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

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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 etiological 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 (21); the SARS-CoV spike (S) protein, a surface glycoprotein that mediates coronavirus entry into receptor-bearing cells, is also distinct from those of other 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 (HIV-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.

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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

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the mouse monoclonal antibody 1D4 (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 cytoplasmic domain (amino acid sequence, NRVRQGYS) (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 Dimiter Dimitrov) (25). ACE2-Ig was generated by ligating the PCR product encoding the ectodomain of ACE2 into a previously described vector encoding the Fc domain of human immunoglobulin G1 (IgG1) (10). ACE2-NN-Ig was generated from ACE2-Ig by altering the codons of ACE2 active-site histidines 374 and 378 to those of asparagines, using the QuikChange method. Plasmids encoding ACE2-Ig and ACE2-NN-Ig were fully sequenced in their coding regions.

Comparison of native and codon-optimized S-protein gene expression. HEK293T cells were transfected, using Polyfect transfection reagent (QIAGEN), with a plasmid encoding codon-optimized S protein or virally encoded (native) S protein or with vector only. Four hours posttransfection, cells were infected, or not, with recombinant vaccinia virus VTF7.3 encoding T7 polymerase and incubated at 31°C (9, 25). Two hours later, the cells were washed, radiolabeled with [^{35}S]cysteine and -methionine, and incubated for 20 h at 31°C. The cells were washed and lysed with phosphate-buffered saline (PBS) containing 0.5% NP-40 and a protease inhibitor cocktail (Sigma). S protein was precipitated from cell lysates by using ACE2-Ig and ACE2-NN-Ig bound to protein A-Sepharose beads and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Relative expression of codon-optimized and virally encoded S proteins was quantified by phosphorimaging.

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 cells 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.

Generation of cells stably expressing ACE2. HEK293T cells were cotransfected by the calcium phosphate method with a pcDNA3.1 plasmid encoding ACE2 and pcDNA3.1-puro, which expresses a puromycin resistance gene. Transfected cells were selected in growth medium containing 0.75 μg of puromycin per ml. Puromycin-resistant clones were expanded, and a clone with a high level of expression of ACE2 (HEK293T-ACE2) was compared with Vero E6 and HEK293T cells for ACE2 expression by flow cytometry using goat anti-ACE2 antibody (R&D Systems) and S1-Ig, which contains S-protein residues 12 to 672 fused to the Fc domain of human IgG1 (16).

Production of and infection by S-protein-pseudotyped retroviruses. S-protein-pseudotyped lentiviral stocks were produced by cotransfecting HEK293T cells with a plasmid encoding S-protein variants or vesicular stomatitis virus protein 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 by the calcium phosphate method with ACE2 or ACE-NN plasmid or with vector alone were incubated with pseudotyped SIV-GFP, normalized to 8×10^5 cpm of [^{32}P] reverse transcriptase activity in a total volume of 2 ml in 12-well plates. Cells were photographed 48 h postinfection and then detached with trypsin, washed once with PBS, and fixed in PBS containing 2% formaldehyde. GFP fluorescence was measured by flow cytometry.

MLVs pseudotyped with S-protein variants were generated by cotransfecting the pQCXIX vector (BD Sciences) expressing GFP and plasmids encoding the indicated S-protein variants or pcDNA3.1 vector into the retroviral packaging cell line GP2-293 (BD Sciences). Forty-eight hours posttransfection, unconcentrated cell supernatants were normalized for reverse transcriptase activity and incubated with HEK293T-ACE2 cells. Forty-eight hours postincubation, GFP fluorescence of infected cells was measured as described above.

