Characterization of the Ca\textsuperscript{2+}-binding Sites of Annexin II Tetramer*

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Annexin II heterotetramer (AIIt) is a multifunctional Ca\textsuperscript{2+}-binding protein composed of two 11-kDa subunits and two annexin II subunits. The annexin II subunit contains three type II and two type III Ca\textsuperscript{2+}-binding sites which are thought to regulate the interaction of AIIt with anionic phospholipid, F-actin, and heparin. In the present study we utilized site-directed mutagenesis to create AIIt mutants with inactive type III (TM AIIt), type II (CM AIIt), and both type II and III Ca\textsuperscript{2+}-binding sites (TCM AIIt). Surprisingly, we found that in the presence of Ca\textsuperscript{2+}, the TM, CM, and TCM AIIt bound phospholipid and F-actin with similar affinity to the wild type AIIt (WT AIIt). Furthermore, the TCM mutant, and to a lesser extent the TM and CM AIIt displayed dose-dependent Ca\textsuperscript{2+}-independent phospholipid aggregation and binding. While the TM and CM AIIt demonstrated Ca\textsuperscript{2+}-dependent binding to F-actin, the binding of the TCM AIIt was Ca\textsuperscript{2+}-independent. These results suggest that the type II or type III Ca\textsuperscript{2+}-binding sites do not directly participate in anionic phospholipid or F-actin binding. We therefore propose that in the absence of Ca\textsuperscript{2+}, the type II and type III Ca\textsuperscript{2+}-binding sites of AIIt stabilize a conformation of AIIt that is unfavorable for binding phospholipid and F-actin. Ca\textsuperscript{2+} binding to these sites, or the inactivation of these Ca\textsuperscript{2+}-binding sites by site-directed mutagenesis, results in a conformational change that promotes binding to anionic phospholipid and F-actin. Since the TM, CM, and TCM AIIt require Ca\textsuperscript{2+} for binding to heparin, we also propose that novel Ca\textsuperscript{2+}-binding sites regulate this binding event.

The annexins are a family of about 13 proteins that bind to cellular membranes and anionic phospholipids in a Ca\textsuperscript{2+}-dependent manner (1–3). All annexins have four repeating, highly homogenous amino acid sequences (eight repeats in the case of annexin VI), known as the annexin repeat (4). The annexin repeat contains 70–80 conserved amino acids, in particular the highly conserved 17-amino acid endonexin fold. Each repeat comprises one compact domain that consists of five \( \alpha \)-helices wound into a right-handed super-helix. The sum of the annexin repeats make up the protein core, which contains the binding sites for common annexin ligands such as Ca\textsuperscript{2+} and anionic phospholipids. Preceding the protein core is the pro-
tese-sensitive N-terminal tail, which varies in sequence among annexins and is therefore thought to be of regulatory importance. There are many suggested functions of annexins including transmembrane ion transport, inhibition of phospholipase \( \alpha_2 \), inhibition of blood coagulation, signal transduction for differentiation and mitogenesis, regulation of cell-matrix interactions, and regulation of cell-cell adhesion (reviewed in Refs. 2 and 5–7). The in vivo functions of annexins have not been well established.

X-ray crystallographic analysis of annexin II has established in the presence of a single type II Ca\textsuperscript{2+}-binding site in each of the second, third, and fourth domains and two type III Ca\textsuperscript{2+}-binding sites in the first domain of the protein. Interestingly, these Ca\textsuperscript{2+}-binding sites, along with the phospholipid-binding sites, are located on the convex surface of the molecule whereas the F-actin and heparin-binding sites are located on the opposite side of the molecule, on the concave surface. Annexin II is unique among the annexins in that its N-terminal 14 amino acids comprise a high affinity-binding site for a member of the S100 family of Ca\textsuperscript{2+}-binding proteins, known as p11 (2). The heterotetrameric complex formed by the interaction of the annexin II and p11 subunits is called annexin II heterotetramer (AIIt). Since the quaternary structure of AIIt is not known, the orientation or conformation of the annexin II subunits within AIIt is unknown. Therefore, it is also unclear whether or not all the Ca\textsuperscript{2+}-binding sites of the annexin II subunits within AIIt are functional.

