A short C-terminal peptide in Gγ regulates Gβγ signaling efficacy

Mithila Tennakoon, Kanishka Senarath, Dinesh Kankanamge, Deborah Chadee, and Ajith Karunarathne

Corresponding author(s): Ajith Karunarathne, University of Toledo

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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.)
Dear Dr. Karunarathne,

Your manuscript has been evaluated by two experts in the field. As you will see from their review reports, they are mildly positive, and have several points that need attention. Therefore, I have to decline the manuscript in its present form, but aim willing to handle a revised version carefully addresses all points raised by the reviewers.

Thank you for submitting your work to Molecular Biology of the Cell.

With kind regards,
Peter van Haastert

Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Karunarathne,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

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Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors
Reviewer #1 (Remarks to the Author):

The paper entitled, "A short C-terminal peptide in Gγ regulates Gβγ signaling efficacy" by the authors Mithila Tennakoon, Kanishka Senarath, Dinesh Kankanamge, Deborah N. Chadee, and Ajith Karunarathne is an excellent example of hypothesis driven research. The rationale and hypothesis that the gamma-subunit of the G protein beta-gamma complex plays an important role in membrane affinity and effector activation is clearly laid out. The experimental plan is logical and presented in a clear manner. I believe this research has significant contribution of our understanding of G protein function.

The manuscript and work should be acceptable with some minor changes/considerations.

Primary issue: The figure legends in some ways are not sufficient for the reader to understand the content or the meaning of the results. Additional information on how the experiments were performed by referencing the Methods section would be useful. Some needs. Fig. 1 and 4, meaning of the arrows is not addressed; Fig with FIM and F PM these terms are not identified (they can be understood with some looking but best to have it directly in the legend), Fig. 5 labels of A, B need to be corrected. Should be A, B, C in the legend as well.

2. I feel the authors need to provide a clear rationale for choosing gamma-9 over generylgermyl gamma subunits (7, 10, or 12) in their comparison to gamma-3. The addition of a second variable (in addition to the FF changes) in the comparison between gamma-3 and gamma-9 seems to complicate the conclusions drawn.

2. Is the tissue distribution known for gamma 1-12, should be addressed

3. Description of F side chain as a methyl-phenyl side chain is somewhat unusual, referring to the side chain of phenylalanine may be a better means to express this.

4. page 15 "... to eliminate unnecessary Gβγ signaling." Since a reference to internal Gβγ signaling is made, should this be edited to say ...to eliminate unnecessary plasma membrane Gβγ signaling.

5. p15 "This complexity is exacerbated by the fact that the majority of organs expressing Gγ types with moderate PM-affinities." "express'
6. Palmitoylated Gα also shows rapid turnover at PM based on their depalmitoylation and internalization. How this fits into your ideas could be explored/suggested/mentioned.

Reviewer #2 (Remarks to the Author):

In this study Tennakoon et al., identified a short C-terminal peptide in Gγ that regulates Gβγ signalling efficacy. The authors generated a set of mutants and analysed these for PM affinity, PI3K activation and adaptation of PIP2 hydrolysis. Although the overall findings are interesting, additional control experiments are needed to justify the main conclusions. Furthermore, the PIP2 hydrolysis experiments are done based on another manuscript that is currently under review somewhere else. It is therefore difficult to judge the novelty and importance of these data.

Specific concerns:

1. Although in general mutations of Gγ3 lead to increase in both rate and extent of inner-membrane translocation of the respective Gβγ complexes, the detailed kinetics of the translocation is not always consistent with PIP3 generation. For example the mutant Gγ3-KK->GG in FF shifted, its translocation rate and extent are both significantly different from the WT, while the extent of PIP3 generation shows no significant difference.

2. The same discrepancy can also be found in partial adaptation of PIP2 hydrolysis. Gγ3-FF shifted has a significant increase in translocation rate when compared to the WT and FF--->GG mutant, however, the PIP2 re-synthesis rate is similar to WT but not having the same trend as FF--->GG mutant, which is increase in the rate of PIP2 re-synthesis. The authors should address and explain the discrepancies in their manuscript.

