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Identification of Major Histocompatibility Complex Class I C Molecule as an Attachment Factor That Facilitates Coronavirus HKU1 Spike-Mediated Infection▽

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Human coronavirus HKU1 (HCoV-HKU1) is a recently discovered human coronavirus associated with respiratory tract infections worldwide. In this study, we have identified the major histocompatibility complex class I C molecule (HLA-C) as an attachment factor in facilitating HCoV-HKU1 spike (S)-mediated infection. HCoV-HKU1 S pseudotyped virus was assembled using a human immunodeficiency virus type 1-derived reporter virus harboring the human codon-optimized spike of HCoV-HKU1. We identified human alveolar epithelial A549 cells as the most susceptible cell line among those tested to infection by HCoV-HKU1 S pseudotypes. A549 cells were shown to bind purified soluble HCoV-HKU1 $S_{1\text{-}600}$ glycopeptide. To search for the functional receptor for HCoV-HKU1, an A549 cDNA expression library was constructed and transduced into the nonpermissive, baby hamster kidney cells line BHK-21. Transduced cells that bind soluble HCoV-HKU1 $S_{1\text{-}600}$ glycoprotein with C-terminal FLAG were sorted. Sequencing of two independent clones revealed cDNA inserts encoding HLA-C. Inhibition of HLA-C expression or function by RNAi silencing and anti-HLA-C antibody decreased HCoV-HKU1 S pseudotyped virus infection of A549 cells by 62 to 65%, whereas pretreatment of cells with neuraminidase decreased such infection by only 13%. When HLA-C was constitutively expressed in another nonpermissive cell line, NIH-3T3, quantitative PCR showed that the binding of HCoV-HKU1 S pseudotyped virus to cell surfaces was increased by 200-fold, but the cells remained nonsusceptible to HCoV-HKU1 S pseudotyped virus infection. Our data suggest that HLA-C is involved in the attachment of HCoV-HKU1 to A549 cells and is a potential candidate to facilitate cell entry. However, other unknown surface proteins on A549 cells may be concomitantly utilized by S glycoprotein of HCoV-HKU1 during viral entry. Further studies are required to elucidate other putative receptors or coreceptors for HCoV-HKU1 and the mechanism of HCoV-HKU1 S-mediated cell entry.

The genus of *Coronavirus* consists of three groups of coronaviruses, which are enveloped single-stranded positive-sense RNA viruses with a genome size of about 30 kb. They are known to cause respiratory or intestinal infections in human and other animals. Human coronavirus HKU1 (HCoV-HKU1), a recently identified coronavirus associated with human respiratory tract infections first discovered in Hong Kong, is classified as a group 2 coronavirus (36, 38) At least three genotypes of HCoV-HKU1 have been found and shown to have arisen from intergenotype recombination (37, 39).

Coronaviruses may overcome the entry or interspecies barrier or develop additional host-receptor interactions, through mutations or incorporation of foreign sequences into the spike (S) protein. This might explain the diversity of receptor usage among coronaviruses, which allows them to exploit different strategies in gaining host-cell entry by utilizing a range of cellular proteins and/or coreceptors. A number of group 1 coronaviruses utilize species-specific aminopeptidase N (APN), a family of metalloproteases, as functional receptors. Indeed, feline APN can serve as a common receptor for group 1 coronaviruses affecting feline, canine, porcine, and human species (11, 20, 30, 41). However, HCoV-NL63, a newly discovered group 1 coronavirus, was found to utilize angiotensin-converting enzyme 2 (ACE2) as an entry receptor (26). The receptor used by some members of group 1 coronavirus, such as porcine epidemic diarrhea virus and type I feline infectious peritonitis virus, has not been identified. The sialic acid *N*-acetyl-9-*O*-acetylneuraminic acid was shown to be the functional receptor for group 2 coronaviruses, such as HCoV-OC43 and bovine coronaviruses (BCoV) (13, 27, 33). But mouse hepatitis virus (MHV), also a group 2 coronavirus, has evolved to use a carcinoembryonic antigen-cell adhesion molecule (CEACAM1) as the major receptor and heparan sulfate, which may function either as the receptor or as an attachment factor, depending on the strain (10, 29, 34). Severe acute respiratory syndrome coronavirus (SARS-CoV), a distantly related group 2 coronavirus, uses ACE2 independently with or without DC-SIGN and related proteins to mediate infection (22). For group 3 coronaviruses, the reported use of sialic acids as the receptor is considered controversial, and heparan sulfate has been reported to act as an attachment factor for infectious bronchitis virus (IBV) (23). Feline APN had been ruled out as a functional receptor for avian IBV (5).

The HCoV-HKU1 S protein contains a predicted furin cleav-

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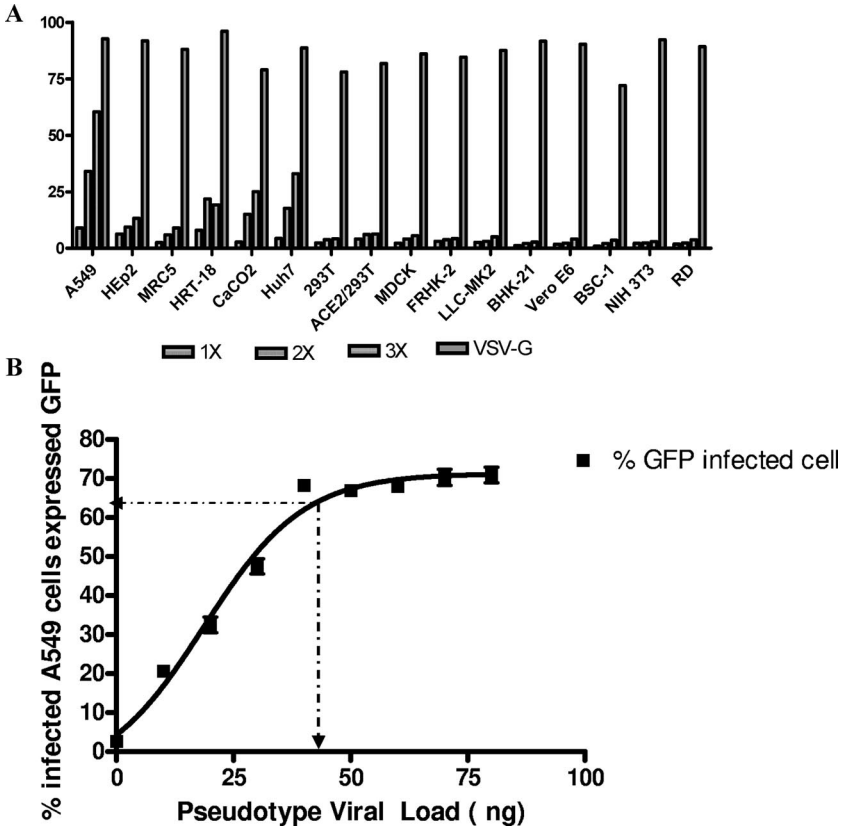

