Bin/Amphiphysin/Rvs (BAR) family members bend membranes in cells

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We provide direct evidence that Bin/Amphiphysin/Rvs (BAR) family members bend the steady state membrane architecture of organelles in intact cells. In response to inducible BAR molecular actuators, organelles exhibit distinct changes to the orientation and degree of their membrane curvature. This rapidly inducible system may offer a mechanism by which to better understand the structure-function relationship of intracellular organelles.

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**Results**

The CID technique relies on two proteins, FKS06 binding protein (FKBP) and FKBP-rapamycin binding domain (FRB), binding to one another upon introduction of a chemical dimerizer such as rapamycin. Using this system, we have previously anchored FRB to the cytosolic face of various individual organelles to which FKBP, now fused to a protein-of-interest, was rapidly recruited. In the present study, we constructed a new series of FKBP fusion proteins that incorporate BAR domains with different intrinsic curvature properties (left panels, Fig. 1). These BAR domains are taken from the human Amphiphysin 1 (N-BAR), formin binding protein 17 (F-BAR), and Missing-in-Metastasis (I-BAR) as well as their respective mutants impaired in curvature-inducing activity: amphipathic helix deletion mutant (ΔN-BAR) or electrostatic mutants (F-BAR-QQ and I-BAR(12,15)). We then transfected
COS-7 fibroblast-like cells with two sets of constructs: one from the FKBP-BAR fusion library and one from the FRB-anchor library that constitutively localizes either to plasma membrane, mitochondria, endoplasmic reticulum (ER), or Golgi (Supplementary Table S1). Organelle morphology was visualized under confocal and epifluorescence microscopes as shown by organelle markers (Figs. 1–2) and recruitable BARs (Supplementary Figs. S1–2), while the cells were simultaneously treated with rapamycin. Any material observations consistent with strict criteria (Supplementary Table S2) were quantified and recorded as the percentage of cells displaying the phenotype (Table 1). We describe the results categorized by BAR domain subtype.

**N-BAR.** Rapid recruitment of N-BAR promoted the appearance of tubulations at the plasma membrane ranging from 2.1 to 33.6 μm in length, and the percentage of cells displaying this phenotype was 36.0% ± 13.1%. As expected, AN-BAR mutant and FKBP negative controls did not result in plasma membrane tubulation. To further investigate the observation at an ultrastructural level, we performed cryo-immunogold electron microscopy where an antibody against a naturally occurring tubules that extend from the Golgi stacks as previously described11. Recruitment of N-BAR showed similar degrees of labeling of the surface membrane, filopodia-like protrusions extended 3.1 to 5.1 μm in length at a frequency of 74.0% ± 4.1%. In contrast, I-BAR(12,15) reduced the frequency of the phenotype (14.9% ± 2.1%). At the mitochondria, unusual protrusions elongated at a rate of more than 0.2 μm/sec at a frequency of 60.9% ± 5.1% as a result of I-BAR recruitment, which is statistically significant relative to I-BAR(12,15) (9.9% ± 4.0%). Recruitment of I-BAR to the ER resulted in punctate structures 1 μm² in size at a frequency of 100.0% ± 0.0%, morphologically distinct from those induced by N-BAR and F-BAR. The resulting phenotype appeared similar to the fusion of ER and the plasma membrane (Supplementary Fig. S3). Recruitment of I-BAR(12,15) resulted in unique punctate structures 50 to 60 μm² at a frequency of 79.7% ± 5.8% and occurred largely at sheet-like ER (Fig. 2 and Supplementary Fig. S3). Recruitment of I-BAR to Golgi did not show any detectable morphological changes.

To test whether the relative expression level or percentage of overexpressed FKBP-BAR translocation to organelles influenced the frequency of the observed phenotypes, we further analyzed the condition of N-BAR induced tubulations at the plasma membrane. Specifically, we quantified the appearance of tubules at the plasma membrane following N-BAR recruitment versus the fluorescence intensity of N-BAR overexpression or percentage of FKBP-BAR translocation per unit area of the cell (Supplementary Fig. S4). Equations could not be fitted to the data to show a trend, suggesting that BAR concentration has no relationship with the resulting
membrane phenotype, at least within the range of BAR expression levels in our experiments.

