Calcium and Membrane-Binding Properties of Monomeric and Multimeric Annexin II†

Thomas C. Evans, Jr., and Gary L. Nelsestuen*

Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108

Received June 21, 1994; Revised Manuscript Received August 24, 1994®

ABSTRACT: The calcium-dependent interaction of several annexins with membranes was studied. A novel technique was developed that allowed estimation of calcium binding to aggregating systems. This consisted of immobilized phospholipids on phenyl-Sepharose. Proteins associated with this affinity gel in the presence of radioactive calcium and were eluted with the same buffer containing excess EGTA. This produced an elution profile with a peak of excess calcium. Protein extinction coefficients were estimated in order to quantitate the protein more accurately. Association of annexin II with the membrane was of very high affinity and involved a calcium stoichiometry of 11 ± 1 at 12.5 μM free Ca^{2+} and 10 ± 2 at 50 μM free Ca^{2+}. $(AII)_2(p11)_2$, a heterotetramer of two annexin II and two p11 subunits, bound 12 ± 1 Ca^{2+} at 12.5 μM Ca^{2+} and 15 ± 1 Ca^{2+} at 50 μM Ca^{2+}. These stoichiometries showed a pattern of "all or none" calcium binding where the number of calcium ions bound to a protein−membrane complex was virtually independent of the free calcium concentration or the density of protein on the membrane. $(AII)_2(p11)_2$ contains two annexin II subunits so that calcium stoichiometry was not directly related to the number of potential sites. $(AII)_2(p11)_2$ required less calcium to support membrane binding than did annexin II. Thus, dimerization of the membrane binding unit may be needed for annexins to function at intracellular calcium levels. Annexin VI contains twice as many putative calcium binding units as annexin V and the same pattern of behavior occurred for this pair of proteins. At 25 μM free calcium, $(AII)_2(p11)_2$ alone bound no detectable calcium (<0.1 mol of calcium/mol of protein) and annexin II bound only 0.3−0.6 calcium ions. These results suggested a general pattern of behavior for annexin proteins where each component, protein and membrane, provides a part of the many calcium binding sites. Partial calcium binding sites on the individual components may not correlate precisely with calcium binding sites of the complex.

The annexins are a family of proteins that bind acidic phospholipids in a calcium-dependent manner [for reviews, see Klee (1988), Raynal and Pollard (1994), and a book edited by Moss (1992)]. Although their functions are not clearly known, they show impact on subcellular endosome distribution (Harder & Gerke, 1993), exocytosis (Drust & Creutz, 1988), inhibition of phospholipase A_2 (Brugge, 1986; Davidson et al., 1987), calcium release in sarcoplasmic reticulum (Diaz et al., 1990), bundling of actin (Gerke & Weber, 1984), and inhibition of blood coagulation (Reutelingsperger et al., 1985). They also bind DNA (Boyko et al., 1994) and may form voltage gated ion channels (Burns et al., 1989; Pollard & Rojas, 1988; Rojas et al., 1990).

While most annexins contain four homologous repeats of a 70 amino acid sequence, annexin VI and lipocortin 85, now known as $(AII)_2(p11)_2$[1] each contain eight. Each repeating unit contains a 17 amino acid segment referred to as the endonexin fold and which may be involved in calcium association (Kretsinger & Creutz, 1986).

Even though annexins are normally found within the cell, the calcium required for protein binding to membranes can range from 6 to 150 μM or higher, depending on the annexin and membrane used (Bazzi et al., 1992). The lower value seems relevant for intracellular calcium while the larger does not. Although the use of phosphatidylethanolamine (PE) as the neutral phospholipid lowers the calcium required for membrane association (Bazzi et al., 1992), some annexins still show a calcium requirement that appears to be in excess of intracellular calcium. Results for annexin V and VI show that the latter has a lower calcium requirement than the former. This may correlate with the presence of eight repeating units in annexin VI but four in annexin V. The relationship of annexin II and $(AII)_2(p11)_2$ provides a unique opportunity to test this correlation since the latter contains eight repeating units made from two annexin II subunits.

The relationship between the number of homologous repeats and calcium binding stoichiometry is not known. Early studies gave values of about two calcium ions/annexin II molecule, and it was assumed that $(AII)_2(p11)_2$ would give exactly twice that value (Glenney, 1986; Glenney et al., 1987). X-ray crystallography shows up to five calcium sites on annexin V (Huber et al., 1990) and six sites on annexin I (Weng et al., 1993). However, sequence analysis suggests at least two other potential sites in annexin I (Weng et al., 1993) so that the potential calcium binding stoichiometry for a molecule such as $(AII)_2(p11)_2$ could be 16. Further-

† Supported by Grant GM 38819 from the National Institutes of Health.

® Abstract published in *Advance ACS Abstracts,* October 1, 1994.

[1] Abbreviations: SUVs, small unilamellar vesicles; LUVs, large unilamellar vesicles; HUVs, huge unilamellar vesicles; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Tris, tris-(hydroxymethyl)aminomethane; PS, phosphatidylserine; PE, phosphatidylethanolamine; dansyl-PE, *N*-dansyl-L-α-dipalmitoylphosphatidylethanolamine; PC, phosphatidylcholine; cpm, counts per minute; PL, phospholipid; EDTA, ethylenediaminetetraacetic acid; O-PE, *O*-phosphorylethanolamine; $(AII)_2(p11)_2$, a heterotetramer of two annexin II and two p11 subunits (Gerke & Weber, 1984).

0006-2960/94/0433-13231$04.50/0

more, other sites with different sequence characteristics may exist so that calcium binding to the protein alone may not detect all the calcium binding sites present in a protein−membrane complex.

This study was undertaken to further characterize the membrane-association behavior and calcium binding stoichiometries of several annexins. These properties may detect general patterns of behavior that suggest relevant cellular characteristics of the proteins. Due to the tendency of some annexins to cause aggregation of membranes it was necessary to develop novel methodology. The results showed common patterns of behavior for the annexins during protein−membrane interaction. All annexins tested bound a very large number of calcium ions (10−15 depending on the individual protein). Calcium binding displayed an "all or none" behavior that implied a large Hill coefficient (equal to the calcium stoichiometry). Proteins with eight repeating units bound only about 30% more calcium than those with four. They also required lower calcium concentration for membrane interaction. Assembly of annexins into complexes with eight repeating units may be necessary for function at intracellular calcium levels.

