Interfacial Basic Cluster in Annexin V Couples Phospholipid Binding and Trimer Formation on Membrane Surfaces*

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Yuede Mo‡, Begona Campos§, Tanya R. Mealy‡, Lois Commodore‡, James F. Head‡, John R. Dedman†, and Barbara A. Seaton‡||

From the ‡Department of Physiology and Biophysics, Boston University School of Medicine, Boston, Massachusetts 02118 and the Departments of §Obstetrics and Gynecology and ¶Molecular and Cellular Physiology, University of Cincinnati, College of Medicine, Cincinnati, Ohio 45220

Annexin V is an abundant eukaryotic protein that binds phospholipid membranes in a Ca²⁺-dependent manner. In the present studies, site-directed mutagenesis was combined with x-ray crystallography and solution liposome binding assays to probe the functional role of a cluster of interfacial basic residues in annexin V. Four mutants were investigated: R23E, K27E, R61E, and R149E. All four mutants exhibited a significant reduction in adsorption to phospholipid membranes relative to the wild-type protein, and the R23E mutation was the most deleterious. Crystal structures of wild-type and mutant proteins were similar except for local changes in salt bridges involving basic cluster residues. The combined data indicate that Arg23 is a major determinant for interfacial phospholipid binding and participates in an intermolecular salt bridge that is key for trimer formation on the membrane surface. Together, crystallographic and solution data provide evidence that the interfacial basic cluster is a locus where trimerization is synergistically coupled to membrane phospholipid binding.

Annexins comprise a large family of homologous eukaryotic proteins with a common core region that promotes Ca²⁺-dependent binding to phospholipid membrane surfaces (for a review, see Refs. 1–5). This property underlies many proposed in vivo annexin functions, including membrane trafficking, cell signaling, and roles in inflammatory and coagulation processes. Annexin V is an abundant protein that binds preferentially to acidic phospholipid membranes in the presence of Ca²⁺. The annexin-phospholipid association is of high affinity but is reversible with the removal of Ca²⁺. When bound to the membrane surface, annexin V molecules assemble laterally into well organized trimers and higher order arrays of trimers (6). This lateral assembly promotes spontaneous two-dimensional crystallization of annexin V on membrane surfaces, a process that is inhibited by extreme membrane surface curvature (7, 8). Annexin-coated membrane surfaces undergo changes in their properties, becoming markedly rigid as the crystallization domains increase in size (9–11). This membrane-bound layer of annexin V, which has anticoagulant properties, is believed to play a functional role in processes associated with the protein. In placenta, reduced levels of annexin V occur with anti-phospholipid syndrome, which causes hypercoagulation and recurrent miscarriage. Rand and co-workers (12, 13) have proposed a mechanism for anti-phospholipid syndrome in which the disease process strips off the protective layer of annexin V at the maternal-fetal interface, permitting excessive thrombosis to occur.

The mechanism of membrane adsorption of annexin V has generated considerable interest from many disciplines, from clinical to structural biology. The protein provides an excellent model for elucidating complex interfacial behavior by peripheral membrane proteins. The molecular structure of the 35-kDa protein has been well characterized in both soluble and membrane-bound forms by x-ray crystallography (14–18) and cryo-electron microscopy (19–21), respectively. Monomeric annexin V possesses four internally homologous domains, arranged in pairs. The molecule possesses an extensive membrane binding surface, from which calcium-binding loops protrude. Ca²⁺ bridges, in which the Ca²⁺ ion is jointly coordinated by protein and phospholipid oxygen ligands, form at these loops (22). Formation of annexin V homo trimers occurs only on membrane surfaces, suggesting that the interfacial surface provides the correct orientation of the bound monomers for oligomerization. The forces that drive this process have not been characterized at the atomic level. The present studies provide evidence that an interfacial cluster of basic residues synergistically couples membrane phospholipid binding and trimerization on the membrane surface.

