Experimental Evidence for Enhanced Receptor Binding by Rapidly Spreading SARS-CoV-2 Variants

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<https://doi.org/10.1016/j.jmb.2021.167058>

Edited by Eric O. Freed

Abstract

Rapidly spreading new variants of SARS-CoV-2 carry multiple mutations in the viral spike protein which attaches to the angiotensin converting enzyme 2 (ACE2) receptor on host cells. Among these mutations are amino acid changes N501Y (lineage B.1.1.7, first identified in the UK), and the combination N501Y, E484K, K417N (B.1.351, first identified in South Africa), all located at the interface on the receptor binding domain (RBD). We experimentally establish that RBD containing the N501Y mutation results in 7-fold stronger binding to the hACE2 receptor than wild type RBD. The E484K mutation only slightly enhances the affinity for the receptor, while K417N attenuates affinity. As a result, RBD from B.1.351 containing all three mutations binds 3-fold stronger to hACE2 than wild type RBD but 2-fold weaker than N501Y. However, the recently emerging double mutant E484K/N501Y binds even stronger than N501Y. The independent evolution of lineages containing mutations with different effects on receptor binding affinity, viral transmission and immune evasion underscores the importance of global viral genome surveillance and functional characterization.

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Introduction

Since its emergence in late 2019, SARS-CoV-2 has rapidly spread across the globe, resulting in more than 160 million confirmed COVID-19 cases and more than 3 million confirmed casualties as of May 15th, 2021 (coronavirus.jhu.edu). As revealed by the GISAID initiative (gisaid.org), SARS-CoV-2 slowly but continuously mutates, resulting in some instances in variants that become dominant in the population due to increased transmission and/or immune evasion. A number of these variants carry mutations in the spike protein, which is located on the viral surface and interacts with the angiotensin-converting enzyme (ACE2) on host

cells, resulting in membrane fusion and viral entry. One such mutation is D614G, which confers increased infectivity and transmissibility and has rapidly become the dominant global variant.^{1,2} Another common mutation is N439K, which is located in the receptor binding domain and enhances affinity for the hACE2 receptor by creating a new salt-bridge across the binding interface.³ SARS-CoV-2 N439K retains fitness and causes infections with similar clinical outcome, but also shows immune evasion.

In December 2020, new variants of concern have been identified in the UK (B.1.1.7⁴), South Africa (B.1.351⁵) and Brazil (P.1 and P.2, both descendants from B.1.1.28^{6,7}). These strains carry multiple

mutations in the spike protein and form the dominant variants in multiple countries. B.1.1.7 contains a change from asparagine to tyrosine at position 501 (N501Y) in the receptor binding motif of the receptor binding domain (RBD) (Figure 1(C)). A high-throughput deep mutational scan using yeast, as well as recent protein interaction analysis, shows this change increases binding affinity for the hACE2 receptor.^{8–10} Furthermore, N501Y was identified as adaptive mutation during serial passaging of a clinical SARS-CoV-2 isolate in mice.¹¹ The N501Y mutation is accompanied by two additional changes at the receptor binding interface in two of the other strains (Figure 1(C)); glutamate to lysine at position 484 (E484K) and lysine to asparagine or threonine at position 417 (K417N in B.1.351; K417T in P.1). P.2 contains only the E484K change in its receptor

binding domain. While for B.1.1.7 increased transmission has been established,¹² increased prevalence of the other lineages may (also) be due to immune escape.^{5,13}

Results

Here we experimentally analyze the effects of different combinations of these RBD mutations on hACE2 receptor binding affinities. Residues N501, E484 and K417 are located relatively far apart on the receptor binding motif (Figure 1(A)) and mutations will directly impact molecular interactions across the interface (Figure 1(B)). Asparagine 501 in the spike RBD forms a single hydrogen bond across the interface with hACE2 tyrosine 41. Mutation of the asparagine into

