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Unraveling the mechanism of dual-specificity GAPs

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1st Editorial Decision 13 December 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. Please excuse the delay in getting back to you with a decision, due to restricted reviewer availabilities at this time at the turning of the years. We have now received the comments of three reviewers, which I am enclosing below. As you will, two referees find your results on the mechanism used by a dual-specificity GAP to target the mechanistically distinct Rap1 GTPase in addition to Ras GTPase interesting and important and also well-supported by the presented experimental evidence, while referee 1 expresses interest in principle but feels that the data fall short of conclusively demonstrating the proposed mechanisms. In light of these reports, I am inclined to follow the majority recommendation of referees 2 and 3 and would therefore like to invite you to prepare a revised version of the manuscript, taking into account the referees' specific (and mostly minor) comments. From an editorial point of view, I would like to add that it would be helpful to try and make the results section somewhat less technical and more easily accessible also for a more general readership, as also indicated by referee 1. When preparing your letter of response to the referees' comments, please also 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. In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

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 REPORTS:

Referee #1 (Remarks to the Author):

The manuscript by Sot et al describes a detailed investigation of the molecular mechanism that rationalizes the observed dual specificity of two GTPase activating proteins (GAPS), IP4BP and RASAL, which act on both Ras and Rap GTPases. The authors convincingly demonstrate the importance of C2 domains, flanking the GAP domain in the two regulatory proteins, for their GTPase activity towards Rap and to a much lesser extent - Ras. The authors expand on this observation to probe the molecular mechanism that allows the two proteins to regulate both Rap and Ras GTPases, even though the two typically require GAPs operating on quite different principles. Time resolved FTIR is the key method in this study, although admittedly it does not provide a complete description of the kinetics of enzyme-substrate interaction. A set of mutants is studied, and the results are consistent with a model which stipulates that the Rap GTPase is forced by the C2 domain to assume a distinctly unique conformation, in which Gln63 is shifted into the position that Gln61 occupies in Ras. In this way, the two GAP proteins can act on Rap using the same arginine finger mechanism that is used for Ras.
Much of the paper is technical, dealing with the details of the FTIR experiments, which are probably not of interest to broader audience. The mechanism which is postulated is very interesting, but remains to a significant degree hypothetical as no structural data are available. Thus, the manuscript in its present form is more appropriate for specialized journals.

Referee #2 (Remarks to the Author):

In this interesting manuscript, the authors use time-resolved FTIR to analyze the formation and decay of conformational and hydrolytic intermediates in the Rap and Ras GTP hydrolysis reactions catalyzed by full length as well as truncated forms of the dual specificity GAP1 family proteins GAP1-IP4BP and RASAL. The most striking observation is that Gln63 in Rap1 (corresponding to Glu63 in Ras) plays a critical catalytic role equivalent to Ras Gln61 in the context of dual specificity GAPs even though it does not contribute to the reactions catalyzed by RapGAP. This result is unexpected but clearly demonstrated. The study further provides convincing evidence for distinct Ras and Rap catalytic modes in which the C2 and PH domains of the dual specificity GAPs either enhance binding (Ras mode) or are necessary for catalysis (Rap mode), presumably by stabilizing a catalytically competent conformation of switch II that properly orients Gln63. The latter point is somewhat speculative though plausible in light of the presently available structural information.

Overall, the manuscript is well written and provides substantial new insight into the distinct catalytic modes utilized by dual specificity GAP1 family proteins. In my view, it would be appropriate for publication with only minor modification.

Minor points:

Figure 4 - key residues (e.g. Q63) are obscured by the labels.

Figure 5 - do the F64A, T65A and F64A/T65A mutants also confer catalytic activity with respect to RASAL?

Referee #3 (Remarks to the Author):

The paper describes interesting new insight into the action of dual specificity GTPase activating

proteins (GAP) on the G proteins Ras and Rap1. The latter interact differently with GAP1-IP4BP as described in the manuscript. Judged from the spectra in Fig 3, the quality of the data is superb and the reproducibility excellent. I would have liked to see also the Rap1 spectra. My comments refer essentially to minor issues, mainly to the readability of the text.

While the text is well structured, it is still complicated to follow for an outsider because several proteins and constructs are compared for both interaction partners (G-protein and GAP). Any attempt to simplify the text will increase the readability. Simple measures could be:
- list all proteins studied in this work at the end of the introduction
- additional subtitles
- try to minimize switching between different proteins
- my impression was that the terminology is not entirely consistent, for example Rap, RapGAP and RasGAP should be used only when the whole protein family is meant and Rap1 (not Rap) should always be used when this particular protein is meant. It would also help to specify the protein when amino acids are discussed, i.e. Rap-Q63 instead of just Q63.
- explain use of the words intrinsic and extrinsic
- avoid discussion of previous data in the Results section (e.g. p 11)
- specify name of protein instead of using just "protein" (e.g. p12, lines 4,19)

Other points:
GAP activity should be defined. Is this identical to GTP hydrolysis activity, or does it refer to a rate increase by GAP? It seems that k2 has been used to measure activity, and not the return to the off state, which seems more logical.