3. The expression of different Gγ proteins are assumed to be equal but it is not always the case. This may hamper the interpretation of results. Moreover, the initial distribution of different Gγ proteins is found to be different (e.g. Gγ3 WT vs Gγ3 FF->GG), however the authors did not comment about it.

4. There are also no evidence showing the Gγ3 and Gγ9 mutants have a similar degree of prenylation and interaction to Gβ subunit. Since prenylation of CAAX proteins is critical for their PM localization regulation, evidence demonstrating proper prenylation of the mutants is hence necessary to explain the results obtained in this manuscript is because of the composition of residues in the pre-CAAX box of Gγ.

5. Tissue-specific expression of Ggamma is shown in panel 5B. However, for me it was unclear where these data are coming from. Where they generated by the authors themselves and/or coming from other sources? This is especially of importance, since based on these data the authors make strong claims, both in the results section (e.g. last sentence on page 13) and in the discussion (last paragraph page 14, continuing on page 15).
We immensely thank the reviewers for taking the time to provide us with valuable comments. We have answered all of them point by point below after performing more experiments. We have revised the manuscript accordingly. Our answers are in a colored font.

Thank you

Authors

Reviewer #1 (Remarks to the Author):

The paper entitled, "A short C-terminal peptide in Gγ regulates Gβγ signaling efficacy" by the authors Mithila Tennakoon, Kanishka Senarath, Dinesh Kankanamge, Deborah N. Chadee, and Ajith Karunarathne is an excellent example of hypothesis driven research. The rationale and hypothesis that the gamma-subunit of the G protein beta-gamma complex plays an important role in membrane affinity and effector activation is clearly laid out. The experimental plan is logical and presented in a clear manner. I believe this research has significant contribution of our understanding of G protein function.

The manuscript and work should be acceptable with some minor changes/considerations.

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We appreciate the reviewer’s comment. We have made necessary changes and corrections to the figure labels and legends in the revised manuscript to improve clarity, as the reviewer suggested.

1. I feel the authors need to provide a clear rationale for choosing gamma-9 over geranylgeranyl gamma subunits (7, 10, or 12) in their comparison to gamma-3. The addition of a second variable (in addition to the FF changes) in the comparison between gamma-3 and gamma-9 seems to complicate the conclusions drawn.

This is a very thoughtful comment, and we have now added a statement to the results section addressing the comment as follows: Since we explore the unique designs of membrane-interacting pre-prenylation (pre-CaaX) residues in Gγ as a major source for Gγ-type-dependent Gβγ signaling, we selected one member from geranylgeranylated (Gγ3) and one from farnesylated (Gγ9) Gγs for the study, especially since these two Gγs represent the two extreme ends of the PM affinity range. When we examined tissue-specific expression, Gγ types such as Gγ5, 10, and 12 show dominant expression in most tissues (Tennakoon, M. et al., Cellular Signalling, 2021). These Gγ types provide moderate-PM affinities to Gβγ, though they are geranylgeranylated. The primary difference between the moderate-Gγs and Gγ3 is that moderates do not possess the Phe-duo (FF) in the pre-CaaX region. Compared to Gγ3-WT, Gγ3-FF→GG exhibited translocation properties similar to moderates than to Gγ3, i.e., compared to T1/2 values of 200 s in Gγ3-WT and 70-100 s in moderate-Gγs, Gγ3-FF→GG showed 93 s. On the contrary, GG→FF mutation in Gγ9 changed T1/2 from 12 s to 171 s. Interestingly, newly added data also show that this Gγ9-GG→FF mutant
remains farnesylated (Fig. S2), while G\(\gamma\)3 mutants are geranylgeranylated. Therefore, the use of G\(\gamma\)3 and G\(\gamma\)9 and their mutants was necessary and allowed us to demonstrate the significant role of pre-CaaX region of G\(\gamma\) in modulating the PM affinity of G\(\beta\gamma\).

