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REVIEW

Annexins as organizers of cholesterol- and sphingomyelin-enriched membrane microdomains in Niemann-Pick type C disease

Magdalena Domon · Mehmet Nail Nasir · Gladys Matar · Slawomir Pikula · Françoise Besson · Joanna Bandorowicz-Pikula

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Abstract Growing evidence suggests that membrane microdomains enriched in cholesterol and sphingomyelin are sites for numerous cellular processes, including signaling, vesicular transport, interaction with pathogens, and viral infection, etc. Recently some members of the annexin family of conserved calcium and membrane-binding proteins have been recognized as cholesterol-interacting molecules and suggested to play a role in the formation, stabilization, and dynamics of membrane microdomains to affect membrane lateral organization and to attract other proteins and signaling molecules onto their territory. Furthermore, annexins were implicated in the interactions between cytosolic and membrane molecules, in the turnover and storage of cholesterol and in various signaling pathways. In this review, we focus on the mechanisms of interaction of annexins with lipid microdomains and the role of annexins in membrane microdomains dynamics including possible participation of the domain-associated forms of annexins in the etiology of human lysosomal storage disease called Niemann-Pick type C disease, related to the abnormal storage of cholesterol in the lysosome-like intracellular compartment. The involvement of annexins and cholesterol/sphingomyelin-enriched membrane microdomains in other pathologies including cardiac dysfunctions, neurodegenerative diseases, obesity, diabetes mellitus, and cancer is likely, but is not supported by substantial experimental observations, and therefore awaits further clarification.

Keywords Annexins · Membrane microdomains · Cholesterol · Niemann-Pick type C disease

M. Domon · S. Pikula
Laboratory of Lipid Biochemistry, Department of Biochemistry, Nencki Institute of Experimental Biology, 3 Pasteur Street, 02-093 Warsaw, Poland

M. Domon · M. N. Nasir · G. Matar · F. Besson
Université de Lyon, 69622 Villeurbanne, France

M. Domon · M. N. Nasir · G. Matar · F. Besson
Université Lyon 1, 69622 Villeurbanne, France

M. Domon · M. N. Nasir · G. Matar · F. Besson
INSA de Lyon, 69622 Villeurbanne, France

M. Domon · M. N. Nasir · G. Matar · F. Besson
CPE Lyon, 69616 Villeurbanne, France

M. Domon · M. N. Nasir · G. Matar · F. Besson
ICBMS CNRS UMR 5246, 69622 Villeurbanne, France

J. Bandorowicz-Pikula (✉)
Laboratory of Cellular Metabolism, Department of Biochemistry, Nencki Institute of Experimental Biology, 3 Pasteur Street, 02-093 Warsaw, Poland
e-mail: j.bandorowicz-pikula@nencki.gov.pl

Annexins as calcium sensors and membrane structure organizers

The role of cholesterol in signal transduction, immune response, cell infection, and cell surface polarity has recently been gaining the attention of many investigators [1–3]. It has been proposed that misregulated cholesterol trafficking and intracellular distribution appear to accompany development and/or sustenance of various unrelated pathologies such as neurodegenerative diseases (Alzheimer's disease, Parkinson's disease), cardiac dysfunctions, diabetes mellitus, diabetes and lysosome-storage diseases such as the Niemann-Pick type A/B and type C diseases, Gaucher type I disease, Krabbe disease, and perhaps other lipidoses [4]. Moreover, many protein families were

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described as affecting cholesterol transport or as being affected by cholesterol. An example is annexins, which were found to co-localize with cholesterol at the plasma membrane and to follow cholesterol trafficking throughout the endocytic pathway [5, 6]. These proteins provide a link between calcium signaling and cholesterol transport [5, 6] and were suggested to participate in the formation of cholesterol-rich membrane domains either in the plasma membrane or membranes of the intracellular organelles such as endoplasmic reticulum, endosomes, lysosomes, and the Golgi apparatus [7–10].

As mentioned above, changes in intracellular [Ca^{2+}] are the most powerful factors regulating the function of proteins and their interactions with biological membranes [11]. Eukaryotic cells contain a wide variety of Ca^{2+}-sensing proteins [12], including annexins [13], that participate, as effectors, in mediating cellular responses to changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). These membrane-interacting proteins are characterized by the presence of globular domains allowing for specific binding to membranes, e.g., pleckstrin homology domain, C2 domain, and the annexin motif [14]. Understanding how these domains are distributed, structured, and how they contribute to membrane–protein interactions is crucial for understanding the localization, function, and mechanism of action of certain proteins.

The annexins are a family of calcium-dependent membrane-binding proteins that are present in all eukaryotes. There are currently 12 identified human annexins, all of which contain unique calcium-binding sites, embedded in the highly conserved annexin repeat motifs within the C-terminal core [13, 15, 16]. In addition to the C-terminal, core annexins contain a significantly more variable N-terminal head. Annexins, due to their ability to bind biological membranes in a calcium-dependent manner, provide a link between calcium signaling and membrane-related cellular functions, including various signaling pathways, cell differentiation, and migration [13]. Members of the annexin protein family are ubiquitously expressed and function as intracellular Ca^{2+} sensors. Most cells contain multiple annexins. It is strongly believed that annexins exert their biological function through influencing membrane dynamics, promoting membrane segregation, and membrane fusion [13]. Moreover, annexins were found to participate in plasma membrane repair mechanisms by reacting to the influx of extracellular Ca^{2+} evoked by mechanical stress, toxins, and pathogens, etc. [16]. The combination of the presence of various annexins in a given cell type together with their individual Ca^{2+}-sensitivity allow for spatially confined, graded responses to membrane injury [16].

