

# Roles of Individual Domains of Annexin I in Its Vesicle Binding and Vesicle Aggregation: A Comprehensive Mutagenesis Study<sup>†</sup>

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**ABSTRACT:** To understand the mechanism by which annexin I induces membrane aggregation, a comprehensive mutagenesis of all six Ca<sup>2+</sup>-binding sites was performed. When the cap residues of type II Ca<sup>2+</sup>-binding sites were systematically mutated to Ala, a type II site in domain II was shown to be essential for Ca<sup>2+</sup>-dependent vesicle binding of annexin I. Domain II was not, however, directly involved in vesicle aggregation. Instead, type II sites in domains III and IV, respectively, and type III sites in domains I and IV were involved in vesicle aggregation. When all type II sites were deactivated, three type III sites provided residual vesicle binding and aggregating activities. Their contributions to these activities in the presence of type II sites were, however, relatively insignificant. To further investigate the role of each domain harboring a type II site, a set of mutants containing only a specific type II site(s) were generated and their activities measured. These measurements again underscored the importance of domain II in vesicle binding of annexin I and the involvement of domains III and IV in vesicle aggregation. The roles of individual domains in vesicle binding and aggregation can be accounted for by the conformational change of membrane-bound annexin I involving modular rotation of domains (I/IV) following the initial membrane adsorption of domains (II/III). In conjunction with mutagenesis studies on other annexins, these results show that individual domains of annexins, although structurally homologous, have distinct functions and that different annexins might interact with membranes via different domains.

Annexins are a family of cellular proteins that reversibly bind the membrane containing anionic phospholipids in a Ca<sup>2+</sup>-dependent manner (1–3). Although exact physiological functions of annexins have not been defined yet, they have been shown to have a wide variety of *in vitro* activities. In particular, some annexins including annexins I, II, IV, and VII can promote membrane aggregation (3). The mechanism of membrane aggregation by annexins is still not understood. Also, the protein residues of these annexins involved in membrane aggregation are not identified. All annexins have highly conserved 4 (or 8 for annexin VI) core 70-amino acid domains and variable amino-terminal residues. Spread over these repeating domains are multiple Ca<sup>2+</sup>-binding sites which can be classified into type II and III sites (4). A typical type II site consists of 3 peptide oxygens from the AB loops with the (K,R)-(G,R)-X-G-T sequence and the bidentate ligands from either Asp or Glu (so-called cap residue) that is 39 residues downstream in the sequence (Figure 1). In contrast, a typical type III site includes only three ligand-donating residues: two peptide carbonyl oxygens from the DE loop and Asp or Glu from the E helix. Since essentially all *in vitro* activities of annexins depend on their Ca<sup>2+</sup>-dependent membrane-binding activity, several mutagenesis studies have been performed to identify domains critically involved in various activities of annexins by

selectively deactivating the Ca<sup>2+</sup>-binding sites located in individual domains (5–9). These studies, however, have yielded conflicting results as to the roles of individual domains in annexin activities.

In the present study, we performed a comprehensive and systematic structure–function analysis of annexin I. Annexin I has a type II Ca<sup>2+</sup>-binding site in each of domains II, III, and IV and also contains two type III sites in domain I and one in domain IV, totaling three type II and three type III sites. To evaluate the relative contribution of domains harboring these Ca<sup>2+</sup>-binding sites to the membrane-binding and -aggregating activities of annexin I, we systematically deactivated one or more domain(s) by mutating these sites and measured the effects on vesicle binding and aggregation by annexin I. Herein we describe results from these studies which define the roles of individual domains of annexin I in its membrane binding and membrane aggregation.

## MATERIALS AND METHODS

**Materials.** 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS),<sup>1</sup> 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine were purchased from Avanti Polar

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<sup>1</sup> Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; LUV, large unilamellar vesicles; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; SDS, sodium dodecyl sulfate.

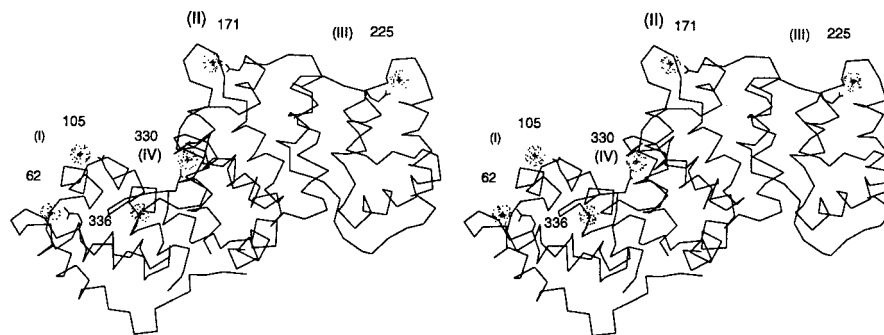


FIGURE 1: Stereodiagram of the annexin I molecule (4). Numbers indicate the cap residues of individual  $\text{Ca}^{2+}$ -binding sites. 62 and 105, type III sites in domain I; 171, 255, and 330, type II sites in domains II, III, IV, respectively; 336, type III site in domain IV. Roman numerals in parentheses indicate domain numbers. Six bound calcium ions are shown as dotted circles.

