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Mg2+-dependent gating of MgtE channel underlies Mg2+ homeostasis

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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 24 April 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 find your study interesting, appreciate the high quality of your data and are supportive of publication. However, there are some issues that would first need to be addressed. A major point, raised by all three referees, concerns the mutations generated within the pore of MgtE: you do not demonstrate whether these mutant proteins are expressed and properly folded, thus precluding conclusions from being drawn as to their functionality. Reviewer 1 also questions the nature of the solutions used for the electrophysiological assays, which do not represent the physiological situation. It would be important to address both these criticisms before we are able to consider publication.

In addition, referee 2 highlights concerns with the quality of the figures. I have to say that I agree with this, and find figures 5 and 6 in particular difficult to follow. I should remind you that the figures will be significantly reduced in size for publication, and it is important that you take this into consideration when preparing your revised manuscript.

Given the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of major revision. Therefore, acceptance of your paper will depend on your ability to fully answer the points raised by the referees.

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

Yours sincerely,
Katherine Brown, PhD
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Mg2+-dependent gating of MgtE channel underlies Mg2+ homeostasis; Hattori et al.

Summary: In this ms., the authors have performed a strucural and molecular analysis of gating of the prokaryotic T. thermophilus MgtE magnesium channel. The studies include structures with increased resolution in different ionic conditions allowing definition of some additional detail, including an additional divalent cation binding site between the N/CBS domain regions of two subunits. In addition, methods for electrophysiologic characterization of currents carried by the T. thermophilus MgtE channel in bacteria were developed, as well as methods for analysis of Mg transport activity via complementation of a Mg auxotrophic strain of bacteria. These were used for in depth biophysical and cell biological analyses of the WT channels, as well as many site-directed structural mutants.
Many of the structural mutants were noted to have lost Mg conduction capacity as evidenced by lack of ability to complement a Mg deficient strain of E. coli - the interpretation of these results was that the residues involved were crucial for Mg transport capacity - this interpretation is clearly correct, but these results are not particularly mechanistically informative.
Analyses of Mg-dependent gating of the WT channel by electrophysiology showed that the channel was inactivated by increased internal Mg - over the 200 micromolar to 10 millimolar range, but not other cations in the same range. Monovalent cations reportedly had no effect, but I do not see this data presented anywhere. In addition, see comment below, the solutions used are far from physiologic, somewhat diminishing my confidence in how to interpret this data. Remarkably, electrophysiological analysis of a variety of cytosolic Mg binding site/sensor mutants showed altered or abolished gating effects of internal Mg, consistent with their roles as intracellular Mg sensors. In addition, an N-deletion mutant showed an increased open probability, and the authors propose a "clamp" model in which the N-domain acts to stabilize the CBS domain in a closed state to explain its function. Using these data in conjunction with the transport and structural data, the authors propose a detailed model for regulation of the T. Thermophilus MgtE channel via cytoplasmic calcium.
Overall, the authors have performed a careful and elegant structural and molecular analysis of T. Thermophilus MgtE protein function, and their data are fully consistent with a role for these proteins in intracellular Mg homeostasis. The reported data are of high quality, and generally support the authors conclusions and structural model. The one major criticism I have is the nature of the solutions used for electrophysiology - I think the complete reliance on these very non-physiologic solutions does not allow a reader a sufficiently accurate picture of channel permeation properties, and I would like to see the addition of data showing permeation properties under more physiological solutions, so that a reader can judge how and to what extent the channel behavior reported is related to how it might function within a cell. With this additional data, this manuscript would represent an important contribution to the field.

Comments:
1) I can't comment on the structural analyses, as it outside of my area of expertise. However, the conclusions drawn from the new structural data seem reasonable.
2) the proteins analyzed are exclusively expressed in bacteria, and this should be made clear in the title and throughout the text, since many higher organism homologues appear not to have the same regulatory mechanism, based on the fact that they completely lack the N-terminal and CBS domains. The introduction and parts of the discussion are similarly unclear - reading through the manuscript, many readers would never guess that there are major structural differences between mammalian and prokaryotic MgtE proteins

Referee #2 (Remarks to the Author):

This paper reports an impressive experimental analysis which used a broad range of complementary techniques to investigate the function of MgtE , a bacterial Mg transport protein which functions as an ion channel. The main findings in the paper are

(i) a higher resolution (2.94 Å) structure which reveals important local differences in pore structure and a new Mg binding site (ion 7), combined with Co anomalous difference maps used to identify 6/7 Mg binding sites.

