



Structural basis of the American mink ACE2 binding by Y453F trimeric spike glycoproteins of SARS-CoV-2

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Abstract

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) enters the host cell by binding to angiotensin-converting enzyme 2 (ACE2). While evolutionarily conserved, ACE2 receptors differ across various species and differential interactions with Spike (S) glycoproteins of SARS-CoV-2 viruses impact species specificity. Reverse zoonoses led to SARS-CoV-2 outbreaks on multiple American mink (*Mustela vison*) farms during the pandemic and gave rise to mink-associated S substitutions known for transmissibility between mink and zoonotic transmission to humans. In this study, we used bio-layer interferometry (BLI) to discern the differences in binding affinity between multiple human and mink-derived S glycoproteins of SARS-CoV-2 and their respective ACE2 receptors. Further, we conducted a structural analysis of a mink variant S glycoprotein and American mink ACE2 (mvACE2) using cryo-electron microscopy (cryo-EM), revealing four distinct conformations. We discovered a novel intermediary conformation where the mvACE2 receptor is bound to the receptor-binding domain (RBD) of the S glycoprotein in a “down” position, approximately 34° lower than previously reported “up” RBD. Finally, we compared residue interactions in the S-ACE2 complex interface of S glycoprotein conformations with varying RBD orientations. These findings provide valuable insights into the molecular mechanisms of SARS-CoV-2 entry.

KEYWORDS

cryo-EM structures, mink ACE2, SARS-CoV-2 spike

Hyunjun Ahn, Brenda M. Calderon, Xiaoyu Fan, and Yunrong Gao contributed equally to this study.

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1 | INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes the respiratory illness coronavirus disease 19 (COVID-19), which escalated to a global pandemic in 2020.¹ SARS-CoV-2 is a positive-sense single-stranded RNA virus made up of four structural proteins, which include nucleocapsid (N), envelope (E), membrane (M), and spike (S) glycoproteins.² S glycoprotein, decorated on the surface of the virus, mediates the virus entry process by binding to different host cell receptors, which includes the human angiotensin-converting enzyme 2 (hACE2), making it an important target for developing vaccines and therapeutics.^{3,4}

S glycoprotein on the mature virion is a homotrimer, with each monomer comprising two subunits: the S1 subunit containing the N-terminal domain (NTD) and the receptor-binding domain (RBD) that binds to the hACE2 receptor, and the S2 subunit containing the fusion peptide that mediates the membrane fusion of the virus and host cells.^{3,5} Rapid advances in the structural biology of the SARS-CoV-2 S glycoprotein have occurred since its initial outbreak, including pre- and postfusion S glycoprotein, RBD-ACE2 complex, and trimeric spike-ACE2 complex.^{5–14}

While *Rhinolophus affinis* represents the natural reservoir of precursors to SARS-CoV-2,¹⁵ multiple species, in addition to humans, are susceptible to infection.^{16–28} In April 2020, outbreaks of SARS-CoV-2 on American mink (*Mustela vison*) farms were first reported in the Netherlands, with later outbreaks observed in mink farms throughout Europe and North America.^{25,26,29,30} The virus was transmitted to mink by infected human workers and was capable of transmission among American mink and back to humans.³¹ In Denmark, sequence analysis of human samples identified five related clusters with different mutations in the S glycoprotein, and one of these, cluster 5, raised alarms internationally for harboring five mutations ($\Delta 69-70$, Y453F, D614G, I692V, M1229I) in the S glycoprotein.^{31,32} A notable mutation present in this and other clusters is Y453F, located in the RBD of the S glycoprotein, which arose via parallel evolution in independent mink outbreaks and has been shown to increase the binding and entry efficiency into cells expressing American mink ACE2 (mvACE2) while retaining its affinity to hACE2.^{8,32}

Previous studies reported the crystal and cryo-EM structures of mink variant spike RBDs in complexes with mink ACE2, providing key insights into how the Y453F mutation affects the binding of the S glycoprotein to ACE2.^{8,9} However, the structure of Y453F trimeric S glycoprotein in complex with mvACE2 has yet to be solved, and many conformational stages remain to be elucidated. In this study, we presented the cryo-EM structures of the trimeric S glycoprotein containing the cluster 5 S1 mutations ($\Delta 69-70$, Y453F, D614G) complexed with mvACE2 receptors in various conformational stages. Notably, our study captured an intermediate step in which mvACE2 is bound to the RBD of the S glycoprotein at a lower angle than previously reported,^{13,14,33} facilitating the further opening of the RBD. Collectively, our results provide further structural insights into the initial residue interactions between the S glycoprotein and

mvACE2 and the molecular mechanisms involved in the conformational changes of the S glycoprotein upon binding to the host mvACE2 receptor.

2 | MATERIALS AND METHODS

2.1 | Expression and purification of the S glycoprotein and ACE2

SARS-CoV-2 S glycoprotein expression plasmids were constructed to encode the ectodomain of S glycoprotein (residues 1–1208, with a mutated furin cleavage site and two stabilizing proline substitutions K986P/V987P) followed by a T4 foldon trimerization domain and a polyhistidine purification tag, as described previously.³⁴ For functional assays, different constructs were designed to encode S with mutations found in wild-type human viruses or viruses from American mink and are referred to throughout the text as S^{D614G}, S^{D614G+Y453F}, and S^{D614G+ $\Delta 69-70$ +Y453F}. For structural analysis, a SARS-CoV-2 S glycoprotein expression plasmid with four additional stabilizing proline mutations (F817P, A892P, A899P, A942P)³⁵ was constructed and is referred to throughout the text as mink S glycoprotein for simplicity.

ACE2 expression plasmids were also constructed as described previously to encode the ectodomain of dimeric ACE2 followed by a human IgG1 Fc purification tag, referred to throughout the text as hACE2-Fc and mvACE2-Fc.³⁴ Additionally, a monomeric mink ACE2 construct, where the IgG1 Fc purification tag was replaced with a polyhistidine purification tag, was constructed for structure determination and is referred to throughout the text as mvACE2. The American mink ACE2 sequence was determined previously.³⁶

Expi293F cells (Thermo Fisher; A14527) were cultured in Expi293 Expression Medium at 37°C and 8% CO₂ in the Orbi-Shaker (Sigma; Z763438) at 130 rpm. The cells were transiently transfected at a 3.0 × 10⁶ cells/mL density using an Expifectamine 293 transfection kit (ThermoFisher; A14524). For polyhistidine-tagged proteins, the supernatant was harvested by centrifugation after 72 h and supplemented with 20 mM Imidazole, followed by affinity chromatography with Ni²⁺-NTA agarose beads (GoldBio; H-350). The sample was washed three times with 5 column volumes (CV) of wash buffer (30 mM Na₂HPO₄ pH 7.4, 500 mM NaCl, 30 mM Imidazole) and then eluted three times with 5 CVs of elution buffer (20 mM Na₂HPO₄ pH 7.4, 500 mM NaCl, 500 mM Imidazole). For Fc-tagged proteins, the supernatant was harvested by centrifugation after 72 h, followed by affinity chromatography using HiTrap protein A columns (Cytiva). The sample was washed with 10 CVs of wash buffer (20 mM sodium phosphate, pH 7.4) and eluted with 5 CVs of elution buffer (0.1 M citric acid, pH 3). Sample collection tubes were preloaded with neutralization buffer (1M Tris HCl, pH 9).

