Structural Determinant of the Vesicle Aggregation Activity of Annexin I†

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ABSTRACT: Some annexins, including annexins I, II, IV, and VII, can promote membrane aggregation. To identify amino acids involved in annexin I-mediated membrane aggregation, we generated truncated mutants of human annexin I lacking various parts of the amino terminus. The in vitro vesicle binding and aggregation activities of these mutants indicated that both the amino-terminal region of annexin I spanning residues 26−29 and the carboxy-terminal core are involved in membrane aggregation. This notion was further supported by the finding that a chimera composed of residues 24−35 of annexin I and the core of annexin V has vesicle aggregation activity that is significantly higher than that of annexin V but lower than that of annexin I. Further site-specific mutations in the amino-terminal region of annexin I indicated that Lys-26 and Lys-29 are essential for its membrane aggregation activity. The comparison of tryptic digest patterns of free and vesicle-bound wild type and K29E mutant suggests that a primary role of Lys-26 and Lys-29 is to induce and stabilize an active conformation of annexin I for vesicle aggregation.

Annexins are a family of cellular proteins that reversibly bind membranes containing anionic phospholipids in a Ca^{2+}-dependent manner (*1*−*3*). Some annexins, including annexin I, II, IV, and VII, can promote membrane aggregation (*1*). The mechanism of membrane aggregation by annexins and the structural determinants of the membrane aggregation activity of these annexins are not fully understood. Two topological models have been suggested to account for the membrane aggregation by annexin I and other annexins. The first model envisages the simultaneous interaction of an annexin molecule (or a single layer of membrane-bound annexin molecules) with two opposing membranes (*4*−*7*), whereas the second model assumes the involvement of protein−protein interactions of annexin molecules adsorbed to the opposing membranes (*4*, *5*, *8*). Both models thus postulate the presence of two independent interaction sites on the annexin I molecule. Annexins contain a conserved carboxy-terminal core region consisting of four homologous domains (I−IV) and a variable amino-terminal region (*2*). X-ray crystallographic analyses (*9*−*13*) and mutagenesis studies (*14*−*17*) have shown that the site responsible for the initial membrane binding of annexins is composed of Ca^{2+}-binding sites located in the core domains of annexins. Calcium ions bound to these sites act as bridges connecting the protein with anionic lipid headgroups (*13*). The identity of the secondary interaction site has not been determined largely because of the lack of high-resolution structural information for membrane-associated annexins. The amino-terminal region of annexin I has been implicated in the secondary interactions (*7*, *18*). Human annexin I has 41 residues in the amino-terminal region, including four potential phosphorylation sites (Tyr-21, Thr-24, Ser-27, and Ser-28) (see Figure 1) (*2*, *19*, *20*). The in vitro phosphorylation of these residues reduces the membrane aggregation activity of annexin I to varying degrees (*19*, *21*), suggesting the involvement of this region in membrane aggregation and the potential regulation of physiological activities of annexin I by phosphorylation. Also, several truncated forms of annexin I lacking the first 12, 26, and 29 amino-terminal residues were shown to have altered membrane aggregation properties (*18*, *22*). Finally, different chimeric constructs of annexin I and annexin V were generated to identify the region of annexin I responsible for its membrane-aggregating activity (*7*, *23*). These studies have not, however, clearly identified the residues essential for the membrane aggregation activity of annexin I. Furthermore, the potential contribution of the conserved carboxy-terminal core of annexin I to its membrane-aggregation activity has not been fully investigated. To systematically assess the contributions of the core and the amino-terminal region of annexin I to the membrane aggregation activity and to identify the essential residues for the activity, we performed extensive structure−function analysis of annexin I. Results indicate that the core of annexin I has considerable membrane aggregation activity, which is greatly augmented by the amino-terminal residues 24−41.

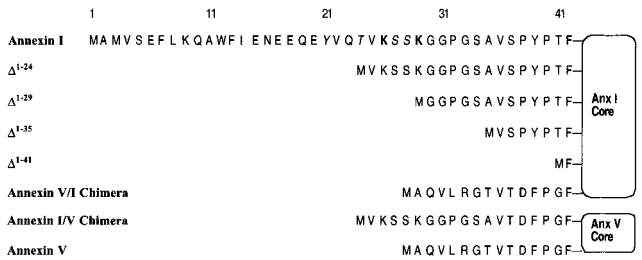

FIGURE 1: Amino acid sequences of amino-terminal regions of human annexin I, its truncated mutants, human annexin V, and chimeras. Four potential phosphorylation sites and mutated residues of wild-type annexin I are shown in italic and boldface characters, respectively. The sequence alignment is based on the method of Raynal and Pollard (*2*).