(ii) Demonstration using single channel recording that MgtE is a Mg and Co permeable ion channel, which is impermeable to Ni, Mn and Ca, with in vivo complimentation.

(iii) Demonstration that MgtE open probability is regulated by [Mg]i and that this is abolished by mutations of structurally identified Mg binding sites, and by deletion of a cytosolic regulatory N domain .

In contrast, the negative data in Fig 4 are entirely unconvincing without direct experimental demonstration that the inactive mutant proteins are both expressed, and correctly folded. If the proteins are not expressed, or are not correctly folded, then we learn nothing about mechanism from the loss of function.

With this one exception the major findings are supported by convincing experimental data, the paper is easy to follow, but the Figures could be substantially improved.

1) In addition further tests regarding the data in Figure 4 there are several other issues the authors need to consider: Although a number of Mg binding site mutants were tested, the newly identified site in which E59 has a unique role was not. It would be appropriate to do this in view of the fact that overall, the new structures are mostly confirmatory.

2) The yhiD gene is an important new finding, but is not documented. Is a paper in press?

3) Many details of the physiology need clarification. When conductances are reported are these chord or slope conductance? The potential at which the conductance was measured should be given; currently its described as 'at negative membrane potentials' which is not acceptable given that the conductance varies with potential. In Fig 3B the 2nd trace of the Empty vector pair should be labeled Triple knockout, since from my reading of the legend this is a different experiment than that in the upper trace.

The open probability measurements are barely adequate, and analysis of open and closed time histograms should be performed. Some of traces e.g. D259N in Fig 6 panel A appear to show large changes in open time.

In Figure 4E the data for 10 mM Mg appears to be meaningless and should be deleted, since if the channel never opens, its not possible to measure its IV relationship. The same applies to panel F: if it never opens the Po is too low to measure. Simply state this without plotting a row of zeros.

4) page 11 para 1: How much of the effect on current size from raising internal Mg arises drom change in driving force? This should be discussed, since without considering this, any other cause of amplitude change is difficult to interpret.

5) Many of the Figures have fonts which are too small, or other composition problems. In Fig 3A the panels will be almost too small to see at final publication size. In Fig 5 panel I has a different font size from the rest of the assembly. The open circle symbol is too fat.

Overall, Figs 4-5 and especially Fig 6 present an overwhelming amount of raw data and IV plots, which could be better replaced by illustration of a few key examples, with succinct text descriptions for the rest of the results.

Minor critique: note that the text numbering jumps from 1-3, back to 1 again after the abstract, following numbering in the version supplied for review.

1) page 3 line 2 delete: Our.

2) page 1 line 1 delete ion (Mg2+) so that the text reads: Magnesium is an essential metal

3) page 1 para 1 last 4 lines reorder reference, so that Hmiel et al comes after Mg2+ transporter (Hmiel et al 1986), which provided the first ...

4) page 1 para 2 lines 2-5 reorder: In Bacillus subtilis the gene expression of MgtE ...

5) page 2 line 6: delete the: acchitecture consisteing of N-terminal ...

6) page 2 line 8-9: delete text [formerly referred ...renamed as "plug helices" and move reference to end of sentence

7) page 3 line 3: change proved to established

8) page 4 line 7 change prove to test: To test the Mg2+ ...

9) page 7 line 8: change reasonable to candidate.

10) page 8 para 2 lines 6-7: delete the words consecutive and axpressed, and add channel after MgtE .

