ca. 70-kDa as well as a 240-kDa protein was detected by S2 subunit-specific antibodies. On the other hand, only the 240-kDa protein was seen on the pseudotype VSV/wt-S by both antibodies. The 150-kDa and 70-kDa proteins detected on pseudotype VSV/cl-S by anti-S1 and S2 antibodies, respectively, were thought to be cleavage products from their antigenicity and sizes. These results indicate that VSV pseudotypes with cl-S or wt-S protein were successfully formed, although the former is a mixture of pseudotypes with cl-S and uncleaved S protein (Fig. 3A). To obtain a preparation containing a higher proportion of pseudotype with cl-S protein, we expressed furin and ACE2 in 293T cells transfected with pCAG/cl-S because the production of a 70-kDa S2 subunit during synthesis was observed only in the presence of ACE2 (Fig. 2B). However, this
treatment did not increase the amount of cl-S proteins on the pseudotypes (data not shown).

We then examined the cell entry pathway of the viruses with cl-S and uncleaved SARS-CoV S proteins by using the above-mentioned preparation of two different pseudotype VSVs. To see whether the virus with cl-S protein bypassed an endosomal pathway, we evaluated the effect on the virus infection of the lysosomotropic agent bafilomycin A1 (Baf) in HeLa-ACE2 cells. The cells seeded at a concentration of 2 × 10^4 cells per well in a 96-well culture plate (Smilon, Tokyo, Japan) the day before infection were incubated in Dulbecco’s minimum essential medium (DMEM; Nissui, Tokyo, Japan) containing 5% fetal bovine serum (FBS; Sigma) and 100 nM Baf (Sigma) for 1 h at 37°C before infection. Then, ca. 500 infectious units of GFP gene-positive pseudotype VSV bearing wt-S, VSV/cl-S, or VSV/G was inoculated and incubated at 37°C for 1 h. Those cells were further incubated for 23 h in DMEM plus 5% FBS containing 100 nM Baf. GFP gene-positive cells were photographed by Keyence fluorescence microscopy (Keyence Corporation, Osaka, Japan), and cell numbers were calculated by using the image measurement and analyzing software VH-H1A5 version 2.6 (Keyence). As shown in Fig. 3B, the infection by VSV/wt-S was reduced by more than 90% by Baf treatment, similar to the suppression seen with VSV/G infection, which takes the endosomal pathway, while the suppression of VSV/cl-S infection was ca. 60%. This finding indicated that a proportion of VSV/cl-S infection was not affected by low pH in the endosome, which suggested that pseudotypes with cl-S protein do not take the endosomal pathway. About 60% of the reduction in infection of VSV/cl-S could be accounted for by the pseudotypes bearing uncleaved S protein, which was contaminated in the pseudotype with cl-S protein, as shown in Fig. 3A. We then examined whether the infection of pseudotypes is affected by treatment with protease inhibitors in a manner similar to that seen with SARS-CoV infection (33). Cells were treated with DMEM containing 100 μM of CPL inhibitor MDL (Sigma), 50 μM of CPL inhibitor EST (Carbiochem, Darmstadt, Germany), 200 nM of cathepsin B inhibitor CA-074 (Sigma), or 50 μM of cysteine protease inhibitor leupeptin (Roche Diagnostics) in a manner similar to that with Baf treatment and then infected with pseudotype VSVs that express GFP, adsorbed at 4°C for 1 h, and then incubated in the presence of Baf and/or HRP at 37°C for 24 h. The infection was evaluated as described above.