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In particular, Lys-26 and Lys-29 in this region are shown to play an important role in membrane aggregation, presumably by inducing and stabilizing the active conformation of annexin I for membrane aggregation.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS),[1] 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. Restriction endonucleases and enzymes for molecular biology were obtained from either Boehringer Mannheim (Indianapolis, IN) or New England Biolabs (Beverly, MA). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and used without further purification. EGTA and $CaCl_2$ were from Fluka (Ronkonkoma, NY) and Aldrich (Milwaukee, WI), respectively.

Preparation of Site-Directed and Truncated Mutants. cDNA of human annexin I, which was previously cloned into pET21d vector by use of *Nco*I and *Hin*dIII (pET-AnxI) (*17*), contains another *Hin*dIII site located at positions 841−846. This site was removed by introducing a silent mutation to Lys-241 (AA*G* to AA*A*) to avoid complications during further gene manipulations. All point mutations were introduced directly to the resulting pET-AnxI′ plasmid with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following manufacturer's instructions. Truncated mutants lacking amino-terminal 24, 29, 35 and 41 residues (Δ^{1-24}, Δ^{1-29}, Δ^{1-35}, and Δ^{1-41}), respectively, were produced by polymerase chain reaction (PCR) with *Pfu* DNA polymerase (Stratagene) with a 5′-end primer introducing an *Nco*I site. This site introduced an amino-terminal Met and also limited our choice of truncated constructs to the ones starting with Gly, Ala, Val, Asp, or Glu, whose codons begin with G. For Δ^{1-41}, whose amino-terminal residue is Phe, site-directed mutagenesis was performed to reintroduce Phe after the truncation of the amino-terminal 41 residues. Annexin I/V chimera was produced by PCR of annexin V cDNA with a 5′-end primer introducing an *Nco*I site and the codons for residues 24−35 of annexin I and a 3′-end primer introducing a *Hin*dIII site. PCR products were purified chromatographically on QIAquick spin columns (Qiagen) and digested with *Nco*I and *Hin*dIII, and resulting fragments were then subcloned into the pET21d vector (Novagen). Similarly, the annexin V/I chimera was produced by PCR of annexin I cDNA with a 5′-end primer introducing an *Nco*I site and the codons for residues 1−14 of annexin V and a 3′-end primer introducing a *Hin*dIII site. DNA sequences of constructs were verified with a Sequenase 2.0 kit (Amersham−Pharmacia).

Expression and Purification of Protein. The expression and purification of annexin mutants was performed as described previously (*17*) with minor modifications. For truncated mutants, 50 mM Tris-HCl buffer, pH 8.0, containing 1 mM EGTA was used for the equilibration and elution of a Q-Sepharose fast flow column (Amersham−Pharmacia). For K26E, K29E, and K26/29E mutants, proteins were eluted with a linear gradient of 0−0.5 M NaCl in the same buffer. The annexin V/I chimera was purified as described for annexin I (*17*). Annexin V and the annexin I/V chimera were purified as described previously (*24*).

Vesicle Aggregation and Binding Assays. Assays were performed for wild-type annexin I and mutants as described previously (*17*). All Ca^{2+} buffers were prepared in 10 mM HEPES−KOH, 0.1 M KCl, and 1 mM EGTA, pH 7.0, according to the method of Bers (*25*). Vesicle aggregation assays were carried out in a quartz cuvette with 1 cm light path using 2 mL of appropriate buffers preequilibrated for ca. 1 min with phospholipid vesicles (0.2 μmol) prior to protein addition (0−6 nmol). Turbidity changes were followed continuously at 450 nm on a Lambda 6 spectrophotometer (Perkin-Elmer). Initial background absorbance of vesicles was ca. 0.02 absorbance unit, and maximal turbidity signals, which were achieved within first 3 min, were typically 10−20 times higher than the background value. The maximal turbidity signals were plotted as a function of protein and Ca^{2+} concentrations. To determine the amount of protein bound to vesicles, the mixtures were centrifuged at 100000*g* at 25 °C for 15 min in a Sorvall RCM120EX microultracentrifuge immediately after turbidity measurements. Supernatants and pellets were separated and pellets were redissolved in 15 μL of 10 mM HEPES−KOH buffer, pH 7.0, containing 100 mM KCl and 1 mM EGTA. These samples were stored on ice until they were used for SDS−polyacrylamide gel electrophoresis. For electrophoresis, 1.5 mm, 10-well 9% polyacrylamide gels (Novex) that could accommodate up to 50 mL of sample were used. The amount of protein in each band was quantified with an IS-1000 digital imaging system (Key Scientific, Mt. Prospect, IL). To convert the protein band density to protein concentration, a standard curve was constructed from density values of varying amounts of annexin I samples (0.5−10 μg).

