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The B cell antigen receptor signals through a preformed transducer module of SLP65 and CIN85

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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 15 March 2011

Thank you for submitting your manuscript to the EMBO Journal. I have now heard back from two of three reviewers who I had asked to review the paper. I am still waiting for a third report, but given the present recommendation I can make a preliminary decision now to save time. This decision is still subject to change should the third referee offer strong and convincing reasons for doing so.

As you can see below, both referees find the analysis interesting. Referee #1 raises a number of relative minor concerns while referees #2 has 2 concerns that has to be addressed before further consideration here. The first concern pertains to the SILAC analysis and the second one concerns figure 4 where more precise quantitation is needed. Should you be able to address the concerns raised then we would consider a revised manuscript. I would like to ask you to start thinking about making the requested changes and additions to the manuscript that would render the paper suitable for publication in the view of these two reviewers. It is EMBO Journal policy to allow a single major round of revision only and that is therefore important to address the concerns raised at this stage. I will forward the comments of the third referee to you as soon as I receive them, together with our final editorial decision.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

best wishes

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In this manuscript Oelerrich et al. have identified the in vivo interactome of the Slp65 adaptor molecule that is crucial for B cell receptor signaling. They successfully applied a novel technique, SILAC (stable isotope with amino acids in cell culture) in resting and activated chicken DT40 B cells, in combination with mass spectrometry. They mainly focus on the constitutive association of Slp65 with the CIN85 adaptor protein, dependent on "atypical" SH3 motifs in Slp65. Although the interaction between the two proteins was described before, this manuscript presents a major conceptual advance. The authors provide evidence that the association (i) is BCR-stimulation independent, (ii) is essential for Slp65 phosphorylation, and (iii) is required for BCR Ca++ flux and NF-kappaB activation. They nicely show that R-to-A mutations within the Slp65 SH3 motifs preclude Slp65 membrane translocation. Importantly, they go on to demonstrate that when Slp76, the T cell homologue of Slp65 (that cannot substitute for Slp65 in B cells and does not have the "atypical" SH3 motifs) is equipped with these CIN85-binding motifs it becomes phosphorylated and B cells mount a robust Ca++ response.

This is an elegant study, especially because of the identification of the interactome of about 30 proteins of Slp65, which is a central adaptor molecule in BCR signalling. The experiments are well-conducted and the paper reads well. Particularly, their focus on the kinetics of the interactome, distinguishing transient, BCR-dependent interactions and constitutive binding, provides new insight in the field of BCR signal transduction. I only have a couple of minor comments.
1. In Figure 1B-D the authors show kinetics of interaction for most of the molecules identified in their interactions screen. It is not clear to me, why important signalling proteins, such as Syk, Btk and IgH chain binding protein are missing.
2. It would be nice if the authors would include a schematic drawing of the structure of Slp65, CD2AP and CIN85 in a suppl. Figure.
3. Figure 2D shows that also Grb2 interacts with the "atypical" SH3 motifs. Although the binding is found to be weaker, this does not mean that it is not important. It may depend on protein availability and kinetics. In the subsequent experiments this finding is ignored. It is conceivable that disrupted Grb2 binding contributes to e.g. the absence of Ca++ flux of NfkappaB induction. This should be discussed. Is Grb2 required for Slp65 phosphorylation?
4. In Figure 3E, which nicely shows the quantification of the effects of R-to-A mutation within the Slp65 SH3 motif, Syk and Btk are missing. Since these are crucial molecules for BCR signalling, it would be important to include them.

Referee #2 (Remarks to the Author):

This is an important study that demonstrates the importance of an interaction involving SLP65 and CIN85 in DT40 B cells in the basal state as well as subsequently BCR-activated state. The studies are well conceived and, in general, support the authors' conclusions. There are two issues that the author should address related to: (1) the technical aspects of their SILAC analysis and subsequent interpretation and (2) confocal analysis shown in figure 4.

1. SILAC analysis. I may have missed the data, though I could not find it in the text, figures or supplemental figures. The authors should report the labeling efficiency in SILAC medium achieved with both heavy and light conditions. Even though the authors are labeling the same clone, labeling efficiency of heavy and light under similar conditions can result in differential efficiency and provide misleading results.
2. Figure 1. The authors normalize the fold change from basal levels and categorize the associated proteins into three categories- early ligands, late ligands and steady ligands. I presume that these analysis were not performed utilizing AQUA based peptide comparisons (as these were not

described in the Methods). As such, these analysis are not very precise in quantitation. This should be pointed out in the text unless the authors have performed quantitative mass spectroscopy.
3. Figure 4- The authors should perform more precise quantitation of cytosolic to membrane-associated pixel intensity as many of the panel shown are not convincing in the primary pdf files to support membrane translocation.

Additional Correspondence 17 March 2011

We have now received the third report on your study and I have provided it below. As you can see Referee #3 finds the analysis interesting, but s/he also raises a number of different concerns with the analysis. In particular further data is need to support the significance of the reported SLP65/CIN85 interaction. Lot of work is needed, but I suspect that you have the tools on hand to address many of the raised concerns. Please also take into consideration the concerns listed in the general comments such as does loss of CIN85 affect SLP65 function?