Lastly, we tested if molecular crowding itself drives membrane bending by recruiting FKBP alone (sans BARs) to each organelle. As a result, we did not observe any of the above changes driven by BARs (Fig. 2), suggesting that properties unique to BARs promote the observed organelar membrane phenotypes. BARs were also recruited to other organelles such as endosomes, lysosomes, and inner nuclear membrane. However, no obvious structural differences emerged (data not shown). These organelles may retain sufficient structural rigidity and lipid packing so as not to be receptive to our technique. Morphological changes may have also been missed due to the limited spatial resolution of fluorescence microscopy.

Discussion

Our results unambiguously demonstrated that BAR domains induce membrane curvature in an organelle-specific manner in live cells on a timescale of seconds. This curvature is dependent on the unique characteristics of each BAR subtype. These differential effects are intriguing in terms of how precisely this bending occurs and how the resulting morphology relates to endogenous cellular events. A number of different mechanisms are responsible for the resulting morphology of cells and organelles. Lipid packing, lipid composition, integral membrane protein localization or wedge-like insertion of peripheral membrane proteins, protein crowding, protein scaffolding, and cytoskeletal-based mechanisms contribute to curvature of intracellular membranes. Previous studies on BAR-induced membrane curvature performed in vitro, in vivo, and in silico also demonstrated that tubule diameters vary based on factors ranging from BAR concentration, type of BAR, oligomerization status, and the dielectric constant of the lipid membrane to the local environment. Given the complexity in the lipid and protein composition of organelles, we thus expect that BAR recruitment to these locations would result in a variety of morphological effects. Indeed, we observed a range of phenotypes occurring at different organelles, whose frequency is not directly correlated with the fluorescence intensity of overexpressed FKBP-BARs.

Our study has indicated the characteristics of FKBP-BAR-induced phenotypes as being reminiscent of actual cellular morphologies, both typical and aberrant. At the plasma membrane, endocytosis exemplifies a cellular process of curvature generation where membrane tubulation and vesicle budding is essential. Using the CID system, we observed membrane tubules forming upon N-BAR recruitment in both live-cell epifluorescence images and electron micrographs. In contrast, F-BAR recruitment to the plasma membrane did not result in tubulations, in contrast to previous studies in vivo and in vitro. It is possible that the FKBP-fusion proteins alter or even diminish the curvature inducing properties of the individual BAR domains themselves. Next, in processes related to cell migration, finger-like projections such as filopodia arise from outward growing filamentous actin pushing against the membrane, to which I-BAR proteins are recruited. Similarly, recruitment of I-BAR via the CID system to the plasma membrane resulted in filopodia-like structures. At the mitochondria, membrane sculpting proteins such as Dmn1/Drp1 regulate the dynamic network of tubules that undergo fusion and fission. Along a similar theme, I-BAR recruitment with our system resulted in dynamic protrusions of the mitochondria. The ER is composed of a tubular and sheet-like network shaped by architectural proteins such as reticulins and atlastins. Mutations in the reticulin family members deform the ER into punctate-like structures along the tubular network. Interestingly, CID-based recruitment of N-BAR and F-BAR produced puncta along the ER network, while I-BAR and the I-BAR(12,15) produced other distinctive punctate structures. The Golgi undergoes broad changes in morphology as a key organelle in the secretory pathway. A variety of tubules extend throughout the
Table 1 | Intracellular locations of BAR recruitment and phenotypes. Organellar anchor unit, BAR domain recruitment, and percentage of cells showing phenotypes. Statistical analysis was performed on a total of at least 30 cells from 3 independent experiments. Mean ±/− SD were recorded. P values were calculated from unpaired, two-tailed Student’s t-tests. P values less than 0.01 are considered significant.