Lipids, Inc. (Alabaster, AL), and used without further purification. Tritiated POPC ( $[^3\text{H}]\text{POPC}$ ) was synthesized from 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine and tritiated oleic acid (American Radiolabeled Inc., St., Louis, MO) as described elsewhere (10). Restriction endonucleases and enzymes for molecular biology were obtained from either Boehringer Mannheim (Indianapolis, IN) or New England Biolabs (Beverly, MA). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). EGTA (>99%) and  $\text{CaCl}_2$  (99.99% pure) were from Fluka (Ronkonkoma, NY) and Aldrich (Milwaukee, WI), respectively.

**Expression and Purification of Recombinant Human Annexin I.** An *EcoRI* fragment of annexin I cDNA subcloned into pUC 13 vector was a generous gift from Dr. J. Browning of Biogen. This construct corresponds to a truncated form of annexin I missing the first six amino acids. For protein expression, the full-length annexin I cDNA was subcloned into pET-21d vector (Novagen, Madison, WI). This was performed by first introducing a double-stranded synthetic linker encoding the missing six amino acids to pET-21d using restriction sites for *NcoI* and *EcoRI* and then subcloning the *EcoRI*–*HindIII* cDNA fragment of annexin I into the resulting plasmid. Linkers used were 5'-CAT GGC AAT GGT ATC AG-3' and 5'-AAT TCT GAT ACC ATT GC-3'. The identity of the final construct (pET-AnxI) was verified by restriction digestion and DNA sequencing using a Sequenase 2.0 kit (Amersham, Arlington Heights, IL).

For protein expression, pET-AnxI plasmid was transformed into *E. coli* strain BL21(DE3)pLysS, and freshly transformed colonies were used to inoculate 5 mL of Luria broth supplemented with 100  $\mu\text{g}/\text{mL}$  ampicillin and the culture was grown in a thermostated shaker (250 rpm) at 37 °C overnight. One milliliter of the overnight culture was used to inoculate 1 L of Luria broth containing 100  $\mu\text{g}/\text{mL}$  ampicillin and 34  $\mu\text{g}/\text{mL}$  chloramphenicol. This culture was grown (300 rpm) at 37 °C for 3.5–4.0 h until the optical density at 600 nm reached 0.8–1.0. At this point, 0.12 g of isopropyl  $\beta$ -D-thiogalactopyranoside (Boehringer Mannheim) was added to the culture to give a final concentration of 0.5 mM, and the culture was grown for another 3 h at 37 °C. The culture was then chilled on ice for 15–30 min, and cells were harvested by centrifugation at 5000g at 4 °C for 8 min. Pellets were resuspended in 20 mL of 50 mM Tris-HCl, 5 mM EGTA, pH 7.4, supplemented with freshly added phenylmethanesulfonyl fluoride to give a final concentration of 1 mM and either used immediately for protein purification

or frozen in an ethanol–dry ice bath and stored at –70 °C. Recombinant annexin I was purified according to the reported procedure with some modifications (11). Cells were lysed by ca. five freeze/thaw cycles, and the lysate was centrifuged at 100000g at 4 °C for 1 h. The supernatant was filtered through a 0.2  $\mu\text{m}$  cellulose acetate syringe filter (Nalgene) and applied onto a Q-Sepharose Fast Flow (Pharmacia) column (2.5  $\times$  18 cm) equilibrated with 50 mM Tris-HCl, 1 mM EGTA, pH 7.6, buffer at a flow rate of 2–2.5 mL/min. A significant amount of essentially pure annexin I was eluted in the void volume peak. Appropriate fractions were pooled and concentrated in a 50 mL ultrafiltration chamber using a YM-30 membrane (Amicon). The buffer was exchanged during ultrafiltration to 10 mM HEPES–KOH, 0.16 M KCl, pH 7.4, or 50 mM MES–NaOH, 1 mM EGTA, pH 6.4, if further purification was necessary. The second purification step was performed using a Resource S cation exchange column (Pharmacia) with 1 mL bed volume preequilibrated with 50 mM MES–NaOH, 1 mM EGTA, pH 6.4, at a flow rate of 3 mL/min. Annexin I was eluted with a linear gradient of 0–0.5 M NaCl in the same buffer. Eluted fractions were collected, concentrated, and desalted in an ultrafiltration chamber using a YM-30 membrane. The buffer was exchanged in this step to 10 mM HEPES–KOH, 0.16 M KCl, pH 7.4, and glycerol was added to a final concentration of 10% (v/v). Addition of glycerol to protein solution was necessary to avoid precipitation. The protein solution was stored at –20 °C. Annexin I mutants were purified by essentially the same protocol. This purification scheme was designed to minimize the purification period because a significant degree of protein degradation (possibly involving the amino-terminal region) was seen by SDS–polyacrylamide gel electrophoresis after elongated purification (e.g., storage of protein solution overnight at 4 °C). Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL).

**Site-Directed Mutagenesis.** Specific mutations were introduced directly into pET-AnxI plasmid using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer's protocol. Briefly, appropriate complementary synthetic oligonucleotides introducing the desired mutations were used as primers for pseudo-PCR reactions performed in a DNA thermal cycler (Perkin-Elmer) using Pfu DNA polymerase (Stratagene). The subsequent transformation of *DpnI*-treated linear double-stranded DNA into supercompetent Epicurian Coli<sup>®</sup> XL1-Blue Cells (Stratagene) yielded clones the nucleotide se-

Table 1: Nomenclature of Annexin I Mutants

OFF mutants		ON mutants	
name	mutations	name	mutations
(II)-OFF	D171A	(II)-ON	E62A/E105A/E255A/E330A/E336A
(III)-OFF	E255A	(III)-ON	E62A/E105A/D171A/E330A/E336A
(IV)-OFF	E330A	(IV)-ON	E62A/E105A/D171A/E255A/E336A
(II,III)-OFF	D171A/E225A	(II,III)-ON	E62A/E105A/E330A/E336A
(II,IV)-OFF	D171A/E330A	(II,IV)-ON	E62A/E105A/E255A/E336A
(III,IV)-OFF	E255A/E330A	(III,IV)-ON	E62A/E105A/D171A/E336A
(II,III,IV)-OFF	D171A/E255A/E330A	(II,III,IV)-ON	E62A/E105A/E336A
All-OFF	E62A/E105A/D171A/E255A/E330A/E336A		

quences of which were verified by DNA sequencing using a Sequenase 2.0 kit (Amersham).

