Crystal structures of annexin V have shown up to 10 bound calcium ions in three different types of binding sites, but previous work concluded that only one of these sites accounted for nearly all of the membrane binding affinity of the molecule. In this study we mutated residues contributing to potential calcium binding sites in the AB and B helices in each of the four domains (eight sites in total) and in DE helices in the first, second, and third domains (three sites in total). We measured the affinity of each protein for phospholipid vesicles and cell membranes by quantitative calcium titration under low occupancy conditions (<1% saturation of available membrane binding sites). Affinity was calculated from the midpoint and slope of the calcium titration curve and the concentration of membrane binding sites. The results showed that all four AB sites were essential for high affinity binding, as were three of the four B sites (in domains 1, 2, and 3); the DE site in the first domain made a slight contribution to affinity. Mutations with different contributions additively and independently to binding affinity; in contrast, AB and B sites within the same domain were interdependent. The number of functionally important sites identified was consistent with the Hill coefficient observed in calcium titrations. This study shows an essential and previously unappreciated role for B-helix calcium binding sites in the membrane binding of annexin V and indicates that all four domains of the molecule are required for maximum membrane binding affinity.

The annexins are a family of calcium-dependent membrane-binding proteins with diverse functions (1–4). Annexin V is a typical member of this protein family, which is becoming important as a means to measure cell death in vivo in cancer chemotherapy, organ transplant rejection, and myocardial infarction (5–10), creating a need to better understand structural and functional properties relevant to its clinical use. Novel engineered forms of annexin V are being developed to improve its utility for imaging (11, 12), and there has been a recent report of a single-domain version of annexin V that may be useful for imaging cell death in vivo (13).

Calcium binding sites in annexins are of three structural types: AB (or Type II), DE (or Type III), and B or AB' (14). In these binding sites, calcium ligands consist of one to three carbonyl oxygens from the protein backbone, and one carboxyl group from an Asp or Glu residue; sites are often named based on the domain (1 through 4) and α-helices (A through E), which contribute the backbone carbonyl ligands. However, the number of calcium ions observed in different structures varies widely; for example, anywhere from 2 or 3 (15, 16) to 10 (17) calcium ions have been observed in different crystal forms of annexin V. Thus, it has been difficult to determine which calcium binding sites are functionally important based on structural data alone. Previous work with annexin V mutants indicated that the AB site in domain 1 was the most important and perhaps the sole determinant of the calcium-dependent membrane binding affinity: a triple mutant lacking AB sites in domains 2, 3, and 4 had essentially the same apparent affinity as the wild-type protein (18). Mutagenesis studies of AB sites in annexins I, II, and IV have concluded that between one and three AB sites determine the membrane binding affinity of the protein; the DE sites make little or no contribution to binding affinity (19–21). The potential significance of the B sites has apparently not been investigated for any annexin.

In contrast to earlier studies with annexin V (18, 22, 23), our recent work (24) suggested indirectly that there may be many more calcium binding sites that are important for annexin V-membrane binding: the Hill coefficient with respect to calcium was eight for binding to cells and vesicles, suggesting involvement of many more sites than just the first-domain AB site, or even all four AB sites. We therefore decided to systematically investigate the number of functionally important calcium binding sites in annexin V. In this study, we examined four AB sites, four B sites, and three DE sites in annexin V by preparing individual mutants and combinations of mutants in either the same domain or different domains. We measured their membrane binding properties with newly developed assays (24) that provide better quantitative measures of the affinity and cooperativity of binding than most of the earlier methods. We find that three of the B sites play an essential and previously unappreciated role in determining membrane binding affinity. All four AB sites are also required for optimum membrane binding. These findings will help to guide the design of improved imaging agents based on annexin V to detect cell death during organ transplant rejection, cancer chemotherapy, and myocardial infarction.

EXPERIMENTAL PROCEDURES

Site-specific Mutagenesis—A base plasmid (pJ128) was first constructed in the pETBlue-1 vector background (Novagen, Madison, WI) to express a 325-amino acid protein (named annexin V-128) similar to wild-type human annexin V but with these changes: 1) an N-terminal extension of (Met)-Ala-Gly-Gly-Cys-Gly-His; 2) deletion of the initiator Met at position 1 of wild-type annexin V; and 3) a point mutation Cys-316 → Ser, to eliminate the single Cys residue present in wild-type annexin V. The wild-type human annexin V is engineered forms of annexin V are being developed to improve its utility for imaging (11, 12), and there has been a recent report of a single-domain version of annexin V that may be useful for imaging cell death in vivo (13).