The eluted proteins were concentrated with 50-kDa or 100-kDa MWCO centrifugal filters. Protein samples used for bio-layer interferometry (BLI) assays were used after affinity chromatography. Protein samples used for structure determination were further

purified by size exclusion chromatography using the Superose 6 increase 10/300 GL column (Cytiva) in buffer (20 mM Tris pH 8.0, 200 mM NaCl). The peak fractions were collected and verified through SDS-PAGE gel analysis. The samples were concentrated and flash-frozen in liquid nitrogen and stored at -80°C .

2.2 | Bio-layer interferometry assay

The binding affinity between the various trimeric S glycoproteins and dimeric ACE2 receptors was evaluated using the Octet RED96 instrument at 30°C with a shaking speed of 1000 RPM (ForteBio), as described previously.³⁷ Antihuman IgG Fc biosensors (ForteBio) were used. Following 10 min of prehydration of antihuman IgG Fc biosensors and 1 min of sensor check, 7.5 nM of hACE2-Fc or 15 nM of mvACE2-Fc in 10X kinetic buffer (ForteBio) were loaded onto the surface of anti-human IgG Fc biosensors for 5 min. We used 10X undiluted buffer here to minimize nonspecific binding, as advised by Sartorius staff and shown in previous literature.^{38,39} After 1.5 min of baseline equilibration, 5 min of association was conducted at 10–100 nM S glycoprotein, followed by 5 min of dissociation in the same buffer used for baseline equilibration. For binding assays using mvACE2-Fc, the association was conducted with 25–200 nM S glycoprotein. The data were corrected by subtracting the signal from the reference sample, and a 1:1 binding model with the global fit was used to determine equilibrium dissociation constants (Kd). Statistical analysis was performed using GraphPad Prism 9 software. Two-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test was used to compare the binding affinity (Kd) of S glycoprotein variants with hACE2-Fc or mvACE2-Fc glycoproteins, and significance was defined as $p < 0.05$. We ran a 4%–12% Bis-Tris gel with MOPS-SDS running buffer, verifying the purity of S^{D614G} , $\text{S}^{\text{D614G}+\text{Y453F}}$, $\text{S}^{\text{D614G}+\Delta 69-70+\text{Y453F}}$, hACE2-Fc receptor, and mvACE2-Fc receptor. The proteins were visualized using Colloidal Blue stain (Figure 1D).

2.3 | Electron microscopy specimen preparation and data acquisition

To prepare cryo-EM samples, $3\ \mu\text{L}$ of $\sim 1.5\ \text{mg/mL}$ mink S glycoprotein-mvACE2 complex (1:2.2 molar ratio S to mvACE2) were applied to glow-discharged holey carbon grids (Quantifoil Cu R1.2/1.3). The grids were blotted for 3 s at 100% relative humidity and flash-frozen in liquid ethane using a Vitrobot Mark IV system (Thermo Fisher Scientific). The cryo grids were imaged using a Krios cryogenic transmission electron microscope operated at 300 kV. The microscope was equipped with a Gatan K3 camera with a slit width of 20 eV, and a total of 11 392 micrographs were collected at $\times 81\ 000$ magnification. The image was exposed for 4.0 s fractionated with 40 frames with an accumulated dose of $49.98\ \text{e}^{-}/\text{\AA}^2$. The pixel size was $1.11\ \text{\AA}$ with a defocus range of -0.75 to $-1.75\ \mu\text{m}$.

2.4 | Cryo-EM data processing

All data processing was conducted in CryoSPARC v4.1.1.⁴⁰ Patch motion correction and CTF estimation were performed before manually curating exposures to exclude 1746 micrographs with statistical outliers. 4 458 674 particles were initially extracted via blob particle picking from the remaining 9646 micrographs, followed by template particle picking. Extracted particles underwent a series of 2D classifications and 3D classifications for refinement. Local refinement was further performed with a mask covering the American mink S glycoprotein RBD interacting with mvACE2. The full workflow is summarized in Supporting Information: Figure S1.

2.5 | Model building and visualization

Initial model building of the different conformations of mink S glycoprotein and the mvACE2 complex was performed in UCSF ChimeraX using PDB 6ACJ and 7LWI for global mink S-mvACE2 complex and PDB 7F5R for mink S glycoprotein RBD-mvACE2 complex after local refinement as a starting model. Several rounds of refinement in Phenix and manual building in Coot were performed until the final reliable models were obtained. Model and map statistics are summarized in Supporting Information: Tables 1 and 2.

Figures were generated using UCSF ChimeraX and PyMol. UCSF ChimeraX was used to measure the angles between the RBD and spike protein.³³ The "Define" function was used to generate the horizontal plane of the American mink S glycoprotein and long axis across the RBD. The "angle" function was used to calculate the angles between the plane and the axis.

Cryo-EM reconstructions and atomic models generated in this study are available at the Protein Data Base (PDB) under the following accession codes: mink S glycoprotein alone (8T21), mink S glycoprotein bound to one mvACE2 with downRBD (8T22) or upRBD (8TAZ), mink S glycoprotein bound to two mvACE2 (8T20), and local refinement of the RBD-ACE2 interface of downRBD (8T25) and upRBD (8T23). All of the complexes, with the mvACE2 removed, were compared to apo-mink S glycoprotein (8T21) by determining the RMSD in Pymol using the "align to molecule" function.

