Location of Tryptophans in Membrane-Bound Annexins†

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ABSTRACT: The annexins are a novel class of calcium-dependent membrane binding proteins with highly homologous sequences and similar binding characteristics. In order to better define structural parameters of the membrane-bound form, the localization of tryptophan residues in several of these proteins was studied by use of quenchers of their intrinsic fluorescence. Lipocortin I contains a single tryptophan located near its N-terminus, while the single tryptophan in lipocortin V is located in a repeated consensus sequence. Calcium-dependent binding to vesicles composed of 50% egg phosphatidylcholine and 50% bovine brain phosphatidylserine was accompanied by an increase in emission intensity resulting from a relief of internal quenching. The tryptophan fluorescence of bound lipocortin I was nearly unaffected by substituting the quencher 1-palmitoyl-2-(5-doxylstearoyl)-*sn*-glycero-3-phosphocholine (5-PC) for egg phosphatidylcholine, while that of the lipocortin V tryptophan was quenched significantly. With the quenching doxyl spin-label located deeper in the bilayer at the 12- and 16-positions of the acyl chain, the quenching was progressively weaker, suggesting an interfacial location for the tryptophan of lipocortin V. The same experiments with lipocortin I show almost no quenching in any case, suggesting that this tryptophan near the amino terminus is protected or oriented away from the membrane surface. Data on the bovine liver calelectrins are also presented showing that endonexin also has a tryptophan residue that interacts strongly with phospholipids.

The annexins are a class of Ca^{2+}-dependent membrane binding proteins found in many tissues (Klee, 1988). Membrane binding activity may be involved in membrane fusion (Creutz et al., 1978; Hong et al., 1981; Drust & Creutz, 1988; Shahid et al., 1989), cytoskeletal interactions (Gerke & Weber, 1984; Glenney, 1986a), antiphospholipase activity (Flower et al., 1984), anticoagulation (Funakoshi et al., 1987a), or intracellular signaling as a kinase substrate (Glenney & Tack, 1985; Sawyer & Cohen, 1985; Isacke et al., 1986; Creutz et al., 1987). All annexins contain four or eight 60–80 amino acid homologous repeat sequences with approximately 50% amino acid identity between any two annexin sequences. Each protein also contains a strongly conserved 17 amino acid consensus sequence appearing in each repeat (Geisow et al., 1986). It has been suggested that each repeat or pair of repeats may represent one Ca^{2+} and/or phospholipid binding portion of the protein on the basis of the observation of two or four phospholipid-dependent Ca^{2+} binding sites (Glenney, 1986b; Glenney et al., 1987; Schlaepfer & Haigler, 1987; Schlaepfer et al., 1987). No structural data have yet suggested how such binding may occur.

The N-terminal sequence is variable and is thought to determine the tissue- or cell-specific function in each case. The N-terminus of the 36-kDa subunit (p36) of calpactin is known to bind to the 10-kDa (p10) subunit by virtue of the fact that a 33-kDa fragment missing the N-terminus does not bind to p10 (Johnsson et al., 1986; Glenney, 1986b). Phosphorylation (Sawyer & Cohen, 1985; Glenney & Tack, 1985; Isacke et al., 1986; Creutz et al., 1987; Schlaepfer & Haigler, 1987; Powell & Glenney, 1987; Varticovski et al., 1988) or cleavage (Drust & Creutz, 1988; Ando et al., 1989) of the N-terminus also appears to modulate the Ca^{2+} sensitivity of some of the annexins. However, the mechanism of this modulation and the function of the N-terminus remain essentially unknown.

Others facts suggest that phospholipid binding capacity resides in the 90% of the protein remaining after N-terminus removal. For instance, the 33-kDa fragment of calpactin retains its ability to bind to phospholipid, although the Ca^{2+} dependence is higher than it is for the complete protein. Removal of the N-terminus of lipocortin I increases the Ca^{2+} sensitivity for membrane binding (Ando et al., 1989). Some large fragments of lipocortin I required for phospholipase A_2 inhibition and presumably for phospholipid binding have been identified in this part of the molecule (Huang et al., 1987).

The sequenced annexins do not contain a classical Ca^{2+} binding site such as the EF-hand (Kretsinger & Creutz, 1986; Geisow & Walker, 1986). While a structural model has been suggested for the membrane-bound annexin (Geisow et at., 1987), it is based on scarce structural data.

