Volume 314, number 2, 159–162 FEBS 11852 December 1992


# Annexin V forms calcium-dependent trimeric units on phospholipid vesicles

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Received 1 september 1992; revised version received 18 October 1992

The quaternary structure of annexin V, a calcium-dependent phospholipid binding protein, was investigated by chemical cross-linking. Calcium was found to induce the formation of trimers, hexamers, and higher aggregates only when anionic phospholipids were present. Oligomerization occurred under the same conditions as annexin–vesicle binding. A model is proposed in which cell stimulation leads to calcium-induced organization of arrays of annexin V lining the inner membrane surface, thus altering properties such as permeability and fluidity.

Calcium; Annexin; Cytoskeleton; Cross-linking; Fluidity

## 1. INTRODUCTION

Annexins constitute a family of proteins proposed to mediate the intracellular calcium signal through their common property of calcium-dependent binding to phospholipid membranes. Specific members of the annexin family have been shown to produce voltage-dependent calcium channel activity (for review see [1]), alter the gating of the sarcoplasmic reticulum calcium release channel [2], bind and bundle actin [3], inhibit $PLA_2$ [4], inhibit protein kinase C [5] and regulate the budding of clathrin-coated vesicles [6].

Several previous studies have considered the membrane-associative properties of annexins. From their ellipsometric measurements, Andree and co-workers proposed that calcium-specific adsorption of annexin V to phospholipid monolayers induces polymerization of the protein in the plane of adsorption [7]. Such a coating of the membrane by annexin molecules could exert a biological effect on either the membrane itself or other membrane-bound proteins. Electron image analysis revealed that annexin V molecules crystallize as trimers in a triskelion pattern on a lipid monolayer [8]. Through the same technique, it was determined that annexins IV [9] and VI [10] crystallize similarly on lipid monolayers. In solution, annexins IV, VI, and VII were found to self-associate when bound to membranes in a $Ca^{2+}$-dependent manner, although the quaternary structure was not identified [11].

The studies described herein were initiated to probe the specific calcium-dependent oligomerization properties of annexin V molecules in the presence of phospholipid vesicles under solution conditions. Chemical cross-linking studies were performed to investigate the quaternary structure of annexin V when bound to phospholipid vesicles. Binding studies were also performed to evaluate whether oligomerization occurred under similar conditions as those associated with annexin–vesicle binding. Results from these experiments are discussed in the context of related studies and of the possible physiological implications for annexin function.

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*Abbreviations.* EGTA, ethylene glycol bis (beta-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DMS, dimethyl suberimidate; PS, phosphatidylserine; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; DOPA, dioleoylphosphatidic acid; CL, cardiolipin; HEPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid.

## 2. EXPERIMENTAL

### 2.1. *Materials*

Porcine lung annexin V was purified as described previously [12]. DMS was from Pierce. Bovine brain extract Type III containing >80% phosphatidylserine (PS) was obtained from Sigma Chemical Co. Bovine heart cardiolipin (CL), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), and dioleoylphosphatidic acid (DOPA) were purchased from Avanti Polar Lipids. Highly purified water was provided by Hydro Services, Inc.

### 2.2. *Vesicles*

Large unilamellar phospholipid vesicles (LUVs) were prepared from either pure or mixed phospholipids by the method of Reeves and Dowben [13] as modified by Mueller, Chien and Rudy [14]. Vesicles were characterized by trapped-volume measurements and by direct observation using video light microscopy with Differential Interference Contrast. LUVs prepared in this manner appeared reasonably homogeneous, ranging in diameter from 0.5 to 3 microns. Vesicles

were prepared fresh for each experiment and used within 12–18 h of preparation.

2.3 *Calcium minielectrodes*

Calcium-sensitive minielectrodes were prepared according to Affolter and Sigel [15] using the neutral carrier ETH 1001. Calcium standard solutions used to calibrate the electrodes were prepared according to Tsien and Rink [16]. The electrodes were prepared and used within 24 h. Before measuring the free calcium concentration in the samples, the electrodes were equilibrated for 20–30 min or until they showed a stable response. The electrodes were selected only if they showed a Nernstian response. Electrodes used were very stable, showing a drift of less than 6 mV in 12 h

2.4. *Assays*

SDS-PAGE was performed according to Laemmli [17]. Protein concentration was measured according to Lowry et al [18], as modified by Stauffer [19], using bovine serum albumin as standard Phospholipid phosphorus was determined following the method of Ames and Dubin [20] The gels were stained with Coomassie brilliant blue as described by Weber and Osborn [21].

