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Annexins

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The annexins are a family of proteins that bind anionic phospholipid surfaces in a Ca^{2+}-dependent manner (general reviews include Raynal & Pollard 1994, Swairjo & Seaton 1994, Seaton 1996, Mollenhauer, 1997). Due to this functional property, individual annexins have been discovered independently by numerous laboratories with diverse experimental goals. Ca^{2+} characteristically causes the annexins to shift from a soluble to membrane associated state. This shift is believed to be the mechanism that underlies annexin cellular function.

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The predicted primary sequence of the annexins is well-established. There are ten unique genes in mammals; homologues exist in Drosophila, C. elegans, Hydra, Dictyostelium and several plant species (Nevid & Horseman 1996; Morgan & Fernández 1997). All of the annexins display domains of repeating sequence of ~70 amino acids. These highly conserved domains have sequence similarities of 40–70% and represent the protease-resistant phospholipid binding core of each protein. A distinctive feature of each annexin is the amino-terminal region which varies in sequence and length and may dictate cellular function. For example, the amino-terminal domains of several annexins are excellent substrates for protein kinase C and tyrosine kinase phosphorylation (Dubois *et al.* 1996; Rothhut 1997). Phosphorylation alters *in vitro* properties, including phospholipid and actin binding. In the case of annexin II, the amino terminal domain binds to a p11 subunit which allows for heterotetramer complex formation (Waisman 1995). Formation of this heterotetramer is inhibited by protein kinase C phosphorylation, which may spatially interfere with the annexin- p11 site of interaction (Jost & Gerke 1996).

The E-F hand motif in calmodulin, troponin C and their homologs are not present in annexins. Instead, the annexins possess a conserved Ca^{2+} binding motif that contains the sequence [(Leu/Met)-Lys-Gly-X-Gly-Thr] followed, after a gap of ~40 residues, by an acidic residue. In soluble form, annexins bind Ca^{2+} more weakly than E-F hand Ca^{2+}-signalling proteins like calmodulin. However, membrane binding enhances Ca^{2+} binding by annexins. The Ca^{2+}-dependent binding to phospholipid membranes in platelets is of high affinity, with dissociation constants in the low nanomolar range (Tait *et al.* 1989).

Annexins share a novel protein fold that comprises the conserved Ca^{2+}/phospholipid membrane-binding core (Fig. 1). Each domain contains a four-helix bundle and a perpendicular -helix. In the four-domain annexins (e.g. annexins I-V), these domains are approximately coplanar and arranged symmetrically around a two-fold axis. The domains are paired as half-molecule modules that make a hydrophilic pore or cleft between them. The eight-domain annexin VI resembles two tetrad annexin structures that are approximately perpendicular to each other and linked by a long, flexible -helical segment (Benz *et al.* 1996; Kawasaki *et al.*, 1996). The relative orientation of the two tetrad portions may change in the presence of membranes. While the conserved annexin core is well-characterized, the more extensive N-terminal domains that exist in several annexins remain poorly characterized in terms of tertiary structure.

