# Annexins – unique membrane binding proteins with diverse functions

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## Summary

**Annexins are a well-known multigene family of $Ca^{2+}$-regulated phospholipid-binding and membrane-binding proteins. Recent work employing annexin-knockdown or -knockout models has provided new insights into the biological functions of different annexin proteins. Transient annexin depletion by RNA interference and the expression of dominant-negative mutant proteins has revealed roles for the proteins in membrane processes ranging from the control of membrane structure to certain membrane transport phenomena. Although such functions correlate well with the ability of annexins to interact with cellular membranes in a reversible and regulated manner, some activities are membrane independent, probably because annexins can also engage in specific protein-protein interactions. Among other things, this is evident in annexin A1- and A2-knockout mice, which show impaired regulation of neutrophil extravasation and defects in plasmin generation, respectively.**

Key words: Calcium, Cell migration, Cytoskeleton, Endocytosis, Exocytosis, Membrane

## Introduction

The annexins are a family of $Ca^{2+}$/lipid-binding proteins that differ from most other $Ca^{2+}$-binding proteins in their $Ca^{2+}$-binding sites. These have a unique architecture that allows them to dock onto membranes in a peripheral and reversible manner. The conserved $Ca^{2+}$- and membrane-binding module is the annexin core domain, which consists of four so-called annexin repeats, each of which is 70 residues in length. It is highly α-helical and forms a compact, slightly curved disc that has a convex surface harboring the $Ca^{2+}$- and membrane-binding sites and a concave side that points away from the membrane and is thereby available for other types of interaction/regulation (Fig. 1). The N-terminal region precedes the core domain and is diverse in sequence and length. It mediates regulatory interactions with protein ligands and regulates the annexin-membrane association (reviewed by Gerke and Moss, 2002; Raynal and Pollard, 1994). Although the N-terminal domain has long been considered a separately folded entity, recent crystal structures reveal that, at least in annexin A1, part of it can integrate into the folded core. $Ca^{2+}$ (and probably membrane) binding can then trigger exposure of the N-terminal region, making it available for additional interactions/activities (Fig. 1) (Rosengarth and Luecke, 2003). The activity of the exposed N-terminal region could thus be tightly controlled through $Ca^{2+}$/membrane binding.

The annexin family comprises >500 different gene products expressed in most phyla and species (reviewed by Morgan and Fernandez, 1997). In vertebrates, 12 annexin subfamilies (A1-A11 and A13), which have different splice variants, have been identified. These have different N-terminal domains and differently positioned $Ca^{2+}$/membrane-binding sites within the core domain. Analyses of the biochemical properties and subcellular localizations of annexins, and later studies of the effects of anti-annexin antibodies and annexin mutants, mainly in permeabilized cell systems, have allowed several potential physiological functions to be assigned to different annexins. Most of these take into account their regulated binding to membranes and a scaffold role at certain membrane domains is a common theme.

Proposed to act as membrane-membrane or membrane-cytoskeleton linkers, annexins have been implicated in $Ca^{2+}$-regulated exocytotic events, certain aspects of endocytosis and stabilization of specific domains of organelle membranes and the plasma membrane. However, other potential functions have been put forward – for example, those taking into account the RNA-binding capacity of some annexins (Filipenko et al., 2004; Vedeler and Hollas, 2000), their regulated nuclear localization (Eberhard et al., 2001; Mizutani et al., 1992; Tomas and Moss, 2003) or specific nucleotide-binding activities (Banderowicz-Pikula et al., 2001; Caohuy et al., 1996). Because some annexins occur extracellularly, they might also function outside the cell, although their (direct or indirect) secretion is not well understood. Detailed discussions of postulated annexin functions and their structure and biochemistry can be found elsewhere (Gerke and Moss, 2002; Bandorowicz-Pikula, 2003). Here, we focus on recent developments that have employed, among other techniques, knockdown and knockout approaches to address annexin function. We concentrate on the involvement of annexins in membrane organization and membrane traffic but also review some potential extracellular activities.

