nature portfolio

Peer Review File

Structural Basis for the Activation of Proteinase-Activated Receptors PAR1 and PAR2

Corresponding Author: Dr Aaron McGrath

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript “Structural Basis for the Activation of Proteinase-Activated Receptors (PARs) by Endogenous Ligands” provides a detailed analysis of the structural mechanisms underlying the activation of Proteinase-Activated Receptors (PARs), specifically PAR1 and PAR2, by their endogenous ligands. By using cryo-EM technique, the authors elucidated the orthosteric binding pockets and receptor activation mechanisms. The discussion on the structural differences between active and inactive states of PAR1 and PAR2 adds depth to the understanding of these receptors. I have some specific comments:

Major issues:
1. Extended Data Figure 2 provides two density maps treated by DeepEMhancer that are not mentioned in the manuscript. It is necessary to truthfully display the density maps obtained after refinement and other processing such as DeepEMhancer, and clarify which density map is used for the final model construction. Additionally, each structure requires a density map colored according to resolution to assess the quality of the TM region.

2. For PAR1, the authors describe hydrogen bonds mentioned in Figure 2B and van der Waals interactions in Figure 2C. Can the authors provide more detail on the role of specific residues in the tethered ligand that are crucial for receptor activation? Are there additional mutagenesis experiments that support the critical role of these residues?

3. For PAR2 pocket, “Mutations of these residues to alanine result in a significant reduction in agonist potency by more than 10-fold, highlighting the critical nature of these interactions for receptor functionality”. The author use reference to support structural findings. While in the reference literature, three ligands were used and the conclusion is “Nineteen of twenty-eight PAR2 mutations reduced the potency of at least one ligand by >10-fold.” If no mutation assay is examined in this work, the authors should make a conclusion to describe the importance of key residues found in this structure on tethered ligand.

4. For both structures, the clash score typically needs to be kept below 10.

5. “unlike other Class A and B GPCRs, which typically exhibit significant activation-associated movements in TM6 (such as a 9 Å outward shift in rhodopsin[19] and about 16 Å in GLP1R when engaged with G proteins[20]), PAR1 shows a more modest 4.7 Å outward movement of TM6 in the active form compared to the vorapaxar-bound state.”
The authors suggest that milder movement of TM6 upon receptor activation is characteristic of PAR1, but whether this distinction is caused by binding of different G protein isoforms to the receptor and whether extensive comparisons of multiple receptor G protein complex structures were performed? Is this unique to PARs, or are there other receptors with similar characteristics?

6. “Both residues are critical for activation, as shown in mutagenesis experiments (reference needed).”
The author should provide reference here.

Other minor issues:
1. In Supplemental Table 1，under Ramachandran plot, there should be three parameters: “Favored”, “Allowed” and “Outlier”.
PAR1-Gαq have a Map resolution of 2.74 Å and Model resolution of 3.1 Å while PAR2-Gαq has a Map resolution of 3.1 Å and Model resolution of 2.7 Å. That seems confusing.

2. “Tethered Ligand Binding and the Orthosteric Binding Pocket”
This subtitle reads a bit confusing.

Reviewer #2

(Remarks to the Author)
The manuscript by Lyu et al. provides structural insights into the activation mechanisms of PAR1 and PAR2 through cryo-EM analysis.
While the manuscript offers some new structural information on PAR1 and PAR2, it suffers from significant flaws in terms of novelty, data interpretation, and scientific rigor. Many of the findings are not sufficiently distinguished from previously published work, and several statements in the manuscript are either incorrect or speculative. To improve the manuscript, the authors must engage more thoroughly with prior structural studies, revise the speculative claims, and provide a clearer, more detailed analysis of their data.

Below are detailed criticism:
1. Vague novelty and insufficient Framing:
• The abstract fails to clearly delineate the novelty of the work. The phrases like "shallow and constricted orthosteric binding pockets" are mentioned as a new findings but is not sufficiently contextualized against previous structural studies of PARs. Similar features have been observed and described in prior studies, such as Guo et al., 2024 which also investigated the active structures of PAR1 with its endogenous ligand. The authors should address how their findings are distinct from those of Guo et al.
• The claim of "minimal TM6 movements" as a novel observation is misleading. Minimal TM6 movement is a common feature in Gi-coupled GPCRs such as the μ-opioid receptor and cannabinoid receptors, so this observation is neither unique nor sufficiently novel. A more in-depth exploration of this phenomenon, especially in the context of other Class A GPCRs, is crucial for interpreting its significance.
• Several structures of PARs have been resolved, including active structures of PAR1 with endogenous ligands. The authors must acknowledge these prior studies, particularly those already deposited in the PDB.
• Another problematic claim is that the findings "provide a structural template for designing novel antagonists." This conclusion is premature and speculative without functional data to support the therapeutic implications. There is no direct evidence in the manuscript linking the structural findings to drug development. This claim should either be supported by additional data or removed entirely.
3. Poorly defined research question:
• The introduction lacks a clear research question or hypothesis, making it difficult to understand the motivation behind the study. It outlines the importance of PAR1 and PAR2 in human physiology but does not clearly define the specific gap in knowledge that this research addresses. What specific aspect of PAR activation or structure is this study intended to clarify?
4. Lack of context and missing references:
• The introduction and throughout the manuscript, there is no adequate discussion of already available structural data on PARs. For example, the authors neglect to reference previous studies that resolved the inactive state of PAR1 and active structures with G proteins in the introduction. These omissions weaken the manuscript's contribution to the field, as it appears the authors have not fully engaged with prior work.
• In terms of functional relevance, the manuscript includes vague sentences like "Notably, PAR1 and PAR2 are crucial for their roles in human physiology and pathology [2-4]." This is an overly broad statement that applies to all GPCRs and adds little specific information about PARs. More precise references are needed to back up such claims.
5. Incomplete analysis of binding pockets:
• The manuscript repeatedly emphasizes the shallow and narrow orthosteric binding pockets of PAR1 and PAR2 but fails to analyze how these structural features might affect ligand specificity or receptor activation. A comparison with other GPCRs that have deeper pockets would provide important context, yet this is missing. Additionally, previous structural studies on PAR1 have also described a shallow binding site for its native ligand, thrombin, which the authors fail to acknowledge.
6. Global structural comparison lacking:
• While the manuscript does mention TM movements and specific residue interactions in the binding pocket, it lacks a comprehensive discussion of common GPCR motifs, such as DRY, PIF, and NPxxY. These motifs are crucial for GPCR activation and signaling but are not addressed in the text.
7. Misinterpretation of dynamics:
• The statements like "We have resolved the structures of these receptors in complex with their endogenous tethered ligands, providing clear illustrations of receptor activation dynamics" are misleading. No dynamics were measured in the study, as cryo-EM provides static structural snapshots.
8. Flawed terminology and typos through the text:
• The manuscript contains several instances of incorrect terminology, such as "cryo-EM mapping," which is not the correct phrase. More accurate terms such as "cryo-EM reconstruction" or "density map" should be used.
9. Missing citations:
• Several key citations need to be included throughout the manuscript, e.g., sentese «The HiBiT was fused to the c-terminus of human Gβ1 and cloned into pFastBac plasmid as described in the VIP1R paper.» does not contain any reference.
10. Cryo-EM data processing description:
• The cryo-EM data processing pipeline is poorly explained, leaving questions about how the authors achieved the quality and accuracy of their reconstructions. More details are needed regarding how they handled particle heterogeneity and refinements, e.g., parameters. Additionally, from the EM density of PAR2, an associated lipid can be seen, yet this lipid was not built into the model.