EXPERIMENTAL PROCEDURES

Materials. Bovine brain phosphatidylserine (PS), PE, and egg yolk phosphatidylcholine (PC) were purchased from either Sigma Chemical Co. or Avanti Polar Lipids, Inc., and were of the highest purity available (≥98%, suppliers estimate). Dipalmitoyl-*N*-dansyl-L-α-phosphatidylethanolamine (dansyl-PE) was purchased from Sigma Chemical Co. $^{45}CaCl_2$ (34 mCi/mg) was purchased from Du Pont NEN Research Products. Phenyl-Sepharose CL-4B was purchased from Sigma Chemical Co. Bovine brain annexin V and VI were kindly provided by Mohammed Bazzi and Doug Plager, purified as described previously (Bazzi & Nelsestuen, 1991b). Bovine prothrombin fragment I (residues 1−156 of prothrombin) was kindly provided by Ruth Schwalbe and was purified as described (Nelsestuen, 1984). Other chemicals and reagents were purchased from Sigma Chemical Co. and were of the highest grade available. Annexin II and $(AII)_2(p11)_2$ were purified from bovine lung as described previously (Khanna et al., 1987). Unless otherwise indicated experiments were performed at 22 ± 2 °C, and all aqueous solutions were buffered with 50 mM Tris, pH 7.5, containing 100 mM NaCl and 0.01% NaN_3.

Determination of Protein Extinction Coefficients. The refractive index increment for each protein was measured with a Phoenix differential refractometer (Brice & Halwer, 1951) that had been calibrated with a solution of KCl (11.0 mg/mL); the latter has a refractive index increment of 0.1336 Δn/g/mL. Protein concentration was 1−3 mg/mL. At least five readings were averaged at each of two wavelengths (436 and 546 nm). The absorbance readings for the proteins were measured with a 2-nm bandpass in a Beckman DU-70 spectrophotometer and were corrected for light scattering by the following relationship:

$$A_{280corr} = A_{280} - A_{320}(320^4/280^4) \qquad (1)$$

$A_{280corr}$ is the corrected absorbance at 280 nm, A_{280} is the measured absorbance at 280 nm, A_{320} is the measured absorbance at 320 nm, and the term $320^4/280^4$ estimates the scattering intensity at 280 nm from the value at 320 nm. Extinction coefficients were then calculated assuming a refractive index increment for proteins of 0.185 and 0.191 Δn/g/mL at 546 and 436 nm, respectively (Brice & Halwer, 1951).

Preparation of Phospholipid Vesicles. Two methods were used to produce vesicles of different sizes. The appropriate mixtures of phospholipid were first dried from organic solution under a stream of nitrogen gas. For the production of huge unilamellar vesicles (HUVs), the dried phospholipids were dissolved in ether and then injected into a solution of warm buffer as described previously (Deamer & Bangham, 1976). Small unilamellar vesicles (SUVs) were prepared by dispersing the dried phospholipids in buffer followed by sonication and gel filtration as described previously (Bazzi & Nelsestuen, 1987; Huang, 1969). The concentration of phospholipid vesicles were determined using a phosphorus assay (Chen et al., 1956) and assuming a phosphorus to phospholipid weight ratio of 1:25. The diameter of each vesicle preparation was measured using quasielastic light scattering on an LSA 2 instrument with a Langley Ford Model 1096 Correlator. Although individual preparations were heterogeneous, the average diameters were typically 40−50 nm for SUVs and 260−480 nm for HUVs. This diameter was constant during the time period that the vesicles were used (within 5 days).

Protein Binding to Phospholipid Vesicles. Light scattering at 90° to the incident light and 320 nm was used as described previously (Nelsestuen & Lim, 1977). In a typical experiment, vesicles and protein were added to the standard buffer. Binding of the protein to SUVs was detected as an increase in the weight average molecular weight of the scattering species in solution. This was quantitated using the following:

$$I_2/I_1 = (M_2/M_1)^2[(\partial n_2/\partial c_2)/(\partial n_1/\partial c_1)]^2 \qquad (2)$$

where I_2 is the light scattering intensity of the protein phospholipid complex, I_1 is the light scattering intensity of the vesicles alone. These values were corrected for light scattering by buffer and free protein and for dilution by additions to the solution. M_1 and M_2 are the weight average molecular weights of the vesicles before and after protein binding, respectively, and $(\partial n_2/\partial c_2)/(\partial n_1/\partial c_1)$ is the ratio of refractive index increments of the two species calculated as described (Nelsestuen & Lim, 1977). This latter term has a relatively small impact on the total signal. In general, these measurements gave signal to noise of at least 20:1. Replicate experiments gave a similar level of reproducibility for the same protein and vesicle preparations.

Phospholipid Affinity Column. The affinity gel was prepared by a previously described method (Kaetzel et al., 1989) with slight modifications. The phenyl-Sepharose was washed with chloroform prior to use and was equilibrated in 20 mM Tris buffer, pH 7.5. Phospholipids were dried from a chloroform solution under a stream of nitrogen gas. The phospholipids were resuspended in 20 mM Tris buffer, pH 7.5, and the solution was sonicated for 3 min with intermittent bursts. The washed phenyl-Sepharose was mixed with the sonicated vesicles (about 2 mg of phospholipid per mL of phenyl-Sepharose), and the mixture was gently agitated overnight. Phospholipids that were not associated with the matrix were removed by washing the

column with at least 10 column volumes of standard buffer containing 50 μM $CaCl_2$.

Determination of Calcium Binding Stoichiometry. In a typical experiment protein (300−500 μg) was loaded onto a column (5 cm × 0.7 cm) of the phospholipid affinity gel in the presence of a high calcium concentration (1 mM). The column was then equilibrated in standard buffer which contained calcium of known specific radioactivity (about 0.5 mCi/mmol). Calcium concentration and specific activity of the stock solutions were determined as described by Bazzi and Nelsestuen (1991b). The procedures ensured a low level ($\leq$1 μM) of contaminating calcium in the buffers and protein (Bazzi & Nelsestuen, 1990). Standard calcium chloride solutions were made from solid $CaCO_3$ which had been heated overnight at 140 °C and the pH adjusted to 5.5 with HCl. Column fractions were collected and assayed for $^{45}Ca^{2+}$ and protein. The same buffer containing EGTA (10 mM) was then used to elute the protein from the column. The elution profile therefore showed a constant calcium background with a peak that eluted with the EGTA. The peak represented calcium which had been bound to the column. Calcium that was bound to the protein was obtained by subtracting the calcium elution profile of an identical experiment that was conducted without protein. This experiment was always run with the same column and immediately before or after the experiment run in the presence of protein. Protein eluted from the column was determined with the dye binding assay described by Bradford (1976) that had been standardized to the specific protein used. Calcium binding stoichiometry was determined for the various proteins by dividing the calcium bound to the protein by the amount of protein eluted from the column by the EGTA-containing buffer. The molecular weights used to calculate stoichiometries were as follows: annexin II, 36 000 g/mol; $(AII)_2(p11)_2$, 85 000 g/mol (Gerke & Weber, 1984); annexin V, 32 000 g/mol (Bazzi & Nelsestuen, 1991b); and annexin VI, 68 000 g/mol (Davies et al., 1984).