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anxin V. The calculated molecular mass of annexin V-128 is 36,272, assuming post-translational removal of the initiator methionine. (Annexin V-128 has the same amino acid sequence as annexin V-117 (11), except for deletion of the initiator Met residue of wild-type annexin V.) Point mutations to delete one or more calcium binding sites were then performed in the pJ128 vector background with the QuikChange Multi site-directed mutagenesis kit (Stratagene, La Jolla, CA). Sequence of the oligonucleotides used are available from the authors. After construction and cloning, each mutant vector was sequenced through the entire protein-coding region to verify the presence of the desired mutation(s) and the absence of unintended mutations. Each annexin V mutant protein was named with an arbitrary numerical suffix.

Expression, Purification, and Lactodactamidofluorescine Labeling of Annexin V Mutants—For protein expression, plasmids were transformed into Escherichia coli strain Tuner (DE3) (PlacI (Novagen, Madison, WI), single colonies isolated, and glycerol stocks were prepared. T broth (500 ml) containing 50 µg/ml carbenicillin was inoculated with 50 µl of glycerol stock, and the culture was grown for 19 h at 37°C with shaking. The cells were harvested by centrifugation, plasmid DNA was prepared from an aliquot of these cells and resequenced to verify that the mutant plasmid had not been lost from the cells during culture. Proteins were purified by a modification of the method of a previous study (11). The cells were lysed by sonication in a buffer consisting of 50 mM Tris HCl, pH 7.2, 1 mM 2-mercaptoethanol, with 10 mM calcium chloride for wild-type and single site mutants, 20 mM calcium for double AB site mutants, and 40 mM calcium for triple and quadruple AB site mutants. Cells were pelleted by centrifugation, and the annexin was released from the pellet with 50 mM Tris HCl, pH 7.2, 1 mM 2-mercaptoethanol, containing EDTA at twice the concentration of calcium used in the previous step. The supernatant was dialyzed overnight against 3 liters of 20 mM Tris HCl, pH 8.0, 1 mM 2-mercaptoethanol. Protein was then treated with Benzonase nuclease (10 units/ml for 6–8 h in the presence of 5 mM MgCl2) to digest DNA and RNA, then dialyzed against two changes of 20 mM Tris HCl, pH 8.0, with 1 mM 2-mercaptoethanol, then three to five changes of the same buffer without 2-mercaptoethanol. The protein solution was concentrated by ultrafiltration to 3 mg/ml by ultrafiltration. The average yield at this purification step was 40 µg/ml. The mutants were then concentrated as needed and stored at −30°C.

Production and Characterization of Mutant Proteins—A total of eleven potential calcium binding sites were selected for study: four AB sites, four B sites, and three DE sites. There is crystallographic evidence (17) for calcium binding to all of these sites except the fourth-domain B site. In most cases, the calcium binding site was inactivated by replacing the capping aspartate or glutamate residue with asparagine or glutamine (Fig. 1), because this change would be nearly isosteric and would therefore have the least potential effect on protein structure. One exception was the 2DE site, where the wild-type residue is already glutamine, which was therefore replaced with alanine. In addition, alanine mutants were also prepared for the 3AB, 1DE, and 3DE sites to verify results obtained with the corresponding glutamine mutants at these sites.

The mutant proteins developed for this study are summarized in Tables 1–III. The mutant proteins were built on a base molecule (annexin V-128), which has a short N-terminal extension containing a single Cys residue to allow site-specific labeling with fluorescein. The addition of the short N-terminal extension did not affect binding affinity (Table II, wild-type versus annexin V-128), as expected based on previous work (11, 12). Each protein was expressed in E. coli, purified, and labeled stoichiometrically with IAF at the single N-terminal Cys residue. Several lines of evidences showed there were no gross structural changes in the mutant proteins. The expression

\[ K = \log K - \log(\text{EC}_{50}) = \log[\text{Membrane}] 
\]

\[ K = \text{EC}_{50} + \log[\text{Membrane}] 
\]

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\]
Differences between wild-type and mutant proteins was evaluated with a one-tailed t-test assuming unequal variances. Underlined residues at the N terminus, deletion of the initiator Met of wild-type annexin V, and mutation of Cys-316 to Ser (overlined with dots) were mutated to destroy calcium binding sites in various mutant proteins.