FIG. 3. Analysis using pseudotype VSV/cl-S or VSV/wt-S protein. (A) The incorporation of S protein into the pseudotype VSV. Pseudotype VSV was prepared by transfection of expression plasmids harboring the cl-S gene, the wt-S gene, or no gene and infection of VSVΔG*G. The pseudotypes in the culture fluids were concentrated by spinning at 45,000 rpm for 2 h using the SW50.1 rotor of a Beckman ultracentrifuge. The resultant pellets were dissolved in lysis buffer and analyzed by Western blot analysis using chicken antisera against S1 and S2 subunits (anti-S1 no. 20 and anti-S2 no. 1125, respectively). (B) Effect of Baf, protease inhibitors CA-074, EST, MDL, or leupeptin on the infection of VSV/G (white column), VSV/cl-S (gray column), or VSV/wt-S (black column) protein. HeLa-ACE2 cells prepared in 96-well plates were treated with those reagents at 37°C for 1 h, and ca. 500 PFU/50 μl of pseudotype VSVs were challenged. The infection was evaluated by measuring the number of GFP gene-positive cells in the wells at 24 h after infection as determined by Keyence fluorescence microscopy. (C) Blockade of pseudotype VSV infection by HRP. HeLa-ACE2 cells prepared in 96-well plates were treated with either Baf and/or HRP (5 μM) for 30 min at 37°C, infected with pseudotype VSVs that express GFP, adsorbed at 4°C for 1 h, and then incubated in the presence of Baf and/or HRP at 37°C for 24 h. The infection was evaluated as described above.
To further confirm that VSV/cl-S enters directly from the cell surface, we utilized a heptad repeat peptide (HRP) that was recently shown to efficiently block SARS-CoV infection from the cell surface but not the infection via the endosomal pathway (38). HeLa-ACE2 cells were treated with a final concentration of 100 nM of Baf and/or 5 μM of HRP-SR9, a peptide of 35 amino acids that corresponds to positions 1151 to 1185 of the SARS-CoV S protein (38), for 30 min at 37°C. Then, pseudotype VSVs were allowed to attach to cells at 4°C for 1 h in the presence of those agents. Then, cells were cultured at 37°C for 24 h in their presence, and the number of GFP gene-positive cells was calculated. As shown in Fig. 3C, Baf treatment alone thoroughly blocked infection with VSV/wt-S and that of VSV/cl-S by 40%, which seemed to indicate that some population of VSV/cl-S could bypass the endosomal pathway as shown in Fig. 3B. When treated with HRP, infection with VSV/wt-S was not at all blocked, which was similar to the results obtained previously (38), while infection with VSV/cl-S was partially blocked. The fraction of virus inhibited by HRP treatment may represent the VSV/cl-S. Furthermore, when treated with Baf and HRP, infection with VSV/cl-S was blocked almost completely, which suggested that the infection of Baf-resistant VSV/cl-S was blocked by HRP treatment. Another HRP (SR9EK1), previously shown to not prevent cell surface entry (38), failed to inhibit VSV/cl-S infection (data not shown). These results suggest that VSV/cl-S enters directly from the cell surface.

Two different pathways for cell entry are known for enveloped viruses, one from the cell surface and the other via an endosome (12). HIV and influenza virus are representative of the former and latter, respectively. Following binding to its receptor/coreceptor, gp160 of HIV is fusogenically activated, and fusion of the viral envelope and plasma membrane takes place, which facilitates the entry of the virus directly from the cell surface. In contrast, activation by hemagglutinin of influenza virus requires a low pH environment in the endosome to which virions are trafficked, following binding to its receptor. A strain of murine CoV, MHV-JHM, is believed to enter cells from the cell surface (16), while another strain, namely, MHV-A59, uses an endosomal pathway for entry due to its similarity to the entry mechanism of influenza virus (13). Although SARS-CoV takes an endosomal pathway, the molecular mechanism of entry is quite different from that of MHV-A59 or influenza virus. SARS-CoV S protein activation is not induced under a low pH environment but by proteolytic cleavage by CPL in the endosome that provides a low pH environment, which is optimal for CPL activity (5, 33). This entry mechanism is unique, though Ebola virus was initially shown to enter into cells in a similar fashion (9). Based on these molecular events of SARS-CoV infection, it can be postulated that SARS-CoV bearing a cl-S protein with fusion activity is able to enter cells directly from the cell surface, such as HIV and MHV-JHM do. The present study showed that cl-S protein along with the cell fusion activity of SARS-CoV facilitated viral entry into cells directly from the cell surface and strengthened the premise concerning the cell entry mechanism of SARS-CoV, as proposed by previous findings from different laboratories (27, 32, 33).

