Supplementary Information

Amphipathic motifs in BAR domains are essential for membrane curvature sensing

Vikram Kjøller Bhatia\textsuperscript{1}, Kenneth L. Madsen\textsuperscript{2}, Pierre-Yves Bolinger\textsuperscript{1}, Andreas Kunding\textsuperscript{1}, Per Hedegård\textsuperscript{3}, Ulrik Gether\textsuperscript{2}, Dimitrios Stamou\textsuperscript{1}\textsuperscript{*}

\textsuperscript{1} Bio–Nanotechnology Laboratory, Department of Neuroscience and Pharmacology & Nano–Science Center, University of Copenhagen, Universitetsparken 5, 2100 Copenhagen, Denmark

\textsuperscript{2} Molecular Neuropharmacology Group and Center for Pharmacogenomics, Department of Neuroscience and Pharmacology, The Panum Institute, University of Copenhagen, Denmark

\textsuperscript{3} Nano–Science Center, Niels Bohr Institute, University of Copenhagen, Denmark

\textsuperscript{4} Present address: Credit Suisse, Uetlibergstrasse 231, 8045 Zürich ZH, Switzerland

*To whom correspondence should be addressed. E–mail: stamou@nano.ku.dk
Supplementary Information

Statistical analysis of fractional binding data
As described in the main text, the liposomes do not admit proteins immediately after the proteins are added to the solution. There is a lag time of several minutes. Once the proteins decide to bind to a given liposome, however, the binding process is very fast. The lag time depends on the size of the liposome and the concentration of proteins. This is illustrated in figure 1. By dividing the number of liposomes of a given size having proteins after 60 minutes with the total number of liposomes of that size, we get a measure of the probability that a liposome has admitted proteins after the 60 minutes. We see, that the larger the liposome the higher is that probability.

Figure 1. A) The diagram shows the size distribution of liposomes, size being measured by the liposome diameter. B) The diagram shows the number of liposomes having admitted proteins after 60 minutes as a function of liposome size. C) The ratio of number of liposomes with proteins to the total number of liposomes is shown as a function of liposome diameter. Intuitively the shape looks like a parabolic function. The data are taken for a protein concentration of 40 nM.

In order to obtain more quantitative results we have performed a Bayesian statistical analysis. This proceeds as follows. First, we model the lag time with simple rate kinetics: the probability that a given liposome with no proteins at time \( t \) will admit proteins in the next \( \Delta t \) seconds is given by \( \gamma \Delta t \). The rate \( \gamma \) is a function of liposome size and of protein concentration. With this, the probability, that a given liposome labelled \( i \) has no proteins after a time \( t \) is given by

\[
P_i(t) = e^{-\gamma_i t}.
\]

where \( \gamma_i \) is the rate for that liposome, while the probability that it has admitted proteins is

\[
1 - P_i(t) = 1 - e^{-\gamma_i t}
\]

The data, \( D \), of the experiment is a number of liposomes with known diameters, some of which have proteins after 60 minutes in a given number of different protein concentrations. We are going to consider models for the rate of the form;

\[
\gamma_i = k r_i^\alpha c^\beta,
\]

where \( c \) is the concentration of proteins, \( r_i \) is the radius of the liposome, and the powers \( \alpha \) and \( \beta \) and the constant \( k \) are to be determined in the experiment.
Straightforward application of Bayes' theorem now gives, that the probability of a
given model with specific parameters given the data, is given by

\[ P(M(\alpha, \beta, k)|D) \propto \prod_{ia} (1 - P_i(t)) \prod_{in} P_i(t), \]

where the first factors, labelled \(ia\), are from the liposomes having admitted proteins, while
the latter factors, labelled \(in\) are from liposomes without proteins.

We have data for a number (several hundreds) of liposomes for 4 different protein
concentrations (4 nM, 40 nM, 400 nM and 4000 nM). In figure 2 is shown a surface plot of
the probability of different values of the two parameters powers \(\alpha\) and \(\beta\).