![Image](structure_of_annexin_v-128_and_its_mutants.png)

**Figure 1. Structure of annexin V-128 and its mutants.** The protein is based on human annexin V (26), with addition of six amino acids (underlined) at the N terminus, deletion of the initiator Met of wild-type annexin V, and mutation of Cys-316 to Ser (underlined). Residues overlined with dots were mutated to destroy calcium binding sites in various mutant proteins.

**Table 1. Stoichiometry of calcium binding to mutant proteins**

| Mutant domain | Mutation | Protein suffix | Mol Ca²⁺/mol protein | S.D. | Number of assays | p value versus wild-type |
|---------------|----------|----------------|----------------------|------|------------------|-------------------------|
| None          | None     | 128            | 13.8                 | 0.97 | 20               |                         |
| 1AB           | E72Q     | 138            | 12.6                 | 0.17 | 3                | 0.005                   |
| 2AB           | D144N    | 134            | 12.9                 | 0.15 | 3                | 0.037                   |
| 3AB           | E228Q    | 132Q           | 11.4                 | 0.35 | 3                | <0.001                  |
| 3AB           | E228A    | 132A           | 10.9                 | 0.15 | 3                | <0.001                  |
| 4AB           | D303N    | 154            | 11.9                 | 0.21 | 2                | <0.001                  |
| 1B            | E35Q     | 156            | 11.5                 | 0.31 | 4                | <0.001                  |
| 3B            | E191Q    | 157            | 12.4                 | 0.86 | 2                | 0.139                   |
| 4B            | D266N    | 159            | 11.6                 | 0.14 | 2                | <0.001                  |
| Mean AB and B |          |                |                      |      |                  |                         |
| single mutants|          | 12.0           |                      |      |                  |                         |
| 1AB,1B        | E35Q,E72Q| 160            | 12.1                 | 0.62 | 3                | 0.008                   |
| 3AB,3B        | E191Q,E228Q| 162           | 12.3                 | 0.98 | 3                | 0.045                   |
| Mean AB/B     | double mutants| 12.2         |                      |      |                  |                         |
| 1DE           | E78A     | 191            | 12.0                 | 1.27 | 3                | 0.070                   |
| 2DE           | Q150A    | 192            | 12.7                 | 0.86 | 3                | 0.073                   |
| 3DE           | E254A    | 193            | 12.3                 | 0.78 | 3                | 0.029                   |

yields of all proteins were comparable to that of the wild-type control within +5%, implying that the one or more mutations did not cause gross structural changes leading to instability or aggregation in vitro or in vivo. All proteins showed very similar chromatographic behavior on the MonoQ column, with a single major sharp peak. Crystallographic analysis of the annexin V-128 protein and the two proteins with mutations in the third-domain AB site (E228A and E228Q mutants) showed that all three proteins had the same global conformation as wild-type annexin V. Previous published work has also shown that E72Q, D144N, E228A, and D303N mutants have the same circular dichroism spectrum as wild-type protein (22), and the E78Q mutant has the same three-dimensional structure as wild-type protein (27). Thus, we concluded that the mutations introduced into these proteins did not cause instability or global conformational or structural changes.

To verify that the mutations had destroyed a calcium binding site, we measured the stoichiometry of calcium binding to wild-type and mutant proteins under conditions in which calcium is the limiting factor in formation of the protein-membrane-calcium complex (Table I). For all mutant proteins tested, there was a decrease in calcium binding compared with the wild-type control, confirming that all mutations were capable of decreasing calcium uptake in the protein-membrane complex. For the AB and B single site mutations as a group, the average reduction in calcium binding was 1.8 ± 0.27 mol/mol (mean ± S.E.), suggesting that disruption of a single AB or B site may decrease calcium binding at another site, probably the adjacent AB or B site in the same domain (see binding affinity measurements below). Consistent with this view, analysis of two double mutants with mutations in the AB and B sites within the same domain also showed about the same reduction in calcium binding (1.6 ± 0.40 mol/mol, mean ± S.E.) as the single site mutants.

