Aggregation of Phospholipid Vesicles by a Chimeric Protein with the N-Terminus of Annexin I and the Core of Annexin V†

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ABSTRACT: A chimeric protein was produced with the N-terminal domain (amino acids 1–45) of annexin I and the core of annexin V (amino acids 19–320). This protein, annexin I_N–V_C, has a similar Ca^{2+} requirement for binding to phospholipid bilayers of 20% phosphatidylserine (PS)/80% phosphatidylcholine (PC) as annexin V. In contrast to annexin V, this protein has a strong potency to aggregate phospholipid vesicles as is shown by turbidimetric measurements and cryo-electron microscopy. Ellipsometry was employed to study quantitatively the phenomenon of phospholipid vesicle adhesion to annexin I_N–V_C bound to a planar phospholipid bilayer. The amount of phospholipid vesicles bound by annexin I_N–V_C on the planar bilayer is proportional to its surface coverage and can be inhibited by coadsorption of annexin V on the planar bilayer or by shielding the phospholipid surface of the vesicles with blood coagulation factor Va. Annexin I_N–V_C, like annexin V, does not bind to pure PC bilayers, but its adsorption on anionic phospholipid bilayers brings about the capacity to bind pure PC vesicles. This suggests that annexin I_N–V_C generates or exposes after binding to anionic phospholipids another phospholipid binding site, that differs from the annexin V phospholipid binding site. Collectively, the data suggest that two-dimensional cluster formation of annexin I_N–V_C on a bilayer with anionic phospholipids is involved in vesicle adherence.

The annexins belong to a family of phospholipid binding proteins, that share structural homology in a conserved core, with 4 or (in the case of annexin VI) 8 internal repeats of about 70 amino acids containing the annexin consensus sequence, and a variable N-terminal tail (Geisow, 1986; Geisow et al., 1986).

Several observations support the notion that the N-terminal part of the annexin molecule can influence the Ca^{2+} requirement for binding to phospholipids. Phosphorylation of the N-terminus of annexin I (De et al., 1986; Pepinsky & Sinclair, 1986) and annexin II (Glenney & Tack, 1985) decreases the amount of Ca^{2+} required for binding (Glenney, 1986; Schlaepfer & Haigler, 1987). Limited proteolysis of the N-terminal tail of annexin I also decreases the Ca^{2+} requirement for binding (Ando et al., 1989). Finally, monoclonal antibodies against the N-terminal domain of annexin I did not inhibit phospholipid binding and even decreased the amount of Ca^{2+} required for binding (Glenney & Zokas, 1988).

Aggregation of phospholipid vesicles has been reported for several annexins. Aggregation of chromaffin granules, purified from adrenal medulla, by annexin VII was already described in 1978 (Creutz et al., 1978). Vesicle aggregation and under certain conditions followed by vesicle fusion has been described for annexins I, II, and VII (Creutz et al., 1978; Blackwood & Ernst, 1990; Nir et al., 1987; Drust & Creutz, 1988; Powel & Glenney, 1987). Ernst et al. (1991) constructed a chimera of annexins I and V with aggregating activity and concluded that this activity comes forth from structures that are included in the first repeat of annexin I plus eight residues of its amino-terminal tail. Annexin II restores secretion in permeabilized cells, whereas secretion is inhibited by immunoaffinity-purified antibodies against annexin II (Ali et al., 1989). Some annexins are, therefore, thought to play a role in exocytosis.

Annexin V in the presence of Ca^{2+} ions binds with high affinity to anionic phospholipids (Andree et al., 1990; Tait et al., 1989; Schlaepfer et al., 1987), but, compared to the other annexins, it displays the highest Ca^{2+} requirement for binding (Blackwood & Ernst, 1990). Furthermore, annexin V does not promote vesicle aggregation, and in fact impairs vesicle aggregation by other annexins (Blackwood & Ernst, 1990; Oshry et al., 1991).

In this paper, we describe a chimeric protein, annexin I_N–V_C,[1] with the N-terminus of annexin I and the C-terminal core with the four repeats of annexin V, constructed for further exploration of the relationship between primary structure and phospholipid binding properties of annexins. Ellipsometry, a sensitive optical technique for the measurement of protein adsorption on reflecting surfaces, was used to show that annexin V and the chimera have identical lipid binding properties. Turbidity measurements and cryo-electron microscopy revealed that annexin I_N–V_C, in contrast to annexin V, promotes aggregation of small unilamellar vesicles and causes the formation of large aggregates. The interaction of the chimera with lipid vesicles was further characterized by ellipsometric measurement of vesicle adsorption to planar bilayers covered with annexin I_N–V_C. The results indicate that the lipid-bound chimera exposes a new phospholipid binding site that is absent or masked in annexin V.

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[1] Abbreviations: annexin I_N–V_C, chimeric protein with N-terminal domain of annexin I and the core of annexin V; PS, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine; PC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine.

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MATERIALS AND METHODS

Preparation of Phospholipid Vesicles and Planar Bilayers. 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylserine (PS) was purchased from Avanti Polar Lipids and the corresponding phosphatidylcholine (PC) from Sigma Chemical Co. (P-1013). All experiments were performed with phospholipid mixtures of 20% PS/80% PC (mol/mol) or pure PC. Small unilamellar vesicles (SUV, 20–40 nm) were prepared according to de Kruijff et al. (1975) by sonicating a nitrogen-dried phospholipid mixture in buffer at 0 °C. Planar phospholipid bilayers were deposited on silicone slides by dipping hydrophilic silicone slides in a stirred vesicle solution (Giessen et al., 1991).