MATERIALS AND METHODS

Recombinant Rat Annexin V Proteins—Site-directed mutagenesis was performed using the Clontech transformer kit as described previously (23). To produce the R23E, R61E, and R149E mutants, the GGG codon was changed to GAG. For the K27E mutant, the AAA codon was changed to GAA. Each mutation was verified by double-stranded DNA sequence analysis. Proteins were expressed from cultures of Escherichia coli strain JM101 and purified using lipid affinity chromatography as described (23). Protein concentration was measured using the Pierce BCA protein assay.

Solution Assays—The fluorescence resonance energy transfer (FRET) assay, which is based upon self-quenching of the NBD chromophore that results from annexin-induced clustering of phospholipids (24), was performed as described previously (23) using large (100-nm) unilamellar vesicles containing 85 mol % phosphatidylcholine and 15

1 The abbreviations used are: FRET, fluorescence resonance energy transfer; NBD, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl); DOPS, dioleoylphosphatidylserine; DOPC, dioleoylphosphatidylcholine.
then centrifuged. The Centricon-filtered pellet and its filtrate were
membranes (annexin V binds only to the DOPS component). Samples
contain any buffering agent. Gels were stained with Coomassie Blue.

After destaining, the gels were dried, scanned, and imaged using the
same buffer solutions are used for all the protein samples, provid-
ing buffer.

Briefly, mixtures of annexin V and phospholipid vesicles were incu-
bated together under a range of CaCl2 concentrations, where CaCl2 is
prepared as described, even with no CaCl2 added to the EGTA-contain-
ing, also was performed as described previously using small unilamellar
vesicles of 1:1 mixtures of DOPS/DOPC (Avanti Polar Lipids) (23).

The final protein and CaCl2 concentrations after equilibration were 14
mol % 1-18:1:2-C12-NBD-PS. Measurements were obtained using a
95% confidence level (p = 0.05). The sedimentation assay, which mea-
sures the Ca2+-induced conversion required for half-maximal liposome bind-
ing, was observed in their binding curves, so the apparent dissociation
constants, \( K_{d} \), was evaluated as (1 + \( PD_{1/2} \)), where \( P_{i} \) is the total amount of protein). The bound fraction, \( \frac{P_{b}}{P_{i}} \), was evaluated as (1 – \( P_{i}/P_{b} \)). The wild-type data fit a standard “noncooperative” Langmuir adsorption isotherm (26), which yielded the dissociation constant, \( K_{d} \), and the number of lipid binding sites per annexin molecule. For the mutants, significant sigmodicity was observed in their binding curves, so the apparent dissociation constants, \( K_{d,app} \), defined as the lipid concentration giving rise to half-
maximal membrane binding of the protein, were estimated graphically
yielded the dissociation constant, \( K_{d} \) to yield \( K_{d,app} \) and the Hill coefficient, \( h \). For all three liposome binding assays, meas-
ments were obtained in triplicate.

The four recombinant rat annexin V mutants, R23E, R27E, and R149E, crystal-
ized under the same conditions as the wild-type protein (i.e. vapor
diffusion against a reservoir of 34−40% saturated ammonium sulfate, 
50 mM HEPES buffer, pH 8.2, 2 mM dithiothreitol, and 10 mM CaCl2).
The final protein and CaCl2 concentrations after equilibration were 14
mg/ml and 20 mM, respectively. The R61E mutant failed to crystallize. X-
ray data were collected at room temperature on an R-Axis IIc imaging
plate detector mounted on a Rigaku RU-300 rotating anode generator. 
Data were indexed, analyzed, and scaled using DENZO and SCALE-
PACK (27). The mutant proteins produced R crystals isomorphous 
with wild-type rat annexin V (16), with one molecule per asymmetric
unit. Partial merohedral twinning was detected (28) in the R23E mu-
tant data. A twin fraction of 0.213 was obtained using the twinning
operator h, -h, -k, -l, and the data were detwinned using CNS (29). Initial
rigid body refinement using the four domains as separate bodies pro-
duced R-free values of ~0.3. Manual model building and refinement
were carried out iteratively using O (30) and CNS (29), respectively,
using simulated annealing protocols, difference electron density maps,
and simulated annealing omit maps (31). Model quality was assessed
troughout refinement using PROCHECK (32) and by monitoring R-
free (33). Data collection and refinement statistics for the mutant struc-
tures are presented in Table I.