I would thus like to confirm our decision and ask you to submit a suitably revised manuscript for our consideration.

Best wishes

Editor
The EMBO Journal

REFEREE REPORT

Referee #3

SLP65 is a scaffold protein playing a crucial role in BCR-proximal signaling. However, it remains unknown how SLP65 is recruited to the plasma membrane immediately after BCR stimulation, how the complex formed by SLP65 with other proteins is spatially organised, and its location relative to the BCR. Ideally, knowledge of all SLP65 interacting partners in space and time before and after BCR stimulation should help answer those questions.

Towards this goal, Oellerich et al. have attempted to define the dynamic interactome of SLP65 using SLP65-deficient DT40 chicken B cell line reconstituted with tagged-SLP65. Proteins interacting with SLP65 before and after activation were detected using SILAC-based quantitative mass spectrometry. This approach identified a number of proteins, including the adaptors CIN85 and CD2AP, equally associated with SLP65 before and after stimulation, while others (e.g., PLCg2 and Vav3) interacted with SLP65 only after stimulation. Although few new partners were found, the authors focused on the adaptor proteins CIN85 and CD2AP. These two similar adaptors contain three SH3 domains in series, a proline-rich region and a C-terminal coil-coiled region that mediates oligomerization. Constitutive association between CIN85 with SLP65 was already reported in 2000 by Watanabe et al., who provided in vitro evidence for CIM85 SH3 domains mediating the binding, likely via atypical proline-arginine (PxxxPR) motifs of SLP65. CIN85 (and CD2AP) have been reported to bind to > 100 proteins and because they form oligomers they may tend to associate with several partners at once. Some of these interactions have been proven to be direct. However, some interactions are suspected to be indirect while the authenticity of others has been questioned (due to poor specificity of SH3 domains and overexpression experiments). Thus, any interaction with CIN85 (and CD2AP) should be treated with caution and studied carefully to assess whether it is direct and real. CIN85 and CD2AP are often seen bound to proteins associated to membranes (endocytotic and recycling) and plasma membrane and/or to the cytoskeleton. They have been implicated in regulating membrane remodelling, associated with vesicle-mediated transport, adhesion and migration (in lamellipodia and filopodia). However, given CIN85 and CD2AP binding promiscuity, implication in other cellular functions cannot be excluded.

To understand the functional significance of this interaction, SLP65-deficient DT40 cells were used here to express SLP65 wt or a SLP65 mutated at three proline-rich sites (R-to-A mutant) supposed to bind to CIN85 and CD2AP. The results show that these motifs can mediate such an interaction.

Importantly, DT40 cells expressing this mutant showed dramatic a defect in BCR-induced SLP65 phosphorylation, loss of most interactors and their tyrosine phosphorylation (including, Grb2, PLCg2 and Vav3), calcium rise and NFkB. Other correlative data are presented using confocal and TIRF microscopy in SLP65-deficient cells (primary mouse B cells and DT40) in an attempt to support the claim of a functional SLP65-CIN85/CD2AP association. However, no evidence by fluorescence microscopy is shown of co-localization of SLP65 with CIN85 or CD2AP. Moreover, no evidence is reported to show the effect of CIN85 deficiency (e.g., by siRNA silencing) on SLP65 function (e.g., phosphorylation, association to various components).
From these data the authors deduce that CIN85 could be the adaptor that recruits SLP65 to the membrane in proximity of the BCR and it is required for SLP65 phosphorylation by Syk. The work is interesting and original in proposing this new mechanism for SLP65 recruitment. Technically, the experiments are well performed, especially the SILAC proteomics, with only some minor defects and lacunae in data presentation (see below). However, the experiments remain correlative and a close look at the evidence does not allow drawing unambiguous conclusions as to the role of CIN85 binding to SLP65. The weakest point of this investigation is that by introducing all three R-A mutations in SLP65 does not allow to evaluate if interactions with other partners (e.g., directly or due to local disturbancy of the mutation) have been disrupted, leading to defective SLP65 recruitment and phosphorylation. If CIN85 were the mediator of SLP65 membrane recruitment and/or phosphorylation one would expect that most SLP65 is associated to it. The work does not provide this important piece of information. Moreover, from all the evidence presented, it is uncertain whether the two molecules associate in the cytoplasm or at the plasma membrane or both

Considering at all the data, it remains possible that SLP65 and CIN85 may associate for other raisons and not necessarily for membrane recruitment. In this respect, the work does not provide solid evidence for a mechanism as to how SLP65 associates to the plasma membrane to be phosphorylated by Syk.

The following critics/suggestions are addressed to the authors.
1 - Figure 1A-D. Was the level of tagged-SLP65 expressed in SLP65-defective DT40 cells comparable to that of wt DT40 cells? This is not mentioned and it is important as overexpression of SLP65 could generate spurious interactions.

