Trypsin Enhances SARS-CoV-2 Infection by Facilitating Viral Entry

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Abstract

Coronavirus infects the cell by cytoplasmic or endosomal membrane fusion driven by the spike (S) protein, which must be primed by proteolytic cleavage at the S1/S2 furin cleavage site (FCS) and S2' site by cellular proteases. Exogenous trypsin as a medium additive facilitates isolation and propagation of several coronaviruses *in vitro*. Here, we showed that trypsin mediated the enhancement of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in cultured cells and that SARS-CoV-2 entered the cells via either a non-endosomal or an endosomal fusion pathway depending on the presence of trypsin. Interestingly, trypsin enabled viral entry at the cell surface and led to more efficient infection efficiency than trypsin-independent endosomal entry, suggesting that trypsin production in the target organs may trigger high replication of SARS-CoV-2 and cause severe tissue injury. Extensive syncytia formation and enhanced growth kinetics were observed only in the presence of exogenous trypsin for the cell-adapted SARS-CoV-2 strains. During 50 serial passages without trypsin addition, a specific R685S mutation occurred in the S1/S2 FCS (⁶⁸¹PRRAR⁶⁸⁵) that was completely conserved but with several mutations in the S2 fusion subunit in the presence of trypsin. These findings demonstrate that S1/S2 FCS is essential for S protein proteolytic priming and fusion activity for SARS-CoV-2 entry but not for SARS-CoV-2 replication. Our data can contribute to the improvement of SARS-CoV-2 production to develop vaccines or antivirals and motivate further investigations into the explicit functions of cell adaptation-related genetic drift in SARS-CoV-2 pathogenesis.

Introduction

In December 2019, an outbreak of mysterious pneumonia was reported in Wuhan, Hubei province, China [1, 2]. A novel coronavirus, called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is closely related to SARS-CoV-1, was detected in patients and subsequently identified as the causative agent of the new respiratory disease in January 2020 [2, 3]. SARS-CoV-2 causes coronavirus disease 2019 (COVID-19), which has become a global pandemic since March 11, 2020. The rapid national and international spread of SARS-CoV-2 has threatened human health and led to the global economic recession [4].

SARS-CoV-2 is the seventh identified human coronavirus that belongs to the subgenus *Sarbecovirus* of the genus *Betacoronavirus* in the family *Coronaviridae* [5]. SARS-CoV-2 is a large, enveloped RNA virus that possesses a single-stranded positive-sense RNA genome of approximately 30 kb. The genome of SARS-CoV-2 encodes 16 nonstructural proteins (nsp1–nsp16); four canonical coronaviral structural proteins, named spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins; and several accessory proteins [6]. The external S protein anchored on the viral envelope binds to the angiotensin-converting enzyme 2 (ACE2) receptor to mediate viral entry into host cells during SARS-CoV-2 infection [7, 8].

Like other coronaviruses, the S protein of SARS-CoV-2 can be functionally divided into two subunits (S1 and S2) that harbor the receptor-binding domain and the fusion domain, respectively [9]. In general, coronavirus enters the target cells via either cytoplasmic or endosomal membrane fusion, which is a primary determinant of viral infectivity. Therefore, efficient proteolytic priming of S at S1/S2 and S2' sites is essential for exposing the fusion peptide and mediating the membrane fusion to complete the viral entry process [10]. To accomplish S protein priming, SARS-CoV-2 uses either endosomal cysteine proteases or transmembrane protease serine 2 (TMPRSS2) [10]. In an infection experiment with SARS-CoV-2, a Vero E6 cell line overexpressing TMPRSS2 produced higher titer virus than the parental cells, indicating the importance of this protease for the fusion capacity and infectivity of SARS-CoV-2 [11]. Trypsin is another protease shown to facilitate the fusion and infection of human and animal coronaviruses [12–15] and is essential for the propagation of swine enteric coronaviruses, such as porcine epidemic diarrhea virus (PEDV) and porcine deltacoronavirus (PDCoV), *in vitro* [16–20]. Therefore, we attempted to investigate whether exogenous trypsin treatment enhances SARS-CoV-2 infection in Vero E6 cells and how it promotes viral replication in cultured cells.

Materials And Methods

Cells and virus

Vero E6 cells (ATCC CRL-1586) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and penicillin-streptomycin (100×; Invitrogen). The cells were maintained at 37°C in an atmosphere of humidified air containing 5% CO₂. The SARS-CoV-2 KCDC03 strain (BetaCoV/South Korea/KCDC03/2020) was provided by the Korea Centers for Disease Control and Prevention (KCDC) and propagated in Vero E6 cells, as described previously [21]. Virus stock was prepared from the KCDC03-infected Vero E6 cell cultures by freezing/thawing, as described previously [19, 22]. The clarified supernatants were aliquoted and stored at –80°C as the 'passage 2 (P2)' viral stock for the next passage. In the same manner, 50 serial passages were performed in Vero E6 cells. The titers of SARS-CoV-2 were measured by limiting dilution on Vero E6 cells in triplicate under the indicated conditions, and then 50% tissue culture infectious dose (TCID₅₀) per ml was calculated as described below. All virus infection experiments were conducted in a biosafety level 3 facility at the Gyeongbuk Veterinary Service Laboratory according to the KCDC laboratory biosafety guidelines.

Reagents and antibody

Trypsin was obtained from USB (Cleveland, OH). Elastase and the lysosomal acidification inhibitor BafA1 A1 (BafA1) were purchased from Sigma-Aldrich (St. Louis, MO). Trypsin and elastase were dissolved in distilled water. BafA1 was dissolved in dimethyl sulfoxide (DMSO). All

reagents were used at the indicated concentrations in DMEM. The Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody was acquired from Invitrogen.

Monoclonal antibody production

The SARS-CoV-2 N gene was amplified by RT-PCR using a primer set (forward primer 5'-GCCGCATATGTCTGATAATGGACCCC-3' and reverse primer 5'-CTTCCTCGAGGGCCTGAGTTGAGTC-3') and inserted into the pET30a expression vector (Life Science Research, Millstone, NJ). The recombinant N protein was expressed in *E. Coli* BL21 (DE3) transformed with pET30a-N under isopropyl-β-D-thiogalactopyranoside induction as described previously [23]. After centrifugation and ultrasonication, the rN protein was purified using a HiTrap TALON crude column (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. The purified protein was concentrated using Amicon Ultra centrifugal filters (30 kDa MWCO; Millipore, Billerica, MA) and the final product was used as the antigen. SARS-CoV-2 N-specific monoclonal antibodies (MAbs) were produced as described previously [23]. Four-week-old female BALB/c mice were immunized four times with 100 µg antigen emulsified in Freund's complete (first injection) or incomplete adjuvant (Sigma, St. Louis, MO) at 1-week intervals. Mouse splenocytes were fused with SP2/0 myeloma cells using 50% polyethylene glycol 1500 (Roche Diagnostics, Mannheim, Germany). The supernatant of the hybridomas was screened by indirect enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay as described previously [23]. The positive hybridomas secreting SARS-CoV-2 N-specific MAbs were subcloned three times by limiting dilution and maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, penicillin-streptomycin (100×), and 10 mM HEPES (Invitrogen).