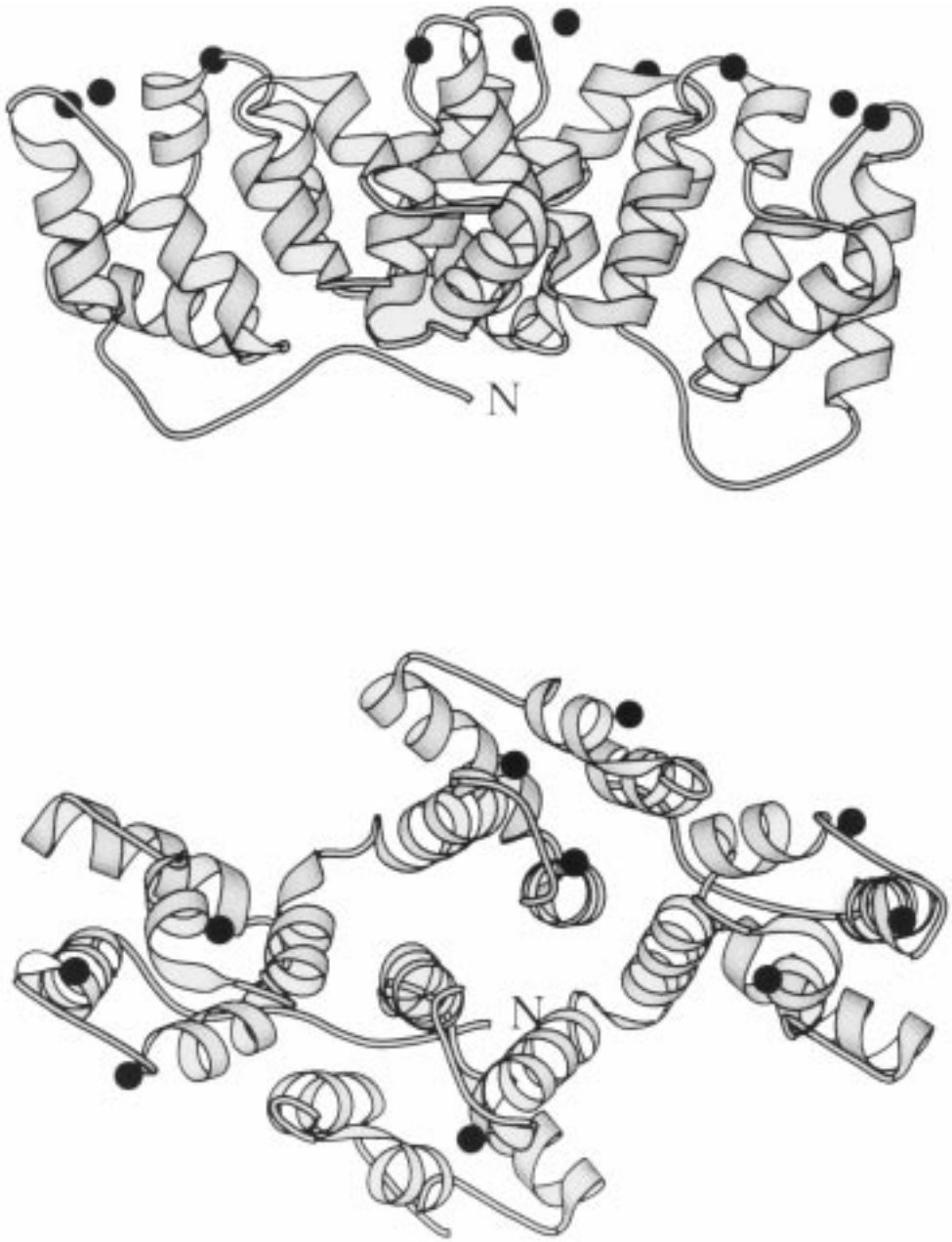

Figure 1. Ribbon diagram of molecular structure of rat annexin V (Swairjo et al. 1995). Dark spheres indicate Ca^{2+} ions, N-terminus is labelled. Top view shows annexin from the side, with membrane binding surface facing upwards. Bottom view (obtained by rotating the molecule 90% around the x axis) looks down at the membrane binding surface.

Annexin binding and alteration of membrane properties.

Annexin V has emerged as a structural paradigm for a Ca^{2+}-dependent, peripheral membrane-binding mechanism. Multiple Ca^{2+} ions bind at sites corresponding to the conserved sequence motif as well as several weaker sites. The bound Ca^{2+} ions are localized to interhelical loops along one surface of the protein. A Ca^{2+}-dependent conformational transition occurs along this surface that translocates a tryptophan residue from a buried to a surface-exposed environment (Concha *et al.* 1993; Sopkova *et al.* 1993), where the side chain contacts the lipid membrane and stabilizes binding (Meers & Mealy 1993; Campos *et al.* 1998). Structures of annexin-phospholipid analogs have identified at least one site of 'Ca^{2+}- bridging,' where the ternary complex