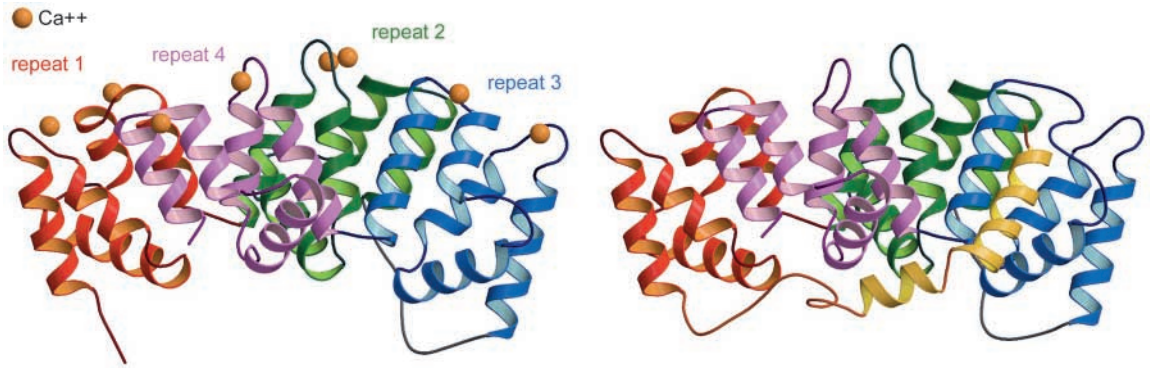


**Fig. 1.** Molecular structure of annexin A1. Ribbon presentation showing the three-dimensional fold of the Cα backbone of annexin A1 in the presence (left) or absence (right) of $Ca^{2+}$ ions (Rosengarth et al., 2001; Rosengarth and Luecke, 2003). The N-terminal domain (residues 1-40) is disordered in the X-ray structure of the $Ca^{2+}$-bound annexin A1 and integrates into repeat 3 of the folded core domain in the $Ca^{2+}$-free protein (depicted in yellow in the right-hand structure). Thus, upon $Ca^{2+}$ binding, the N-terminal α helix is expelled from the protein core and most likely becomes accessible for other interactions (Rosengarth and Luecke, 2003). In the $Ca^{2+}$-bound conformation, the annexin can attach to membranes through its convex (upper) side, with the $Ca^{2+}$ ions serving a bridging function. Figure kindly provided by Anja Rosengarth (University of California, Irvine).

## Annexins as membrane scaffold proteins

The central biochemical characteristic of annexins is their $Ca^{2+}$-regulated binding to the periphery of membranes containing acidic phospholipids. This could allow them to organize the interface between the cytoplasm (or cytoskeleton) and the cytoplasmic face of cellular membranes. In fact, cryoelectron and atomic force microscopy of annexins bound to model membranes have provided compelling images of the proteins forming two-dimensional lattices or certain domains on the surface of membranes (Janshoff et al., 2001; Lambert et al., 1997; Reviakine et al., 2000; Lambert et al., 2004). Although the coating of target membranes to establish and/or regulate lateral membrane domains is likely to be a key element of annexin function, a few questions remain. First, what are the domains stabilized by such annexin activity? Second, do all annexins share this function, and which membranes do they interact with? Third, how is this activity regulated? Recent work employing several different approaches has shed light on some of these questions, particularly in the case of annexin A2.

Sites of actin assembly at cellular membranes have been identified in different cell systems as points at which some annexins are specifically recruited. For example, in smooth muscle cell membranes, the organization of raft (here used to refer to glycosphingolipid- and cholesterol-rich membrane areas resistant to extraction with Triton X-100 in the cold) and non-raft (glycerophospholipid-rich) microdomains is regulated by annexin A2 and annexin A6. Both associate with rafts and appear to mediate interaction with the cytoskeleton (Babiychuk and Draeger, 2000; Draeger et al., 2003). Other structures providing examples of recruitment of annexins to actin-associated membrane areas are (i) endothelial adherens junctions, which recruit annexin A2, together with the Shp2 tyrosine phosphatase, in a cholesterol-dependent manner, (ii) epithelial adherens junctions, at which annexin A2 associates with Rac1-containing complexes and (iii) the adhesion molecule CEACAM (Burkart et al., 2003; Hansen et al., 2002; Kirshner et al., 2003). In polarizing epithelial cells, annexin A2 and its intracellular protein ligand S100A10 (see below) are targeted to actin-rich apical junctions, where they function in a complex with the large actin-binding protein AHNAK in organizing the cortical actin cytoskeleton during polarization (Benaud et al., 2004). Moreover, annexin A2 localizes to highly dynamic membrane domains that serve as F-actin assembly platforms. These include attachment sites for non-invasive enteropathogenic *Escherichia coli* (EPEC), which manipulate the host cell cytoskeleton to form actin-rich pedestals underneath the adherent bacteria, and the membrane-F-actin interfaces of motile pinosomes, which assemble actin comet tails for intracellular movement (Goosney et al., 2001; Merrifield et al., 2001; Zobiack et al., 2002). In the latter case, expression of a dominant-negative annexin A2 mutant abolishes the formation of motile pinosomes, providing strong evidence for a role for the protein in mediating actin assembly at certain membrane sites (Merrifield et al., 2001).

