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Structure of a double ubiquitin-like domain in the talin head: a role in integrin activation.

Benjamin T. Goult, Mohammed Bouaouina, Paul R. Elliott, Neil Bate, Bipin Patel, Alexandre R. Gingras, Günter Grossman, Gordon C.K. Roberts, David A. Calderwood, David R. Critchley and Igor L. Barsukov

Corresponding author: Igor L. Barsukov, University of Liverpool

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1st Editorial Decision 28 October 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. Your manuscript has now been evaluated by three referees and I enclose their reports below. As you will see from their comments the majority of the referees are, in general, positive regarding the study and require some additional experimental analysis for the manuscript to be further considered for the EMBO Journal. These include further support for the helical transition within F1 and also more physiological phospholipids association assays. Should you be able to address these issues, we would be wiling to consider a revised manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments 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 your revision.

Yours sincerely,

Editor

The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors describe structural analysis of the previously uncharacterized N-terminal region of the tail head domain. They report individual NMR structures of the F0 and F1 domains, as well as a structure of the tandem domains. Structure/function analysis shows that a large loop within the F1 domain is important for integrin activation, but not via direct binding to integrin tails. Rather, the authors show that basic residues in this loop mediate binding to acidic phospholipids, and that the loop likely adopts a helical conformation. They propose that this membrane interaction is the basis for the contribution of the F1 region to integrin activation.

There is a tremendous amount of structural work presented here, all technically of high quality and integrated with insightful and informative functional studies. The manuscript is clearly written and illustrated, and is acceptable for publication in its current form.

One minor suggestion: Can the authors comment on whether the F0-F1 unit is likely to have a defined orientation with respect to the F2F3 lobes? If so, a schematic diagram or "cartoon" model of the entire head unit indicating interactions with the membrane and integrin tail would help provide an overall orientation/context for the reader.

Referee #2 (Remarks to the Author):

The manuscript entitled "Structure of a double ubiquitin-like domain in talin head: a role in integrin activation" by Goult et al employs NMR, phospholipid and integrin binding experiments as well as integrin activation assays, with the Talin head domain and isolated sub-domains from the Talin FERM domain, to elucidate the role of the F0 and F1 domains in talin-mediated integrin activation. It has previously been shown that the F3 domain of the Talin FERM domain binds to ß-integrin tails, and this interaction is believed to promote integrin activation by interfering with a salt bridge between integrin ß and ß tails that maintains the integrin in a low affinity state. However, other domains in the talin head, i.e., F0 and F1 sub-domains, are suggested to be involved in integrin activation. The focus of this study is to elucidate the role of the F0 and F1 FERM sub-domains in integrin activation.

As the F0 domain is not conserved in many proteins that contain a FERM domain, it is postulated that this additional sub-domain may prove important for Talin-specific function(s). The authors have solved the solution structures of the F0, F1, F0-F1 (minus insert) and F1 insert (in TFE) sub-domains by NMR, to generate molecular information that may aid in elucidating the role of the F0/F1 sub-domains. These structural efforts have revealed that both the F0 and F1 domains contain ubiquitin-like folds and interact in a fixed orientation due to multiple intermolecular interactions between the F0 and F1 domains. Although the F0 shows homology with the Ras binding Domain from RalGDS, binding to Ras and Rap proteins were found to be of low affinity. Thus, the role of this domain in Talin function remains elusive. As the F0 domain forms a fixed orientation with the F1 domain, perhaps the dual domain is required for recognition of Talin ligands, rather than the separate domains alone? The authors also find that the F1 domain contains an unstructured insert that is predisposed to helix formation and propose that helix formation induced by membrane interactions may promote interactions between basic residues with acidic phospholipids and aid in integrin activation. Although mutation of the insert affects acidic phospholipid binding and integrin activation, the authors fall short of establishing its role in phospholipid mediated integrin activation. Specific major and minor concerns are delineated below:

Major points
1) Although the F1 inert peptide adopts helical structure in the presence of TFE, this does not provide strong evidences that the insert becomes helical upon ligand (possibly membrane) binding. The authors may want to consider mutations that disrupt helix formation to further assess this

hypothesis.