Cell viability assay

The cytotoxic effects of all reagents used in this study were analyzed using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich) to detect cell viability as described previously [24]. Briefly, Vero E6 cells were grown at 1×10^4 cells/well in a 96-well tissue culture plate and treated with each chemical for 24 h. After incubation, 50 µl of MTT solution (1.1 mg/ml) was added to each well, and the samples were incubated for an additional 4 h. The supernatant was then removed from each well, followed by the addition of 150 µl DMSO to dissolve the colored formazan crystals produced by MTT. The absorbance of the solution was measured at 540 nm using an ELISA plate reader. All MTT assays were performed in triplicate.

Immunofluorescence assay (IFA)

Vero E6 cells grown in 96-well tissue culture plates were mock-infected or infected with SARS-CoV-2 (P3) at a multiplicity of infection (MOI) of 1 for 1 h and cultured under the indicated conditions. At 24 h post-infection (hpi), SARS-CoV-2-infected cells were fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS at room temperature for 10 min. The cells were blocked with 1% bovine serum albumin in PBS at room temperature for 30 min and then stained with SARS-CoV-2 N-specific MAb. After washing five times in PBS, the cells were incubated at room temperature for 1 h with corresponding Alexa Fluor-conjugated secondary antibodies, followed by counterstaining with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). The coverslips were mounted on glass microscope slides in a mounting buffer, and the stained cells were visualized under a fluorescent Leica DM IL LED microscope (Leica, Wetzlar, Germany).

Virus titration

Vero E6 cells were infected with SARS-CoV-2 in the presence or absence of each protease. The culture supernatant was collected at different time points (3, 6, 12, 24, 36, 48, 60, and 72 hpi) and stored at -80°C. The SARS-CoV-2 titer was measured by limiting dilution on Vero E6 cells in triplicate, and then the TCID₅₀ per ml was calculated using the Reed-Muench method [25].

Time course of trypsin treatment

Vero E6 cells were infected with SARS-CoV-2 at an MOI of 1. At 0, 1, 2, 4, 6, and 10 hpi, trypsin was added to achieve the indicated final concentration over the remainder of the time course of the experiment. The culture supernatants were harvested at 24 hpi and subjected to virus titration to evaluate the magnitude of SARS-CoV-2 infection, as described above (section 2.6).

Virus binding and internalization assay

A proteinase K infectious assay was conducted to assess the efficiency of proteinase K treatment as described previously [26]. Vero E6 cells were grown in 6-well culture plates and incubated with SARS-CoV-2 at an MOI of 1 at 4°C for 1 h. The cells were washed with PBS and treated with proteinase K (0.5–1 mg/ml) at 4°C for 45 min. After treatment, the supernatant was removed, and total RNA was extracted from the cell lysates using the TRIzol reagent (Invitrogen) and then treated with DNase I (TaKaRa, Otsu, Japan) according to the manufacturer's instructions. The concentrations of the extracted RNA were measured using a NanoVue spectrophotometer (GE Healthcare). Quantitative real-time RT-PCR was performed using a One Step SYBR PrimeScript RT-PCR Kit (TaKaRa) and primers (forward primer 5'-GACCCCAAAATCAGCGAAAT-3' and reverse primer 5'-TCTGGTTACTGCCAGTTGAATCTG-3'). A primer set targeting monkey β-actin included forward primer 5'-GGTCTTCTGTACCTGATTT-3' and reverse primer 5'-CATCCACCTCCACTTCTCTAAC-3'. The reaction was performed using a Thermal Cycler Dice Real-Time System (TaKaRa),

according to the manufacturer's protocol. The amounts of viral RNA were determined in 10-fold serially diluted SARS-CoV-2 (P3) from 10^6 to 10^1 TCID₅₀ by real-time RT-PCR, and a standard curve for each PCR plate was generated. Genome copy numbers were calculated based on this standard curve and normalized to β -actin gene copy numbers by using the comparative cycle threshold values determined in parallel.

Binding and internalization assays were performed as described previously with some modifications [24]. Vero E6 cells grown in 6-well culture plates were incubated with SARS-CoV-2 at an MOI of 1 at 4°C for 1 h. After removing unbound viruses by washing with PBS, the cells were incubated at either 4°C (allowing virus binding only) or 37°C (permitting both virus binding and internalization) in the presence of trypsin for 1 h; in the latter case, the cells were further treated with proteinase K (0.5 mg/ml) at 37°C for 45 min to remove the bound but uninternalized virus particles. The SARS-CoV-2-infected cells were then serially diluted in DMEM and inoculated onto fresh Vero E6 cell monolayers in 96-well tissue culture plates. At 24 h post-incubation, bound or internalized viruses were titrated as described above (section 2.6), and the TCID₅₀ was determined.

Quantitative real-time RT-PCR (qRT-PCR)

Vero E6 cells in 96-well culture plates were treated with DMEM containing 1 μ M BafA1 at 37°C for 30 min and then chilled at 4°C for 10 min as described previously [13]. The cells were infected with SARS-CoV-2 at an MOI of 1 at 4°C. After 30-min adsorption, the virus was removed, and infected cells were treated with each protease in the culture medium plus BafA1 at room temperature for 5 min. After protease was removed, the cells were maintained in the presence of BafA1 at 37°C for 6 h. Viral RNA was extracted from the cell lysates using the TRIzol reagent and prepared, as described above (section 2.8). The RNA concentrations were measured, and SARS-CoV-2 N gene-based quantitative real-time RT-PCR was performed using a One Step PrimeScript RT-PCR Kit (TaKaRa) and a primer set (forward primer 5'-GACCCCAAATCAGCGAAAT-3', reverse primer 5'-TCTGGTTACTGCCAGTTGAATCTG-3', and a probe 5'-FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1-3'). The reaction was performed using a Thermal Cycler Dice Real Time System (TaKaRa) according to the manufacturer's protocol under the following conditions: 1 cycle of 45°C for 30 min, 1 cycle of 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The results were analyzed using the system software as described previously [22, 27]. To measure the amounts of virus that entered into cells, we infected cells with 10-fold stepwise diluted SARS-CoV-2 (P3) from 10^6 to 10^1 TCID₅₀. The quantities of viral RNA were determined by qRT-PCR, and a standard curve for each PCR plate was generated. The virus concentrations (genomic copies/ml) in the samples were calculated based on this standard curve.

