Nature of curvature coupling of amphiphysin with membranes depends on its bound density

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Our approach shows that the strength of curvature sensing and range, protein was enriched on the tube as compared to the vesicle. As a consequence, the formation of a scaffold around the tube. At low densities of protein on the nearly flat vesicle, the action of the protein depends directly on its density on the membrane. At high densities, the radius is independent of membrane tension. In the dilute limit, when practically no proteins were present on the vesicle, no mechanical effects were detected, but strong protein enrichment proportional to curvature was seen on the tube. At high densities, the radius is independent of tension and vesicle protein density, resulting from the formation of a scaffold around the tube. As a consequence, the scaling of the force with tension is modified. For the entire density range, protein was enriched on the tube as compared to the vesicle. Our approach shows that the strength of curvature sensing and mechanical effects on the tube depends on the protein density.

In cells, the formation of closed membrane carriers needed for membrane trafficking requires a panoply of proteins that transiently interact with the lipid membrane to control the shape, size, and composition of the nascent bud. Recently, membrane shaping and remodeling by proteins has attracted attention (1), motivated by the role membrane curvature plays in intracellular trafficking. Indeed, to generate membrane intermediates that transport cargo between organelles, many proteins have acquired through evolution the ability to sense and/or to generate membrane curvature (1).

Among these proteins are those in the BAR (Bin-Amphipathic-Rvs) family, implicated in endocytosis and in membrane-triggered actin polymerization (2). They contain a membrane-binding module, known as the BAR domain, consisting of a crescent-shaped dimer (3), and in the most common case of the N-BAR, is combined with N-terminal amphipathic helices (1). The first studies on N-BAR domain proteins showed that they strongly deformed membranes, usually forming tubular structures (4). Since then, several biochemical studies have explored the sensing and membrane-deforming functions of N-BAR proteins by measuring curvature-dependent binding (5) and by studying the effect of mutation or deletion of the N-terminal helices on tubulation (4). Meanwhile, inspired by earlier theoretical work on the effect of bound proteins and inclusions on lipid membranes (6–10), simulation work has focused on membrane curvature generated by oligomerized N-BAR proteins (11, 12), and modeling based on elastic theory has looked at the membrane curvature induced by helix insertion (13).

Despite these contributions, there is still not a comprehensive picture of the physical parameters that govern the functioning of the BAR domain. Importantly, theoretical work to date focused only on the interaction between protein and isolated curved membrane patches, without considering the connection of the curved patch to a membrane reservoir as encountered in vivo. In this study, we quantitatively evaluated the mechanical action of amphiphysin 1 when binding to the membrane as a function of parameters such as membrane tension, curvature, and bound protein density.

We chose amphiphysin 1 as a paradigm for the study of N-BAR domains, which is enriched in the necks of clathrin-coated pits prior to endocytic bud fission (14, 15). It is thought that amphiphysin 1 assists in recruiting dynamin to the neck in dynamin-dependent endocytosis (4). We have used an in vitro system in which the tension, curvature, and protein density can be varied, encompassing the entire range of physiological conditions for these parameters. This system consists of membrane nanotubes pulled by optical tweezers from giant unilamellar vesicles (GUVs) aspirated into a micropipette; the initial tube diameter (10–100 nm) is directly controlled by the tension of the GUV (16, 17). Once formed, the GUV and nanotube are exposed to a solution of full length, Alexa 488 labeled human amphiphysin 1 (referred to as amph1*). By measuring the protein densities on the GUV and tube, the pulling force, and the tube radius in the presence of bound protein, we identified how the interplay between protein and curved membrane depends on the bound protein density on the GUV. The first result is that the amphiphysin 1 density on the tube is always enriched compared to that on the GUV, for all protein densities on the GUV. The mechanical effects on the tube of amphiphysin 1 binding strongly depend, however, on the GUV density: At low densities (less than
1.000 μm⁻² on the GUV), amphiphysin amplifies curvature, but also is strongly enriched on curved membranes. Based on a theoretical model, we show that simultaneous curvature sensing and inducing at low densities results from the spontaneous curvature of the membrane generated by protein. In the extreme case of vanishing densities on the GUV, no tube deformation was detected, despite a clear enrichment of protein on the tube proportional to its curvature, in agreement with theory. At high densities (greater than 1.000 μm⁻² on the GUV), amphiphysin has a strong mechanical effect, as the tube radius no longer depends on tension, which is consistent with the formation of a protein scaffold forming around the tube. These results agree with simple modeling based on membrane bending energy, protein–membrane–protein–protein interactions. Our work provides an understanding of how the action of amphiphysin 1 depends on its bound density.

Results

Amphiphysin 1 requires the presence of negatively charged lipids to bind to the membrane due to the positive residues on the side of the BAR domain in contact with the membrane (3). We found that the simplest lipid mixture yielding GUVs on which amph1* binding was observed, and being easy to produce using an adapted electroformation procedure (SI Text), was an equimolar lipid mixture of dioleoylphosphatidylcholine (DOPC): dioleoylphosphatidylethanolamine (DOPE): dioleoylphosphatidylserine (DOPS) (1:1:1), supplemented with 0.5% (mol/mol) of a red lipid dye [Bodipy TR-ceramide (Cer*)]. All the experiments discussed below were carried out using GUVs with this composition.

Two Regimes of Amphiphysin-Membrane Interaction. To characterize the amphiphysin interaction with the membrane, we first evaluated amphiphysin 1 binding on our GUVs as a function of bulk concentration. Evolution of bound amph1* on the GUVs was calibrated using a method adapted from ref. 18; see SI Text and Fig. S1. We have measured the amph1* fluorescence signal on GUVs incubated in buffers containing increasing concentrations of amph1*. The density of bound proteins on the GUV, Φ, was found to increase nonlinearly with concentration of amph1* in bulk, Cbulk. Results are shown in Fig. 1A, in which we fit the data to a Langmuir isotherm, Φ = Cmax/(1 + Kd/Cbulk), where Kd is the dissociation constant (19). The fit gives Kd ≈ 35 nM, a small value that reflects fast protein adsorption but slow desorption (see SI Text and Fig. S2). Φ reaches a saturation density of about 3,000 μm⁻² for Cbulk in the micromolar range. This value can be compared with the area per N-BAR domain on a flat membrane, equal to 50 nm² (3), corresponding to a close-packing (CP) density ΦCP = 20,000 μm⁻². The plateau density of 3,000 μm⁻² we measured in these conditions thus corresponds to 15% of ΦCP. Based on the isotherm, we divided our study of amphiphysin-membrane interaction into two regimes: (i) For Φ ≤ 1,000 μm⁻² (Cbulk < Kd), no apparent membrane deformations were detected on the GUV, referred to as the low-density regime. In the dilute limit of this regime, where no bound protein can be detected on the GUV, we observed that, once a tube was pulled, the protein was found to be clearly enriched on tubes (Fig. 1B), suggesting curvature-sensing behavior. More generally in this regime, in addition to protein enrichment on the tube, we will investigate whether preexisting tube curvature is amplified by bound protein. (ii) For Φ ≥ 1,000 μm⁻² (Cbulk > Kd), unaspirated GUVs with bound protein frequently exhibit extensive tubular deformations, indicating a strong mechanical effect (see Fig. 1C), referred to as the high-density regime.

Based on these observations, we quantitatively study how the curvature-sensing and amplifying capabilities of amphiphysin 1 depend on the GUV density. We first consider low protein densities.

