Heptad repeat-derived peptides block the protease-mediated direct entry from cell surface of SARS coronavirus but not entry via endosomal pathway

Makoto Ujike[1, †], Hiroki Nishikawa[2, †], Akira Otaka[3], Naoki Yamamoto[4], Norio Yamamoto[4], Masao Matsuoka[5], Eiichi Kodama[5], Nobutaka Fujii[2, 5]* and Fumihiro Taguchi[1, *].

[1]Department of Virology III, National Institute of Infectious Disease, Gakuen 4-7-1, Musashi-murayama, Tokyo 208-0011, Japan. [2]Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan. [3]Graduate School of Pharmaceutical Sciences, The University of Tokushima, Tokushima 770-8505, Japan. [4]Department of Molecular Virology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. [5]Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan.

Corresponding author. Mailing address: Department of Virology III, National Institute of Infectious Disease, Gakuen 4-7-1, Musashi-murayama, Tokyo 208-0011, Japan. Phone: +81-42-561-0771, ext. 533. Fax: +81-42-567-5631. E-mail: ftaguchi@nih.go.jp, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan, nfujii@pharm.kyoto-u.ac.jp

[†] M.U. and H.N. contributed equally to this work.

Running title: HRP blocks SCoV infection from cell surface

Abstract

The peptides derived from the heptad repeat (sHRP) of severe acute respiratory syndrome (SARS) coronavirus (SCoV) spike (S) protein 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 infection via a previously-known endosomal entry. To compare the inhibitory effect of sHRP in each pathway, we selected two sHRP, 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 it efficiently inhibited SCoV infection of the protease-mediated cell-surface pathway, but had little effect on the endosomal pathway. This finding suggests that sHRP may effectively prevent the 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 the virus that takes endosomal pathway and one that enters directly from 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 future possible 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 anti-parallel 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 protein have been shown to block the interaction of HR1-HR2 complexes, resulting in the inhibition of a number of viral infections, including 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 HIV/SCoV pseudotyped virus infection (1, 18, 24, 45). However, these inhibitory effects were significantly lower than those of one of the most effective HRPs from 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 S protein activated for fusion by 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 cell surface is over 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 described the inhibitory effects of sHRP on SCoV infection via the endosomal pathway (1, 18, 24, 45), little is known about their effects on the protease-mediated cell-surface pathway. Thus, in this study, we re-evaluated the inhibitory effects of the sHRP on the 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 HR2 peptide consists of two extended regions and one α-helical region (35, 40). Since we have found that the HRPs with the replacement by the X-EE-XX-KK sequence into 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 prepare the control peptide SR9EK1 without sequence relatedness (Fig. 1B). 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 S protein: 892-964) and Glutathione S-Transferase (GST)-HR2 (1141-1192)] were expressed using *Escherichia Coli* and purified using Amylose Resin (New England Biolabs, MA, USA) and Glutathione Sepharose 4B (GE Healthcare, Bucks, UK), respectively. GST-HR2 dissolved in sodium carbonate buffer (pH 8.5), 3.6 μg/ml in concentration was coated on ELISA plate by incubating at 4 ºC for 8 h. After BSA blocking (1 mg/ml) 4 ºC for 2.5 h, GST-HR2 on the plate was allowed to bind MBP-HR1 protein (8.8 μg/ ml) by incubating at 37 ºC for 1.5 h in the presence of various concentrations of sHRPs to be examined for inhibition activity. After washing the plate, inhibiting potency of the

