THE EMBO JOURNAL

Manuscript EMBO-2009-72275

Structure of an integrin with an αI domain, complement receptor type 4

Can Xie, Jianghai Zhu, Xing Chen, Lizhi Mi, Noritaka Nishida, and Timothy A. Springer

Corresponding author: Timothy Springer, Immune Disease Institute, and Program in Cellular and Molecular Medicine, Children's Hospital

Review timeline:

Submission date:	18 August 2009
Editorial Decision:	14 September 2009
Revision received:	15 October 2009
Additional Correspondence:	04 November 2009
Accepted:	05 November 2009

Transaction Report:

(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 14 September 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three referees show significant interest in your study and recommend publication pending satisfactory revision; primarily involving text changes rather than any additional experimental data. In particular, all three referees find that some of your statements are not well justified given the relatively low resolution of the structure, and therefore highlight the need to tone down some of the stronger conclusions.

In the light of the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript.

I would also like to remind you of the need to submit the coordinates of your structures to PDB before acceptance of your manuscript, and to include the relevant PDB accession codes in the text. Particularly since I think it unlikely that we will need to send your manuscript out for re-review, I would ask you to include these when you submit your revised version.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This manuscript reports the first crystal structure of a β2 integrin and the first showing an αI domain in the context of a full-length structure. It is well written and illustrated and presents an interesting model for transmission of allostery between the αI and βI domains. Overall this paper is likely to be of significant interest and value to the community.

The structure is of fairly low resolution and as such the authors must make clear to the readers the limitations that this places on their interpretations.

The authors note that they confirmed the sequence register at the two junctions between the β-propeller domain and the α-I domain in several ways but only data for the 4 SeMet anomalous signals is shown. Please also show the density for the Cys-97-126 disulfide bond and Phe-328 sidechain and the SeMet-332 anomalous signal described in the text.

Legends for the supplementary figures were missing from my version of the text.

Referee #2 (Remarks to the Author):

The manuscript describes the first crystal structure of the ectodomain of an integrin (αxβ2) that contains an I-domain in the α subunit. The integrin is in a dormant (low affinity) state similar to that of αIIbβ3 (an integrin lacking an αI domain), yet the structure of αxβ2 reveals noticeable variations in the inter-domain arrangements that suggest specialization within the αI-containing subfamily of integrins. Analysis of the structure in the context of previous data on activating mutations, and activation-associated epitopes and antibodies supports that activation of αxβ2 involves extension of the integrin's legs and opening of the headpiece. On the other hand, the structural data does not support other models for activation (disulfide reduction and deadbolt release). A major finding is the lack of specific interactions between the αx-I domain and the β-propeller and β2-I domains. Finally, based on the structure of the low-affinity (bent) state, a revised model for the activation of integrins that contain an αI-domain is proposed.

The data presented by Xie et al provide valuable novel information on the structure of an important subfamily of integrin receptors. The study makes a significant contribution to the understanding of integrin regulation.

Major comments:

1) This group has previously published an excellent paper (Nishida et al [2006] Immunity 25:583-594) showing a detailed EM analysis of αxβ2 in multiple states (bent, extended-closed, extended-open). The αxβ2 construct used in the current work has a slightly different C-terminal clasp, which includes an additional disulfide bridge that is maintained after TEV digestion (but can be broken by

reduction). Nevertheless, it is not clear what does the current EM data add to that of the 2006's paper. It seems that the variability in the position of the αI domain could be deduced from the inspection of the published data.

2) The model for activation of the αI domain proposes that αx Glu318 only binds to the activated βI MIDAS when the αI domain is in the intermediate or open states. The model implies that Glu318 does not bind to the βI MIDAS when the αI domain is in the low affinity conformation. It should be explained why this can be unequivocally ruled out. The results of this work do not exclude this possibility. In fact, Glu318 is part of the C-terminal linker between the αI domain and the propeller, which seems to have high conformational freedom based on the weak electron density observed. Moreover, Glu318 is accessible (in the αI-low affinity conformation) to the MIDAS site in the βI domain. It is plausible that the lack of binding of Glu318 (or other putative internal ligand) to the βI MIDAS is due to the α2I domain being looked in a low affinity state by the bent conformation of the ectodomain; not a consequence of the low affinity state of the αI domain. Therefore, the statement "αx Glu-318 does not bind the β2I MIDAS, consistent with the finding that both the αx and β2 I domains are in the closed, low affinity conformations" might need to be reconsidered. In summary, the current model of αI-containing integrins should include all possible steps and modes of activation.

Minor comments:

3) The first sentence of the paragraph should indicate that the paper describes the structure of the "ectodomain".

4) The results and discussion section would be clearer for the general reader if it would be divided by subheadings.