On the basis of growing evidence it can be assumed that annexins are indeed well suited to perform their specific biological activities due to the presence of a unique N-terminal domain that enables each annexin to perform unique functions in a diverse range of cellular processes including cytoskeleton regulation, membrane conductance, and organization as well as exo- and endocytosis [17]. Given their involvement in such a variety of processes, annexins have also been implicated in a range of pathologies, such as the progression of cancer, diabetes, the autoimmune disorder anti-phospholipid syndrome, and others frequently related to dysregulated vesicular traffic [18–20].

In this review, we will discuss the participation of annexins in the formation, stabilization, and cell distribution of cholesterol, and sphingomyelin-enriched microdomains. These lipid microdomains were identified on the basis of their ability to remain insoluble in cold non-ionic detergents such as Triton X-100. Their properties are briefly described in the next paragraph. We will also describe evidence suggesting that intracellular trafficking of such domains and their presence not only in the plasma membrane but also in membranes of the intracellular organelles may accompany cholesterol-related pathologies with the focus on the Niemann-Pick type C disease.

Lipid microdomains at plasma membrane and membranes of intracellular organelles

Biological membranes are assumed to encompass a plethora of protein–lipid and protein–protein interactions that compartmentalize the bilayer into temporarily formed ordered structures called membrane microdomains. These laterally organized entities are characterized by various half-life times and chemical composition, and therefore posses different biophysical and biochemical properties. Lipid-based membrane domains, frequently called lipid rafts, constitute an important group of structures of the plasma membrane [21, 22]. These microdomains are believed to be small (10–200 nm) and, under normal conditions, cannot be resolved by light microscopy [23]. They are most often functionally identified by their resistance to solubilization by cold non-ionic detergents; hence, their alternate name is detergent-resistant membranes (DRMs). Domain separation can be visualized microscopically when the cellular cholesterol concentration is experimentally lowered, leading to a coalescence of the previously dispersed rafts [24]. Domains or rafts have also been reported to exist in membranes of the intracellular organelles. Among many microdomains identified so far, some are enriched in specific lipids, such as cholesterol and sphingolipids, as well as specific proteins, and were suggested to be involved in the regulation of various cellular processes [25, 26].

Some investigators suggest that proteins, both peripheral and transmembrane ones, postranslationally modified with saturated lipids are recruited to DRMs while those with short, unsaturated, and/or branched hydrocarbon chains are not [27]. Indeed, a relatively large number of observations support the association of glycophosphatidylinositol (GPI)-anchored proteins, the most widely studied group of saturated lipid-modified extracellular peripheral proteins, with DRMs [28, 29]. Such preferential interactions between specialized membrane domains and GPI-anchored proteins were also shown using biomimetic membranes such as the supported bilayers [30]. In the case of transmembrane and peripheral membrane proteins, their DRM partitioning is mediated via *S*-acylation with saturated fatty acids [31].

The mechanism of membrane microdomain formation is not well understood. In fact, the whole concept of lipid-based microdomains [26, 32] is still a matter of scientific dispute. Uncertainty still exists as to the raft existence, but also their size, mechanism of formation, stability, as well participation in vital cellular functions [33, 34]. Furthermore, arguments are raised against the use of detergents to isolate lipid rafts due to the possibility of creating experimental artifacts. In relation to that, the detergent-free methods are being developed to avoid the unwanted effects of detergents on the membrane structure [35, 36]. In addition, the existence of membrane microdomains in the cholesterol-poor membranes was reported, suggesting the role of polyunsaturated fatty acid moieties of phospholipids [37] or ceramides [38] in membrane microdomain formation. Finally, the role of electrostatic interactions in sequestration of proteins into membrane local heterogeneities with different chemical composition, for example enriched in phosphatidylinositol-4,5-bisphosphate, has been reported [39] thus minimalizing the role of hydrophobic interactions between lipids and/or lipids and proteins in the formation and stability of membrane microdomains.

Despite what is mentioned above, many investigators agree that protein sorting and assembly during membrane biogenesis is accompanied by the appearance of ordered domains of lipids. Some of these microdomains are composed of phospholipids, glycosphingolipids, and cholesterol. It has been shown that cholesterol interacts with sphingomyelin to form a liquid-ordered bilayer phase; how other lipid molecules are participating in the formation of rafts is, however, not well characterized. The observations accumulated recently suggest that the order created by the quasicrystalline phase may provide an appropriate scaffold for the organization and assembly of raft proteins on both sides of the membrane [40]. Atomic-scale molecular dynamics simulations revealed that ordering and the associated packing effects in membranes largely result from the unique features of the cholesterol molecule that distinguish it from other sterols. Cholesterol molecules prefer to be located in the second coordination shell, avoiding direct cholesterol–cholesterol contacts, and form a three-fold symmetric arrangement with the proximal cholesterol molecules. At larger distances, the lateral three-fold organization is broken by thermal fluctuations. For other sterols, with less structural asymmetry, the three-fold arrangement is considerably lost. In conclusion, cholesterol molecules act collectively in lipid membranes. This is the main reason why the liquid-ordered phase only emerges at cholesterol concentrations well above 10 mol%, when the collective self-organization of cholesterol molecules arises spontaneously [41].

Concerning another important component of the lipid rafts, i.e., sphingomyelin, systematic analysis of the effect of the headgroup size on membrane properties and interactions with cholesterol revealed that an increase in the headgroup size resulted in a decrease in the main phase transition. Atom-scale molecular-dynamic simulations have shown that the molecular areas increased and the acyl chain order decreased with increasing headgroup size. Furthermore, the transition temperatures were constantly higher for sphingomyelin headgroup analogs compared to corresponding phosphatidylcholine headgroup analogs. Analysis of the affinity of cholesterol for phospholipid bilayers revealed that an increased headgroup size increased sterol affinity for the bilayer, with a higher sterol affinity for sphingomyelin analogs as compared to phosphatidylcholine analogs. Moreover, the size of the headgroup affected the formation and composition of cholesterol-containing ordered domains [42]. Other results emphasized that the interfacial electrostatic interactions are important for stabilizing cholesterol interactions with sphingomyelins [43]. These processes were also studied using giant unilamelar vesicles [44].