peptide was assessed by colorimetric analyses using the anti-MBP antibody-alkaline phosphatase (ALP) conjugate (SIGMA, MO, USA) with 1: 1000 dilution incubating at 4 ºC for 1 h, and then stained with BluePhos Microwell Phosphatase (KPL, MD, USA). As shown in Fig.1B, the 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 as these sHRPs were found to have a strong binding inhibition activity along with control peptide SR9EK1. We examined their effects on both the endosomal and the protease-mediated cell surface entry processes. Viral entry via endosome was examined as described previously with a slight modification (20). In brief, VeroE6 cells were pretreated with each sHRP at 37 ºC for 30 min, then inoculated with SCoV (multiplicity of infection = 1.0) and incubated on ice for 30 min to allow viral attachment to ACE2 but not allow viral entry. After removal of unattached viruses, the cells were incubated at 37 ºC for 6 h. Viral entry was measured by quantifying the newly synthesized mRNA9 using real-time PCR (20). To evaluate the entry via cell-surface, the cells were pretreated with 1 μM bafilomycin (Baf), which blocks SCoV endosomal entry at 37 ºC 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 indicated 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 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 severe SARS patients in which some proteases were produced in the 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 the previous studies that sHRPs have low inhibitory effect on endosomal infection of native SCoV. The reported 50% effective dose (EC_{50}) was 3.68 - 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 (Fig. 2B and Fig. 3). Cell surface infection of SCoV is anticipated to occur in the lung of SARS patients, since various types of inflammatory cells infiltrate in the lung of the patients (25) and thus elastase, a protease produced in the 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 effect of sHRP on cell-surface infection may help prevent severe damage by SCoV infection in the major target organ. Thus, 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 sHRP in the infection via endosome. 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 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 an acidic environment and a neutral pH is different and sHRP fails to bind to the S protein in the former environment, even if six-helix bundles with intramolecular HR2 are formed in both conditions. We are currently studying which of those possibilities, or even another could be attributed to the inefficient inhibition of virus entry into cells.

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 (ALSV) that takes endosomal pathway was reported to inhibit the infection fairly efficiently (EC_{50} = 25 - 170 nM) (5, 23). The inhibition was, however, executed during the conformational rearrangement of the envelope protein that occurs on cell surface following the attachment to the receptor and facilitates the

exposure of HRs, but not later than the transport into endosome where ALSV genome enters into cell cytoplasm by its envelope and endosomal membrane fusion in low pH environment (19, 22, 23). These observations together with 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 endosome and stable in the environment, it may be new antiviral candidates against those viruses that take endosomal entry pathway, such as influenza virus, Ebola virus, and SCoV. Thus, the detailed molecular studies on SCoV and sHRP will provide a good model for the development and evaluation of such endosome-philic antiviral peptide inhibitors.

Recent studies have reported that the lower inhibitory effect of the SCoV sHRP compared to 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 of SCoV could be due to different entry pathways between SCoV and MHV, rather than the weaker interaction of HRP and SCoV-S. To further explore this possibility, studies are currently ongoing to determine the effect of MHV sHRPs on the infection of MHV-2 which, like SCoV, also utilizes an endosomal infection pathway (29).

Acknowledgements

We thank Miyuki Kawase for her excellent technical assistance and Shutoku Matsuyama for his valuable discussions. This work was financially supported by grants from the Ministry of Education, Culture, Sports, Science and Technology.

References

1. **Bosch, B. J., B. E. Martina, R. Van Der Zee, J. Lepault, B. J. Haijema, C. Versluis, A. J. Heck, R. De Groot, A. D. Osterhaus, and P. J. Rottier.** 2004. Severe acute respiratory syndrome coronavirus (SARS-CoV) infection inhibition using spike protein heptad repeat-derived peptides. Proc Natl Acad Sci U S A **101:**8455-60.
2. **Bosch, B. J., R. van der Zee, C. A. de Haan, and P. J. Rottier.** 2003. The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. J Virol **77:**8801-11.
3. **Chan, W. E., C. K. Chuang, S. H. Yeh, M. S. Chang, and S. S. Chen.** 2006.

Functional characterization of heptad repeat 1 and 2 mutants of the spike protein of severe acute respiratory syndrome coronavirus. J Virol **80:**3225-37.