FIG. 1. Tissue tropism demonstrated by infectivity of HCoV-HKU1 S-bearing pseudotyped virus in different cell lines. (A) Three different doses of pseudotyped viruses infected different cell lines at a cell density of 1×10^5 per well in a 24-well plate. Infectivity was measured by expression of the reporter eGFP by flow cytometry. VSV-G was included as a positive control. A total of 1× HKU1 pseudotyped virus is equivalent to 12.5 ng, quantified by detection of p24. Percentage of infection is measured by GFP expression of infected cells over the total cell population. (B) Infectivity of A549 cells by CoV-HKU1 S pseudotyped virus was viral load dependent, and saturation was achieved at ~40 ng. The ACE2-transduced 293T cell line is a kind gift from M. Farzan (22).

age site between the S1 and S2 subdomains. Inhibition of the cleavage of recombinant HCoV-HKU1 S protein by a furin inhibitor is concentration dependent in a cell-based proteolysis assay (3). The S1 subdomain (residues 14 to 760) presumably contains the receptor-binding region (36). However, the identity of the host receptor is still unknown. As HCoV-HKU1 cannot be maintained in cell culture yet, the identification of the receptor will be critical in understanding the biology and entry mechanism of this elusive virus. In this study, we identified human lung epithelial cell line A549 to be highly susceptible to HCoV-HKU1 S-bearing pseudotyped virus. By adopting an expression/cloning approach, we transduced an A549-derived retroviral cDNA library into nonsusceptible hamster kidney (BHK-21) cells. Transduced cells that bound soluble, codon-optimized, C-terminally FLAG-tagged HCoV-HKU1 S glycoprotein (amino acids 1 to 600) were sorted by flow cytometry. The HCoV-HKU1 S binding cells were revealed to have incorporated a cDNA transcript identical to that of human HLA-C molecules, which were subsequently confirmed to function as an attachment factor by enhancing virus binding onto cell surfaces.

MATERIALS AND METHODS

Cell lines and cultures. A panel of cell lines was tested for susceptibility to infection by HCoV-HKU1 pseudotyped virus, including A549 (human alveolar basal epithelial adenocarcinoma), HEp-2 (human larynx carcinoma), MRC-5 (human lung fibroblast), Huh-7 (human hepatoma), CaCO2 (human colon adenocarcinoma), HRT-18 (human rectum-anus adenocarcinoma), RD (human rhabdomyosarcoma embryonic muscle), NIH 3T3 (mouse embryonic fibroblast), 293T (human embryonic kidney fibroblast), ACE2/293T (ACE2 stably expressed in 293T; a kind gift from M. Farzan) (22), BSC-1 (African green monkey kidney epithelial), Vero E6 (African green monkey kidney fibroblast), MDCK (canine kidney epithelial), LLC-Mk2 (rhesus monkey kidney epithelial), and BHK-21 (hamster kidney fibroblast) (Fig. 1). Cell lines were propagated in Dulbecco modified Eagle medium (DMEM) (Invitrogen) containing 10% fetal calf serum (FCS), 20 mM HEPES and 1% penicillin-streptomycin (Invitrogen).

Plasmid construction. The synthetic human codon-optimized S gene was used as a PCR template for all S plasmid constructions. For pcDNA-S, forward primer (5′-CGCGGATCC*ACC*ATGCTGCTGATCATCTTCATCCTG), containing an N-terminal signal sequence with a BamHI site and Kozak sequence, and reverse primer (5′-CGGAATTCCTAGTCATCATGGGAGGTCTTGAT), containing a C-terminal cytoplasmic domain with an EcoRI site, were used to generate full-length S in pcDNA 3.1(+). For the construction of S1, the same 5′ forward sequence was used together with the S1 reverse primer (5′-GCGGATCCCTAGTTGATGCCAT TCAGG) with a BamHI site. It was then subcloned, with the C terminus fused in-frame with the FLAG sequence (DYKDDDDK), into the BamHI site of the pSFV1 vector (kindly provided by P. Liljestrom), resulting in plasmid pSFV-S1-FLAG. For the construction of HLA-C into the pSFV-1 vector, forward primer (5′-CGCGGATCC*ACC*ATGCGGGTCATGGCGCCCCG) and reverse primer (5′-CGCGGATCCTCAGGCTTTACAAGTGATGAG) containing BamHI sites were used, resulting in pSFV-HLA-C. For the construction of HLA-C into pFB-Neo (Stratagene) for stable expression, forward primer (5′-CCGGAATTC*ACC*ATGC GGGTCATGGCGCCCCG) with an EcoRI site and reverse primer (5′-TACGCC

TCGAGTCAGGCTTTACAAGTGATGAG) with an XhoI site were used, resulting in HLA-C-pFB-Neo.

Production of HCoV HKU1 pseudotyped virus by cotransfection. 293FT cells were cultured in DMEM containing 10% FCS, 20 mM HEPES, and 1% penicillin-streptomycin. 293FT cells were maintained separately with the addition of 0.1 mM MEM nonessential amino acids and 500 μg/ml Geneticin (Invitrogen).

Lentivirus-based HCoV-HKU1-S pseudotypes were generated by cotransfection of 293FT cells with pcDNA-S in combination with the pHIV backbone plasmid bearing green fluorescent protein (GFP) reporter gene, pNL4-3-deltaE-eGFP, using Lipofectamine 2000 agent as suggested by the supplier (Invitrogen) (42). pcDNA-S was replaced with pHEF-VSVG to produce pseudotyped virus bearing vesicular stomatitis virus G glycoprotein (VSV-G) envelopes as control (4). Both pNL4-3-deltaE-eGFP and pHEF-VSVG were obtained through the NIH AIDS research and reference reagents program.

Cells transfected overnight were replenished with fresh medium and supplemented with 1 mM MEM sodium pyruvate (Invitrogen). The viral particles in supernatant were harvested 48 h posttransfection and filtered through a 0.45-μm-pore-size syringe filter. Viral particles were concentrated by high-speed centrifugation at 50,400 × *g* for 4 h (Beckman rotor JA-21). The p24 concentrations from different batches of pseudotyped virus produced were quantified by the p24 enzyme-linked immunoassay kit (bioMérieux) and stored in aliquots at −80°C.

HCoV-HKU1 S pseudotyped virus infection assay. Different doses of HCoV-HKU1 S retroviral-based pseudotyped viruses equivalent to 12.5, 25, and 37.5 ng HIV-p24 were used to infect tested cell lines cultured in 24-well plates with 10^5 cells/well. Viruses and cells were incubated at 37°C for 1 h in FCS-free DMEM containing Polybrene (Sigma) at a concentration of 8 mg/ml. The medium was replaced with fresh medium with 10% FCS after 1 h, and cells were cultured for another 40 h. Cells were detached and washed, and GFP expression was detected by FACSCalibur flow cytometry (Becton Dickinson).

Soluble HCoV-HKU1 S1 protein expression and binding. The soluble HCoV HKU1 S1 fragment (amino acid positions 1 to 600) was expressed in Semliki Forest virus expression system (22a). HCoV-HKU1 S1 FLAG protein was immunoprecipitated from supernatant cleared from cell debris by using anti-FLAG M2 monoclonal antibody-conjugated agarose beads (Sigma) overnight at 4°C with gentle rocking. Bound proteins were pelleted at 8,000 × *g* for 1 min, washed three times in 1× washing buffer (10 mM Tris [pH 7.5], 150 mM NaCl) and eluted with 3× FLAG peptide according to the supplier's instructions (Sigma). Eluted proteins were analyzed by running them on a NuPAGE 4-12% sodium dodecyl sulfate-polyacrylamide gel (Invitrogen) under reducing conditions.

