An alternative N-terminal fold of the intestine-specific annexin A13a induces dimerization and regulates membrane-binding

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Annexin proteins function as Ca\textsuperscript{2+}-dependent regulators of membrane trafficking and repair that may also modulate membrane curvature. Here, using high-resolution confocal imaging, we report that the intestine-specific annexin A13 (ANX A13) localizes to the tips of intestinal microvilli and determined the crystal structure of the ANX A13a isoform to 2.6 Å resolution. The structure revealed that the N terminus exhibits an alternative fold that converts the first two helices and the associated helix-loop-helix motif into a continuous α-helix, as stabilized by a domain-swapped dimer. We also found that the dimer is present in solution and partially occludes the membrane-binding surfaces of annexin, suggesting that dimerization may function as a means for regulating membrane binding. Accordingly, as revealed by in vitro binding and cellular localization assays, ANX A13a variants that favor a monomeric state exhibited increased membrane association relative to variants that favor the dimeric form. Together, our findings support a mechanism for how the association of the ANX A13a isoform with the membrane is regulated.

Annexins (ANXs) comprise a large superfamily of proteins that promote Ca\textsuperscript{2+}-dependent modulation of membrane curvature (1). This fundamental process underlies biological activities such as membrane organization, membrane repair, the separation of membranes during cell division, and formation of the multivesicular body (2, 3). A mechanism for how annexin proteins influence membrane curvature was suggested by the crystal structure of ANX A5 (4), which revealed a core fold of four separate four-helix bundles that form a distinctively curved, convex surface. Subsequent annexin structures identified that each of the four helical bundles is associated with up to three Ca\textsuperscript{2+} ions on the convex surface (5, 6). These Ca\textsuperscript{2+} ions bind to conserved clusters of backbone carbonyls and acidic side chains (7, 8). Cocryrstallization of ANX A5 with Ca\textsuperscript{2+} and phosphoserine further identified a cleft within the C-terminal helical bundle that could accommodate lipid head groups, with Ca\textsuperscript{2+} bridging the interaction between phosphoserine and the annexin protein (5). The interaction between the membrane and this convex surface was proposed to physically adjust the membrane curvature to conform to the annexin surface (9), a function proposed to be conserved across annexin proteins.

ANX A13\textsuperscript{a} is transcribed as two isoforms (A13a and A13b) that were originally identified in a screen for myristoylated intestine-specific genes (10). Cellular localization studies of ANX A13 homologs from rabbit (11) and fluke (12) confirmed the narrow intestine-specific expression pattern. More recently, RT-PCR of two human colorectal carcinoma cell lines showed that both the ANX A13a and ANX A13b isoforms are expressed in cells (13). The enrichment of ANX A13 in vesicles shedding from the luminal tips of intestinal microvilli (14) suggests a possible biological role in the creation of these exosome-like vesicles.

In exploring the function of ANX A13, one unusual biochemical property particularly stands out. Although ANX A13 is proposed as the common ancestor of mammalian annexins (15), it is the only annexin to be N-terminally myristoylated. As assessed in the ANX A13b isoform, this post-translational lipidation allows constitutive, Ca\textsuperscript{2+}-independent association with membranes (16). This result initially seemed paradoxical because it implied that ANX A13 isoforms localize to the membrane without using the canonical membrane-remodeling surface. An alternative suggestion is that the membrane-remodeling surface of ANX A13 does not function in high-affinity...
membrane binding. However, it is also possible that both the myristoylation and the canonical membrane-binding surface facilitate membrane interactions cooperatively and/or under different biological situations. The interplay between these two possible mechanisms of membrane interaction has not been evaluated. Here, we combine X-ray crystallography and biochemical measurements with localization studies in tissues and cells to explore structure-function relationships in ANX A13a. These findings identify new mechanisms controlling annexin membrane association and support a model where the canonical membrane-binding surface is functionally relevant in ANX A13 isoforms.

Results

In vivo localization of ANX A13 in mouse intestine shows preference toward brush border

Prior studies used immunolabeling and fluorescence microscopy to reveal that ANX A13 exhibits prominent enrichment at the apical surface of intestinal epithelial cells (10–12). We used high-resolution confocal imaging of tissue sections to more closely examine the distribution of ANX A13 in this tissue; the antibody used for these labeling studies does not differentiate between ANX A13a and ANX A13b. Confocal imaging showed a strong signal corresponding to the apical brush border (Fig. 1), with striking punctate enrichment that mapped to the highly curved membranes at the tips of microvilli. Microvillar tip labeling makes biological sense given that a previous proteomic analysis of brush border–derived luminal vesicles (14) identified ANX A13 as a major cargo. Because these luminal vesicles are predicted to have intestinal alkaline phosphatase (IAP) as a major cargo, we asserted that ANX A13 and IAP colocalize, which we confirmed with costaining (Fig. 1).

Structure of an ANX A13a dimer reveals an elongated α-helix

Crystals of the nonmyristoylated ANX A13a were grown in the absence of Ca\(^{2+}\) and diffracted to 2.6 Å resolution (Fig. 2 and Table 1). Reasonable electron density was apparent for the residues corresponding to the annexin core fold (residues 13–316). In contrast, the N-terminal regulatory region, including residues 2–12 and the canonical site of myristate attachment at Gly-2, was disordered (Fig. 2, A and B).

