Supplementary data to accompany paper:

Mechanism of endophilin N-BAR domain-mediated membrane curvature

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Analytical Ultracentrifugation

Sedimentation equilibrium experiments were performed in a Beckman Optima XL-A analytical ultracentrifuge with an An60-Ti rotor, with 39, 78 and 156 μM in 150 mM NaCl, 20 mM HEPES pH 7.4, 1 mM TCEP. Sedimentation was at 11,000 rev/min, 4.0 °C, with initial overspeeding at 18,000 rev/min for 6 hr, to reduce the time to reach equilibrium (S9). Long sample columns were used. Scans (averaging 10 readings) were taken at 280 nm at 24 hr intervals, until no movement of the distribution was visible, when final scans (averaging 100 readings) were taken and assumed to be operationally at equilibrium. The rotor was then accelerated to sediment the macromolecule away from the meniscus, and further scans taken to provide initial estimates of the baseline for each cell. Data were analyzed as described in detail in the supplementary data of previous paper (Mills et al., 2003).

Sedimentation velocity runs were performed at the speeds indicated using a single cell and taking scans as quickly as possible (~90sec intervals). All components were tested at 40 and 150 μM protein in 150 mM NaCl, 20 mM HEPES pH 7.4, 2 mM DTT unless otherwise stated. Data were analysed initially by plotting g(s*) against s* (where g(s*) is the fraction of material sedimenting between s* and (s*+ds*)) using the DCDT+ software package (Version 1.05)(Philo, 2000). This software was also used for the direct fitting of simple gaussian functions to dc/dt versus s curves to test for the number of components and estimate their molecular mass. Partial specific volumes and solvent densities were calculated as previously described in Peter et al. (2004). For the plots above, every tenth scan was taken to give visual separation of the traces. In these experiments we calculate the partial specific volume but anything that broadens the boundary during sedimentation (slight unfolding, microheterogeneity, a part of the protein that flips around a bit etc) will give a

larger estimate for the density (d) and as this is inversely related to the apparent mass the result can be slightly smaller than the calculated molecular weight.

CD spectroscopy

Measurements were made using a CD6 Jobin Yvon CD spectroscope. Proteins were diluted to a concentration of 0.2 mg/ml to give a buffer concentration of 30 mM NaCl, 5 mM HEPES pH 7.4, 0.4 mM DTT. Readings were an average of 5 scans from 190-260 nm. Buffer reading were subtracted and the data smoothed using the moving average of 2.5 nm. Fractional helical content was calculated as described previously (Chen et al., 1972). Ultracentrifugation methods have been described previously (Peter et al., 2004).

Electron paramagnetic resonance (EPR)

Spin labels were introduced onto each residue from 2-16 of GST-tagged rat endophilin-A1 N-BAR domain. First we introduced a cysteine residue at each position on a cysteine negative mutant (C108S). This native cysteine is on the loop between helices 1 and 2 and the mutant functions normally on liposome binding and tubulation assays (data not shown). Proteins were reacted in 150 mM NaCl, 20 mM HEPES pH 7.4 with an MTSL nitroxide spin label ([1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl]-methanethiosulfonate) to generate the new side chain R1 using a previously described protocol (Jao et al., 2004). 2 µM protein was incubated with 1.4 mg/ml liposomes and centrifuged to separate bound from unbound. Measurements were made in the presence and absence of 400 nm Folch liposomes. EPR experiments were recorded in a Bruker EMX spectrometer fitted with a dielectric resonator. The Bruker HS cavity was used for endophilin labelled at position 227. Power saturation experiments to determine the oxygen and NiEDDA accessibility (Π) were performed as previously described (Altenbach et al., 1994; Jao et al., 2004) with a 10 mM concentration of NiEDDA and oxygen as in air in equilibrium with buffer. The Φ parameter was calculated by the relationship $\Phi = \ln[\Pi (O2)/ \Pi (NiEDDA)]$. The immersion depth was calibrated using 1-palmitoyl-2-stearoyl-(n-DOXYL)-sn-glycero-3-phosphocholine (Avanti Polar Lipids) as described previously (Altenbach et al., 1994; Jao et al., 2004). We obtained the following relationship between immersion depth (d) and Φ: d[Å]= 6.3*Φ-3.9. This immersion depth represents the depth of the nitroxide moiety. For peripherally bound helices, the lipid facing nitroxides are 7-10 Å deeper in the membrane than the centre of the helix (Jao et al., 2004; Langen et al., 2000). Based on Φ values of 1.5 to 2 obtained here, the centre of the helix is located near the level of the phosphates. Distances were determined following previously established protocols (Altenbach et al., 2001) using a simulation program kindly provided by

Dr. Altenbach (UCLA). The simulations require a reference spectrum without strong dipolar interactions, which was generated by adding three fold excess (6 µM) of unlabelled cysteine-free protein. Very similar spin dilution results were obtained using endophilin labelled at position 227 with a non-paramagnetic analogue of the spin label (Gross et al., 1999) (data not shown).

FRET assay of membrane fusion

250 µg/ml liposomes comprising 98:1:1 molar ratio of phosphatidylserine (PS), NBD-phosphatidylethanolamine (PE) and rhodamine-PE were incubated for 5 min with a range of N-BAR and mutant domain concentrations ± unlabelled liposomes (at 100% PS). The excitation wavelength was 450 nm and emission spectrum taken from 480-700 nm with slits of 5 nm using the Fluoromax-2 fluorimeter (Jobin Yvon). 1% triton was added to obtain a value for donor fluorescence in Figure 7C. In Figure 7D, 55 µM protein was used and the ratio of emission peaks was taken within a single measurement to decrease the systematic error. 5 experiments and controls for each experiment were averaged separately and the difference plotted ± SEM, except the KKK-EEE mutant (2 repeats). The major source of error is differences between batches of liposomes.

