nature portfolio

Peer Review File

Structural basis for receptor-binding domain mobility of the spike in SARS-CoV-2 BA.2.86 and JN.1

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This study addresses the structural basis of receptor recognition of emerging SARS-CoV-2 sub-variant BA.2.86 by determining the Cryo-EM structures of BA.2.86 S proteins alone and complexed with cellular receptor ACE2. They found ACE2-bound “down” RBD, indicating a trigger structure before the RBD-up conformation. Fourthermore, they found the N354-linked glycan contributes to the neutralizing antibody evasion in BA.2.86.
Previous research has indicated the dynamic nature of CoV S proteins, where ACE2 can induce the transition from the "down" to the "up" state of the RBD (PMID: 28393837; 38112468). In this manuscript, the authors claim to capture the two RBD-up–one RBD-down conformation. However, current data are insufficient to support this claim. Regarding immune evasion, only K356T, V445H, and P621S residues were tested, which may not fully represent the immune escape mechanisms of BA.2.86. Although K356T showed reduced evasion of neutralizing antibodies, it remains unclear whether this is due to residue substitution or the presence of an N354-linked glycan.
Major Concerns
1. Line 169: The authors claimed that "this region was not visible in S proteins of other variants". Actually, it has been observed in B.1.1.7 spike protein (PMID: 34168070).
2. If the two RBD-up–one RBD-down conformation is, as the author speculated, an intermediate state wherein the two RBD-up transitions to three RBD-up, one may assume that the ACE2-bound "down" RBD is flexible as the intermediate state is likely transient. However, in the determined structure, the "down" RBD is actually more stable. How will the author explain such observation?
3. Comparison of the opening angle presented in Fig. 2C is disorganized and confusing. First, on Line 199 and Line 204 the author stated that the comparison is conducted on the two "up" RBDs of both S-ACE2 complexes. However, in Fig. 2C and corresponding description the angle is formed by "G139 of the "up" RBD, A624 of the S1 subunit, and H444 of the "down" RBD". In that case, the comparison is illogical as in the "2RBD-up" S protein the angle is formed between two "up" RBDs but in the "two RBD-up-one RBD-down" S protein the angle is formed between "up" RBD and "down" RBD. Second, according to Fig. 2C G139 seems to belong to ACE2 but not the "up" RBD. Please re-organize this part of the manuscript and specify exactly what the author is comparing.
4. There was a previous study reporting the varying erecting angle of SARS-CoV RBDs bound by ACE2, with the angle between long axes of RBD and horizontal plane as low as 51.2º (PMID: 30102747). It would be favorable for the author to compare this structure with two RBD-up–one RBD-down and examine whether the one RBD is actually "down".
5. How does the conclusion "the "down" RBD established interactions with a neighboring protomer" came up and how does such interaction result in partial distinction from "up" RBD? (Line 230) Please further develop this argument.
6. The authors mentioned about the difference of H34 from the ACE2 binding surface, would it be a coincidence of dynamic particles, or a consequence related to RBD up/down conformations?
7. Line 258: As stated in the Introduction, “BA.2.86 acquired over 30 amino acid substitutions in the spike (S) protein compared with those of the previously predominant lineage XBB and its parent

BA.2." Why were only the K356T, V445H, and P621S residues selected to assess cellular entry and neutralization? I don't believe they can adequately represent the infection and immune evasion capabilities of BA.2.86, even though they may influence the conformation of the S protein.
8. It is unclear that the increased immune evasion brought by K356T is due to residue substitution or N354-linked glycan. Additional experiments are needed for such validation.
9. Lines 250-254: Please added the structural analyses for the decreased binding of JN.1 compared to BA.2.86.

Minor Concerns
1. Line 93: There is an additional right parenthesis.
2. Line 99: Reference should be cited for the statement of ACE2 binding to RBDs in the "up" conformation.
3. Fig. 2A, B: Please try rearranging the figure so that the down RBD is more visible. It is hard to see the conformation of down RBD in the current figure. Perhaps a side-view will help?
4. Lines 309-310: Please add reference.
5. Line 778: There is an additional right parenthesis.
6. Acronyms for amino acids need to be uniform. Eg: Line 228 His34; Line237 H34.

