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Review

Annexin A6—Linking Ca^{2+} signaling with cholesterol transport☆

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ABSTRACT

Annexin A6 (AnxA6) belongs to a conserved family of Ca^{2+}-dependent membrane-binding proteins. Like other annexins, the function of AnxA6 is linked to its ability to bind phospholipids in cellular membranes in a dynamic and reversible fashion, in particular during the regulation of endocytic and exocytic pathways. High amounts of AnxA6 sequester cholesterol in late endosomes, thereby lowering the levels of cholesterol in the Golgi and the plasma membrane. These AnxA6-dependent redistributions of cellular cholesterol pools give rise to reduced cytoplasmic phospholipase A2 ($cPLA_2$) activity, retention of caveolin in the Golgi apparatus and a reduced number of caveolae at the cell surface. In addition to regulating cholesterol and caveolin distribution, AnxA6 acts as a scaffold/targeting protein for several signaling proteins, the best characterized being the Ca^{2+}-dependent membrane targeting of p120GAP to downregulate Ras activity. AnxA6 also stimulates the Ca^{2+}-inducible involvement of PKC in the regulation of HRas and possibly EGFR signal transduction pathways. The ability of AnxA6 to recruit regulators of the EGFR/Ras pathway is likely potentiated by AnxA6-induced actin remodeling. Accordingly, AnxA6 may function as an organizer of membrane domains (i) to modulate intracellular cholesterol homeostasis, (ii) to create a scaffold for the formation of multifactorial signaling complexes, and (iii) to regulate transient membrane–actin interactions during endocytic and exocytic transport. This article is part of a Special Issue entitled: 11th European Symposium on Calcium.

1. Introduction

Annexins are a dynamic and multifunctional family of structurally related calcium and membrane-binding proteins that have been identified in a large variety of species ranging from protists to higher vertebrates and are classified into five groups (A–E), with the 12 human and vertebrate annexins representing group A [1,2]. All annexins are composed of two domains: a conserved core that is responsible for Ca^{2+}- and phospholipid-binding, and a variable N-terminal tail. Although synthesized as cytosolic proteins, annexins are predominantly associated with different intracellular membranes as well as the plasma membrane primarily due to their ability to bind negatively charged phospholipids in a Ca^{2+}-dependent manner. This provides a connection between Ca^{2+} signaling and membrane functions and enables annexins to participate in the organization of membrane domains or signaling platforms and the formation of complex protein networks. Their dynamic nature, together with the high amounts and multiple locations of annexins found in most cells and tissues, probably accounts for annexins being implicated in a wide variety of otherwise unrelated events ranging from membrane dynamics to cell differentiation and migration.

Crystallographic studies and experimental approaches utilizing model membranes provided the biophysical framework to understand the topology, details of the membrane-binding side, interactions with other proteins (exemplified by the S100 family), ligand binding sites and affinities to various phospholipids (and cholesterol) [3]. Furthermore, *in vivo* studies using annexin knockout-mice and knockdown approaches in cell culture have further identified processes that depend on annexins, including certain membrane-trafficking steps, aspects of Ca^{2+} signaling, but also extracellular events.

In this review, we will focus on the largest member of the annexin family, annexin A6 (AnxA6). AnxA6 is the only annexin that contains two annexin cores within a single physical entity, which probably arose from the duplication and fusion of the genes encoding for

Abbreviations: AA, arachidonic acid; AnxA, annexin group A; BCC, breast cancer cells; cav-1, caveolin-1; $cPLA_2$, cytoplasmic phospholipase A_2; CHO, Chinese Hamster Ovary; DRM, detergent resistant membranes; EGFR, epidermal growth factor receptor; eNOS, endothelial nitric-oxide synthase; Erk, extracellular signal-regulated kinase; HDL, high density lipoprotein; LDL, low density lipoprotein; MAPK, mitogen-activated protein kinase; NPC1, Niemann-Pick C1; NRK, Normal Rat Kidney; p120GAP, p120 GTPase-activating protein; PI(4,5)P2, phosphatidylinositol (4,5)-biphosphate; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PS, phosphatidylserine; SNARE, SNAP (soluble NSF Attachment Protein) receptor; SOCE, store-operated Ca^{2+} entry; TGN, trans-Golgi-network