Calcium Binding by the Hummel−Dreyer Technique. Gel filtration chromatography according to Hummel and Dreyer (1962) was performed on annexin II and $(AII)_2(p11)_2$ in the absence of phospholipid vesicles. The columns were composed of Sephacryl S-100 HR (1 cm × 25 cm) and were equilibrated with standard buffer containing 25 μM $CaCl_2$, plus an appropriate amount of $^{45}CaCl_2$. Approximately 400 μg of protein was diluted to give buffer and $^{45}CaCl_2$ concentrations equivalent to that of the column and a final protein concentration of 0.5−2 mg/mL. The sample was applied to the column and eluted with the same buffer. The calcium concentration in each fraction was determined by scintillation counting in a Beckman LS 5000 TD liquid scintillation counter. Protein concentration was measured using the dye binding assay described above.

Annexin Exchange Rate between Vesicles. The rate at which the annexins dissociated from vesicles to which they were bound was measured with a technique described previously (Bazzi & Nelsestuen, 1991a). In a typical experiment, 20 μg of protein was bound to 20 μg of HUVs (PS/dansyl-PE/PC, 25:5:70) in the presence of calcium and in a volume of 2 mL. Aromatic groups present in the protein were excited at 284 nm, and fluorescence energy transfer to the dansyl group in the membrane was monitored at 520 nm. Fluorescence intensity due to energy transfer was estimated from the following relationship:

$$\text{fluorescence energy transfer intensity} = [(I - I_f)100]/(I_i - I_f) \quad (3)$$

where I is the measured intensity, and I_f is the final fluorescence intensity when all protein had dissociated from the initial vesicle population. This was obtained after EGTA addition or from samples where the excess unlabeled vesicles were mixed before protein addition. Both methods gave the same value for I_f. I_i is the intensity at time zero, immediately after addition of the unlabeled vesicles. The latter was corrected for dilution and background from the added vesicles. At zero time, a 20-fold excess of unlabeled vesicles (PS/PC, 30:70) was added, and the decrease in fluorescence energy transfer was monitored as a function of time. Another cuvette with the same reactants, but no protein, was monitored simultaneously to detect changes due to photobleaching. The signal drift due to photobleaching was less than 6.5% of the original signal.

RESULTS

Protein Extinction Coefficients. In order to quantitate protein concentrations and allow accurate stoichiometry estimations, protein extinction coefficients at 280 nm were determined as described under Experimental Procedures. Values obtained for annexin II and $(AII)_2(p11)_2$ were 0.54 ± 0.02 and 0.68 ± 0.04 mL/mg cm^{-1}, respectively. The extinction coefficients of annexin V and annexin VI at 280 nm were 0.59 ± 0.03 and 0.70 ± 0.02 mL/mg cm^{-1}, respectively. The error estimates are standard deviations of at least five replicate readings at the two wavelengths used. These values were used to determine protein concentrations and to standardize the dye binding assay described by Bradford (1976). This assay gave indistinguishable color yields for the various annexins and bovine serum albumin. Consequently, the latter could be used as the standard for this assay.

Characteristics of Protein−Membrane Binding. The extent of annexin II and $(AII)_2(p11)_2$ binding to phospholipid vesicles was followed by 90° light scattering or fluorescence energy transfer. Both annexin II and $(AII)_2(p11)_2$ are known to cause aggregation of phospholipid vesicles, a process that greatly complicated data interpretation. Aggregation was easily distinguished from initial protein−membrane association. The latter gave a rapid increase in light scattering intensity that was stable. Aggregation appeared as a continuous and nonsaturable increase of intensity. Fortunately, aggregation required several minutes and was very sensitive to concentration. Aggregation was minimized by use of dilute solutions (less than 30 μg/mL), very large vesicles (greater than 250 nm diameter), and short time intervals.

Calcium titration of annexin II and $(AII)_2(p11)_2$ binding to vesicles was measured in dilute solution and at short time intervals that avoided aggregation (Figure 1). The calcium concentration at the midpoints of these titrations were 15 μM for $(AII)_2(p11)_2$ and 45 μM for annexin II. Titrations such as those shown in Figure 1 arise from a complex set of equilibria so that these midpoints do not represent single equilibrium constants. However, the titrations are useful for purposes of comparison. The differences between $(AII)_2(p11)_2$ and annexin II were made more apparent by the inset (Figure 1) which shows that $(AII)_2(p11)_2$ may bind to

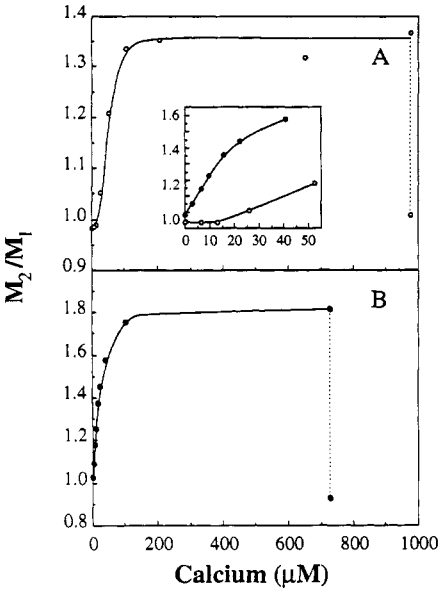

FIGURE 1: Calcium titration of annexin II and $(AII)_2(p11)_2$ interaction with membranes. (Panel A) Annexin II (3.5 μg) was mixed with SUVs (13.4 μg, PS/PC/PE, 30:30:40) in a final volume of 1.5 mL. Light scattering intensity of the solution was measured (I_1, eq 2). Calcium was added, and the intensity was measured (I_2, eq 2) immediately (≤0.5 min.). Excess EDTA was added to show complete reversibility (illustrated for the highest calcium concentration only). Protein–membrane binding was quantitated by M_2/M_1, the molecular weight ratio of the protein vesicle complex (M_2) to that of the vesicle alone (M_1). This was obtained from the light scattering measurements as described under Experimental Procedures. The midpoint of the titration occurred at 45 μM calcium. (Panel B) Single additions of calcium were made to solutions containing $(AII)_2(p11)_2$ (13.5 μg) and SUVs (15 μg, PS/PC/PE, 30:20:50) in 1.5 mL of buffer. In every case the addition of excess EDTA caused complete dissociation of the protein from the membrane (shown for the last determination). The titration midpoint was 15 μM. (Panel A, inset). Results for annexin II (●) and $(AII)_2(p11)_2$ (○) are compared at low calcium concentrations. In both titrations, replicate experiments gave values that were within the dimensions of the symbols.

membranes at intracellular calcium levels (≤10 μM) while annexin II may not.