5) The manuscript would benefit from a more detailed description of the crystallographic results in the "Results and discussion" section (page 4). For example, three crystal lattices are mentioned but they are not described in the main text (space group, cell dimensions, etc... are only presented in the supplementary files). This information is relevant to understand the crystallographic analysis (e.g. the lattices seem to be highly related). Along these lines, table S1 could be shown in the main text to help evaluate the quality of the crystallographic data.

6) The limits of the outermost resolution shells should be included in Table S1. In addition, the table would benefit from including: the number of unique reflections in the work and test sets, and the average multiplicity.

7) It seems that the resolution cutoff for the crystallographic data was done based on the I/sI (perfectly acceptable criteria). But given the high Rmerge values in the outermost resolution shell (not very unusual for weak data), the authors could include the Rwork and Rfree values for the outermost shell to support that those data contain valuable information.

8) Page 5, last paragraph. "Density for a metal ion is present at the αx MIDAS (Fig.2e and S4)". The quality of figure S4 is insufficient to asses the electron density around the metal. Moreover, the 2Fo-Fc map shown seems to have been calculated using phases obtained form a refined model that contains the metal. Given that the data extend only to "relatively low resolution" and that there is a reasonable risk of model bias, omit maps (preferably "simulated-annealing" or "kick" maps) should be shown. Finally, since fig 2e does not show the electron density I suggest showing such omit map(s) in fig 2e.

9) Figure S1. Panel S1a: because the conformation of helix α7 is a major determinant of the activation state of the αI domain, it would be very useful to label helix α7 in figure S1a. As previously mentioned, showing an omit map of the αI domain (after multi crystal averaging if necessary) would be much more convincing for the reader. Finally, I think that image quality is better in panel S1b than in S1a, it may be due to the use of a white background.

Referee #3 (Remarks to the Author):

Springer and co-workers report the x-ray structure of an integrin ectodomain containing an aI domain. The ms is a very valuable addition to the known structures of integrin ectodomains and has important implications for the understanding of integrin function. Nonetheless, certain issues remain:

a)´How may the artificially introduced C-terminal disulphide bridge influence the structure and/or dynamics of the integrin? It has not been discussed in the text how this restraint might lead to alterations in the tertiary structure.

b) How may the lacking membrane influence the structure/dynamics of the complex? Although it has been argued that a roughly perpendicular orientation of the calf-2 domains relative to the membrane might allow enough space for the aI domain to be positioned above the membrane (pg 7-8), it has not been elaborated if such a perpendicular orientation is in line with the recent structures solved by Ulmer and how the membrane might further influence tertiary structure and dynamics. Are there indications of membrane binding patches in the structure?

c) What is the expected role of glycosylation? Integrins in mammalian cells are heavily glycosylated. Is it possible that this glycosylation alters the structures and dynamics of the system, in particular given the highly flexible nature of the linkage of the aI domain to the remaining of the integrin ectodomain?

d) Given the points above and the poor resolution of the data, the strong statements about the deabolt model (abstract and page 11) are not well founded. Although it appears as if this structure is indeed in favour of an extension model, the low quality of the data as well as the nature of the construct used (lacking membrane, artifical disulphide bridge) argue for a more moderate discussion of the different activation models. In particular, the strong statement in the abstract must be removed.

1st Revision - Authors' Response 15 October 2009

Referee #1 (Remarks to the Author):

This manuscript reports the first crystal structure of a β_2 integrin and the first showing a βI domain in the context of a full-length structure. It is well written and illustrated and presents an interesting model for transmission of allostery between the αI and βI domains. Overall this paper is likely to be of significant interest and value to the community. The structure is of fairly low resolution and as such the authors must make clear to the readers the limitations that this places on their interpretations. The authors note that they confirmed the sequence register at the two junctions between the β-propeller domain and αI domain in several ways but only data for the 4 SeMet anomalous signals is shown. Please also show the density for the Cys-97-126 disulfide bond and Phe-328 sidechain and the SeMet-332 anomalous signal described in the text. Legends for the supplementary figures were missing from my version of the text.

Response. All of this information has been added as Fig. 1. Supplementary legends are in the supplementary text.

Referee #2 (Remarks to the Author):

The manuscript describes the first crystal structure of the ectodomain of an integrin ($\alpha_X\beta_2$) that contains an I-domain in the α subunit. The integrin is in a dormant (low affinity) state similar to that of $\alpha_{IIb}\beta_3$ (an integrin lacking an αI domain), yet the structure of $\alpha_x\beta_2$ reveals noticeable variations in the inter-domain arrangements that suggest specialization within the αI-containing subfamily of integrins. Analysis of the structure in the context of previous data on activating mutations, and activation-associated epitopes and antibodies supports that activation of $\alpha_x\beta_2$ involves extension of the integrin's legs and opening of the headpiece. On the other hand, the structural data does not support other models for activation (disulfide reduction and deadbolt release). A major finding is the lack of specific interactions between the α_x-I domain and the β-propeller and β_2-I domains. Finally, based on the structure of the low-affinity (bent) state, a revised model for the activation of integrins that contain an αI-domain is proposed. The data presented by Xie et al provide valuable novel information on the structure of an important subfamily of integrin receptors. The study makes a significant contribution to the understanding of integrin regulation.