Reviewer #2 (Remarks to the Author):

A triggering structure of SARS CoV-2 BA2.86 spike upon ACE2 binding for receptor-binding domain up

Hisano Yajima1, Yuki Anraku2, Yu Kaku3, Kanako Terakado Kimura1, Arnon Plianchaisuk3, Kaho Okumura3,4, Yoshiko Nakada-Nakura1, Shunsuke Kita2, Jiei Sasaki1, Hiromi Sumita5, The Genotype to Phenotype Japan (G2P-Japan) Consortium, Jumpei Ito3,6, Katsumi Maenaka2,7,8,9, Kei Sato3,10,11,12,13,14,15*, Takao Hashiguchi1,12,16*

In 2023, a SARS-CoV-2 Omicron variant appeared with 30+ mutations in its spike glycoprotein. This variant termed BA2.86 likely stemmed from BA.2. It was initially feared that the development of 30+ new mutations relative to BA.2 may lead to increased transmissibility and escape from antibody responses. Yajima et al present a series of cryoEM structures of the SARS-CoV-2 BA2.86 spike glycoprotein bound and unbound to its receptor, ACE2. They observed an ACE2-bound S protein in 2RBD-up/1RBD-down and 3RBDup states. The 2RBD-up/1RBD-down is thought to be a "pre-triggered" conformation and based on the orientation of the RBD in the up-bound state, the authors suggest the wider and mobile angle of the RBD allows for a third ACE2 receptor to engage and transition to the 3RBD-up states. Lastly, the authors analyzed the impact of three BA.2.86 substitutions (K356T, V445H and P621S) on infectivity and neutralization; K356T and P621S increased infectivity whereas V445H did not.

Overall, the manuscript is well written and thorough in terms of experimentation and analysis. The

findings are sound and provide new characterization of an important Omicron variant.
Comments:

Initially it was feared that BA2.86 would be able to partially evade earlier immunity, but people who were previously immunized with the Omicron mRNA vaccines were still protected, thus suggesting that antibodies were not rendered ineffective with the new mutations observed in BA.2.86. Does the author's BA2.86 structure explain how this is the case? Where are the 30+ mutations located in relation to where neutralizing antibodies are binding in the RBD and S1 subunits? A figure showing this would be helpful. Can the authors analyze existing SARS-CoV-2 S-antibody structures in the PDB and see how neutralizing antibodies can still bind in BA2.86? Including a discussion in the paper would be beneficial.

The authors use the term trigger structure for the ACE2-bound "down" RBD. Would this not be more accurately described as a "pre-triggered" structure? Along this line, does the transition of the last RBD-down to the RBD-up have to be triggered by ACE2? Or can there be dynamic sampling of multiple conformational states that puts the third RBD-down in equilibrium with RBD-up? For example, do the authors observe any RBD-ACE2-2up/RBD- up but ACE2 unbound conformation in their cryoEM dataset? If so, this would suggest that there is no triggering involved and rewording of the term triggered should be considered.

The title "A triggering structure of SARS CoV-2 BA2.86 spike upon ACE2 binding for receptor-binding domain up" is awkwardly written. I would encourage the authors to rewrite.

Throughout the manuscript, the authors use the term "EM map" when describing the unknown and unmodeled regions of the cryoEM map. Please consider calling this "EM density" instead of "EM map".

Reviewer #3 (Remarks to the Author):

This manuscript by by Yajima et al., reports structures of the SARS-CoV-2 BA.2.86 spike protein, unliganded and in complex with the ACE2 receptor. In particular, for the ACE2 receptor bound spikes, they identified a structure that had two RBDs in the up position and 1 in the down position with all there RBDs bound to ACE2, thus providing a unique structural definition of ACE2 bound to a down RBD.

