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FG/FxFG- as well as GLFG repeats form a selective permeability barrier with self-healing properties

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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 13 February 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see while referee 2 is more positive referee 1 is not convinced yet that as it stands the study is strong enough and provides a sufficient advance over your earlier publications to support publication here at this stage of analysis. Still, he/she puts forward a number of suggestions how to improve the paper. Referee 3 is also hesitant whether the advance provided by the paper is sufficient, at least according to the overall rating and his/her comments to the editor, but also puts forward a suggestion how the paper could be strengthened. He/she also feels that the existing literature should be presented in a more balanced manner. Taking together all issues raised and given that the study addresses an important and very basic issue we have come to the conclusion that we would be able to consider a revision if you can take the study further along the lines suggested by the referees. However, you need to persuade referees 1 and 3 that their concerns have been addressed in an adequate manner.

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 as well as on the final assessment by the referees.

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 manuscript by Frey and Gorlich represents an extension of previous studies by this group examining the properties of gels formed in vitro using regions of FG-containing nucleoporins. In the current work, passive diffusion of 'inert' molecules into gel derived from FG/FXFG-nups was examined in the presence and absence of nuclear transport factors (NTFs). On the basis of their data, the authors conclude that the presence of NTFs does not increase the permeability of the gel to inert molecules but rather further restricts passive diffusion into the gel. In addition to the FG/FXFG-hydrogels, the present study also examines the characteristics of hydrogels formed using fragments of GLFG-containing nucleoporins and combinations of these and regions of FG/FXFG-nucleoporins. The authors present data showing these gels also exclude inert proteins but allow entry of NTF at a faster rate then FG/FXFG-hydrogels. Other differences include the effects of the transport inhibitor importin ß45-462, which have a greater inhibitory effect on importin ß/cargo entry into the GLFG-hydrogel.

As with the accompanying manuscript, the data in this paper are of high quality. This manuscript provides further insight into the nature of the hydrogels and, in certain regards, their potential relationships to the in vivo situation. I do, however, believe that certain additional experiments would further enhance this study and make it a significant advancement over their previously published studies. In some cases, addressing the questions posed below will provide a clearer view of the properties of the hydrogels and their functional similarities to the NPC.

Major concerns.

1) There are alternative explanations for the inhibition of MBP-Cherry entry in the FG-hydrogel in the presence of the IBB-MBP-mEGFP/ importin ß complex. It is, for example, possible that the 'channels' created by the IBB-MBP-mEGFP/ importin ß complex are not resealed, but rather the greater affinity of the importin ß for the FG repeats allows the complexes to follow one another into a growing channel while excluding the MBP-Cherry. These, and potentially other, interpretations of their data should be discussed.

2) The authors show that preincubation of the FG-hydrogel with the IBB-MBP-mEGFP/ importin ß complex suppresses gel entry of mCherry. Based on these results, they suggest that the IBB-MBP-mEGFP/ importin ß complex 'tightens' the diffusion barrier of the FG-hydrogel and, by analogy, NTFs would have similar effects on the NPC. I am concerned with the interpretation of these data, as certain controls are missing that would address the specificity of the effects of the IBB-MBP-mEGFP/ importin ß complex on the permeability of the hydrogel. For instance, would any protein with an affinity for the FG-hydrogel also clog the diffusion barrier and, if so, how can a conclusion be drawn about the physiological significance of their observations? Would, for example, incubation of the FG-hydrogel with an antibody directed against Nsp1p also reduce diffusion into the gel?

3) Would the authors please clarify why they chose to use a fusion of the repeat domains of Nup49p and Nup57p rather than using them individually, or in a mixture of the two nups, to form the GLFG-hydrogel. This latter approach would be more reflective of the state of these nups in the NPC.

4) The authors show that NTFs rapidly enter the FG/FXFG- or GLFG-hydrogel. An important tenet of current models for transport through the NPC is that NTFs can also exit the FG-containing environment of the NPC. If soluble NTFs are removed from the adjoining chamber (to create a concentration gradient), can the NTFs also diffuse out of the hydrogels?

Minor points

1) Please adjust sentence three of the Results section. As you are discussing in vitro FG-hydrogel properties in this sentence, it is inaccurate to refer to intermixing of 'nuclear and cytoplasmic contents' in the context of this sentence.

2) As requested in the review of the accompanying manuscript, please recheck grammar and sentence structure in this manuscript as well.