Tryptic Digest. Hydrolytic cleavage of annexin I wild type and mutants by trypsin was performed at room temperature in 500 μL of 10 mM HEPES−KOH, pH 7.0, containing 0.1 M KCl, 0.1 mM EDTA, and 200 μg of protein (5.3 nmol). For the tryptic digest of vesicle-bound annexin I and mutants, 1 mM Ca^{2+} and 0.1 mM POPC/POPE/POPS (2:5:2 in mole ratio) vesicles were added to the reaction mixture. To reaction mixtures was added TPCK-treated trypsin (Sigma) to a final concentration of 0.4 μg/mL. Aliquots (25 μL) were taken at regular intervals, mixed with 15 μL of the gel loading buffer [0.125 M Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% SDS, and 0.005% bromophenol blue], boiled for 5 min, and analyzed on 9% polyacrylamide gels.

RESULTS

Amino-Terminal Region versus Carboxy-Terminal Core. To determine the relative contribution of the amino-terminal region and the carboxy-terminal core to the vesicle aggregation activity of annexin I, we first created a truncated mutant lacking the entire amino-terminal region (Δ^{1-41}; see Figure 1) and compared its vesicle binding and aggregation activities with those of wild-type annexin I. We also measured

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PCR, polymerase chain reaction; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; SDS, sodium dodecyl sulfate.

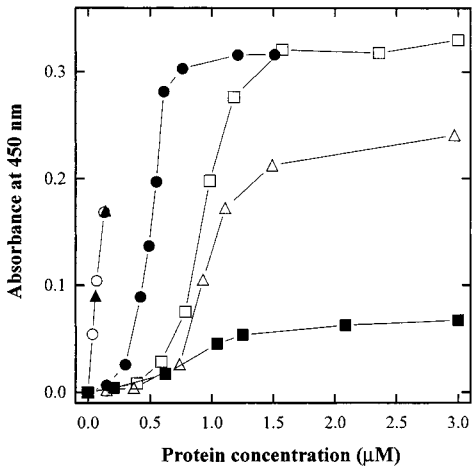

FIGURE 2: Aggregation of phospholipid vesicles by annexin I (○), Δ^{1-41} (●), Δ^{1-35} (□), Δ^{1-29} (△), Δ^{1-24} (▲), and annexin V (■) as a function of protein concentration. The aggregation was initiated by adding protein to the assay buffer (10 mM HEPES−KOH and 0.1 μM KCl, pH 7.0) containing 0.1 mM POPC/POPE/POPS (2:5:2 in mole ratio) vesicles and 1 mM Ca^{2+}. Vesicle aggregation was followed by continuously monitoring turbidity changes at 450 nm. For Δ^{1-24}, 0.1 mM Ca^{2+} was used because Ca^{2+} at higher concentrations was inhibitory for this mutant (see Figure 4).

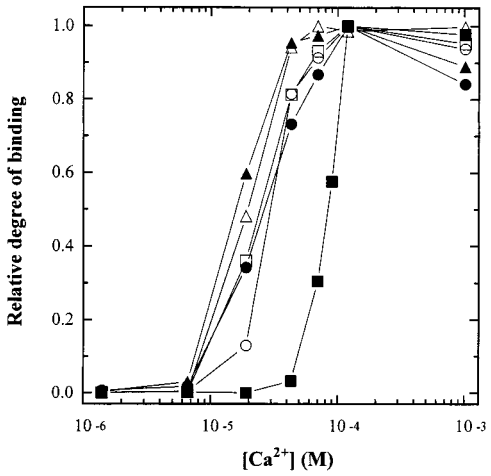

FIGURE 3: Vesicle binding of annexin I (○), Δ^{1-41} (●), Δ^{1-35} (□), Δ^{1-29} (△), Δ^{1-24} (▲), and annexin V (■) as a function of Ca^{2+}. Assay mixtures contained 0.13 μM protein, 0.1 mM POPC/POPE/POPS (2:5:2) vesicles, and varying concentrations of free Ca^{2+} in the assay buffer (10 mM HEPES−KOH and 0.1 mM KCl, pH 7.0). Pellets separated by centrifugation were redissolved in 15 μL of 10 mM HEPES−KOH buffer, pH 7.0, containing 0.1 mM KCl and 1 mM EGTA, and the amount of protein was quantified by densitometric determination of the annexin band on an SDS−polyacrylamide gel (see under Materials and Methods). Relative degree of binding was calculated by dividing the amount of vesicle-bound protein by total protein.

