Cryo-EM structures and binding of mouse and human ACE2 to SARS-CoV-2 variants

of concern indicate that mutations enabling immune escape could expand host range

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ABSTRACT

Investigation of potential hosts of the severe acute respiratory syndrome coronavirus-2

(SARS-CoV-2) is crucial to understanding future risks of spillover and spillback. SARS-

CoV-2 has been reported to be transmitted from humans to various animals after requiring

relatively few mutations.[1] There is significant interest in describing how the virus interacts

with mice as they are well adapted to human environments, are used widely as infection

models and can be infected.[2] Structural and binding data of the mouse ACE2 receptor with

the Spike protein of newly identified SARS-CoV-2 variants are needed to better understand

the impact of immune system evading mutations present in variants of concern (VOC).

Previous studies have developed mouse-adapted variants and identified residues critical for

binding to heterologous ACE2 receptors.[3,4] Here we report the cryo-EM structures of

mouse ACE2 bound to trimeric Spike ectodomains of four different VOC: Beta, Omicron

BA.1, Omicron BA.2.12.1 and Omicron BA.4/5. These variants represent the oldest to the

newest variants known to bind the mouse ACE2 receptor. Our high-resolution structural data

complemented with bio-layer interferometry (BLI) binding assays reveal a requirement for a

combination of mutations in the Spike protein that enable binding to the mouse ACE2

receptor.

AUTHOR SUMMARY

The SARS-CoV-2 virus can infect different types of animals beyond humans. The virus uses

its Spike protein on its surface to bind to cells. These cells have a protein called ACE2 that

the Spike protein recognizes. Animals have slightly different ACE2 receptors compared to

humans. Mice are widely used as a research animal and live in the same environments as

humans so scientists are particularly interested. Understanding how Spike proteins binds to

the mouse ACE2 receptor allows us to understand the impact of immune evading mutations

found in new variants. We use a high resolution imaging technique called cryo-electron

microscopy to look at how different Spike variants bind to the ACE2 receptor from mouse at

a resolution where we can see the amino acids. We can see directly the individual amino

acids and mutations on the Spike protein that interact with the mouse ACE2 receptor. Many

of the mutations found in variants of concern also increase the strength of binding to the

mouse ACE2 receptor. This result suggests that mutations in the Spike protein of future

variants may have an additional effect in influencing how it binds to not only human ACE2

receptors but to mice and also different animals.

INTRODUCTION

Severe Acute Respiratory Syndrome (SARS) is a zoonotic disease caused by SARS-CoV

infection, and its symptoms can range from mild to severe.[5] SARS-CoV was first identified

in 2003 and it sparked a major global public health crisis.[6,7] Covid-19 is caused by SARS-

CoV-2 infection, which was first reported in Wuhan in late 2019 and has led to a global

pandemic.[8–10] Both viruses are of animal origin and their intermediate hosts prior to the

jump to humans have not been precisely identified, however, it is accepted that like the

SARS-CoV virus, the original host of the SARS-CoV-2 virus are most likely Rhinophilus

bats .[11–14] It is well understood that angiotensin-converting enzyme 2 (ACE2) is a major

receptor for host cell invasion by a range of coronaviruses, including SARS-CoV-2.[15–19]

The Spike glycoprotein embedded in the viral envelope binds specifically to human or animal

ACE2 on the cell surface, where it undergoes further membrane fusion and ultimately enters

and infects the host cell. Due to the conserved nature of ACE2 in animals and constant

selection of Spike variants, particularly ones that evade the human immune system, SARS-

CoV-2 may exhibit a high degree of spillover and spillback.[20–22] Several animal infections

or exposures have occurred during the covid-19 outbreak, including cats, dogs, rats, minks

and American white-tailed deer.[23–26]

SARS-CoV-2 evades the human immune system in several ways, such as accumulating

mutations on the Spike protein that reduce binding to host antibodies.[27–31] Significantly,

many sites of mutations in VOC are located on the receptor binding domain (RBD) that

engages ACE2. Mutations that endow the ability for the Spike protein to evade antibodies in

humans may also play an additional role by influencing ACE2 binding specificity and host

range. The availability of 3D structures of variant RBDs interacting with the receptor is

crucial for understanding how they continue to bind not only human ACE2 but potentially

those of other animals. Indeed, there has been speculation and critical debate that the

Omicron variant could have arisen in a mouse reservoir.[32–35] There have been structural

studies of animal ACE2 receptors with the SARS-CoV-2 Spike protein but few focusing on

mouse.[36–41] To better inform the relationship between these variants and potential species

tropism, there is a need to examine the structural details between animal ACE2 receptors and

the variant Spike proteins.

