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Osmotically-Induced Tension and the Binding of N-BAR Protein to Lipid Vesicles

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ABSTRACT: The binding affinity of a curvature-sensing protein domain (N-BAR) is measured as a function of applied osmotic stress while the membrane curvature is nearly constant. Varying the osmotic stress allows us to control membrane tension, which provides a probe of the mechanism of binding. We study the N-BAR domain of the Drosophila amphiphysin and monitor its binding on 50-nm-radius vesicles composed of 90 mol% DOPC and 10 mol% PIP. We find that the bound fraction of N-BAR is enhanced by a factor of approximately 6.5 when the tension increases from zero to 2.6 mN/m. This tension-induced response can be explained by the hydrophobic insertion mechanism. From the data we extract a hydrophobic domain area that is consistent with known structure. These results indicate that membrane stress and strain could play a major role in the previously reported curvature-affinity of N-BAR.

Introduction

The membrane of a cell is the site of a wide variety of essential biological processes including passive transport, signal transduction, cell-cell communication, and membrane fusion. Although the cell membrane's composition is complex and heterogeneous, it has nonetheless proved very informative to describe the membrane in terms of continuum concepts such as elasticity, curvature, and mechanical tension. Tension, in particular, is important for regulation of membrane trafficking and is regulated in cells.[1-4] Tension contributes to the balance between open and closed states of ion channels or pores because the open state requires work against the tension.[5,6] However, the effect of tension on the reversible binding of peripheral membrane proteins has not been explored. Owing to the fact that peripheral membrane proteins can alter the local shape of the membrane, it is likely that binding may be strongly coupled to applied tension. For instance, tension should present an energy barrier if protein binding deforms the membrane because the deformation requires gathering membrane area into the binding site, which requires work against the tension. By contrast, tension should favor binding if it is accompanied by insertion of the protein, which increases the membrane area and allows the tension to do work (lower energy).

Proteins that are members of the BAR (Bin/Amphiphysin/Rvs) superfamily have become a canonical example of shape-dependent protein binding.[7-9] Previous studies in cells and *in vitro* show that BAR proteins display striking membrane-curvature effects that are thought to support their role in cell migration, membrane trafficking and synaptic vesicle endocytosis.[7,10,11] Specifically, BAR proteins show a clear tendency to bind to membranes of high curvature,[8,12,13] to partition to highly curved regions of a given vesicle[14-17] or wild-type cell,[13] and even in some

cases to remodel the membrane shape.[8,17-23] Two key structural motifs have been identified as mechanisms for the curvature affinity and induction.[11,24,25] First, the BAR domain forms homodimers with a curved, banana shape with positively charged residues along the inside of the curve,[8] where the protein binds to the membrane.[26] Computer simulations indicate that this curved structure could lead to curvature 'sensing' or remodeling by a scaffolding mechanism, in which the membrane adopts a shape that complements the shape of the BAR domain.[10,27] The second important structural motif is an amphipathic helix, a sequence stretch at the N-terminal end of the BAR domain. (This helix and the BAR together are called N-BAR.) The helix contains positively charged residues that interact with anionic lipids[28] and a hydrophobic region that can insert into the hydrophobic region of a lipid bilayer membrane. There is evidence that hydrophobic insertion is the major mechanism for sensing or inducing curvature.[10,12,29-33] A third mechanism by which BAR or N-BAR might induce curvature is protein-protein crowding, where a high concentration of bound protein on one side causes the membrane to bend.[34] Thus, a connection between membrane curvature and BAR proteins is established by experiments, but reports in the literature give contradictory indications of which of these mechanisms predominates. Experiments using a combination of pipette aspiration (to control tension) and laser tweezers (to pull highly curved tubules) show that the N-BAR domain[14,16] and N-BAR-containing amphiphysin1[17] partition to the highly curved tubules. In these cases, the applied force plays an interesting dual role: it both modifies the tension within the membrane and also increases the curvature of the tubule. Knowing how tension affects vesicles of *constant curvature* is needed to isolate these two effects and distinguish the binding mechanism.