Intriguingly, the first α-helix (residues 13–44) of all three copies of ANX A13a in the asymmetric unit deviates significantly from the structure observed in all other annexin crystal structures reported to date (Fig. 3A). Here, ANX A13a lacks the helix–turn–helix motif that normally allows these 30 residues to participate in the core fold. It instead adopts an extended conformation, which results in the first α-helix doubling its length from \(\sim 20\) to \(\sim 40\) Å (Figs. 2, A and B, and 3A). In this extended conformation, the N terminus of the first helix now completes the first four-helical bundle of a second ANX A13a molecule, such that the molecule forms a domain swapped dimer (Fig. 2). The dimer buries 1,850 Å\(^2\) of surface area per protomer, a value consistent with the formation of a stable biological assembly. We further investigated the potential relevance of this interaction by calculating shape complementarity of the dimer interface. A mean value of 0.74 is consistent with physiological protein oligomer interfaces, which range from 0.7 to 0.76 (17). Finally, the dimer does not appear to be influenced by crystal packing interactions, which could conceivably support either the N-terminal helix in either the canonical conformation or the extended conformation (Fig. S1, A and B).

In evaluating the side chains that mediate dimerization, many are buried hydrophobic residues (Fig. 2C). However, near the center of the extended helix is an interaction of note that involves Glu-34, Glu-71, and Asp-306. Glu-34 is positioned
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Figure 2. Domain swapped dimer of ANX A13a. In the ribbon diagram, one protomer is colored blue, one protomer is colored green, and the N and C termini are labeled. Putative ethylene glycol molecules from the cryoprotectant that are bound to the predicted lipid sites are shown as space-filling models. The two views are separated by 90°. A, view of the domain-swapped dimer perpendicular to the molecular 2-fold axis. The membrane-binding surface is marked with a curved line. Access to the predicted membrane-binding surface is occluded by dimer formation. B, rotation of the view by 90° shows the domain-swapped dimer down the molecular 2-fold axis. C, interactions of the extended helices. The view is from the protein interior such to highlight buried side chains. This identifies hydrophobic interactions (black circles) and six unusual hydrogen bonds between acidic residues (three in each of the red circles). The acidic hydrogen bonding would suggest that Glu-34 and Asp-306 share at least two protons. The inset highlights these hydrogen-bonds; the view of the inset is rotated 30° around the y axis as compared with the rest of the panel.

Table 1
Crystallographic data collection and refinement statistics

| Data collection | PDB entry | 6B31 |
|----------------|-----------|------|
| SBGrid entry* | 353       |      |
| Beamline      | 21-ID-G   |      |
| Resolution    | 50.0–2.6 Å (2.64–2.60 Å) |      |
| Completeness  | 98.5% (98.5%) |      |
| I/σ           | 15.6 (1.9) |      |
| Rsym          | 0.053 (0.448) |      |
| Rfree         | 0.039 (0.334) |      |
| CC1⁄2         | 0.758     |      |
| Number of reflections | 126,525 |      |
| Unique reflections | 49,419 |      |
| Rfree, reflections | 2,485 |      |

| Refinement | Resolution | 50.0–2.6 Å (2.68–2.60 Å) |
|------------|------------|--------------------------|
| Rcryst     | 0.187 (0.283) |
| Rfree      | 0.225 (0.316) |
| Number of atoms | 7,144 |      |
| RMS bonds  | 0.002 Å    |      |
| RMS angles | 0.49°      |      |
| Average B-factor (protein) | 79.5 Å² |      |
| Average B-factor (solvent) | 66.1 Å² |      |
| Ramachandran |          |
| Most favored | 97.1% |      |
| Additionally allowed | 2.3% |      |
| Outliers   | 0.6%       |      |

* Raw data can be accessed at doi: 10.15785/SBGRID/353.

require that the Glu-34, Glu-71, and Asp-306 side chains are protonated. This may have been facilitated by the mildly acidic crystallization conditions, may be a result of altered pKₐ values of these side chains, or may be a combination of these two possibilities.

The sequence motifs that bind to Ca²⁺ and phospholipids are biochemically well-defined in homologous annexins, and the electrostatic surface potential is consistent with the presence of Ca²⁺-binding sites (Fig. S1, C and D). This allows us to propose putative binding sites for these molecules on the convex surface of ANX A13a. For example, in ANX A5, side chains analogous to Glu-34 and Glu-71 are among those that contribute to the coordination sphere of three Ca²⁺ ions (5, 18). The conformational change of ANX A13a that extends the N-terminal helix relocates Glu-34 and Glu-71 such that these putative Ca²⁺-binding sites are now eliminated in the ANX A13a dimer (Fig. 3A and Table 2). Comparisons between the structure of ANX A13a and ANX A5 bound to phosphoserine as bridged by Ca²⁺ (5, 18) identify that four of the seven remaining Ca²⁺-binding loops differ in conformation despite conservation of the Ca²⁺-coordinating residues. Thus, this ANX A13a structure would require conformational adjustment to support Ca²⁺ binding. Analysis of the three copies of ANX A13a in the asymmetric unit suggests that flexibility of these Ca²⁺-binding regions could allow them to sample a conformation capable of interacting with Ca²⁺. Specifically, whereas the RMS deviation in pairwise comparisons of each of the chains in the asymmetric unit is ~0.3 Å, there are significant conformational differences in the loops comprising residues 184–189 and 255–263, which are putative Ca²⁺-binding regions. These loops have a maximal displacement of 10.0 and 10.6 Å, respectively (Fig. 3A). Elevated temperature factors of these loops also suggest that they are flexible in the absence of Ca²⁺ (Fig. 3B).