In order to better define the structure of these proteins, I have studied the localization of tryptophan residues within the membrane-bound forms of lipocortin I and V and the mammalian calelectrins. Lipocortin I was originally isolated from rabbits and identified as a phospholipase A_2 inhibitor (Hirata et al., 1980). It has also been identified as calpactin II (Glenney, 1986a), p35 (Fava & Cohen, 1984), and chromobindin 9 (Creutz et al., 1987). Lipocortin V has been identified as an anticoagulant protein, PAP (Funakoshi et al., 1987a), as well as endonexin II (Schlaepfer et al., 1987), chromobindin 5 (Creutz et al., 1987), and lipocortin (Rothhut et al., 1987). The sequences of lipocortin I and V are highly homologous (Funakoshi et al., 1987b) with 42% identity. Both proteins exhibit Ca^{2+}-dependent membrane binding. Lipocortin I contains a single tryptophan in the N-terminal region of the molecule, while lipocortin V contains a single tryptophan in one of the consensus sequences. These fluorescent amino acids constitute ideal probes for localization of portions of the bound annexins. The fluorescence quenching method presented below (Chattopadhyay & London, 1987) is used for such a localization for these proteins and for both mammalian calelectrins (Südhof et al., 1984), referred to here as endonexin and the 67-kDa calelectrin.

Materials and Methods

1-Palmitoyl-2-(5-doxylstearoyl)-*sn*-glycero-3-phosphocholine

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(5-PC),[1] 1-palmitoyl-2-(12-doxylstearoyl)-*sn*-glycero-3-phosphocholine (12-PC), 1-palmitoyl-2-(16-doxylstearoyl)-*sn*-glycero-3-phosphocholine (16-PC), bovine brain phosphatidylserine (PS), and egg phosphatidylcholine (PC) were purchased from Avanti Polar Lipids (Birmingham, AL). 1-Palmitoyl-2-[10-(1-pyrenyl)decanoyl]-*sn*-glycero-3-phosphocholine (pyrene-PC) was from Molecular Probes (Eugene, OR). Human serum albumin and 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid (TES) were obtained from Sigma (St. Louis, MO). $CaCl_2$ (>99%) and NaCl (>99%) were from Fisher (Pittsburgh, PA). Polycarbonate filters were from Nuclepore (Pleasanton, CA).

Vesicle Preparation. Large unilamellar vesicles were prepared by the reverse-phase evaporation method (Szoka & Papahadjopoulos, 1978) as modified by Wilschut et al. (1980). Liposomes were extruded through 0.2-μm and then 0.1-μm polycarbonate filters during this procedure (Szoka et al., 1980). Phospholipid concentrations were determined according to a phosphate assay by Bartlett (1959) as modified by Morrison (1964).

All samples were maintained at 25 °C in 100 mM NaCl, 5 mM TES, and 0.1 mM EDTA at pH 7.4 (buffer A). Additions of materials to intitiate binding, such as Ca^{2+}, were made with small aliquots of a concentrated stock. All Ca^{2+} concentrations are expressed as the excess free Ca^{2+} over the initial EDTA concentration. With a Ca^{2+} electrode and standard solutions, this assumption for free Ca^{2+} concentration was found to hold for approximately 10 μM Ca^{2+} and higher. In all experiments, Ca^{2+} was added last to a solution containing the appropriate vesicles and protein.

Protein Preparation. Lipocortin I and lipocortin V were kindly provided by Dr. R. Blake Pepinsky of Biogen Corp. The 67- and 32-kDa calelectrins were purified from bovine liver as previously described (Südhof & Stone, 1987) with small modifications. Protein solutions were assayed by the bicinchoninic acid method (Smith et al., 1985). Purity of all proteins was assessed as 95% or greater by single-dimension polyacrylamide gel electrophoresis prior to use.

Fluorescence Measurements. Fluorescence measurements were made on a Spex Fluorolog or Fluorolog 2 fluorometer. Excitation for tryptophan was at 295 nm. For kinetic experiments, emission was monitored at 350 nm for all proteins except the 67-kDa calelectrin, which had an apparent emission maximum at 340 nm. All experiments were maintained at 25 °C with a jacketed cuvette holder and circulating water. Samples were continuously stirred in the cuvette with a Castle-type stir bar. All spectra presented were smoothed with a five-point moving average, and then the spectrum of buffer was subtracted, elmiminating a constant background and a significant water Raman peak at approximately 330 nm.

