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Design and biological activities of novel inhibitory peptides for SARS-CoV spike protein and angiotensin-converting enzyme 2 interaction

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Abstract
Severe acute respiratory syndrome (SARS) is an emerging infectious disease caused by a novel coronavirus (SARS-CoV). The binding of SARS-CoV spike (S) protein to cellular angiotensin-converting enzyme 2 (ACE2) is the first step in SARS-CoV infection. Therefore, we assayed the inhibitory effects of small peptides derived from S protein on the binding of S protein to ACE2 and on the S-protein-pseudotyped retrovirus infectivity. SP-4 (residues 192–203), SP-8 (residues 483–494), and SP-10 (residues 668–679) significantly blocked the interaction between S protein and ACE2 by biotinylated enzyme-linked immunosorbent assay, with IC50 values of 4.30 ± 2.18, 6.99 ± 0.71, and 1.88 ± 0.52 nmol, respectively. Peptide scanning suggested the region spanning residues 660–683 might act as a receptor-binding domain. SP-10 blocked both binding of the S protein and infectivity of S protein-pseudotyped retrovirus to Vero E6 cells. In conclusion, this is the first report of small peptides designed to disrupt the binding of SARS-CoV S protein to ACE2. Our findings suggest that SP-10 may be developed as an anti-SARS-CoV agent for the treatment of SARS-CoV infection.

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Keywords: SARS-CoV; Spike protein; Angiotensin-converting enzyme 2; Peptide; Vero E6 cells; Pseudovirus

1. Introduction
Severe acute respiratory syndrome (SARS) is a new emerging human disease, resulting in progressive respiratory failure and death in close to 10% of infected individuals (Ksiazek et al., 2003; Peiris et al., 2003). A novel coronavirus was identified as the etiological agent of SARS and designated as SARS coronavirus (SARS-CoV) (Drosten et al., 2003; Fouchier et al., 2003). The full-length genome sequence of SARS-CoV was elucidated within weeks after the identification of this novel pathogen (Marra et al., 2003; Rota et al., 2003). The SARS-CoV spike (S) protein is a large type I transmembrane glycoprotein projected from viral envelope (Bosch et al., 2003). SARS-CoV S protein consists of two functional domains S1 (residues 1–667) and S2 (residues 668–1255) (Wu et al., 2004). S1 contains the receptor-binding site and is responsible for binding to cellular receptors on the target cells (Li et al., 2003). S2 contains two heptad repeat regions (HR1 and HR2), which assemble into a six-helix bundle and result in membrane fusion (Liu et al., 2004; Tripet et al., 2004). Moreover, S protein contains important virus-neutralizing epitopes that elicit neutralizing antibody in the host species (Chen et al., 2005; Hofmann et al., 2004; Sui et al., 2004). Several reports indicated that blocking the binding of the S protein to cellular receptors can prevent virus entry. For examples, antibody against S protein efficiently neutralizes SARS-CoV and inhibits syncytial formation between S protein and ACE2 (Keng et al., 2005; Sui et al., 2004). Furthermore, soluble S or ACE2 protein blocks S protein mediates infection (Hofmann et al., 2004; Moore et al., 2004; Wong et al., 2004). These finding suggested that S protein might be an attractive target for drug development.

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Recently, small peptides derived from the H1 regions of SARS-CoV S protein have been shown to inhibit SARS-CoV infection by the interference of SARS-CoV fusion with target cells (Liu et al., 2004; Ni et al., 2005; Zhu et al., 2004; Yuan et al., 2004). We designed a series of peptides corresponding to SARS-CoV S protein to evaluate the inhibitory potential of small peptides on both the binding of S protein and the infectivity of S-protein-pseudotyped retrovirus to Vero E6 cells in this study. This approach of utilizing compounds that block receptor interaction has proven useful in other viral systems, including study. This approach of utilizing compounds that block receptor interaction has proven useful in other viral systems, including study. This approach of utilizing compounds that block receptor interaction has proven useful in other viral systems, including study. This approach of utilizing compounds that block receptor interaction has proven useful in other viral systems, including this study indicated that SP-10 (residues 668–679) was a potent inhibitor and abolished binding of the S protein to ACE2 and Vero E6 cells.