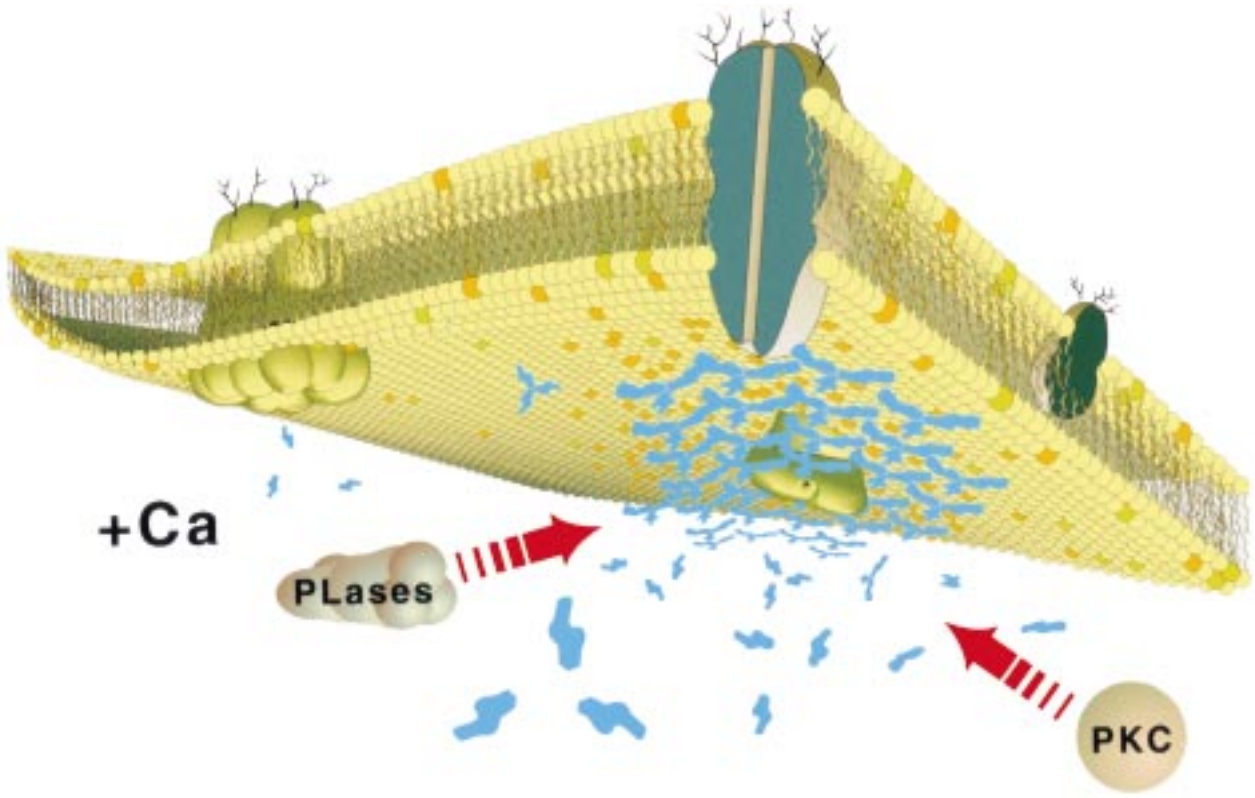

Fig. 2: Model of Ca^{2+}-dependent binding of annexin to the inner membrane leaflet, with formation of annexin trimers and extended triskelion-based arrays around membrane target proteins, protein kinase C (PKC) and phospholipases (PLases). Reproduced with permission (Kaetzel & Dedman, 1995).

contains a Ca^{2+} ion coordinated by oxygen ligands from both the protein and phospholipid head group (Swairjo *et al.* 1995).