Furthermore, it has been reported that efficient depletion of sphingolipids in two different cell lines did not abrogate the ability to isolate DRMs from these cells, suggesting that even extensive sphingolipid depletion does not affect lipid raft integrity or the function of the lipid-raft-associated proteins, as for example MRP1 [45]. Sphingolipids constitute a diverse array of lipids in which fatty acids are linked through amide bonds to a long-chain base and, structurally, they form the building blocks of eukaryotic membranes. Ceramide is the simplest one and serves as a precursor for the synthesis of the three main types of complex sphingolipids: sphingomyelins, glycosphingolipids, and gangliosides. Sphingolipids are no longer considered as mere structural spectators, but as bioactive molecules with functions beyond providing a mechanically stable and chemically resistant barrier to a diverse array of cellular processes.

Protein sorting into membrane lipid microdomains is tightly regulated [46]. Furthermore, it has been evidenced that the function of membrane proteins may depend on their

residence at the lipid rafts. For example, in the case of Mrp1 (ABCC1), the disruption of cortical actin resulted in a loss of Mrp1 from DRMs and its internalization [47]. Localization of many proteins at lipid microdomains is considered as an important factor regulating their biological activity, as it was shown for adenosine receptors [48], NADPH oxidase sub-units and related proteins [49], many types of ion channels [50] including human ether-à-go–go-related gene (HERG) potassium channels [51], calcium-activated chloride channels (CACCs) [52], peroxisomal membrane proteins required for peroxisome biogenesis [53], human herpesvirus-6 (a lipid raft-associated mechanism of entry into cell [54]), protein complexes involved in calcium entry Orai1, TRPCs and STIM1 [55], caveolin 1 (Cav-1 may contribute to persistent infection in macrophages [56]), cadherins [57], and supervillin. The latter is an F-actin- and myosin II-binding protein that tightly associates with signaling proteins in cholesterol-rich domains [58].

Above we described evidence that lipid microdomains, as revealed by existence of DRMs, predominantly reside in the plasma membrane. However, they can be identified in the intracellular compartments of the cellular secretory pathway as well. In this pathway, the membranes of the Golgi complex represent a transition stage between the cholesterol-poor membranes of the endoplasmic reticulum and the cholesterol-rich plasma membrane. DRMs isolated from HT29 cells were characterized by the presence of the Golgi-resident SPCA1 Ca^{2+}/Mn^{2+} pump and the raft-resident, flotillin-2, while SERCA2b was detergent-soluble. Furthermore, cholesterol depletion of these cells resulted in redistribution of flotillin-2 and SPCA1d to the detergent-soluble fractions of the density gradient and inhibited the activity of SPCA1d, while SERCA2b activity was not altered [59].

It is accepted that the endoplasmic reticulum is poor in lipid rafts [46], and that these microdomains are present in the lipid biosynthetic pathway in the Golgi [60]. The reason for that is that although cholesterol and ceramide (the precursor of sphingolipids) are both synthesized in the endoplasmic reticulum, most of the head groups of the sphingolipids are added only upon reaching the Golgi, and then rafts can begin to form. It must be borne in mind that some proteins characteristic for the DRM fraction were also found in endoplasmic reticulum. Cholesterol and sphingomyelin associate in membrane microdomains and are metabolically co-regulated. Such coordinate regulation occurs in the Golgi apparatus where oxysterol binding protein (OSBP) mediates sterol-dependent activation of the ceramide transport protein (CERT) and sphingomyelin synthesis. CERT transfer activity is dependent on its phosphatidylinositol 4 phosphate-specific pleckstrin homology domain [45, 61].

Several endoplasmic reticulum (ER) proteins including the sigma-1 receptor chaperone were identified at lipid raft-like microdomains of the ER membrane. The sigma-1 receptor chaperone, which is highly expressed at a subdomain of the ER membrane directly apposing mitochondria, known as the mitochondria-associated ER membrane or MAM, has been shown to associate with steroids as well as cholesterol. The sigma-1 receptor has been implicated in ER lipid metabolism/transport, lipid raft reconstitution at the plasma membrane, trophic factor signaling, cellular differentiation, and cellular protection against beta-amyloid-induced neurotoxicity. Recent studies on the sigma-1 receptor chaperone and other ER proteins clearly suggest that cholesterol may regulate several important functions of the ER including folding, degradation, compartmentalization, segregation of ER proteins, and the biosynthesis of sphingolipids [62].

Annexins in organization and stabilization of membrane microdomains enriched in cholesterol

One of the intriguing features of annexins is their ability to participate in the lateral organization of artificial lipid membranes. Thus, the question arises whether annexins might act in a similar manner also in vivo, affecting lateral organization of lipids, especially cholesterol, and of other membrane components and to contribute, in this way, to biogenesis, function and sustenance of cholesterol-enriched microdomains. In other words, whether annexins may contribute to the organization, stabilization, and dynamics of lipid rafts by affecting their membrane lateral distribution. Furthermore, is this idea consistent with observations that annexins are able to attract other proteins and signaling molecules onto lipid rafts and, by influencing soluble versus membrane protein interactions, may serve as regulatory molecules in various signaling pathways?