2. Materials and methods

2.1. Design and synthesis of peptides

The hydrophilicity, surface probability, and chain flexibility of SARS-CoV S protein were calculated by ProteinStructure program of Genetics Computer Group according to Kyte and Doolittle plots, Emini prediction, and Karplus and Schulz prediction, respectively (Emini et al., 1985; Karplus and Schulz, 1985; Kyte and Doolittle, 1982). The peptides of 12 residues from SARS-CoV S protein were synthesized by solid-phase method (Cytosol Corp., Union City, CA, USA). The purity (>95%) and composition of peptides were assessed by high-pressure liquid chromatography and electrospray mass spectrometry. The peptides were dissolved in water to a final concentration of 50 μg/ml and stored at −20°C.

2.2. Expression and purification of recombinant SARS-CoV S protein

The SARS-CoV S gene was cloned into pET-28b(+) (Novagen, Madison, WI, USA) to create the pET-spike expression plasmid (Ho et al., 2004). Recombinant S protein was expressed in Escherichia coli (E. coli) BL21(DE3) pLysS strain by transforming the pET-spike to produce an N-terminal fusion protein containing six histidine residues. The expression and purification of recombinant SARS-CoV S protein were performed as described previously (Ho et al., 2004). Protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and quantified with a Bradford assay (Bio-Rad, Hercules, CA, USA). Recombinant S protein produced by baculovirus expression system was purchased from Protein Science (Meriden, CT, USA).

2.3. Biotinylation of recombinant S protein

Recombinant E. coli-expressed or baculovirus-expressed S protein was mixed with Sulfo-NHS-LS-biotin (Pierce, Rockford, IL, USA) in a ratio of 1:10. After a 2-h-incubation on ice, the unincorporated biotin was removed by centrifugal 10-kDa filtration (Millipore, Bedford, MA, USA), and the biotinylated S protein was stored at 4°C until further analysis. Sulfo-NHS-LS-biotin should be prepared freshly by dissolving in water.

2.4. Biotinylated enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (MaxiSorp Nunc-Immum™ plates, Nunc, Denmark) were coated at 4°C overnight with 50 μl of spike, ACE2 (R&D Systems, Minneapolis, MN, USA), or bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA), which was diluted in 0.05 M carbonate buffer (16 mM Na2CO3, 34 mM NaHCO3, pH 9.6). The wells were rinsed with 200 μl washing buffer (0.5% Tween 20 in phosphate-buffered saline (PBS) (137 mM NaCl, 1.4 mM KH2PO4, 4.3 mM Na2HPO4, 2.7 mM KCl, pH 7.2)), and blocked with 200 μl blocking buffer (5% BSA in washing buffer) by incubating at 37°C for 30 min. The absorbed protein in each well was challenged with 50 μl diluted biotinylated protein and incubated at 37°C for 1 h. After three washes with washing buffer, 50 μl diluted peroxidase-conjugated avidin was added to each well and incubated at 37°C for 1 h. Following three washes, 50 μl chromogenic substrate, 2,2'-azinobis(3-ethylbenzothiazoline-sulfonic acid) (Sigma, St. Louis, MO, USA), was added to each well and incubated at 37°C for 15 min. The absorbance was read at 405 nm in an ELISA plate reader (Anthos Labtec Instruments, Austria).

2.5. Immunofluorescence assay (IFA)

Vero E6 cells (10⁴ cells) were seeded in 24-well plates containing glass coverslips and incubated at 37°C for 1 day. The coverslips were then rinsed with PBS, fixed with 3.7% PBS-buff formaldehyde at room temperature for 30 min, and blocked with 1% BSA at 37°C for 1 h. After four washes with PBS, biotin-labeled S protein was added to each coverslip and incubated at 4°C overnight. Following four washes with PBS, diluted fluorescence-conjugated streptavidin (Chemicon, Temecula, CA, USA) was added and incubated at 37°C for 90 min in the dark. The coverslips were then washed four times with PBS, placed onto glass slides, mounted with fluorescent mounting G (Electron Microscopy Sciences, Hatfield, PA, USA), and observed under a confocal microscope (Leica, Germany).

