Heptad Repeat-Derived Peptides Block Protease-Mediated Direct Entry from the Cell Surface of Severe Acute Respiratory Syndrome Coronavirus but Not Entry via the Endosomal Pathway[▽]

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The peptides derived from the heptad repeat (HRP) of severe acute respiratory syndrome coronavirus (SCoV) spike protein (sHRPs) are known to inhibit SCoV infection, yet their efficacies are fairly low. Recently our research showed that some proteases facilitated SCoV's direct entry from the cell surface, resulting in a more efficient infection than the previously known infection via endosomal entry. To compare the inhibitory effect of the sHRP in each pathway, we selected two sHRPs, which showed a strong inhibitory effect on the interaction of two heptad repeats in a rapid and virus-free in vitro assay system. We found that they efficiently inhibited SCoV infection of the protease-mediated cell surface pathway but had little effect on the endosomal pathway. This finding suggests that sHRPs may effectively prevent infection in the lungs, where SCoV infection could be enhanced by proteases produced in this organ. This is the first observation that HRP exhibits different effects on virus that takes the endosomal pathway and virus that enters directly from the cell surface.

Severe acute respiratory syndrome (SARS) coronavirus (SCoV) is a causative agent of life-threatening SARS (4, 7, 15, 31). Although the first outbreak of SARS was stamped out, an effective antiviral drug is still required for the treatment and prevention of possible future outbreaks. SCoV is an enveloped virus and enters cells via fusion between the cellular membrane and its envelope. SCoV membrane fusion is mediated by the spike (S) protein, which is classified as a class I fusion protein. One of the most important features of class I fusion proteins is the conserved heptad repeat regions (HR1 and HR2) which play an essential role in virus-cell fusion activities (3, 6, 10, 28). In the fusion process, HR1 forms an interior, trimeric coiled-coil structure to which HR2 binds in an antiparallel fashion, resulting in the formation of a six-helix bundle. This structure brings viral and cellular membranes into close proximity to facilitate membrane fusion. Synthetic short peptides derived from the HR (HRP) of class I fusion proteins have been shown to block the interaction of HR1-HR2 complexes, resulting in the inhibition of a number of viral infections, including those of

retroviruses (11, 14, 21, 23, 32, 38, 39), paramyxoviruses (12, 16, 30, 36, 42–44), filovirus (37), and coronavirus (2). Similarly, HRP of SCoV S (sHRP) could also inhibit SCoV and human immunodeficiency virus (HIV)/SCoV-pseudotyped virus infection (1, 18, 24, 45). However, these inhibitory effects were significantly less than those of one of the most effective HRPs from HIV type 1 (HIV-1) (39) and even those from the same family, murine coronavirus mouse hepatitis virus (MHV) (2).

The major organs targeted by SCoV are the lungs and intestines, although the virus grows in a variety of tissues that express angiotensin-converting enzyme 2 (ACE2). Recently we and others showed that SCoV uses two distinct entry pathways depending on the presence of proteases (20, 33, 34). In the absence of proteases, SCoV enters the cell via an endosomal pathway (9, 26, 41), with the S protein activated for fusion by the cathepsin L protease, which is active only under acidic conditions in the endosome (8, 33). In contrast, in the presence of protease, SCoV virion S proteins attach to ACE2 on the host cell surface and are activated for fusion by proteases such as trypsin or elastase, which leads to envelope-plasma membrane fusion and direct entry from the cell surface (20, 33, 34). Infection via the cell surface is more than 100 times more efficient than infection via the endosomal pathway (20). These results suggested the possibility that the severe illnesses in the lung and intestine could be due to the enhancement of direct SCoV cell surface entry mediated by proteases produced in these organs (20).

Although previous studies have described the inhibitory effects of the sHRP on SCoV infection via the endosomal path-