Nucleotide sequence analysis

The full-length genomes of SARS-CoV-2-P2 and cell-adapted -P3, -P10, -P20, -P30, -P40, and -P50 strains were sequenced by traditional Sanger methods as described previously with some modifications [19, 22]. Viral RNA was extracted from each virus stock serially passaged in Vero E6 cells and was used as a template to amplify 11 overlapping cDNA fragments encompassing the entire genome of each virus strain using RT-PCR. The individual amplicons were gel purified, cloned into a pGEM-T Easy Vector System (Promega, Madison, WI), and sequenced in both directions using two commercial vector-specific T7 and SP6 primers and viral gene-specific primers. The 5' and 3' ends of the genomes of each strain were determined by rapid amplification of cDNA ends (RACE), as described previously [28]. The complete genomic nucleotide sequences of each passage derivatives were deposited in the GenBank database under accession numbers (MZ930250 to MZ930252, MZ930392 to MZ930396, and MZ995184 to MZ995188).

Statistical analysis

All values are expressed as mean $\pm$ standard deviation of the mean difference (SDM). Statistical analyses were conducted using the GraphPad Prism 7 software package (GraphPad Software, San Diego, CA). P-values of ≤ 0.05 were considered to be statistically significant.

Results

Contribution of trypsin to SARS-CoV-2 infection

To explore the impact of trypsin on viral replication, Vero E6 cells were incubated with trypsin for 1 h at the following concentrations (0, 1.25, 2.5, 5, and 10 μ g/ml) and then mock-infected or SARS-CoV-2 (P3)-infected at an MOI of 1 for 1 h. The infected cells were washed once with PBS and cultivated in the cell growth medium deprived of trypsin. According to the MTT cell viability assay, none of the trypsin dosages examined in this study resulted in measurable degrees of cell death (data not shown). SARS-CoV-2 replication was initially measured by monitoring the intensity of cytopathic effect (CPE) and was confirmed by IFA using the anti-N protein MAb at 24 hpi. As shown in Fig. 1A, treating cells with trypsin before virus infection had no effect on SARS-CoV-2 infectivity at any concentration tested compared to the untreated control. We further quantified viral production using virus titration at 24 hpi. The virus yields remained unchanged, with mean titer ranges of $10^{6.53}$ – $10^{6.73}$ TCID₅₀/ml regardless of the presence or absence of trypsin before infection (Fig. 1B). Furthermore, SARS-CoV-2 propagation in cell culture was unaffected by adding exogenous trypsin during the 1 h-inoculation period (Fig. 1C and D). These results revealed that trypsin treatment before or during infection did not influence SARS-CoV-2 replication.

Next, we investigated whether SARS-CoV-2 infectivity is enhanced by supplementing trypsin after SARS-CoV-2 infection. Vero E6 cells were subjected to SARS-CoV-2 infection at an MOI of 1 for 1 h without trypsin and then propagated in the FBS-free medium with trypsin at the following concentrations (0, 1.25, 2.5, 5, and 10 $\mu\text{g/ml}$). Unless otherwise specified, trypsin was present throughout the infection period. CPE observations and IFA analyses revealed that when the virus-infected cells were cultivated in the presence of trypsin, the virus infectivity increased (Fig. 1E). When exogenous trypsin was added at post-inoculation, virus titers were considerably enhanced compared to viral propagation without trypsin (Fig. 1F). The highest viral titer ($10^{7.52}$ TCID₅₀/ml) was achieved in the presence of 5 $\mu\text{g/ml}$ trypsin, which was 1-log higher than the control mean titer ($10^{6.67}$ TCID₅₀/ml). Additionally, Vero E6 cells were inoculated with ten-fold serially diluted SARS-CoV-2 and maintained in the presence or absence of trypsin (5 $\mu\text{g/ml}$), and IFA was used to visualize the infected cells (Fig. 2). The results revealed that Vero E6 cells propagated with trypsin displayed a >10-fold higher number of SARS-CoV-2-infected cells than those cultured without trypsin addition. The virus growth medium (high-glucose DMEM supplemented with penicillin-streptomycin and 5 $\mu\text{g/ml}$ trypsin) was then used in all subsequent experiments unless otherwise indicated. We also tested the effect of another protease, elastase, on SARS-CoV-2 under the same experimental conditions. However, no apparent effect of elastase on SARS-CoV-2 replication was detected in cell culture when cells were treated with elastase at non-cytotoxic concentrations (5–10 $\mu\text{g/ml}$) before, during, or after infection (Fig. 3). These data indicated that the presence of trypsin at post-virus inoculation promotes the replication of SARS-CoV-2 in a protease type-dependent manner.

To establish the point at which trypsin is present during SARS-CoV-2 infection, we added trypsin to Vero E6 cells at different intervals post-infection. At 24 hpi, the extent of viral replication was assessed directly by virus titration. As presented in Fig. 4, the addition of trypsin at up to 2 hpi (i.e., 3 h after inoculation) resulted in a considerable enhancement of SARS-CoV-2 infectivity. However, when trypsin was introduced at or after 4 hpi, little or no increase in SARS-CoV-2 propagation was observed. These findings established that trypsin must be present during the early stages of viral infection in order to effectively enhance SARS-CoV-2 infection. Taken together, our results suggest that trypsin participates in the entry pathway of SARS-CoV-2.

Involvement of trypsin in the virus internalization

We next explored whether trypsin facilitates the entry process of SARS-CoV-2 in cultured cells. To accurately corroborate the parameters of the viral internalization assay necessary for monitoring SARS-CoV-2 entry, we used a proteinase K infectious assay that assesses the rate of virus attachment and penetration by quantifying productive bound or internalized virus particles, respectively. To determine the effectiveness of proteinase K treatment in removing associated viruses, Vero E6 cells were infected with SARS-CoV-2 for 1 h at 4°C and then treated with proteinase K at the specified doses for 45 min at 4°C. The quantity of virus bound was measured using qRT-PCR to determine the SARS-CoV-2 genome copy number. As demonstrated in Fig. 5A, proteinase K treatment had a substantial effect on the quantity of bound virions at the cell surface, suggesting that virus linked to the cells is efficiently removed by proteinase K treatment.

We then studied the two phases of viral entrance (attachment and penetration) in the presence of trypsin by virus binding and internalization assays. Vero E6 cells were inoculated with SARS-CoV-2 at 4°C for 1 h to enable only virus attachment and then maintained at 4 or 37°C in the presence of trypsin to limit or permit virus internalization, respectively. Following that, proteinase K was used to remove any remaining virus particles from the cell surface. Serial dilutions of infected cells were performed on fresh Vero E6 cell monolayers, and the viral titers were determined (Fig. 5B). Regardless of the presence or absence of trypsin, the viral titers were equivalent among cells treated at 4°C to allow virus binding but inhibit penetration. However, virus production was considerably enhanced in cells maintained at 37°C with trypsin to allow virus internalization to progress, compared to the vehicle control without trypsin under the same condition. These findings suggest that trypsin accelerates SARS-CoV-2 internalization.

