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Cargo surface hydrophobicity is sufficient to overcome the nuclear pore complex selectivity barrier

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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 25 February 2009

Thank you for submitting your research manuscript to our editorial office. I have now had the opportunity to carefully read it, and I have also discussed it with the other members of the editorial team. I am afraid that our conclusion is not a positive one, as we find that your manuscript is not well suited for publication in The EMBO Journal.

We appreciate that have demonstrated that hydrophobicity of proteins is a criterion for nuclear transport through the NPC. Using a neat approach where you modify the non-passively transported protein BSA with different hydrophobic analogues, you show that increased hydrophocity independent of the analogue used enables the nuclear import of modified BSA. You show that addition of only four hydrophobic residues increases nuclear import and that further addition does not have a dramatic effect, suggesting that limited number of interactions with hydrophobic Nup repeats are required for import. Finally, you show that import of modified BSA occurs at a similar rate to import receptors. However, while we appreciate the systematic approach used in the study we find that previous work has shown that hydrophobic interactions are required for the nuclear import of receptors and that different hydrophobic sequences can bind to the protein receptors, it has also been shown that limited interactions occur via the receptor and Nups. Therefore, while we appreciate that you have demonstrated that hydrophobicity is a key determinant of nuclear import, we find that overall this does not provide a sufficient conceptual advance to be further considered fro 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 only subject those manuscripts to

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external review which are likely to receive enthusiastic responses from our reviewers and readers. As in our carefully considered opinion, this is not the case for the present submission, I am afraid our conclusion regarding its publication here cannot be a positive one. I am sorry to have to disappoint you on this occasion.

Yours sincerely,

Editor
The EMBO Journal

Rebuttal Letter 26 February 2009

While I appreciate the fact that you have read the manuscript carefully, and you clearly have expertise in its subject matter, I would like to ask you to reconsider your decision.

Our results show conclusively that surface hydrophobicity is not merely a criterion but is the determining physical trait that separates cargoes that will from those that will not go through the NPC. This has not been proven experimentally before but rather was inferred. Regarding the valance of the interactions between FG repeats and transport receptors, the only information that exists comes from in vitro analyses using isolated FG fragments or from simulations, using again short FG peptides. Clearly, these isolated, fragmented repeats cannot mimic either the structure or the concerted action of the complex network formed by many different polypeptides confined within the pore. Indeed, an active debate regarding the valance of these interactions still exists in the field with the number of interactions cited in the literature varying between 2 and 14. Our work provides the first in vivo measurement of this valance, which as detailed in the manuscript is to understanding how NPCs manage to transport efficiently while maintaining selectivity.

Prior to submitting the manuscript, we sent it out to several colleagues in the field of NPC transport and have had the opportunity of discussing its results in person with others including Prof. Günter Blobel. The response was overwhelmingly enthusiastic. One colleague stated that our results completely changed the way he thinks about transport through NPC's and another said it was an extremely important experiment.

We would therefore like to ask you to reconsider your decision and allow our manuscript to go through the review process.

Response to Rebuttal 2 March 2009

Thank you for your letter regarding my original decision on your nuclear import manuscript. I have had the opportunity to discuss the matter with one of our Editorial Board Members who finds that it is a potentially interesting study that may, as you suggest, provide further insight into the mechanism of nuclear import. Therefore, based on this discussion I am willing to change my decision and send your manuscript out for in depth review.

Sincerely,

Editor
The EMBO Journal

2nd Editorial Decision 25 March 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. Your manuscript has now been evaluated by three referees whose comments are enclosed below. As you will see from their reports while one referee finds that the study confirms the NPC functions through hydrophobic interactions, the majority of referees are quite positive about it and express potential

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interest in the findings, however, it is clear that further experimental analysis is required to make it suitable for publication in the EMBO Journal.