For the binding assay, 1 μg purified HCoV-HKU1 S1 protein was added to 10^5 A549 cells suspended in 0.1 ml fluorescence-activated cell sorter (FACS) buffer (2% FCS in phosphate-buffered saline [PBS]) and incubated at 4°C for 1 h. The cell-protein mixture was washed and resuspended in 0.1 ml FACS buffer containing 1 μg anti-FLAG fluorescein isothiocyanate (FITC)-conjugated antibody (Sigma) and incubated at 4°C for 1 h. HCoV-HKU1 S1 protein-bound cells were measured by FACSCalibur flow cytometry. To verify the specificity of binding, HCoV-HKU1 S1 was preincubated with convalescent serum of HCoV-HKU1-infected patients and serum of normal donors (1:50 dilution) for 1 h at 4°C prior to cell binding.

Construction of the A549 cDNA library. Total RNA was extracted from A549 cells by using an RNeasy kit (Qiagen). Poly(A) RNA was then isolated using an Oligotex column (Qiagen). A total of 5 μg mRNA was used to prepare a cDNA library by using the Uni-ZAP XR library construction kit (Stratagene). A cDNA library with cDNA sizes ranging from 1 to 5 kb, flanked with 5′ EcoRI and 3′ XhoI adapters, was ligated into a prelinearized pFB retroviral vector (Stratagene). The ligated cDNAs were transformed into *Escherichia coli* XL10-Gold competent cells (Stratagene).

Expression library cloning and flow cytometry sorting. A total of 10 μg cDNA plasmids were cotransfected with 10 μg ViraPort gag-pol expression vectors and 5 μg env-expressing VSV-G vectors (Stratagene) using the Lipofectamine 2000 agent (Invitrogen) for every 10^7 293FT cells (Invitrogen). Cells transfected overnight were replenished with fresh DMEM containing 10% FCS and supplemented with 1 mM minimum essential medium with nonessential amino acids and sodium pyruvate. Culture supernatant was harvested 48 h later and filtered through a 0.45-μm-pore-size syringe filter. Viral particles were concentrated by high-speed centrifugation at 50,400 × *g* for 4 h. Production of pseudotyped virus was quantified by the p24 enzyme-linked immunosorbent assay kit and the multiplicity of infection (MOI) was determined by comparing to pseudotypes containing the ViraPort pFB-GFP control vector (Stratagene), measured by FACSCalibur flow cytometry.

Pseudotypes carrying the cDNA library were used to transduce 3×10^6 BHK-21 cells at MOIs of 1 to 2 in the presence of Polybrene (8 mg/ml). Pseudotypes were incubated for 4 h at 37°C to be adsorbed onto cells. An S1 binding assay was performed 48 h postinfection. Transduced cells bound to S1 were selected by FACS sorting (FACSVantage SE; Becton Dickinson). Untransduced BHK-21 cells stained against S1-FLAG-FITC were included as background control.

DNA sequencing of cDNA inserts encoding putative attachment factor. Fluorescent cells recovered by FACS sorting were cultured in DMEM containing 10% FCS for 5 days. Genomic DNA was isolated using a DNeasy kit (Qiagen). PCRs of proviral cDNA inserts from sorted transduced cells were performed using pFB vector-specific primers flanking a multiple cloning site (Stratagene). PCR products were ligated to a TA TOPO vector (Invitrogen). cDNA inserts were sequenced using M13 forward and reverse primers.

HLA-C coimmunoprecipitation. A total of 2 μg S1-FLAG was preadsorbed onto M2 affinity agarose beads (Sigma) and incubated with BHK-21 cell lysate transfected with pSFV-HLA-C for 2 h at 4°C with gentle rocking. Beads were washed four times with lysis buffer (10 mM Tris buffer [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100). Precipitant complexes were resolved on a NuPAGE 4–12% gel and detected with anti-FLAG M2-HRP conjugates (1:500) (Sigma) and goat anti-human HLA-C antibodies (1:200) (Santa Cruz) and goat anti-human immunoglobulin G (IgG) (H+L; 1:5,000) (Invitrogen). Controls were included by using 2.5 μg *E. coli* bacterial alkaline phosphatase (BAP)-FLAG (Sigma) preadsorbed onto M2 affinity beads and 50 μg untransfected BHK-21 cell lysate.

HLA-C knockdown in A549 cells and constitutive expression of HLA-C in NIH-3T3 cells. Stealth RNAi (siRNA) targeted against human HLA-C was purchased from Invitrogen (catalog number 1299003). A total of 6 pmol of stealth RNAi duplex was used to transfect 0.5×10^5 A549 cells by using RNAi Max (Invitrogen). After 24 h posttransfection, A549 cells were checked for reduction in HLA-C level by reverse transcription-PCR (RT-PCR) (HLA-C forward primer 5′-GGACAAGAGCAGAGATACACG and reverse primer 5′-GAGAGACTCATCAGAGCCCT), Western blot analysis, immunofluorescence, and flow cytometry.

For stable expression of HLA-C, ViraPort pseudotypes carrying HLA-C-pFB-Neo were used to infect NIH-3T3 cells. Infected cells were selected under Geneticin (Invitrogen) at 500 μg/ml. Selected cells were verified for HLA-C expression.

Immunofluorescence microscopy for HLA-C expression. To evaluate the surface expression of HLA-C expression in transduced NIH-3T3 cells and siRNA-treated A549 cells, cells grown on coverslips were fixed in PBS containing 4% paraformaldehyde for 15 min and quenched in PBS containing 50 mM NH_4Cl for 10 min at room temperature. Unpermeabilized cells were blocked for 1 h at room temperature in PBS containing 10% FCS and 5% normal goat serum (Invitrogen). Cells were incubated for 1 h with goat anti-human HLA-C (200 μg/ml; Santa Cruz Biotechnology) (1:200) in PBS with 2% goat serum and then washed and stained with anti-human FITC-conjugated secondary antibodies (1:500; Invitrogen) for 30 min. Coverslips were washed and mounted onto slides by using antifade medium containing DAPI (4′,6-diamidino-2-phenylindole; Invitrogen) prior to image analysis by fluorescence microscopy (Eclipse 80i Nikon).