SARS-CoV-2 entry at the cell surface facilitated by trypsin

To test whether SARS-CoV-2 enters cells by a pH-dependent endosomal pathway, we first evaluated the effect of BafA1, a lysosomotropic agent, on SARS-CoV-2 replication by virological analyses. Vero E6 cells were treated with BafA1 at concentrations of 0.1 and 0.5 μM or DMSO (0.5%) as a vehicle control before, during, or after infection. The MTT assay revealed that cellular cytotoxicity did not occur in drug-treated cells at the concentrations used in the study (data not shown). Viral production was measured by IFA using anti-N protein MAb and confirmed by virus titration at 24 hpi (Fig. 6). Treating cells with BafA1 before or during infection significantly attenuated SARS-CoV-2 N expression (Fig. 6A and C) and further suppressed viral infection (Fig. 6B and D) in a dose-dependent manner. By contrast, virus propagation remained unchanged, regardless of the presence or absence of BafA1 after infection (Fig. 6E and F). Nevertheless, these results suggest that SARS-CoV-2 entry occurs through an endosomal pathway.

We further investigated the effectiveness of trypsin in facilitating SARS-CoV-2 entry directly at the cell surface. Vero E6 cells treated with BafA1 at a concentration of 0.5 μM were inoculated with SARS-CoV-2 at an MOI of 1 and adsorbed at 4°C for 30 min to block the virus entry into cells. The cells were then treated with different proteases at room temperature for 5 min and maintained at 37°C for 6 h, and virus internalization was calculated by qRT-PCR (Fig. 7). Trypsin greatly facilitated SARS-CoV-2 entry, whereas elastase did not influence viral entry (Fig. 7A). Trypsin treatment of cells prior to viral infection had no influence on SARS-CoV-2 internalization (Fig. 7B), showing that the effects of trypsin on cells are

irrelevant for this infection. These results imply that SARS-CoV-2 entry occurs via a non-endosomal, direct fusion in the presence of trypsin, which cleaves the fusion-inducing S protein.

Treatment with high concentrations (10–75 µg/ml) of trypsin mediated the enhancement of virus entry or replication compared with the normal infection without trypsin (Fig. 7A; compare bars 6–8 with bar 1 from left). The replication kinetics of SARS-CoV-2 were then compared in cells treated with BafA1 (0.5 µM) and a high concentration (75 µg/ml) of trypsin to those in virus-infected cells maintained in the absence of BafA1 and trypsin. At any point during the early stage of infection, trypsin-treated cells produced substantially more virus (Fig. 7C).

Phenotypic and genotypic characterizations of SARS-CoV-2 serially passaged in the presence or absence of trypsin

We serially propagated viruses *in vitro* up to 50 passages in the presence or absence of trypsin and generated virus stocks every ten passages labeled P10, P20, P30, P40, and P50. Like the parental virus (P3), all SARS-CoV-2 strains cultured in the absence of trypsin produced CPE typical of viral infection, such as cell rounding, clumping together in clusters, and detachment, in infected Vero E6 cells (Fig. 8, left panels). However, trypsin-adapted strains could induce different patterns of CPE that included cell fusion and multi-nucleated cells or syncytia formation in infected cells (Fig. 8, right panels). The vacuolation and syncytia were larger and more predominant in cells infected with SARS-CoV-2 strains that were more consecutively passaged in the presence of trypsin. As a consequence, the size of syncytia increased progressively with the serial passage number, and the high-passage P50 virus generated the prevalent syncytia with many more nuclei than the low-passage P10 virus in the presence of trypsin (Fig. 8; compare panel n with panel j).

To examine the phenotypic characteristics of serially passaged SARS-CoV-2 strains *in vitro*, we evaluated the one-step growth rates of representative P3 and P10 strains cultured with or without trypsin (Fig. 9). The parental P3 virus without trypsin addition had the highest level, showing a peak titer of $10^{5.57}$ TCID₅₀/ml at 24 hpi, after which its growth declined (Fig. 9A). By contrast, the growth kinetics of the P3 virus were significantly promoted by trypsin. At 12 hpi, the P3 virus in the presence of trypsin caused a rapid increase in virus titers, which were maintained until 48 hpi, ranging from $10^{6.33}$ to 10^7 TCID₅₀/ml. Furthermore, the cell-adapted viruses serially passaged under the trypsin-free condition showed similar growth curves and virus titers to those of the P3 virus, with an apex at 24 hpi (Fig. 9B). Despite the comparable growth curves of the P3 and P10 strains, the trypsin-adapted virus grew faster and produced higher titers as the passage number advanced. In particular, cells infected with the trypsin-adapted P10 virus reached virus titers of $10^{7.93}$ TCID₅₀/ml at 12 hpi and continued to produce up to 48 hpi, which was maximally 100-fold higher than that of the parental or passaged strain without trypsin addition. Compared to the P10 virus cultured in the presence of trypsin, the growth patterns were comparable among further trypsin-adapted P20–P50 strains passaged under trypsin addition (data not shown). These results demonstrated that trypsin is able to promote SARS-CoV-2 infection by facilitating cell-to-cell fusion.

To evaluate the genomic alterations that may have occurred during *in vitro* serial passages in Vero E6 cells in the presence or absence of trypsin, we determined the full-length nucleotide sequences of the parental P2 and its derived passages, P3–P50, using Sanger sequencing and RACE. The sequence data divulged no mutations in the P3 virus propagated in the presence (+) or absence (–) of trypsin compared with the original KCDC03 P2 strain. Although the 5'- and 3'-untranslated regions (UTRs) remained unchanged during *in vitro* serial passages in Vero E6 cells, virus mutations emerged in the protein-coding regions and increased gradually over time. At the nucleotide level, the genomes of P3(+) or P3(–) shared a high degree of similarity (99.94–99.99%) with the respective cell-adapted strain. In comparison to the parental P3(+) or P3(–) strain, the numbers of nucleotide/amino acid substitutions increased in direct proportion to the number of the *in vitro* passages, resulting in the highest and lowest identities with each respective P10 and P50 (Table 1).