### RESULTS

**Solution Liposome Binding Assays**—The four mutants and 
the wild-type annexin V were assayed in the presence of calcium

| Parameter | Value |
|-----------|-------|
| Data collection | R23E | R27E | R149E |
| Unit cell dimensions, a and c (Å) | 157.76, 36.76 | 157.60, 37.06 | 157.54, 36.37 |
| Maximum resolution (Å) | 2.70 | 2.00 | 2.00 |
| No. of reflections measured | 8460 | 21924 | 22384 |
| Completeness (%) | 93.8 (89.8) | 96.7 (92.0) | 99.7 (99.5) |
| Rmerge,overall (highest resolution shell) | 12.6 (23.4) | 6.4 (23.6) | 7.7 (29.2) |
| Resolution range (Å) | 50–0.3 | 50–2.1 | 50–2.1 |
| Rmerge | 0.1601 | 0.1870 | 0.1927 |
| Rmerge* | 0.2412 | 0.2294 | 0.2285 |
| No. of protein atoms (mean B in Å²) | 2502 (27.2) | 2502 (34.7) | 2502 (40.1) |
| No. of atoms total (mean B in Å²) | 2514 (27.3) | 2717 (35.7) | 2662 (40.1) |
| No. of ions (Ca²⁺/SO₄²⁻) | 2/2 | 53 | 53 |
| No. of water molecules | 0/0 | 195/140 | 140/140 |
| r.m.s.d.* angles (Å²) | 0.009649 | 0.005531 | 0.005859 |
| r.m.s.d.d bonds (degrees) | 1.52800 | 1.0184 | 1.11217 |

* Rmerge = \( \Sigma [I_i] − [I] \) / \( \Sigma I_i \times 100 \), where \( I_i \) is the intensity of an individual reflection and \( [I] \) is the mean intensity of that reflection.

**Crystalllographic Analysis of Annexin V Mutants**—Three of the four
recombinant rat annexin V mutants, R23E, R27E, and R149E, crystall-
lized under the same conditions as the wild-type protein (i.e. vapor
diffusion against a reservoir of 34−40% saturated ammonium sulfate, 
50 mM HEPES buffer, pH 8.2, 2 mM dithiothreitol, and 10 mM CaCl2).
The final protein and CaCl2 concentrations after equilibration were 14
mg/ml and 20 mM, respectively. The R61E mutant failed to crystallize. X-
ray data were collected at room temperature on an R-Axis IIc imaging
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Data were indexed, analyzed, and scaled using DENZO and SCALE-
PACK (27). The mutant proteins produced R crystals isomorphous 
with wild-type rat annexin V (16), with one molecule per asymmetric
unit. Partial merohedral twinning was detected (28) in the R23E mu-
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and simulated annealing omit maps (31). Model quality was assessed
troughout refinement using PROCHECK (32) and by monitoring R-
free (33). Data collection and refinement statistics for the mutant struc-
tures are presented in Table I.
and 1:1 DOPS/DOPC liposomes. In FRET measurements, which reflect the extent of annexin-induced clustering of acidic phospholipids following membrane adsorption, the mutants produced less quenching than wild-type annexin V. The order of quenching in the FRET experiments was as follows: wild type > R149E > K27E > R61E > R23E (Fig. 1). A similar hierarchy was apparent from the liposome sedimentation assay measuring the half-maximal Ca\(^{2+}\) concentrations required for membrane binding (Fig. 2), except that R61E was more similar to the K27E than to the R23E phenotype. In this assay, higher half-maximal Ca\(^{2+}\) concentrations reflect lower intrinsic affinity for membranes (i.e. higher levels of Ca\(^{2+}\) offset weaker membrane binding affinity due to the synergism between Ca\(^{2+}\) and membrane binding in this system). Taken together, these assays demonstrate that the mutants exhibit lower membrane affinity than wild-type protein, with R23E showing the weakest interaction.