Referee #2 (Remarks to the Author):

It is generally accepted that the essential fundamental components of the nuclear pore complex are nucleoporin FG repeats. Previously, the authors have presented an in vitro biophysical model system of the NPC that mimicked cargo selectivity and nuclear transport receptor mediated entry into the NPC. In this manuscript the authors go further in analysis of the in vitro model and find that different types of FG repeats can form a selectice permeability barrier with self-healing properties. In other words, receptor/cargo permeation does not induce barrier leakiness.

This is an interesting manuscript that provides a plausible model of how the NPC can be an effective block in diffusion of reasonably small proteins, yet can alow passage of certain very large macromolecules at high capacity.

However, cargoes used to demonstrate that cargo/receptor entry does not lead to higher influx of inert molecules are relatively small (IBB-MBP-mEGFP), and it would be interesting to see whether this would hold up with a larger cargo, e.g. the tertrameric RedStar protein fused to IBB.

In the discussion, the authors state that the virtual gate model of the NPC (Rout et al., 2003) did not so far generate any testable predictions or convincing evidence (p.17). This is a sweeping statement that in my opinion requires more explanation to be acceptable. For example, the authors could refer to evidence and predictions made by the model and explain why they are not convincing or testable, respectively. Alternatively, the authors could limit themselves to the statement that no testable predictions were made about the size selectivity.

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In this paper, Frey and Gorlich extend their analysis of FG peptide hydrogels and continue their comparison between the properties of in vitro formed gels and native nuclear pore complexes. The novelties of this manuscript are:

1. GLFG repeats and mixed GLFG/FxFG peptides can form selective hydrogels that are comparable in their properties to the previously reported FxFG hydrogels.
2. The selectivity of the hydrogels stays intact even in the presence of high concentrations of transport substrates. In fact, transport receptors appear to tighten the selectivity
3. A dominant negative importin ß fragment Impß45-462 blocks the entry of nuclear transport receptors and also lowers the exclusion limit of passive cargoes.
Most of the results that are presented in this paper are solid but the paper would benefit from a more balanced discussion of the previous literature.

Specific point:

Interestingly, the Impß45-462 mutant does not only block the entry of transport receptors but it also freezes their movement within the gel. To explain this, the authors argue that this mutant multimerizes (in data not shown) and that a subsequent increase in avidity can explain this behavior. These results need to be included and it should be tested whether this mutant indeed binds more tightly to certain FG repeats than the wild-type, full-length protein

1st Revision - authors' response 05 May 2009

Point-by-point answers to the reviewers' comments.

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As with the accompanying manuscript, the data in this paper are of high quality. This manuscript provides further insight into the nature of the hydrogels and, in certain regards, their potential relationships to the in vivo situation. I do, however, believe that certain additional experiments would further enhance this study and make it a significant advancement over their previously published studies. In some cases, addressing the questions posed below will provide a clearer view of the properties of the hydrogels and their functional similarities to the NPC.

Major concerns.

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In this alternative scenario, channels would not close behind a translocating species, but remain permanently open. Sealing of those static channels would be achieved by filling them with following translocating species. This is an interesting thought, but for the following reasons we regard it as unlikely:

(A) NTRs enter "virgin" hydrogels very efficiently and are thus able to introduce new perforations. The kinetics suggests that this happens with high probability upon the first contact between NTR and gel. We cannot see how NTRs could loose this potential of de novo perforating the gel during the experiment and what could force them to follow exactly the path of NTRs that entered the gel previously.

(B) The suggestion of the reviewer could also be interpreted such that NTRs use only pre-existing channels. This pre-existing channels would have to be large enough to accommodate large cargo·NTR complexes. We can rule out this possibility, because large inert objects are essentially excluded also from NTR-free hydrogels.

(C) We could calculate that during the course of an typical influx experiment, each point of the gel-surface is hit ~3000 times by an NTR·cargo complex. If new channels were constantly produced without re-forming inter-repeat contacts behind the translocating species, then the gel would rapidly disintegrate. This is, however, not the case.

(D) The suggested scenario requires that "following species" fit the dimensions of the channel created by the "pioneer" nuclear transport receptor. For that, all translocating species should have the same size. This is, however, not the case. Imagine an NTF2 dimer (28 kDa) "following" a large ribosomal subunit (≈3 MDa) through the permeability barrier. How should NTF2 (5 nm diameter) be able to seal a static channel that is large enough for transporting ribosomes (30 nm diameter) against passive influx? The fact that NPCs are highly selective for GFP-sized as well as for ribosome-sized objects strongly argues for an adaptive barrier that closes behind each translocating species.