Both referee #1 and #3 would like to see further control experiments by looking at BSA modified using non-hydrophobic amino acid analogues such as Ala and Tyr. In addition referee #3 would like to see a detailed characterization of the conjugation reactions to determine the distribution of labeling. Should you be able to address the referees concerns we would be willing to consider a revised 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 COMMENTS

Referee #1 (Remarks to the Author):

The authors report that surface modified BSA can enter the nucleus throught NPCs without the requirement of nuclear transport receptors. This confirms that the NPC simply operates through hydrophobic exclusion.

However, a few points need to be taken into account.

First, a better control is required than NLS-conjugated BSA to control for aspecific effects through the amino acid conjugation procedure. The authors should use at least two non-hydrophobic amino acids such as alanine or tyrosine.

The datapoints in Figure 2A and B do not look like averages of 20-30 measurements, because of their discontuniety. It seems that the primary data must be extremely noisy and thus low quality, considering the simplicity of the assay. Could the authors provide the raw numerical data for these experiments?

No quantitation is presented for Fig 1F and G.

Referee #2 (Remarks to the Author):

This study focuses on the specific permeation mechanism of proteins through nuclear pore complexes (NPCs). In order to know that hydrophobic interaction between hydrophobic patches on nuclear transport factors and FG repeats on the NPC is crucial for the permeation, the authors nicely designed and prepared model molecules. That is, they conjugated different hydrophobic amino acid analogues to the surface of bovine serum albumin (BSA). Using these modified proteins, they demonstrated that the proteins conjugated with as few as four hydrophobic amino acid homologues enter the nucleus without soluble factors in permeabilized semi-intact cells, indicating that the presence of a small number of hydrophobic spots on the cargo surface is sufficient for efficient passage through the NPCs. They also showed that the permeation does not depend on the nature and density of the hydrophobic amino acids, suggesting that a non-specific, limited and pliant interactions between hydrophobic patches on transporting molecules and FG repeats are involved in the permeation through the NPCs. These findings are interesting and provide new insights into the issue of how NPCs allow the passage of large molecules when bound to transport factors. Furthermore, I am sure that the use of model molecules used in this work allows systematic analysis of the effects of hydrophobicity on macromolecules passing through NPCs and can provide a new tool. Collectively, this work is appropriate for publication in EMBO Journal. But, the following point should be addressed before publication.

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In order to confirm that the modified protein passes through the NPCs by the same pathway as importin beta-family molecules, the authors should test whether the permeation of the modified BSA is competed with importin beta alone.

Referee #3 (Remarks to the Author):

In this short manuscript, Naim et al. address the question of how the hydrophobicity of a transport cargo affects its translocation through the nuclear pore complex (NPC). Overall this is an important question and the authors use an experimental approach that could provide novel and interesting insight into the problem of how macromolecules are translocated through the NPC channel. However, there are several issues that need to be addressed before I could recommend publication.

1. The authors have to include a negative control, i.e. a hydrophilic amino acid residue, that can be coupled in the identical manner as the 3 hydrophobic residues used in the study.
2. The description of the experiments is at times woefully incomplete and several key controls need to be included:
(a) What is Phe7, Phe21, etc? I assume this is the average number of conjugations but that is nowhere described in the paper
(b) The authors must show the experiments in which they determined the average number of conjugations on their modified BSA. What are the labeling rations used? What is the spread of the number of labels. They should include mass spec, CD, gel filtration analysis for all the conjugates
3. In Fig. 1, there is a huge variation in the size of the nuclei. Why? Are these taken with the same magnification? Scale bars need to be included. Also, more than 1 cell needs to be shown in the panels E-G.
4. The authors should include a model of BSA (using the human SA structure and should show the structures of the amino acid analogues.
5. The authors should discuss their findings with respect to the prevalent models for NPC function (, i.e. the Rout and the Gorlich models)
6. Minor point: In the discussion, the authors state that their experiments are conducted 'in vivo'. That is certainly not true.

1st Revision - authors' response 27 May 2009

Detailed list of changes to the manuscript:

1. p. 6, line 2. "in-vivo" was replaced by “in live cells."