Table 1
The number of nucleotide and amino acid changes between the parental KNU-SARS-CoV-2 P3 and cell-adapted viruses

UTRs/ORFs	Encoded proteins	No. of nucleotide/amino acid changes (No. of amino acid deletion)									
		P10(- ^a)	P20(-)	P30(-)	P40(-)	P50(-)	P10(+ ^b)	P20(+)	P30(+)	P40(+)	P50(+)
5' UTR		_c	-	-	-	-	-	-	-	-	-
ORF1a	nsp1	-	-	3/1 ^d	3/1	3/1	-	-	-	-	-
	nsp2	-	-	-	-	-	-	-	-	-	-
	nsp3	-	-	-	-	-	-	-	-	-	-
	nsp4	-	-	-	-	-	-	-	1/1	1/1	-
	nsp5	-	-	-	-	-	-	-	1/1	-	-
	nsp6	-	-	-	-	-	-	-	-	1/1	1/1
	nsp7	-	-	-	-	-	-	-	-	-	1/0
	nsp8	-	-	-	-	-	-	-	-	-	-
	nsp9	-	-	-	-	-	-	-	-	-	-
	nsp10	-	-	-	-	-	-	-	-	-	-
	nsp11	-	-	-	-	-	-	-	-	-	-
ORF1b	nsp12	-	-	-	-	-	-	-	-	-	-
	nsp13	-	-	-	-	-	-	-	-	-	1/1
	nsp14	-	-	-	-	-	-	-	-	-	-
	nsp15	-	-	-	-	-	-	-	-	-	-
	nsp16	-	-	-	-	-	-	-	-	-	-
ORF2	S	2/2	3/3	5/5	5/5	5/5	2/2	6/5	4/3	4/3	6/5
ORF3a	ORF3a	-	-	-	-	-	-	-	-	-	-
ORF4	E	1/1	1/1	1/1	1/1	1/1	-	-	-	-	-
ORF5	M	1/1	1/1	-	-	-	-	-	-	1/1	1/1
ORF6	ORF6	-	-	-	-	-	1/1	1/1	1/1	1/1	1/1
ORF7a	ORF7a	-	-	-	-	-	-	-	-	-	-
ORF7b	ORF7b	-	-	-	-	-	-	-	-	4/13 ^e	4/13
ORF8	ORF8	-	-	-	-	-	-	-	1/1	1/1	1/1
ORF9	N	1/2 ^f	1/2	1/2	1/2	1/2	-	-	-	-	-
ORF10	ORF10	-	-	-	-	-	-	-	-	-	-
3' UTR		-	-	-	-	-	-	-	-	-	-

^aSARS-CoV-2 strains passaged in the absence of trypsin

^bSARS-CoV-2 strains passaged in the presence of trypsin

^cNo mutations

^dThe deletion of three nucleotides (ATG) covering the codon for one amino acid (methionine)

^eThe deletion of 13 amino acid residues by an early termination due to a C92A substitution in ORF7b

^fThe deletion of two amino acid residues by an early termination due to a C1252T substitution in N

UTRs/ORFs	Encoded proteins	No. of nucleotide/amino acid changes (No. of amino acid deletion)									
		P10(- ^a)	P20(-)	P30(-)	P40(-)	P50(-)	P10(+ ^b)	P20(+)	P30(+)	P40(+)	P50(+)
Total number		5/6	6/7	10/9	10/9	10/9	3/3	7/6	8/7	13/21	16/23
		(2)	(2)	(3)	(3)	(3)				(13)	(13)
^a SARS-CoV-2 strains passaged in the absence of trypsin											
^b SARS-CoV-2 strains passaged in the presence of trypsin											
^c No mutations											
^d The deletion of three nucleotides (ATG) covering the codon for one amino acid (methionine)											
^e The deletion of 13 amino acid residues by an early termination due to a C92A substitution in ORF7b											
^f The deletion of two amino acid residues by an early termination due to a C1252T substitution in N											

Interestingly, the number and location of the amino acid (aa) changes differed between the cell culture-passage strains in the presence or absence of trypsin (Fig. 10). The 50th-passage strain without trypsin addition contained 9 aa mutations, including 3 aa deletions (DELs), whereas the P50(+) virus had 23 aa variations, including 13 aa DELs. The 9 aa mutations in the P50(-) virus were distributed in open reading frames (ORFs) 1a, 2, 4, and 9 encoding nsp1, S, E, and N, respectively. Among those, 5 aa changes were accumulated in the S protein of P50(-). Notably, 1 aa mutation (R685S) was found in an S1/S2 furin cleavage site (FCS) consisting of multiple basic amino acids (⁶⁸¹PRRAR⁶⁸⁵) (Table 2), which was found since P10(-). One (M) and two (QA) DELs emerged independently in nsp1 and N during the cell culture passages in the absence of trypsin, the former in P30(-) and the latter in P10(-) (Table 2). The M-DEL at position 85 in nsp1 resulted from a three-nucleotide (AUG) DEL covering the codon for methionine (M) at positions 253–255 in ORF1a (490–492 at the genome level). At positions 418 and 419 in N, the QA-DEL arose from a C to T substitution (C1252T) at position 1252 in ORF9 (29,497 at the genome level). This change resulted in the modification of CAG coding for glutamine (Q) to TAG terminator sequence at positions 1252–1254 in ORF9 (29,497–29,499 at the genome level), causing a premature termination directly removing two QA residues from the C-terminal end of N.

Table 2
Summary of amino acid mutations during *in vitro* serial passages

ORFs	Encoded proteins (aa length)	aa position ^a	Mutation at the indicated passage number										
			P3	P10(- ^b)	P20(-)	P30(-)	P40(-)	P50(-)	P10(+ ^c)	P20(+)	P30(+)	P40(+)	P50(+)
ORF1a	nsp1 (180)	85	M			DEL	DEL	DEL					
	nsp4 (500)	106	L								F	F	
	nsp5 (306)	302	G								R		
	nsp6 (290)	192	M									R	R
ORF1b	nsp13 (601)	413	T										I
ORF2	S (1273)	68	I							R	R	R	R
		178	D	N	N	N	N	N					
		185	K						N	N			
		186	F						V	V			
		484	E		D	D	D	D					
		655	H			Y	Y	Y					
		685	R	S	S	S	S	S					
		813	S			I	I	I					
		814	K										R
		949	Q										R
		960	N							I	I	I	I
		991	V							A	A	A	A
ORF4	E (75)	22	A	V	V	V	V	V					
ORF5	M (222)	125	H	Y	Y							Y	Y
ORF6	ORF6 (61)	2	F						L	L	L	L	L
ORF7b	ORF7b (43)	31–43	S–A ^d									DEL	DEL
ORF8	ORF8 (121)	67	S								F	F	F
ORF9	N (419)	418/419	QA	DEL	DEL	DEL	DEL	DEL					
^a Amino acid position numbering on the basis of the genomic sequence of SARS-CoV-2 P3 strain													
^b SARS-CoV-2 strains passaged in the absence of trypsin													
^c SARS-CoV-2 strains passaged in the presence of trypsin													
^d Deleted amino acid residues SLELQDHNETCHA in ORF7b													