2 - Figure 1E and D. These figures show the SLP65-CIN85 (and CD2AP) interaction only in B cell lines. However, this should be shown in primary B cells as well. The evidence for an interaction of SLP-65 with CIN85 is in J558L is made feeble by the presence of identical but weaker bands with control Ab ip and also by the fact that there is no evidence that using anti-CIN85 one can co-precipitate SLP65. Also, in Ramos cells the SLP65-CIM85 association is not shown. For clarity, all these experiments should be shown.

3 - Figure 2B. Does the level of SLP65-GFP match physiological levels of expression?
Although the data show that the R-to-A mutant does not bind, it is important to exclude that those interactions are not driven by overexpression.

4 - Figure 2D. The peptide used for pool-down does not contain the R-to-A mutations that were shown throughout figure 2 being important for the binding.

5 - Figure 3A. The authors should ascertain the effect of mutations of the individual PxxxPR site and see if they affect binding of Vav3 and PLCg2... One critical point is the fact that Grb2 binding is affected by the mutation in the three atypical SH3 binding sites. Does this mean that also Grb2 may bind or that such mutation affects local stability of SLP65 and this affects interactions with PLCg2 and/or Vav3?

6 - What is the temporal relationship between SLP65 translocation and phosphorylation.
Translocation by confocal is recorded at 3 min, quite late as compared to the phosphorylation that happens within less than a minute.

7 - Commenting the experiment in Figure 4A the authors claim that "in both types of B cells, wild-type SLP65 quantitatively translocated from the cytosol to the plasma membrane in response to BCR activation". This is unlikely as it is well established that only a fraction of signaling effectors is used at a given time under normal stimulatory conditions.

Concerning Figure 4A the images shown weakly support the claim that activation results in CIN85-mediated SLP65 translocation to the plasma membrane. The panel of wt in primary B cells shows only two cells; in the lower one it is possible to see a speckle-type pattern at the plasma membrane before stimulation while in the other SLP65 is apparently diffused with little discernible membrane association. Does this mean that part of SLP65 is already recruited to the membrane? After 3 min stimulation one cell shows a better signal/noise (most cytoplasm background - free-SLP65? - seem to have dramatically decreased). However, in the other cell it is difficult to say if there has been an induced translocation, the background before stimulation being very high and the speckle-type pattern at the membrane is hardly visible. Can the author provide a better image?

In the R-to-A panel, one sees two cells; one with very low fluorescence in which nothing happens. The second cell instead shows some weak but visible reinforcement of signal at the plasma membrane. In the DT40 panel one can see a weak but definitive reinforcement of the fluorescence in the R-to-A panel. This same panel shows (upper part at 3 min) some bits of membrane with definitive strong fluorescence. Does the mutation result in a quantitative change in SLP65 translocation?
This way of representing imaging data is not adequate and may be misleading. Rather a quantification of the images should be provided. The authors should classify various patterns and provide statistics on a minimum number of cells (e.g. 50). With the current software capacity in imaging this should be not a problem to such an experienced group.

8 - Figure 4. CIN85 and CD2AP have two different patterns in unstimulated cells.
CD2AP seems in good part to be contained in vesicles in the cytoplasm and not at the PM as claimed by the authors.

9 - The authors state, "In the case of CIN85, deletion of the three SH3 domains trapped the molecule in the cytosol (Figure 4D)". However, Figure 4C and D cannot be compared. There is very high total fluorescence in DT40 expressing the CIN85ΔSH3 mutant (over-expression?) that could not allow seeing the actual pattern of the mutant distribution. This is indeed the case for CD2APΔSH3, which is not diffused by still show a speckle-type pattern and surprisingly shows some increased membrane distribution after stimulation. Indeed, in all this analysis a rigorous comparison should be done only using cells that express reasonably comparable expression levels of the constructs.

10 - Figure 5 and movies. Co-localization analysis. In addition to showing representative image (by indicating the time at which they were taken (12 min?) the authors should show quantification of colocalization. Each colocalized pixel can be plotted on a scatter diagram for producing correlation plots with colocalizing pixels falling around a diagonal line and calculate the Pearson's correlation coefficient (Rr) as mean {plus minus} SE p values (two-tailed) determined with Student's t test. What is missing is one evidence in intact live cells that CIN85 (or CD2AP) co-localises with or directly interact (FRET experiments) with SLP65.

Minor points

1 - The notion of preformed complexes put forward by Wienands and Reth states that once expressed on the surface, the BCR organizes protein tyrosine phosphatases, PTKs, and their substrates into a transducer complex that can be activated by pervanadate in the absence of BCR crosslinking. As a consequence signaling complexes are partially and dynamically pre-assembled ready to form larger and more stable ones. However, this is not the case for the association of SLP65 and CIN85 and CD2AP because such activities exist even in absence of tyrosine phosphrylation and BCR expression. The authors should temperate a claim for such a parallel.