Major comments:

1) This group has previously published an excellent paper (Nishida et al [2006] Immunity 25:583-594) showing a detailed EM analysis of $\alpha_x\beta_2$ in multiple states (bent, extended-closed, extended-open). The $\alpha_x\beta_2$ construct used in the current work has a slightly different C-terminal clasp, which includes an additional disulfide bridge that is maintained after TEV digestion (but can be broken by reduction). Nevertheless, it is not clear what does the current EM data add to that of the 2006's paper. It seems that the variability in the position of the αI domain could be deduced from the inspection of the published data.

Response. We did notice I domain flexibility previously, and in fact had thought the αI domain was not flexible, as emphasized by a quote from that article incorporated in the text. We are not sure of the reason that we now noticed the flexibility of the αI domain. Our image classification procedure has improved. Everyone says EM is not as mature as crystallography, and that is true. Dr. Nishida was using a script for k-means classification that was handed down from the Walz lab. Later, when my lab members got more active in designing and understanding these scripts, we discovered that the k-means seed particles were not randomly selected in each successive iteration, but were the same as in previous cycles. In the current manuscript, the k-means classification is implemented correctly. This could have contributed to our seeing I domain differences. It does not seem to make a large difference for our integrin particles, but it makes a huge difference for another protein we work on, EGFR. Another difference could just be better analysis. For example, the k-means classification does not align different classes one to another. We now do this as an extra last step, and it makes it much easier to see subtle differences (Fig. S2). In any event, I want to keep the EM, and have added a new paragraph on the effect of different clasps, and how the preparation we use in crystals can adopt all three major integrin conformational states.

2) The model for activation of the αI domain proposes that α_x Glu318 only binds to the activated βI MIDAS when the αI domain is in the intermediate or open states. The model implies that Glu318 does not bind to the βI MIDAS when the αI domain is in the low affinity conformation. It should be explained why this can be unequivocally ruled out. The results of this work do not exclude this possibility. In fact, Glu318 is part of the C-terminal linker between the αI domain and the propeller, which seems to have high conformational freedom based on the weak electron density observed. Moreover, Glu318 is accessible (in the αI-low affinity conformation) to the MIDAS site in the βI domain. It is plausible that the lack of binding of Glu318 (or other putative internal ligand) to the βI MIDAS is due to the αI domain being looked in a low affinity state by the bent conformation of the ectodomain; not a consequence of the low affinity state of the βI domain. Therefore, the statement "α_x Glu-318 does not bind the β_2I MIDAS, consistent with the finding that both the α_x and β_2I domains are in the closed, low affinity conformations" might need to be reconsidered. In summary, the current model of αI-containing integrins should include all possible steps and modes of activation.

Response. We have added an extra paragraph, which corresponds to a though that was omitted in the previous version. The gist is that the Glu-318 cannot move much relative to the αI domain because the preceding residue is in its hydrophobic core, and rigid body motion of the αI domain

could not enable Glu-318 to bind to the MIDAS without introducing clashes. So, some α-helix extension and unwinding has to occur.

Minor comments:

3) The first sentence of the paragraph should indicate that the paper describes the structure of the "ectodomain".

Response. Done.

4) The results and discussion section would be clearer for the general reader if it would be divided by subheadings.

Response. We have carried out this excellent suggestion.

5) The manuscript would benefit from a more detailed description of the crystallographic results in the "Results and discussion" section (page 4). For example, three crystal lattices are mentioned but they are not described in the main text (space group, cell dimensions, etc... are only presented in the supplementary files). This information is relevant to understand the crystallographic analysis (e.g. the lattices seem to be highly related). Along these lines, table S1 could be shown in the main text to help evaluate the quality of the crystallographic data.

Response. We have added all the requested information.

6) The limits of the outermost resolution shells should be included in Table S1. In addition, the table would benefit from including: the number of unique reflections in the work and test sets, and the average multiplicity.

Response. Done.

7) It seems that the resolution cutoff for the crystallographic data was done based on the I/sI (perfectly acceptable criteria). But given the high Rmerge values in the outermost resolution shell (not very unusual for weak data), the authors could include the Rwork and Rfree values for the outermost shell to support that those data contain valuable information.

Response. Done.