Recently, it was reported that a bacterially expressed mutant of annexin II, composed of inactivated type II Ca\textsuperscript{2+}-binding sites displayed a higher Ca\textsuperscript{2+} requirement for phospholipid binding. Furthermore, the inactivation of both the type II and type III Ca\textsuperscript{2+}-binding sites resulted in the complete loss of phospholipid binding by the mutant annexin II (8). It was therefore concluded that functionally intact type II and type III Ca\textsuperscript{2+}-binding sites were required for the binding of phospholipid. However, these studies utilized a truncated annexin II in which the N-terminal 30 residues were removed proteolytically. It was therefore unclear if the results obtained from these studies would be applicable to the native protein. Since the truncated annexin II could not bind the p11 subunit, it was also not possible to investigate the function of the type II and type III Ca\textsuperscript{2+}-binding sites of AIIt.

We have previously reported the purification and character-

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‡ The abbreviations used are: AIIt, annexin II tetramer; TCM AIIt, recombinant AIIt composed of wild type p11 subunit and mutant annexin II subunit (Glu-52 → Ala and Glu-95 → Ala, Asp-161 → Ala, Glu-246 → Ala, and Asp-321 → Ala); p11, p11 light chain of annexin II tetramer; WT AIIt, recombinant AIIt composed of wild type annexin II and wild type p11 subunits; TM AIIt, recombinant AIIt composed of wild type p11 subunit and mutant annexin II subunit (Glu-52 → Ala and Glu-95 → Ala); CM AIIt, recombinant AIIt composed of wild type p11 subunit and mutant annexin II subunit (Asp-161 → Ala, Glu-246 → Ala, and Asp-321 → Ala); PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid.
ization of bacterially expressed human annexin II and p11 subunits (9). The recombinant AIIt, composed of the wild type recombinant subunits of annexin II and p11, possessed identical biological activity to that of native AIIt purified from bovine lung. In the present article, we report the first characterization of AIIt in which the type II and type III Ca²⁺-binding sites have been inactivated (TCM AIIt). Interestingly, inactivation of the type II and type III Ca²⁺-binding sites of annexin II result in the Ca²⁺-independent association of AIIt with both phospholipid and F-actin. Furthermore, since the binding of the TCM AIIt to heparin requires Ca²⁺, we postulate that Ca²⁺-binding sites other than the five characterized type II and type III Ca²⁺-binding sites mediate the AIIt-heparin interaction.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis of Annexin II—**Bacterial expression vectors (pDS10) containing the cDNA sequences of human annexin II with the appropriate mutations for inactivation of type III Ca²⁺-binding sites (E52A and E95A), type II Ca²⁺-binding sites (D161A, E246A, and D321A), and both the type III and type II Ca²⁺-binding sites (E52A, E95A, D161A, E246A, and D321A) were kindly provided by Dr. Volker Gerke (8, 10). Consistent with Dr. Gerke’s nomenclature, we have referred to these mutants as the TM, CM, and TCM mutants, respectively. These vectors were originally derived from the pAE65 vector, which contains the Annexin II sequence to provide an epitope for antibody recognition (10). This site was reverted to the wild type sequence (E66A) in all three mutants. The annexin II DNA sequences were excised from the pDS10 vector using polymerase chain reaction. The polymerase chain reaction resulted in blunt ended DNA and therefore encoded the exact sequence of wild type annexin II (11, 12). A BamHI site was added to the 3’ end using polymerase chain reaction with the appropriate primers. This cDNA sequence was then ligated into a NdeI-cut, Klenow filled-in, BamHI-digested pAE94.91 vector. These vectors were then transformed into Escherichia coli BL21(DE3) and grown as described previously (9).

**Purification of Recombinant Wild Type (WT), TM, CM and TCM Annexin II—**After 4 h of induction with isopropyl-1-thio-β-D-galactopyranoside, bacteria were collected by low speed centrifugation. They were subsequently sonicated in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT) containing protease inhibitors. This cell lysate was then centrifuged at 100,000 × g, followed by overnight dialysis against 20 mM Tris-HCl, pH 7.5, 5 mM CaCl₂, 1 mM DTT containing protease inhibitors. The resulting cell lysate supernatant was purified by hydroxylapatite chromatography, followed by heparin affinity chromatography, and gel permeation chromatography as reported previously (9).

**Purification of Recombinant p11—**The production of the recombinant p11 construct and the subsequent protein purification scheme has been previously reported (9). Briefly, bacteria were grown and induced with isopropyl-1-thio-β-D-galactopyranoside as described above. The resultant cell lysate supernatant was applied to Fast S ion-exchange chromatography, followed by affinity heparin chromatography, and gel permeation chromatography as reported previously (9).