Overall, this is a well-done study with carefully characterized structures with many important insights, such as the visualization of the N354 glycan in BA.2.86. The main concern I have here is regarding the interpretation of the ACE2-bound down state RBD as the "triggering structure". The third ACE2 bound to the down RBD as consequence of the two up RBDs moving away substantially. While an interesting structure, the data presented do not indicate whether or not this is the trigger required for third RBD to go to the up position. Highlighting this in the title and the abstract is,

therefore, misleading. The authors should rephrase and reword these conclusions that are not supported by the data presented, while discussing the implications of the structural observations and considering their biological significance in the discussion section.

Other points:

Lines 250-255: Reduced affinity of JN.1 for ACE2 (relative to BA.2.86) was reported here: https://www.thelancet.com/journals/laninf/article/PIIS1473-3099(23)00744-2/
The authors should reference this study.

Lines 281-282: “S proteins exclusively adopting the closed-1 state, exemplified by BA.2; and those exclusively displaying the closed-2 state, as demonstrated by BA.2.86 in this study, all within the context of RBD all-down states.” Because the inability to identify a specific state within a cryo-EM dataset can often be a function of the data processing workflow, it would be better if this sentence was tempered to reflect this.

Figure 1: Between the figure and the legend, panels D and E are transposed.
Figure 2: Panel A: the legend says: “2 RBD-up state (left), and 3 RBD-up state (right).”
although in the fugure the structures are shown in up and down organization.
Figure 2: Panel D: not clear which RBD it is referring to - the up or the down, both should be added for comparison
Figure 2: Panel C: Not clear which structure is the 2RBD-up and which is the 2-RBD-up 1-RBD-down. Should not the 2-RBD-up structure have its third RBD down? Nomenclatures should be clarified.

Residue G139 is part of the NTD, not RBD.

We thank all the reviewers for their insightful and constructive feedback. We have addressed each comment, and we hope that the revised manuscript, together with this detailed response, resolves both major and minor concerns.

All reviewers raised a concern regarding the term “a triggering structure,” therefore, this point is tempered in the related conclusions and no longer present in the title and abstract. We instead employed the term “pre-triggered structure” in the text as suggested by reviewer #2.

Reviewer #1 (Remarks to the Author):

This study addresses the structural basis of receptor recognition of emerging SARS-CoV-2 sub-variant BA.2.86 by determining the Cryo-EM structures of BA.2.86 S proteins alone and complexed with cellular receptor ACE2. They found ACE2-bound “down” RBD, indicating a trigger structure before the RBD-up conformation. Fourthermore, they found the N354-linked glycan contributes to the neutralizing antibody evasion in BA.2.86.
Previous research has indicated the dynamic nature of CoV S proteins, where ACE2 can induce the transition from the "down" to the "up" state of the RBD (PMID: 28393837; 38112468). In this manuscript, the authors claim to capture the two RBD-up–one RBD-down conformation. However, current data are insufficient to support this claim. Regarding immune evasion, only K356T, V445H, and P621S residues were tested, which may not fully represent the immune escape mechanisms of BA.2.86. Although K356T showed reduced evasion of neutralizing antibodies, it remains unclear whether this is due to residue substitution or the presence of an N354-linked glycan.

Response: We thank the reviewer for references that ACE2 can induce the transition from the "down" to the "up" state of the RBD in CoV S proteins. We tempered in the “triggering structure” related conclusions in the BA.2.86 S protein, which is removed the term “triggering structure,” form the manuscript. The title and abstract were also modified.

Regarding the selection criteria for point mutants examined in the infectivity and the immune evasion, we examined the point mutants in the BA.2.86 S protein, where the structural alterations were obvious compared to that in XBB.1.5. We have chosen this selection criterion to match the variant, XBB.1.5,

of the sera used in the neutralization assay with the variant of the structural comparison. To fill in the missing mutations in the present study, we mapped the mutations acquired by BA.2.86 that have been reported to be important for changes in neutralizing potential onto the determined BA.2.86-S structure (**Supplementary Fig. 7A**). However, there are differences from our experiments in the sera used and the panel of monoclonal antibodies, which described in the DISCUSSION section.