2) It will be interesting to discuss the transient kinetics of protein complex formation in conjunction with known kinetics of protein phosphorylation; reported kinetics (Figure 1B) resemble that of tyrosine phosphorylation.

3) Two biological replicates were carried out to define the reported kinetics of complex formation. It is not easy to find in the supplementary list which list corresponds to which experiment. Lists should be better annotated for clarity. It is crucial to report in a figure the variability observed between biological replicates so that the reader has the sense of appreciation and judgement; e.g. kinetics

from both experiments of some binders should be shown.

4) Page 9, 'reverse proteomics' is unclear and likely misleading.

5) Figure 1 legend: two series of activation time points with resting cells as common time point for normalization are not two 'approaches' but simply two complementary experiments to obtain continuous kinetics.

In conclusion, the evidence provide does not convincingly supports the role of CIN85 (or CD2AP) for SLP65 translocation to the plasma membrane. Could instead such an interaction be relevant for recycling/internalization of SLP65 together with the BCR?

1st Revision - authors' response 16 May 2011

On behalf of all authors I thank the reviewers for critical evaluation of our manuscript about the identification of the SLP65 interactome and the key role of the ligand CIN85 as an integral part of the BCR transducer complex. We were pleased to read that the reviewers 1 and 2 judged our work as "an elegant study" that is "well conceived" and "presents a major conceptual advance". As outlined in detail below, we have addressed all comments of reviewers 1 and 2. We also rectified most of the concerns of reviewer 3 but some of which we disagree with.

Response to reviewer #1

ad 1. We could not analyze the interaction kinetics of some SLP65 ligands such as Syk and Btk in our triple SILAC approach although these interaction partners were detected before in our conventional SILAC approach. The main reason for those gaps is the increased sample complexity in experiments with three mixed samples of differentially labeled proteins (instead of two in the “conventional” SILAC approach) that increases the number of peptides eluting into the MS because even a single peptide species is represented by three isotopically labeled "isoforms". During the mass spectrometric analysis, the MS instrument always selects the most six abundant peptides to be sequenced in each scanning round irrespectively whether these are labeled or non-labeled peptides. As a consequence only the highly abundant fractions of labeled and non-labeled peptide pairs are predominantly selected for subsequent sequencing within the MS whereas peptides from ligands that only transiently or weakly interact (for example Syk and Btk) are less abundant and unfortunately are not necessarily selected by the instrument. Currently this instrument-based *per se* can not be circumvented and this limitation is also described in other proteomic approaches. We thank the referee for pointing out this discrepancy that indeed requires an explanation. Accordingly, we have described this in the manuscript.

ad 2. A schematic drawing of the domain architecture of SLP65 and CD2AP/CIN85 adaptors will be indeed helpful to the reader. They are now depicted in Figure 2 A and B, respectively.

ad 3. We agree that although binding of Grb2 to the atypical SH3 binding motifs in SLP65 was found to be weaker than that of CIN85 or CD2AP, the Grb2 ligand might be functionally relevant "dependent on protein availability". We have included this valid statement in the Result section as well as in the Discussion. In fact, this statement is supported by our newly added gene targeting experiments in which expression of CD2AP and CIN85 was ablated or reduced, respectively (see new Figure 6). In this situation, the observed residual Ca^{2+} mobilization might be supported by increased binding of Grb2 whose SH3 domains may partially substitute a conformational impact that is otherwise provided by the SH3 domains of CIN85 (or CD2AP). In the physiological situation, Grb2 is not required for SLP65 phosphorylation (Stork et al. (2004) *Immunity*, Stork et al. (2007) *EMBO J.*).

ad 4. Lack of quantification of Syk or Btk binding to the R-to-A mutant of SLP65 is due to the same technical limitations of the MS instrument described above (see ad 1).

Response to reviewer #2

ad 1. We agree that labeling efficiency is a key issue in our SILAC-based interactome analysis. The original supplementary table 1 provided the raw MS data demonstrating that the labeling efficiency of proteins derived from cells that were grown in medium containing amino acids with heavy isotopes was almost 100%. However, it is indeed somewhat challenging to extract this information from the EXCEL file. We are sorry for this inconvenience. We have now described the labeling efficiency in the result section and provide a separate Figure (supplementary Figure 1) to illustrate that fact. Briefly, the MS spectra of supplementary figure 1, reveal that in the case of affinity-purified SLP65 only the "heavy" peptide (peptide containing amino acids with incorporated stable isotopes) was visible and no signal of a light peptide could be detected. The same was true for all SLP65 peptides. These quantitative MS results proof unambiguously that exclusively heavily labeled SLP65 was purified. Moreover, the absence of any signal derived from light peptides further proof that SLP6 is labeled almost to 100 % (if not completely at all) with amino acids that contain the stable isotopes (^{13}C and ^{15}N). The same scenario appears for a peptide derived from the known interaction partner of SLP65, i.e. Grb2.