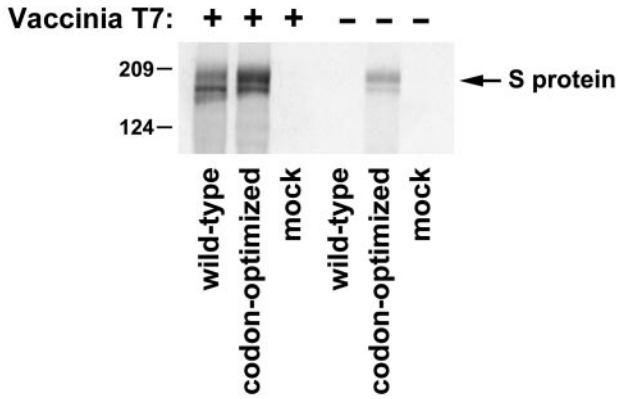

FIG. 1. Efficient expression of codon-optimized S protein. A plasmid encoding native S protein or codon-optimized S protein or vector alone was transfected into HEK293T cells and infected or not, as indicated, with recombinant vaccinia virus expressing T7 polymerase. Cells were radiolabeled with [^{35}S]cysteine and -methionine. Lysates were precipitated with ACE2-Ig and analyzed by SDS-PAGE.

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 glycoprotein. Transfected cells were radiolabeled with [^{35}S]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 2×10^5 cpm of [^{32}P] 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 a mixture of antisera 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).

RESULTS

Codon optimization enhances S-protein expression. The gene encoding the S protein of SARS-CoV contains many codons used infrequently 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

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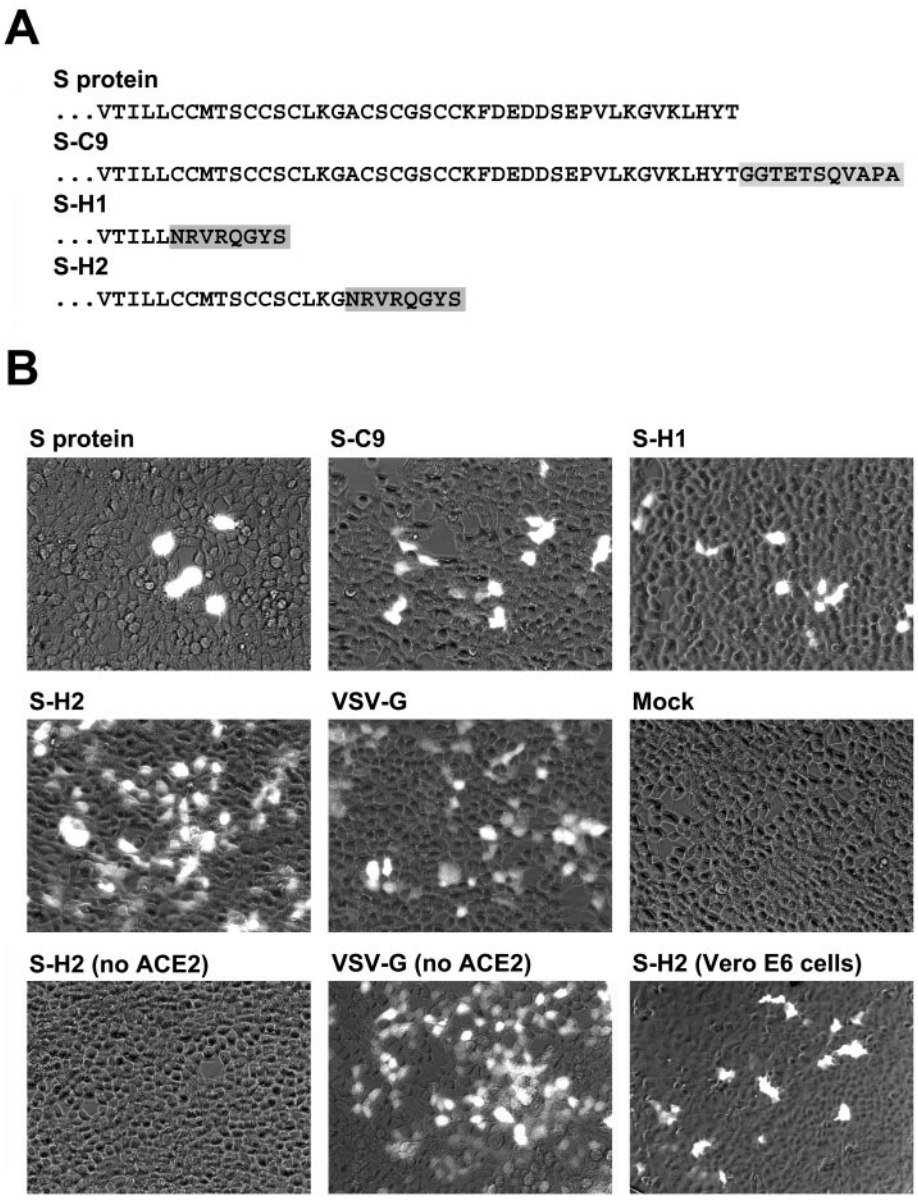