Molecular structures of membrane-bound annexins on phospholipid monolayers, obtained at high resolution by image-enhanced cryoelectron microscopy, generally resemble those in x-ray crystal structures (Berendes *et al.* 1993; Voges *et al.* 1994; Olofsson *et al.* 1995). However in the membrane-bound state, the protein molecules reorient themselves so that their Ca^{2+}-binding sites become more closely coplanar with the membrane. Less is known about interactions that occur on the protein surface facing away from the membrane, i.e. toward the intracellular or extracellular milieu. This surface contains the N-terminus, where forms a junction with the conserved C-terminal annexin core, and the C-terminus. Biochemical data link this region of the molecule to sites of protein-protein interaction as well as phosphorylation. Recent crystal structures show a binding pocket for certain drugs in this locale (Kaneko *et al.* 1997; Hofmann *et al.* 1998).

Chemical cross-linking (Concha *et al.* 1992; Kirsch *et al.* 1997) and cryoelectron microscopic studies (Pigault *et al.* 1994) indicate that annexin V self-oligomerizes into trimeric or hexameric ordered arrays on acidic phospholipid membrane surfaces. These and other experimental data have led to the following model to describe the mechanism of action of many of the annexins (Fig. 2). In the resting state, where intracellular free Ca^{2+} concentrations are low, annexins exist primarily in the soluble, unaggregated state. During cell stimulation, where Ca^{2+} concentrations rise subadjacent to the membrane, annexins bind to the membrane surface and self-associate. The presence of organized arrays of surface-bound annexins alters membrane properties, such as fluidity and segregation of certain phospholipids, and the properties of other membrane-bound proteins and enzymes. Mechanisms of extracellular annexin function may be similar except that exposure of certain phospholipids, particularly acidic, is likely to be the trigger rather than a rise in free Ca^{2+}.

Oligomerization on membrane surfaces may explain how annexins with fusogenic activity bring two membranes together prior to fusion (Gerke & Moss 1997). Cryo-electron microscopy studies of several annexins show that the fusogenic annexins

self-associate so that each protein molecule can interact with the outer lipid leaflet of opposing membranes (Lambert *et al.* 1997). In addition, phospholipid sequestration through extensive coverage of the membrane surface is considered a likely basis for inhibition of several membrane-associated enzymes by annexins (Andree *et al.* 1992; Dubois *et al.* 1998). Indeed the behavior of annexins on membrane surfaces may be general enough to account for many of the apparent functions of annexins.

Investigation of annexin cellular function

Initial studies with each annexin have been to identify an *in vitro* function, i.e., membrane fusion, ion conductance, cytoskeletal binding, enzyme inhibition or coagulation inhibition. Additional data are the expression and location of the individual annexin within cells of defined physiological function. Kaetzel *et al.* (1994) identified annexin IV concentrated along the apical membrane of fluid-secreting epithelia. Whole-cell patch clamp measurements determined that the annexin regulated Cl^- efflux. Similarly, Naciff *et al.* (1996a) found annexin VI associated with the plasma membrane of motor neurons. Infusion of affinity-purified anti-annexin VI antibodies into cultured neurons demonstrated activation of inward Ca^{2+} currents (Naciff *et al.* 1996b).

Two structure-based hypotheses have been presented to explain the annexin-induced voltage-gated Ca^{2+} channel activity observed *in vitro*. In the 'microscopic electroporation' model described for annexin V, the peripheral binding of the protein changes the local permeability of the membrane. As a result, Ca^{2+} ions are translocated across the membrane and gated peripherally through central pore of the annexin. The angle between the two annexin half-molecule modules can vary, altering the local environment and gating properties of the pore (Demange *et al.* 1994; Voges *et al.* 1995). In an alternative model of Ca^{2+} channel activity, based on the crystal structure of hydra annexin XII, a hexameric form of the protein inserts into the bilayer and creates a transmembrane structure resembling an inverted micelle (Luecke *et al.* 1995).

