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Clathrin-Dependent Entry of Severe Acute Respiratory Syndrome Coronavirus into Target Cells Expressing ACE2 with the Cytoplasmic Tail Deleted▽

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The penetration of various viruses into host cells is accomplished by hijacking the host endocytosis machinery. In the case of severe acute respiratory syndrome coronavirus (SARS-CoV) infection, viral entry is reported to require a low pH in intracytoplasmic vesicles; however, little is known about how SARS-CoV invades such compartments. Here we demonstrate that SARS-CoV mainly utilizes the clathrin-mediated endocytosis pathway for its entry to target cells by using infectious SARS-CoV, as well as a SARS-CoV pseudovirus packaged in the SARS-CoV envelope. The SARS-CoV entered caveolin-1-negative HepG2 cells, and the entry was significantly inhibited by treatment with chlorpromazine, an inhibitor for clathrin-dependent endocytosis, and by small interfering RNA-mediated gene silencing for the clathrin heavy chain. Furthermore, the SARS-CoV entered COS7 cells transfected with the mutant of ACE2 with the cytoplasmic tail deleted, SARS-CoV receptor, as well as the wild-type ACE2, and their entries were significantly inhibited by treatment with chlorpromazine. In addition, ACE2 translocated into EEA1-positive early endosomes immediately after the virus attachment to ACE2. These results suggest that when SARS-CoV binds ACE2 it is internalized and penetrates early endosomes in a clathrin-dependent manner and that the cytoplasmic tail of ACE2 is not required for the penetration of SARS-CoV.

Severe acute respiratory syndrome (SARS) is an emerging infectious disease with high mortality caused by infection of the respiratory system by SARS coronavirus (SARS-CoV). SARS-CoV is a human enveloped coronavirus containing a helical nucleocapsid structure composed of a single-stranded, positive-polarity RNA of approximately 29 kb plus the poly(A) tail (44). Phylogenetic analysis classifies the coronaviruses into three groups based on their genetic and serological relationships.

The first essential step in virus infection is the entry of viruses into host cells. After their attachment to their respective cell surface receptor, most viruses make use of cellular endocytosis machineries, such as clathrin-dependent and -independent pathways, for their entry (22, 29). Clathrin-dependent endocytosis has been well characterized using growth factor receptors such as the transferrin receptor (23), epidermal growth factor receptor (EGFR) (39), and the keratinocyte growth factor receptor (3). The endocytosed receptors are translocated into endosomes, where they are degraded or recycled to the cell surface. Similarly, various viruses, among them Semliki Forest virus, vesicular stomatitis virus (VSV), and influenza virus, enter into host cells via the clathrin-dependent endocytosis pathway and translocate into endosomes, where they are uncoated (10, 35, 40). The clathrin-dependent endocytosis is initiated by the binding of adaptor protein 2 (AP2) complexes to the cytoplasmic tail of the cell-surface receptors, which recruits clathrins (27, 38). Subsequently, the receptors are invaginated to form "pits," which are surrounded by a spherical cage-like structure made of clathrin triskelions. Viruses bound to the receptors are endocytosed similarly and then transported to vesicles called early endosomes. It is well known that early endosomes are somewhat acidic (pH 6.5 to 6.0) and become more acidic as they mature to form late endosomes (pH 6.0 to 5.5). The acidification of endosomes is required for incorporated viruses to establish an infection (22, 29).

On the other hand, the clathrin-independent pathways include a caveola-dependent pathway. Caveolae are relatively smaller vesicles of 50 to 100 nm in diameter, formed by membrane invagination at the cell surface, and coated by caveolin-1 (12, 15, 28). Simian virus 40 (SV40), for example, utilizes caveolae to be internalized into the "caveosomes" under a neutral condition (30). In contrast, effective internalization of SV40 was also found in cells that do not express caveolin-1, suggesting that SV40 utilizes not only the caveola-dependent pathway but also the lipid-raft-dependent and caveola-independent pathway (6). Other viruses utilizing the caveola-dependent pathway include some of the picornaviruses (21), papillomaviruses (4), filoviruses (8), and retroviruses (2).

The binding and subsequent entry of SARS-CoV into the host cells are primarily mediated by a viral spike glycoprotein, called S protein, which binds to its receptor, angiotensin-converting enzyme 2 (ACE2) (16). ACE2 is a cell-surface-bound enzyme of the type I membrane protein topology, with its

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catalytic site exposed to the extracellular surface. ACE2 along with its related family protein ACE is indispensable for blood pressure homeostasis via the renin-angiotensin system (31). However, despite the 40% amino acid identity shared by ACE and ACE2, ACE does not act as a SARS-CoV receptor (25). The broad expression profile of ACE2, which includes the gastrointestinal tract and lungs, matches well with the affected organs in patients with SARS (9). Although accumulating evidence has documented a physical and functional interaction between SARS-CoV and ACE2, little is known about how the ACE2-mediated entry of SARS-CoV is linked to cellular endocytosis machineries.

In the present study, we investigated the internalization mechanisms of SARS-CoV after binding to ACE2. We found that SARS-CoV hijacks the clathrin-dependent machinery for endocytosis via ACE2 with the cytoplasmic tail deleted, as well as the wild-type ACE2.

MATERIALS AND METHODS

Cell lines. The cell lines used here were human hepatoma HepG2 and monkey kidney COS7 cell lines, which were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (FCS) and antibiotics, under 5% CO_2 in a humidified incubator.

