Cell entry mechanisms of SARS-CoV-2

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A novel severe acute respiratory syndrome (SARS)-like coronavirus SARS-CoV-2 is causing the global coronavirus disease 2019 (COVID-19) pandemic. Understanding how SARS-CoV-2 enters human cells is a high priority for deciphering its mystery and curbing its spread. A virus surface spike protein mediates SARS-CoV-2 entry into cells. To fulfill its function, SARS-CoV-2 spike binds to its receptor human ACE2 (hACE2) through its receptor-binding domain (RBD) and is proteolytically activated by human proteases. Here we investigated receptor binding and protease activation of SARS-CoV-2 spike using biochemical and pseudovirus entry assays. Our findings have identified key cell entry mechanisms of SARS-CoV-2. First, SARS-CoV-2 RBD has higher hACE2 binding affinity than SARS-CoV RBD, supporting efficient cell entry. Second, paradoxically, the hACE2 binding affinity of the entire SARS-CoV-2 spike is comparable to or lower than that of SARS-CoV spike, suggesting that SARS-CoV-2 RBD, albeit more potent, is less exposed than SARS-CoV RBD. Third, unlike SARS-CoV, cell entry of SARS-CoV-2 is preactivated by proprotein convertase furin, reducing its dependence on target cell proteases for entry. The high hACE2 binding affinity of the RBD, furin preactivation of the spike, and hidden RBD in the spike potentially allow SARS-CoV-2 to maintain efficient cell entry while evading immune surveillance. These features may contribute to the wide spread of the virus. Successful intervention strategies must target both the potency of SARS-CoV-2 and its evasiveness.

Significance

A key to curbing SARS-CoV-2 is to understand how it enters cells. SARS-CoV-2 and SARS-CoV both use human ACE2 as an entry receptor and human proteases as entry activators. Using biochemical and pseudovirus entry assays and SARS-CoV as a comparison, we have identified key cell entry mechanisms of SARS-CoV-2 that potentially contribute to the immune evasion, cell infectivity, and wide spread of the virus. This study also clarifies conflicting reports from recent studies on cell entry of SARS-CoV-2. Finally, by highlighting the potency and the evasiveness of SARS-CoV-2, the study provides insight into intervention strategies that target its cell entry mechanisms.

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In addition to receptor binding, protease activators for SARS-CoV-2 entry have been examined. It has been shown that TMPRSS2 and lysosomal proteases are both important for SARS-CoV-2 entry (33, 34). In avian influenza viruses, proprotein convertase (PPC) motif in the surface glycoprotein is a hallmark of high pathogenesis (35). However, although SARS-CoV-2 spike contains a PPC motif at the S1/S2 boundary, it was reported that PPC cleavage of the spike protein did not enhance SARS-CoV-2 entry into cells (31), challenging the well-established concept on the role of PPC motif. This raised questions about the role of PPC motif in SARS-CoV-2 entry.

Here we investigate the receptor binding and protease activations of SARS-CoV-2 spike, using SARS-CoV spike as a comparison. Our results identify important cell entry mechanisms of SARS-CoV-2 that potentially contribute to the immune evasion, cell infectivity, and wide spread of the virus. The findings reconcile conflicting recent reports on cell entry of SARS-CoV-2. By revealing the surprising strategies that SARS-CoV-2 adopts to infect humans while evading immune surveillance, the findings provide insight into possible intervention strategies targeting cell entry of the virus.

**Results**

Through examining the sequence of SARS-CoV-2 spike, we identified a putative cleavage site for PPCs at the S1/S2 boundary (Fig. 1 C and D). Curiously, this putative PPC site is absent in the spikes of SARS-CoV and SARS-like bat coronaviruses. In this study, we investigated the role of PPC, along with other proteases, in SARS-CoV-2 entry. To this end, we established a pseudovirus entry assay for SARS-CoV-2. More specifically, replication-deficient lentiviruses were pseudotyped with SARS-CoV-2 spike (i.e., SARS-CoV-2 pseudoviruses) and used to enter target cells. This type of pseudovirus assay separates viral entry from other steps of the viral infection cycle (e.g., replication), enabling us to focus on the viral entry step that is mediated by SARS-CoV-2 spike. Three types of target cells were used: HeLa cells (human cervical cells) exogenously expressing hACE2, Calu-3 cells (human lung epithelial cells) endogenously expressing hACE2, and MRC-5 cells (human lung fibroblast cells) endogenously expressing hACE2.