**Purification of Recombinant Annexin II Tetramer—**Equimolar amounts of recombinant annexin II (WT, TM, CM, or TCM) and recombinant p11 were incubated at 4 °C for 30 min and then subjected to gel permeation chromatography. Proteins were subsequently stored in buffer A at −80 °C until use.

**45Ca Binding via Equilibrium Dialysis—**A 300-μl aliquot of each AIIt (WT, TM, CM, or TCM) or annexin II (WT or TCM) at a concentration of 5 mg/ml were placed into 12-mm EDTA-treated dialysis tubing (dissolved in chloroform) and were shelled by N₂ gas. The resultant residue of lipids was resuspended in 1 ml of phospholipid aggregation buffer (30 mM HEPES, pH 7.5, 50 mM KCl) and sonicated at 75 W for 30 min. The resultant liposomes were then subjected to gel permeation chromatography and the bulk of the liposomes were collected in the void volume and concentrated to 1 ml using a Millipore Ultrafree centrifugal filtrator (Millipore, Bedford, MA). Protein was quantified using the bicinchoninic acid (BCA) method with BSA as the standard. Protein concentrations were determined in a 1-ml aliquot by densitometric analysis as described above for the phospholipid binding assay.

**Fluorescence Spectroscopy—**A 100-μl sample of WT, TM, CM, or TCM AIIt (in buffer A) was excited at 292 nm, and the emission spectra was recorded from 300–350 nm using a PerkinElmer Life Sciences LS-50B spectrophotometer (slit widths were 10 nm). Each protein sample was scanned in the presence of buffer A, and rescanned following addition of 2 mM Ca²⁺. All measurements were collected in at least triplicate, and the λ_max was recorded for each protein in presence of Ca²⁺ or EGTA.

**Heparin Binding Assay—**Heparin binding was assessed as described previously (17). Briefly, AIIt was incubated with 17-kDa bovine lung heparin (Calbiochem) for 20 min in the presence of 500 μg Ca²⁺ or 10 μg EGTA, followed by high speed centrifugation at 400,000 × g for 30 min in a Beckman Optima TLX Ultracentrifuge. The pellets were then resuspended in SDS-PAGE buffer, followed by densitometric analysis as described above for phospholipid binding.

**RESULTS**

**Mutagenesis of Annexin II and Formation of Mutant AIIt—**X-ray crystallographic analysis of annexin II has established that each of three acidic amino acid residues (Asp-161, Glu-246, and Asp-321) provide two carboxylate oxygens for the formation of three separate type II Ca²⁺-binding sites in domains 2, 3, and 4, respectively. Two glutamic acid residues (Glu-52 and Glu-95) are similarly involved in the formation of the two type
III Ca\(^{2+}\)-binding sites in domain 1 of the protein (8, 19). Site-directed mutagenesis studies of annexin II have shown that replacement of these critical acidic residues with alanine results in the inactivation of the type II and type III sites (12). In accordance with the previous nomenclature, we have referred to these annexin II mutants as the TM mutant (type III sites inactivated), CM mutant (type II sites inactivated), and TCM mutant (type II and III sites inactivated).

WT annexin II as well as TM, CM, and TCM annexin II were expressed in our bacterial expression system and purified as described previously (9). The elution profiles of the recombinant wild type (WT) and mutant annexin II on hydroxyapatite, heparin affinity, and gel permeation chromatography were indistinguishable (data not shown). The recombinant wild type or mutant annexin II was combined with recombinant p11 subunit and subjected to gel permeation chromatography as described previously (9). The elution profiles, as well as the circular dichroism spectra of the recombinant WT and TM, CM, and TCM AIIt were also indistinguishable (data not shown). This suggests that a gross change in the conformation of the mutant AIIt has not occurred as a result of the mutations in the Ca\(^{2+}\)-binding sites.