3 | RESULTS

3.1 | The binding of S glycoprotein homotrimers to hACE2-Fc and mvACE2-Fc

To identify potential differences in the binding affinity of S glycoproteins to hACE2-Fc or mvACE2-Fc glycoproteins, we performed bio-layer interferometry (BLI) assays. We determined the equilibrium dissociation constants (Kd) for trimeric S stabilized by two proline substitutions (K986P and V987P)³⁵ and harboring either the human (S^{D614G}) or American mink-associated mutations ($\text{S}^{\text{D614G}+\text{Y453F}}$ or $\text{S}^{\text{D614G}+\Delta 69-70+\text{Y453F}}$) from immobilized dimeric hACE2-Fc

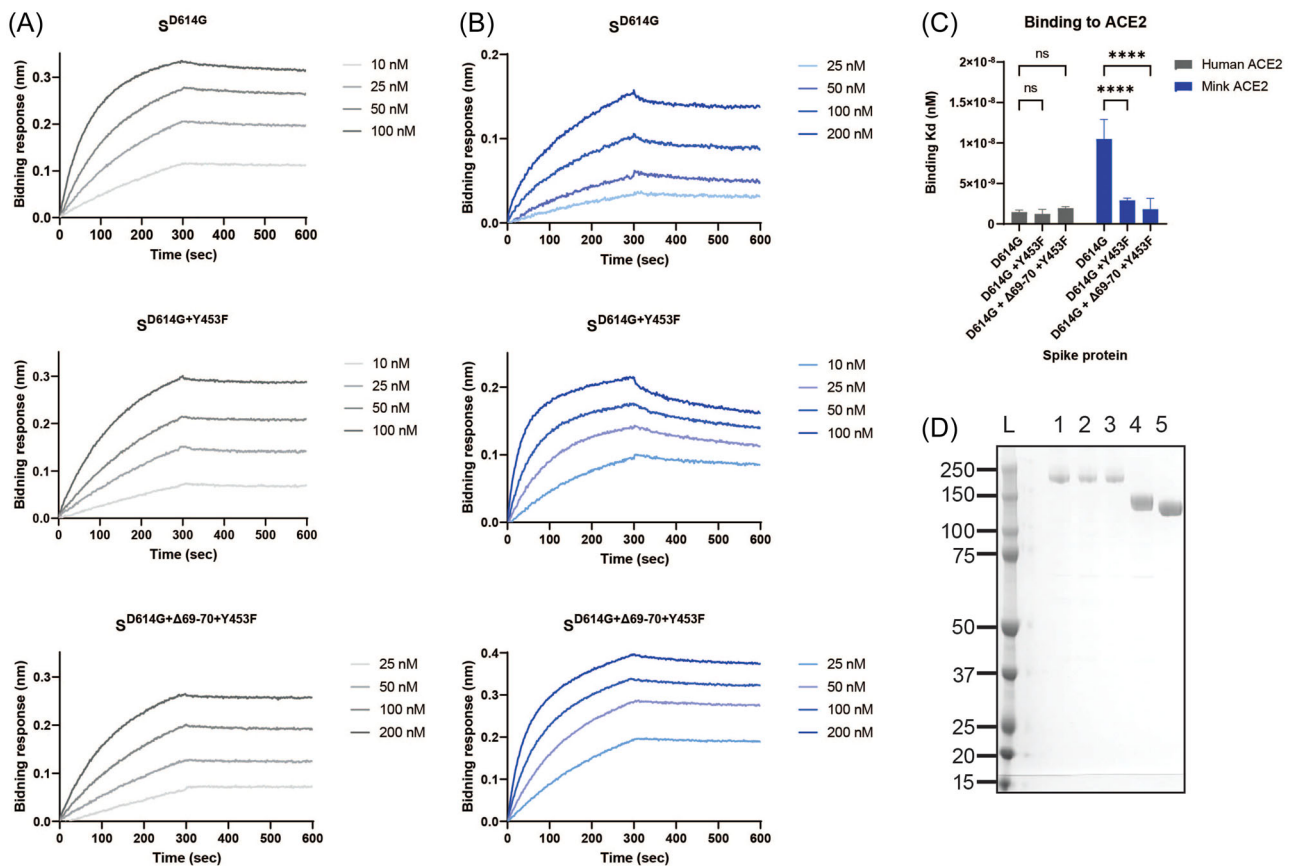


FIGURE 1 Binding of S glycoprotein trimers to hACE2-Fc and mvACE2-Fc receptors. (A) Representative binding profiles of S glycoprotein trimers to the hACE2-Fc receptor. The hACE2-Fc receptor was immobilized to biosensors, and binding to S glycoprotein trimers (S^{D614G} , $S^{D614G+Y453F}$, and $S^{D614G+\Delta 69-70+Y453F}$) was measured by bio-layer interferometry. (B) Representative binding profiles of S glycoprotein trimers to dimeric mvACE2-Fc receptors. The mvACE2-Fc receptor was immobilized to biosensors, and binding to S glycoprotein trimers (S^{D614G} , $S^{D614G+Y453F}$, and $S^{D614G+\Delta 69-70+Y453F}$) was measured by bio-layer interferometry. (C) Comparison of binding affinity (Kd) measured by bio-layer interferometry of S glycoprotein trimers to hACE2-Fc or mvACE2-Fc receptors. (D) SDS-PAGE analysis of proteins used in binding assays. The proteins S^{D614G} (1), $S^{D614G+Y453F}$ (2), $S^{D614G+\Delta 69-70+Y453F}$ (3), hACE2-Fc receptor (4), and mvACE2-Fc receptor (5) were loaded on a 4%–12% Bis-Tris gel and run in MOPS-SDS running buffer. Proteins were visualized using Colloidal Blue stain.

or mvACE2-Fc receptors. The S^{D614G} was chosen as it represents the predominant SARS-CoV-2 viruses circulating in humans when the variants infecting American mink emerged. The trimeric S glycoproteins S^{D614G} , $S^{D614G+Y453F}$, and $S^{D614G+\Delta 69-70+Y453F}$ all bound to dimeric hACE2-Fc receptor with similar affinity, and no statistically significant differences were observed (Figure 1). However, the S^{D614G} displayed a 5-fold reduction in affinity when the hACE2-Fc was substituted with mvACE2-Fc in the assay. The affinity of S for mvACE2-Fc was restored to S^{D614G} -hACE2-Fc levels by the Y453F mutation in the spike, as both $S^{D614G+Y453F}$ and $S^{D614G+\Delta 69-70+Y453F}$ showed a >4-fold increase of affinity to mvACE2-Fc, compared to S^{D614G} ($p < 0.0001$). The $\Delta 69-70$ deletion in NTD didn't further increase the binding affinity to mvACE2-Fc beyond the effect of the Y453F RBD mutation (Figure 1).

3.2 | Cryo-EM structures determination

To facilitate structure determination, we prepared a trimeric mink S glycoprotein, harboring some of the mutations ($\Delta 69-70$, Y453F,

D614G) found in mink cluster 5, with six proline substitutions that are reported to have increased protein yields and stability in the prefusion state.³⁵ We mixed the mink S glycoprotein with monomeric mvACE2 at a molar ratio of 1:2.2 and incubated it for 15 min before subjecting the complex to vitrification for EM analysis.