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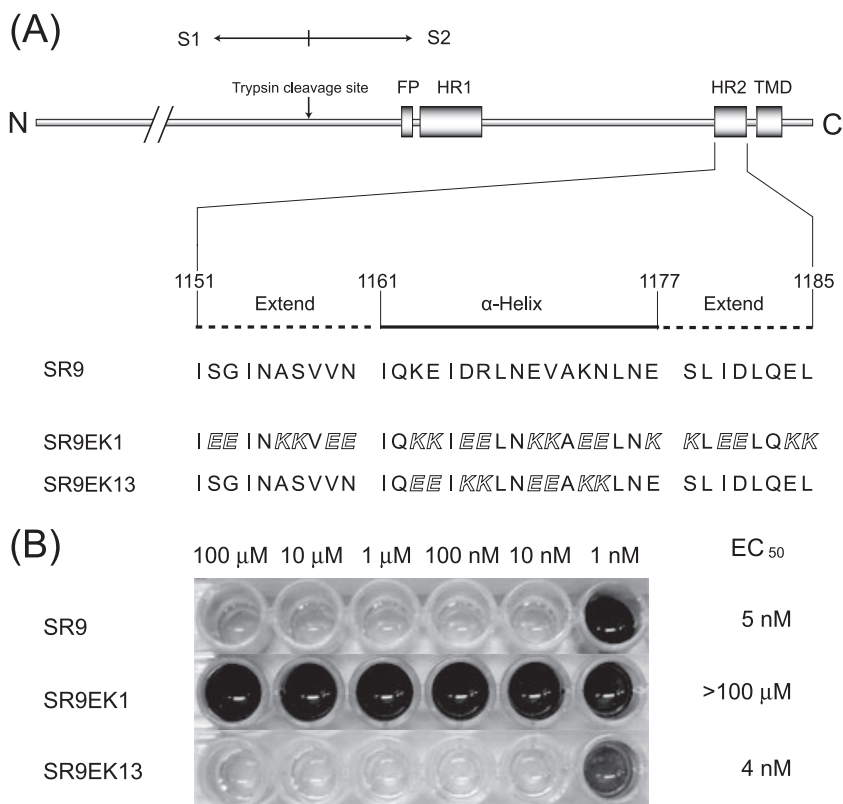▽ Published ahead of print on 17 October 2007.

FIG. 1. (A) Schematic of SCoV S protein and sequences of native sHRP (SR9) and its EK substitution derivatives. The S protein contains two α-helical heptad repeats (HR1 and HR2), a putative fusion peptide (FP), a transmembrane domain (TMD), and a trypsin cleavage site (17). The expanded region shows the amino acid sequence of HR2 (SR9), which consists of two extended parts (1151 to 1160 and 1178 to 1185) and one α-helix part (1161 to 1177). Substituted EKs are shown with italic white letters. (B) In vitro binding inhibition assay of HRPs. GST-HR2-coated plates were incubated with MBP-HR1 in the presence of various concentrations (1 nM to 100 μM) of sHRP. Inhibitory potency of the peptide was assessed using the anti-MBP antibody-alkaline phosphatase conjugate and staining with 5-bromo-4-chloro-3-indolylphosphate.

way (1, 18, 24, 45), little is known about their effects on the protease-mediated cell surface pathway. Thus, in this study, we reevaluated the inhibitory effects of the sHRP on infection via the two distinct pathways of SCoV entry.

Recent studies of the X-ray crystal structure of the SCoV S HR1-HR2 complex have shown that the HR2 peptide consists of two extended regions and one α-helical region (35, 40). Since we have found that HRPs with replacement by the X-EE-XX-KK sequence in the HIV-1 HR2 region exhibited potent anti-HIV-1 activity (27), we chose to modify the α-helical region of HRP derived from SCoV S HR2 (sHRP) and also to prepare the control peptide SR9EK1 without sequence relatedness (Fig. 1A). To estimate these sHRPs, we established a rapid and virus-free in vitro novel assay system based on the inhibition of HR1-HR2 complex formation. Two fusion proteins (maltose binding protein [MBP]-HR1 [amino acid residues of the S protein, 892 to 964] and glutathione S-transferase [GST]-HR2 [1141 to 1192]) were expressed using Escherichia coli and purified using amylose resin (New England Biolabs) and glutathione Sepharose 4B (GE Healthcare, Bucks, United Kingdom), respectively. An enzyme-linked immunosorbent assay plate was coated with GST-HR2 dissolved in sodium carbonate buffer (pH 8.5), 3.6 μg/ml in concentration, by incubation at 4°C for 8 h. After bovine serum albumin blocking (1 mg/ml) at 4°C for 2.5 h, GST-HR2 on the plate was allowed to

bind the MBP-HR1 protein (8.8 μg/ml) by incubation at 37°C for 1.5 h in the presence of various concentrations of sHRPs to be examined for inhibition activity. After the plate was washed, the inhibiting potency of the peptide was assessed by colorimetric analyses using the anti-MBP antibody-alkaline phosphatase conjugate (Sigma) with a 1:1,000 dilution with incubation at 4°C for 1 h and then staining with BluePhos microwell phosphatase (KPL). As shown in Fig. 1B, SR9 and SR9EK13 showed significant binding inhibition in a nanomolar range, whereas the control, SR9EK1, without sequence relatedness, had no inhibitory effect at a concentration of 100 μM.