An unusual property of annexin V and VI was that the saturation level of the membrane depended on the calcium concentration of the solution (Bazzi & Nelsestuen, 1991a). This unique behavior was "apparent" and was proposed to arise from a high calcium stoichiometry and cooperativity coupled with anticooperativity with respect to protein on the membrane. Annexin II behaved in a similar manner and attained an apparent membrane saturation that differed with the concentration of calcium in the solution (Figure 2). Below the saturation level, the amount of protein bound equaled the theoretical limit (dashed line, Figure 2), indicating that there was virtually no free protein. It was not possible to perform the same experiment with $(AII)_2(p11)_2$ because of aggregation at higher protein to phospholipid ratios.

The rate of dissociation of annexin II and $(AII)_2(p11)_2$ from membrane vesicles was determined by the exchange experiment described under Experimental Procedures. This exchange rate was extremely slow and protein complexed to SUVs always precipitated within the required time frame. Larger membrane vesicles of about 120 nm in diameter and produced by the extrusion technique (Hope et al., 1985) also aggregated over this time frame. However, annexins did not

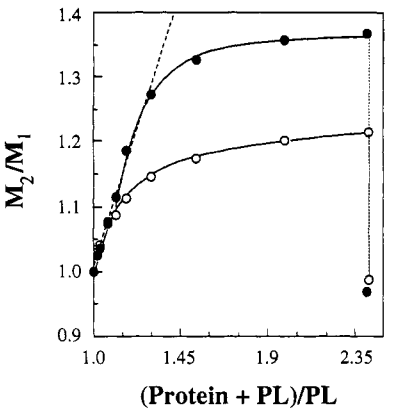

FIGURE 2: Protein titration of membranes at different calcium concentrations. Annexin II was mixed with SUVs (44 μg, PS/PC, 30:70) in 1.5 mL to produce the weight ratios shown. The light scattering intensity (I_1, eq 2) was measured. Calcium was added to a final concentration of 0.95 mM (●) or 0.225 mM (○), and the light scattering intensity (I_2) was measured again. Excess EDTA then caused complete dissociation of the complex as illustrated for the final determination in each titration. Each data point represents a separate experiment. The extent of association is reported as M_2/M_1, obtained as described under Experimental Procedures and in the legend to Figure 1. The dashed line represents the expected value if all the protein added to the solution were associated with the membrane. Replicate experiments gave indistinguishable results.

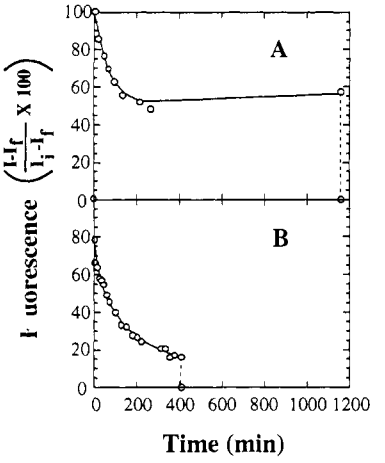

FIGURE 3: Dissociation of annexin II and $(AII)_2(p11)_2$ from vesicles. (Panel A) Annexin II (20 μg) was mixed with HUVs (20 μg, PS/dansyl-PE/PC, 25:5:70) in 2 mL of buffer containing 1 mM calcium. Fluorescence intensities were measured to obtain fluorescence due to energy transfer $[(I - I_f/I_i - I_f)100]$ as described in eq 3. This was adjusted to a value of 100 and is a measure of protein associated with the initial vesicle population. A large excess of unlabeled vesicles (420 μg of HUV, PS/PC 30:70) were added at zero time, and dissociation of protein from the initial vesicle population was monitored by decreased fluorescence energy transfer. (Panel B) The experiment was conducted as in panel A except that $(AII)_2(p11)_2$ (15 μg) was mixed with HUVs (15 μg of PS/dansyl-PE/PC, 25:5:70) in 2 mL of buffer containing 1 mM calcium. Appropriate fluorescence intensity measurements were made, and dissociation of the complex was monitored after addition of 175 μg of unlabeled HUVs (PS/PC, 30:70). Half of the $(AII)_2(p11)_2$ had dissociated after about 70 min. The dashed line shows the effect of adding EGTA to the samples. Replicate experiments gave similar results.

cause aggregation of HUVs at these low concentrations and over the time course of the experiment. These proteins exchanged between HUV membranes extremely slowly, and about 50% of annexin II had not redistributed after 12 h (Figure 3A). $(AII)_2(p11)_2$ exchanged at a somewhat faster rate, so that about 80% had exchanged after 6 h (Figure 3B). This very slow dissociation rate ($k_{off} \leq 10^{-4}$ s^{-1}) coupled

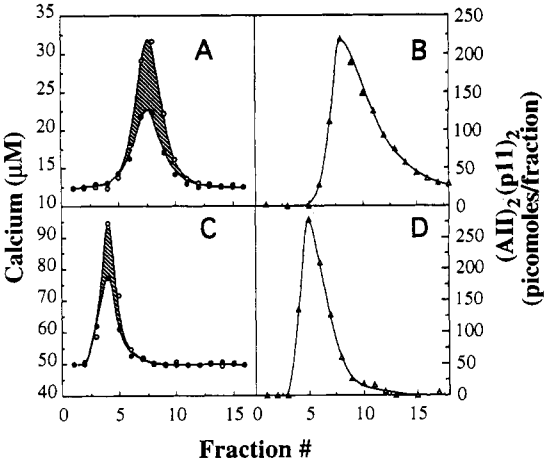

FIGURE 4: Calcium binding to $(AII)_2(p11)_2$. $(AII)_2(p11)_2$ was bound to a column composed of phenyl-Sepharose beads coated with phospholipids (PS/PE, 50:50). (Panel A) $(AII)_2(p11)_2$ (240 μg) was applied to the column (0.7 × 5 cm) which was washed with buffer containing calcium (12.5 μM calcium, 0.9 mCi/mmol). It was finally eluted with buffer containing calcium and EGTA (10 mM, applied at fraction 1). Calcium elution from the same column without protein (●) or with protein (○) was determined. The shaded region represents the calcium associated with the protein. (Panel B) The protein eluted from the column described in panel A is shown. (Panels C and D) Results of experiments run as in panels A and B, respectively, are shown. The difference was that the buffer contained 50 μM calcium (0.2 mCi/mmol) and 280 μg of $(AII)_2(p11)_2$ was applied to the column. The column size was (0.7 × 4.1 cm). Fraction size was 0.54 mL in all cases.