![Figure 2. Surface plot of the probability distribution P(M(\alpha,\beta)|D), given the data.](image)

We see, that the most probable values for \(\alpha\) is very close to 2, while the most probable
value for \(\beta\) is very close to 0.50 . The value for \(\alpha\) is not surprising, since this implies that
the rate is proportional to the surface area of the liposome. This suggests that whatever the
process initiating the admission of proteins is, it has to be some local process on the surface
of the liposome. More surprising, is the result for the power \(\beta\). The found value indicates a
square root dependence on protein concentration, however the significance of this
discovery remains open for future studies.
**Discrepancies with existing literature**

To the best of our knowledge there are three types of discrepancies with existing literature. First, we find that several BAR domain proteins (aNBAR, eNBAR and oFBAR) are capable of sensing curvature in our SLiC assay. Previously published results using a pull down assay (Peter *et al.*, 2004; Gallop *et al.*, 2006; Henne *et al.*, 2007) have shown that these BAR domains are incapable at sensing membrane curvature. However, these results were measured at membrane curvature inducing conditions – thus the contradicting results are explained by the differences in the assay conditions.

Secondly, other proteins such as Centaurin β2, Oligophrenin, SNX1 and SNX2 have been shown to be sensitive to membrane curvature due to the presence of a BAR domain (Peter *et al.*, 2004; Carlton *et al.*, 2004; Carlton *et al.*, 2005). In agreement with this we measure that Centaurin β2 and SNX1 are capable of sensing membrane curvature. However, we conclude that sensing is due to the presence of a putative AH or other hydrophobic insertion motifs and indeed our bioinformatics search resulted in the identification of N-terminal peptide sequences for all proteins revealing amphipathic helical nature (Figure S7). Our interpretation is further supported by the recent finding that SNX9 contain an AH (Pylypenko *et al.*, 2007) and that Arfaptin binding to liposomes is not due to a BAR domain, but rather due to a peptide stretch preceding the BAR domain (Peter *et al.*, 2004).

Third, previous studies have shown that deletions of NBARs that removed the N-terminal AH to isolate the BAR domain are capable of sensing membrane curvature (Peter *et al.*, 2004; Gallop *et al.*, 2005). We have tried repeatedly and under different conditions to purify such deletion constructs ourselves and reproduce these experiments. We never succeeded in purifying aBAR. We succeeded in purifying eBAR but our data, in contradiction to the results of Gallop *et al.* (Gallop *et al.*, 2005), showed the eBAR is not a sensor of membrane curvature. Attempts to purify more deletion constructs of BAR domains (arfaptin, PICK1) showed systematically these constructs to be unstable after GST cleavage. We caution against their use and therefore decided to proceed with point mutations that clearly did not affect the solubility of the proteins. Another lab has reported similar problems (Nie *et al.*, 2006).
Supplementary Figures

Figure S1

S1. Membrane curvature sensing using the SLiC assay. 
(A) Single immobilized fluorescent liposomes are imaged using confocal fluorescence microscopy. Most particles appear as diffraction limited spots. 
(B-C) Close-up and surface plot of liposomes. Liposome size is calculated by tracking every particle and integrating its intensity. Scale bars are 10, 2 and 1 µm respectively. 
(D) Histogram of liposome sizes for a sample extruded at 800 nm. A bulk pulldown assay of such a sample includes severe ensemble averaging error. Approx. 90 % of the liposomes are below 300 nm.
S2. Membrane sensing and deformation by eNBAR.
(A) At certain conditions, e.g. low concentration eNBAR is only a curvature sensor. Thus no deformation is observed at 400 nM. (B) At certain conditions, e.g. high concentration the sensor can turn into a membrane deformer. We observe that membrane deformation happens at concentrations close to the BAR dimer $K_d$ estimated to be $\sim 10 \mu M$ (Gallop et al., 2006). At 6.4 $\mu M$ eNBAR we see a small fraction of liposomes starting to deform (red arrows). Black arrows indicate no change. Scale bars are 4 $\mu m$. 