4. **Drosten, C., S. Gunther, W. Preiser, S. van der Werf, H. R. Brodt, S. Becker, H. Rabenau, M. Panning, L. Kolesnikova, R. A. Fouchier, A. Berger, A. M. Burguiere, J. Cinatl, M. Eickmann, N. Escriou, K. Grywna, S. Kramme, J. C. Manuguerra, S. Muller, V. Rickerts, M. Sturmer, S. Vieth, H. D. Klenk, A. D. Osterhaus, H. Schmitz, and H. W. Doerr.** 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med **348:**1967-76.
5. **Earp, L. J., S. E. Delos, R. C. Netter, P. Bates, and J. M. White.** 2003. The avian retrovirus avian sarcoma/leukosis virus subtype A reaches the lipid mixing stage of fusion at neutral pH. J Virol **77:**3058-66.
6. **Follis, K. E., J. York, and J. H. Nunberg.** 2005. Serine-scanning mutagenesis studies of the C-terminal heptad repeats in the SARS coronavirus S glycoprotein highlight the important role of the short helical region. Virology **341:**122-9.
7. **Fouchier, R. A., T. Kuiken, M. Schutten, G. van Amerongen, G. J. van Doornum, B. G. van den Hoogen, M. Peiris, W. Lim, K. Stohr, and A. D. Osterhaus.** 2003. Aetiology: Koch's postulates fulfilled for SARS virus. Nature **423:**240.
8. **Huang, I. C., B. J. Bosch, F. Li, W. Li, K. H. Lee, S. Ghiran, N. Vasilieva, T. S. Dermody, S. C. Harrison, P. R. Dormitzer, M. Farzan, P. J. Rottier, and H. Choe.** 2006. SARS coronavirus, but not human coronavirus NL63, utilizes cathepsin L to infect ACE2-expressing cells. J Biol Chem **281:**3198-203.
9. **Inoue, Y., N. Tanaka, Y. Tanaka, S. Inoue, K. Morita, M. Zhuang, T. Hattori, and K. Sugamura.** 2007. Clathrin-Dependent Entry of Severe Acute Respiratory Syndrome Coronavirus into Target Cells Expressing ACE2 with the Cytoplasmic Tail Deleted. J Virol **81:**8722-9.
10. **Jahn, R., T. Lang, and T. C. Sudhof.** 2003. Membrane fusion. Cell **112:**519-33.
11. **Jiang, S., K. Lin, N. Strick, and A. R. Neurath.** 1993. HIV-1 inhibition by a peptide. Nature **365:**113.
12. **Joshi, S. B., R. E. Dutch, and R. A. Lamb.** 1998. A core trimer of the paramyxovirus fusion protein: parallels to influenza virus hemagglutinin and HIV-1 gp41. Virology **248:**20-34.
13. **Kawabata, K., T. Hagio, and S. Matsuoka.** 2002. The role of neutrophil elastase in acute lung injury. Eur J Pharmacol **451:**1-10.

14. **Kilby, J. M., S. Hopkins, T. M. Venetta, B. DiMassimo, G. A. Cloud, J. Y. Lee, L. Alldredge, E. Hunter, D. Lambert, D. Bolognesi, T. Matthews, M. R. Johnson, M. A. Nowak, G. M. Shaw, and M. S. Saag.** 1998. Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry. Nat Med **4:**1302-7.

15. **Ksiazek, T. G., D. Erdman, C. S. Goldsmith, S. R. Zaki, T. Peret, S. Emery, S. Tong, C. Urbani, J. A. Comer, W. Lim, P. E. Rollin, S. F. Dowell, A. E. Ling, C. D. Humphrey, W. J. Shieh, J. Guarner, C. D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Y. Yang, N. Cox, J. M. Hughes, J. W. LeDuc, W. J. Bellini, and L. J. Anderson.** 2003. A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med **348:**1953-66.

16. **Lambert, D. M., S. Barney, A. L. Lambert, K. Guthrie, R. Medinas, D. E. Davis, T. Bucy, J. Erickson, G. Merutka, and S. R. Petteway, Jr.** 1996. Peptides from conserved regions of paramyxovirus fusion (F) proteins are potent inhibitors of viral fusion. Proc Natl Acad Sci U S A **93:**2186-91.

17. **Li, F., M. Berardi, W. Li, M. Farzan, P. R. Dormitzer, and S. C. Harrison.** 2006. Conformational states of the severe acute respiratory syndrome coronavirus spike protein ectodomain. J Virol **80:**6794-800.

