Combinatorial Mutagenesis of the Four Domains of Annexin IV: Effects on Chromaffin Granule Binding and Aggregating Activities†

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ABSTRACT: This study addresses the roles of individual annexin IV domains in calcium-dependent membrane binding and aggregation through an analysis of the activities of mutant annexin IV proteins in which critical residues in one or more domains have been altered. The consensus sequence for high-affinity Ca^{2+}-binding pockets obtained from the annexin V crystal structure is GXGT-38 residues-D/E [Huber, R., et al. (1992) *J. Mol. Biol. 223*, 683−704]. Site-directed mutagenesis was used to change the conserved acidic residues (D/E) in these sequences to alanine residues in each of the four domains of bovine annexin IV, singly or in combinations. Fourteen mutants with one, two, three, or four mutated domains were constructed and expressed in *Escherichia coli.* Purified recombinant product was evaluated for Ca^{2+}-dependent binding to and aggregation of bovine chromaffin granules. Increases in the number of mutated domains resulted in increased Ca^{2+} requirements for both granule binding and aggregation. Further analysis revealed that mutations in individual domains had preferential effects on the binding or aggregating activities of the protein. For example, mutation of the first or fourth domains had a greater effect on membrane binding than aggregation, while, conversely, mutation of the second domain had a more dramatic effect on membrane aggregation. Mutation of the third domain was largely silent in these assays. An additional mutation was made in the fourth domain to substitute a serine for a highly conserved arginine residue (Arg274) present at the C-terminus of the fourth endonexin fold. This mutation increased the calcium requirement for membrane binding 2-fold and for membrane aggregation 3-fold. This mutant protein was found to be an effective inhibitor of membrane aggregation by native annexin IV at intermediate levels of calcium.

The annexins compose a distinct class of calcium-binding proteins in that they do not possess the well-characterized EF hand motif, yet they do contain unique internally repeating domains (Geisow & Walker, 1986). All annexins possess four (or eight for annexin VI) core 70-amino acid domains that contain several conserved sequence elements. The repeating domain structure led to the hypothesis that the annexins are capable of coordinating multiple calcium ions. Biochemical evidence from equilibrium dialysis with $^{45}Ca^{2+}$ and the apparent cooperativity demonstrated in calcium-dependent binding to lipids and aggregation of chromaffin granules support this hypothesis (Creutz et al., 1978; Glenney 1986; Schlaepfer & Haigler, 1987; Ando et al., 1989; Junker & Creutz, 1994). Although precise physiological roles have yet to be defined for the annexins, suggested roles include the mediation of membrane fusion in exocytosis, phospholipase A_2 inhibition/anti-inflammation, anticoagulation, oncogene and growth factor receptor signal transduction, interaction with the cytoskeleton, ion channel formation, cell surface receptor regulation, extracellular matrix interaction, glucocorticoid response, and inositol phosphate metabolism [reviewed in Creutz (1992), Moss (1992), and Raynal and Pollard (1994)].

Annexin IV (endonexin I) is a 32 kDa polypeptide of 318 amino acids. The complete amino acid sequence was first reported for porcine annexin IV after direct protein sequencing (Weber et al., 1987). Human placenta and bovine liver cDNA clones have also been sequenced (Grundmann et al., 1988; Hamman et al., 1988). Like other annexins, annexin IV contains four homologous 70-amino acid core domains, each containing the 17-amino acid endonexin fold sequence. These domains in annexin IV are surrounded by relatively short amino and carboxy termini that do not possess any identified functional properties.

Crystal structures have been solved for annexins I and V, providing valuable three-dimensional structural information (Huber et al., 1990a,b, 1992; Weng et al., 1993; Concha et al., 1993; Sopkova et al., 1993). The annexin core consists of a planar array of the four tightly coupled 70-amino acid domains, each containing five α-helices with short connecting loops. The data suggest that a calcium-binding pocket (type II; Weng et al., 1993) is formed by the consensus sequence GXGT(in the endonexin fold or the loop between the 1st and 2nd α-helices)-38 residues-D/E(in the loop between the 4th and 5th α-helices). This consensus sequence is found within each first, second, and fourth domain of most annexins. In the third domain, there is a substitution (or insertion) for the initial glycine, although the downstream acidic residue is still present, and calcium binds to this domain in association with a major conformational change (Concha et al., 1993; Sopkova et al., 1993; Burger et al., 1994).

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Table 1: Oligonucleotides Used To Create D/E→A Mutations and the R4 Mutation[a]

oligonucleotide	5′→3′ oligonucleotide sequence	restriction site introduced
DE1	CCTGATGGACGACTTGAAAAGCGCGCTGAGTGGCAACTTCG	*Bss*HII
DE2	GATGATATTCGCTCTGCGACGTCATTCATGTTCC	*Aat*I
DE3	CAGAGTATTAAATCTGCGACGTCTGGCAGCTTTGAAG	*Aat*I
DE4	CTCCTTCATCAAGGGCGCCACATCCGGAGATAC	*Nar*I
R4	GTGATGGTTTCTTCTGCAGAGATTGACATG	*Pst*I

[a] Underlined bases represent mismatches from the wild-type sequence. Bases shown in boldface type represent the new restriction site introduced to facilitate screening and recombination to form additional mutants.

In order to examine the relationship between these calcium-binding motifs and the calcium-dependent activities of annexin IV, a strategy was employed that functionally isolates single and multiple domains. Two of the Ca^{2+}-coordinating ligands provided by the annexin V type II binding pockets in each domain are contributed by the conserved acidic residue carboxyl group (Huber et al., 1992). Therefore, these acidic residues were changed to alanines. A neutral residue was chosen in order to remove the Ca^{2+}-coordinating, negatively charged carboxyl group. Introduction of a basic residue was avoided due to the possibility that a fixed positive charge in the binding pocket might mimic the presence of a calcium ion and result in a constitutively active domain, as has been reported in the case of modifications of the calcium-binding sites in calmodulin (Gao et al., 1993). Similar alterations of the homologous residues in annexin II were made in the recent mutagenesis studies of Jost and colleagues (1992, 1994). Their results also implied that these sites play an important role in promoting the interactions of annexin II with membranes and calcium.

A partial library of acidic residue mutants was constructed by oligonucleotide-directed mutagenesis in phage M13. Mutations in single or multiple domains were made with the intention of creating constructs that have functionally deleted domains, but a three-dimensional structure that is comparable to wild-type annexin IV. The mutants are referred to here as DE*wxyz* mutants, where *wxyz* represents numbers referring to the domains that have been altered. For example, the DE24 mutant has the acidic residues involved in binding calcium in domains 2 and 4 changed into alanines.

A conserved arginine residue is located at the carboxy terminus of each endonexin fold. The crystal structure data for annexin V suggests that these conserved arginines (R45, R117, R201, R276) form internal salt bridges with conserved aspartate residues (D20, D92, D175, D280) (Huber et al., 1992) within the same domain. These salt bridges are not all in homologous positions: in domains 1 and 3 they are on the protein surface, while in domains 2 and 4 they are located in the internal pore region that forms an ion channel. In the present study, an attempt was made to construct a library of mutant annexins IV in which these arginines were replaced with serines, singly or in combinations. Most of these constructs proved to be only poorly soluble when expressed in bacteria. However, an annexin IV construct with the fourth arginine (Arg274) replaced by serine (the R4 mutant) was isolated in soluble form and found to be significantly altered in its ability to interact with chromaffin granules.

MATERIALS AND METHODS

Materials. Reagents were obtained from the following sources: antibiotics, Sigma; IPTG,[1] Sigma; restriction enzymes, Promega, Boehringer-Mannheim, and Bio-Rad; nitrocellulose, Schleicher & Schuell; Prime Time labeling kit, IBI; radionucleotides, Dupont-NEN; buffers/salts/chemicals, Sigma unless designated otherwise; pET11d vector and BL21DE3 and BL21DE3pLysS cells, Novagen; protein molecular weight standards, Bio-Rad. A rabbit muscle sarcoplasmic reticulum fraction containing calsequestrin was generously provided by Howard Kutchai; the bovine cDNA clone was generously provided by Helen C. Hamman (Hamman et al., 1988); and native bovine annexin IV isolated from liver was generously provided by Matt Junker (Junker & Creutz, 1993).