In this study, using cryo-electron microscopy single particle analysis (cryo-EM SPA) we

resolved a series of high-resolution structures of the SARS-CoV-2 variant Spikes in complex

with mouse ACE2, including the Beta, BA.1, BA.2.12.1 and BA.4/5 variants. We compared

the binding affinity of different variants Spike to the receptors. We further analyzed the high-

resolution structure of the widespread variant, BA.4/5, with human ACE2 using cryo-EM.

Together, these suggest that there is a level of structural plasticity in the RBD of the Spike

protein that is implicated in the evolution of both adaptation for animal hosts and immune

escape.

RESULTS

Mouse ACE2 binds to Spike variants of concern Alpha, Beta, Gamma and Omicron

subvariants

We performed a BLI binding screen between SARS-CoV-2 variants of concern Alpha, Beta

and Gamma against a set of animal ACE2, including mouse ACE2 (mACE2), compared to

human ACE2 (hACE2). Interestingly, unlike cat, dog and mink ACE2 that could bind to the

wild-type Spike protein, mACE2 only showed a substantial binding signal against the VOC,

similar to other reports.[22,42,43] (S1 Fig) We decided to focus on and better characterize

mACE2 binding affinities against these Spike VOC and performed full BLI binding assays at

three concentrations. The wild-type Spike protein bound robustly hACE2 but not strongly to

mACE2, with only appreciable signal at the highest concentration of 300 nM (Fig 1). The

emergence of the N501Y mutation found in the receptor binding domain (RBD) of the Spike

protein of Alpha, Beta and Gamma variants (S2 Fig) has been associated with mice

susceptibility to infection.[44–47] In accordance with these results we found that Alpha, Beta

and Gamma Spikes had a higher affinity (K_d between 35, 45 and 25 nM, respectively) for

mACE2 compared to the ancestral Spike protein (K_d above 100nM) (Fig 1A). From the end

of 2021, Omicron strains have caused successive waves of infections worldwide.[48] While

affinity for hACE2 of Omicron BA.1 and BA.2 Spikes was 2.5 times increased compared to

the ancestral Spike, affinity for mACE2 was increased more than 40 times (Fig 1A). The

Spike protein from the more recent Omicron BA.2.12.1 and BA.4/5 variants, containing

respectively 1 and 3 mutations in the RBD compared to BA.2, retain a strong affinity for

mACE2 (Fig 1A and S2 Fig).

Cryo-EM structure of Spike variants of concern bound to mouse ACE2 and BA.4/5 to

human ACE2

To probe how variants of concern gained high affinity mACE2 binding we used cryo-EM to

determine the structures of trimeric Spike variants in complex with human and mouse ACE2

receptors. We used Spikes from Beta, Omicron BA.1, BA.2.12.1, and BA.4/5, and obtained

resolutions of 3.9 Å, 2.7Å, 2.9 Å and 2.9Å, respectively (Fig 2 and S3-S7 Figs). We also

solved the human ACE2/BA.4/5 Spike complex at 2.8Å for comparison (Fig 3). Our cryo-

EM structures determined with dimeric Fc-ACE2 and a trimerized Spike ectodomain

recapitulates the hACE2/wild-type complexes observed in other studies done by x-ray

crystallography (PDB 6M0J) or cryo-EM (PDB 7A94), with monomeric ACE2 and RBD or

monomeric ACE2 and trimerized Spike, respectively. All of our structures show that

mACE2/hACE2 bind the RBD of the Spike protein in the same manner at the binding

interface of a RBD in the up-position. The RBD is highly mobile and its transition between

the down- to its up- state reveals the ACE2 binding site and allows for it to bind ACE2. The

process is dynamic, and the proportion of RBD-down and -up has been shown to be

dependent on many factors such as mutations, glycans, pH and temperature.[49–56] During

cryo-EM data-processing we observed all the Spikes with a mixture of 2-up or 3-up

conformation, with each up-RBD having an ACE2 bound (S3-S7 Figs). To gain structural

information at side-chain level resolution of the binding interface, we performed image

processing of the cryo-EM data by location-focused refinement on an ACE2-bound RBD that

was the most well resolved. This yielded maps at higher quality for each variant's