**Binding Affinity of Single Site Mutants**—The binding affinities of single site mutants were measured in calcium titrations performed with the RBC binding assay under conditions of low membrane occupancy (Fig. 2 and Table II). Mutation of any one of the four AB sites caused a reduction in binding affinity (Fig. 2A). Similarly, single mutations in the B sites in domains 1, 2, or 3 decreased binding affinity substantially; only the mutation of the DE site in domain 4 was silent (Fig. 2C). In contrast, mutation of the DE site in domain 1 had only a very slight effect on binding affinity, and mutations of DE sites in domains 2 and 3 were silent (Fig. 2C). These results were confirmed with the vesicle binding assay (Table III for AB mutants; data not shown for B and DE mutants), indicating that the mutations had the same effect on binding to both cellular membranes (containing phospholipids, cholesterol, and other substances) and artificial membranes (containing only phospholipid). Thus, a total of eight calcium binding sites were identified that affected membrane binding affinity under low occupancy conditions.

Because a previous mutagenesis study of annexin V used alanine instead of glutamine as the substituting residue in the
third-domain AB site (22), we also created an alanine mutant (E228A). This protein had the same binding properties as the glutamine mutant (E228Q), thus indicating that any differences with the earlier study were not due to subtle differences in the domain-3 mutant proteins. We also prepared E78A and E234A mutants for the 1DE and 3DE sites; these proteins had the same binding affinity as the E78Q and E234Q mutants, indicating that the side-chain carbonyl group of glutamine in the domain-3 mutant proteins. We also prepared E78A and E234Q mutants (E228Q), thus indicating that any differences with the earlier study were not due to subtle differences in the protein.

**Mutational Analysis of Calcium Binding Sites**

To determine whether calcium binding sites interacted affecting the properties of the other. Thus, the AB and B sites within a domain are interdependent, with a change in one affecting the properties of the other.

**Mutation of Multiple AB and B Sites in Different Domains**—To determine whether calcium binding sites interacted between domains, multisite AB mutants were prepared and assayed in the vesicle binding assay (Table III and Fig. 4). (The vesicle binding assay was used primarily because the triple and quadruple mutants had affinities too weak to measure reliably in the RBC binding assay, where dissociation of weakly binding proteins can occur during the centrifugation and wash steps.) There is a progressive decrease in the pK value as the number of mutant sites increases. For the whole set of AB-mutant proteins, there is an excellent linear correlation ($r^2 = 0.94$) between the number of binding sites mutated and the logarithm of the equilibrium constant (Fig. 4). The slope of the fitted line indicates that on average removal of each binding site decreases the pK value by about 7.4. There was also a progressive decrease in the Hill coefficient as more AB binding sites were mutated (Table II). These results were also confirmed with the RBC assay for the double AB and double B mutants (Table II). Overall, these results showed that each domain contributed additively and independently to binding affinity.

**DISCUSSION**

**Role of Individual Sites in Membrane Binding**—This study shows that at least eight calcium binding sites in annexin V are involved in controlling membrane binding affinity: all four AB sites, three B sites (in domains 1, 2, and 3), and one DE site (in domain 1). Although the involvement of at least one AB site was expected based on previous work with annexin V and other annexins (18–21), the critical role of the B sites has not been previously appreciated. The present results are also in marked agreement with these previous studies.