2. p. 6, line 9 and p. 7 line 1. We replaced "amino acid side-chain analogues" for "amino acid analogues "

3. A description of the additional control experiments requested by the referees was included on pages 7 (line 14-17) & 8 (starting at line 23).

4. p. 10, line 20. we added the number of leucine molecules attached to the surface of the cargo (66) to the sentence. The definition of the notation then follows from this on line 21. The number was also added to all superscripts denoting this mimic on page 11, lines: 7, 9, 12, 16 and 21.

5. p. 12, line 19. "in-vivo" was replaced by “in live cells."

6. p. 17, line 6-12. We added a note on the relevance of our results to prevalent models of NPC transport in the discussion.

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7. p. 22, line 4. A description of the notation used in the figures for the conjugates was added to the legend to Figure 1 following the request of reviewer 3.

8. To accommodate the new data from the additional control experiments, it became necessary to split Figure 1 into two figures. The new Figure 1 now contains data on nuclear entry of NTR mimics by themselves, whereas the new Figure 2 shows controls in which agents known to inhibit NPC passage were used as controls. The legends to figures 1 and 2 were corrected to reflect the changes made in the figures themselves.

9. Following the request of reviewer 1 we added a quantification of the data that was previously in Figure 1F and G in Figure 2D.

10. We added scale bars to the images in figures 1 and 2 (as per reviewer 3ís request).

11. The images in Fig. 2 were replaced with ones showing two cells in each field.
12. We added a figure to the supplementary material (Fig. 1S) with the modelled structure of BSA.

13. A figure showing mass spectra of representatives of the conjugates used in the study was added (Fig. S2).

14. A figure showing far-UV CD spectra and size exclusion chromatograph modified to the largest extend by each of the amino acid side chain analogues was added (Fig. S3).

Replies to Referees

Referee #1:

"The authors report that surface modified BSA can enter the nucleus throught NPCs without the requirement of nuclear transport receptors. This confirms that the NPC simply operates through hydrophobic exclusion.

“However, a few points need to be taken into account."

"First, a better control is required than NLS-conjugated BSA to control for aspecific effects through the amino acid conjugation procedure. The authors should use at least two non-hydrophobic amino acids such as alanine or tyrosine."

We thank the referee for her/his comments. Following the request of the referee (which was also made by reviewer 3), we performed two additional experiments, in which non-hydrophobic moieties were added to the BSA surface, using the same conjugation procedure employed for the derivatization with hydrophobic amino acid side-chain analogues. In the first, we modified BSA with a side-chain analogue of the hydrophilic amino acid serine, which has previously been used as a control for the interactions between FG repeats and themselves or with NTRs (Frey S. et al., Science 314, 815-817, 2006). In the second, we derivatized BSA with a nuclear localization signal peptide. In both cases, the modified BSA failed to enter the cell nuclei in the absence of cytosolic extract. [As expected, the NLScarrying BSA was able to enter the cell nucleus when the latter was added together with an energy regenerating system]. This, together with the fact that all

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hydrophobic BSA derivates were able to enter the nucleus confirms that the trait which allows our NTR mimics to enter the nucleus is indeed surface hydrophobicity.

"The datapoints in Figure 2A and B do not look like averages of 20-30 measurements, because of their discontuniety. It seems that the primary data must be extremely noisy and thus low quality, considering the simplicity of the assay. Could the authors provide the raw numerical data for these experiments?"

The numerical data for Figure 2A and B in the original manuscript (Figure 3 in the revised manuscript) is attached to the Email in a separate Excel file marked "RefereeOnlyData4Fig3.xls". As the referee will see, the primary data is not particularly noisy, nor is it of low quality as he/she suggested. Rather, it has a variability entirely within that characteristic of live cells, differing in shape, local density, and, probably, extent of permeabilization. We and others have observed similar variability in different cell types supplemented with different cargoes. We also note that the variability in nuclear entry rates has absolutely no bearing on our conclusions, as the difference in t1/2 between BSA modified by hydrophobic moieties and innate BSA is about two orders of magnitudes, well above the variance.