**Preparation of Vesicles.** Chloroform solutions of appropriate lipid mixtures were dried under a stream of nitrogen in a 20 mL glass vial. The dried lipid film was hydrated in an appropriate volume (500  $\mu$ L) of 10 mM HEPES–KOH, 0.2 M sucrose, pH 7.0, for 10 min at room temperature and then vortexed vigorously for 2 min. Large unilamellar vesicles (LUV) with 100 nm diameter were prepared by multiple extrusion of phospholipid dispersions through a 0.1  $\mu$ m polycarbonate filter (Millipore) in a microextruder Liposfast (Avestin; Ottawa, Ontario). Vesicles were used within 2 days of preparation. Phospholipid concentrations were determined by phosphate analysis (12).

**Preparation of  $\text{Ca}^{2+}$  Buffers.** To maintain reproducible free  $\text{Ca}^{2+}$  concentrations for all assays performed in this study, we prepared calcium–EGTA buffers according to the method of Bers (13). All  $\text{Ca}^{2+}$  solutions were prepared in 10 mM HEPES–KOH, 0.1 M KCl, 1 mM EGTA, pH 7.0, buffer. Seven  $\text{Ca}^{2+}$  buffers with free  $\text{Ca}^{2+}$  concentrations in the range of 2.4  $\mu$ M to 1.02 mM were prepared. The buffers with different free  $\text{Ca}^{2+}$  levels in this range were prepared by appropriate dilution of these buffers.

**Vesicle Aggregation and Binding Experiments.** Vesicle aggregation assays were carried out in a quartz cuvette with 1 cm light path using 2 mL of appropriate  $\text{Ca}^{2+}$  buffers preequilibrated for ca. 1 min with phospholipid vesicles (0.2  $\mu$ mol) prior to protein addition (0–0.3 nmol). The dilution of  $\text{Ca}^{2+}$  buffers during mixing was minimized by adding small volumes of concentrated stock solutions of vesicles (20 mM in total lipid concentration) and of proteins (3–8 mg/mL). Turbidity changes were followed continuously at 450 nm using a Lambda 6 Spectrophotometer (Perkin-Elmer) for 500 s. The initial background absorbance of liposomes was about 0.02 AU, and maximal turbidity signals which were achieved within the first 2–3 min were typically 7–10 times the background values. The maximal turbidity signals were plotted as a function of free  $\text{Ca}^{2+}$  concentration, and the concentrations of  $\text{Ca}^{2+}$  giving rise to a half-maximal aggregation ( $[\text{Ca}^{2+}]_{1/2}$ ) were determined. To determine the amount of protein bound to vesicles, the mixtures with different calcium concentrations were centrifuged at 100000g at 25 °C for 15 min using a Sorvall RCM120EX micro-ultracentrifuge immediately after turbidity measurements. Supernatants and pellets were separated, and pellets were redissolved in 15  $\mu$ L of 10 mM HEPES–KOH buffer, pH 7.0, containing 100 mM KCl, 1 mM EGTA. In control experiments, vesicles were doped with 0.1 mol % [ $^3\text{H}$ ]POPC, and scintillation counting revealed that <5% of the vesicles remained in the supernatant after centrifugation. Also, the

degree of vesicle aggregation did not significantly affect the pelleting efficiency under our experimental conditions. These samples were stored on ice until used for SDS–polyacrylamide gel electrophoresis. For the electrophoresis, 1.5 mm, 10 well 9% SDS gels (Novex) which could accommodate up to 50  $\mu$ L of sample were used. The amount of protein in each band was quantified using an IS-1000 Digital Imaging System (Key Scientific, Mt. Prospect, IL). To convert the protein band density to the protein concentration, a standard curve was constructed from density values of varying amounts of annexin I samples (0.5–10  $\mu$ g).

## RESULTS

**Design and Expression of Mutant Proteins.** Annexin I has a type II  $\text{Ca}^{2+}$ -binding site in each of domains II, III, and IV and also contains two type III sites in domain I and one in domain IV, totaling three type II and three type III sites (4). We systematically deactivated one or more domain(s) by mutating the cap residue of type II sites in each domain to Ala (these mutants will be referred to as OFF mutants hereafter). Also, we generated a series of multisite mutants retaining only specified type II  $\text{Ca}^{2+}$ -binding site(s) with all type III sites deactivated (ON mutants). For instance, (II)-ON refers to the E62A/E105A/E255A/E330A/E336A mutant whose entire  $\text{Ca}^{2+}$ -binding sites except a type II site in domain II are removed. A majority of ON and OFF mutants used in these studies are listed in Table 1. Bacterial expression of annexin I and all its mutants generally yielded more than 15 mg of pure protein per liter of medium, indicating that none of mutants has significantly lower thermodynamic stability or altered folding pathway. The CD spectra of all mutants (in the absence of  $\text{Ca}^{2+}$ ) were essentially indistinguishable from that of wild type, indicating a lack of significant changes in secondary structure due to mutations (data not shown).