Although the putative Ca²⁺ and phospholipid-binding sequence motifs could conceivably bind Ca²⁺ with conforma-
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We further wanted to identify whether there was a dynamic equilibrium between monomers and dimers. Again, using size-exclusion chromatography, we measured the monomer–dimer equilibrium at multiple concentrations of ANX A13a protein. The relative quantities of the monomer and dimer appear to be concentration-dependent (Fig. 4A), with an increase in dimer observed upon an increase in protein concentration. Reinjection of either the isolated monomer peak or an isolated dimer peak resulted in a similar monomer–dimer distribution (Fig. S2). Taken together, this suggests that the protein is in a dynamic monomer–dimer equilibrium at slightly basic pH and in solution.

The dimer interface of the crystal structure involved unusual protonation of acidic side chains. Protonation of these residues is anticipated to increase as pH values are decreased; thus, we anticipate increased dimer formation at lower pH values. To test the influence of pH on the monomer–dimer equilibrium of ANX A13a protein, we again used size-exclusion chromatography (Fig. 4B). Increasing the pH to 8.5 resulted in a near complete conversion of ANX A13a to a monomer, whereas decreasing the pH to 7.5 increased the dimeric population. Decreasing the pH further resulted in protein insolubility (not shown) that prevented additional analysis. These observations indicate that ANX A13a oligomerization is pH-dependent.

Because the dimer interface also involved restructuring of the putative Ca²⁺-binding sites, we assessed whether the addition of Ca²⁺ affected dimerization. At the ANX A13a concentrations necessary to evaluate this effect, the addition of Ca²⁺ resulted in protein precipitation under the conditions tested. Solubility could not be restored through the addition of detergent (not shown).

Oligomerization of AnxA13a influences liposome binding

The dimer observed in the crystal structure brings two copies of ANX A13a together in a manner that occludes a significant portion of the membrane-binding surface. There are two reasonably plausible competing hypotheses for how this dimer might contribute to the regulation of membrane remodeling (Fig. 6). One possibility is that the dimer inhibits association between the membrane remodeling surface of ANX A13a and the membrane; in this scenario, the ANX A13a dimer could retain membrane association via the N-terminal myristoylation. Disassociation of the dimer into monomers would allow the membrane-remodeling surface to be available for interaction with the membrane. An alternative possibility is that the dimer would bind to the membrane across the two monomers; in this latter scenario, the membrane would be pinched between the two protomers of the dimer, potentially inducing an opposite membrane curvature than the annexin monomer.

To distinguish between these possibilities, we constructed variants of ANX A13a that we predicted would bias the equilibrium toward monomers or dimers and tested liposome binding in vitro. We used several strategies to develop a designed monomer, including targeted mutagenesis and chimeragenesis. Of these variants, ANX A13a with Thr-32, Asn-33, and Glu-34 replaced by Pro–Gly–Pro expressed, folded, and exclusively formed monomers when analyzed by size-exclusion chromatography (Figs. 4C and S5). The rationale behind this ANX

ANX A13a exists as a pH-dependent monomer–dimer in solution

Because the dimer observed in the crystal structure requires an alternative fold that is unprecedented in crystal structures of ANX family proteins, we tested whether ANX A13a dimerizes in solution and whether this occurs at physiological pH. To do this, we monitored the average Stokes radius of purified ANX A13a using size-exclusion chromatography (Fig. 4A). At pH 8, ANX A13a eluted as a doublet, with calculated molecular masses of 37.9 ± 3.7 and 74.5 ± 6.4 kDa. These values are consistent with the theoretical molecular masses of an ANX 13a monomer (35.4 kDa) and dimer (70.8 kDa). The eluted protein from each of the two peaks was evaluated as >95% pure using SDS-PAGE (Fig. 5) and validated as ANX A13a by MS.

Figure 3. Ca²⁺-binding sites of ANX A13a. A, overlay of the structure of the three molecules of ANX A13a in each asymmetric unit (black with red, cyan, and magenta Ca²⁺-binding loops) with ANX A5 (Protein Data Bank entry 1A8A (5); gray backbone with yellow loops) bound of Ca²⁺ (yellow spheres) and phosphoserine (yellow sticks). The positions of the Ca²⁺-binding loops are marked and are among the regions of greater structural dissimilarity. B, relative temperature factors of ANX A13a. The structure of ANX A13a (A chain) is colored according to temperature factor, with the lowest temperature factor in blue and ramping via green, yellow, and orange to the highest temperature factor in red. Sequence regions containing the highest temperature factors are labeled and correlate with the Ca²⁺-binding loops.
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Table 2
Summary of putative Ca\(^{2+}\)-binding sites in the ANX A13a dimer