Crystallisation and structure solution

A large number of different constructs and conditions produced crystals which diffracted poorly. Successful crystallisation used a construct of residues 1-247 of rat endophilin A1 with an N-terminal hexahistidine tag and expressed in *E.coli* strain BL21. Crystals were grown by vapour diffusion from 100mM TrisHCl pH 8.0, 25% butane-1,4-diol at 4°C, cryoprotected at 4°C by increasing concentrations of butane-1,4-diol and ethylene glycol in 5% steps to 40% butane-1,4-diol, 25% ethylene glycol, and plunged into liquid nitrogen for data collection. Crystals grown from SeMet-substituted protein diffracted a little better than native crystals.

Crystals belonged to spacegroup $I4_1$ with unit cell dimensions a = b = 126.5Å, c = 101.1Å. Three datasets were collected on beamline ID29 at ESRF on a single crystal around the Se absorption edge, from the peak and inflection point of the fluorescence curves, and at a "remote" high-energy point, in that order. At each wavelength, two opposed 90° wedges were collected, and the initial wedge at the peak energy was recollected at the end to show that there was no significant radiation damage. Intensities were integrated to 2.9Å resolution using Mosflm (Leslie, 1992) and scaled with Scala (Collaborative Computational Project, 1994). Data collection and refinement statistics are given below.

Of the eleven methionine residues in the sequence, seven Se sites were located with the program Solve (Terwilliger and Berendzen, 1999), and three more were located during phasing with Sharp (de la Fortelle and Bricogne, 1997): the weakest of the ten sites was not on a Se-methionine residue, and two of the sites represent alternative conformations of Met133. MAD phases were improved by solvent-flattening with Solomon (Abrahams, 1997) using a solvent content of 77%. The model was built using O (Jones et al., 1991), refined with Refmac (Murshudov et al., 1997) and the model was updated during refinement with Coot (Emsley and Cowtan, 2004). For refinement, data from the three wavelengths were merged together, to improve the weak measurements at the high resolution limit. The final model included residues 25 to 67 and 87 to 247, ie the hexahistidine tag and the N-terminal amphipathic helix are disordered, as well as a loop in the middle of the first helix which contains two methionine residues. Crystals soaked in Ins(1,4,5)P_3 and acetyl-CoA, because of binding to PtdIns(4,5)P_2 liposomes and a reported lysophosphatidic acid acyl transferase activity, gave no additional density.

Data collection, phasing and refinement statistics for crystal structure of rat endophilin A1 BAR domain.

Data collection statistics				
	Merged data (outer shell)	**Peak**	**Inflection Point**	**Remote**
Space Group	$I4_1$			
Resolution (Å)	2.9 (3.06-2.9)			
Completeness (%)	99.9 (100)	99.9(100)	99.9(100)	99.9(100)
Multiplicity	22.1 (22.3)	11.1 (11.2)	7.4 (7.4)	7.4 (7.4)
R_{merge}	0.155 (0.951)	0.130(0.942)	0.110(0.801)	0.124(0.891)
R_{meas} (within I+/I-)	0.163 (0.966)	0.143(1.038)	0.128(0.934)	0.145(1.040)
$R_{p.i.m}$ (within I+/I-)	0.048 (0.209)	0.058 (0.436)	0.063 (0.482)	0.072 (0.537)
$\langle I/\sigma \rangle$ outer shell	3.6	2.5	2.3	2.1
Anomalous completeness	99.7(99.8)	99.7(99.7)	99.7(99.8)	99.7(99.8)

Phasing	**N Acentric**	**FOM Acentric**	**N Centric**	**FOM Centric**
Overall	16913	0.474	596	0.368
Phasing powers	**Dispersive Acentric**	**Centric**	**Anomalous Acentric**	
Peak	1.047	1.021	1.725	
Inflection point	1.237	1.251	0.69	
Remote	0	0	0.85	

Refinement statistics			
Resolution Å (outer shell)	89 - 2.90 (2.97)		
R_{factor} (working set)	0.247 (0.36)	Ramachandran plot:	
R_{free}	0.266 (0.36)	% in favoured region	95.5
R_{free} test set size (%)	5.1	% outliers	0.5
$\langle B \rangle$ (Å^2)	69		
$N_{reflections}$	16875		
N_{atoms} (non-hydrogen)	1653		
R_{msd} bond length (Å)	0.021		
R_{msd} bond angle (º)	0.940		

$R_{merge} = \Sigma\Sigma_j|I_{hj} - \langle I_h \rangle|/\Sigma\Sigma_j I_{hj}$

$R_{meas} = \Sigma\sqrt{(n/(n-1))}\Sigma_j|I_{hj} - \langle I_h \rangle|/\Sigma\Sigma_j I_{hj}$ multiplicity-weighted R_{merge}

$R_{p.i.m} = \Sigma\sqrt{(1/(n-1))}\Sigma_j|I_{hj} - \langle I_h \rangle|/\Sigma\Sigma_j I_{hj}$ precision indicating R_{merge}

(Diederichs and Karplus, 1997; Weiss, 2001; Weiss and Hilgenfeld, 1997)

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