ad 2) The statement that we did not utilized AQUA-based peptide comparisons is correct. As established by the Gygi lab the AQUA-based strategy is for absolute quantification and is mainly used for determination of i) total amount of a protein in a sample (in terms of mol) and/or ii) for determination of protein stoichiometries of proteins (or protein copy numbers) within a protein complex. Importantly, we have not performed an absolute quantification in our study. We have preformed a so-called relative quantification in which the amount of one protein in sample A (e.g. non-stimulated cells) is compared to the–amount of the same protein present in sample B (e.g. stimulated cells). This approach of relative quantification is highly accurate (in particular as metabolic labeling via. SILAC has been applied). In this manner, normalization of the quantitative MS data from all the experiments of the different time points was performed with the software MaxQuant upon referring to the control, i.e. non-stimulated.

ad 3. We have now quantified our microscopic images and included the corresponding bar diagrams into Figure 4. In fact the statistic data support our conclusions even more strikingly. We thank the reviewer for her/his recommendation. Note, that we refrained from providing a statistic calculation for the retroviral transfection experiments with primary SLP65-ngeative B cells for two reasons. First, *slp65*$^{-/-}$ mutant mice possess only few mature splenic B cells as a result of severely compromised B cell development (Jumaa et al., 1999). Second, the efficiency of retroviral transduction experiments is generally weak and further reduces the number of cells are available for the microscopic analysis. Together these limitations resulted in a very low number of positive transductants which complicated a statistical calculation. We would like to emphasize the fact that the images presented are of course representative.

Response to reviewer #3

Response to general comments

The reviewer is correct in that *"although few new partners* [of SLP65] *were found the authors focused on the adaptor proteins CIN85 and CD2AP"*. In fact the comprehensive and unbiased identification of the SLP65 interactome was a main objective of our study. Another central aim, however, was the identification of BCR-proximal signal effector molecules that couple BCR ligation to SLP65 activation and hence are integral components of the primary BCR transducer complex. Based on our kinetic interaction analysis, the steady SLP65 ligands CIN85 and CD2AP appeared likely candidates to fulfill such a function. As described, we therefore concentrated our functional analysis on these two ligands.

It is correct that Watanabe *et al.* (2000) reported on the *"constitutive association between CIN85 and SL65 ... likely via atypical proline-arginine (PXXXXPR) motifs of SLP65"*. We have acknowledged that finding by referring to the original publication throughout the manuscript. As the reviewer cites these data and in light of our numerous biochemical, genetic, imaging and finally mass spectrometric data that confirm this interaction, we wonder why she/he nonetheless requests

additional experiments such as FRET to demonstrate the CIN85/SLP complex once another time. However additional data are included into the revised version of our manuscript (see below).

We agree with the reviewer that CIN85 and CD2AP *"bind >100 proteins"* and *"have been implicated in regulating membrane remodelling, associated with vesicle-mediated transport, adhesion and migration (in lamellipodia and filopodia)",* and *therefore, "implication in other cellular functions* [than those reported in our study] *cannot be excluded"*. At no point in our manuscript we have excluded this possibility but we believe that it is beyond the scope of single publication to test a putative role of the SLP65/CIN85 complex in all these cellular processes.

We strongly disagree with the reviewer's statement *"no evidence by fluorescence microscopy is shown of co-localization of SLP65 with CIN85 or CD2AP"*. TIRF microscopy and corresponding movies (Figure 5) demonstrate that following BCR activation SLP65 as well as CIN85 translocate into BCR microcluster. Hence both proteins (and to a lesser degree CD2AP) reside in the same subcellular plasma membrane compartments.

The reviewer comments that no RNA interference experiments had been conducted. Although this issue is not explicitly taken up again or even requested in the reviewer's specific comments, we generated CD2AP-deficient B cells by gene targeting and reduced CIN85 expression in wild-type as well as in the *cd2ap*$^{-/-}$ mutant cells. Investigating the BCR signaling capacities of these cells directly supported our conclusion. The data have been incorporated into the revised manuscript (Figure 7).

The reviewer states that *"the weakest point of this investigation is that by introducing all three R-A mutations in SLP65 does not allow to evaluate if interactions with other partners ... have been disrupted"*. In fact, we directly assessed this issue by our Reverse Proteome approach in which we determined the complete interactome of the R-to-A mutant in B cells by SILAC-based quantitative mass spectrometric (Figure 3E). The reviewer may have overlooked this part of the manuscript, but to our best of knowledge such an approach to quantitatively investigate how a given mutation in a cellular protein affects the complete network of its interacting ligands has not been reported to date.

The reviewer argues that *"if CIN85 were the mediator of SLP65 membrane recruitment and/or phosphorylation one would expect that most SLP65 is associated to it"*. Conversely, she/he later comments on our microscopic recruitment studies in that *"it is well established that only a fraction of signaling effectors is used at a given time under normal stimulatory conditions"*. These are contradictory statements. What signaling scenario does she/he thinks is correct?

