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Key residues on microtubule responsible for activation of kinesin ATPase

Seiichi Uchimura , Yusuke Oguchi, You Hachikubo, Shin'ichi Ishiwata and Etsuko Muto

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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 15 July 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. I have now had a chance to read it carefully and to discuss it with my colleagues and I am sorry to say that we cannot offer to publish it.

We appreciate that, following on from your 2006 paper in which you identified and characterised a number of mutants in beta-tubulin that affect kinesin binding and motility, you have extended this study and identified residues in alpha-tubulin that are also required for the kinesin interaction. Specifically, you identify a number of acidic residues in the H11-12 loop of alpha-tubulin that appear to be important for the initial, weak, interaction between ADP-bound kinesin and MTs. Mutating these residues also affects the ATP-hydrolysis rate of kinesin, suggesting a role for this binding site in stimulating ADP release upon binding. The residues in beta-tubulin, conversely, seem to be primarily involved in the strong binding of the nucleotide free or ATP-bound kinesin, as you previously showed. You therefore propose a two-step model for kinesin binding, whereby interactions with the alpha subunit mediate the initial interaction, which is further strengthened upon ADP release by interactions with the beta subunit. While we do appreciate your study identifies novel mutations that disrupt the interaction, the concept of two-step binding, regulated by ADP release, is not new. Moreover, the interaction interface between MTs and kinesin has been mapped by EM studies, where the H11-12 loop of alpha-tubulin was shown to be in close contact with kinesin. Therefore, while we do recognise that your work will be of interest to the immediate field, I am afraid we do not feel that the manuscript provides the kind of conceptual advance required for us to consider it further for publication in the EMBO Journal.

Please note that we publish only a small percentage of the many manuscripts that we receive at the EMBO Journal, and that the editors have been instructed to subject only those manuscripts to external review which are likely to receive enthusiastic responses from our reviewers and readers. I am sorry to have to disappoint you on this occasion, but I hope that this negative decision will not prevent you from considering the EMBO Journal for publication of future studies.

Yours sincerely,

Editor
The EMBO Journal

Appeal Letter 17 July 2009

Thank you for your e-mail, informing us of the editorial board's decision on our manuscript entitled 'Key residues on microtubule responsible for activation of kinesin ATPase" (MS# EMBOJ-2009-71865-T). We feel compelled to disagree with the reviewing editor's comment that the editorial board does not appreciate the significant conceptual advances in our work. Let us clarify the potential misunderstandings below.

(1) The first criticism: The concept of two-step binding is not new.
It is not our intention to claim "nucleotide-dependent two-step reaction scheme" as our original finding. The authors are concerned that the reviewing editor may have missed the crucial discovery of this study. The key finding in our study is that in this two-step reaction, only the first step is coupled with the reaction of ATPase, and the residue E415 in H11-12 loop of -tubulin is essential for this specific binding interaction. The original "two-step reaction scheme" is based on the results of the kinetic studies. Its structural basis, at the molecular level, is uncovered for the first time by analyzing the structure-function relationship of kinesin-microtubule interaction.
How the microtubule binding induces ADP release from kinesin has been a key question in understanding the mechanism of motility, because ADP release is the rate-limiting step in a cycle of ATP hydrolysis, critical for the stepping motion. Nevertheless, the question remained unresolved for a long time, because of the technical difficulties in tubulin molecular biology. As explained below, EM studies cannot provide definitive information. By clarifying the structural basis for microtubule-dependent ADP release, we believe that the contribution of our work is significant and that the insight provided will be of interest to those studying the mechanism of allosteric activation.
(2) The second criticism: EM studies have shown that the H11-12 loop of α-tubulin is in proximity to kinesin.
The EM studies can only "suggest" the potential interface for kinesin. As explained in our Introduction and Discussion, H11-12 loop is not the only site located in proximity to kinesin and therefore, without mutational analysis of tubulin, it is impossible to identify which site of tubulin molecule is "actually" involved in the interaction with kinesin. As explained in the cover letter, none of the previous works have succeeded in identifying which microtubule site is responsible for the interaction with kinesin and for ATPase activation.
In fact, based on the crystal structure of kinesin and the cryo-EM map of kinesin-microtubule complex, Hirokawa's group has suggested that kinesin binding to H4 of β-tubulin, but not H11-12 loop of α-tubulin, triggers ADP ejection from nucleotide pocket (Nat. Struct. Mol. Biol. 15, 1067-1075). However, our experiment using the mutated microtubules with charged-to-alanine mutation at H4 did not support their hypothesis (see p. 12 in the manuscript). The model based on the EM studies is led, in part, by speculation, and thus cannot be conclusive without mutational analysis of tubulin.
If our description on the background of the work (in Introduction) and the relevance to the structural studies (in Discussion) misled the editor into believing that our work only confirmed the fact which had been regarded obvious from the EM studies, we are willing to rewrite these parts so that the readers can understand this work uncovered the structure-function relationship of kinesin-microtubule interaction for the first time, using a novel technique of tubulin molecular biology.

