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# Endocytosis of the receptor-binding domain of SARS-CoV spike protein together with virus receptor ACE2

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ABSTRACT

Cell entry of severe acute respiratory syndrome coronavirus (SARS-CoV) is mediated by the viral spike (S) protein. Amino acids 319–510 on the S protein have been mapped as the receptor-binding domain (RBD), which mediates binding to the SARS-CoV receptor angiotensin converting enzyme 2 (ACE2) on SARS-CoV susceptible cells. In this study, we expressed a fusion protein containing the human codon-optimized RBD of the SARS-CoV spike protein linked to the Fc portion of human IgG1 (named RBD-Fc) in HEK293 cells. The RBD-Fc protein was purified by affinity chromatography. The flow cytometry assay showed that the purified RBD-Fc protein could bind to ACE2. We demonstrated that the RBD spike protein alone could be internalized into SARS-CoV susceptible cells together with ACE2. We also showed that the removal of N-glycans from the RBD spike protein did not abolish this phenomenon. Our discoveries may have some implications for the development of the SARS vaccine.

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## 1. Introduction

In 2003, severe acute respiratory syndrome (SARS) emerged as a deadly global threat (Lee et al., 2003; Poutanen et al., 2003; Tsang et al., 2003). The pathogen was identified as severe acute respiratory syndrome coronavirus (SARS-CoV) (Drosten et al., 2003; Ksiazek et al., 2003; Marra et al., 2003; Rota et al., 2003), which is an enveloped, single-strand plus-sense RNA virus. Spike (S), nucleocapsid (N), membrane (M) and envelope (E) are its major structural proteins (Drosten et al., 2003; Marra et al., 2003; Rota et al., 2003).

Like other coronaviruses, SARS-CoV entry is mediated by the S protein (Hofmann et al., 2004; Inoue et al., in press; Simmons et al., 2004; Yang et al., 2004). The S protein consists of 1255 amino acids that forms typical petal-shaped spikes on the surface of SARS-CoV (Ksiazek et al., 2003). There is no direct evidence that the S protein of SARS-CoV is processed proteolytically into the S1 receptor-binding subunit and the S2 membrane fusion subunit, but the two subunits can be predicted by sequence alignment with other coronavirus S proteins (Rota et al., 2003; Spiga et al., 2003). Angiotensin converting enzyme 2 (ACE2) has been demonstrated to be a functional receptor for SARS-CoV in vitro and in vivo (Kuba et al., 2005; Li et al., 2003) by binding to the receptor-binding domain (RBD, amino acids 319–510) of the S protein (Chakraborti et al., 2005; Wong et al., 2004). Additionally, there are 23 potential N-linked glycosylation sites in the SARS-CoV S protein (Rota et al., 2003), and two are in the RBD.

Usually, ligand binding induces endocytosis of the receptors. Our previous study demonstrated that the binding of the S protein to endogenous ACE2 in mice resulted in down-regulation of ACE2 surface expression (Kuba et al., 2005), implying ACE2 internalization. Therefore, we would like to explore whether RBD, the minimal receptor-binding domain on the S protein, could induce endocytosis of the receptor.

To test this hypothesis, we used the recombinant RBD spike protein as a defined model system, which avoided possible effects of other fragments on the S protein. We constructed a new vector using a human codon-optimized RBD DNA sequence, and created a stable RBD-Fc-expressing cell line. The RBD spike protein could then be secreted into culture medium and easily purified by Protein A affinity chromatography. The flow cytometry assay and immunostaining experiments demonstrated the endocytosis of the RBD spike protein by susceptible cells together with ACE2. At the same time, the removal of N-glycans from the RBD spike protein could still induce ACE2 internalization. To our knowledge, this is the first report showing that the receptor-binding domain of SARS-CoV alone can trigger the endocytosis of susceptible cells.

*Abbreviations:* SARS-CoV, severe acute respiratory syndrome coronavirus; RBD, receptor-binding domain; ACE2, angiotensin converting enzyme 2.

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## 2. Materials and methods

### 2.1. Construction of the recombinant plasmid

The amino acids 319–510 of the SARS-CoV spike protein are mapped as the minimal ACE2-binding domain (RBD) (Chakraborti et al., 2005; Wong et al., 2004). The cDNA fragment encoding the RBD was amplified by PCR using a plasmid, PUC18-S as the template, which contains the human codon-optimized SARS-CoV (Urbani strain) spike protein (GenBank accession no. AAP13441) coding sequence synthesized by Generay Inc., and the primers (forward: 5′-GGCGCTAGCCATCACCAACCTGTGCCCC-3′, containing NheI recognition site; reverse: 5′-CGCGGATCCGTCACGGTGGCGGGGGCGTTC-3′, containing BamHI recognition site). The PCR product was digested with NheI and BamHI, and then cloned in-frame downstream of the leader peptide of human CD5 antigen (CD5L), and upstream of the Fc portion of human IgG1 (Fc) in the Peak13 expression vector (provided by B. Seed, Harvard Medical School, Boston, MA), which was also digested by NheI and BamHI. The resulting recombinant plasmid was named Peak13-RBD-Fc.

### 2.2. Cell cultures

VeroE6 cells (African green monkey kidney cell line), HEK293 cells (human embryo kidney cell line) and a HEK293 cell line stably expressing RBD-Fc (RBD-Fc-293) or human ACE2-GFP (ACE2-GFP-293) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C with 5% $CO_2$.