properties of annexin V for comparison. Figure 2 illustrates the vesicle aggregation activity of these proteins as a function of their concentration. As reported previously (*23*, *26*), annexin I showed high vesicle aggregation activity, whereas annexin V caused insignificant aggregation even at high concentrations. For annexin I, we could not evaluate the extent of vesicle aggregation at protein concentrations above 0.15 μM, because under such conditions annexin I caused extensive precipitation of vesicles, which resulted in a decrease in turbidity signal. Interestingly, the truncated mutant, Δ^{1-41} induced significant vesicle aggregation at high protein concentrations (i.e., >0.7 μM). The protein concentration required to achieve the same degree of vesicle aggregation was about 5 times higher for Δ^{1-41} than for wild type. This indicated that the core of annexin I has a low but definite ability to induce vesicle aggregation. To determine whether the altered vesicle aggregation activity of the truncated mutant derives from decreased vesicle binding or reduced vesicle aggregation per se, we measured the binding of wild type and the mutant to vesicles. We previously showed that the relative vesicle binding affinity of annexin I mutants could be readily assessed from the Ca^{2+} dependency of vesicle binding (*17*). As illustrated in Figure 3, the wild type and the mutant have essentially the same Ca^{2+} dependency of vesicle binding, showing that they have comparable vesicle affinity. Together, these data show that the core of annexin I has full vesicle affinity and a limited ability to induce vesicle aggregation.

Recovery of Vesicle Aggregation Activity. To determine which subregion of the amino-terminal region of annexin I is important in the annexin-induced vesicle aggregation, we incrementally added 5−6 amino acid extensions to the core to create Δ^{1-35}, Δ^{1-29}, and Δ^{1-24}. Then we measured their vesicle aggregation activity and vesicle affinity. Interestingly, Δ^{1-29} and Δ^{1-35} were significantly less active than Δ^{1-41}, as indicated by their higher protein requirements (Figure 2). This suggests that residues 29−41 might be inhibitory to the residual vesicle aggregation activity of the core of annexin I. The Ca^{2+} dependency of vesicle binding (Figure 3) indicates that Δ^{1-29} and Δ^{1-35} have vesicle affinities comparable to that of wild type. Thus, residues 29−41 appear to have an inhibitory effect only on vesicle aggregation. In contrast to these truncated mutants, Δ^{1-24} fully recovered the vesicle aggregation activity of wild-type annexin I (Figure 2). Since both wild type and Δ^{1-24} induced extensive aggregation of vesicles at high concentrations, we measured the Ca^{2+} dependency of vesicle aggregation at a given protein concentration (i.e., 0.13 μM) to better assess the relative vesicle aggregation activity. As shown in Figure 4, the Ca^{2+} concentration giving rise to half-maximal vesicle aggregation activity was slightly lower for Δ^{1-24} than for wild type. With the further increase of Ca^{2+}, however, the vesicle aggregation by this mutant gradually decreased. This was not due to extensive vesicle precipitation as it was not detected by visual inspection of the assay mixtures. None of the other truncated mutants (Δ^{1-41}, Δ^{1-35}, and Δ^{1-29}) caused any detectable vesicle aggregation even at high Ca^{2+} concentrations under the same conditions. The Ca^{2+} dependence of vesicle binding (Figure 3) shows that Δ^{1-24} has slightly higher vesicle affinity than does wild type in the Ca^{2+} concentration range of 10−100 μM, which is consistent with its slightly higher vesicle aggregation activity. The reduction of aggregation activity at higher Ca^{2+} levels does not, however, correlate with the vesicle binding affinity, which remains constant with the increase in Ca^{2+} concentration (Figure 3). Taken together, these results indicate that the subregion of annexin I containing residues 25−29 is important for its vesicle aggregation activity.

Identification of a Structural Determinant of Vesicle Aggregation. The subregion of annexin I containing residues 25−29 (see Figure 1) harbors two cationic residues, Lys-26 and Lys-29, and two serines, Ser-27 and Ser-28, whose in vitro phosphorylation by protein kinase C was shown to

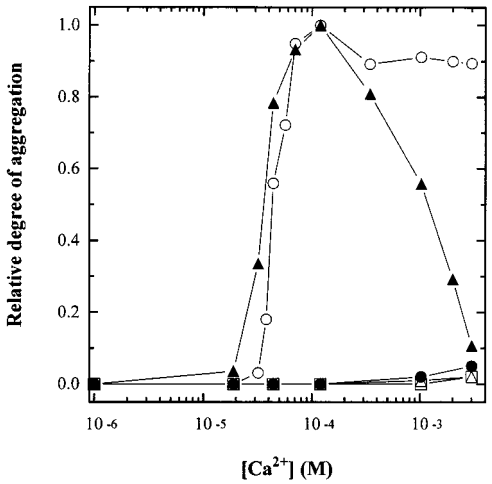

FIGURE 4: Aggregation of phospholipid vesicles by annexin I (○), Δ^{1-41} (●), Δ^{1-35} (□), Δ^{1-29} (△), and Δ^{1-24} (▲) as a function of Ca^{2+}. Assay mixtures contained 0.13 μM protein, 0.1 mM POPC/POPE/POPS (2:5:2) vesicles, and varying concentrations of free Ca^{2+} in the assay buffer (10 mM HEPES−KOH and 0.1 mM KCl, pH 7.0). Vesicle aggregation was followed by continuously monitoring turbidity changes at 450 nm. Relative degree of aggregation was calculated by dividing observed turbidity change by maximal turbidity change. Data points indicate means of duplicate determinations, an average standard error of which was about 5%.

