Retroviruses Pseudotyped with the Severe Acute Respiratory Syndrome Coronavirus Spike Protein Efficiently Infect Cells Expressing Angiotensin-Converting Enzyme 2

Michael J. Moore,1 Tatyana Dorfman,1 Wenhui Li,1 Swee Kee Wong,1 Yanhan Li,2 Jens H. Kuhn,1,3 James Coderre,4 Natalya Vasilieva,5 Zhongchao Han,2 Thomas C. Greenough,4 Michael Farzan,1* and Hyeryun Choe 5*

Partners AIDS Research Center, Brigham and Women’s Hospital, and Department of Medicine (Microbiology and Molecular Genetics),1 and Perlmutter Laboratory, Children’s Hospital, and Department of Pediatrics,2 Harvard Medical School, Boston, and Program in Molecular Medicine, University of Massachusetts Medical School, Worcester,4 Massachusetts; State Key Laboratory of Experimental Hematology, Institute of Hematology and Hospital of Blood Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin, China5; and Department of Biology, Chemistry, Pharmacy, Freie Universität Berlin, Berlin, Germany3

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Infection of receptor-bearing cells by coronaviruses is mediated by their spike (S) proteins. The coronavirus (SARS-CoV) that causes severe acute respiratory syndrome (SARS) infects cells expressing the receptor angiotensin-converting enzyme 2 (ACE2). Here we show that codon optimization of the SARS-CoV S-protein gene substantially enhanced S-protein expression. We also found that two retroviruses, simian immunodeficiency virus (SIV) and murine leukemia virus, both expressing green fluorescent protein and pseudotyped with SARS-CoV S protein or S-protein variants, efficiently infected HEK293T cells stably expressing ACE2. Infection mediated by an S-protein variant whose cytoplasmic domain had been truncated and altered to include a fragment of the cytoplasmic tail of the human immunodeficiency virus type 1 envelope glycoprotein was, in both cases, substantially more efficient than that mediated by wild-type S protein. Using S-protein-pseudotyped SIV, we found that the enzymatic activity of ACE2 made no contribution to S-protein-mediated infection. Finally, we show that a soluble and catalytically inactive form of ACE2 potently blocked infection by S-protein-pseudotyped retrovirus and by SARS-CoV. These results permit studies of SARS-CoV entry inhibitors without the use of live virus and suggest a candidate therapy for SARS.

A distinct coronavirus (SARS-CoV) has been identified as the etiologic agent of severe acute respiratory syndrome (SARS), an acute pulmonary syndrome characterized by an atypical pneumonia that results in progressive respiratory failure and death in close to 10% of infected individuals (8, 11, 14, 15). SARS-CoV is not closely related to any of the three previously defined genetic and serological coronavirus groups, although it may be distantly related to group 2 coronaviruses (18, 20). SARS-CoV is not closely related to any of the three previously defined genetic and serological coronavirus groups, although it may be distantly related to group 2 coronaviruses (18, 20). Reflecting this difference, SARS-CoV does not utilize any previously identified coronavirus receptors to infect cells. Rather, as our group have recently demonstrated, angiotensin-converting enzyme 2 (ACE2) serves as a functional receptor for this coronavirus (16, 24, 25).

A quantitative system utilizing a well-characterized retroviral vector (1) for measuring SARS-CoV S-protein-mediated infection would obviate the need for specialized biosafety facilities for many studies, including those assessing humoral responses to potential vaccines. Here we show that simian immunodeficiency virus (SIV) pseudotyped with several codon-optimized S-protein variants could efficiently infect Vero E6 cells and HEK293T cells transiently or stably expressing ACE2. One such variant, truncated at its cytoplasmic tail and bearing instead a region of the tail of the human immunodeficiency virus type 1 envelope glycoprotein (17), was especially efficient at mediating infection. Murine leukemia virus (MLV) pseudotyped with this S-protein variant also infected ACE2-expressing cells more efficiently than MLV pseudotyped with other S-protein variants. We used this system to show quantitatively that the enzymatic activity of ACE2 does not contribute to S-protein-mediated infection. We also show that a catalytically inactive form of soluble ACE2 can potently inhibit infection by S-protein-pseudotyped virus and by SARS-CoV and therefore may be useful in the treatment of SARS.