We fundamentally disagree with the statement that *"from all the evidence presented, it is uncertain whether the two molecules associate in the cytoplasm or at the plasma membrane or both"*. With our mass spectrometric approach as well as in all of our biochemical binding studies (including co-immunoprecipitation experiments) we directly and unambiguously show that SLP65 that resides in the cytosol of resting B cells (see microscopic imaging) associates with CIN85. Our quantitative mass spectrometric data show that in activated B cells no loss of SLP65 ligands occurs and TIRF microscopy directly supports an association of SLP65 with CIN85 at the plasma membrane. Hence, we do not accept the reviewer's concern.

We also fundamentally disagree with the final conclusion of the reviewer, *"considering at all the data, it remains possible that SLP65 and CIN85 may associate for other raisons* (means 'reason'?) *not necessarily for membrane recruitment"*. She/he may have overlooked the complete Figure 6 of the manuscript (which is Figure 7 of the revised version). It shows that transfer of the CIN85 binding sites confers membrane translocation to the T cell molecule SLP76 and subsequently enables the engineered SLP76 molecule to participate in BCR signal transduction. We do not exclude that the SLP65/CIN85 complex performs several functions (see above) but subcellular targeting of SLP65 is clearly one of which.

Response to specific comments

ad 1) We agree that the expression level of tagged SLP65 is an important aspect. As determined by immunoblot analysis and as shown in Figure 1 of the revised manuscript, tagged SLP65 was expressed by the reconstituted cells in similar amounts compared to endogenous SLP65 in wild-type-cells. This is also described in the text.

ad 2) Despite the fact that a CIN85/SLP65 interaction had been described by Watanabe *et al.* in 2000 and in light of the large body of evidence that this complex is present in various types of B cells (see above) the reviewer asks for additional co-immunoprecipitation experiments in additional cell lines and primary B cells. The later issue is indeed valid and we now demonstrate co-immunoprecipitation of endogenously expressed wild-type proteins in primary B cells (see novel Figure 3A of the revised manuscript). We do not see that additional co-immunoprecipitation experiments add further information to this topic. In particular we are not aware of any example that for demonstrating a given protein-protein interaction co-immunoprecipitations were requested to be conducted in 'both orientation' i.e. in our case with anti-SLP65 antibodies as well as with anti-CIN85 antibodies. We have confirmed the direct interaction between CIN85 and SLP65 using recombinantely expressed proteins (see novel Figure 2B, right panel).

ad 3) As mentioned above we agree that expression levels are critical issues of signaling studies. However the expression level of a GFP fusion protein (here SLP65-GFP) can not be compared to that of the endogenous protein (here SLP65) as the two versions possess distinct molecular masses and hence discrete molarities. It is thus impossible to determine molar expression rates of the proteins by conventional immunoblot analyses. In such cases the critical control experiment is to separately express wild-type and mutant fusion proteins and to confirm their equal expression by the corresponding transfectants. This necessary control was indeed performed in our study (see Figure 2B, left panel).

ad 4) The comment that *"the peptide used for pool-down* (should read 'pull-down') *does not contain the R-to-A mutations"* is correct. This does however not interfere with our conclusions as in both, the biochemical and genetic experiments, the wild-type sequences of the SH3 domain-binding sites were always included as control. We assume that the reviewer accepts this set-up because she/he does not comment further on that issue.

ad 5) The requested experiment *"to ascertain the effect of mutations of the individual PxxxPR site and see if they affect binding of Vav3 and PLCg2"* had been already performed and was incorporated into the original manuscript (Figure 3E). The reviewer may have overlooked this fact. Her/his question about the role of Grb2 is answered in detail in the Result as well as in the Discussion part of the manuscript.

ad 6) The *"temporal relationship between SLP65 translocation and phosphorylation"* is indeed a so-far weakly understood signaling aspect of SLP65 as well as of other cytosolic effector proteins in immune and non-immune cells. As mentioned by the reviewer, maximal SLP65 phosphorylation can be measured earlier by biochemical techniques than maximal membrane anchoring visualized by imaging techniques. However that does not necessarily mean that phosphorylation precedes translocation as the two techniques to measure these processes are different. We assume that the reviewer is aware of this technical limitation because she/he does not comment further on that issue.

ad 7) In our experiment depicted in Figure 4A wild-type SLP65 quantitatively translocated from the cytosol to the plasma membrane in response to BCR activation of primary as well as DT40 B cells". The reviewer's comment that *"this is unlikely as it is well established that only a fraction of signaling effectors is used at a given time under normal stimulatory conditions"* is unacceptable and an inappropriate judgment of experimental data. First of all, such a general statement is incorrect. Secondly, nobody has ever quantified that process for SLP65. Thirdly, this claim contradicts the reviewer's own assumption stated earlier that *"if CIN85 were the mediator ... one would expect that most SLP65 is associated to it"*. Lastly and most importantly, questioning the scientific accurateness of presented experimental data by such a general believe is not a scientific argument.

ad 8) We appreciate the reviewer's comment that *"CIN85 and CD2AP have two different* [staining] *patterns in unstimulated cells"*. This conclusion is in line with our conclusion that the adaptors seem to perform different cellular functions.

ad 9) We have now provided statistical quantification of the images which support our conclusions. We have exchanged the upper panel of figure 4D to present cells that express lower levels of the CIN85 mutant. Again there was no CIN85 membrane recruitment detected in the absence of its SH3 domains

ad 10) The reviewer may have missed that quantifications of the colocalization studies shown in Figure 5 were already included into the original manuscript according to the standards of TIRF experiments and as described in numerous publication before (for examples see Treanor et al. (2010) *Immunity*, **32**:187-199, Depoil et al. (2008) *Nat Immunol*, **9**:63-72).