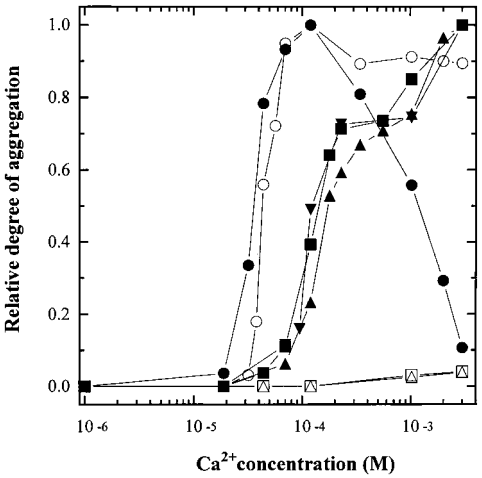

FIGURE 5: Aggregation of phospholipid vesicles by annexin I (○), Δ^{1-24} (●), K26E (▼), K29E (■), K26/29E (▲), Δ^{1-24}/K26E (□), and Δ^{1-24}/K29E (△) as a function of Ca^{2+}. Measurements were performed as described for Figure 4.

decrease the vesicle-aggregating activity of annexin I (*21*, *27*). To assess their importance in vesicle aggregation, we first mutated Lys-26 and Lys-29 of Δ^{1-24} to glutamates to generate Δ^{1-24}/K26E and Δ^{1-24}/K29E. As in the case with Δ^{1-24}, we measured the Ca^{2+} dependency of vesicle aggregation for these mutants to assess their relative vesicle aggregation. As shown in Figure 5, these mutants caused no detectable vesicle aggregation activity with Ca^{2+} concentrations up to 3 mM. They also showed no vesicle aggregation activity with protein concentrations up to 5 μM at 1 mM Ca^{2+} (data not shown). The two mutants, however, bound vesicles as tightly as wild type (Figure 6). These results clearly indicate that Lys-26 and Lys-29 are essential for the vesicle aggregation activity of Δ^{1-24} but not for vesicle binding affinity. To see if the two lysines are also important for the vesicle aggregation activity of full-length

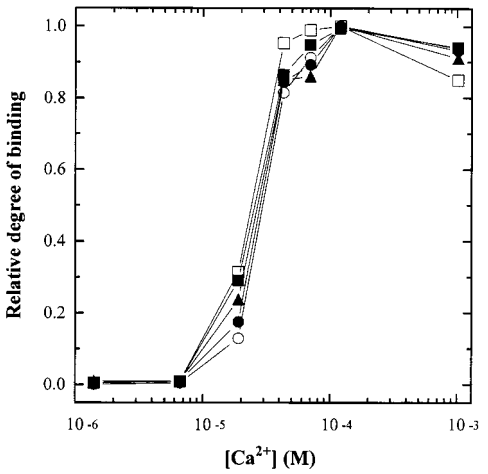

FIGURE 6: Vesicle binding of annexin I (○), K26E (●), K29E (△), K26/29E (▲), Δ^{1-24}/K26E (□), and Δ^{1-24}/K29E (■) as a function of Ca^{2+}. Measurements were performed as described for Figure 3.

annexin I, we generated three mutants, K26E, K29E, and K26E/K29E, and measured their vesicle binding and aggregation activities. As shown in Figure 5, these mutants exhibited much reduced vesicle aggregation activity at lower Ca^{2+} concentrations, but unlike Δ^{1-24}/K26E and Δ^{1-24}/K26E, they were able to induce vesicle aggregation at higher Ca^{2+} concentrations. The Ca^{2+} concentration required for half-maximal vesicle aggregation was 3−4 times higher for these mutants than for wild type. In contrast, all three mutants have essentially the same vesicle binding affinity as the wild type (Figure 6). Overall, these results indicate the importance of Lys-26 and Lys-29 in the vesicle aggregation activity of annexin I.

Characterization of Annexin Chimeras. To corroborate the notion that the residues 24−29 are essential for the vesicle aggregation activity of annexin I, we prepared a chimera (annexin I/V chimera) with these residues attached to the core of annexin V and measured its vesicle aggregation activity. To generate an active chimera with the addition of a minimal length of amino-terminal extension to annexin V, we inspected the primary and tertiary structures of annexins. The inspection revealed that the amino-terminal regions are anchored to the core of the annexins at the boundary of domains I and IV via Val or Ile residing in the hydrophobic pocket. The Val (residue 36 for annexin I and residue 9 for annexin V) is located 6 residues ahead of another conserved residue, Phe-42 (see Figure 1), which is considered to be the first residue of domain I (*2*). The BLAST search on the amino-terminal regions of annexin I from different species also showed that the sequence surrounding the Val is highly conserved. In fact, residues between the conserved Val and Phe are similar for annexins I and V (*V*SPYPT*F* and *V*TDFPG*F*, respectively). On the basis of this structural information, residues 24−35 (VKSSKGGPGSA) of annexin I were linked to the annexin V core containing residues 9−320 (see Figure 1). The Ca^{2+} dependency of vesicle binding (Figure 7) showed that the resulting chimera has essentially the same vesicle affinity as annexin V, which requires higher Ca^{2+} levels than does annexin I for full activity. We then measured the vesicle aggregation activity of these proteins as a function of protein concentration at a fixed Ca^{2+} concentration (i.e., 1 mM) that allows for their complete vesicle binding. Under this condition, the chimera