18. **Liu, S., G. Xiao, Y. Chen, Y. He, J. Niu, C. R. Escalante, H. Xiong, J. Farmar, A. K. Debnath, P. Tien, and S. Jiang.** 2004. Interaction between heptad repeat 1 and 2 regions in spike protein of SARS-associated coronavirus: implications for virus fusogenic mechanism and identification of fusion inhibitors. Lancet **363:**938-47.

19. **Matsuyama, S., S. E. Delos, and J. M. White.** 2004. Sequential roles of receptor binding and low pH in forming prehairpin and hairpin conformations of a retroviral envelope glycoprotein. J Virol **78:**8201-9.

20. **Matsuyama, S., M. Ujike, S. Morikawa, M. Tashiro, and F. Taguchi.** 2005. Protease-mediated enhancement of severe acute respiratory syndrome coronavirus infection. Proc Natl Acad Sci U S A **102:**12543-7.

21. **Medinas, R. J., D. M. Lambert, and W. A. Tompkins.** 2002. C-Terminal gp40 peptide analogs inhibit feline immunodeficiency virus: cell fusion and virus spread. J Virol **76:**9079-86.

22. **Melikyan, G. B., R. J. Barnard, R. M. Markosyan, J. A. Young, and F. S. Cohen.** 2004. Low pH is required for avian sarcoma and leukosis virus Env-induced hemifusion and fusion pore formation but not for pore growth. J Virol **78:**3753-62.

23. **Netter, R. C., S. M. Amberg, J. W. Balliet, M. J. Biscone, A. Vermeulen, L. J. Earp, J. M. White, and P. Bates.** 2004. Heptad repeat 2-based peptides inhibit avian sarcoma and leukosis virus subgroup a infection and identify a fusion intermediate. J Virol **78:**13430-9.

24. **Ni, L., J. Zhu, J. Zhang, M. Yan, G. F. Gao, and P. Tien.** 2005. Design of recombinant protein-based SARS-CoV entry inhibitors targeting the heptad-repeat regions of the spike protein S2 domain. Biochem Biophys Res Commun **330:**39-45.

25. **Nicholls, J. M., L. L. Poon, K. C. Lee, W. F. Ng, S. T. Lai, C. Y. Leung, C. M. Chu, P. K. Hui, K. L. Mak, W. Lim, K. W. Yan, K. H. Chan, N. C. Tsang, Y. Guan, K. Y. Yuen, and J. S. Peiris.** 2003. Lung pathology of fatal severe acute respiratory syndrome. Lancet **361:**1773-8.

26. **Nie, Y., P. Wang, X. Shi, G. Wang, J. Chen, A. Zheng, W. Wang, Z. Wang, X. Qu, M. Luo, L. Tan, X. Song, X. Yin, J. Chen, M. Ding, and H. Deng.** 2004. Highly infectious SARS-CoV pseudotyped virus reveals the cell tropism and its correlation with receptor expression. Biochem Biophys Res Commun **321:**994-1000.

27. **Otaka, A., M. Nakamura, D. Nameki, E. Kodama, S. Uchiyama, S. Nakamura, H. Nakano, H. Tamamura, Y. Kobayashi, M. Matsuoka, and N. Fujii.** 2002. Remodeling of gp41-C34 peptide leads to highly effective inhibitors of the fusion of HIV-1 with target cells. Angew Chem Int Ed Engl **41:**2937-40.

28. **Petit, C. M., J. M. Melancon, V. N. Chouljenko, R. Colgrove, M. Farzan, D. M. Knipe, and K. G. Kousoulas.** 2005. Genetic analysis of the SARS-coronavirus spike glycoprotein functional domains involved in cell-surface expression and cell-to-cell fusion. Virology **341:**215-30.

29. **Qiu, Z., S. T. Hingley, G. Simmons, C. Yu, J. Das Sarma, P. Bates, and S. R. Weiss.** 2006. Endosomal proteolysis by cathepsins is necessary for murine coronavirus mouse hepatitis virus type 2 spike-mediated entry. J Virol **80:**5768-76.