Results

The fluorescence spectra of lipocortin I, lipocortin V, and human serum albumin are shown in Figure 1. The emission intensity of the single tryptophan of human serum albumin was much greater than that of either lipocortin I or lipocortin V on an equimolar basis. When either 0.1% SDS or 7.8 M urea was added to these proteins, the fluorescence of the lipocortins increased greatly while that of human serum albumin decreased by a small amount. The fluorescence of lipocortin V and lipocortin I was the same as that of human serum albumin under these conditions. Therefore, the relatively low

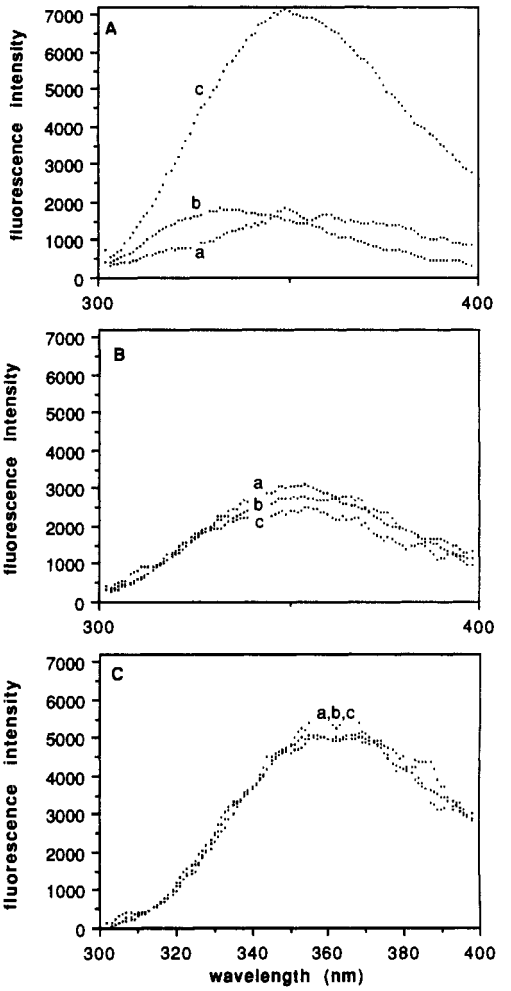

FIGURE 1: Effect of denaturation on tryptophan fluorescence of (a) lipocortin I, (b) lipocortin V, and (c) human serum albumin. Samples contained approximately 10 μg/mL protein in buffer A with (A) no addition, (B) 0.1% sodium dodecyl sulfate, or (C) 7.8 M urea. All spectra were obtained at 25 °C. Spectra were normalized to compare equimolar amounts of each protein. Fluorescence intensity is in arbitrary units.

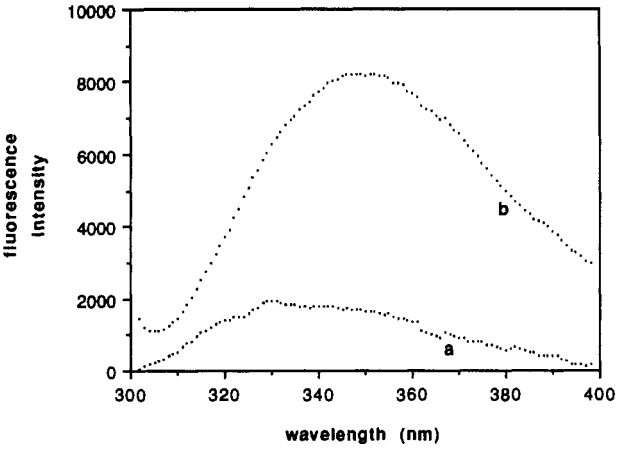

FIGURE 2: Effect of membrane binding on the fluorescence of tryptophan 186 in lipocortin V. Spectrum a represents protein and vesicles in the absence of Ca^{2+}. In spectrum b, Ca^{2+} was added to a final free concentration of 100 μM. Both solutions contained 10 μg/mL lipocortin V and PS/PC (1/1) vesicles at a total phospholipid concentration of 50 μM in buffer A. The temperature was maintained at 25 °C. Fluorescence intensity is in arbitrary units.

[1] Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; 5-PC, 1-palmitoyl-2-(5-doxylstearoyl)-*sn*-glycero-3-phosphocholine; 12-PC, 1-palmitoyl-2-(12-doxylstearoyl)-*sn*-glycero-3-phosphocholine; 16-PC, 1-palmitoyl-2-(16-doxylstearoyl)-*sn*-glycero-3-phosphocholine; pyrene-PC, 1-palmitoyl-2-[10-(1-pyrenyl)decanoyl]-*sn*-glycero-3-phosphocholine; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; EDTA, ethylenediaminetetraacetate.