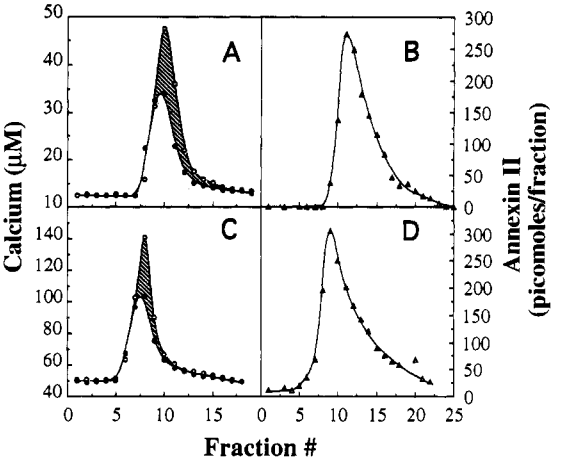

FIGURE 5: Calcium binding to annexin II. Experiments were conducted as in Figure 4 except that annexin II (297 μg) was used instead of $(AII)_2(p11)_2$. (Panels A and B) Experiments were conducted with buffer containing 12.5 μM calcium (0.7 × 5.3 cm column), and the experiment was conducted as described in Figure 4 (panels A and B). (Panels C and D) These experiments were conducted as in panel A and B, except that 50 μM calcium was used (0.7 × 5 cm column). Calcium associated with the column in the presence (●) and absence (○) of annexin II was determined. The shaded regions of panels A and C represent calcium bound to the protein. Annexin II concentrations (△) were obtained from the dye binding assay described under Experimental Procedures.

with rapid initial binding (≤15 s half-life at 0.1 μM protein corresponded to $k_{on} \geq 1 \times 10^5$ $M^{-1}s^{-1}$) gave a very high binding affinity ($K_A \geq 10^9$ M^{-1}) for both annexins. These numbers indicate high affinity but are not presented as a single equilibrium constant for all binding sites. As reported previously (Bazzi & Nelsestuen, 1991a), it is probable that the K_{eq} of individual protein−membrane binding events vary with the protein density on the membrane.

Calcium-Binding Measurements. Aggregation precluded the estimation of calcium binding by most common methodologies. However, immobilization and sequestering the phospholipid in a gel matrix prevented the aggregation. Figures 4 and 5 illustrate typical results of the affinity chromatography method that was developed. Figure 4A shows calcium elution in the presence and absence of $(AII)_2(p11)_2$. The shaded region represents the excess calcium which was assumed to have been bound to protein−membrane complexes. Figure 4B shows the elution of protein from the column. Trailing of the protein peak sometimes occurred (Figures 4B and 5D). However, this accounted for less than 15% of the protein eluted from the column and was completed 10−15 fractions beyond the main peak. Calcium binding stoichiometry was calculated from the protein content of all fractions that contained detectable protein. Total yields of the protein applied to the affinity column ranged from 40% to 80%. Neither the percent yield nor the extent of protein trailing seemed to influence the estimated calcium binding stoichiometry. Control experiments detected no protein interaction with a gel matrix column that did not contain phospholipids (not shown).

The peak of excess calcium, divided by the total protein eluted from the column, gave a calcium to protein ratio for $(AII)_2(p11)_2$ of 12 ± 1 at 12.5 μM calcium (Figure 4A,B and Table 1) and 15 ± 1 (Figure 4C,D and Table 1) at 50 μM calcium. Two different calcium concentrations were used to determine whether calcium stoichiometry varied with free calcium. Multiple experiments showed that a 30% difference in the amount of annexin bound per milliliter of gel did not significantly affect the stoichiometries. The calcium concentrations were chosen from titration curves (Figure 1) to represent one point that was well below and one that was above apparent saturation. The concentrations of calcium therefore differed for the various annexins (Table 1). The results suggested that calcium binding per protein was essentially "all or none" and that membrane association was always accompanied by binding the full complement of calcium. This property was consistent with the model of

Table 1: Calcium Binding Stoichiometries

protein	[calcium] (μM)[a]	calcium binding stoichiometry (mol of Ca^{2+}/ mol of protein)	reported values
		I. Protein−Membrane Complex[b]	
annexin II	12.5	11 ± 1 (3)	2 (Glenney, 1986; Glenney
	50	10 ± 2 (3)	et al., 1987)
lipocortin 85	12.5	12 ± 1 (3)	
	50	15 ± 1 (3)	
annexin VI	20	15 ± 2 (3)	9 (Bazzi & Nelsestuen, 1991b)
	100	16 (1)	
annexin V	12.5	12 ± 1 (3)	5 (Huber et al., 1990)
			8 (Bazzi & Nelsestuen, 1991b)
prothrombin fragment I	200	12 (1)	8−11 (Sommerville et al., 1986)
calmodulin	12.5	4.7 ± 0.5 (3)	4 (Lin et al., 1974)
		II. Free Protein[c]	
annexin II	25	0.3 ± 0.01	0 (Glenney, 1986)
+ 5 mM Mg		0.6 ± 0.01	
lipocortin 85	25	0 ± 0.1	

[a] The calcium concentation at which the affinity column was equilibrated. [b] Stoichiometry obtained as described under Experimental Procedures, error represents the standard deviation of the number of experiments given in parentheses. [c] Stoichiometry were determined as described in the legend to Figure 6.