Low-Density Regime. We consider here Φ < 1,000 μm⁻². The nanotube curvature was controlled using a technique developed recently in our laboratory that enables measuring the tube pulling force at a given tension, σ, fixed by micropipette aspiration (20, 21), and the tube composition using confocal microscopy (16, 17). For a single-component membrane in the absence of protein, the tube radius Rv is given by Rv = f/2πσ, where f = 2πνσ is the pulling force on the tube and ν is the bending rigidity (22). Rv ranges from 7–10 nm up to a few 100 nm. A GUV was aspirated at low tension (ca. 10⁻⁵ N/m) and a tube was pulled with an optically trapped bead bound to the GUV. The tension was then increased step by step. The force f was found to vary linearly with √σ (open symbols in Fig. 2A), consistent with ref. 22. From the slope, we deduced ν = 12 ± 2 kBT, where kB is Boltzmann’s constant and T the temperature. Once at high tension (ca. 10⁻⁴ N/m), the injection pipette containing the amph1* solution was brought close to the GUV; the injection flow was low enough not to perturb the force measurement (see SI Text and Fig. S3). The aspiration pressure was kept constant until the amph1* fluorescence signal on the membrane equilibrated (typically 5–10 min). The tension was then decreased stepwise under continuous protein injection, with a waiting period of 1–2 min to reach mechanical equilibrium. The fluorescence intensities and force were then recorded.

Changes in the forces are a first way to probe mechanical action of the protein on the membrane. From Fig. 2A, we see that the force drops in the presence of protein, but still varies linearly with √σ, as it does in the absence of protein; in addition, the slope decreases and there is a nonzero intercept, σ*. As a second way to probe the effect of protein on membrane mechanics, we measured the tube radius in the presence of bound amph1*. Our set-up provides two methods to determine the radius. First, one can calculate it from the force and tension measurement (see above). This relation, however, should be used with caution because it might be modified in the presence of protein; see below. The second method is a direct, model-independent determination based on the lipid fluorescence of the tube, which is proportional to the radius, Rv. By normalizing the lipid fluorescence of the tube by the
fluorescence of the GUV, and considering that the dye is homogeneously distributed in the membrane, we can estimate directly the tube radius (see SI Text). We first verified that, with no protein, both measurement techniques give, within experimental error, the same value of the radius at several different tensions (Fig. S4). In Fig. 2B, we show that, after protein injection, the radius is lowered, but still decreases with increasing tension. Thus, we find that the reductions of the force and the radius in the presence of amphiphysin are clear indications of the membrane-deforming abilities of this protein.

Next, to quantify the curvature-sensing ability of amphiphysin, we measured the protein sorting ratio, $S = \Phi_s$, where $\Phi$ is the protein density on the tube (see Materials and Methods). Based on Fig. 1B, we expect a dependence of $S$ on curvature ($1/R$). In Fig. 2C, we observe a strong protein enrichment on the tube, which increases with curvature.

The affinity of amphiphysin 1 for curved membranes results from the lowering of the membrane bending energy upon binding to the outer leaflet, which can be described in terms of membrane spontaneous curvature (7, 10, 13). At low protein densities, we observe a strong protein enrichment on the tube, which increases linearly with $1/R$. Based on our model, we now discuss quantitatively the mechanical effects of amphiphysin binding on tubes. First, the spontaneous curvature has an effect on the force at low densities. Theory predicts a linear relation between the force and $\sqrt{\sigma}$, in agreement with our experiments (see Fig. 2A). From a fit of Fig. 2A to Eq. 2, we extract $\kappa_{\text{eff}}$, and $\kappa_{\text{eff}}$. Indeed, from Eq. 2, we expect that for small $\phi$, the force vanishes at $\sigma = \frac{\kappa_{\text{eff}}}{R}$. In Fig. S6A, we plot $\sigma$ versus $\phi$; a linear fit yields $C_0^{-1} = 1.3 \pm 0.6$ nm. For small values of $\phi$, we expect that the effective bending rigidity varies as $\frac{\kappa_{\text{eff}}}{R} = 1.3 \pm 0.6$ nm. We note that characterizes the background noise intensity for an overestimate of $\phi$, yielding $C_0^{-1} = 6 \pm 3$ nm. Second, we see from Fig. 2D how the tube radius is modified by protein binding. Fig. 2D shows that, at fixed tension, the radius decreases with $\Phi$. Based on our model, the spontaneous curvature causes a lowering of the radius. Consistently, for small values of $\phi$, Eq. 2 gives $R = \sqrt{\frac{\kappa_{\text{eff}}}{\kappa_{\phi}}}(1 - \frac{\kappa_{\phi}}{\kappa_{\text{eff}}})$. We use this expression to fit the radius data in Fig. 2D for $\Phi < 600 \mu m^{-2}$, giving $C_0^{-1} = 5 \pm 2$ nm.

We next compared our theoretical prediction of the protein sorting with the experimental data, for $\Phi = 280 \pm 100 \mu m^{-2}$. We have fitted Eq. 3 to Fig. 2C, which provides another value of the spontaneous curvature, $C_0^{-1} = 1.9 \pm 0.4$ nm, for which we have used $\rho = 1/50 \text{ nm}^{-2}$ and have neglected the entropic part of $\chi$. Thus, at low protein densities, we see that protein enrichment on the tube and mechanical effects are simultaneously observed. By comparing our theory with experiments, we are able to extract an estimate of the mechanical parameter $C_0$ that characterizes the deformations caused by amphiphysin 1.

To further understand the low-density regime, we investigated the dilute limit, corresponding to densities on the GUV indistinguishable from the background level (we used the amplitude of the background noise intensity for an overestimate of $\Phi$, ref. 24, $\leq 100 \mu m^{-2}$). In this case, Fig. 1B shows that amphiphysin is enriched on the tube, but is undetectable on the GUV. We observed furthermore that upon protein injection and adsorption there was no detectable change in the dependence of $f$ on $\sqrt{\sigma}$ (see Fig. 3D, filled symbols). Because our force resolution is limited to 1 pN, any changes in the force due to protein binding must be less than this value. This result on the force is consistent with the dilute limit of Eq. 2, in which the protein effect on the force vanishes. We also see from our fluorescence data (Fig. 3B) that, within experimental error, protein binding in the dilute limit has
no detectable influence on the radius. In addition, there is no measurable difference between the radius calculated from the force \( R_{\text{pwm}} \) and from direct fluorescence measurements \( \langle R_{\text{pwm}} \rangle \) (Fig. 3B). Thus, we find that, in this limit, the binding of amph1* has no detectable effect on membrane tube mechanics, as predicted by a limiting case of our model.

In contrast, the dilute limit of our model predicts a finite relative enrichment of protein on the tube versus that on the GUV. In this limit, \( S \) (see Eq. 3) is given by

\[
S = 1 + \frac{\kappa}{k_B T \rho R_t}. \tag{4}
\]

An important consequence of Eq. 4 is that protein sorting is independent of \( \Phi_t \), in contrast with the mechanical effects of protein binding, which vanish as \( \Phi_t \) tends to zero.

In Fig. 3C, we find a linear dependence of \( \Phi_t \) on tube curvature, and importantly in Fig. 3D a strong sorting signal that is linear with curvature. We have fitted the theoretical sorting expression, Eq. 4, to data compiled from five different vesicles (Fig. 3D). To estimate the parameter \( C_0 \) from Fig. 3D, we used \( k = 12 \ k_B T \), which gives \( C_0^{-1} = 0.8 \pm 0.4 \ \text{nm} \). We remark that this value is of the same order of magnitude as that obtained theoretically for \( \alpha \)-helix insertions in bilayers (13).

We thus conclude that, in the dilute limit, bound amph1* density is sensitive to curvature, yet has no detectable mechanical effect on the tube; it behaves mainly as a curvature sensor.

**High-Density Regime.** We consider next \( \Phi_t \gtrsim 1.000 \ \mu \text{m}^{-2} \). In this regime, we observe significant labeling of amph1* on both the tube and the GUV (Fig. S7A). Similar to the low-density regime, after protein injection there is a downward shift in the force compared with that in the absence of protein; see Fig. 4A, where \( \Phi_t = 1.100 \pm 200 \ \mu \text{m}^{-2} \). However, in striking contrast with the force at low densities, the force scales linearly with \( \sigma \), instead of with \( \sqrt{\sigma} \) (Fig. 4A and Fig. S8). The tension at which the force vanishes, \( \sigma^* \), reaches values up to four times higher than in the low-density regime, (see Fig. S9 in which \( \Phi_t \approx 2.000 \ \mu \text{m}^{-2} \), \( \sigma^* = 6 \times 10^{-5} \ \text{N/m} \). When the tension is decreased below \( \sigma^* \) and the optical trap is turned off, the tube does not retract (Fig. S7B).