\(^{45}\)Ca Binding by Mutant AIIt—Although x-ray crystallographic analysis of annexin II has established that the protein has two type III Ca\(^{2+}\)-binding sites and three type II Ca\(^{2+}\)-binding sites (3), it is not clear if all of these sites are occupied at physiological Ca\(^{2+}\) concentrations. Previous studies using equilibrium dialysis have shown that annexin II binds only two Ca\(^{2+}\) ions in the presence of 1 mM Ca\(^{2+}\) (20). As shown in Table I, we found that in the presence of 2 mM Ca\(^{2+}\), annexin II binds about 4 mol of Ca\(^{2+}\)/mol of protein. In contrast, the TCM annexin II bound about 1 mol of Ca\(^{2+}\)/mol of protein. We also compared the Ca\(^{2+}\)-binding capacities of the AIIt mutants. The wild type AIIt bound about 6 mol of Ca\(^{2+}\)/mol of protein while the TM, CM, and TCM mutants bound about 5, 4, and 2–3 mol of Ca\(^{2+}\)/mol of protein, respectively (Table I). The ability of the TCM annexin II or AIIt to bind Ca\(^{2+}\) suggested the existence of Ca\(^{2+}\)-binding sites other than those previously characterized.

\(^{45}\)Ca binding by WT, TM, CM, and TCM AIIt

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mol (^{45})Ca bound/mol of protein</th>
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<tbody>
<tr>
<td>WT annexin II</td>
<td>3.63 ± 0.47</td>
</tr>
<tr>
<td>TCM annexin II</td>
<td>1.11 ± 0.41</td>
</tr>
<tr>
<td>WT AIIt</td>
<td>5.92 ± 0.73</td>
</tr>
<tr>
<td>TM AIIt</td>
<td>5.04 ± 1.50</td>
</tr>
<tr>
<td>CM AIIt</td>
<td>4.35 ± 1.43</td>
</tr>
<tr>
<td>TCM AIIt</td>
<td>2.53 ± 1.14</td>
</tr>
</tbody>
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\(^{45}\)Ca binding by WT, TM, CM, and TCM AIIt

<table>
<thead>
<tr>
<th>Protein</th>
<th>(\text{Ca}^{2+})</th>
<th>EGTA (in buffer A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT AIIt</td>
<td>315.94 ± 0.16</td>
<td>324.37 ± 0.13</td>
</tr>
<tr>
<td>TM AIIt</td>
<td>315.84 ± 0.14</td>
<td>323.97 ± 0.04</td>
</tr>
<tr>
<td>CM AIIt</td>
<td>325.62 ± 0.08</td>
<td>325.83 ± 0.14</td>
</tr>
<tr>
<td>TCM AIIt</td>
<td>323.73 ± 0.29</td>
<td>323.87 ± 0.12</td>
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Phospholipid Aggregation and Binding by Mutant Annexin II

FIG. 1. \(\text{Ca}^{2+}\)-dependence of annexin II-mediated phospholipid vesicle aggregation by WT and TCM Annexin II. Phospholipid vesicles consisting of phosphatidylserine, phosphatidylethanolamine, and cholesterol were prepared as described under “Experimental Procedures” and incubated at 20 °C with 30 mM HEPES, pH 7.5, 50 mM KCl and either 200 \(\mu\)M CaCl\(_2\) (black bars), 20 \(\mu\)M CaCl\(_2\) (light gray bars), or 3 mM EGTA (dark gray bars). Immediately after taking the first absorbance reading, 1.4 \(\mu\)M WT or TCM annexin II was added to the reaction mixture, and the \(A_{400}\) was continuously recorded for 15 min. Results are shown as a percentage of starting \(A_{400}\) (no AIIt added), and are expressed as mean ± S.D. \((n = 3)\). Inset, \(\text{Ca}^{2+}\)-dependence of annexin II-mediated phospholipid vesicle binding by WT and TCM annexin II. After completion of the aggregation reactions, the reaction mixtures were centrifuged at 14,000 \(\times\) g and phospholipid associated protein determined by densitometric scanning of SDS-PAGE as described under “Experimental Procedures.” Results are representative of three independent experiments.
Annexin II is present in cells as a monomer or complexed with the p11 subunit as a heterotetramer called AIIt. The interaction of these subunits alters their conformation and it is therefore unclear if the type II and type III Ca\(^{2+}\)-binding sites of AIIt are equivalent in structure or function to those of annexin II monomer. We found, in contrast to the Ca\(^{2+}\)-dependent aggregation of phospholipid vesicles by TCM annexin II (Fig. 1), that the aggregation of phospholipid vesicles by TCM AIIt was not Ca\(^{2+}\)-dependent (Fig. 2, A and B). Furthermore, half-maximal aggregation of the phospholipid liposomes by TCM AIIt was similar regardless of the presence or absence of Ca\(^{2+}\). In the absence of AIIt, we did not observe any nonspecific phospholipid aggregation. In addition, in the absence of phospholipid vesicles, AIIt did not contribute to the turbidity nor did it pellet (data not shown). This data provided evidence that Ca\(^{2+}\) binding at the type II and type III Ca\(^{2+}\)-binding sites does not play a direct role in the phospholipid aggregating activity of AIIt. We also observed that the phospholipid aggregating activity of the TM and CM AIIt did not require Ca\(^{2+}\) (Fig. 2B), although the extent of Ca\(^{2+}\)-independent phospholipid aggregation by TM AIIt was not as high as CM AIIt. Therefore, inactivation of either the type III (TM AIIt) or type II (CM AIIt) Ca\(^{2+}\)-binding sites of AIIt resulted in Ca\(^{2+}\)-independent phospholipid aggregating activity. Furthermore, since TCM AIIt did not aggregate or bind phospholipid vesicles composed of phosphatidylcholine (data not shown), it was concluded that the inactivation of the type II or type III Ca\(^{2+}\)-binding sites of AIIt did not alter the phospholipid specificity of the protein.