We greatly appreciate the reviewer's point about whether K356T itself or N354-linked glycan is actually important. We performed additional experiments to distinguish this point and were able to conclude that glycosylation is not related to neutralizing antibody evasion, but that the K356T itself is important.

Major Concerns

1. Line 169: The authors claimed that "this region was not visible in S proteins of other variants". Actually, it has been observed in B.1.1.7 spike protein (PMID: 34168070).

Response: We thank the reviewer for this comment and have modified the text. Line 173-174.

2. If the two RBD-up–one RBD-down conformation is, as the author speculated, an intermediate state wherein the two RBD-up transitions to three RBD-up, one may assume that the ACE2-bound "down" RBD is flexible as the intermediate state is likely transient. However, in the determined structure, the "down" RBD is actually more stable. How will the author explain such observation?

Response: We thank the reviewer for this comment. For the two RBD-up–one RBD-down conformation, we assume that the “up” RBDs become very flexible/mobile in exchange for the stabilization of the “down” RBD, and that the balance between the “up” and “down” conformations has led to this non-canonical conformation. To this end, we performed additional experiments. We showed that “two-RBD-up–one-RBD-down$_{\text{three-ACE2}}$” can be observed in BA.2.86-S protein treated at 37 °C for 1 hour, but not in BA.2.86 S protein treated at 42 °C for 1 hour (**Supplementary fig. 5**). These results would suggest that an intermediate structure, two-RBD-up–one-RBD-down$_{\text{three-ACE2}}$, has been thermally eliminated, which indicate unstable as an S trimer bound to ACE2. Although the data is not shown in the manuscript, no “two-RBD-up-one-RBD-down$_{\text{three-ACE2}}$” structure was similarly identified after 3 hours of treatment at 42 °C. Further, we found the same

“two-RBD-up–one-RBD-down$_{three\text{-}ACE2}$” structure in the JN.1-S, which has only one amino-acid substitution compared to BA.2.86-S. Therefore, this structural conformation may be a shared feature in the BA.2.86 lineage (**Supplementary fig 6**). Line 217-231 and 275-304.

3. Comparison of the opening angle presented in Fig. 2C is disorganized and confusing. First, on Line 199 and Line 204 the author stated that the comparison is conducted on the two "up" RBDs of both S-ACE2 complexes. However, in Fig. 2C and corresponding description the angle is formed by "G139 of the "up" RBD, A624 of the S1 subunit, and H444 of the "down" RBD". In that case, the comparison is illogical as in the "2RBD-up" S protein the angle is formed between two "up" RBDs but in the "two RBD-up-one RBD-down" S protein the angle is formed between "up" RBD and "down" RBD. Second, according to Fig. 2C G139 seems to belong to ACE2 but not the "up" RBD. Please re-organize this part of the manuscript and specify exactly what the author is comparing.

Response: We thank the reviewer for this comment and apologize for our confusing description. For the first point, the initial manuscript presented the angles formed in the monomer, which was confusing. Therefore, we have rewritten the sentences to compare the angles formed by the” two RBD-up” in the two S-ACE2 complexes. The corresponding figure 2C has also been revised. Line 206-209 and **Fig. 2C**.

For the second point, G139 was an amino-acid residue in ACE2 in our initial manuscript and we apologize for this typo. The amino acid residues forming the angle have been corrected along with the above.

4. There was a previous study reporting the varying erecting angle of SARS-CoV RBDs bound by ACE2, with the angle between long axes of RBD and horizontal plane as low as 51.2º (PMID: 30102747). It would be favorable for the author to compare this structure with two RBD-up–one RBD-down and examine whether the one RBD is actually "down".