Buffer. Experiments were performed in a buffer of 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 3 mM $CaCl_2$, and 0.5 g/L bovine serum albumin (Sigma Chemical Co., A-7030) at 20 °C, unless stated otherwise.

Proteins. Annexin V was purified from human placental tissue as described (Reutelingpserger et al., 1985). Purity of annexins was checked by SDS–polyacrylamide gel electrophoresis according to Schägger and Jagow (1987) in the presence of 2% 2-mercaptoethanol, stained with Coomassie Blue.

Production of Annexin I_N–V_C. A plasmid fragment, obtained after cutting plasmid pRH291 (Maurer-Fogy et al., 1988) with restriction enzymes *Pvu*II and *Sph*I (*Pvu*II–*Sph*I fragment), contained part of the phoA promoter, the bacterial ribosomal binding site, the entire coding region for annexin V, and a nontranslated part. It was subcloned into the commercially available plasmid M13mp18 doubly-cut with *Sma*I and *Sph*I. Using the mutagenesis primer EBI-977 (CAACAGAGTCAGGATCGATTCCTCATCTGTGCCC) complementary to annexin V from nucleotides 221–254 (Maurer-Fogy et al., 1988), and Amersham's in vitro mutagenesis system, mutated cDNA was produced with the indicated *Cla*I site at the codon for serine-36, without affecting the amino acid sequence. It was sequenced, and a large *Eco*RI–*Hin*dIII insert was isolated and recloned in Bluescribe M13+ (Stratagen), resulting in pGN31.

Coding oligonucleotide strands of the N-terminus of annexin I, EBI-982 (CGATTCCTCATCTGTGCCCAAGCCTTTCATAGCCTTCCGAAGAGTTTCTGCATCCGAGGATGGATTGAAGGTAGGATATAGGGGCTCACCGCTGATCCGG) and EBI-988 (GACCACCTTTGGATGACTTCACAGTTTGAACATATTCCTGCTCTTCATTTTCAATAAACCAGGCCTGCTTGAGGAATTCTGATACCATTGCCATAAAATCACCTCAACCTC, were phosphorylated using T4 polynucleotide kinase (New England Biolabs). The reaction was stopped by heating at 95 °C for 10 min, and 12 μL of water was added, containing 1 pmol of the noncoding strands EBI-972 (TCGAGAGGTTGAGGTGATTTTATGGCAATGGTATCAGAATTCCTCAAGCAGGCCTGGTTTATTGAAAATGAAGAGCAGGAATATGTTCAAACTGTGAAGTCATCCAA) and EBI-978 (AGGTGGTCCCGGATCAGCGGTGAGCCCCTATCCTACTTCAATCCATCCTCGGATGCAGAAACTCTTCGGAAGGCTATGAAAGGCTTGGGCACAGATGAGGAAT) and approximately 1 μg of the *Xho*I–*Cla*I fragment of pGN31. After being annealed, the fragments were ligated using T4 ligase (New England Nuclear). Competent *Escherichia coli* JM 101 cells were transformed. A correctly expressed plasmid, checked by sequencing, was selected from the clones and named pGN32.

The *Xho*I–*Hin*dIII insert of pGN32, containing the coding regions of the hybrid annexin, was cloned into pRH284, cut with *Xho*I and *Hin*dIII (Maurer-Fogy et al., 1988). The resulting plasmid, pGN38, expressed annexin I_N–V_C (4 mg of annexin I_N–V_C/L of fermentation) in *E. coli* JM 101 cells, grown as described (Maurer-Fogy et al., 1988).

Purification of Annexin I_N–V_C. The fermentation of pGN38-transformed *E. coli* 101 was submitted to a French press to break open the *E. coli* cells. The solution was adjusted to 5 mM $CaCl_2$ and 0.5% poly(ethylenimine) (w/v) and was then centrifuged. Under these conditions, the chimera bound to the *E. coli* membranes. The membrane pellet was washed thoroughly. The final wash step contained EDTA to liberate the chimera from the membranes. The resulting supernatant was loaded on a TSK-DEAE column equilibrated with 20 mM Tris-HCl, pH 8.4. Elution from the column was achieved using a linear gradient from 0 to 500 mM NaCl. The fractions containing the chimera were concentrated and loaded on a S-200 gel permeation column, equilibrated with 20 mM Tris-HCl, pH 7.5, and 200 mM NaCl. Finally, the chimera-containing fractions were pooled, dialyzed against 50 mM Tris-HCl, pH 7.9, and applied to a Mono Q column from which it was eluted by a NaCl gradient of 0–250 mM NaCl. The purified chimera was checked by sequencing on a pulsed liquid-phase sequenator (ABI, Model 477 A) using Edman chemistry. Identification of the cleaved amino acids was done by on-line reverse-phase HPLC.

^{125}I-Annexin I_N–V_C. Annexin I_N–V_C (100 μg) was added to a vial containing two Iodo-beads (Pierce) and 1 mCi of ^{125}I in 1 mL of buffer (50 mM Tris-HCl, pH 7.5, and 100 mM NaCl) and incubated for 15 min under continuous stirring. Free ^{125}I was removed with a gel filtration (G-25) column. This resulted in a specific activity of 4×10^6 cpm/μg of annexin I_N–V_C.