Here we report measurements of the binding affinity of the N-BAR domain of *D. melanogaster* amphiphysin as a function of the membrane tension, which is adjusted by means of osmotic pressure. We use large unilamellar vesicles (LUVs) composed of a mixture of 90 mol% unsaturated zwitterionic lipids (DOPC) and 10 mol% anionic lipid (PIP). The mean LUV radius is 52 nm. We find that when the vesicles are under tension by osmotic stress, the binding of N-BAR is substantially enhanced, by a factor of more than six when the tension is increased from 0 to 2.6 mN/m. We also find that the results are indistinguishable whether we use glucose or glucose-salt mixture, which implies a nonspecific and non-electrostatic mechanism. The binding affinity also increases modestly under hyperosmotic conditions, when the vesicles are partially deflated. We interpret the positive-tension results in light of the scaffolding and hydrophobic insertion mechanisms and conclude that the latter is the predominant mechanism for sensing tension. Furthermore, the results indicate that membrane strain may be the fundamental origin of the previously reported curvature affinity, since the curved leaflet of a bilayer is strained. Finally, we show that the response to applied osmotic stress depends on how long the vesicles are maintained in their buffer prior to exposure to the protein, which we attribute to the time needed for the vesicles to reach their equilibrium spherical shape (as reported previously[35]); this result shows the importance of equilibrating vesicles for several hours prior to binding experiments. We propose that these stress-controlled measurements will be a useful tool in understanding how protein binding couples to membrane shape.

Materials and methods

Proteins, lipids and vesicles

Residues 1-256 of amphiphysin from Drosophila melanogaster were obtained from the H. T. MacMahon laboratory. These residues were expressed as His-tagged proteins from a pETite Kanamycin vector in E.coli HI-Control™ BL21(DE3) cells, which were obtained from Lucigen (Middleton, WI). The cysteine residue at position 66 was previously mutated to alanine to facilitate attachment of a maleimide dye only at position 82, however, all protein used in these experiments was unlabeled. Before use in binding experiments, N-BAR in buffer A was diluted with deionized water to achieve a final protein concentration of 4 μM and buffer concentration of 200 mOsm, and gently centrifuged 10 min at 10,000 rpm to remove large protein aggregates. (Details are given in the online supplementary information.)

Vesicles were made from a mixture of unsaturated zwitterionic phosphocholine lipid and a minority of anionic phosphatidylinositol with a 9:1 molar ratio. The lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and L-α-phosphatidylinositol-4-phosphate (Brain, Porcine) (PIP) were purchased in powder form and dissolved in chloroform (Avanti cat. nos. 850375 and 840045). Vesicles were formed as described previously:[36,37] in brief, lipids were mixed in solution, dried under N_2 gas and then placed under vacuum for at least 2 h to remove residual solvent. A suspension of multilamellar vesicles (MLVs) was formed by rehydrating with 1 mL of 200 mM glucose and vortexing. The solution was then passed 15 times through a polycarbonate membrane with a pore size of 100 nm (Whatman, Piscataway, NJ, cat. No 7060-4701). The resulting LUV suspension was used for experiments either directly after preparation or, alternatively, after incubation at 37°C for at least 24 h. As we will show later (and as pointed out previously[35]) a 24 hour incubation is a crucial step in obtaining repeatable and interpretable results.

Dynamic light scattering (DLS) was used to measure the mean size of the incubated LUVs (Zen3600 Zetasizer, Malvern Instruments, Worcestershire, UK; 632.8 nm wavelength). The number-averaged radius for the vesicles used in these experiments was r_0 = 52 nm and the standard deviation was 10 nm. In control experiments, we found that the size of the LUVs did not change between 24 h and 75 h after preparation.