Given that the force on the tube is strongly affected by amph1* in the high-density regime, we expected that the tube radius should also change markedly. We measured the radius in the high-density regime using the lipid fluorescence method described above. After injection, as the tension was then lowered, the radius remained constant, at roughly 7–10 nm (full circles in Fig. 4B), in contrast with the increase in radius with decreasing tension for bare tubes (empty symbols in Fig. 4B). Our fluorescence measurements on seven different tubes in this regime, corresponding to different values of \( \Phi_t \), show that, on average, the radius is equal to \( 7 \pm 2 \ \text{nm} \); see Fig. 4C.

In this regime, the density of amph1* bound to the tube, \( \Phi_t \), is also quasi-constant: It rapidly saturates with tension (Fig. S9C) and reaches values higher than the saturation density on the GUV, \( \Phi_{t,\text{GUV}} \), of the order of 4,000–6,000 \( \mu \text{m}^{-2} \). These density values are summarized in the *SI Text* (Table S1), together with the densities on the GUVs, showing an enrichment of amph1* on the tube relative to the GUV, but weaker than for the narrowest tubes in the dilute limit; see Fig. 3C.

The scaling of the force with tension and our radius measurements in the high-density regime cannot be explained with the model used in the low-density one, in which protein–protein interactions were neglected; this suggests that they play an important role in this regime, as in ref. 17. The importance of protein–protein interactions is supported by several lines of evidence. First, electron microscopy has revealed that N-BAR domains

![Fig. 3](image-url)

**Fig. 3.** The dilute limit. The results presented in this figure correspond to a single GUV with \( \Phi_t < 50 \ \mu \text{m}^{-2} \). (A) No difference between the tube force as a function of \( \sqrt{\sigma} \) with protein (□) or without (●). The linear fit to the force, \( f = 2 \pi \sqrt{2} \kappa \sigma \), gives \( k = 12 \ \k_B T \). (B) The radius, \( R_t \), versus tension, \( \sigma \), with protein (empty symbols) or without (full symbols). Radius is deduced either from fluorescence (round symbols) or from force (square symbols) measurements. (C) Amphiphysin density on the tube, \( \Phi_t \), versus tube curvature, \( 1/R_t \). \( R_t \) was found from force measurements. A linear fit yields \( \Phi_t = A/R_t^{\mu} \mu \text{m}^{-2} \), where \( A = 29 \pm 2 \ \mu \text{m}^{-1} \). (D) Linear variation of the sorting ratio as a function of \( 1/R_t \). Data correspond to five independent experiments. A fit using Eq. 4 gives \( C_0^{-1} = 0.8 \pm 0.4 \ \text{nm} \).

![Fig. 4](image-url)

**Fig. 4.** High-density regime. The experiments presented in A to D correspond to a single GUV with \( \Phi_t = 1,100 \pm 100 \ \mu \text{m}^{-2} \). (A) The force is lower with protein (●) than without (□). Force data without protein are fitted to \( f = A/f_0 \ \text{pN} \), where \( A = 56 \pm 1 \ \text{pN}/\text{fN}^{1/2} \), and without to \( f = B(\sigma - \sigma^*) \), where \( B = 67 \pm 4 \ \text{nm} \) and \( \sigma^* = 1.0 \pm 0.9 \times 10^{-5} \ \text{N/m} \) (the asterisk denotes \( \sigma^* \)). (B) \( R_t \) versus \( \sigma \) with no protein (empty symbols) and with protein (full symbols). The radius is found from fluorescence (round symbols) or from force (square symbols) measurements. With no protein, the radius was determined from the force according to \( R_t = f/2 \pi \sigma \). (C) \( R_t \) as a function of \( \Phi_t \), as measured by fluorescence. (D) Tension at zero force, \( \sigma^* \), versus \( \Phi_t \). Data were fitted to Eq. 6, neglecting the logarithmic term, \( \sigma^* = A \Phi_t + B \), where \( A = (4 \pm 1) \times 10^{-6} \ \text{Nm}^{-1} \mu \text{m}^{-2} \) and \( B = (2.7 \pm 1.4) \times 10^{-5} \ \text{N/m} \).
form striations on tubules, suggestive of higher-order oligomerization (4). Polymerization mediated by interactions between N-terminal helices has been observed in F-BAR domains, a related family of protein module (25). It is thus expected that these N-terminal helix-mediated interactions are important in proteins containing the N-BAR domain, as predicted by simulations (12).

In our experiments, the independence of tube radius on tension (Fig. 4B) suggests that proteins reorganize into a scaffold-like structure whose radius is imposed on the tube. To verify this hypothesis, we performed fluorescence recovery after photobleaching (FRAP) experiments on tubes coated with amphiphysin in the high-density regime. Results are shown in Fig. S10. On the tube, no recovery is seen 5 min after bleaching, confirming interactions between amphiphysin dimers. On the contrary, a rapid recovery is seen when FRAP was performed on the same GUV, indicative of different organization of proteins on the GUV than on the tube, associated with the difference in area density. Based on this evidence, we have developed a theoretical model of tube mechanics at high densities in which the radius is fixed by protein–protein interactions, an approach which has been successfully applied to dynamin polymerization around membrane tubules (17).

Here, we assume that the bulk concentration is high enough that all negatively charged lipids on the GUV and tube are protein bound; thus, as the tension is varied during the experiment, there may be exchange of proteins between the tube and the GUV, but no exchange with the bulk solution. We also assume that proteins in the tube are in a condensed phase with a well-defined bound area fraction, \( \phi_t \). In this regime, the tube free energy is

\[
F_t = 2\pi R_t L_c \left[ \sigma + W(\phi_t) + f_m(\phi_t) \right] - fL_t, \tag{5}
\]

where \( R_t \) is the constant tube radius, fixed by the protein scaffold; \( W \) is the enthalpy density that includes membrane bending and protein–protein interactions; and \( f_m(\phi_t) \) is the protein mixing entropy density; see SI Text. The force can be found by minimization of the free energy with respect to tube length; the system consisting of the pipette-aspirated tongue, the vesicle, and the tube is subjected to the constraints of total membrane area and total number of bound proteins (see SI Text). When the length of the tube is altered, it is assumed that the concentration on the tube remains unchanged. We may show then that (see SI Text) the force may be expressed as

\[
f = 2\pi R_t \left( \sigma - \sigma^* \right), \quad \text{where} \quad \sigma^* = \kappa C_0 \phi_t \phi_0 - W + \rho k_B T \phi_t \ln \left( \frac{\phi_t}{\phi_0} \right), \tag{6}
\]

defines the tension below which the tube is stable under no force. In this expression, the first term comes from the gain in elastic energy in transferring proteins from an energetically unfavorable situation on the vesicle to the tube; the second comes from the gain in enthalpy; and the last term comes from the entropy penalty in further enriching protein on the tube. From this analysis, for tensions significantly greater than \( \sigma^* \), it follows that the radius is \( R_t \approx \frac{f}{\pi \sigma} \). In Fig. 4B, we show the radius as a function of tension as measured by fluorescence and as calculated from the pulling force, and both are in good agreement. Importantly, we are able to measure the protein’s ability to stabilize membrane tubules as a function of protein density on the GUV, as given by \( \sigma^* \); see Fig. 4D. Neglecting the small logarithmic term in Eq. 6, a linear fit to Fig. 4D yields \( C_0 \approx 4.0 \pm 1.5 \) nm, where the value of \( \phi_t \) used is the average over all vesicles, equal to 5.000 \( \mu \text{m}^{-2} \) (See Table S1). We thus see that, although the tube radius is fixed by the protein scaffolding, the pulling force is influenced by the quantity of protein on the vesicle.