Reviewer #3

(Remarks to the Author)
The article is clearly written and contains 2 new cryo-EM GPCR structures. The structural models give insights into the binding modes of the agonist peptides and the resulting rearrangements associated with receptor activation.

That said, there are several points that needs to be addressed before the manuscript is suitable for publication in Nature Communications.

My copy of the manuscript did not contain any line numbering so I will refer to different sections in the manuscript with quotations marks.

Major

The paper claims to be “revealing their activation mechanism in unprecedented details” and “also identifies residues that are crucial for receptor activation”. To make theses statements one would normally expect structures to be complemented with functional data of mutated protein constructs to provide support for the structural models. For PAR1 and PAR2 there are already several mutational studies available in literature. To that end, please expand section and detail on how the structural models fit the existing mutational studies where specific interactions are implied (e.g. Nanevicz et al JBC 1995, Cheng et al Nature 2017, Kennedy et al ACS Pharmacol Transl Sci. 2018 etc.) to validate the structures. For example, Nanevicz et al demonstrate that the arginine of SFLLRN peptide likely interacts directly with Glu260 through a rescue mutation strategy. How does this fit the models presented here. Also, how do the peptides transmit the activating signals to drive the rearrangements observed in the paper?

“Comparative analysis with the inactive vorapaxar-bound PAR1 reveals key structural differences from the tethered ligand-bound active state of PAR1.” Please specify how the structural overlay was generated and write so that it is unambiguous that the same structural overlay was used throughout the analysis. Also do this in the section describing the comparison in between the activated PAR2 receptor and the AZ8838 complex structure.

Minor

“Surprisingly, comparisons with antagonist-bound structures show minimal conformational changes in the TM6 helix, a typical of GPCR activation, with large movements of TM7 observed upon activation.” Written as the minimal conformational change is the typical hallmark of receptor activation. Please rephrase to make clearer.

“This strategy promises highly selective therapeutic options, enhancing treatment efficacy while minimizing off-target effects” Why would targeting the orthosteric pocket enhance efficacy? The tethered agonist has a very high local concentration (which suggests that allosteric ligands should be more effective). Also, what evidence is there thar targeting the orthosteric pocket would minimize off-target effects? Are there unique features in this pocket compared to all other pockets in the proteome? Please, phrase the enthusiasm for this strategy at a more appropriate level.

The manuscript claims to observe several hydrogen bonds. Unless the experimental data enable you to observe the hydrogen bonds directly (i.e. the actual position of the hydrogens) you only observe heteroatoms at distances consistent with hydrogen bonds and should describe them as putative hydrogen bonds.

In the section comparing the active PAR1 structure to the vorapaxar complex structure I would suggest that you first fully describe the extracellular side and then discuss the rearrangements on the cytoplasmic side (i.e. move the discussion starting with “Thirdly, both vorapaxar ...”.

Figure 3A. The arrow to the left (signifying a 2.6Å movement) appear to be pointing in the wrong direction? On that note, is the statement “TM5 does not undergo dramatic extensions but does exhibit a 2.6 Å outward movement in the ligand-bound state compared to the inactive state” correct?

Figure 3B Mark the peptide residue numbers.

Figure 3B-D. Please be consistent in how you denote vorapaxar and the tethered peptide. I would suggest using coloured text rather that coloured boxes with black text.

Figure 3D The article text refers to a movement in TM6 (“leading to the downward movement of TM6 and activating the receptor (Figure 3D).” but the figure only denotes TM7? Please change/add.

Figure 4A Please be consistent in how you present the helices in figures 3A and 4A.

Figure 4B-C Please be consistent in how you denote the peptide and AZ8838.

“The tethered ligand exhibits a different binding model from AZ8838,”. Please write ”The tethered ligand exhibits a different binding mode from AZ8838,”

“Both residues are critical for activation, as shown in mutagenesis experiments (reference needed).” You should add a

reference. Also, please expand the section when you compare with structures with literature mutations (see comment above).

Figure 4D The main text states “The movement of Y326 of TM7 results in Y156 of TM3 moving away from H227 of ECL2, breaking an H-bond and forming a new stabilizing H-bond with the hydroxyl group of Y156 of TM3. Once stabilized, Y326 of TM7 sterically pushes TM6 outwards through interactions with L306 and L305 of TM6, resulting in activation of G-protein binding (Figure 4D).”. Please denote both TM6 and TM7 in the figure. Also, is it possible to also show L306 and L305 from this angle (as they are key to the argument)?

Figure 5. Please denote key PAR helices.

Figure 7. Is the movement in helix 5 consistent for both receptors. Figures 3 and 4 seem to indicate that it moves in opposite direction for the two receptors.