The 23 aa variations in the 50th passage in the presence of trypsin were dispersed randomly in ORFs 1a, 1b, 2, 5, 6, 7b, and 8 encoding nsp6, nsp13, S, and M, as well as accessory proteins, respectively (Table 1). Although the number (five) of aa mutations in the S protein of P50(+) was identical to that in P50(-), their positions in the P50(+) S protein were completely distinct from those in P50(-) (Fig. 10). Intriguingly, 13 aa DELs occurred at positions 31–43 in ORF7b of P40(+) and were maintained until the 50th cell culture passage in the presence of trypsin (Table 2). The S–A-DEL resulted from a C to A substitution (C92A) at position 92 in ORF7b (27,819 at the genome level). This mutation altered the sequence

TCA, encoding serine (S), to a TAA termination codon at positions 91–93 in ORF7b (27,818–27,820 at the genome level), leading to an early termination eliminating 13 aa residues from the C-terminus of ORF7b.

Discussion

The receptor binding and subsequent proteolytic priming of the S protein are prerequisites for coronavirus entry into host cells. The priming event is mediated by numerous host cell proteases, including trypsin, TMPRSS2, furin, and cathepsin [29]. Among those, trypsin can promote the replication of porcine coronaviruses, including the emerging/re-emerging swine coronaviruses PEDV and PDCoV and is therefore commonly used in their isolation and cultivation [15, 17, 19]. Furthermore, protease-mediated enhancement of infection is known for SARS-CoV-1, as well as other viral families, such as influenza and parainfluenza viruses [13]. In this study, we demonstrated that trypsin mediates the enhancement of SARS-CoV-2 infection in cultured cells, and its addition at the early time points of infection (at least until 2 hpi) is indispensable for its contribution to viral replication.

The entry process of SARS-CoV-2 begins with the attachment of viral particles to the plasma membrane through the interaction between the viral S protein and its cellular receptor ACE2 [30]. The S protein consists of two functional subunits, S1 and S2; the former is responsible for ACE2 binding, and the latter is involved in the fusion between the viral and cellular membranes. Following endocytic uptake of SARS-CoV-2, pH-dependent S1/S2 proteolytic cleavage of the S protein is executed by the cellular serine protease TMPRSS2, thereby activating the entire fusion process [10]. Several other proteases, including trypsin, are known to facilitate the entry of SARS-CoV-1 at the cell membrane, indicating that SARS-CoV-1 can enter cells via either an endosomal or non-endosomal pathway, depending on the presence of proteases [13]. Similarly, the present study showed trypsin-mediated augmentation of SARS-CoV-2 entry at the cell surface. Thus, like SARS-CoV-1, SARS-CoV-2 can also exploit two different entry pathways depending on the presence of trypsin. Moreover, the non-endosomal or direct entry of SARS-CoV-2 at the cell surface, mediated by trypsin, resulted in a >1-log more efficient infection than the endosomal entry pathway. Trypsin treatment before or during viral inoculation for 1 h failed to manifest its enhancing effect on SARS-CoV-2 infection. Thus, we suppose that virus necessitates at least 1 h for trafficking from the cell surface to bind to ACE2, suggesting the role of trypsin in a post-attachment step (i.e., internalization). Given that the S protein bound to ACE2 and then cleaved by proteases could achieve fusion activity, the binding of the S protein to ACE2 appears to cause its conformational modification that is unavoidable for the direct fusion process by proteases. Therefore, trypsin can successfully trigger the fusion activity of the S protein after ACE2 binding.

SARS-CoV-2 can replicate in the respiratory (upper and lower) and gastrointestinal tracts [9]. The present study implies that the trypsin-like proteases secreted in the airways and the lungs may play a role in enhancing the replication of SARS-CoV-2 in those target organs, thereby causing severe tissue damage. In particular, SARS-CoV-2 infection of pneumocytes could induce an inflammatory response that produces numerous proteases, provoking a strong infection and the high-level growth of SARS-CoV-2 in the lungs, causing severe tissue damage. Although severe COVID-19 pneumonia accompanied by lung injury in COVID-19 patients is associated with high plasma concentrations of multiple cytokines, a so-called cytokine storm [31], a higher replication could contribute to a cytokine storm by destroying a greater number of cells infected with SARS-CoV-2. However, we found that elastase, a major protease generated during pneumonia, had no enhancing effect on SARS-CoV-2 infection, which conflicts with a previous study showing elastase-mediated enhancement of SARS-CoV-1 infection [13]. This discrepancy was attributable to a >10-fold higher concentration of elastase used in that study than compared to our study. In addition, it is tempting to assume that the coinfection with non- or low-pathogenic bacteria in respiratory organs favors the production of the proteases, which, in turn, results in severe lung illness in SARS-CoV-2-infected patients. Conversely, the small intestines, another major target organ, express ACE2 and TMPRSS2 and secrete a variety of proteases [10, 32]. The presence of trypsin might also trigger the high multiplication of SARS-CoV-2 in the small intestines, which could be associated with the high incidence rate of diarrhea in patients with COVID-19 [33].

We obtained cell culture-adapted strains of SARS-CoV-2 by 50 passages in Vero E6 cells in the presence or absence of trypsin and ascertained their phenotypic and genetic characteristics. The virological assays revealed that the cell-adapted viruses without trypsin addition grew comparably and formed CPE (cell rounding and clumping) similarly to the parental virus in cultured cells. By contrast, the trypsin-adapted strains replicated more efficiently and produced larger syncytia than the P3(+) virus in Vero E6 cells. Moreover, SARS-CoV-2 strains passaged in the presence of trypsin showed higher multiplication kinetics than those passaged in the absence of trypsin. Altogether, these findings indicated that the trypsin-adapted strains had a superior viral fitness in the cultured cells and that trypsin supplementation positively influenced *in vitro* viral growth.

Subsequently, we sequenced the entire genomes of the cell culture-adapted strains to decode genetic mutations that emerged during serial cell passages in the presence or absence of trypsin. Upon cell adaptation without trypsin addition, a total of six non-synonymous mutations arose, and among them, 5 aa substitutions were accumulated in the S protein. Interestingly, all cell-adapted P10(–) through P50(–) strains contained an R685S change in the S1/S2 FCS (⁶⁸¹PRRAR⁶⁸⁵), resulting in the altered S1/S2 FCS (⁶⁸¹PRRAS⁶⁸⁵). Although biochemical studies are needed to investigate whether their S proteins with the R685S mutation are cleaved or not, this genetic mutation suggests that the proteolytic priming of the S protein is dispensable for virus replication *in vitro*. Considering that none of the P10(–) through to P50(–) strains induced syncytia formation, the R685S mutation might make the S1/S2 FCS non-functional and, accordingly, result in the failure of the S cleavage process, which, in turn,

attenuates the membrane fusion activity of the S protein. In addition to point mutations, we found the genesis of two different DEL signatures in nsp1 and N of the cell-adapted strains without trypsin addition, indicating that the DELs are associated with virus adaptation in the cell culture system in the absence of trypsin.

