Regulation by metal ions and the ADMIDAS of integrin α5β1 conformational states and intrinsic affinities

Jordan Anderson, Jing Li, and Timothy Springer

Corresponding author(s): Timothy Springer, Boston Children's Hospital/Harvard Medical School

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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.)

RE: Manuscript #E21-11-0536
TITLE: Regulation by metal ions and the ADMIDAS of integrin α5β1 conformational states and intrinsic affinities

Dear Dr. Springer:

Your manuscript, entitled "Regulation by metal ions and the ADMIDAS of integrin α5β1 conformational states and intrinsic affinities" has been seen by two referees whose verbatim comments are enclosed. Both referees felt that your findings, in principle, would be of interest to our MBoC readership. However, they raised some important points that need to be addressed. We look forward to receiving your revised manuscript and a letter indicating your response to the referees in the near future.

Sincerely,

Diane Lidke
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Springer,

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 haveopted out of publishing the review history.

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In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

The manuscript entitled "Regulation by metal ions and the ADMIDAS of integrin a5b1 conformational states and intrinsic

affinities by Jordan M. Anderson, Jing Li, and Timothy A. Springer examines the granular details of Mn2+ induced integrin affinity regulation. The paper is well written and provides important mechanistic details based on integrin a5b1. The most significant contribution to integrin literature concerns the idea that Mn2+ affects discrete population ensembles. It would be beneficial in the discussion to comment on factors that regulate the selection of integrin populations activated by Mn2+ relative to ones that are refractory to Mn2+ activation. Also, a recent manuscript (PMID: 34650161) incidentally showed that Mn2+ induced higher affinity in 20% of a cell population. Presumably, this activation distribution was applicable to different integrin types. Because the present study focused on the integrin a5b1 model, could the authors comment on the applicability of their study to other integrins?

Reviewer #2 (Remarks to the Author):

Anderson et al. analyze the influence of divalent ions, i.e. magnesium, calcium and manganese, on the conformational ensemble of α5β1 integrin ectodomains, ligand affinity of α5β1 integrin ectodomains and α5β1 integrins on K562 cells. The authors find that manganese decreases the energy barrier between integrin conformations and hence, increases the probability of integrin α5β1 to adopt the active, extended-open conformation. They also show that manganese increases the affinity of α5β1 for RGD peptides in all conformations, manganese and magnesium compete with calcium for the ADMIDAS resulting in an increase of integrin activation, and mutation of ADMIDAS inhibits ion coordination leading to increased intrinsic affinities of the inactive, closed integrin conformations and a decreased intrinsic affinity of the active, open integrin conformation. The findings are interesting, however, their novelty is limited, as assays and approach have been published by the same lab (Li et al.; EMBO J, 2017) and the finding that manganese increases affinity of α5β1 integrin for RGD-containing ligands has been published by Schuhmacher et al. (Sic. Adv., 2021). The analysis of ADMIDAS for integrin activation is novel and of high interest and should receive more emphasis in the manuscript.

Major points:
(1) Put emphasis on ADMIDAS. Furthermore, put own findings into context of the Schuhmacher findings. The two data sets contradict each other. In contrast to the Anderson et al. paper, Schuhmacher and colleagues did not observe significant changes of the conformational ensemble upon manganese addition in single-particle EM approach. As Anderson et al. assumes that Fabs shift the conformational ensemble of integrins to 100% EO, which has to my knowledge not been demonstrated yet, the contradiction and limitations of this approach have to be discussed. Such a discussion is rewarding and interesting for integrin afficionados.
(2) The authors state that "Ca2+ is present in laboratory de-ionized water at concentrations of ~5 to 10 μM" which hampers an accurate titration in Fig. 2. This can be avoided by filtering applied buffers and solutions with chelex. They may try this.
(3) The accuracy of the approach may suffer from ill-defined fits in Fig. 3D (8E3 and HUTS4), 4E, 4F (basal), 5E, F. These fits can and should be improved.
(4) It is not clear whether the ensembles reported in Table 1 describe free ectodomains, as claimed in the text, or ectodomains in presence of RGD peptide, as noted on the side of Table 1. It should be clarified whether ensembles of ectodomains were also measured in the absence of RGD ligand.