| Location       | Anchor unit | Anchor unit abbr. | BAR-induced stack | Recruited unit CFP-FKBP-BAR domain | Phenotype     | Frequency % cells | P value FKBP | P value Mutant BAR |
|----------------|-------------|-------------------|-------------------|-----------------------------------|---------------|------------------|-------------|-------------------|
| Plasma membrane| Lyn         | Lyn               | Filopodia         | N-BAR                             | 0%            | NA               | NA          | NA                |
|                | ΔN-BAR      |                  |                   | F-BAR                             | 0%            | NA               | 0.37        | NA                |
|                | F-BAR-QQ    |                  |                   | iBAR                              | 2.0 +/− 3.4%  | 0.37             | NA          |       |
|                | iBAR(12,15) |                  |                   | FKBP only                         | 14.9 +/− 2.1% | 0.00027*         | NA          |       |
|                | Inward tubulation |          |                   | N-BAR                             | 36.4 +/− 13.1%| 0.00088*         | 0.00088*    |       |
|                | ΔN-BAR      |                  |                   | F-BAR                             | 3.7 +/− 6.4%  | 0.37             | NA          |       |
|                | F-BAR-QQ    |                  |                   | iBAR                              | 2.6 +/− 4.4%  | 0.37             | 0.86        |       |
|                | iBAR(12,15) |                  |                   | FKBP only                         | 2.0 +/− 3.4%  | 0.37             | NA          |       |
| Mitochondria   | Mono-amine  | MoA               | Protrusion         | N-BAR                             | 5.3 +/− 4.7%  | 0.27             | 0.15        |       |
|                | Oxidase A   |                   |                   | ΔN-BAR                            | 20.5 +/− 14.2%| 0.35             | NA          |       |
|                |             |                   |                   | F-BAR                             | 2.2 +/− 3.9%  | 0.11             | 0.20        |       |
|                |             |                   |                   | F-BAR-QQ                          | 15.6 +/− 14.5%| 0.64             | NA          |       |
|                |             |                   |                   | iBAR                              | 60.9 +/− 5.1% | 0.00043*         | 0.00017*    |       |
|                |             |                   |                   | iBAR(12,15)                       | 9.9 +/− 4.0%  | 0.79             | NA          |       |
|                |             |                   |                   | FKBP only                         | 11.1 +/− 6.2% | 0.37             | NA          |       |
| Endoplasmic     | Cyto-chrome  | Cb5               | Punctate structures at tubular ER | N-BAR                            | 55.1 +/− 14.5%| 0.0083*         | 0.17        |       |
| Reticulum      | b5          |                   |                   | ΔN-BAR                            | 36.9 +/− 11.8%| 0.027            | NA          |       |
|                |             |                   |                   | F-BAR                             | 48.9 +/− 1.9% | 0.00099*         | 0.0038*     |       |
|                |             |                   |                   | F-BAR-QQ                          | 17.0 +/− 8.9% | 0.30             | NA          |       |
|                |             |                   |                   | iBAR                              | 100.0 +/− 0.0%| 3.5 × 10−5*      | 0.0037*     |       |
|                |             |                   |                   | iBAR(12,15)                       | 79.7 +/− 5.8% | 0.00023*         | NA          |       |
|                |             |                   |                   | FKBP only                         | 8.9 +/− 7.8%  | 0.64             | NA          |       |
|                |             |                   |                   | N-BAR                             | 47.0 +/− 2.6% | 0.00020*         | 0.012       |       |
|                |             |                   |                   | ΔN-BAR                            | 25.0 +/− 8.3% | 0.21             | NA          |       |
|                |             |                   |                   | F-BAR-QQ                          | 19.4 +/− 5.9% | 0.63             | 0.63        |       |
|                |             |                   |                   | iBAR(12,15)                       | 17.5 +/− 2.9% | 1.0              | NA          |       |
|                |             |                   |                   | iBAR only                         | 29.8 +/− 11.4%| 0.21             | 0.12        |       |
|                |             |                   |                   | iBAR(12,15)                       | 12.1 +/− 6.9% | 0.28             | NA          |       |
| Golgi          | Giantin     | Giantin           | Large tubulation   | N-BAR                             | 7.3 +/− 2.9%  | 0.37             | NA          |       |

