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A hexapeptide of the receptor-binding domain of SARS corona virus spike protein blocks viral entry into host cells via the human receptor ACE2

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ABSTRACT

In vitro infection of Vero E6 cells by SARS coronavirus (SARS-CoV) is blocked by hexapeptide Tyr-Lys-Tyr-Arg-Tyr-Leu. The peptide also inhibits proliferation of coronavirus NL63. On human cells both viruses utilize angiotensin-converting enzyme 2 (ACE2) as entry receptor. Blocking the viral entry is specific as alpha virus Sindbis shows no reduction in infectivity. Peptide ^{438}YKYRYL^{443} is part of the receptor-binding domain (RBD) of the spike protein of SARS-CoV. Peptide libraries were screened by surface plasmon resonance (SPR) to identify RBD binding epitopes. ^{438}YKYRYL^{443} carries the dominant binding epitope and binds to ACE2 with K_D = 46 μM. The binding mode was further characterized by saturation transfer difference (STD) NMR spectroscopy and molecular dynamic simulations. Based on this information the peptide can be used as lead structure to design potential entry inhibitors against SARS-CoV and related viruses.

1. Introduction

The SARS-associated corona virus (SARS-CoV) has been identified as the causative agent of severe acute respiratory syndrome (SARS) which emerged as an alerting epidemic in winter of 2002–2003 resulting in over 8000 infected cases with approximately 10% deaths (Drosten et al., 2003; Ksiazek et al., 2003; Marra et al., 2003; Peiris et al., 2003; Rota et al., 2003; WHO, 2004). SARS-CoV infects human host cells by an initial interaction of its spike glycoprotein (S) and the receptor on human cells, angiotensin-converting enzyme 2 (ACE2) (Dimitrov, 2003; Holmes, 2003; Li et al., 2003). Functional characterization of the S protein suggests that the receptor-binding domain (RBD) is located between amino acid residues 303 and 537 (Xiao et al., 2003). Flow cytometry indicated that amino acids 270–510 are the minimal receptor binding region of the S glycoprotein (Babcock et al., 2004). Further studies located the RBD from residues 318 to 510. The RBD fused to the Fc region of human IgG_1 (RBD-Fc) binds ACE2 with higher affinity ($K_D \sim 10$ nM) than does the full length S1-Ig chimera (Li et al., 2005b; Wong et al., 2004). The crystal structure of residues 306–527 of the S1 in complex with the receptor ACE2 reveals that a loop within the RBD (residues 424–494) makes all the contacts to ACE2 and is referred to as the receptor-binding motif (RBM). Six tyrosine residues are involved in direct binding to the receptor (Li et al., 2005a). Studying the virus adaptation to humans the spike protein also seems to play a major role in the species specificity of coronavirus infection. Especially, the introduction of a threonine residue at position 487 and an asparagine instead of a charged lysine residue at position 479 of the spike protein seem to be responsible for its high affinity to human ACE2 (Holmes, 2005; Li et al., 2005b; Qu et al., 2005). Yi et al. demonstrated that a single amino acid substitution (R441A) in a full-length spike protein DNA vaccine failed to induce neutralizing antibodies (NAbs) and that the same mutation yielded pseudoviruses that were unable to enter the human cells (Yi et al., 2005). Furthermore, the RBD-Fc bearing the same R441A mutation shows no affinity to ACE2 and is not capable of blocking S protein-mediated pseudovirus entry (He et al., 2006). The interaction of SARS-CoV with its receptor ACE2 is an attractive drug target as epitopes of the RBD on the spike protein may serve as leads for the design of effective entry inhibitors (Du et al., 2009). Another drug target is the fusion process of the spike protein with the host cell membrane that is characterized by the presence of two heptad repeat (HR) regions, HR1 and HR2, which are postulated to form a fusion-active conformation similar to those of other typical viral fusion proteins (Sainz et al., 2006; van der Hoek et al., 2004; Yuan et al., 2004).

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2. Material and methods

2.1. Peptide synthesis

Peptides were synthesized on solid phase using a Fmoc-protecting group strategy on a Fmoc-PAL-PEG-PS resin (Applied Biosystems) with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU, Iris Biotech) as activator. A MOS Ω496 synthesizer (Advanced ChemTech) and a Liberty microwave synthesizer (CEM) were used for peptide syntheses starting with 20 μmol amino groups each. After each coupling step the growing peptide was capped with an acetyl residue by 10% acetic anhydride in DMF. Cysteine residues were substituted by serines to avoid dimerization. Using trifluoroacetic acid (TFA), triisopropylsilan and H_2O (95:5:2, v/v), peptides were cleaved off the resin leaving an amide at the C-terminus. The cocktail was applied twice for 90 and 60 min, respectively. Preparative RP-HPLC was carried out on a BIOCAD 700E instrument (PerSeptive Biosystems) using a H_2O/acetonitrile gradient (0.1% TFA) on a VP250/21 Nucleodur C18 Pyramid 5 μ column (Macherey & Nagel). Peptides were characterized by MALDI-TOF mass spectrometry on a BIFLEX III instrument (Bruker Daltonics) in reflector mode using 2,5-dihydroxybenzoic acid (DHB) or α-cyano-4-hydroxycinnamic acid (CCA) as a matrix. Peptide RBD-11b (YKYRYL, Y438-L443) and related peptides were further characterized by 1D- and 2D-NMR spectroscopy (not shown).