**Optimization of Aggregation Assay Condition.** The aggregation assay used herein continuously monitors the turbidity change at 450 nm in a  $\text{Ca}^{2+}$  buffer containing annexin I (or its mutant) and phospholipid LUV (100 nm diameter). Aggregation assays were carried out using sucrose-loaded LUV in order to enhance the pelleting efficiency of vesicles in ensuing binding measurements. To optimize the assay condition in terms of sensitivity and reproducibility, the total amount of protein and phospholipid, the molar protein:phospholipid ratio, the free  $\text{Ca}^{2+}$  concentration, and the size and composition of liposomes were varied. When 150  $\mu$ g of POPS (ca. 0.2  $\mu$ mol) was used to prepare LUV in 2 mL of buffer, ca. 10  $\mu$ g (0.3 nmol) of annexin I gave a saturated signal at a saturating  $\text{Ca}^{2+}$  concentration (e.g., 20  $\mu$ M) (data not shown). As the phospholipid



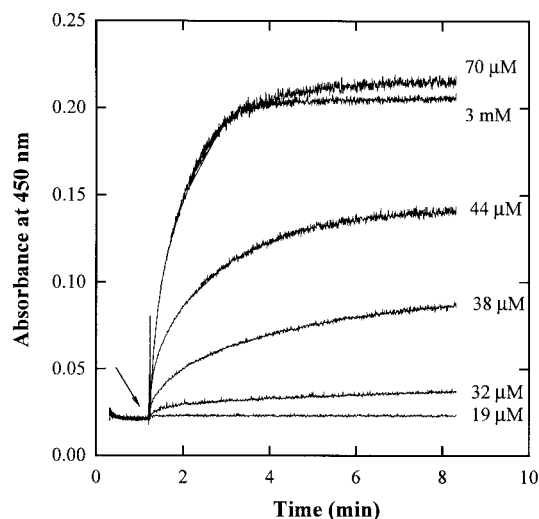


FIGURE 2: Aggregation of phospholipid vesicles by annexin I as a function of  $\text{Ca}^{2+}$  concentration. The aggregation was initiated (indicated by an arrow) by adding  $0.13 \mu\text{M}$  (final concentration) of protein to the assay buffer (10 mM HEPES–KOH, 0.1 M KCl, pH 7.0) containing 0.1 mM POPC/POPE/POPS (2:5:2) LUV and varying concentrations of free  $\text{Ca}^{2+}$  ( $7 \mu\text{M}$  to 3 mM).

composition of liposomes was varied, dramatic changes in the  $\text{Ca}^{2+}$  requirement for aggregation were observed. For instance, the  $[\text{Ca}^{2+}]$  needed for half-maximal aggregation of POPC/POPS liposomes with 15–100% POPS composition varied from  $900 \mu\text{M}$  to  $5 \mu\text{M}$ . As reported previously (14, 15), the addition of PE into these liposomes also significantly decreased the  $\text{Ca}^{2+}$  requirement for binding and aggregation. Based on these results, we selected POPC/POPE/POPS LUV (2:5:2) for a standard vesicle aggregation assay. For this vesicle,  $43 \mu\text{M}$   $\text{Ca}^{2+}$  was required for half-maximal aggregation by annexin I (Figure 2).

**Vesicle-Binding and Vesicle-Aggregating Properties of OFF Mutants.** First, the relative vesicle-binding affinity and vesicle-aggregating activity of annexin I and its type II  $\text{Ca}^{2+}$ -binding site OFF mutants were evaluated in terms of  $\text{Ca}^{2+}$  concentrations required for half-maximal binding and aggregation. Calcium dependencies of vesicle binding and aggregation by annexin I and selected OFF mutants are shown in Figure 3 and Figure 4, respectively, and the  $[\text{Ca}^{2+}]_{1/2}$  values for these processes are summarized in Table 2. Comparison of  $[\text{Ca}^{2+}]_{1/2}$  values for vesicle binding of these single-site mutants showed that domain II plays a more important role than domains III and IV in vesicle binding. A 7.5-fold increase in the  $[\text{Ca}^{2+}]_{1/2}$  value for (II)-OFF indicated that the reduced  $\text{Ca}^{2+}$  affinity of this binding site greatly affected the  $\text{Ca}^{2+}$ -dependent vesicle binding of annexin I. In contrast, (III)-OFF and (IV)-OFF showed essentially the same  $\text{Ca}^{2+}$  requirement as wild type. For (II)-OFF, the weaker vesicle-binding was well correlated with its reduced vesicle aggregating activity, as seen from comparable  $[\text{Ca}^{2+}]_{1/2}$  values for vesicle binding and vesicle aggregation. Since vesicle aggregation would occur after (or coincidentally with) vesicle binding of annexin I, no additional effect of the (II)-OFF mutation on vesicle aggregation implied that domain II does not play a direct role in vesicle aggregation. On the other hand, two other single mutants, (III)-OFF and (IV)-OFF, had twice higher  $[\text{Ca}^{2+}]_{1/2}$  values for vesicle aggregation than expected from their respective  $[\text{Ca}^{2+}]_{1/2}$  values for vesicle binding. Thus, it