30. **Rapaport, D., M. Ovadia, and Y. Shai.** 1995. A synthetic peptide corresponding to a conserved heptad repeat domain is a potent inhibitor of Sendai virus-cell fusion: an emerging similarity with functional domains of other viruses. Embo J **14:**5524-31.

31. **Rota, P. A., M. S. Oberste, S. S. Monroe, W. A. Nix, R. Campagnoli, J. P. Icenogle, S. Penaranda, B. Bankamp, K. Maher, M. H. Chen, S. Tong, A. Tamin, L. Lowe, M. Frace, J. L. DeRisi, Q. Chen, D. Wang, D. D. Erdman,**

T. C. Peret, C. Burns, T. G. Ksiazek, P. E. Rollin, A. Sanchez, S. Liffick, B. Holloway, J. Limor, K. McCaustland, M. Olsen-Rasmussen, R. Fouchier, S. Gunther, A. D. Osterhaus, C. Drosten, M. A. Pallansch, L. J. Anderson, and W. J. Bellini. 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science **300:**1394-9.

32. **Sagara, Y., Y. Inoue, H. Shiraki, A. Jinno, H. Hoshino, and Y. Maeda.** 1996. Identification and mapping of functional domains on human T-cell lymphotropic virus type 1 envelope proteins by using synthetic peptides. J Virol **70:**1564-9.
33. **Simmons, G., D. N. Gosalia, A. J. Rennekamp, J. D. Reeves, S. L. Diamond, and P. Bates.** 2005. Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. Proc Natl Acad Sci U S A **102:**11876-81.
34. **Simmons, G., J. D. Reeves, A. J. Rennekamp, S. M. Amberg, A. J. Piefer, and P. Bates.** 2004. Characterization of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) spike glycoprotein-mediated viral entry. Proc Natl Acad Sci U S A **101:**4240-5.
35. **Supekar, V. M., C. Bruckmann, P. Ingallinella, E. Bianchi, A. Pessi, and A. Carfi.** 2004. Structure of a proteolytically resistant core from the severe acute respiratory syndrome coronavirus S2 fusion protein. Proc Natl Acad Sci U S A **101:**17958-63.
36. **Wang, E., X. Sun, Y. Qian, L. Zhao, P. Tien, and G. F. Gao.** 2003. Both heptad repeats of human respiratory syncytial virus fusion protein are potent inhibitors of viral fusion. Biochem Biophys Res Commun **302:**469-75.
37. **Watanabe, S., A. Takada, T. Watanabe, H. Ito, H. Kida, and Y. Kawaoka.** 2000. Functional importance of the coiled-coil of the Ebola virus glycoprotein. J Virol **74:**10194-201.
38. **Wild, C., T. Oas, C. McDanal, D. Bolognesi, and T. Matthews.** 1992. A synthetic peptide inhibitor of human immunodeficiency virus replication: correlation between solution structure and viral inhibition. Proc Natl Acad Sci U S A **89:**10537-41.
39. **Wild, C. T., D. C. Shugars, T. K. Greenwell, C. B. McDanal, and T. J. Matthews.** 1994. Peptides corresponding to a predictive alpha-helical domain of human immunodeficiency virus type 1 gp41 are potent inhibitors of virus infection. Proc Natl Acad Sci U S A **91:**9770-4.
40. **Xu, Y., Z. Lou, Y. Liu, H. Pang, P. Tien, G. F. Gao, and Z. Rao.** 2004. Crystal structure of severe acute respiratory syndrome coronavirus spike protein fusion core. J Biol Chem **279:**49414-9.

41. **Yang, Z. Y., Y. Huang, L. Ganesh, K. Leung, W. P. Kong, O. Schwartz, K. Subbarao, and G. J. Nabel.** 2004. pH-dependent entry of severe acute respiratory syndrome coronavirus is mediated by the spike glycoprotein and enhanced by dendritic cell transfer through DC-SIGN. J Virol **78:**5642-50.

42. **Yao, Q., and R. W. Compans.** 1996. Peptides corresponding to the heptad repeat sequence of human parainfluenza virus fusion protein are potent inhibitors of virus infection. Virology **223:**103-12.