Figure 1. Effects of amino acid mutations on molecular interactions and strength of the interface between hACE2 and the SARS-CoV-2 receptor binding domain. (A) Location of residues N501, E484 and K417 indicated by blue spheres at the interface of SARS-CoV-2 receptor binding domain (white cartoon representation) and the hACE2 ectodomain (orange surface representation) in 6m0j.pdb (24). (B) Details of the interactions with RBD N501 forming a hydrogen bond with hACE2 Y41, RBD E484 forming an ion-pair with hACE2 K31, and RBD K417 forming a salt-bridge (ion-pair plus hydrogen bond) with hACE2 D30. (C) Amino acid changes in RBDs of different SARS-CoV-2 lineages. (D) Cartoon visualizing SPR setup using Biacore T100. (E) Sensorgrams of increasing concentrations of RBD binding to immobilized hACE ectodomain (colored datapoints) with fit of a 1:1 binding model (thin black lines) for wild type and mutant RBDs. (F) Affinity of RBD mutants relative to wild type RBD.

tyrosine will result in loss of this hydrogen bond. *In silico* structure analysis suggests that the aromatic tyrosine 501 sidechain might be able to stack onto hACE2 tyrosine 41 and form favorable van der Waals interactions using its pi-electron orbitals (Figure S1(A) and (B)). Furthermore, the Y501 hydroxyl group may form a hydrogen bond with hACE2 lysine 353. Verification of these predictions awaits the determination of a high-resolution structure of the mutant complex.

E484K is a charge reversal mutation, resulting in the loss of an ion-pair across the interface with hACE2 lysine 31 (Figure 1(B)). *In silico* predictions suggest that a new ion-pair might be formed with neighboring glutamate 35 in the same hACE2 alpha-helix (Figure S1(C) and (D)), probably accompanied by local rearrangements of flexible side chains to avoid energetically unfavorable electrostatics with lysine 31. According to deep mutational scanning analysis, the polar rearrangements upon mutation of glutamate to lysine at position 484 slightly increase affinity for the receptor.⁸ The K417N mutation is expected to reduce affinity as replacement of the lysine with a shorter asparagine (in B.1.351, or threonine in P.1) will disrupt the salt-bridge across the interface (Figure S1(E) and (F)). A similar mutation (K417V) was experimentally shown to reduce affinity 2-fold.³

To experimentally determine the effect of different combinations of these mutations on binding affinity for the hACE2 receptor, we purified the hACE2 ectodomain and different SARS-CoV-2 RBD variants from human cells (Figure S2(A)–(D)), and determined rate and affinity constants for complex formation using surface plasmon resonance (Figure 1(D)–(F)). Complex formation is accurately described by a 1:1 binding model (Figure S2(E)). Obtained rate and affinity constants for wild type RBD (Table 1) are similar to previously published values.^{3,9,10} The increase in affinity for the N501Y variant is 7.1-fold ($K_D = 2.4$ nM instead of 17 nM; Figure 1(F), table 1). This is relatively large for a single amino acid change and typical for mutations that improve the hydrophobic effect, in this case possibly due to the ring stacking of the two tyrosine side chains across the interface. The change in affinity is predominantly caused by a reduction in the dissoci-

ation rate constant, indicating the N501Y spike protein remains bound to the receptor for a longer time period than wild type RBD, increasing the chance to undergo the proper conformational change and induce membrane fusion and cell entry.

Compared to the effect of N501Y, the effect of the single E484K mutation on binding affinity is minor (1.4-fold; Figure 1(F), table 1). Obviously, plasticity at the receptor binding interface allows rearrangements of the polar interactions into a new local conformation that is energetically at least as favorable as for the original RBD. Interestingly, while the increase in affinity for N501Y is due to decreased dissociation, increased affinity for E484K is accomplished through faster association. The single K417N mutation destabilizes the interaction with hACE2 4-fold through a combination of slower binding and faster dissociation (Figure 1(F), table 1).

The combination of all three mutations, as present in strain B.1.351 (first identified in South Africa), is predominantly additive and results in a 2.4-fold less stable complex than for N501Y alone due to the effect of K417N, but still 3-fold more stable than with wild type RBD (Figure 1(F), table 1). As the K417T mutation in strain P.1 (first identified in Brazil) will likewise disrupt the intramolecular salt bridge, we expect a similar intermediate affinity for this variant. Double mutant E484K/N501Y forms a slightly more stable complex with hACE2 than the N501Y single mutant (1.4 nM instead of 2.4 nM, Table 1, Figure 1(F)). The increase in affinity for the double mutant (12-fold) is 1.3-fold higher than expected based on the additive effect of the single mutants (10-fold), which could reflect experimental variation or minor positive cooperativity.