To detect the cleavage state of SARS-CoV-2 spike on the surface of pseudoviruses, we packaged SARS-CoV-2 pseudoviruses in HEK293T cells (human embryonic kidney cells) and performed Western blot on the pseudoviruses. The result showed that SARS-CoV-2 spike had been cleaved during viral packaging (Fig. 2A). We then mutated the putative PPC site in SARS-CoV-2 spike to the corresponding sequence in SARS-CoV spike; the mutant SARS-CoV-2 spike was no longer cleaved during viral packaging (Fig. 2A). Further, we performed pseudovirus entry assay using both wild-type SARS-CoV-2 pseudoviruses and PPC site mutant SARS-CoV-2 pseudoviruses. The result showed that SARS-CoV-2 pseudoviruses efficiently entered all three types of target cells (Fig. 2B). In contrast, the mutant SARS-CoV-2 pseudoviruses demonstrated significantly reduced efficiency in entering the same cells (Fig. 2B). The remaining cell entry of the mutant SARS-CoV-2 pseudoviruses was likely due to the activation from other host proteases that play partially overlapping and cumulative roles with PPCs (see below). Therefore, we have identified and confirmed the PPC cleavage site in SARS-CoV-2 spike, and shown that PPC cleavage of SARS-CoV-2 spike during viral packaging is critical for SARS-CoV-2 to enter three different types of target cells.

To provide further evidence for the role of prior PPC cleavage in SARS-CoV-2 entry, we treated HEK293T cells with PPC inhibitor (PPCI) during packaging of wild-type SARS-CoV-2 pseudoviruses, and then subjected the PPCI-treated SARS-CoV-2 pseudoviruses to entry into the aforementioned three types of target cells. The result showed that PPCI treatment inhibited PPC cleavage of SARS-CoV-2 spike on pseudoviruses, and that the
PPCi-treated SARS-CoV-2 pseudoviruses demonstrated significantly reduced cell entry efficiency (Fig. 3A). In comparison, SARS-CoV spike was not cleaved during packaging of SARS-CoV pseudoviruses, and PPI treatment during virus packaging had no effect on the subsequent cell entry process (Fig. 3B). These results further confirm that the efficiency of SARS-CoV-2 entry into target cells can be enhanced by the prior PPI cleavage of the SARS-CoV-2 spike during viral packaging. In contrast to SARS-CoV whose cell entry does not depend on PPI activation.

Since the PPI used above is a broad-spectrum PPI, we further investigated which specific PPI activates SARS-CoV-2 spike using small interfering RNA (siRNA) assay. To this end, we packaged SARS-CoV-2 pseudoviruses in HEK293T cells that were treated with furin-targeting siRNA. Furin was selected in our study because it is the prototypic PPI and it preactivates the entry of many other viruses, including some coronaviruses (22, 23). The result showed that, after furin-targeting siRNA treatment, the spike molecules on the packaged SARS-CoV-2 pseudoviruses were intact (Fig. 3C), revealing that furin is the PPI that preactivates SARS-CoV-2 spike. To rule out the possibility that furin-dependent activation of matrix metalloproteinases (MMPs) led to indirect activation of SARS-CoV-2 spike, we treated HEK293T cells with MMP inhibitor during packaging of SARS-CoV-2 pseudoviruses. The result showed that, after MMP inhibitor treatment, the spike molecules on the packaged SARS-CoV-2 pseudoviruses were still cleaved (Fig. 3D), demonstrating that MMP is not involved in the activation of SARS-CoV-2 spike. Taken together, these findings show that furin is the PPI that preactivates SARS-CoV-2 spike (1, 2).