Aggregation of Phospholipid Vesicles Measured with Turbidity. Aggregation of phospholipid vesicles by annexins was measured essentially as described by Creutz et al. 1978. The optical density (OD) at 405 nm was recorded every 20 s for 10 min in a Cobas-Bio (Hoffmann-La Roche) autoanalyzer at 37 °C, which yielded the same results as aggregation measured in a conventional photometer. The optical density was corrected for the blank value of the cuvette, and the accuracy of the Cobas-Bio photometer signal was better than 0.5 mOD. The turbidity of solutions of small vesicles (20–40 nm) used in this study is very small (<1 mOD). The formation of small aggregates will therefore induce only small absolute changes in light scattering, which are not easily detectable. The observed large changes in optical density reflect the formation of large aggregates, as was confirmed with electron microscopy.

Ellipsometric Determination of Protein and Phospholipid Vesicle Adsorption to a Planar Phospholipid Bilayer. Ellipsometry is an optical technique for the measurement of the change of the polarization state of light caused by reflection (Azzam & Bashara, 1977). These changes are strongly influenced by the presence of very thin phospholipid and protein films on the reflecting surface. The changes in polarization are measured by null-ellipsometry: The He–Ne laser beam is transformed to circular polarized light, is polarized by a polarizing prism, P, and passes through the compensator, a quarter-wavelength retarder plate with the fast axis at 45° to the plane of incidence. Then the light is reflected on a silicone slide and finally passes through a second polarizing prism, A. The final light intensity, measured by a photodiode after prism A, is kept minimal by rotation of prisms P and A by computer-steered stepping motors, and every 5–10 s, null positions P(t) and A(t) are obtained. The adsorbed mass of protein and phospholipid can be determined from the null positions of

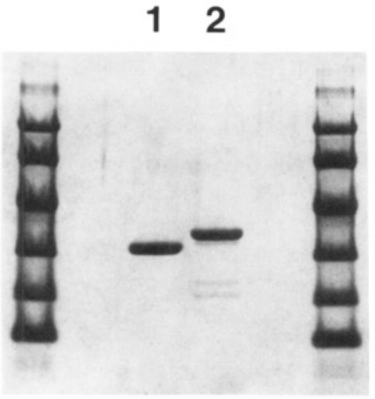

FIGURE 1: SDS–PAGE of annexin I_N–V_C and annexin V. Five micrograms of annexin V (lane 1) and 5 μg of annexin I_N–V_C (lane 2) in the presence of 2% 2-mercaptoethanol were applied to the gel. The gel was stained with Coomassie Blue. Low molecular mass markers (Pharmacia) of 94, 67, 43, 30, 20.1, and 14.4 kDa were used as standards.

P(t) and A(t) using the Lorentz–Lorenz relation. The instrument and analysis of data have been described (Cuypers et al., 1983; Corsel et al., 1986). For the optical constants of silicone and an angle of incidence equal to 68°, the mass calculation can be simplified to $\Gamma(t) = 0.085[P(t) - Ps]$, where $\Gamma(t)$ is the adsorbed mass at time t, expressed in micrograms per centimeter squared, $P(t)$ is the null position of P, expressed in degrees, and Ps is the starting position of P. Adsorption experiments were performed in a trapezoidal quartz cuvette, with walls perpendicular to the light beam. The cuvette was treated with Sigmacote (Sigma Chemical Co., SL-2) in order to avoid protein depletion and was filled with 5 mL of buffer. The solution was stirred with a magnetic stirring bar (8 × 2 mm) rotating at 2400 rpm on the bottom of the cuvette in front of the reflecting surface, and the adsorption was measured on a spot behind the center of the stirrer. Annexin adsorptions to silicone slides covered with a phospholipid bilayer were started by addition of the protein. Vesicle adherence was also monitored by ellipsometry. First the desired amount of annexin was preadsorbed; then free protein was removed from the cuvette by flushing with 50 mL of buffer. Vesicles were added to the cuvette, and the adsorption of vesicles was measured by ellipsometry.

Visualization of Phospholipid Vesicles with Cryo-Electron Microscopy. Phospholipid vesicles (1 mM PS/PC) were incubated for at least 10 min with (20 μM) annexin in buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 3 mM $CaCl_2$; T = 20–22 °C). Thin aqueous films were prepared on 700-mesh (hexagonal) specimen grids without any supporting film. These thin films were vitrified in liquid ethane and observed at −170 °C using a Gatan 626 cyro-holder and a Philips CM12 microscope (Frederik et al., 1991a,b). Electron micrographs were taken at low dose conditions, and 1–2 μm defocus was used to enhance contrast. They show the suspended material in the hydrated state without any staining.

RESULTS

Annexin I_N–V_C. A mutant protein with amino acids 1–45 of the N-terminal tail of annexin I and amino acids 19–320 of the core of annexin V was constructed, expressed in *E. coli*, and purified. The chimera, called annexin I_N–V_C, had a molecular mass of 39 kDa, as was calculated from its amino acid sequence. Figure 1 shows SDS–polyacrylamide gel electrophoresis of annexin V and annexin I_N–V_C. The protein bands correspond to apparent molecular masses of 31 and 34 kDa, respectively, consistent with earlier reports (Reutelingsperger et al., 1985). Its first 10 N-terminal amino acids were checked by Edman chemistry and were identical to the first 10 N-terminal amino acids of annexin I. No evidence for dimerization of annexin V or annexin I_N–V_C was found.