2.6. Infection with S-protein-pseudotyped retrovirus

Recombinant retroviruses expressing a luciferase reporter gene and pseudotyped with S proteins were produced as described previously (Li et al., 2005; Sui et al., 2005). Briefly, 293T cells were cotransfected with a plasmid pcDNA-spike
Forty-eight hours later, viral supernatants were harvested and reporter gene under control of the HIV-1 long terminal repeat. Gag-Pol, and a plasmid pHIV-Luc encoding the firefly luciferase were added to ACE2-expression Vero E6 cells in a 96-well plate. Forty-eight hours postinfection, luciferase activity was measured and normalized to protein content. Relative infectivity was presented as comparison with the control protein BSA (data not shown). The results demonstrated the specificity of biotinylated ELISA. S protein bound to ACE2 in a dose-dependent manner. The interaction between S protein and ACE2 displayed a sigmoidal curve between 0.001 and 10 μg/ml S protein, indicating that S protein bound to ACE2 cooperatively. Moreover, the binding ability of S protein to ACE2 was saturated when the concentration of S protein exceeded 10 μg/ml, suggesting that one molecule of recombinant S protein probably bound to 1.15 molecules of ACE2. These results revealed the specificity of biotinylated ELISA in analyzing the receptor-binding ability of S protein. It also suggested that recombinant S protein could serve as a probe to analyze the interaction between SARS-CoV and cellular receptors.

3. Results

3.1. Recombinant S protein binds to ACE2 in a dose-dependent manner

In order to investigate the binding ability of S protein to ACE2, we expressed and purified the S protein from E. coli. The 138-kDa recombinant S protein was expressed in soluble form, and the amount of recombinant S protein recovered was approximately 0.2–0.3 mg/100 ml of bacterial culture after purification. Biotinylated ELISA and Western blot show that recombinant S protein can bind to Vero E6 cell lysate (Ho et al., 2004). Since ACE2 is the functional receptor for SARS-CoV (Li et al., 2003), we designed an ELISA assay with ACE2 instead of Vero E6 cell lysate to determine the binding ability of S protein to ACE2. The binding interaction between SARS-CoV and cellular receptors is mostly determined by hydrophobic interaction, surface charge distribution, and geometry (Dimitrov, 2004). To design small peptides, we selected 14 synthetic peptides derived from S protein containing 12 residues each. The hydrophilic index of 14 small peptides ranged from 1.3344 to 0.3012, with an average of 0.042. We employed the Chou-Fasman method which suggested that SP-4, SP-8, and SP-10 form an α-helical structure while SP-4, SP-8, and SP-10 form a β-sheet (Chou and Fasman, 1978).

To evaluate the inhibitory effects of the 14 small peptides on the interaction between S protein and ACE2, we mixed 10 nmol of each peptide with 1 nmol of biotin-labeled S protein, incubated the mixture at 37 °C for 2h, and added it to ACE2-coated wells. As shown in Fig. 2, pre-incubation of biotinylated S protein with BSA slightly inhibited the binding of S protein to ACE2, with the inhibitory percentage of 7%. Pre-incubation of biotinylated S protein with the peptide (endG, EGWRRREDRADAR) derived from human endonuclease G inhibited the binding of S protein to ACE2, with the inhibitory percentage of 30%. However, SP-4, SP-8, SP-10, SP-11, and SP-12 significantly blocked the binding of S protein to ACE2, exhibiting 50–90% inhibition at 10 nmol. Therefore, these results suggested that SP-4, SP-8, SP-10, SP-11, and SP-12 were potential inhibitors of the interaction between S protein and ACE2.

3.3. A novel putative receptor-binding region of S protein is identified by a peptide-scanning method

SP-4, SP-8, and SP-10 blocked the ACE2 and S protein interaction in a dose-dependent manner (Fig. 3). The IC50 values of SP-4, SP-8, and SP-10 were 4.3 ± 2.18, 6.99 ± 0.71, and
Table 1
Sequences and properties of synthetic peptides used in this study