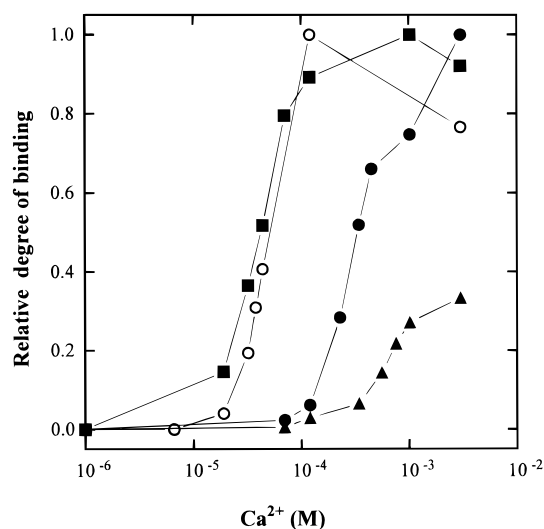


FIGURE 3: Vesicle binding of annexin I (○) and selected mutants including (II)-OFF (●), (III)-OFF (■), and (II,III,IV)-OFF (▲). Assay mixtures contained  $0.13 \mu\text{M}$  protein, 0.1 mM POPC/POPE/POPS LUV, and varying concentrations of free  $\text{Ca}^{2+}$  in the assay buffer (10 mM HEPES–KOH, 0.1 M KCl, pH 7.0). Pellets separated by ultracentrifugation were redissolved in  $15 \mu\text{L}$  of 10 mM HEPES–KOH buffer, pH 7.0, containing 100 mM KCl and 1 mM EGTA, and the amount of bound protein was quantified by densitometric determination of the annexin band on a SDS–polyacrylamide gel (see Materials and Methods). The relative degree of binding was calculated by dividing the amount of vesicle-bound protein by the total protein. A slight decrease in vesicle binding was observed for annexin I at high calcium concentrations. Data points indicate means of duplicate determinations, an average standard error of which was about 10%.  $[\text{Ca}^{2+}]_{1/2}$  values were determined graphically.

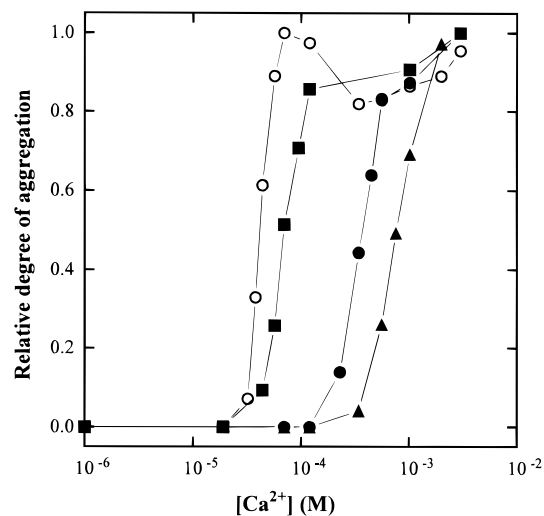


FIGURE 4: Vesicle aggregation by annexin I (○) and selected mutants including (II)-OFF (●), (III)-OFF (■), and (II,III,IV)-OFF (▲). Assay mixtures contained  $0.13 \mu\text{M}$  protein, 0.1 mM POPC/POPE/POPS LUV, and varying concentrations of free  $\text{Ca}^{2+}$  in the assay buffer (10 mM HEPES–KOH, 0.1 M KCl, pH 7.0). Vesicle aggregation was followed by continuously monitoring turbidity changes at 450 nm as shown in Figure 1. The relative degree of aggregation was calculated by dividing the observed turbidity change by the maximal turbidity change (at saturating  $\text{Ca}^{2+}$  concentration). Data points indicate means of duplicate determinations, an average standard error of which was about 5%.  $[\text{Ca}^{2+}]_{1/2}$  values were determined graphically.

appeared that domain II is essential for vesicle binding of annexin I and that domains III and IV might be involved in vesicle aggregation. This notion is further supported by the

Table 2: Calcium Requirements for the Binding and Aggregation of Vesicles by Annexin I and Its *OFF* Mutants

annexin I mutants <sup>a</sup>	Ca <sup>2+</sup> required for half-maximal binding ( $\mu$ M) <sup>b</sup>	Ca <sup>2+</sup> required for half-maximal aggregation ( $\mu$ M) <sup>b</sup>
wild type	48 $\pm$ 5	43 $\pm$ 3
(II)- <i>OFF</i>	360 $\pm$ 30	410 $\pm$ 50
(III)- <i>OFF</i>	41 $\pm$ 5	80 $\pm$ 7
(IV)- <i>OFF</i>	41 $\pm$ 5	91 $\pm$ 4
(II,III)- <i>OFF</i>	360 $\pm$ 40	450 $\pm$ 30
(II,IV)- <i>OFF</i>	360 $\pm$ 20	610 $\pm$ 30
(III,IV)- <i>OFF</i>	81 $\pm$ 4	130 $\pm$ 30
(II,III,IV)- <i>OFF</i>	630 $\pm$ 20 <sup>c</sup>	850 $\pm$ 90
All- <i>OFF</i>	nd <sup>d</sup>	nd <sup>d</sup>

<sup>a</sup> See Table 1 for the nomenclature of mutants. <sup>b</sup> Determined with 100 nm LUV composed of POPS/POPE/POPC (2:5:2 mole ratio). Data indicate mean  $\pm$  SD from at least two sets of measurements. <sup>c</sup> Reached only ca. 30% saturation (see Figure 3). <sup>d</sup> Not detected with up to 3 mM Ca<sup>2+</sup>.