General Methods. All ligations, restriction digests, and agarose gel electrophoreses were conducted according to standard molecular biology techniques (Ausubel et al., 1989; Sambrook et al., 1989). All cultures were grown at 37 °C, unless stated otherwise. The elution of DNA bands from agarose gels was performed using the freeze−fracture method (Huff et al., 1991). SDS−PAGE was conducted using the method of Laemmli (1970). One-dimensional isoelectric focusing was performed using a discontinuous buffer method in an SE 600 vertical slab gel unit (Hoefer Scientific Instruments) and pH 4−6 ampholytes (protocol of Gary Giulian, University of Wisconsin, described in the Hoeffer 1992−1993 catalog). Western blots were performed as previously described using a horseradish peroxidase color-developing system (Creutz et al., 1987). Minilysate plasmid preparations and major plasmid preparations utilizing poly(ethylene glycol) precipitations were conducted according to standard molecular biology techniques (Ausubel et al., 1989). The Sanger method of DNA sequencing was performed using Sequenase II (Tabor & Richardson 1987; Sanger et al., 1977). Protein concentration was determined by the Bradford (1976) method, using bovine serum albumin as the standard. Calcium concentrations were obtained by direct measurement with a calcium electrode (Orion).

Site-Directed Mutagenesis. A partial library of annexin IV DE mutants was constructed using two methods of mutagenesis. Primary mutants were obtained by oligonucleotide-directed mutagenesis (Kramer et al., 1984). The oligonucleotides listed in Table 1 annealed to heteroduplex M13 DNA containing the wild-type annexin IV cDNA insert, creating M13mp9Endo1DEx derivatives (x = domains containing D/E→A mutations). The derivatives possess multiple restriction sites at the 3′-terminal polylinker, as well as the newly created restriction sites at the mutated positions (*Bss*HII, *Aat*II, or *Nar*I). Mutant clones were identified by restriction digest analysis of cDNA obtained from transformed cells or by hybridization of ^{32}P-end-labeled DE

[1] Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Table 2: Construction of the DE Mutants[a]

construct[b]	mutagenesis[c] method	substrates[d] domains supplied	substrates[d] source
pMNEndo1DE2	ligation	1	pMNEndo1[e]
		2, 3	M13mp9Endo1DE12[f]
		4	pMNEndo1
pMNEndo1DE3	ligation	1	pMNEndo1
		2, 3	M13mp9Endo1DE134
		4	pMNEndo1
pMNEndo1DE4	ligation	1, 2, 3	pMNEndo1
		4	pMNEndo1DE234
pMNEndo1DE12	ligation	1	pMNEndo1DE1234
		2, 3	M13mp9Endo1DE12
		4	pMNEndo1
pMNEndo1DE13	ligation	1	pMNEndo1DE1234
		2, 3	M13mp9Endo1DE134
		4	pMNEndo1
pMNEndo1DE14	mutagenesis		M13mp9Endo1 oligo-DE1,DE4
pMNEndo1DE23	ligation	1	pMNEndo1
		2, 3	pMNEndo1DE234
		4	pMNEndo1
pMNEndo1DE24	ligation	1	pKKEndo1[g]
		2, 3	M13mp9Endo1DE12
		4	pMNEndo1DE234
pMNEndo1DE34	mutagenesis		M13mp9Endo1 oligo-DE3,DE4
pMNEndo1DE123	ligation	1	M13mp9Endo1DE134
		2, 3	pMNEndo1DE234
		4	pMNEndo1
pMNEndo1DE124	ligation	1	M13mp9Endo1DE134
		2, 3	M13mp9Endo1DE12
		4	pMNEndo1DE234
pMNEndo1DE134	mutagenesis		M13mp9Endo1 oligo-DE1, DE3, DE4
pMNEndo1DE234	mutagenesis		M13mp9Endo1 oligo-DE2,DE3,DE4
pMNEndo1DE1234	mutagenesis (two rounds)		M13mp9Endo1 oligo-DE1,DE2,-DE3,DE4

[a] The names of the expression plasmids containing the mutant cDNAs and the methods of construction of each are summarized. [b] Construct: The name of the expression plasmid, incorporating the designation of the residues altered, e.g., pMNEndo1DE2 expresses a protein that has the acidic residue in the second domain changed to alanine. [c] Mutagenesis method: Either ligation of altered domains or direct mutagenesis of the acidic residues. [d] Substrates: The source of each domain supplied to form the final mutant expression plasmid is given. These include other expression plasmids, M13 constructs, or direct application of oligonucleotide-directed mutagenesis, as listed. [e] pMNEndo1 is the expression plasmid pET11d with the native bovine annexin IV cDNA. [f] M13mp9ENDo1DE*xy* is the phage M13mp9 with the annexin IV cDNA mutated at domains *x* and *y*. [g] pKKENdo1 is the expression plasmid pKK233-2 with the native bovine annexin IV cDNA (Nelson & Creutz, 1994).

mutant oligonucleotides to isolated phage DNA. Five of the fifteen possible acidic residue combination mutants were obtained in a single mutagenesis reaction and screen. A second round of mutagenesis was performed to obtain the DE1234 mutant. All of the mutations were confirmed by sequencing the corresponding regions.

Further mutants were then obtained by the ligation of annexin IV coding segments from selected mutants (Table 2). The presence of an *Xho*I (or *Ava*I) site preceding the fourth-domain aspartate and an *Ava*I site between the first-domain glutamate and second-domain aspartate enabled the ligation of two or three fragments to yield the desired full-length mutant constructs. Eight mutants not obtained by oligonucleotide-directed mutagenesis were constructed in this manner. The 15th possible mutant (DE1) was not obtained in this study.

The R4 mutant (Arg274 to Ser) was similarly obtained by site-directed mutagenesis of M13mp9Endo1 using the R4 oligonucleotide described in Table 1.

Expression of Constructs. *Nco*I/*Eco*RI-digested wild-type or mutant annexin IV inserts were subcloned into the T7 vector pET11d (Studier et al., 1990) to create pMNEndo1 and pMNEndo1DE*x* constructs [*x* = domain(s) where D/E→A mutation has been made] and the pMNEndo1R4 construct. *Escherichia coli* strain BL21DE3 was then transformed with purified plasmid DNA. Successful transformants were identified by (1) restriction digest analysis of minilysates and/or (2) a miniexpression screen consisting of the growth of 2 mL cultures induced with 0.4 mM IPTG, followed by SDS−PAGE and Western blot analysis of cell pellets containing 50−100 μg of total protein.

Recombinant Protein Purification. Purification of recombinant protein was conducted using reversible Ca^{2+}-dependent binding to purified bovine brain lipids, as previously described (Creutz et al., 1991, 1992; Nelson & Creutz, 1995). Briefly, cell cultures were induced with 0.4 mM IPTG for 3−4 h, and pelleted at 6500 rpm in a Sorvall GSA rotor. Cell pellets were suspended in buffer A (10 mM EGTA, 50 mM HEPES (pH 7.4), and 150 mM NaCl) and lysed by sonication 3 × 20 s with a probe sonicator. Soluble proteins in the supernatant were mixed with Ca^{2+} (12 mM final concentration) and purified bovine brain lipids. The annexin-coated lipid vesicles were pelleted at 184000*g* for 45−60 min. The vesicle pellet containing reversibly bound annexin constructs was then washed in calcium-containing buffers B^+ and B^- (50 mM HEPES (pH 7.4), 10 mM $CaCl_2$, ± 150 mM NaCl), followed by extraction with 10−12 mL of buffer D10 (50 mM HEPES (pH 7.4) and 10 mM EGTA) and centrifuged as before. The supernatant (E1) contained soluble, recombinant annexin IV. In some cases this pellet was reextracted with additional buffer D10 (E2 and E3). Protein in these extracts was then precipitated by the addition of ammonium sulfate (0.6 g/mL). Pellets obtained after ultracentrifugation at 53000*g* in an SW28 rotor were resuspended in 750 μL of aggregation buffer (50 mM HEPES (pH7.4), 240 mM sucrose, and 30 mM KCl) and chromatographed on an FPLC Superose 12 gel filtration column (Pharmacia) equilibrated with aggregation buffer.