FIG. 2. Lentiviruses pseudotyped with S-protein variants infect ACE2-expressing cells. (A) C-terminal sequences of S-protein variants used in these studies are shown for comparison. Gray boxes indicate residues from a C-terminal tag (S-C9) or from a membrane-proximal region of the cytoplasmic domain of HIV-1 gp160 (S-H1 and S-H2). (B) SIV-GFP pseudotyped with S-protein variants or VSV-G or lacking any fusion protein (mock) was normalized for reverse transcriptase activity and used to infect HEK293T cells transiently transfected with ACE2 (top two rows) or with vector alone (bottom row, left and center panels) or untransfected Vero E6 cells (bottom row, right panel). Images were taken 48 h after infection. (C) Cells shown in panel B were detached, and the mean fluorescence was measured by flow cytometry. Bars indicate averages and error bars show the ranges of results for three independent infections. (D) ACE2 expression levels (vertical axis) of HEK293T cells stably expressing ACE2, Vero E6 cells, or untransfected HEK293T cells were analyzed by flow cytometry with S1-Ig, a fusion protein containing the S1 domain of the S protein (residues 12 to 672) and the Fc portion of human Ig1. In parallel, cells were infected with SIV-GFP pseudotyped with S-H2, and the percentages of GFP-expressing cells (horizontal axis) were determined by flow cytometry.

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

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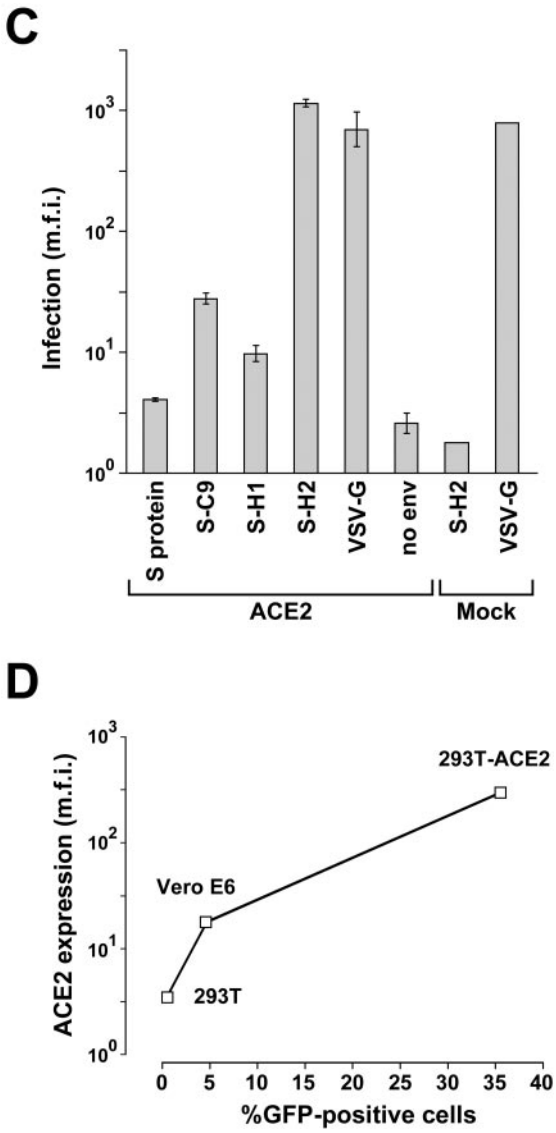

FIG. 2—*Continued*.