Quantitative analysis of the binding of annexin V proteins to DOPS/DOPC vesicles showed the wild-type protein bound most tightly, with a \(K_d\) of 20 nM. The wild-type data fit well to a Langmuir isotherm and yielded a value of 4.2 \(\pm\) 0.3 for \(n\), the number of lipid molecules bound per annexin V molecule. In contrast, the binding isotherms for the mutants were distinctly sigmoidal (Fig. 3), consistent with their weaker binding and greater dependence upon clustering of acidic lipids for adsorption. Hill coefficients were similar (\(h = 3\)–4) in all four mutants. The apparent \(K_d\) values were in the range of 1–5 \(\mu\)M and showed a trend: R149E < K27E < R61E = R23E (Table II). The apparent \(K_d\) values measured at this fixed protein concentration are all higher than the apparent \(K_d\) extracted for the wild-type protein (<1 \(\mu\)M; data not shown).

Crystal Structures of Annexin V Mutants—In crystallization trials, K27E and R149E mutants behaved nearly as well as wild-type annexin V, forming crystals that grew quickly and diffracted well (2.0-Å resolution versus 1.8 Å for wild type), whereas the R23E and R61E mutants performed very poorly during crystallization trials. The R61E mutant failed to crystallize, and the R23E mutant produced over several months only a single, merohedrally twinned crystal. This crystal produced no observable diffraction beyond 2.7-Å resolution, and data were only usable to 3.0 Å.

The tertiary structures of the R23E, K27E, and R149E mutants are essentially identical to that of the wild-type protein, with root mean square deviations in C-\(\alpha\) atoms of 0.494, 0.356, and 0.344 Å\(^2\), respectively. Two to five Ca\(^{2+}\) ions are observed in the structures. The Ca\(^{2+}\) ion bound in the IAB loop is present in all of the structures. Another shared feature is a Ca\(^{2+}\) ion found in the IIAB loop (residues 182–188) and a sulfate ion coordinating that Ca\(^{2+}\).

Structural differences between the wild-type and mutant structures are localized to the immediate vicinity of the basic cluster (Fig. 4). 1) In R149E, the Arg\(^{63}\) forms a strong salt bridge with the mutated Glu\(^{149}\) side-chain (residues from the adjacent subunit are designated with a prime). To form this new bridge, the Arg\(^{63}\) side chain moves 0.5 Å away from
Arg_{61}^{23}-2) In K27E, the mutated Glu_{27}^{23} interacts only with solvent. The substitution removes the intramolecular Lys_{27}^{23}-Glu_{190}^{23}-salt bridge. 3) In R23E, more significant alterations are observed: (i) Arg_{61}^{23} forms a new intramolecular salt bridge with the mutated Glu_{27}^{23} side chain; (ii) neither Arg_{23}^{23} nor Arg_{61}^{23} bind the sulfate ion, which remains associated with the protein only via the Gly_{28}^{23} main chain; and (iii) the Arg_{23}^{23}-Glu_{190}^{23} interaction is lost, and Glu_{190}^{23} forms an intermolecular salt bridge only with Lys_{27}^{23}.

**DISCUSSION**

Surface clusters of basic amino acids occur infrequently in proteins, where they usually play specialized roles. Proteins that utilize basic clusters for their association with phospholipid membranes include some forms of secretory phospholipase A_{2} (34, 35), pp60^c-src (36), calcineurin B (37), and type II beta phosphatidylinositol phosphate kinase (38). In those studies, the roles of membrane interfacial cationic residues were investigated using site-directed mutagenesis and membrane binding and enzyme activity assays. The present study takes this approach a step further by additionally evaluating the structural consequences of each mutation at the atomic level with x-ray crystallography to make detailed structure-function correlations. Membrane adsorption properties for annexin V mutants were determined through liposome binding assays.