**Discussion**

Prior studies of amphiphysin 1 binding to membranes assessed the deforming ability of the protein by comparing wild-type and mutated forms (3–5), often with curvature sensing and curvature inducing separately occurring (26). We have shown in this study that amphiphysin 1 can act as a curvature sensor and a curvature inducer simultaneously, for a wide range of densities. Moreover, by varying the protein density, and without performing any mutation, the relative strength of curvature sensing and amplifying can be tuned: At very low densities, this protein mainly senses curvature, whereas at high densities, it strongly deforms membranes by forming a scaffold-like structure around tubes. We note that similar scaffolding behavior on tubes has been measured for dynamin (17).

In the high-density regime, we have measured the tube radius, \( R_t \), using fluorescence and force measurements to be 7 ± 2 nm. Within experimental precision, this measurement compares well with the internal radius of amphiphysin-coated membrane tubules studied in vitro by electron microscopy (4). Though our measurement of \( R_t \) is close to the radius of curvature of the N-BAR dimer, in general the radius depends strongly on the type of organization that occurs on the membrane; simulations find radii varying between 13 and 80 nm, depending on the packing arrangement (12).

With our experimental approach, we have been able to evaluate, from five independent measurements, the effective spontaneous curvature, \( C_0 \), of amphiphysin 1. We emphasize here that \( C_0 \) is not the same as the tube radius \( R_t \) measured in the high-density regime, nor is it the spontaneous curvature measured in ref. 27. This parameter determines the relative enrichment of protein on the tube in the dilute limit. Furthermore, it is the mechanical parameter of the protein that controls the curvature amplification by the protein in the low-density regime, in which interactions between proteins can be ignored. In this regime, the deformation of the tube by proteins can be described completely by \( C_0 \), without recourse to a detailed knowledge of the structure of the protein. We found that, even in the high-density regime, the pulling force depends on \( C_0 \) because the tube is connected to a large vesicle on which the interaction between protein and membrane is governed by \( C_0 \). We found that the five values of \( C_0 \), obtained using force and fluorescence measurements, spanned over three decades in GUV density, were all of the same order of magnitude (on the order of 3 nm).

The exact role of the N-BAR domain has been intensively discussed in the past due to its particular structure. The crescent shape was thought to either mold the membrane or bind to highly curved membranes, in both cases the membrane curvature matching the shape of the protein. Our study gives a different view of the membrane–N-BAR domain interaction: That amphiphysin 1 binds to a wide range of membrane curvatures and that the degree of membrane deformation depends on the protein density argues against the protein solely deforming the membrane through a sculpting action. Instead, at low densities, the mechanical action of the protein on the membrane depends on \( C_0 \) and on the GUV density \( \Phi_t \). Beyond a certain protein density, the tube radius no longer depends on the GUV density, and interestingly, the corresponding density on the tube is well below the closest packing value, suggesting that lateral interactions between the protein terminal helices play an important role in creating the scaffold-like structure on the tube. The existence of such lateral interactions between the N-terminal amphiphatic helices of N-BAR domain proteins has been demonstrated recently using Förster resonance energy transfer in ref. 28.

Our in vitro study can help shed light on in vivo results. When moderately overexpressed, most BAR proteins do not bind strongly to the plasma membrane and have a diffuse cytosolic staining (3, 29, 30). However, if the protein density on the membrane is increased under strong overexpression, numerous tu-
bules are observed (3), pointing to a membrane-shaping function. A specific case of membrane shaping is that of muscular amphiphysin 2 (M-Amph2), a splice variant of amphiphysin 1 with an additional membrane-binding domain, which has been reported to play a critical role in the formation of tubular invaginations (T-tubules) in striated muscle cells (29, 31). Interestingly, when wild-type fluorescent M-Amph2 is expressed in nonmuscle cells, strong labeling on the plasma membrane and tubule formation are observed; however, deletion of a specific domain binding to PIP2 (exon10), a lipid concentrated in the plasma membrane, suppresses plasma membrane labeling and tubulation (29). This suggests that this domain induces stronger membrane-protein interactions, thus a higher protein density on the plasma membrane suggests that this domain induces stronger membrane-protein interactions: Insights from multi-scale simulation. 91:138102.

Materials and Methods

Giant Unilamellar Vesicles. All the data presented in this study have been obtained with GUVs having the same lipid composition: DOPC: DOPE: 1:1:1 + 0.33% distearoyl phosphatidyl ethanolamine-PEG(2000)-Biotin (Avanti Polar Lipids, Inc.) and 0.5% BODIPY TR ceramide (Cer* Invitrogen). Giant Unilamellar Vesicles.

To obtain yields with this composition containing a high amount of negatively charged lipids, we adapted the electroformation protocol described in ref. 33. Ten microliters of lipid mix at 0.5 mg/mL was dried on conductive indium-tin oxide coated glass (Präzisions Glas und Optik, GmbH) for a few minutes at 60 °C then under high vacuum for at least 1 h. The lipid film was then rehydrated in a sucrose solution (osmolarity 100–300 mOsM) and GUVs were allowed to grow for 30 min under a sine voltage (850 mV, 10 Hz).

Force and Tension Measurements. Our experimental setup has been described elsewhere (16). The force f exerted by the tube was found from the displacement of the bead x – x0 from its equilibrium position x0 in the optical trap using the linear relationship f = k(x – x0), where k is the trap stiffness. The bead position was measured off line by video tracking (21) and k was determined by the viscous drag method (34). The membrane tension σ was determined using σ = ΔPΓRpip, where Rpip is the pipette radius, Rpip the vesicle radius, and ΔP is the difference of hydrostatic pressure caused by the vertical displacement of the water reservoir connected to the pipette (20).

Protein Density on the Tube. To measure the protein densities on the membrane, we used a fluorescence calibration method adapted from ref. 18. The amphiphysin density on the GUV, Φpip, is given by Φpip = cal × I, where I is the fluorescence signal on the GUV coming from labeled amphiphysin, and cal is an experimentally measured conversion constant. The protein density on the tube, Φt, is given by Φt = Φpip × (Rpip/Rt)2, where Rt and Rpip are the fluorescence intensities per pixel of amphiphysin on the tube, of the lipid Cer* on the GUV. The lipid intensities are used here to eliminate geometric factors (see SI Text and Fig. S1).

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Supporting Information

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SI Text

**Giant Unilamellar Vesicles (GUVs) Preparation. Reagents.** Lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), DSPE (di-stearoyl phosphatidyl ethanolamine)-(PEG2000)-Biotin, dioleoylphosphatidylserine (DOPS), and dioleoylphosphatidylethanolamine (DOPE) were purchased from Avanti Polar Lipids. BODIPY-FLCS-hexadecanoyl phosphatidylcholine (HPC*) and BODIPY-TR-C5-ceramide (Cer*) were obtained from Molecular Probes.

**Electroformation technique.** All the data presented have been obtained with GUVs having the same lipid composition: DOPC:DOPE:DOPS (1:1:1) + 0.03% DSPE-PEG(2000)-Biotin and 0.5% BODIPY TR ceramide. To obtain good yields with this composition containing a high amount of negatively charged lipids, we had to adapt the original electroformation protocol described in refs. 1 and 2. Ten microliters of lipid mix at 0.5 mg/mL were deposited on conductive indium-tin oxide (ITO) coated glass (Präzisions Glas and Optik). The lipid film was dried for a few minutes at 60°C to obtain better homogeneity in the lipid film (3) and subsequently dried under high vacuum for at least 1 h. The lipid film was then rehydrated in a sucrose solution (osmolarity 100–300 mOsm) in a growth chamber made of two ITO electrodes separated by 1 mm. We observed that charged vesicles grew much faster than those with zwitterionic lipids. As a consequence, only 30 min of growth under a sinusoidal voltage of 850 mV rms amplitude and 10 Hz frequency were sufficient to obtain a large number of vesicles.