2. Is the tissue distribution known for gamma 1-12, should be addressed
At the mRNA level, several databases with a considerable agreement show the tissue-specific expression of the 12 G\(\gamma\) types (Fagerberg, L. et al., Mol. Cell. Proteomics, 2014, Syrovatkina, V. et al., J Mol Biol, 2016). The presented RNA data is extracted from the FANTOM5 repository in the human protein atlas database, which also confirms the tissue-specific distribution of 12 G\(\gamma\) types (Uhlén, M. et al., Science, 2015, Thul, P.J. et al., Science, 2017, Uhlén, M. et al., Science, 2017). From the same atlas, we extracted tissue microarray-based immunohistochemistry protein data, which shows that, even in the same tissue, different cell types show distinct G\(\gamma\)-expression profiles (Normal tissue data in the human protein atlas database) (Fig. S5 and S6). This data show an elevated expression of G\(\gamma\)13 in retinal bipolar cells, while rod and cone photoreceptor are the only cells with a prominent expression of G\(\gamma\)1 and G\(\gamma\)9. Additionally, our RNAseq data show that RAW264.7 macrophages and HeLa cells express substantially different G\(\gamma\) expression profiles (Senarath, K. et al., Journal of Biological Chemistry, 2018). In this manuscript, we demonstrated that specific G\(\gamma\) profiles are associated with the functional specifications of cells. As an example, we showed the need for G\(\gamma\)3 in RAW264.7 macrophages to supports PIP3 production and cell migration. We have included a discussion about this under Fig. 5B, S5, and S6 description.

3. Description of F side chain as a methyl-phenyl side chain is somewhat unusual, referring to the side chain of phenylalanine may be a better means to express this.
In the revised manuscript, following the literature (Das, T. et al., Biomaterial Science, 2018), we have now changed the term “methyl-phenyl” to “benzyl” side chain.

4. page 15 "... to eliminate unnecessary G\(\beta\gamma\) signaling." Since a reference to internal G\(\beta\gamma\) signaling is made, should this be edited to say ...to eliminate unnecessary plasma membrane G\(\beta\gamma\) signaling. In the revised manuscript, we have corrected it as suggested.

5. p15 "This complexity is exacerbated by the fact that the majority of organs expressing G\(\gamma\) types with moderate PM-affinities." "express"
In the revised manuscript, we have made the correction as suggested.

6. Palmitoylated G\(\alpha\) also shows rapid turnover at PM based on their depalmitoylation and internalization. How this fit into your ideas could be explored/suggested/mentioned.
Unlike protein palmitoylation (Huang, C. et al., PNAS, 1999, Wedegaetner, P. B., Subcellular Biochemistry, 2018), protein prenylation is an irreversible process (Wang, J. et al., MeChemComm, 2017, Palsuledesai, C. C. and Distefano, M. D., ACS Chemical Biology, 2015). Further, prenylation requires -aaX residues of the CaaX motif to proceed. Upon prenylation, -aaX is cleaved by RCE1, followed by ICMT induced cysteine carboxymethylation. Even if de-prenylation were to occur, re-prenylation of such proteins could not occur due to the permanent modification of the prenyltransferase-recognizing-region on G\(\gamma\). Further, the shuttling of the farnesylated small G protein, KRas, is mediated by the molecular chaperone PDE\(\delta\)6, and KRas does not indicate any involvement of a process like de-farnesylation. PDE\(\delta\)6 masks the farnesyl
group of KRas, and allow its shuttling through the cytosol to reach internal membranes (Zimmermann, G. et al., Nature, 2013). We have briefly discussed this in the discussion.

Reviewer #2 (Remarks to the Author):

In this study Tennakoon et al., identified a short C-terminal peptide in $G_{\gamma}$ that regulates $G_{\beta\gamma}$ signalling efficacy. The authors generated a set of mutants and analysed these for PM affinity, PI3K activation and adaptation of PIP2 hydrolysis. Although the overall findings are interesting, additional control experiments are needed to justify the main conclusions. Furthermore, the PIP2 hydrolysis experiments are done based on another manuscript that is currently under review somewhere else. It is therefore difficult to judge the novelty and importance of these data.