Figure S2
Figure S3

S3. Membrane sensing curves for eNBAR at different concentrations. (A-B) Concentrations 40, 400 and 4000 nM with dbl. exp. fits to guide the eye. Increasing the concentration increases the density of protein bound to the liposomes. (C) Log-log plot of figure 1F. We record identical membrane curvature sensing for eNBAR at different concentrations. To compare quantitatively sensing at different concentrations we extract the a-value (slope from the linear fit) following (Hatzakis et al., 2009) and observe that all sensing graphs collapse and within uncertainty can be fitted to the same value. Typically, the uncertainties of the fits are 0.1 and between experiments 0.2 - the average a-value for all concentrations was 1.3 ± 0.25. (D) Membrane curvature sensing fits measured for all eNBAR concentrations, protein density increases as a function of concentration. By extracting the concentration dependent density values for one particular diameter (gray columns), binding curves for a particular curvature can be achieved. Values from a diameter of 100 nm, 200 nm and 650 nm is extracted from the graph and plotted in Figure 3A.
S4. Cryo TEM images and oligo-lamellar test of brain lipid liposomes used in this study. (A-C). Under the preparation condition used, the unilamellarity of the liposomes is strongly favored. (D) No multilamellar and a low percentage ~10% of liposomes containing 1-2 smaller liposomes are present. Scale Bars are 100 nm. (E) Quenching assay adapted from (Mcintyre and Sleight, 1991; Meers et al., 2000) to determine amount of accessible bilayer in the bulk liposome solution. Quenching of outer bilayer is achieved using collisional quenching (KI) of lipidprobe DiI(5)c18ds. (F) Fluorescence of liposome solution before and after addition of quencher (Q), close to complete quenching of outer bilayer (0.56) was achieved which upon calculation translates into 88 % of the theoretical possible available lipid mass. Thus, the 12% that are un-quenched are in excellent agreement with the ~10% oligo-lamellarity reported by means of CryoTEM.
S5. Folding of endophilin N-helix and helix mutant F10E.
It has previously been established that disruption of the N-terminal amphipathic helix is crucial in terms of liposome binding and tubulation (Farsad et al., 2001; Masuda et al., 2006). We measured by Circular Dichroism (CD) if the F10E mutation disrupts helix formation in the presence of liposomes and verify that this mutation disables the N-part of the NBAR. CD spectra of rat endophilin A1 N-helix (residues 1-33) and helix F10E in the presence or absence of brain lipid liposomes. A) The helix-peptide alone in solution shows dominantly a random coil signature. Upon addition of liposomes the alpha-helical content increases indicated by the two local minima at 208 and 222 nm. B) The helix 1-33 F10E shows a complete random coil signature alone in solution. The insertion of a glutamic acid in the hydrophobic ridge hinders α-helix formation and addition of liposomes confers in this case no structural changes. Folding and thereby binding to liposomes is evidently hindered by the F10E mutation.
Figure S6

(A) The isolated BAR domain of endophilin eNBAR-Δ33 (eBAR) at 6 µM does not show any curvature sensing properties. The residual binding, probably attributed to the positive charges on the concave face of dimer, shows binding at equal densities for all sizes. We caution against the use of isolated BAR domains as they show limited stability. (B) Amphiphysin mutant with two charges changed on the concave interface of the BAR domain (aNBAR 159/161 2KE) does not impair sensing by the wt aNBAR. (C) Point mutation in Amphiphysin N-helix (F9E) does not impair membrane curvature sensing as seen for endophilin. This indicates that the N-helix is not fully disabled and that insertion of a hydrophobic region still is possible. Binding was measured at 600 nM protein. To completely impair sensing by the N-helix, 3 mutations (3xE) were introduced (see Figure 4F). (D) Membrane curvature sensing curves for Sortin Nexin 1 (SNX1) and Centaurin β2 (also known as ACAP2). These proteins possessing a BAR domain have previously been shown to be curvature selective (Peter et al., 2004; Carlton et al., 2004). In our assay, in line with endophilin results, we measure a significant increased size selectivity effect due to exclusion of bulk ensemble smearing. Remarkably the membrane curvature sensing curves resemble the one of eNBAR.