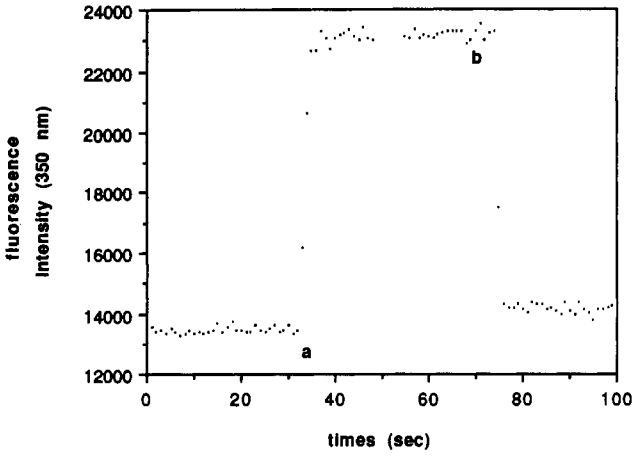

FIGURE 3: Kinetics of association and dissociation of lipocortin V with phospholipid vesicles. Ca^{2+} at a final free concentration of 100 μM was added at (a) to a solution of 10 μg/mL lipocortin V and PS/PC (1/1) (total phospholipid concentration 50 μM) in buffer A. At (b) 2.5 mM EDTA was added. Tryptophan was excited at 295 nm, and emission at 350 nm was monitored. The temperature was maintained at 25 °C. Fluorescence intensity is in arbitrary units. Each dot represents a 1-s time point.

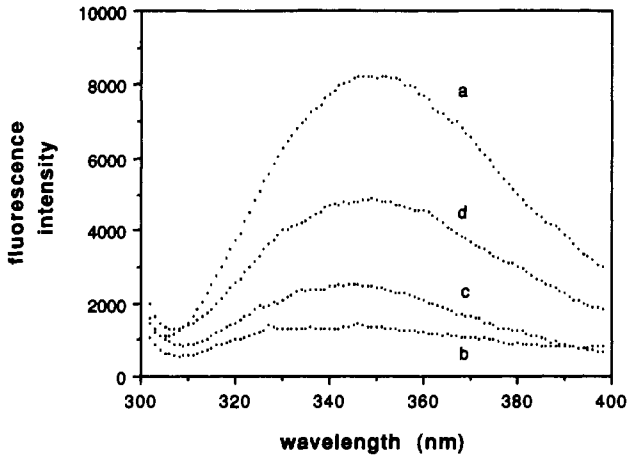

FIGURE 4: Effect of phospholipid quenchers on the fluorescence of tryptophan in lipocortin V. All samples consisted of 10 μg/mL lipocortin V and 50 μM total phospholipid in 100 μM free Ca^{2+}, 100 μM EDTA, 100 mM NaCl, and 5 mM TES, pH 7.4 at 25 °C. The phospholipid compositions were (a) PS/PC (1/1), (b) PS/5-PC (1/1), (c) PS/12-PC (1/1), and (d) PS/16-PC (1/1).

fluorescence of the lipocortins is due to internal quenching of tryptophans that is relieved by denaturation.

The response of tryptophan fluorescence to Ca^{2+}-dependent binding to PS/PC vesicles is shown in Figure 2. The fluorescence emission spectrum of the single tryptophan at position 186 in lipocortin V was monitored. In order to avoid aggregation and fusion of vesicles by maintaining some residual surface charge, yet induce significant annexin binding, a relatively low Ca^{2+} concentration and a relatively nonfusogenic phospholipid composition were chosen. Thus, under the conditions of these experiments, annexins bound predominantly to a single bilayer. When 100 μM Ca^{2+} was added to lipocortin V in the presence of PS/PC (1/1) vesicles, a large increase in fluorescence was observed. The characteristic blue shift of a hydrophobic environment was not observed. Instead, there appeared to be a small red shift of the peak compared to that of the free form. Lipocortin I displayed a similar increase in fluorescence. In the absence of either vesicles or Ca^{2+}, this increase in fluorescence was not observed for either protein. When excitation was at 280 nm instead of 295 nm, a similar increase in fluorescence was observed, indicating that a slight shift of the absorption spectrum near 295 nm could not account for the increased emission intensity.