Substantial evidence has thus revealed a connection between annexin A2, which is an F-actin-binding protein (Filipenko and Waisman, 2001; Gerke and Weber, 1984), and certain sites of actin attachment at cellular membranes. However, these appear to be quite divergent, ranging from EPEC-induced actin pedestals to actin comet tails propelling membrane vesicles and actin structures at specialized sarcolemmal domains. What is the unifying principle in these actin-membrane interactions that requires annexin A2? Given that the protein does not associate with cytosolic actin-containing structures such as stress fibers or the actin tails that propel intracellular *Listeria* (Merrifield et al., 2001), the specificity for annexin A2 recruitment must lie in the cellular membranes that serve as the actin assembly platform. Indeed, when we compare the membrane domains involved, it becomes apparent that they share raft characteristics. Moreover, in several of the examples listed above, disturbing raft structure by cholesterol depletion or sequestration precludes interaction with annexin A2 (and also interferes with the specific actin assembly) (Babiychuk and Draeger, 2000; Benaud et al., 2004). This indicates that annexin A2 is specifically recruited to membrane rafts that serve as platforms for actin assembly.

Specificity for annexin A2 could be provided by cholesterol itself, because it increases the binding of annexin A2 to

negatively charged phospholipid liposomes, and annexin A2 protects membrane cholesterol from extraction with methyl-β-cyclodextrin (Ayala-Sanmartin et al., 2001; Mayran et al., 2003). In vitro experiments employing solid-supported model membranes do not reveal a direct interaction of annexin A2 with cholesterol (Ross et al., 2003), although it should be stressed that solid-supported membranes behave differently from liposomes. However, direct binding to and colocalization of annexin A2 with phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)$P_2$] has been observed (Hayes et al., 2004; Rescher et al., 2004). Since PtdIns(4,5)$P_2$ can cluster with raft markers and the assembly of actin comets behind motile pinocytic vesicles requires PtdIns(4,5)$P_2$ (Rozelle et al., 2000), this lipid might serve as the prime membrane anchor for raft-associated annexin A2, possibly acting in conjunction with cholesterol. Thus, we propose that annexin A2 is first targeted to membranes by a general binding to negatively charged phospholipids and then recruited to raft structures by the specific interaction with PtdIns(4,5)$P_2$. By engaging in homophilic lateral interactions, annexin A2 could then induce and/or stabilize raft clustering, which is likely to be a prerequisite for actin assembly at such sites (Fig. 2). Given that other annexins form lateral assemblies on membranes in vitro, they might function similarly to annexin A2, possibly in different membrane domains.

How is such a scaffold function regulated? Given that most annexins reside in the cytosol of resting cells, at least in culture, their domain-organizing capability must be activated somehow. The prime candidate for the activator is intracellular $Ca^{2+}$. Intracellular $Ca^{2+}$ mobilization induced by several stimuli triggers recruitment of annexins to membranes in several cell models, the free $Ca^{2+}$ concentration required for membrane translocation differing between different annexins (reviewed by Gerke and Moss, 2002; Raynal and Pollard, 1994). Thus, depending on the mode of $Ca^{2+}$ mobilization, the location of the rise in intracellular $Ca^{2+}$ concentration and the nature of the $Ca^{2+}$ signal, different annexins can probably be recruited independently from one another to their respective target membrane. This could enable cells to respond to different stimuli by undergoing a range of dynamic membrane reorganizations supported by annexin-induced membrane scaffolds. However, we must consider variations of this theme because some annexins can interact with membranes in the absence of $Ca^{2+}$ (reviewed by Gerke and Moss, 2002). Moreover, some annexins, such as annexin A9, annexin A10 and the *Caenorhabditis elegans* annexin NEX4, lack high-affinity $Ca^{2+}$-binding sites, which indicates that they are probably not affected by intracellular $Ca^{2+}$ fluctuations (Goebeler et al., 2003; Morgan and Fernandez, 1998; Morgan et al., 1999).

## Membrane/protein transport steps involving annexins

The link between annexins and vesicle traffic dates back to the first isolation of an annexin, annexin A7 (synexin), as a protein participating in $Ca^{2+}$-regulated chromaffin granule exocytosis (reviewed by Creutz, 1992; Raynal and Pollard, 1994). Although annexin $A7^{+/-}$ mice have a defect in insulin secretion (Srivastava et al., 1999), more-detailed characterization has revealed that they exhibit altered expression of the inositol (1,4,5)-trisphosphate [Ins(1,4,5)$P_3$] receptor and a resulting failure of Ins(1,4,5)$P_3$-induced $Ca^{2+}$ release from internal stores, which is typically required for the secretion of insulin from pancreatic islet cells (Srivastava et al., 1999). Thus, the insulin secretion defect is probably caused by altered $Ca^{2+}$