During 50 cell passages in the presence of trypsin, a total of 20 non-synonymous mutations and 13 DELs were identified, all of which completely differed from those identified in the cell-adapted strains without trypsin addition. These results suggest that trypsin may be relevant to the genetic modification of SARS-CoV-2 during *in vitro* growth. Unlike the P10(–) through to P50(–) strains, all trypsin-adapted P3(+) through to P50(+) strains retained the intact S1/S2 FCS (⁶⁸¹PRRAR⁶⁸⁵) in the S protein. The S protein induces fusion of the viral envelope with host cell membranes, and this fusion activity is most likely conferred by the internal hydrophobic sequences in the S2 subunit [34, 35]. The trypsin-adapted strains formed more extensive syncytia, leading to an increase in vacuolated areas, which were proportional to the number of cell culture passages. Given the proteolytic cleavage of the S protein by the functional S1/S2 FSC, the mutations in S2 appear to be involved in the formation of syncytia in cells infected with the trypsin-adapted virus. Accordingly, N960I and V961A mutations were identified in S2 of the P20(+) through to P50(+) strains, and additional K849R and Q949R emerged in the S2 fusion domain of P50(+), suggesting that these genetic drifts may contribute to cytopathology. Interestingly, the trypsin-adapted P40(+) and P50(+) viruses had a large 13-aa DEL in a short ORF7b protein composed of 43 aa residues. Although the precise role of this accessory protein is unknown, we cannot exclude the involvement of ORF7b in SARS-CoV-2 propagation *in vitro*.

In conclusion, the present study showed that trypsin enhances the replication of SARS-CoV-2 in cultured cells and facilitates viral entry by promoting a direct fusion process at the cell surface. Our findings indicate that SARS-CoV-2 has the potential to admit distinct pathways, by which it can enter cells depending on the presence of trypsin. Remarkably, the trypsin-triggered SARS-CoV-2 non-endosomal entry at the cell surface facilitated more efficient infection than the endosomal pathway in the absence of trypsin. Moreover, the Vero E6 cell-adapted SARS-CoV-2 strains with trypsin addition exhibited clear syncytia formation and robust infection in cultured cells, whereas the strains without trypsin addition failed to form syncytia in infected cells and to enhance viral infection. These results suggest that trypsin or other proteases produced in the lungs or small intestines may be responsible for the high multiplication of SARS-CoV-2, leading to severe tissue damage. Conversely, these data may provide practical methodology to improve the isolation and propagation of SARS-CoV-2 for the development of vaccines and other therapeutic agents. We also identified genetic mutations during serial *in vitro* passages in the presence or absence of trypsin, which might be involved in the proteolytic priming and further fusion activity of the S protein. Thus, our genetic data will provide fundamental insights into future research involving reverse genetics to address the specific roles of mutations and other DELs, occurring during cell adaptation, in SARS-CoV-2 replication and pathogenesis.

Declarations

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Compliance with Ethical Standards

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Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: All animal procedures were carried out in accordance with the guidelines established by Institutional Animal Care and Use Committee.

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Figures

Fig. 1

Figure 1

Trypsin-mediated enhancement of SARS-CoV-2 infection in cultured cells. (A and B) Vero E6 cells were preincubated with trypsin for 1 h before infection and then mock infected or infected with SARS-CoV-2 (P3) at an MOI of 1 for 1 h without trypsin addition. The virus-infected cells were further maintained in the absence of trypsin. (C and D) Vero E6 cells were mock infected or infected with SARS-CoV-2 (P3) at an MOI of 1 for 1 h in the presence of trypsin. The virus-infected cells were further maintained in the absence of trypsin. (E and F) Vero E6 cells were mock infected or infected with SARS-CoV-2 (P3) at an MOI of 1 for 1 h without trypsin addition. The virus-infected cells were further maintained in the presence of

trypsin. SARS-CoV-2-specific CPE was observed daily, and cells were photographed at 24 hpi using an inverted microscope at a magnification of 200× (top panels). For immunostaining, infected cells were fixed at 24 hpi and incubated with MAb against the SARS-CoV-2 N protein, followed by incubation with Alexa green-conjugated goat anti-mouse secondary antibody (middle panels). Then, the cells were counterstained with DAPI (bottom panels) and examined under a fluorescence microscope at 200× magnification (A, C, and E). The virus supernatants were collected at 24 hpi, and viral titers were determined (B, D, and F). Values shown are representative of the mean of three independent experiments, and error bars denote mean ± SDM. *P = 0.001 to 0.05.

Fig. 2

Figure 2

Comparison of SARS-CoV-2 production in the presence and absence of trypsin. Vero E6 cells were mock infected or infected with 10-fold serially diluted SARS-CoV-2 and further cultivated in the presence (A) or absence (B) of trypsin. The virus infected cells were fixed at 24 hpi and incubated with MAb against the SARS-CoV-2 N protein, followed by incubation with Alexa green-conjugated goat anti-mouse secondary antibody (top panels). The cells were then counterstained with DAPI (bottom panels) and examined under a fluorescence microscope at 200× magnification.

Fig. 3

Figure 3

Effect of elastase on SARS-CoV-2 infection. (A and B) Vero E6 cells were preincubated with elastase for 1 h before infection and then mock infected or infected with SARS-CoV-2 (P3) at an MOI of 1 for 1 h without elastase addition. The virus-infected cells were further maintained in the absence of elastase. (C and D) Vero E6 cells were mock infected or infected with SARS-CoV-2 (P3) at an MOI of 1 for 1 h in the presence of elastase. The virus-infected cells were further maintained in the absence of elastase. (E and F) Vero E6 cells were mock infected or infected with SARS-CoV-2 (P3) at an MOI of 1 for 1 h without elastase addition. The virus-infected cells were further maintained in the presence of elastase. For immunostaining, infected cells were fixed at 24 hpi and incubated with MAb against the SARS-CoV-2 N protein, followed by incubation with Alexa green-conjugated goat anti-mouse secondary antibody (top panels). The cells were then counterstained with DAPI (bottom panels) and examined under a fluorescence microscope at 200× magnification (A, C, and E). The virus supernatants were collected at 24 hpi, and viral titers were determined (B, D, and F). Values shown are representative of the mean of three independent experiments, and error bars denote mean $\pm$ SDM.