Chromaffin Granule Binding and Aggregation Assays. The membrane aggregation assay was conducted as previously described (Creutz et al., 1978). Chromaffin granules were isolated by differential centrifugation from homogenates of adrenal medullary tissue (Pazoles & Pollard, 1978). Binding and aggregation data were accumulated from the same experiment: at time 0, aggregation buffer, protein, and granules were added to a disposable plastic cuvette and placed in a Beckman DU-70 spectrophotometer. Turbidity at a wavelength of 540 nm was monitored as an index of granule aggregation (Creutz et al., 1978). The initial absorbance was 0.14−0.16 OD. After 1.33 min, a calcium buffer consisting of 2.5 mM EGTA and various amounts of Ca^{2+} (as $CaCl_2$) was added, and turbidity changes were monitored for an additional 8.67 min. Each 10 min experimental trial consisted of one control sample (no annexin protein) and five experimental samples. After the granule aggregation assay was complete, the reaction mixture was centrifuged at 13000*g* for 6 min at 4 °C. The pellet was suspended in 100 μL of Laemmli buffer, and SDS−

Table 3: Nomenclature of the Annexin IV Acidic Residue Mutants

name	domains altered	residues changed to alanine[a]
DE2	2	Asp142
DE3	3	Glu226
DE4	4	Asp301
DE12	1, 2	Glu70, Asp142
DE13	1, 3	Glu70, Glu226
DE14	1, 4	Glu70, Asp301
DE23	2, 3	Asp142, Glu226
DE24	2, 4	Asp142, Asp301
DE34	3, 4	Glu226, Asp301
DE123	1, 2, 3	Glu70, Asp142, Glu226
DE124	1, 2, 4	Glu70, Asp142, Asp301
DE134	1, 3, 4	Glu70, Glu226, Asp301
DE234	2, 3, 4	Asp142, Glu226, Asp301
DE1234	1, 2, 3, 4	Glu70, Asp142, Glu226, Asp301

[a] Numbering of residues is from the N-terminal alanine as residue 1 (initiating methionine, residue 0) (Hamman et al., 1988).

PAGE analysis was performed (Laemmli, 1970). Scanning densitometry of Coomassie-stained 10% or 12.5% polyacrylamide gel regions corresponding to the appropriate molecular weights for annexin IV yielded relative density units that were used to calculate the fraction of maximum annexin IV binding.

Polyacrylamide Gel Characterization. Ruthenium red (Charuk et al., 1990) and Stains-All (Campbell et al., 1983) staining, Western blots (Creutz et al., 1987), and $^{45}Ca^{2+}$ overlays (Maruyama et al., 1984) were conducted as previously described.

RESULTS

Construction of a Partial Library of Annexin IV DE Mutants

To examine the significance of domain structure in annexin IV, point mutations were made in conserved residues thought to be critical for calcium-dependent binding to and aggregation of bovine chromaffin granules (Table 3). Each mutant construct consisted of one or more alterations of conserved acidic (aspartate or glutamate) residues located in the loop between helices 4 and 5 in the carboxy-terminal half of each domain (E70, D142, E226, and D301, responsible for forming the high-affinity, type II calcium-binding sites). The library included all possible combinations of these mutations, with the exception of the DE1 mutant, which was not obtained.

Nearly all mutant constructs were purified with yields of 4−5 mg/L of bacterial culture by calcium-dependent binding to lipid vesicles (see Materials and Methods). However, the DE1234 mutant protein, with all four acidic residues replaced by alanines, was not purified in comparable amounts (<200 μg/L). This construct was expressed to percentages of total bacterial protein comparable to wild-type and other DE mutants, as judged by SDS−PAGE analysis of induced whole-cell lysates. The low yield was expected if indeed the calcium-binding pockets involved in calcium-dependent binding to lipid membranes were partially inactivated by the mutations.

Since a small amount of the DE1234 mutant nonetheless was isolated by binding to phospholipid membranes, the mutant-binding pockets may retain a three-dimensional structure such that the remaining coordinating oxygens of this loop bind calcium with a low affinity. The 10 mM Ca^{2+} concentration present during purification may be sufficient for a suboptimal binding event. The aspartate or glutamate alteration to an alanine removes coordinating oxygen moieties, but does not provide steric hindrance that might have prevented calcium binding. Alternatively, some degree of calcium-dependent membrane binding may have been mediated by the type III calcium-binding sites, which are of lower affinity (Jost et al., 1994; see Discussion). In addition, some degree of protein self-association, independent of lipid binding (Zaks & Creutz, 1991), may have been induced by the high level of calcium used during the protein preparation, resulting in sedimentation of the protein with the lipid vesicles.

Recombinant mutant proteins were analyzed by SDS−PAGE and stained with Ponceau S, for total protein, with anti-annexin IV antiserum, with $^{45}Ca^{2+}$, and with ruthenium red (Figure 1). Panel A of Figure 1 shows the Ponceau S staining, demonstrating that the recombinant proteins have been purified to homogeneity. Panel D is a Western blot demonstrating that the constructs are all recognized by an annexin IV-specific polyclonal antibody (Creutz et al., 1987). The time for development of all of the bands in the Western blot was similar, suggesting that the major epitopes recognized by this antiserum were not altered. Panel C is a $^{45}Ca^{2+}$ overlay. From the 2-day exposure shown, it is clear that mutants with at least one intact domain bind some calcium, although far less than is seen with the classical calcium-binding protein, calsequestrin. Relative differences in the densities of the autoradiographs for most of the different mutants were variable, so that it was not possible to determine whether there were differences in calcium-binding affinity that correlated with alterations in particular domains. However, the DE1234 mutant appeared to consistently bind the lowest amount of calcium. Long exposure (>7 days) revealed only limited association of the radioligand with the DE1234 protein and also with the protein molecular weight standards, which likely represented low-affinity or nonspecific binding. Ruthenium red has been described as a sensitive dye that can be used to stain calcium-binding proteins, either in acrylamide gels or on nitrocellulose following transfer (panel B; Charuk et al., 1990). Under the reaction conditions used, ruthenium red stained all constructs. Consistent with studies of other annexins, all annexin IV constructs stain orange with the carbocyanin dye, Stains-All (data not shown; Campbell et al., 1983).

Further characterization of recombinant products was obtained by isoelectric focusing. For every acidic residue that was changed to an alanine, a negative charge was removed, which caused a shift in the position of the protein band. Figure 2 illustrates several isoelectric variants. Products focused into zones corresponding to 0 (wild type), 1 (DE2), 2 (DE12, DE13, DE24, DE34), or 3 (DE134) mutations. Within these zones, some of the mutants with the same number of mutations focused in slightly different positions (e.g., compare DE23 and DE12). Apparently, the exact location of the charge removed by mutation had additional subtle effects on the p*I* of the protein, either through disruption of charge−charge interactions or minor conformational changes unique to each domain. Locations of bands for other constructs, including DE1234, were also consistent with the number of mutated domains (data not shown).

Although sequence and charge alterations have been verified, it was imperative to confirm that the types of alteration made tended not to grossly disturb the overall