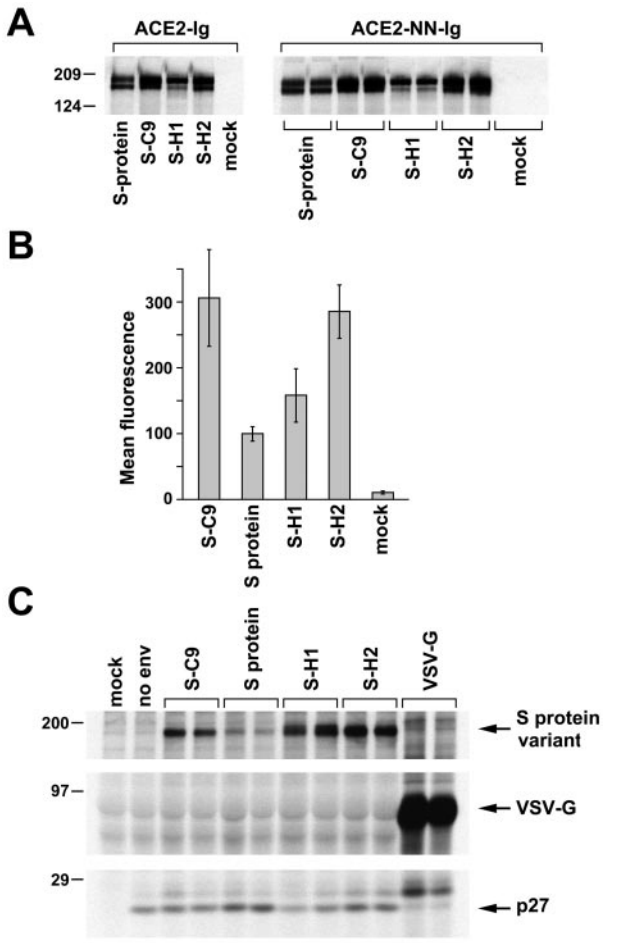

FIG. 3. Efficient surface expression and virion incorporation of S-H2. (A) HEK293T cells were transfected with plasmids encoding S-protein variants or with vector alone and radiolabeled with [^{35}S]cysteine and -methionine. Lysates were precipitated with ACE2-Ig or ACE2-NN-Ig and analyzed by SDS-PAGE. (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 [^{35}S]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.

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

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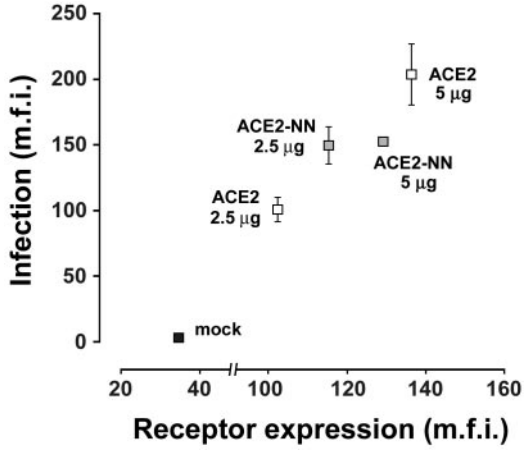

FIG. 4. ACE2 enzymatic activity does not contribute to S-protein-mediated infection. HEK293T cells were transfected with 2.5 and 5 μg of plasmids encoding ACE2 or a catalytically inactive form of ACE2 (ACE2-NN) or with vector alone (5 μg, mock). Aliquots of transfected cells were analyzed by flow cytometry with S1-Ig (vertical axis) to determine receptor expression or incubated with SIV-GFP pseudotyped with S-H2 (horizontal axis). Infection by SIV-GFP was quantified by flow cytometry.

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).