The osmotic stress on the LUV membranes was tuned by diluting the suspension 1:1 in either hyperosmotic or hypoosmotic solutions of either NaCl or glucose. Osmolalities of all solutions were measured by a vapor pressure osmometer at room temperature. The uncertainty of these measurements, estimated from repeated measurements, is 3 mOsm/L. The concentration of the swelling solution was 200 mM and the exterior solution was tuned over the range of 130 to 270 mM. Following the addition of hyper- or hypoosmotic solution, the lipid concentration was 45 mg/mL. The LUVs were left for 30 min before proceeding with the addition of protein. This waiting time is more than sufficient for the water to permeate the vesicles and balance the osmotic pressure,[38] yet far too little to allow significant solute permeation.[35]

Bound fraction measurements

Sedimentation assays were used to quantify the amount of N-BAR bound to the vesicles, following a standard procedure.[39] In brief, the LUVs were buoyant in suspension but became denser than the solution when N-BAR bound to them. For sedimentation assays, a fixed amount (25.7 μL) of protein was incubated between 5 and 10 minutes with a known quantity of LUVs in salt or glucose buffers of known osmolarity to achieve a final protein concentration of 1 μM and a protein:lipid molar ratio of 1:40. After centrifugation at 160,000 g for 30 min at 25°C, the

fraction of NBAR in the sediment (pellet) and the supernatant were then quantified by ImageJ gel analysis software.[40]

The fraction of the total protein that binds, which we hereafter call f_b, was defined as the ratio of the integrated intensity of the pellet to the sum of the integrated intensities of the pellet and of the supernatant. Variations in defining a baseline in the ImageJ gel analysis tool resulted in < 1% variation in the percent of bound protein. Uncertainty in the bound fraction f_b was estimated by comparing the results of four measurements made under nearly the same osmolarity conditions (closest to zero). The standard deviation of these four measurements was 10% of the mean and we assumed that the fractional uncertainty is the same for all our data.

Results and discussion

Binding of N-BAR to LUVs

Figure 1 shows how the fraction of membrane-associated N-BAR changes with osmotic stress on the membrane. We plot the fraction of N-BAR that binds to membrane (f_b) as a function of Δc, defined as the difference of initial solute osmolarity inside and outside the vesicles ($\Delta c \equiv c_{\rm in}^{\rm initial} - c_{\rm out}^{\rm initial}$), where $c_{\rm in}^{\rm initial}$ = 200 mM. The horizontal axis includes measurements under hypoosmotic and hyperosmotic conditions (Δc > 0 and < 0, respectively). These measurements were repeated using salt (NaCl, △) rather than glucose (■) to adjust osmolarity of the exterior solution. While the data points with Δc > 10 mOsm/L show scatter, the trend of sharply increasing f_b is quite clear for the glucose and salt data. In all cases, f_b is minimum when Δc = 0 and rises sharply when Δc > 0 and gradually for Δc < 0.

The upper axis of Fig. 1 shows the tension on the membrane, which we calculated from Δc. When Δc > 0 (hypoosmotic conditions), the membrane is stretched as osmotic pressure causes water to permeate the membrane. During the duration of these measurements (~30 min), glucose and salt ions permeate the membrane in negligible quantity,[35] so that the number of solute molecules in the vesicles is approximately fixed. Water permeates the membrane rapidly[35,41] and is drawn into the vesicles to reduce the interior solute concentration. The influx of water also stretches the membrane and creates a mechanical tension τ. We calculate the tension on the membrane in the standard way, using Laplace's Law: $\tau = \frac{1}{2}\, r\, \Delta P$, where ΔP is the osmotic pressure difference between the inside and the outside of the vesicle ($P_{\rm in}$–$P_{\rm out}$), and r is the vesicle radius.[35] Here, ΔP is approximated by the ideal-gas law, $\Delta P = (c_{\rm in} - c_{\rm out})\, RT$, where $c_{\rm in}$ and $c_{\rm out}$ are the osmolarities of the solute (glucose , Na^+, Cl^-)) inside and outside the vesicle, R is the ideal gas constant (8.314 J mol^{-1} K^{-1}), and T is the temperature in Kelvins. In these experiments, $|\Delta P|$ ranged from zero to approximately 1.9×10^5 Pa and τ ranged from 0 to approximately 5 mN/m. Assuming that stretching modulus of the membrane is approximately the same as that of DOPC (k = 265 mN/m [42]), the vesicle radius should change by approximately 1% or less and we can use the initial values of the radius r and the interior solute concentration $c_{\rm in}$. (Details are given in the online supplementary information.) Under hyperosmotic conditions (Δc < 0), the membrane is not under compression; instead it deforms and the vesicle becomes non-spherical.[35]