Response: We thank the reviewer for this comment and the suggested reference. We have appended the sentences regarding this comparison. Line 417-429 and **Supplementary Fig. 7EFG**.

5. How does the conclusion "the "down" RBD established interactions with a

neighboring protomer" came up and how does such interaction result in partial distinction from "up" RBD? (Line 230) Please further develop this argument.

Response: We thank the reviewer for this comment. We have deleted this sentence because what we would like to state is “No significant differences were detected in the overall structures of both ACE2–RBDs, but slight variations were observed at the interface between ACE2 and the "up" or "down" RBD”.

6. The authors mentioned about the difference of H34 from the ACE2 binding surface, would it be a coincidence of dynamic particles, or a consequence related to RBD up/down conformations?

Response: We think this would be a consequence related to RBD up/down conformations. H34 in ACE2, along with E35 in some variants, interacts with RBD Q/R493, but the interactions are different form each variant. Compared to other interactions in the ACE2-RBD interface, this interaction via H34 in ACE2 is particularly reoriented well. Therefore, even slight difference in the interaction between ACE2 and “up” or “down” RBDs may be reflected in the different orientation of H34 in ACE2. We have appended the sentences regarding this possibility in the DISCUSSION section: Line 403-407.

7. Line 258: As stated in the Introduction, “BA.2.86 acquired over 30 amino acid substitutions in the spike (S) protein compared with those of the previously predominant lineage XBB and its parent BA.2.” Why were only the K356T, V445H, and P621S residues selected to assess cellular entry and neutralization? I don't believe they can adequately represent the infection and immune evasion capabilities of BA.2.86, even though they may influence the conformation of the S protein.

Response: We apologize for the lack of explanation. Regarding the selection criteria for point mutants examined in the infectivity and the immune evasion, we examined the point mutants in the BA.2.86 S protein, where the structural alterations were obvious compared to that in XBB.1.5. We have chosen this selection criterion to match the variant, XBB.1.5, of the sera used in the neutralization assay with the variant of the structural comparison. We have appended this explanation in the text. Line 308-312.

To fill in the other substitutions in the present study, we mapped the substitutions acquired by BA.2.86 that have been reported to be important for

changes in neutralizing potential onto the determined BA.2.86-S structure (**Supplementary Fig. 7A**). However, there are differences from our experiments in the used sera and the panel of monoclonal antibodies, which described in the DISCUSSION section. Line 373-396 and **Supplementary fig. 7A-C**.

8. It is unclear that the increased immune evasion brought by K356T is due to residue substitution or N354-linked glycan. Additional experiments are needed for such validation.

Response: We greatly appreciate the reviewer's point about whether K356T itself or N354-linked glycan is actually important. We performed additional experiments to distinguish this point and were able to conclude that glycosylation is not related to neutralizing antibody evasion, but that the K356T itself is important (**Fig. 4BD**). Line 328-339 and 360-364.

9. Lines 250-254: Please added the structural analyses for the decreased binding of JN.1 compared to BA.2.86.

Response: Structural analysis of the JN.1-ACE2 complex was not straightforward, but we have determined the structure and have created a new paragraph entitled “Structures of the ACE2-bound JN.1 S protein” (**Fig. 3DE, Supplementary fig. 6**). Based on the determined structures, we calculated the shape complementarity (Sc) between RBD and ACE2 in BA.2.86 and JN.1. As the results, the Sc (0.42) of JN.1 S RBD–ACE2 was lower than that (0.52) of BA.2.86. The binding free energy (dG= –17.88) of JN.1 S RBD-ACE2 was increased compared to that (dG= –24.30) of BA.2.86. These values indicate that JN.1 S is less favorable for binding to ACE2 than that of BA.2.86. Line 275-304.

Minor Concerns

1. Line 93: There is an additional right parenthesis.

Response: We thank the reviewer for this comment. We have deleted it.

2. Line 99: Reference should be cited for the statement of ACE2 binding to RBDs in the "up" conformation.

Response: We thank the reviewer for this comment. We have cited a reference (PMID: 32075877).