We also examined the ability of the mutant AIIt to bind to phospholipid vesicles. In the presence of Ca\(^{2+}\), the WT, TM, CM, and TCM AIIt bound phospholipid with similar affinity and capacity (Fig. 3A). Likewise, WT and TCM annexin II had similar binding affinities for phospholipid vesicles in the presence of Ca\(^{2+}\) (Fig. 3, inset). Thus, the inactivation of the type II or type III Ca\(^{2+}\)-binding sites did not affect the affinity of annexin II or AIIt for phospholipid vesicles. However, in the presence of EGTA, all three mutant AIIt show Ca\(^{2+}\)-independent binding to phospholipid vesicles (Fig. 3B), whereas WT AIIt shows characteristic Ca\(^{2+}\)-dependent binding. The TCM AIIt is able to pellet equally in the presence or absence of Ca\(^{2+}\). As well, about 40 and 70% of the TM and CM AIIt, respectively, pelleted in the absence of Ca\(^{2+}\), compared with the values obtained in the presence of Ca\(^{2+}\).

**F-actin Bundling and Binding by Mutant Annexin II and AIIt—** AIIt binds to F-actin in the presence of Ca\(^{2+}\), which results in the formation of large anisotropic F-actin bundles. The Ca\(^{2+}\)-dependent formation of anisotropic F-actin bundles is rapid and readily reversed by the addition of excess EGTA (14, 23). Light scattering measurements of F-actin bundle formation by AIIt revealed that the WT and TM AIIt have an identical half-maximal Ca\(^{2+}\) requirement for bundling (\(K_{0.5} = 287 \pm 12 \mu M\)), whereas CM AIIt has a higher Ca\(^{2+}\) requirement for half-maximal bundling (\(K_{0.5} = 490 \pm 17 \mu M\)) (Fig. 4). Moreover, TCM AIIt demonstrated Ca\(^{2+}\)-independent F-actin bundling. Examination of the Ca\(^{2+}\) dependence of F-actin binding by the mutant AIIt revealed that WT, TM, and CM AIIt bound to F-actin in a Ca\(^{2+}\)-dependent manner, whereas TCM AIIt did not require Ca\(^{2+}\) for binding to F-actin (Fig. 4, inset). In the absence of F-actin, pelleting of AIIt was not observed (data not shown).

We also examined the F-actin binding affinity of the WT, TM, CM, and TCM AIIt. As shown in Fig. 5, the WT and mutant AIIt showed similar binding affinity for F-actin. Likewise, the F-actin binding affinity of WT and TCM annexin II was similar (Fig. 5, inset). Thus, the inactivation of the type II or type III
Ca\textsuperscript{2+}-binding sites did not affect the affinity of annexin II or AIIt for F-actin.

Ca\textsuperscript{2+} Dependence of t-PA-dependent Plasminogen Activation—Recently, our laboratory reported the dramatic stimulation of t-PA-dependent plasminogen activation by AIIt (15, 16). We also reported that the stimulation of t-PA-dependent plasminogen activation by AIIt was Ca\textsuperscript{2+}-independent. As shown in Fig. 6, the WT, TM, CM, and TCM AIIt stimulated t-PA-dependent plasminogen activation with similar potency. This suggested that the type II or type III Ca\textsuperscript{2+}-binding sites did not regulate the stimulation of t-PA-dependent plasminogen activation by AIIt.