**TABLE II**

*Binding parameters for mutant proteins in RBC assay*

| Mutant domain(s) | Mutation | Protein suffix | EC$_{50}$ (mM) | Hill coefficient | Binding affinity (nM) |
|------------------|----------|----------------|----------------|-----------------|---------------------|
| None             | None     | wt             | 1.20 ± 0.31    | 8.0 ± 0.67      | 30.1 ± 2.16         |
| None             | E78Q     | 155,191        | 5.5 ± 1.25     | 8.0 ± 1.25      | 29.3               |
| None             | E228Q    | 158,193        | 1.05 ± 0.50    | 8.0 ± 1.05      | 29.8               |
| Mean, wt         |          |                | 1.23 ± 0.23    | 8.0 ± 0.23      | 30.4               |
| 1DE              | E78Q     | 155,191        | 1.50 ± 0.25    | 8.0 ± 1.25      | 29.3               |
| 2DE              | E228Q    | 158,193        | 1.05 ± 0.50    | 8.0 ± 1.05      | 29.8               |
| Mean, single DE  |          |                | 1.26 ± 0.26    | 8.0 ± 0.26      | 30.0               |
| 1AB              | E72Q     | 138            | 2.69 ± 0.19    | 6.1 ± 0.58      | 22.4 ± 1.47         |
| 2AB              | D144N    | 134            | 3.12 ± 0.12    | 5.1 ± 0.12      | 19.5               |
| 3AB              | E228Q    | 132,159        | 2.16 ± 0.16    | 5.3 ± 0.36      | 20.7               |
| Mean, single AB  |          |                | 2.68 ± 0.56    | 5.6 ± 0.56      | 21.3               |
| 1B               | E35Q     | 154            | 1.77 ± 0.17    | 6.1 ± 0.61      | 23.5               |
| 2B               | E107Q    | 156            | 1.91 ± 0.19    | 6.4 ± 0.64      | 24.1               |
| 3B               | E144Q    | 157            | 2.90 ± 0.29    | 4.4 ± 0.44      | 17.9               |
| 4B               | D266N    | 159            | 1.23 ± 0.23    | 7.8 ± 0.58      | 29.4               |
| Mean, single B   |          |                | 1.95 ± 0.62    | 6.2 ± 0.62      | 23.8               |
| 1B and 1B        | E35Q,E72Q| 160            | 2.65 ± 0.65    | 6.2 ± 0.62      | 22.7               |
| 2AB and 2B       | E107Q,E144Q| 161    | 4.27 ± 0.42    | 4.2 ± 0.42      | 16.7               |
| 3AB and 3B       | E191Q,E228Q| 162  | 2.42 ± 0.51    | 5.1 ± 0.51      | 20.1               |
| 4AB and 4B       | D266N,D303N| 163 | 2.40 ± 0.54    | 6.4 ± 0.54      | 23.5               |
| Mean, AB and B   |          |                | 2.94 ± 0.55    | 5.5 ± 0.55      | 20.7               |
| 1AB and 2AB      | E72Q,D144N| 142    | 8.69 ± 1.21    | 4.0 ± 0.53      | 14.9 ± 1.23         |
| 1AB and 3AB      | E72Q,D303N| 140    | 8.44 ± 1.24    | 2.8 ± 0.84      | 12.6               |
| 1AB and 4AB      | E228Q,D303N| 135  | 7.13 ± 1.27    | 4.2 ± 0.42      | 15.8               |
| 1B and 2B        | E35Q,E107Q| 164   | 3.34 ± 0.63    | 6.1 ± 0.61      | 21.8               |
| 1B and 3B        | E35Q,E191Q| 165   | 5.93 ± 0.83    | 4.4 ± 0.64      | 16.5               |
| 1B and 4B        | E107Q,E191Q| 167  | 5.85 ± 0.83    | 4.3 ± 0.63      | 16.3               |
| 1AB and 2AB      | E72Q,D144N| 162   | 3.16 ± 0.60    | 4.9 ± 0.69      | 19.0               |
| Mean, double AB  |          |                | 4.98 ± 0.45    | 4.5 ± 0.45      | 17.3               |
contrast to previous studies with annexin V, which concluded that only the domain 1 AB site was important for membrane binding affinity (18, 22, 23). There are probably several reasons for the differences with earlier studies. First, the binding assays used here have higher sensitivity and precision than most previous methods, allowing relatively modest differences in EC50 values to be measured precisely. The vesicle assay also does not require a step to physically separate bound and free ligand, which allows more quantitative measurements with lower affinity mutant proteins that can partly or completely dissociate from the membrane during the time required for the separation steps in centrifugation assays. Second, we have compared mutants on the basis of equilibrium constants calculated from a model that takes into account both EC50 and Hill coefficient values. Comparisons based solely on EC50 values may not fully use the information in the binding data and may underestimate the differences between mutants. This is particularly true when cooperativity of binding is very high, as it is for the single and double mutants. Third, we have measured binding parameters under conditions of low membrane occupancy; measurements performed at higher protein-membrane ratios overestimate EC50 and underestimate Hill coefficients due to the negative cooperativity of binding with respect to protein concentration (24, 28, 29).

From a practical standpoint these findings are important in designing novel variants of annexin V for imaging of cell death in disease states. It has been proposed that a single-domain “mini-annexin” may be suitable for imaging of cell death in vivo (13). Although such a molecule may have other advantages, our results indicate that single-domain annexins are likely to face severe disadvantages with regard to affinity for the target in vivo. Indeed, we have recently found that in vivo uptake of annexin V mutants in apoptotic tissues is completely abolished by mutation of AB sites in as few as two domains (30). However, modest, graded reductions in binding affinity, which can be achieved by mutation of a single DE or B site, may be useful in the design of annexin V variants with shorter residence times on targets in vivo, which may be useful in applications involving repetitive imaging over short periods of time.