3. Fig. 2A, B: Please try rearranging the figure so that the down RBD is more

visible. It is hard to see the conformation of down RBD in the current figure. Perhaps a side-view will help?

Response: We thank the reviewer for this comment. We have modified the **Fig. 2AB**.

4. Lines 309-310: Please add reference.

Response: We have added references. Line 399-400 (Three references, 36-38, were cited).

5. Line 778: There is an additional right parenthesis.

Response: We thank the reviewer for this comment. We have deleted it.

6. Acronyms for amino acids need to be uniform. Eg: Line 228 His34; Line237 H34.

Response: We have corrected them to be uniform.

Reviewer #2 (Remarks to the Author):

In 2023, a SARS-CoV-2 Omicron variant appeared with 30+ mutations in its spike glycoprotein. This variant termed BA2.86 likely stemmed from BA.2. It was initially feared that the development of 30+ new mutations relative to BA.2 may lead to increased transmissibility and escape from antibody responses. Yajima et al present a series of cryoEM structures of the SARS-CoV-2 BA2.86 spike glycoprotein bound and unbound to its receptor, ACE2. They observed an ACE2-bound S protein in 2RBD-up/1RBD-down and 3RBDup states. The 2RBD-up/1RBD-down is thought to be a "pre-triggered" conformation and based on the orientation of the RBD in the up-bound state, the authors suggest the wider and mobile angle of the RBD allows for a third ACE2 receptor to engage and transition to the 3RBD-up states. Lastly, the authors analyzed the impact of three BA.2.86 substitutions (K356T, V445H and P621S) on infectivity and neutralization; K356T and P621S increased infectivity whereas V445H did not.

Overall, the manuscript is well written and thorough in terms of experimentation and analysis. The findings are sound and provide new characterization of an

important Omicron variant.

Response: We thank reviewer's positive comments for our manuscript. We tempered in the "triggering structure" related conclusions in the BA.2.86 S protein, which is removed the term "triggering structure," form the manuscript. The title and abstract were also modified.

Comments:
Initially it was feared that BA2.86 would be able to partially evade earlier immunity, but people who were previously immunized with the Omicron mRNA vaccines were still protected, thus suggesting that antibodies were not rendered ineffective with the new mutations observed in BA.2.86. Does the author's BA2.86 structure explain how this is the case?

Response: We explained that the overall structure of the BA.2.86 S protein was not substantially altered with most amino-acid substitutions dispersed throughout the S1 structure and not concentrated in the RBM to the point of escaping the variety of neutralizing antibodies at serum levels. Line 373-396 (**Fig. 1F and Supplementary Fig. 7D**).

Where are the 30+ mutations located in relation to where neutralizing antibodies are binding in the RBD and S1 subunits? A figure showing this would be helpful.

Response: We have added as a figure (**Supplementary Fig. 7D)** along with our response to your first comment. Line 373-396 and **Supplementary Fig. 7D**.

Can the authors analyze existing SARS-CoV-2 S-antibody structures in the PDB and see how neutralizing antibodies can still bind in BA2.86? Including a discussion in the paper would be beneficial.

Response: We employed 34 antibodies registered in the PDB, which are either therapeutic antibodies (in clinical trials or on the market) or neutralizing antibodies that have been reported. Analyzed result was exhibited as a figure and discussed (Line 384-396 and **Supplementary Fig. 7BC)**. In summary, a significant reduction in the binding of neutralizing antibodies was observed for the BA.2.86-S protein compared to that of the ancestral strain, but no significant difference was found for the BA.2.86-S protein compared to that of XBB.1.5.

The authors use the term trigger structure for the ACE2-bound "down" RBD. Would this not be more accurately described as a "pre-triggered" structure? Along this line, does the transition of the last RBD-down to the RBD-up have to be triggered by ACE2? Or can there be dynamic sampling of multiple conformational states that puts the third RBD-down in equilibrium with RBD-up? For example, do the authors observe any RBD-ACE2-2up/RBD- up but ACE2 unbound conformation in their cryoEM dataset? If so, this would suggest that there is no triggering involved and rewording of the term triggered should be considered.