8) Page 5, last paragraph. "Density for a metal ion is present at the α_x MIDAS (Fig.2e and S4)". The quality of figure S4 is insufficient to assess the electron density around the metal. Moreover, the 2Fo-Fc map shown seems to have been calculated using phases obtained form a refined model that contains the metal. Given that the data extend only to "relatively low resolution" and that there is a reasonable risk of model bias, omit maps (preferably "simulated-annealing" or "kick" maps) should be shown. Finally, since fig 2e does not show the electron density I suggest showing such omit map(s) in fig 2e.

Response. We have qualified this statement in the revision, explained limitations on metal ions in the first section in Results and Discussion, and have omitted the panel.

9) Figure S1. Panel S1a: because the conformation of helix α_7 is a major determinant of the activation state of the αI domain, it would be very useful to label helix α_7 in figure S1a. As previously mentioned, showing an omit map of the αI domain (after multi crystal averaging if necessary) would be much more convincing for the reader. Finally, I think that image quality is better in panel S1b than in S1a, it may be due to the use of a white background.

Response. We have added another view to show the α-7 helix of the I domain in Fig. 1. All density is now with multi-crystal averaging, not 2Fo-Fc.

Referee #3 (Remarks to the Author):

Springer and co-workers report the x-ray structure of an integrin ectodomain containing an αI domain. The ms is a very valuable addition to the known structures of integrin ectodomains and has important implications for the understanding of integrin function. Nonetheless, certain issues remain:

a) How may the artificially introduced C-terminal disulphide bridge influence the structure and/or dynamics of the integrin? It has not been discussed in the text how this restraint might lead to alterations in the tertiary structure.

Response. We have now added a section on the ectodomain-transmembrane domain linkers that includes this information.

b) How may the lacking membrane influence the structure/dynamics of the complex? Although it has been argued that a roughly perpendicular orientation of the calf-2 domains relative to the membrane might allow enough space for the αI domain to be positioned above the membrane (pg 7-8), it has not been elaborated if such a perpendicular orientation is in line with the recent structures solved by Ulmer and how the membrane might further influence tertiary structure and dynamics. Are there indications of membrane binding patches in the structure?

Response. We have elaborated on all this in the same section. The inner face of the plasma membrane contains negatively charged phospholipids, and basic patches such as in talin can bind them. However, all external lipids are zwitterionic, and specific binding patches on ectodomains have not been identified.

c) What is the expected role of glycosylation? Integrins in mammalian cells are heavily glycosylated. Is it possible that this glycosylation alters the structures and dynamics of the system, in particular given the highly flexible nature of the linkage of the aI domain to the remaining of the integrin ectodomain?

Response. This is an interesting question, but I do not know the answer. The endoH-resistant glycans we have identified might interact with the αI domain and modulate that, but I hesitate to speculate. Also, even if there is an interaction, the mass of the glycan is so much smaller than that of the αI domain its effect on dynamics would be small.

d) Given the points above and the poor resolution of the data, the strong statements about the deadbolt model (abstract and page 11) are not well founded. Although it appears as if this structure is indeed in favour of an extension model, the low quality of the data as well as the nature of the construct used (lacking membrane, artifical disulphide bridge) argue for a more moderate discussion of the different activation models. In particular, the strong statement in the abstract must be removed.

Response. We have added panels with electron density showing strong support for the location of the β-tail domain and the location of the CD loop. I thank the referee for challenging us on this, as we have been able to provide more information that supports the points being made. We have removed the word "refute" from the abstract but not the text. Since scientists always test "hypotheses", it must sometimes be possible to "refute" them, although I do not believe we have ever done that more thoroughly than in this case, or ever used this word before.

Additional Editorial Correspondence 04 November 2009

Many thanks for submitting the revised version of your manuscript EMBOJ-2009-72275R. It has now been seen again by referee 2, whose comments are enclosed below. As you will see, he/she is happy with the revised version, and I am therefore pleased to be able to tell you that we will be able to accept your manuscript for publication in the EMBO Journal. However, the referee does point out a couple of errors in the referencing of the manuscript that first need to be corrected. I would therefore ask that you modify the text file accordingly and to send an updated version (as a .doc file) by e-mail - we can then upload this in place of the original file. Once we have received this, we should be able to formally accept the manuscript without further delay.

Many thanks,

Yours sincerely,
Editor
The EMBO Journal

Referee 2 comments:

The authors have satisfactorily addressed the comments and doubts that I had to the initial manuscript. I consider that the new information (e.g. more detailed crystallographic data) and descriptions added (e.g. effects of the C-terminal clasps and the role of Glu318) have improved the clarity of the manuscript both the people specialized in the integrin field and to the general readers.

Finally, I have observed a couple of minor typos. In the "Negative stain EM of αxβ2" (Materials and methods, page 23) the references at the end of the paragraph lack the publication year (these references are properly presented in the reference list, though).