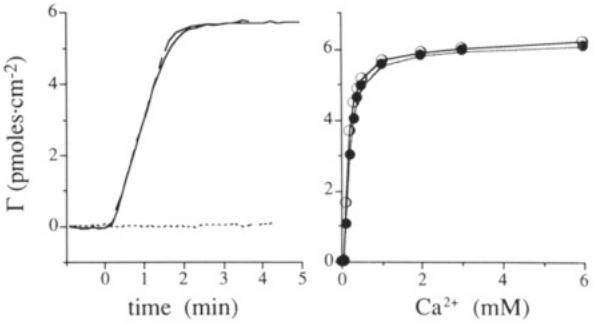

FIGURE 2: Binding of annexin I_N–V_C to phospholipid bilayers. Left panel: Adsorptions of 50 nM annexin V (- - -) or 50 nM annexin I_N–V_C (—) to a 20% PS/80% PC bilayer are shown. Annexin I_N–V_C adsorption to a bilayer of 100% PC (···) is negligible. Ca^{2+} concentration was 3 mM. Right panel: Ca^{2+}-dependent adsorption of 30 nM annexin I_N–V_C (○) or 30 nM annexin V (●) to a 20% PS/80% PC bilayer. Buffer: 50 mM Tris-HCl, pH 7.5, and 100 mM NaCl; T = 20 °C.

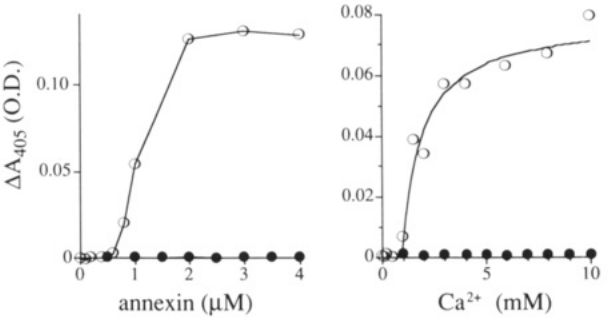

FIGURE 3: Vesicle aggregation measured by change of turbidity. Left panel: Aggregation of 100 μM phospholipid vesicles as a function of the final annexin I_N–V_C (○) or annexin V (●) concentration was measured by the change in optical density at 405 nm. Ca^{2+} concentration was 3 mM. Right panel: Effect of Ca^{2+} concentration on the change in turbidity of 50 μM vesicles was measured in the presence of 2 μM annexin I_N–V_C (○) or 2 μM annexin V (●). Buffer: 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.5 g/L bovine serum albumin; T = 37 °C.

Phospholipid Binding of Annexin V and Annexin I_N–V_C. Figure 2 presents the binding of annexin V and annexin I_N–V_C measured with ellipsometry. The adsorption kinetics and maximal binding (Γ_{max} = 5.7 pmol·cm^{-2}) of these two proteins to a bilayer of 20% PS/80% PC were nearly identical. No adsorption of annexin I_N–V_C to a 100% PC bilayer was measurable. The right panel of Figure 2 shows the effect of Ca^{2+} concentration on the binding of annexin V and annexin I_N–V_C to 20% PS/80% PC bilayers. Half-maximal Ca^{2+} concentrations for binding were similar, 0.22 ± 0.02 and 0.17 ± 0.04 mM (mean ± SD, n = 3), for annexin V and annexin I_N–V_C, respectively. Addition of EDTA showed that lipid binding of both proteins was completely reversible.

Turbidity Measurements of Vesicle Aggregation. Although annexin V and annexin I_N–V_C have identical phospholipid binding properties to a single phospholipid layer, Figure 3 shows that annexin I_N–V_C in contrast to annexin V was able to aggregate vesicles. The aggregation was monitored by changes in optical density (405 nm). It appeared that addition of annexin I_N–V_C resulted in a rapid increase in optical density which reached a steady-state value within 6 min, and Figure 3 shows this total change in optical density. The left panel

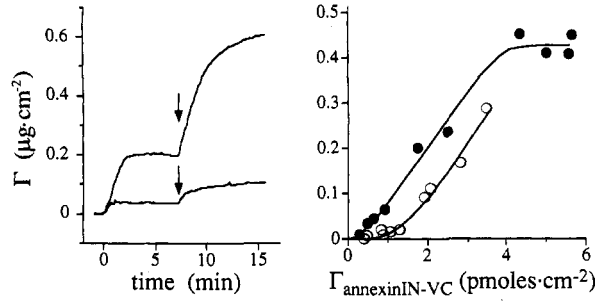

FIGURE 4: Phospholipid vesicle aggregation as a function of surface coverage with annexin I_N–V_C. Phospholipid vesicle aggregation was measured by ellipsometry as described. Left panel: The planar 20% PS/80% PC bilayer on the silicone slide was covered with 0.037 $\mu g \cdot cm^{-2}$ (=0.95 $pmol \cdot cm^{-2}$, lower curve) or 0.19 $\mu g \cdot cm^{-2}$ (=4.9 $pmol \cdot cm^{-2}$, upper curve) annexin I_N–V_C. After removal of free annexin I_N–V_C, 5 μM phospholipid vesicles was added (indicated by the arrow), and adsorption was followed with time. Right panel: The experiment presented in the left panel was carried out at different coverages of the planar phospholipid surface with annexin I_N–V_C, and the adsorbed mass was measured 8 min after addition of the phospholipid vesicles (●). Similar experiments were performed with bilayers nearly completely covered with a mixture of annexin V and annexin I_N–V_C. Open circles (○) show the dependence of the final vesicle adherence on the surface concentration of annexin I_N–V_C. Buffer: 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.5 g/L bovine serum albumin; T = 20 °C.