To investigate the role of other proteases in SARS-CoV-2 entry, we performed pseudovirus entry assay in the presence of inhibitors that specifically target these other proteases. First, SARS-CoV-2 pseudovirus entry into all three types of target cells was reduced in the presence of TMPRSS2 inhibitor camostat (Fig. 4A), suggesting that these cells endogenously express TMPRSS2 and that these TMPRSS2 molecules activate SARS-CoV-2 entry. Second, SARS-CoV-2 pseudovirus entry into all three types of target cells was reduced in the presence of lysosomal cathepsin inhibitor E64d (Fig. 4A). Hence, lysosomal cathepsins activate SARS-CoV-2 entry. Similarly, SARS-CoV entry can also be activated by TMPRSS2 and lysosomal cathepsin (Fig. 4B). Moreover, prior treatment of pseudovirus-packaging cells with PPI, combined with treatment of pseudovirus-targeted cells with either camostat or E64d, further reduced the efficiency of SARS-CoV-2 pseudovirus entry into HeLa cells (Fig. 4A). Thus, TMPRSS2 and lysosomal cathepsins both have cumulative effects with furin on activating SARS-CoV-2 entry. In contrast, neither camostat nor E64d has cumulative effects with PPI on activating SARS-CoV entry (Fig. 4B). Overall, these results demonstrate that cell surface proteases and lysosomal proteases can both activate SARS-CoV-2 entry; in addition, furin and these other proteases have cumulative effects on activating SARS-CoV-2 entry.

Discussion
With mounting infections, fatalities, and economic losses caused by SARS-CoV-2, it is imperative that we understand the cell entry mechanisms of SARS-CoV-2. However, recent studies have presented puzzling and sometimes conflicting findings on how SARS-CoV-2 enters cells, raising pressing scientific questions (30–32, 34). For example, which virus binds to hACE2 more tightly, SARS-CoV-2 or SARS-CoV? What is the role of furin in SARS-CoV-2 entry? How does SARS-CoV-2 successfully evade human immune surveillance while maintaining its high cell infectivity? The current study addresses these questions by detailing the cell entry mechanisms of SARS-CoV-2.

Receptor recognition is an important determinant of coronavirus infection and pathogenesis. It is also one of the most...
important targets for host immune surveillance and human intervention strategies. The current study and other recent studies have revealed two patterns of results on the hACE2 binding affinity of SARS-CoV-2. First, with regard to the RBD, SARS-CoV-2 RBD has significantly higher hACE2 binding affinity than SARS-CoV RBD does. This was shown in our recent study using SPR assay as well as structural and mutagenesis analyses (30). In addition, using protein pull-down assay, the current study confirmed that SARS-CoV-2 RBD has higher hACE2 binding affinity than SARS-CoV RBD does. Second, despite the potency of its RBD’s binding to hACE2, the entire SARS-CoV-2 spike does not bind to hACE2 any more strongly than SARS-CoV spike does. Using protein pull-down assay, the current study showed that SARS-CoV-2 spike binds to hACE2

Fig. 3. Effect of PPCs on SARS-CoV-2 spike-mediated cell entry. (A) SARS-CoV-2 pseudovirus entry into three types of target cells in the presence of PPCi. The pseudoviruses were packaged in the presence of different concentrations of PPCi before they were subjected to cell entry; (-) control: no pseudovirus was added. Also shown is the Western blot result of the corresponding pseudoviruses (packaged in the presence of different concentrations of PPCi). The entry efficiency of SARS-CoV-2 pseudoviruses without any treatment was taken as 100%. Error bars indicate SD (n = 4). ***P < 0.001; **P < 0.01; *P < 0.05. (B) SARS-CoV pseudovirus entry into three types of target cells in the presence of PPCi. The experiments were performed in the same way as in A, except that SARS-CoV spike replaced SARS-CoV-2 spike in pseudoviruses. The entry efficiency of SARS-CoV pseudoviruses without any treatment was taken as 100%. (C) Western blot result of SARS-CoV-2 pseudoviruses packaged in cells treated with siRNA. (Left) Pseudoviruses packaged in cells treated with siRNA-negative control. (Right) Pseudoviruses packaged in cells treated with furin-targeting siRNA. (D) Western blot result of SARS-CoV-2 pseudoviruses packaged in cells treated with MMP inhibitor. (Left) Pseudoviruses packaged in cells not treated with MMP inhibitor. (Right) Pseudoviruses packaged in cells treated with MMP inhibitor.