**Protein Purifications.** Human amphiphysin 1 was purified using the standard GST fusion expression protocol as detailed in the manufacturer literature (Glutathione Sepharose 4B from GE Healthcare). Bacteria (BL21 Escherichia coli) were transfected with a plasmid expressing GST fusion of the proteins of interest under the control of an IPTG sensitive inducer, a generous gift from P. de Camilli (Yale, New Haven, CT). Protein production was induced by addition of 1 mM IPTG in the culture medium during 3 h at 37°C or overnight at 30°C. GST fusion amphiphysin 1 was purified from cell lysate using GST beads (GE Healthcare). The protein was eluted with 25 mM reduced glutathione in PBS at pH 7.8. GST was cleaved by GST-linked precision enzyme during overnight dialysis in experiment buffer (20 mM Hapes, 100 mM NaCl). The free GST and GST-linked precision enzyme were removed with another round of binding to GST beads. Amphiphysin 1 concentrations were measured using Bradford assay (Biorad) and light absorption at 280 nm (nanodrop). Our purification yielded 1 mL of 4.5 μM amphiphysin solution. An average number of three Alexa 488 bound was found per amphiphysin dimer, using extinction coefficient at 488 nm (nanodrop). Tubulating activity of the protein was checked using the flat membrane sheets tubulation assay (4).

**Experimental Protocol for Tube Pulling Experiments.** In order to study the impact of a soluble membrane-deforming protein on membrane mechanical properties, the tube pulling protocol that we have used in our previous studies (5, 6) had to be slightly modified: Fluorescent Alexa 488-labeled amphiphysin 1 (amph1*) was not present in bulk in the experiment chamber. Instead, it was injected near the GUV under study, using the microinjection protocol presented in the following.

Microinjection was chosen for three reasons: (i) This protocol permits us to reach high local amph1* concentration while using only a small amount of protein. (ii) When amph1* is present everywhere in solution prior to micromanipulation, GUV are often found to be already tubulated (see Fig. 1C), which is not compatible with tube pulling experiments. Microinjection allows us to control the initiation of amph1* binding. (iii) Finally, before injection of amph1*, the mechanical characteristics of each GUV in the absence of amph1* can be measured, providing a reference for each experiment. We used large pipettes (about 10 μm in diameter). To control the injection pressure, we used a hydrostatic system similar to the one we used to control the membrane tension (see Materials and Methods in text). The pipette was first filled by the tip to prevent jamming with a few microliters of protein solution and then backfilled with mineral oil. Oil prevented diffusion of our protein into the water of the pressure control system. A pressure as low as 10 Pa was sufficient to inject protein only around the vesicle under study, with flow speed low enough to avoid force measurement perturbation. This protocol allowed us to monitor in real time the effects of protein binding on membrane mechanical properties.

A typical experiment went as follows. A GUV, made of DOPC:DOPE:DOPS (1:1:1), initially under low membrane tension (showing optically visible fluctuations) was grabbed with the aspiration pipette. A tube was pulled with an optically trapped bead, and the membrane tension (σ) was increased step by step by rising micropipette aspiration. For each membrane tension, the force necessary to hold the tube was recorded. Once at high membrane tension, the injection pipette containing amph1* in solution was brought in the field of view, close to GUV (typically 10–20 μm). The injection flow was slow enough (<μm·s⁻¹) not to perturb the force measurement and the osmolarity of the injection buffer was matched to the experiment buffer to avoid any osmotic shock during injection. The membrane tension was kept constant until the amph1* fluorescence signal on the GUV was stabilized (5–10 min). The membrane tension was then decreased step by step. The protein solution was continuously injected during the course of the experiment, keeping the bulk concentration constant near the GUV. At both low and high concentrations, we observed that amph1* binding to the tube was homogeneous along the length of the tube (Fig. S7), and took approximately 1 min to reach steady state. For each value of the tension, the force was recorded and an image was acquired. We checked that, when a buffer solution without protein was injected, the forward and backward curves were superimposed and that the force (f) was linear with √σ (see Fig. S3). By fitting these curves, we obtained a value of the bending rigidity of κ = 12 ± 4 k_B T for our DOPC:DOPE:DOPS mix.

**Tube Radius Measurement.** Our experimental setup provides two independent ways to measure the tube radius. The first method consists in using the well-established equilibrium relationship between the tube radius (R), the force necessary to hold the tube (f), and the membrane tension (σ): (7)

\[ R = \frac{f}{4\pi\sigma} \]  

where f and σ are measured quantities in our system. This expression might not, however, be valid in cases where the membrane is covered by proteins.

We used a second, model-independent method to measure R that takes advantage of the fact that, in most experiments, the tube diameter is smaller than the thickness of the confocal...
volume. The fluorescence signal coming from fluorescently labeled lipids in the membrane in the tube ($I_v$), normalized by the same intensity in the GUV ($I_l$), is proportional to the tube surface and thus to the tube radius. As a consequence, the fluorescence signal should be proportional to the radius value deduced from force and tension measurements:

$$R = F \times \left( \frac{I_v}{I_l} \right). \quad [S2]$$

The prefactor value ($F = 200 \pm 50$ nm, based on six independent measurements) has been calibrated experimentally using a linear fit of the radii values measured with force measurements in the absence of protein on the membrane, ($R = \frac{I_v}{I_l}$) vs. $I_v/I_l$; see Fig. S4.

**Measurement of Protein Density on the Membrane.** The fluorescence signal measured using confocal microscopy is proportional to the number of fluorescent molecules in the confocal volume; it is therefore possible to transform the fluorescence values given by the microscope (in gray levels, arbitrary units) into a density of bound molecules per unit area, if an appropriate calibration method is used. We have adapted the “supported lipid bilayer standard” method proposed by ref. 6. The standard used is a lipid bilayer containing a controlled amount of a lipid dye emitting in the same channel as the labeled protein of interest. In ref. 8, the standard used is a flat supported bilayer because the object of their study has a flat geometry (adherent cells). Here, we used GUVs containing BodipyFL-C5-HPC (HPC*), a green fluorescent labeled lipid, at various concentrations. An image analysis procedure used to extract fluorescence values from images has been described elsewhere (5). We found that the measured fluorescent signal ($HPC^*$) from GUV was linear with HPC* for area density $\Phi_{HPC}$ up to 14,000 HPC* per $\mu$m$^2$ (ca. 0.5 mol %); see Fig. S1A:

$$\Phi_{HPC} = A_{\text{gain}} \times \frac{I_v}{I_l}. \quad [S3]$$

The conversion constant $A_{\text{gain}}$ was measured for various gains of the confocal photomultiplier tube detector (PMT). For the example presented in Fig. S1A (PMT gain = 110), we found $A_{\text{gain}} = 3.80 \pm 0.15$ (based on two independent measurements). Eq. S3 is however only valid to quantify HPC* fluorescence in the membrane. Because amph1* will have a different efficiency than HPC*, the calibration must be corrected to take into account the spectral differences of the two fluorophores and how those spectra are affected by the microscope optics:

$$\Phi_1 = A_{\text{gain}} \times \frac{I_v}{I_l}. \quad [S4]$$

where $F_1 = I_p/\text{emiss} \times I_l$ is the ratio of fluorescence intensities of amph1* and HPC*, respectively, at a given concentration in solution, and $I_p$ is the fluorescence signal coming from amph1* measured on the GUV. $F_1$ is deduced from the evolution of the intensity ratio measured in solution, as a function of the concentration of labeled molecules in bulk. In the case of HPC*, which is an insoluble molecule, we measured the bulk fluorescence of a solution of small unilamellar vesicles (SUVs) which have a size below optical resolution. We found that both signals of amph1* and HPC* in SUVs in bulk were linear with labeled molecule concentration (Fig. S1 B and C, respectively). $F_1$ is the ratio of the slope of the linear fits to graphs C and B in Fig. S1. Ultimately, we used a value of $F_1 = 1.8 \pm 0.1$, this value is independent on the PMT gains. Because there is an average of three Alexa 488 molecules on each amph1* dimer, corresponding to a relative efficiency of Alexa 488 to BodipyFL-HPC of 0.6, a value comparable to those found by Galush et al. (8). Finally, for a given value of PMT gain, the area density of amph1* on the GUV ($\Phi_1$), in number of molecules bound per $\mu$m$^2$, is then

$$\Phi_1 = \text{cal} \times I_v. \quad [S5]$$

where cal = $A_{\text{gain}}/F_1$ is the conversion constant between fluorescence signal due to amph1* and its density on the membrane, for a given value of the PMT gain of the confocal.