43. **Young, J. K., D. Li, M. C. Abramowitz, and T. G. Morrison.** 1999. Interaction of peptides with sequences from the Newcastle disease virus fusion protein heptad repeat regions. J Virol **73:**5945-56.

44. **Yu, M., E. Wang, Y. Liu, D. Cao, N. Jin, C. W. Zhang, M. Bartlam, Z. Rao, P. Tien, and G. F. Gao.** 2002. Six-helix bundle assembly and characterization of heptad repeat regions from the F protein of Newcastle disease virus. J Gen Virol **83:**623-9.

45. **Yuan, K., L. Yi, J. Chen, X. Qu, T. Qing, X. Rao, P. Jiang, J. Hu, Z. Xiong, Y. Nie, X. Shi, W. Wang, C. Ling, X. Yin, K. Fan, L. Lai, M. Ding, and H. Deng.** 2004. Suppression of SARS-CoV entry by peptides corresponding to heptad regions on spike glycoprotein. Biochem Biophys Res Commun **319:**746-52.

FIGURE LEGENDS

FIG. 1. (A)Schematic of SCoV-S protein and sequences of native sHRP (SR9) and its EK substituted 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 (1151-1160, 1178-1185) and one α-helix part (1161-1177). (B) In vitro binding inhibition assay of the HRPs. GST-HR2 coated plate was incubated with MBP-HR1 in the

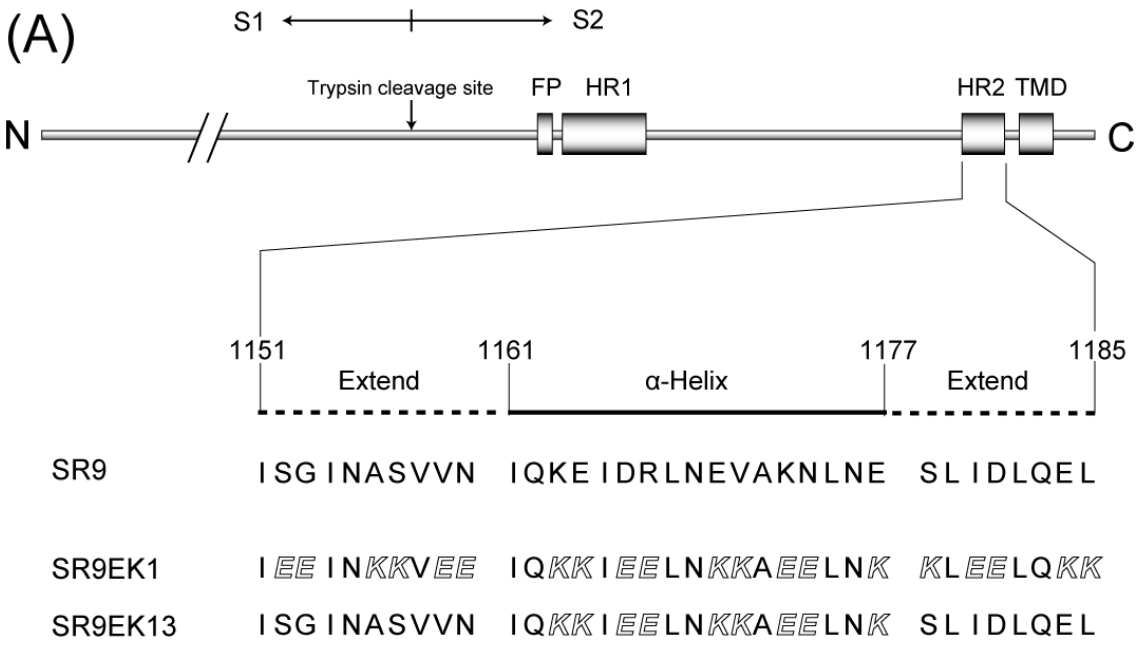
presence of various concentrations (1 nM to 100 μM) of sHRP. Inhibitory potency of the peptide was assessed using the anti-MBP antibody-ALP conjugate and stained with BCIP.