Discussion

Taken together, our results show that receptor binding domains from rapidly spreading variants of SARS-CoV-2 bind with increased affinity to the hACE2 receptor and that this is predominantly caused by the N501Y mutation. This is in agreement with recent experimental observations

Table 1 Rate and affinity constants for binding of variants of the SARS-CoV-2 RBD to the human ACE2 ectodomain determined using surface plasmon resonance. Relative affinity of the mutant variants corresponds to the fold decrease in equilibrium binding constant relative to wild type. Values are mean $\pm$ range of two independent experiments

RBD variant	k_{on} $\times 10^5 \text{ M}^{-1} \text{ s}^{-1}$	k_{off} $\times 10^{-3} \text{ s}^{-1}$	K_D nM	Relative affinity (fold more stable)
Wild type	4.5 ± 0.2	7.8 ± 0.5	17 ± 0.6	1.0
N501Y	5.7 ± 0.9	1.3 ± 0.001	2.4 ± 0.4	7.1
E484K	8.9 ± 0.07	11 ± 0.2	13 ± 0.4	1.4
K417N	3.5 ± 1	24 ± 0.9	75 ± 20	0.23
E484K/N501Y	11 ± 2	1.5 ± 0.1	1.4 ± 0.2	12
K417N/ E484K/N501Y	7.6 ± 1	4.3 ± 0.04	5.8 ± 0.8	3.0

of a similar set of RBD mutations.^{9,14,15} The large increase in receptor binding strength of variants carrying N501Y, and a reported overall positive correlation between the stabilizing effect of mutations on receptor binding and their incidence in the population,^{8,16} indicate that affinity for the receptor may be one of the factors that determine viral transmission.

The use of the minimal, highly purified system we describe here allows accurate determination of the intrinsic kinetic and thermodynamic parameters for 1:1 complex formation between a single RBD and single receptor subunit under controlled circumstances. During viral infection, actual binding of virus particles to host cells will be further influenced by the biochemical environment in the host, as well as avidity effects due to the receptor being dimeric when embedded in the cell membrane, the spike protein being a trimer, and the presence of multiple copies of the receptor and the spike oligomers on the cell and viral surface. Synergy between the N501Y and E484K mutations in a cellular multivalent system seems to be more prominent¹⁶ than in our 1:1 system, and it would be worthwhile to unravel if this is a result of differences in experimental conditions or protein homogeneity or if this is caused by avidity. In addition, within the spike trimer, the receptor binding domains are in a dynamic equilibrium between up and down conformations, with only the up conformation being capable of receptor binding.¹⁷ If the mutations influence this equilibrium, this will affect the association rates for viral attachment.

While the E484K mutation does not have a large effect on affinity for the receptor, it seems to have a significant effect on immune response. Mutation of glutamate to lysine at this position abrogates binding to certain antibodies and results in immune evasion, reinfection and reduced efficacy of vaccines.^{9,13,18–21} It is therefore possible that the increased prevalence of lineage P.2, carrying only E484K in its RBD, is due to immune escape rather than increased transmissibility. The recently reported observation of the independent emergence of the E484K mutation into the more transmissible B.1.1.7 strain in for example the UK⁹ is of particular concern, as it combines the immune evasion properties of the E484K mutation with N501Y's high affinity, as shown here for the E484K/N501Y double mutant. Additional adaptation seems to have occurred in certain global regions with relatively high previous exposure; mutation of K417 in P.1 and B.1.351 confers additional immune evasion¹⁸ at the expense of RBD-receptor complex stability due to loss of the salt bridge across the interface. The K417N mutation in B.1.351 has occurred as an independent event onto the E484K/N501Y combination before rapidly spreading into the population.⁵ As herd immunity will build

up due to increased exposure and vaccination, continuous genomic and functional characterization will be crucial to tailor restrictive guidelines, vaccine composition and vaccination strategies towards optimal control of variants with increased receptor affinity, transmissibility and/or immune evasion.