Real-time PCR quantitation of HCoV-HKU1 pseudotyped virus attached on cell surfaces. A total of 40 ng HCoV-HKU1 pseudotypes were inoculated in A549 cell culture, with and without HLA-C knockdown, and in NIH-3T3 cell culture, with and without HLA-C transfection, at cell densities of 10^5/well in a 24-well plate for 1 h at 4°C. After three washes, total RNA was recovered from cells in each well by using RNeasy (Qiagen), the copy number of attached virus was estimated by real-time PCR using primers and conditions adapted from Brussel and Sonigo (2) for detection of the human immunodeficiency virus (HIV) RNA viral genome matching the pseudoviral backbone vector pNL4-3-delta E-eGFP positions 557 to 690 (42). For normalization of the mRNA amount, human and mouse β-actins in each sample were quantified. All PCRs were performed under the recommended conditions for LightCycler FastStart Sybr green (Roche), as follows: 1 cycle at 95°C for 10 min, then 40 cycles at 95°C for 10 s, 50°C for 5 s, and 72°C for 10 s and 1 cycle for the melting curve. The copy number of the HCoV-HKU1 pseudotype viral genome was compared to that from a serial dilution of HIV standard templates in the TA vector (Invitrogen). Primers for amplifications were as follows: HIV forward primer 5′-TGTGTGC CCGTCTGTTGTGT and reverse primer 5′-GATCTCTCGACGCAGGACTC; human β-actin forward primer 5′-CGTACCACTGGCATCGTGAT and reverse primer 5′-GTGTTGGCGTACAGGTCTTTG; mouse β-actin forward primer 5′-CGTGGGCCGCCCTAGGCACCA and reverse primer 5′-TTGGCCTTAG GGTTCAGGGGGG.

Quantitation of surface HLA-C molecule expression by measuring MEFL. Tested cells were surface stained with goat anti-human HLA-C (200 μg/ml; Santa Cruz) at a 1:150 dilution and counterstained with rabbit anti-goat IgG

(H+L) FITC conjugate (1.5 mg/ml; Invitrogen) at a 1:100 dilution. Purified goat IgG was used as the isotypic controls (Invitrogen). The number of fluoresceins binding to the cells, measured in terms of molecules of equivalent fluorescein (MEFL), was cross-matched with the eight-peak Sphero rainbow calibration particles (Spherotech, Inc.), determined by flow cytometry (FASCalibur; Becton Dickinson). Net MFEL of tested cells were calculated by subtraction of their isotypic controls. FITC-stained cells were also observed by immunofluorescence microscopy.

Neuraminidase treatment and HCoV-HKU1 pseudotyped virus infection. A549 cells grown in 24-well plate to a confluence of 1×10^5 were washed twice with 1× PBS (Invitrogen) and incubated with neuraminidase from *Clostridium perfringens* (Sigma), using DMEM free of FBS as diluent. After incubation at 37°C with 5% CO_2 for 1 h, cells were washed three times with DMEM and infected with 40 ng HCoV-HKU1 S pseudotypes, with or without preincubation with polyclonal HLA-C antibody as described in previous section. Infectivity was measured for GFP expression by flow cytometry (FASCalibur; Becton Dickinson).

RESULTS

Range of target cells susceptible to HCoV-HKU1 S-dependent infection. The establishment of pseudotyped virus provided a surrogate model for studying the viral tropism of HCoV-HKU1. For this analysis, we employed HIV-1-derived reporter virus encoding the human codon-optimized S protein of the HCoV-HKU1 to investigate the range of cells permissive to HCoV-HKU1 S protein-mediated entry (Fig. 1A). Pseudotypes encoding the G protein of the amphotropic VSV were used as positive control. Efficiency of infection with pseudotyped viruses can be conveniently quantified by the expression of a reporter gene encoded by the proviral genome. Among the tested cell lines, A549 (human lung epithelial cells) showed the highest susceptibility to pseudotyped virus, which was five to sixfold higher than those of the other two lung cell lines, Hep-2 and MRC-5, suggesting stronger tropism of HCoV-HKU1 to lower-respiratory-tract epithelial cells. Two other intestinal cell lines (CaCO-2 and HRT-18) and one liver cell line (Huh7) exhibited susceptibility to HCoV-HKU1 pseudotypes but at lower levels. Kidney-derived cell lines and ACE2-overexpressed cell line were uninfected, which showed that HCoV-HKU1 S-mediated entry was achieved using distinct sets of cellular receptors unrelated to SARS-CoV. The pattern of tissue tropism indicated by the pseudotyped virus correlated with the detection of the virus in nasopharyngeal aspirate and stool specimens (12, 21, 32). Another notable observation was that the infectivity is viral load dependent. The infectivity was six- to sevenfold higher when the viral load used is threefold higher when infecting the A549 cells. The relationship between viral load and pseudotype infectivity in A549 cells is demonstrated in Fig. 1B. Infectivities in A549 cells increased as viral load was increased from 5 ng and started to saturate at 40 ng. This signified that the infection is not arbitrary. All cell lines were infected readily by VSV-G pseudotypes, which served as the control.

Infectious entry of HCoV-HKU1 S-bearing pseudotypes into A549 cells could be blocked by serum of a convalescent patient recovering from HKU1 infection but not by control sera from patients infected with SARS or influenza, indicating the specificity of the of S neutralizing activity (data not shown).

Expression of soluble $S_{1\text{-}600}$ glycoprotein and binding assay. S glycoprotein of coronaviruses plays a pivotal role in mediating viral entry (14). We have evidence that HCoV-HKU1 S will undergo furin cleavage into N-terminal S1 and C-terminal S2 subdomains as a classical example of group 2 coronavirus S in which the S1 subunit contains the receptor binding domain (RBD). Expression of full-length secretion protein, $S_{1\text{-}760}$, is not efficient (3). Studies of RBD structure in SARS-CoV (amino residues 318 to 510) have revealed that it consists of two subdomains (1, 35, 40). The first subdomain that contains the RBD is similar to the analogous regions of other group 2 coronaviruses. The receptor binding motif (residues 424 to 494) is unique to SARS-CoV and is critical in docking with ACE2. To determine whether the soluble HCoV-HKU1 $S_{1\text{-}600}$ domain can interact with A549 cells, we purified the secreted protein from the medium and confirmed that it was expressed as an 88-kDa N-linked Endo H-resistant glycoprotein (Fig. 2A). The binding of this protein to A549 cells was detected by anti-FLAG antibody as shown by flow cytometry. We found that HCoV-HKU1 $S_{1\text{-}600}$ can bind to A549 cells but not to nonpermissive BHK-21 cells (Fig. 2B and E, respectively), while the blocking of binding to A549 cells was observed when HCoV-HKU1 S1 was preincubated with serum from a HCoV-HKU1-infected patient but not when preincubated with serum of a healthy donor (Fig. 2C and D, respectively). These indicate that $S_{1\text{-}600}$ contains the RBD which can interact with and bind to potential receptor(s) present on the surface of A549 cells.

cDNA library screening and transduced cell sorting by flow cytometry. The A549 cDNA library was constructed and expressed in Viraport system to identify the HCoV-HKU1 receptor. Library transduced BHK-21 cells were bound to soluble S1 glycoprotein as an affinity ligand and sorted by flow cytometry. The primary library contained 2×10^6 to 3×10^6 individual cDNA clones in the pFB retroviral vector, which achieve more than a complete representation of mRNA molecules of mammalian cells, and was packaged in VSV-G enveloped pseudotypes to mediate infectious entry into BHK-21 cells. BHK-21 cells were transduced at an MOI of <2 to avoid multiple integration in each cell if infected by multiple viral particles.