Referee #3 (Remarks to the Author):

This manuscript follows on the determination of a low-resolution structure of the eubacterial magnesium channel MgtE. The manuscript is exceptionally clear and synthesizes a number of disparate methods in an elegant fashion to gain mechanistic insight into how this molecule functions. In addition to obtaining higher resolution crystallographic data (2.94 Å versus 3.5 Å from the original report) the authors develop and employ both genetic complementation and sphereoplast patch clamping methods to assess function. The higher resolution crystallographic data resolve some key features of the pore that were absent in the original report. The use of divalent cation soaking with cobalt and nickel to verify the assignment of various magnesium binding sites is an important additional step. The functional analysis by both genetic complementation and patch clamp clearly demonstrates the channel function of MgtE and gives important new insights into the likely mechanisms of action and modulation. This manuscript is very well suited for publication in EMBO J. There are only two issues that should be addressed.

1) The mutations at the ion binding site in the central cavity (D423A and D432N) are non-functional. I was left wondering whether these mutations impair channel assembly. Did the authors examine whether these proteins were made and actually present in the membranes of the bacteria/sphereoplasts? It is well established that the permeant ions of potassium channels have a very important role in stabilizing the channel assembly. It would be good to know whether the loss of function that correlates with the loss of the putative binding site of the permeant ion in MgtE also has deleterious effects on the stability/assembly of the channel complex. The authors have done such a thorough job on other aspects of this manuscript that the lack of this important detail really stands out.

2) The authors use the word 'prove' frequently in the manuscript. (for example p.4 'To prove the Mg2+ dependent gating model...' I believe the intent is 'test' not 'prove'. No mechanisms are proven here. Likely mechanisms are suggested and supported by the analysis, but definitely not proven. This is an important linguistic distinction and should be corrected.

Print Email

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1st Revision - authors' response 20 August 2009

Specific comments by Referee 1
1. "Monovalent cations reportedly had no effect, but I do not see this data presented anywhere."

Based on the previously reported molecular dynamics simulation of the cytosolic domain of MgtE, Na+ binding to the cytosolic domain did not induce a structural change of the closed form in silico (Ishitani et al., 2008). Therefore, we think that the MgtE cytosolic domain would not sense monovalent cations, such as Na+ ions. However, there were no "biological" data to support this. Now we have newly performed the limited proteolysis and the patch clamp analysis with a high concentration of monovalent cation (Na+ or K+) (Figure S11). These results indicated that these monovalent cations would not have any effect on the stabilization of the closed state of the MgtE channel at the low intracellular monovalent cation concentrations, but they do have an effect on the stabilization of the closed conformation at high concentrations (200-300 mM), which are, however, significantly higher than the physiological concentration (lines 10-22 on page 16).

2. "The one major criticism I have is the nature of the solutions used for electrophysiology - I think the complete reliance on these very non-physiologic solutions does not allow a reader a sufficiently accurate picture of channel permeation properties, and I would like to see the addition of data showing permeation properties under more physiological solutions, so that a reader can judge how and to what extent the channel behavior reported is related to how it might function within a cell."

We agree with the referee's comment. The problems about the nature of the solution can be mainly divided into two points: the use of N-methyl-D-glucamine (NMDG) and the high concentration of sucrose.
First, NMDG is a well-known sodium substitute often used in patch clamp analysis. NMDG-gluconate blocks the current derived from the endogenous Na+ or Cl- channels. In the presence of NaCl or Na-gluconate without NMDG, the current derived from the endogenous Na+ or Cl- channels would interfere with the detection of Mg2+ conductance by MgtE. Therefore, NMDG must be included in the experimental solution. Actually, in the patch clamp analysis of the Mrs2p Mg2+ channel from yeast, NMDG-gluconate was also used to clearly detect the Mrs2p activity (Schindl et al., 2007).
Second, 300 mM sucrose in the measurement condition causes high osmolarity, which might make the solution differ considerably from the physiological conditions in E. coli as well as mammalian cells. The referee might think this is the most problematic point. However, to prepare the giant spheroplasts, it was necessary to maintain the high osmolarity. Since patch clamp analyses with bacterial cells are not frequently employed, we would like to explain the details of the giant spheroplast preparation, as described below. First of all, the present methodology of patch clamp analysis of E. coli giant spheroplasts was established and authorized since 1987 (Martinac et al., 1987), and has been improved. We referred to the patch clamp analysis of the bacterial cyclic nucleotide-gated K+ channel (Kuo et al., 2007) for our MgtE patch clamp analysis.
First, we added the antibiotic (cephalexin) to the E. coli cell culture, which inhibited the division of the E. coli cells, allowing them to become long, filamentous cells. Then, we disrupted the cell wall of the E. coli cells by osmotic shock, using a high concentration of sucrose, which enables the E. coli cells to become sufficiently large and spherical for the patch clamp experiment (giant spheroplasts). In contrast, once the sucrose concentration is reduced after the spheroplast preparation, the cells become very fragile, and are no longer suitable for the patch clamp experiment.
Especially, in this study, to exclude the effects of the other E. coli Mg2+ transporters, we established