In the present study, we designed a mutant S protein to be cleaved during its maturation at the 798 amino acid position by inserting a recognition site for the furin-like protease. The detection of a 70-kDa fragment by anti-S2 antibody in Western blot analysis suggests that cleavage takes place at an expected position. Follis et al. produced a mutant with a cleavage site at amino acid position 667 (14). An S2 subunit of this mutant S protein was shown to be ca. 100 kDa and fusogenic, although insertion of extra amino acids in a newly appearing cleavage site is required for enhanced fusogenicity (14). We also produced and expressed the S protein without an extra amino acid insertion, as reported by Follis et al. (14), although it failed to induce cell-to-cell fusion in the absence of trypsin but successfully induced fusion when treated with trypsin, similar to the wt-S protein (data not shown). In our system, S protein with a cleavage site at amino acid 798 is only an S protein competent in the induction of cell-to-cell fusion without trypsin treatment.

It is clear that there is no stringency in the cleavage site of the SARS-CoV S protein for the fusion activity. Cleavage of the S protein by trypsin and CPL resulted in the fusion activity (5, 27, 32), even if the cleavage site was slightly different (5, 22). Also, our present study showed that cleavage at 120 amino acids downstream compared with the sites of trypsin and CPL conferred the fusion activity to the S2 protein. This is a unique feature of CoV S protein that was not found in the envelope protein of other viruses having a class I fusion protein, like HIV or influenza virus.

The finding that cl-S2 cleaved at position 798 has fusion activity implies that this molecule has a fusion peptide together with two different heptad repeats (HRs), motifs critical for fusion activity of class I fusion protein of the virus. This suggests that the fusion peptide on SARS-CoV S protein would locate downstream of amino acid position 798. Although there are controversial reports on the localization of a fusion peptide in SARS-CoV S protein, present study suggests that the fusion peptide locates immediately upstream of the HR1 (18) rather than in the 770 to 788 region (30), because this region is located outside the fusogenic 70-kDa S2 subunit produced by cl-S protein expression.

We reported that SARS-CoV infection was enhanced in cultured cells in the presence of proteases, such as trypsin and elastase, and that this enhancement was facilitated by virus entry from the cell surface (27). Elastase is a major protease produced by neutrophils during lung inflammation and may be relevant to the high growth of SARS-CoV in the lung, a major target organ (20). Based on these findings, we have established an exacerbated fatal pneumonia of mice with high pathogenic similarity to human SARS by the coinfection of SARS-CoV and nonpathogenic respiratory bacteria that stimulates elastase secretion (1). Severe pneumonia of mice was attributed to an enhanced SARS-CoV infection in the lung that could be facilitated by the elastase (1). We have not evaluated yet whether this enhanced infection is due to the cell surface infection. If this is the case, HRP may be a good candidate for a therapeutic tool, since HRP efficiently blocks SARS-CoV infection from the cell surface, as shown in the present and previous studies (38). Most MHV strains induce cell-to-cell fusion in infected cells, yet a highly hepatopathogenic strain, MHV-2, fails to induce fusion (41). MHV-2 shares a common feature with SARS-CoV in terms of its S protein. MHV-2 was revealed to enter cells in...
a fashion highly similar to that of SARS-CoV, which is dependent on the proteases in the endosomal compartment having a low pH environment (28). A mutant virus with fusogenic S protein was isolated from wild-type MHV-2 bearing unclaved S protein, although isolation was extremely low in efficiency, ca. 1 mutant out of half a million to 1 million (41). This could suggest the possibility that SARS-CoV with fusogenic cL-S protein exists among a large number of viruses with uncleaved S protein. It is of interest to see the effects of SARS-CoV with a cL-S protein in terms of cell entry mechanisms as well as in pathogenesis for animals.

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