Annexin A6 as a cholesterol and nucleotide binding protein involved in membrane repair and in controlling membrane transport during endo- and exocytosis

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ABSTRACT

Annexins, calcium- and membrane-binding proteins, have been extensively studied at the Nencki Institute since early 1990s, in terms of their structure, potential ligands and functions in the organism, with emphasis on mineralization processes in norm and pathology. The results of recently performed studies have revealed that annexins are playing essential roles in membrane organization. In this review we characterize the largest member of the annexin family of proteins, annexin A6 (AnxA6), in respect to its cholesterol and nucleotide binding properties, as well as intracellular pH sensing and ability to change membrane permeability to ions. Furthermore, we discuss biological functions of AnxA6 such as participation in membrane lateral organization, cell membrane repair and regulation of vesicular transport.

INTRODUCTION – THE ANNEXINS

The vertebrate annexin (AnxA) superfamily consists of 12 members of calcium and phospholipid binding proteins which share high structural homology [Morgan *et al*. 2004; 2006; Gerke *et al*. 2005; Bandorowicz-Pikula 2003; Bandorowicz Pikula *et al*. 2001; 2012; Domon *et al*. 2012; Kodavali *et al*. 2014; Grewal *et al*. 2016]. In keeping with this hallmark feature, annexins have been implicated in Ca^{2+}-controlled regulation of a broad range of membrane related events, which may suggest its potential therapeutic value, namely, the regulation of immune response and control of tissue homeostasis [Schloer *et al*. 2018]. Recent highlights concerning transgenic (knockout animals) are summarized in table 1.

In this review, we focus on one of the main subjects of our investigations, annexin A6 (AnxA6), its properties and potential functions in addition to major achievements made by us in this field since 1997, we discuss recent advances related to hypothetical functions of AnxA6, including organization of biological membranes, or membrane repair mechanisms. The latter may be controlled in response to a disrupted cellular hemostasis and vesicle-related cellular processes, such as biomineralization [Balcerzak *et al*. 2008; Kapustin & Shanahan 2016; Minashima & Kirsch 2018; Bottini *et al*. 2018], transport and storage of cholesterol [Enrich *et al*. 2011; 2017; Reverter *et al*. 2011].

ANNEXIN A6 AT THE NENCKI INSTITUTE

Annexins were first introduced to the Nencki Institute as one among several experimental subjects of the Laboratory of Plasma Membrane Receptors at early 1990s [Bandorowicz *et al*. 1992; 1996; Sobota *et al*. 1993], and since 1997 further explored at the Laboratory of Lipid Biochemistry and Laboratory of Cellular Metabolism, but also since 1991 by the members of Laboratory of Calcium Binding Proteins [Filipek *et al*. 1991; 1995; Filipek & Wojda 1996]. During this time the important discoveries have been made concerning the annexin structure, potential ligands and finally their cellular and organismal functions. They are listed in table 2. It should be underlined that structural features, biochemical and biophysical properties of AnxA6 together with its functions have been well characterized *in vitro* and in cellular systems mainly on the basis of two fundamental discoveries – nucleotide binding properties of AnxA6 [Bandorowicz-Pikula & Awasthi 1997; Bandorowicz-Pikula *et al*. 1997a; 1997b; 1999; Bandorowicz-Pikula 1998; Bandorowicz-Pikula & Pikula 1998; Danieluk *et al*. 1999] and its interaction with cholesterol and cholesterol enriched biological membranes in a calcium- and pH-dependent manner [Sztolsztener *et al*. 2010; 2012; Domon *et al*. 2010; 2011; 2013a,b]. It should be stressed that cholesterol binding properties of AnxA6, characterized by us on the basis of a series of *in vivo* and *ex vivo* experiments, have been extensively studied in many other laboratories around the world. The obtained results suggest that AnxA6 acts as a multifunctional scaffold protein and is able to recruit vast number of sig-

Table 1. Properties of mammalian annexins revealed on the basis of analyses of knockout animals.

Annexin	Gene encoding	MW (Da)	Total aa	C-terminal core domain	Some proposed functions*
Annexin A1	ANXA1	38,714	346	4 repeat domains	anti- or pro-inflammatory responses, wound closure, epithelial motility, cancer cell metastasis, insulin secretion, cell fusion, vesicular transport, cell signaling, uptake of viruses
Annexin A2	ANXA2	40,41138,604	357 339	4 repeat domains	cancer cell metastasis, fibrinolysis, pathogen recognition, defense against bacterial infection
Annexin A3	ANXA3	36,375	323	4 repeat domains	nd
Annexin A4	ANXA4	35,883	321	4 repeat domains	cardiomyocyte signaling, integrity of urothelium
Annexin A5	ANXA5	35,937	320	4 repeat domains	biomineralization, thrombosis, angiogenesis, recognition of apoptotic cells
Annexin A6	ANXA6	75,873 72,423	673 641	8 repeat domains and linker	calcium homeostasis, plasma membrane organization, membrane repair, gluconeogenesis, biomineralization, chondrocyte differentiation
Annexin A7	ANXA8	52,739 50,316	488 466	4 repeat domains	cardiac contraction and remodeling, insulin secretion, cell proliferation
Annexin A8	ANXA9	36,881	327	4 repeat domains	nd
Annexin A9	ANXA10	38,364	345	4 repeat domains	nd
Annexin A10	ANXA11	37,278	324	4 repeat domains	nd
Annexin A11	ANXA12	54,390	505	4 repeat domains	nd
Annexin A13	ANXA13	35,415 39,744	316 357	4 repeat domains	nd