Fig. 4

Figure 4

Effect of proteases on SARS-CoV-2 propagation at early time points post-infection. Vero E6 cells were mock infected or infected with SARS-CoV-2 (P3) at an MOI of 1. At the indicated time points post-infection, trypsin (A) or elastase (B) was added to achieve a final concentration of 5 or 10 µg/ml, respectively. At 12 hpi, the culture supernatant was collected, and viral production was quantified by virus titration. Results are presented as the mean values of three independent experiments, and error bars denote mean ± SDM. *P < 0.05; **P < 0.001.

Fig. 5

Figure 5

Effect of trypsin on SARS-CoV entry. (A) Vero E6 cells were incubated with SARS-CoV-2 at an MOI of 1 at 4°C for 1 h, after which the unbounded virus was removed and then treated with proteinase K (0.5–1 mg/ml) at 4°C for 45 min. The cells were collected in TRIzol for RNA isolation and SARS-CoV-2 RNA copy number determination. Results are shown as a percentage of SARS-CoV-2 RNA copy number compared with controls (ctrl), in which PBS was substituted for proteinase K. (B) Vero E6 cells were infected with SARS-CoV-2 (MOI of 1) at 4°C for 1 h and washed with cold PBS. The infected cells were then incubated in the presence or absence of trypsin (5 µg/ml) either at 4°C (binding; blue bars) or 37°C (internalization; red bars) for an additional 1 h. The virus-infected cells incubated at 37°C were further treated with proteinase K (0.5 mg/ml) at 37°C for 45 min. The infected cells were then serially diluted and plated onto fresh Vero E6 cells. At 24 h post-incubation, bound or internalized viruses were titrated. Data are expressed as the mean values from three independent experiments performed in triplicate, and error bars represent mean ± SDM. *P < 0.05; **P < 0.001.

Fig. 6

Figure 6

Effect of BafA1 on SARS-CoV-2 infection. (A and B) Vero E6 cells were preincubated with BafA1 for 1 h before infection and then mock infected or infected with SARS-CoV-2 (P3) at an MOI of 1 for 1 h without BafA1 addition. The virus-infected cells were further maintained in the absence of BafA1. (C and D) Vero E6 cells were mock infected or infected with SARS-CoV-2 (P3) at an MOI of 1 for 1 h in the presence of BafA1. The virus-infected cells were further maintained in the absence of BafA1. (E and F) Vero E6 cells were mock infected or infected with SARS-CoV-2 (P3) at an MOI of 1 for 1 h without BafA1 addition. The virus-infected cells were further maintained in the presence of BafA1. For immunostaining, infected cells were fixed at 24 hpi and incubated with MAb against the SARS-CoV-2 N protein, followed by incubation with Alexa green-conjugated goat anti-mouse secondary antibody (top panels). The cells were then counterstained with DAPI (bottom panels) and examined under a fluorescence microscope at 200× magnification (A, C, and E). The virus supernatants were collected at 24 hpi, and viral titers were determined (B, D, and F). Values shown are representative of the mean of three independent experiments, and error bars denote mean $\pm$ SDM. *, $P = 0.001$ to 0.05 .

Fig. 7

Figure 7

Entry of SARS-CoV-2 at the cell surface facilitated by trypsin. (A) Effect of proteases on SARS-CoV-2 into Vero E6 cells treated with BafA1. Vero E6 cells cultured in 6-well plates were treated with BafA1 at a concentration of 0.5 μM at 37°C for 30 min, placed at 4°C for 30 min and infected with SARS-CoV-2 at MOI of 1 for 30 min. Then, cells were treated with various concentrations of trypsin or elastase at room temperature for 5 min and maintained in the presence of BafA1 for an additional 6 h. The amount of SARS-CoV-2 was measured quantitatively by real-time PCR. Cells not treated with BafA1 or those treated with BafA1 but not treated with trypsin or elastase were used as controls. (B) Effect of trypsin treatment before or after inoculation on SARS-CoV infection in the presence of BafA1. Vero E6 cells were treated with BafA1 (0.5 μM) at 37°C for 30 min and then treated with trypsin (10 and 30 μg/ml) at room temperature for 5 min before (pre) or after (post) virus inoculation. Viral infectivity was estimated quantitatively by real-time PCR. (C) SARS-CoV-2 kinetics after treatment of trypsin. Vero E6 cells were treated with BafA1, infected with SARS-CoV-2, and treated with 75 μg/ml trypsin, as described in the legend to Fig. 7A. The production of SARS-CoV was measured by real-time PCR at 3–6 h after virus inoculation. Vero E6 cells without any treatment were also infected as a control (untreated). The viral titers were expressed as genomic copies/ml. Data are expressed as the mean values of three independent experiments, and error bars denote mean ± SDM. *P < 0.05; **P < 0.001.

Fig. 8

Figure 8

Cytopathic changes in virus-infected cells cultured in the presence or absence of trypsin. Fifty subsequent passages were performed in Vero E6 cells in the presence or absence of trypsin. Vero E6 cells were mock infected or infected with each representative cell-adapted SARS-CoV-2 strain (P3, P10, P20, P30, P40, and P50) and maintained in the presence or absence of 5 µg/ml trypsin. SARS-CoV-2 specific CPE was observed daily, and cells were photographed at 24 hpi using an inverted microscope at a magnification of 200×.

Fig. 9

Figure 9

One-step growth kinetics of SARS-CoV-2 strains passaged in the presence or absence of trypsin. Vero E6 cells were infected with each representative cell-adapted SARS-CoV-2 strain P3 (A) and P10 (B) and maintained in the presence or absence of 5 µg/ml trypsin. At the indicated time points post-infection, culture supernatants were harvested, and virus titers were determined. Results are expressed as the mean values of three independent experiments performed in triplicate, and error bars represent mean ± SDM. *P < 0.05; **P < 0.001.

Fig. 10

Figure 10

Schematic diagram of the amino acid differences between SARS-CoV-2 (P3) and its cell-adapted decedents (P10–P50). The SARS-CoV-2 genomic organization of approximately 29.8 kb is shown at the top. The first diagram represents the genomic regions, with blue arrows indicating the nonstructural proteins (nsp1–16). The second illustration represents the genomic regions, with orange bars symbolizing the identified ORFs. Light gray arrows represent each 5'- and 3'-untranslated region. Lightly shaded areas are identical to those of SARS-CoV-2 (P3), and the vertical black bars represent one amino acid sequence that is divergent from the P3 virus. Thin horizontal dashed lines indicate deletions. S1/S2 FCS represents an S1/S2 furin cleavage site (FCS) consisting of multiple basic amino acids (681PRRAR685), and the vertical red bars indicated an R685S mutation at the S1/S2 FCS.