**MATERIALS AND METHODS**

Plasmids encoding ACE2 and S-protein variants. A gene encoding the entire SARS-CoV S protein, except its signal sequence (residues 12 to 1255), was constructed de novo by recursive PCR and subcloned into a previously described pcDM8-derived vector that encodes the signal sequence of CD5 and a nine-residue C-terminal tag (C9; amino acid sequence, TETSQVAPA) recognized by...
the mouse monoclonal antibody ID4 (National Cell Culture Center) (5, 19, 20). Three additional variants of this gene (S-C9) were generated by the QuikChange method (Stratagene). The first was modified to exclude the C9 tag. The second and third were modified to include the eight most membrane-proximal residues of the HIV-1 envelope glycoprotein transmembrane domain (amino acid sequence: YNRRQGYS) (17) after residue 1216 (S-H1) or 1228 (S-H2) of the S protein. Plasmids encoding S-C9, S protein, S-H1, and S-H2 were sequenced within their entire coding regions. The codon-optimized S-protein gene was also subcloned into the vector pcDNA3.1 (Invitrogen) for direct comparison with the virally encoded S-protein gene (also in this vector; generously provided by Dimitar Dimitrov) (25). ACE2-Ig was generated for later use. Vero E6 cells were infected with recombinant vaccinia virus expressing T7 polymerase, which can transcribe message in the cytoplasm, and incubated at 31°C for later use. Vero E6 cells, HEK293T-ACE2 cells, or HEK293T cells transiently transfected with the calcium phosphate method with ACE2 or ACE-NN plasmid or with vector alone were incubated at 37°C with medium alone preincubated for 30 min at 37°C C for later use.

Comparison of native and codon-optimized S-protein gene expression.

Generation of cells stably expressing ACE2. HEK293T cells were transduced by the calcium phosphate method with a plasmid encoding S, S-C9, S-H1, or S-H2 or with vector alone. Forty-eight hours posttransfection, cells were detached with PBS containing 5 mM EDTA, split into two aliquots of approximately 10^6 each, and incubated for 45 min with 10 μg of S1(12-327)-Ig per ml, previously shown not to bind ACE2 (16), or of ACE2-NN-Ig. Cells were washed once in PBS containing 0.5% bovine serum albumin (BSA), stained with goat anti-human IgG-fluorescein isothiocyanate conjugate, and analyzed by flow cytometry.

Surface expression of S-protein variants. HEK293T cells were transfected by the calcium phosphate method with a plasmid encoding S, S-C9, S-H1, or S-H2 or with vector alone. Forty-eight hours posttransfection, cells were detached with PBS containing 5 mM EDTA, split into two aliquots of approximately 10^6 each, and incubated for 45 min with 10 μg of S1(12-327)-Ig per ml, previously shown not to bind ACE2 (16), or of ACE2-NN-Ig. Cells were washed once in PBS containing 0.5% bovine serum albumin (BSA), stained with goat anti-human IgG-fluorescein isothiocyanate conjugate, and analyzed by flow cytometry.

Production of and infection by S-protein-pseudotyped retroviruses. S-protein-pseudotyped lentiviral stocks were produced by cotransferring HEK293T cells with a plasmid encoding S-protein variants or vesicular stomatitis virus glycoprotein G (VSV-G) or with vector alone, together with a previously described plasmid (1) containing the SIV genome with the env gene deleted and the nef gene replaced by that for green fluorescent protein (GFP). Forty-eight hours posttransfection, viral supernatants were harvested, cleared of cell debris by centrifugation and filtration through a 0.45-μm-pore-size filter, and concentrated by ultracentrifugation to one-fifth their original volume. Viral stocks were quantified by measuring reverse transcriptase activity in the concentrated supernatant (4). Stocks were aliquoted and frozen at −80°C for later use. Vero E6 cells, HEK293T-ACE2 cells, or HEK293T cells transiently transfected with the calcium phosphate method with ACE2 or ACE-NN plasmid or with vector alone were incubated with pseudotyped SIV-GFP, normalized to 2 × 10^6 cpm of [35S] cysteine and -methionine per ml containing 0.2% sodium deoxycholate, and 0.05% SDS and analyzed by SDS-PAGE.