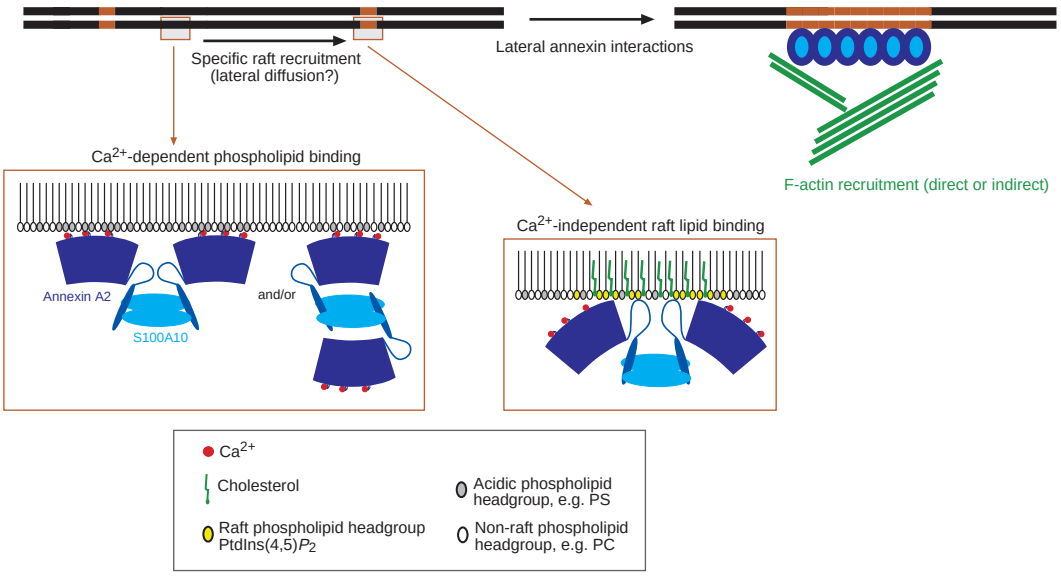


**Fig. 2.** Model of membrane domain stabilization mediated by annexin A2. The model takes into account the $Ca^{2+}$-dependent binding of annexin-A2–S100A10 to acidic phospholipids, which is mediated through the annexin core domain (left). Lateral diffusion could then direct the complex to raft membrane domains rich in cholesterol, glycosphingolipids and certain phosphatidylinositol phosphates, in particular phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)$P_2$]. This could result in an annexin-A2–S100A10 fraction not requiring external $Ca^{2+}$ for raft lipid binding. Following this raft recruitment, lateral annexin-annexin interactions, possibly regulated by $Ca^{2+}$, could lead to the formation of a protein scaffold beneath the membrane and the concomitant clustering of rafts and recruitment of F-actin.

signaling rather than loss of some granule docking or fusion activity, which annexin A7 can provide in vitro (reviewed by Creutz, 1992; Raynal and Pollard, 1994). Interestingly, homozygous annexin A7$^{-/-}$ mice, described by Pollard and co-workers, exhibit an embryonic lethal phenotype (Srivastava et al., 1999), whereas annexin A7$^{-/-}$ mice generated by Noegel and co-workers (Herr et al., 2001) are viable. The difference could be due to a different genetic background. However, even in the latter (viable) knockout mice, altered $Ca^{2+}$ signaling is observed in some cells. Cardiomyocytes derived from these mice show disturbances in $Ca^{2+}$-dependent and frequency-induced shortening (Herr et al., 2001). Moreover, astrocytic $Ca^{2+}$ waves exhibit an increased velocity in primary astrocytes isolated from the viable annexin A7$^{-/-}$ mice (Clemen et al., 2003). Collectively, the data suggest that annexin A7 is involved in $Ca^{2+}$ signaling and/or $Ca^{2+}$ homeostasis in these cells and thereby could affect electromechanical properties (Herr et al., 2001). The annexin A7$^{-/-}$ astrocytes also show a significantly increased proliferation rate (Clemen et al., 2003), which is in line with the finding that annexin A7$^{+/-}$ mice are cancer prone and suggests annexin A7 functions as a tumor suppressor gene (Srivastava et al., 2003).

Other annexin-knockout models (mice lacking annexins A1, A2, A5 or A6, and DT40 chicken pre-B cells lacking annexin A5) also show no obvious phenotype related to a primary defect in vesicle docking and/or fusion events (Brachvogel et al., 2003; Hannon et al., 2003; Hawkins et al., 1999; Hawkins et al., 2002; Ling et al., 2004; Song et al., 2002). This indicates that the annexins targeted in these mice do not serve as essential factors in vesicle docking and/or fusion or that such functions are redundant or taken over by another member of the family during mouse development. Given the sequence and structural homology among the annexins and their overlapping tissue distributions, such compensatory mechanisms have to be considered.