Fig. 4. Effect of other protease inhibitors on SARS-CoV-2 entry. (A) SARS-CoV-2 pseudovirus entry into three types of target cells in the presence of protease inhibitors. For pseudoviruses treated with PPCi, the pseudoviruses were packaged in the presence of PPCi (5 μM) before they were subjected to cell entry. For pseudoviruses treated with TMPRSS2 inhibitor camostat or lysosomal protease inhibitor E64d, pseudovirus entry was performed in the presence of camostat (50 μM) or E64d (50 μM). The cleavage state of SARS-CoV-2 spike was the same as in Fig. 3A (5 μM PPCi condition). The entry efficiency of SARS-CoV-2 pseudoviruses without any treatment was taken as 100%. Error bars indicate SD (n = 4). ***P < 0.001; *P < 0.05. (B) SARS-CoV pseudovirus entry into three types of target cells. The treatments were done in the same way as in A.
To maintain its high infectivity while keeping its RBD less accessible, SARS-CoV-2 relies on a second strategy—host protease activation. Host protease activation is a significant determinant of coronavirus infection and pathogenesis, and a significant target for host immune surveillance and human intervention strategies. Using a combination of mutagenesis, protease inhibitors, and siRNA approaches, here we showed that furin preactivation enhances SARS-CoV-2 pseudovirus entry into different types of hACE2-expressing cell lines, including lung epithelial and lung fibroblast cell lines. We also showed that cell surface protease TMPRSS2 and lysosomal cathepsins activate SARS-CoV-2 pseudovirus entry and that both TMPRSS2 and cathepsins have cumulative effects with furin on SARS-CoV-2 entry. In comparison, SARS-CoV-2 entry is activated by TMPRSS2 and cathepsins, but not furin. Furin preactivation allows SARS-CoV-2 to be less dependent on target cells, enhancing its entry into some target cells, particularly cells with relatively low expressions of TMPRSS2 and/or lysosomal cathepsins. This has also been observed with furin-preactivated avian influenza viruses (32). However, a recent study showed that furin preactivation enhances SARS-CoV-2 pseudovirus entry into BHK cells (baby hamster kidney fibroblast cells), but reduces SARS-CoV-2 pseudovirus entry into Vero cells (African green monkey kidney epithelial cells) (31). These seemingly conflicting results can be explained by how coronavirus entry is regulated by proteases. Protease activation of coronavirus spikes potentially leads to the final structural change of coronavirus S2 needed for membrane fusion; this process is irreversible and needs to be tightly regulated (13). Indeed, it has been shown that, on SARS-CoV-2 virus particles, many spike molecules have already undergone the final structural change (36). Hence, in principle, virus particles preactivated by furin may have unchanged or reduced entry efficiency in some types of cells with high expressions of TMPRSS2 and/or lysosomal proteases; this may particularly be the case in vitro for virus particles that are not fresh, as the final conformational change of spike molecules may occur slowly spontaneously or be facilitated by environmental factors (e.g., high temperature, physical force, or some chemicals) (37). Overall, furin preactivation can facilitate SARS-CoV-2 to enter some types of cells (particularly those with low expressions of TMPRSS2 and/or lysosomal cathepsins) (Fig. 6d).