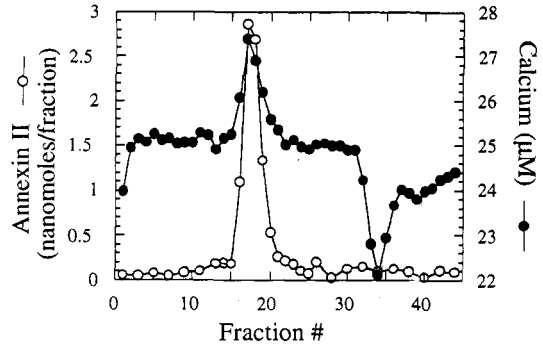

FIGURE 6: Calcium binding to annexin II in the absence of phospholipid. Annexin II (525 μg) was applied to a Sephacryl S-100 HR column (1 × 26 cm) equilibrated in standard buffer containing 5 mM $MgCl_2$ and 25 μM $^{45}CaCl_2$ (0.5 mCi/mmol). Protein was measured by the dye binding assay. The elution profiles of protein (○) and calcium (●) are shown. A baseline for calcium was obtained from the average of fractions 2−13 and 24−31. This was subtracted from the calcium in the four peak fractions (16−19) to determine excess calcium associated with the protein. Excess calcium divided by the protein concentration in each of the four fractions gave four calcium to protein ratios. The average value was 0.60 ± 0.01.

protein−membrane binding which consists of a sequence of individual equilibria, each of which is highly cooperative ($n \geq 10$) with respect to calcium (Bazzi & Nelsestuen, 1991a). In effect, this model predicts that virtually all membrane-bound protein will be saturated with calcium even though the membrane can accommodate more protein molecules.

The same experiment was performed on three other annexins. Annexin II yielded a calcium stoichiometry of 11 ± 1 at 12.5 μM calcium and 10 ± 2 at 50 μM calcium (Table 1). The stoichiometries for annexin VI were 15 ± 2 at 20 μM calcium and 16 at 100 μM calcium (Table 1). Annexin V gave a stoichiometry of 12 ± 1 at 70 μM calcium (Table 1). These results showed a high calcium binding stoichiometry and the "all or none" calcium binding property for all annexins tested.

Other proteins of known calcium binding stoichiometry were also examined. At a free calcium concentration of 200 μM, membrane-bound prothrombin fragment 1 had 12 calcium ions per protein, a value that agreed well with previous studies (Table 1). Calmodulin was also amenable to this method since it binds to phenyl-Sepharose in a calcium-dependent manner (Gopalakrishna & Anderson, 1982). The stoichiometry of 4.7 ± 0.5 calcium ions per protein (Table 1) was in good agreement with the known stoichiometry of four (Lin et al., 1974).

Calcium binding to annexin II and $(AII)_2(p11)_2$ was also measured in the absence of phospholipids. At 25 μM calcium, annexin II bound (0.3−0.6) ± 0.01 calcium ions per protein (Figure 6 and Table 1). In contrast, calcium binding to $(AII)_2(p11)_2$ was not detectable with a limit of <0.1 calcium ions per protein molecule (Table 1). These results were in good agreement with previous studies showing that the annexins by themselves bind little or no calcium at micromolar calcium concentrations (Bazzi & Nelsestuen, 1991b; Glenney, 1986; Shadle et al., 1985).

Previous studies showed that magnesium did not support annexin−membrane binding. In fact, magnesium increased the calcium required to support membrane interaction (Bazzi & Nelsestuen, 1991b), possibly by ionic shielding of the polyanionic membrane surface, thereby decreasing calcium attraction. In contrast, the calcium bound to free annexin II not only was very specific for calcium but was not inhibited by a 200-fold excess of magnesium (5 mM) in the buffer (Table 1). This suggested that the calcium binding site(s) on the protein may not be polyionic so that they were not shielded to a significant degree by polyvalent cations in the solution. In fact, several calcium binding sites identified on annexin V include a single carboxyl group (Huber et al., 1990).

DISCUSSION

This study compared two pairs of annexin proteins which are related by having four or eight homologous repeating units. General patterns of behavior became evident, which may be important for biological functions. New methods were developed that avoided the problems presented by annexin-induced vesicle aggregation.

Analysis of an interaction by equilibrium expressions requires a nonaggregated system. Aggregation produces a two-phase mixture where a simple equilibrium may not apply. Furthermore, variable characteristics of aggregates prevent systematic characterization. In the course of these investigations, measurement of calcium binding to annexin II and $(AII)_2(p11)_2$ was initially attempted by gel filtration using the method of Hummel and Dreyer (1962). However, protein and vesicle mixtures did not pass through the column due to aggregation. While use of low concentrations and very large vesicles (10 μg of annexin II and 10 μg of HUVs/mL) gave no detectable aggregation in 2 h (Figure 3), determination of metal ion binding by gel filtration required much higher concentrations which always aggregated quickly. In order to circumvent aggregation, a phospholipid affinity column was developed. Although alleviating many of the experimental difficulties associated with aggregation in solution, it raised other concerns. For example, the phospholipids could be present as a monolayer that coated the gel beads, or they could be present as bilayer vesicles that were trapped in the gel matrix. Nevertheless, the calcium binding stoichiometries of several known proteins were consistent with results obtained for protein and vesicles in solution, and the binding of the annexins to the column was dependent on the presence of phospholipids and calcium. These properties implied that the phospholipids in the column were similar to vesicles with respect to annexin binding.

The most striking features of calcium binding to the annexins was the large stoichiometry and the almost complete dependence on the presence of phospholipids. A popular model shows annexins binding to phospholipid vesicles through calcium ions (Huber et al., 1990), and this large number of contact points should produce very high affinity. In fact, the results showed no appreciable free annexin II as long as the membrane was below its apparent saturation level for the calcium concentration used (Figure 2). Furthermore, exchange rates for both annexin II and $(AII)_2(p11)_2$ occurred over a time course of hours (Figure 3). If the calcium contacts are treated as electrostatic interactions, each should stabilize membrane binding by about 1−2 kcal/mol (Mosior & McLaughlin, 1992), and 7−13 calcium contacts per protein would be sufficient to provide the tight binding to vesicles.

The structure of annexin I, determined by X-ray crystallography (Weng et al., 1993), showed six calcium ions per protein with two more potential sites for a possible stoichi-

ometry of eight calcium ions. The larger value is still slightly less than stoichiometries of 10−12 obtained for annexin V and II in this study but may be within the error limits of the measurements. Alternatively, it is conceivable that some calcium binding sites at the protein−phospholipid interface utilize the acidic phospholipids in the membrane as the major donator of ligands, so that structural studies with the free protein may not show all the calcium sites.