Methods: “The first 41 residues (residue 1-41) of PAR1 and the first 36 residues (residue 1-36) were chopped off to mimic the protease cleavage.” Please add “of PAR2”

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
While the authors have reasonably addressed my specific comments, I am not fully convinced by the reliance on mutagenesis results from published studies to support the structural findings. To strengthen the impact of their results, I believe the authors should perform mutagenesis and functional assay for key mutations with their ligands. Without this additional experimental validation, it remains unclear what NOVEL contributions their findings bring to the field, given that similar structures have already been published.

Reviewer #2

(Remarks to the Author)
The authors extensively revised their manuscript and addressed all the concerns raised. I recommend the manuscript for publication in Nature Communications.

Reviewer #3

(Remarks to the Author)
Thanks to the writers for responding to all the comments. I think the added material provides valuable background and relevance to the findings within the paper. However, I also think that the added material is often unclearly written and sometimes outright wrong, which reduces the precision of the paper. In addition, I still think that the figures need improvements to make it easier to follow the argumentation (especially now that the discussion adds more detail). As a result, I would request another round of editing before I can recommend publication of the paper in Nature Communication.

Examples of unclear passages:

Fig 1:
• Main text (e.g. row 117-117) refers to TM5, TM5, TM7 etc. Please find a way to incorporate labels into Figure.
• In this figure it appears as if the full N-termini is modelled for the receptors. Are the models different from the ones presented in figure 2 or is it just the angle?
• The colour for the tethered PAR1 agonist is poorly chosen. It is difficult to differentiate in between oxygens and carbons. Please change.

Fig 2:
• Again, main text refers to TM1, TM5 etc (e.g. row 141). Please find a way to incorporate labels into figure (e.g. to avoid confusion with amino acid labels you can add white text within the helices or lighter grey next to the helices).
• Putative hydrogen bonds are marked in the same way as VDW interactions. Please differentiate (e.g. “putative hydrogen bonds (heteroatoms within 3.2 Å ... or whatever cutoff used) are shown as black dotted lines whereas VDW interaction are shown as yellow dotted lines”).
• Minor Fig2F denote helices as tubes. Please be consistent.
Row 145: “while L38’s side chain is involved in polar interaction L230”. It should be the main chain carbonyl.

Row 146: “and forms van der Waals interactions with S320 and Y323 of TM7 as well as forming an H-bond with D228”. The first part should be referring to the side chain and the second part should be referring to the main chain nitrogen. Either exchange “L38’s side chain” with “L38” or make sure you reference side chain and main chain atoms correctly. Also, please describe hydrogen bonds as “putative” or “potential” hydrogen bonds as you do not observe the hydrogens.

Row 168: “after activation in this region”. Unclearly written (there is no local activation). Receptor activation leads to a certain movement in this region.

Figure 3: Again, modify ligand colours (difficult to differentiate in between tethered agonist and vorapaxar in 3B). Also, it appears as you have also modelled the asparagine after Arg46 in figure 3A. Could you include that also in Figure 2A?

Row 186: “Y350 is located at a position that is poised to form a hydrogen bond with vorapaxar”. I do not see that in figure 3. Is it the backbone carbonyl of Y350 you refer to (not shown)?

Row 202: “activated PAR2 exhibits minimal movement on TM6, with an 3.4Å outward shift and an 2Å shift to the intracellular side”. Please describe a bit clearer (e.g. “ , with a 3.4Å outward tilt and a 2Å downwards movement towards the intracellular side”).

Row 210: “Upon binding at the orthosteric pocket, the sidechain of L38 sterically pushes Y323 of TM7 downwards”. Can you find an angle that shows this (L38 is not in the figure).

Row 211: “forming a stabilizing bond with D228”. Please label D228 in the figure. You can use slightly smaller font if needed.

Row 213: “movement of Y326 results in Y156 of TM3 moving away from H227 of ECL2, breaking an H-bond and forming a new stabilizing H-bond with the hydroxyl group of Y156 of TM2”. This is written as if Y156 forms an H-bond with itself? Please write clearer. Also, here I do not believe that you need to show H227 to make the arguments but whenever you talk about a residue that us not shown please state that so that the reader do not look for it (“moving away from H227 of ECL2 (not shown)”.

Figure 4D. Use the arrows more consistently. Some arrows signify a movement in between 2 structural states (not all residues are shown with that representation though) whereas other arrows signify a push that a residue exerts to neighbouring residues (Y326 to L307 and L330). You can for example use black arrows to signify movements of residues and red arrows to signify the push. Please also include description of the different arrows in the figure legend.

Figure 5: “PAR1-Gaq (blue and green cartoons) ad PAR2-Gaq (coral and green)” One Gaq is white, please modify. Also, in images like this I find it helpful with more labels (e.g. include PAR1 in blue text, PAR2 in skin coloured text and then you can denote Gaq as GaqPAR1 GaqPAR2 in the respective colour.)

Figure 6: I appreciate the schematic but want more details to clearly follow the arguments in the main text. Label the tyrosines (e.g. T350/323 and T353/326). “(L38 in PAR1 and F43 in PAR2)” should state “(L38 in PAR1 and F43 in PAR2, not shown)”. Please highlight the PIF/Y motif in figure (it might be busy to add all side chains, but you could potentially colour the corresponding part of the TM helices). Similarly, please add nomenclature for DPxxY and DRF motifs.

Figure 7B.
• please change colour for GB88 so that it is visible in figure.
• Please add some label for key TMs.
• It does not seem like GB88 interact with H227 as stated?

Row 266: You mention interactions to V229 and Y322 but do not show them. Either include amino acids or write “now shown”.

Row 269: “the isoxazole oxygen accepts an H-bond from H310”. This does not appear to be true. It looks like H310 forms interaction with the amide oxygen?

Row 271: “pushing L69” should read “pushing L69 (not shown)”

Row 273: “almost identical interaction with the activation residue Y323”. Would be useful to show this (otherwise write “not shown”).

Extended Data Figure 4, caption: “B. Structural superposition of the tethered peptide...” should be written as “B. Position of the PAR1 tethered peptide ...” as I assume that the superposition was done on the full receptors.

Extended Data Figure 5. Title “Structural comparison of active and inactive state of PAR1” is incorrect. The figure only displays one receptor structure. The comparison is in between the position of the tethered ligand and vorapaxar. Also, when you select a different colour for the tethered ligand, please use a colour that is more distinct from that of vorapaxar (e.g. if you use magenta for vorapaxar you could use the lighter orange for the PAR1 tethered peptide).