We note that the relative error on protein density, $\Phi_1$, is the sum of the relative error on the fluorescence calibration (10%, see above) and the relative error on the fluorescence measurements from a single GUV. In a single experiment, the amph1* fluorescence on the GUV is measured typically 5–10 times. The error on the fluorescence measurement is thus given by the standard deviation.

**Amphiphysin density on the tube.** Amphiphysin density on the tube $\Phi_t$ is given by

$$\Phi_t = S \times \Phi_1. \quad [S6]$$

where $S$, the sorting ratio, is defined as the relative enrichment of amphiphysin on the tube compared to the GUV: $S = \frac{F_v}{F_l}$, where $F_v$, $F_l$, and $I_v$ are the fluorescence intensities per pixel of amph1* on the tube, of the lipid Cer* on the tube, and of Cer* on the GUV, respectively. It follows that the amphiphysin density on the tube is given by

$$\Phi_t = \frac{\text{cal} \times I_v}{I_l} \times I_v. \quad [S7]$$

The lipid intensities are used here to eliminate geometric factors; we note that no sorting of Cer* occurs, as it is equally distributed on the tube and on the GUV.

**Langmuir isotherm.** The density of bound protein on the GUV follows a Langmuir binding isotherm (see Fig. 1A) when the amph1* bulk concentration is increased: $\Phi_t = \Phi_{\text{max}}/(1 + (K_d/C_{\text{bulk}}))$. We found $K_d = 35$ nM for the binding of amph1* on DOPE:DOPS (1:1:1) GUVs. This value will change depending on the nature and the density of charged lipids in the membrane (9). This low $K_d$ value reflects a strong asymmetry between binding and unbinding processes. Indeed, we have measured that for $C_{\text{bulk}} \approx 1$ mM, it takes about 1 min to reach steady state when amph1* is injected, which corresponds to an on-rate $k_{\text{on}} \approx 1/60$ M$^{-1}$ s$^{-1}$, and therefore an off-rate $k_{\text{off}} = k_d = k_{\text{on}} \approx 1/(30$ min). In fact, when a GUV is coated with amph1*, it takes more than 30 min (see Fig. S2) to desorb half of them, confirming the very slow desorption process. Our experiments are performed 2 or 3 min after injection, and usually last less than 10 min, implying that, in the high-concentration regime, the protein density on the GUV $\Phi_t$ is at steady state and constant during the measurement time, as confirmed experimentally. Moreover, as $\Phi_1 \approx \Phi_{\text{max}}$, we can consider that all DOPS molecules are saturated by proteins. In the second limiting regime corresponding to very low bulk concentrations, the time evolution of $\Phi_t$ is very slow, being proportional to $k_{\text{on}} = C_{\text{bulk}} \approx 10^{-3}$ s$^{-1}$ for $C_{\text{bulk}} \approx 10$ nM. Thus, the protein density on the membrane will be changed only by a factor 2 in 20 min.

**Model of Amphiphysin Binding to Membrane Tubes.** In this section, we present a theoretical model of the adsorption of amphiphysin to membrane tubes. In Free energy (below), we propose a free energy for a membrane tube connected to a vesicle. In Protein binding in the dilute regime, we consider the dilute regime and calculate the protein sorting, the tube radius, and the force.
needed to hold the tube. In Protein binding in the concentrated regime, we consider the concentrated regime and show how the pulling force can vanish at a sufficiently low tension. We show furthermore how this critical tension depends on the protein density on the vesicle.

**Free energy.** The membrane free energy that we use consists of the Helfrich bending energy and energies that account for protein mixing entropy and protein–protein interactions. We consider the system consisting of a GUV, a tongue aspirated in a pipette, and a long membrane tube, as shown in Fig. S5.

The free energy of this system is written as

\[
F = F_p + F_v + F_t = PV_{tot} + \sigma A_{tot} - \mu N_{tot},
\]

where \(F_p\), \(F_v\), and \(F_t\) are the free energies of the pipette-aspirated tongue, the vesicle, and the tube. The quantities \(P\), \(\sigma\), \(\mu\) are Lagrange multipliers introduced to keep the total volume, \(V_{tot}\), the total area, \(A_{tot}\), and the total number of proteins, \(N_{tot}\), constant. The total volume of the system is

\[
V_{tot} = V_p + V_v + V_t,
\]

where

\[
V_p = \pi R_p^2 L_p,
\]

\[
V_v = \frac{4}{3} \pi R_v^3,
\]

\[
V_t = \pi R_t^2 L_t,
\]

and where \(R_p\) is the pipette radius, \(L_p\) is the tongue length, \(R_v\) is the vesicle radius, \(R_t\) is the tube radius, and \(L_t\) is the tube length. The total area of the system is

\[
A_{tot} = A_p + A_v + A_t,
\]

where

\[
A_p = 2\pi R_p L_p,
\]

\[
A_v = 4\pi R_v^2 - \pi R_v^2,
\]

\[
A_t = 2\pi R_t L_t.
\]

We note that we have neglected the extremities of the tongue and the tube. In fact, when we minimize the free energy with respect to \(L_p\) and \(L_t\), these constant terms vanish, and hence are not important here. Finally, the total protein number is

\[
N_{tot} = \rho (\phi_p A_p + \phi_v A_v + \phi_t A_t),
\]

where \(\phi_p\), \(\phi_v\), and \(\phi_t\) are the protein area fractions on the tongue, vesicle, and tube. The constraint on the protein number is an approximation. In effect, in the dilute regime, we assume that the adsorption of proteins from the bulk solution is a much slower process than diffusion between the tube and vesicle. In the concentrated regime, we assume that all available charged receptor lipids are saturated, so that no new adsorption occurs over the time scale of the experiment; see Langmuir isotherm.

In the dilute regime, we neglect interactions between proteins and the effect of binding of individual proteins to the membrane is modeled by a concentration-dependent spontaneous curvature. We assume that the concentration of proteins on the pipette and vesicle is always low enough so that protein–protein interactions there may be neglected. In this regime, the individual energies of the pipette tongue, vesicle, and tube are

\[
F_p = P_p V_p + A_p \left\{ \kappa \left( \frac{1}{R_p^2} - C_0(\phi_p) \right)^2 + f_m(\phi_p) \right\},
\]

\[
F_v = P_v V_v + A_v \left\{ \kappa \left( \frac{2}{R_v^2} - C_0(\phi_v) \right)^2 + f_m(\phi_v) \right\},
\]

\[
F_t = P_t V_t + A_t \left\{ \kappa \left( \frac{1}{R_t^2} - C_0(\phi_t) \right)^2 + f_m(\phi_t) \right\} - f L_t,
\]

First, in Eq. S18, \(P_p\) is the pressure in the pipette, \(C_0(\phi)\) is the protein area fraction-dependent spontaneous curvature, and \(f_m(\phi)\) is the mixing free energy, given by

\[
f_m(\phi) = k_B T \rho \mu \ln \phi + (1 - \phi) \ln (1 - \phi),
\]

where \(k_B\) is Boltzmann’s constant and \(\rho\) is the inverse area per protein. Second, in Eq. S19, \(P_v\) is the pressure outside the vesicle. Finally, in Eq. S20, \(f\) is the force exerted on the free end of the tube in the concentrated regime, the only change to the above is the form of the tube free energy. In this regime, we assume that a dense, scaffold-like structure of proteins forms around the tube, imposing a particular radius, \(R_s\). The tube free energy is then given by

\[
F_t = P_v V_t + A_t \left\{ \kappa \left( \frac{1}{R_t^2} - C_0(\phi_t) \right)^2 + f_m(\phi_t) \right\} - f L_t,
\]

where \(W(\phi_t)\) is the enthalpy density, which includes membrane bending and protein–protein interactions. The protein fraction \(\phi_t\) is assumed to be fixed in the concentrated regime by the protein–protein interactions existing within the scaffold.