### 2.3. Establishment of a stable RBD-Fc expressing cell line

The day before transfection, HEK293 cells were trypsinized and plated into 6-well plate at a density of $2 \times 10^5$ cells per well. The next day, 0.5 μg Peak13-RBD-Fc plasmid linearized by AvrII or an equal volume of $H_2O$ was transfected into the HEK293 cells using lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. After 48 h, aliquots of the transfected cells were transferred into selection medium containing increasing concentrations of puromycin (0.3, 0.4, 0.5, 0.6 and 0.7 μg/ml). After 3 days, the transfected HEK293 cells were selected in suitable puromycin concentration, under which some cells transfected with the Peak13-RBD-Fc plasmid survived, but those transfected with $H_2O$ all died, and were transferred into 96-well plate using a limited dilution method. After about 10 days, 10 μl of culture supernatant from a single confluent cell colony in the 96-well plate was assayed for RBD-Fc expression by Western blot using anti-human IgG (whole molecule) peroxidase conjugated antibody (Sigma). The highest RBD-Fc expression clone was selected by an ELISA assay for recombinant protein production.

### 2.4. ELISA

$2 \times 10^5$ cells from each RBD-Fc-293 cell clone were seeded in 6-well plates and cultured with 2 ml culture medium (DMEM containing 10% FBS) for 72 h. The concentration of the RBD-Fc protein in culture supernatant was quantified with an ELISA assay, which was performed using BD OptEIA™ Reagent Set A (BD Biosciences) as described by the manufacturer. Briefly, 96-well flat bottom EIA/RIA plates (Corning, NY) were coated overnight at 4 °C with 100 μl culture medium, the human IgG standards serial diluted in culture medium (10 ng to 30 μg/ml) or the cell culture supernatant of each RBD-Fc-293 cell clone. Then, wells were washed once with Washing Solution, blocked with Assay Diluent for 1 h at room temperature and washed with Washing Solution three times. Next, anti-human IgG (whole molecule) peroxidase conjugated antibody (Sigma) diluted in Assay Diluent (1: 5000) was added to each well, and gently agitated at room temperature for 1 h. After being washed eight times, 100 μl of Substrate Reagent was added to each well and wells were incubated at room temperature in the dark for 30 min. Finally, the reaction was stopped using Stop Solution and color development was monitored at a wavelength of 450 nm.

### 2.5. Protein purification

The cell culture supernatant of RBD-Fc-293 cells was harvested and dialyzed with a solution (20 mM sodium phosphate and 1 mM EDTA, pH 7.0) over 8 h. After centrifugation at 4000 rpm for 30 min, the supernatant was filtered through 0.45 μm Durapore membrane filters (Millipore, Ireland). The purification of RBD-Fc protein was performed using HiTrap Protein A HP 5 ml column (GE healthcare Amersham Biosciences AB) and Econo™ Gradient Pump Tubing Kit (Bio-Rad, USA) according to the manufacturer's protocol. In brief, the column was first washed with 10 column volumes of the binding buffer (20 mM sodium phosphate, pH 7.0) at a flow rate of 5 ml/min. Then the cell culture supernatant was pumped onto the column. Next, the column was washed with 20 column volumes of the binding buffer followed by the supernatant. Finally, the RBD-Fc protein was eluted with 2–5 column volumes of the elution buffer (0.1 M glycine, pH 3.1). Neutralization buffer (60–200 μl, 1 M Tris–HCl, pH 9.0) was added to each collection tube. The RBD-Fc protein was further purified using a HiTrap Protein A HP 1 ml column (GE healthcare Amersham Biosciences AB). The protein concentration was determined using Protein Assay Dye Reagent Concentrate (Bio-Rad) according to the manufacturer's protocol, in which bovine serum albumin (BSA) was used as a standard.

### 2.6. SDS-PAGE and Western blot analysis

Samples were mixed 1:1 with 2× loading buffer (2% SDS, 5% sucrose, 0.1% bromphenol blue and 5% mercaptoethanol) and heated at 95 °C for 5 min. SDS-PAGE was performed using 5% stocking and 10% or 12% separating acrylamide gels (Laemmli, 1970). A broad range of pre-stained protein marker kit (6–175 kDa, New England Biolabs) was used to determine approximate molecular mass. Proteins in gels were visualized using 0.25% Coomassie Brilliant Blue R-250 staining in 45% methanol and 10% acetic acid. The images of gels were captured using Furi FR-200 equipment with Smartview software (China). For Western blot analysis, proteins in gels were electrophoretically transferred onto a nitrocellulose membrane with a buffer of 20 mM Trisbase, 153 mM glycine and 20% (v/v) methanol at 300 mA for 2 h. After being blocked in 2% chicken egg white albumin and 0.1% TritonX-100 at room temperature for 1 h, the membrane was incubated with anti-human IgG (whole molecule) peroxidase conjugated antibody (1:10,000 dilution, Sigma) for 1 h and washed three times with TBST buffer (50 mM Trisbase, 150 mM NaCl and 0.1% TritonX-100, pH 7.4). Finally, the membrane was developed with a Western blotting luminal reagent (Santa Cruz Biotechnology) to visualize positive signals.

### 2.7. Cell binding and internalization assay by flow cytometry

We detached $1 \times 10^6$ VeroE6 cells using a 2 mM mixture of EDTA and PBS, and then incubated them with 350 nM purified RBD-Fc or Fc protein diluted in DMEM for 3 h at 4 °C or 37 °C, respectively. Next, the cells were washed with ice-cold PBS and incubated with a FITC-conjugated affinipure goat anti-human IgG (H + L) antibody (1: 100, Jackson ImmunoResearch) at 4 °C in the dark for 30 min.

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