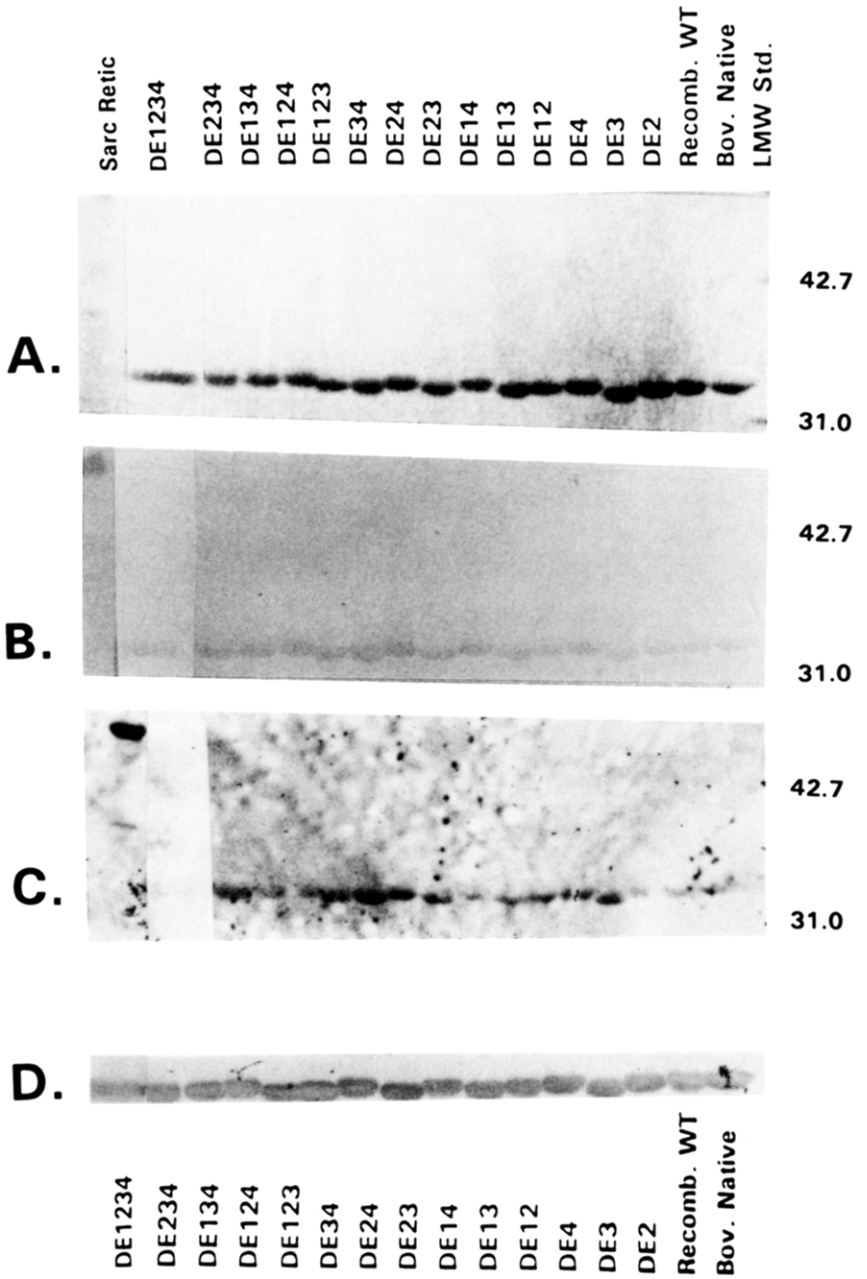

FIGURE 1: Staining properties of recombinant annexin IV constructs transferred to nitrocellulose. Proteins expressed by the DE mutant constructs were separated on 10% polyacrylamide gels and transferred to nitrocellulose. 10 μg of native bovine annexin IV, wild-type annexin IV, or various DE mutants or 5 μg of a rabbit muscle sarcoplasmic reticulum fraction containing calsequestrin was loaded in each lane. The top legend applies to panels A−C. The bottom legend applies to panel D. (A) Ponceau S stain: all proteins are stained. (B) Ruthenium red stain: all bands are faintly stained, including sarcoplasmic reticulum proteins (Sarc Retic). (C) $^{45}Ca^{2+}$ overlay (2-day exposure for all except DE1234, for which a 7-day exposure was used): native, wild-type, and DE mutants were moderately labeled. There was intense labeling of calsequestrin (Sarc Retic). (D) Western blot showing comparable recognition of native and mutant proteins.

three-dimensional structure of annexin IV. Circular dichroism analysis of protein isolated from bovine liver, recombinant wild-type protein, and the DE3 and DE124 mutants was conducted and revealed no detectable differences between these four proteins (Nelson & Creutz, 1995).

Chromaffin Granule Binding and Aggregating Activity of Mutant Annexins

The role of individual domains in the calcium-dependent membrane-binding and -aggregating activities of annexin IV was addressed by analyzing the activity of DE mutant constructs in *in vitro* assays. The low yield of the DE1234 annexin IV mutant obtained by reversible calcium-dependent binding to PS vesicles provided an indication that specific binding was either eliminated or required higher Ca^{2+} concentrations. Under the conditions of the assay, the D1234 mutant also failed to bind to or aggregate chromaffin granules. These observations suggest that the alterations made to the calcium-binding sites were sufficient to inactivate them, at least at the levels of calcium examined (<12.5 mM). Table 4 summarizes the membrane-binding and -aggregating activities of the other mutants in terms of the concentrations of calcium (EC 50%) that give a half-maximal response in the binding or aggregation assays. The 10 μg of protein used in all experiments, unless otherwise noted, is below the maximum binding capacity of the granules and is sufficient to give a maximum response in the aggregation assay. The numbers in Table 4 represent the averages of values obtained from 2−5 titrations with each protein. When titrations were repeated with similarly prepared materials, half-maxima

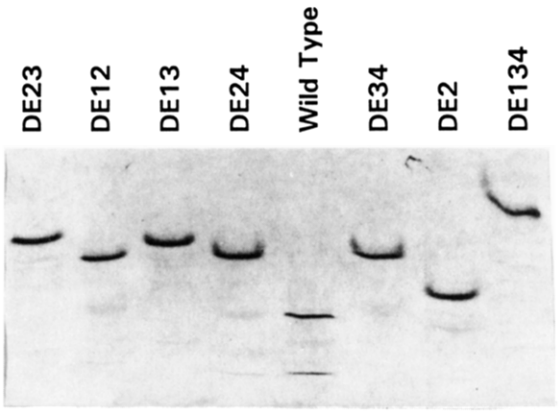

FIGURE 2: Isoelectric focusing analysis of native annexin IV and several DE mutants. The pH of the gel decreases from top (pH 6) to bottom (pH 4). DE mutants are shifted upward with respect to wild-type protein. Gel was stained with Coomassie Blue.

Table 4: Summary of the Calcium Dependence of Chromaffin Granule Binding and Aggregation by the DE Mutant Annexins IV[a]

construct	binding EC50 (M)	aggregation EC50 (M)
annexin IV	5×10^{-6}	1.4×10^{-4}
DE2	1.2×10^{-5}	2.7×10^{-4}
DE3	5×10^{-6}	6.2×10^{-5}
DE4	7.7×10^{-5}	2.4×10^{-4}
DE12	4.0×10^{-5}	3.2×10^{-4}
DE13	4.6×10^{-5}	6.5×10^{-5}
DE14	2.5×10^{-4}	4.5×10^{-4}
DE23	7×10^{-6}	1.2×10^{-4}
DE24	1.4×10^{-4}	1.2×10^{-3}
DE34	4.9×10^{-5}	5.1×10^{-4}
DE123	4.6×10^{-5}	2.5×10^{-4}
DE124	5.5×10^{-4}	4.1×10^{-3}
DE134	2.6×10^{-4}	6.0×10^{-4}
DE234	1.1×10^{-4}	2.3×10^{-3}

[a] The calcium concentrations (EC50) giving half-maximal binding or aggregation of chromaffin granules are given for wild-type (annexin IV) and the various DE mutant constructs.

occasionally varied up to 50%, with the greatest variability seen in the case of constructs requiring the highest levels of calcium for activity. Therefore, some of the values presented in the table differ slightly from the half-maxima apparent in the specific, representative titrations illustrated and discussed in the following.

By comparing the lack of activity of DE1234 with the activities of other mutants in the library, it is clear that a single domain is sufficient for binding to granule membranes. However, there was an up to 50% reduction in total DE134 and DE124 protein bound at a Ca^{2+} concentration of 12.5 mM, as judged by the visualization of SDS−PAGE gels. Saturation may not have been reached for these constructs, and thus the reported EC50's may be underestimates. Assays were not conducted in the presence of higher concentrations of Ca^{2+} (>12.5 mM) in order to avoid direct effects of calcium on chromaffin granule aggregation.

In general, an increase in the number of mutated domains led to an increase in the required calcium concentration for half-maximal binding. For example, while Ca^{2+} titration curves of similar overall shape were obtained for wild-type, DE4, DE14, and DE124 binding to lipid membranes, there was a shift to the right with each added mutation (Figure 3). A similar relationship was seen for a Ca^{2+} titration of granule aggregation mediated by these constructs (Figure 4). Similar trends were also observed with other progressions from single

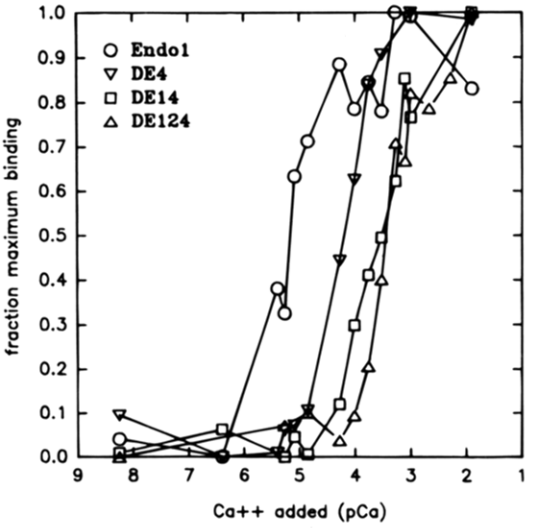

FIGURE 3: Effect of increasing the number of DE mutations on the calcium dependence of annexin IV binding to chromaffin granules. Samples containing 10 μg of wild-type (Endo1) or DE mutant protein, chromaffin granules, and varying concentrations of Ca^{2+} were incubated for 10 min at room temperature, subjected to SDS−PAGE, and then analyzed by densitometry.

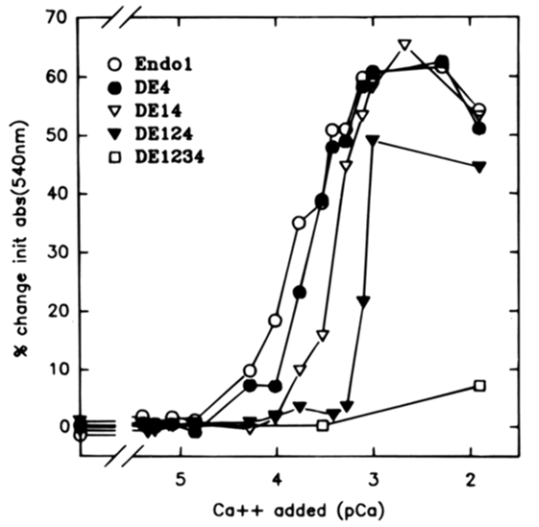

FIGURE 4: Effect of increasing the number of DE mutations on the calcium dependence of aggregation of chromaffin granules by annexin IV. Samples containing 10 μg of wild-type (Endo1) or DE mutant protein, chromaffin granules, and varying concentrations of Ca^{2+} were incubated for 7 min at room temperature. The percent change in absorbance, reflecting the aggregation of granules, is plotted as a function of calcium concentration.

to triple mutations for both binding and aggregation (Table 4). Note that constructs with a single DE mutation, in general, approximate wild-type annexin IV in Ca^{2+} dependence of binding and aggregation compared to double- and triple-mutant constructs.