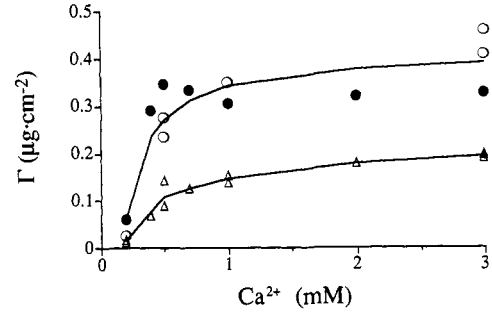

FIGURE 5: Ca^{2+}-dependent adherence of vesicles to a bilayer coated with annexin I_N–V_C. At the indicated Ca^{2+} concentrations, annexin I_N–V_C was adsorbed to a planar 20% PS/80% PC bilayer. After completion of the adsorption, free annexin was removed, and the amount of annexin I_N–V_C retained at the planar bilayer was measured by ellipsometry (△). Then 5 μM 20% PS/80% PC (○) or 100% PC (●) vesicles were added, and vesicle adsorption was measured. Indicated is the vesicle adsorption after 8 min. Buffer: 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.5 g/L bovine serum albumin; T = 20 °C.

of Figure 3 shows that aggregation of 100 μM phospholipid vesicles started at 0.6 μM annexin I_N–V_c and half-maximal change in optical density occurred at a concentration of 1.2 μM. The concentrations of annexin I_N–V_C that resulted in a half-maximal change in turbidity and the maximal change in turbidity are proportional to the phospholipid concentration from 20 to 100 μM (data not shown). The right panel of Figure 3 shows the aggregation of 50 μM vesicles by 2 μM annexin I_N–V_C plotted as function of the Ca^{2+} concentration. There exists an apparent threshold concentration of 1 mM Ca^{2+} for aggregation, and this threshold probably reflects the high Ca^{2+} requirement for binding of annexin I_N–V_C to small vesicles (see Discussion). Half-maximal light scattering occurred at 2 mM Ca^{2+}. No change in optical density was observed in these experiments if annexin V instead of annexin I_N–V_C was added.

Vesicle Aggregation Measured with Ellipsometry. The lipid–annexin I_N–V_C–lipid interactions were further investigated by ellipsometric measurement of vesicle binding to planar bilayers covered with annexin. This system affords easy control of the protein surface coverage of the planar bilayer and allows coverage of the vesicle surface with other proteins and the use of PC vesicles as a probe for the lipid interaction to annexin-covered planar bilayers. The left panel of Figure 4 shows the experimental procedure. The experiment was started by annexin I_N–V_C adsorption to a planar 20% PS/80% PC bilayer. The adsorption was complete after about 3 min, and free protein was removed by flushing after 5 min (upper curve). Flushing caused only a minimal desorption of less than 10% of the adsorbed mass. Then 5 μM 20% PS/80% PC vesicles was added, and a substantial extra vesicle adsorption was observed. The initial adhesion rate of the vesicles was (2.6 ± 0.3) × 10^{-3} $\mu g \cdot cm^{-2} \cdot s^{-1}$ (mean ± SD), which approximates the diffusion limit (see Discussion). No vesicle adsorption was observed to bilayers covered with annexin V (data not shown). In the lower curve (Figure 4, left panel), a planar bilayer with 0.03 $\mu g \cdot cm^{-2}$ annexin I_N–V_C was prepared by removing the protein from solution after about 30 s, far before completion of the adsorption. Both the final mass and the rate of vesicle adherence were decreased. The right panel of Figure 4 shows the vesicle adsorption after 8 min as a function of the surface concentration of annexin I_N–V_C. Also shown is the vesicle adherence to planar bilayers fully covered (Γ = 0.17–0.20 $\mu g \cdot cm^{-2}$) with a mixture of annexin V and annexin I_N–V_C prepared by allowing complete adsorption of a mixture of these proteins. Again, free protein was removed by flushing the cuvette. The surface concentration of annexin I_N–V_C was estimated in a separate series of experiments using mixtures of annexin V and ^{125}I-annexin I_N–V_C, containing fraction X = 0, 0.1, 0.2, 0.25, 0.3, and 1.00 (mol/mol) of ^{125}I-annexin I_N–V_C. After completion of the adsorption and removal of free protein, the total protein adsorption, $\Gamma_{t,X}$ ($\mu g \cdot cm^{-2}$), was measured by ellipsometry. Then the slide was removed, and ^{125}I-annexin I_N–V_C was brought in solution by addition of EDTA and counted in a Wallac 1470 Wizard γ-counter ($counts_X$). The amount of ^{125}I-annexin I_N–V_C, I-N,X ($\mu g \cdot cm^{-2}$), on the bilayer was calculated by $\Gamma_{I\text{-}N,X} = (counts_X/counts_{1.00})\Gamma_{t,100}$. Linear regression showed that $\Gamma_{I\text{-}N,X}/\Gamma_{t,X} = (1.00 \pm 0.08)X - 0.02 \pm 0.01$ (regression line ± SE), and this last relation was used to estimate the surface concentration in the right panel of Figure 4. It appeared that coadsorption of annexin V results in a substantially decreased vesicle adhesion compared to adhesion to planar bilayers containing the same amount annexin I_N–V_C without annexin V. This reduction in vesicle adsorption was most striking for annexin I_N–V_C surface concentrations below 1.5 $pmol \cdot cm^{-2}$. Regression analysis revealed that in this range the vesicle adhesion Γ ($\mu g \cdot cm^{-2}$) was a linear function (with zero intercept) of the annexin I_N–V_C surface concentration, $\Gamma_{annexin\ IN\text{-}VC}$ ($pmol \cdot cm^{-2}$). The following relations were found (regression coefficient ± SE): $\Gamma = (0.061 \pm 0.0036)\Gamma_{annexin\ IN\text{-}VC}$ without coadsorption of annexin V; $\Gamma = (0.018 \pm 0.0061)\Gamma_{annexin\ IN\text{-}VC}$ with coadsorption of annexin V. This difference in regression coefficient is significant ($p < 10^{-6}$). Incorporation of annexin I_N–V_C in the annexin V matrix nearly completely abolished vesicle adhesion.