The use of x-ray crystallography in the present work provides not only high resolution structural details but also a relative measure of annexin V trimer formation capabilities for the mutants. Since annexin V trimer formation is reversible and occurs only on membrane surfaces, trimerization is difficult to measure directly in solution, and no reliable assay has been developed. However, the ability to form stable trimers is requisite to form R3 crystals. Studies combining high resolution cryoelectron microscopy with x-ray crystallography have demonstrated that for annexin V, the trimers obtained from two-dimensional crystals on lipid monolayers or three-dimensional crystals from solution have the same structure (21). Three-dimensional crystallization thus can provide a useful comparative measure of the propensity of wild-type and mutant annexin V proteins to form trimers and higher order aggregates.

**The Basic Cluster in the Wild-type Annexin V Crystal Structure**—In wild-type annexin V, the basic cluster is situated between trimer subunits at the largely hydrophilic subunit interface (Figs. 4a and 5). The cationic side chains of the cluster reside in a cleft slightly above the plane of the Ca^{2+}-binding loops that comprise the membrane surface. The cationic functional groups of Lys_{27}^{23}, Arg_{23}^{23}, Arg_{61}^{23}, and Arg_{149}^{23} form a closely spaced row, each separated by 4-5 Å (Fig. 4a). Arg_{23}^{23}, Lys_{27}^{23}, and Arg_{61}^{23}, all from domain I, each participate in one or more electrostatic interactions, mostly strong salt bridges with lengths of <3 Å (Table III). An intermolecular salt bridge is made between Arg_{61}^{23} and Asp_{98}^{23}, whereas Glu_{190}^{23} from the adjacent subunit makes intermolecular salt bridges with Arg_{23}^{23} and Lys_{27}^{23} side chains. Arg_{23}^{23}, Lys_{27}^{23}, and Glu_{190}^{23} are from domains II and III, respectively. Arg_{149}^{23} does not form any salt bridges, although it makes up part of the cluster.

**Table II**

| Mutant | Apparent $K_{d}$ | Hill coefficient |
|--------|-----------------|------------------|
| R149E  | 1.56 ± 0.07     | 3.75 ± 0.46      |
| K27E   | 3.32 ± 0.17     | 3.20 ± 0.43      |
| R61E   | 4.76 ± 0.23     | 3.35 ± 0.35      |
| R23E   | 4.73 ± 0.32     | 3.90 ± 0.75      |

The Basic Cluster as a Binding Site for Sulfate and Phospho-ryl Oxygen Anions—Bound sulfate ions in several annexin V crystal structures have usefully located potential phospholipid binding sites. In structures of wild-type annexin V crystallized from ammonium sulfate, a strongly bound sulfate ion forms one of seven ligands in the primary coordination shell of the Ca^{2+}-ion in the IIIAB loop (15, 17). In the crystal structures of two ternary complexes formed between annexin V, Ca^{2+}, and phospholipid head group analogs, this sulfate ion site is occupied by a phosphoryl group (21). Another significant binding site for sulfate ion is observed at the basic cluster in wild-type annexin V (Fig. 4e). This sulfate ion forms several interactions with the protein: salt bridges with Arg_{61}^{23} and Arg_{23}^{23} side chains and a hydrogen bond to the amide nitrogen of Gly_{28}^{23} in the IAB Ca^{2+}-binding loop (residues 26-32). Despite its close proximity (6 Å) to the IAB Ca^{2+}-ion, there is no direct coordination between the metal and sulfate ions as occurs in the IIIAB loop. Instead, Gly_{28} coordinates the metal ion via its carbonyl oxygen while forming a bond to the sulfate ion through its amide nitrogen. This bridging arrangement is an example of the synergy between Ca^{2+} and phospholipids in the annexin system.