Cryo-EM analysis of the complex determined four distinctive species: mink S glycoprotein alone (13.8%), mink S glycoprotein bound to one mvACE2 with downRBD (16.0%) or upRBD (14.3%), and mink S glycoprotein bound to two mvACE2 in the upRBD position (55.8%) (Figure 2). After 3D classification and refinement, apo-mink S glycoprotein was resolved at 3.60 Å, while three conformational states of the mink S glycoprotein-mvACE2 complex were captured and determined at 3.83, 3.75, and 3.36 Å. The RBDs were flexible with respect to the horizontal plane of the spike protein, as shown by the 2D and 3D classification analysis, capturing four different RBD angles: 27.1°, 28.9°, 62.8°, and 69.2° (Supporting Information: Figures S1F and S2).

Cryo-EM structural analysis of the mink S glycoprotein shows that its overall organization of the trimer is similar to other variants,

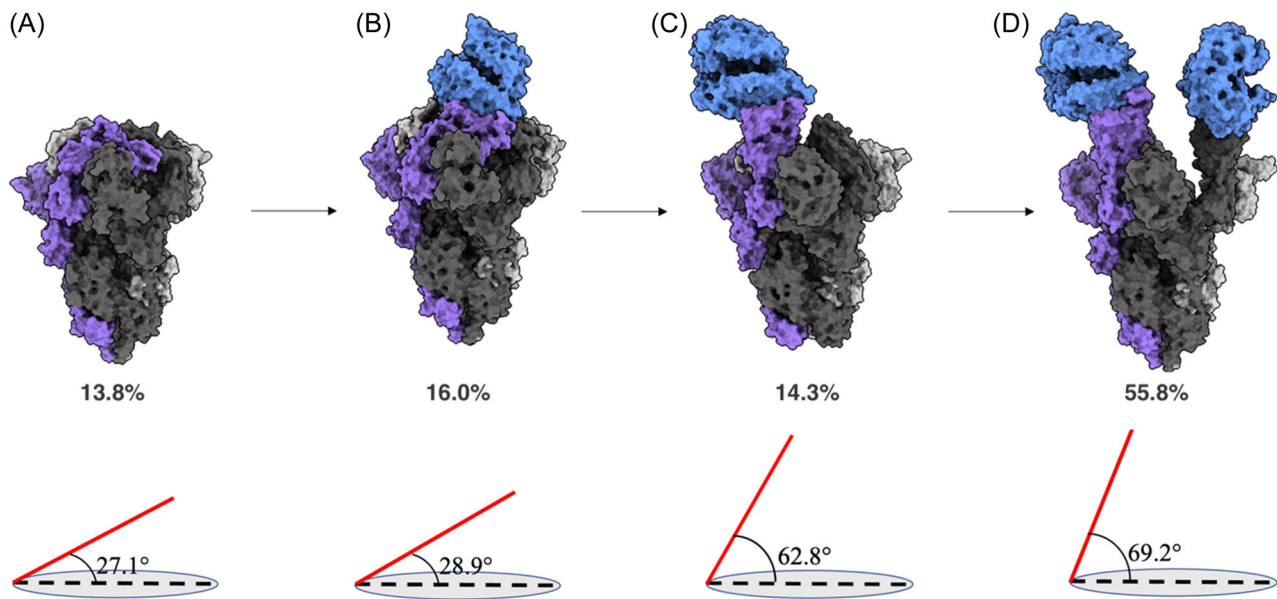


FIGURE 2 Electron density map of the mink ACE2-free and mvACE2 bound SARS-CoV-2 mink S glycoprotein. Diagram illustrating the density map of ACE2-free mink S glycoprotein [PDB: 8T21], one mvACE2-bound mink S glycoprotein [PDB: 8T22 and 8TAZ], and two mvACE2-bound mink S glycoproteins [PDB: 8T20]. The relative percent sample of each population is 13.8% (A), 16.0% (B), 14.3% (C), and 55.8% (D). The angle between the long axes of RBD and the horizontal plane is also shown below each species.

including Alpha, Delta, Omicron, and previously published mink S glycoprotein (Figure 3). As will be discussed in our structural analysis in later sections, and is shown in the provided sequence alignment of the RBD, many residues involved in the interaction between mink S protein and mvACE2 are well conserved in different conformations across variants, except some residues of Omicron (PDB: 7T9J), which has extensive mutation of the RBD. As seen in the overall secondary structure of the shown S proteins, mink, and Alpha appear more similar to each other than mink is to Delta and Omicron (Figure 3). A notable mutation of the mink S glycoprotein is the Y453F mutation, which does not occur in any other spike variants compared here. Our captured apo-mink S glycoprotein was determined to have three receptor-binding domains in the down position. The RBDs of the mink S glycoprotein structures are less well-resolved, most likely due to its flexibility in the region compared to the rest of the protein chain. However, it is clear that the overall architecture of the different S variants is conserved, which is consistent with the conserved high affinity for hACE2 receptor.

3.3 | Structure of SARS-CoV-2 S glycoprotein trimer with downRBD bound to mvACE2

In our cryo-EM analysis, we found that 30.3% of the species were composed of the S glycoprotein bound to a single mvACE2 receptor (Figure 2B,C). Sixteen percent of the single mvACE2 bound species had strong density for mvACE2 bound to an RBD at an angle of 28.9° (“downRBD”) (Figure 2B), while the other 14.3% (Figure 2C) had strong density at an angle of 62.8° (“upRBD”). The mvACE2 bound to

the downRBD-spike (Figure 2B) represents a novel intermediary conformation that has not been previously captured. There is some overlap in the density between the mvACE2 and neighboring RBD; however, the resolution is too low to determine any interaction between specific mvACE2 and neighboring RBD mink S glycoprotein residues. Previous studies have suggested that the spike protein can only accommodate ACE2 binding when its RBD is up at an angle of at least 50° with respect to the horizontal plane of the spike protein.^{14,33,41}

To further elucidate the mechanism of this conformation, we resolved the complex structure of mvACE2 bound to downRBD-spike at 3.75 Å (Figure 4A, Supporting Information: Figure S1C,E). The structure was further improved using local refinement covering the mink S glycoprotein RBD-mvACE2 interface, returning a 3.62 Å resolution of the density map (Figure 4B, Supporting Information: Figure S1H,J). To analyze the binding interface between the complex, we selected residues located less than 4.0 Å apart⁴² (Figure 4C). Structural analysis (Figure 4C–F) revealed that the binding of the two molecules in the conformation is mediated by π -cation interaction between S R403 and mvACE2 Y34 and between S Y505 and mvACE2 R393, π - π stacking between S F486 and mvACE2 H79 and between S Y505 and mvACE2 H354, and ionic interaction between S K417 and mvACE2 E30. Multiple sequence alignments of different S glycoprotein variants show that these participating residues are mostly well conserved (Figure 3). Interestingly, we found that S F453 does not form hydrophobic, π - π stacking or any contact interaction with the mvACE2 Y34 (Figure 4C,E), contrary to when the mvACE2 is bound to upRBD of the spike, as it was previously reported^{8,9} and as will be demonstrated in the following section.