The problem of compensatory upregulation of alternative annexins in a given annexin knockout, which has for example been observed in annexin A1$^{-/-}$ mice (Hannon et al., 2003), can be overcome by transient downregulation (e.g. through RNA interference) or the expression of dominant interfering mutants. Both have indeed proven successful in several cases. For example, a dominant-negative annexin A2 mutant not only blocks the formation of motile pinosomes (see above) but also leads to aberrant membrane clustering of the raft-associated hyaluronate receptor CD44 (Merrifield et al., 2001; Oliferenko et al., 1999). Similarly, truncation of annexin A6 generates a dominant interfering mutant that inhibits cysteine-protease-dependent budding of coated pits. Moreover, it markedly reduces internalization and/or transport of low-density lipoproteins (LDLs) to late endosomes/lysosomes, the function of annexin A6 in endosomal transport probably depending on membrane-cholesterol-dependent recruitment of the protein (de Diego et al., 2002; Kamal et al., 1998; Pons et al., 2001).

Annexin A13 is N-terminally myristoylated and mutants to which the N-terminal fatty acid cannot be attached, as well as anti-annexin antibodies, reveal a role for the protein in the apical delivery of raft-enriched membranes in polarized epithelial cells. Interestingly, the two splice variants of annexin A13 – 13a and 13b – have non-overlapping functions. Variant 13b acts primarily in transport of vesicles to the apical membrane surface, whereas 13a also participates in basolateral delivery (Lafont et al., 1998; Lecat et al., 2000).

Recently, Mayran et al. (Mayran et al., 2003) and Zobiack et al. (Zobiack et al., 2003) used RNA interference (RNAi) to downregulate the expression of annexin A2 in HeLa cells. This efficiently depleted the intracellular annexin A2 pool, although at least in one study some annexin A2 remained in the cortex underneath the plasma membrane, presumably because of a significantly longer half-life (Zobiack et al., 2003). This could explain why the RNAi had no effects on plasma-membrane-related events, such as fluid-phase and receptor-mediated internalization. However, the transient knockdown did produce defects in the generation of multivesicular endosomal carrier vesicles on early endosomes (Mayran et al., 2003) and in the morphology and distribution of recycling endosomes (Zobiack et al., 2003), which is consistent with the localization of an intracellular annexin A2 pool on early/recycling endosomes. Mayran et al. (Mayran et al., 2003) observed a block in carrier vesicle formation and a concomitant inhibition of the transport of internalized epidermal growth factor (EGF) receptor and fluid-phase tracers to late endosomes/lysosomes. Zobiack et al. (Zobiack et al., 2003) found that annexin A2 depletion affected the transferrin-recycling pathway: Rab11-positive, perinuclear recycling endosomes appeared more condensed, and characteristic endosomal tubules were often bent to form circles. Although generating somewhat different phenotypes, both knockdown studies are in line with the idea that annexin A2 (presumably in complex with S100A10) has a structural/scaffolding function on endosomal membranes. Once this is disturbed, characteristic endosomal morphologies, such as the tubular extensions on recycling endosomes and the multivesicular carrier vesicles, cannot be generated and/or maintained.

In addition to being present on endosomes and the plasma membrane, annexin A2 is also present on structures of the biosynthetic pathway. The protein is found on secretory granules of chromaffin cells (Creutz, 1992) and a subset of exocytotic transport vesicles. These carry the raft-associated enzyme sucrase isomaltase (SI) from the trans-Golgi network (TGN) to the apical plasma membrane in polarized epithelial cells (Jacob et al., 2004). The SI-containing vesicles, but not a different set of apical transport vesicles containing the non-raft-associated enzyme lactase phlorizin hydrolase (LPH), acquire annexin A2 on their membranes, and their transport to the apical surface is inhibited by RNAi-mediated depletion of annexin A2 (Jacob et al., 2004). Interestingly, the SI- but not the LPH-carrying vesicles require F-actin for efficient transport, revealing another link between annexin A2 and actin-dependent membrane events. Finally, RNAi and interfering antibodies indicate that annexin A2 also participates in the insulin-stimulated plasma membrane translocation of the glucose transporter GLUT-4 (Huang et al., 2004). Moreover, annexin A2, like annexin A1, interacts with dysferlin, the product of the gene whose mutation causes limb girdle muscular dystrophy type 2B. This interaction might be required for $Ca^{2+}$-dependent sarcolemmal wound repair mediated by exocytotic membrane transport (Lennon et al., 2003).