a new E. coli Mg2+ auxotrophic triple mutant strain (corC-, MgtA-, yhiD-), and prepared giant spheroplasts of the strain, which have distinct membrane features and are even more fragile than the WT giant spheroplasts. Therefore, it was necessary to maintain the high sucrose concentration for the patch clamp analysis. Actually, we tried to perform MgtE patch clamp analyses without sucrose and with much less sucrose, but we failed due to the cell fragility. In the previous patch clamp analysis of the bacterial cyclic nucleotide-gated K+ channel (Kuo et al., 2007), which we referred to for the present MgtE patch clamp analysis, a high concentration of sucrose (500mM sucrose) was also used.
Therefore, to judge how and to what extent the reported channel behavior is related to how it might function within a cell, the patch clamp data using the giant spheroplasts should be compared with those from other genetic and biochemical experiments to demonstrate their validity. Actually, Kuo's group performed phenotypic analyses by genetics, in parallel with the patch clamp analysis, which produced consistent results. We also performed other genetic (Figures 3A and 4, and Figure S13) and biochemical (Figure S14) analyses. For the Mg2+ transport, the results of the in vivo complementation (Figure 3A), the measurement of the Mg2+ influx in the giant spheroplasts using a Mg2+ indicator (Figure S5) and the patch clamp analysis (Figure 3B) are consistent. For the mutational analysis of the ion-conducting pore, the results of the in vivo complementation (Figure 4B,C), the in vivo 57Co2+ transport experiment (Figure S9) and the patch clamp analysis (Figure 4D) are consistent. For the Mg2+ dependent gating of MgtE, the results of the patch clamp analysis (Figure 5A-D) and the Mg2+-dependent protease protection analysis of MgtE (Figure S14) are consistent. Especially, the threshold for MgtE inactivation by [Mg2+]in is essentially consistent with the results from the Mg2+-dependent protease protection analysis of MgtE (Figure S14) (Ishitani et al., 2008). The solution used for the limited proteolysis does not include a high concentration of sucrose (See Materials and Methods), and would more closely resemble the physiological conditions than the current patch clamp conditions. For the effect of the monovalent cations, the results of the patch clamp analysis and the limited proteolysis (Figure S11) are consistent. For the cytosolic Mg2+ binding site mutants, the in vivo complementation (Figure S13A), Co2+ sensitivity (Figure S13B), patch clamp analysis (Figures 6 and S12) and limited proteolysis (Figure S14) results are all consistent. Therefore, the reported channel behavior would be related to how it functions within a cell, at least qualitatively, and partially quantitatively, although the current nature of the solution in the patch clamp analysis was not always close to the physiological conditions, due to the intrinsic technical problem. We have added these descriptions about the nature of the solution for the patch clamp analysis in the main text (lines 11-15 on page 22).
Furthermore, we have finally added a fluorescence experiment to confirm the normal behavior of the giant spheroplasts used in the patch clamp analysis. We could observe the Mg2+ influx in the giant spheroplasts using a Mg2+ indicator, mag-fluo4, under the more "physiological condition" only with 10 mM sucrose as well as under the previous 300 mM sucrose condition (Figure S5). This result also supports that the channel properties reported in this study would reflect the physiological events.