We understand that there is a great demand to publish in EMBO J, and that a strict review process is thus essential. However, we feel that your review of our submission may have missed the crucial finding of the paper, and the relevance of this finding to the past studies in the field. We respectfully

request that you kindly reassess the work, taking the above appeal into consideration.

I thank you in advance for reviewing this request, and remain available to answer any questions you may have.

Yours sincerely,
Etsuko Muto

Response to Appeal 28 July 2009

I am sorry for the delay in responding to your appeal on this manuscript. As I explained, I wanted to get advice from one of our external advisors on your manuscript, and this took some time. Following the discussions with this advisor, we are willing to send your manuscript out for in-depth review. I should stress, however, that our willingness to reconsider at this stage is obviously no guarantee for the outcome of the review process.

In your appeal letter, you mentioned that you could re-write parts of the manuscript to stress the novel findings of your study. Both we and the external advisor think this would be useful. Therefore, I would ask you to revise your manuscript accordingly. If you only need to make changes to the main body of the text, you can just e-mail a revised word document, which we will upload in place of the original.

However, if you also wish to make changes to the figures, please let me know so that we can allow you to do this through the online system.

Many thanks for your patience, and I look forward to receiving your modified manuscript.

Yours sincerely,

Editor
The EMBO Journal

Author Correspondence 30 July 2009

Thank you for your careful reassessment of our paper. I am happy to know that you have decided to send our manuscript to the peer review.

With regards to the revision of the manuscript, we will make changes only in the text. I will send the modified manuscript as soon as possible (by early next week at the latest).

Thank you again for your time and effort on this matter.

Sincerely,
Etsuko Muto

2nd Editorial Decision 28 August 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 appreciate the high quality of your study, but vary somewhat in their overall assessment. While referees 1 and 3 are broadly supportive of publication, referee 2 feels that the manuscript would be more appropriate for the specialised literature.

Given the majority opinion, we are prepared to overlook this negative recommendation, and to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I would in particular like to draw your attention to the comments of referee 3 regarding the need to provide better evidence concerning the effects of the various tubulin mutants on ADP binding and release. 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.

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 is an important piece of work for the kinesin field, establishing exactly which tubulin residues are important for interaction with kinesin. It is particularly useful to have shown which residues are most involved in the strongly-binding versus the weakly-binding stages in the cycle. The authors correctly conclude that the work provides a basis for a better understanding the two main stages of the binding interaction.
My only question is why what seems to me to be an important part of the discussion has been relegated to the Supplementary file? Also, R380 seems quite likely to be directly important for the interaction with kinesin rather than affecting tubulin structure sigificantly. It might, for example, help to put the neck linker in its correct position during forwards movement. If it acts as a repellent rather than an attractant (unlike most of the vital residues), this might explain the anomaly?