| Residues  | Ca\(^{2+}\) bound to ANX A5 | Comments |
|-----------|-----------------------------|----------|
| 25–34     | 2                           |          |
| 69–74     | 1                           |          |
| 97–106    | 2                           |          |
| 142–146   | 1                           |          |
| 180–190   | 2 + p-Ser                   |          |
| 226–230   | 1                           |          |
| 257–261   | 1                           |          |
|           |                             | Eliminated in A13a dimer by the elongation of helix 1 |
|           |                             | Eliminated in A13a dimer by contacts to the adjacent protomer |
|           |                             | Conformation in A13a differs from that of Ca\(^{2+}\)-bound ANX A5 |
|           |                             | Conformation in A13a appears competent to bind Ca\(^{2+}\) |
|           |                             | Ca\(^{2+}\)-binding loop is flexible |
|           |                             | Conformation in A13a appears competent to bind Ca\(^{2+}\) |
|           |                             | Ca\(^{2+}\)-binding loop is flexible |

A13a\(^{T32P/N33G/E34P}\) variant is that the introduced prolines should prevent the first helix–loop–helix motif from adopting the extended helix.

We also designed several variants intended to stabilize the dimer. Although we were not able to develop a variant that fully converted ANX A13a to the dimeric form, ANX A13a\(^{S73C}\) expressed, folded, and modestly increased the population of the dimer as compared with the WT protein under the conditions tested (Figs. 4C and 5). The ANX A13a\(^{S73C}\) variant is proposed to introduce a disulfide between protomers of the dimer, which would lock the dimeric state. We considered ANX A13a\(^{T32P/N33G/E34P}\) (designed monomer) and ANX A13a\(^{S73C}\) (enhanced dimer) suitable for additional analyses.

To identify whether dimerization impacts the ability of the membrane-remodeling surface of ANX A13a to interact with membranes, we measured Ca\(^{2+}\)-dependent binding of nonmyristoylated WT, ANX A13a\(^{T32P/N33G/E34P}\), and ANX A13a\(^{S73C}\) to liposomes in vitro. Because myristoylation promotes constitutive membrane binding in ANX A13b (16), we used nonmyristoylated protein. This now allows membrane association to be a reporter for the interaction of the membrane-remodeling surface with liposomes. We used three types of liposomes. The first is pure 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), which is not anticipated to bind annexin proteins, even in the presence of Ca\(^{2+}\). We also used two different lipid mixtures that each had 70% DOPC but also contained either 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) or 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA). Prior studies show that these acidic phospholipids support Ca\(^{2+}\)-dependent binding of ANX proteins via the membrane-remodeling surface (16).

Nonmyristoylated ANX A13a (dynamic monomer–dimer equilibrium) exhibited Ca\(^{2+}\)-dependent binding to liposomes containing acidic phospholipids (Fig. 7 and Fig. S3). Under the conditions that we tested, membrane recruitment was incomplete, consistent with previously published studies (13, 16). The ANX A13a\(^{S73C}\) (enhanced dimer) had subtly decreased membrane association relative to WT, which may be anticipated because this mutation only modestly increased the percentage of dimer (Fig. 4C). In contrast, ANX A13a\(^{T32P/N33G/E34P}\) (designed monomer) appeared to be fully recruited to liposomes containing acidic phospholipids in the presence of Ca\(^{2+}\). Similar treatment of purified WT and variant AnxA13a proteins in the absence of lipid resulted in all protein remaining in solution, indicating that the amount of pelleted protein was not influenced by a population of protein that was insoluble. Taken together, these studies indicate that the ANX A13a monomer has an improved capacity to interact with acidic phospholipids in the presence of Ca\(^{2+}\).

Designed variants of ANX A13 isoforms dictate membrane association in epithelial cells

To understand how ANX A13a oligomerization affected membrane association in cells, we heterologously expressed WT and variant EGFP-tagged ANX A13a in LS174T-W4 epithelial cells. These LS174T-W4 cells exhibit a low surface area to volume ratio, making it easy to assess the extent of plasma membrane targeting for expressed proteins. ANX A13a-EGFP was strongly enriched on the plasma membrane with a periphero–cytoplasmic intensity ratio of 3.5:1 (Fig. 8 and Table 3). Conversely, nonmyristoylated ANX A13a\(^{G2A}\)-EGFP appeared to be purely cytoplasmic, with little observable enrichment at the plasma membrane (Fig. 8 and Table 3). The effect of myristoylation can be inferred by the direct comparison between ANX A13a-EGFP and ANX A13a\(^{G2A}\)-EGFP, where the loss of myristoylation reduces ANX A13a at the phospholipid membrane of the cell.

To test how oligomerization affects annexin localization, we next expressed the constitutive monomer ANX A13a\(^{T32P/N33G/E34P}\)-EGFP and enhanced dimer ANX A13a\(^{S73C}\)-EGFP. ANX A13a\(^{T32P/N33G/E34P}\)-EGFP (designed monomer) distinctly favors localization at the cell membrane, with a peripheral–cytoplasmic intensity ratio similar to WT A13a-EGFP. The slight shift in localization of this monomeric variant to the cytoplasm as compared with WT might reflect that the designed monomer mutagenizes residues of a Ca\(^{2+}\)-binding site, which may reduce phospholipid-binding activity. In contrast, the A13a\(^{S73C}\)-EGFP (enhanced dimer) exhibited a reduced peripheral–cytoplasmic intensity ratio. These results strengthen the premise that ANX A13a dimerization inhibits interaction between the membrane-remodeling surface and the phospholipid bilayer.