At 48 h postinfection, transduced cells bound to S1 protein were recovered by FACS sorting. Recovered cells were grown in medium to expand into cell clusters and then lysed for PCR. Amplified cDNA proviral inserts were ligated into the TOPO vector (Invitrogen) and sequenced. DNA sequences from each fragment were queried against the NCBI nr database by using BLAST. Among the sequenced clones, HLA-C, a surface molecule, was identified from two individual clones of different insert sizes with 99% match to GenBank data (accession number NM_002117).

In vitro interaction of S1-FLAG with HLA-C. To confirm the authenticity of CoV-HKU1 $S_{1\text{-}600}$ protein binding to HLA-C-expressing cDNA-transduced BHK-21 cells, in vitro coimmunoprecipitation was performed (Fig. 3). HLA-C was transiently expressed in BHK-21 cells transfected with recombinant plasmid HLA-C/pSFV-1. The result confirmed the specificity of interaction between immunopurified recombinant CoV-HKU1 $S_{1\text{-}600}$ preadsorbed on beads, as HLA-C was able to pull down expressed HLA-C from the lysate, while the control protein BAP-FLAG failed to bind HLA-C.

Verifying the role of HLA-C in HCoV-HKU1 S-bearing pseudotype entry. To determine the influence of HLA-C on HCoV-HKU1 S-mediated entry into A549 cells, HLA-C ex-

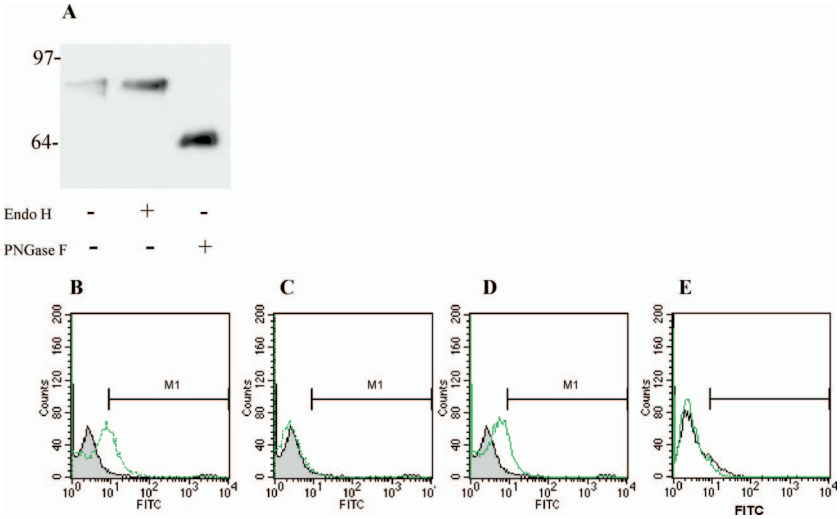

FIG. 2. Expression of secreted S1 which can bind to A549 cells. (A) Secreted $S_{1\text{-}600}$ was expressed as an 88-kDa N-linked glycoprotein which is Endo H resistant. +, test performed; −, test not performed. (B) S1-bound A549 cells were measured by flow cytometry using M2 anti-FLAG-FITC against C-terminally tagged FLAG (FACSCalibur; Becton Dickinson). Binding is inhibited by preincubated $S_{1\text{-}600}$ with convalescent serum from a CoV-HKU1-infected patient (C), but not by normal-donor serum (D). (E) $S_{1\text{-}600}$ does not bind to nonpermissive BHK-21 cells. Black line with gray-shaded area, A549 cell line; black line with white area, BHK-21 cell line; green line, S1 binding.

pression was neutralized by siRNAs and antibody blocking independently. Two nonoverlapping stealth RNAi duplexes can suppress 80% of HLA-C expression as detected by flow cytometry (Fig. 4C). The efficiency of HLA-C knockdown was also confirmed by Western blot analysis and immunofluorescent microscopy (Fig. 4A and B, respectively). HLA-C knockdown and HLA-C antibody-preblocked A549 cells were challenged by HCoV-HKU1 pseudotyped viral infection and compared with untreated A549 cells. GFP expression was used as a surrogate marker for entry and determined by FACS (Fig. 4D and E). The two strategies were found to have similar levels of effectiveness and resulted in a 62 to 65% reduction in viral entry. The saturation effect in the inhibition of entry by blocking HLA-C with increasing concentrations of antibody indicated that the pseudotyped virus may enter cells by another receptor(s).

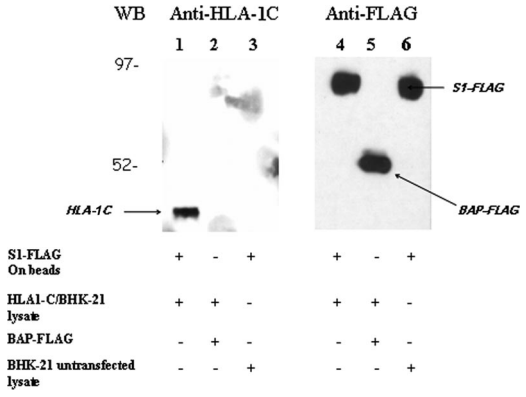

FIG. 3. In vitro interaction of CoV-HKU1 S1-FLAG with HLA-C by coimmunoprecipitation. Purified $S_{1\text{-}600}$-FLAG preadsorbed onto anti-FLAG M2 affinity beads was incubated with HLA-C-transfected BHK-21 cell lysate. Precipitant complexes were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed with anti-human HLA-C (lanes 1 to 3) and anti-FLAG-M2-HRP (lanes 4 to 6) by Western blotting (WB). BAP-FLAG (Sigma) and untransfected BHK-21 cell lysate were used as control. HLA-C-transfected BHK-21 cell lysate was recognized, pulled down by S1-FLAG, and coprecipitated and detected by both antibodies (lanes 1 and 4). No cross-reaction was observed between HLA-C and BAP-FLAG, and only BAP-FLAG was detected (lanes 2 and 5), while S1-FLAG did not show nonspecific recognition with untransfected cell lysate (lanes 3 and 6). +, components added; −, components not added.

Real-time PCR quantitation of HCoV-HKU1 pseudotypes on the cell surface. HLA-C cDNA insert of A549 cells was ligated to the pFB-Neo vector (Stratagene). The construct was cotransfected with ViraPort retroviral vector and packaged in VSV-G pseudotypes. Infected NIH-3T3 cells were selected under Geneticin and tested for the HLA-C expression. Over 96% of the stably transfected cell population was stained strongly by anti-human HLA-C antibody, as detected by flow cytometry and immunofluorescent microscopy (Fig. 5A and C, respectively). The cells were challenged with HCoV-HKU1 S pseudotyped virus as described in the previous section. HLA-C-expressed NIH-3T3 cells were shown to have lower infection efficiency for HCoV-HKU1 S pseudotyped virus than A549 cells (Fig. 5D). We then tested whether HLA-C enhances HCoV-HKU1 S pseudotype infection by attachment. Transduced NIH-3T3 and A549 cells were challenged with HCoV-HKU1 S pseudotypes as in previous assays with incubation temperature changed to 4°C to enhance viral attachment. After three washes to remove unbound viruses, total RNA was recovered from cell lysis. The copy number of the HIV RNA genome in pseudotyped viruses was determined by real-time PCR quantification. Results showed that HCoV-HKU1 S pseudotypes can attach to cell surfaces more efficiently in transduced NIH-3T3 and A549 cells by 100-fold and 200-fold, respectively, than in untransduced NIH-3T3 and A549 cells with HLA-C knockdown by siRNAs. Besides, A549 cells can retain 72-fold more viral copies than HLA-C-transduced NIH-