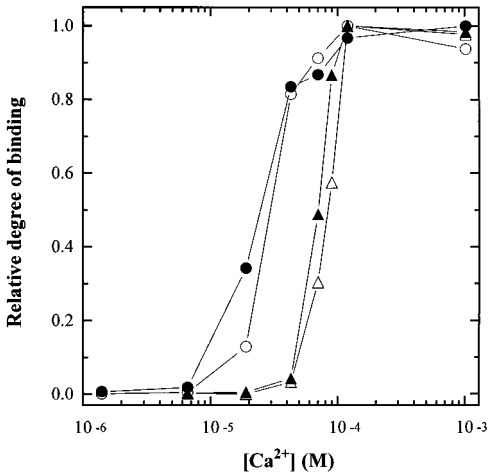

FIGURE 7: Vesicle binding of annexin I (○), annexin V/I chimera (●), annexin I/V chimera (△), and annexin V (▲) as a function of Ca^{2+}. Measurements were performed as described for Figure 3.

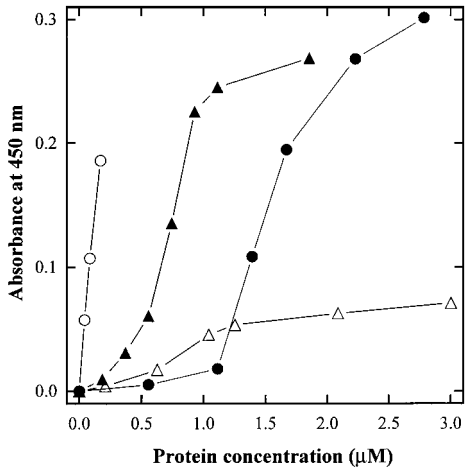

FIGURE 8: Aggregation of phospholipid vesicles by annexin I (○), annexin V/I chimera (●), annexin I/V chimera (▲), and annexin V (△) as a function of protein concentration. Measurements were performed as described for Figure 2.

exhibited significantly higher aggregation activity than annexin V (Figure 8), but a higher concentration of the chimera was required to achieve the vesicle aggregation activity of annexin I. The vesicle aggregation activity of the chimera was roughly similar to that of Δ^{1-35}. These results thus corroborate the notion that the amino-terminal subregion of annexin I spanning residues 24−35 is essential for its vesicle aggregation activity. The reduced vesicle aggregation activity of the chimera compared to that of annexin I also suggests that the interplay of the core and the amino-terminal region of annexin I is important for its vesicle aggregation activity.

We also prepared another chimera (annexin V/I chimera) containing the amino-terminus (residues 1−15) of annexin V and the carboxy-terminal core of annexin I (see Figure 1). The vesicle aggregation activity of the chimera was measured to further test whether the specific amino-terminal sequence of annexin I is essential for its activity. Figure 7 shows that this chimera and annexin I have comparable vesicle binding affinity, consistent with the notion that the core of annexin I is responsible for its vesicle affinity. When we measured the vesicle aggregation activity of the chimera as a function of protein concentration (Figure 8), we found

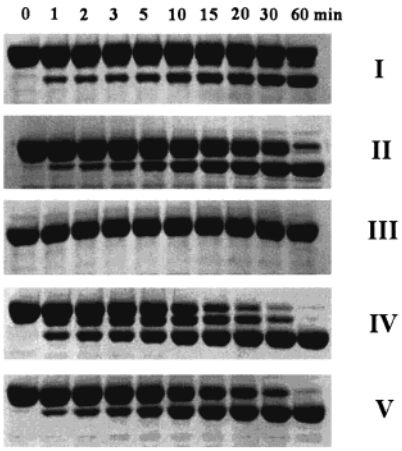

FIGURE 9: Tryptic digest patterns of annexin I and mutants. Row I, free wild type; row II, free K29E; row III, free K26E/K29E; row IV, wild type with 1 mM Ca^{2+} [and 0.1 mM POPC/POPE/POPS (2:5:2) vesicles]; row V, K29E with 1 mM Ca^{2+} (and 0.1 mM vesicles). For rows IV and V, Ca^{2+} alone had the same effect as Ca^{2+} and vesicles.

that the chimera had lower activity than the core of annexin I (i.e., Δ^{1-41}, see Figure 2) and behaved like Δ^{1-35}. This inhibitory effect of the amino-terminal sequence of annexin V on the vesicle aggregation activity of annexin I again underscores the importance of the specific nature of vesicle aggregation induced by the amino-terminal extension of annexin I.