ACE2/RBD interface, allowing us to better understand the effects of local mutations in these

regions (Figs 2B and 3 and S3-S8 Figs). Each of the mACE2/RBD complex structures were

highly superimposable, in particular the RBD domain and the two helices of mACE2 that

interact with it, with 0.5-0.8 Å RMSD compared to the mACE2/BA.1 complex (Fig 2C). The

hACE2/BA.4/5 complex is also highly superimposable with that of the crystal structure of the

isolated wildtype RBD in complex with monomeric hACE2 (PDB: 6M0J) with an RMSD of

0.9 Å and with movements principally at the ACE2 lobe distal to the interaction site with the

RBD (Fig 3B). The hACE2/BA.4/5 complex is very similar to the mACE2/BA.4/5 complex

with an overall RMSD of 0.6 Å (Fig 2D). We were also able to build most mutations and

residues present in the variants highlighting the high-quality of our cryo-EM reconstructions

(Fig 4 and S9 Fig).

The combination of the N501Y and E484K mutation allow for Spike binding to mACE2

The first mutation reported to be crucial for mACE2 engagement and shared by the Alpha, Beta and Gamma and the more recent Omicron variants is the N501Y mutation. This mutation is located within the RBD and is one residue that has been identified to enable higher affinity interactions with the mACE2 receptor.[4,57,58] In the mACE2/Beta structure, we observe that the Spike N501Y mutation which replaces a polar asparagine (N) residue with a polar and aromatic tyrosine (Y) residue, allows for π-π interactions with Y41 of mACE2, and enables a new cation-π and hydrogen bond interaction with histidine H353 of mACE2 (Figs 1C and 4B). These new interactions stabilize the interface between mACE2 and the Beta RBD. Beta and Gamma both also share the E484K and K417N/T substitutions in the Spike RBD (S2 Fig). These two residues are unfortunately not resolved in our cryo-EM maps. However, *in vitro* BLI experiments showed that while the introduction of either N501Y, K417N or the E484K mutations alone are not sufficient to confer binding to mACE2, at the concentration of Spike protein tested here (75 nM), the combination of Y501 and K484 led to appreciable binding of the mutant Spike to mACE2 (S10 Fig).

BA.1 mutations Q493R and Q498R further increase binding to mACE2

We next investigated the impact of additional mutations found in Omicron variants BA.1, BA.2, BA.2.12.1 and BA.4/5. These variants contain up to 34 mutations scattered across the entire Spike protein, with the 15 of those concentrated on the RBD, including N501Y and E484A (S2 Fig). In particular, some Omicron RBD mutations (K417N, G446S(BA.1), E484A, F486S (BA.4/5) Q493R, G496S(BA.1), Q498R (BA.1, BA.2, BA.2.12.1) and N501Y) have been shown to be associated with immune evasion, with a loss of antibody binding or higher affinity ACE2 binding.[31,59–62] The accumulation of these mutations at once prompted an ongoing discussion whether the Omicron variant may have arisen in a

chronically infected COVID-19 person who is immunocompromised, or possibly spilled over

from a mouse reservoir.[34] Indeed, the mACE2/BA.1 structure reveals multiple new

interactions localized in two different patches between the RBD and mACE2 (Fig 4A, B). In

patch 1, the longer arginine (R) sidechain of the BA.1 Q493R mutation allows for new

hydrogen bonds with mACE2 residues N31 and Q34. These were not possible with the

shorter glutamine (Q) residue present in the original Spike protein. In patch 2, Y501 is

observed to form cation-π interactions with H353 from mACE2, as in the mACE2/Beta

complex, and new BA.1 mutations G496S and Q498R form a new set of hydrogen bonds and

electrostatic interactions with mACE2's aspartic acid D38 (Fig 4B). This also reorients Y449

of the Omicron BA.1 RBD to form a new hydrogen bond with Q42 of mACE2. Furthermore,

mutation of Y505 to a smaller histidine residue now allows for better packing at the interface

and potentially a CH- π T-shape interaction.[63] Glutamate (E) 37 also flips away from the

interface and is now sandwiched between H353 and R393, stabilizing the overall

conformation of the interface. Overall, Omicron BA.1 mutations Q493R and Q498R form a

cascade of new interactions alongside the N501Y mutation present in preceding variants.