We thank the reviewer for taking the time to review and provide comments for our manuscript. The manuscript that describes the molecular regulation of PIP2 hydrolysis by $G_{\beta\gamma}$ is just published in the Journal of Biological Chemistry. We have cited the work in the revised manuscript (Kankanamge, D. et al., Dissociation of the G protein $\beta\gamma$ from the Gq-PLC$\beta$ complex partially attenuates PIP2 hydrolysis, Journal of Biological Chemistry, 2021). We, therefore, believe this addresses the above reasonable concern.

Specific concerns:

1. Although in general mutations of $G_{\gamma}3$ lead to increase in both rate and extent of inner-membrane translocation of the respective $G_{\beta\gamma}$ complexes, the detailed kinetics of the translocation is not always consistent with PIP3 generation. For example the mutant $G_{\gamma}3$-KK$\rightarrow$GG in FF shifted, its translocation rate and extent are both significantly different from the WT, while the extent of PIP3 generation shows no significant difference.

2. The same discrepancy can also be found in partial adaptation of PIP2 hydrolysis. $G_{\gamma}3$-FF shifted has a significant increase in translocation rate when compared to the WT and FF$\rightarrow$GG mutant, however, the PIP2 re-synthesis rate is similar to WT but not having the same trend as FF$\rightarrow$GG mutant, which is increase in the rate of PIP2 re-synthesis. The authors should address and explain the discrepancies in their manuscript.

This is an important concern, and it allowed us to discuss this as follows in the discussion. $G_{\beta\gamma}$ translocation is a direct indicator of the $G_{\beta\gamma}$ PM affinity (Senarath, K. et al., ACS Analytical Chemistry, 2016). Therefore, it likely captures even minor changes in the PM-affinity of $G_{\beta\gamma}$. PIP3 generation upon PI3K activation, however, is one of the downstream processes of $G_{\beta\gamma}$ and is additionally regulated by processes such as recruitment of cytosolic PI3Ks to the PM and the efficacy of cellular phosphatases that consume PIP3. Such a complex regulation could easily make PIP3 production sensitive only to drastic changes in the $G_{\beta\gamma}$ availability at the PM. The reduced sensitivity of PIP2 hydrolysis adaptation process for the subtle changes in PM affinity of $G_{\beta\gamma}$ can also be similarly explained. Our recently published work shows that this process is also regulated by multiple and parallel downstream mechanisms (Kankanamge, D. et al., Dissociation of the G protein $\beta\gamma$ from the Gq-PLC$\beta$ complex partially attenuates PIP2 hydrolysis, Journal of Biological Chemistry, 2021).

3. The expression of different $G_{\gamma}$ proteins are assumed to be equal but it is not always the case.
This may hamper the interpretation of results. Moreover, the initial distribution of different Gγ proteins is found to be different (e.g. Gγ3 WT vs Gγ3 FF->GG), however the authors did not comment about it.

We have previously demonstrated that (Senarath, K. et al., Journal of Biological Chemistry, 2018) the transfected Gγ type becomes the most prominent and the dominant Gγ over the endogenous Gγs in a cell line. This observation agrees with Gγ-specific distinct signaling changes observed in cells upon Gγ transfection. While the expression level differences of Gγ among cells can influence the extent of their effects, we mitigate this by considering cells with only a defined GFP-Gγ expression range for translocation and signaling measurements. For instance, we use a constant excitation intensity and only consider cells with ~mean±30% emission range. This range is selected because these cells show a predominantly PM-bound Gγ with a minor presence at the IMs (Thul, P.J. et al., Science, 2017). As shown in sample images in Fig. 1D, the selection of cells with a defined range of fluorescence intensities allows us to have cells with near-similar Gγ WT and mutant expressions for signaling quantification. We have discussed this in the results section. We have commented on the initial distribution differences of Gγs (and mutants) as heterotrimeric in the 1.1 results section.