It may also be helpful to determine whether membrane localization of Talin variants, that lack the insert and/or have mutations in basic residues, is altered.

2) A more physiological phospholipid association assay is required to establish that the insert may be involved in acidic phospholipid binding. In Figure 4D, the authors present COSY data showing the effect of POPS on the peptide resonances. The NMR studies are conducted with an unstructured peptide corresponding to the F1 insert. As this peptide contains several basic residues, the broadening observed may reflect non-specific electrostatic interactions between basic residues of the peptide and the acidic phospholipid headgroups. Moreover, a COSY spectrum of the F1 loop peptide with POPC containing SUVs is required to exclude possible line broadening due to "background" lipids such as POPC. Overlaying HSQC spectra of F1 domain, F1 with POPC SUVs, and F1 with 1:4 POPS:POPC will aid in assessing whether the arginine residues (146, 149, 153, and 156) are perturbed upon associating with POPS. As PIP2 has been implicated in talin-mediated integrin activation, it is unclear why the NMR studies were conducted with PS rather than PIP2. While additional experiments are conducted with phosphatidyinositol phosphate strips, ligand interactions with these phospholipid strips does always correlate with physiologically relevant phospholipid binding and specificity. Rather, the authors should consider liposome co-sedimentation assays, with liposomes containing lipids with composition and concentration that reflect those in the membrane (i.e., PE, PC and either PS or PI, with PIP2 replacing PS at the concentration employed). The phospholipid co-sedimentation studies should be conducted on the talin head domain and compared with data obtained with the isolated F1 domain (with and without the insert and/or mutations in basic residues) for comparison.

Minor points
1) The first paragraph of the results is actually not results and belongs in the background.
2) In the NMR structure calculation section descrinbed in Methods, the authors note that "initial models" are used in Aria, but it is not revealed what these are.
3) A minor point is that Table 1, 2, and 3 are not part of the supplementary data, but in the main body of manuscript.
4) Figure S7 a) RDC correlation, add the unit (Hz) to both axes
5) In Figure S10, an overlay of a) and b) will help compare the chemical shift perturbation for F1 resonances in the presence of POPS.

Referee #3 (Remarks to the Author):

The manuscript fills another gap in the talin structures by reporting the NMR structure of the F0 and F1 domains of the talin head. The results further the understanding of signalling events involving talin and integrin. The authors report that secondary effects stemming from interactions of the talin F0F1 domains with the membrane influence integrin mediated signalling. This is a very important result that merits publication. The manuscript is clearly written and understandable for a broad audience ranging from structural biologists to cell biologists. The experiments were conducted skillfully. The main conclusions are well founded by experiment. In conclusion, I strongly recommend publication

1st Revision - authors' response 10 December 2009

Reviewer #1.

Minor suggestion: Can the authors comment on whether the F0-F1 unit is likely to have a defined orientation with respect to the F2F3 lobes?
We are currently analysing the structure of the full talin head containing all domains F0-F3 by a range of biophysical methods. Small angle X-ray scattering (SAXS) shows that in the talin head the

individual domains are arranged in an open configuration that is distinctly different from a compact clover-leaf-like structure of the reported FERM domains of other proteins. While the SAXS data are insufficient for an accurate modelling of the talin head structure, they clearly demonstrate that the homology modelling will be unreliable. For these reason we cannot make reliable comments on the relative orientation of the F0F1 and F2F3 units until more experimental data become available.

Reviewer #2.

Major points

1). The helical structure in the presence of TFE does not provide strong evidence that the insert becomes helical upon ligand (possibly membrane) binding. Helix disruption mutants and membrane localization experiments may be helpful.