The most striking result of Fig. 1 is that the bound fraction f_b increases dramatically when membranes are made tense (Δc and τ > 0). To quantify this effect, we note that f_b is constant

(within error) near $\Delta c = 0$, which allows us to define the stress-free bound fraction (f_b^0) as the mean of the 4 data points with Δc between −12 mOsm and +5 mOsm: $f_b^0 = 0.110 \pm 0.005$. In the data with glucose as osmolyte (■), we find a maximum $f_b = 0.72 \pm 0.07$ when $\Delta c = 40$ mOsm/L and $\tau = 2.6$ mN/m. This bound fraction is greater than f_b^0 by a factor of 6.5 ± 0.7.

The fraction of the vesicles' outer surface that is covered by bound N-BAR is estimated from the known protein:lipid molar ratio and assuming that the surface area of N-BAR is approximately 3600 Å^2, [12] and the area per molecule of DOPC is 72.5 Å^2. [43] For the stress-free reference bound fraction f_b^0, we find that approximately 12% of the vesicle's surface is covered by bound N-BAR. The peak $f_b = 0.72$ corresponds to an area fraction of approximately 0.8, which approaches close packing of BAR on the membrane.

With still larger applied osmotic stress ($\Delta c > 60$ mOsm/kg, $\tau > 4$ mN/m), f_b decreases to approximately f_b^0. It may be that greater membrane strain suppresses binding. However, it seems more likely that this reduction of f_b arises from rupture (lysis) of the vesicles, during which the vesicle releases a portion of its contents and reseals at a lower tension.[35] The dynamics of this process are not known from our data, but we can safely assume that the concentration difference after lysis must be smaller than Δc because of solute exchange. Hence, binding should decrease toward f_b^0, which is consistent with the data. While a lysis tension of 4.5 mN/m is lower than the reported lysis tension of 9.9 mN/m for pure DOPC LUVs,[38] this reduction might arise from the addition of the anionic lipid[44] (PIP) or from the interaction of N-BAR with the membrane (analogous to the 'molecular harpoon' mechanism, whereby surfactants or lysolipids with a wedge-shaped hydrophobic region can reduce the lysis tension[45-47]).

Under hyperosmotic conditions ($\Delta c < 0$), Fig. 1 shows quite different behavior: the bound fraction f_b increases smoothly as Δc becomes more negative. Under the most hyperosmotic conditions probed with glucose, f_b increased to about 0.23 ± 0.04, which is approximately a two-fold enhancement relative to the stress-free reference f_b^0. In these vesicles, we expect that water should flow outward until c_{in} matches c_{out}. For example, when $\Delta c = -70$ mOsm/L, the interior volume should decrease by 26% in order to balance osmotic pressure. Under these conditions, the vesicles change shape from spherical to undulating discoid or ellipsoid. Hence, we would qualitatively expect that some regions of the membrane are more highly curved than the average. The two-fold enhancement of binding might arise from the already established tendency for N-BAR to bind to regions of higher curvature.[7,8,10-16]

Reversibility of Binding

To test the reversibility of N-BAR binding, we first incubated N-BAR with vesicles in hypoosmotic solution ($\Delta c > 0$; tense vesicles) then transferred to hyperosmotic solution ($\Delta c < 0$; floppy). In one experiment, LUVs were exposed to $\Delta c = 37$ mOsm/kg (glucose), f_b was measured, and then the LUVs and N-BAR were incubated in $\Delta c = -25$ mOsm/L (glucose) for 30 min and f_b was measured again. After the first step, we found $f_b = 0.11 \pm 0.01$, however, in this case the initial molarity of N-BAR in solution was 2 μM rather than 1 μM so that this result should not be directly compared to the data in Fig. 1. After the second incubation step, we found $f_b = 0.040 \pm 0.004$; in this case the concentration of N-BAR was again 1 μM, the same as that of the other measurements of Fig. 1. This latter measurement is represented in Fig. 1 by the star. This bound fraction is substantially less than that of the initial Δc of 37 mOsm/L, which indicates

that N-BAR re-equilibrated by desorbing from the vesicles during the second 30-min incubation period.