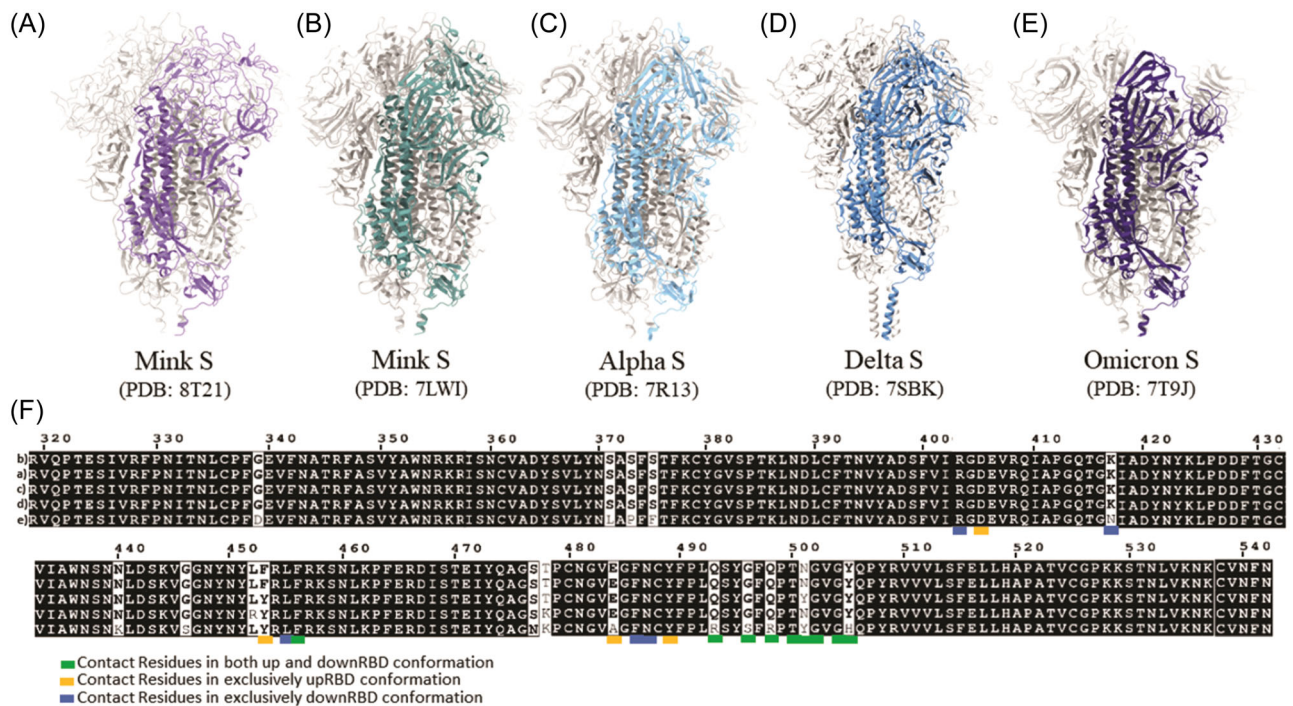


FIGURE 3 Comparison of different *apo* spike glycoprotein variants. Structural comparison of the *apo*-mink S glycoprotein determined in this study [PDB: 8T21] (A) with previously reported *apo*-mink S glycoprotein [PDB ID: 7LWI] (B), Alpha S [PDB: 7R13] (C), Delta S [PDB: 7SBK] (D), and Omicron S [PDB: 7T9J] (E). The sequence alignment of the S glycoproteins RBD (F) has the conserved residues indicated in black. Residues of mink S glycoprotein interacting with mvACE2 are indicated using colored tags. Mink S glycoprotein residues interacting with mvACE2 at both upRBD and downRBD conformation are indicated with green, exclusively downRBD conformation with blue, and exclusively upRBD conformation with yellow. Notable mutations in the mink S glycoprotein include substitution mutation Y453F.

3.4 | Structure of SARS-CoV-2 S glycoprotein trimer with upRBD bound to mvACE2 receptor

One of the three mvACE2-bound spike trimers captured had a strong density for mvACE2 bound to two of the RBD at 69.2°. After multiple rounds of additional 2D and 3D classification analysis, we determined the complex structure of mink S glycoprotein bound to two mvACE2 at 3.36 Å (Figure 5A, Supporting Information: Figure S1B,D). The local resolution of the protein interface between the complex was low due to its flexibility in the region. Nonetheless, the overall architecture of the mvACE2-mink S glycoprotein complex was comparable to the previously published structure. To visualize the key interaction at the protein interface between the complex, we performed local refinement with the mask covering the RBD-ACE2 interface, returning the improved local resolution of the density map at 3.82 Å (Figure 5B, Supporting Information: Figure S1G,I). To analyze the binding interface between the complex, we selected residues located less than 4.0 Å apart⁴² (Figure 5C).

Overall, our structure largely agreed with the previous Y453F RBD-mink ACE2 in interface residues^{8,9} (Figure 5D-F). Notable interactions include a salt bridge between mink S D405 with mvACE2 H354 and S E484 with mvACE2 H79; hydrogen bond between S T500 with mvACE2 Y41, S G502 with mvACE2 K353, and S Y505 with mvACE2 R393; π -cation interaction between S Y489 with mvACE2 K31; and π - π stacking between S F453 with mvACE2 Y34 and S Y505 with mvACE2

H354. F453 in S glycoprotein is unique to the mink variant among other S variants (Figure 3), and as indicated in our structural and binding affinity studies, as well as noted in previous studies,^{8,9} Y453F mink S glycoprotein enhances the interaction with mvACE2 Y34.

