THE EMBO JOURNAL

Manuscript EMBO-2012-81959

Secretome Protein Enrichment Identifies Physiological BACE1 Protease Substrates in Neurons

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Review timeline:

Submission date:	04 May 2012
Editorial Decision:	29 May 2012
Revision received:	04 June 2012
Accepted:	05 June 2012

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 29 May 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. You will be pleased to see that all three referees are very positive and support publication after minor revision. I would thus like to invite you to submit a revised version of the manuscript that addresses the points put forward by the referees in an adequate manner.

In addition, there are two editorial issues that need further attention:

* Please include an author contributions section into the main body of the manuscript text right after the acknowledgements section.

* As a new initiative, we now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

I should add that it is EMBO Journal policy to allow only a single round of revision and that acceptance of your manuscript will therefore depend on the completeness of your responses in this

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

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

Kuhn et al. report a novel proteomics strategy to identify the "secretome" of primary cells such as neurons. The method is based on "click" chemistry that metabolically labels sugars on glycoprotein ectodomains that are secreted or shed and subsequent pull-down following biotinylation. Using this approach, the team identified a number of novel candidate BACE1 substrates in primary cultured neurons treated with a BACE1 inhibitor or that were genetically null for BACE1. Seizure-protein 6, L1, CHL1 and contactin-2 were among the major novel BACE1 substrates identified and were validated in brains of BACE1 inhibitor-treated and BACE1 knock-out mice. These proteins have roles in neurite outgrowth and synapse formation and suggest that BACE1 may regulate their function through proteolytic processing.

This report is significant because it not only allows better understanding of BACE1 function and the phenotypes of BACE1 knock-out mice, but it also is useful for predicting potential mechanism-based toxicity of therapeutic BACE1 inhibition for AD and for developing biomarkers for monitoring these side-effects in clinical trials. Overall, the data are robust and the manuscript is well written. I have only a few minor comments:

1. A few previously identified BACE1 substrates in neurons did not emerge from the current analysis. It is possible that some may have been below the level of detection, e.g, neuregulin, as discussed by the authors. However, others are likely abundant in neurons, such as LRP and the beta subunit of the voltage gated sodium channel. It would be informative for the authors to comment why they think BACE1 cleaved ectodomains from these abundant neuronal proteins may have escaped detection in their assay.

2. Please include the key to the shading of the histogram bars in Figure 3B.

3. Please describe the procedure used to synthesize LY-2811376

4. Please define STE (Brain Fractionation Methods)

5. Sigma antibody T7451 recognizes acetylated alpha tubulin, not beta tubulin.

Referee #2

Kuhn et al describe a novel and innovative approach to probe cell surface shedding. Using metabolic incorporation of click-reactive sugars, their approach removes the need for serum free cell cultivation in order to probe secreted or shed proteins. The authors use this approach to investigate shedding by the BACE1. Selected BACE1 substrates are validated in vivo.

Cell surface proteolysis is a highly important process. However research in this field has been impeded by the need for serum-free cell cultivation for the generation of cell conditioned medium. Serum starvation alters cellular behavior; hence the approach by Kuhn et al is a major technical advance and opens a plethora of novel possibilities for the analysis of shed and secreted proteins.

The authors cleverly designed their strategy based on previous work, e.g. by the group of Bertozzi. In the present work they identify a number of established or novel BACE1 substrates. Both the number of protein identifications and stringency of protein quantification might be improved in future applications; in the present work however the authors provide sufficient, biologically significant data to demonstrate the usefulness and broad applicability of their approach.

This is an important work which should be published by the Journal. My only criticism relates to the length of the discussion: the discussion section should be considerably shortened.

Referee #3

Based on the technical limitations of prior techniques - low detectability; contamination with albumin, other serum proteins, and cytosolic proteins; incompatibility of serum-free or protein-free medium with culture systems; and mislocalization of gene overexpression - the authors developed a novel technique for quantitative proteomics of cell culture supernatants using secretome protein enrichment with click sugars (SPECS). The SPECS technique bypasses the need to overexpress specific secretases, which often leads to false positive target identification, and allows the use of serum proteins and/or albumin in culture medium for culturing primary neurons. The authors used SPECS to identify novel, physiological BACE1 substrtes in primary neurons, some of which (seizure-protein 6, L1, CHL1, and contactin) were then validated in the brains of BACE1 inhibitor-treated and BACE1 knock-out mice.

While there are some limitations to the technique, which are appropriately noted by the authors, the novel SPECS technique allows for the identification of a high proportion of physiological sheddome peptides by mass spectrometry and within 6 days (2 days of metabolic labeling, 2 days of processing, and 2 days of mass spectrometric analysis).