In Figure 3 is shown the rate of the Ca^{2+}-dependent increase in fluorescence. Under these conditions, the fluorescence increase occurred in just approximately a few seconds and was almost fully reversible by EGTA, also in a few seconds. In some cases, a very small amount of remaining fluorescence appeared to decrease slowly to the prebound level, indicating slow exchange or an unknown artifact.

The effect of annexin binding on the lateral distribution of phospholipids was determined with 1-palmitoyl-2-[10-(1-pyrenyl)decanoyl]-*sn*-glycero-3-phosphocholine (pyrene-PC). This phospholipid probe has been used previously to detect protein-mediated lateral phase separation of mixed phospholipids as manifested by an increase in the excimer to monomer ratio (Jones & Lentz, 1986). In our experiments, vesicles composed of PS/PC (1/1) with 5 mol % pyrene-PC and a total phospholipid concentration of 50 μM were exposed to lipocortin I or lipocortin V followed by 100 μM free Ca^{2+}. No increase in the excimer to monomer ratio was observed under these conditions (now shown). Instead, a 3% increase in the intensity of the monomer peak at 377 nm was observed with the same rise time as that for the change in tryptophan fluorescence. Therefore, there is no indication of lateral phase separation of phospholipids under these conditions.

Table I: Effect of Membrane-Bound Spin-Labels on Fluorescence Emission of Tryptophan from Lipocortins I and V

sample[a]	Ca^{2+} effect, intensity ratio	F/F_0
lipocortin I, PS/PC (1/1)	2.45 ± 0.09	1.0
lipocortin I, PS/5-PC (1/1)	2.08 ± 0.05	0.91
lipocortin I, PS/12-PC (1/1)	2.04 ± 0.27	0.83
lipocortin I, PS/16-PC (1/1)	2.37 ± 0.07	0.97
lipocortin V, PS/PC (1/1)	4.89 ± 0.05[b]	1.0
lipocortin V, PS/5-PC (1/1)	0.764[b]	0.16
lipocortin V, PS/12-PC (1/1)	1.40 ± 0.03	0.29
lipocortin V, PS/16-PC (1/1)	2.68	0.55

[a] Samples contained 100 μM free Ca^{2+}, 50 μM total phospholipid, and 0.28 μM lipocortin V or 0.15 μM lipocortin I in buffer A. Ratios represent the emission intensity of each sample at 350 nm divided by the emission intensity of the same sample without Ca^{2+}. F/F_0 is the fluorescence of annexins bound to the spin-labeled samples divided by the fluorescence of the annexins bound to vesicles without spin-labels. Experiments were performed at 25 °C. [b] Nearly identical data were obtained with lipocortin V at 0.15 μM.

Because of the lack of lateral phase separation, it was possible to study the interaction of annexins with PS/PC membranes by use of PC probes. Therefore, the interaction of these proteins with membranes was monitored by employing fluorescence quenching by membrane-localized spin-labels. In Figure 4 is shown the effect of replacing 50% phosphatidylcholine with phosphatidylcholine analogues containing a fatty acyl chain with a doxyl spin-label at some position along the chain. It can be seen that the doxyl label at the 5-position very strongly, quenched fluorescence of the tryptophan of lipocortin V. The effect was less pronounced for the 12 label and least for the 16 label.

Such experiments for both lipocortin I and lipocortin V are summarized in Table I. The second column shows the ratio of fluorescence intensity for each protein at 350 nm in the presence and absence of Ca^{2+}. The numbers show the variation of this effect with vesicle phospholipid composition. The large intensity increase of tryptophan 186 of lipocortin V upon binding to PS/PC vesicles is reflected in these numbers. When spin-labeled PC's were substituted for egg PC, the Ca^{2+}-induced increase in fluorescence of lipocortin V was greatly attenuated as in Figure 4. On the other hand, there was almost no effect of any of the spin-labels on tryptophan 12 of lipo-