2.2. SPR screen of peptide libraries

SPR studies were carried out using a BIACORE 3000 or Biacore T100 instrument. For all experiments a temperature of 25 °C, flow rate of 5 μL/min (BIACORE 3000) or 30 μL/min (T100), and a TBS running buffer (25 mM Tris, 0.2 M NaCl, 5 μM $ZnCl_2$ at pH 8) were used. The carboxymethylated sensorchip surface of a CM5 chip (Biacore) was activated by NHS/EDC followed by immobilization of rhACE2 (R&D Systems) in acetate-buffer (pH 3.5, Biacore). rhACE2 was obtained in TBS that had to be changed for the immobilization of the enzyme to PBS containing additional 5 μM $ZnCl_2$ (pH 7.4) using a Slide-A-Lyzer MINI Unit (Pierce Biotechnology) with a Molecular Weight Cut-off of 3500 at 4 °C for at least 12 h. Sensorgrams of RBD-11 and RBD-14 were recorded with a chip that had 54 fmol of ACE2 immobilized, that of RBD-15 on a chip with 74 fmol, those of RBD-11b and of the RBD-11b related peptide library on a chip with 100 fmol, respectively. Carboxyl groups of the activated chip surface that had not reacted with the protein were capped with ethanolamine (Biacore).

2.3. Saturation transfer difference (STD) NMR spectroscopy

2.3.1. Sample preparation

NMR samples were prepared in deuterated Tris-buffered saline (d-TBS) containing 25 mM perdeuterotris(hydroxymethyl)aminomethane (Tris-d_{11}), 0.2 M NaCl and 5 μM $ZnCl_2$ (pH 7.8) in deuterium oxide (D_2O, 99.9%). TBS of the commercial rhACE2 (R&D Systems) was changed to d-TBS in Slide-A-Lyzer MINI Units (Pierce Biotechnology) with a Molecular Weight Cut-off of 3500 twice for at least 12 h at 4 °C. YKYRYL was added from 3 mM stock solution in d-TBS with sample volume adding up to 100 μL in a 3 mm Shigemi NMR Micro Tube with c(ACE2) = 0.83 μM and peptide concentration between 14.9 and 222 μM (18–288 fold excess over ACE2).

2.3.2. Acquisition of NMR Spectra

All STD spectra were recorded at a temperature of 295 K with a spectral width of 15 ppm on a Bruker Avance DRX 700 MHz spectrometer equipped with a 5 mm inverse triple resonance cryoprobe. Selective saturation of the protein was achieved by a train of Gauss-shaped pulses of 50 ms length each, truncated at 1%, and separated by a 1 ms delay leading to a total length of saturation time of 4 s. The on-resonance irradiation of the protein was performed at a chemical shift of −0.5 ppm. Off-resonance irradiation was set at 40 ppm. Total scan number in the STD experiments was 4096. NMR spectra were multiplied by an exponential line-broadening function of 1.0 Hz prior to Fourier transformation. Water suppression was achieved by an excitation sculpting pulse sequence. Spectra processing was performed using Topspin 2.1 software (Bruker).

2.4. SARS-CoV infection assay

The SARS-CoV inhibition assay was performed as described previously (Vassilatis et al., 2003). In brief, Vero cells in 24-well plates were infected in the biosafety level 4 laboratory (BNI Hamburg) with SARS-CoV (Frankfurt isolate) at a multiplicity of infection (MOI) of 0.01. The inoculum was removed after 1 h and replaced with fresh medium complemented with different concentrations of compound. The virus RNA concentration in the supernatant was measured by real-time PCR after 2 days. RNA was prepared from 140 μL supernatant using diatomaceous silica (Pfaff et al., 1994). Quantitative real-time reverse transcription-PCR (RT-PCR) was performed with the purified RNA according to a published protocol (Drosten et al., 2003). *In vitro* transcripts of the target region were used in the PCR to generate standard curves for quantification of the virus RNA.

2.5. Molecular dynamic (MD) simulation

The MD simulations were carried out with the software Maestro/Desmond on an HP Z600 workstation (one Quadcore CPU), using the OPLS_AA/2005 force field. The starting structure was placed in a water box with orthorhombic boundary conditions and salt concentration of 200 mmol/L (SPC solvent model, 18,145 water molecules, $78 \times 88 \times 102$ Å). MD simulations over 11,000 ps were performed to equilibrate the system at 300 K. The simulation in equilibrium was performed over 3000 ps at 300 K, with the Nose-Hoover thermostat method and a relaxation time of 2.0 ps. The recording interval was 0.12 ps. Before starting MD simulations the system was minimized three times over 2000 steps, respectively. The period of the MD after equilibration with constant potential energy was used for the analysis.

Table 1
Synthetic peptide library of sixteen 12mer peptides comprising RBD-residues N318-T509 of SARS-CoV spike protein. The peptides RBD-11, RBD-14 and RBD-15 show binding to ACE2.

Peptide	Residues of the spike protein (S)	Amino acid sequence
RBD-1	N318-F329	NITNLSPFGEVF
RBD-2	N330-E341	NATKFPSVYAWE
RBD-3	R342-S353	RKKISNSVADYS
RBD-4	V354-K365	VLYNSTFFSTFK
RBD-5	S366-L377	SYGVSATKLNDL
RBD-6	S378-V389	SFSNVYADSFVV
RBD-7	K390-Q401	KGDDVRQIAPGQ
RBD-8	T402-P413	TGVIADYNYKLP
RBD-9	D414-T425	DDFMGSVLAWNT
RBD-10	R426-N437	RNIDATSTGNYN
RBD-11	Y438-R449	YKYRYLRHGKLR
RBD-12	P450-S461	PFERDISNVPFS
RBD-13	P462-N473	PDGKPSTPPALN
RBD-14	S474-T485	SYWPLNDYGFYT
RBD-15	T486-V497	TTGIGYQPYRVV
RBD-16	V498-T509	VLSFELLNAPAT