Specific Roles of Individual Domains

Although an increase in the number of mutations led to increased calcium requirements for binding and aggregation activities, there were also obvious differences among mutants with the same number of mutations. By comparing DE123, DE124, DE134, and DE234 membrane binding and aggregation (Table 4), the DE123 mutant has Ca^{2+} titration half-maxima (4.6×10^{-5} M for binding and 2.5×10^{-4} M for aggregation) up to an order of magnitude less than the other three. In the case of double mutants, the range of half-

maxima also extends over an order of magnitude from 7×10^{-6} (DE23) to 2.5×10^{-4} M (DE14) for membrane binding and from 6.5×10^{-5} (DE13) to 1.2×10^{-3} M (DE24) for aggregation. These large ranges and the fact that four different mutants provided the extremes of the half-maxima are indications that different domains play different roles in these activities.

Domain 3. As in all other annexins, the third domain of annexin IV lacks the complete consensus sequence, GXGT-38 residues-E/D, that has been identified in the crystal structure of annexin V as a calcium-binding pocket in domains 1, 2, and 4 (Huber et al., 1990a,b). Although the acidic residue is present, a substitution for the initial glycine distorts the structure of the pocket. For this reason, it was first proposed that the pseudopocket might not bind calcium. However, additional crystallographic and spectroscopic data suggest that this domain, in annexin V, may bind calcium and undergo a conformational change at high concentrations of calcium (Concha et al., 1993; Sopkova et al., 1993; Burger et al., 1994; Meers & Mealy, 1993). This conformational change results in the expulsion of a tryptophan in the third domain from the body of the protein toward the putative position of the membrane, where it might be involved in membrane anchoring. However, there is virtually no difference in the EC50's for binding chromaffin granules between DE3 and wild-type annexin IV (Table 4 and Figure 5). The data thus suggest that the third domain does not participate directly in calcium-dependent binding to chromaffin granule membranes. This was further supported by pairing all DE3-containing mutants with the corresponding constructs containing other mutations. For example, DE23 vs DE2, DE123 vs DE12, and DE134 vs DE14 all reveal similar calcium-dependent binding and aggregation activities (Table 4 and Figure 5). Unexpectedly, removal of the acidic residue in the third domain alone slightly enhanced the calcium sensitivity of the protein in the aggregation assay when compared with the native protein (Table 4). It is also of note that the DE124 mutant, which has only the third domain unaltered, displayed weak binding and aggregation activities (Table 4) due to either the presence of the native third domain or the residual activity of the altered domains.

Domain 4. A list of mutants exhibiting severely defective membrane binding and aggregation includes DE14, DE24, DE124, DE134, and DE234 (as well as DE1234). All of these annexin IV derivatives contain mutated fourth-domain aspartate residues.

The fourth domain is the most conserved among annexins and is seen to bind Ca^{2+} in the annexin V structure (Barton et al., 1991; Huber et al., 1992). Ca^{2+} titration curves for both membrane binding and aggregation are shifted to the right for constructs containing the DE4 mutation. Figure 6 illustrates this for the DE4 mutant compared to wild type, with the largest shift occurring in calcium-dependent binding to granule membranes. Figure 6 also graphically displays the ratios of the EC50's for membrane binding and aggregation for constructs with or without DE4 mutations. Addition of the DE4 mutation in every case results in a dramatic increase in the EC50 for binding. Consistent increases of this magnitude were not seen with mutations of other domains. There is also an increase in the EC50 for granule aggregation in each case compared to wild type. However, these increases are less than that for membrane binding. This is apparent in the widened gap between the open (binding) and closed (aggregation) bars upon the addition of the DE4 mutation in Figure 6.

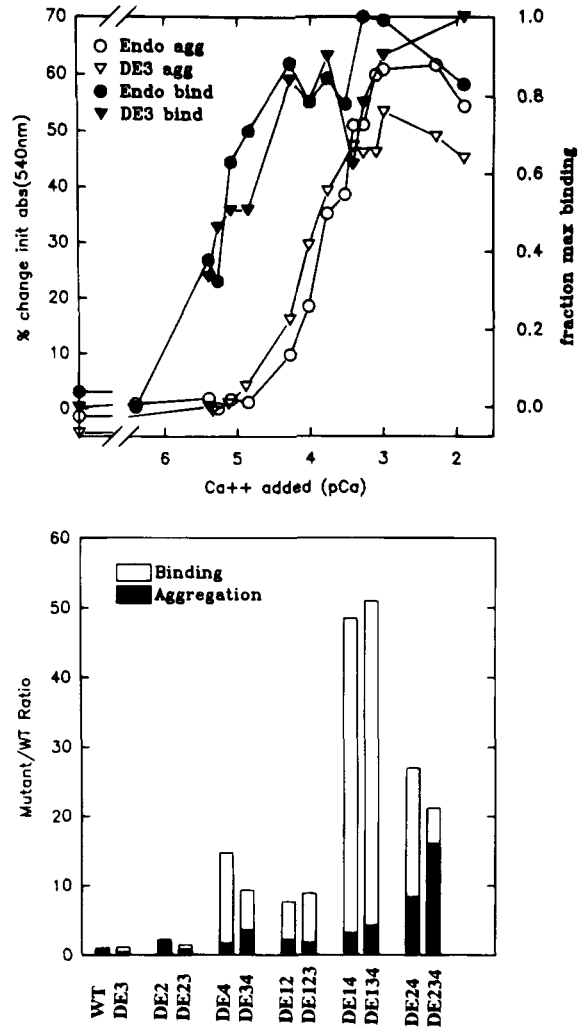

FIGURE 5: Effect of the DE3 mutation on the calcium dependence of annexin IV binding to and aggregation of chromaffin granules. (Top) Binding and aggregating activity were assayed as described in the legends to Figures 3 and 4, respectively: Endo agg, aggregation by wild-type protein; DE3 agg, aggregation by DE3 mutant; Endo bind, binding of wild-type protein; DE3 bind, binding of DE3 mutant. (Bottom) Comparison of the calcium sensitivities of mutant annexins IV with or without the DE3 mutation. Data are presented as the ratios of the EC50's for each mutant and the wild-type protein. The solid bars represent the aggregation data, and the open bars represent the binding data.

Also supporting a role for the fourth domain in the attachment to membranes is the fact that the DE4 mutant has a lower apparent affinity (calcium sensitivity) than even the DE123 mutant, which presumably has only one functional domain (the fourth domain).

Domain 1. Change of the first-domain acidic residue (glutamate) to an alanine also results in rightward shifts of Ca^{2+} titration curves for binding (Figure 7). Changes in the calcium requirements for aggregation above that for the wild type are negligible, except for the DE24/DE124 pair (Figure 7 (bottom), similar closed bars and widened open/closed bar gap). Alteration of the first-domain calcium-binding pocket, therefore, also has more of an effect on binding to membranes than on membrane aggregation. This pocket also might serve as a substitute in binding to membranes in the presence of a mutation in the fourth domain. This is shown by the DE234 mutant, which does exhibit membrane-binding (EC50 = 1.1×10^{-4} M) and -aggregating (EC50 = $2.3 \times$