Discussion

Annexin proteins have long been recognized for their roles in membrane remodeling. However, each of the 12 mammalian isoforms of annexin (ANX A1–13, with ANX A12 unassigned) performs distinct biological roles associated with specific biochemical properties (3, 19). The location of ANX A13a at the apical brush border of intestines (Fig. 1) is consistent with a biological role in forming luminal vesicles (14).

The structures of ANX A13 isoforms have been proposed to center around a highly conserved core of helical bundles common to all characterized annexins (13, 16). Surprisingly, the crystal structure of ANX A13a reveals only three of the four
expected helical bundles because the N-terminal helical bundle exhibits an alternative fold. Here, a unique extended $\alpha$-helix at the N terminus mediates dimerization (Fig. 2).

An annexin oligomer is not uncommon. Indeed, one biophysical property that differs in the various annexin isoforms is its oligomerization. For example, both ANX A5 and ANX B12 form trimers (20, 21). The trimers of ANX A5 pack into two-dimensional arrays on membranes (22, 23), with this close association proposed to assist in local membrane organization. However, a domain-swapped dimer has not previously been reported. Many annexins can also form heteromeric assemblies facilitated by a partner protein (24). This strikingly different type of assembly is exemplified by ANX A2 where the N-terminal sequence forms a complex with an oligomeric partner protein, S100 (25–29). Because S100 proteins can self-associate with multiple geometries, heteromeric annexin oligomerization can allow one annexin protein to approach multiple membrane surfaces at different angles, which is anticipated to induce different membrane curvatures.

In stark contrast to these oligomers that assist in membrane remodeling, the ANX A13a dimer (Figs. 2 and 4) appears to negatively regulate remodeling (Figs. 7 and 8). Notably, the monomer is sufficient to interact with liposomes in vitro (Fig. 7), but membrane interaction also requires myristoylation in cells (Fig. 6).

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**Figure 7. Ca²⁺-dependent binding of ANX A13a to phospholipids.** Phospholipids with varied compositions (100% DOPC, 70% PC/30% PA, 70% PC/30% PS) were incubated with purified WT ANX A13a, the designed monomer (ANX A13aR32P/N33G/E34P), and the enhanced dimer (ANX A13aS73C) at a ratio of 500:1 and in the presence or absence of 0.5 mM CaCl₂ (± Ca²⁺). Samples were centrifuged at 100,000 × g, and the resultant supernatant (S) and pellet (P) were separated by SDS-PAGE. The assay was performed in triplicate, with a representative gel shown.

In contrast, the dimer reduces lipid association both in vitro and in cells (Fig. 7 and 8). The apparently contradictory finding that nonmyristoylated protein can still associate with the membrane in vitro (Fig. 7) but has significantly reduced membrane interaction in cells (Fig. 8) suggests that the monomer–dimer ratio (and/or membrane association) may be additionally regulated in cells. There are several possibilities for how this could occur. Our finding that the monomer–dimer ratio is concentration-dependent (Fig. 4A) suggests that one possibility is via local annexin concentrations. Indeed, a high local concentration of myristoylated ANX A13a could shift the equilibrium toward dimerization as occurs in vitro (Fig. 4A), and one interpretation of our results is that the local concentration of ANX A13a on the cell membrane is higher than was used in our in vitro assays. An alternative possibility for regulating membrane association in cells could be via an interacting protein. Prior proteomics analysis of intestinal exosomes identified that in addition to ANX A13a, these vesicles contain a range of proteins that could serve this function (14). Finally, the ANX A13a structure shows that the dimer interface involves unusual hydrogen-bonding interactions of glutamates and aspartates (Fig. 2C), an observation that indicates that protonation likely accompanies the formation of the dimer. Because these same residues are predicted to form a Ca²⁺-binding site in the canonical annexin fold, one may posit that a local decrease in either pH or Ca²⁺ concentrations could convert monomers to dimers. Conversely, a local increase in pH and/or Ca²⁺ concentration could convert dimers to monomers by deprotonating the acidic residues and exposing the membrane-remodeling Ca²⁺-binding sites. Combinations of these mechanisms are also possible.

Although data support the dimeric form of ANX A13a as a negative regulator of membrane remodeling, they do not exclude other physiological functions for the dimer or other alternatively folded ANX proteins. One speculative possibility arises from the consideration of early studies of annexins, which showed that purified annexins can insert into lipid bilayers and conduct Ca²⁺ current under mildly acidic conditions (30–33). In the context of the canonical annexin architecture, a plausible mechanism for Ca²⁺ channel activity is difficult to envision. However, site-directed spin labeling combined with EPR spectroscopy in *Hydra* ANX B12 (34) identified that acidic conditions induced a large conformational change for α-helix 5 and its associated helix–loop–helix motif that converted these into a continuous α-helix. Extrapolation of this finding to all helices allowed a proposal where the annexin sequence supports two distinct alternative folds: a membrane-remodeling fold represented by the available crystal structures, and a Ca²⁺ channel fold that involves the straightening and extension of alternative helix–loop–helix motifs into seven continuous membrane-spanning α-helices (34).