Materials and Methods

Construction of expression clones

The hACE2 ectodomain (residues 18–615) was fused with the HA signal sequence, a linker containing a 8*HIS-tag, a Twin-Strep-tag and TEV protease cleavage site into vector pCEP4 using Gibson assembly.²² The same strategy was used to create the expression plasmid for wild type SARS-CoV-2 spike receptor binding domain (reference genome Wuhan-HU-1; residues 333–529). Templates used to create the PCR fragments were pTwist-EF1alpha-SARS-CoV-2-S-2xStrep (gift from Nevan Krogan; Addgene plasmid #141382), pCEP4-myc-ACE2 (gift from Erik Procko; Addgene plasmid #141185). Oligonucleotides for Gibson assembly and site-directed mutagenesis were obtained from IDT (Integrated DNA Technologies, Coralville, USA). Single amino acid changes in the RBD (N501Y, E484K, K417N) and combinations thereof were introduced using QuikChange (XL Site-Directed Mutagenesis Kit, Agilent, Santa Clara, USA). Resulting expression plasmids were purified using Nucleobond Xtra maxi, (Macherey-Nagel, Dueren, Germany) and sequence verified.

Cell culture

Human embryonic kidney (HEK) 293T cells were adapted to suspension culture in Gibco Freestyle 293 medium (Thermo Fisher Scientific, Waltham USA) supplemented with 20 U/L penicillin, 100 mg/L streptomycin (Sigma Aldrich, Saint Louis USA) and 1% FCS (Capricorn Scientific, Ebsdorfergrund Germany) at 37 °C, 5% CO₂, 150 RPM. Cell densities were kept between 0.3–3 *10⁶ cells/ml during propagation. For transfections, cells were seeded to a density of 0.7 *10⁶ cells/ml and incubated for 24 h. Plasmid DNA (1 mg) in 25 ml Gibco Hybridoma-SFM (Thermo Fisher Scientific, Waltham USA) was mixed by dropwise addition of 3 mg linear polyethylenimine M_w 25,000 (Polysciences Inc, Warrington USA) in 25 ml Hybridoma SFM, incubated for 30 min, added to HEK293T cells (50 ml/1 liter cell culture) and incubated for 96 h.

Protein purification

Cell culture medium was cleared by centrifugation at 1000g for 15 min and imidazole was added to a final concentration of 20 mM. Medium was stirred

for 1 h at 4 °C in the presence of 5 ml Ni-NTA Agarose beads (Qiagen, Venlo, The Netherlands) washed with 25 mM Hepes-KOH pH 7.5, 150 mM NaCl (buffer A). The beads were collected in a gravity flow column, and washed 3 times with buffer A supplemented with 20 mM imidazole. Protein was eluted with buffer A supplemented with 250 mM imidazole. For hACE2, eluate was loaded on a 1 ml StrepTrap HP column (Cytiva Marlborough USA) operated by an ÄKTA pure (Cytiva, Marlborough USA). hACE2 was eluted with 2.5 mM desthiobiotin in buffer A. Eluate containing RBD variants was incubated with 1 ml Streptactin XT superflow beads (IBA GmbH, Göttingen, Germany) for 1 h at 4 °C. Beads were collected by centrifugation for 2 min at 1000g, washed 3 times with buffer A and incubated over night at 4 °C in the presence of 0.3 mg reducing agent-free TEV protease. Eluate was supplemented with 20 mM imidazole and TEV protease was removed using Ni-NTA agarose beads. hACE2 and RBD eluates were buffer exchanged against buffer A using a 5 ml HiTrap desalting column (Cytiva, Marlborough, USA) operated by ÄKTA pure. Aliquots were snap frozen in liquid nitrogen and stored at −80 °C. Protein concentrations were determined using OD^{280 nm} measured on a Nanodrop 2000 (Thermo Scientific, Waltham USA) and extinction coefficients of $\epsilon_m^{280\text{ nm}} = 101,170\text{ M}^{-1}\text{ cm}^{-1}$ for tagged hACE2, $\epsilon_m^{280\text{ nm}} = 33,850\text{ M}^{-1}\text{ cm}^{-1}$ for wild type, E484K and K417N RBD, and $\epsilon_m^{280\text{ nm}} = 35,340\text{ M}^{-1}\text{ cm}^{-1}$ for N501Y, double and triple mutant RBD. Protein purity and concentration were verified using 15% SDS-PAGE (Figure S2(A) and (B)).