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annexin A5 and A10. Crystal structures of AnxA6 in solution and in association with artificial membranes show that the two core modules can probably orient themselves in a flexible manner relative to one another. This allows the molecule to bind with its core domains attached to either one membrane or to two separate membranes [4–7] (Fig. 1). AnxA6 binds to phosphatidylserine (PS), phosphatidylinositol, phosphatidic acid [2], and earlier reports also identified affinity towards phosphatidylethanolamine and arachidonic acid [8]. Besides the Ca^{2+}-dependent targeting of AnxA6 to membranes, acidic pH and cholesterol also regulate the targeting and trafficking events for this annexin [9–14].

In addition to its membrane binding properties, AnxA6 interacts with members of the actin cytoskeleton and multiple signaling proteins. In this review we will make an attempt to characterize the features that enable AnxA6 to regulate membrane trafficking and signal transduction: the ability of AnxA6 (1) to regulate caveolin-1 transport, cellular cholesterol distribution and $cPLA_2$ activity, but also (2) to organize the cytoskeleton and (3) the formation of multifactorial, PKC and HRas containing signaling complexes.

2. AnxA6 and cholesterol homeostasis

Cholesterol is an essential structural constituent of cellular membranes in eukaryotes. Mammalian cells synthesize their own cholesterol but also receive exogenous cholesterol through lipoprotein uptake. The intracellular routes for the transport of cholesterol—vesicular and non-vesicular—between membrane compartments are complex and their regulation is still not fully understood [15,16]. One important consequence of these various transport routes is that cholesterol is not uniformly distributed in biological membranes, but enriched in specific membrane domains. Certain proteins are found in these cholesterol-rich domains (membrane rafts), and thus influence the functioning of the plasma membrane.

As outlined above, annexins interact with negatively charged membrane phospholipids in a Ca^{2+}-dependent manner and up to date, have generally been considered not to bind cholesterol. However, the mutational analysis of the annexin core and N-terminal tail showed that cholesterol can increase the Ca^{2+}-dependent membrane binding and aggregation of AnxA2. Similarly, AnxA5 and AnxA6, two annexins with unrelated N-terminal tails but homologous core domains, also showed a Ca^{2+}-dependent binding to membranes that was cholesterol-sensitive [17,18]. Smart et al. [19,20] further challenged the concept of annexins as non-cholesterol binding proteins and isolated AnxA2 from a cytosolic complex together with cholesterol and caveolin. Further pointing at the possibility of AnxA2 to interact with cholesterol, AnxA2 translocates to cholesterol-rich late endosomes in fibroblasts from patients with the cholesterol-storage disorder Niemann-Pick C [21]. From these studies it was speculated that cholesterol potentially affects the membrane binding affinity of other annexins as well, and eventually, their intracellular localization. Hence, annexins could indeed contribute to cholesterol-related events in biological membranes through the re-arrangement of microdomains [17,22].

In support of this hypothesis, we identified two different pools of AnxA6 in Chinese Hamster Ovary (CHO) cells. While the majority (70–80%) of AnxA6 bound to membranes in a Ca^{2+}-dependent manner, the remaining AnxA6 proteins associated with endosomal membranes even in the absence of Ca^{2+}. This pool of Ca^{2+}-independent AnxA6 proteins is strongly influenced by the amount and distribution of cholesterol. Cholesterol depletion using methyl-β-cyclodextrin reduced the membrane binding of Ca^{2+}-independent AnxA6 proteins to early and late endosomes. Conversely, cholesterol-loading of late endosomes, using low density lipoproteins (LDL) or the amphipatic steroid U18666A, stimulated the binding of AnxA6 to late endosomal membranes [13,14]. Since this translocation of AnxA6 was also observed in drug-induced, cholesterol-rich late endosomes of NRK fibroblasts, we proposed that cholesterol is a general and additional modulator of AnxA6-membrane binding and intracellular distribution. In line with these data, recent studies suggest Ca^{2+}-independent and cholesterol-, but not cholesteryl acetate, dependent membrane-binding properties of recombinant AnxA6, implicating the hydroxyl group of cholesterol in AnxA6-membrane interaction [11].