Virus and infection. SARS-CoV (Vietnam/NB-04/2003) was maintained in Vero E6 cell cultures as described previously (43). In brief, the SARS-CoV titer of the stock virus was determined by infection of Vero E6 cells by a 50% tissue culture infective dose. Cells were inoculated by SARS-CoV at a multiplicity of infection of 1 and allowed to adsorb the virus for 1 h at 4°C. Subsequently, the cells were washed with phosphate-buffered saline (PBS) and cultured for the indicated times. All experiments using infectious SARS-CoV was done in a laboratory certified with biosafety level 3.

RNA extraction and RT-PCR. Total RNA from HepG2 cells infected with SARS-CoV was extracted with the TRIzol reagent (Invitrogen Corp.) by the following the protocol. Reverse transcription-PCR (RT-PCR) for the detection of viral RNA was performed with Titan One-Tube RT-PCR kit (Roche Molecular Systems) by following the manufacturer's protocol. The primer sequences for SARS-CoV detection were gained from World Health Organization network laboratories. The sequence of BNIoutS2 (sense) is 5′-ATGAATTACCAAGTCAATGGTTAC-3′, and the sequence of BNoutAs (antisense) is 5′-CATAACCAGTCGGTACAGCT AC-3′. For an internal control, a primer set of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used. The sequence of GAPDH-F (sense) is 5′-AGTC AGCCGCATCTTCTTTTGC-3′, and the sequence of GAPDH-R (antisense) is 5′-CTCCTGGAAGATGGTGATGGGA-3′.

Pseudoviruses. The pseudoviruses were SARS-CoV(HIV), VSV(HIV), and A-MLV (amphotropic murine leukemia virus)(HIV), which consist of the viruses' respective envelope glycoprotein, the HIV Gag/Pol proteins, and a luciferase plasmid. The pseudoviruses were produced with methods described previously (41). In brief, 293T cells were transfected with 7 μg of pCMVΔR8.1 (HIV Gag/Pol plasmid) and 7 μg of pHR′-luciferase, along with an expression vector for the respective viral envelope glycoprotein: 800 ng of pCMV-SARS-S (SARS-CoV S plasmid), 7 μg of pMD.G (VSV G plasmid), or 7 μg of pDJ (A-MLV envelope glycoprotein plasmid). The 293T cells were cultured in 10-cm dishes, and transfection was performed by the calcium phosphate method. At 48 h posttransfection, the culture supernatants were harvested and filtered through 0.45-μm-pore-size screens. These filtered supernatants were used to infect host cells with the pseudoviruses.

Preparation of ACE2 mutants. Three mutants of ACE2 were prepared from pcDNA ACE2 expression vector (20). ACE2-Δtail mutant has a stop codon at the end of the transmembrane domain, ACE2-Δtail-TM mutant is ACE2-Δtail mutant replaced with the transmembrane domain derived from EGFR, and ACE2-sol consists of the extracellular domain of ACE2. The extracellular domains of ACE2-sol from pcDNA ACE2 were amplified by PCR and introduced into pCXN2 after XhoI digestion. These plasmids were transfected with FuGeneHD according to the manufacturer's instructions (Roche Molecular Systems).

Luciferase assay. Cells were infected with pseudovirus for 12 h, and the culture medium was then replaced with virus-free Dulbecco modified Eagle medium. After further 48 h of incubation, the cells were lysed for luciferase assays. The Dual-Luciferase Reporter Assay System (Promega) was used according to the manufacturer's protocol. The luciferase activities of the samples were measured with a Lumat LB 9507 (Berthold).

Inhibition of endocytosis and endosomal acidification. Extraction of cholesterol from the plasma membranes was performed by using methyl-β-cyclodextrin (MBCD; Sigma), as described elsewhere (7). HepG2 cells seeded in a 24- or a 96-well plate 1 day prior to the experiments were treated with serially diluted MBCD for 30 min. After extensive washes with ice-cold PBS, pseudoviruses were added in the absence of MBCD for an additional 12 h. For the inhibition of clathrin-mediated endocytosis, cells were incubated with the respective doses of chlorpromazine (Sigma) for 1 h and then infected with the pseudovirus for an additional 12 h in the presence of chlorpromazine. After extensive washes with PBS, cells were further incubated in the absence of chlorpromazine for 48 h. To determine the pH dependency of the pseudoviruses, the cells were pretreated with serial dilutions of an endosome acidification inhibitor, either NH_4Cl (Sigma) for 1 h, and then infected with pseudoviruses in the presence of the inhibitor. Luciferase activities were determined for cell extracts prepared from these cultures 48 h after the infection.

Immunoblotting. Immunoblotting assays were performed as described previously (17). In brief, cells were lysed in NP-40 lysis buffer (1% Nonidet P-40, 40 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 20 μg of aprotinin/ml). The cell lysates were precleared of cellular debris by centrifugation (10,000 × g) for 30 min at 4°C and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were electrotransferred onto polyvinylidene difluoride membranes (Millipore). The membranes were first blocked with 5%% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 and then probed with the indicated primary antibodies. The antibodies used were anti-human CHC (Santa Cruz), anti-α-tubulin (Sigma), and anti-ACE2 (R&D Systems). After three washes, the membranes were probed with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology). Signals were visualized with a Super Signal Pico Detection kit (Pierce), and digital images were collected with a Lumi-Imager F1 (Roche Molecular Systems).