RESULTS

S-protein incorporation into virions. HEK293T cells were transfected as described above to produce S-, S-C9-, S-H1-, S-H2-, and VSV-G-pseudotyped SIV-GFP, as well as SIV-GFP lacking an envelope glycopeptide. Transfected cells were radiolabeled with [35S] cysteine and -methionine 1 day after transfection. Forty-eight hours posttransfection, viral supernatants were harvested, cleared of cell debris by centrifugation and filtration, and layered over a 20% sucrose cushion in PBS. Viral particles were pelleted by ultracentrifugation. The supernatant was removed, and virions were lysed in buffer containing 1% NP-40, 0.2% sodium deoxycholate, and 0.05% SDS and analyzed by SDS-PAGE.

ACE2-NN-Ig and patient serum inhibition of S-protein-mediated infection. HEK293T cells stably expressing ACE2 were plated in 24-well plates and infected with S-H2- and VSV-G-pseudotyped SIV-GFP normalized to 2 × 10^6 cpm of [35S] reverse transcriptase activity in the presence of various concentrations of ACE2-NN-Ig or S1(12-327)-Ig or in the presence of various dilutions of plasma from 12 patients who had recovered from SARS. At 72 h postinfection cells were detached, washed, and analyzed by flow cytometry. In some cases MLV-GFP was used instead of SIV-GFP. In parallel, Vero E6 cells were infected with 2,100 tissue culture infective doses of SARS-CoV per ml or with medium alone preincubated for 30 min at 37°C at neutral pH with various concentrations of ACE2-NN-Ig or of BSA and washed at 1 h postinfection (16). Three days postinfection, SARS-CoV-induced cytopathicity was measured by reading absorbance at 492 nm of cells incubated with CellTiter96 (Promega).

Codon optimization enhances S-protein expression. The gene encoding the S protein of SARS-CoV contains many codons used inefficiently in mammalian genes for efficiently expressed proteins (12, 18, 20). We therefore generated a codon-optimized form of the S-protein gene and compared its expression with the S-protein gene of the native viral sequence. S protein was readily detected in HEK293T cells transfected with a plasmid encoding the codon-optimized S protein (Fig. 1). No S protein was detected in cells transfected with a plasmid encoding the native S-protein gene. When transfected cells were infected with recombinant vaccinia virus expressing T7 polymerase, which can transcribe message in the cytoplasm, S protein was efficiently produced from plasmids containing either codon-optimized or native genes (9, 25). However, the codon-optimized gene expressed more than twice as much S protein as the native viral sequence. Because S protein could be efficiently expressed from the codon-optimized plasmid.
without T7 polymerase, we used this plasmid in our subsequent studies.

**Infection with SIV pseudotyped with S-protein variants.** HEK293T cells transfected with a plasmid encoding ACE2, but not mock-transfected HEK293T cells, form large multinucleated syncytia with cells expressing SARS-CoV S protein and support efficient replication of SARS-CoV (16). We investigated whether an SIV variant (SIV-GFP) whose nef gene has been replaced by that for GFP, lacking a functional env gene, and pseudotyped with S protein could infect ACE2-expressing cells (1). SIV was initially chosen because it readily infects Vero E6 cells, commonly used to propagate SARS-CoV, whereas HIV-1 does not (2). The complete S protein and three additional S-protein variants, differing in their cytoplasmic domains, were assayed (Fig. 2A). One variant, S-C9, includes at its C terminus nine amino acids derived from the rhodopsin C

![Fig. 2. Lentiviruses pseudotyped with S-protein variants infect ACE2-expressing cells.](http://jvi.asm.org/)
terminus and recognized by the antibody 1D4 (19). Two additional variants, truncated after S-protein residue 1216 (S-H1) or 1228 (S-H2), were also studied. In both these variants, an additional eight residues—the most membrane-proximal of the HIV-1 envelope glycoprotein cytoplasmic domain—were included. An HIV-1 envelope glycoprotein truncated after these residues has been shown to be better expressed on the cell surface and better incorporated into MLV and HIV-1 virions than is wild-type HIV-1 envelope glycoprotein (17).