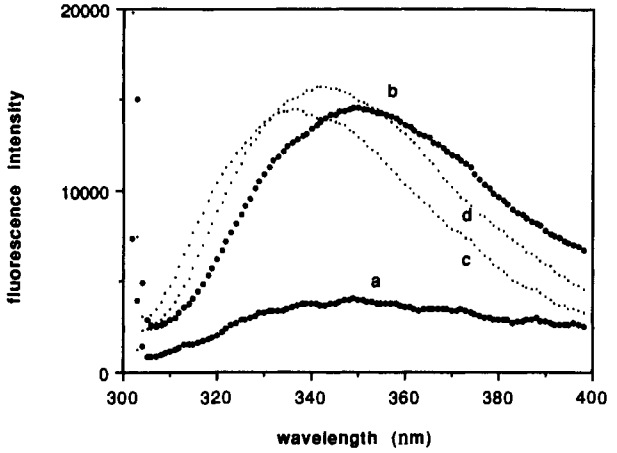

FIGURE 5: Effect of membrane binding on the fluorescence of endonexin and the 67-kDa calelectrin. Experiments contained PS/PC (1/1) vesicles and (a) 10 μg/mL endonexin, (b) 10 μg/mL endonexin and 100 μM free Ca^{2+}, (c) 10 μg/mL 67-kDa calelectrin, or (d) 10 μg/mL calelectrin and 100 μM free Ca^{2+}. All spectra were taken at 25 °C in buffer A.

cortin I. In order to rule out the possibility that less lipocortin I than lipocortin V was membrane bound under these conditions, the binding was measured by vesicle sedimentation at approximately 240000*g* for 3 h. In agreement with previous studies (Schlaepfer & Haigler, 1987), approximately 95% of lipocortin I remained with the pelleted vesicles at this Ca^{2+} concentration. Thus, tryptophan 12 of lipocortin I must be inaccessible to the phospholipid spin-labels.

In order to estimate the depth of penetration of the tryptophan residue into the membrane, the relative strength of the effects of the spin-labels was used. In Table I, the values of F/F_0 directly compare the effects of the spin-labels by setting the fluorescence of the protein bound to PS/PC as F_0 and the fluorescence of the protein bound to vesicles containing spin-label quenchers as F (Chattopadhyay & London, 1987). The effects of the spin-labels on F/F_0 of lipocortin V reflect the data in Figure 4. This pattern indicates qualitatively that the tryptophan does not penetrate into the membrane and may reside at the interfacial region. The actual values for F/F_0 correspond almost exactly to those observed by Chattopadhyay and London (1987) for quenching of *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE) by 50 mol % of the 5 and 12 spin-labels. Their calculations using these values put this fluorophore at about 14.2 Å from the center of the bilayer, that is, at the interfacial region of the membrane. Therefore, an interfacial location for tryptophan 186 is inferred.

The tryptophan fluorescence of bovine endonexin or 67-kDa calelectrin also exhibited interesting behavior upon membrane binding (Figure 5). The endonexin tryptophan behaved in a manner similar to that of the tryptophan in lipocortin V. There was a strong Ca^{2+}-dependent increase in emission intensity upon binding and a small red shift in the peak. The 67-kDa calelectrin is unique in that its fluorescence only increased slightly upon membrane binding. A similar red shift was also observed. Vesicle sedimentation experiments gave an estimate that at least 50% of this protein bound to the membrane under these conditions. Approximately 70% of the endonexin appeared to bind under these conditions.

The accessibility of tryptophans in endonexin and the 67-kDa calelectrin to phospholipids is shown in Table II. As for lipocortin V, the tryptophan in endonexin had high accessibility to phospholipid spin-labels. The dependence on the depth of the quencher was also similar to that of lipocortin V. The

Table II: Effect of Membrane-Bound Spin-Labels on Fluorescence Emission of Tryptophan from Mammalian Calelectrins

sample[a]	Ca^{2+} effect, intensity ratio	F/F_0
endonexin, PS/PC (1/1)	3.63	1.0
endonexin, PS/5-PC (1/1)	1.03	0.28
endonexin, PS/12-PC (1/1)	2.13	0.59
endonexin, PS/16-PC (1/1)	2.60	0.71
67-kDa calelectrin, PS/PC (1/1)	1.10	1.0
67-kDa calelectrin, PS/5-PC (1/1)	0.96	0.88
67-kDa calelectrin, PS/12-PC (1/1)	1.10	1.0
67-kDa calelectrin, PS/16-PC (1/1)	1.07	0.98

[a] Samples contained 100 μM free Ca^{2+}, 50 μM total phospholipid, and 10 μg/mL protein (0.28 μM endonexin or 0.15 μM 67-kDa calelectrin) in buffer A. Ratios represent the emission intensity with Ca^{2+} divided by the emission intensity of the same sample without Ca^{2+}. F/F_0 is the fluorescence of the spin-labeled samples divided by the fluorescence of the annexins bound to vesicles without spin-labels. Experiments were performed at 25 °C. Emission intensity was measured at 350 nm for endonexin and at 340 nm for 67-kDa calelectrin.