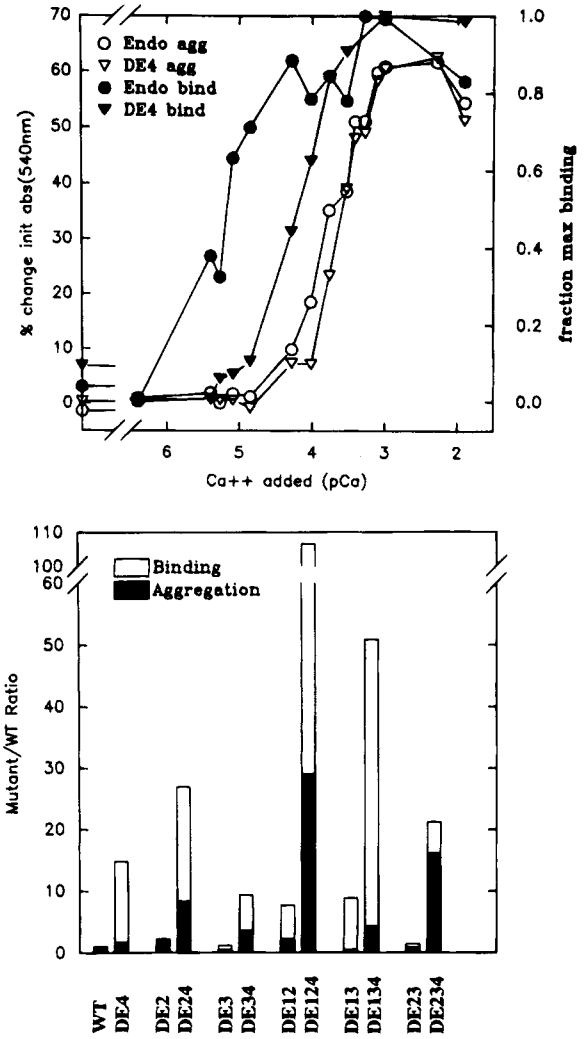

FIGURE 6: Effect of the DE4 mutation on the calcium dependence of annexin IV binding to and aggregation of chromaffin granules. (Top) Binding and aggregating activity were assayed as described in the legends to Figures 3 and 4, respectively: Endo agg, aggregation by wild-type protein (from Figure 5, top); DE4 agg, aggregation by DE4 mutant; Endo bind, binding of wild-type protein (from Figure 5, top); DE4 bind, binding of DE4 mutant. (Bottom) Comparison of the calcium sensitivities of mutant annexins IV with or without the DE4 mutation. Data are presented as the ratios of the EC50's for each mutant and the wild-type protein. The solid bars represent the aggregation data, and the open bars represent the binding data.

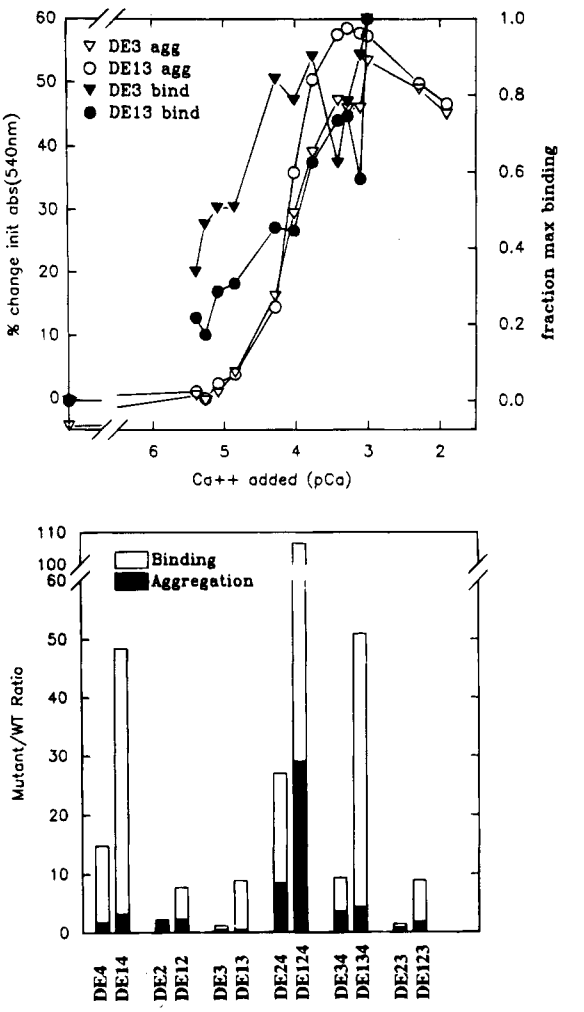

FIGURE 7: Effect of the DE1 mutation on the calcium dependence of annexin IV binding to and aggregation of chromaffin granules. (Top) Binding and aggregating activity were assayed as described in the legends to Figures 3 and 4, respectively: DE3 agg, aggregation by the DE3 mutant (from Figure 5, top); DE13 agg, aggregation by the DE13 mutant; DE3 bind, binding of the DE3 mutant (from Figure 5, top); DE13 bind, binding of the DE13 mutant. (Bottom) Comparison of the calcium sensitivities of mutant annexins IV with or without the DE1 mutation. Data are presented as the ratios of the EC50's for each mutant and the wild-type protein. The solid bars represent the aggregation data, and the open bars represent the binding data.

10^{-3} M) activities, albeit with increased Ca^{2+} requirements compared to the D123 mutant (Table 4).

Domain 2. In direct contrast to the DE1 and DE4 mutations, second-domain acidic residue (aspartate) mutants exhibit relatively small changes in calcium-dependent binding, but they show increases in calcium requirements for aggregation. Figure 8 (top) is a comparison of the DE234 and DE34 mutants, and Figure 8 (bottom) compares mutants with and without the DE2 mutation. The largest effect is seen in the calcium dependence of granule aggregation. This is shown by a greater rightward shift of the DE234 Ca^{2+} titration curve for aggregation with respect to DE34 than the titration curve for granule binding (Figure 8, top). A predominant effect on granule aggregation calcium requirements was demonstrated by a greater increase in the mutant/wild type ratio for constructs with the DE2 mutation compared to constructs without the DE2 mutation. This is seen in Figure 8 (bottom) as an increased proportion of the open bar for binding being shadowed by the solid bar for aggregation (or a narrowed open/closed bar gap) for mutants with the DE2 mutation.

Characterization of the R4 (Arg274 to Ser) Mutant

The structural integrity of the R4 mutant was analyzed by circular dichroism spectroscopy and found to be indistinguishable from recombinant protein of native sequence or protein isolated from tissue (Nelson & Creutz, 1995). This result suggests that there are probably no gross structural changes in the three-dimensional structure of this mutant.

A Ca^{2+} titration of the binding of the R4 mutant to membranes was performed (Figure 9, top). Half-maximal binding occurred at an EC50 of approximately 11 μM Ca^{2+}, which is about twice the calcium requirement exhibited by wild-type annexin IV (5 μM Ca^{2+}). These results were reproduced with three additional titrations for each construct. The Ca^{2+} requirement for chromaffin granule aggregation by this mutant exceeded that of wild-type annexin IV by 3-fold (Figure 9, bottom). Half-maximal aggregation oc-

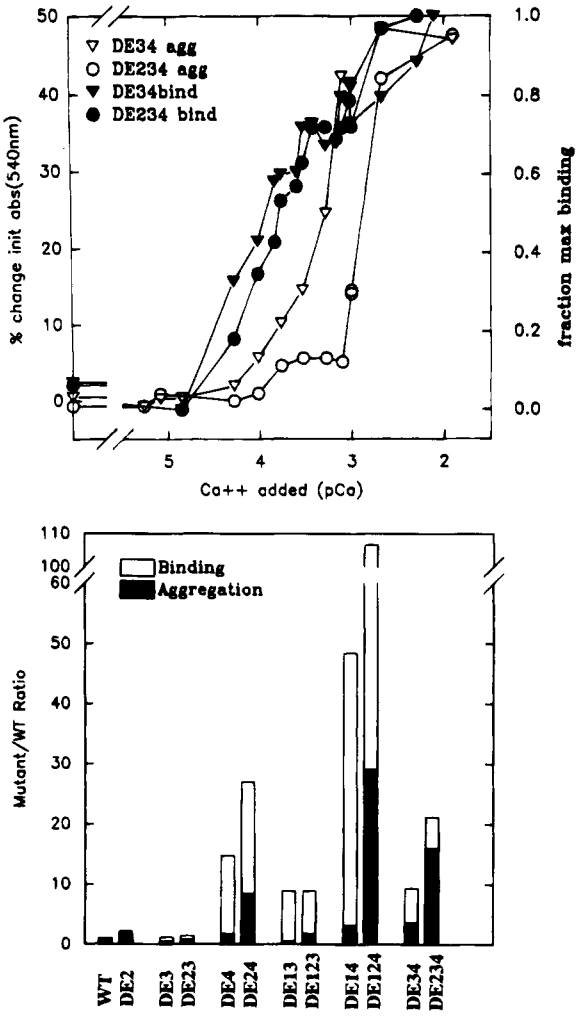

FIGURE 8: Effect of the DE2 mutation on the calcium dependence of annexin IV binding to and aggregation of chromaffin granules. (Top) Binding and aggregating activity were assayed as described in the legends to Figures 3 and 4, respectively: DE34 agg, aggregation by the DE34 mutant; DE234 agg, aggregation by the DE234 mutant; DE34 bind, binding of the DE34 mutant; DE234 bind, binding of the DE234 mutant. (Bottom) Comparison of the calcium sensitivities of mutant annexins IV with or without the DE2 mutation. Data are presented as the ratios of the EC50's for each mutant and the wild-type protein. The solid bars represent the aggregation data, and the open bars represent the binding data.