Minor points
(1) Move line 104-112 to materials and methods.
(2) Rephrase first paragraph of result section: move FP theory to introduction or remove, explain the motivation for why performing the experiments.
(3) Rephrase line 159-160 "do not conform to the common notion in the integrin field that Mn2+ completely stabilizes the high affinity integrin state". This has already been disproved by Schuhmacher et al. (2021).
(4) Line 171: typo: "the large increase intrinsic affinity".
(5) Line 179: please explain, what the mutant does and what MS is.
(6) Line 207/208: why are the numbers underlined?
(7) Line 242: change a5b1 to α5β1.
(8) Move Fig. 2A to supplement.
(9) Move Fig. 3 to supplement; was shown already in Li et al. (2017) EMBO J.

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Timothy A. Springer, Ph.D.
Latham Family Professor

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Department of Biological Chemistry
and Molecular Pharmacology
Department of Medicine

January 11, 2022

Re: Manuscript #E21-11-0536 Rebuttal

Reviewers,

Thank you for your comments. Please note your comments addressed below:

Reviewer #1 (Remarks to the Author):

The manuscript entitled "Regulation by metal ions and the ADMIDAS of integrin a5b1 conformational states and intrinsic affinities by Jordan M. Anderson, Jing Li, and Timothy A. Springer examines the granular details of Mn2+ induced integrin affinity regulation. The paper is well written and provides important mechanistic details based on integrin a5b1. The most significant contribution to integrin literature concerns the idea that Mn2+ affects discrete population ensembles. It would be beneficial in the discussion to comment on factors that regulate the selection of integrin populations activated by Mn2+ relative to ones that are refractory to Mn2+ activation. Also, a recent manuscript (PMID: 34650161) incidentally showed that Mn2+ induced higher affinity in 20% of a cell population. Presumably, this activation distribution was applicable to different integrin types. Because the present study focused on the integrin a5b1 model, could the authors comment on the applicability of their study to other integrins?

We have revised the paragraph in Discussion beginning with to "It is conceptually important" to address this comment. This paragraph reads in part "Also, "population" is just a measure of the percentage of molecules in a particular state, and does not imply any difference between the molecules other than what conformation they are in. Indeed, it is essential to the validity of our thermodynamic formalism that all of the molecules are equally able to equilibrate from one state to another."

The recent manuscript (PMID: 34650161) cited is not in the mainstream of what we are addressing on integrins in cell adhesion, because it addresses entry by SARS-CoV2. The system is also not well defined, as entry is inhibited both by antagonizing LFA-1, which does not recognize RGD, and by antagonizing RGD-recognizing integrins.

Reviewer #2 (Remarks to the Author):

Anderson et al. analyze the influence of divalent ions, i.e. magnesium, calcium and manganese, on the conformational ensemble of α5β1 integrin ectodomains, ligand affinity of α5β1 integrin ectodomains and α5β1 integrins on K562 cells. The authors find that manganese decreases the energy barrier between integrin conformations and hence, increases the probability of integrin α5β1 to adopt the active, extended-open conformation. They also show that manganese increases the affinity of α5β1 for RGD peptides in all conformations, manganese and magnesium compete with calcium for the ADMIDAS resulting in an increase of integrin activation, and mutation of ADMIDAS inhibits ion coordination leading to increased intrinsic affinities of the inactive, closed integrin conformations and a decreased intrinsic affinity of the active, open integrin conformation. The findings are interesting, however, their novelty is limited, as assays and approach have been published by the same lab (Li et

al.; EMBO J, 2017) and the finding that manganese increases affinity of α5β1 integrin for RGD-containing ligands has been published by Schuhmacher et al. (Sic. Adv., 2021). The analysis of ADMIDAS for integrin activation is novel and of high interest and should receive more emphasis in the manuscript.