We tested the inhibitory effects of SR9 and SR9EK13 on SCoV entry, since these sHRPs were found to have a strong binding inhibition activity, along with the control peptide SR9EK1. We examined their effects on both the endosomal and protease-mediated cell surface entry processes. Viral entry via the endosome was examined as described previously with a slight modification (20). In brief, VeroE6 cells were pretreated with each sHRP at 37°C for 30 min and then inoculated with SCoV (multiplicity of infection = 1.0) and incubated on ice for 30 min to allow viral attachment to ACE2 but not viral entry. After removal of unattached viruses, the cells were incubated at 37°C for 6 h. Viral entry was measured by quantifying the newly synthesized mRNA9 using real-time PCR (20). To evaluate entry via the cell surface, the cells were pretreated with 1
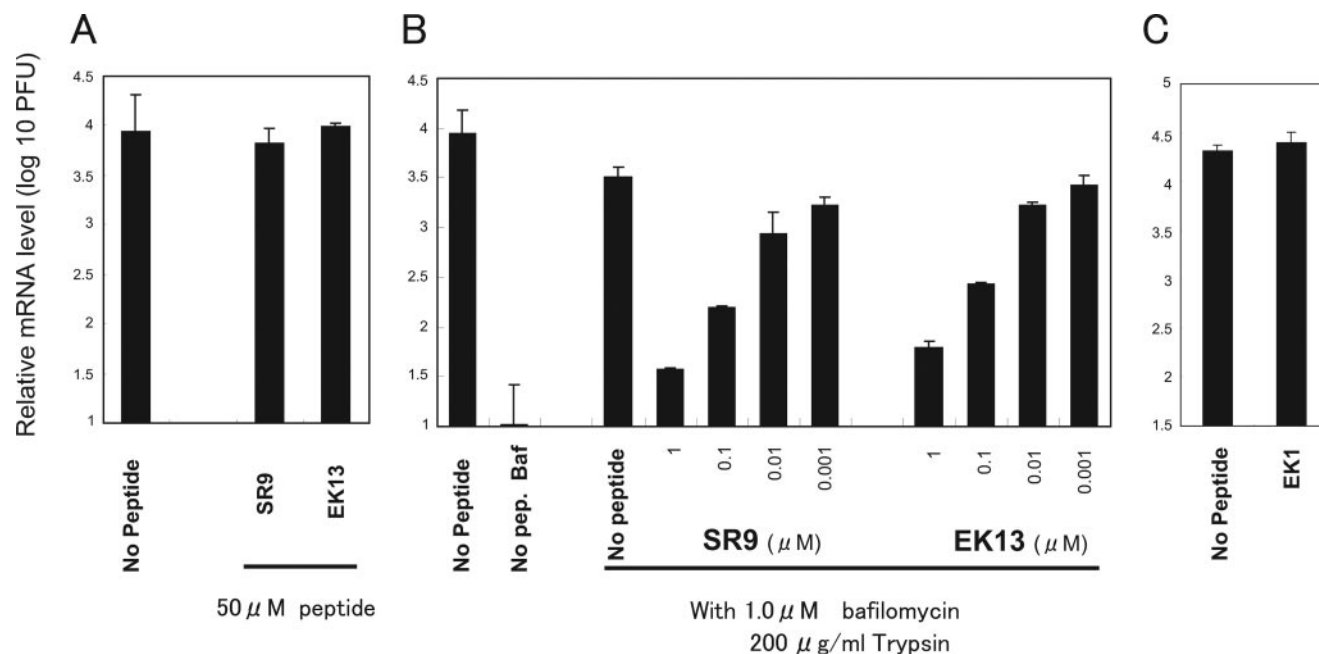
FIG. 2. Inhibitory effect of sHRPs on SCoV infections via the endosomal pathway (A) or protease-mediated cell-surface pathway (B). (A) VeroE6 cells were pretreated with 50 μM sHRPs at 37°C for 30 min, placed on ice for 10 min, and then inoculated with SCoV at a multiplicity of infection of 1.0 on ice for 30 min. After the removal of unbound virus, the cells were incubated in medium containing 50 μM sHRPs at 37°C for 6 h. (B) Cells pretreated with 1 μM Baf and sHRPs at the indicated concentrations were inoculated with SCoV as described above. After the removal of unbound virus, the cells were treated with 200 μg/ml L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin at room temperature for 5 min and incubated at 37°C for 6 h. sHRP and Baf were present in the media in all steps at indicated concentrations. To measure amounts of viruses that entered cells, cells were infected with 10-fold-stepwise-diluted SARS-CoV from 10^6 to 10^2 PFU without Baf and trypsin and the amounts of mRNA9 were quantified by real-time PCR. Amounts of viral entry in this study were calculated from a calibration line obtained as described above and are shown as relative mRNA levels (20). (C) EK1 has no sequential similarity to sHRP and showed no inhibitory effect in vitro. Cells were treated with 1 μM EK1 as a control peptide, and other procedures were performed as described for panel B.