Extended Data Figure 6. This appears to be identical to Figure 4C??? Also, there does not appear to be a reference to neither extended data figure 6 nor 7 in the main text.

Minor corrections:

Row 38: exchange “which” for “and”.

Row 127: “that binds into the core of PAR1”. In the rest of the article the pocket is described as shallow. Please exchange for something like “a shallow pocket at the centre of the 7 TM bundle”.

Figure 4: Please be consistent on how you represent ligand.

Row 235: “and competitive analysis with antagonists”. Exchange “competitive” with something like “comparative” or rewrite passage.

Row 255: “physiochemical nature”. I would exchange that with something like “functional profile”.

Row 280-281: “pocket formed by between V326”. Please remove “by”.

RESPONSE TO REVIEWER COMMENTS

We sincerely thank the reviewers for their feedback on our manuscript. In response to the reviewers' insightful comments, we have undertaken significant revisions that include:

1. **The structure of GB88 bound to PAR2:** Most notably, the revised manuscript introduces the structure of PAR2 bound to the agonist, and pathway-selective antagonist, GB88. The structural elucidation of GB88 bound to PAR2 demonstrates how this small molecule mimics key interactions of the endogenous tethered agonist, allowing it to effectively occupy the PAR2 orthosteric pocket.
2. **Improved Structural Analysis and Discussion**: We have expanded the discussion on the structural insights related to the activation mechanisms of PAR1 and PAR2, providing clearer details about helical movement upon receptor activation and the critical residues involved. Figures 2, 3 and 4 have been updated to reflect these changes. We have included a more comprehensive discussion on the implications of our findings, particularly related to the insights garnered from our GB88 structure.
3. **Expanded Abstract, Introduction and Results**: Enhanced details on the roles of PAR1 and PAR2 in physiology, including a better explanation of their activation mechanisms and physiological implications. The revised version includes more detailed supplemental tables and references, indicating the inclusion of re-refined models and associated improvements in validation metrics.
4. **Improved Clarity in Experimental Procedures:** There are refinements in the methods section, particularly in the procedures related to cryo-EM data collection and processing, ensuring clarity and reproducibility.

A point-by-point response to each reviewer comment is below, illustrating the changes we have made. Additionally, all edits are clearly tracked in the revised document.

Reviewer #1 (Remarks to the Author):

The manuscript "Structural Basis for the Activation of Proteinase-Activated Receptors (PARs) by Endogenous Ligands" provides a detailed analysis of the structural mechanisms underlying the activation of Proteinase-Activated Receptors (PARs), specifically PAR1 and PAR2, by their endogenous ligands. By using cryo-EM technique, the authors elucidated the orthosteric binding pockets and receptor activation mechanisms. The discussion on the structural differences between active and inactive states of PAR1 and PAR2 adds depth to the understanding of these receptors. I have some specific comments:

Major issues:
1. Extended Data Figure 2 provides two density maps treated by DeepEMhancer that are not mentioned in the manuscript. It is necessary to truthfully display the density maps obtained after refinement and other processing such as DeepEMhancer, and clarify which density map is used for the final model construction. Additionally, each structure requires a density map colored according to resolution to assess the quality of the TM region.

DeepEMHancer was used to improve the map quality. We have included the use of DeepEMhancer in the methods and included the reference. We have also included the resolution-colored maps for all structures in extended data Figure 7.

2. For PAR1, the authors describe hydrogen bonds mentioned in Figure 2B and van der Waals interactions in Figure 2C. Can the authors provide more detail on the role of specific residues in the tethered ligand that are crucial for receptor activation? Are there additional mutagenesis experiments that support the critical role of these residues?

We have provided additional discussion in the Results and Discussion section. We have updated Figure 2 and cited additional available mutagenesis data from the literature, which is in support of our model. Most notably, a recent publication from the Xu lab that reports extensive PAR1 mutagenesis data to understand receptor activation.

3. For PAR2 pocket, “Mutations of these residues to alanine result in a significant reduction in agonist potency by more than 10-fold, highlighting the critical nature of these interactions for receptor functionality”. The author use reference to support structural findings. While in the reference literature, three ligands were used and the conclusion is “Nineteen of twenty-eight PAR2 mutations reduced the potency of at least one ligand by >10-fold.” If no mutation assay is examined in this work, the authors should make a conclusion to describe the importance of key residues found in this structure on tethered ligand.

We thank the reviewer for highlighting this misleading statement. We have reworded the passage and also include additional reference that report supporting mutagenesis data.

4. For both structures, the clash score typically needs to be kept below 10.

We have extensively re-refined our structures and significantly improved the related statistics. The clash score is now well below 10 for all three structures. PAR1-tethered ligand is 6.12, PAR2 tethered ligand is 7.74 and the newly included GB88 structure is 4.83.

5. “unlike other Class A and B GPCRs, which typically exhibit significant activation-associated movements in TM6 (such as a 9 Å outward shift in rhodopsin[19] and about 16 Å in GLP1R when engaged with G proteins[20]), PAR1 shows a more modest 4.7 Å outward movement of TM6 in the active form compared to the vorapaxar-bound state.”
The authors suggest that milder movement of TM6 upon receptor activation is characteristic of PAR1, but whether this distinction is caused by binding of different G protein isoforms to the receptor and whether extensive comparisons of multiple receptor G protein complex structures were performed? Is this unique to PARs, or are there other receptors with similar characteristics?

During the preparation of the manuscript, a Cell Research paper reported cryoEM structures of PAR1 bound to both G_q G_i . According to this publication, the conformation and activation of GPCR is not G protein dependent according to this study. Many of the observations and conclusions of this study are consistent with our discussions. We have included a discussion at the end of our manuscript.

6. “Both residues are critical for activation, as shown in mutagenesis experiments (reference needed).”
The author should provide reference here.

Added references.

Other minor issues:
1. In Supplemental Table 1, under Ramachandran plot, there should be three parameters: “Favored”, “Allowed” and “Outlier”.

Done

PAR1-Gαq have a Map resolution of 2.74 Å and Model resolution of 3.1 Å while PAR2-Gαq has a Map resolution of 3.1 Å and Model resolution of 2.7 Å. That seems confusing.