**Effects of the Mutations on Phospholipid Binding**—The membrane binding behavior of annexin V is complex, with many interrelated variables, including calcium concentration, lipid composition, and membrane curvature. We used three different liposome binding assays to probe the effect of mutation on annexin-membrane interactions. In our studies, all four mutants exhibit significantly reduced adsorption to acidic membranes, compared with wild type, confirming the importance of the basic cluster in membrane binding. Since these are charge reversal mutations, some loss of affinity in binding to acidic membranes would be expected simply from reducing the overall net positive charge of the cluster from +4 to +2. However, the varied impairment of the different phenotypes demonstrates that the mutated residues are not functionally interchangeable. In all three binding assays, R149E is the most similar to wild type, followed by K27E, and the R23E mutant is the most deleterious, whereas the properties of R61E range between those of K27E and R23E, depending upon the assay. In terms of calcium dependence, R61E behaves more like K27E; in the FRET assay, it falls in between K27E and R23E, and its $K_{d}$ for DOPS binding is equivalent to that of R23E. The structural basis of the R61E properties could not be determined since R61E failed to crystallize.

For the structurally characterized mutants, a direct correlation can be made between membrane binding affinity (R149E > K27E > R23E) and the number of bonds formed between the sulfate ion and Arg_{61}^{23} (R149E, 2; K27E, 1; R23E, 0). The number of Arg_{61}^{23}-sulfate bonds appears to be less critical but has sufficient influence to reduce membrane binding affinity of R149E relative to wild-type annexin V. In wild-type protein, the sulfate ion is held by two sets of interactions: (i) a hydrogen bond with Gly_{28}^{23} and (ii) salt bridges to the Arg_{23}^{23} and Arg_{61}^{23} guanidinium groups, each providing two bonds to the sulfate. In the mutants, the sulfate-Gly_{28}^{23} hydrogen bond is unaffected, but interactions with the basic cluster vary by phenotype (Table III, Fig. 4). For R149E, the least deleterious mutant phenotype, the Arg_{61}^{23}-sulfate bond is unperturbed. However, the Arg_{61}^{23} guanidinium group shifts 0.5 Å (centroid-centroid distance) away from the sulfate ion, removing one bond to the sulfate and lengthening the bond distance for the other. In K27E, the Arg_{23}^{23} guanidinium group reorients its position to form a salt bridge with the mutated Glu_{27}^{23}. This shift removes one Arg_{23}^{23}-sulfate bond and lengthens both Arg_{23}^{23}-sulfate bonds. For R23E, contact is lost completely between the sulfate ion and the basic cluster; the mutation has removed the Arg_{23}^{23}.
Fig. 4. Basic cluster residues in annexin V wild type (a), R23E (b), K27E (c), R149E (d), and annexin IV (e), shown in stereo view. The Ca$^{2+}$ ion in the IAB loop is depicted as a green sphere, and mutated residues in b–d are shown in cyan. The IAB loop backbone is shown only for residues 27 and 28. Dotted lines represent salt bridges and hydrogen bonds enumerated in Table III.
side chain and shifted the Arg$^{61}$ guanidinium group $>2\text{ Å}$ away from the sulfate.

Promotion of Subunit Interactions by Basic Cluster Residues—Apart from distinctions in their membrane adsorption properties, the mutants also exhibit varying abilities to form quaternary structure through self-association. In this property, Arg$^{23}$ again appears to play a key role, this time through formation of an intermolecular salt bridge with Glu$^{190}$. The mutational analysis suggests that the Arg$^{23}$-Glu$^{190}$ ion pair is a key feature in promoting oligomerization and crystallization. In wild-type annexin V, the importance of having an intermolecular bridge at this site is supported by its apparent functional redundancy; both Arg$^{23}$ and Lys$^{27}$ form salt-bridges to Glu$^{190}$. The arginine guanidinium group makes more interactions with Glu$^{190}$ than does the lysine ε-amino group, giving the Arg$^{23}$-Glu$^{190}$ bridge greater stabilizing power. The combined results show that if Lys$^{27}$ is removed but Arg$^{23}$ is retained, as in the K27E mutant, crystallization is not much different from wild type. However, in the reverse case, when Arg$^{23}$ is removed, leaving only Lys$^{27}$, as in the R23E mutant, crystallization is severely impaired. The number of intermolecular salt bridges, surprisingly, is less important to crystallization. The R149E mutant forms one more salt bridge (Arg$^{23}$-Glu$^{149}$) than is found in wild-type, yet R149E crystals are more similar to those of K27E, diffraction to resolution than wild-type. The effects of the R61E mutation on trimer formation can only be speculative in the absence of crystallographic data. In the wild-type protein, Arg$^{61}$ makes no intermolecular bonds and would seem to have little influence on protein-protein interactions. However, it is possible that in R61E, the mutated Glu$^{61}$ competes with Glu$^{190}$ for the Arg$^{23}$-Glu$^{190}$ group, thereby interfering with Arg$^{23}$-Glu$^{190}$ salt bridge formation and inhibiting crystallization.