properties of double-site mutants. (II,III)-*OFF* and (II,IV)-*OFF* had essentially the same [Ca<sup>2+</sup>]<sub>1/2</sub> for vesicle binding as (II)-*OFF* but had higher [Ca<sup>2+</sup>]<sub>1/2</sub> for vesicle aggregation than (II)-*OFF*. This trend was more pronounced for (II,IV)-*OFF*. Another double mutant, (III,IV)-*OFF*, further demonstrated that type II Ca<sup>2+</sup>-binding sites in domains III and IV are more important in vesicle aggregation than in vesicle binding. When all three type II Ca<sup>2+</sup>-binding sites were mutated, i.e., (II,III,IV)-*OFF*, vesicle-binding and -aggregating activities of annexin I were greatly reduced but not fully abolished. As shown in Figures 3 and 4, at saturating Ca<sup>2+</sup> concentration (3 mM), (II,III,IV)-*OFF* showed ca. 30% of wild-type vesicle-binding activity while exhibiting essentially full vesicle-aggregating activity, which suggested that it has an altered vesicle-binding mode which is also conducive, albeit less effective, to vesicle aggregation. More importantly, the residual vesicle-binding and -aggregating activities of (II,III,IV)-*OFF* indicated that lower-affinity type III Ca<sup>2+</sup>-binding sites might also contribute to these activities. We therefore mutated three type III Ca<sup>2+</sup>-binding sites to assess their contributions. None of the three mutants, E62A, E105A, and E336A, showed any significant change in vesicle-binding and -aggregating activities when compared to wild type (data not shown). When all six Ca<sup>2+</sup>-binding sites were mutated, i.e., E62A/E105A/D171A/E255A/E330A/E336A (All-*OFF*), however, the mutant showed no detectable vesicle-binding and -aggregating activities even in the presence of 3 mM Ca<sup>2+</sup> (data not shown). These data indicate that type III Ca<sup>2+</sup>-binding sites make small but definite contributions to the vesicle-binding and -aggregating activities of annexin I which are relatively insignificant when compared to those from higher affinity type II sites.

**Vesicle-Binding and Vesicle-Aggregating Properties of *ON* Mutants.** Having established that All-*OFF* mutant had essentially no vesicle-binding and -aggregating activities, we reactivated one or more type II Ca<sup>2+</sup>-binding site(s) from this mutant to systematically analyze the contribution of each Ca<sup>2+</sup>-binding site (and the domain harboring each site) to vesicle-binding and -aggregating activities of annexin I. This was done by systematically mutating cap alanine residues of the All-*OFF* mutant back to the original glutamate or aspartate. Calcium dependencies of vesicle binding and of aggregation by resulting *ON* mutants are shown in Figures 5 and 6, respectively, and [Ca<sup>2+</sup>]<sub>1/2</sub> values for vesicle binding

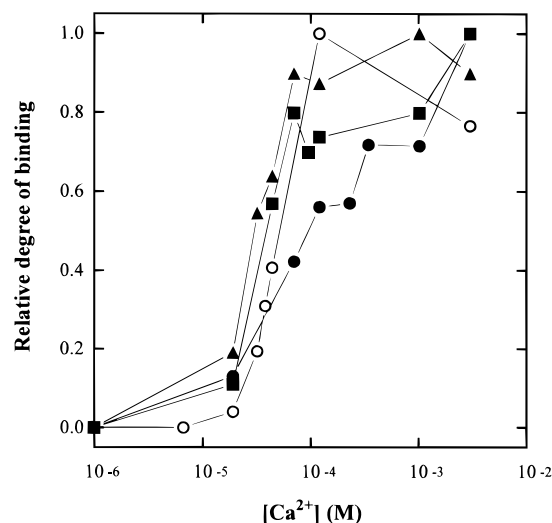


FIGURE 5: Vesicle binding of annexin I (○) and selected mutants including (II)-*ON* (●), (II,III)-*ON* (■), and (II,III,IV)-*ON* (▲). Measurements were performed as described in Figure 3.

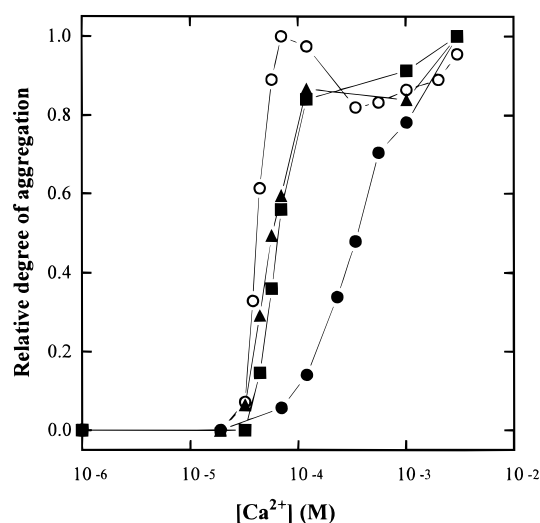


FIGURE 6: Vesicle aggregation by annexin I (○) and selected mutants including (II)-*ON* (●), (II,III)-*ON* (■), and (II,III,IV)-*ON* (▲). Measurements were performed as described in Figure 4.