Fixed.

2. “Tethered Ligand Binding and the Orthosteric Binding Pocket”
This subtitle reads a bit confusing.

Fixed.

Reviewer #2 (Remarks to the Author):

The manuscript by Lyu et al. provides structural insights into the activation mechanisms of PAR1 and PAR2 through cryo-EM analysis.
While the manuscript offers some new structural information on PAR1 and PAR2, it suffers from significant flaws in terms of novelty, data interpretation, and scientific rigor. Many of the findings are not sufficiently distinguished from previously published work, and several statements in the manuscript are either incorrect or speculative. To improve the manuscript, the authors must engage more thoroughly with prior structural studies, revise the speculative claims, and provide a clearer, more detailed analysis of their data.

We appreciate the reviewer’s comments. In response, we have provided an improved version that engages more thoroughly with prior structural studies, revises the speculative claims, and provides a clearer, more detailed analysis of the data.

Below are detailed criticism:
1. Vague novelty and insufficient Framing:
• The abstract fails to clearly delineate the novelty of the work. The phrases like "shallow and constricted orthosteric binding pockets" are mentioned as a new findings but is not sufficiently contextualized against previous structural studies of PARs. Similar features have been observed and described in prior studies, such as Guo et al., 2024 which also investigated the active structures of PAR1 with its endogenous ligand. The authors should address how their findings are distinct from those of Guo et al.

Thank you for the comment. We hope that our thoroughly revised manuscript has addressed the reviewers’ concerns.

• The claim of "minimal TM6 movements" as a novel observation is misleading. Minimal TM6 movement is a common feature in Gi-coupled GPCRs such as the μ-opioid receptor and

cannabinoid receptors, so this observation is neither unique nor sufficiently novel. A more in-depth exploration of this phenomenon, especially in the context of other Class A GPCRs, is crucial for interpreting its significance.

After carefully analyzing these structures again, we found μ-opioid receptor TM6 movement to consistently be ~9-10 angstroms:

"Some insight into the structural underpinnings of μOR activation and μOR mediated G protein signaling is provided by high-resolution structures. The G_i C-terminal helix binds to an opening within the cytoplasmic surface of the 7-transmembrane (TM) bundle, which is formed upon an ~10 Å outward movement of the intracellular end of TM6"
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10168371/

Similarly, in the agonist bound cannabinoid receptor CB2 TM6 movements of ~ 11 Å were observed:

"While there is an outward movement of TM6 between both receptors, CB2 undergoes minor conformational changes compared with CB1. CB2 activation results in an 11 Å outward movement of the intracellular part of TM6, allowing for G protein binding.":

https://portlandpress.com/biochemsoctrans/article/51/4/1533/233363/Structural-and-functional-insights-into-the-G#:~:text=While%20there%20is%20an%20outward,allowing%20for%20G%20protein%20binding.

These movements are much larger than the ~4.7 Å and ~3.4 Å movements we observe at the cytoplasmic end of TM6 for PAR1 and PAR2, respectively.

• Several structures of PARs have been resolved, including active structures of PAR1 with endogenous ligands. The authors must acknowledge these prior studies, particularly those already deposited in the PDB.

Added. Please know that the active PAR1 study was published after we submitted this manuscript to BioRxiv and for consideration to be published.

• Another problematic claim is that the findings "provide a structural template for designing novel antagonists." This conclusion is premature and speculative without functional data to support the therapeutic implications. There is no direct evidence in the manuscript linking the structural findings to drug development. This claim should either be supported by additional data or removed entirely.

To further strengthen our claim that this work will "provide a structural template for designing novel antagonists.", we have included an additional structure of PAR2 bound to the small molecule GB88. GB88 is a biased antagonist of PAR2 that selectively inhibits the PAR2/Gq/11/Ca^{2+}/PKC signaling pathway. It is of note that this is the first structure reported of a PAR bound to a small molecule in the true orthosteric site. We believe the inclusion of this structure significantly improves the novelty of the work and will provide a frame for additional ideation around small molecule activation, and inactivation, of PARs. In addition, we have included an in-depth discussion comparing this structure to peptide bound PARs, PAR1 bound to vorapaxar and to structures of PAR2 bound to AZ8838 and AZ3451.

3. Poorly defined research question:
• The introduction lacks a clear research question or hypothesis, making it difficult to understand the motivation behind the study. It outlines the importance of PAR1 and PAR2 in human physiology but does not clearly define the specific gap in knowledge that this research addresses. What specific aspect of PAR activation or structure is this study intended to clarify?

We have extensively revised manuscript to clearly communicate the study's objectives and significance. Our study is intended to elucidate the structural basis of PAR1 and PAR2 activation mechanisms by directly visualizing how the endogenous tethered ligands bind and activate these receptors. Additionally, our manuscript now also explores the selective inhibition mechanism of PAR2 by the antagonist GB88, offering insights for small molecule engagement and design. These findings contribute significantly to our understanding of how the PARs functions and open avenues for therapeutic interventions. We hope we have addressed the reviewer's difficulty in understanding.

4. Lack of context and missing references:
• The introduction and throughout the manuscript, there is no adequate discussion of already available structural data on PARs. For example, the authors neglect to reference previous studies that resolved the inactive state of PAR1 and active structures with G proteins in the introduction. These omissions weaken the manuscript's contribution to the field, as it appears the authors have not fully engaged with prior work.

We have added more introduction and discussion in relation to prior work, including the recent publication by Gua et al. As stated above, we submitted this manuscript prior to agonized PAR1 structure publication.

• In terms of functional relevance, the manuscript includes vague sentences like "Notably, PAR1 and PAR2 are crucial for their roles in human physiology and pathology [2-4]." This is an overly broad statement that applies to all GPCRs and adds little specific information about PARs. More precise references are needed to back up such claims.

We have made corrections accordingly.

5. Incomplete analysis of binding pockets:

• The manuscript repeatedly emphasizes the shallow and narrow orthosteric binding pockets of PAR1 and PAR2 but fails to analyze how these structural features might affect ligand specificity or receptor activation. A comparison with other GPCRs that have deeper pockets would provide important context, yet this is missing. Additionally, previous structural studies on PAR1 have also described a shallow binding site for its native ligand, thrombin, which the authors fail to acknowledge.