Depletion of CHC by siRNA. To transfect small interfering RNA (siRNA) designed to target the clathrin heavy-chain gene (Invitrogen Corp.), a reverse transfection method was used by using Lipofectamine RNAiMAX (Invitrogen Corp.) according to the manufacturer's instructions. In brief, 5 pmol of stealth siRNA duplex-Lipofectamine RNAiMAX complexes were used for 10^5 HepG2 cells in a 24-well plate. Gene silencing efficiency was assessed 48 h after the transfection by immunoblotting. The siRNA (clathrin heavy chain [CHC]) target sequence was 5′-UGAAGUAUUGACAUCAAAUUUCCGG-3′. A nonfunctional oligonucleotide was used for the control (Invitrogen Corp.).

Immunofluorescence staining. HepG2 cells were grown in 35-mm glass-bottom dishes (Iwaki) at a density of 4×10^4 cells/dish. After 24 h, the cells were washed twice with PBS, fixed with 4%% paraformaldehyde for 15 min, washed with the wash buffer (0.1% Triton X-100–PBS), and blocked with the same buffer containing 10% FCS. For immunostaining, samples were incubated overnight with the primary antibodies, including anti-ACE2 and anti-EEA1 (Santa Cruz). After three washes, the samples were incubated with the secondary antibodies (Molecular Probes; Alexa 488-labeled and Alexa 594-labeled antibodies) for 1 h. Confocal imaging was performed by using the 510 META microscope with a 60×/1.30-0.60 oil immersion objective lens (Carl Zeiss).

RESULTS

Effects of endocytosis inhibitors on SARS-CoV entry. To investigate the entry mechanism of SARS-CoV, we used SARS-CoV(HIV), because it allows safe, rapid, and quantitative analyses. Two other pseudoviruses, the VSV(HIV) and A-MLV(HIV), were used as control viruses, because VSV(HIV) utilizes the pH-dependent and clathrin-dependent entry mechanisms (40), while A-MLV(HIV) utilizes the pH-independent and clathrin-independent entry mechanisms (2, 25, 40).

HepG2 cells were infected with pseudoviruses, SARS-CoV-(HIV), VSV(HIV), or A-MLV(HIV) in the presence of an endocytosis inhibitor, chlorpromazine. Chlorpromazine is a cationic amphiphilic drug that disrupts clathrin-mediated endocytosis by inhibiting the relocation of clathrin and AP2 from the cell surface (42). HepG2 cells were pretreated with various