FIG. 2. Inhibitory effect of sHRPs on SCoV infections by endosomal pathway (A) and protease-mediated cell-surface pathway (B). (A) VeroE6 cells were pretreated with 50 μM sHRPs at 37 ºC for 30 min, placed on ice for 10 min, then inoculated with SCoV at moi = 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 ºC for 6 h. (B) The 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 TPCK-trypsin at room temperature for 5 min, and incubated at 37 ºC for 6 h. sHRP and Baf were present in the media in all steps at indicated concentrations. To measure the amounts of viruses that entered into 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. The amounts of viral

entry in this study were calculated from a calibration line obtained as above and was shown as relative mRNA level (20). (C) The EK1 has no sequential similarity to sHRP and showed no inhibitory effect *in vitro*. The cells were treated with 1 μM EK1 as a control peptide, and other procedures were performed as described in (B).

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 Fig. 2. After the removal of unbound virus, the cells were treated with 200 μg/ml TPCK-trypsin at room temperature for 5 min, and incubated at 37 ºC for 6 h. sHRP were present in the media in all steps at indicated concentrations. The relative viral mRNA9 was measured quantitatively by real-time PCR as described in Fig.2. In this assay, cells were not treated with Baf throughout the experiment.

Fig. 1.


(A)
S1
S2
Trypsin cleavage site
FP
HR1
HR2
TMD
N
C
1151
1161
1177
1185
Extend
α-Helix
Extend
SR9
ISG INASVVN IQKE IDRLNEVAKNLNE SL IDLQEL
SR9EK1
IEE INKKVEE IQKK IEELNKKAEELNK KLEELQKK
SR9EK13
ISG INASVVN IQKK IEELNKKAEELNK SL IDLQEL