We have expanded on our comparative analysis of the tethered ligand orthosteric pocket observed in our new structures and the previous antagonist bound crystal structures (vorapaxar for PAR1 and AZ8838 for PAR2). We have included the reference to: Gandhi, P.S., Z. Chen, and E. Di Cera, Crystal structure of thrombin bound to the uncleaved extracellular fragment of PAR1. J Biol Chem, 2010. ***285****(20): p. 15393-15398.*

6. Global structural comparison lacking:
• While the manuscript does mention TM movements and specific residue interactions in the binding pocket, it lacks a comprehensive discussion of common GPCR motifs, such as DRY, PIF, and NPxxY. These motifs are crucial for GPCR activation and signaling but are not addressed in the text.

We now include a discussion on these critical motifs in the "G protein coupling and activation mechanism" section.

7. Misinterpretation of dynamics:
• The statements like "We have resolved the structures of these receptors in complex with their endogenous tethered ligands, providing clear illustrations of receptor activation dynamics" are misleading. No dynamics were measured in the study, as cryo-EM provides static structural snapshots.

Clear conformational changes were observed from the structures of PAR2 in complex with tethered ligand, to the antagonist bound structures. Therefore, we believe that the dynamics of the PARs were observed to some degree. We measured the distance change of critical elements in different structures.

We acknowledge that no motion can be observed directly in cryoEM single particle structures, and we did not intend to suggest capable of doing so. We have removed one mention of the word dynamic, and tined down the language around the concept in the second instance.

8. Flawed terminology and typos through the text:
• The manuscript contains several instances of incorrect terminology, such as "cryo-EM mapping," which is not the correct phrase. More accurate terms such as "cryo-EM reconstruction" or "density map" should be used.

Corrected.

9. Missing citations:
• Several key citations need to be included throughout the manuscript, e.g., sentese «The HiBiT was fused to the c-terminus of human Gβ1 and cloned into pFastBac plasmid as described in the VIP1R paper.» does not contain any reference.

Added reference.

10. Cryo-EM data processing description:
• The cryo-EM data processing pipeline is poorly explained, leaving questions about how the authors achieved the quality and accuracy of their reconstructions. More details are needed regarding how they handled particle heterogeneity and refinements, e.g., parameters. Additionally, from the EM of PAR2, an associated lipid can be seen, yet this lipid was not built into the model.

We have added a more detailed description of the cryo-EM data processing pipeline. Unfortunately, we were unable to satisfactorily include a lipid into this density during re-refinement of the PAR2 model.

Reviewer #3 (Remarks to the Author):

The article is clearly written and contains 2 new cryo-EM GPCR structures. The structural models give insights into the binding modes of the agonist peptides and the resulting rearrangements associated with receptor activation.

That said, there are several points that needs to be addressed before the manuscript is suitable for publication in Nature Communications.

My copy of the manuscript did not contain any line numbering so I will refer to different sections in the manuscript with quotations marks.

Major

The paper claims to be “revealing their activation mechanism in unprecedented details” and “also identifies residues that are crucial for receptor activation”. To make theses statements one would normally expect structures to be complemented with functional data of mutated protein constructs to provide support for the structural models. For PAR1 and PAR2 there are already several mutational studies available in literature. To that end, please expand section and detail on how the structural models fit the existing mutational studies where specific interactions are implied (e.g. Nanevicz et al JBC 1995, Cheng et al Nature 2017, Kennedy et al ACS Pharmacol Transl Sci. 2018 etc.) to validate the structures. For example, Nanevicz et al demonstrate that the arginine of SFLLRN peptide likely interacts directly with Glu260 through a rescue mutation strategy. How does this fit the models presented here. Also, how do the peptides transmit the activating signals to drive the rearrangements observed in the paper?

We thank the reviewer for these very helpful suggestions. We have made adjustment to the phrase “revealing their activation mechanism in *unprecedented details”. We have also cited and acknowledged, where applicable, the work described in Nanevicz et al JBC 1995, Cheng et al Nature 2017, Kennedy et al ACS Pharmacol Transl Sci. 2018. We have also included a more thorough description of the transmission of ligand binding activation to the G-protein coupling interface. Please see the section “G protein coupling and activation mechanism” and associated Figures.*

Only in the PAR1 structure is the position 5 arginine visible. While it is plausible that there are potential interactions with residues from extracellular loop 2, no evidence is observed in our maps. This observation by Nanevicz is cited in the updated version of the manuscript.

“Comparative analysis with the inactive vorapaxar-bound PAR1 reveals key structural differences from the tethered ligand-bound active state of PAR1.” Please specify how the structural overlay was generated and write so that it is unambiguous that the same structural overlay was used throughout the analysis. Also do this in the section describing the comparison in between the activated PAR2 receptor and the AZ8838 complex structure.

We have included: “All structure alignments described were done by aligning the GPCR chains only instead of the whole complex in UCSF Chimera”. Additionally, the section “Structural Comparison of Active and Inactive States of PAR2”, now describes the comparison between the activated PAR2 receptor and the AZ8838 complex structure.

Minor

“Surprisingly, comparisons with antagonist-bound structures show minimal conformational changes in the TM6 helix, a typical of GPCR activation, with large movements of TM7 observed upon activation.” Written as the minimal conformational change is the typical hallmark of receptor activation. Please rephrase to make clearer.

Modified as:
“Large conformational change in the TM6 helix is a typical signature of GPCR activation. Surprisingly, comparisons with antagonist-bound structures show minimal conformational changes in the TM6 helix and larger movements of TM7 upon activation.”

“**This strategy promises highly selective therapeutic options, enhancing treatment efficacy while minimizing off-target effects**” Why would targeting the orthosteric pocket enhance efficacy? The tethered agonist has a very high local concentration (which suggests that allosteric ligands should be more effective). Also, what evidence is there thar targeting the orthosteric pocket would minimize off-target effects? Are there unique features in this pocket compared to all other pockets in the proteome? Please, phrase the enthusiasm for this strategy at a more appropriate level.

Corrected.