Sequence Conservation of the Basic Cluster among Annexins—The basic cluster is not invariant in annexins but appears to be restricted to the subset that have been shown to form trimers on membrane surfaces: annexins IV (39), V (21), and VI (40). This distribution suggests that the basic cluster plays a specialized role in these particular annexins (i.e., stabilizing trimer subunit interactions). The crystal structure of annexin IV (Thr$^6 \rightarrow$ Asp mutant; PDB code 1I4A) crystallized from ammonium sulfate presents the four conserved basic residues in a subtly different spatial arrangement than in annexin V. However, the cluster appears to be functionally similar in the two proteins. In annexin IV (Fig. 4e), the Arg$^{23}$, Lys$^{27}$, Arg$^{61}$, and Arg$^{149}$ side chains form a circular cationic patch that binds a sulfate ion, forming numerous strong salt bridges with Arg$^{23}$, Lys$^{27}$, and Arg$^{149}$. The Arg$^{61}$ side chain does not interact with the sulfate ion but may contribute to the net cationic character of the cluster, as does Arg$^{149}$ in annexin V. Glu$^{190}$, the important salt bridge partner of Arg$^{23}$ and Lys$^{27}$ in annexin V, is not conserved in annexin IV and is replaced by valine. Instead of the Arg$^{23}$-Glu$^{190}$ salt bridge, the sulfate ion cross-links the two subunits by forming a complex network of strong salt bridges with Arg$^{23}$, Lys$^{27}$, and Arg$^{149}$. In serving as a subunit cross-linker, the sulfate ion moves away from the calcium binding loop to take a position spatially analogous to the carboxylate group of Glu$^{190}$ in annexin V. This alternate but effective arrangement in annexin IV provides another example of how phospholipid binding and trimerization are coupled at the basic cluster site.

In annexin VI, the spatial arrangement of basic cluster residues more closely resembles that of annexin V than IV. Annexin VI is a unique, eight-repeat annexin in which each half of the molecule (designated VIA or VIB) corresponds to the core

![Fig. 5. Location of sulfate ion (space-filling representation) bound to the basic cluster in wild-type annexin V (ribbon representation), relative to membrane and subunit interfaces.](image)

**TABLE III**

| Electrostatic interactions at the annexin V basic cluster |
|---------------------------------------------------------|
| **Wild type** | **K27E** | **R149E** | **R23E** |
| Arg$^{23}$ | SO4 O1 (NH1) 3.28 | SO4 O2 (NH1) 3.15 | Mutated to Glu |
| SO4 O2 (NH2) 2.85 | Mutated to Glu | SO4 O2 (NH1) 3.15 | |
| E190' OE1 (NH2) 3.07 | E190' OE1 (NH2) 3.13 | E190' OE1 (NH2) 3.46 |
| E190' OE1 (NE) 2.93 | E190' OE2 (NE) 3.03 | E190' OE2 (NE) 3.15 |
| Arg$^{61}$ | SO4 O1 (NH2) 3.59 | Mutated to Glu | |
| SO4 O2 (NH2) 3.98 | | |
| D66 OD1 (NH1) 3.86 | D66 OD1 (NH2) 3.34 | Mutated to Glu | |
| D66 OD2 (NH1) 3.98 | D66 OD1 (NE) 2.96 | E190' OE2 (NH2) 2.66 |
| Lys$^{27}$ | E190' OE2 (NZ) 2.97 | E190' OE1 (NZ) 2.48 | E190' OE1 (NZ) 3.76 |
| Gly$^{28}$ | SO4 O3 (N) 2.31 | SO4 O4 (N) 2.81 | E23 OE1 (NHR) 3.63 |
| Mutated to Glu | | E25 OE2 (NH1) 2.36 |