Genetic approaches have contributed to the investigation of individual annexin function. Harder and Gerke (1993) designed a 'dominant-negative' mutant p11 gene that, when expressed, would specifically aggregate cellular annexin II and p11 subunits and disrupt the cellular function of the heterotetramer. The mutant gene was expressed in polarized cultured MD CK cells. The removal of annexin 11/p11 from the cortical region of the cells disrupted endocytosis yet had no effect on microfilament or late endosome organization. These studies demonstrate that annexin II/p11 binds early endosomes at the plasma membrane and facilitates vesicle aggregation, fusion and cellular targeting.

One approach to analyzing annexin function in the context of an intact animal is with transgenic mice. Ca^{2+} is a primary regulator of cardiac function including contraction/relaxation cycles, gene transcription and mitochondrial enzyme function. To evaluate the role of annexin VI in cardiac function, the protein was overexpressed 10-fold using a heart-specific promoter (Gunteski-Hamblin *et al.* 1996). Indeed, myocyte overexpression markedly reduced the basal Ca^{2+} levels and the stimulated Ca^{2+} transients. This cellular phenomenon resulted in the development of congestive heart failure. It appears that annexin VI plays a critical role in the regulation of Ca^{2+} homeostasis which, in turn, affects the physiological status of the intact animal.

While many putative annexin functions are intracellular, others occur outside the cell. Cell-surface annexins act as receptors for many polypeptide ligands (Upton *et al.* 1996; Siever & Erickson 1997), including viruses (De Meyer *et al.* 1997; Pietropaolo & Compton 1997) and tissue plasminogen activator (Hajjar *et al.* 1998). Annexin V has been identified as a collagen-binding protein, known in this context as anchorin CII (von der Mark & Mollenhauer 1997). Other extracellular roles for annexins may include cell adhesion, cell signalling, and anti-inflammation (Ahluwalia *et al.* 1996) or anticoagulation activities (Reutelingsperger & van Heerde 1997).

Clinical relevance of the annexins

The mammalian plasma membrane is maintained in an ATP-dependent asymmetric manner: phosphatidylcholine (PC) is primarily on the outer leaflet, and the acidic and aminophospholipids, phosphatidic acid (PA), phosphatidylserine (PS) and phosphatidylethanolamine (PE), are segregated to the inner leaflet. The exposure of PS to the outer leaflet is known to be a biological signal. Platelet activation and endothelial cell injury, for example, causes rapid translocation of PS to the cell membrane outer surface. This signal initiates the clotting cascade. Similarly, an early event of apoptosis and erythrocyte aging is extracellular PS exposure. The final stage in these processes is macrophage recognition and clearance by phagocytosis.

Several autoimmune disorders, including Lupus and Antiphospholipid Syndrome (APS), display circulating antiphospholipid antibodies which compete with naturally occurring plasma PS binding proteins. These autoantibodies cause inappropriate thrombi resulting in infarcts throughout the cardiovascular system. Extracellular annexins would compete for these antibodies and prevent the inappropriate autoimmune response. Several studies have shown infused annexin V to be effective in reducing thrombosis in animal models (Romisch *et al.* 1991; VanRyn-McKena *et al.*, 1993; Chollet *et al.* 1992; Yokoyama *et al.*, 1994) Radiolabeled annexin V may prove valuable in medical imaging. Tait *et al.* (1994) demonstrated concentration of radiolabeled annexin V to acute occlusive arterial thrombosis. The anionic phospholipid binding of annexins has the potential to target thrombolytic agents, such as streptokinase, urokinase and tissue plasminogen activator, to sites of activated platelets and injured endothelium. Tait *et al.* (1995) have created a prourokinase annexin V chimera which demonstrates phospholipid surface binding and clot-lysing activity *in vitro*. The anionic phospholipid binding property of the annexins makes them excellent agents in development of diagnostic and treatment strategies for hemologic disorders. The usefulness of annexin V in this regard has been established in flow cytometric assays for apoptosis that are based on detection of phospholipid asymmetry (van Engeland *et al.* 1998). Other applications that take advantage of the annexins' distinctive membrane-binding properties are likely to follow.

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