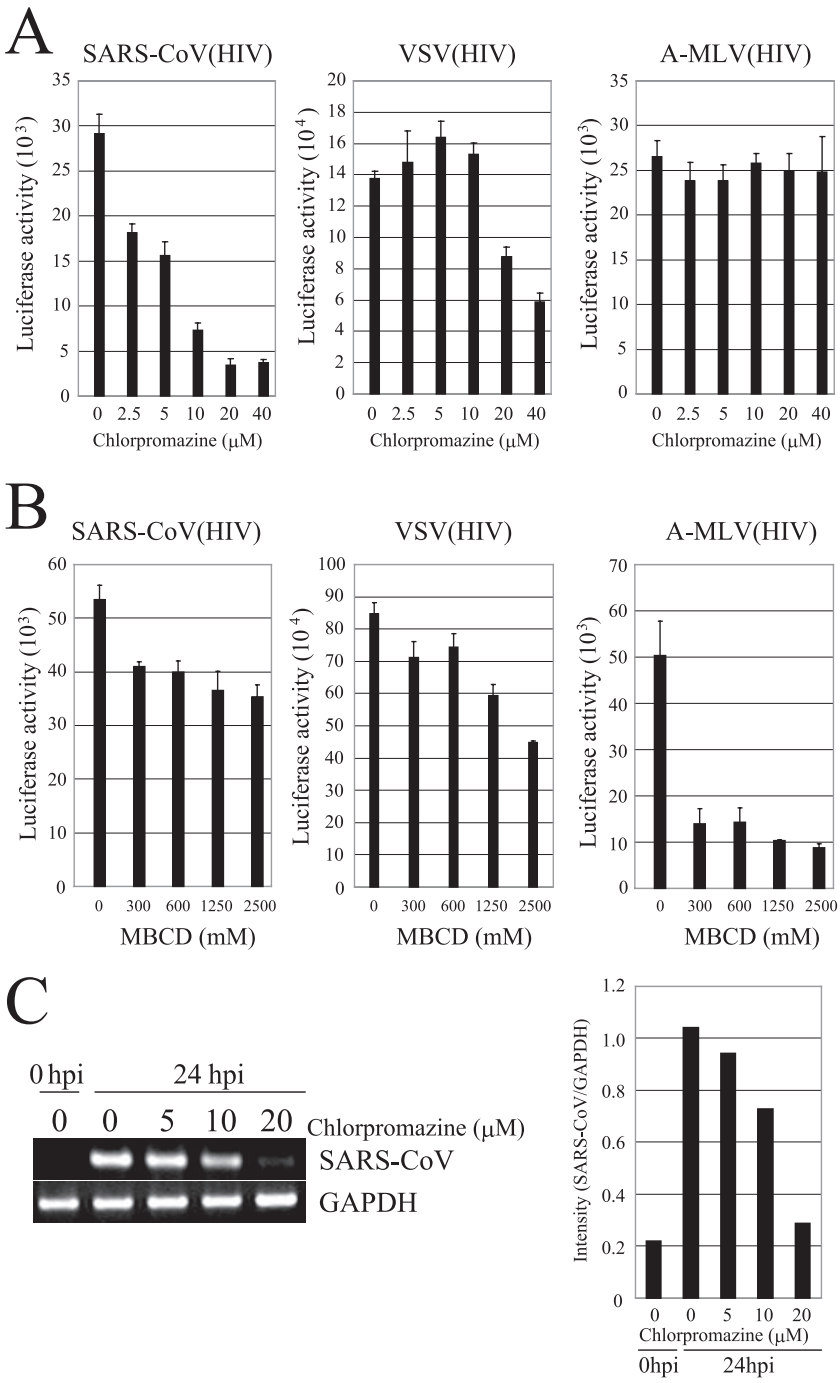

FIG. 1. Effects of chlorpromazine and MBCD on pseudoviruses and SARS-CoV infection. (A and B) HepG2 cells were treated with the indicated doses of chlorpromazine or MBCD and then infected with SARS-CoV(HIV), VSV(HIV), or A-MLV(HIV) for overnight. The effects of chlorpromazine and MBCD on the infectivity of each pseudovirus were evaluated by measuring the luciferase activities. The columns represent the mean values of triplicate experiments; bars indicate maximum values. (C) HepG2 cells treated with the indicated doses of chlorpromazine were infected with SARS-CoV (Vietnam/NB-04/2003) for 24 h, and their expressions of viral RNA were measured by RT-PCR.

doses of chlorpromazine for 1 h and then infected with the indicated pseudovirus in the presence of chlorpromazine (Fig. 1A). Chlorpromazine significantly inhibited the infection efficiency of SARS-CoV(HIV); only 14% infectivity was observed with 20 μM chlorpromazine. Infection by VSV(HIV) was also inhibited, but less so; 42% infectivity was still seen with 20 μM chlorpromazine. Unlike the other two pseudoviruses, infection by A-MLV(HIV) was largely unaffected by any concentration of chlorpromazine.

We also examined the effects of another endocytosis inhibitor, MBCD, on infections by the pseudoviruses, because MBCD disrupts cholesterol-rich microdomains, resulting in