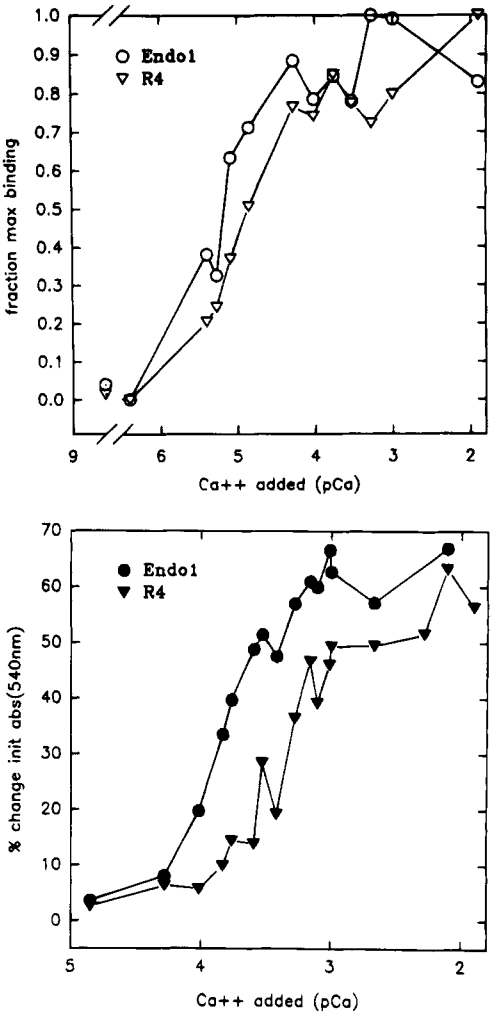

FIGURE 9: Effect of the R4 (Arg274 to Ser) mutation on the calcium dependence of annexin IV binding to and aggregation of chromaffin granules. (Top) Binding to chromaffin granules: ○, wild-type annexin IV (Endo1); ▽, R4 mutant. Samples containing 10 μg of R4 or wild-type protein, chromaffin granules, and varying concentrations of Ca^{2+} were incubated for 7 min at room temperature, subjected to SDS−PAGE, and then analyzed by densitometry. (Bottom) Aggregation of chromaffin granules: ●, wild-type annexin IV (Endo1); ▼, R4 mutant. 10 μg of R4 or wild-type protein, chromaffin granules, and varying concentrations of Ca^{2+} were incubated for 7 min at room temperature. The percent change in absorbance at 540 nm as a function of Ca^{2+} concentration is shown.

curred at Ca^{2+} concentrations of 4.8×10^{-4} M for the R4 mutant and 1.4×10^{-4} M for wild-type annexin IV.

Since the R4 mutant requires more Ca^{2+} than wild-type annexin IV to promote granule aggregation, the inhibition of wild-type annexin IV-mediated aggregation by the R4 mutant was investigated. Figure 10 reveals that the R4 mutant can inhibit normal aggregation in a dose-dependent manner at an intermediate concentration of Ca^{2+}, 250 μM. This concentration of Ca^{2+} falls significantly below the half-maximum for R4-induced aggregation, but above that for wild-type annexin IV. Inhibition is not complete at this concentration because it is above the threshold level for the intrinsic aggregating activity of the R4 mutant. Dose-dependent inhibition was also observed at all other calcium concentrations tested (250, 300, 350, and 400 μM) (data not shown). The inhibitory activity of the R4 mutant was destroyed by heating at 100 °C for 10 min, suggesting that the activity depends on the structure of the mutant protein.

The nature of the inhibition was then examined by varying the sequence of reaction substrate additions (Figure 11, Table 5). If the mechanism of inhibition were merely the occupation of membrane-binding sites, it would be expected that preadsorption of wild-type annexin IV to granules prior to the addition of the R4 mutant would preclude the inhibition, since the exchange rate for bound annexins in the presence of calcium is immeasurably slow on the time scale of these experiments (Junker & Creutz, 1994). Likewise, preadsorption of the R4 mutant should completely the inhibit wild-type-aggregating activity to the level of the membrane-aggregating activity of R4.

The experiment is made possible by the difference between the binding and aggregation calcium dependencies, so that protein can be bound at one level of calcium and aggregation assessed at a higher level of calcium. For reference, the aggregating activity of R4 alone at a total Ca^{2+} concentration of 300 μM resulted in a 10% change in initial absorbance (Figure 9). It is apparent that, regardless of the order of addition, the aggregation of chromaffin granules in the presence of both proteins occurs at a level very near the

Table 5: Experimental Protocol To Detect Inhibition of Chromaffin Granule Aggregation by the R4 Mutant[a]

trace	$t = 0$ min	$t = 2$ min	$t = 4$ min	$t = 6$ min
A	buffer, 585 μL	Ca^{2+}, 50 μM	buffer, 15 μL	Ca^{2+}, 250 μM final, 300 μM
B	buffer, 563 μL wt, 10 μg, 22 μL	Ca^{2+}, 50 μM	buffer, 15 μL	Ca^{2+}, 250 μM final, 300 μM
C	buffer, 563 μL wt, 10 μg, 22 μL	Ca^{2+}, 50 μM	R4, 10 μg, 15 μL	Ca^{2+}, 250 μM final, 300 μM
D	buffer, 578 μL	Ca^{2+}, 250 μM	buffer, 22 μL	Ca^{2+}, 50 μM final, 300 μM
E	buffer, 578 μL	Ca^{2+}, 250 μM	wt, 10 μg, 22 μL	Ca^{2+}, 50 μM final, 300 μM
F	buffer, 563 μL R4, 10 μg, 15 μL	Ca^{2+}, 250 μM	wt, 10 μg, 15 μL	Ca^{2+}, 50 μM final, 300 μM

[a] Table summarizes the order of addition of components to the aggregation assay cuvettes (A–F). Optical density traces from the experiment are shown in Figure 11. At the indicated times (0, 2, 4, and 6 min), aggregation buffer, calcium, wild-type protein (wt), or R4 mutant protein (R4) is added.

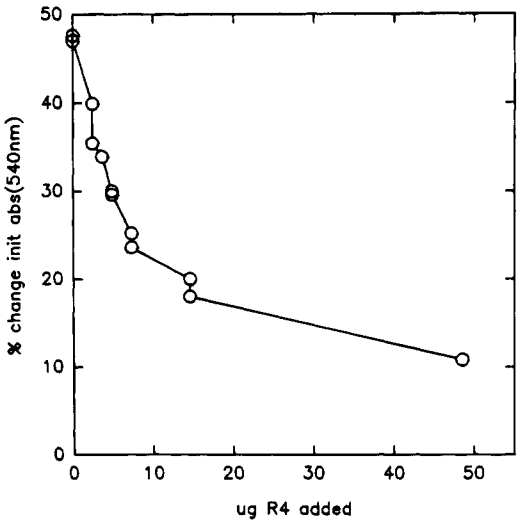

FIGURE 10: R4 (Arg274 to Ser) mutant inhibition of the aggregation of chromaffin granules by wild-type annexin IV. Samples containing 10 μg of recombinant wild-type annexin IV, chromaffin granules, and varying amounts of R4 were incubated at room temperature for 7 min in the presence of 250 μM Ca^{2+}.

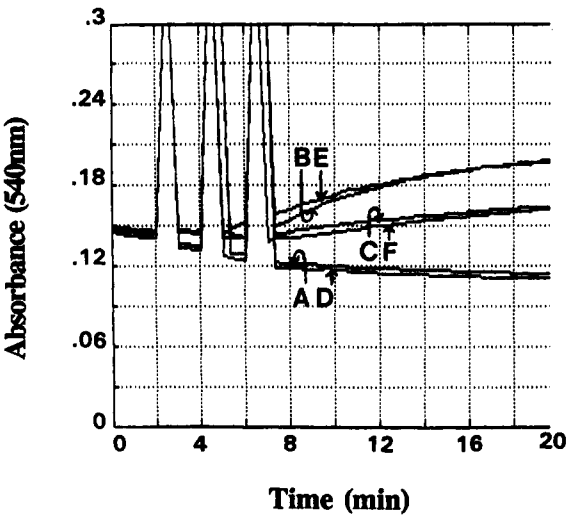

FIGURE 11: Inhibition of granule aggregation by the R4 (Arg274 to Ser) mutant is independent of the order of addition of wild-type or mutant annexin IV (see Table 5 for the composition of each reaction (A–F). Large deflections at 2, 4, and 6 min represent opening of the spectrophotometer chamber for the addition of substrates. A and D: No protein. B and E: Wild type alone. C and F: R4 was added after (C) or before (F) wild type.

intrinsic R4-aggregating activity. This is in agreement with the inhibition data obtained following the simultaneous addition of the two proteins presented earlier (Figure 10).