Referee #2 (Remarks to the Author):

The manuscript of Uchimura et al describes the in vitro characterisation of point mutations in yeast tubulin and their effects on the kinesin-1 binding and mechanochemistry. This work extends a study published by the same group (Uchimura et al (2006)) in which engineered S. cerevisae was used to express and purify single tubulin isoform dimers and mutants thereof. This is particularly useful because of tubulin's notorious resistance to bacterial recombinant expression and the difficulty of isolating single isoform tubulin from other sources. A total of 36 charged residues in the C-terminal region of both a-and b-tubulin were individually mutated to alanine and those which caused slow growth or lethality in vivo (12 of the 36) were examined biochemically in this study. Several of the b-tubulin mutants were characterised in the previous work (under slightly different experimental conditions) and are included here for comparison and to emphasise the different roles for a- and b-tubulin in the kinesin interaction. The authors therefore have a unique and extensive set of experimental tools to probe the role of individual tubulin residues in the kinesin mechanism, an important question for molecular motor research. The manuscript is clearly written, the thorough experiments are carefully described, the data support their principal conclusions and are largely consistent with other reports. [For example, observation of shift in kinesin (albeit kinesin-13) on the tubulin dimer on ADP release see: Regulation of KinI kinesin ATPase activity by binding to the microtubule lattice. Moores CA, et al J Cell Biol. 2003 Dec 8;163(5):963-71; the role of a-tubulin E415 was also very recently reported elsewhere: Glu415 in the a-tubulins plays a key role in stabilizing the microtubule-ADP-kinesin complexes. Gaspar I, Szabad J. J Cell Sci. 2009 Aug 15;122(Pt 16):2857-65. Epub 2009 Jul 21.] However, while throwing light on some of the details of the molecular mechanism of the kinesin-tubulin interaction, this study might be more suited to a more specialised audience.

Detailed suggestions for clarification and further insight:

1) Much of the technical detail about generation of the tubulin mutants has been omitted from the current manuscript, with reference made instead to Uchimura et al (2006). However, it would be helpful if at least some of these details (particularly how lethal mutant tubulins are separated from the co-expressed non-lethal tubulins required to maintain cell viability) were restored to the Supplementary Materials of this paper. Interpretation in the current manuscript depends on confidence that the data presented arise from the introduced mutations and readily accessible experimental details are essential for this. In particular, the use of the Tub2tax mutant as the "wild type" makes sense but should be stated explicitly.

2) The consensus in the field is that kinesin motor domains bind tubulin dimers as depicted in Figure 5, i.e. centred above the intra-dimer interface, but it would be interesting to know how the authors would interpret their data with respect to the other formal possibility, i.e. that kinesin binds on the inter-dimer interface.

3) Tubulin is the nucleotide exchange factor for kinesin. Can any structural parallels be drawn between the mechanisms of classic GEFs for G-proteins and the likely mechanism by which tubulin induces nucleotide release from kinesin-1?

4) All the experiments described are performed on kinesin-1 (aka conventional kinesin or Kinesin Heavy Chain). Throughout the paper, the more generic term "kinesin" is used but it would be more precise to note that tubulin mutants could influence different aspects of the kinesin-tubulin interaction in other kinesin families [see for example: The depolymerizing kinesin MCAK uses lattice diffusion to rapidly target microtubule ends. Helenius J, et al Nature. 2006 May 4;441(7089):115-9; Docking and rolling, a model of how the mitotic motor Eg5 works. Rosenfeld SS, et al J Biol Chem. 2005 Oct 21;280(42):35684-95.

5) Recently, a number of point mutations in human a-tubulin, including in the C-terminal region, have been linked to brain developmental disorders. Although the correlation between in vivo phenotype and the detailed in vitro work described in the current manuscript is speculative, it should be mentioned. [Mutations in alpha-tubulin cause abnormal neuronal migration in mice and lissencephaly in humans. Keays DA, et al Cell. (2007) Jan 12;128(1):45-57. Large spectrum of lissencephaly and pachygyria phenotypes resulting from de novo missense mutations in tubulin alpha 1A (TUBA1A). Poirier K, et al Hum Mutat. (2007) Nov;28(11):1055-64.]

6) The discussion contained in the Supplementary Discussion should be in the body of the text accompanying the relevant data.

7) There is a slight discrepancy between the number of tubulin mutants generated in the study and those high-lighted in Figure 1a; 36 mutants are mentioned in the Legend to Figure 1 and Supplementary Table 1 but 38 residues are coloured in Figure 1a.