Table 3: Calcium Requirements for the Binding and Aggregation of Vesicles by Annexin I and Its *ON* Mutants

annexin I mutants <sup>a</sup>	Ca <sup>2+</sup> required for half-maximal binding (mM) <sup>b</sup>	Ca <sup>2+</sup> required for half-maximal aggregation (mM) <sup>b</sup>
wild type	48 $\pm$ 5	43 $\pm$ 3
(II)- <i>ON</i>	125 $\pm$ 2	350 $\pm$ 20
(III)- <i>ON</i>	nd <sup>c</sup>	nd <sup>c</sup>
(IV)- <i>ON</i>	nd <sup>c</sup>	nd <sup>c</sup>
(II,III)- <i>ON</i>	40 $\pm$ 10	90 $\pm$ 20
(II,IV)- <i>ON</i>	47 $\pm$ 2	200 $\pm$ 20
(III,IV)- <i>ON</i>	470 $\pm$ 30 <sup>d</sup>	760 $\pm$ 50
(II,III,IV)- <i>ON</i>	32 $\pm$ 2	70 $\pm$ 10

<sup>a</sup> See Table 1 for the nomenclature of mutants. <sup>b</sup> Determined with 100 nm LUV composed of POPS/POPE/POPC (2:5:2 mole ratio). Data indicate mean  $\pm$  SD from at least two sets of measurements. <sup>c</sup> Not detected with up to 3 mM Ca<sup>2+</sup>. <sup>d</sup> Reached only ca. 30% saturation (see Figure 5).

and aggregation are summarized in Table 3. The comparison of single-site *ON* mutants again demonstrated the critical role of the type II Ca<sup>2+</sup>-binding site in domain II in binding of annexin I to phospholipid vesicles. (II)-*ON* showed signifi-

cant vesicle binding affinity whereas (III)-*ON* and (IV)-*ON* displayed no significant aggregating and binding activity even in the presence of 3 mM  $\text{Ca}^{2+}$ . Interestingly, (II)-*ON* required much higher  $\text{Ca}^{2+}$  for vesicle aggregation than for vesicle binding, indicating again that domain (II) is not directly involved in vesicle aggregation. When compared to single-site mutations, double-site *ON* mutations clearly showed the synergism between domains in both vesicle binding and aggregation. (II,III)-*ON* and (II,IV)-*ON* essentially recovered the binding affinity of wild type, and, furthermore, the former mutant showed large improvement in vesicle-aggregating ability compared to (II)-*ON*. As expected from the properties of (III,IV)-*OFF* mutants, (III,IV)-*ON* showed the weakest vesicle-binding and aggregating activities among double-site *ON* mutants. Interestingly, this mutant behaved similarly to (II,III,IV)-*OFF* in that its vesicle-binding reached only 30% of wild-type activity while showing full vesicle aggregating activity at high  $\text{Ca}^{2+}$  concentrations (e.g., 3 mM). Again, this mutant might have an altered vesicle binding mode which is conducive to vesicle aggregation. Finally, (II,III,IV)-*ON* showed slight further improvement in aggregation activity when compared to (II,III)-*ON* and (II,IV)-*ON*, which suggests a minor synergism between domains III and IV in vesicle aggregation.

## DISCUSSION

This paper describes the first comprehensive mutagenesis of  $\text{Ca}^{2+}$ -binding sites of annexin I aimed at evaluating the roles of individual domains in its vesicle binding and aggregation. The mechanism by which annexins induce vesicle aggregation is still not fully understood. Several hypothetical models have been proposed for the vesicle aggregation by annexins which can be classified into two main categories. The first model assumes that vesicle aggregation results from the association of annexin molecules bound to different vesicles via protein-protein interactions (16, 17). The second model holds that annexins induce vesicle aggregation by directly binding two vesicles in a bidentate mode (18–20). In general, the latter model assumes that the binding of a primary binding site of annexin to a vesicle induces the binding of a secondary site to another vesicle (18). Both models assume that vesicle binding of annexins is a prerequisite for vesicle aggregation and that the two processes are discrete steps. The membrane binding of annexins is generally believed to take place through the formation of annexin- $\text{Ca}^{2+}$ -phospholipid(s) complex in which the  $\text{Ca}^{2+}$ -binding sites are directly involved (21, 22). Thus, one can positively identify the  $\text{Ca}^{2+}$ -binding site(s) and domain(s) that play an essential role in membrane binding by assessing the effect of mutations of  $\text{Ca}^{2+}$ -binding site on vesicle binding. The membrane aggregation might not, however, directly involve  $\text{Ca}^{2+}$ -binding sites especially if it is mediated by protein-protein interactions. Thus, the effect of mutations of  $\text{Ca}^{2+}$ -binding sites on membrane aggregation would only provide indirect information as to whether the membrane binding of these sites is linked to subsequent membrane aggregation.

Our results clearly indicate that the type II  $\text{Ca}^{2+}$ -binding site in domain II plays an essential role in anchoring the annexin I molecule to vesicle surfaces. The critical role of domain II in vesicle binding can be most clearly highlighted