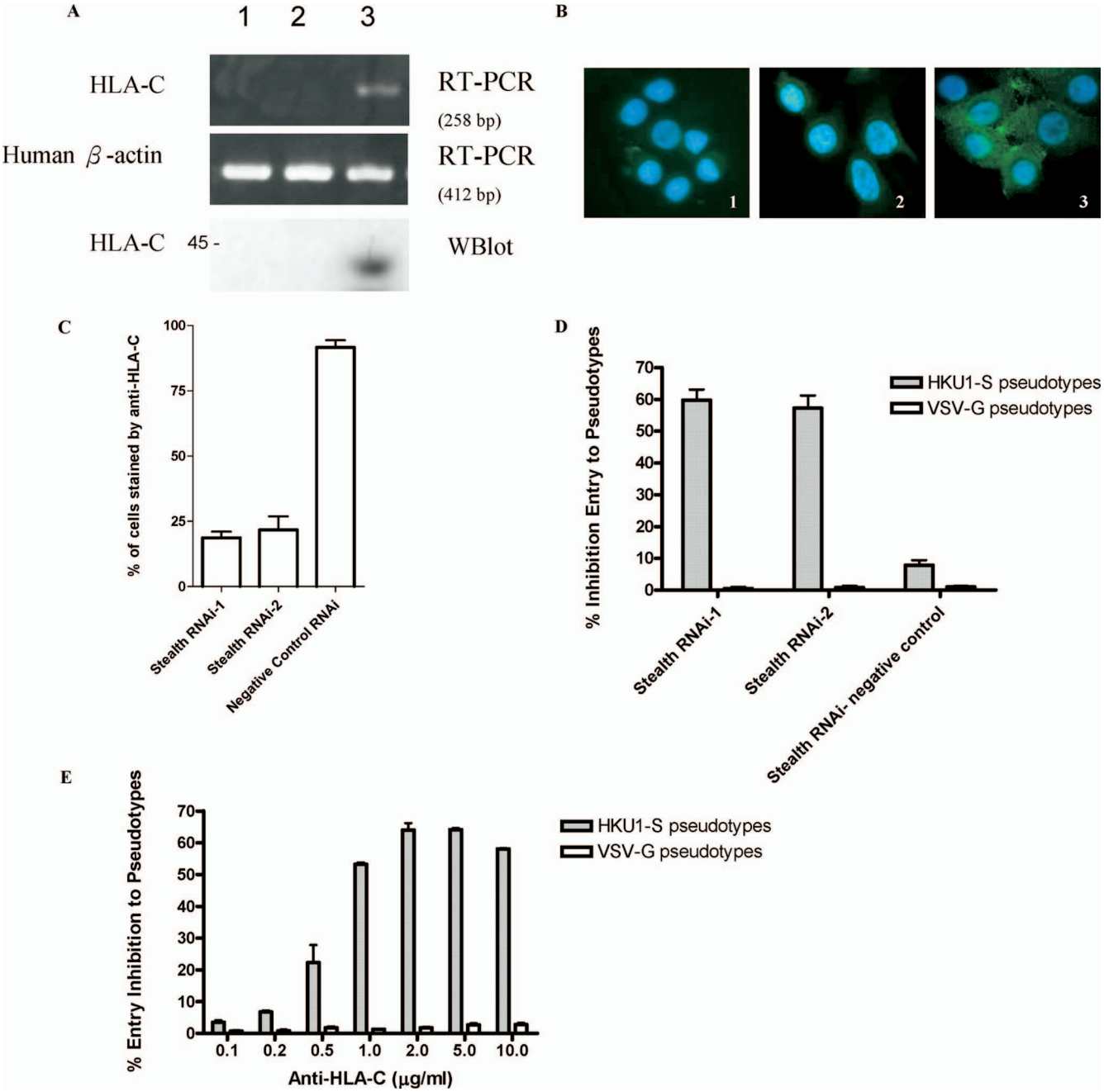

FIG. 4. Inhibition of HCoV-HKU1 S-pseudotyped virus entry into A549 cells by knockdown of HLA-C expression by siRNAs or by anti-HLA-C antibody. (A) A549 cells were transfected with siRNA1 (lane 1), siRNA2 (lane 2), and siRNA negative control (lane 3) and harvested for RT-PCR after 24 h. (B and C) A549 cells treated by stealth RNAi-1 (lane 1), stealth RNAi-2 against HLA-C (lane 2), and RNAi negative control (lane 3) for 24 h were fixed and surface stained by goat anti-human HLA-C (200 μg/ml; Santa Cruz) (immunofluorescence [IF], 1:100 and 1:150 for flow cytometry) and rabbit anti-goat IgG (H+L) FITC (IF, 1:500 and 1:100 for flow cytometry) and analyzed by immunofluorescent microscopy (Eclipse 80i Nikon) (B) and flow cytometry (FACSCalibur; Becton Dickinson) (C). (D and E) Effects on entry of CoV-HKU1 S pseudotyped virus. The nucleus was stained by DAPI in blue. A549 cells were either transfected with two individual duplexes of siRNAs for 24 h (D) or preincubated with polyclonal HLA-C antibodies at various concentrations as indicated for 1 h at 37°C (E). A total of 40 ng of CoV-HKU1 pseudotyped virus was inoculated onto 1×10^5 treated or untreated A549 cells in 24-well plates and incubated for 1 h at 37°C after three washes and being replenished with fresh medium. Percentage of infection was indicated by GFP expression and determined by flow cytometry. VSV-G pseudotypes were included as positive control.

3T3 cells by quantitative PCR, suggesting that some other surface receptors or coreceptors might also participate in the entry process (Fig. 5E).

Quantities of surface HLA-C molecules not directly correlated to HCoV-HKU1 S pseudotype entry. The amounts of surface HLA-C molecules in cell lines permissive to HCoV-HKU1 S pseudotype infection quantified in terms of MEFL are compared in Table 1. The fluorescence intensities in the tested cell lines were also shown by immunofluorescence microscopy and correlated well with each other (Fig. 6). The most susceptible