| Peptide | Amino acid sequence | Molecular weight (Da) | Average of hydrophilic index per residue | Predicted secondary structure |
|---------|---------------------|-----------------------|-----------------------------------------|-----------------------------|
| SP-1 (23–34) | DDVQAPNYTQHT | 1388.53 | 1.2319 | – |
| SP-2 (34–45) | TSSMRGYVVYDE | 1404.62 | 0.7761 | – |
| SP-3 (84–95) | KDQGFYATAEKS | 1329.67 | 0.0608 | – |
| SP-4 (192–203) | GFLYVYKGYQPI | 1447.86 | 0.2798 | α-Helix |
| SP-5 (292–303) | SFEIDGKGYQTS | 1387.69 | 0.6203 | – |
| SP-6 (426–437) | RNIDATSTGNYN | 1325.43 | 1.2273 | – |
| SP-7 (444–455) | RHGKLRPFERDI | 1524 | 1.3344 | – |
| SP-8 (483–494) | FTTTYGQYPQ | 1410.68 | 0.4155 | β-Sheet |
| SP-9 (581–592) | VSVITPGTNASS | −0.3012 | – |
| SP-10 (668–679) | STSQKSIV AYTM | 1315.6 | 0.0427 | β-Sheet |
| SP-11 (740–751) | SFCTQLNRALSG | −0.1002 | – |
| SP-12 (1027–1038) | CGKGYHLMSFPQ | 1367.86 | 0.1773 | – |
| SP-13 (1066–1078) | HEGKAYFPREGV | 1389.75 | 0.3499 | – |
| SP-14 (1125–1136) | PELDSFKEELDK | 1449.81 | 1.2488 | α-Helix |

Numbers in the brackets are the beginning and end residues for each peptide corresponding to S protein.

The hydrophilic index is calculated according to Kyte and Doolittle plots.

The secondary structure of peptide is calculated according to Chou–Fasman prediction.

1.88 ± 0.52 nmol, respectively. SP-11 and SP-12 were excluded because they bound not only S protein but also BSA (data not shown). To further identify the biologically active peptides, the peptide-scanning method that involved synthesizing overlapping peptides of 12 residues covering additional amino acids on both the amino and carboxy ends of SP-10 was used. The inhibitory potential of small overlapping peptides on the SARS-CoV S protein and ACE2 interaction was analyzed by competitive biotinylated ELISA. SP-10-2 (residues 660–671), SP-10-3 (residues 664–675), SP-10-4 (residues 672–683) had inhibitory activities, with the IC50 values of 6.21 ± 2.13, 5.47 ± 0.41, and 2.07 ± 1.01 nmol, respectively (Table 2). However, SP-10-1 (residues 648–659) and SP-10-5 (residues 676–687) did not block the ACE2 and S protein interaction. These findings suggested that the region spanning residues 660–683 of SARS-CoV S protein may interact with ACE2.

3.4. SP-10 blocks both the binding of S protein and the infectivity of S-protein-pseudotyped retrovirus to Vero E6 cells

The inhibitory potential of SP-10 on the SARS-CoV S protein and Vero E6 cell interaction was further analyzed by IFA and S protein-pseudotyped retrovirus infectivity. Vero E6 cells were treated with BSA, biotin-labeled S protein or SP-10/biotin-labeled S protein mixture, and stained with fluorescence-conjugated streptavidin. BSA-treated cells showed negative result, whereas the biotinylated S protein-treated Vero E6 cells showed the strong fluorescence (Fig. 4A). Treatment of Vero E6 cells with SP-10/biotin-labeled S protein mixture diminished the cell-associated fluorescence in a dose-dependent manner. Because the S protein expressed in E. coli is not glycosylated, we further analyzed the inhibitory potential of SP-10 using glycosylated S protein purified from baculovirus. Similar results were obtained by using the recombinant S protein produced by baculovirus expression system (Fig. 4B). These results indicated that SP-10 was capable of blocking the binding of S protein to Vero cells.

Table 2
The IC50 values of overlapping peptides covering additional amino acids on both the amino and carboxy ends of SP-10

| Peptide | Amino acid sequence | IC50 (nmol) |
|---------|---------------------|-------------|
| SP-10-1 (648–659) | CDPICAGICAS | >20 |
| SP-10-2 (660–671) | YHTVSILRSTSQ | 6.21 ± 2.13 |
| SP-10-3 (664–675) | SLLRSTSQKSV | 5.47 ± 0.41 |
| SP-10 (668–679) | STSQKSVATMY | 1.88 ± 0.52 |
| SP-10-4 (672–683) | KSVATMSLGA | 2.07 ± 1.01 |
| SP-10-5 (676–687) | AYTMSSLGA | >20 |

Numbers in the brackets are the beginning and end residues for each peptide corresponding to S protein.