2) The authors show that preincubation of the FG-hydrogel with the IBB-MBP-mEGFP/ importin ß complex suppresses gel entry of mCherry. Based on these results, they suggest that the IBB-MBP-mEGFP/ importin ß complex 'tightens' the diffusion barrier of the FG-hydrogel and, by analogy, NTFs would have similar effects on the NPC. I am concerned with the interpretation of these data, as certain controls are missing that would address the specificity of the effects of the IBB-MBP-mEGFP/ importin ß complex on the permeability of the hydrogel. For instance, would any protein with an affinity for the FG-hydrogel also clog the diffusion barrier and, if so, how can a conclusion be drawn about the physiological significance of their observations? Would, for example, incubation of the FG-hydrogel with an antibody directed against Nsp1p also reduce diffusion into the gel?

First of all, NTRs do not clog NPCs non-specifically. Instead, they tighten the permeability barrier against passive influx, while still allowing facilitated passage. This is evident both for in vitroassembled FG-hydrogels (this study) and for intact NPCs (accompanying paper). Given the high cellular concentration of NTRs, it is plausible that this tightening of the passive diffusion barrier also occurs in living cells.

The prediction that any protein with an affinity for FG-repeats tightens the diffusion barrier is probably correct, at least if such protein interacts multivalently with the repeats. Here, the lectin WGA is a prototypical example (this is a major point in the accompanying manuscript). It binds sugar residues within certain FG-repeats of vertebrate NPCs, blocks facilitated translocation and suppresses passive passage. Since WGA does not occur inside vertebrate cells (unless being microinjected), this effect has, of course, no physiological relevance.

As far as we know, nuclear transport receptors and other nucleoporins are the only physiological ligands of FG-repeats, probably because evolution selected against other intracellular binders. Thus, there is currently no evidence for the reviewer's concern that FG-binders distinct from Nups or NTRs would clog NPCs in an uncontrolled way.

3) Would the authors please clarify why they chose to use a fusion of the repeat domains of Nup49p and Nup57p rather than using them individually, or in a mixture of the two nups, to form the GLFGhydrogel. This latter approach would be more reflective of the state of these nups in the NPC.

We fused the FG-repeat domains to each other to prevent a local unmixing, i.e. to guarantee a stoichiometric ratio between the components at all points of the gel.

4) The authors show that NTFs rapidly enter the FG/FXFG- or GLFG-hydrogel. An important tenet of current models for transport through the NPC is that NTFs can also exit the FG-containing environment of the NPC. If soluble NTFs are removed from the adjoining chamber (to create a concentration gradient), can the NTFs also diffuse out of the hydrogels?

We addressed this question in the new Figure 9. Indeed, we see efflux of importin ß out of the gel, when Phenyl-Sepharose beads are placed as local sinks in front of the gel. This is really a nice experiment, so thank you for the suggestion.

Figure 9 also shows that GTP-Gsp1p (Yeast Ran) accelerates the efflux of cargo and importin ß from the gel, which recapitulates a key feature of the classical nuclear import pathway.

Minor points

1) Please adjust sentence three of the Results section. As you are discussing in vitro FG-hydrogel properties in this sentence, it is inaccurate to refer to intermixing of 'nuclear and cytoplasmic contents' in the context of this sentence.

Agreed and done. The new sentence reads: "If such perforations would remain open or persist for too long in authentic NPCs, then the permeability barrier would break down and nuclear and cytoplasmic contents would intermix."

2) As requested in the review of the accompanying manuscript, please recheck grammar and sentence structure in this manuscript as well.

Agreed and done.

Referee #2 (Remarks to the Author):

It is generally accepted that the essential fundamental components of the nuclear pore complex are nucleoporin FG repeats. Previously, the authors have presented an in vitro biophysical model system of the NPC that mimicked cargo selectivity and nuclear transport receptor mediated entry into the NPC. In this manuscript the authors go further in analysis of the in vitro model and find that different types of FG repeats can form a selectice permeability barrier with self-healing properties. In other words, receptor/cargo permeation does not induce barrier leakiness.