The cell entry mechanisms of SARS-CoV-2 have implications for understanding clinical features of coronavirus disease 2019 (COVID-19) (Fig. 6d). The hidden RBD can evade immune surveillance, potentially leading to insufficient immune responses and prolonged recovery time. Granted, there are other immune evasion strategies for coronaviruses. For example, some coronavirus nonstructural proteins can help evade the host innate immune responses (38, 39). Importantly, viruses commonly hide their RBD or other critical parts of their spike proteins from host adaptive immune responses using two main strategies (40). The first is conformational masking, where viruses conceal their RBDs in locations like canyons (as in the case of picornaviruses) (41) or recessed pockets (as in the case of HIV) (42). The second is glycan shielding, where viruses conceal critical parts of their spike proteins behind glycan clusters (as in the case of Hepatitis C virus) (43). Our finding about the discrepancy in hACE2 binding affinity between SARS-CoV-2 RBD and spike, combined with other groups’ observation of the lying-down RBD in SARS-CoV-2 spike, suggests that the hidden RBD contributes to the immune evasion of SARS-CoV-2 as one of the conformational masking strategies. Indeed, a recent study showed that SARS-CoV RBD-induced mouse sera bind SARS-CoV-2 RBD with high affinity, but poorly neutralize SARS-CoV-2 pseudovirus entry into host cells;
cellular proteases as entry activators, inhibitor mixtures against protease activators (47). Because SARS-CoV-2 uses several proteases to gain entry, inhibitors that target these proteases can block SARS-CoV-2 entry. SARS-CoV-2 can be blocked using inhibitors that target the protease activators and furin, which is involved in the proteolytic processing of the spike. Thus, multiple approaches to developing drugs that target SARS-CoV-2 entry mechanisms are possible.

The success of this approach for vaccine development, however, may be limited because the S2 subunit is less immunogenic than the RBD (15). Last, the cell entry process of SARS-CoV-2 is more complex than that of SARS-CoV. Understanding the cell entry mechanism of SARS-CoV-2 can inform intervention strategies. The RBD is the most immunogenic region of the whole spike (15, 45). Hence, the hidden RBD of SARS-CoV-2 presents a major challenge to both vaccination and antibody drug therapy due to the limited access of neutralizing antibodies to the target. Correspondingly, there are several approaches for intervention strategies, with some caveats. First, antibody drugs can be developed to bind to the RBD very tightly, preferably with both a high on rate and a low off rate, such that, during the limited exposure of RBD, the drugs can latch onto the RBD quickly and keep a strong hold on it. It was recently shown that recombinant ACE2 can inhibit SARS-CoV-2 infection in artificial human tissues (46), suggesting that blocking the RBD is feasible. Thus, an antibody drug with significantly higher RBD binding affinity than ACE2 can dominate over cell surface ACE2 in latching onto the RBD, blocking viral attachment. Second, RBD vaccines can be developed. Because neutralizing antibodies elicited by RBD vaccines may have limited access to the RBD, structure-guided engineering will be needed to significantly enhance the efficacy of RBD vaccines (45). Third, vaccines and drugs can be developed to target the membrane fusion S2 subunit. The success of this approach for vaccine development, however, may be limited because the S2 subunit is less immunogenic than the RBD (15).

Materials and Methods

Cell Line and Plasmids. HEK293T, HeLa, Calu-3, and MRC-5 cells were obtained from the American Type Culture Collection and cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies).

Full-length SARS-CoV-2 spike (GenBank accession number QHD43416.1), SARS-CoV Spike (GenBank accession number APRS8740.1), MERS-CoV spike (GenBank accession number A5SB936.1), and human ACE2 (GenBank accession number NM_021804) were synthesized (GenScript Biotech) and subcloned into the pDNA3.1 (+) vector (Life Technologies) with a C-terminal C9 tag. SARS-CoV-2 RBD (residues 319 to 535), SARS-CoV RBD (residues 306 to 521), MERS-CoV RBD (residues 367 to 588), and human ACE2 peptidase domain (residues 1 to 615) were subcloned into pFastBac vector (Life Technologies) with an N-terminal honey bee melittin signal peptide and a C-terminal His tag. For human ACE2 peptidase domain, a construct was also made containing a C-terminal Fc tag instead of the C-terminal His tag.