Much of our paper is focused on the the ADMIDAS, including both with the WT and ADMIDAS mutant integrin preparations. With respect to "novelty is limited, as assays and approach have been published by the same lab (Li et al.; EMBO J, 2017)", we do not claim that the assays and approaches are novel, so we believe that this criticism if not valid. The reviewer might say that it was not novel for our group to publish that a4b1 and a5b1 had different energy landscapes, because we had already published similar work on a5b1. However, the reviewers of our paper published in JCB, which used the same assays and approaches previously published on a5b1 and extended them to a4b1, together with cell adhesion assays, "Li, J. and T. A. Springer (2018) Energy landscape differences among integrins establish the framework for understanding activation." J Cell Biol 217(1): 397-412", raised no such objections. Assays and approaches only have to be novel for papers reporting new methods. Assays and approaches do not have to be novel to report new findings. One could make the argument that the many structures with GPCR are not novel, because the assays (GPCR binding to ligands) and approaches (crystallography and cryoEM) have already been published, yet many such structures, >50, have already been published in Cell, Nature, and Science. We also disagree that "the finding that manganese increases affinity of α5β1 integrin for RGD-containing ligands has been published by Schuhmacher et al. (Sic. Adv., 2021)." Many authors have made affinity measurements in Mn2+; however, Schumacher et al. made none.

This group only complexed integrins with Fab and ligands in Mg and Mn, and looked at negative stain class averages. They also misreported several findings in the literature, which are discussed in a paper in eLife: Li, J., et al. (2021). "Low affinity integrin states have faster ligand binding kinetics than the high affinity state." eLife **in press**.

Major points:

(1) Put emphasis on ADMIDAS.

As pointed out above, much of our paper is focused on the ADMIDAS, including both with the WT and ADMIDAS mutant integrin preparations.

Furthermore, put own findings into context of the Schuhmacher findings. The two data sets contradict each other. In contrast to the Anderson et al. paper, Schuhmacher and colleagues did not observe significant changes of the conformational ensemble upon manganese addition in single-particle EM approach.

Their result is actually consistent with our own. We found that on cell surfaces, where the integrin TM and cytoplasmic domains can associate, as in the nanodisc system, only a small fraction (4.9%) of the integrin shifted to EO in the absence of ligand. Schumacher found in their Fig. 6 that among class averages from cryoEM, the integrin remained predominantly bent, consistent with our finding that on cell surfaces, integrin a5b1 is 93% bent-closed in Mn (they did not look for small subsets and only showed five class averages). In negative stain EM, Schumacher found a much higher proportion of extended integrins both in Mg and Mn than we find. Both the round nanodisc and the extended integrin are seen en face, as if the integrin extends from the rim of the nanodisc rather than from the membrane-like middle of the nanodisc, where it belongs. This is a common artifact in negative stain EM, where particles orient with the largest surfaces in contact with the grid. Also, like us, in presence of ligand, almost all integrin shifted to the EO state in Mn2+. So our results are highly consistent. We have discussed this with the Naoka Mizuni group.

As Anderson et al. assumes that Fabs shift the conformational ensemble of integrins to 100% EO, which has to my knowledge not been demonstrated yet, the contradiction and limitations of this approach have to be discussed. Such a discussion is rewarding and interesting for integrin afficionados.

This is not an assumption; it has been demonstrated for all of the Fabs used here. When these Fabs bind, the integrin is always in the intended conformation, as shown with multiple EM class averages. It is also demonstrated that those Fabs specific for the EO state induce the same high affinity for ligand; this would vary if the % that they stabilized varied. We have not used TS2/16 in this work, which is rather mysterious and a special case we hope to write up in the next year or two.

We have revised the first paragraph of Discussion to point out the assumptions of our model and why they are valid.

(2) The authors state that "Ca2+ is present in laboratory de-ionized water at concentrations of ~5 to 10 μM" which hampers an accurate titration in Fig. 2. This can be avoided by filtering applied buffers and solutions with chelex. They may try this.

Thanks, this is a good point.

(3) The accuracy of the approach may suffer from ill-defined fits in Fig. 3D (8E3 and HUTS4), 4E, 4F (basal), 5E, F. These fits can and should be improved.