These additional contacts greatly increase the binding between mACE2 and Omicron Spike

proteins with measured affinities by BLI up to 14-20-fold stronger compared to Beta (Fig

1A). Interestingly, the BA.1 Q493R mutation eliminates a favorable interaction with the

human ACE2 residue K31 due to charge repulsion with R493 and thus is better adapted for

binding to mACE2 than to hACE2 (Fig 4B and S10 Fig).

L452Q/R, F486V and the R493Q mutations of Omicron subvariants do not impact

mouse or human ACE2 binding

Both the BA.2.12.1 and BA.4/5 Omicron subvariants carry the immune-evading L452Q/R

mutation first seen in the Delta variant (S2 Fig). The site of mutation is not within the RBD-

ACE2 binding interface and, as expected, the binding affinities for both hACE2 and mACE2

binding to BA.2.12.1 are unchanged compared to BA.2 (Fig 1A,B and S9 Fig). In the

mACE2/BA.2.12.1 structure, the critical interactions made by Spike residues R498 and Y501

with mACE2 within patch 2 are conserved as in BA.1 (Fig 4B). Surprisingly, compared to

BA.1, R493 of BA.2.12.1 forms an alternative hydrogen bonding network with mACE2

residues N31 and E35, instead of Q34, that now forms a new hydrogen bond with Y453 of

BA.2.12.1 suggesting a plasticity in its interactions (Fig 4B).

The Omicron subvariants BA.4 and BA.5 that were the principal variants during an infection

wave in the summer of 2022 also share the L452Q/R mutation. Additionally, BA.4 and BA.5

has gained an additional immunity evading F486V mutation, and the reversion of R493 back

to the wild-type Q493 (S2 Fig). The structure of the mACE2/BA.4/5 complex show crucial

changes at patch 1, where the BA.4/5 mutation F486V loses Van der Waals interactions with

adjacent mACE2 residues F83 and F28 compared to BA.1 (Fig 4B). Q493 on the Spike RBD

also no longer forms interactions with mACE2 due to the loss of the longer favorable

arginine side-chain.

It has been predicted by modelling and then demonstrated by deep mutation scans of the

RBD that the F486V mutation reduces the affinity of the RBD for hACE2 and before the

appearance of the BA.4/5 subvariant, mutation at this site was observed at very low

rates.[64,65] The hACE2/BA.4/5 complex confirms this loss of hydrophobic contacts for

F486V mutation with hACE2 residues F28 and Y83, as seen with mACE2/BA.4/5, however,

Q493 now reestablishes a hydrogen bond interaction with hACE2 K31 residue, an interaction

previously first lost with the appearance of the Omicron lineage due to the charge repulsion

between R498 and K31 (Fig 4C and S10 Fig). The combination of these two mutations

allows for the BA.4/5 Spike to maintain high affinity interactions with both hACE2 and

mACE2. This is an example of the interplay between balancing immune evasion, while

maintaining high-affinity receptor binding. The other principal interactions between mACE2

and Spike's BA.4/5 in patch 2 are comparable to those observed between mACE2 and

Omicron subvariants BA.1 or BA2.12.1.

DISCUSSION

New variants of the SARS-CoV-2 virus have emerged posing a challenge to vaccine/antibody

efficacy and global pandemic management. We report here that since the appearance of the

first variant of concern Alpha, the SARS-CoV-2 Spike glycoprotein has serendipitously

gained the ability to also bind the mouse ACE2 receptor potentially broadening its host

range.[44–47] Here we demonstrate by a structural approach that this ability to bind the

mouse ACE2 receptor has further been maintained even with immune evading mutations in

the Omicron lineage.

Our BLI assays with early variants of concern and mutants agree with earlier findings that

N501Y and E484K are critical residues that allow for the Spike protein to bind to other

mammalian ACE2 homologs. To our knowledge, there has been no reported structure of the

Beta Spike bound to a non-human ACE2. Our cryo-EM structure illuminates how a single

mutation, N501Y, allows for new interactions especially with the mACE2-unique residue

H353 via cation- π interactions, analogous to K353 as observed with Alpha, Beta and Gamma

Spike bound hACE2.[66].