There is still some controversy among investigators as to why several members of the annexin family, such as AnxA2, AnxA5, AnxA6, and AnxA13, appear to be associated with membrane microdomains, as evidenced by the analysis of protein composition of DRMs, while others seem to be excluded from the raft territory. The reason for this could be related to the different calcium and pH sensitivity of various annexins, their lipid specificity, as well as phosphorylation [63, 64] and membrane partners with whom annexins can interact [9, 10, 13, 15]. Nevertheless, proteomic and immunochemical studies revealed the presence of certain annexins at DRMs fractions enriched in membrane lipid rafts [65–67], suggesting that annexins may participate in biogenesis, stabilization, and dynamics of these membrane microdomains.

Experimental evidence along with the analysis of primary structures favors the idea that some annexins, especially AnxA2, AnxA6, and AnxA13 may resemble

genuine cholesterol-interacting proteins, and that intracellular localization and membrane binding of annexins at low pH is determined by cholesterol (Fig. 1) [68–71]. Furthermore, experimental data suggest that certain functions of annexins may be regulated by cholesterol and, last but not least, that annexins may participate in the cholesterol traffic and storage. Factors were identified that play a role in regulation of annexin-membrane interactions, including calcium, pH, and membrane lipid composition. Growing evidence, coming mostly from in vitro experiments, suggests that cholesterol may affect the affinity constants of annexins binding to artificial lipid membranes such as liposomes of various chemical composition [72, 73] or solid supported lipid membranes [74].

Below we summarize the evidence suggesting that annexins residing at membrane microdomains enriched with cholesterol may participate in various membrane functions. We also provide supportive information coming from in vitro experiments.

Annexins in lateral organization and function of biological membranes

One of the annexins present in mammalian cells and tissue that has been localized to the membrane lipid rafts is AnxA2 [75]. Moreover, it has been demonstrated that catecholamine-evoked formation of lipid rafts in the plasma membrane, essential for exocytosis, can be attributed to the AnxA2 tetramer. On the basis of this finding, it was proposed that AnxA2 may act as a calcium-dependent promoter of lipid microdomains required for structural and spatial organization of the exocytotic machinery [76]. Furthermore, there is growing evidence suggesting that cholesterol is a very important factor influencing AnxA2 interactions with membranes of cellular organelles [77, 78]. AnxA2 was shown to be associated with chromaffin granules in the presence of EGTA [79]. This bound AnxA2 was released from the membranes by methyl-β-cyclodextrin (MβCD), which depleted cholesterol from the membranes. Restoration of the cholesterol content of chromaffin granule membranes with cholesterol/MβCD complexes restored the Ca^{2+}-independent binding of AnxA2 [79]. The core domain of AnxA2 was found to be responsible for the cholesterol-mediated effects [77]. A similar phenomenon was previously described [7]. Even a low concentration of cholesterol sequestering agents, such as filipin or digitonin, quantitatively released AnxA2 from the membranes of early endosomes of BHK cells [77]. It is proposed that AnxA2 forms cholesterol-rich platforms that organize the membranes of early endosomes [7, 8]. It was suggested that in the presence of Ca^{2+}, AnxA2 binds to and possibly promotes the lateral association of glycosphingolipid- and cholesterol-rich lipid microdomains (lipid rafts) [9, 10, 80].

As for AnxA6, the largest member of the family of annexins, it has been demonstrated that it is implicated in processes related to vesicular transport such as endo- and exo-cytosis, membrane aggregation, and membrane fusion [78, 81–83]. Recently, for example, the experimental evidence has been provided that AnxA6 may affect localization and functioning of the target membrane SNAP receptors (t-SNAREs), SNAP23 and syntaxin-4, along the exocytic pathway [84].

AnxA6 has been shown to be predominantly associated with membranes of the late endosome and prelysosomal compartments of NRK fibroblasts, WIF-B hepatoma, and rat kidney cells [78, 85, 86]. Although the majority of AnxA6 is most likely targeted to membranes via Ca^{2+}-dependent binding to negatively charged phospholipids [68], it was also demonstrated that its binding to the membranes depends on the cholesterol content. In addition, it has been shown that AnxA6 is a molecule linking calcium signaling with cholesterol transport, working as a scaffold/targeting protein for several signaling proteins [6].

Upon cell activation, AnxA6 was observed to be recruited to the plasma membrane, endosomes, and membrane rafts to interact with signaling proteins, the endocytic machinery and actin cytoskeleton in order to inhibit epidermal growth factor receptor and Ras signaling. In addition, AnxA6 associated with late endosomes to regulate cholesterol export leading to reduced cytoplasmic phospholipase A_2 activity and caveolae formation [5, 87].

Many researchers observed a Ca^{2+}-dependent translocation of AnxA6 to Triton-$\times$100 insoluble caveolin- and cholesterol-enriched membrane fractions (DRMs). On the basis of this and the above-mentioned evidence, it might be assumed that AnxA6 is implicated in the organization of membrane domains, in particular in their association with cytoskeleton in smooth muscle cells [9, 88]. Since AnxA6 can bind phospholipids, actin [89, 90], and signaling proteins, it is also presumed that it could stabilize and regulate the assembly of lipids and proteins during caveolae/membrane raft formation [5, 87, 91].

Other annexins, like AnxA4, AnxA8, and AnxA13, due to their cellular localization along the endocytic pathway or in the membrane microdomains, and their sensibility to cholesterol sequestering agents, are supposed to interact with cholesterol. This is consistent with the existence in their structure of cholesterol-binding motifs (Fig. 1). The impact of these annexins on the organization of lipid rafts depends on how their membrane association is determined by changes in the cytosolic conditions (Ca^{2+} or pH) and on the membrane lipid content (cholesterol, acidic phospholipids) [15]. Other annexins in particular AnxA13, much like AnxA2, may define specific platforms on other cellular membranes [92]. AnxA13b has been assigned to raft-dependent and -independent apical traffic in MDCK cells [93].