Response: We could not observe any RBD-ACE2-2up/RBD-up but ACE2 unbound conformation in our cryoEM dataset. Therefore, the two-RBD-up–one-RBD-down$_{three\text{-}ACE2}$ structure would be considered somehow contribute a functional roll in the "pre-triggered" of the S protein.

The title "A triggering structure of SARS CoV-2 BA2.86 spike upon ACE2 binding for receptor-binding domain up" is awkwardly written. I would encourage the authors to rewrite.

Response: We thank the reviewer for this comment. We have corrected the title as "**Structural basis for receptor-binding domain mobility of the spike in SARS-CoV-2 BA.2.86 and JN.1"**

Throughout the manuscript, the authors use the term "EM map" when describing the unknown and unmodeled regions of the cryoEM map. Please consider calling this "EM density" instead of "EM map".

Response: We thank the reviewer for this comment. We used the term "EM density" for the unknown and unmodeled regions of the cryo EM map throughout the manuscript.

Reviewer #3 (Remarks to the Author):

This manuscript by Yajima et al., reports structures of the SARS-CoV-2 BA.2.86 spike protein, unliganded and in complex with the ACE2 receptor. In particular, for the ACE2 receptor bound spikes, they identified a structure that had two RBDs in the up position and 1 in the down position with all there RBDs bound to ACE2, thus providing a unique structural definition of ACE2 bound to a down RBD.

Overall, this is a well-done study with carefully characterized structures with many important insights, such as the visualization of the N354 glycan in BA.2.86. The main concern I have here is regarding the interpretation of the ACE2-bound down state RBD as the "triggering structure". The third ACE2 bound to the down RBD as consequence of the two up RBDs moving away substantially. While an interesting structure, the data presented do not indicate whether or not this is the trigger required for third RBD to go to the up position. Highlighting this in the title and the abstract is, therefore, misleading. The authors should rephrase and reword these conclusions that are not supported by the data presented, while discussing the implications of the structural observations and considering their biological significance in the discussion section.

Response: We thank reviewer's positive comments for our manuscript. We tempered in the "triggering structure" related conclusions in the BA.2.86 S protein, which is removed the term "triggering structure," form the manuscript. The title and abstract were also modified. The implications of the structural observations and considering their biological significance have discussed in the DISCUSSION section. Line 428-433.

Additional experiments (**Supplementary fig. 6**) confirmed that the "two-RBD-up-one-RBD-down$_{three\text{-}ACE2}$" structure is also precent in the JN.1-S. Therefore, we have now proposed that the increase in RBD mobility/flexibility associated with ACE2 binding is a common property of both S proteins in BA.2.86 and JN.1. Line 275-304.

To experimentally confirm that the "two-RBD-up-one-RBD-down$_{three\text{-}ACE2}$" structure is unstable, we performed additional experiments. We showed that "two-RBD-up–one-RBD-down$_{three\text{-}ACE2}$" can be observed in BA.2.86-S protein treated at 37 °C for 1 hour, but not in BA.2.86-S protein treated at 42 °C for 1 hour (**Supplementary fig. 5**). These results would suggest that an intermediate structure, two-RBD-up–one-RBD-down$_{three\text{-}ACE2}$, has been thermally eliminated, which indicate unstable as an S trimer bound to ACE2. Although the data is not shown in the manuscript, no "two-RBD-up-one-RBD-down$_{three\text{-}ACE2}$" structure was similarly identified after 3 hours of treatment at 42 °C. Line 217-231.

Other points:

Lines 250-255: Reduced affinity of JN.1 for ACE2 (relative to BA.2.86) was reported here: https://www.thelancet.com/journals/laninf/article/PIIS1473-3099(23)00744-2/
The authors should reference this study.
Response: We thank the reviewer for this comment. We have cited this reference. Line 270-271.