2.5. *Binding assays*

Binding of annexin V to phospholipid vesicles was determined by a method based on that described by Boustead et al. [22]. Phospholipid vesicle preparations containing 0.1 μmol of phospholipids were mixed with 10 μg of protein in 25 mM HEPPS, pH 8.0, 100 mM KCl and the indicated calcium concentration in a final volume of 500 μl. The mixture was incubated for 15 min at room temperature. After the incubation period, the tubes were centrifuged at 12,000 × *g* for 10 min. The pellets were washed once or twice by resuspending them with 450 μl of buffer and centrifuging each time. 50 μl samples of the resuspended pellets and the supernatants were analyzed by SDS-PAGE.

2.6. *Chemical cross-linking*

Cross-linking of annexin V in the absence of phospholipids was performed in 25 mM HEPPS, pH 8.0, containing final concentrations of 100 mM KCl, 0.05 mM EGTA, the indicated concentration of $CaCl_2$, 5% (v/v) 2-mercaptoethanol, and 1 mg/ml protein in a final volume of 50 μl, unless otherwise stated A 50 mM DMS stock solution was freshly prepared in 25 mM HEPPS buffer, pH 8.0 containing 100 mM KCl and added to the protein solution to give the stated desired final concentration [23,24]. The cross-linking reaction proceeded for 3 h at room temperature and the products were analyzed by SDS-PAGE

For the cross-linking experiments the protein was incubated with the phospholipid vesicles, as described above for the binding studies, and then centrifuged. 10 μl of 25 mM HEPPS, pH 8.0, 100 mM KCl buffer and 5 μl of 200 mM DMS were added to the pellet containing the vesicle-bound protein to give a final volume of approximately 30 μl. The suspension was mixed, incubated for 3 h at room temperature and the reaction products analyzed by SDS-PAGE. For experiments performed using vesicles prepared solely from PC, the protocol was changed to reflect the fact that annexin V does not bind to PC For these samples, the vesicles were first pelleted without protein, then mixed with protein, calcium, and DMS and used without further pelleting.

## 3. RESULTS AND DISCUSSION

Annexin V is a single 35 kDa polypeptide chain. To investigate whether oligomeric states exist, either free in solution or in association with membranes, chemical cross-linking studies using the bifunctional reagent, dimethylsuberimidate, were performed under various conditions. Two key observations were made in these experiments (see Fig. 1). First, annexin V forms cross-linked trimers, hexamers, and higher aggregates in the presence of vesicles but not in their absence. Vesicles containing DOPG, DOPA, or cardiolipin promoted this cross-linking, while DOPC did not. Annexin V, like other annexins, does not bind to PC, although it will bind to PG, PA, cardiolipin. Second, calcium was required in all cases for cross-linking; magnesium could not substitute for calcium. It is apparent from these results that annexin binding to the vesicles was a requisite for trimer/hexamer formation, since even high levels of calcium could not induce annexin oligomerization when pure (100%) PC vesicles were used.