μM bafilomycin (Baf), which blocks SCoV endosomal entry, at 37°C for 30 min before SCoV inoculation. After removal of unattached viruses, the cells were treated with trypsin (0.2 mg/ml) for 5 min at room temperature and viral entry was measured as described above. Each sHRP and/or Baf was present in the media in all steps at various concentrations. In the absence of proteases, these sHRPs showed no measurable inhibitory effect on SCoV endosomal infection even at concentrations as high as 50 μM, despite showing a potent inhibitory effect in vitro (Fig. 2A). This lack of inhibition is consistent with previous observations that the same or homologous-sequence sHRPs had no inhibitory effect on SCoV infection at high concentrations of 10 μM (45) or 50 μM (1), respectively. In contrast, when SCoV was allowed to enter cells via the cell surface by treatment with protease and Baf, these sHRPs showed a strong inhibitory effect on SCoV infection in a dose-dependent manner (Fig. 2B). At a concentration of 0.1 μM, the SR9 sHRP reduced newly synthesized mRNA9 levels by about 10-fold, while an sHRP concentration of 1 μM saw a 50-fold decrease. The control sHRP, SR9EK1, did not inhibit SCoV cell-surface-mediated infection even at the concentration of 1 μM, indicating that the inhibition is peptide sequence specific (Fig. 2C). We finally evaluated the inhibitory effect of sHRPs in the presence of trypsin but without Baf treatment. These conditions may resemble the situation of patients with severe SARS, in which some proteases were produced in infected lung and intestinal tissue. Under these conditions,

these sHRPs also showed a potent inhibitory effect on SCoV infection (Fig. 3).

The present study indicates that our sHRPs fail to inhibit endosome-mediated SCoV infection. This finding is consistent with those of previous studies indicating that sHRPs have a low inhibitory effect on endosomal infection of native SCoV. The reported 50% effective dose (EC_{50}) was 3.68 to 19.0 μM (1, 18, 45). However, our results suggest that sHRPs, which showed no measurable inhibitory effect on SCoV endosomal infection, have a very strong inhibitory effect on protease-mediated cell surface SCoV infection; the EC_{50} was less than 100 nM (Fig. 2B and 3). Cell surface infection of SCoV is anticipated to occur in the lungs of SARS patients, since various types of inflammatory cells infiltrate the lung of the patients (25), and thus elastase, a protease produced in lung inflammation (13) and shown to enhance SCoV infection in cultured cells (20), could enhance SCoV infection in the lung by facilitating the infection from cell surface. Inhibitory effects of sHRPs on cell surface infection may help prevent severe damage by SCoV infection in the major target organ. Thus, the sHRPs shown in this study would be effective anti-SARS therapeutic drugs.