Second Phospholipid Binding Site. Figure 5 shows Ca^{2+}-dependent adherence of vesicles of 20% PS/80% PC and vesicles of 100% PC to bilayers covered with annexin I_N–V_C. At low Ca^{2+} concentrations, the removal of annexin I_N–V_C from the cuvette (by flushing with buffer) resulted in considerable desorption of annexin I_N–V_C from the planar bilayer. This explains that for Ca^{2+} concentrations below 1 mM the annexin I_N–V_C binding was lower than in Figure 2. The reduced vesicle adherence at 0.2 mM Ca^{2+} is in agreement with the decreased surface concentration of annexin I_N–V_C

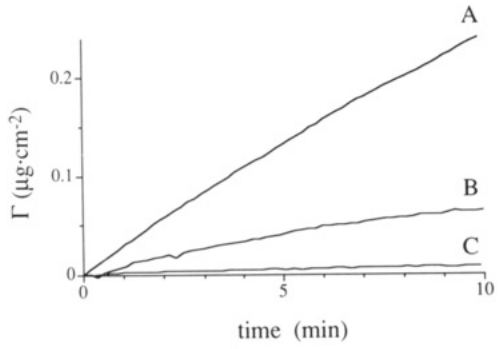

FIGURE 6: Inhibition of aggregation by factor Va. Approximately 5 pmol·cm^{-2} annexin I_N–V_C was adsorbed to a planar bilayer of 20% PS/80% PC. Unbound annexin I_N–V_C was removed from the cuvette. 1 μM phospholipid vesicles (20% PS/80% PC) was incubated for 5 min either with buffer (A), with 5 nM factor Va (B), or with 10 nM factor Va (C). The incubated suspension was then added to the cuvette, and adherence to the planar bilayer covered with annexin I_N–V_C was recorded with time by ellipsometry. Buffer: 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 g/L bovine serum albumin, and 3 mM $CaCl_2$; T = 20 °C.

and the relationship between vesicle adherence and surface concentration of annexin I_N–V_c, indicating a much lower Ca^{2+} requirement for vesicle adherence than for lipid binding of annexin I_N–V_C. Unexpected was the finding that vesicles consisting of pure PC vesicles showed the same adherence as PS/PC vesicles. These observations indicated that annexin I_N–V_C once bound to the lipid membrane exposed a second lipid binding site distinct from the annexin V binding site.

The necessity of an uncovered opposing membrane surface for bound annexin I_V–V_C to interact with this surface was shown by using blood coagulation factor Va. Ellipsometry allowed the separate covering of two phospholipid surfaces with different proteins and measurement of the interactions between the two covered surfaces. Figure 6 illustrates that shielding of the phospholipid surface of the vesicles by factor Va resulted in a more than 95% decrease of vesicle binding to the planar phospholipid bilayer that was covered with the chimera.

Visualization of Vesicle Aggregation. In Figure 7, sonicated vesicle preparations without annexin (A), with annexin V (B), and with annexin I_N–V_C (C) are visualized with cryo-electron microscopy. Control vesicles were stable and showed no significant increase in size for at least 7 h (not shown). Adsorption of annexin V to the vesicles did not change size or appearance. The presence of annexin I_N–V_C (C) induced large multilamellar aggregates, with loss of intervesicular membranes, indicating membrane fusion.