Although the ANX A13a dimer is clearly not a channel, the alternative fold of the N terminus (Fig. 2) provides direct evidence that the first helical bundle can undergo the elongation proposed to be necessary for conversion to a channel. Because the structural conversion to a channel likely requires protonation of acidic residues (Fig. 2C), it should be enhanced by lowered pH, as previously shown (30–33); however, the observation of a monomer–dimer equilibrium at pH 8 in solution (Fig. 4A) suggests that this structural rearrangement is partially achievable at neutral pH in ANX A13a. The intimate contacts observed in the dimer allow us to further posit that a channel form of ANX proteins could involve a symmetric, intercalated dimer with each monomer containing seven transmembrane helices (14 transmembrane helices for the dimer). An alternative fold associated with a distinct function is observed in other moonlighting proteins. For example, an alternative fold of the RfaH protein converts it from a transcription factor to a translation factor (35). The structural results on ANX A13a further support the general possibility that some moonlighting proteins have more than one fold.

These studies reveal that the ANX A13 domain-swapped dimer in the crystal structure converts a helix–turn–helix motif into an uninterrupted, extended helix, providing direct evidence that the annexin sequence can support two architectures in this region. Our results determine that ANX A13 localizes to the intestinal brush border and suggest that both myristoylation and an annexin monomer (canonical fold) are required for efficient membrane binding. Conversely dimerization (alternative N-terminal fold) prevents the membrane-remodeling surface of the annexin protein from interacting with acidic phospholipids in a Ca²⁺-regulated fashion.
pellets were stored at (100 mM KCl, 20 mM Tris, pH 8.5) supplemented with 4% glyceral 500 ml of LB supplemented with 100 ampicillin, and a single colony was grown at 30 °C with shaking.

debris was removed by centrifugation (1 h at 37,000 g). The cells were lysed by sonication, and the cellular DNAse, 5 then cells were harvested by centrifugation at 6000 g.

Figure 8. Localization of ANX A13a variants expressed in LS174T-W4 cells. A, single plane confocal images of individual LS174T-W4 cells expressing EGFP-tagged ANX A13a, A13aG2A (nonmyristoylated), ANX A13aT32P/N33G/E34P (designed monomer), and A13aS73C (enhanced dimer). Higher intensities are encoded with warmer colors. WT ANX A13a is robustly localized at the cell surface, whereas nonmyristoylated ANX A13a (ANX A13aG2A) is distinctly localized in the cytoplasm. Expression of the designed monomeric form of ANX A13a (ANX A13T32P/N33G/E34P) results in a subtle decrease in surface levels compared with WT ANX A13a. The enhanced dimeric form of ANX A13a exhibits increased cytoplasm localization relative to WT ANX A13a. All boxes are 25 × 25 μm. B, peripheral:cytoplasmic intensity ratios derived from 11–13 cells representing two biological replicates. Pair-wise p values are listed in Table 3.

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**Table 3**
p values and significance for pair-wise comparisons of peripheral:cytoplasmic intensity ratios

|          | WT  | G2A | T32P/N33G/E34P | S73C |
|----------|-----|-----|----------------|------|
| WT       | —   | *** |                |      |
| G2A      | <0.001 | *** | 0.06          |      |
| T32P/N33G/E34P | 0.02 | <0.001 | 0.06      |      |
| S73C     | <0.001 |      |                |      |

A Student’s t test was used to compare the peripheral:cytoplasmic intensity ratios in Fig. 6. Numeric p values are given in the lower left, and the levels of significance are listed in the upper right. NS, not significant; *, p < 0.05; ***, p < 0.001.

**Experimental procedures**

**Protein expression and purification**

The cDNA encoding human ANX A13a was obtained from the Mammalian Gene Collection (National Institutes of Health). Three single nucleotide polymorphisms (SNPs) associated with human ANX A13a are reported; sequencing identified that this clone contained two of the three SNPs that encode the minor variant (R86H and V272I). These SNPs were retained for this study and are referred to as WT ANX A13a in the text. ANX A13a was subcloned into the pPro ExHTa vector, encoding for this study and are referred to as WT ANX A13a in the text. ANX A13a was crystallized using the hanging-drop vapor diffusion method by equilibrating 1.5 μl of ANX A13a (5 mg/ml in annexin buffer) and 1.5 μl of reservoir solution (0.8% w/v PEG 8000, 40 mM 1,2-butanediol, 0.1 M sodium acetate, pH 5.05) against 700 μl of reservoir solution and equilibrating the reactions at 20 °C. Crystals formed in space group C2, with unit cell dimensions of a = 190.6 Å, b = 93.8 Å, c = 94.9 Å, β = 106.0°, and three annexin protomers in each asymmetric unit. The crystals were cryoprotected in a solution containing all of the crystallization conditions plus 10% glycerol and 10% ethylene glycol and were cryocooled by plunging into liquid nitrogen.