Protein Expression and Purification. All of the proteins were expressed in sf9 insect cells using the Bac-to-Bac system (Life Technologies). Briefly, His-tagged proteins were harvested from cell culture medium, and were purified sequentially on Ni-NTA column and Superdex200 gel filtration column (GE Healthcare) as described previously (30). The Fc-tagged protein was purified in the same way, except that protein A column replaced Ni-NTA column (30). Purified proteins were stored in a buffer containing 20 mM Tris pH 7.2 and 200 mM NaCl for later use.

Coronavirus Spike-Mediated Pseudovirus Entry Assay. Retroviruses pseudotyped with SARS-CoV-2 spike or SARS-CoV spike were generated in HEK293T cells, and pseudovirus entry assay was performed as previously described (48). Briefly, HEK293T cells were cotransfected with a plasmid carrying an Env-defective, luciferase-expressing HIV-1 genome (pNL4-3.lucR-E-) and pDNA3.1 (+) plasmid encoding one of the indicated spikes.
Pseudoviruses were harvested 72 h after transfection, and were used to enter target cells. Six hours after incubation with pseudoviruses, cells were transferred to fresh medium. After another 6 h, cells were washed and lysed for detection of luciferase signal (relative luciferase units or RLU). Target cells for pseudovirus entry assay included HEK293 cells expressing human ACE2, and Calu-3 and MRC-5 cells endogenously expressing human ACE2.

For pseudoviruses treated with PPCi or matrix MMP inhibitor, PPCi chloromethylketone (Enzo Life Sciences) was added to the medium at indicated concentrations 6 h after transfection for pseudovirus packaging began. Pseudoviruses were harvested after an additional incubation time of 66 h. Pseudoviruses were then used to enter target cells.

For pseudoviruses treated with siRNA, siRNA furin and siRNA negative control (Thermo Fisher Scientific) were transfected separately into HEK293 cells 6 h after transfection for pseudovirus packaging began. Pseudoviruses were harvested after an additional incubation time of 66 h. Pseudoviruses were then subjected to Western blot analysis.

For pseudoviruses treated with other protease inhibitors, target cells were pretreated with camostat (50 μM) (Sigma-Aldrich) or E64d (50 μM) (Sigma-Aldrich) for 1 h and then subjected to pseudovirus entry assay as described above.

**Protein Pull-Down Assay.** Protein pull-down assay was performed using a Dynabeads immunoprecipitation kit (Invitrogen) as previously described (30). Briefly, 80 μL of Dynabeads, either for His-tagged proteins or for Fc-tagged proteins, were washed with phosphate-buffered saline (PBS) buffer and then were incubated with either 5 μg hACE2-His6 (human ACE2 with a C-terminal His6 tag) or 5 μg hACE2-Fc (human ACE2 with a C-terminal Fc tag), respectively, on a roller at room temperature for 30 min. Subsequently, hACE2-bound beads were washed three times with 1 mL of PBS buffer plus 0.05% Tween-20 (PBST) on a roller for 10 min and then were aliquoted into different tubes for later use. To prepare cell-associated coronavirus spike protein, HEK293T cells were transfected with pcDNA3.1(+)-plasmid encoding coronavirus spike protein (containing a C-terminal C9 tag); 48 h after transfection, the spike-expressing cells were lysed using a sonicator in assay buffer and centrifuged at 12,000 × g for 2 min. The supernatants containing solubilized SARS-CoV-2 spike (for spike pull-down assay) or purified recombining coronaviruses (for Fc or His tag pull-down assay) were incubated with the hACE2-bound beads in 2-mL tubes (spike or RBD was in excess of hACE2) on a roller at room temperature for 1 h. Then beads were washed three times with PBST buffer, and the bound proteins were eluted using elution buffer. The samples were then subjected to Western blot analysis and detected using an anti-C9 tag antibody or anti-His tag antibody.

**Statistical Analysis.** All experiments were repeated at least four times. Statistical analyses were performed using t tests. A P value < 0.05 was considered statistically significant; **P < 0.01. ***P < 0.001. **P < 0.005.
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