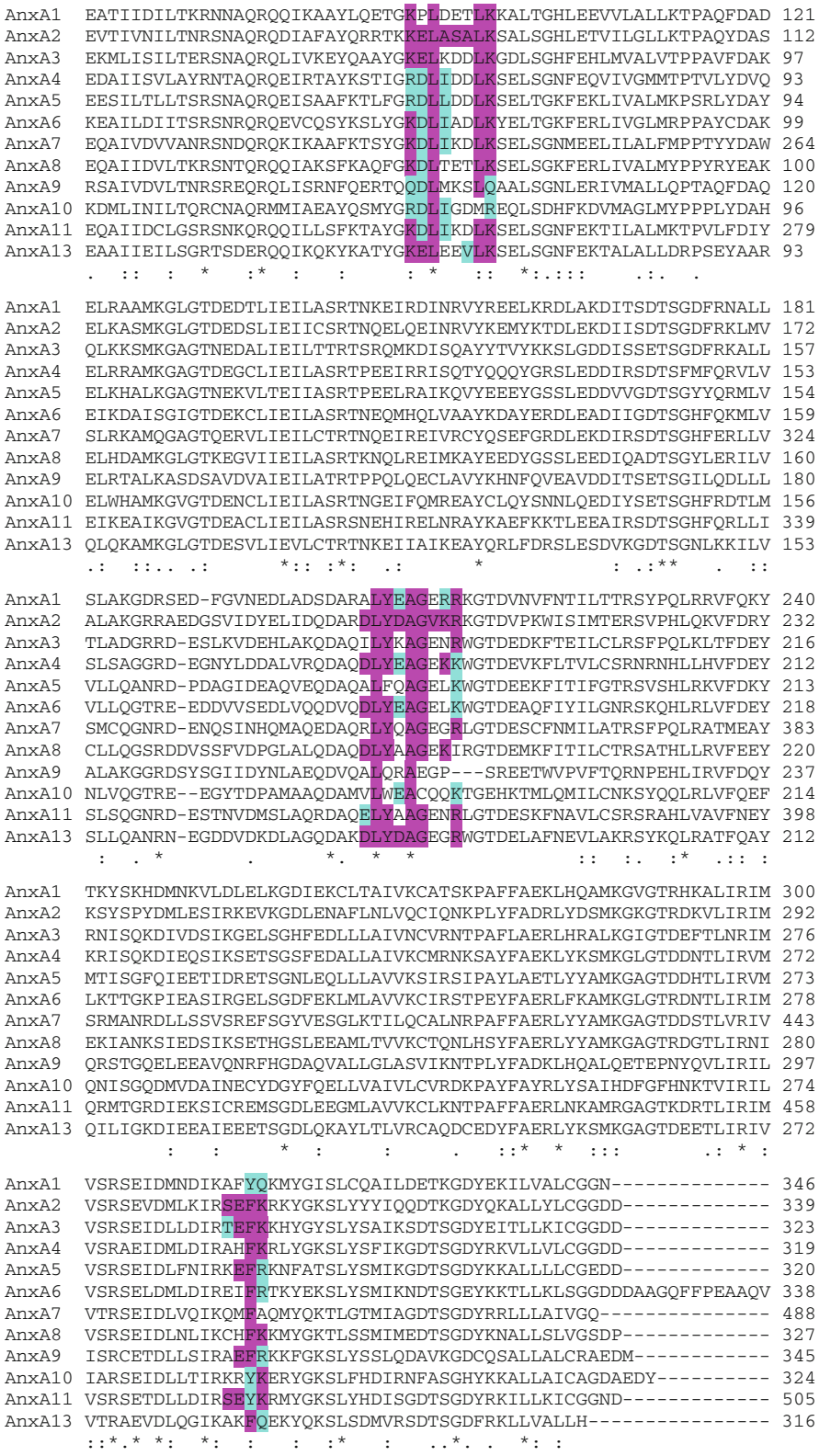

Fig. 1 Sequence alignment of different human annexins. Sequences were retrieved from the UniProt knowledgebase by a sequence accession code for each annexin. Multiple sequence alignment was made using the ClustalW algorithm. The symbols: asterisk (*), colon (:) and dot (.) indicate identity, or strong or weak similarity of residues, respectively. Three regions of AnxA2 identified as important in the annexin–membrane interactions [77] are in purple. Identical or similar residues found in the corresponding regions of other annexins are in purple or blue, respectively. Local similarity calculations using the BLOSUM matrix revealed that the KELASALK motif of AnxA2 was found to be conserved also in AnxA3, AnxA6, AnxA8, AnxA11, and AnxA13, while the DLYDAGVKR motif was conserved in AnxA1, AnxA4, AnxA6, AnxA8, and AnxA13. In the case of the SEFK motif of AnxA2, it is too short to draw any conclusions. It is worth underlying that the KELASALK and DLYDAGVKR motifs were identified by Lambert et al. [69] as membrane-interacting regions of AnxA2, by using a hydrophobic probe, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl) diazirine (^{125}I-TID), both at pH 4.5 in the absence of calcium and at pH 7.0 in the presence of calcium. The authors suggested that calcium-independent interactions of AnxA2 with membranes at pH 4.5 are mediated mostly by cholesterol. The membrane-interacting regions of AnxA2 are not conserved within the whole annexin family suggesting a different impact of cholesterol on the membrane binding of each annexin type. However, it is worth mentioning that similar conclusions have been reached concerning the molecular mechanism of pH-dependent interactions of AnxA6 with artificial lipid membranes [68, 70]. Other explanations are in the text

Evidence from in vitro experiments: Various experimental designs and techniques, including liposomes, monolayers, supported lipid films, as well as atomic force microscopy, polarization modulation infrared reflection adsorption spectroscopy, and Brewster angle microscopy have been used to study annexin–membrane interactions. It was found for example that membranes formed from palmitoyloleylphosphatidylcholine/palmitoyloleylphosphatidylserine (POPC/POPS) exhibit phase separation into POPC- or POPS-enriched domains in a [Ca^{2+}]-dependent manner. AnxA1 was found to interact with these membranes in the presence of calcium and to form irreversible complexes only with POPS-enriched microdomains; the attachment of AnxA1 to POPC/POPS-enriched membrane regions was fully reversible [94]. Appearance of microdomains has also been observed when AnxA6 was interacting with PC/PS interfacial monolayers deposited onto a calcium-containing subphase [68].