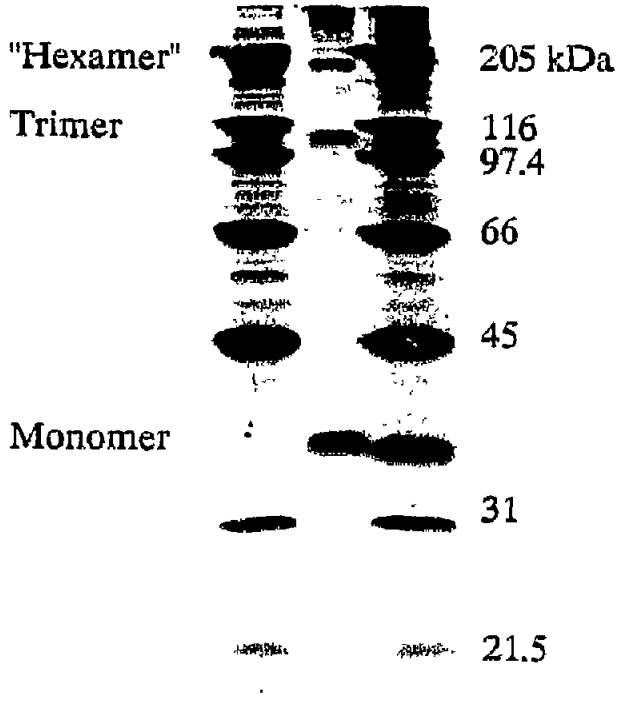


Fig. 1. Molecular weight measurements of annexin V monomer, trimer, and higher aggregates using SDS gel standards *Left lane*: molecular weight standards, 205, 116, 97.4, 66, 45, 31, 21.5 kDa. *Middle lane:* annexin V cross-linked by DMS in presence of 0.1 mM $CaCl_2$ and DOPC:DOPG vesicles. *Right lane*: samples of the left and middle lanes combined.

To ascertain whether annexin V oligomerization correlates with binding to membranes, free calcium measurements were determined in parallel for both binding and cross-linking experiments. Free calcium measurements were made using a calcium-sensitive minielectrode. Annexin–vesicle binding was determined by a centrifugation-separation assay in which the mixture was centrifuged, and the supernatant and pellet analyzed by SDS-PAGE. Bound protein remained with vesicles in the pellet. The free calcium concentration for half-maximal binding occurred within a range of 13–16

$\mu$M for DOPG/DOPC vesicles, 5.5–6.9 $\mu$M for DOPA/DOPC and CL/DOPC vesicles, and 24–47 $\mu$M for brain PS vesicles. These levels are reached during the course of calcium transients within the cell [25]. Oligomerization appeared at similar free calcium concentrations as the respective binding assays (Fig. 2). The cross-linking pattern was consistent with all the anionic phospholipids tested.

Our results indicate that annexin V in solution self-associates into trimers and higher aggregates when bound to vesicles. Thus annexin V monomers in the presence of phospholipid vesicles may exhibit organizational behavior in solution similar to that observed in the 2D crystalline state [8]. Multimer formation depends upon formation of the annexin-$Ca^{2+}$-phospholipid membrane ternary complex. The system requires that when the free calcium level is appropriately elevated and a compatible phospholipid surface is present, multimers can form. Binding to the membrane may orient annexin monomers in order to facilitate polymerization.

We propose the following model for the molecular mechanism of annexin V action. In the resting cell, when free calcium concentrations are below $10^{-6}$ M, annexin V exists primarily as a monomer. During cell stimulation, free calcium concentrations subadjacent to the cell membrane rise to micromolar levels [25]. Calcium promotes the binding of the protein to anionic phospholipid surfaces, where it further organizes into trimers, hexamers and higher aggregates to form an extended hexagonal array. This submembranous network would have a more profound influence on membrane properties than would individually bound annexin molecules. The apparent affinity of annexin V for different membranes at a given free calcium concentration depends on the phospholipid composition of the membrane. This may result in the targeting of annexin V to specific membrane sites during transient rises in intracellular free calcium levels following stimulation. Thus, calcium-dependent formation of such an annexin network would give rise to locally altered membrane properties.

Membrane surface curvature appears to influence the formation of two-dimensional arrays of annexin molecules. In a recent study using cryoelectron microscopy [26], annexin V binding to large vesicles was observed to promote the formation of planar facets on the vesicles, where the surface is flattened by large clusters of annexin V molecules. High surface curvature was found to hinder formation of the two-dimensional protein arrays. Studies using low-angle neutron scattering of annexin V bound to small unilamellar vesicles [27] therefore may not be observing the aggregated state but rather extensive binding of monomeric annexin V. In contrast, our study and the electron microscopy studies have used either lipid monolayers or large vesicles, and these have detected significant organization of annexin V into trimeric unit aggregates. Our cross-linking results are in agreement with the neutron scattering studies [27] in that annexin V is monodisperse in the absence of vesicles.