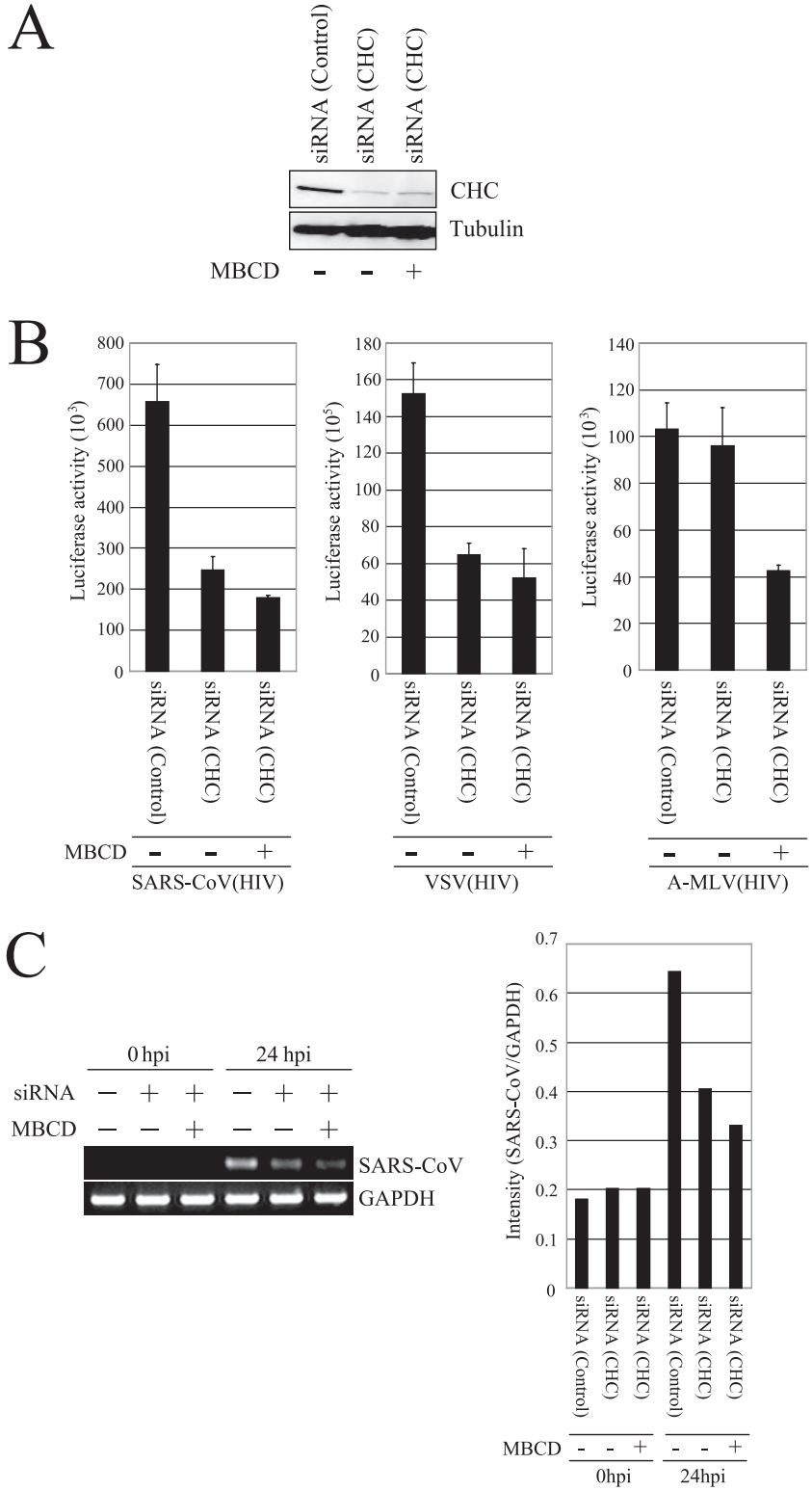

FIG. 2. Effects of CHC-specific siRNA on pseudoviruses and SARS-CoV infection. (A) HepG2 cells were transfected with the CHC-specific siRNA or the control siRNA and analyzed by immunoblotting with anti-CHC or anti-tubulin monoclonal antibody after 48 h of transfection. (B) The cells were treated with 2.5 mM MBCD for 30 min and then infected with each pseudovirus. The infectivities of the pseudoviruses are represented as luciferase activities. The experiment was performed in triplicate. (C) The cells were also infected with SARS-CoV (Vietnam/NB-04/2003) for 24 h, and their expressions of viral RNA were measured by RT-PCR.

the inhibition of both the caveola-dependent endocytosis and caveola-independent lipid-raft-dependent endocytosis (18, 32). HepG2 cells were treated with various concentrations of MBCD for 30 min and thereafter infected with the pseudoviruses in the absence of MBCD to avoid any potential effect of MBCD on the viral envelopes (Fig. 1B). MBCD treatment inhibited the susceptibility of HepG2 cells to A-MLV(HIV), to 18% of the control level at 2500 μM MBCD, whereas only a modest reduction in the cells' susceptibility to the SARS-CoV-(HIV) and VSV(HIV) was seen, to 66 and 53% of the control, respectively (Fig. 1B).

We next used SARS-CoV (Vietnam/NB-04/2003). HepG2 cells were treated or untreated with indicated concentrations of chlorpromazine for 1 h and then infected with SARS-CoV for 24 h. Infection efficiency of SARS-CoV was determined by RT-PCR. It was significantly inhibited by chlorpromazine treatment (Fig. 1C). Collectively, these results suggest that SARS-CoV entry into HepG2 cells is mostly mediated by the clathrin-dependent pathway, although some SARS-CoV pseudovirus entry appears to be dependent on caveolae and/or lipid rafts.

Effect of CHC depletion on SARS-CoV entry. To examine whether clathrin-mediated endocytosis is required for SARS-CoV(HIV) entry, we used siRNA-mediated gene silencing against the major component of the clathrin triskelion, CHC. In addition, under this clathrin knockdown condition, we simultaneously inhibited the clathrin-independent pathways with MBCD. This method was designed to determine whether the pseudoviruses entered the cells via the clathrin-dependent or -independent pathways, or both.

Transfection of a specific siRNA for CHC reduced the CHC protein expression to less than 10% of that in the control siRNA-transfected HepG2 cells, and we observed no effect of MBCD treatment on the CHC knockdown (Fig. 2A). CHC-depleted MBCD-treated cells, CHC-depleted mock-treated cells, and control cells were infected with the SARS-CoV(HIV), VSV(HIV), and A-MLV(HIV). CHC depletion reduced the SARS-CoV-(HIV) infectivity to 38% and the infectivity of VSV(HIV) to 42% of that in the control cells. Cholesterol depletion by MBCD in the CHC knockdown cells reduced the SARS-CoV(HIV) infectivity to 28% and the infectivity of VSV(HIV) to 36% of their infectivity in the control cells (Fig. 2B). On the other hand, there was little effect on the A-MLV(HIV) infectivity in cells expressing the siRNA. However, MBCD treatment markedly reduced the A-MLV(HIV) infectivity to 41% (Fig. 2B). The *t* test revealed the statistical significances in combinations between MBCD-treated and -untreated CHC knockdown cells infected with the pseudoviruses.

We also examined the effect of clathrin knockdown on SARS-CoV (Vietnam/NB-04/2003) infection to HepG2 cells under a similar condition to the pseudoviruses. CHC depletion reduced the SARS-CoV infectivity to 65% of that in the control cells at 24 h postinfection. Cholesterol depletion by MBCD in the CHC-knockdown cell slightly inhibited to this effect (Fig. 2C). These results support the evidence described above that the clathrin-mediated pathway of endocytosis is required for an efficient SARS-CoV entry into HepG2 cells.

Receptor activities of ACE2 mutants for SARS-CoV infection. To further investigate the clathrin-dependent endocytosis of SARS-CoV, we sought to determine whether the cytoplasmic domain of ACE2 is required for interaction with AP2/clathrin complexes. We prepared an ACE2 mutant (ACE2-Δtail) that lacks the cytoplasmic domain by introduction of the stop codon at the end of the transmembrane domain of ACE2. The virus receptor activity of ACE2 mutant was examined with COS7 cells because COS7 cells are negative for ACE2 expression detected by RT-PCR and immunoblotting but positive for caveolin-1 (data not shown) (34). COS7 cells transfected with ACE2-Δtail were infected with SARS-CoV(HIV) equally well to the cells transfected with the wild-type ACE2 (Fig. 3A and