The data were collected at the Advanced Photon Source Beamline 21-ID-G at −180 °C using a Mar CCD detector. The data were processed and scaled using HKL2000 (36) and handled using programs within the CCP4 suite (37). The structure of ANX A13a was determined by molecular replacement using the program MOLREP (38) and the structure of ANX A8 as the search model (Protein Data Bank entry 1W3W (39)). Model building was performed using COOT (40). The structure was refined using REFMAC (41), PHENIX (42), and CNS (43). Crystallographic data collection and statistics are listed in Table 1.

**Assessment of oligomerization**

Oligomerization of WT and variant ANX A13a was assessed in triplicate by injecting 0.5 ml of purified protein onto a Superdex S200 Increase column (GE Healthcare) equilibrated with 25 mM Tris, pH 8.0, and 50 mM KCl and a flow rate of 0.25 ml/min. For the pH dependence studies, ANX A13a at 1 mg/ml was injected into 25 mM Tris, 50 mM KCl at either pH 7.5, 8, or 8.5. Elution volumes were compared with a standard curve that was developed from proteins of known molecular mass.

**Crystallization, data collection, and structure determination**

ANX A13a was crystallized using the hanging-drop vapor diffusion method by equilibrating 1.5 μl of ANX A13a (5 mg/ml in annexin buffer) and 1.5 μl of reservoir solution (0.8% w/v PEG 8000, 40 mM 1,2-butanediol, 0.1 M sodium acetate, pH 5.05) against 700 μl of reservoir solution and equilibrating the reactions at 20 °C. Crystals formed in space group C2, with unit cell dimensions of a = 190.6 Å, b = 93.8 Å, c = 94.9 Å, β = 106.0°, and three annexin protomers in each asymmetric unit.

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**Annexin A13a dimerization regulates membrane binding**

**Liposome binding assays**

Large unilamellar vesicles composed of DOPC (100 mol%), DOPC/DOPS (70/30 mol%), or DOPC/DOPA (70/30 mol%) were prepared as follows. Lipids (Avanti Polar Lipids) in chloroform (CHCl₃) were mixed, evaporated under a nitrogen stream, dried in a vacuum overnight, and then rehydrated in 10 mM HEPES, pH 7.2, and 100 mM KCl to a final concentration of 1 mg/ml. Lipid mixtures were vortexed, exposed to three cycles of freeze thaw using a dry ice/ethanol bath and 37 °C water bath, and then extruded 11 times through an 800-nm filter using an Avanti extruder. Large unilamellar vesicles were then frozen at −80 °C until further use.

Liposome binding assays (300 μl in annexin buffer) were performed with a constant phospholipid to protein ratio of 500:1; negative controls lacked liposomes. A final concentration of 0.5 mM CaCl₂ was added to samples containing Ca²⁺. Negative controls lacked liposomes. A final concentration of 0.5 mM CaCl₂ was added to samples containing Ca²⁺. Samples were incubated at 22 °C for 30 min. The mixture was centrifuged at 100,000 g for 45 min at 4 °C, and the supernatant was separated from the phospholipid–protein pellet. The pellet was reconstituted in 300 μl of annexin buffer. The supernatant and the pellet were then separated by SDS-PAGE.

**Assessing localization of ANX A13a variants**

EGFP-tagged variants of ANX A13a were created using standard molecular biological techniques to insert the ANX A13a coding sequence into pEGFP-N2 (Clontech). Maxiprep plasmid DNA was prepared with an endotoxin free method (Qiagen) and then used to transfected LS174T-W4 epithelial cells. LS174T-W4 cells were maintained and transfected according to our previously published protocols (44). For visualization of construct targeting, the cells were plated on no. 1.5 coverslips at single cell densities, thus reducing the prevalence of cell–cell contacts that ultimately confound plasma membrane enrichment analyses. LS174T-W4 cells were imaged using a Nikon A1r laser scanning confocal microscope equipped with a 100×/1.49 NA oil immersion objective. Peripheral:cytoplasmic ratios were derived from intensities measured using ImageJ (National Institutes of Health) as previously described (45).