With the aid of the quartz crystal microbalance (QCM) technique, in combination with solid-supported lipid bilayers used to monitor the interaction of AnxA1 with lipid membranes, the affinity constants were determined for the binding of AnxA1 to lipid membranes of different compositions. These experiments revealed that at low calcium ion concentration, the presence of cholesterol increases the binding affinity of AnxA1 to lipid membranes, stressing the fact that cholesterol might be important for forming a high-affinity interface for the attachment of the protein [74].

As for AnxA2, its tetrameric form bound to liposomes containing phosphatidylserine in the absence of Ca^{2+}, and addition of cholesterol to these liposomes increased the binding. Also, liposomes containing phosphatidylserine and cholesterol were aggregated by the tetrameric form of AnxA2 at submicromolar Ca^{2+} concentrations [79]. In the case of liposomes containing phosphatidic acid, supplementation with cholesterol in the absence of Ca^{2+} increased AnxA2 binding and this binding was only marginally affected by MβCD [95]. These results are in contrast with other studies [96] in which, using QCM with dissipation monitoring (QCM-D) and liposome techniques, it has been demonstrated that the AnxA2 heterotetramer does not bind in a Ca^{2+}-independent manner to cholesterol-containing membranes. These contraries may be due to the fact that the tests [96] were performed using AnxA2 heterotetramer purified from porcine intestine while the other researchers used recombinant AnxA2. It is possible that a specific membrane structure [97] facilitates localization of AnxA2 at cholesterol-rich membranes in vivo.

Cholesterol seems to also play a regulatory role in interactions of annexins with other lipid constituents of membranes. Analysis performed in [76] revealed that in the absence of Ca^{2+}, AnxA5 was unable to bind to phosphatidylcholine/phosphatidylserine (PC/PS) (75/25 by weight) or PC/PS/cholesterol (50/25/25, by weight) liposomes, however, in the presence of calcium, the amount of AnxA5 bound to liposomes was significantly higher for the PC/PS/cholesterol than for the PC/PS liposomes at pH 7.4. Other in vitro tests (including surface plasmon resonance analysis) with AnxA5, suggest that although phosphatidylserine plays a dominant role in AnxA5 binding to liposomal membranes [98], the binding increased with an increase in the cholesterol content. This suggests that cholesterol in the liposomes may act as a "phospholipid arrangement factor". It was also shown that AnxA5 can induce formation of large PS domains, only in the presence of cholesterol [99]. It is worth noting that in the absence of PS, cholesterol did not exert the binding-enhancement effect. Stability of AnxA5 binding was significantly improved by the increase in cholesterol content. In vitro experiments using reconstituted systems confirmed that annexins may affect lipid phase behavior and protein partitioning into giant liposomes, as in the case of AnxA5 [100].

In the absence of Ca^{2+}, AnxA6 was unable to associate with liposomes made of PC/PS (75/25 by weight) or PC/PS/cholesterol (50/25/25 by weight) [76]. However, in the presence of Ca^{2+}, the amount of bound annexin was significantly higher for the cholesterol-containing liposomes. This demonstrates that cholesterol also stimulates binding of AnxA6 to liposomes in vitro [76]. Recent findings using membrane-mimicking systems (such as Langmuir monolayers or air–water interface imaging using Brewster angle microscopy) also confirm the importance of cholesterol in AnxA6–membrane interactions [101]. In the absence of Ca^{2+}, at pH 7.4, no insertion of AnxA6 to the monolayer composed of dipalmitoylphosphatidylcholine (DPPC) was observed. The addition of cholesterol promoted the insertion of AnxA6 to the monolayer in a concentration-dependent manner. At pH 5.0, insertion of AnxA6 into the monolayer composed of DPPC was observed, however, the addition of cholesterol significantly increased AnxA6 incorporation. Interestingly, the replacement of cholesterol by cholesteryl acetate significantly diminished AnxA6 incorporation, suggesting that the –OH group of cholesterol is implicated in AnxA6–cholesterol interactions [101].

Annexins and membrane microdomains in etiology of Niemann-Pick type C disease

Cholesterol and sphingomyelin-enriched microdomains at the plasma membrane that probably coordinate and regulate a variety of signaling processes were implicated in various pathologies including cardiac dysfunctions [63, 102, 103], invasion of pathogenic *Escherichia coli* [104], cell-to-cell HIV-1 transmission [105], Alzheimer's disease

[106], and other neurodegenerative diseases (Parkinson's, amyotrophic lateral sclerosis, Huntington's, those caused by prions) [107], obesity and diabetes mellitus [108], tumorigenesis and malignant tumors [109–111], as well as in physiological processes such as brain plasticity [112, 113], neuroprotection [114], cholesterol homeostasis [115], cell survival, and apoptosis [116]. In addition, sphingolipids, although they account for a minor component of the total cellular lipid pool, when accumulated in excess in certain cells, may be a cause of many diseases [117]. Accumulating evidence suggests that large ceramide-enriched platforms that are formed due to the activation of sphingomyelinase and generation of ceramide, and through which transmembrane signals are transmitted and/or amplified, are involved in the modulation of the cell and intracellular membrane ion channels, cell proliferation and apoptotic cell death, neutrophil adhesion to the vessel wall, vascular tone, and in the development of cardiovascular diseases, to name some important examples [25, 118].