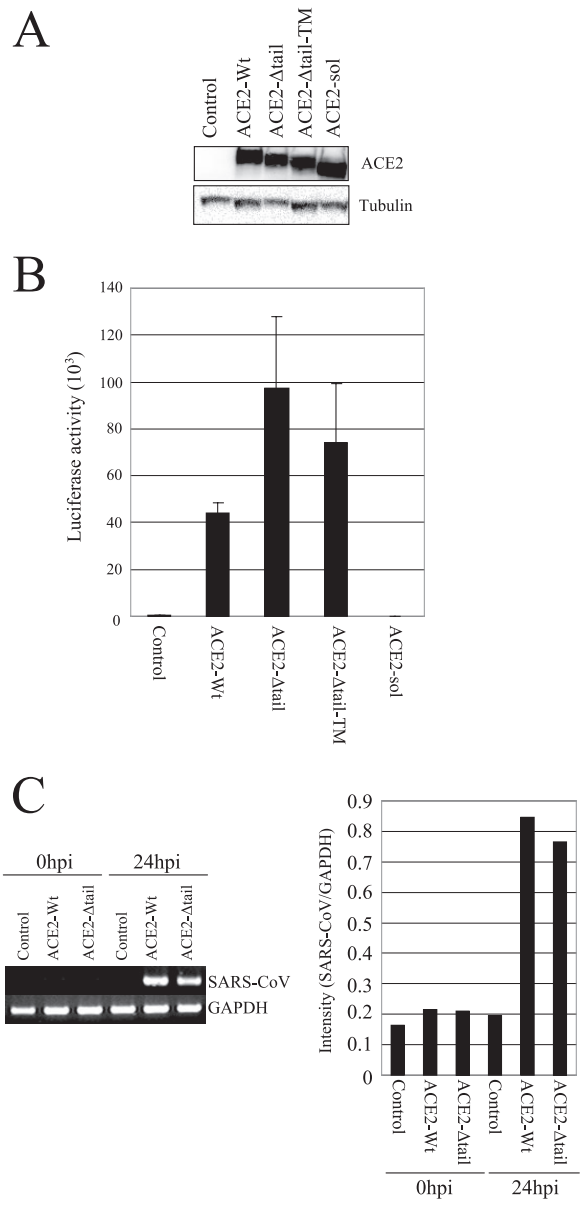

FIG. 3. Receptor activity of ACE2 mutants for pseudoviruses and SARS-CoV infection. (A) COS7 cells were transiently transfected with ACE2-wt, ACE2-Δtail, ACE2-Δtail-TM, ACE2-sol or control plasmids, and after 48 h of transfection their lysates were tested by immunoblotting with anti-ACE2 or anti-tubulin monoclonal antibody. (B and C) The transfected cells were infected with SARS-CoV(HIV) (B) or SARS-CoV (Vietnam/NB-04/2003) (C). Their luciferase activities were measured in triplicate, and their expressions of viral RNA were measured by RT-PCR.

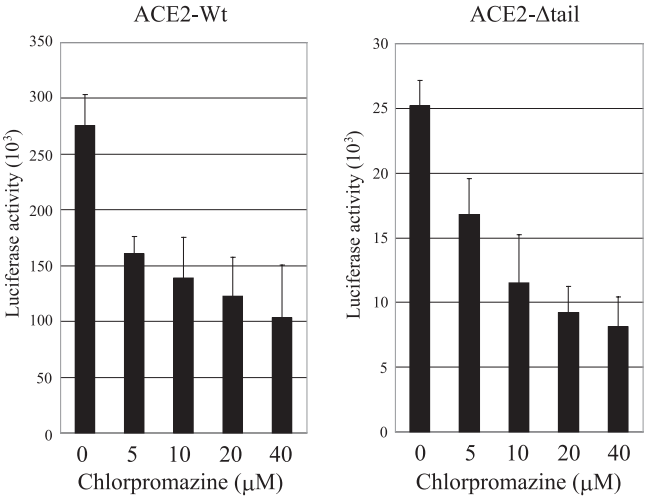

FIG. 4. Effects of chlorpromazine on pseudovirus infection to cells expressing ACE2-Δtail. COS7 cells were transiently transfected with ACE2-wt or ACE2-Δtail and then treated with the indicated amounts of chlorpromazine. Subsequently, the cells were infected with SARS-CoV(HIV). Their luciferase activities were measured in triplicate.

B). We further examined the receptor activity of ACE2-Δtail-TM, which lacks the cytoplasmic domain and replaces the transmembrane domain with that derived from EGFR. ACE2-Δtail-TM also showed a receptor activity for SARS-CoV(HIV) (Fig. 3B). We also confirmed that ACE2-sol, a soluble form of the ACE2 extracellular domain, has no receptor activity for SARS-CoV(HIV). Next, we used SARS-CoV (Vietnam/NB-04/2003). COS7 cells transfected with ACE2-Δtail were also infected with SARS-CoV equally well to the cells transfected with the wild-type ACE2 at 24 h postinfection (Fig. 3C). These results suggest that the cytoplasmic domain of ACE2 is not essential for its receptor activity and that there is no specificity of the transmembrane domain for its receptor activity.

We next confirmed that the ACE2-Δtail-mediated infection of SARS-CoV(HIV) is also clathrin dependent. COS7 cells transfected with the wild-type ACE2 or ACE2-Δtail were pretreated with chlorpromazine and infected with SARS-CoV-(HIV). The chlorpromazine treatment induced suppression of SARS-CoV(HIV) infection to COS7 cells expressing ACE2-Δtail, as well as the wild-type ACE2 (Fig. 4).