We agree that HUTS4 titration data in Fig. 3D is not well fitted. We redid the fit and updated Fig. 3D. A number of the Fabs showed little effect and as explained in the legends, we did not fit those data. That is why the points in Fig. 3D for 8E3 show no fit curve. The lower affinity of the mutant to linear RGD peptide makes it difficult to accurately determine the affinity in Fig. 4E&F, but only under basal conditions. The data is shown here for direct comparison to Fig. 4B and C, which are measurements on WT. We were careful to not use these values to calculate the energy landscape for the mutant (no population or deltaG values for the ADMIDAS mutant are shown in Table 1), so it does not influence the accuracy of our approach.

Data in Fig. 5E and 5F on basal affinity are well fitted, and they agree well with the measurement shown in Figure 5B and C under basal condition. The basal condition affinities for 12G10 Fab shown in Fig. 5H are the average values from measurements in Panel B and E, or panels C and F, as footnoted. These affinities were determined with two different Fab reporters, and the excellent fits are attested by the agreements between these independent measurements: the means ± the difference from the means are 67±8 and 17.4±2.4.

(4) It is not clear whether the ensembles reported in Table 1 describe free ectodomains, as claimed in the text, or ectodomains in presence of RGD peptide, as noted on the side of Table 1. It should be clarified whether ensembles of ectodomains were also measured in the absence of RGD ligand.

Thank you for pointing this out. We now make this clearer in the Discussion. All of our data report values for the unliganded integrin. The populations that pre-exist before ligand is added determine the affinity that is measured. The ligands added to the side of the Table were only the ligands used to make these measurements. The affinities measured, but not the populations or free energies, depend on the ligand. In the new Table 1, RGD and cRGD are used as subscripts for the Kd values to avoid this confusion.

<u>Minor points</u>

(1) Move line 104-112 to materials and methods.

Our judgement is that the purity of the integrin is important for the validity of our results. We also believe that how the metal ions were exchanged in or out of the integrin preparations makes a difference, as we experienced while attempting to set this system up.

(2) Rephrase first paragraph of result section: move FP theory to introduction or remove, explain the motivation for why performing the experiments.

We believe that the Reviewer's method of presentation has merit, as does the one we chose, which we prefer. This is a matter of style in our opinion. The "motivation for why performing the experiments" has just been explained in the Introduction and does not need repetition.

(3) Rephrase line 159-160 "do not conform to the common notion in the integrin field that Mn2+ completely stabilizes the high affinity integrin state". This has already been disproved by Schuhmacher et al. (2021).

Rephrased as suggested.

(4) Line 171: typo: "the large increase intrinsic affinity".

Thank you, revised.

(5) Line 179: please explain, what the mutant does and what MS is.

Thank you, revised.

(6) Line 207/208: why are the numbers underlined?

Thank you, revised.

(7) Line 242: change a5b1 to α5β1.

Thank you, revised.

(8) Move Fig. 2A to supplement.

We prefer to keep it in main text. The results are completely dependent on the purity of the preparation. If impurities are 50% of the protein, the affinity will go down 2-fold. It takes little room in the main text. It surprises me that many investigators use integrins that they purchase commercially and do not show that they are monodisperse in gel filtration or pure.

(9) Move Fig. 3 to supplement; was shown already in Li et al. (2017) EMBO J.

Only Fig.3A was previously published and we noted that in the figure legend. A in Mg is shown for comparison to results in Mn in D.

Sincerely,

Timothy A Springer, Ph.D.
E-mail: springer@crystal.harvard.edu

RE: Manuscript #E21-11-0536R
TITLE: "Regulation by metal ions and the ADMIDAS of integrin α5β1 conformational states and intrinsic affinities"

Dear Dr. Springer:

Thank you for revising your manuscript in response to the referees' recommendations. I have read the revised manuscript carefully along with your responses to the referees and find that you have addressed their major concerns. I am pleased to accept your manuscript for publication in MBoC. Congratulations to you and your colleagues on this insightful study.

Sincerely,
Diane Lidke
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Springer:

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.

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