Several lines of evidence suggest that annexins may also participate in the pathologies mentioned above, including obesity and type II diabetes mellitus [119], by exerting their biological function through interaction with membrane constituents of the lipid microdomain/rafts. Studies carried out on cell lines from patients suffering from the Niemann-Pick type C (NPC) disease have shown that intracellular distribution of annexins matches cholesterol distribution in these cells. On the basis of these findings, we propose a hypothesis that some annexins may play a role in intracellular cholesterol storage, including in the aberrant storage occurring in NPC disease [120, 121].

Niemann-Pick type C (NPC) disease

The NPC disease (OMIM 257220) is a fatal, autosomal recessive disorder characterized by progressive neurodegeneration and hepatosplenomegaly [122]. The NPC disease results from dysfunctions of the NPC1 or NPC2

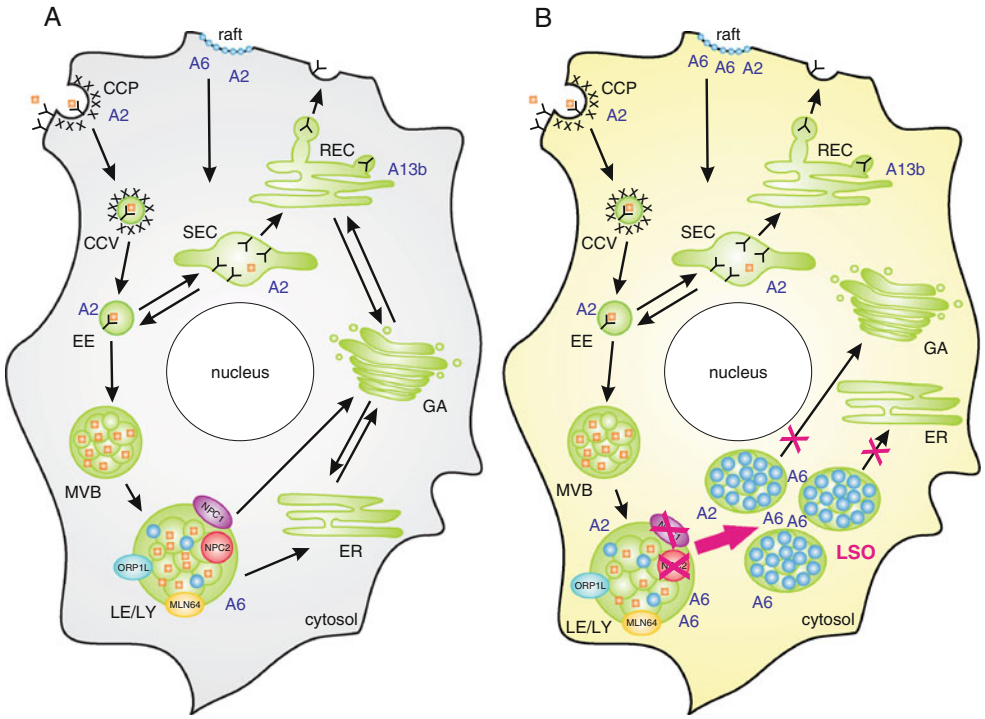

Fig. 2 Schematic representation of vesicular transport of light density lipoproteins (LDL) via receptor endocytosis in normal (**a**) and NPC cells (**b**) characterized by mutation in the *NPC1* gene encoding the NPC1 protein or in the *HE1/NPC2* gene encoding the NPC2 protein. Low level or malfunction of mutated NPC1 or NPC2 proteins lead to disturbances of intracellular cholesterol transport (depicted in **b** by *crossed darts*) and an overnormative storage of cholesterol in lysosome-like storage organelles (LSO) corresponding to the late endosome/lysosome compartment (LE/LY, pH_{in} 5.0-6.0). Intracellular localization of annexins (A2, A6, A13b) is shown. Rafts, cholesterol-enriched membrane microdomains, are identified as possible targets for annexins. Other explanations are in the text. Abbreviations and symbols: *CCP* clathrin-coated pit; *CCV* clathrin-coated vesicle (pH_{in} 7.2–7.4); *EE* early endosome; *SEC* sorting endosome compartment (pH_{in} 5.9–6.0); *REC* recirculating endosome compartment; *MVB* multivesicular body; *GA* Golgi apparatus; *ER* endoplasmic reticulum (pH_{in} 7.2–7.4); *LE* late endosome (pH_{in} 5.0–6.0); *LY* lysosome (pH_{in} 5.0–5.5); NPC1/NPC2 –Niemann-Pick type C1 or C2 proteins; ORP1L and MLN64 LE proteins participating in the transport of cholesterol; *orange squares* LDL particles; *black crosses* clathrin network; *blue dots* cholesterol

proteins (Fig. 2). In approximately 95% of NPC patients, development of the disease is associated with the existence of mutations in the *NPC1* gene encoding a large, transmembrane, heavily glycosylated late endosome NPC1 protein [123]. Only in 5% of patients mutations in the *HE1/NPC2* gene encoding a small soluble NPC2 protein that resides in the lumen of late endosomes/lysosomes have been described [124]. Both NPC1 and NPC2 proteins, as cholesterol binding proteins, were implicated in the transport of lipids from the late endosome/lysosome compartment to other cellular compartments such as plasma membrane, endoplasmic reticulum, and Golgi [125–127]. Due to the dysfunction described above, accumulation of abnormal amounts of cholesterol and other lipids in the late endosome/lysosome compartment, also called the lysosome-like storage organelles, is observed [128–130].

