[redacted]

Sent:
To:
Subject:

[redacted]

M8:01166

[redacted]

Dear Dr.

Your paper, [redacted] 1D and is reduced in laminin alpha2 chain [redacted] has been reviewed by a member of the Editorial Board with the assistance of three expert referees, and their critiques are enclosed. Unfortunately, the reviewers did not recommend publication of the paper in the Journal of Biological Chemistry. The reviewers felt that the identification of Cib2 as a binding protein for integrin alpha7Bbeta1D is interesting, but that the study does not provide underlying mechanistic information. Two reviewers felt that key findings in figure 4 experiments are compromised by the detergent extraction methods used to isolate integrins. Three reviewers note problems with lack of correlation between Northerns and RT-PCR data and the variability of the GAPDH controls in some of the experiments. One reviewer felt that more direct data are needed to define in vivo interactions of the integrin and Cib2. Another reviewer felt that the muscle types used need to be defined, that protein specific reagents in mouse are needed to verify conclusions and that controls are needed for the peptide binding experiments. Other points for clarification are noted by the reviewers. For these reasons, the Journal must decline the paper at this time.

Thank you for allowing the Journal to review your work.

Sincerely,

[redacted]

Comments for author:

Critique #1

The authors present evidence that Cib2 is a calcium binding protein that interacts with alpha7Bbeta1D integrin. However there are no data to show the putative functional consequences of this interaction and no analysis of the mechanism(s) that underlie that potentially altered function.

Critique #2

Laminins have been shown to be ligands for the alpha7beta1 integrin, and

2008-06-10

We are resubmitting our manuscript [redacted]
for publication in *Journal of Biological Chemistry*.

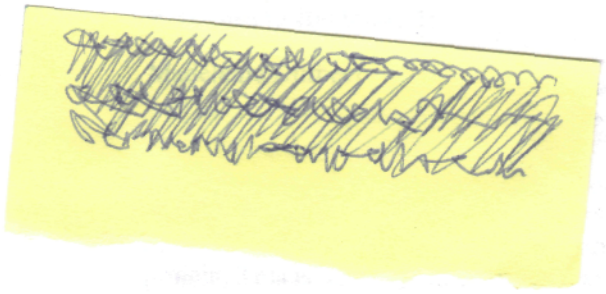
We would like to thank you and the reviewers for the valuable comments on our manuscript. Although the reviewers found the questions at issue interesting and important, each reviewer did list several concerns. We have now carefully considered the reviewers' comments and have specifically addressed each issue raised, both in the manuscript and in the "Response to reviewers". We have also added new data to satisfy the questions raised by the reviewers. For example, three of the reviewers noted lack of correlation between Northern blot and RT-PCR data and the variability of the GAPDH controls in Figure 1. Since the GAPDH mRNA expression in fact is quite variable between tissues, we have instead monitored ribosomal RNAs 28S and 18S. Moreover, we now show that the Northern blot and RT-PCR data are congruent (by novel semi-quantitative RT-PCR experiments; new Figure 1A) and we have added a Supplemental Figure (2) in which quantitative real-time PCR was used to clearly show that Cib2 mRNA is mainly expressed in skeletal muscle. Two of the reviewers felt that the key findings in Figure 4 were compromised by the detergent extraction methods to isolate integrins. To address this, we have added a Supplemental Figure (3), in which we show that the integrins extracted clearly bind to laminin-111 (which does not bind Cib2). Thus, the extraction method does not accidentally affect the activity of integrins. We have also shown that a control peptide (α7A) does not bind Cib2 (new Figure 4D). Furthermore, we have developed antibodies that recognize mouse Cib2 and verify the gene expression data at the protein level. We also demonstrate that the expression of Cib2 and integrin α7B subunit overlaps, which provides additional support for an interaction between Cib2 and integrin α7B (new Figure 3A and 5B-C).

In the pages that follow, we have summarized our changes to the manuscript and responded to each of the reviewer's comments point by point.

[redacted] Telephone Int. +4[redacted]5

We feel that the changes made have significantly improved the manuscript, and hope that it is now acceptable for publication. We look forward to hearing your decision on this matter.

Best Regards,



Response to Reviewer 1:

We have now revised the paper extensively with major additional experimentation.