SARS-CoV(HIV) is transported into EEA1-positive early endosomes. Accumulating evidence suggests that cell surface molecules internalized by the clathrin-dependent pathway are transferred into early endosomes. We used confocal microscopy to examine whether ACE2 is internalized in early endosomes upon SARS-CoV(HIV) binding. After a 3 h period of serum starvation, HepG2 cells expressed ACE2 predominantly on the cell surface (Fig. 5, upper panels). The cells were then infected with SARS-CoV(HIV) concentrated 10-fold by ultracentrifuge. By 10 min after the infection, the ACE2 localization had changed dramatically, from the cell surface to EEA1-positive early endosomes (Fig. 5, lower panels). We also confirmed that the SARS-CoV(HIV) entry was affected by acidification inhibitors such as ammonium chloride (NH_4Cl) and chloroquine (data not shown). Furthermore, we examined the effect of ammonium chloride on SARS-CoV(HIV) infection into COS7 cells expressing ACE2-

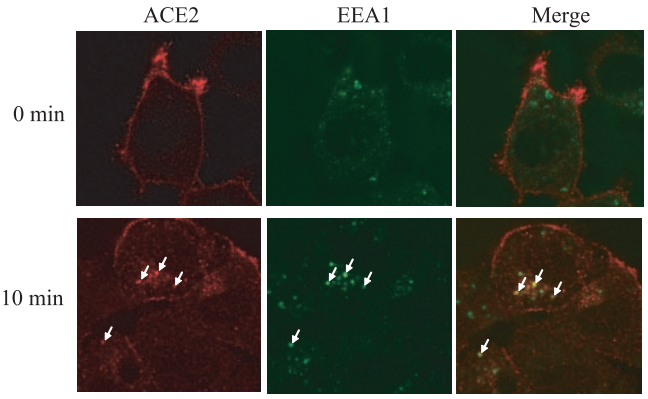

FIG. 5. Immunohistochemical localization of ACE2 after pseudovirus infection. HepG2 cells were cultured in the FCS-free medium to induce ACE2 on cell surfaces and incubated with concentrated SARS-CoV-(HIV) for 10 min at 37°C. They were then stained for ACE2 and EEA1 by immunofluorescence.

Δtail (Fig. 6). The ammonium chloride treatment induced inhibition of SARS-CoV(HIV) infection in a manner similar to that for HepG2 cells. These results suggest that the binding of the SARS-CoV(HIV) to ACE2 induces rapid internalization of the ACE2/pseudovirus complex into EEA1-positive early endosomes, where a low pH condition is required for it to establish an infection and that the cytoplasmic tail of ACE2 is not required for the internalization of SARS-CoV(HIV) into endosomes.

DISCUSSION

Productive infection of target cells by animal viruses requires their access to highly specific entry pathways that allow

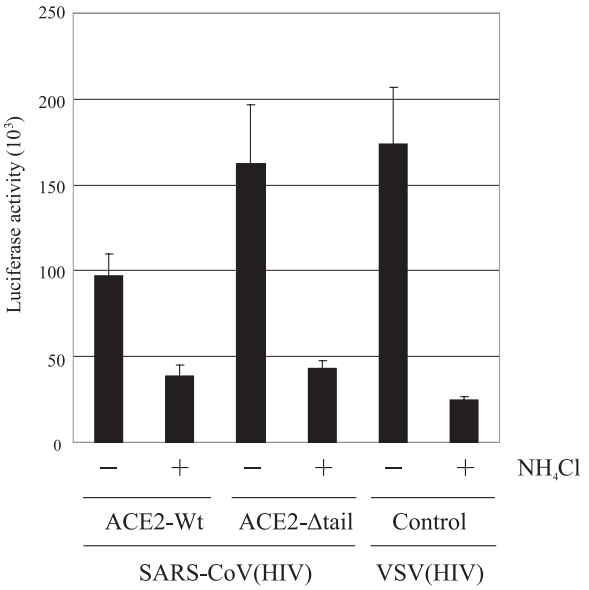

FIG. 6. Dependence on acidic environment for infection by the pseudoviruses. COS7 cells transiently transfected with ACE2-wt, ACE2-Δtail, or control plasmids were treated with 20 mM NH_4Cl and then infected with SARS-CoV(HIV) and VSV(HIV). Their infectivities were evaluated by measuring the luciferase activity. The experiment was performed in triplicate.

critical virion components to be introduced into the cytoplasm for subsequent processes, including uncoating, gene expression, and replication. The present study documented that the main pathway of SARS-CoV entry into host cells is dependent on clathrin. Chlorpromazine is a cationic amphiphilic agent that inhibits the formation of clathrin-coated pits (24). The use of chlorpromazine has established that a number of other viruses, including VSV (40) and influenza virus (36), use clathrin for entry into the host cell. We found that chlorpromazine inhibited the infection of HepG2 cells by SARS-CoV(HIV) more significantly than by VSV(HIV). We also confirmed the inhibitory effect of chlorpromazine on the SARS-CoV (Vietnam/NB-04/2003) infection to HepG2 cells. Moreover, HepG2 cells used here are unable to form caveolae because of no expression of caveolin-1 (data not shown). These results suggest that the SARS-CoV entry is mainly mediated by clathrin-coated pits. The specificity of the chlorpromazine effect was supported by the observation that the chlorpromazine treatment had little effect on the entry of the clathrin-independent A-MLV(HIV). To verify SARS-CoV's dependence on clathrin for host cell entry, we used CHC knockdown HepG2 cells as the target for SARS-CoV infection. The treatment of HepG2 cells with CHC siRNA induced significant suppression of the SARS-CoV(HIV) infection, as well as the SARS-CoV infection. Furthermore, we found that the ACE2 is colocalized with the CHC out of the lipid raft. Hence, although we cannot exclude a minor contribution of the clathrin-independent pathway to SARS-CoV(HIV) entry, the clathrin-dependent pathway is probably the main one used by SARS-CoV for host cell entry.