A few possibilities are conceivable for the explanation of an inefficient inhibitory effect of sHRPs in infection via the endosomal pathway. One is the failure of sHRPs to be trafficked to the endosome vehicles from culture medium. Thus, their concentration in the endosome is not sufficient to prevent SCoV infection. Alternatively, sHRPs may be sufficiently transported
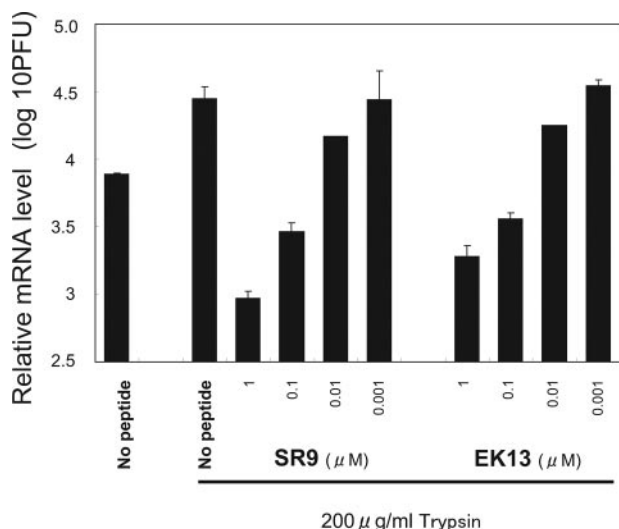
FIG. 3. Effective inhibition by HRPs of SCoV infection in the presence of exogenous trypsin. VeroE6 cells pretreated with sHRPs at the indicated concentrations were inoculated with SCoV as described in the legend to Fig. 2. After the removal of unbound virus, the cells were treated with 200 μg/ml L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin at room temperature for 5 min and incubated at 37°C for 6 h. sHRPs were present in the media in all steps at the indicated concentrations. The relative viral mRNA9 was measured quantitatively by real-time PCR as described in the legend to Fig. 2. In this assay, cells were not treated with Baf throughout the experiment.

to the endosome but are inactivated by the low-pH environment or are degraded or digested with proteases present in the endosome. Another possibility is that the conformation of the cleaved S protein in the acidic environment of the endosome is different from that in a neutral pH and the sHRP fails to bind to the S protein in the former environment even if six-helix bundles with intramolecular HR2 are formed under both conditions. We are currently studying whether the inefficient inhibition of virus entry into cells could be attributed to one of those possibilities, or even another.

Interestingly, the EC$_{50}$ (approximately 680 μM) of HRP of Ebola virus (37), which is thought to enter cells via an endosomal pathway, is remarkably higher than those of other viruses which enter cells directly from the cell surface. The inhibition with HRP of influenza virus infection, which also uses an endosomal pathway, has not yet been reported, even though its hemagglutinin protein is the prototype class I fusion protein and its cell entry mechanism has been extensively studied. In contrast, HRP of avian leucosis sarcoma virus, which uses the endosomal pathway, was reported to inhibit the infection fairly efficiently (EC$_{50}$ = 25 to 170 nM) (5, 23). The inhibition, however, was executed during the conformational rearrangement of the envelope protein that occurs on the cell surface following attachment to the receptor and facilitates the exposure of HRs but not later than the transport into the endosome, where the avian leucosis sarcoma virus genome enters the cytoplasm by its envelope and endosomal membrane fusion in a low-pH environment (19, 22, 23). These observations together with those of the present study and others (1, 18, 24, 45) suggest that the HRPs have very low or little inhibitory effect in the endosome. If the above assumption is correct and

the HRPs were designed to be efficiently transferred into the endosome and to be stable in the environment, they may be new antiviral candidates against those viruses that take the endosomal entry pathway, such as influenza virus, Ebola virus, and SCoV. Thus, detailed molecular studies on SCoV and the sHRP will provide a good model for the development and evaluation of such endosome-philic antiviral peptide inhibitors.

Recent studies have reported that the low inhibitory effect of the SCoV sHRP compared to that of the MHV HRP could be attributed to the weaker interaction of the SCoV S HR1-HR2 complex versus that of MHV S (1, 2). However, SCoV infection was efficiently blocked by sHRP under certain conditions, as revealed in this study; the concentration of sHRPs needed to inhibit SCoV infection is even lower than that required for MHV inhibition (1, 2). The apparent difference between MHV and SCoV infection is the pathway used to enter cells; the former enters directly from the cell surface, whereas the latter takes an endosomal pathway. Both MHV and SCoV infections were efficiently blocked when these viruses utilized the cell surface pathway for entry. These observations suggest that the lower HRP inhibitory effect on SCoV could be due to different entry pathways between SCoV and MHV rather than the weaker interaction of the HRP and SCoV S. To further explore this possibility, studies are ongoing to determine the effect of MHV sHRPs on infection by MHV-2, which, like SCoV, utilizes an endosomal infection pathway (29).

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