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Molecular Basis for Activation of G protein-Coupled Receptor Kinases

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 10 June 2010

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by two referees and their comments to the authors are provided below. As you can see both referees find the analysis interesting, well executed and appropriate for the journal. They raise a few issues that should be addressed before publication here. Given these comments, I'd like to invite you to submit a revised manuscript, taking these issues into account. When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

Boguth et al have determined the crystal structure of GRK6 bound to either AMP or the adenosine analog sangivamycin, and find that these structures adopt closed states of the kinase catalytic domain consistent with the active conformation of the kinase. The key findings of this report is that in this nearly-active state (it is still not GPCR-activated), the extreme amino and carboxyl terminal regions of GRK6 adopt stable conformations that can be seen in the crystals, and these regions both help to stabilize the closed state of the kinase catalytic site and form part of the putative activated GPCR-interacting site. A structure-based model is proposed for how GRK6 binds to activated rhodopsin.

Comments:

There are several minor concerns about clarity of description and data presentation.

Figures 1a, 1b, 2a, 2b, 3a, 3c, 5a-d, and 6 all appear to use the same color-coding system (NT green, kinase CT extension magenta, GRK CT orange). However, the color key being used is not mentioned explicitly in the legends to Figures 1b, 2b, 3a, 3c, 5a-d, or 6. These should be clarified.

In Fig 1b, it would be helpful to indicate explicitly the axes and directions of the domain rotations in the new more closed state compared to the previous more open state structure.

In the results description for Fig 5 (p 11), it is stated that "<B>ecause the C-terminal regions of the three GRK subfamilies are not conserved, they are not expected to play a direct role in binding GPCRs." This statement assumes that all GRKs recognize all GPCRs and that there is no GRK-subclass specifity/selectivity for receptor types, which appears untrue. While the conclusion may be true, this is not a good reason to support it. Structure-based evidence that the extreme C-tail is likely not near the conformationally-sensitive regions of the activated receptor is a more compelling argument.

Also in the description of Fig 5 (p 11-12), the issue of CT palmitoylation is mentioned, and it is pointed out that the palmitoylation site, just past the end of the stable CT structure, is quite far from the presumed membrane-interacting surface of the kinase. One observation not mentioned here is that the GRK6c variant, which ends naturally at almost this position and which lacks palmitoylation, is not only membrane associated but also fully active as a GPCR kinase (ref 25). Palmitoylation is clearly dispensible for membrane targeting of GRK6, and thus may indeed play a regulatory role by binding to the GRK6 surface instead.

In the model in Fig 6, the basic putative PIP2-binding surface should be indicated and the end of the CT (where the palmitate would likely be) should also pointed out (much as the active site cleft is shown). The legend should also remind the reader that the opsin structure lacks the longer 3rd loop and particularly the opsin CT where the kinase substrate sites are located, so it becomes very clear that these longer peptide chains would be able to wriggle into the active site quite readily.

Referee #2 (Remarks to the Author):

This manuscript describes the crystal structure of an activated form of GRK6 co-crystallised with either AMP or sangivamycin. The authors deduce that this is an activated state mainly from the closed conformation of the kinase domain, reminiscent of a transition-like conformation of PKA, and the ordering of the C-terminal tail of GRK6 which is also similar to other activated AGC kinase such as PKA and PKB. The very interesting feature of this structure is the ordering of an N-terminal α-helix (and the C-terminal kinase domain tail) which spans the N- and C-lobes of the kinase. The ordered αNT helix is proposed to recognise activated GPCRs, suggesting that reciprocally GPCRs act to allosterically activate GRK6 through ordering of αNT helix. The authors test their model by introducing mutations into αNT. Mutation of exposed residues predicted to disrupt GPCR recognition ablates phosphorylation of GPCRs but not short synthetic peptides, whereas mutation of buried αNT residues, predicted to disrupt interactions with the N and C lobes causes loss of kinase activity in general. Finally, the authors identify a basic region close to αNT that is predicted to recognise phospholipids. Overall this is a high quality and significant study of considerable general interest. The text is well written and the figures are clear. Only a few minor corrections are required

prior to acceptance.