stacks of cis-, medial-, and trans- Golgi. Recruitment of N-BAR resulted in larger tubulations than those already occurring. It is notable that BAR recruitment to these different intracellular locations resulted in phenotypes already observed naturally under specific conditions. The rapidly recruitable BAR technique developed in the present study may thus offer a powerful method to investigate cellular events associated with the morphological changes under both physiological and pathophysiological conditions.

In summary, organellar shape has long been thought to be fundamental to cellular function. Yet, it has been difficult to study the structure-function relationships of individual organelles because structural perturbations have been primarily driven by genetic manipulations where the “architectural” roles of proteins are not easily distinguishable from their “signaling” ones. The molecular actuators introduced here perturb organellar shape on a second timescale in live cells where we can simultaneously measure biologically relevant cellular activity. Their use permits insights into how specific conditions. The rapidly recruitable BAR technique developed resulted in larger tubulations than those already occurring. It is significant.

**Table 1 | Intracellular locations of BAR recruitment and phenotypes.**

Intracellular locations of BAR recruitment and phenotypes. Organellar anchor unit, BAR domain recruitment, and percentage of cells showing phenotypes. Statistical analysis was performed on a total of at least 30 cells from 3 independent experiments. Mean ±/− SD were recorded. P values were calculated from unpaired, two-tailed Student’s t-tests. P values less than 0.01 are considered significant.

**Methods**

**DNA constructs and other reagents.** We received the constructs encoding human Amphiphysin 1 N-BAR and human FBP17 F-BAR from Dr. Toshiko Itoh (Kobe University), and human MIM F-BAR from Dr. Petka Lappalainen (University of Helsinki). BARs were subcloned into FKBP vectors between BamHI and EcoRI restriction sites. These DNA constructs include BARs encoding the N-BAR from human Amphiphysin 1 (1–248 AA), curvature-inducing deficient mutant AN-BAR (26–248 AA), F-BAR from human forming-binding protein (FBP17) (1–300 AA), lipid binding deficient mutant F-BAR (K113Q) + K114Q), F-BAR from human mitrochondria (MIM) (1–254 AA), and I-BAR (12,15) mutant (K146A, K149A, K150A, K158A, K139A). The anchor units Lyn-fluorescent protein-FRb at the plasma membrane, fluorescent protein-FRb-MoA at the mitochondria, fluorescent protein-FRb-Cb5 at the ER, or fluorescent protein-FRb-Giantin at the cis- and medial- Golgi were previously described.

**Live-cell imaging.** Live-cell imaging of ER, Golgi, and the plasma membrane was conducted using the Axiovert 155 TV epifluorescence microscope (Zeiss) with 63× oil magnification. Twenty-four hours after transfection, cells were imaged by the CCD camera (QImaging) driven by Metamorph 7.5 imaging software (Molecular Devices) at 15 second time interval for at least 15 minutes. Mitochondria were also imaged on the Leica SPS inverted confocal microscope, with resonant scanner, HCX PL APO 40× objective lens. NA = 0.25 at 15 second time interval for at least 15 minutes. Mitochondria were also imaged on the spinning-disc Axiovert 200 confocal microscope (Zeiss). YFP and mCherry excitations were conducted with an argon laser (CVI-Melles Griot) with 40× objective (Zeiss) and an additional 1.6× magnification and NA = 1.30. 100 nm of raphymycin (Tecollad) was added after approximately 2 min and 30 seconds of imaging.