Referee #3 (Remarks to the Author):

This manuscript by Uchimura et al is a follow up of previous report from the same group (Uchimura et al EMBOJ 2006); based on the cryo-EM models the authors extended the charge-null mutations to both alpha- and beta-tubulin. Using biophysical and biochemical studies, the authors identified a cluster of charged residues in both alpha- and beta-tubulin responsible for interaction with the microtubule at different nucleotide states.

Overall this study presents a model in which the ADP release of kinesin takes place upon initial binding to microtubule at the alpha-tubulin site. In the nucleotide free and ATP bound state the kinesin-MT interaction is mainly via beta-tubulin. To this reviewer, the manuscript seems publishable when the authors address the following concerns appropriately.

1. Although most of the derived model majorly fits the experimental results, the role of alpha-E415 is still not clearly explained and in many places it is ambiguous, for example to quote a sentence from their abstract;

"Mutation in the α-tubulin binding site results in a deceleration of ATP hydrolysis (kcat)...." and in the discussion the authors go on to claim " The α-E415 residue might be a key residue indispensable to the initial recognition of the MT by kinesin, which triggers the cascade of structural changes leading to ADP release..."

Since alpha-E415 is the only catalytically critical residue identified, it needs to be clarified whether the lower kcat value is due to its direct involvement in ATP hydrolysis or its inability to release ADP. It would be preferable if the authors could measure ADP release data for at least this mutant. Alternatively the authors should clearly suggest their view on this residue unambiguously.

2. Apart from the alpha-E415A mutant, the rest of the mutants show a weak affinity for microtubules. Based on the unbinding studies of the mutants, the authors suggest (in the abstract) that Kinesin-ADP mainly interact with alpha-tubulin, whereas the nucleotide free and ATP bound kinesin occurs both alpha- and beta-tubulin. However, the unbinding measurements with ADP show only minor differences between alpha and beta-tubulin mutants, so this claim is not strongly supported.

To add extra evidence, the authors should show that indeed only the alpha-tubulin mutants are affected in ADP state using regular microtubule binding assays (spin down assays). This assay will make their claim much stronger.

3. The authors should report more experimental details. For example, there is little information regarding the yield amounts from yeast tubulin preparations.

1st Revision - Authors' response 25 January 2010

Response to Referee #1

Thank you for reviewing the manuscript entitled “Key residues on microtubule responsible for activation of kinesin ATPase” (EMBOJ-2009-71865R-A). Following the comments of three reviewers, in the revised manuscript, changes have been made to the main text and the Supplementary Information. Figure 5 and Table 2 have been added. We appreciate the comments of the reviewers, as they have helped us to improve our paper.

Reviewer’s comments:
My only question is why what seems to me to be an important part of the discussion has been relegated to the Supplementary file? Also, R380 seems quite likely to be directly important for the interaction with kinesin rather than affecting tubulin structure significantly. It might, for example, help to put the neck linker in its correct position during forwards movement. If it acts as a repellent rather than an attractant (unlike most of the vital residues), this might explain the anomaly?

Authors’ response:
The authors understand the reviewer’s intention that ‘Interpretation of motility and ATPase data’ is important and, therefore, should be included in the main text. However, upon reconsideration, it has become clear to us that comparing MT-activated ATPase and the results of the single molecule motility assay does not bring us any significant insight into the mechanism of mechano-chemical coupling. Since the single-molecule motility of kinesin becomes very poor by mutation, any effort to relate the influence of mutation on the motility of kinesin to that on ATPase activity is not rewarding. Because of this, we have abandoned our previous Discussion which has been placed in the Supplementary Information. In the revised version of the manuscript, we simply compare ATPase activity and MT velocity in the gliding assay (p.8, line 8- and p.11, line 29-).