Additional reversibility experiments were performed with an initial protein concentration of 1 μM and a final concentration of 0.5 μM. Two samples incubated initially at Δc = 40 mOsm/L showed bound fractions f_b = 0.72 and 0.54 (1 μM). After incubation in Δc = -38 mOsm/L for 5 min (much less than our standard 30 min incubation time), f_b decreased to 0.48 and 0.25 for the same samples, respectively (0.5 μM). Again, these latter data are not shown in Fig.1 because of the different protein concentrations. They do, however, demonstrate that binding is at least partially reversible via the following simple argument. If binding were *ir*reversible then the amount of bound protein and hence the amount of protein found in the pellet would not change, but the amount of protein found in the fixed-volume aliquot of supernatant would decrease two-fold. Hence, the f_b (as defined by our measurement) would increase. By contrast, our data show clearly that f_b decreases. While we could not perform a reversibility experiment with constant protein concentration throughout, these three data sets nevertheless indicate that N-BAR binding is at least partially reversible over 5 min and likely fully reversible after 30 min.

Proposed mechanism for the tension response

The similarity of response using only glucose or glucose and NaCl together implies that the effect of Δc comes from osmotic pressure rather than from a specific interaction with glucose (which in any case was not expected). In particular, the lack of a systematic difference between NaCl and glucose solutions suggests that the electrostatic interaction between the N-BAR and lipids is not essential for the tension response. The salinity at the peak binding with added NaCl was 125 mM, which corresponds to a Debye length of about 8 Å.[48] Henne *et al.*[49] (using amphiphysin BAR) and Gallop *et al.*[30] (using endophilin N-BAR) found that the binding affinity of N-BAR was unaffected (or modestly enhanced) with increasing [NaCl] up to 250 mM, which is consistent with our results. We therefore turn our attention to a non-specific role of the solute, *i.e.*, osmotic stress and mechanical tension applied to the membrane.

Because N-BAR binding is reversible, we can use Boltzmann statistics to relate bound fraction to free energy: $f_b \propto \exp(-F_b/k_BT)$, where F_b is the free energy change from binding to the membrane. According to the data in Fig. 1, f_b increases by a factor of approximately 6.5 when τ changes from 0 to 2.6 mN/m, which indicates that the binding energy becomes larger (more negative) by approximately $k_BT \ln(6.5) \sim 2\ k_BT$ or -8×10^{-21} J. Because the binding free energy increases with mechanical tension on the membrane, we interpret this change as a mechanical effect, where binding is accompanied by an increase of total membrane area owing to partial insertion of an amphiphilic region of the protein.[32] This area increase allows work to be done by the applied tension τ. The associated work energy is $\Delta F_b = -\tau \Delta a$, where Δa is the net change of membrane area induced by binding of a single N-BAR dimer. (A conceptually similar approach has been used to describe the energy of pore formation.[6]). By this argument, our data gives $\Delta a \sim 2k_BT/(2.6\text{mN/m})$, which is approximately 3 nm^2. This value of Δa is remarkably similar to the area of the N-terminal amphipathic helix of N-BAR, which, according to simulations, is 4-6 nm^2.[50] This semi-quantitative similarity suggests that the insertion of the amphipathic helix is responsible for the positive effect of tension.

This proposed model should be considered as approximate because we have not accounted for the fact that the inner leaflet of the bilayer is not penetrated by N-BAR (as pointed out by

Campelo *et al.*).[32] Moreover, we have ignored interactions among the bound N-BAR proteins, which might arise if the membrane shape is perturbed by the binding.[51-55]