3. "the proteins analyzed are exclusively expressed in bacteria, and this should be made clear in the title and throughout the text, since many higher organism homologues appear not to have the same regulatory mechanism, based on the fact that they completely lack the N-terminal and CBS domains. The introduction and parts of the discussion are similarly unclear - reading through the manuscript, many readers would never guess that there are major structural differences between mammalian and prokaryotic MgtE proteins"

As the referee pointed out, the eukaryotic MgtE homologues lack the N-terminal and CBS domains. Thus, many higher organism homologues appear not to have the same regulatory mechanism. According to the comment, we have added a description about this in the introduction (lines 1-2 on page 5) and in the discussion (lines 17-19 on page 22), and also added the phrase "bacterial" in the title.

Specific comments by Referee 2

1. *"In contrast, the negative data in Fig 4 are entirely unconvincing without direct experimental demonstration that the inactive mutant proteins are both expressed, and correctly folded. If the proteins are not expressed, or are not correctly folded, then we*

learn nothing about mechanism from the loss of function."

According to the referee's comment, we checked the expression of the T. thermophilus MgtE mutant proteins as well as that of the wild type in the membrane fraction of E. coli cells by anti-His tag western blotting (Figure S3). We confirmed the expression of all of the mutants in the membrane fraction, which would indicate the integration of the proteins into the membrane.

2. "Although a number of Mg binding site mutants were tested, the newly identified site in which E59 has a unique role was not. It would be appropriate to do this in view of the fact that overall, the new structures are mostly confirmatory."

According to the reviewer's request, we performed the mutational analysis of the E59A mutant (Figure S17). These results indicated that the Mg7 binding site also contributes to the regulation of the Mg2+ transport by MgtE, but it plays a less important role than those revealed by the other regulatory domain mutants (from lines 1-16 on page 21).

3. "The yhiD gene is an important new finding, but is not documented. Is a paper in press?"

As the referee pointed out, the yhiD gene is a new finding. We presently have no plans to submit another paper describing this finding. Therefore, we have added a description about it, as follows: the result newly indicated that the yhiD gene would be an essential component for the Mg2+ uptake system in E.coli (lines 11-12 on page 10).

4. "Many details of the physiology need clarification. When conductances are reported are these chord or slope conductance? The potential at which the conductance was measured should be given; currently its described as 'at negative membrane potentials' which is not acceptable given that the conductance varies with potential. In Fig 3B the 2nd trace of the Empty vector pair should be labeled Triple knockout, since from my reading of the legend this is a different experiment than that in the upper trace."

It is the slope conductance that is reported. The potential is -40mV. We have added the description in lines 18-20 on page 11. In Figure 3B, the 2nd trace of the empty vector pair is not a different experiment from that in the upper trace (we previously showed two traces, just as examples). To avoid repetition of the data and potential misunderstanding, we have deleted the 2nd traces from Figures 3B and 3C.

5. "The open probability measurements are barely adequate, and analysis of open and closed time histograms should be performed. Some of traces e.g. D259N in Fig 6 panel A appear to show large changes in open time."

According to the referee's request, we performed the analysis of the open and closed time histograms (Figure S15), which actually showed large changes in the open times of the 1-129 and D259N mutants (lines 11-13 on page 19 and lines 6-7 on page 20).

6. "In Figure 4E the data for 10 mM Mg appears to be meaningless and should be deleted, since if the channel never opens, its not possible to measure its IV relationship. The same applies to panel F: if it never opens the Po is too low to measure. Simply state this without plotting a row of zeros."

According to the referee's comment, we have deleted the data for 10 mM [Mg2+]in in Figures 5E and 5F, and added a description of the data for 10 mM MgCl2 to simply state this in the Figure legend of Figure 5, without plotting a row of zeros.

7. "4) page 11 para 1: How much of the effect on current size from raising internal Mg arises drom change in driving force? This should be discussed, since without considering this, any other cause of amplitude change is difficult to interpret."