The dimeric S100 protein subunit of the annexin-A2–S100A10 complex, S100A10 (also known as p11), links two annexin A2 chains to form what appears to be a highly

symmetrical heterotetrameric entity (Lewit-Bentley et al., 2000; Rety et al., 1999). It also participates in certain transport steps. S100A10 binds directly to several plasma membrane ion channels, and this correlates with their incorporation into the plasma membrane. The tetrodotoxin-resistant sodium channel $Na_v$ 1.8, the 2P-domain potassium channel TASK-1 and the epithelial calcium channels TRPV5 and TRPV6 all interact with S100A10 through a cytoplasmic domain of the channel and most likely in a manner that does not interfere with binding of annexin A2 to S100A10 (Girard et al., 2002; Okuse et al., 2002; van de Graaf et al., 2003). This could allow annexin A2 in a channel–S100A10–annexin-A2 complex to interact with membrane lipids, possibly the raft lipids discussed above, thereby supporting the transport of the channel to the plasma membrane (Fig. 3). In fact, downregulation of S100A10 by antisense oligonucleotides or RNAi, or disruption of the channel-S100A10 interaction by mutations in the S100A10-binding sites of the channels, interferes with transport of the channels to the plasma membrane (Girard et al., 2002; Okuse et al., 2002; van de Graaf et al., 2003).

S100A10 also binds to the NS3 protein of bluetongue virus. NS3 is a nonstructural protein that is thought to play a role in virus egress from some types of cell. Following synthesis it is transported through the Golgi to the plasma membrane, where it colocalizes with virus-like particles. By interacting with both the viral capsid protein VP2 and S100A10, NS3 could represent a bridging molecule that connects the assembled virus with the cellular export and/or membrane-targeting machinery. Interestingly, the S100A10-binding site on NS3 maps to a 13-residue peptide that mimics the S100A10-binding site on annexin A2, thereby allowing NS3 to compete with annexin A2 for S100A10. Exactly how this relates to the cellular export of assembled virus remains to be determined (Beaton et al., 2002).

## The ultimate puzzle: extracellular annexin activities

Annexins are soluble cytosolic proteins lacking signal sequences that could direct them into the classical secretory pathway. Nevertheless, some members of the family have been identified consistently in extracellular fluids. Alternative pathways for the secretion of annexins A1 and A2 have been proposed (Castro-Caldas et al., 2002; Chapman et al., 2003; Danielsen et al., 2003; Faure et al., 2002; Zhao et al., 2003), but release from lysed cells could also account for the amount of extracellular annexins present in the vasculature. Binding sites for extracellular annexins exist on the cell surface and several possible extracellular functions for these proteins have been proposed. They include a role of annexin A5 as an anti-coagulant protein, a function of annexin A2 as an endothelial cell-surface receptor for plasminogen and tissue-type plasminogen activator (tPA), and anti-inflammatory activities of annexin A1, which are mediated through an interaction with chemoattractant receptors on leukocytes (reviewed by Rand, 2000; Kim and Hajjar, 2002; Perretti and Gavins, 2003). Annexin A5, whose intracellular activities have been linked to the induction of apoptosis (Cardo-Vila et al., 2003; Hawkins et al., 2002), binds to phosphatidylserine exposed on the surface of syncytiotrophoblasts. Such binding could provide a protective shield, which could become disrupted by anti-phospholipid antibodies. A resulting exposure of coagulation-promoting surfaces could lead to pregnancy failure observed in patients suffering from antiphospholipid syndrome (Rand, 2000; Wang et al., 1999).

As mentioned above, annexin A2 on the surface of endothelial cells and leukocytes can function as a receptor for plasminogen and tPA, thereby acting as a positive modulator in the fibrinolytic cascade (reviewed by Kim and Hajjar, 2002). In line with this activity, overexpression of annexin A2 on the surface of leukemic cells derived from acute promyelocytic leukemia (APL) patients correlates with the clinical manifestation of bleeding (Menell et al., 1999). Additional support for the profibrinolytic activity of annexin A2 stems from annexin-A2-knockout mice, which show a marked decrease in tPA-dependent plasmin generation at the endothelial cell surface and an incomplete clearance of injury-induced arterial thrombi (Ling et al., 2004). However, the proposed profibrinolytic function of annexin A2 is somewhat controversial, as annexin A2 in complex with the S100A10 subunit can also inhibit plasmin activity and thereby fibrinolysis (Choi et al., 1998; Fitzpatrick et al., 2000). Moreover, S100A10 has also been identified as a plasminogen