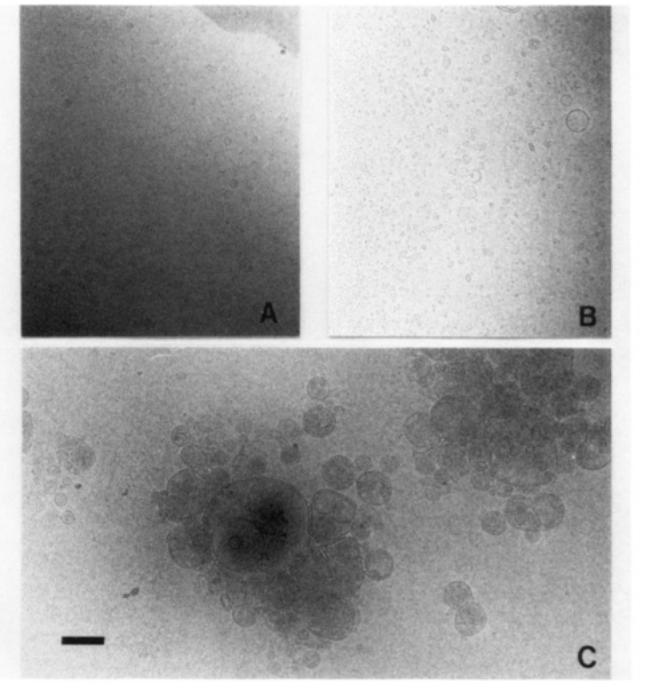

FIGURE 7: Cryo-electron micrographs of aggregated vesicles. 1 mM 20% PS and 80% PC vesicles (A) with 20 μM annexin V (B) or with 20 μM annexin I_N–V_C (C) was vitrified by rapid plunging into liquid ethane and viewed with cryo-electron microscopy. Bar represents 200 nm. Buffer: 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 3 mM $CaCl_2$; T = 20 °C.

DISCUSSION

Ellipsometry as a New Method To Study Vesicle Aggregation. Ellipsometry permits accurate determination of the adherence of vesicles to a macroscopic surface, both for low vesicle concentrations and for low protein surface concentrations. It is possible to control and measure protein adsorption and relate surface coverage to vesicle adherence. The immobilized protein offers the advantage of separation of events occurring in free solution and at the surface. The presence of multiple phospholipid binding sites on annexin I_N–V_C was thus easily demonstrated. This approach also allowed the study of interaction of pure PC vesicles with membrane-bound annexin I_N–V_C.

Adsorption Kinetics of Vesicles. The mean vesicle diameter was 25 nm, and from the Stokes–Einstein relation, a diffusion constant $D = 1.7 \times 10^{-7}$ cm^2·s^{-1} can be calculated for these vesicles. The theoretical transport rate limit of vesicles, for the stirring conditions used in the ellipsometer, was determined by comparison with transport rate-limited prothrombin adsorption. The transport-limited adsorption rate, $d\Gamma/dt$ (μg·cm^{-2}·s^{-1}), is proportional to the mass-transfer coefficient, Δ (cm·s^{-1}), times the bulk concentration, C (μg·cm^{-3}), and Δ is proportional to $D^{2/3}$ (Levich, 1962). With the stirring conditions used in this paper, the mass-transfer coefficient of prothrombin was 15×10^{-4} cm·s^{-1}. Using values of $D = 6.2 \times 10^{-7}$ cm^2·s^{-1} for prothrombin (Lim et al., 1977) and $D = 1.7 \times 10^{-7}$ cm^2·s^{-1} for vesicles, one obtains a value $\Delta = 6.3 \times 10^{-4}$ cm·s^{-1} for the mass-transfer coefficient of vesicles. This would imply a value of $d\Gamma/dt = 2.5 \times 10^{-3}$ μg·cm^{-2}·s^{-1} for diffusion-limited adsorption of a 5 μM vesicle solution. The initial adsorption rate of vesicles to a bilayer fully covered with annexin I_N–V_C was 2.6×10^{-3} μg·cm^{-2}·s^{-1}, i.e., equal to the diffusion limit. After flushing of the cuvette, less than 1 molecule of annexin I_N–V_C was present to every 30 vesicles. This excludes a contribution of annexin I_N–V_C adsorption to the observed vesicle adsorption rate.

Protein–Protein Interactions versus Multiple Phospholipid Binding Sites. It could be hypothesized that vesicle aggregation occurred through interaction of annexin I_N–V_C present on both adhering phospholipid surfaces. Planar bilayers covered with annexin I_N–V_C could, however, bind vesicles without substantial amounts of annexin I_N–V_C in the buffer, indicating that aggregation occurred through direct interaction of annexin I_N–V_C bound to the planar lipid bilayer with the opposing phospholipid. An alternative explanation is offered by the adsorption of annexin I_N–V_C dimers. Annexin VII shows Ca^{2+}-dependent self-association, which was proposed as a mechanism for its aggregation (Creutz et al., 1979). Annexins show weak monomer–dimer equilibria with K_d values of (0.3–1.3) $\times 10^{-3}$ M (Ahn et al., 1988), which exceed by 3–6 orders of magnitude the protein concentrations used in the present study. Annexin V was shown to adsorb as a protein

monolayer (Andree et al., 1990; Mosser et al., 1991), and in view of the similar values observed for Γ_{max}, this was also the case of annexin I_N–V_C. Therefore, it is concluded that formation of annexin I_N–V_C dimers, in solution or at the surface, is not a plausible model for vesicle aggregation. This leads to the model that a second phospholipid binding site was formed on a phospholipid surface covered with annexin I_N–V_C. Our observation that vesicle adherence was strictly proportional to the annexin I_N–V_C surface concentration (see Figure 4) indicates that annexin I_N–V_C bound to the surface expressed this binding site.