S6. Membrane curvature sensing graphs for eBAR, aNBAR 2KE, aNBAR F9E, SNX1 and Centaurin β2. (A) The isolated BAR domain of endophilin eNBAR-Δ33 (eBAR) at 6 µM does not show any curvature sensing properties. The residual binding, probably attributed to the positive charges on the concave face of dimer, shows binding at equal densities for all sizes. We caution against the use of isolated BAR domains as they show limited stability. (B) Amphiphysin mutant with two charges changed on the concave interface of the BAR domain (aNBAR 159/161 2KE) does not impair sensing by the wt aNBAR. (C) Point mutation in Amphiphysin N-helix (F9E) does not impair membrane curvature sensing as seen for endophilin. This indicates that the N-helix is not fully disabled and that insertion of a hydrophobic region still is possible. Binding was measured at 600 nM protein. To completely impair sensing by the N-helix, 3 mutations (3xE) were introduced (see Figure 4F). (D) Membrane curvature sensing curves for Sortin Nexin 1 (SNX1) and Centaurin β2 (also known as ACAP2). These proteins possessing a BAR domain have previously been shown to be curvature selective (Peter et al., 2004; Carlton et al., 2004). In our assay, in line with endophilin results, we measure a significant increased size selectivity effect due to exclusion of bulk ensemble smearing. Remarkably the membrane curvature sensing curves resemble the one of eNBAR.
Figure S7

S7. Amphiphatic helical peptide stretches N-terminal to BAR domains.
AHs identified upstream to BAR domains and their amino acid sequence. From sequence alignment of BAR domain containing proteins to arfaptin (Habermann, 2004), we identify amino acid sequences N-terminus to the BAR domain that are new putative AHs by examining these regions on a helical wheel. A wheel representation is displayed of all proteins covering the major phylogenetic BAR groups identified by Habermann (Habermann, 2004). The endophilin and amphiphysin helices show similarity in the presence of a net positive charge on the polar side of the helix. However endophilin have serine and threonine bridging the hydrophobic and hydrophilic side, which may imply a different intrinsic binding strength. All AHs have interfacial lysines on or next to the border between the polar and non-polar edge of the helix, which is well suited for membrane interactions. Centaurin β2 and Oligophrenin 1 are peculiar exceptions compared to other identified helices since they have a net negative charge on the polar side and no lysines near the boarder. It's possible that the target membrane lipid composition might be different compared to other NBARs.

The N-terminal part of endophilin has been reported to contain the highest region of similarity when aligned with amphiphysin (Farsad et al., 2001). Thus, it is not surprising that the membrane curvature sensing ability may be conserved in this structural motif. Recently it was reported that SNX9 binds liposomes in a curvature selective manner and identified a correspondingly putative AH (Pylypenko et al., 2007).
Figure S8

S8. Heterogenous binding of eNBAR to liposomes. 
(A-B) Fractional binding recorded for liposomes of various sizes (red, A) – only a low percentage of liposomes (white arrows) shows eNBAR (green, B) binding above background. A remarkable number of liposomes do not show binding of protein above background. Scale bar is 5 µm and applies to both images. 
(C-D) The fractional binding was reproduced for giant liposomes (blue) in solution. eNBAR (green) only binds above background to one out of two liposomes, and not equally on both. Note that eNBAR did not induce deformation of the liposomes, the circular integrity persist.
Electrostatics tune the membrane binding probability. Potential plots for eNBAR and eNBAR 3KE. PDB file 2C08 was used to plot potentials of the rEndophilin A1 monomer and 3KE mutant. The potentials were generated using Swiss PDB-viewer v.3.7 (SP5), by applying colours red (negative) to blue (positive) in the range of -0.5 to 0.5 kT/e. The overall potential of the monomer is negative and suggests repulsion from negative membranes. A positive potential is present at the tip of the NBAR monomer, which in space includes the amphipathic helix. In the eNBAR 3KE mutant this positive area is lost making it more difficult for the amphipathic helix to approach and bind a negative charged membrane. Thus, the barrier of binding (created by two net negative entities) is increased in the 3KE mutant.

Thus, this illustrates that charge mutations in the BAR domain tune the long-range electrostatic forces promoting binding.

Supplementary references