Structural Role of Lys-26 and Lys-29 of Annexin I in Vesicle Aggregation. The essential Lys-26 and Lys-29 might either be directly involved in vesicle aggregation or induce conformational changes of annexin I for vesicle aggregation. To explore the latter possibility, we performed the tryptic digest of wild type and mutants including K26E, K29E, and K26E/K29E in the absence and presence of Ca^{2+} and vesicles. It has been shown that annexin I is prone to tryptic digest and that the cleavage patterns for free and vesicle-bound annexin I are different (*28*). The tryptic digest of free annexin I yielded a single product resulting from the hydrolysis at Lys-26, whereas that of vesicle-bound annexin I produced two cleavage products resulting from the hydrolysis at Lys-26 and, presumably, Lys-9. This finding was interpreted as evidence for conformational changes of annexin I in the course of vesicle binding and aggregation (*28*). We found that wild type annexin I followed a distinct time course of tryptic cleavage in the absence and presence of vesicles. As shown in Figure 9, free annexin I yielded primarily a single cleavage product after 60 min under our cleavage condition, whereas annexin I in the presence of Ca^{2+} and vesicles produced triplet bands with two cleavage products under the same condition. It appears that the difference derives mainly from the rate of secondary cleavage. Interestingly, the tryptic digest in the presence of 1 mM Ca^{2+} alone in the buffer produced the same pattern as that in the presence of Ca^{2+} and vesicles, suggesting that the high concentration of Ca^{2+} might also induce conformational changes of annexin I. As expected from the putative site of tryptic cleavage (i.e., Lys-26), neither K26E nor K26E/K29E was cleaved by trypsin in both the presence and absence of Ca^{2+}/vesicles. Another mutant, K29E, was, however, hydrolyzed by trypsin as shown in Figure 9. In the absence of Ca^{2+} and vesicles, the tryptic digest of K29E yielded the

same pattern as wild type and the rate of hydrolysis of K29E was faster than that of wild type. The difference in rate might be due to minor differences in local structure or electrostatics near Lys-26. Importantly, K29E exhibited essentially the same cleavage pattern in both the presence and absence of Ca^{2+}/vesicles. This suggests that, unlike wild-type annexin I, K29E does not undergo a significant degree of conformational changes when it binds to vesicles. Thus, these results suggest that Lys-29 (and Lys-26) might induce and/or stabilize the conformation of vesicle-bound annexin I that is conducive to vesicle aggregation.

DISCUSSION

Since the membrane aggregation activity of annexin I was first reported (*26*, *29*, *30*), extensive effort has been made to identify the structural determinant of this activity. The sequence comparison of annexins suggested that the variable amino-terminal region of annexin I might be involved in membrane aggregation. Further studies of annexin I, however, have failed to unambiguously identify the structural determinant of its membrane aggregation activity. For instance, several reports on annexin I−annexin V chimeras have yielded conflicting results: Some suggested that domain I of the carboxy-terminal core of annexin I is essential for membrane aggregation (*23*), whereas others indicated that the amino-terminal region is responsible (*7*). Also, measurements of membrane aggregation activity of annexin I mutants lacking the first 12, 26, and 29 residues yielded conflicting results. One study reported that the mutant missing the first 29 residues has the same aggregation activity as the full-length annexin I (*18*), whereas the other study found that the same mutant has significantly lower activity (*22*).

To systematically assess the contribution of the amino-terminal region and the carboxy-terminal core to membrane aggregation activity of annexin I and to pinpoint the essential residues, we first truncated the whole amino-terminal region (i.e., Δ^{1-41}) and then measured the effect of incremental addition of the amino-terminal extension on vesicle aggregation activity. As reported previously, we established a sensitive vesicle aggregation and binding assay for annexin I (*17*). To rigorously determine the effect of mutations on vesicle aggregation activity of annexin I, we performed the vesicle aggregation assay under various conditions; i.e., different concentrations of lipid, protein, and Ca^{2+}. This approach led us to find that Δ^{1-41}, which shows no detectable vesicle aggregation activity under our typical assay conditions (i.e., at 0.1 μM protein), has unexpectedly high activity at high protein concentrations (see Figure 2). This is in sharp contrast to annexin V, which does not induce significant vesicle aggregation in the same protein concentration range, and thus disputes the notion that the difference in vesicle aggregation activity between annexins I and V derives mainly from the amino-terminal extension of annexin I. Since annexin I, Δ^{1-41}, and annexin V are fully vesicle-bound under the conditions of the vesicle aggregation assay (i.e., 1 mM Ca^{2+}; see Figure 3), the observed differences largely reflect different intrinsic aggregation activity of vesicle-bound proteins. These results thus suggest that the carboxy-terminal core domains of annexin I contain at least a part of the interaction site for vesicle aggregation. The recovery of vesicle aggregation activity by the addition of amino-terminal residues is not incremental. The addition of residues 29−41 to the carboxy-terminal core lowers the residual activity of the core, whereas the addition of five extra residues (25−29) fully restores the wild-type activity. Also, the addition of an amino-terminal extension of annexin V to the core of annexin I inhibited its residual vesicle aggregation activity. Thus, it appears that the delicate interplay of the amino-terminal region, Lys-26 and Lys-29 in particular, and the core of annexin I is essential for its full vesicle aggregation activity.