HEK293T cells were transfected with a plasmid encoding SIV-GFP and with plasmids encoding each of the S-protein variants or VSV-G or with vector alone. Cell supernatants were concentrated, normalized for reverse transcriptase activity, and incubated with HEK293T cells transfected with ACE2 or with vector alone. Fluorescent cells were observed in cultures of all ACE2-transfected cells infected with SIV-GFP pseudotyped with S-protein variants or with VSV-G (Fig. 2B and C). No fluorescence was observed in cultures of ACE2-transfected cells infected with SIV-GFP lacking a fusion protein (Fig. 2B, right panel, middle row), nor was any observed in cultures of mock-transfected HEK293T cells infected with virus pseudotyped with any S-protein variants (Fig. 2B, left panel, bottom row, and results not shown). SIV-GFP pseudotyped with S-H2, consistently the most efficient of the S-protein variants (Fig. 2B, left panel, middle row), also infected Vero E6 cells (Fig. 2B, bottom right panel). Figure 2D shows that HEK293T cells stably expressing ACE2 were more efficiently infected by S-H2-pseudotyped SIV-GFP than were Vero E6 cells, consistent with higher ACE2 levels in the

**FIG. 2**—Continued.

![Graph](image1)

**FIG. 3.** Efficient surface expression and virion incorporation of S-H2. (A) HEK293T cells were transfected with plasmids encoding S-protein variants or with vector alone and radiolabeled with [35S]cysteine and -methionine. Lysates were precipitated with ACE2-Ig or ACE2-NN-Ig and analyzed by SDS-PAGE. (B) HEK293T cells were transfected with plasmids encoding S-protein variants or vector alone, incubated with ACE2-NN-Ig, and stained with a goat anti-human IgG-fluorescein isothiocyanate conjugate. Fluorescence was measured by flow cytometry. Error bars indicate the ranges of results for three independent transfections. (C) HEK293T cells were transfected with plasmids encoding SIV-GFP with vector only, S-protein variants, or VSV-G and radiolabeled with [35S]cysteine and -methionine. Forty-eight hours posttransfection, supernatants were applied to a 20% sucrose cushion and ultracentrifuged. Viral pellets were lysed and analyzed by SDS-PAGE. p27, encoded by SIV gag, indicates relative virion production levels of pseudotyped variants.
former cells. Again, no infection was observed in HEK293T cells lacking exogenous ACE2.

S-H2 incorporates efficiently into retroviral virions. We investigated possible reasons for the substantially higher efficiency of infection observed with SIV-GFP pseudotyped with S-H2 compared to virus pseudotyped with the other S-protein variants. Total expression, cell surface expression, and virion incorporation of these variants were compared (Fig. 3). Lysates of metabolically labeled HEK293T cells transfected with plasmids encoding S-protein variants were precipitated with the ectodomain of ACE2 fused to human IgG1 (ACE2-Ig) or with a catalytically inactive variant of ACE2-Ig whose active-site histidines have been replaced by asparagines (ACE2-NN-Ig). Substantially more S-C9 and S-H2 than S protein or S-H1 were precipitated by both forms of ACE2-Ig (Fig. 3A). No significant differences were observed in the ability of ACE2-Ig and ACE2-NN-Ig to precipitate S-protein variants, indicating that modification of the ACE2 active site did not detectably alter the ACE2 affinity for S protein. We therefore used the catalytically inactive ACE2-NN-Ig for subsequent studies. The amount of each S-protein variant precipitated from cell lysates was also reflected in its cell surface expression, as measured by flow cytometry using ACE2-NN-Ig (Fig. 3B). In contrast, incorporation of each S-protein variant into the SIV virion did not correlate with its total or surface expression levels (Fig. 3C). Rather, those variants (S-H1 and S-H2) with shorter cytoplasmic domains and containing residues from the cytoplasmic tail of the HIV-1 envelope glycoprotein were preferentially incorporated. We conclude that the greater efficiency with which S-H2-pseudotyped virions infect ACE2-expressing cells compared to virions pseudotyped with S protein or S-C9 is likely due to preferential incorporation of S-H2. The observed difference between S-H1 and S-H2 may be due to the absence in S-H1 of cysteines critical to the function of the S proteins of other coronaviruses (3).