**Protein binding in the dilute regime.** To determine the protein sorting, tube radius, and force in the dilute regime, we treat the pipette and vesicle as flat membrane surfaces: \(1/R_p = 1/R_v = 0\). Next, following refs. 10 and 11, we assume that the spontaneous curvature is a linear function of protein area fraction:

\[
C_0(\phi) = \tilde{C}_0 \phi,
\]

where \(\tilde{C}_0\) is the effective spontaneous curvature; see main text. Making these assumptions and minimizing \(F\) with respect to \(\phi_p\) and \(\phi_v\), we find \(\phi_p = \phi_v = \phi\), and

\[
\rho \mu = f_m'(\phi) + \kappa \tilde{C}_0^2 \phi,
\]

where \(f_m'\) denotes a derivative of \(f_m\) with respect to \(\phi\). Minimization of \(F\) with respect to \(L_p\) and \(R_v\) then yields the Laplace law

\[
P_0 - P_p = \frac{2\pi}{R_p} \left( 1 - \frac{R_v}{R_p} \right),
\]

where

\[
\tilde{\sigma} = \tilde{\sigma} + \kappa \frac{\tilde{C}_0^2 \phi}{2} + f_m(\phi) - \rho \mu \phi_v - \frac{\tilde{\sigma} - \kappa \tilde{C}_0^2 \phi}{2} + k_B T \rho \ln (1 - \phi_v)
\]

is the physically controlled membrane tension. We may then write the effective tube free energy \(F_t'\) as \(F_t' = F_t - \rho \mu \phi_v A_t + \sigma A_t\), and expand it to second order in \(\Delta \phi_t \equiv \phi_t - \phi_v\), obtaining

\[
F_t' = 2\pi \rho A_t L_t \left\{ \frac{1}{R_t^2} \left( \frac{2\tilde{C}_0 \phi_t}{R_t} \right) + \tilde{\sigma} + \frac{1}{2} \kappa \Delta \phi_t^2 \right\} - f L_t.
\]
where $\chi = f'(\phi_r) + \kappa \bar{C}_0$ is the effective osmotic susceptibility. Because the protein density on the vesicle is assumed to be always small, in the main text we use the $\phi_r = 0$ limit of $\chi$, namely $\chi = k_B T p / \phi_r + \kappa \bar{C}_0$. We note that we have neglected the $(P_0 - P) V_t$ term in Eq. S27 because it is of order $R_t / R_s$ smaller than the other terms.

We now calculate $\phi_r$, $R_t$, and $f$. Minimizing Eq. S27 with respect to $\phi_r$ yields

$$\Delta \phi = \frac{\kappa \bar{C}_0}{2 \bar{R}_t}, \quad [S28]$$

which is a linear function of the tube curvature. Next, minimizing Eq. S27 with respect to $R_t$ yields the tube radius

$$R_t = \sqrt{\frac{\kappa_{\text{eff}}}{2 \bar{\sigma}}} \quad [S29]$$

where $\kappa_{\text{eff}} = \kappa (1 - \kappa \bar{C}_0 / \bar{\chi})$ is the renormalized bending modulus.

Finally, minimizing $F_t$ with respect to $L_t$ gives the force

$$f = 2 \pi \sqrt{2 \kappa_{\text{eff}} \bar{\sigma}} - 2 \pi \kappa \bar{C}_0 \phi_r. \quad [S30]$$

We see that the tube radius and tube force scale with tension in the same way in the dilute regime as in the absence of proteins.

**Protein binding in the concentrated regime.** We next consider the mechanical effects of protein binding on the membrane tube in the concentrated regime. It is assumed that, in this regime, all charged receptor lipids are bound to proteins. As before, the tube force is obtained by minimizing the effective tube free energy $F_t^* = F_t + \alpha A_t - \mu p A_t$, with respect to $L_t$, but now $F_t$ is given by Eq. S22. The protein density on the tube, $\phi_r$, is fixed, yet because of protein number and membrane area conservation, as the tube length is varied, the density $\phi_r$ will vary. This effect is captured here by introducing the Lagrange multiplier $\mu$. Performing $\partial F_t^* / \partial L_t = 0$, and using Eqs. S24 and S26, we obtain

$$f = 2 \pi R_\phi \left\{ \bar{\sigma} + W(\phi_r) + \kappa \bar{C}_0 \phi_r \left( \frac{\phi_r}{2} - \phi_r \right) \right\}$$

$$+ k_B T p \left[ \phi_r \ln \left( \frac{\phi_r}{\phi_r - \phi_r} \right) + \ln \left( \frac{1 - \phi_r}{1 - \phi_r} \right) \right].$$

where $\sigma^*$ is the tension at which the pulling force vanishes, given by

$$\sigma^* = -W(\phi_r) - \kappa \bar{C}_0 \phi_r \left( \frac{\phi_r}{2} - \phi_r \right)$$

$$+ k_B T p \left[ \phi_r \ln \left( \frac{\phi_r}{\phi_r - \phi_r} \right) + \ln \left( \frac{1 - \phi_r}{1 - \phi_r} \right) \right].$$

For $\phi_r \gg \phi_r$ and $\phi_r, \phi_r \ll 1$ this expression simplifies to

$$\sigma^* = -W(\phi_r) + \kappa \bar{C}_0 \phi_r \phi_r + k_B T p \phi_r \ln \left( \frac{\phi_r}{\phi_r} \right).$$

We note the dependence of $\sigma^*$ on $\phi_r$: As shown in the main text, the linear term, $\kappa \bar{C}_0 \phi_r \phi_r$, when fitted to the experimental results, allows a determination of the intrinsic curvature $\bar{C}_0$.

Fig. S1. Fluorescence calibration. (A) Fluorescence measured with confocal in the green channel, $\Phi_{\text{v}}^{\text{HPC}}$, from GUVs membrane containing increasing amount of HPC*, $\Phi_{\text{v}}^{\text{HPC}}$, expressed in number of molecules per micron squared. Linear fit $\Phi_{\text{v}}^{\text{HPC}} = A_{\text{gain}} \times I_{\text{HPC}}^{\text{v}}$ gives the conversion constant, $A_{\text{gain}} = 3.8 \pm 0.1$ (six GUVs) in these acquisition conditions. (B) Fluorescence measured in bulk ($I_{\text{HPC}}^{\text{bulk}}$) using SUVs, as a function of the volume concentration of HPC* ($C_{\text{HPC}}^{\text{bulk}}$). Linear fit to $I_{\text{HPC}}^{\text{bulk}} = a_{\text{HPC}} \times C_{\text{HPC}}^{\text{bulk}}$ gives $a_{\text{HPC}} = 150 \pm 2$. (C) Amph1* fluorescence signal ($I_{\text{bulk}}^{\text{amph}}$) measured in bulk using SUVs, as a function of the volume concentration of amph1* ($C_{\text{bulk}}^{\text{amph}}$). Linear fit to $I_{\text{amph}}^{\text{bulk}} = a_{\text{amph}} \times C_{\text{bulk}}^{\text{amph}}$ gives $a_{\text{amph}} = 280 \pm 10$. The efficiency ratio $F_1$ is defined as $F_1 = \frac{a_{\text{amph}}}{a_{\text{HPC}}} = 1.8 \pm 0.1$. All the data presented in this figure were acquired using the same excitation/acquisition conditions.
Fig. S2. (A). Confocal image of experimental setup with schematic illustration of the protein solution injection. (Scale bar: 5 μm.) (1) Aspiration pipette holding the GUV and used to set membrane tension. (2) GUV. (3) Tube pulled from the GUV. (4) Polystyrene bead held in an optical trap that is used to pull the tube and to measure the tube pulling force. (5) Pipette used to inject labeled amphiphysin; direction of flow is indicated by arrows (roughly to scale). (B) Absence of desorption of amph1*. A single GUV aspirated in a micropipette is flushed with a second pipette containing a solution of 2.5 μM amph1* for 750 s (0–750 s on the graph). The second pipette is then removed (approximately 750 s on the graph). The fluorescence intensity was obtained from the maximum of the fluorescence profile over a cross-section passing across the vesicle, with an averaging window of three images (one image every 1.5 s). The fluorescence of the protein stays roughly constant during 2,000 s, indicating very slow desorption of amph1*.
Fig. S3. Control experiment: Comparison of the force as a function of $\sqrt{\sigma}$ between experiments in the presence of an injected solution containing buffer but no protein (●) and in the absence of injection (□). Linear fit according to $f = 2\pi \kappa \sqrt{\sigma}$ gives $f = 1,900 \pm 100 \times \sqrt{\sigma}$, ($\kappa = 11 \pm 1 \text{k}_B T$).