The manuscript claims to observe several hydrogen bonds. Unless the experimental data enable you to observe the hydrogen bonds directly (i.e. the actual position of the hydrogens) you only observe heteroatoms at distances consistent with hydrogen bonds and should describe them as putative hydrogen bonds.

Corrected.

In the section comparing the active PAR1 structure to the vorapaxar complex structure I would suggest that you first fully describe the extracellular side and then discuss the rearrangements on the cytoplasmic side (i.e. move the discussion starting with “Thirdly, both vorapaxar ...”.

Corrected.

Figure 3A. The arrow to the left (signifying a 2.6Å movement) appear to be pointing in the wrong direction? On that note, is the statement “TM5 does not undergo dramatic extensions but does exhibit a 2.6 Å outward movement in the ligand-bound state compared to the inactive state” correct?

Corrected.

Figure 3B Mark the peptide residue numbers.

Corrected.

Figure 3B-D. Please be consistent in how you denote vorapaxar and the tethered peptide. I would suggest using coloured text rather that coloured boxes with black text.

Corrected.

Figure 3D The article text refers to a movement in TM6 ("leading to the downward movement of TM6 and activating the receptor (Figure 3D)." but the figure only denotes TM7? Please change/add.

TM6 is out of the view in this orientation. We have improved labeling and cited Figure 3A here.

Figure 4A Please be consistent in how you present the helices in figures 3A and 4A.
Corrected.

Figure 4B-C Please be consistent in how you denote the peptide and AZ8838.

Corrected.

"The tethered ligand exhibits a different binding model from AZ8838,". Please write "The tethered ligand exhibits a different binding mode from AZ8838,"

Corrected

"Both residues are critical for activation, as shown in mutagenesis experiments (reference needed)." ***You should add a reference***. Also, please expand the section when you ***compare with structures with literature mutations*** (see comment above)

.
We appreciate this comment and have adjusted and added references accordingly.

Figure 4D The main text states "The movement of Y326 of TM7 results in Y156 of TM3 moving away from H227 of ECL2, breaking an H-bond and forming a new stabilizing H-bond with the hydroxyl group of Y156 of TM3. Once stabilized, Y326 of TM7 sterically pushes TM6 outwards through interactions with L306 and L305 of TM6, resulting in activation of G-protein binding (Figure 4D).". ***Please denote both TM6 and TM7 in the figure. Also, is it possible to also show L306 and L305 from this angle (as they are key to the argument)?***

To address this important comment, we have re-written this section and generated Figure 4D.

Figure 5. Please denote key PAR helices.

Corrected.

Figure 7. Is the movement in helix 5 consistent for both receptors. Figures 3 and 4 seem to indicate that it moves in opposite direction for the two receptors.

Helix 5 in the two activated PAR structures is very similar upon activation. Please note the overlays comparing activated PAR1 and PAR2 in Figures 5 and extended data Fig4. The reason the direction of movement of helix 5 appears different in Figures 3 and 4 is because of the different antagonist states being compared with each activated receptor.

Methods: “The first 41 residues (residue 1-41) of PAR1 and the first 36 residues (residue 1-36) were chopped off to mimic the protease cleavage.” Please add “of PAR2”

Corrected.

REVIEWER COMMENTS

We are sincerely grateful for Reviewer #3's constructive feedback, which has significantly enhanced our manuscript. Following their suggestions, we have meticulously revised both the text and figures to improve clarity and precision. Unclear passages have been rewritten for accuracy, and we have improved the figures to ensure easier comprehension of the scientific arguments. Minor textual errors, highlighted in the detailed comments, have been corrected for overall coherence and consistency. We believe these revisions significantly enhance the quality and impact of our manuscript and address all concerns raised during the re-review process.

Reviewer #3 (Remarks to the Author):

Thanks to the writers for responding to all the comments. I think the added material provides valuable background and relevance to the findings within the paper. However, I also think that the added material is often unclearly written and sometimes outright wrong, which reduces the precision of the paper. In addition, I still think that the figures need improvements to make it easier to follow the argumentation (especially now that the discussion adds more detail). As a result, I would request another round of editing before I can recommend publication of the paper in Nature Communication.

We would like to sincerely thank the reviewer for their meticulous review, the comments are thoughtful and have help us improve our manuscript immensely.

Examples of unclear passages:

Fig 1:
• Main text (e.g. row 117-117) refers to TM5, TM5, TM7 etc. Please find a way to incorporate labels into Figure.

Please see the new Figure 1 where we have included an additional panel and TM labels throughout.

• In this figure it appears as if the full N-termini is modelled for the receptors. Are the models different from the ones presented in figure 2 or is it just the angle?

As in our final structure, the N termini is truncated in all figures, we believe this is the viewing angle. Please note that we have remade several panels, and this hopefully helps with the angle.

• The colour for the tethered PAR1 agonist is poorly chosen. It is difficult to differentiate in between oxygens and carbons. Please change.

We have chosen a more suitable color and remade the PAR1 figures.

Fig 2:

• Again, main text refers to TM1, TM5 etc (e.g. row 141). Please find a way to incorporate labels into figure (e.g. to avoid confusion with amino acid labels you can add white text within the helices or lighter grey next to the helices).

Please see the new Figure 2 where we have included an additional panel and TM labels throughout.

• Putative hydrogen bonds are marked in the same way as VDW interactions. Please differentiate (e.g. “putative hydrogen bonds (heteroatoms within 3.2 Å ... or whatever cutoff used) are shown as black dotted lines whereas VDW interaction are shown as yellow dotted lines”).

This was an error. We have amended the figure to show putative H-bonds only.

• Minor Fig2F denote helices as tubes. Please be consistent.

We have remade this figure.

Row 145: “while L38’s side chain is involved in polar interaction L230”. It should be the main chain carbonyl.

Fixed.

Row 146: “and forms van der Waals interactions with S320 and Y323 of TM7 as well as forming an H-bond with D228”. The first part should be referring to the side chain and the second part should be referring to the main chain nitrogen. Either exchange “L38’s side chain” with “L38” or make sure you reference side chain and main chain atoms correctly. Also, please describe hydrogen bonds as “putative” or “potential” hydrogen bonds as you do not observe the hydrogens.

Fixed.

Row 168: “after activation in this region”. Unclearly written (there is no local activation). Receptor activation leads to a certain movement in this region.