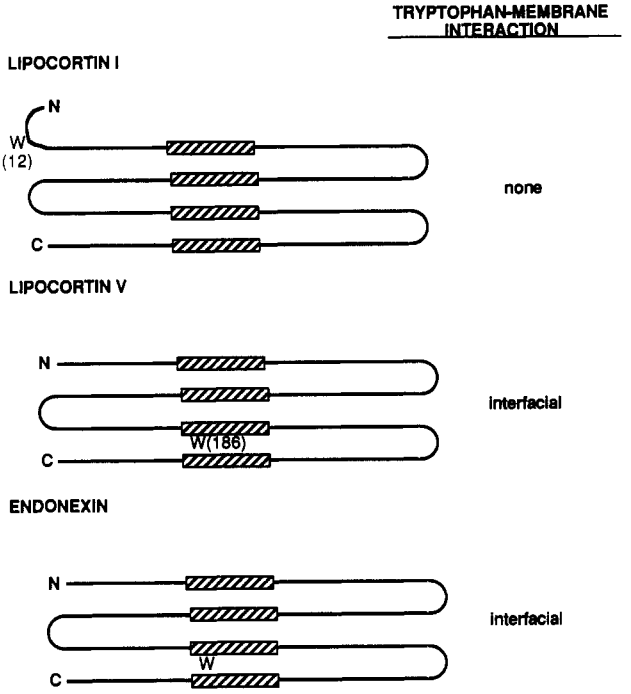

FIGURE 6: Schematic representation of annexin sequences. Hatched regions represent consensus sequences. Positions of tryptophans are shown by the one-letter amino acid representation.

effect on endonexin is quite strong compared with that of the 67-kDa calelectrin tryptophan, which showed little interaction with phospholipids.

Discussion

These data are the first spectroscopic results that clearly demonstrate significant differences in the interactions of various parts of annexins with phospholipids. As depicted in Figure 6, each 30–40-kDa annexin contains four repeated homologous segments with a smaller consensus sequence within each repeat. The close contact of tryptophan 186 from the third repeat unit of lipocortin V with membrane phospholipids supports the hypothesis that consensus sequence repeats are involved in phospholipid binding (Geisow et al., 1986). Though the first two repeat units of lipocortin I are sufficient and necessary for inhibition of phospholipase A_2 activity (Huang et al., 1987), this activity may not directly correlate to membrane binding of each fragment. It is still quite possible that the third repeat is also involved in membrane binding of the whole protein at the Ca^{2+} concentrations used here. The lack

of membrane interaction of tryptophan 12 from lipocortin I is consistent with the observation that the N-terminus is not necessary for phospholipid binding (Johnsson et al., 1986; Glenney, 1986b; Huang et al., 1987; Drust & Creutz, 1988; Ando et al., 1989).

Results from studies with the bovine annexins also support these conclusions. Bovine endonexin contains a tryptophan in a position almost identical with that in lipocortin V (Figure 6), on the basis of homology of bovine peptides and the porcine sequence (Geisow et al., 1986; Weber et al., 1987; Pepinsky et al., 1988). Like tryptophan 186 in lipocortin V, this tryptophan appears to make intimate contact with membrane phospholipids. Although endonexin (lipocortin IV) may also contain some lipocortin III with a second tryptophan in the N-terminal region (Pepinsky et al., 1988), both have a tryptophan at an equivalent position in the third repeat unit. Hence, the similar behavior of endonexin and lipocortin V is understandable and confirms the validity of assumptions concerning equivalence of binding by various annexins. On the other hand, the tryptophans of the 67-kDa calelectrin show little interaction with phospholipids. It is possible that these differences may bear some relationship to the differences in fusion-promoting activity of these two proteins. The 67-kDa protein is inhibitory to Ca^{2+}-dependent fusion of all phospholipid vesicles tested, while endonexin promotes fusion of some phospholipid compositions (P. Meers, unpublished observation).

In order to reach these conclusions, it was assumed that all annexins bind to phospholipid vesicles in an equivalent manner. While various annexins may not bind in an identical manner, their high homology, their similar binding characteristics, and the similarity of endonexin and lipocortin V results suggest that this assumption is generally valid. Equivalence between individual molecules of a given bound annexin is supported by the fact that binding of lipocortin V (Tait et al., 1989; Meers et al., unpublished results) and binding of lipocortin I (Meers et al., unpublished results) have been described by a simple monomer binding mechanism. Some data suggest that annexin monomers may organize into polymeric structures on the surface of membranes (Creutz et al., 1979; Meers et al., 1988; Newman et al., 1989), but no evidence for nonequivalent membrane-bound subunits exists.