With regards to the discussion headed ‘Interpretation of the unbinding force in the β-R380A mutant’, the authors could not concur with the reviewer’s hypothesis that the possible role of β-R380 is to guide the kinesin neck linker into proper position. If this were actually the case, we expect that a charged-to-alanine mutation at this site would have made kinesin less processive or

slow (because of the shaky guidance). However, our results showed the mutation affects neither the run length nor the velocity of single kinesin movement (Figure 2). In addition, in the structure of the kinesin-MT complex (PDB-2HXF), this residue is positioned more than 3 nm away from the position of the neck linker. When viewed from kinesin, it is located behind the shield of highly negatively-charged H12. Given this information, it is hard to imagine that electrostatic repulsion between β-R380 and the neck linker has a substantial effect.

In the end, we deleted this part of the discussion previously contained in the Supplementary Discussion section. The greater part of this discussion is already included in the main text (p.7, line 16-20) and a more detailed discussion would digress from the main point.

Response to Referee #2

Thank you for reviewing the manuscript entitled “Key residues on microtubule responsible for activation of kinesin ATPase” (EMBOJ-2009-71865R-A). Following the comments of three reviewers, in the revised manuscript, changes have been made to the main text and the Supplementary Information. Figure 5 and Table 2 have been added. We appreciate the comments of the reviewers, as they have helped us to improve our paper.

Reviewer’s comments:
1) Much of the technical detail about generation of the tubulin mutants has been omitted from the current manuscript, with reference made instead to Uchimura et al (2006). However, it would be helpful if at least some of these details (particularly how lethal mutant tubulins are separated from the co-expressed non-lethal tubulins required to maintain cell viability) were restored to the Supplementary Materials of this paper. Interpretation in the current manuscript depends on confidence that the data presented arise from the introduced mutations and readily accessible experimental details are essential for this. In particular, the use of the Tub2tax mutant as the "wild type" makes sense but should be stated explicitly.

Authors’ response:
Following the reviewer’s suggestion, the methods for generation of tubulin mutants (including the construction of the *TUB2*^{tax} mutant) and for the purification of tubulin have been included in the Supplementary Materials. The use of the *TUB2*^{tax} mutant as the "wild type" is stated in both Materials and Methods and the Methods section in Supplementary Information.

Reviewer’s comments:
2) The consensus in the field is that kinesin motor domains bind tubulin dimers as depicted in Figure 5, i.e. centred above the intra-dimer interface, but it would be interesting to know how the authors would interpret their data with respect to the other formal possibility, i.e. that kinesin binds on the inter-dimer interface.

Authors’ response:
If we attempt to dock the kinesin motor domain on the inter-dimer interface, we have to exchange the assignment of α- and β-tubulin in the cryo-EM map. In such a situation, the critical residues α-E415 and β-D417 will be remote from the kinesin interface, thus the model is inconsistent with our observation that the mutation in these residues disrupts kinesin motility. On the other hand, docking the kinesin motor domain on tubulin intra-dimers will allow the major structural elements on the kinesin interface (L11/α4/α6, L12/α5, L8) to be situated in close proximity to the critical residues on tubulin, as described in the Discussion (p. 12-13). Overall, the inter-dimer model is incompatible with our results and the majority of the results reported in the existing literature. We did not mention this argument in the manuscript, since kinesin binding on the intra-dimer interface is well established.

Reviewer’s comments:
3) Tubulin is the nucleotide exchange factor for kinesin. Can any structural parallels be drawn between the mechanisms of classic GEFs for G-proteins and the likely mechanism by which tubulin induces nucleotide release from kinesin-1?

Authors' response:
This is an important point to note. We addressed the reviewer's question in the Discussion (p.13, first paragraph).

Reviewer's comments:
4) All the experiments described are performed on kinesin-1 (aka conventional kinesin or Kinesin Heavy Chain). Throughout the paper, the more generic term "kinesin" is used but it would be more precise to note that tubulin mutants could influence different aspects of the kinesin-tubulin interaction in other kinesin families [see for example: The depolymerizing kinesin MCAK uses lattice diffusion to rapidly target microtubule ends. Helenius J, et al Nature. 2006 May 4;441(7089):115-9; Docking and rolling, a model of how the mitotic motor Eg5 works. Rosenfeld SS, et al J Biol Chem. 2005 Oct 21;280(42):35684-95.