Role of B Sites in Membrane Binding: Interdependence of AB and B Sites—This study appears to be the first to show that calcium binding sites in the B helices of annexins are important for membrane binding. Crystal structures have sometimes shown calcium or lanthanum atoms in one or more B-helix sites for annexin V (16, 17, 27, 31) and other annexins (32), but these sites have generally been considered to be of low affinity and unimportant for membrane binding. However, our results show that, in the context of a membrane, these sites are critical for calcium-dependent binding. The residues that we mutated to destroy B sites tend to be well conserved in other annexins, especially in the first and second domains (33), suggesting that these sites may be important in other annexins as well. It is also evident that the AB and B sites within a given domain interact to form the membrane binding site in that domain; the binding energies of these two sites are not independent and additive. There are several mechanisms by which this might occur. The structural data of Swairjo et al. (17) obtained with the model compound glycerophosphoserine show that a single glycerophosphoserine molecule binds to the AB calcium (via the phosphoryl oxygen) and the B calcium (via the serine carboxyl). Thus, loss of one of the two adjacent calcium ions could lower the overall affinity of the site sufficiently to prevent binding of a PS molecule to that domain. In another mechanism, the presence of calcium in either the AB or B site could serve to maintain the adjacent B or AB site in the optimum conformation for calcium-dependent membrane binding. In this regard, it has been shown that the mutation E231A in annexin III, which removes the capping residue in the AB calcium binding site in domain 3, also prevents calcium binding in the B and DE sites in the same domain due to a large-scale conformational rearrangement of domain 3 (34).

Independence of Domains—In contrast to the interactions between sites within a domain, calcium binding sites in different domains do not interact appreciably. The four AB calcium
binding sites and three B calcium binding sites (in domains 1, 2, and 3) act in a largely independent and additive fashion: affinity is progressively reduced as the number of mutated sites in different domains increases. As a corollary, any one site makes only a modest contribution to the overall membrane binding affinity of the native protein. Furthermore, the binding affinity of the multisite mutants can be well predicted from the affinity of the single site mutants. These results are consistent with structural studies of annexin V-membrane complexes showing that all four domains of annexin V are adjacent to the membrane and are thus capable of interacting directly and independently with membrane constituents (35, 36). From a structural standpoint, the independence of binding sites in different domains seems reasonable, because the sites are much further separated in space than are binding sites within the same domain, and each domain forms a fairly autonomous folding unit.

The free energy associated with membrane binding mediated by a single-domain AB plus B site can be estimated from the data of this study. Deletion of a single AB site reduces the logarithm of the equilibrium constant by an average of 7.4 (Fig. 4), corresponding to a free energy change at room temperature of \(-10\ \text{kcal/mol}\). Deletion of AB calcium binding sites in all four domains created a molecule with minimal binding activity that could only be detected under conditions of very low ionic strength and very high membrane PS content. The residual activity detected under these conditions could be due to the remaining calcium binding sites (in the DE helices or elsewhere), or perhaps due to ionic interactions with certain positively charged residues that may be involved in regulating binding affinity and/or trimer formation (18, 37).

Calcium Uptake in the Annexin-membrane Complex—In this and our previous study (24), we measured Hill coefficients of about eight under low occupancy conditions, implying that at least eight calcium ions are involved in the formation of the annexin V-membrane complex. This number agrees well with the mutagenesis results that identify eight calcium binding sites that affect binding affinity under these conditions. We identified an additional three sites that, when mutated, decreased the stoichiometry of calcium binding when measured under conditions that will detect even loosely bound calcium. The total stoichiometry of calcium binding under these conditions is about 13.8 (this study) or 13.43 (38) for annexin V, and 11–13 for annexin 12 (39), indicating that perhaps two or three additional calcium binding sites may still be unidentified. There may be one or more calcium ions bound loosely to the “central pore” in annexin V that is responsible for the observed calcium-channel activity of this protein, because mutagenesis of residue Glu-95 decreases calcium conductance (40). Additional low affinity calcium binding sites could also be formed in the interstices between adjacent protein molecules when the membrane surface becomes relatively crowded, as it does under the conditions of the calcium stoichiometry assay. For example,
a novel intermolecular calcium binding site was observed in the annexin 12 hexamer structure (41). Thus, in addition to the eight calcium ions required for high affinity binding under all conditions, there may be four to six additional calcium ions taken up loosely at several locations in the annexin-membrane complex under high occupancy conditions.

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