We have assumed that the vesicles remained spherical during binding (following Bhatia *et al.*[12]), though in fact a conversion of spherical LUVs to cylindrical tubules could occur and would not be detected by our centrifugation assay. If the vesicles did tubulate, then the increase of binding affinity with tension must be even larger than our estimate, in order to overcome the additional energy cost of membrane deformation. While a full analysis of the minimal-energy shape is beyond our scope, an analysis of simple limiting cases shows that tubulation is unlikely for 50-nm-radius LUVs. For example, even if the membrane area increased by 10% during tubulation owing to the hydrophobic insertion, then the interior volume of the vesicle must still decrease in order to change from sphere to tubule; this change would be accomplished by expelling water, which requires work against the interior osmotic pressure. This work can be estimated by assuming that the tubule has a diameter of approximately 40 nm.[8,56] A sphere with r_0 = 50 nm would become a cylinder of length of approx. 275 nm and interior volume that is approx. 35% less than that of the initial sphere. For the case of Δc = 40 mOsm/L, the work associated with this volume reduction is on the order of 10^3 k_BT per vesicle, or a roughly 6-k_BT energy penalty per N-BAR. On the other hand, if the interior volume were conserved then the area would expand by approx. 70% during tubulation, which would greatly exceed the lysis strain. The data show no evidence of lysis (when Δc < 70 mOsm/L), so we can rule out this possibility for the LUVs. In giant unilamellar vesicles (GUVs, approx. 10-20 μm diam.), we have seen tubules formed by N-BAR, as have other researchers studying other N-BAR proteins.[14,16,17,23] Sorre *et al*. found that in GUVs with N-BAR-containing amphiphysin1, tubule formation is suppressed when τ exceeds approximately 0.06 mN/m;[17] this threshold is well below the tension magnitudes where we found the increase in bound fraction..

Results with as-made LUVs (not pre-swelled)

We found that the response of N-BAR binding to LUVs used within 60 min after extrusion is different from the binding to LUVs that were incubated in glucose for 24 h. Figure 2 shows the measured f_b *vs*. Δc (by glucose) with LUVs measured soon after extrusion. (This plot is the equivalent of Fig. 1, except that Fig. 1 was obtained after 24 incubation). These data do not show the dramatic increase in binding at $\Delta c \sim 20$ mOsm/L, which appeared in the pre-incubated LUVs of Fig. 1. We attribute this change to the fact that the as-made extruded vesicles were not spherical. In earlier experiments, Mui *et al*. showed that extruded LUVs in sugar or salt solutions are mostly discoid in shape, in contrast to the common assumption of spherical shape.[35] Those authors reported that when as-made LUVs are exposed to a hypoosmotic solution they "round up" to spheres without experiencing an osmotic pressure or tension. Indeed, Mui *et al*. showed that as-made 100 nm LUVs composed of egg phosphatidylcholine and cholesterol withstood Δc as large as 1780 mOsm/L without experiencing any tension. Over time, however, glucose slowly permeates the membrane so that the vesicles maintain zero osmotic pressure difference while also adjusting their interior volume to adopt a spherical shape, thereby minimizing energy of both bending and membrane stretching. Hence, incubating in glucose ensures that the initial shape of the LUVs is spherical; subsequent exposure to hypoosmotic conditions then results in tension in the membrane.

The data of Fig. 2 show considerable scatter, more so than with the pre-incubated LUVs of Fig. 1. The origin of the scatter is not known, but it might arise from the distribution of vesicle

shapes that are found immediately after extrusion. We do not draw quantitative conclusions from the data of Fig. 2 but include them here to emphasize the importance of an extended incubation of the LUVs following the extrusion process.