Response to Reviewer 2:

1. The reviewer asks why laminin α2 chain mRNA is only reduced 2.65 fold as shown in the microarray analyses (and not completely absent). It has been shown by Guo et al. (2003) that dy^{3K}/dy^{3K} animals have no detectable laminin α2 chain, in contrast to other mouse models for laminin α2 chain deficiency (dy^{W}/dy^{W}, *dy/dy*). Thus, it appears that the remaining mRNA in dy^{3K}/dy^{3K} animals (which may not be correctly spliced) does not to encode a protein. This is now explained in the text.

2. The reviewer had concerns about Figure 1. First, the RT-PCR data does not always correlate with the Northerns. The PCR-reaction shown in Figure 1A was not quantitative. Now, we have included a semi-quantitative RT-PCR where the Cib2 PCR reactions were run for 24 cycles and clearly muscle contains most Cib2 mRNA and lower amounts are seen in brain and lung (new Figure 1A). We have also performed quantitative LightCycler PCR and again we show that the major tissue of Cib2 mRNA expression is muscle, whereas lower amounts are seen in brain (Supplemental Figure 2). These data are now matching the Northern blot data.
We agree that the GAPDH mRNA expression is variable. In fact, GAPDH mRNA is differentially expressed in many tissues. Therefore, it might not be an adequate loading control. We have repeated the Northern blot analysis. To ascertain equal loading and RNA integrity, 18S and 28S rRNAs were monitored instead (new Figure 1C).
The reviewer was concerned about the specificity of the human Cib2 antibody used for the IF image and suggested the production of a mouse antibody to compare Cib2 in WT and dy^{3K}/dy^{3K} muscles. We have now generated Cib2 antibodies that work well in mouse tissues. In a new Figure 3A, we demonstrate that Cib2 is expressed at the sarcolemma and enriched at the neuromuscular- and myotendinous junctions. These are all locations where integrin α7B is also enriched (see co-expression of Cib2 and integrin α7B in Figure 3). In the same figure, we show that Cib2 expression is reduced in dystrophic laminin α2 chain deficient muscle. The same antibodies are also used in Western blot analyses (new Figure 5B and 5C) to demonstrate that the Cib2 gene encodes a 22 kDa protein that is reduced in laminin α2 chain deficient muscle.

3. The reviewer thought that the key findings in Figure 4A were compromised by the detergent extraction methods to isolate integrins. To address this, we have added a Supplemental Figure 3, where it is shown that the integrins extracted (α7A, α7B, β1A, β1D) clearly bind to laminin-111 (which *per se* does not bind Cib2) as expected. Thus, it does not seem that the extraction method accidentally affects activity of integrins. We have

clarified in the figure legend 4B that it is saturation, not sigmoid appearance that helps to establish significance of binding. In Figure 5D, we have shown that a peptide corresponding to α7A does not bind Cib2 specifically. Hence, the data presented in panel D matches data presented in panel A.

Response to Reviewer 3:

1. The reviewer wonders if the authors can explain why no transcriptional change in integrin α7 expression was detected in the expression profiling considering that previous studies have demonstrated a significant decrease in integrin α7 gene expression in laminin α2 chain deficient muscle. By quantitative real time PCR we have previously noted an approximately 2-fold upregulation of integrin α7B mRNA in muscles of dy^{3K}/dy^{3K} mice. A similar upregulation was also noted at the protein level by Western blot analyses. Intriguingly, integrin α7B can not be detected at the sarcolemma of dy^{3K}/dy^{3K} muscles despite this upregulation (Gawlik et al., 2006). Notably, the microarray analyses confirm the previous findings that integrin α7 mRNA is in fact upregulated in dy^{3K}/dy^{3K} muscles. However, in the paper, we only present differentially expressed genes with an average fold change > 2 and integrin α7 mRNA appears to be upregulated approximately 1.8-fold.