We fully agree that the high helical propensity, as demonstrated by the TFE experiments, does not prove that the helix is formed in the bound state. However, indirect support for this comes from the positive charge clustering on one face of the helix that is optimal for the binding to negatively charged membrane surfaces. In agreement with this the F1 loop region interacts specifically with negatively charged but not neutral membranes. In follow up experiments, we have analysed the interaction between the F1 loop peptide and negatively charged membranes by CD spectroscopy. We observed a striking change of the peptide CD spectra on additions of SUVs that corresponds to a large increase in the proportion of the helical structure in the F1 loop peptide. This provides direct evidence that the membrane interaction in the loop region is mediated through a stabilisation of the helical structure. The CD data are presented in Figure 4E and analysed in the text (page 15).

Following the referee's suggestion, we designed a set of mutants that destabilise the transient helix of the F1 loop. For this we used two types of mutation ñ residue substitutions with Gly or Ser that reduce helical propensity, and introduction of Pro that prevents helix formation through sterical clashes. For the first type of mutants we observed small reduction of the membrane binding affinity that can be detected by NMR (Supplementary Figure S10A, C), while for the Pro mutations the effect was much more pronounced and was detected both by NMR and pull-down experiments (Supplementary Figure S10B, D and E). This demonstrates the importance of helix formation in the loop region within the full F1 domain, connecting the data for the isolated peptide with the behaviour of the complete domain. In addition, the membrane binding effect was observed only on a small localised region of the loop for the Pro mutants (Supplementary Figure S10F), demonstrating that the disruption of the helix traps the complex at the intermediate stage ii of the model shown in Figure 5C. The full analysis of the data is presented in text (pages 16, 17 and 20).

2) More physiological phospholipid association assay is required to establish that the insert may be involved in acidic phospholipid binding. The phospholipid co-sedimentation studies should be conducted on the talin head domain and compared with data obtained with the isolated F1 domain (with and without the insert and/or mutations in basic residues) for comparison.

To complement our original NMR data we performed lipid co-sedimentation assays on the wild type F1 and its mutants (Figure 5A, Supplementary Figure S10E). These data fully agree with the NMR experiments and demonstrate that the loop region is essential for the F1 interaction with negatively charged membranes. No interaction was detected with non-charged membranes. The F1 interaction is specific and selective for the negatively charged membrane surface, but not for specific lipid charged groups, as we observed similar effects for POPS and PIP2-containing membranes. In the context of cell adhesion, PIP2 is expected to be the most relevant lipid since it is thought to be enriched at adhesion sites. The data are analysed in pages 16 and 17.

In addition, we conducted membrane co-sedimentation assays with the full talin head. In this case we observed a higher affinity interaction in agreement with reported additional acidic phospholipid binding sites in the F2 and F3 domains. These additional sites masked the effect of the F1 loop region, although we could detect a small reduction in the binding upon loop deletion. Because of this, we did not use the full talin head for subsequent studies and in any event, the current report is focussed on the properties of the F0F1 polypeptide.

As PIP2 has been implicated in talin-mediated integrin activation, it is unclear why the NMR studies

were conducted with PS rather than PIP2. The authors should consider liposome co-sedimentation assays, with liposomes containing lipids with composition and concentration that reflect those in the membrane (i.e., PE, PC and either PS or PI, with PIP2 replacing PS at the concentration employed).

The PS and PIP2 lipids have been used interchangeably to study membrane interactions that depend on charge rather than the nature of the lipid head group. The reported comparison between these types of lipids showed that lower concentration of PIP2 than PS was required to achieve similar binding. Talin head, in contrast to some other FERM domain, does not contain a PIP2-specific binding sites. Rather, the protein surface includes a number of positively charged clusters that are important for the association with negatively charged membrane surfaces. The F1 loop region is highly dynamic and can only form a transient helix - we speculate that this is sufficient for the selection of the negatively charged surface, but not a specific lipid head group. For these reasons we originally restricted the binding studies to the 1:4 PS:PC mixtures that have been used with other FERM domains. In response to the comments we extended the experiment to PIP2-containing vesicles (Figures 4D, 5A and S10). Our additional data show that the F1 interaction with 1:4 PS:PC and 1:19 PIP2:PC vesicles is similar, as reported for other FERM domains. Both NMR and co-sedimentation assays demonstrate that for talin, the association with the membrane is controlled through the membrane surface charge, with a lower proportion of PIP2 than PS required for a similar effect, reflecting the charge density rather than head group specificity. Considering the similarity in the effect, the higher chemical stability of PS in comparison to PIP2 offers more stable experimental conditions. In the revised version, we present both PS and PIP2 data, described in pages 15-17.