Response to minor points

ad 1) The reviewer instructs us on our own publication that provided genetic evidence for the existence of preformed signaling complexes in B cells, and in turn wants us to temperate our notion that the CIN85/SL65 complex is a part of that machinery. We have compared our newly described findings to those published in Proc. Natl. Acad. Sci., USA (1996) and see no reason to weaken our conclusion that the CIN85/SLP65 module resembles a preformed BCR transducer complex.

ad 2) A discussion on "the transient kinetics of protein complex formation in conjunction with known kinetics of protein phosphorylation" is indeed interesting. As stated by the reviewer the basic conclusion is that "reported kinetics (Figure 1B) resemble that of tyrosine phosphorylation." That correlation had been pointed out in our original manuscript.

ad 3) To determine the kinetics of SLP65-mediated complex formation we performed three independent SILAC-based experiments. We have calculated the standard deviations of each SILAC ratio (representing the fold change in figure 1) for most of the binding partners at the given time points. The standard deviations are outlined in supplementary table 2. For a few interactors we could not calculate the standard deviations as we have determined their SILAC ratios only in two independent experiments. The reason for that is the high complexity of the samples in triple SILAC experiments. Due to this fact the mass spectrometer is not able to identify all peptide species as it selects the six most abundant peptides to be sequenced per scanning round. This is a common limitation of large-scale quantitative proteomic analyses.

ad 4) The term Reverse Proteomics has been clearly defined in the text as a novel and comprehensive proteomic method to determine the effect of a given mutation on the composition of the interactome of the protein-of-interest. Thus there is no reason to change this nomenclature.

ad 5) In the legend to Figure 1 the term 'approaches' has been changed to 'complementary experiments'.

2nd Editorial Decision 20 June 2011

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original three referees to review the revised manuscript and I have now received the comments back. While referee #1 and 2 are supportive of the study, referee #3 raises significant concerns with the analysis. After further discussion with the other two referees, I have come to the conclusion that the remaining issues can be addressed with appropriate text changes in the article file or in the point-by-point response as you see fit. If you have data on hand to address point #2 (-one important point is also that ...) you can include that, but no further experiments are needed at this stage.

Once we receive the revised version, we will proceed with its acceptance here.

Thank you for submitting your interesting study to the EMBO Journal.

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

It is an obvious limitation that important interactions, as those by Syk and Btk, cannot be studied in the triple SILAC technique used (related to my comments 1 and 4). Nevertheless, many interactions can be analysed with this method and the authors have now stated this point clearly in the manuscript.
The experiments described in Figure 6 (with B cells in which CD2AP is absent or CIN85 expression is reduced) are a valuable addition.
Taken together, I feel that the authors have sufficiently addressed my concerns.

Referee #2 (Remarks to the Author):

The authors have addressed all of my concerns

Referee #3 (Remarks to the Author):

I have re-examined very carefully this manuscript, the rebuttal letter and the modification added to respond to my suggestions/critics.

Nobody can deny that the authors have carefully confirmed and consolidated previous data of CIM85/CD2AP interacting with SLP65. However, it still appears that a rigorous evidence of a direct interaction of SLP65 and CIM85/CD2AP is lacking and as well as a consistent demonstration that CIM85/CD2AP allow SLP65 recruitment and phosphorylation at the plasma membrane after BCR stimulation.

- In vitro pool-downs with peptides or purified proteins do not prove a direct interaction via an SH3/proline motive, for the reasons I had already mentioned in my previous comments.

-One important point is also that Fig 3A lacks immunoprecipitation with anti-SLP65 of SLP65 mutant followed by probing with anti-CIN85. Also, the evidence that mutation of the two C-terminal docking sites abolishes binding to the SH3 domains of CIN85 and CD2AP (to correlate with the calcium defect) is not shown (page 9 of the manuscript). Moreover, it is unclear if the authors here refer to pool-down experiments or to SLP65-deficient cells reconstituted with this mutant.

- Partial co-localization of CIM85 with the BCR clusters (as shown in Fig. 5) does not mean co-localization with SLP65. Co-localisation in live cells is not shown. And, as I pointed out in my comments to the previous version, the imaging data of Fig. 4 and 5 render the picture of CIM85/CD2AP role with respect to SLP65 function, at the most suggestive of some correlation, but quite confusing (the representative images are not very convincing).