3.5 | The conformational changes of mink S glycoprotein with mvACE2 receptor

Herein, we performed a simultaneous structural comparison of mvACE2-unbound and mvACE2-bound to spike at various RBD angles to investigate the different conformational changes induced by binding. Previous studies have reported that spike glycoprotein could only accommodate ACE2 binding when its RBD is up at an angle of at least 50° with respect to the horizontal plane of the S glycoprotein.^{14,33,41} However, this data shows that about half of the mink S glycoprotein bound to one mvACE2 accommodated the mvACE2 binding with the RBD at a lower angle than previously reported^{13,14,33} (Figures 2 and 6A).

Further structural comparison of our density map shows that the mvACE2-free conformation had its RBD at 27.1° in relation to the horizontal plane of mink S glycoprotein (Figure 2A). In comparison, one mvACE2 and two mvACE2 bound spikes had their RBD rotate outward with an up position at 62.8° and 69.2°, respectively (Figure 2C,D). The angle at which mvACE2 is bound to the upRBD

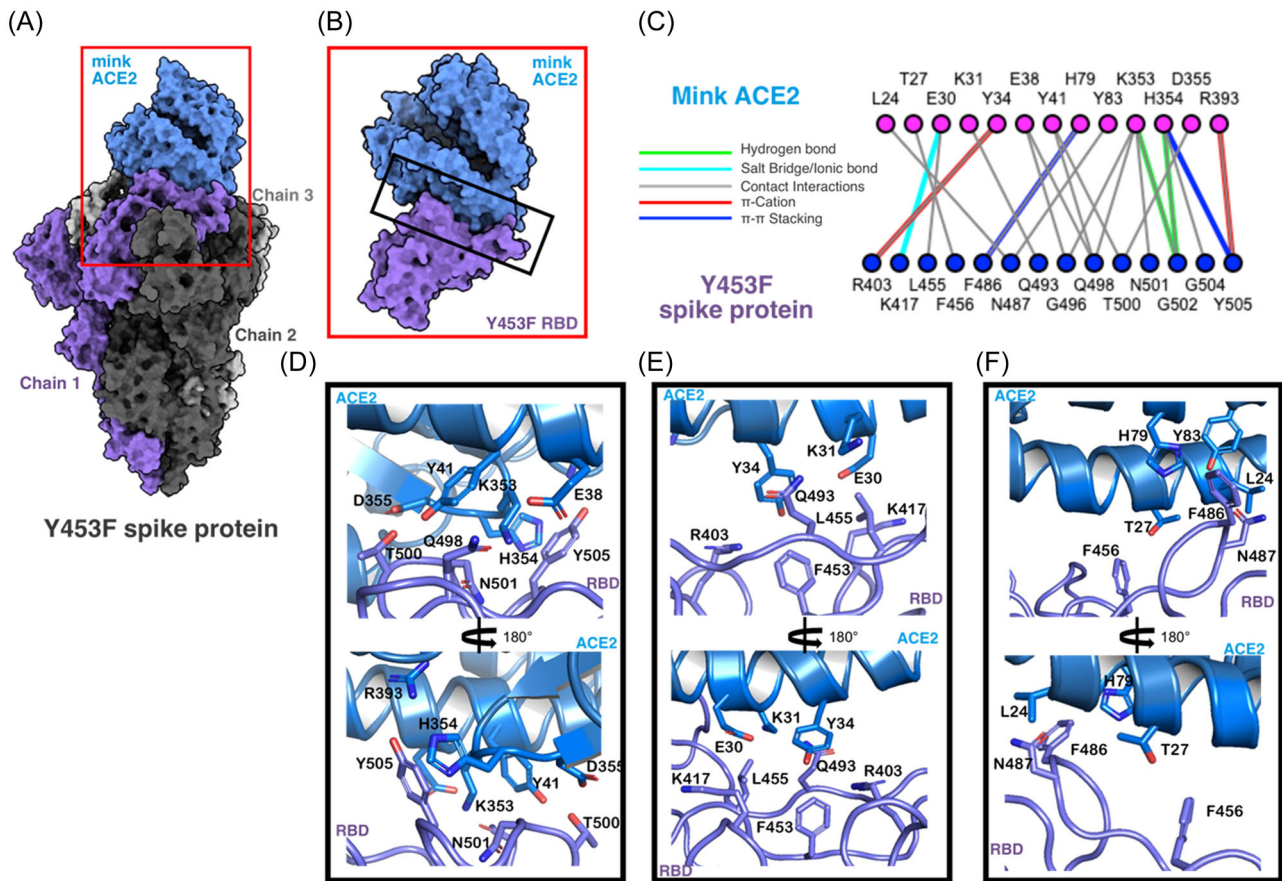


FIGURE 4 Cryo-EM structure of mvACE2 bound downRBD in SARS-CoV-2 mink S glycoprotein. (A) Cryo-EM map of downRBD spike glycoprotein-mvACE2 complex at 3.75 Å resolution. (B) Cryo-EM map of the Mink S glycoprotein RBD with mvACE2 after local refinement at 3.62 Å [PDB: 8T25] (C) Contacting residues less than 4 Å are labeled, and lines indicate the interactions. Hydrogen bonds, contact, pi-cation, and pi-pi stacking interactions are indicated by green, cyan, magenta, red, and blue, respectively. (D–F) Close-up view of the RBD-ACE2 interface, with key interacting residues labeled.

in our study is comparable to the angles previously reported^{14,33,41} (Figure 6B,C). The intermediate stage of the mvACE2-bound mink S glycoprotein with downRBD captured was at an angle of 28.9°, similar to the angle of mvACE2-free conformation and approximately 34°–40° lower than when it had upRBD (Figure 6A). The RMSD of the complexes with ACE2 removed are 0.789 Å (downRBD), 1.114 Å (1 upRBD), and 1.295 Å (2 upRBD) when compared to apo-Mink Spike. This shows that the overall conformation of the mink S glycoprotein is more similar between downRBD and apo-Mink than either of the upRBD and apo-Mink. These findings challenge the previously reported notion that ACE2 binding may only occur when the RBD is up at a high angle as well as provide insight into the conformational changes that are induced upon ACE2 binding.

4 | DISCUSSION

Our *in vitro* binding studies demonstrate a weaker binding affinity of the S^{D614G} protein to the mvACE2 receptor compared to the hACE2 receptor, and this suboptimal fitness in a new host may be overcome

by selecting the virus with host-adaptive mutations. Indeed, mutations were frequently observed in SARS-CoV-2 isolates derived from infected mink.^{26,43} We show that the addition of the mink-associated S glycoprotein mutations $\Delta 69-70$ and Y453F enhances binding to mvACE2 while having little impact on binding to the hACE2 receptor, suggesting they are host-adaptive mutations to American mink. The similarity in binding affinity between S^{D614G+Y453F} and S^{D614G+ $\Delta 69-70$ +Y453F} with the mvACE2 receptor suggests that the Y453F change is the main driver of the observed enhanced binding to mvACE2. Y453F has been shown by others to enhance mustelid ACE2 usage.^{8,9,44} Residue 453 is located in the S glycoprotein RBD and interacts with hACE2 receptor residue H34, which is a Y34 in mvACE2. To elucidate the enhanced mvACE2 receptor binding mechanism, we solved the structure of the American mink S glycoprotein trimer bound to the mvACE2 receptor.