This is an interesting manuscript that provides a plausible model of how the NPC can be an effective block in diffusion of reasonably small proteins, yet can alow passage of certain very large macromolecules at high capacity.

However, cargoes used to demonstrate that cargo/receptor entry does not lead to higher influx of inert molecules are relatively small (IBB-MBP-mEGFP), and it would be interesting to see whether this would hold up with a larger cargo, e.g. the tertrameric RedStar protein fused to IBB.

We did the experiment using the tetrameric ZsGreen protein fused to IBB. The resulting $(IBBZsGreen)_4{\cdot}Imp\beta_4$ complex has a mass of ≈500 kDa. Pre-incubation of an FG-hydrogel with this complex did not increase, but instead suppressed, the passive influx of mCherry (29 kDa). We included these data as new Supplementary Figure S4.

In the discussion, the authors state that the virtual gate model of the NPC (Rout et al., 2003) did not so far generate any testable predictions or convincing evidence (p.17). This is a sweeping statement that in my opinion requires more explanation to be acceptable. For example, the authors could refer to evidence and predictions made by the model and explain why they are not convincing or testable, respectively. Alternatively, the authors could limit themselves to the statement that no testable predictions were made about the size selectivity.

Agreed. The paragraph now reads: " The "virtual gate model" assumes that entropic exclusion by Brownian motion of the extended FG-repeat domains is sufficient to explain the suppression of passive fluxes through NPCs and that NTRs overcome this "entropic barrier" by binding the repeats (Rout et al., 2003). Although peripheral, non-interacting FG-repeat domains might indeed enlarge the target area of NPCs and "feed" NTRs into the actual permeability barrier, this model could so far not explain the characteristic size-selectivity of NPCs."

Minor point:

1. p.9 "pre-incubation of the gel lowered the gel" not clear what is meant here.

To improve clarity, we changed it to read: " Instead, the pre-incubation of the gel lowered the partition coefficient of the passive species between gel and buffer from 0.2 in an untreated gel to ≤ 0.02."

2. Materials and Methods are scetchy in places and purity of recombinant protein should be described. It would be helpful to have plasmid sequences deposited.

We extended the methods and include a new Supplementary Figure (S5) that documents the purity of the proteins used. We would be happy to deposit the plasmid sequences. Perhaps the Editor could suggest how this should be done for an EMBO Journal publication.

Referee #3 (Remarks to the Author):

In this paper, Frey and Gorlich extend their analysis of FG peptide hydrogels and continue their comparison between the properties of in vitro formed gels and native nuclear pore complexes. The novelties of this manuscript are:

1. GLFG repeats and mixed GLFG/FxFG peptides can form selective hydrogels that are comparable in their properties to the previously reported FxFG hydrogels.

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3. A dominant negative importin ß fragment Impß45-462 blocks the entry of nuclear transport receptors and also lowers the exclusion limit of passive cargoes.

Most of the results that are presented in this paper are solid but the paper would benefit from a more balanced discussion of the previous literature.

We went through the text and changed several passages to a more balanced wording, in particular in the discussion.

Specific point:

Interestingly, the Impß45-462 mutant does not only block the entry of transport receptors but it also freezes their movement within the gel. To explain this, the authors argue that this mutant multimerizes (in data not shown) and that a subsequent increase in avidity can explain this behavior. These results need to be included and it should be tested whether this mutant indeed binds more tightly to certain FG repeats than the wild-type, full-length protein.

We agree, these are important data. We included them in the accompanying manuscript (new Figure 6), because in this ms, we already arrived at 9 figures. The data set includes gel filtration experiments showing that Impß$^{45-462}$ appears monomeric at low concentrations (0.5 M), but forms associates corresponding to at least dimers and tetramers at higher concentration. We also present a competition experiment that shows that Impß$^{45-462}$ binds stronger to FG-repeats from Nsp1p than full length importin ß.

2nd Editorial Decision 05 June 2009

Thank you for sending us your revised manuscript. Our original referees 1 and 2 have now seen it again. In general, both referees are now positive about publication of your paper. Still, referee 1 feels that before we can ultimately accept your manuscript there is one issue that still needs to be addressed in respect to negative controls that he/she feels are needed in figures 3, 4, 5 (see below). I recognise that he/she had brought this up already in his/her initial report and that you have responded to this point already in your point-by-point response. Yet, he/she is not satisfied by your response and feels rather strongly that this issue needs to be addressed by performing the controls. I would thus like to ask you to address this request prior to acceptance of this manuscript.