The Second Phospholipid Binding Site Differs from the Annexin V Phospholipid Binding Site. Pure PC membranes showed no annexin I_N–V_C binding, while vesicles of pure PC adhered to preadsorbed annexin I_N–V_C. This indicates a different phospholipid specificity of the second phospholipid binding site compared to the annexin V binding site. Similar binding of PC vesicles to rabbit lung annexins I and 33 000 phopholipid binding protein (=annexin VIII) adsorbed on anionic liposomes was demonstrated earlier (Tsao, 1990). We observed only an indirect effect of Ca^{2+} concentration on vesicle adherence as the Ca^{2+} concentration appeared only to control annexin I_N–V_C adsorption. Therefore, it is concluded that the second phospholipid binding site probably had a reduced Ca^{2+} requirement compared to the first phospholipid binding site of annexin V.

Aggregation by Another Chimera of Annexins I and V. Ernst and co-workers constructed a chimeric protein with amino acids 41–118 of annexin I, containing the first repeat plus 8 residues of the N-tail, and amino acids 92–320 of annexin V (Ernst et al., 1991). This chimera aggregated phospholipid vesicles, suggesting a specific function of the first repeat of annexin I for vesicle aggregation. The chimera of the present study shows that the first repeat of annexin I was not mandatory to confer aggregating potency to annexin V. The structural basis for this property might be beared by amino acid residues 41–46 of annexin I, which are shared by both chimerae. In light of the work of Huber and co-workers (Huber et al., 1990a,b), the possibility arises that the aggregating activity was modulated by a more global conformation of annexin V, which in turn was regulated by the N-terminal tail. Huber et al. (1990a,b) showed by 3-D structure analysis of annexin V crystals that the repeat organization of the primary structure was reflected in the 3-D conformation. Domains 1, 2, and 4 each contained a Ca^{2+} binding site, and all three sites were located at the convex face of the molecule, which is directed toward the phospholipids during membrane binding (Huber et al., 1990b; Meers, 1990). The Ca^{2+} binding sites are believed to be directly involved in membrane attachment. The N-terminal tail was clamped by noncovalent interactions to domains 1–4. In the case of the chimeric protein, this interaction could be disturbed, resulting in more rotational freedom for domains 1 and 4. This again could give rise to the apical exposure of a phospholipid binding site by the phospholipid-bound chimera, a phenomenon that was suppressed in annexin V.

Clustering of Annexin I_N–V_C. Considerably more Ca^{2+} was required for aggregation of vesicles measured with turbidity (Figure 3) than for adherence of vesicles measured in the ellipsometer (Figure 5). In a previous paper (Andree et al., 1992), we reported that annexin V required 3 mM Ca^{2+} for half-maximal binding to small vesicles. This was 10–15 times more than required for half-maximal binding to the planar bilayers (0.2 mM). We also showed that lipid-bound annexin V formed planar rigid clusters on phospholipid membranes with low curvature. It was inferred that high curvature counteracts the formation of such clusters and that this explains the higher Ca^{2+} requirement for binding to high-curvature surfaces. The increased Ca^{2+} concentration required for aggregation, compared to the adherence of vesicles to the bilayer, probably reflects the Ca^{2+} requirement for binding to high-curvature membranes.

Vesicle adherence was proportional to the surface coverage of annexin I_N–V_C. If one single annexin I_N–V_C (surface area in plane of adsorption = 25 nm^2) would bind one vesicle (surface area = 600 nm^2), maximal binding of vesicles would occur at approximately 5% of the maximal surface coverage of annexin I_N–V_C. Since considerably more annexin I_N–V_C was required, it was suggested that multiple annexin I_N–V_C molecules were involved in adhering a single vesicle either by increasing the affinity for the vesicles or by formation of large (>25 molecules) protein islands. Annexin V coadsorption next to annexin I_N–V_C presumably resulted in a random "spacing" of annexin I_N–V_C on the surface, interfering with annexin I_N–V_C clustering. The inhibitory effect of coadsorption of annexin V, therefore, indicates that clusters of annexin I_N–V_C were involved in vesicle adherence.

Membrane Fusion. Membrane fusion was also observed for several members of the annexin family (annexins, I, II, and VII). These proteins required low Ca^{2+} concentrations to induce fusion (Nir et al., 1987; Drust & Creutz, 1988). In these resports, vesicles were used with high percentage of PS or PE, and mixtures were perturbed with arachidonic acid, or experiments were performed at low pH. Vesicle fusion could be inhibited by addition of phosphatidylcholine. It is not clear whether addition of PC inhibited vesicle fusion or just binding of the annexin. The size of vesicles in the present study was stable for at least 7 h as determined with cryo-electron microscopy. Vesicles with 80% PC at neutral pH were aggregated by annexin I_N–V_C, although high (>1 mM) Ca^{2+} concentrations were required. These aggregates grew up to 1 μm in size and became multilamellar. These observations indicate specific membrane fusion by annexin I_N–V_C, which is presently under further investigation.

Conclusions. A chimera of amino acids 1–45 of annexin I and amino acids 19–320 of annexin V was constructed (annexin I_N–V_C). This protein bound to single phospholipid bilayers at identical Ca^{2+} concentrations as annexin V, but aggregated and fused vesicles. Annexin I_N–V_C contained a second phospholipid binding site capable of binding pure PC vesicles. Aggregation probably required two-dimensional clusters of adsorbed annexin I_N–V_C.

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