DISCUSSION

In this study, two characteristics of the annexins, their monovalent (membrane-binding) and bivalent (membrane-aggregating) activities, have been examined for each member of the partial library of structural mutants. Both of these activities were influenced by alterations of domains 1, 2, and 4, all of which contain a canonical sequence motif associated with calcium binding in the crystal structures of annexins (Huber et al., 1992). Neither activity was influenced significantly at low calcium concentrations by alteration of the 3rd domain, in spite of the reported ability of this domain in annexin V to bind calcium and undergo a conformational change associated with the exposure of a hydrophobic residue in the direction of the lipid membrane (Concha et al., 1993; Sopkova et al., 1993; Burger et al., 1994).

The alterations of domains 1 and 4 had a greater impact on the membrane-binding activity of annexin IV at low calcium concentrations than on the bivalent (membrane-aggregating) activity of the protein. These domains may be particularly important in interactions with membranes at low calcium levels. However, alteration of the second domain had a greater effect on the membrane-aggregating activity of annexin protein than on the membrane-binding activity. Possibly, in these experiments membrane binding was mediated by the unmodified first or fourth domains, while aggregation at higher levels of calcium required the cooperative action of the second domain. It is unknown how annexin molecules on a single membrane, or on both membranes, might cooperate to drive membrane aggregation. However, it appears the second domain may play an important role in such cooperative interactions.

A recent analysis of electron microscopic images of annexin V bound to lipid monolayers suggests that all four domains participate in membrane attachment (Voges et al., 1994). Apparently, as a consequence of attachment to the membrane, there is a conformational change in the molecule from the state seen in three-dimensional crystal structures determined in the absence of membranes. Domains 2 and 3 shift so that the third domain is moved closer to the membrane and the second domain is bent away from the membrane. As indicated by the bulk exclusion of negative stain, domain 2 appears farthest from the membrane (Voges et al., 1994), and in our study with annexin IV, the

homologous domain seemed less important than domains 1 and 4 for promoting membrane attachment at low levels of calcium. However, domain 3 was found to be closer to the membrane (Voges et al., 1994), yet mutation of this domain in annexin IV had little influence on the membrane attachment seen in our study. The EM image possibly represents a conformation that annexin V (and possibly annexin IV) adopts after attachment is promoted by other domains.

It might have been anticipated that as individual domains in the annexin IV core were inactivated by mutagenesis the binding of the protein to membranes or the aggregation of membranes would display lower degrees of cooperativity with respect to calcium. However, the calcium titration curves remained steep for the mutants (e.g., Figures 3, 4, 6, and 8). This apparent cooperativity therefore might be due to interactions between annexin molecules as they self-associate on the membrane surface (Zaks & Creutz, 1991), rather than between calcium-binding sites within individual molecules.

The arginine residues present at the end of the endonexin fold motif in each domain were an attractive target for mutagenesis because of their high degree of evolutionary conservation (Barton et al., 1991). However, crystallographic data now suggest that these residues may be important for maintaining overall protein structure through salt bridges, and they may not be directly involved in membrane or calcium binding (Huber et al., 1992). This structural role for these residues may explain our failure to obtain soluble mutant proteins with alterations in three of these residues. However, the one mutant obtained, the R4 (Arg274 to Ser) mutant protein, was soluble and of largely unaltered structure as determined by circular dichroism. In annexin V, the corresponding arginine forms an intradomain salt bridge with a nearly adjacent residue (D280) near the hydrophilic channel in the center of the protein (Huber et al., 1992). Possibly this mutant is more stable than some of the others since, in the pore region, there are more charged residues that may take part in stabilizing interactions, whereas this may not be the case for the surface salt bridges. In annexin V, salt bridges formed in the pore region by Glu112 as well as by Glu95 could be disrupted by mutagenesis, yielding soluble proteins (Demange et al., 1994; Burger et al., 1994). However, the arginine in the second domain that we altered is also located in the pore region, but mutation of this residue failed to yield a soluble protein. The arginine in the second domain may be in a microenvironment that does not provide suitable supporting interactions after mutagenesis. The effects of mutations on the aggregation of recombinant proteins produced in bacteria have recently been reviewed (Wetzel, 1994).

The R4 mutant protein displayed altered calcium sensitivities in both types of activity assays. Therefore, this sequence alteration may be translated into structural or flexibility changes in one or more of the calcium-binding loops. The ability of the R4 mutant to inhibit the wild-type protein appears to include mechanisms that extend beyond simple competition for membrane-binding sites, since prior binding of the wild-type protein to the membrane did not block the inhibition. The R4 protein might act as an inhibitor by associating with membrane-bound wild-type protein and blocking the protein−protein associations necessary for membrane aggregation.

Several other recent studies have employed the strategy of mutagenesis to define the functional roles of annexin structural features. Studies of the N-terminal domains of annexins II and XI have established the role of these domains in providing the binding sites for small protein ligands (Becker et al., 1990; Watanabe et al., 1993). Formation of a chimeric protein composed of the N-terminus of annexin I and the core of annexin V revealed that the N-terminus of annexin I plays an essential role in membrane aggregation (Andree et al., 1993). Mutagenesis of the phosphorylation sites in the annexin I N-terminus indicated that this role in aggregation may be regulated by phosphorylation (Wang & Creutz, 1994). Mutagenesis of residues in the third domain of annexin V that had been postulated to play a role in the inhibition of blood coagulation revealed that these residues are not directly involved in calcium or lipid binding (Tait & Smith, 1991). Alteration of a residue seen to form a salt bridge in the apparent hydrophilic channel in the center of annexin V results in changes in the ion selectivity of channels formed by this protein on synthetic lipid bilayers (Berendes et al., 1993).

After crystallographic data indicated the importance of certain acidic residues of annexin V in binding calcium, homologous residues were targeted for mutagenesis in annexin II (Jost et al., 1992, 1994). In general, it appears that the 70-amino acid annexin repeat has the potential to form two types of calcium-binding site, which have been referred to as type II and type III sites (Weng et al., 1993). The type II sites involve the consensus sequence GXGT-38 residues-D/E that begins in the highly conserved endonexin fold motif. Mutagenesis demonstrates that these sites have the highest affinity for calcium (Jost et al., 1992, 1994), and they are occupied in repeats 1, 2, and 4 in crystals of annexin V (Huber et al., 1992) and in repeats 2, 3, and 4 in crystals of annexin I (Weng et al., 1993). In addition, a type II site in domain 3 of annexin V can be occupied at high calcium concentrations, coincident with a major conformational change (Concha et al., 1993; Sopkova et al., 1993; Burger et al., 1994). The type III sites involve residues in the C-terminal portion of the 70-amino acid repeat, near the D/E residues of the type II site. These sites show various degrees of occupancy in crystals of annexins I and II (Huber et al., 1992; Weng et al., 1993). Only alteration of the type II sites was found to influence the calcium- and lipid-binding properties of annexin II at low calcium levels (Jost et al., 1992, 1994). Similar to our findings, an increase in the number of mutated type II sites progressively decreased the calcium sensitivity of annexin II in a lipid-binding assay. Furthermore, alteration of the type II sites, but not the type III sites, led to alterations in the intracellular distribution of annexin II (Jost et al., 1994). It is therefore a plausible hypothesis that the type II sites are the most important for the physiological, intracellular activities of the annexins.

Our study has focused exclusively on the type II sites of annexin IV. The D/E residues that were changed to alanines are the cap residues that complete these calcium-binding pockets (Huber et al., 1992). However, because of the high calcium concentrations to which some of the titrations were extended, it is possible that the type III sites contributed to some of the residual activities seen in these mutants.

Trave and colleagues (1994) mutated a conserved lysine residue in the endonexin fold in the second domain of annexin I into a glutamate. Crystallographic data previously had led to the speculation that this basic residue might interact with the negative charge of a phospholipid headgroup when the annexin binds to a membrane. The alteration

resulted in a change in the lipid specificity of annexin I, as the mutant protein showed better aggregation of mixed phosphatidylserine and phosphatidylethanolamine vesicles than that seen with the wild-type protein, which preferred pure phosphatidylserine vesicles. This result emphasizes the possibility that there may be differences in the lipid specificities of the different 70-amino acid domains. Our study has utilized a natural membrane with mixed lipid composition. However, the availability of the partial library of DE mutants created in this study may allow a systematic evaluation to now be made of the specificities of the four annexin domains for membranes of defined lipid compositions.

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