by comparing the properties of (II)-*ON* and (II)-*OFF* mutants, whose functional  $\text{Ca}^{2+}$ -binding sites are only the type II site in domain II and all but this site, respectively. Both mutants have a comparable  $\text{Ca}^{2+}$  requirement for vesicle aggregation, but (II)-*OFF* requires 3 times more  $\text{Ca}^{2+}$  than (II)-*ON* for half-maximal vesicle binding. Thus, the type II site in domain II alone is much more effective than all other sites combined in bringing annexin I to vesicle surfaces. However, vesicle binding of this site (and domain) does not appear to be directly involved in vesicle aggregation. This notion is further supported by the finding that (II)-*OFF* has the same  $\text{Ca}^{2+}$  requirement for vesicle binding and vesicle aggregation. Since vesicle binding and vesicle aggregation of annexin I are most likely discrete steps, the finding that domain II is important in vesicle binding but not in vesicle aggregation points to the involvement of other domain(s) in vesicle aggregation. Although these mutational analyses could not unambiguously identify the domains involved in vesicle aggregation, evidence suggests that type II  $\text{Ca}^{2+}$ -binding sites in domains III and IV are involved in vesicle aggregation. For instance, all mutants with deactivated type II sites in domains III and IV have higher  $\text{Ca}^{2+}$  requirements for vesicle aggregation than for vesicle binding. Interestingly, combinatorial mutations of these sites show more pronounced effects on vesicle aggregation than expected from individual mutations [compare, for example, (III)-*ON* and (III,IV)-*ON* in Table 3], implying synergism between the two domains. Finally, type III sites in domains I and IV also appear to have some effect on vesicle aggregation when comparing  $[\text{Ca}^{2+}]_{1/2}$  values for aggregation of (II)-*ON* and (III,IV)-*OFF*: the former containing only the active type II site in domain II requires ca. 3 times more  $\text{Ca}^{2+}$  than the latter with the active domain II site and all type III sites. Since vesicle binding of annexin I must precede vesicle aggregation, it is difficult to assess the contributions of all these sites to the vesicle-aggregating activity separately from the vesicle-binding activity. Qualitatively speaking, however, it is evident from these studies that annexin I has two functionally distinct domains: one involved in membrane binding (domain II) and the other involved in vesicle aggregation (domains III/IV and possibly domain I).

Related mutagenesis studies have been performed on three other annexins, including annexin II (5, 6), annexin IV (7), and annexin V (9), to evaluate the relative contributions of individual domains to their *in vivo* and *in vitro* activities. These studies were all based on the same experimental approach of mutating cap residues of type II  $\text{Ca}^{2+}$ -binding sites in individual domains. Despite high structural homology among annexins, these studies yielded conflicting results as to the relative importance of individual domains. For instance, it was shown that for annexin IV the  $\text{Ca}^{2+}$ -binding site in domain IV plays a significant role in binding to isolated chromaffin granules and the site in domain I synergistically complements this binding (7). Domains II and III do not contribute significantly. On the other hand, the mutagenesis of annexin II revealed that all three domains (i.e., II, III, and IV) make essentially the same contributions to vesicle binding and aggregation when assayed using pure POPS vesicles (5, 6). However, a more recent cell study using annexin II mutants indicated that domain II plays a more direct role in binding to human endothelial cell surfaces (8). Finally, the characterization of annexin V mutants by



a phospholipase A<sub>2</sub> inhibition assay indicated that domain I is essential for membrane binding while domain IV also plays some minor role (9). Taken together with our results, these studies all point to the following common themes. (1) Four domains (or eight for annexin VI) of annexins, although structurally homologous, have distinct functions. (2) Different annexins might interact with membranes via different domains.

Structural analyses of annexin V provide some clue to the differential roles of individual domains of annexin I in its membrane binding and membrane aggregation (23). For annexin V bound to phospholipid monolayers, it was shown that the protein initially trimerizes on phospholipid monolayers and then trimers aggregate to form polymers which grow into two-dimensional crystals. The comparison of structures of free and monolayer-bound annexin V revealed a large degree of rotation of the module made of domains II and III with respect to the module containing domains I and IV for monolayer-bound annexin V molecules. For annexin V, the module (I/IV) plays a key role in its initial binding to membranes and the modular rotation moved the less important module (II/III) closer to membrane surfaces (9). Based on this information, we propose the following model to account for our results. Annexin I would make initial contact with membranes via the module containing domains II and III. This notion is based on the finding that the (II,III)-ON mutant behaves much like wild type in terms of vesicle binding and aggregation (Table 3). In particular, its type II Ca<sup>2+</sup>-binding site in domain II makes immediate contact with membrane surfaces through the formation of an annexin–Ca<sup>2+</sup>–phospholipid(s) complex. The role of domain III is likely to ensure tight contact of module (II/III) with the membrane surface by providing extra stabilizing interactions. It appears from the properties of the (II,III)-ON mutant that once this initial positioning of the protein on the membrane has been achieved, subsequent steps leading to vesicle aggregation are much more energetically favorable than the initial binding. These steps might include a conformational change of the annexin I molecule involving the rotation of the module (I/IV) to achieve approximately coplanar positioning of all domains on the membrane surface, as seen with annexin V (9). The modular rotation would allow extra protein–Ca<sup>2+</sup>–membrane interactions via lower affinity type III Ca<sup>2+</sup>-binding sites in domains I and IV. The conformational change might also lead to the proper positioning of domains I and IV for either self-association of membrane-bound annexin I molecules or binding of membrane-bound annexins to other vesicles, either of which would result in membrane aggregation. The apparent synergism between domains (II/III), (II/IV), and particularly (III/IV) might be simply due to the fact that vesicle binding of module (II/III) is prerequisite for and tightly coupled with the rotation of module (I/IV). It is noteworthy that the amino-terminal region of annexin I has been shown to be important in its vesicle-aggregating activity (20, 24). Thus, it is possible that the proposed modular migration of domain (I/IV) might be important in properly locating the amino-terminal extension

of domain I. Although this hypothetical model could explain much of our mutagenesis data, further studies are needed to understand exactly how membrane-bound annexin I undergoes a conformational change, how the change leads to membrane aggregation, and which protein residues are involved in these processes. These studies pave the way toward better understanding of these aspects of annexin I actions.

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