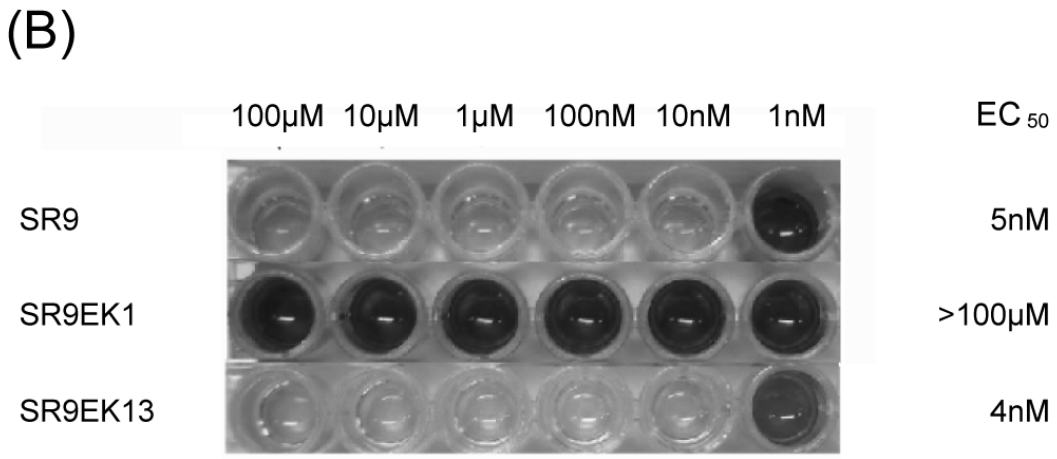

(B)
100μM
10μM
1μM
100nM
10nM
1nM
EC 50
SR9
5nM
SR9EK1
>100μM
SR9EK13
4nM

Fig. 2

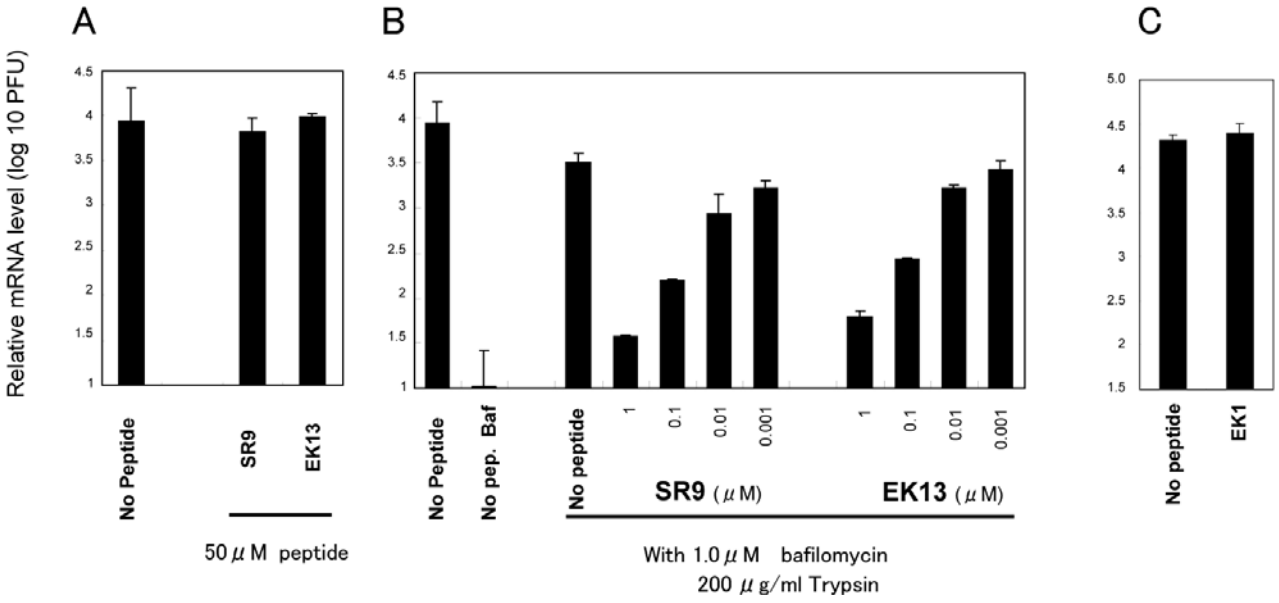
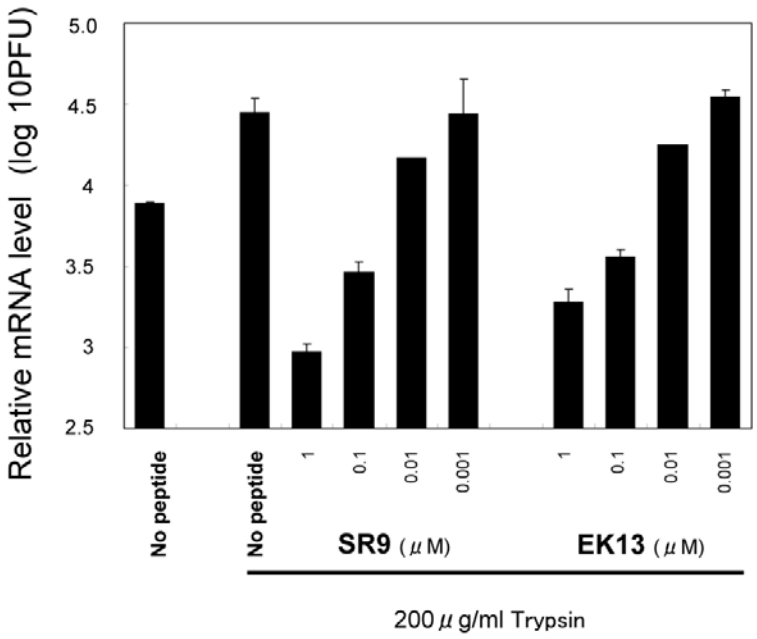

A
B
C
Relative mRNA level (log 10 PFU)
No Peptide
SR9
EK13
50 μM peptide
No Peptide
No pep. Baf
No peptide
SR9 (μM)
EK13 (μM)
With 1.0 μM bafilomycin
200 μg/ml Trypsin
No peptide
EK1

Fig. 3.


Relative mRNA level (log 10PFU)
No peptide
No peptide
SR9 (μM)
EK13 (μM)
200 μg/ml Trypsin