A previous report demonstrated that an ACE2 mutant partially deleted of the cytoplasmic domain sustains a receptor activity for SARS-CoV infection (11). Since we found that the ACE2-mediated infection of SARS-CoV is mainly clathrin dependent, we then definitively sought to determine whether the cytoplasmic domain of ACE2 is required for the clathrin-dependent pathway of SARS-CoV infection. We prepared an ACE2 mutant, ACE2-Δtail, with the cytoplasmic domain completely deleted, and demonstrated that the SARS-CoV infection via the ACE2-Δtail is mainly clathrin dependent because chlorpromazine inhibits the ACE2-Δtail-mediated entry of SARS-CoV(HIV) into COS7 cells. These results indicate that the cytoplasmic domain of the ACE2 is not essential for the clathrin-dependent entry of SARS-CoV, which suggests that there is a possible coreceptor for the ACE2, which interacts with the AP2/clathrin complex. The replacement of the transmembrane domain of ACE2 with that of EGFR showed no effect on the susceptibility of the cells to SARS-CoV. Since the ACE2 extracellular domain alone is unable to induce the receptor activity, the extracellular domain containing the transmembrane domain is indispensable for the receptor activity for SARS-CoV but the cytoplasmic tail of ACE2 is dispensable.

SARS-CoV infection was previously shown to be suppressed partially by treatment of the cholesterol-depleting reagent, MBCD (19). We also observed lower but significant levels of MBCD-mediated suppression of the SARS-CoV(HIV) and VSV(HIV) infection compared to the A-MLV(HIV) infection. The difference in MBCD's suppressive effects between A-MLV(HIV) and the other two pseudoviruses may be explained by the possibility that the SARS-CoV(HIV) entry might be partially mediated by a clathrin-independent pathway corresponding to the lipid raft-mediated pathway. Alternatively, in a previous report, MBCD partially inhibited the clathrin-mediated endocytosis of transferrin receptor and EGFR, as well as completely inhibiting their lipid raft- and/or caveola-mediated endocytosis (14, 33), suggesting that MBCD might have some unexpected effect on the clathrin-mediated pathway for the SARS-CoV infection. To address these possibilities, we investigated the additive or synergistic effect of MBCD with CHC-siRNA on the SARS-CoV infection to HepG2 cells. MBCD induced a weak but significant additive suppression on CHC-siRNA-treated HepG2 cell infection by SARS-CoV, suggesting a minor clathrin-independent entry pathway for SARS-CoV.

The SARS-CoV S protein is cleaved into S1 and S2 proteins by an acidic protease, cathepsin L, in endosomes, which is essential for fusion between the viral envelope and the endosome vesicular membrane (1, 13, 37). We demonstrated here the translocation of ACE2 to the EEA1-positive endosomes upon SARS-CoV(HIV) infection and showed the suppressive effects of acidification inhibitors, NH_4Cl and chloroquine, on SARS-CoV(HIV) infection to HepG2 cells. Furthermore, the SARS-CoV(HIV) infection to COS7 cells expressing ACE2-Δtail was significantly suppressed by NH_4Cl treatment, suggesting that SARS-CoV infection via ACE2-Δtail is not different from that via the wild-type ACE2.

Based on the present study, we propose a model for SARS-CoV's internalization by target cells. SARS-CoV attaches the cell surface through an interaction between the envelope spike glycoprotein and its receptor, ACE2. Clathrin-coated pits are then formed by interactions between the ACE2/virus complex and the AP2/clathrin complex via a possible coreceptor in a non-lipid-raft portion of the plasma membrane. The ACE2/virus complex is then translocated to endosomes, where the virus is uncoated by the help of endosomal acid protease, such as cathepsin L (13, 37).

Among the *Coronaviridae*, human coronavirus 229E (HCoV-229E) utilizes aminopeptidase N (CD13) as its receptor, which is localized in the lipid rafts, and HCoV-229E infection is inhibited by the treatment of target cells with MBCD and the transfection of an siRNA specific for caveolin-1, suggesting that HCoV-229E utilizes the caveola-mediated endocytosis pathway for its entry into target cells (26). Since CD13 is a common viral receptor for other group 1 coronaviruses, such as porcine transmissible gastroenteritis virus, porcine epidemic diarrhea virus, feline infectious peritonitis virus, and canine coronavirus, the caveola-mediated endocytosis pathway seems to be conserved among them. In contrast, murine hepatitis virus, which is a group 2 coronavirus, was previously shown to utilize the lipid raft-mediated endocytosis pathway (5). Hence, SARS-CoV, which is also classified into the group 2 coronavirus, seems to be unique among the *Coronaviridae* because it utilizes the clathrin-mediated pathway for its entry into HepG2 and COS7 cells.

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