With the appearance of the Omicron variant and its subvariants, many epitopes on the surface of the Spike glycoprotein have mutated leading to reduced or abolished antibody binding. The BA.1 variant was first reported with increased resistance to antibodies and significantly stronger binding to hACE2.[59,67] We also observe that mACE2 also shows drastically increased binding (almost 10-fold) to the Omicron BA.1 Spike compared to all other variants that preceded it. The cryo-EM structure of mACE2/BA.1 reveals residues on the Spike protein that increase the number of interactions with mACE2: N501Y, Q493R, G496S and Q498R. This structural observation is consistent with literature reports that reported Spike mutations N501Y, Q493H and K417N increases binding to ACE2 from mice and other species.[3,4,68] We further observe that Spike proteins from Omicron subvariants with significant antibody evasion abilities such as BA.2.12.1 and BA.4/5 still bind to mACE2 demonstrating that the SARS-CoV-2 virus can balance both evasion of the human humoral immune response and its ability to bind to different host species. Notably, this is highlighted at position 493, where in the BA.1 Spike, the glutamine (Q) to arginine (R) mutation allows for new hydrogen bonds with mACE2 residues. However, in humans, it has been shown that this position favored antibody evasion at the cost of an interaction with hACE2 residue K31. Further Omicron subvariants such as BA.4/5 have reverted the arginine back to glutamine while picking up the F486V mutation that reduces van der Waals at the ACE2 binding interface. This compensatory mechanism allowed for further immune evasion while maintaining adequate binding affinity for the hACE2 Spike. Importantly, binding to mACE2 was unaffected even with the loss of two interactions.

Our structural study of human and mouse ACE2 bound to variant Spikes is not without limitations. The ability to bind mACE2 does not necessarily imply that it is able to infect and replicate in mouse.[69] However, numerous *in vivo* mouse studies with early-pandemic,

271 Omicron and mouse-adapted variants demonstrate that these variants are able to infect and

272 cause disease[35,44,45,57,70] In particular, our cryo-EM models shed light on the structural

273 basis of mACE2 binding to these variants. Importantly, our data complements previous

274 studies that also identified mutations that enable mACE2 binding, such as N501Y.

276 In summary, our structural data and binding analysis of mACE2 to SARS-CoV-2 variants of

277 concern highlight how mutations acquired to evade the immune response in humans may

278 have an impact on the binding to ACE2 receptors of other species thus possibly increasing

279 host susceptibility. Our binding assays and structural analysis identified 4 of the mutations

280 found in SARS-CoV-2 Spikes and known to be associated with immune escape, N501Y,

281 E484A, Q493R and Q498R, as critical mutations involved in high-affinity binding of VOC

282 Spikes to the mouse ACE2 receptor, and as such potentially allowing for expansion of SARS-

283 CoV-2 host range.

285 **FIGURE LEGENDS**

286 **Fig 1**. **mACE2 and hACE2 binding to variants of concern. (A)** BLI binding assays of

287 captured dimeric mouse ACE2 versus various concentrations of Spike variants of concern.

288 Data curves are colored by concentration and the black line indicates the 1:1 fit of the data.

289 **(B)** BLI binding assays of captured dimeric human ACE2 versus various concentrations of

290 Spike variants of concern. Data curves are colored by concentration and the black line

291 indicates the 1:1 fit of the data. **c.** Sequence alignment of human ACE2 with mouse ACE2

292 and other selected species. Red boxes highlight critical differences between human and

293 mouse ACE2 residues.

294 **Fig 2**. **Cryo-EM structures of mACE2 bound to variants of concern**

(A) Cryo-EM densities of the full mACE2/Spike variant of concern complexes. Each

protomer of the Spike trimer is colored separately with mACE2 colored in grey. **(B)** Focused

refinement of the RBD-mACE2 interface of each complex as in (A). **(C)** Superposition of

RBD-mACE2 complexes on to the BA.1-mACE2 complex show mACE2 binds similarly for

all variants. **(D)** Superposition of the BA.4/5 RBD-hACE2 complex with the BA.4/5 RBD-

mACE2 complex.