Deglycosylation

Glycosylation status of the purified hACE2 and RBD variants was investigated using PNGase (New England Biolabs, Ipswich, USA) under denaturing conditions according to the manufacturers protocol. Proteins (2 µg) were denatured in 10 µl Glycoprotein Denaturing Buffer for 10 min at 100 °C and chilled on ice. Glycobuffer 2 and NP-40 (1% final concentration) were added to a total volume of 20 µl. PNGase F (500 units) was added (except for control reactions) and the mixture was incubated for 60 min at 37 °C. Reactions were analyzed using 15% SDS-PAGE (Figure S2(B)).

Size exclusion chromatography

Immediately prior to surface plasmon resonance (SPR) analysis, aggregates and oligomers were removed from stored protein fractions using size exclusion chromatography (Figure S2(C) and (D)). ACE2 was injected onto a Superdex 200 Increase 3.2/300, while RBD variants were injected onto a Superdex 75 Increase 3.2/300. Columns were

equilibrated with assay buffer (25 mM Hepes-KOH pH 7.5, 150 mM NaCl, 10 µM ZnCl₂ to stabilize hACE2, 0.05% Tween 20) and operated using an ÄKTA micro mc (Cytiva, Marlborough, USA). The central peak fraction (50 µl) was used for SPR analysis.

Surface plasmon resonance

SPR spectrometry was performed at 25 °C on a Biacore T100 (Cytiva, Marlborough USA). A Series S CM5 sensor chip surface was derivatized with 1500 response units (RU) of Streptactin XT (Twin-Strep-tag® Capture Kit; IBA GmbH, Göttingen, Germany) using the amine coupling kit (Cytiva, Marlborough USA). Flow cell 1 was used for reference subtraction, flow cell 2 was derivatized with 50 RU hACE2 using the capture procedure. Increasing concentrations of RBD variants (0, 7.81, 15.6, 31.3, 62.5, 125 and 250 nM) in assay buffer were injected across the chip at 50 µl/min. Flow cells were regenerated using 3 M GuHCl (Sigma Aldrich, Saint Louis USA). Sensorgrams were fit with a 1:1 binding model using BiaEvaluation software (Cytiva, Marlborough USA), and visualized using Prism (GraphPad Software, San Diego, USA). Two independent experiments (including size exclusion chromatography) were performed for each RBD variant.

Structure analysis

An optimized model for the complex between hACE2 receptor and the SARS-CoV-2 receptor binding domain was created from 6m0j.pdb²³ using PDB-redo.²⁴ This resulted, amongst others, in a side chain flip for asparagine 501 in the RBD such that it forms a hydrogen bond across the complex interface with Tyrosine 41 in ACE2. Models for mutant RBDs N501Y and E484K were created using Missense3D.²⁵ Models were analyzed and figures were created using PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

Acknowledgments

We are grateful to Bart Haagmans and Marion Koopmans for valuable comments and critical reading of the manuscript. We are grateful for infrastructural support from the Josephine Nefkens Precision Cancer Treatment Program. Funding: this work is supported by the gravitation program CancerGenomics.nl from the Netherlands Organisation for Scientific Research (NWO), part of the Oncode Institute, which is partly financed by the Dutch Cancer Society, and NWO grant OCENW.XS.055.

Author contributions

R.K and J.L supervised the project. K.K, C.L and J.L created expression clones. K.K and C.L purified proteins. C.L performed SPR analysis. C.L and J.L analyzed SPR data. J.L performed structure analysis and visualized results. C.L and J.L wrote draft, C.L, J.L and R.K edited manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jmb.2021.167058>.

Received 6 April 2021;

Accepted 17 May 2021;

Available online 21 May 2021

Keywords:

coronavirus;

spike receptor binding domain;

mutations;

angiotensin converting enzyme 2 receptor;

surface plasmon resonance

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