Role of Type II or III Ca\textsuperscript{2+}-binding Sites in Heparin Binding—Annexin II contains a classic Cardin-Weintraub heparin-binding consensus sequence. Furthermore, the binding of heparin by annexin II or AIIt is Ca\textsuperscript{2+}-dependent (17). Interestingly, shown in Fig. 7, the WT, TM, CM, and TCM AIIt all demonstrated Ca\textsuperscript{2+}-dependent heparin binding. This suggests
that a Ca\(^{2+}\)-binding site, distinct from the type II or type III Ca\(^{2+}\)-binding sites, is involved in the regulation of heparin binding by AIIt.

**DISCUSSION**

AIIt is a multifunctional protein that interacts in a Ca\(^{2+}\)-dependent manner with anionic phospholipid, F-actin, and heparin. The binding sites for these ligands are located within the annexin II subunit of AIIt. In the present study we utilized site-directed mutagenesis to inactivate the type III (the TM mutant), type II (the CM mutant), and both type II and III Ca\(^{2+}\)-binding sites of annexin II (the TCM mutant). The strategy for inactivation of the annexin II Ca\(^{2+}\)-binding sites was initially proposed by Dr. Gerke’s (8, 11, 12) laboratory, who confirmed that the substitution of the key acidic residue of each Ca\(^{2+}\)-binding site with alanine resulted in the inactivation of the Ca\(^{2+}\)-binding site. However, in contrast to that study, which utilized a chymotrypsin-digested annexin II, we generated a full-length recombinant annexin II. Therefore, for the first time, we were able to form the TM, CM, and TCM mutant forms of AIIt by combining the mutant recombinant annexin II with wild type recombinant p11 subunit. In the present report we have used these mutant forms of AIIt to examine the role of the type II and III sites of AIIt in the Ca\(^{2+}\)-dependent interaction with phospholipid, F-actin, heparin, and plasminogen.

Since the WT and three mutant forms of annexin II had identical elution profiles after chromatography on hydroxylapatite, heparin-Sepharose, and Sephacryl S-100, we concluded that, as reported earlier, these mutations did not substantially perturb the conformation of annexin II. As well, the mutant annexin II monomers were able to bind to p11 and form AIIt, a further indication that annexin II was in its native conformation. Furthermore, since the WT recombinant AIIt and the TM, CM, and TCM mutants of AIIt had identical gel permeation chromatographic profiles and identical CD spectra, we have concluded that the TM, CM, and TCM mutants of AIIt were not conformationally compromised. The ability of the three mutants to bind heparin and stimulate t-PA-dependent plasminogen conversion to plasmin was further evidence that the conformation of these proteins was not perturbed.

Our analysis of the Ca\(^{2+}\)-binding capacity of the WT, TM, CM, and TCM AIIt has produced several unexpected results. First, we found that in the presence of 2 mM Ca\(^{2+}\), WT annexin II and WT AIIt bound only about 4 or 6 mol of Ca\(^{2+}\)/mol of AIIt, respectively. Since the crystal structure of annexin II established the presence of two type III Ca\(^{2+}\)-binding sites and three type II Ca\(^{2+}\)-binding sites, it was expected that annexin II should bind 5 mol of Ca\(^{2+}\)/mol and AIIt would bind at least 10 mol of Ca\(^{2+}\)/mol of AIIt. Previous equilibrium dialysis experiments performed in the presence of 1 mM Ca\(^{2+}\) showed that annexin II and AIIt bound 2 and 4 mol of Ca\(^{2+}\)/mol of protein, respectively (20). One explanation for the discrepancy between the equilibrium dialysis results and the predicted results is that full occupancy of the Ca\(^{2+}\)-binding sites may require greater than 2 mM Ca\(^{2+}\). However, extended incubation of AIIt in the presence of greater than 2 mM Ca\(^{2+}\) leads to precipitation of the protein. It is therefore not possible to perform equilibrium dialysis experiments at concentrations of Ca\(^{2+}\) greater than 2 mM. It is also possible that AIIt binds less Ca\(^{2+}\) than expected because the conformation or orientation of the annexin II subunits is such that not all Ca\(^{2+}\)-binding sites of the annexin II subunit are functional. However, without the elucidation of the x-ray crystallographic structure of AIIt, it is not possible to evaluate this possibility.