Fig 3. **Cryo-EM structures of hACE2 bound to the BA.4/5 Spike**

(A) Cryo-EM densities of the full hACE2/BA.4/5 Spike. Each protomer of the Spike trimer is

colored separately with hACE2 colored in light green. Inset shows focused refinement of the

RBD-hACE2 interface. **(B)** Superposition of BA.4/5 RBD-hACE2 complex with the BA.1

RBD-hACE2 complex (PDB 7T9L).

Fig 4. **Structural basis of mACE2 binding to variants of concern and of hACE2/BA.4/5**

(A) Zoomed view of the binding interface between mACE2 and RBD of variants of concern

with cryo-EM densities. **(B)** Highlighted views of specific interaction sites of patch 1 and

patch 2 as indicated in (A). **(C)** Zoomed view of the binding interface between hACE2 and

RBD of variants of concern with cryo-EM densities with highlighted views of specific

interaction sites.

DATA AVAILABILITY

Cryo-EM maps for the Spike variants in complex with mouse ACE2 were deposited in the

Electron Microscopy Data Bank (EMDB) under the access codes EMD-15541 (full map,

Beta, two mACE2 bound), EMD-15589 (local map, mACE2/Beta), EMD- 15580 (full map,

BA.1, two mACE2 bound), EMD-15581 (full map, BA.1, three mACE2 bound), EMD-15590

(local map, mACE2/BA.1), EMD-15584 (full map, BA.2.12.1, two mACE2 bound), EMD-

15585 (full map, BA2.12.1, three mACE2 bound), EMD-15591 (local map,

mACE2/BA.2.12.1), EMD-15586 (full map, BA.4/5, two mACE2 bound) and EMD-15592

(local map, mACE2/BA.4/5).

Maps for Spike the human ACE2/Omicron BA.4/5 complex were deposited in the EMDB

under the access codes EMD-15587 (full map, BA.4/5, three hACE2 bound) and EMD-15588

(local map, hACE2/BA.4/5).

Atomic models were deposited in Protein Data Bank (PDB) under the access codes of PDB-

8AQS (hACE2-BA.4/5), PDB-8AQT (mACE2/Beta), PDB-8AQU (mACE2/BA.1), PDB-

8AQV (mACE2/BA.2.12.1) and PDB-8AQW (mACE2/BA.4/5).

Raw electron microscopy image data were deposited at the Electron Microscopy Public

Image Archive (EMPIAR) under access codes EMPIAR-11181 (mACE2/Beta), EMPIAR-

11179 (mACE2/BA.1), EMPIAR-11180 (mACE2/BA.2.12.1), EMPIAR-11177

(mACE2/BA.4/5), EMPIAR-11176 (hACE2/BA.4/5).

CONTRIBUTIONS

D.N., K.L., P.T., F.P., D.T. designed the project. P.T. designed and cloned the Spike variants.

K.L purified the Omicron Spike and ACE2 and performed BLI assays. B.B., S.N., E.U.,

A.M., froze and screened cryo-EM grids, collected data, and performed on-the-fly

processing. D.N. processed cryo-EM data and built models. D.N. and K.L. analyzed data and

prepared figures. K.L., D.N., wrote the manuscript and P.T. contributed to the editing of the manuscript. F.P., H.S., and D.T. supervised the project.

ACKNOWLEDGEMENTS

Cryo-EM data collection and initial image processing was performed at the Dubochet Center for Imaging, a common initiative of the EPFL, UNIL and UNIGE, with data collection performed in both Geneva and Lausanne. We thank Yashar Sadian for data collection on the Talos Arctica. We thank Rosa Schier, Laurence Durrer, Soraya Quinche and Michael François of the Protein Production and Structure Core Facility of EPFL for helping in the production of Spike Omicron and ACE2 and Charlene Raclot for cloning of the Spike variants. This work was supported in part by NCCR TransCure, a National Centre of Competence in Research, funded by the Swiss National Science Foundation (grant number 185544)