As the referee pointed out, the addition of Mg2+ to the bath solution will change the driving force for Mg2+ transport. This means that the equilibrium potential will be shifted to a more positive voltage, thus reducing the current. The reduction of the current due to the change of driving force is estimated to be about 17% for 1mM [Mg2+]in, as compared to 0.2 mM [Mg2+]in, 25% for 5 mM [Mg2+]in and 42% for 10 mM [Mg2+]in at -40 mV, with a conductance of 96 pS. This means that currents of about 5.8 pA for the 1 mM condition, about 5.3 pA for the 5 mM condition and about 4 pA for the 10 mM condition should be measured at -40 mV (around 7 pA was measured at -40mV in 0.2 mM MgCl2). For the 1 mM and 5 mM conditions, the measured amplitudes are quite consistent with the predicted ones. However, for 10 mM [Mg2+]in, we could not measure any current, which supports the intracellular Mg2+-dependent transport suppression against the driving forces. We have added a description, from line 16 on page 14 to line 8 on page 15.

8. "5) Many of the Figures have fonts which are too small, or other composition problems. In Fig 3A the panels will be almost too small to see at final publication size. In Fig 5 panel I has a different font size from the rest of the assembly. The open circle symbol is too fat."

According to the referee's comment, we have enlarged the font size throughout the Figures, enlarged the size of the panels in Figure 3A, and made the open circle symbol thinner. In addition, we have also changed the font size in panel I of Figure 5 to be consistent with the rest of the assembly.

9. "Overall, Figs 4-5 and especially Fig 6 present an overwhelming amount of raw data and IV plots, which could be better replaced by illustration of a few key examples, with succinct text descriptions for the rest of the results."

According to the referee's comment, we have moved the I-V and Po plots about the effect of Ca2+ in Figure 5 to the Supplemental Figure S10, and the raw data and I-V plots of the E258Q, D259N and 1-129 mutants to the Supplemental Figure S12, with succinct descriptions for the rest of the results in their respective figure legends.

10. "Minor critique: note that the text numbering jumps from 1-3, back to 1 again after the abstract, following numbering in the version supplied for review."
According to the referee's comment, we have revised the numbering of the manuscript.

11. "1) page 3 line 2 delete: Our."
According to the referee's comment, we have deleted the term "Our" as in line 3 on page 3.

12. "2) page 1 line 1 delete ion (Mg2+) so that the text reads: Magnesium is an essential metal"
According to the referee's comment, we have deleted the term "ion (Mg2+)" in line 2 on page 4.

13. "3) page 1 para 1 last 4 lines reorder reference, so that Hmiel et al comes after Mg2+ transporter (Hmiel et al 1986), which provided the first ..."
According to the referee's comment, we have reordered the reference in line 9 on page 4.

14. "4) page 1 para 2 lines 2-5 reorder: In Bacillus subtilis the gene expression of MgtE ..."
According to the referee's comment, we have reordered the sentence as in line 14 on page 4.

15. "5) page 2 line 6: delete the: architecture consisting of N-terminal ..."
According to the referee's comment, we have deleted the "architecture consisting of N-terminal ..." in lines 7-8 on page 5.

16. "6) page 2 line 8-9: delete text [formerly referred ...renamed as "plug helices" and move reference to end of sentence"
According to the referee's comment, we have revised this in lines 9-10 on page 5.

17. "7) page 3 line 3: change proved to established"
According to the referee's comment, we change the term "prove" to "established" in line 4 on

page 6.

18. "8) page 4 line 7 change prove to test: To test the Mg2+ ..."
According to the referee's comment, we have changed the term "prove" to "test" in line 7 on page 7.

19. "9) page 7 line 8: change reasonable to candidate."
According to the referee's comment, we have changed the term "reasonable" to "candidate" in line 8 on page 10.

20. "10) page 8 para 2 lines 6-7: delete the words consecutive and axpressed, and add channel after MgtE ."
According to the referee's comment, we have deleted the words in lines 13-14 on page 11.

Specific comments by Reviewer 3

1. "1) The mutations at the ion binding site in the central cavity (D423A and D432N) are non-functional. I was left wondering whether these mutations impair channel assembly. Did the authors examine whether these proteins were made and actually present in the membranes of the bacteria/sphereoplasts?"

According to the comment, we checked the expression of the T. thermophilus MgtE mutant proteins and the wild type in the membrane fraction of E. coli cells, as well as that of the giant spheroplasts, by western blotting using an anti-His tag antibody (Figure S3). We have confirmed the expression of all of the mutants in the membrane fraction, which would indicate the integration of the proteins into the membrane.