Please let us have a suitably amended manuscript as soon as possible.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors have made several changes that have improved the manuscript. However, an issue still remains regarding point 2 of my previous review and the response of the authors. First of all, to respond to the authors, I am keenly aware that the NPC is not non-specifically clogged by NTRs. But as the authors should note, I was not discussing the properties of NPCs in this point but rather the hydrogel. (In this regard, the authors should use caution when referring to properties of NPCs and the hydrogels interchangeably.) What is in question here is whether the effects of NTRs on the diffusion of mCherry into the hydrogel (e.g. Fig.4) are specific. Importantly, what are your negative controls? If everything that binds the hydrogel blocks diffusion in, what can you conclude about the significance of the effects of the NTRs. Without such data it is difficult to draw meaningful

conclusions and comparisons to NPCs.

2nd Revision - authors' response 18 June 2009

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Our answer to this point:

1. The key issue here is that specificity is achieved at the level of binding. Many labs looked hard to identify cellular binding partners for FG-repeats, the result being that only two types of ligands were found: nuclear transport receptors (NTRs) and other nucleoporins. It is reasonable to assume that evolution selected against non-specific binders to FG-repeats and that this selectivity of binding to FG-repeats is crucial for nuclear pore complex (NPC) function.

It is well possible that artificial ligands to FG-repeats also tighten the permeability barrier against passive influx (in fact, if we had a glycosylated FG-hydrogel, we would assume that wheat germ agglutinin does). Given, however, that such artificial ligands never see NPCs of undisturbed cells, this would not be an argument against the specificity of the effects of the nuclear transport receptors.

Let me illustrate this for another example. Imagine you were studying a neutralising antibody that prevents entry of a particular virus into cells. What would be a good negative control? Control antibodies that do not recognise the virus or the specific antibody blocked by an antigenic peptide. A different antibody that binds to the same or a similar site of the virus is expected to have similar effects, but this does not mean that the first antibody would act in a non-specific manner.

2. Of course, we performed negative controls for the specificity of the NTR-effects, namely pre-incubation of the FG-gel with buffer or with inert proteins. Such control was included in every experiment.

A strict "negative control ligand" for the NTR effects on the FG hydrogel should have the same size, accumulate to the same local concentration and penetrate as deeply into the gel as an NTR. The combination of these criteria can, however, only be fulfilled by nuclear transport receptors. Antibodies, in contrast, bind only to the surface of the gel and do not show facilitated entry into the gel (see below).

3. The FG-hydrogel experiments reproduced the behaviour of authentic NPCs, where NTRs or the dominant-negative importin beta mutant makes NPCs tighter against passive influx. This parallel is a strong argument for specificity. Furthermore, this correlation is consistent with the assumption that the permeability barrier of NPCs is indeed an FG-hydrogel.

4. The pre-incubation of the FG-hydrogel with nuclear transport receptors improves the selectivity of the gel. It has only a moderate effect on facilitated entry, but makes the barrier considerably tighter towards passive influx. The magnitude of the effect depended on which type of gel, which passive and which receptor species were used. With some combinations an

up to 100-fold increase in selectivity was observed. We feel it is inappropriate to describe an increase of selectivity as a non-specific effect.

5. We followed the request of this reviewer from the first round of review and compared the effects of anti-FG-repeat antibodies on the permeability of the FG hydrogel with the effects of importin ß . The behaviour of the two ligands was as different as it could be: While importin ß penetrated deep into the gel, the antibodies remained stuck at the buffer gel interface. We include these new data as an additional supplemental figure. This finding confirms that a mere binding of macromolecules to an FG hydrogel is not sufficient for allowing facilitated transport.

We already showed that importin ß improves the selectivity of the barrier, i.e. it suppresses passive influx more strongly than facilitated influx (see Figure 4). In contrast, the anti repeat antibodies lowered the selectivity of the FG gel by a large factor. They inhibited receptormediated gel entry strongly (at least 100-fold inhibition for a monomeric importin ß·cargo and more than 1000-fold inhibition for a tetrameric importin ß·cargo complex), while leaving passive influx of mCherry essentially unaffected. This clearly indicates that the interaction of an FG/FxFG repeat hydrogel with importin ß is fundamentally different from its interaction with an anti-FG repeat antibody. We include these new data as an additional supplemental figure.