Lines 281-282: "S proteins exclusively adopting the closed-1 state, exemplified by BA.2; and those exclusively displaying the closed-2 state, as demonstrated by BA.2.86 in this study, all within the context of RBD all-down states." Because the inability to identify a specific state within a cryo-EM dataset can often be a function of the data processing workflow, it would be better if this sentence was tempered to reflect this.
Response: We thank the reviewer's suggestion. We removed "exclusively" from the text and tempered/added the sentences. Line 345-351.

Figure 1: Between the figure and the legend, panels D and E are transposed.
Response: We greatly appreciate this comment. We have corrected them.

Figure 2: Panel A: the legend says: "2 RBD-up state (left), and 3 RBD-up state (right)." although in the fugure the structures are shown in up and down organization.
Response: We appreciate this comment very much. We have corrected them.

Figure 2: Panel D: not clear which RBD it is referring to - the up or the down, both should be added for comparison
Response: We thank the reviewer's comment. We have added labels to make it easier to identify which RBD is the one.

Figure 2: Panel C: Not clear which structure is the 2RBD-up and which is the 2-RBD-up 1-RBD-down. Should not the 2-RBD-up structure have its third RBD down? Nomenclatures should be clarified.
Response: We thank the reviewer's comment. To clearly distinguish between these two, we have rewritten as "two-RBD-up$_{\text{two-ACE2}}$; one RBD is down state and not bound to ACE2" and two-RBD-up–one-RBD-down$_{\text{three-ACE2}}$: all three RBDs bound to ACE2". Line 197 and 203-204.

Residue G139 is part of the NTD, not RBD.

Response: We thank the reviewer's comment. We have changed the amino acid residues forming the angle.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The revised manuscript has been significantly improved by addressing the concerns raised by the reviewers. There is just some minor suggestions:
1. The main idea of this manuscript is that they observed the ACE2-bound “down” RBD, suggesting an intermediate structure prior to the RBD-up conformation. I recommend incorporating Fig S7 E-G into the main figure.
2. Please check if Fig 2C is incorrectly labeled.
3. Please standardize the formatting of amino acids throughout the article. For example: Fig S4D, “V445H, R403K, Phe514 to Thr522”.

Reviewer #2 (Remarks to the Author):

The authors have addressed my comments satisfactorily. I appreciate the authors' additional analysis on the neutralizing antibodies, as well as tempering the conclusions about a 'triggering structure'. The manuscript is a solid contribution to the understanding of SARS-CoV-2 S mechanisms of receptor engagement and neutralization.

We thank two reviewers for their insightful and constructive feedback. We have addressed each comment, and we hope that the revised manuscript, together with this detailed response, resolves minor concerns.

Reviewer #1 (Remarks to the Author):

The revised manuscript has been significantly improved by addressing the concerns raised by the reviewers. There is just some minor suggestions:

Response: We appreciate this reviewer's comment.

1. The main idea of this manuscript is that they observed the ACE2-bound "down" RBD, suggesting an intermediate structure prior to the RBD-up conformation. I recommend incorporating Fig S7 E-G into the main figure.

Response: We thank the reviewer for this comment. We have transferred the Supplementary Fig. 7E-G to the Main Fig. 5A-C.

2. Please check if Fig 2C is incorrectly labeled.

Response: We thank the reviewer for this comment. We have corrected.

3. Please standardize the formatting of amino acids throughout the article. For example: Fig S4D, "V445H, R403K, Phe514 to Thr522".

Response: We thank the reviewer for this comment. We have standardized.

Reviewer #2 (Remarks to the Author):

The authors have addressed my comments satisfactorily. I appreciate the authors' additional analysis on the neutralizing antibodies, as well as tempering the conclusions about a 'triggering structure'. The manuscript is a solid contribution to the understanding of SARS-CoV-2 S mechanisms of receptor engagement and neutralization.

Response: We appreciate this reviewer's comment.