The authors were thorough in their use of the SPECS technique to study well-known BACE1 substrates (APP, APLP1, APLP2), as well as to validate novel BACE1 substrates that were identified through this assay (seizure-protein 6, L1, CHL1, and contactin) in the brains of BACE1-knockout and BACE1 inhibitor-treated mice.

The manuscript is well written, easy to understand and read, and represents a novel contribution of the SPECS technique to the field. The authors were thorough in the use of appropriate controls and in discussion of the limitations and interpretability of the technique. Overall, the manuscript is interesting, complete, and of high quality. My only minor criticism is that several of the Western blots in Figure 3A (most notably APPfl Lys, sAPLP2 DEA, APLP2 Lys, sAPLP1 DEA) are of relatively low quality. If higher quality blots are available, then the authors ought to replace these within the panel.

1st Revision - authors' response 04 June 2012

Thank you very much to all three reviewers for the very encouraging reviews. All three reviewers requested only minor changes. In the following we give a point-by-point response to each of the reviewers.

Reviewer 1

Overall, the data are robust and the manuscript is well written. I have only a few minor comments:

1. A few previously identified BACE1 substrates in neurons did not emerge from the current analysis. It is possible that some may have been below the level of detection, e.g, neuregulin, as discussed by the authors. However, others are likely abundant in neurons, such as LRP and the beta subunit of the voltage gated sodium channel. It would be informative for the authors to comment why they think BACE1 cleaved ectodomains from these abundant neuronal proteins may have escaped detection in their assay.

In fact, we did detect the ectodomain of LRP1 in the conditioned medium of the neurons. However, in one of the five biological replicates there was one strong outlier – probably due to technical limitations. As a result the standard deviation for LRP was so large that LRP did not meet our stringent criteria for inclusion into the hit list. Given this limitation, we decided not to discuss LRP in the manuscript. The data, however, are given in supplementary table 5 (line 281).
In the SPECS method a 30 kDa cut-off filtration step was used, such that ectodomains with a mass lower than 30 kDa are lost. The ectodomain of voltage gated sodium channels is below this cut-off, giving a possible explanation for why this protein was not detected by SPECS. This information is now included in the discussion on top of page 13. In future experiments, the use of filters with a lower molecular weight cut-off should enable detection of the voltage gated sodium channels.

2. Please include the key to the shading of the histogram bars in Figure 3B.

The key to the shading has been included into revised figure 3B.

3. Please describe the procedure used to synthesize LY-2811376

We included into the methods' section the reference to the patent by Eli Lilly (WO2009134617) where the synthesis of LY2811376 is described in detail (page 55, example 5). This is exactly the protocol used in our study for the synthesis.
In the meantime the compound is also commercially available, for example at
http://www.sun-shinechem.com/cp_view.asp?id=1383
http://www.pharm-intermediates.com/info/1194044-20-6.html

4. Please define STE (Brain Fractionation Methods)

This has been included into the methods' section. The new sentence (page 16) reads: "Pellet was lysed in STE buffer (150 mM NaCl, 50 mM TRIS, 2 mM EDTA)". Thus, STE stands for Sodium chloride, TRIS, EDTA.

5. Sigma antibody T7451 recognizes acetylated alpha tubulin, not beta tubulin.

This has been changed accordingly in the methods' section (page 15) and now reads: "mAb Acetylated tubulin (Sigma Aldrich, T7451)".

Reviewer 2

This is an important work which should be published by the Journal. My only criticism relates to the length of the discussion: the discussion section should be considerably shortened.

The discussion has been considerably shortened (by about one page). In particular in the last part of the discussion we deleted the background information on SEZ6 and contactin-2 (which was partly redundant with the results' section) and the speculative part on the role of BACE1 in the function of both proteins.

Reviewer 3

My only minor criticism is that several of the Western blots in Figure 3A (most notably APPfl Lys, sAPLP2 DEA, APLP2 Lys, sAPLP1 DEA) are of relatively low quality. If higher quality blots are available, then the authors ought to replace these within the panel.

We replaced the sAPLP1 panel in Figure 5 by one of a better quality. All other blots are of the same quality as the ones displayed. The original blots from the different experiments are found in the "source data" file. In particular for APLP1 and APLP2 the available antibodies against the ectodomain (to detect soluble APLP) are the limiting factor for blot quality, in particular when brain samples are used.

Additional change
We replaced Figure 3C with new pictures from the same kind of experiment. Instead of only showing the contactin-2 staining, the new panel also shows staining of nucleus (TOPRO) and of neuronal processes (Tau) and, thus, is more informative than the old pictures. The main message is exactly the same as before: inhibition of BACE1 increases cell surface levels of contactin-2.