Conclusions

In published reports, the curvature sensing or curvature affinity of N-BAR and related proteins is usually attributed to either or both of two possible mechanisms: scaffolding or hydrophobic insertion.[10,24,29,33] In measurements of the protein binding affinity via sedimentation assays, Peter *et al* found that BAR displays enhanced binding on spherical vesicles of smaller radius (done with 5 μM BAR – without the N-terminal amphipathic helix; LUVs of brain bovine lipid Folch fraction 1).[8] Those authors also found that N-BAR displays no detectable curvature preference at low concentration, though this might be explained by N-BAR inducing a curvature of the membrane in its immediate vicinity so that the global shape has a negligible effect. Fernandes *et al.* report that the amphipathic helix of N-BAR by itself is insufficient to cause tubulation and conclude that the scaffold effect must be predominant in tubulation.[28] Likewise, computer simulations of BAR and N-BAR bound to membranes indicate that efficient membrane deformation requires scaffolding via the electrostatic interactions between the BAR and lipid, and that the amphipathic helix plays a secondary role.[27,50,57,58] By contrast, in measurements of binding affinity via fluorescence microscopy, Bhatia *et al.* found that endophilin BAR displays no preference for smaller vesicles, whereas endophilin N-BAR displays a clearly measurable preference for small vesicles (1 nM – 5 μM protein, LUVs of bovine brain lipid Folch fraction 1).[12] Moreover, Galic *et al*. found that N-BAR of nadrin 2 or amphiphysin 1 (but not BAR) are recruited to curved regions of the plasma membrane of cells.[13] These two studies results imply that hydrophobic insertion, not scaffolding, is essential for the curvature affinity of BAR. Variation of observed phenomena might arise from differences in lipid composition, protein concentration, or tension in the cell membrane.[17] Our work supports the possibility that the variation in prior results with LUVs could arise from differences in membrane tension, which is usually not controlled in LUV experiments.

Our tension-controlled data are consistent with hydrophobic insertion of the N-terminal amphipathic helix: the excess area generated by insertion allows the tension to do work and thereby reduce the free energy. By contrast, scaffolding should cause the opposite result because the protein would have to gather excess membrane area in order to locally curve the membrane. This requires work against the applied tension. It might also be that tension increases the size or concentration of exposed hydrophobic regions in the membrane, known as lipid packing defects,[12,59,60] which are locations where a hydrophobic domain is likely to insert.

Our results with tensed vesicles were obtained with a vesicle curvature that was nearly constant. However, the results are consistent with the previous studies with varying curvature if we note a geometric connection between applied tension τ and membrane curvature. Consider, for example, the vesicles with $\tau = 2.6$ mN/m: prior to addition of N-BAR, the strain in these LUVs is given by τ/k, which is approximately 1%. This tension and strain cause a 6.5-fold increase in N-BAR binding (Fig. 1). For comparison, we note that a 1% strain of the outer leaflet of a bilayer could also be realized by bending a planar membrane into a cylinder of radius 200 nm or a sphere of radius 100 nm, provided that lipids lack sufficient time to flip from one leaflet of the bilayer to the other. Published experiments have shown that the binding affinity is

noticeably enhanced over this range of vesicle radii.[12] Hence, our results with fixed shape and varied tension are at least qualitatively consistent with prior measurements with varied size.

Membrane strain may therefore be the parameter that most effectively explains the behavior of N-BAR binding as a function of both tension (in our experiments) and curvature radius (in previous experiments). Previous reports proposed that curvature-induced strain not only increases binding energy but also increases the concentration of defects in the packing of the lipid molecules, which serve as protein binding sites.[12,33] Computer simulations confirm that increasing the curvature of a lipid membrane increases the density of lipid packing defects.[59] The same mechanism might apply at fixed curvature under increasing tension. In future experiments, simulations, and theory, it would be very illuminating to separate the effects of changing tension and shape.

In summary, we find that N-BAR binding is reversible and is enhanced by osmotically-induced tension in the membrane. This result is consistent with the hydrophobic insertion mechanism. With the approximations that the membrane may be treated as an elastic continuum and that the interactions among bound N-BAR proteins are negligible compared to k_BT, we found that each binding event results in a net increase of effective membrane area by roughly 3 nm^2, which is consistent with insertion of the hydrophobic region of the N-terminal amphipathic helix. Measurements of binding as a function of tension for other proteins would provide useful insight into the connection between protein structure and function on the membrane. At a practical level, our measurements also show that controlling tension is necessary for reliable measurements of binding affinity.