COSY spectra of the peptide may reflect non-specific membrane association. Control experiments using PC vesicles are required.

We performed the control experiment using POPC vesicles in the original manuscript. This experiment was mentioned in the text, although the spectrum was not shown as it is virtually identical to the spectrum of the free peptide. We added the COSY spectrum in the presence of PC to Figure 4D. The lack of the interaction with the non-charged membranes shows that the peptide interaction is specific to negatively charged membrane surfaces.

HSQC spectra are better presented as overlays to assess whether the arginine residues (146, 149, 153, and 156) are perturbed upon associating with POPS.

Following this suggestion, we present the HSQC spectra as overlays for a better comparison. The assessment of the involvement of specific residues from the spectra comparison is impossible as residual signal in the spectra correspond to the strongest signals of a low residual fraction of unbound F1. The broadening of signals from the bound form is too severe to be detected, and is agreement with the "fly-cast" model that has F1 tightly associated with the membrane in the fully bound state. However, the helix destabilising Pro mutations trap the complex in the intermediate state of binding. For such mutants the binding selectively perturbs resonances of the positively charged 146R, 156K, 157K, 164K and residues immediately adjacent to them. This directly demonstrates the involvement of these positively charged residues with the negatively charged lipid head groups. The additional data are analysed in pages 16 and 17.

Minor points

1) The first paragraph of the results is actually not results and belongs in the background.

The first paragraph describes our analysis of the atypical features of the talin head sequence, highlighting the similarity between the F0 and F1 regions and the lack of the fold recognition in the database annotation for the F0 domain. This analysis was performed as part of the reported study and contributes to the results. For these reasons we have included the description in the Results section.

2) In the NMR structure calculation section described in Methods, the authors note that "initial models" are used in Aria, but it is not revealed what these are.

Initial models were generated with CYANA using the CANDID method for NOESY cross-peak assignment and calibration. This is described as part of the structure calculation method in Supplementary page 3.

3) Table 1, 2, and 3 are not part of the supplementary data, but in the main body of manuscript.

In the revised manuscript the tables are relocated to the supplementary data.

4) Figure S7 a) RDC correlation, add the unit (Hz) to both

Corrected in the revised version.

5) In Figure S10, an overlay of a) and b) will help compare the chemical shift perturbation for F1 resonances in the presence of POPS.

The HSQC spectra in the presence of PS and PIP2 (Supplementary Figures S9 and S10 in the revised manuscript) are now presented as overlays for better comparison. The HSQC spectrum in the presence of pure PC (Supplementary Figures S9) is shown as an individual spectrum because the similarity with the spectra of the free F1 leads to nearly complete overlap for the majority of the cross-peaks, and obscures the comparison.

In summary, in response to the referee's suggestions, we have conducted a range of new experiments that further clarify and extend the original results. The new data are integrated into the revised manuscript as detailed above and the requested modifications of the text and figures are incorporated. We trust that these have fully addressed the referee's points and the revised manuscript is now acceptable for publication in EMBO Journal.

2nd Editorial Decision 11 January 2010

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

I have reviewed the revised manuscript and the authors' responses to the referees. I was quite positive about the manuscript in its earlier form, and I remain so now. The authors have very conscientiously addressed the concerns of reviewer 2, the manuscript is suitable for publication in its present form.