Cholesterol is one of the major lipid components of the eukaryotic plasma membrane [131]. Its content in the membrane may reach up to 50% of total lipids and may significantly affect membrane properties such as fluidity, permeability, and distribution of other membrane constituents. The distribution of cholesterol in intracellular membranes strongly depends on the metabolic activity of a given cell type or tissue and it can be dysregulated in certain pathologies. Experimental data demonstrate that altered cholesterol homeostasis changes the physicochemical properties of the plasma membrane in NPC cells. In general, the level of cholesterol in the internal membranes is much lower than in the plasma membrane and is restricted to special microdomains, most probably originating from similar microdomains of the plasma membrane due to the retrograde vesicular transport via the endocytic mechanism. It is suggested that changes in the architecture and composition of biological membranes due to excessive cholesterol accumulation may strongly affect vital cellular processes leading to cell death [132].

Participation of AnxA6 in transport and storage of cholesterol in NPC disease: In the Niemann-Pick type C disease the absence, low level or presence of dysfunctional NPC1 and NPC2 proteins may not be the only cause of the disease. Growing evidence suggests that malfunction and a dysregulated content or intracellular distribution of other proteins should be considered as an important factor of NPC disease etiology. In this regard, annexins (Fig. 2), among them AnxA6, are gaining the attention of many investigators.

Upon cell activation, AnxA6 is recruited to the plasma membrane, endosomes, and caveolae/membrane rafts to interact with signaling proteins, and to the endocytic machinery and actin cytoskeleton to inhibit epidermal growth factor receptor and Ras signaling. In addition, AnxA6 associates with late endosomes to regulate cholesterol export, which in turn leads to reduced cytoplasmic phospholipase A_2 activity and caveolae formation. Investigators suggested that AnxA6 may function as an organizer of membrane domains by creating a scaffold for the formation of multifactorial signaling complexes which regulate transient membrane–actin interactions during endocytic transport, and modulate intracellular cholesterol homeostasis [5]. AnxA6, due to its unique structure, may be able to recruit interacting partners to membrane microdomains and to bridge specialized membrane domains with cortical actin skeleton [133]. The following experimental evidence favors the hypothesis of AnxA6 involvement in the etiology of NPC disease.

First, it has been shown that AnxA6 colocalizes with lysobisphosphatidic acid (LBPA), a maker of cholesterol-rich late endosomal structures, in Chinese hamster ovary (CHO) cells with overexpression of the *ANXA6* gene, suggesting that cholesterol modulates the intracellular distribution of Ca^{2+}-dependent and -independent pools of this protein [78].

Second, in NPC1-null CHO cells, altered distribution of cholesterol and AnxA6 as well as an altered membrane architecture of the endosomal compartment, enriched with glycosphingolipids and cholesterol, has been observed in comparison to control cells. These alterations in membrane composition may be responsible for the deficit in endocytic trafficking found in the NPC disease. This altered intracellular trafficking may, in turn, be the result of mistargeting and disrupted function of proteins associated with membrane microdomains [134].

Third, CHO cells expressing high levels of AnxA6 were characterized by accumulation of caveolin-1 in the Golgi complex. This was associated with the sequestration of cholesterol in the late endosomal compartment and lower levels of cholesterol in the Golgi and the plasma membrane, both likely contributing to the retention of caveolin in the Golgi apparatus and to a reduced number of caveolae at the cell surface. Furthermore, knock down of the AnxA6 gene and the ectopic expression of the Niemann-Pick C1 protein in AnxA6-overexpressing cells restore the cellular distribution of cav-1 and cholesterol, respectively. In summary, this study demonstrates that elevated expression levels of AnxA6 perturb the intracellular distribution of cholesterol and indirectly inhibit the exit of caveolin from the Golgi complex [135, 136].

In addition to what is mentioned above, it has been recently shown that AnxA6 co-purifies with the late endosome/lysosome fraction of membranes isolated from NPC L1 fibroblasts obtained from an NPC patient. A significant pool of AnxA6 was found to interact with cholesterol in a calcium-independent manner. In conclusion, it was postulated that AnxA6 may participate in the formation of cholesterol-rich platforms in the late endosome compartment and therefore may contribute to the pathology of the NPC disease [120]. Furthermore, using the

same cells, it has been evidenced that in the presence of calcium, AnxA6 re-located to the fractions enriched in DRMs only in the NPC cells, suggestive of AnxA6 participation in the organization of cholesterol-enriched microdomains in NPC fibroblasts [121].

Considering other members of the annexin family of proteins, AnxA2 was found to regulate the endogenous low-density lipoprotein receptor levels [137, 138]. Therefore, AnxA2 began to be considered as a target for the treatment of hypercholesterolemia. AnxA2 was also implicated in the Niemann-Pick type C disease [139], since it showed, similarly to AnxA6, distorted distribution, related to the mislocalization of membrane microdomains, in NPC cells [134]. Identification of other members of the annexin family as potential players in the pathophysiology of disorders related to altered cholesterol traffic and storage as well as distorted membrane organization requires further experiments.

Concluding remarks and perspectives

To summarize, growing evidence suggests that certain members of the annexin family of proteins may participate, as membrane-binding proteins, in the mechanism of biogenesis, organization, and maintenance of the cholesterol and sphingomyelin-enriched microdomains, i.e., lipid rafts, and, in consequence, they may regulate the intracellular cholesterol transport and storage. Dysregulation of the annexin–cholesterol interaction and intracellular cholesterol distribution may lead to development and sustenance of fatal disorders such as the Niemann-Pick type C disease. Identification of other diseases in which annexins and cholesterol-enriched microdomains are important is only a question of time.

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