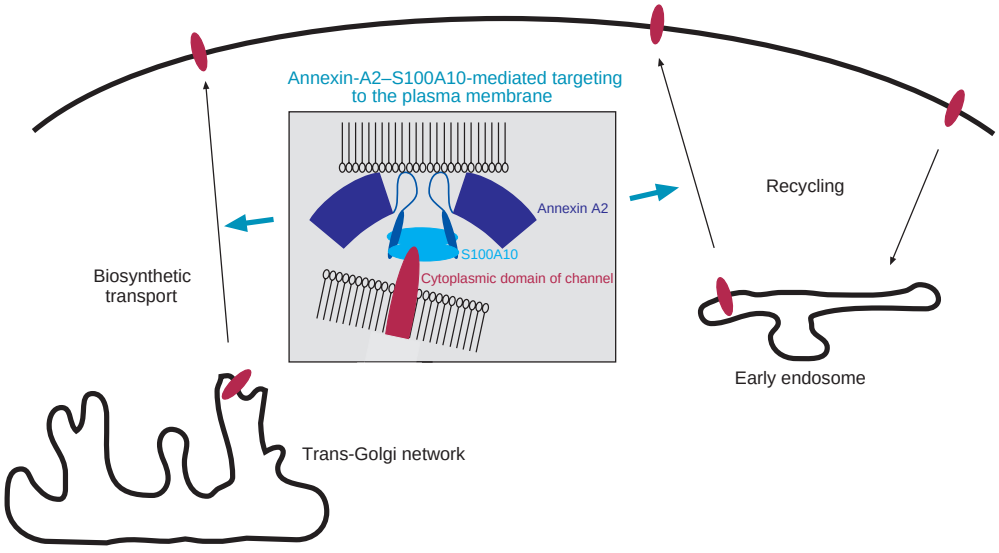


**Fig. 3.** Potential role of annexin-A2–S100A10 in the transport of plasma membrane channels. Plasma membrane ion channels containing a binding site for S100A10 (e.g. the $Ca^{2+}$ channel TRPV5 or the sodium channel $Na_v$ 1.8) can be directed to the cell surface through a S100A10-mediated interaction with the annexin-A2–S100A10 complex and subsequent membrane binding of the annexin A2 subunits of the complex. This could occur within the biosynthetic pathway or upon recycling of the channels through an endosomal recycling organelle.

receptor, and its downregulation by RNAi in colorectal cancer cells attenuates plasmin generation on the surface of these cells (Zhang et al., 2003).

Annexin A1 has the longest history of reported extracellular activity. The most compelling evidence for extracellular annexin A1 is from analysis of seminal plasma. Although annexin A1 and annexin A4 are expressed in the same cells of the ductal prostate epithelium, only annexin A1 is found extracellularly (Christmas et al., 1991). It is also present in human serum, in particular in inflammatory scenarios such as myocardial infarction and experimental colitis (Romisch et al., 1992; Vergnolle et al., 1997). This is consistent with the anti-inflammatory activity displayed by pharmacologically applied annexin A1 in a variety of animal models of inflammation (reviews detailing the anti-inflammatory activity of annexin A1 can be found elsewhere: Flower and Rothwell, 1994; Perretti, 1997; Perretti and Gavins, 2003). In these in vivo systems, as well as in in vitro models, annexin A1 potently and specifically inhibits the transendothelial migration of leukocytes, thereby limiting the degree of inflammation (see, for example, Perretti et al., 1996; Walther et al., 2000). Compelling evidence for an extracellular activity of annexin A1 as a regulator of leukocyte extravasation was the identification of specific annexin A1 receptors on human neutrophils and monocytes. These are members of the formyl peptide receptor (FPR) family of chemoattractant receptors (Perretti et al., 2002; Walther et al., 2000).