Authors' response:
We agree with the reviewer's opinion that we have to take the variable aspects of the kinesin-tubulin interaction in other kinesin families into consideration. This point has been noted in the Discussion (p.13, last paragraph).

Reviewer's comments:
5) Recently, a number of point mutations in human a-tubulin, including in the C-terminal region, have been linked to brain developmental disorders. Although the correlation between in vivo phenotype and the detailed in vitro work described in the current manuscript is speculative, it should be mentioned. [Mutations in alpha-tubulin cause abnormal neuronal migration in mice and lissencephaly in humans. Keays DA, et al Cell. (2007) Jan 12;128(1):45-57. Large spectrum of lissencephaly and pachygyria phenotypes resulting from de novo missense mutations in tubulin alpha 1A (TUBA1A). Poirier K, et al Hum Mutat. (2007) Nov;28(11):1055-64.]

Authors' response:
These papers have been cited in the Introduction (p. 4, line 14). Because the correlation between the observed in vivo phenotype and kinesin motility in these mutants is not known, we have only suggested, as a general hypothesis, the possible involvement of the α-tubulin in the interaction with kinesin.

Reviewer's comments:
6) The discussion contained in the Supplementary Discussion should be in the body of the text accompanying the relevant data.

Authors' response:
With regards to the section dealing with the 'Interpretation of motility and ATPase data', after extensive consideration, we decided to abandon our previous Discussion which has been placed in the Supplementary Information. Since the single-molecule motility of kinesin becomes very poor by mutation, comparing MT-activated ATPase and the results of the single molecule motility assay does not bring us any significant insight into the mechanism of mechano-chemical coupling. In the revised version of the manuscript, we simply compare ATPase activity with MT velocity in the gliding assay (p.8, line 8- and p.11, line 29-).

The discussion headed 'Interpretation of the unbinding force in the β-R380A mutant' has been deleted from the revised version of the manuscript. The greater part of the discussion on this residue is already included in the main text (p.7, line 16-20) and a more detailed discussion would digress from the main point.

Reviewer's comments:
7) There is a slight discrepancy between the number of tubulin mutants generated in the study and those high-lighted in Figure 1a; 36 mutants are mentioned in the Legend to Figure 1 and

Supplementary Table 1 but 38 residues are coloured in Figure 1a.

Authors' response:
The charged residues α-D439 and β-E427, previously colored in Fig. 1a, were not subjected to charged-to-alanine mutation, because these two residues are located in the area of the MT outside H12. In the revised version, these residues are represented by a black font.

Response to Referee #3

Thank you for reviewing the manuscript entitled "Key residues on microtubule responsible for activation of kinesin ATPase" (EMBOJ-2009-71865R-A). Following the comments of three reviewers, in the revised manuscript, changes have been made to the main text and the Supplementary Information. Figure 5 and Table 2 have been added. We appreciate the comments of the reviewers, as they have helped us to improve our paper.

Reviewer's comments:
1.Although most of the derived model majorly fits the experimental results, the role of alpha-E415 is still not clearly explained and in many places it is ambiguous, for example to quote a sentence from their abstract;

"Mutation in the α-tubulin binding site results in a deceleration of ATP hydrolysis (k_{cat})...." and in the discussion the authors go on to claim "The α-E415 residue might be a key residue indispensable to the initial recognition of the MT by kinesin, which triggers the cascade of structural changes leading to ADP release..."

Since alpha-E415 is the only catalytically critical residue identified, it needs to be clarified whether the lower k_{cat} value is due to its direct involvement in ATP hydrolysis or its inability to release ADP. It would be preferable if the authors could measure ADP release data for at least this mutant. Alternatively the authors should clearly suggest their view on this residue unambiguously.