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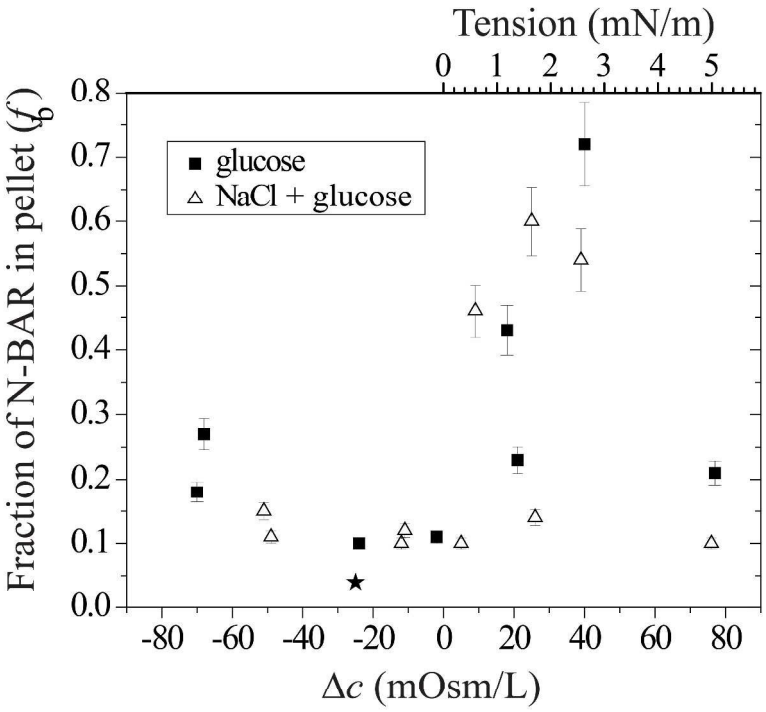

Fig. 1 Plot of the fraction f_b of N-BAR in the pellet as a function of the applied osmotic difference, Δc. For $\Delta c > 0$, the LUVs are under tension and for $\Delta c < 0$ they are relaxed. The upper axis shows the calculated tension. Filled squares: LUVs in glucose solution. Open triangles: LUVs in NaCl+glucose solution. The star corresponds to the final state of the reversibility experiment, in which the LUVS were exposed first to Δc = +37 mOsm/L and then –25 mOsm/L (glucose).

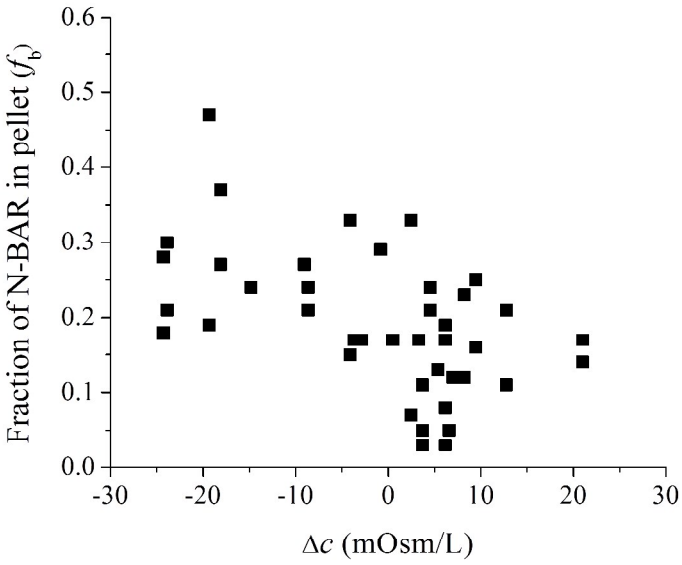

Fig. 2 Plot of the fraction of N-BAR in the pellet as a function of the applied osmotic difference Δc for as-made LUVs (without extended incubation in glucose solution).

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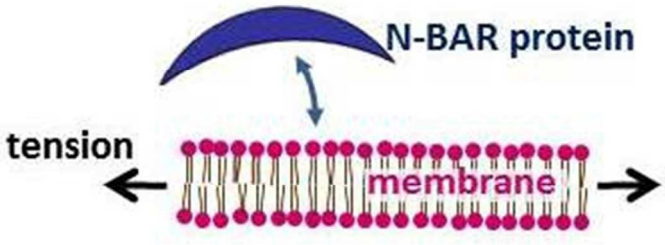

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