Second, it was unexpected that the TCM annexin II and TCM AIIt bound 1 mol of Ca\(^{2+}\)/mol and 2–3 mol of Ca\(^{2+}\)/mol, respectively, because the x-ray crystallographic data had identified the presence of only type II and type III Ca\(^{2+}\)-binding sites on annexin II (3, 22). One possible explanation for this result is that the mutations introduced into annexin II do not provide total inactivation of the type II and type III Ca\(^{2+}\)-binding sites. We feel this is unlikely because the substitution of acidic residues with alanine would result in the loss of two carboxyl groups.

\[2 \text{ N. R. Filipenko and D. M. Waisman, unpublished observation.}\]
oxygen atoms from each Ca$^{2+}$-binding sites. This would mean that in order for the Ca$^{2+}$-binding sites to bind Ca$^{2+}$, the type III Ca$^{2+}$-binding sites of the TM AIIt would be required to coordinate Ca$^{2+}$ binding with only two carbonyl oxygens and five water molecules. This type of geometry for a Ca$^{2+}$-binding site is unlikely. The type II Ca$^{2+}$-binding sites of CM AIIt would require the coordination of Ca$^{2+}$ by only three carbonyl oxygens and four water molecules. At best this would be expected to generate a very weak Ca$^{2+}$-binding site. The loss in the blue shift of the Trp-212 emission spectra for the CM AIIt is strong evidence that the Glu-246 → Ala substitution had inactivated the type II Ca$^{2+}$-binding site of domain III of annexin II. It is therefore reasonable to expect that similar mutations in the other type II sites would result in their inactivation.

Another possible explanation for the ability of the TCM annexin II to bind Ca$^{2+}$ is that novel Ca$^{2+}$-binding sites, other than type II or type III Ca$^{2+}$-binding sites are present on these proteins. Since the TCM annexin II and AIIt mutants show Ca$^{2+}$-dependent binding to heparin, it is likely that the type II or type III Ca$^{2+}$-binding sites are not involved in regulating heparin-binding. It is therefore reasonable to propose that the single Ca$^{2+}$-binding site present on TCM annexin II or the two to three Ca$^{2+}$-binding sites present on the TCM AIIt are novel Ca$^{2+}$-binding sites that are important for regulating the interaction of AIIt with heparin.

If it is assumed that the binding of approximately 2 mol of Ca$^{2+}$/mol of AIIt are due to Ca$^{2+}$-binding sites distinct from the type II and type III Ca$^{2+}$-binding sites then it is possible to determine the number of atoms of Ca$^{2+}$ bound to the type II or type III sites for each mutant AIIt. Since TM AIIt binds 5 mol of Ca$^{2+}$/mol of protein and two of these Ca$^{2+}$ are probably not type II or III Ca$^{2+}$-binding sites, we can assume that three Ca$^{2+}$ atoms remain bound to the type II sites present on TM AIIt. Similarly, the CM AIIt binds four Ca$^{2+}$ and assuming two Ca$^{2+}$ are not type II or III Ca$^{2+}$-binding sites then the CM AIIt binds 2 mol of Ca$^{2+}$/mol of protein to the type III sites present on the protein. Although speculative, the recalculated Ca$^{2+}$-binding data is consistent with the binding of Ca$^{2+}$ to only one

![Fig. 5. Concentration dependence of F-actin binding by WT, TM, CM, and TCM AIIt.](image)

![Fig. 6. Stimulation of tissue plasminogen activator-mediated plasminogen activation by WT, TM, CM, and TCM AIIt. Tissue plasminogen activator (5.6 nM) was incubated at 25 °C in PG buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 5 mM CaCl$_2$), and 104 μM plasmin substrate (Spectrozyme number 251) in the presence of 2 μM WT, TM, CM, or TCM AIIt. The reaction was initiated by the addition of the indicated amounts of [Glu]plasminogen. The amidolytic activity of generated plasmin was monitored by $A_{405}$ as described under "Experimental Procedures." Shown is a comparison of the rates of plasmin generation calculated from plots of $A_{405}$ versus time. The plasmin generation unit is defined as $(A_{405}/\text{min}) \times 10^{-3}$ and is expressed as mean ± S.D. ($n = 3$).](image)
of the annexin II subunits within AIIt. This presents the possibility that only one of the two annexin II subunits of WT AIIt may have type II or type III Ca\(^{2+}\)-binding sites capable of binding Ca\(^{2+}\) in the presence of 2 mM Ca\(^{2+}\). Alternatively, one WT annexin II subunit could have functional type II Ca\(^{2+}\)-binding sites and the other subunit could have functional type III Ca\(^{2+}\)-binding sites.