As previously reported, the RBD was at a range of tilts and angles regarding the horizontal plane of spike.³³ The general consensus was that the RBD of the spike protein undergoes a conformational shift in its RBD from an inactive “down” state to an active “up” state at an angle of at least 50° to access the ACE2 of the host cell.^{14,33,41} Our

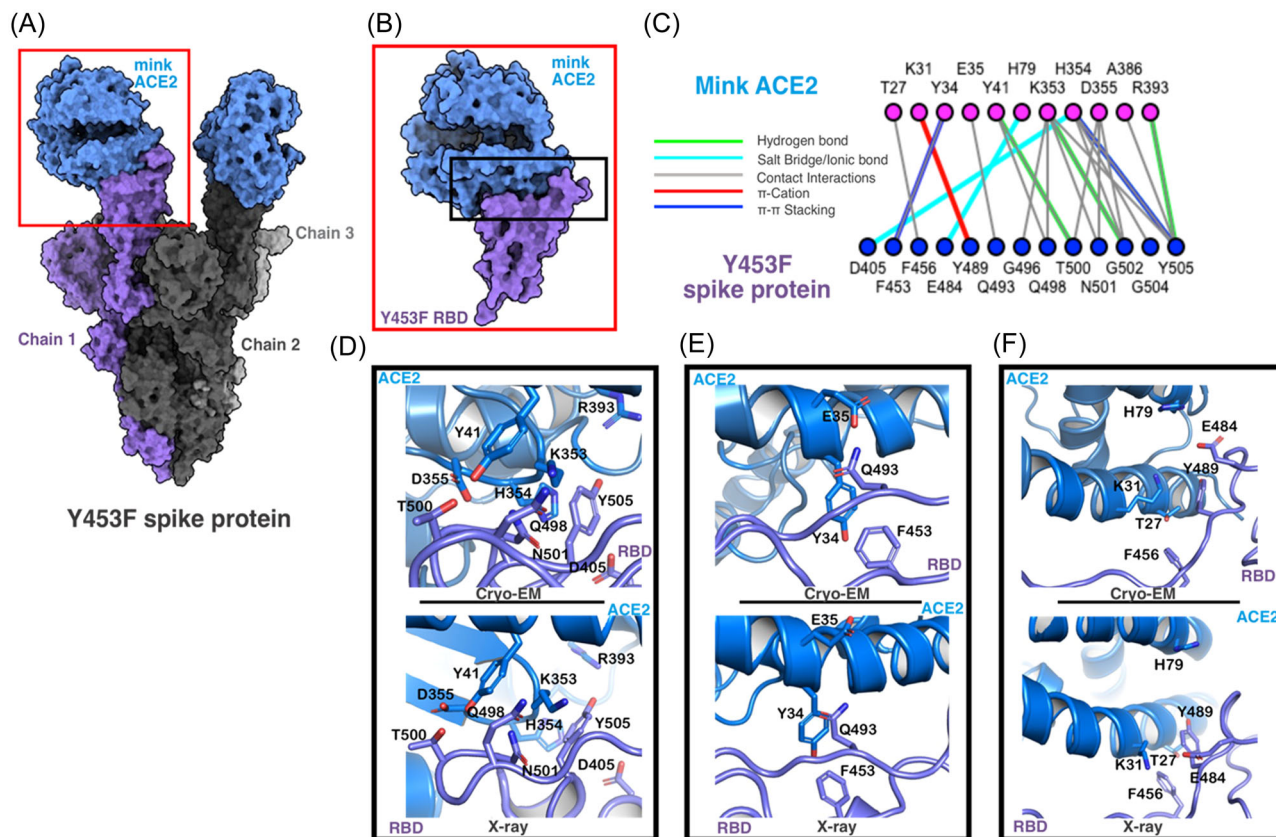


FIGURE 5 Cryo-EM structure of two mvACE2 bound to upRBD in SARS-CoV-2 mink S glycoprotein. (A) Cryo-EM map of upRBD mink S glycoprotein-mvACE2 complex at 3.36 Å. (B) Cryo-EM map of the Mink S glycoprotein RBD with mvACE2 after local refinement at 3.82 Å [PDB: 8T23]. (C) Contacting residues located less than 4 Å are labeled, and lines indicate the interactions. Hydrogen bonds, ionic, contact, pi-cation, and pi-pi stacking interactions are indicated by green, cyan, magenta, red, and blue, respectively. (D–F) Comparison of the close-up view of the RBD-ACE2 interface between the interface of the trimeric spike cryo-EM structure (top panel) and of the Y453F RBD-ACE2 in the X-ray crystal structure [PDB: 7F5R] (bottom panel). The key interacting residues are labeled.

study captured a novel intermediate step in which the mvACE2 binds to mink S glycoprotein with the RBD at a relatively lower angle than previously reported (Figures 4 and 6A). The simultaneous structural comparison suggests that the binding of the mvACE2 receptor facilitates the further opening of the CTD1 of the S glycoprotein. This would expose the fusion cleavage site of S2 in the spike, triggering the release of the S1 subunit from the S glycoprotein trimer.⁴⁵

Overall, our structural analysis of the full trimeric mink S-mvACE2 complex is mainly in agreement with previously published structures^{5–14} in its architecture. Additionally, comparing the molecular interface between previously published mink S RBD-mink ACE2 complex and our full trimeric mink S-mvACE2 complex when RBD is up at $>50^\circ$ revealed comparable interacting residues^{8,9} (Figure 6). This includes the enhanced interaction between S F453 with mvACE2 Y34 via π - π stacking from the Y453F substitution mutation in the mink S glycoprotein. A previous spike variant with Y453, however, is reported to cause a spatial clash with mvACE2 Y34, hindering π - π stacking interactions.⁸