**Electron microscopy.** COS-7 cells were seeded in 15 mm dishes and transfected with Lyn-FRB and GFP-FKBP-N-BAR, Lyn-FRB and YFP-FKBP-N-BAR, or Lyn-FRB and GFP-FKBP-AN-BAR using Amaxa Nucleofector (Lonza) or FuGENE HD (Roche) for transfection. Following 24–48 hours after transfection, rapamycin was added to a final concentration of 100 nM and incubated for 10 minutes at 37°C. Cells were then fixed with 3% formaldehyde and 0.1% glutaraldehyde for 15 minutes at room temperature. Next, cells were incubated in 3% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M sodium cacodylate, 5 mM magnesium chloride, 2.5% sucrose pH 7.4 at 0°C for 1.25 hours. Cells were then washed three times with 0.1 M sodium cacodylate, 5 mM magnesium chloride, and then postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate, 1% potassium ferricyanide, 1% potassium ferrocyanide, 1% potassium permanganate, 1% cupric sulfate for 1.25 hours. Cells were then washed three times with 0.1 M sodium cacodylate, 5 mM magnesium chloride and then dehydrated through a series of ethanol and propylene oxide. Finally, cells were infiltrated with propylene oxide and Epon 812 and then epoxy resin 812. Sections were cut on an ultramicrotome, stained with uranyl acetate and lead citrate, and then imaged on a Leo 912 microscope.
Figure 3 | Electron micrographs of the plasma membrane region. Anti-GFP immunogold labeling of COS-7 cells expressing Lyn-FRB and YFP-FKBP-N-BAR or Lyn-FRB and GFP-FKBP-ΔN-BAR. (a) Immunogold labeling of YFP-FKBP-N-BAR shows localization at the plasma membrane and internal membrane. These labeled internal membrane tubules may reflect the tubules we observe under light microscopy. Less immunogold labeling of internal membrane tubules occurs in the GFP-FKBP-ΔN-BAR negative control sample. The p value calculated from unpaired, two-tailed Student’s t-test is 0.81. (b) Quantification of the ratio of gold particles at the plasma membrane surface to the overall amount of labeling is similar for YFP-FKBP-N-BAR and the negative control condition. YFP-FKBP-N-BAR scaffolds internal membrane tubules, as measured within 100 nm of the internal tubule, while the negative control has significantly less labeling along similar tubules. The p value calculated from unpaired, two-tailed Student’s t-test is less than 0.0001. Scale bar indicates 500 nm.
tubules were identified and the number of gold particles within 100 nm of the tubules was calculated for the N-BAR and AN-BAR negative control. Since gold-labeling of N-BAR occurs on a greater length scale than N-BAR alone, analysis was conducted with respect to the length of 100 nm. This encompasses the summation of lengths of the following components: FKBP, FRB, GFP, N-BAR or AN-BAR, chicken anti-GFP (lgY), and donkey anti-chicken 12 nm gold particle.

**Statistical analysis.** Statistical analysis of epifluorescence images was performed on a total of at least 30 cells from 3 independent trials. Mean $\pm$ 1 SD was recorded in Table 1. P values were calculated from unpaired, two-tailed Student’s t-tests. Experimental data sets were compared to corresponding mutant BAR and FKBP data sets. Statistical analysis of EM images was performed by computing Mean $\pm$ 1 SD of immunogold labeling of intracellular membranes. P values were calculated from unpaired, two-tailed Student’s t-tests of images clearly depicting plasma membrane (8 images analyzed for each N-BAR or AN-BAR conditions comparing plasma membrane surface labeling with total labeling of surface and internal membranes) or clearly showing membrane tubulation (14 images analyzed for N-BAR or AN-BAR conditions comparing immunogold labeling along internal membrane structures). P values were recorded in the Fig. 3 legend. P values less than 0.01 were considered statistically significant.

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**Author contributions**

A.S. and T.U. performed cell biology experiments described in Figures 1 and 2, as well as in Table 1. J.M.M. conducted electron micrograph experiments shown in Figure 3. R.H. made some of the DNA constructs. A.S. and T.I. wrote the manuscript, which was reviewed by all authors.

**Additional information**

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