Moreover, it is now well-documented that acidic pH induces conformational changes in AnxA6 leading to increased hydrophobicity, membrane binding affinity [23–25] and high preference for monolayers containing cholesterol. In model membranes at acidic pH, the tryptophan 343 residue (Trp343) within the linker region of AnxA6 seems to be important for the interaction of AnxA6 with cholesterol in a Ca^{2+}-independent manner [11]. Fig. 2 depicts, in a simplified scheme, the steps within intracellular cholesterol transport pathways and the locations of the various annexins possibly involved.

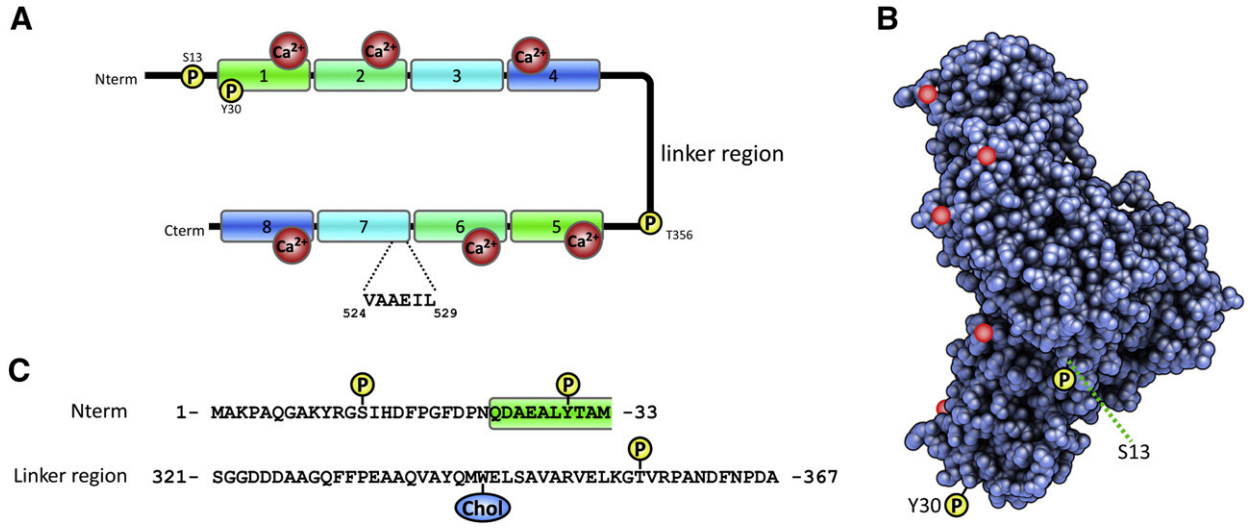

Fig. 1. Annexin A6 protein structure. (A) The eight repeats of AnxA6 are shown, and the localization of the six potential calcium-binding sites in repeats 1, 2, 4, 5, 6 and 8 are indicated [177]. The position of phosphorylation sites at S13, Y30, T356 and the larger splice variant (Pos. 524–529) [173] are highlighted. (B) Atomic space-filling model of bovine AnxA6 [178], where visible Ca^{2+} (red) and putative phosphorylation sites (yellow) are highlighted. (C) Schematic representation of the amino acid sequences in the N-terminal and the linker region of human AnxA6. The putative phosphorylation sites within the N-terminus (S13, Y30) and linker (T356) as well as the potential cholesterol-binding site (chol) in the linker at Trp343 (W343) [11] are indicated.