p 6 The data for GAP1-IP4BP in Fig 1b seem to indicate that the activity is higher for the GAP domain (i.e. without two C2 domains and the C-terminus) than for the variant without the first C2 domain, in contrast to what the text says. Probably the data were omitted.

p7 The authors might consider to discuss an overview infrared spectrum as an introduction to the description of Fig 2.

p7 The assignments should be discussed or a reference given. It should be mentioned here or on p 9 that protein bound Pi is doubly protonated.

p8 line 3 abbreviation fl for full length needs to be defined.

p9 should be antisymmetric not asymmetric PO2 stretching and it should be specified that Pi is meant not the alpha phosphate of GDP.

p11 The section on the switch II region is difficult to follow. Text at the beginning of p 12 says that a model was prepared for the binding of Rap (Rap1) to GAP1-IP4BP. The figure legend says however, that the Rap1 structure was taken from a Rap1-GAP1-IP4BP structure and modeled into the Ras-p120GAP domain structure. So the GAP domain is from p1120GAP. Neither it is clear why the homology model was built, nor whether it is relevant since the Rap1-Q63E results seem to indicate that the homology model does not show the correct structure of the catalytic site.

It seems that "switch II" was not explained in the introduction.

The activity of wt Ras should be included in Fig 5B for comparison.

Why was it not checked with FTIR whether the effects of Ras-Q61 mutation are due to lower affinity or slower catalysis?

30-fold lower activity of Rap1-T61Q seems to be wrong no matter whether k2 or k3 is used for comparison.

From the amide I region it should be possible to judge whether the off state conformation is adopted for the mutants. This should be discussed.

p13 line 13 "similar results" confusing since FTIR data are not reported for RASAL. "similar

inhibition" is better.

p14 line 8 should be Fig 5B

Why not include the data in table 2 in table 1?

p 15 reference needed for NF1 FTIR data

p 16 not clear how the homology model supports the proposed mechanism for Rap1 since Rap-Q63 is further away from the catalytic site than Ras-E63. Is it assumed that the binding site of GTP is different?

p19 305K seems to be wrong, the tables say 280 or 285K. Slower measurements should be specified.

legend of Fig 2 What is meant with artifact and what is its reason? How was it assured that contributions from k2 and k3 do not contain artifacts.

legend of Fig 3C references should be given for data from previous work

table 1 what is meant with RapGAP in the bottom section. If it is a different protein than GAP1-IP4BP, it should not be listed in the same column, or the column heading should be changed.

suppl Fig 2 bands should be labeled

1st Revision - authors' response 28 January 2010

Herewith we are sending the revised manuscript entitled:

Unraveling the mechanism of dual-specificity GAPs
By Begona Sot , Carsten Kötting, Delia Deaconescu1, Yan Suveyzdis, Klaus Gerwert, Alfred Wittinghofer1*

which we have revised following the suggestions of the reviewers. The paper is indeed, as noted by you and one of the reviewers, technical, as an important part of the paper deals with FTIR, which is not a straight forward technique. We have nevertheless tried to change the text as much as possible to make it "less technical", shortening and simplifying some of the descriptions of the FTIR experiments which have been moved to supplements, including parts figure 3 (suppl. Fig. 3).

We have answered all the referees' questions, as outlined in detail below

Referee #2

Minor points:

Figure 4 - key residues (e.g. Q63) are obscured by the labels.

The figure has been changed accordingly

Figure 5 - do the F64A, T65A and F64A/T65A mutants also confer catalytic activity with respect to RASAL?

F64A, F65A and F64A/T64A mutants reduce the GAP activity of both GAP1IP4BP and RASAL. RASAL results are now included in the figure 5A and explained in the text.

Referee #3

Any attempt to simplify the text will increase the readability, terminology ect…

We have added a table in supplementary information which lists all the proteins used in the paper. We would like to stick with the general description of Rap and Ras, otherwise we would very much complicate the nomenclature. We (and others) use consistently truncated versions of H-ras and Rap1B and have clarified the terminology in the Supplementary Table I. Furthermore, we consistently added the protein name to the residues (e.g Rap-Q63) and used the specific name of proteins instead of just ìproteinî where appropriate.

Other points:
GAP activity should be defined. Is this identical to GTP hydrolysis activity, or does it refer to a rate increase by GAP? It seems that k2 has been used to measure activity, and not the return to the off state, which seems more logical.

The GAP activity term is commonly used and a definition has nevertheless been added in introduction (p3), as were the terms intrinsic and extrinsic.

K2 describes the chemical step of hydrolysis, while k3 measures the release of Pi from the G protein. Therefore, it is more appropriate to use k2 in order to better compare the chemistry of the GTPase reaction between slow and fast hydrolyzing proteins. In the slow catalysis mutants, the hydrolysis and the Pi release step occur simultaneously.

p 6 The data for GAP1-IP4BP in Fig 1b seem to indicate that the activity is higher for the GAP domain (i.e. without two C2 domains and the C-terminus) than for the variant without the first C2 domain

The GAP1-IP4BP variant without the first C2 domain could not be isolated due to insolubility, so we do not have this data. We did not mention it in the text, what could cause misunderstandings. Thus, we have changed the text to clarify it. (p6)

p7 The authors might consider to discuss an overview infrared spectrum as an introduction to the description of Fig 2.