2. The reviewer asks us to explain why a strong Cib2 transcript was detected by RT-PCR in heart and not by Northern blot analysis. Actually, 34 cycles were used for the RT-PCR analysis, which is considered to be too many cycles for quantification. Therefore, we have shown by semi-quantitative RT-PCR (24 cycles) that Cib2 mRNA is mainly found in muscle. Lower amounts were seen in brain and lung (new Figure 1A). We have also performed quantitative LightCycler PCR and again we show that the major tissue of Cib2 mRNA expression is muscle whereas lower amounts are seen in brain (Supplemental Figure 2). These data now match the Northern blot data. Moreover, since the GAPDH mRNA expression is quite variable between tissues, we have repeated the Northern blot analysis and instead monitored 18S and 28S rRNAs (new Figure 1C).

3. We have removed the previous Figure 1E and instead we present protein data with antibodies that work in mouse. In a new Figure 3A, we demonstrate that Cib2 is expressed at the sarcolemma and enriched at the neuromuscular- and myotendinous junctions (NMJ and MTJ, respectively). We also show that Cib2 expression is reduced in laminin α2 chain deficient muscle. The same antibodies are also used in Western blot analyses (new Figure 5B and 5C) to demonstrate that the Cib2 gene encodes a 22 kDa protein that is reduced in dy^{3K}/dy^{3K} muscle.

4. The reviewer asks us to explain why the GAPDH control is so light in previous Figure 3B. We changed this figure (now Figure 3C), and show the X-ray film of a membrane that had been exposed longer and stronger GAPDH signals are thus visible.

It has been shown that integrin α7 mRNA expression is increased (approximately 3-fold) in mdx muscle and the reviewer finds it surprising that Cib2 expression is not increased in mdx muscle. The increase in integrin α7 mRNA expression can be attributed to a similar increase in both α7A and α7B expression (Hodges et al. 1997). Thus, it is reasonable to speculate that there is an approximately 1.5 fold increase in integrin α7B expression in mdx muscle and a similar increase of Cib2 expression could therefore be expected. However, it is difficult to quantify a 1.5-fold increase by Northern blot analyses. We have tried to quantify the Cib2 mRNA expression in mdx muscle by real time LightCycler PCR but the results are inconclusive.

5. We now show co-localization of Cib2 and integrin α7B at the sarcolemma, NMJ and MTJ. These data provide additional support for an interaction between Cib2 and integrin α7B.

6. The reviewer suggests that a quantitative graph should be included and statistical tests applied to demonstrate changes in Cib2 expression between WT and dy^{3K}LNα1TG muscles. We have now compared Cib2 protein expression in WT, laminin α2 chain deficient and laminin α1 chain rescued animals (dy^{3K}LNα1TG). We show that Cib2 expression is significantly reduced in dy^{3K}/dy^{3K} muscles and that there is no significant difference in Cib2 expression between WT and dy^{3K}LNα1TG muscles (new Figure 5B-C).

7. The reviewer asks whether Cib2 is expressed in myoblasts and the answer is yes and this is indicated in the discussion section ("Cib2 is indeed expressed in myoblasts; data not shown"). Also, we note a slight increase in Cib2 mRNA expression when myoblasts differentiate to myotubes (this data is not presented in the paper).

Minor concerns:

1. We now write that loss of integrin α7 leads to myopathy (and not dystrophy as previously stated).

2. All loading controls have been defined (e.g. Rps18, ribosomal protein S18).

3. Sm was changed to skm in Figure 1.

4. We have included the references Vachon et al., 1997 and Hodges et al., 1997.

Response to Reviewer 4:

1. We now more clearly indicate (in Experimental Procedures) that all hindlimb muscles were used when comparing gene expression in dy^{3K}/dy^{3K} vs. WT muscles. The dy^{3K}/dy^{3K} animals are severely growth retarded and in order to be able to generate sufficient amounts of RNA for the microarray analyses (and Northern blot analyses), all hindlimb muscles were needed.

The reviewer asks us to comment on the modest decline in laminin α2 chain mRNA expression as shown in the microarray analyses (and why it is not completely absent)? It has been shown by Guo et al. (2003) that dy^{3K}/dy^{3K} animals have no detectable laminin α2 chain, in contrast to other mouse models for laminin α2 chain deficiency (dy^{W}/dy^{W}, *dy/dy*). Thus, it appears that the remaining mRNA seen in dy^{3K}/dy^{3K} animals (which may not be correctly spliced) does not to encode a protein. This is now explained in the text.