For annexin VI, the calcium binding stoichiometry obtained by this method (15−16) was higher than that obtained by equilibrium dialysis (about eight; Bazzi & Nelsestuen, 1991b). It is possible that the affinity column technique may be more accurate. Equilibrium dialysis provides stoichiometry from the total amount of protein present. If some protein were nonfunctional, the calcium stoichiometries would be low. In contrast, the affinity column only detected protein that was functional and which bound to the column. This larger number (16) actually correlated with twice the sum of observed and potential calcium sites found in an annexin containing four repeating units (Weng et al., 1993).

While simple arithmetic would suggest twice as much calcium bound to proteins that contain eight repeating units [annexin VI and $(AII)_2(p11)_2$] versus those with four (annexins II and V), the former bound only about 30% more calcium than the latter. Lack of a simple relationship is not surprising for annexins V and VI which do not have identical sequences, since the number of calcium ions per repeating unit may differ. Crystallographic data suggests that domain 1, the first 70 amino acid repeat of annexin V, binds three calcium ions (Huber et al., 1990), while the equivalent domain of annexin I binds only two (Weng et al., 1993). However, $(AII)_2(p11)_2$ contains two identical annexin II subunits so that the larger structure bound fewer than the full capacity of two monomers. This implied that, upon dimerization, the calcium binding properties of annexin II may change somewhat. Lack of a simple multiplier of calcium and membrane binding properties for $(AII)_2(p11)_2$ versus annexin II was also illustrated by a slower exchange of annexin II, the monomer, from the membrane (Figure 3). Slower exchange implies tighter binding. The dimer did require a lower free calcium concentration to support membrane interaction (Figure 1). Thus, three aspects of annexin−membrane interaction, (1) the equilibrium constant for protein−membrane binding at saturating calcium, (2) the number of calcium ions bound per protein, and (3) the calcium concentration needed to support protein−membrane interaction, did not vary exactly as one would expect for two structures related by a simple multiplier of two. This may suggest that calcium binding at the protein−membrane interface for annexin II may differ significantly from that of $(AII)_2(p11)_2$.

The number of repeating units in an annexin may be significant for its biological function. Annexin VI bound to PS/PE vesicles with a calcium midpoint of about 6 μM, whereas annexin V required about 60 μM calcium to bind to the same membrane (Bazzi et al., 1992). This same behavior was seen with $(AII)_2(p11)_2$ and annexin II which gave titration midpoints of 15 and 45 μM calcium, respectively. In fact, the differences were more pronounced at low calcium concentrations (Figure 1, inset). Expression of function may be regulated by the expression or availability of p11. That is, assembly to a protein with eight repeating units effectively reduced the calcium needed to levels that may be relevant to the intracellular environment during stimulation ($\leq$10 μM; Kretsinger, 1979). Other annexins could be regulated in a similar manner. Others have found that additional S-100 proteins bind to various annexins (Bianchi et al., 1992; Tokumitsu et al., 1992, 1993; Zeng et al., 1993). Annexin VI is unique in that it contains eight repeating units in a single polypeptide. Its membrane binding properties may therefore be independent of further assembly.

Calcium binding by annexin V and VI was previously found to be greatly enhanced by the presence of acidic phospholipids (Bazzi & Nelsestuen, 1991b). Neutral phospholipids will function but require very high calcium concentrations (Andree et al., 1990; Bazzi et al., 1992). In fact, calcium binding to $(AII)_2(p11)_2$ was not detected in the absence of acidic phospholipids (Table 1). In contrast, annexin II bound a detectable amount of calcium (0.3−0.6 mol of calcium/mol of annexin II at 25 μM free calcium) in the absence of phospholipid. This showed a surprising inverse relationship between calcium binding to the free protein versus calcium required to support protein−membrane interaction. That is, calcium binding properties of the annexin in the absence of phospholipids was not a good predictor of subsequent membrane association. It was also found that the presence of magnesium did not inhibit calcium binding to free annexin II. In contrast, the presence of magnesium raised the calcium concentration required to support calcium binding by the protein−membrane complex and to support annexin association to membranes (Bazzi & Nelsestuen, 1991b). Ionic shielding by magnesium should inhibit calcium association with the phospholipids (McLaughlin, 1989). Furthermore, the calcium requirement for membrane binding was found to vary with phospholipid composition by nearly 10^4 (Bazzi et al., 1992). These results suggested that initial contact between calcium and phospholipid may be important for protein−membrane association. Conversely, none of these results indicated that calcium−protein association must precede membrane interaction. Thus, initial interaction of free protein and calcium may or may not be required for membrane binding.

The calcium-binding properties require that, for a single protein−membrane binding event, the cooperativity coefficient for calcium be virtually equal to the calcium binding stoichiometry. For example, at 10 μM calcium, neither $(AII)_2(p11)_2$ nor phospholipid bound significant amounts of calcium. However, approximately 15 calcium ions were bound to the complex. The much lower cooperativity of total protein−membrane binding titrations (Figure 1) arise from anticooperativity with respect to protein density on the membrane surface (Bazzi & Nelsestuen, 1991a). However, in the cell, the annexins constitute about 2.5% of total protein (Hullin et al., 1989; Romisch et al., 1992). A protein to lipid weight ratio of about 3.6:1 (Alberts et al., 1989) would provide an 11:1 weight ratio of lipid to annexin. This low annexin density on the membrane should minimize protein−protein anticooperativity so that the response to calcium could display a cooperativity coefficient of 10 or more. This could produce threshold-type responses to calcium concentration in the cell.

An actual calcium bridge between protein and phospholipid has not been directly observed for the annexins. However, the X-ray crystal structure of serum amyloid P component (Emsley et al., 1994) shows a protein−calcium−phosphate bridge and may provide a relevant comparison.

In this case, simultaneous protein interaction with calcium and the phosphate ligand, phosphorylethanolamine (O-PE), involved a different number of protein−calcium ligands than were found in the protein−calcium complex without O-PE. Thus, if the annexins use protein−calcium−phosphate contacts, it is possible that the calcium ligands provided by the annexins will be somewhat different in the presence and absence of phospholipid.

This study characterized the calcium and membrane-binding properties of several members of a unique family of calcium response proteins. The novel properties displayed by the annexins may be important for cell regulation. For example, annexins can respond quickly to a calcium signal by rapidly associating with membranes. High cooperativity would allow unusual behavior due to the fact that each association event displays "all or none" binding. Protein−membrane complexes would then remain constant and static due to the very slow protein−membrane dissociation rate at constant free calcium. Finally, annexins can be rapidly dissociated from the membrane by removing the free calcium from solution. This type of behavior may provide important functions in cell calcium regulation.