1. The authors don't explain why co-crystallising GRK6 with either AMP or sangivamycin results in a closed active state, whereas the AMP-PNP complex is inactive. Since AMP-PNP is an ATP analogue this is a surprising observation.

2. I have some concern about the resolution and statistics listed in Table 1. Crystals diffract anisotropically to between 3.5 and 4 A along a* and b* and to about 2.7 A along c*, yet the authors quote 100% data completeness to 2.7 A and 2.9 A for the sangivamycin and AMP data sets. I don't understand this or the >100% Rsym quoted for the highest resolution shell.

3. The fit of sangivamycin into density in Fig. 4 isn't compelling and one could imagine an opposite orientation of the ribose. A stereoview should be shown and a comparable figure for AMP.

4. The authors propose that a Ser at residue 328 accounts for GRK6 affinity for sangivamycin. Was this tested?

5. In Fig. 3 label the N- and C-termini of αNT and the C-terminal tail.

6. Page 9, reference to Supp Fig. S1 doesn't seem appropriate.

1st Revision 21 July 2010

Response to Comments:

Thanks to both referees for their positive and constructive comments.

(Referee #1):

Figures 1a, 1b, 2a, 2b, 3a, 3c, 5a-d, and 6 all appear to use the same color-coding system (NT green, kinase CT extension magenta, GRK CT orange). However, the color key being used is not mentioned explicitly in the legends to Figures 1b, 2b, 3a, 3c, 5a-d, or 6. These should be clarified.

We have included a statement to this effect in the caption for Fig. 1a to avoid unnecessary redundancy of text in the other figure captions: “The same color scheme is used for GRK6 in all subsequent figures, unless otherwise indicated.”

In Fig 1b, it would be helpful to indicate explicitly the axes and directions of the domain rotations in the new more closed state compared to the previous more open state structure.

We have added arrows/axes to the figure to help describe changes in the RH and kinase subdomains.

In the results description for Fig 5 (p 11), it is stated that "Because the C-terminal regions of the three GRK subfamilies are not conserved, they are not expected to play a direct role in binding GPCRs." This statement assumes that all GRKs recognize all GPCRs and that there is no GRK-subclass specifity/selectivity for receptor types, which appears untrue. While the conclusion may be true, this is not a good reason to support it. Structure-based evidence that the extreme C-tail is likely not near the conformationally-sensitive regions of the activated receptor is a more compelling argument.

We have decided to avoid this problem entirely by instead stating: “The unique C-terminal regions of the three GRK subfamilies are expected to play roles in membrane targeting and, in at least some cases, allosteric activation”

Also in the description of Fig 5 (p 11-12), the issue of CT palmitoylation is mentioned, and it is pointed out that the palmitoylation site, just past the end of the stable CT structure, is quite far from the presumed membrane-interacting surface of the kinase. One observation not mentioned here is that the GRK6c variant, which ends naturally at almost this position and which lacks

palmitoylation, is not only membrane associated but also fully active as a GPCR kinase (ref 25). Palmitoylation is clearly dispensible for membrane targeting of GRK6, and thus may indeed play a regulatory role by binding to the GRK6 surface instead.

We concur and have altered this paragraph to read: "The palmitoylation sites that occur several residues after the end of aCT (a region disordered in this structure) are thus relatively distant from the expected membrane surface (Fig. 3, 6). However, this observation is consistent with the fact that the GRK6C splice variant, which lacks the palmitoylation sites entirely and whose C-terminus is similar to the end of the observed structure reported here, fully retains its ability to bind membranes and phosphorylate activated GPCRs [25]. Palmitoylation is therefore dispensable for function in GRK6, and the region C-terminal to aCT, and its modifications, may chiefly be involved in auto-regulation of the adjacent kinase domain."