Calcium-dependent membrane binding associated with multimer formation may be common to many annexins. The immunofluorescent localization patterns and high tissue concentrations of the annexins [28] support this possibility. Complex multimer sheets lining the phospholipid inner membrane leaflet would alter properties such as fluidity and sequestration of specific phospholipids, which, in turn, have been shown to alter membrane protein function [26,29]. Such a submembranous scaffolding maintains and stabilizes the membrane [30,31]. For example, dystrophin is localized to the inner surface of the sarcolemma in normal skeletal muscle.

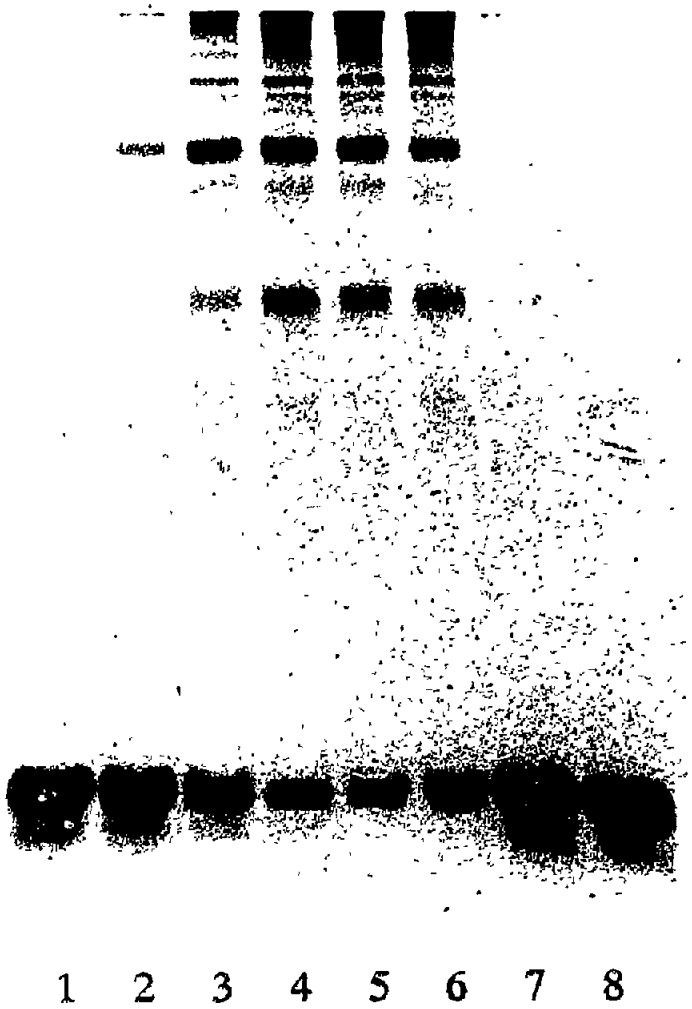


Fig. 2. Effects of calcium, magnesium, phospholipids on annexin V cross-linking. Lanes 1–7 are all in the presence of DOPC:DOPG LUVs. (1) 0.1 mM EGTA, (2) 0.05 mM $CaCl_2$, (3) 0.10 mM $CaCl_2$, (4) 0.20 mM $CaCl_2$, (5) 0.50 mM $CaCl_2$, (6) 1.0 mM $CaCl_2$, (7) 0.1 mM EGTA, 2 mM $MgCl_2$, (8) 1.0 mM $CaCl_2$, but without phospholipid. Calcium but not magnesium causes multimer formation. Cross linking does not occur in the absence of phospholipids.

When absent, as in Duchenne muscular dystrophy, the plasma membrane is unstable and the fibers rapidly turn over [32]. In addition, the $Ca^{2+}$-dependent lining of the membrane would sterically block the translocation of phospholipid binding proteins such as protein kinase C and cellular phospholipases. The calcium-dependent self-association on membrane surfaces therefore may represent a novel mechanism of second messenger-coupled cell regulation.

*Acknowledgements:* We wish to thank M. El-Sayed, P Meers, and M.F. Roberts for useful suggestions and B. Bonavia for technical assistance. This work was supported by National Institutes of Health Grants. R29-GM-44554 (B.A.S ), R01-NS-20357 (J.F.H.), R01-DK-41740 (J.R.D.); and the American Heart Association, MA Division, Research Grant-in-Aid 13-539-878 (B.A.S.). B A S. is the recipient of an American Cancer Society Junior Faculty Research Award (JFRA-215).

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