2. "It is well established that the permeant ions of potassium channels have a very important role in stabilizing the channel assembly. It would be good to know whether the loss of function that correlates with the loss of the putative binding site of the permeant ion in MgtE also has deleterious effects on the stability/assembly of the channel complex. The authors have done such a thorough job on other aspects of this manuscript that the lack of this important detail really stands out."

According to the referee's comment, we performed the gel filtration analysis of the purified D432A mutant in the presence and absence of Mg2+, which showed similar elution profiles to that of the purified wild type MgtE (Figure S8). It should be noted that the Mg2+ concentration of 0.2 mM in the gel filtration condition is significantly higher than the K0.5 value (below 0.1 mM from Figure S16), presumably corresponding to the Mg2+ affinity for the transmembrane domain. The result implies that the D432A mutant, which lost the putative Mg2+ binding site in the TM domain, would still possess the normal channel assembly. We have added these descriptions and a discussion of the comparison with KcsA from line 17 on page 13 to line 1 on page 14.

3. "2) The authors use the word 'prove' frequently in the manuscript. (for example p.4 'To prove the Mg2+ dependent gating model...' I believe the intent is 'test' not 'prove'. No mechanisms are proven here. Likely mechanisms are suggested and supported by the analysis, but definitely not proven. This is an important linguistic distinction and should be corrected."

According to the referee's comment, we have deleted the word "prove" throughout the manuscript. Instead, we have changed the word "prove" to "test" in line 7 on page 7, in line 9 on page 16 and in line 1 on page 20.

References

Ishitani, R., Sugita, Y., Dohmae, N., Furuya, N., Hattori, M. and Nureki, O. (2008) Mg2+-sensing mechanism of Mg2+ transporter MgtE probed by molecular dynamics study. Proc Natl Acad Sci U S A, 105, 15393-15398.

Kuo, M.M., Saimi, Y., Kung, C. and Choe, S. (2007) Patch clamp and phenotypic analyses of a prokaryotic cyclic nucleotide-gated K+ channel using Escherichia coli as a host. J Biol Chem, 282, 24294-24301.
Martinac, B., Buechner, M., Delcour, A.H., Adler, J. and Kung, C. (1987) Pressure-sensitive ion channel in Escherichia coli. Proc Natl Acad Sci U S A, 84, 2297-2301.
Schindl, R., Weghuber, J., Romanin, C. and Schweyen, R.J. (2007) Mrs2p forms a high conductance Mg2+ selective channel in mitochondria. Biophys J, 93, 3872-3883.

Preliminary decision letter 03 September 2009

Many thanks for submitting the revised version of your manuscript EMBOJ-2009-70940R. It has now been seen again by referees 1 and 2 (whose comments are appended below), who are satisfied with the revision, and support publication. However, referee 2 highlights a couple of inaccurate statements that would need to be corrected before we are able to accept the manuscript.
I would therefore ask you to revise the text according to the comments of referee 2. The easiest way to do this would be for you to email me an updated version of the manuscript text (as a .doc file). We can then upload this in place of the original. Once we have received the corrected manuscript text, we should be able to accept without further delay.

With best wishes,

Editor
The EMBO Journal

Referee 1 comments:

I think the authors have done an excellent job responding to the reviewer comments.

Referee 2 comments:

With one exception the revised manuscript adequately addresses the major issues raised in my prior review.

1) On page 15 lines 7-10 There remains an incorrect statement that needs revision. It is stated that

"In contrast, for [Mg2+]in=10 mM, we could not measure any current (Figures 5D), which supports the intracellular Mg2+-dependent transport suppression against the driving force. Quantitatively, an increase in [Mg2+]in from 0.2 mM to 10 mM drastically reduced the conductance and the open probability (Figures 5E and 5F).

The authors appear to confuse changes in driving force for ion current, versus the effects on gating, measured as changes in open probability. The fact that they could not measure current in 10 Mg does NOT occur because of "suppression against the driving force" but instead because the channel does not open.

The statement "Quantitatively, an increase in [Mg2+]in from 0.2 mM to 10 mM drastically reduced the conductance" is also not correct because the single channel conductance could not be measured, because the channel does not open.