4. There are also no evidence showing the Gγ3 and Gγ9 mutants have a similar degree of prenylation and interaction to Gβ subunit. Since prenylation of CAAX proteins is critical for their PM localization regulation, evidence demonstrating proper prenylation of the mutants is hence necessary to explain the results obtained in this manuscript is because of the composition of residues in the pre-CAAX box of Gγ.

In the revised manuscript, we show that the type of prenylation is independent of the pre-CaaX region (Fig. S5). Here, we show both the WT Gγ and its pre-CaaX mutant exhibiting similar sensitivities to their corresponding prenyltransferase inhibitors. However, their prenylation was not inhibited by the other kind. For instance, similar to Gγ9-WT, Gγ9-GG→FF mutant remains farnesylated (sensitive to farnesyl transferase inhibitor, Tipifranib, however not to the geranylgeranyl transferase inhibitor, GGTI286) (Fig. S2). Similarly, both Gγ3-WT and its mutant Gγ3-FF→GG mutant showed geranylgeranylation.

Examining FRET between fluorescently-tagged Gγ and Gβ, we now show that mutations in the pre-CaaX region do not perturb Gγ-Gβ interactions (Fig. S1).

5. Tissue-specific expression of Ggamma is shown in panel 5B. However, for me it was unclear where these data are coming from. Where they generated by the authors themselves and/or coming from other sources? This is especially of importance, since based on these data the authors make strong claims, both in the results section (e.g. last sentence on page 13) and in the discussion (last paragraph page 14, continuing on page 15).

We have now included a better description of the data using in Fig. 5B and S5. At the mRNA level, several databases with a considerable agreement show the tissue-specific expression of the 12 Gγ types (Fagerberg, L. et al., Mol. Cell. Proteomics, 2014, Syrovatkina, V. et al., J Mol Biol, 2016). The presented RNA data is extracted from the FANTOM5 repository in the human protein atlas database, which also confirms the tissue-specific distribution of 12 Gγ types (Uhlén, M. et al., Science, 2015, Thul, P.J. et al., Science, 2017, Uhlén, M. et al., Science, 2017). In our manuscript, we only present the 6 tissue types that exhibit the highest Gγ expression. From the same atlas, we extracted tissue microarray-based immunohistochemistry protein data, which shows that, even in
the same tissue, different cell types show distinct $G_{\gamma}$-expression profiles (Normal tissue data in the human protein atlas database) (Fig. S5). For instance, in the retina, while bipolar cells show an elevated expression of $G_\gamma13$, rod and cone photoreceptor cells show a prominent expression of $G_\gamma1$ and $G_\gamma9$. These data also show that the photoreceptor cells are the only cells expressing $G_\gamma1$ and $G_\gamma9$; the two fastest translocating $G_\gamma$s. Additionally, our RNAseq data show that RAW264.7 macrophages and HeLa cells express substantially different $G_\gamma$ expression profiles (Senarath, K. et al., *Journal of Biological Chemistry*, 2018). We have further demonstrated that the specific $G_\gamma$ profiles are associated with the functional specifications of cells. For instance, we showed $G_\gamma3$ supports PIP3 production and migration of RAW264.7 macrophages. We also showed that knockdown of $G_\gamma3$ abrogates both the above responses. We have included a summary of this discussion under Fig. 5B, S5, and S6 descriptions.
RE: Manuscript #E20-11-0750R
TITLE: "A short C-terminal peptide in Gy regulates Gβγ signaling efficacy"

Dear Dr. Karunarathne:

Your revised manuscript has been evaluated by the two experts. Both reviewers state that you have addressed their remarks very well. I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Thank you for submitting your work to Molecular Biology of the Cell.

Sincerely,
Peter Van Haastert
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Karunarathne:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

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We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
Reviewer #1 (Remarks to the Author):

To the authors: Thank you for your attention to details. Your responses are thorough and improve the publication overall. Your work is very interesting.

Reviewer #2 (Remarks to the Author):

The authors have addresses all my concerns and I now full support publication