Further analysis of the mink S-mvACE2 complex revealed that the interacting residues in the interface of the mink S and mvACE2 molecules differ depending on the angle at which the RBD is bound

to mvACE2 (Figures 5C and 6C, Supporting Information: Figure S2A–C). These results provide insights into the residues in the spike trimer that are important for initiating the binding of the host ACE2 receptor. For example, while we observed that S R403, K417, and F486 may play a role in initiating the binding of the ACE2 receptor, we did not note any interaction in these residues when the ACE2 is bound to S at a higher angle. Similarly, we note that S Y505 plays a role in both initiating and maintaining the binding of host ACE2 receptors. This is congruent with previous reports that Y505 is a critical viral determinant for specific recognition of ACE2 by SARS-CoV-2 RBD and why many potent neutralizing antibodies interact with this specific residue.^{44,46,47} Overall, our sequence alignment indicates that these interacting residues of the spike in downRBD conformation are well-conserved across different variants except the Y505H substitution in the Omicron BA.1 variant (Figure 3). It is reported that while the Y505H mutation in Omicron BA.1 significantly reduced ACE2 binding, other RBD mutations compensated for its decreased binding affinity.^{13,48}

The interactions between SARS-CoV-2 S glycoproteins and ACE2 receptors are of prime interest due to its essential role in species specificity, transmission and pathogenesis. SARS-CoV-2 is

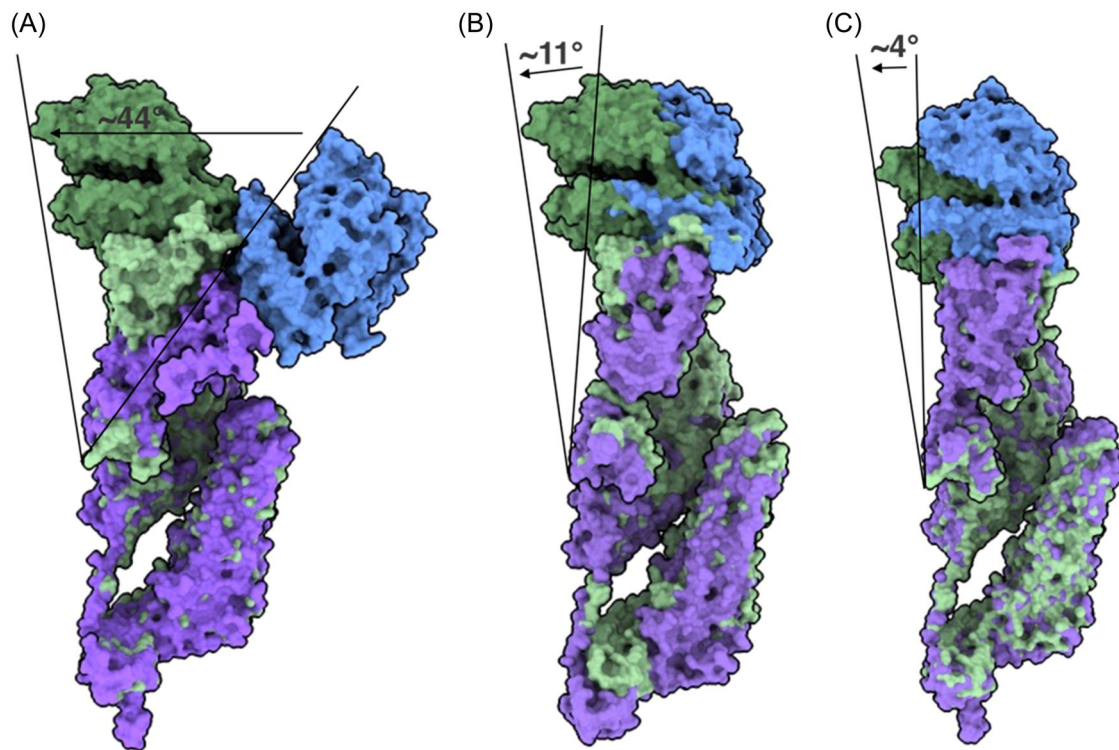


FIGURE 6 Superimposition of the surface density of SARS-CoV-2 mink S glycoprotein bound to mvACE2. (A) Comparison of previously reported spike chain of the trimer bound to mvACE2 complex [green; PDB ID: 6ACJ] with one mvACE2 bound S glycoprotein with downRBD [PDB ID: 8T22], (B) one mvACE2-bound S glycoprotein with upRBD [PDB ID: 8TAZ], and (C) two mvACE2-bound S glycoprotein with upRBD [PDB ID: 8T20].

now endemic in humans and will give rise to periodic epidemics similar to that of influenza A and B viruses and respiratory syncytial virus. Mutations will continue to emerge in the S glycoprotein to escape host immunity and/or optimize interactions with hACE2, and performing similar structural studies on new variants is necessary for understanding the disease and updating vaccines. Monitoring S glycoprotein variants for expanded or altered species specificity will also help assess the risk of zoonosis and reverse zoonosis.

AUTHOR CONTRIBUTIONS

Hyunjun Ahn, Brenda M. Calderon, Xiaoyu Fan, Bin Zhou, and Bo Liang conceived the project. Hyunjun Ahn, Brenda M. Calderon, Xiaoyu Fan, and Natalie L. Horgan contributed to cell culture. Brenda M. Calderon and Xiaoyu Fan designed the gene. Hyunjun Ahn, Brenda M. Calderon, Xiaoyu Fan, and Natalie L. Horgan carried out protein purification. Brenda M. Calderon, Xiaoyu Fan, conducted the bio-layer interferometry assay. Hyunjun Ahn and Natalie L. Horgan prepared cryo-EM grids. Hyunjun Ahn, Natalie L. Horgan, and Bo Liang collected cryo-EM data. Hyunjun Ahn, Natalie L. Horgan, Yunrong Gao, Bo Liang processed cryo-EM data. Yunrong Gao and Bo Liang processed the cryo-EM image, built and refined the model. Hyunjun Ahn analyzed the interaction between the molecules. Natalie L. Horgan conducted multiple sequence alignments and compared spike proteins across the variants. Bo Liang and Bin Zhou supervised and coordinated the project. Hyunjun Ahn, Brenda

M. Calderon, Natalie L. Horgan, Dylan S. Blohm, Bin Zhou and Bo Liang wrote the manuscript. All authors reviewed and revised the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request. The 3D cryo-EM

density maps and atomic coordinates generated in the study are available at the protein DataBank with the following accession codes: apo mink S glycoprotein (8T21), mink S glycoprotein with one mvACE2 at downRBD (8T22), mink S glycoprotein with one mvACE2 at upRBD (8TAZ), mink S glycoprotein with two mvACE2 (8T20), local refinement of mink S glycoprotein RBD with one mvACE2 at downRBD (8T25) and upRBD (8T23). The data that support the findings of this study are openly available in RCSB PDB at <https://www.rcsb.org/>, reference number 8T20, 8T21, 8T22, 8T23, 8T25, 8TAZ.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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