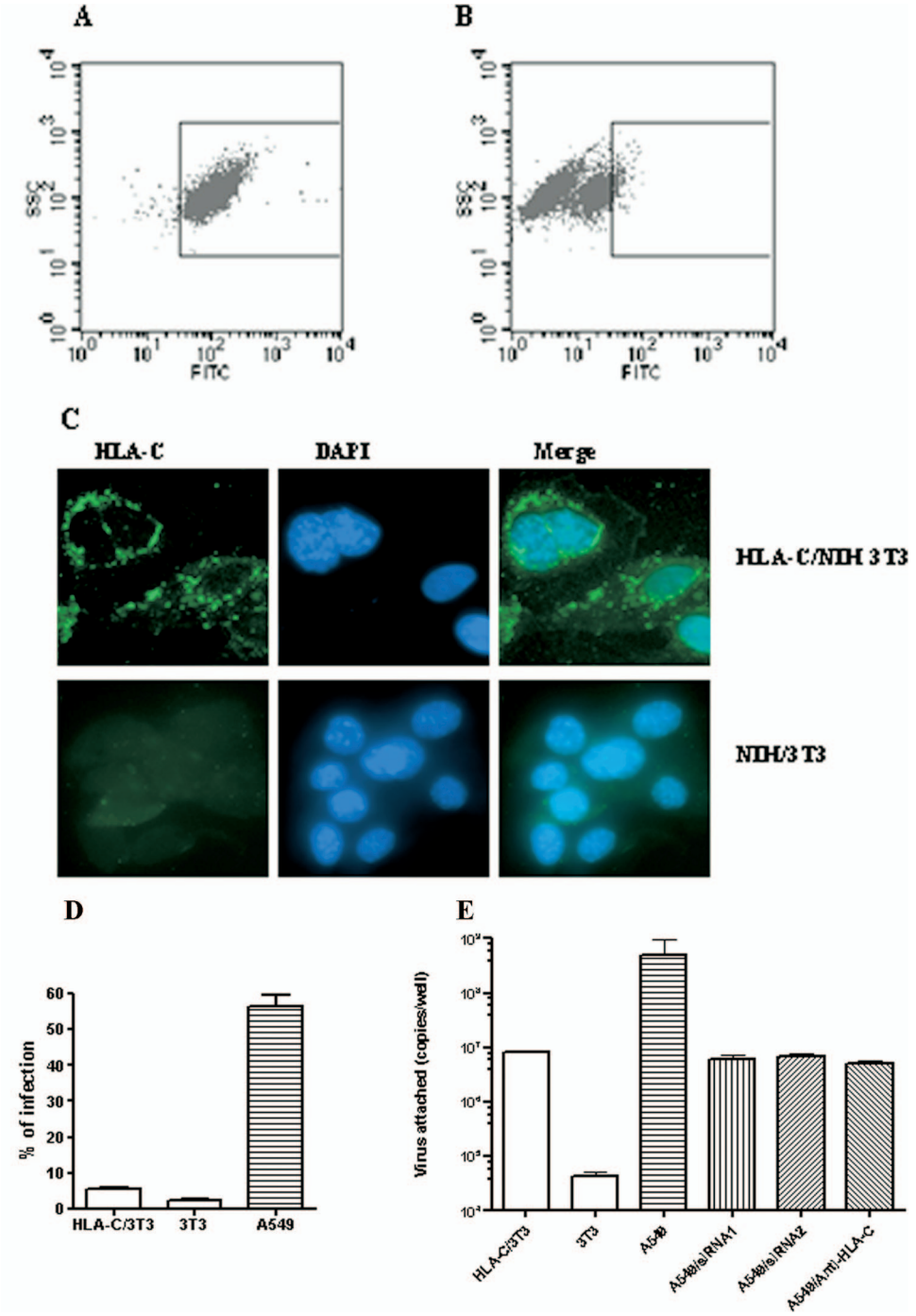

FIG. 5. Expression of HLA-C does not restore CoV-HKU1 infection in nonpermissive cell lines but enhances viral binding to cell surfaces. HLA-C was stably expressed in HCoV-HKU1 nonpermissive cells. NIH-3T3 (3T3) cells were infected with VSV-G pseudotyped virus carrying human HLA-C/pFB-Neo and selected under Geneticin. NIH-3T3 cell lines constitutively expressing HLA-C were surface stained with anti-human HLA-C polyclonal antibodies and determined by flow cytometry (HLA-C/3T3 [A] and NIH-3T3 cells [B], respectively) and immunofluorescence (C). A total of 40 ng CoV-HKU1 pseudotyped virus was inoculated onto cells at the concentration of 1×10^5 cells/well in 24-well plates. For infectivity studies, virus was incubated with cells at 37°C for 1 h and washed and replenished with fresh medium. (D) GFP expression was measured by flow cytometry 48 h postinfection. For quantitation of viral attachment, pseudotyped virus was inoculated onto cells at 4°C for 1 h. After three washes, total RNA was harvested from cell lysis. (E) The number of viral copies was determined by real-time quantitative PCR.

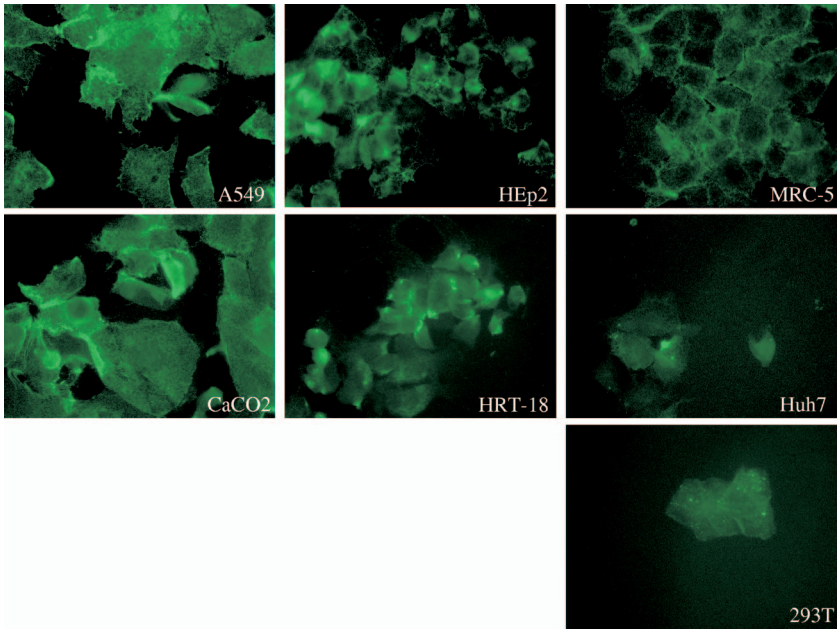

FIG. 6. Immunofluorescence microscopy showing surface staining of HLA-C in tested human cell lines. All tested human cell lines were able to be stained positively for the presence of HLA-C. The differential intensities corroborate well with the relative MEFL ratios shown in Table 1.

cell line, A549, demonstrated in Fig. 1A, did not have the highest abundance of surface expression of HLA-C molecules. CaCO2 and Huh-7 both came second in the order of susceptibility, with a 6.17-fold difference in surface expression of HLA-C. This indicates that Huh-7 might contain other entry receptors, as in A549, despite the lower amount of HLA-C. Similarly, though 293T cells were also stained positive with HLA-C, they were not permissive to infection by HCoV-HKU1 S pseudotyped virus. Despite comparable levels of HLA-C surface expression, A549 and HLA-C/3T3 are distinctive in susceptibilities to infection and viral binding. Altogether, HLA-C was shown to function as an attachment factor which enhances viral entry but may function with only as-yet-unknown receptors or coreceptors.