In the model in Fig 6, the basic putative PIP2-binding surface should be indicated and the end of the CT (where the palmitate would likely be) should also pointed out (much as the active site cleft is shown). The legend should also remind the reader that the opsin structure lacks the longer 3rd loop and particularly the opsin CT where the kinase substrate sites are located, so it becomes very clear that these longer peptide chains would be able to wriggle into the active site quite readily.

We have altered the figure as requested and added the following sentence to the caption: "Furthermore, in this model either a longer IL3 loop (as is common in other GPCRs) or the cytoplasmic tail of rhodopsin (which extends an additional 22 amino acids beyond the last ordered residue in this model) can readily access the kinase active site cleft and serve as phospho-acceptors."

Referee #2 (Remarks to the Author):

1. The authors don't explain why co-crystallising GRK6 with either AMP or sangivamycin results in a closed active state, whereas the AMP-PNP complex is inactive. Since AMP-PNP is an ATP analogue this is a surprising observation.

It was surprising to us as well. We believe that this particular conformation of GRK6 occurs because lattice contacts involving the N-terminal helix stabilize this structure as a helix, much like an activated GPCR would, and therefore allow us to capture a more closed conformation, as described on page 9. Our best theory is that either ATP or an ATP analog would induce further closure of the kinase domain, resulting in an overall conformation that is incompatible with the $P6_1$ crystal lattice. To address this question, we have added the following sentence to the crystallization methods: "We were unable to grow crystals in the presence of ADP or ATP analogs, perhaps because these compounds favor a distinct, more closed conformation of the kinase domain that is incompatible with the lattice of the $P6_1$ crystals."

2. I have some concern about the resolution and statistics listed in Table 1. Crystals diffract anisotropically to between 3.5 and 4 A along a* and b* and to about 2.7 A along c*, yet the authors quote 100% data completeness to 2.7 A and 2.9 A for the sangivamycin and AMP data sets. I don't understand this or the >100% Rsym quoted for the highest resolution shell.

The 100% completeness simply indicates that we collected a complete data set out to 2.7 Å, even though many of the reflections in the higher resolution shells were at background (i.e., if we had an isotropically diffracting crystal). After integration and before scaling, we truncated the data set to remove reflections outside the stated ellipsoid boundaries. The 100% R-factor in the highest shell corresponds to all collected data (before truncation); a high number is expected because the majority of the reflections in the outermost shell are at background.

In Table 1, we give two versions of the relevant data collection statistics, one for if we include the entire, "isotropic" data set, and one for the ellipsoidally truncated set. This is just due diligence-but we have changed the footnotes to hopefully more clearly describe what we are doing.

3. The fit of sangivamycin into density in Fig. 4 isn't compelling and one could imagine an opposite orientation of the ribose. A stereoview should be shown and a comparable figure for AMP.

They are not compelling perhaps because these are omit maps, and not final 2fo-fc maps, which do

not impose model bias. Interpretation of the density at this stage and at this resolution (considering anisotropy) can be less straightforward. However, accommodation of ribose in another conformation than shown is really not possible due to packing interactions (and precedence). We have altered this figure to be a stereo view and now include AMP as panel b.

4. The authors propose that a Ser at residue 328 accounts for GRK6 affinity for sangivamycin. Was this tested?

It was not, nor do we mean to imply that we had. It is simply an observation and a hypothesis for future study.

5. In Fig. 3 label the N- and C-termini of αNT and the C-terminal tail.

We think you are referring to Fig 3B (text is garbled, unfortunately), and we have added the requested labels.

6. Page 9, reference to Supp Fig. S1 doesn't seem appropriate.

We have deleted Supp. Fig. S1 and the reference call.

2nd Editorial Decision 28 July 2010

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referee #2 to take a final look at the paper and as you can see below, this referee is satisfied with the introduced changes. I am therefore very pleased to proceed with the acceptance of the paper for publication here. You will receive the formal acceptance letter shortly.

Thank you for submitting your interesting manuscript to the EMBO Journal.

Sincerely

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2

The authors have addressed my comments