The IC50 value of each peptide was determined as the quantity of peptide required to inhibit the interaction between S protein and ACE2 at 50%. Values are mean ± standard error of four independent assays.
Fig. 3. Inhibitory effects of SP-4, SP-8, and SP-10 on the SARS-CoV S protein and ACE2 interaction by competitive biotinylated ELISA. Biotin-labeled S protein (1 nmol) was mixed with various amounts of SP-4 (A), SP-8 (B) or SP-10 (C), and incubated at 37°C with shaking. After a 2-h incubation, the mixtures were added to wells, which were coated with 1 ng of ACE2, and incubated at 37°C for 1 h. Following three washes, peroxidase-conjugated avidin and chromic substrate were sequentially added. The absorbance was read at 405 nm in an ELISA plate reader. The results are expressed as inhibition described in Section 2. Values are mean ± standard error of six independent assays.

In addition to IFA, Vero E6 cells transfected with the plasmid encoding human ACE2 were infected with vector-pseudotyped retrovirus, S-protein-pseudotyped retrovirus, or SP-10/S-protein-pseudotyped retrovirus mixture. The luciferase activity was assayed 48 h postinfection. SP-10 inhibited the S-protein-pseudotyped retrovirus infectivity in a dose-dependent manner (Fig. 5). Following three washes, peroxidase-conjugated avidin and chromic substrate were sequentially added. The absorbance was read at 405 nm in an ELISA plate reader. The results are expressed as inhibition described in Section 2. Values are mean ± standard error of six independent assays.

Fig. 3. Inhibitory effects of SP-4, SP-8, and SP-10 on the SARS-CoV S protein and ACE2 interaction by competitive biotinylated ELISA. Biotin-labeled S protein (1 nmol) was mixed with various amounts of SP-4 (A), SP-8 (B) or SP-10 (C), and incubated at 37°C with shaking. After a 2-h incubation, the mixtures were added to wells, which were coated with 1 ng of ACE2, and incubated at 37°C for 1 h. Following three washes, peroxidase-conjugated avidin and chromic substrate were sequentially added. The absorbance was read at 405 nm in an ELISA plate reader. The results are expressed as inhibition described in Section 2. Values are mean ± standard error of six independent assays.

In conclusion, this is the first report of small peptides designed to disrupt the interaction of SARS-CoV S protein and ACE2. By analyzing a series of peptides derived from S protein, we
Fig. 4. Inhibitory effect of SP-10 on the SARS-CoV S protein and Vero E6 cell interaction by IFA. Vero E6 cells were cultured on glass coverslips and incubated with 20 μg/ml BSA, 20 μg/ml biotin-labeled S protein, 0.5 nmol SP-10/20 μg/ml biotin-labeled S protein mixture, 2.5 nmol SP-10/20 μg/ml biotin-labeled S protein mixture, or 5 nmol SP-10/20 μg/ml biotin-labeled S protein mixture. Recombinant S protein was purified from E. coli (A) or baculovirus (B). After a 16-h incubation at 4°C, cells were stained with fluorescence-conjugated streptavidin and evaluated under a confocal microscope. Magnification, 400×.

Fig. 5. Inhibitory effect of SP-10 on the SARS-CoV S-protein-pseudotyped retrovirus infectivity. S-protein-pseudotyped retroviruses were mixed with various amounts of SP-10 and incubated at 37°C with shaking. After a 2-h incubation, the mixtures were inoculated with Vero E6 cells transfected with the plasmid encoding human ACE2. The luciferase activity of cell lysate was assayed 2 days postinfection. Relative infectivity is presented as comparison with the RLU relative to untreated cells. Values are mean ± standard error of four independent assays.

identified that peptides spanning residues 192–203, 483–494, and 660–683 of S protein efficiently blocked the binding of S protein to ACE2. A novel putative receptor-binding region of S protein was defined by a peptide-scanning method. Furthermore, SP-10 has been identified as a potent inhibitor for the inhibition of S protein binding to Vero E6 cells. Therefore, SP-10 can be used as a candidate in designing more potent anti-SARS-CoV peptides for drug development in the future.

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