We have now included a Supplemental Figure 1 in which we demonstrate the results of hierarchical clustering of samples. The clustering dendogram revealed that WT samples comprised a distinct subgroup, whereas dy^{3K}/dy^{3K} samples were subgrouped together.

2. The reviewer had concerns about Figure 1. First, the RT-PCR data does not always correlate with the Northerns. The PCR-reaction shown in Figure 1A was not quantitative. Now, we have included a semi-quantitative RT-PCR where the Cib2 PCR reactions were run for 24 cycles and clearly muscle contains most Cib2 mRNA and lower amounts are seen in brain and lung (new Figure 1A). We have also performed quantitative LightCycler RT-PCR and again we show that the major tissue of Cib2 mRNA expression is muscle and lower amounts are detected in brain (Supplemental Figure 2). These data are now matching the Northern blot data.
We agree that the GAPDH mRNA expression is variable. In fact, GAPDH mRNA is differentially expressed in many tissues. Therefore, it might not be an adequate loading control. We have repeated the Northern blot analysis. To ascertain equal loading and RNA integrity, 18S and 28S rRNAs were monitored instead (new Figure 1C).

The reviewer suggested the production of a mouse antibody to compare Cib2 in WT and dy^{3K}/dy^{3K} muscles. We have now generated rabbit Cib2 antibodies that work on mouse tissue. In a new Figure 3A, we demonstrate that Cib2 is expressed at the sarcolemma and enriched at the neuromuscular- and myotendinous junctions. These are all locations where integrin α7B is enriched (see co-expression of Cib2 and integrin α7B in Figure 3). In the same figure, we also show that Cib2 expression is reduced in dystrophic laminin α2 chain deficient muscle. The same antibodies are also used in Western blot analyses (new Figure 5B and 5C) to demonstrate that the Cib2 gene encodes a 22 kDa protein that is reduced in laminin α2 chain deficient muscle.
3. The reviewer asks why a complete developmental series is not shown for the *in situ* hybridization study. We have now done whole-mount *in situ* hybridization on E12.5 embryos and the Cib2 mRNA expression is very

similar to the expression seen in E13.5 embryos. This finding is presented as "data not shown". Whole-mount *in situ* hybridization in embryos older than E14.5 is more difficult to do. We tested whole-mount *in situ* hybridization on E16 embryos but no results were obtained. Cib2 mRNA is mainly expressed in skeletal muscle and brain in the adult mouse and previous whole-mount *in situ* analyses show that developing CNS and muscle also are the main sites of expression in the developing embryo. Thus, we feel that *in situ* hybridization analyses of all developmental stages will not provide additional information that would significantly improve the paper.

4. We agree with the reviewer that we do not present data to support the notion that calcium binding properties of Cib2 might relate to aberrant calcium homeostasis in muscular dystrophy. However, our intentions were to show that 1) Cib2 binds calcium and 2) that Cib2 expression is not affected in other mouse models of muscular dystrophy. Nevertheless, we have slightly re-written this section. Whether Cib2 is involved in regulation of Ca^{2+} signals in muscle might be revealed by the generation and characterization of mice lacking Cib2 (ongoing studies).

5. In Figure 3B (now 3C) it was not clear to the reviewer which muscles were used in the Northern blot analyses. We now clarify in the figure legend that all hindlimb muscles were used.

6. The reviewer thought that the key findings in Figure 4 were compromised by the detergent extraction methods to isolate integrins. To address this, we have added a Supplemental Figure 3, where it is shown that the integrins extracted (α7A, α7B, β1A, β1D) clearly bind to laminin-111 (which *per se* does not bind Cib2) as expected. We also address the concern about lack of control peptides in the tryptophan fluorescence measurements. In Figure 5D, we show that a peptide corresponding to α7A does not bind Cib2 specifically. Hence, the data presented in panel D matches data presented in panel A.

7. It was unclear to the reviewer what the point of the experiment in Figure 5 was. Integrin α7B is reduced at the sarcolemma of dy^{3K}/dy^{3K} muscle, just like Cib2. However, upon transgenic expression of laminin α1 chain, integrin α7B chain is reconstituted. Thus, we reasoned that Cib2 should also reappear if Cib2 indeed interacts with integrin α7B. We previously showed this at the RNA level and in a new Figure 5B-C, we confirm this finding at the protein level.