ACKNOWLEDGMENT

I thank Dr. Mohammed Bazzi and Doug Plager for the generous gifts of annexin V and VI, and Dr. Ruth Schwalbe for the kind gift of bovine prothrombin fragment-1.

REFERENCES

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., & Watson, J. D. (1989) in *Molecular Biology of The Cell* (second ed.) p 88, Garland Publishing, Inc., New York and London.

Andree, H. A., Reutelingsperger, C. P., Hauptmann, R., Hemker, H. C., Hermens, W. T., & Willems, G. M. (1990) *J. Biol. Chem. 265,* 4923.

Bazzi, M. D., & Nelsestuen, G. L. (1987) *Biochemistry 26,* 115.

Bazzi, M. D., & Nelsestuen, G. L. (1990) *Biochemistry 29,* 7624.

Bazzi, M. D., & Nelsestuen, G. L. (1991a) *Biochemistry 30,* 7970.

Bazzi, M. D., & Nelsestuen, G. L. (1991b) *Biochemistry 30,* 971.

Bazzi, M. D., Youakim, A., & Nelsestuen, G. L. (1992) *Biochemistry 31,* 1125.

Bianchi, R., Pula, G., Ceccarelli, P., Giambanco, I., & Donato, R. (1992) *Biochim. Biophys. Acta 1160,* 67.

Boyko, V., Mudrak, O., Svetlova, M., Negishi, Y., Ariga, H., & Tomlin, N. (1994) *FEBS Lett. 345,* 139.

Bradford, M. M. (1976) *Anal. Biochem. 72,* 248.

Brice, B. A., & Halwer, M. (1951) *J. Opt. Soc. Am. 41,* 1033.

Brugge, J. S. (1986) *Cell 46,* 149.

Burns, L. A., Magendzo, K., Shirvan, A., Srivastava, M., Rojas, E., Alijani, M. R., & Pollard, H. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86,* 3798.

Chen, P. S., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28,* 1756.

Davidson, F. F., Dennis, E. A., Powell, M., & Glenney, J. J. R. (1987) *J. Biol. Chem. 262,* 1698.

Davies, A. A., Wigglesworth, N. M., Allan, D., Owens, R. J., & Crumpton, M. J. (1984) *Biochem. J. 219,* 301.

Deamer, D., & Bangham, A. D. (1976) *Biochim. Biophys. Acta 443,* 629.

Diaz, M. M., Hamilton, S. L., Kaetzel, M. A., Hazarika, P., & Dedman, J. R. (1990) *J. Biol. Chem. 265,* 15894.

Drust, D. S., & Creutz, C. E. (1988) *Nature 331,* 88.

Emsley, J., White, H. E., O'Hara, B. P., Oliva, G., Srinivasan, N., Tickle, I. J., Blundell, T. L., Pepys, M. B., & Wood, S. P. (1994) *Nature 367,* 338.

Gerke, V., & Weber, K. (1984) *EMBO J.* 227.

Glenney, J. (1986) *J. Biol. Chem. 261,* 7247.

Glenney, J. R., Jr., Tack, B., & Powell, M. A. (1987) *J. Cell Biol. 104,* 503.

Gopalakrishna, R., & Anderson, W. B. (1982) *Biochem. Biophys. Res. Commun. 104,* 830.

Harder, T., & Gerke, V. (1993) *J. Cell Biol. 123,* 1119.

Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812,* 55.

Huang, C. (1969) *Biochemistry 8,* 344.

Huber, R., Schneider, M., Mayr, I., Romisch, J., & Paques, E. P. (1990) *FEBS Lett. 275,* 15.

Hullin, F., Raynal, P., Ragab-Thomas, J., Fauvel, J., & Chap, H. (1989) *J. Biol. Chem. 264,* 3506.

Hummel, J. P., & Dreyer, W. J. (1962) *Biochim. Biophys. Acta 63,* 530.

Kaetzel, M. A., Hazarika, P., & Dedman, J. R. (1989) *J. Biol. Chem. 264,* 14463.

Khanna, N. C., Tokuda, M., & Waisman, D. M. (1987) *Cell Calcium 8,* 217.

Klee, C. B. (1988) *Biochemistry 27,* 6645.

Kretsinger, R. H. (1979) *Adv. Cyclic Nucleotide Res. 11,* 1.

Kretsinger, R. H., & Creutz, C. E. (1986) *Nature 320,* 573.

Lin, Y. M., Liu, Y. P., & Cheung, W. Y. (1974) *J. Biol. Chem. 249,* 4943.

McLaughlin, S. (1989) *Annu. Rev. Biophys. Chem. 18,* 113.

Mosior, M., & McLaughlin, S. (1992) *Biochemistry 31,* 1767.

Moss, S. E., Ed. (1992) *The Annexins*, Portland Press, London and Chapel Hill.

Nelsestuen, G. L. (1984) *Methods Enzymol. 107,* 507.

Nelsestuen, G. L., & Lim, T. K. (1977) *Biochemistry 16,* 4164.

Pollard, H. B., & Rojas, E. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85,* 2974.

Raynal, P., & Pollard, H. B. (1994) *Biochim. Biophys. Acta 1197,* 63.

Reutelingsperger, C. P. M., Hornstra, G., & Hemker, H. C. (1985) *Eur. J. Biochem. 151,* 625.

Rojas, E., Pollard, H. B., Haigler, H. T., Parra, C., & Burns, L. A. (1990) *J. Biol. Chem. 265,* 21207.

Romisch, J., Schuler, E., Bastian, B., Burger, T., Dunkel, F. G., Schwinn, A., Hartmann, A. A., & Paques, E. P. (1992) *Blood Coagulation Fibrinolysis 3,* 11.

Shadle, P. J., Gerke, V., & Weber, K. (1985) *J. Biol. Chem. 260,* 16354.

Sommerville, L. E., Resnick, R. M., Thomas, D. D., & Nelsestuen, G. L. (1986) *J. Biol. Chem. 261,* 6222.

Tokumitsu, H., Mizutani, A., Minami, H., Kobayashi, R., & Hidaka, H. (1992) *J. Biol. Chem. 267,* 8919.

Tokumitsu, H., Mizutani, A., & Hidaka, H. (1993) *Arch. Biochem. Biophys. 303,* 302.

Weng, X., Luecke, H., Song, I. S., Kang, S. D., Kim, S.-H., & Huber, R. (1993) *Protein Sci. 2,* 448.

Zeng, F. Y., Volker, G., & Gabius, H. J. (1993) *J. Biochem. (Tokyo) 25,* 1019.