"No quantitation is presented for Fig 1F and G. "

The data that was presented in Fig 1F and G in the original manuscript is now presented in Fig 2. We added a panel to this figure (Fig. 2D) which includes quantification of the data in Fig. 2 A-C.

Referee #2

"This study focuses on the specific permeation mechanism of proteins through nuclear pore complexes (NPCs). In order to know that hydrophobic interaction between hydrophobic patches on nuclear transport factors and FG repeats on the NPC is crucial for the permeation, the authors nicely designed and prepared model molecules. That is, they conjugated different hydrophobic amino acid analogues to the surface of bovine serum albumin (BSA). Using these modified proteins, they demonstrated that the proteins conjugated with as few as four hydrophobic amino acid homologues enter the nucleus without soluble factors in permeabilized semiintact cells, indicating that the presence of a small number of hydrophobic spots on the cargo surface is sufficient for efficient passage through the NPCs. They also showed that the permeation does not depend on the nature and density of the hydrophobic amino acids, suggesting that a non-specific, limited and pliant interactions between hydrophobic patches on transporting molecules and FG repeats are involved in the permeation through the NPCs. These findings are interesting and provide new insights into the issue of how NPCs allow the passage of large molecules when bound to transport factors. Furthermore, I am sure that the use of model molecules used in this work allows systematic analysis of the effects of hydrophobicity on macromolecules passing through NPCs and can provide a new tool. Collectively, this work is appropriate for publication in EMBO Journal. But, the following point should be addressed before publication."
In order to confirm that the modified protein passes through the NPCs by the same pathway as importin beta-family molecules, the authors should test whether the permeation of the modified BSA is competed with importin beta alone"

We thank the referee for her/his comments. Due to the nature of our cargoes, they are most likely able to interact with all accessible FG sites present within the pore, making the different pathways apparently redundant. Indeed, as shown in Fig. 3C, BSA modified with luecine side-chain analogues (BSA^{Leu66}) entered the cell

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nucleus with the same rate, regardless of whether the cells were or were not supplemented with cytosolic extract and an energy regenerating system (thus allowing for transport of free and cargo-bound NTRs). We therefore do not expect competition with cargoes making use of any particular pathway and, hence, have not performed a systematic study of this point.

Referee #3 :

"In this short manuscript, Naim et al. address the question of how the hydrophobicity of a transport cargo affects its translocation through the nuclear pore complex (NPC). Overall this is an important question and the authors use an experimental approach that could provide novel and interesting insight into the problem of how macromolecules are translocated through the NPC channel. However, there are several issues that need to be addressed before I could recommend publication.

"The authors have to include a negative control, i.e. a hydrophilic amino acid residue, that can be coupled in the identical manner as the 3 hydrophobic residues used in the study."

We thank the referee for her/his comments. Following the request the referee (which was also made by Reviewer 1), we performed further control experiments, in which non-hydrophobic moieties were added to the BSA surface, using the same conjugation procedure employed for the derivatization with hydrophobic amino acid side-chain analogues. In the first, we modified BSA with a side-chain analogue of the hydrophilic amino acid serine, which has previously been used as a control for the interactions between FG repeats and themselves or with NTRs (Frey S. et al., Science 314, 815-817, 2006). In the second, we derivatized BSA with a nuclear localization signal peptide. In both cases, the modified BSA failed to enter the cell nuclei in the absence of cytosolic extract. [As expected, the NLScarrying BSA was able to enter the cell nucleus when the latter was added together with an energy regenerating system]. This, together with the fact that all hydrophobic BSA derivates were able to enter the nucleus confirms that the trait which allows our NTR mimics to enter the nucleus is indeed surface hydrophobicity.

"2. The description of the experiments is at times woefully incomplete and several key controls need to be included:"

"(a) What is Phe7, Phe21, etc? I assume this is the average number of conjugations but that is nowhere described in the paper"

A definition of the notation was added on lines 20-21 of page 10 as well as in the legend to Figure 1.