METHODS AND MATERIALS

Protein Production and Purification

The Spike trimer was designed to mimic the native trimeric conformation of the protein in vivo and the expression vector was kindly provided by Prof. Jason McLellan, University of Texas, Austin (TX), USA. It encoded the prefusion ectodomain of the original 2019-CoV Spike, containing a native signal peptide, residues 986 and 987 mutated to proline (2P), a mutated putative furin cleavage site (residues 682-685 mutated to GSAS), a C-terminal T4 foldon fusion domain to stabilize the trimer complex, followed by C-terminal 8x His and 2x Strep tags for affinity purification.[19] The trimeric Spike protein was expressed as previously reported.[71–73] transiently expressed in suspension-adapted ExpiCHO cells (Thermo Fisher) in ProCHO5 medium (Lonza) at 5 x10^6 cells/mL using PEI MAX (Polysciences) for DNA delivery. At 1 h

post-transfection, dimethyl sulfoxide (DMSO; AppliChem) was added to 2% (v/v). Following

a 7-day incubation with agitation at 31 °C and 4.5% CO_2, the cell culture medium was

harvested and clarified using a 0.22 µm filter. The conditioned medium was loaded onto

Streptactin XT columns (IBA) washed with PBS and eluted with 50 mM biotin in 150 mM

NaCl, 100 mM HEPES 7.5. Eluted protein was then dialyzed overnight into PBS. The purity

of Spike trimers was determined to be >99% pure by SDS-PAGE analysis. Point mutations

were generated by InFusion-mediated site directed mutagenesis. Variant clones were generated

by gene synthesis (Twist Biosciences, Genscript and IDT) on the 2019-CoV Spike background

as above. All mutants were produced and purified in an identical manner to the original 2019-

Cov S protein.

Cryo-electron microscopy

mACE2/Beta complex

Cryo-EM grids were prepared with a Leica EM GP2 (Leica) plunge-freezing device, using

Quantifoil R2/1 copper 400 grids. 3.0 µL of a sample containing 0.4 µM Beta Spike and 0.7

µM mACE2-Fc was applied to the glow-discharged grids, and backblotted for 2 s with a 10 s

wait time, 80% humidity and 10 °C in the sample chamber, and the blotted grids were

plunge-frozen in liquid nitrogen-cooled liquid ethane.

Grids were screened for particle presence and ice quality on a TFS Talos Arctica transmission

electron microscope (TEM) operated at 200kV. Cryo-EM data was collected using the same

microscope, equipped with a TFS Falcon 3 camera. Movies were recorded at a nominal

magnification of 150kx, corresponding to a 0.9759Å pixel, with defocus values ranging from

-0.8 to -2.5 μm. Exposures were adjusted automatically to 40 e-/Å2 total dose with automatic

collection using EPU.

mACE2/Omicron BA.1 complex

Cryo-EM grids were prepared with a Vitrobot Mark IV (Thermofisher Scientific (TFS)).

Quantifoil R1.2/1.3 Au 400 holey carbon grids were glow-discharged for 120 s at 15mA

using a PELCO easiGlow device (Ted Pella, Inc.). 3.0 μL of a sample containing 9 μM

Omicron BA.1 and 16 μM mACE-Fc was applied to the glow-discharged grids, and blotted

for 6 s under blot force 10 at 100% humidity and 4 °C in the sample chamber, and the blotted

grids were plunge-frozen in liquid nitrogen-cooled liquid ethane.

Grids were screened for particle presence and ice quality on a TFS Glacios TEM (200kV),

and the best grids were transferred to TFS Titan Krios G4 TEM. Cryo-EM data was collected

using TFS Titan Krios G4, equipped with a Cold-FEG and Selectris X energy filter, on a

Falcon IV detector in electron counting mode. Falcon IV gain references were collected just

before data collection. Data was collected using TFS EPU v2.12.1 using aberration-free

image shift protocol (AFIS). Movies were recorded at the nominal magnification of 165kx,

corresponding to the 0.726Å pixel size at the specimen level, with defocus values ranging

from -0.7 to -2.0 μm. Exposures were adjusted automatically to 60 e-/Å^2 total dose.

hACE2/Omicron BA.4/5, mACE2/Omicron BA.4/5, mACE2/Omicron BA.2.12.1

Cryo-EM grids were prepared with a Vitrobot Mark IV (Thermofisher Scientific (TFS)).