Authors' response:
Stopped-flow assay of mant-ADP release revealed that ADP release is actually slowed down in the α-E415A mutant (Fig. 5). Based on this result, we revised the model in the Discussion. The interpretation for the results of the kinetic analysis, and our view on the role of α-E415, are described in the Results (p.8-9) and Discussion (p. 10-11) sections, respectively. To understand the entire picture with regard to the mechanism of MT-dependent ATPase activation, it is preferable to measure the ADP release rate for all the mutants critical for kinesin motility. However, considering the labor involved in stopped-flow measurement (60 L of yeast culture is required to purify a total of 5 mg of MTs), measurement of only the α-E415A mutant and the wild type was the best we could do.

Reviewer's comments:
2. Apart from the alpha-E415A mutant, the rest of the mutants show a weak affinity for microtubules. Based on the unbinding studies of the mutants, the authors suggest (in the abstract) that Kinesin-ADP mainly interact with alpha-tubulin, whereas the nucleotide free and ATP bound kinesin occurs both alpha- and beta-tubulin. However, the unbinding measurements with ADP show only minor differences between alpha and beta-tubulin mutants, so this claim is not strongly supported.

To add extra evidence, the authors should show that indeed only the alpha-tubulin mutants are affected in ADP state using regular microtubule binding assays (spin down assays). This assay will make their claim much stronger.

Authors' response:
The authors admit that in the presence of ADP, the difference in the unbinding force between α- and β-tubulin mutants is not very large (Fig. 3A). Our previous interpretation that "Only interaction via α-tubulin contributes to the ADP state" was excessive.

In our new model (starting from the last paragraph on p. 10), α-tubulin plays a crucial role in transmitting a signal to kinesin to induce ADP release. However, in terms of the mechanical stability of the ADP state, the role of α-tubulin was not particularly highlighted. The authors prefer to abstain from any interpretation of the unbinding force measured in the presence of ADP, because, for the following reasons, this cannot be done simply.

Our previous study showed that the unbinding force measured in the presence of ADP is actually a composite of the unbinding force for the MT K ADP state and MT K states:

$$\mathrm{MT} + \mathrm{K{\cdot}ADP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{MT{\cdot}K{\cdot}ADP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{MT{\cdot}K} + \mathrm{ADP}$$

Thus, the observed reduction in the unbinding force in mutants may reflect either a change in the equilibrium of the two states, or the reduced stability of the MT·K·ADP complex. To rigorously evaluate the effect of mutation on the kinesin-MT interaction in the presence of ADP, we must analyze not only the average value of the unbinding force but also the distribution of binding forces at variable ADP concentrations (an example of such an analysis can be found in Uemura et al, 2003, *Nat. Struct. Biol.* 10, 308-311).

For the same reason, the spin down assay at a fixed concentration of ADP (*e.g.*, 1 mM) is not sufficient to pin down the relationship between tubulin mutation and the dissociation constant (= k_{-1}/k_1). In the spin down assay, what we measure is the proportion of kinesin in the pellet, whether or not kinesin holds ADP. Therefore, if K_d measured at 1 mM ADP is changed by mutation, this means the mutation has modulated either k_2/k_{-2} or k_{-1}/k_1. To genuinely evaluate the effect of mutation on k_{-1}/k_1, it is necessary to measure Kd at variable ADP concentrations, thereby calculating both k_{-1}/k_1 and k_2/k_{-2}.

Though we could not fulfill the request of this referee, we still appreciate his/her comment, as it has prompted us to deliberate extensively on our interpretation of the results.

Reviewer's comments:
3. The authors should report more experimental details. For example, there is little information regarding the yield amounts from yeast tubulin preparations.

Authors' response:
Experimental details regarding the construction of mutants and tubulin purification are described in the Supplementary Methods and in Supplementary Figure S1.

3rd Editorial Decision 10 February 2010

Many thanks for submitting the revised version of your manuscript to the EMBO Journal. It has now been seen again by referee 2, who is happy with your revision and now finds the manuscript suitable for publication (his/her comments are enclosed below). Therefore, I am pleased to be able to tell you that we can now accept your manuscript - you should receive the formal acceptance message shortly.

Yours sincerely,

Editor
The EMBO Journal

Referee 2 comments:

The revised manuscript of Uchimura et al, describing the role of individual tubulin residues in kinesin-1 binding and mechanochemistry, is very clear and interesting.