"(b) The authors must show the experiments in which they determined the average number of conjugations on their modified BSA. What are the labeling rations used? What is the spread of the number of labels. They should include mass spec, CD, gel filtration analysis for all the conjugates."

The average number of conjugates added to the BSA was determined using mass spectrometry, as described in Supplementary Figure 2. The range of protein/modifier stoichiometries used for conjugation is specified in the Materials and Methods along with a detailed description of the conjugation procedure. For molecules whose size is close to that of BSA, the molecular mass measurements, as performed in our study, are accurate to within 0.1-0.5%. Taking as an example

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the most extensively modified BSA we used (BSA^{Leu66}) this will translate to ≤4 hydrophobic moities.

Following the reviewer's request we provided mass spectra of innate BSA, as well as of BSA modified by each of the four different amino acid side-chain analogues we used (Supplementary Figure 2). We also included CD spectra and sizeexclusion chromatographs of BSA and of BSA molecules modified to the largest extent by each of the analogues used in this study (Supplementary Figure 3).

"3. In Fig. 1, there is a huge variation in the size of the nuclei. Why? Are these taken with the same magnification? Scale bars need to be included. Also, more than 1 cell needs to be shown in the panels E-G."

The variation in size between panels did in fact reflect different magnifications. Scale bars have now been added to the images in figures 1 and 2.

Regarding the reviewer's request to present more than one cell in each panel in the control experiments (now presented in Fig. 2). We substituted the images with new ones, each showing two cells. This is the largest number of cells we could find in a single field because this set of images was taken at high magnification. To supplement this, we present quantification of the data from all of the acquired images in Fig. 2D).

"4. The authors should include a model of BSA (using the human SA structure and should show the structures of the amino acid analogues."

As requested by the referee, we added the model structure we used in the analysis (Supplementary Figure 1). To avoid making the figure cumbersome we did not add the structures of the amino acid side-chain analogues added to the BSA surface.

"5. The authors should discuss their findings with respect to the prevalent models for NPC function (, i.e. the Rout and the Gorlich models)"

The main difference between prevalent models for NPC function lies in the way the interaction between FG repeats themselves is treated rather than in the way the interactions between cargo and NPC are perceived. Furthermore, these models are of a qualitative rather than a quantitative nature and thus our data cannot be used to discriminate between them. We hope that our data will be useful in placing constraints on future models attempting to reconstruct the permeability barrier of NPCs. We have added a note to this effect in the discussion. (p. 17, beginning on line 6).

"6. Minor point: In the discussion, the authors state that their experiments are conducted 'in vivo'. That is certainly not true."

We have replaced the "in-vivo" with "live cells" in the text.

3rd Editorial Decision 15 June 2009

Your revised manuscript has been reviewed by two of the original referees and as you can see they both support publication, referee #3 requests the incorporation of some small text changes (please see the comments below). Pending this minor revision, we would be willing to consider publishing

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your manuscript in the EMBO Journal.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.

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

Yours sincerely,

The EMBO Journal

REFEREE COMMENTS

Referee #2 (Remarks to the Author):

I feel that the authors appropriately responded to the criticism raised by the referee. I understood and agreed with their comments written in their letter.

Referee #3 (Remarks to the Author):

The authors have been able to address most of my concerns. The authors insist that their experiments were performed "in live cells" yet most of their experiments were performed with digitonin permeabilized cells, which are no longer alive. They need to change the text to reflect this.

2nd Revision - authors' response 16 June 2009

Thank you for your decision regarding our manuscript entitled "Cargo surface hydrophobicity is sufficient to overcome the nuclear pore complex selectivity barrier" (EMBOJ-2009-70697R-A). We have changed the wording in the text, as per reviewer #3's request. Specifically we changed "live cells" to "intact NPCs within cells" in page 6 line 2 and page 12 at the discussion paragraph on line 8.
We hope that you will find the paper ready for publication.

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