ACE2 enzymatic activity does not contribute to S-protein-mediated fusion. We then investigated whether the enzymatic activity of ACE2 contributes to its ability to promote S-protein-mediated infection. HEK293T cells were transfected with a plasmid expressing full-length ACE2 or a variant thereof (ACE2-NN) altered as described for ACE2-NN-Ig so as to be catalytically inactive. This full-length variant was expressed on HEK293T cells as efficiently as wild-type ACE2 (Fig. 4). Various amounts of each plasmid or a control plasmid were transfected, and expression of ACE2 and ACE2-NN was measured with S1-Ig, using flow cytometry, 24 h posttransfection. An

![FIG. 4. ACE2 enzymatic activity does not contribute to S-protein-mediated infection.](http://jvi.asm.org/)

![FIG. 5. A soluble, catalytically inactive form of ACE2 potently inhibits S-protein-mediated infection.](http://jvi.asm.org/)
aliquot of each set of transfected cells was also incubated at 24 h posttransfection with SIV-GFP pseudotyped with S-H2, and infection was measured by flow cytometry 60 h posttransfection. A plot of receptor expression against S-protein-mediated infection is shown in Fig. 4. Infection was dependent on receptor expression levels but was independent of the ACE2 variant used. These data indicate that the enzymatic activity of ACE2 makes no detectable contribution to S-protein-mediated infection.

ACE2-NN-Ig potently inhibits infection. We also investigated whether S-H2-pseudotyped virions could be useful in evaluating inhibitors of S-protein-mediated fusion. We have previously shown that a protein [S1(12-327)-Ig] bearing an amino-terminal fragment of the S protein does not bind ACE2 (16). As expected, this protein had no effect on the efficiency of S-H2-mediated infection of HEK293T cells stably expressing ACE2 (Fig. 5A). In contrast, ACE2-NN-Ig potently blocked infection, with a 50% inhibitory concentration of approximately 2 nM. In parallel, neutralization assays were performed in which SARS-CoV (400 TCID₅₀ per well) was preincubated with various concentrations of ACE2-NN-Ig or BSA for 1 h before infection of Vero E6 cells (5,000 per well). Three days postinfection, SARS-CoV-induced cytopathicity was measured and half-maximal cell viability was consistently observed at less than 19 nM ACE2-NN-Ig. These data show that quantitative measurements of infection are possible using S-H2-pseudotyped virus and that ACE2-NN-Ig may be a potent inhibitor of SARS-CoV infection.

MLV can be pseudotyped with S-protein variants. Finally, we explored whether MLV pseudotyped with S-protein variants could infect ACE2-HEK293T cells. As shown in Fig. 6, MLV expressing GFP and pseudotyped with S protein, S-H2, or an S-H2 molecule lacking residues derived from HIV-1 (S-TR) could infect ACE2-expressing cells whereas MLV made in the absence of any S-protein variant could not. As with SIV-GFP, MLV pseudotyped with S-H2 infected cells more efficiently than that pseudotyped with S protein. MLV pseudotyped with S-H2 was slightly more efficient than MLV pseudotyped with S-TR, implying that truncation of the S protein makes a substantial contribution to the efficiency of pseudotyping whereas residues derived from HIV-1 may contribute modestly. MLV-GFP pseudotyped with S-H2 was used to assay the potency of a mixture of antisera from individuals who had recovered from SARS (Fig. 6C). These antisera efficiently blocked infection by S-H2-pseudotyped MLV at the highest dilution assayed. Figure 6C also demonstrates that ACE2-NN-Ig blocked MLV-GFP infection with an efficiency comparable to that observed with SIV-GFP. These data show that S-protein-mediated fusion can be studied with convenient vectors and packaging cell lines expressing MLV.

FIG. 6. Efficient pseudotyping of MLV with S-protein variants. (A) C-terminal sequences of S-protein variants used in panel B are shown for comparison. The gray box indicates residues derived from a membrane-proximal region of the cytoplasmic domain of HIV-1 gp160 (S-H2). (B) A packaging cell line (GP2-293) stably expressing MLV gag and pol gene products was transfected with a vector containing the MLV packaging signal and engineered to express GFP and with either vector alone or a plasmid encoding S protein, S-H2, or an S-protein variant (S-TR) truncated, like S-H2, after residue 1228 but lacking the HIV-1-gp160-derived residues present in S-H2. Viruses were normalized for reverse transcriptase activity and incubated with ACE2-HEK293T cells. Numbers in parentheses indicate mean fluorescent as determined by flow cytometry. (C) MLV generated as for panel B and pseudotyped with S-H2 was incubated with ACE2-HEK293T cells and the indicated dilutions of convalescent-phase patient sera or the indicated concentrations of ACE2-NN-Ig or BSA. Infection is reported as mean fluorescence normalized to that observed in the absence of any inhibitor.