Quantifoil R1.2/1.3 Au 400 holey carbon grids were glow-discharged for 120 s at 15mA

using a PELCO easiGlow device (Ted Pella, Inc.). 3.0 μL of a sample containing 14 μM of

the corresponding Spike and 25 µM ACE-Fc was applied to the glow-discharged grids, and

blotted for 6 s under blot force 10 at 100% humidity and 4 °C in the sample chamber, and the

blotted grid was plunge-frozen in liquid nitrogen-cooled liquid ethane.

Grids were screened for particle presence and ice quality on a TFS Glacios TEM (200kV),

and the best grids were transferred to TFS Titan Krios G4 TEM. Cryo-EM data was collected

using the TFS Titan Krios G4 TEM, equipped with a Cold-FEG, on a Falcon IV detector in

electron counting mode. Falcon IV gain references were collected just before data collection.

Data was collected using TFS EPU v2.12.1 using aberration-free image shift protocol (AFIS).

Movies were recorded at nominal magnification of 165kx, corresponding to the 0.83Å pixel

size at the specimen level, with defocus values ranging from -0.7 to -2.5 µm. Exposures were

adjusted automatically to 60 e-/Å2 total dose.

Cryo-EM image processing

On-the-fly processing was first performed during data acquisition for evaluating the data

quality during screening by using cryoSPARC live v3.3.1.4.[74] The obtained ab-initio

structures were used as templates for better particle picking. Motion correction was

performed on raw stacks without binning, using the cryoSPARC implementation of motion

correction. The full data processing workflow is shown in Supplementary information, S3-S8

Figs and processing statistics on S1 Table.

Cryo-electron microscopy model building

The models of a SARS-CoV2 Spike (PDB ID 7QO7), mouse ACE2 (PDB ID 7FDK) and

human ACE2 (PDB ID 7FDG) were re-fit into the cryo-EM maps with UCSF Chimera.[75]

These docked models were extended and rebuilt manually with refinement, using Coot and

Phenix. Statistics reported on S1 Table are from MolProbity as implemented in Phenix real-

space refine.[76,77] Figures were prepared in UCSF ChimeraX.[78]

Biolayer Interferometry (BLI)

All experiments were performed on a Gator BLI system. Running buffer was 150 mM NaCl,

10 mM HEPES 7.5. For binding assays, dimeric Fc-ACE2 was diluted to 10 µg/mL and

captured with MFc tips (GatorBio). Loaded tips were dipped into a 3 or 2-fold serial dilution

series (300 nM, 100 nM, 33.3 nM, or 100 nM, 50 nM, 25 nM) of target analyte Spike protein.

Screening of independent point mutations was done as above at a single concentration of 75

nM. Curves were processed using the Gator software with a 1:1 fit after background

subtraction. Plots were generated in Prism 9. Original data is provided in an excel spreadsheet

as S1 Dataset.

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Supporting Information

S1 Fig: BLI assays of animal ACE2 against various variants of concern or mutants.

S2 Fig: Overview of the domain architecture of selected variants of concern. Mutations are

shown as red lines and labelled for each variant. Specific domains are highlighted: signal

peptide (SP), N- terminal domain (NTD), receptor binding domain (RBD), S1 and S2

domain.

S3 Fig: Cryo-EM processing of the mACE2/Beta complex

S4 Fig: Cryo-EM processing of the mACE2/BA.1 complex

S5 Fig: Cryo-EM processing of the mACE2/BA.2.12.1 complex

S6 Fig: Cryo-EM processing of the mACE2/BA.4/5 complex

S7 Fig: Cryo-EM processing of the hACE2/BA.4/5 complex

S8 Fig: FSC curves indicating resolutions at (FSC 0.143) and final focused refined maps

colored by local resolution.

S9 Fig: View of the 452 residue in various variants of concern with cryo-EM density.

S10 Fig: The BA.1 mutation Q493R abolishes interactions with the hACE2 residue K31

compared to wildtype.

S1 Table: Cryo-EM data collection and refinement statistics

S1 Dataset: BLI Raw data for Figure 1 and S1 Fig.

A

mACE2 +
Beta

mACE2 +
Omicron BA.1

mACE2 +
Omicron BA.2.12.1

mACE2 +
Omicron BA.4/5

mACE2

b

Protomer A

Protomer B

Protomer C

Glycans

B

C mACE2

Beta
Omicron BA.1
Omicron BA2.12.1
Omicron BA.4/5

D mACE2
hACE2

Omicron BA.4/5