- The authors have misinterpreted my comments " if CIN80 were the mediator of SLP65...." . I meant to say that to allow an effective recruitment to a subcellular localization of a partner by an adaptor, the latter should bind to it at high stoichiometry (e.g., SLP76 and Gads). This is not demonstrated in the present work for CIM85/CD2AP and SLP65 and may raise concerns as to the actual role of CIM85/CD2AP in SLP65 function. Thus, only a minor fraction of the SLP76-Gads complex is recruited to LAT (as I intended to say as a logical concept and generalize to other complexes) but the stoichiometry of the SLP76-Gads association must be high; otherwise the only traces of SLP76 will be recruited to LAT. I hope this is clear. May be the authors should correct their wording as quantitatively means completely.

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- The new added data, namely, the correlation between absence of CIN85 or CD2AP or both using siRNA do not well corroborate the data of the SLP65 3xarginine mutant. BCR-induced calcium rise is only partially abolished when correlated with the apparent high degree (unfortunately, not quantified) of double knockdown. Also, one would expect to see data that the double-knockdown affects also SLP65 tyrosine phosphorylation.

The role of the three proline motives individually should be better understood with more thorough studies. With these data alone, one cannot exclude that the SLP65 proline motifs serve for its recruitment to the BCR and phosphorylation but via another mechanism that does not include CIM85/CD2AP.

Again, the data concerning the direct interaction of SLP65 with CIM85/CD2AP and its potential role in regulating SLP65 membrane recruitment and Syk-mediated phosphorylation are correlative and lack rigorous demonstration.

Unfortunately, I still find this work quite weak and correlative and not adequate for publication in EMBO J., as I pointed out already in my previous comments.

2nd Revision - authors' response 04 July 2011

Response to reviewer #3

ad 1. The reviewer still doubts that the interaction between CIN85 and SLP65 is direct. However she/he has apparently overlooked that the new experiment shown in Figure 2B (left panel) was performed with **isolated** SLP65 and GST-CIN85$(SH3)_3$ proteins. Hence, this approach is not a pull-down experiment with B cell lysates but a common method to measure direct protein-protein interactions. The data shown in Figure 2B provide definitive proof that the interaction between SLP65 and CIN85 is a direct one.

ad 2. According to the original request of reviewer #3 we have successfully performed co-immunoprecipitation experiments of CIN85 and SLP65 in primary B cells, and incorporated the results in the revised version of our manuscript (inlay of figure 3A). Most notably, we co-immunoprecipitated the endogenously expressed proteins from wild-type primary B cells. Reviewer #3 now criticizes that we have not additionally immunoprecipitated the mutant version of SLP65 from primary B cells (figure 3A). First, loss of CIN85 binding upon inactivation of the atypical SH3 binding sites has been thoroughly proven by numerous biochemical as well as mass spectrometric approaches and the data were readily accepted upon the first submission of our manuscript. The latter also holds true for the analysis of individual SH3 binding sites in SLP65. Second, the functional analysis of SLP65 mutants in primary B cells requires retroviral transduction which is a difficult technique that is well known to work with low efficiency. In this approach the efficiency is reduced even further owing to the low number of B cells in SLP65-negative mouse mutants. The number of primary B cell transductants is sufficient for flow cytometric or microscopic imaging approaches but generally insufficient for biochemical experiments. In summary the experiment shown in Figure 3A resembles a classical genetic approach to investigate a given mutation in primary B cells. The results are complementary to and consistent with all other data in our manuscript.

ad 3. We wonder why reviewer #3 states that CIN85 only partially colocalizes with BCR microcluster. The time lapse video and the quantification of TIRF microscopic imaging readily shows that the colocalization is almost complete. The same holds true for SLP65. Hence both proteins colocalize with BCR microcluster. As BCR microcluster do not exist in different forms with distinct compositions of signaling elements our live cell imaging approach provides convincing evidence for subcellular colocalization of CIN85 and SLP65. These data are fully consistent with and complementary to our mass spectrometric and biochemical approaches including co-immunoprecipitation of the endogenously expressed proteins from wild-type primary B cells (see

above). Hence, we have demonstrated complex formation between SLP65 and CIN85 with all the currently available techniques.

ad 4. We do not understand as to how predictions about the LAT/SLP76/Gads complex in T cells relate to the CIN85/SLP65 complex in B cells. Why should we test that speculations about the LAT/SLP76/Gads complex in T cells apply to the CIN85/SLP65 complex in B cells (for example the reviewer's assumption about the high stoichiometry)?

ad 5. Reviewer #3 expects that *"double-knockdown* [of CIN85 and CD2AP] *affects also SLP65 tyrosine phosphorylation".* Using a number of biochemical and genetic approaches we have already shown that the steady SLP65/CIN85 complex is instrumental for SLP65 phosphorylation. Moreover, our analysis of the signaling capacity of CIN85/CD2AP-compromised B cells measured BCR-induced Ca^{2+} mobilization that is **downstream** of SLP65 phosphorylation and hence the more meaningful and more significant functional read-out.