**TABLE III Continued**

| Electrostatic interactions at the annexin V basic cluster |
|---------------------------------------------------------|
| **Wild type** | **K27E** | **R149E** | **R23E** |
| Arg$^{149}$ | Mutated to Glu | | |
| Gly$^{28}$ | SO4 O3 (N) 2.31 | Mutated to Glu | |
region of a tetrad repeat annexin. In annexin VI, the two tetrad lobes A and B are rotated about 90° from each other, connected by a linker region. However, the two lobes can adopt different relative orientations (41, 42). In the x-ray crystal structure of annexin VI (PDB code 1AVC) (43), the basic cluster occurs only in the VIB lobe, where Arg377, Lys377, and Arg411 correspond to Arg377, Lys377, and Arg411 in annexins IV and V. The existence of a functionally equivalent residue to Arg411 in annexins IV and V is difficult to predict, since the tertiary and quaternary structure of annexin VI is more complex. Furthermore, in this structure, the crystallization medium did not contain sulfate ions (43). Nonetheless, despite the structural differences, most features of the basic cluster are retained in VI, including the intramolecular salt bridge between Arg377 and Arg411 (equivalent to Arg377-Arg411 in annexins IV and V and an intermolecular salt bridge between Arg377 and Glu277 (equivalent to Arg377-Glu277 in annexin V).

Conclusions—Whereas the precise details may differ among these trimer-forming annexins, their oligomerization mechanism on membrane surfaces may involve the same key elements. The central influence of Ca2+ or its equivalent in mediating and facilitating protein-protein contacts is a common feature in annexins IV, V, and VI. Whereas data are not available yet for annexin VI, annexins IV and V both couple phospholipid binding and subunit stabilization through mechanisms involving the basic cluster; in annexin V, cross-linking of subunits is promoted via the intermolecular Arg23-Glu190 salt bridge and in annexin IV, cross-linking is promoted through a sulfate ion that bridges basic cluster side chains in adjacent subunits.

There are teleological advantages to coupling phospholipid binding and trimer formation in annexins. Regulation may be a primary purpose if the annexin trimer is more biologically active than the monomer. If phospholipid binding is required for trimerization, then annexin activity would be restricted to specific environments where phospholipids are available (i.e. membranes) and prohibited elsewhere. Phospholipid binding also provides additional energetically favorable interactions that contribute to the high affinity of annexins for membranes. In addition, the surface of the phospholipid membrane provides a venue for trimerization and subsequent two-dimensional crystallization or array formation. With intrinsic subunit affinities relatively weak, as might be expected for a system in which trimerization is reversible, proper molecular orientation on the membrane surface is critical to the stable association of subunits and higher order aggregates. Hence, specific interactions between the bound phospholipid and basic cluster residues effectively position monomers in the optimal orientation for oligomerization. The Ca2+ ion bound in the IAB loop is another important component in this complex electrostatic-based network. Ca2+ binding stabilizes the loop proximal to the cluster, whereas Gly28 securely anchors the membrane-bound lipid to the metal-bound loop by making a key hydrogen bond to the phospholipid. Thus, the Ca2+/protein/phospholipid components of this system act in concert to promote and stabilize protein-protein and protein-membrane association in a synergistic manner.
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Youde Mo, Begoña Campos, Tanya R. Mealy, Lois Commodore, James F. Head, John R. Dedman and Barbara A. Seaton

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