Fig. S4. Comparison of the tube radius measurements deduced from the force and from the calibrated fluorescence signal, in the absence of proteins in solution. $R_{\text{force}}$ was deduced from Eq. S1: $R_{\text{force}} = \frac{\sqrt{\sigma}}{2\pi \kappa}$. $R_{\text{fluo}}$ was measured using the calibrated fluorescence ratio ($\frac{I_{\text{tube}}}{I_{\text{GUV}}}$) of lipid dyes in the membrane $R_{\text{fluo}} = 200 \pm 50 \times \frac{50}{100}$. The calibration prefactor was measured using six independent experiments. Line: $R_{\text{force}} = R_{\text{fluo}}$.

Fig. S5. Schematic of the system as used in the theoretical part.

Fig. S6. Intermediate regime. (A) Plot of $\sigma^*$ as a function of the squared protein area fraction on the vesicle, $\phi_v$, in the intermediate density regime. The tension at which the force, $f$, vanishes is $\sigma^*$. Data are fitted to $\sigma^* = (14 \pm 5) \times 10^{-3} \phi_v^2 \text{ N/m}$. (B) Effective bending rigidity as of the protein area fraction on the vesicle, $\phi_v$. Data are fitted to $(\kappa - \kappa_{\text{eff}})/\kappa = 15 \pm 5 \phi_v$. 

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Fig. S7. Confocal fluorescence image of a tube pulling experiment in the high-density regime (A) Image corresponding to experiment presented in Fig. 4. Note the homogeneous distribution of protein on the tube. Membrane is labeled using a red lipid dye (BODIPY TR ceramide, red fluorescence channel) and amphiphysin 1 (amph1*, green channel) is labeled with Alexa 488. In this experiment, $\Phi_v = 1,100 \pm 100 \, \mu m^{-2}$. (Scale bar: 5 \, \mu m.) (B) Stabilization of the tube by amph1* in the high-density regime. At high $\Phi_v$, the tube no longer retracts when optical tweezers are turned off. Confocal fluorescence picture: The membrane is labeled using a red lipid dye (BODIPY TR ceramide, red fluorescence channel) and amphiphysin 1 (amph1*, green channel) is labeled with Alexa 488. Tube appears blurry due to movements between averaged frame acquisitions. (Scale bar: 5 \, \mu m.) (C) Protein density on the tube ($\Phi_t$) versus membrane tension ($\sigma$) for the vesicle presented in Fig. 4.

Fig. S8. Effect of protein binding in the high-density regime. Log–log plot of the force versus tension corresponding to Fig. 4A. It shows the change in scaling of the force with tension upon protein binding in the high-density regime. Linear fit to data taken in the absence of protein in solution $\square$ gives $\log(f) = (0.55 \pm 0.03) \times \log(\sigma) + (3.5 \pm 0.1)$, whereas when amphiphysin is in solution $\bullet$ the fit yields $\log(f) = (1.03 \pm 0.06) \times \log(\sigma) + (4.9 \pm 0.2)$. 

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Fig. S9. Another example of vesicle in the high $\Phi_v$ regime. The experiments presented in this figure correspond to a single GUV with $\Phi_v = 2,100 \pm 500 \ \mu m^{-2}$.

(A) Effect on the tube force of the presence of amphipysin 1 in solution. The force in the presence of amph1* bound to the membrane (●) is lowered as compared to in its absence (□). Following Fig. S5B, data in the absence of protein are fitted to $f = 2370 \times \sqrt{\sigma}$; ($\kappa \approx 17 k_B T$), whereas when amph1* is bound, a linear variation between the force and $\sigma$ is measured together with a shift of the origin of the force to nonzero tension. Fit $f = (4.9 \times 10^{-4}) \times \sigma - 2.5$, $\sigma^* = 5 \times 10^{-5} \ \text{N} \cdot \text{m}^{-1}$. (B) Effect on the tube radius of the presence of amph1* in solution; tube radius $R_t$ versus membrane tension $\sigma$ in the absence (empty symbols, deduced from force or fluorescence) or with amph1* (full symbols deduced from fluorescence) in solution. (C) Variation of amph1* density on the tube $\Phi_t$ with membrane tension. Error bars based on standard deviation on four measurements.
Fig. S10. Fluorescence recovery after photobleaching (FRAP) experiment on amph1*-coated tube and GUV. (A) Images taken during amph1* injection (at 0 s) then again at 2 min, 30 s after injection (approximately 1 min, 30 s after bleaching of the tube (bleached area corresponds to the red box in the 0 s image)), and approximately 5 min after injection, corresponding to 1 min, 30 s after bleach of the vesicle (bleach area corresponds to the red box in the 2 min, 30 s image). On this last image, no recovery is observed on the tube. (B) Fluorescence intensity along the tube in the box shown in the 0 s image in (A) (the bleach time corresponds to the vertical blue line). (C) Fluorescence intensity along the portion of the vesicle in the red box shown in the 2 min, 30 s image in (A). (Scale bar: 10 μm.)

Table S1. Summary of data in the high-density regime

<table>
<thead>
<tr>
<th>$\Phi_v$, μm$^{-2}$</th>
<th>$\sigma^*$, $10^{-7}$ N·m$^{-1}$</th>
<th>$\Phi_t$, μm$^{-2}$</th>
<th>$R_t$, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,100 ± 500</td>
<td>5 ± 2.5</td>
<td>8,100 ± 650</td>
<td>6</td>
</tr>
<tr>
<td>1,400 ± 300</td>
<td>1.5 ± 0.3</td>
<td>7,500 ± 650</td>
<td>5</td>
</tr>
<tr>
<td>1,100 ± 200</td>
<td>1 ± 0.9</td>
<td>3,500 ± 850</td>
<td>6</td>
</tr>
<tr>
<td>1,800 ± 250</td>
<td>4.6 ± 2</td>
<td>3,200 ± 750</td>
<td>4</td>
</tr>
<tr>
<td>800 ± 200</td>
<td>0.5 ± 0.6</td>
<td>4,900 ± 2000</td>
<td>10</td>
</tr>
<tr>
<td>1,450 ± 250</td>
<td>4.7 ± 2</td>
<td>2,700 ± 150</td>
<td>6</td>
</tr>
<tr>
<td>900 ± 150</td>
<td>0.8 ± 0.8</td>
<td>4,200 ± 300</td>
<td>12</td>
</tr>
</tbody>
</table>

For each experiment, $\Phi_v$ is the amph1* density on the GUV, $\sigma^*$ is the tension at which the measured force vanishes (see Fig. 3 and Fig. S7), $\Phi_t$ is the amph1* density measured on the tube, and $R_t$ the value of the tube radius at $\sigma = 2 \times 10^{-7}$ N·m$^{-1}$. 