The FPRs are seven-transmembrane-span, G-protein-coupled receptors initially identified as targets for bacterially derived peptides of the prototype fMet-Leu-Phe (fMLF). They direct migrating leukocytes to sites of bacterial infection by triggering FPR-dependent signaling cascades leading to cytoskeletal rearrangements required for directional cell migration (reviewed by Prossnitz and Ye, 1997). Initially identified as a ligand for the human FPR (Walther et al., 2000), annexin A1 also binds to the FPR-related lipoxin $A_4$ receptor, also known as FPRL1 (Perretti et al., 2002; Ernst et al., 2004), and to murine FPR (Perretti et al., 2001). In each case, the binding is mediated through the N-terminal annexin A1 peptide Ac1-25 (i.e. the first 25 residues containing an N-terminal acetyl group), which is probably generated at sites of inflammation. Receptor activation induces, among other things, shedding of L-selectin from the leukocyte surface and detachment of adherent leukocytes from activated endothelium (Gavins et al., 2003; Walther et al., 2000). Moreover, pretreatment of human neutrophils with the pharmacologically active annexin A1 peptide (Ac1-25) results in a dose-dependent desensitization of the FPR towards subsequent fMLF challenge (Walther et al., 2000). Thus, by interacting at sites of inflammation with the FPR and/or the related FPRL1, annexin A1 can downregulate the extent of leukocyte extravasation, thereby acting in an anti-inflammatory manner (Fig. 4). Strong support for this anti-inflammatory action of extracellular annexin A1 comes from analysis of inflammatory responses in annexin-A1-knockout mice. Neutrophil extravasation in response to a zymosan-induced peritonitis is substantially increased in annexin $A1^{-/-}$ mice compared with their wildtype counterparts, and the knockout mice suffer from a marked exacerbation of antigen-induced arthritis (Hannon et al., 2003; Yang et al., 2004). Moreover, the anti-inflammatory effects of dexamethasone, attributed to the increased expression and secretion of annexin A1, are greatly reduced in the knockout mice. Thus, by activating FPR family members, annexin A1 can serve as an important regulator of leukocyte migration and as an endogenous anti-inflammatory protein. Moreover, since FPR is not restricted to leukocytes, the annexin-A1–FPR interaction might also regulate the migratory behavior of other cells expressing FPR/FPRLs, such as dendritic cells, hepatocytes, astrocytes and alveolar type II cells (McCoy et al., 1995; Sozzani et al., 1998; Le et al., 2000; Rescher et al., 2002).

## Conclusions

Although we have known for a long time that annexins are a multigene family of $Ca^{2+}$-regulated membrane-binding proteins, only in recent years have direct knockout and knockdown approaches revealed functional properties of different annexin proteins. In most mouse knockout models, the phenotypes are rather subtle, possibly because of compensatory actions of other annexin family members. Nevertheless, the phenotypes observed are in line with the previously proposed functions of annexins as $Ca^{2+}$-signal mediators (annexin A6 and A7 in cardiomyocytes), mediators of local fibrinolytic action (annexin A2 on endothelial cells) and anti-inflammatory proteins regulating the extravasation of neutrophils (annexin A1). RNAi technology has overcome at least to some extent the potential problem of compensatory expression and/or activity within the multigene family. In the case of annexin A2, this has led to the identification of several

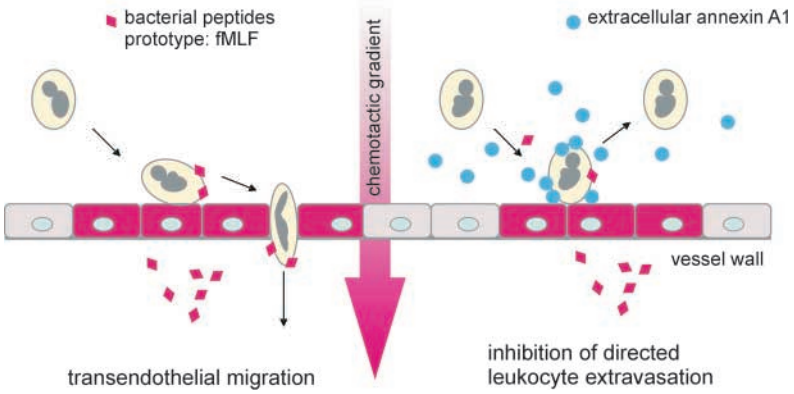


**Fig. 4.** Inhibition of leukocyte extravasation by annexin A1. Directed leukocyte transmigration through activated endothelium (red) into inflamed/infected tissue is mediated by chemoattractant receptors of the FPR family, which are targets of fMLF (left). This directed migration is inhibited in the presence of extracellular annexin A1 and/or N-terminal annexin A1 peptides, which are probably generated at sites of inflammation (right). An interaction of annexin A1 with FPR/FPRLs present on migrating leukocytes can trigger receptor desensitization and/or L-selectin shedding, leading to the observed block in directed leukocyte migration.

cellular processes affected by downregulation of the protein. Cells appear to require annexin A2 as a structural or scaffolding protein that stabilizes and/or regulates the dynamics of certain membrane domains. It is likely that annexin A2 shares such activity with other annexins, which could act as dynamic scaffolds on different target membranes and/or other domains within a given membrane. Future work should be directed towards identifying these scaffolds, and the associated annexins, and describing the molecular basis of lateral annexin assemblies and their regulation in cells. Given the possible compensation that occurs in individual annexin knockouts, it will also be of interest to generate and analyze knockouts of multiple annexins. This will provide additional and possibly still unexpected insights into the physiology of annexins.

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