As discussed above, x-ray crystallographic studies established that annexin II contains two type III Ca\(^{2+}\)-binding sites located in the first domain of the protein and three type II Ca\(^{2+}\)-binding sites located in the second, third, and fourth domains of the protein. The identified consensus amino acid sequence of the type II Ca\(^{2+}\)-binding site is -G-X-G-T-X\(_{38}\)-(D/E). Ca\(^{2+}\) is heptacoordinated by three carbonyl oxygen atoms, two oxygen atoms of the distal acidic residue and two solvent molecules. The x-ray crystallographic structure of the annexin V-glycerophosphoserine complex has been reported and it was concluded from these studies that Ca\(^{2+}\) forms a bridge between the amino acids of the type II Ca\(^{2+}\)-binding sites and the phosphoryl oxygen atom of the phospholipid (22). Furthermore, the carboxyl group of the distal acidic residue of the type II Ca\(^{2+}\)-binding sites hydrogen bonds with the serine amino group of glycerophosphoserine. It was therefore unexpected that TM and CM AIIt bound to phospholipid vesicles and even more surprising that the binding of the CM and TCM mutant was Ca\(^{2+}\)-independent. It was also unexpected that the CM and TCM AIIt had identical affinity for phospholipid in the presence or absence of Ca\(^{2+}\). This is surprising because the substitution of the acidic residues of the CM AIIt with alanine would be expected to substantially weaken the interaction of AIIt with phospholipid. This is likely to occur by preventing the interaction of the carboxyl oxygen of the distal acidic residue with the polar head group of phosphatidylserine and by preventing the interaction of Ca\(^{2+}\) with the phosphoryl oxygen of phosphatidylserine. However, since the affinity of the WT, TM, CM, and TCM AIIt for phospholipid is identical, it is likely that these interactions are not sufficiently strong to influence the interaction of annexin II with phosphatidylserine.

A detailed analysis of the mechanism of the interaction of annexin II or AIIt with phosphatidylserine will not be possible until the x-ray crystallographic structure of the phosphatidylserine-AIIt complex is elucidated.

Our results differ dramatically from those obtained by Dr. Gerke's (8, 11, 12) laboratory who reported that the TCM annexin II did not bind to phospholipid. Since the TCM annexin II mutant used in these experiments was a chymotryptic fragment that lacked the first 29 residues of the N terminus, it is possible that their protein was not in its native conformation. Drs. Drust and Creutz (24) have shown that proteolysis of the N terminus of annexin II dramatically increases the Ca\(^{2+}\) dependence of interaction of AIIt with biological membranes.

It was also interesting that the TM and CM AIIt demonstrated Ca\(^{2+}\)-dependent binding to F-actin. This suggested that the type II or III Ca\(^{2+}\)-binding sites are not involved in the Ca\(^{2+}\)-dependent interaction of AIIt with F-actin. It was therefore unexpected that the TCM AIIt showed Ca\(^{2+}\)-independent binding to F-actin. Although speculative, it is reasonable to propose that in the absence of Ca\(^{2+}\), the distal acidic residues of the type II Ca\(^{2+}\)-binding sites and the acidic residue of the type III Ca\(^{2+}\)-binding sites interact with other residues within annexin II. This interaction results in the stabilization of a conformation of annexin II which does not allow for interaction with phospholipid or F-actin. The binding of Ca\(^{2+}\) to the Ca\(^{2+}\)-binding sites results in the reorientation of these acidic residues such that they can now participate in Ca\(^{2+}\) coordination. The resulting change in the conformation also allows the interaction of annexin II with phospholipid or F-actin. Similarly, removal of these residues by site-directed mutagenesis also prevents these acidic residues from interacting with other amino acid residues and promoting the conformation of annexin II that cannot interact with phospholipid or F-actin. Validation of this experimental model will await elucidation of the x-ray crystallographic structure of AIIt.

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REFERENCES

Additions and Corrections


Characterization of the Ca$^{2+}$-binding sites of annexin II tetramer.

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Dr. Hyoung-Min Kang’s name was inadvertently omitted from the author list. The corrected author list is shown above. Dr. Kang’s present address is: Dept. of Laboratory Medicine, University of Washington, Box 357110, 1959 N. E. Pacific St., Seattle, WA 98195.

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