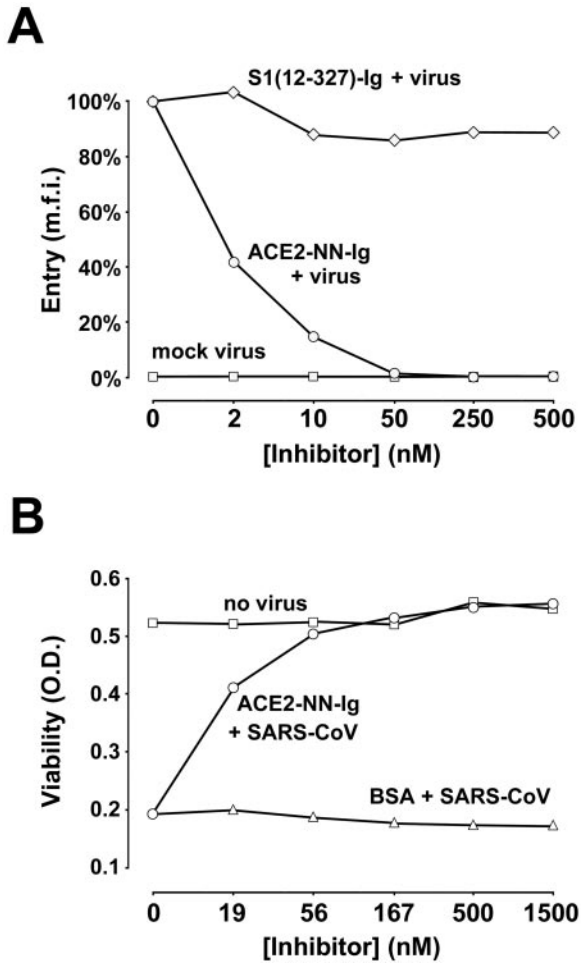

FIG. 5. A soluble, catalytically inactive form of ACE2 potently inhibits S-protein-mediated infection. (A) HEK293T cells stably expressing ACE2 were infected with 2×10^5 cpm of [^{32}P] reverse transcriptase activity of SH-2-pseudotyped SIV-GFP (diamonds and circles) or of virus lacking any fusion protein (squares) in the presence of the indicated concentrations of ACE2-NN-Ig (circles and squares) or S1(12-327)-Ig (diamonds). Forty-eight hours postinfection, GFP fluorescence was measured by flow cytometry. (B) SARS-CoV (circles and triangles) or medium alone (squares) was preincubated with the indicated concentrations of ACE2-NN-Ig (circles and squares) or BSA (triangles) before infection of Vero E6 cells. Cell viability was determined by staining with a dye (CellTiter96) active in living cells and measuring optical density at 492 nm. Zero points for BSA and ACE2-NN-Ig are included for clarity and extrapolated from the average of BSA values. The experiment is representative of two with quantitatively similar results.