By use of quenchers at various depths in the membrane, the depth of penetration of tryptophans into the phospholipid bilayer was estimated. The data presented here indicate that none of the tryptophans studied penetrates beyond an interfacial site on the membrane. Consistent with an interfacial localization is the lack of a blue shift of the lipocortin V tryptophan when it binds to PS/PC vesicles, despite its accessibility to phospholipid spin-labels. Rapid reversibility of binding is also consistent with a lack of membrane penetration. A preferential interfacial location for exposed tryptophans is not without precedent (Feigenson et al., 1977) despite the hydrophobic nature of this amino acid. A lack of hydrophobic penetration by annexins is also consistent with the lack of exposure of hydrophobic calelectrin domains in response to Ca^{2+} (Südhof, 1984) and data suggesting that synexin enhances aggregation but not fusion of vesicles (Meers et al., 1988). On the other hand, synexin has been proposed to exist as a transmembrane protein on the basis of single-channel current measurements (Burns et al., 1989). Our only data consistent with this mode of binding are the possible existence of a small amount of increased tryptophan fluorescence from membrane-bound annexins that is slowly reversible by EDTA (Figure 3). Though existence of transmembrane forms of these proteins cannot be completely precluded by our experiments, these forms cannot comprise a significant portion of the total protein. Under the conditions of these experiments, the vast majority of annexin molecules bind in a rapidly reversible manner to phospholipid membranes with tryptophan moieties at locations no deeper than interfacial.

The interpretations of the results concerning depth of the tryptophan are based on the assumption that binding of annexins to vesicles containing 50% PS/50% spin-labeled PC is the same as binding to 50% PS/50% PC. Since the annexins have been shown to bind to acidic phospholipids such as PS but not to PC alone under the conditions of these experiments, the headgroup composition is probably much more important than acyl chain composition. All experiments contained the same amount of the same PS. Hence, the acyl chain composition of the PC in the membrane is unlikely to exert a major effect on the amount or mode of binding. In support of this assertion are the data from lipocortin I (Table I) indicating approximately the same binding-induced tryptophan fluorescence increase for each phospholipid composition. It has also been demonstrated that normal bilayer vesicles capable of trapping aqueous materials can be formed from these spin-labeled PC's alone (London & Feigenson, 1981).

Results obtained in the absence of spin-labels also provide interesting information. The experiments in Figure I show that lipocortin I and V tryptophans are internally quenched in the aqueous soluble form of the protein. Ca^{2+}-dependent binding of these proteins to PS/PC membranes results in a large increase in tryptophan fluorescence and a shift to longer wavelengths. Quenching of tryptophan 186 of lipocortin V by phospholipid spin-labels blue shifts fluorescence to some extent (Figure 4), confirming that the red-shifted components are associated with the membrane-bound form. Denaturation of either protein also dramatically increases the tryptophan fluorescence emission intensity and shifts it to a longer wavelength. It is tempting to speculate that the red shift and increase in intensity are related to conversion to a less tightly folded form when annexins bind to phospholipids. A relatively flexible molecule could allow an increased rate of collisions between phospholipids away from the protein binding site in situations where the annexin helps bridge two vesicles (Meers et al., 1988).

The rates of binding and dissociation are very rapid as indicated by the fast changes in fluorescence. The highest rate constants for vesicle aggregation observed under similar conditions are in the range of 10^7 M^{-1} s^{-1} (Meers et al., 1988). With this rate constant, the aggregation of vesicles takes much longer than the completion of the binding-induced increase in tryptophan fluorescence. Though vesicle aggregation is minimized in the experiments presented here, it will be of great interest to compare annexin-aggregated systems where new binding modes may appear.

In summary, these data suggest that the third repeat unit of annexins interacts strongly with membrane phospholipids when Ca^{2+} is present. This part of the protein probably does not penetrate deeper than the interfacial part of the membrane though. The N-terminus in membrane-bound annexins is inaccessible to phospholipids. Though limited hydrophobic interaction of some part of the protein is not precluded, there is no evidence for deep penetration of any annexins into the hydrophobic interior of the bilayer under the conditions of these experiments. A more quantitative evaluation of the position of these tryptophans will be possible by further analysis at varying spin-label concentrations. This method may also prove to be very useful for studying other annexins, including

annexins with tryptophans placed in various positions by site mutation.

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