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**References**

1. Creutz, C. E., Pazojes, C. J., and Pollard, H. B. (1978) Identification and purification of an adrenal medullary protein (synexin) that causes calcium-dependent aggregation of isolated chromaffin granules. *J. Biol. Chem.* 253, 2858–2866 Medline
2. Gerke, V., Creutz, C. E., and Moss, S. E. (2005) Annexins: linking Ca²⁺ signalling to membrane dynamics. *Nat. Rev. Mol. Cell Biol.* 6, 449–461 CrossRef Medline
3. Lizarbe, M. A., Barraza, J. I., Olmo, N., Gavilanes, F., and Turnay, J. (2013) Annexin–phospholipid interactions: functional implications. *Int. J. Mol. Sci.* 14, 2652–2683 CrossRef Medline
4. Huber, R., Berendes, R., Burger, A., Luecke, H., and Karshikov, A. (1992) Annexin V–crystal structure and its implications on function. *Behring Inst. Mitt.* 107–125 Medline
5. Swarjo, M. A., Concha, N. O., Kaetzl, M. A., Dedman, J. R., and Seaton, B. A. (1995) Ca²⁺-bridging mechanism and phospholipid head group recognition in the membrane-binding protein annexin V. *Nat. Struct. Biol.* 2, 968–974 CrossRef Medline
6. Favier-Perron, B., Lewit-Bentley, A., and Russo-Marie, F. (1996) The high-resolution crystal structure of human annexin III shows subtle differences with an annexin V. *Biochemistry* 35, 1740–1744 CrossRef Medline
7. Capila, I., Hernández, M. J., Mo, Y. D., Mealy, T. R., Campos, B., Dedman, J. R., Linhardt, R. J., and Seaton, B. A. (2001) Annexin V-heparin oligosaccharide complex suggests heparan sulfate–mediated assembly on cell surfaces. *Structure* 9, 57–64 CrossRef Medline
8. Leow, C. Y., Willis, C., Osman, A., Mason, L., Simon, A., Smith, B. J., Gasser, R. B., Jones, M. K., and Hofmann, A. (2014) Crystal structure and immunological properties of the first annexin from *Schistosoma mansoni*: Insights into the structural integrity of the schistosomal tegument. *FEBS J.* 281, 1209–1225 CrossRef Medline
9. Tait, J. F., and Gibson, D. (1992) Phospholipid binding of annexin V: effects of calcium and membrane phosphatidylserine content. *Arch. Biochem. Biophys.* 298, 187–191 CrossRef Medline
10. Wice, B. M., and Gordon, J. I. (1992) A strategy for isolation of cDNAs encoding proteins affecting human intestinal epithelial cell growth and differentiation: characterization of a novel gut-specific N-myristoylated annexin. *J. Cell Biol.* 116, 405–422 CrossRef Medline
11. Massey-Harroche, D., Mayran, N., and Maroux, S. (1998) Polarized localizations of annexins I, II, VI and XIII in epithelial cells of intestinal, hepatic and pancreatic tissues. *J. Cell Sci.* 111, 3007–3015 Medline
12. Choi, S. H., Kwon, S. R., Lee, E. H., and Kim, K. H. (2009) Molecular cloning, functional characterization and localization of an annexin from a fish gill fluke *Microcystis sebasitic* (Platyhelminthes: Monogenea). *Mol. Biochem. Parasitol.* 163, 48–53 CrossRef Medline
13. Fernández-Lizarbe, S., Lecona, E., Santiago-Gómez, A., Olmo, N., Lizarbe, M. A., and Turnay, J. (2017) Structural and lipid-binding characterization of human annexin A13a reveals strong differences with its long A13b isoform. *Biol. Chem.* 398, 359–371 CrossRef Medline
14. McConnell, R. E., Higginbotham, J. N., Shifrin, D. A., Jr., Tabb, D. L., Coffey, R. J., and Tyska, M. J. (2009) The enterocyte microvillus is a vesicle-generating organelle. *J. Cell Biol.* 185, 1285–1298 CrossRef Medline
15. Iglesias, J. M., Morgan, R. O., Jenkins, N. A., Copeland, N. G., Gilbert, D. J., and Fernandez, M. P. (2002) Comparative genetics and evolution of an–nexin A13 as the founder gene of vertebrate annexins. *J. Cell Sci.* 116, 405–422 CrossRef Medline
16. Coffey, R. J., and Tyska, M. J. (2009) The enterocyte microvillus is a vesicle-generating organelle. *J. Cell Biol.* 185, 1285–1298 CrossRef Medline
17. Lawrence, M. C., and Colman, P. M. (1993) Shape complementarity at protein/protein interfaces. *J. Mol. Biol.* 234, 946–950 CrossRef Medline
18. Sopkova, J., Renouard, M., and Lewit-Bentley, A. (1993) The crystal structure of a new high-calcium form of annexin V. *Nat. Struct. Biol.* 10, 968–974 CrossRef Medline
19. Hatoum, D., Yagoub, D., Ahadi, A., Nassif, N. T., and McGowan, E. M. (2017) Annexin/S100A protein family regulation through p14ARF-p53
Annexin A13a dimerization regulates membrane binding

32. Rojas, E., Pollard, H. B., Haigler, H. T., Parra, C., and Burns, A. L. (1990) Calcium-activated endonexin II forms calcium channels across acidic phospholipid bilayer membranes. *J. Biol. Chem.* **265**, 21207–21215

33. Rojas, E., Pollard, H. B., Haigler, H. T., Parra, C., and Burns, A. L. (1992) Annexin A2 complexes with membranes. *FEBS Lett.* **314**, 159–162

34. Brocklehurst, K. (1989) Purification and biochemical assay of synexin and donexin II and lipocortin I. *Methods Cell Biol.* **31**, 257–278

35. Rezvanpour, A., Santamaria-Kisiel, L., and Shaw, G. S. (2011) The AHNAK peptide in a 1:2:2 complex with S100A10 and an acetylated N-terminal peptide of annexin A2. *Acta Crystallogr. D Biol. Crystallogr.* **67**, 235–242

36. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., et al. (2010) PHENIX: A comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 21207–21215

37. Grega-Larson, N. E., Crawford, S. W., Erwin, A. L., and Tyska, M. J. (2015) Myosin-1A targets to microvilli using multiple membrane binding motifs in the tail homology 1 (TH1) domain. *J. Biol. Chem.* **287**, 13104–13115