Since referee#1 is criticising that there is already too much technical detail of the FTIR experiments shown, we do not want to add another Figure with a spectrum. However, an overview is present in Figure 3 and Supplemental Figure 3. We refer to these Figures now already at this point and added lines into the Figure in order to highlight the position of the marker bands.

p7 The assignments should be discussed or a reference given. It should be mentioned here or on p 9 that protein bound Pi is doubly protonated.

This is now mentioned on page 7.

p8 line 3 abbreviation fl for full length needs to be defined.

Now it is defined.

p9 should be antisymmetric not asymmetric PO2 stretching and it should be specified that Pi is meant not the alpha phosphate of GDP.

We changed the text accordingly (p9).

p11 The section on the switch II region is difficult to follow. Text at the beginning of p 12 says that a model was prepared for the binding of Rap (Rap1) to GAP1-IP4BP. The figure legend says however, that the Rap1 structure was taken from a Rap1-GAP1-IP4BP structure and modeled into the Ras-p120GAP domain structure. So the GAP domain is from p1120GAP. Neither it is clear why the homology model was built, nor whether it is relevant since the Rap1-Q63E results seem to indicate that the homology model does not show the correct structure of the catalytic site.

We have changed the text (p11) in order to clarify how figure 4 was prepared. Furthermore, its relevance comes, as the referee suggests, by the fact that it can not be correct. The alignment of Rap1 with Ras highlights rap1-thr61 as the only Rap1 residue close enough to the -phosphate to be implicated in catalysis. However, the Thr61 mutants results contradict this, pointing out that switch II conformation must be different than the modeled one. It also shows that Rap1-Gln63, although not oriented towards the -phosphate, is not far away, and could be positioned closer to the GTP by conformational rearrangements in switch II. Furthermore, in the model, Rap1-Gln63 is close to helix 6 and the important proline, which therefore could be implicated in this conformational change. We agree that in our first manuscript version it was not enough explained, and we changed the text in pag 12 and 17 in order to make it more clear.

It seems that "switch II" was not explained in the introduction.

Although we think it is a very common term in structure-function studies of GT- and AT-binding proteins, it is explained now in p3.

The activity of wt Ras should be included in Fig 5B for comparison.

It has been included

The effects of Ras-Q61 mutation are due to lower affinity or slower catalysis?

It is established in over 30 years of Ras biology and fully demonstrated that Ras-Q61 is the key catalytic residue from Ras, which orients the water molecule for the hydrophilic attack (Wittinghofer and Pai, Trends Biochem Sci. (1991) 16, 382.) (Scheffzek, et al. Science (1997) 277, 333), and therefore its mutation produce strong effects due to slower catalysis. Some of the Q61 mutants actually bind tighter than wt.
In the FTIR experiments, the proteins concentration are very high, meaning that all the results with slower kinetics than wild type are most probably due to slower catalysis rather than lower affinity.

30-fold lower activity of Rap1-T61Q seems

The referee is completely right, it is a 22-fold stimulation and it has been changed in the text.

From the amide I region it should be possible to judge whether the off state conformation is adopted for the mutants. This should be discussed.

The mutants are no ìoff-stateî mutants. For a discussion we would need to show several additional spectra. In our opinion this point is not important for the general mechanism presented here. We prefer to leave this point out in order to prevent further lengthening of the paper.

p13 line 13 "similar results" confusing since FTIR data are not reported for RASAL. "similar inhibition" is better.

We have substituted it in the text

p14 line 8 should be Fig 5B

It has been substituted it in the text

Why not include the data in table 2 in table 1?

Although it would be a good idea to have all data together, in our opinion it would make table I too dense in information and therefore less clear.

p 15 reference needed for NF1 FTIR data

The reference has been added

p 16 not clear how the homology model supports the proposed mechanism for Rap1 since Rap-Q63

is further away from the catalytic site than Ras-E63. Is it assumed that the binding site of GTP is different?

It is assumed that the switch II conformation is different than the model. It has been explained in a previous point and in discussion in the manuscript.

p19 305K seems to be wrong;

It was wrong, and has been changed accordingly

legend of Fig 2 What is meant with artifact and what is its reason? How was it assured that contributions from k2 and k3 do not contain artifacts.

It is meant that the absorption change is not due to free Pi. The reason is a disturbance by the laser flash. This disturbance leads to small absorbance changes which are usually an order of magnitude smaller compared to our signal. However at 1078 cm-1 the absorption change is unusually large due to a huge absorption band of the buffer. This change, but only this change is also present in an analogues control experiment without any protein (photolysis of pHP-GTP in the same buffer).

Reference in legend of Fig 3C

The reference is now given.

Table 1: what is meant with RapGAP in the bottom section.

It is the Rap-specific GAP RapGAP. We followed referee's advice and we added a column heading in table I.

suppl Fig 2 bands should be labeled

The bands are now labelled.