This now reads “receptor activation leads to rearrangements in the region”.

Figure 3: Again, modify ligand colours (difficult to differentiate in between tethered agonist and vorapaxar in 3B). Also, it appears as you have also modelled the asparagine after Arg46 in figure 3A. Could you include that also in Figure 2A?

We have chosen different colors to fix the issue. N47 is now included in Figure 1D.

Row 186: “Y350 is located at a position that is poised to form a hydrogen bond with vorapaxar”. I do not see that in figure 3. Is it the backbone carbonyl of Y350 you refer to

(not shown)?

This now reads: “In the vorapaxar-bound structure, Y350 is located at a position that is poised to form a hydrogen bond with H255 on ECL2, stabilizing Y350 and helping to maintain TM7 in the inactive conformation (Figure 3C)”.

Row 202: “activated PAR2 exhibits minimal movement on TM6, with an 3.4Å outward shift and an 2Å shift to the intracellular side”. Please describe a bit clearer (e.g. “ , with a 3.4Å outward tilt and a 2Å downwards movement towards the intracellular side”).

This now reads: “Analogous to our PAR1-Gαq complex, activated PAR2 exhibits minimal movement on TM6, with a 3.4 Å outward tilt and a 2 Å downwards movement towards the intracellular side.”

Row 210: “Upon binding at the orthosteric pocket, the sidechain of L38 sterically pushes Y323 of TM7 downwards”. Can you find an angle that shows this (L38 is not in the figure).

We have tried out best to clearly show L38 in Figure 2D. Y323 is shown below L38 to help illustrate this observation.

Row 211: “forming a stabilizing bond with D228”. Please label D228 in the figure. You can use slightly smaller font if needed.

We have labelled D228 and show this bond in Figure 2D.

Row 213: “movement of Y326 results in Y156 of TM3 moving away from H227 of ECL2, breaking an H-bond and forming a new stabilizing H-bond with the hydroxyl group of Y156 of TM2”. This is written as if Y156 forms an H-bond with itself? Please write clearer. Also, here I do not believe that you need to show H227 to make the arguments but whenever you talk about a residue that us not shown please state that so that the reader do not look for it (“moving away from H227 of ECL2 (not shown)”.

We have reworded this paragraph and now include Figure 4D to illustrate this observation.

Figure 4D. Use the arrows more consistently. Some arrows signify a movement in between 2 structural states (not all residues are shown with that representation though) whereas other arrows signify a push that a residue exerts to neighbouring residues (Y326 to L307 and L330). You can for example use black arrows to signify movements of residues and red arrows to signify the push. Please also include description of the different arrows in the figure legend.

We have only included arrows to signify one residue pushing another (in RED). We believe this make the figure clearer to the reader.

Figure 6: I appreciate the schematic but want more details to clearly follow the arguments in the main text. Label the tyrosines (e.g. T350/323 and T353/326). “(L38 in PAR1 and F43 in PAR2)” should state “(L38 in PAR1 and F43 in PAR2, not shown)”. Please highlight the PIF/Y motif in figure (it might be busy to add all side chains, but you could potentially colour the corresponding part of the TM helices). Similarly, please add nomenclature for DPxxY and DRF motifs.

We have included the sidechains for the conserved tyrosine residues. We have also labeled the relative positions of the PIF/Y, DRF/NRY and DPxxY motifs.

Figure 7B.
• please change colour for GB88 so that it is visible in figure.
Fixed.
• Please add some label for key TMs.
A new figure panel (Figure 7C) has been added and TM labels have been included throughout the figure.
• It does not seem like GB88 interact with H227 as stated?

We have corrected this statement in the body text.

Row 266: You mention interactions to V229 and Y322 but do not show them. Either include amino acids or write “now shown”.

We have removed this statement from the body text.

Row 269: “the isoxazole oxygen accepts an H-bond from H310”. This does not appear to be true. It looks like H310 forms interaction with the amide oxygen?
We have included Figure 7E to show this clearly.

Row 271: “pushing L69” should read “pushing L69 (not shown)”

Fixed

Row 273: “almost identical interaction with the activation residue Y323”. Would be useful to show this (otherwise write “not shown”).

We have added “not shown” in the body text.

Extended Data Figure 4, caption: “B. Structural superposition of the tethered peptide...” should be written as “B. Position of the PAR1 tethered peptide ...” as I assume that the superposition was done on the full receptors.

Fixed.

Extended Data Figure 5. Title “Structural comparison of active and inactive state of PAR1” is incorrect. The figure only displays one receptor structure. The comparison is in

between the position of the tethered ligand and vorapaxar. Also, when you select a different colour for the tethered ligand, please use a colour that is more distinct from that of vorapaxar (e.g. if you use magenta for vorapaxar you could use the lighter orange for the PAR1 tethered peptide).

We have removed this figure.

Extended Data Figure 6. This appears to be identical to Figure 4C??? Also, there does not appear to be a reference to neither extended data figure 6 nor 7 in the main text.

We have removed this duplicate.

Minor corrections:

Row 38: exchange “which” for “and”.

Fixed.

Row 127: “that binds into the core of PAR1”. In the rest of the article the pocket is described as shallow. Please exchange for something like “a shallow pocket at the centre of the 7 TM bundle”.

This now reads: “They adopt an extended conformation that binds into a shallow pocket at the center of the 7 TM bundle of PAR1, making extensive contact with TM1, TM5, TM6, TM7, and extracellular loop 2 (ECL2).”

Figure 4: Please be consistent on how you represent ligand.

Fixed.

Row 235: “and competitive analysis with antagonists”. Exchange “competitive” with something like “comparative” or rewrite passage.

This now reads: “Our agonist bound structures and comparison with antagonists bound structures of PAR1 and PAR2 reveals a shared activation mechanism involving the downward movement of TM7 and subsequent displacement of TM6 (Fig. 6).”

Row 255: “physiochemical nature”. I would exchange that with something like “functional profile”.

This now reads: “The true functional profile of the compound has recently been shown to be nuanced, demonstrating partial agonist properties (EC50 3 mM) against several PAR2 signaling arms, including Gαq/11 directed pathways, and the ability to internalize the receptor post activation.”

Row 280-281: “pocket formed by between V326”. Please remove “by”.

Fixed.