There are at least two possible mechanisms that can account for our data. One possibility is that Lys-26 and Lys-29, along with other residues in the core, form the interaction site for vesicle aggregation. This mechanism is not fully consistent with our findings, however. For instance, if the two lysines are directly involved in vesicle-aggregating interactions, the effects of mutations, K26E, K29E, and K26E/K29E, would be equally drastic for both wild type and Δ^{1-24}, which have comparable vesicle aggregation activity. The observed differential effects (see below and Figure 5) imply that the lysines as well as residues 1−24 might be involved in stabilizing the active conformation of annexin I for vesicle aggregation. In other word, Lys-26 and Lys-29 might specifically interact with the core to generate and/or stabilize a spatially separate interaction site for vesicle aggregation. This alternative mechanism can better account for our data, including the tryptic digest patterns of annexin I and mutants. The finding that Δ^{1-29} and Δ^{1-35} have lower vesicle aggregation activity than does Δ^{1-41} indicates that without such specific interactions involving Lys-26 and Lys-29 the amino-terminal extension interferes with vesicle aggregation, presumably by physically blocking a part of interaction site located in the core of annexin I. Also, the amino-terminal residues 1−24 of annexin I appear to play some, albeit not an essential, role in stabilizing the active conformation of annexin I for two reasons. First, Δ^{1-24} has the wild-type activity at low Ca^{2+} (i.e., < 0.1 mM) but drastically loses the activity at higher Ca^{2+} concentrations (see Figure 4), which induce conformational changes of annexin I (see Figure 9). Second, K26E and K29E have higher vesicle aggregation activity than do K26E/Δ^{1-24} and K29E/Δ^{1-24}, indicating that residues 1−24 can partially compensate for the loss of activity caused by the charge reversal.

On the basis of the comprehensive mutagenesis of the core of annexin I, we previously proposed that the membrane-bound annexin I undergoes conformational changes that involve a modular rotation of core domains I and IV (*17*). Our present tryptic digest study indicates that Lys-29 (and Lys-26) plays a central role in the conformational changes of vesicle-bound annexin I. Presumably, the conformational changes juxtapose the two lysines to their counterparts in the core, and the resulting interactions stabilize the conformation that exposes an interaction site for vesicle aggregation. The conformational changes involving the two lysines would be facilitated by the putatively flexible loop spanning residues 30−33 (GGPG; see Figure 1). Phosphorylation of Thr-24, Ser-27, and Ser-28 by protein kinase C has been shown to inhibit the membrane aggregation activity of annexin I (*20*, *21*). The effect of phosphorylation of Thr-24, Ser-27, and Ser-28 on the vesicle aggregation activity of annexin I would be similar to the inhibitory effect of K26E and/or K29E mutations. Note that these mutations increase

the calcium requirement of annexin I for vesicle aggregation activity and that in vitro vesicle aggregation assays typically require a calcium concentration higher than its cellular concentration range. Under the physiological conditions, the charge-reversal mutations of the two lysines would therefore turn off the vesicle aggregation activity of annexin I. Similarly, it is tempting to postulate that the phosphorylation of the amino-terminal residues downregulates the cellular membrane aggregation activity of annexin I by electrostatically neutralizing cationic side chains of Lys-26 and Lys-29, thereby decreasing their interaction with the core to form the interaction site. The BLAST search of annexin I sequences from different species show that the residues 25−33 are conserved; in particular, Lys-26 and Lys-29 are absolutely conserved. Thus, the proposed mechanism should apply to all annexins I from different species. The two lysines and phosphorylation sites are not, however, conserved in the amino-terminal regions of other annexins with membrane aggregation activity, suggesting that they might have different structural determinants of their aggregation activity and distinct regulatory mechanisms. As for annexin I, further research is required to answer questions as to the identity of residues with which Lys-26 and Lys-29 interact, the exact location of the interaction site for membrane aggregation, and whether the secondary site mediates membrane−annexin or annexin−annexin interactions.

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