Effect of neuraminidase treatment on HCoV-HKU1 S pseudotyped virus infection. Some group 2 coronaviruses like BCoV and HCoV-OC43 utilize O-acetylated sialic acid as entry receptor, as they do for influenza C virus (27, 33). To find out whether sialic acid is crucial to HCoV HKU1 S-mediated infection, A549 cells were treated with neuraminidase from *Clostridium perfringens*, a bacterial neuraminidase which can completely remove agglutination to erythrocytes by BCoV, and HCoV-OC43 (33) and then challenged by HCoV-HKU1 S pseudotyped virus (Fig. 7). Pretreatment with neuraminidase alone reduced infection from 5% with 5 mU/ml to 13% with 50 mU/ml and beyond (Fig. 4). This was far less efficient than the blocking by HLA-C antibody, with a reduction in infection by 22% (0.5 μg/ml) to 62.5% (2 μg/ml).

DISCUSSION

In this study, we showed that HLA-C binds HCoV-HKU1 S and serves as the attachment factor involved in HCoV-HKU1 cell entry. Using expression of human lung epithelial cell (A549) cDNAs in the nonpermissive kidney cells (BHK21) from a retroviral library, HLA-C was identified from two of the sequenced clones and confirmed to bind purified soluble HCoV-HKU1 $S_{1\text{-}600}$ glycopeptide. Blocking of HLA-C by siRNA silencing and anti-HLA-C antibody inhibited HCoV-HKU1 S pseudotyped vi-

TABLE 1. The differential surface expression of HLA-C in different cell lines indicated by MEFL

Cell line	Net MEFL[a]	Relative MEFL to A549 cells
A549	12,506	1.0
HEp2	8,017	0.64
MRC-5	6,603	0.53
CaCO2	28,854	2.31
HRT-18	11,120	0.89
Huh7	4,670	0.37
293T	3,816	0.31
NIH 3T3	117	0.01
HLA-C/3T3	14,080	1.12

[a] Net MEFL, the measured MEFL minus the isotypic background control.

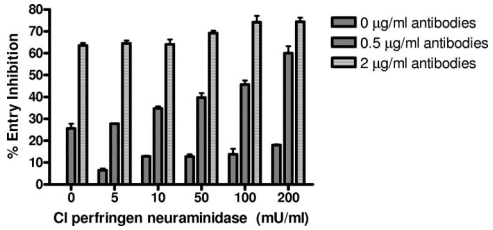

FIG. 7. Effect of neuraminidase treatment of A549 cells on CoV-HKU1 S pseudotyped virus infection. A549 cells grown in 24-well plates were incubated with the indicated concentrations of neuraminidase from *C. perfringens* (Cl perfringen) for 1 h prior to inoculation with 40 ng CoV-HKU1 S pseudotyped virus, with or without 1 h of preincubation with anti-HLA-C polyclonal antibody.

rus infection of A549 cells by 62 to 65%, suggesting that HLA-C serves as an attachment factor that facilitates HCoV-HKU1 S-mediated infection. However, constitutional expression of HLA-C in NIH-3T3 cells failed to render the nonpermissive cell line susceptible to infection, although it increased the binding of HCoV-HKU1 S pseudotyped virus onto cell surfaces. The results indicated that other receptors are involved in the cellular entry of HCoV-HKU1. Neuraminidase treatment has little effect on pseudotyped virus entry into A549, which therefore suggests that sialic acid is less likely to be the key receptor as distinct from the case of other group 2 coronaviruses including the HCoV-OC43 and the BCoV.

HLA class I molecules, encoded by the major histocompatibility complex (MHC), are ubiquitously expressed on all human nucleated cells and function as highly specialized antigen-presenting molecules by forming complexes with processed antigen peptides from endocytosed molecules for recognition by T lymphocytes, thus playing a pivotal role in immune responses. Structurally, all HLA class I molecules are composed of three α chains noncovalently associated with a β-microglobulin molecule. The three α chains are organized into three external domains and followed by a hydrophobic transmembrane domain and hydrophilic cytoplasmic tail. Since the membrane-proximal domain possesses the basic Ig fold structure, class I MHC molecules are classified as members of the Ig superfamily. Although an immunohistological study has shown that HLA-ABC antigens were strongly stained along the epithelial linings of trachea and bronchioles as well as at the alveolar epithelium in the respiratory system (9), the cell tropism and clinical manifestations of HCoV-HKU1 infections cannot be fully explained. In our present study, HLA-C is highly expressed in a human intestinal epithelial cell line (CaCO-2) and also expressed in human hepatocellular epithelial cells (Huh7), which is compatible with reports of finding this virus in diarrheal stool of patients as well as in a patient with hepatitis (12, 32). HLA class I antigen has been reported as one of the receptors for another group 2 HCoV, HCoV-OC43, with infectivity in human rhabdomyosarcoma cells completely blocked by antibody against HLA class I antigen (7, 8). As for other viruses, the class I MHC complex molecule has also been reported as being an essential receptor component for simian virus 40 entry (25). The MHC class I α2 domain on the surface of human epithelial and B lymphoblastoid cells can also bind adenovirus type 5 fiber knob as a high-affinity receptor (17). In the present study, HLA-C was shown to be involved in the attachment of HCoV-HKU1 to A549 cells. However, its surface expression on the nonpermissive cell line NIH-3T3 was not sufficient to confer susceptibility to CoV-HKU1 S pseudotyped virus. Moreover, A549 cells were able to bind CoV-HKU S 72-fold higher than HLA-C-expressing NIH-3T3 cells. In addition, the abundance of HLA-C in the cell lines does not correlate directly with the degree of susceptibility by HCoV-HKU1 S pseudotypes. Although the amount of receptor required for cell entry can be very small, these findings still suggest that an additional surface receptor(s) present on A549 cells might be utilized by S, which facilitates membrane fusion and entry of HCoV-HKU1.

Cell entry by viruses involves two sequential steps: attachment and entry. Interaction with the attachment receptor, though it is not required, increases infectivity by concentrating viruses on the surface of target cells, thereby increasing the chances of receptor docking and engagement leading to subsequent entry (15, 31). Many viruses use multiple alternative receptors and/or coreceptors for the same step, which may facilitate infection of different tissues or hosts. For example, HIV uses CD4 together with coreceptor CCR5 or CXCR4, CCR2b, CCR3, CCR8, and CCR9 to trigger fusion of viral and cellular membranes and confer virus entry into cells (6). The S of coronaviruses is known to be the largest viral S protein, and therefore, it is possible for it to have multiple functional surface domains which exploit multiple receptors to facilitate infection in different cell types (18, 28). For SARS-CoV, the key host-cell receptor attached by S is the ACE2 (22). DC-SIGN and related protein DC-SIGNR may serve as pathogen attachment factors and have been shown to promote SARS-CoV entry through engagement with ACE2 (16, 24), although DC-SIGNR has also been proposed as the receptor for SARS-CoV (18). As for MHV, it has been shown that the hemagglutinin esterase protein enhances the efficiency of infection and promotes viral dissemination in at least some tissues, presumably by serving as a second receptor-binding protein. CEACAM1 has been shown as being the receptor for initiation of infection, and the existence of coreceptors has not been demonstrated (13, 19). Heparan sulfate was once reported as being the entry receptor utilized by MHV; it was subsequently demonstrated to function as binding molecules for MHV and IBV (10, 34). Further studies are required to elucidate other possible receptors or coreceptors for HCoV-HKU1.

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