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

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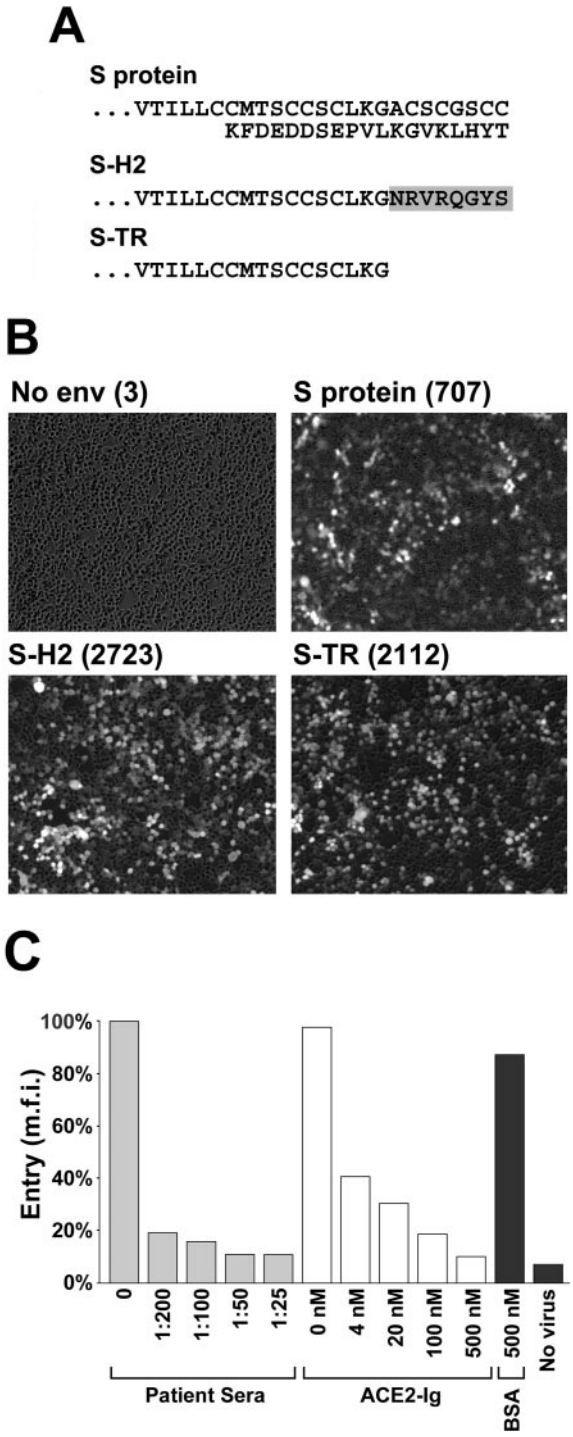

FIG. 6. Efficient pseudotyping of MLV with S-protein variants. (A) C-terminal sequences of S-protein variants used in panel B are shown for comparison. The gray box indicates residues derived from a membrane-proximal region of the cytoplasmic domain of HIV-1 gp160 (S-H2). (B) A packaging cell line (GP2-293) 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 fluorescence 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.

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 $TCID_{50}$s 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.

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DISCUSSION

Here we developed and utilized a system for studying S-protein-mediated infection using commonly used retroviral vectors, thereby making accessible studies of S-protein entry to many investigators without access to Biosafety Level 3 facilities. This system uses multiple approaches to make more efficient and quantitative what might otherwise be prohibitively inefficient. First, we used a codon-optimized form of the S protein, which simplifies its efficient expression. Second, we modified the cytoplasmic tail of the S protein to allow more efficient incorporation into the SIV and MLV virions. This modification likely reduces steric interaction with the retroviral matrix protein and may facilitate S-protein expression on the cell surface, where retroviral but not coronaviral budding occurs. Third, cells stably expressing high levels of the SARS-CoV receptor ACE2 were used for infection. We anticipate that these or similar approaches will be useful in the quantitative assessment of inhibitors of S-protein-mediated infection and of antisera from vaccinated animals (22, 23, 26).

Using this system, we demonstrated quantitatively that the enzymatic activity of ACE2 does not make even a small contribution to the ability of ACE2 to support S-protein-mediated infection. Similar observations have been made for the group 1 coronavirus transmissible gastroenteritis virus, which utilizes the zinc metalloprotease aminopeptidase N (CD13) as a receptor (7). Our data imply that the S-protein-binding site of ACE2 is distinct from its catalytic site and that small molecules screened for their ability to block ACE2 catalysis (6, 13) may not slow viral replication.

We also used this system to document the potency of a soluble form of a catalytically inactive ACE2 in blocking infection. Infection of SIV-GFP pseudotyped with S-H2 was inhibited with a 50% inhibitory concentration of 2 nM ACE2-NN-Ig. Similar concentrations of ACE2-NN-Ig were required to block replication of live SARS-CoV. It is not yet clear whether the soluble receptor irreversibly inactivates the S protein or whether its potency derives from its high affinity for S protein, measured at 1.7 nM by surface plasmon resonance (23). Regardless, these studies suggest that soluble ACE2 may be useful as a therapy for SARS and perhaps that soluble receptors of other coronaviruses can be used to control replication of these viruses in infected animals.

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