Information taken from https://www.uniprot.org, www.ncbi.nlm.nih.gov/protein/. *Some intra and extracellular functions suggested on the basis of the experiments performed using knockout animals, reviewed in [Grewal *et al*. 2016; Schloer *et al*. 2018]. Abbreviations: aa – amino acid residue, nd – not determined.

naling proteins, modulates cholesterol transport and its distribution within the cell, and also regulates membrane transport through actin dynamics. These activities suggest that AnxA6 may contribute to the formation of specific protein complexes and membrane domains relevant in signal transduction, cholesterol homeostasis and endo-/exocytosis [Grewal *et al*. 2017].

MEMBRANE-RELATED FUNCTIONS OF ANNEXIN A6

MEBRANE LATERAL ORGANIZATION

Annexin A6, as a cholesterol binding and multifunctional scaffold protein plays a crucial role in cell motility [Hayes *et al*. 2004; Monastyrskaya *et al*. 2009; Grewal *et al*. 2017] and is implicated also in cell signaling [Koese *et al*. 2013; Hoque *et al*. 2014; Qi *et al*. 2015; Cornely *et al*. 2016; Raouf *et al*. 2018]. Moreover, annexin A6 has been reported to regulate a formation of multifunctional signaling complexes at the membranes, affect membrane lateral organization, or influence cholesterol metabolism and distribution, but also to participate in the vesicular transport both in endo- and exocytosis [Cubells *et al*. 2007; 2008; Enrich *et al*. 2014; Garcia-Melero *et al*. 2016; Cairns *et al*. 2017]. In addition, it has been shown that AnxA6 recruited to the plasma membrane is able to affect membrane remodelling, e.g. upregulated AnxA6 in the cell decreased plasma membrane order through the regulation of cellular cholesterol homeostasis and its interaction with the actin cytoskeleton in the living cells [Alvarez-Guaita *et al*. 2015]. Strong experimental evidence has been accumulated that AnxA6 due to its unique, among annexins, structure affecting the distribution of cell specific surface receptors, recruits the interaction partners and simultaneously bridges specialized membrane domains with cortical actin surrounding activated receptors [Cornely *et al*. 2011].

MEBRANE REPAIR

The features of AnxA6 described above may be further extended to the observations suggesting its participation

Table 2. Properties and potential cellular functions of annexin A6 studied in the Laboratory of Lipid Biochemistry.

Feature	Description	References
Cellular localization	Plasma membrane, lysosomes and endosomes, matrix vesicles	[Bandorowicz *et al*. 1992; Balcerzak *et al*. 2008; Strzelecka-Kiliszek *et al*. 2008; Sztolsztener *et al*. 2010; 2012; Cmoch *et al*. 2011; Bottini *et al*. 2018]
Membrane binding properties and ion channel-like activity	Calcium dependent binding to plasma membrane phosphatidylserine A pH and calcium dependent interaction of AnxA6 with cholesterol, identification of cholesterol binding domain in AnxA6 Mechanism of folding of AnxA6 in membranes at acidic pH	[Bandorowicz-Pikula *et al*. 1996] [Domon *et al*. 2010; 2011; 2013a; 2013b] [Golczak *et al*. 2001; 2004; Pikula 2003; Buzhynskyy *et al*. 2009]
Nucleotide binding properties	Identification of a putative consensus sequence for the nucleotide-binding site in AnxA6 GTP-induced ion channel activity of AnxA6	[Kirilenko *et al*. 2002; 2006; Bandorowicz-Pikula 2003; Bandorowicz-Pikula *et al*. 2003]
Intracellular functions	Lateral organization of plasma membrane – microdomains Transport and storage of cholesterol Catecholamine and interleukin-2 secretion Biomineralization and matrix vesicles biogenesis	[Domon *et al*. 2012] [Bandorowicz-Pikula *et al*. 2012] [Podszywalow-Bartnicka *et al*. 2007; 2010; Strzelecka-Kiliszek *et al*. 2008]
Pathogenesis	Lipid storage in Niemann-Pick type C disease associated with mitochondrial dysfunction	[Wos *et al*. 2016; 2018]

in membrane repair mechanisms. Efficient cell membrane repair mechanisms are essential for maintaining membrane integrity and, thus, for cell life [Lauritzen *et al*. 2015; Demonbreun *et al*. 2016; Boye *et al*. 2017].

Initially, it has been observed that AnxA1 is involved in the repair of plasmalemmal lesions induced by bacterial toxins. Furthermore, highly Ca^{2+}-sensitive AnxA6, that responds faster to $[Ca^{2+}]_i$ elevation than AnxA1, promotes formation of lesions, and therefore is able to react to a limited and sustained membrane injury [Potez *et al*. 2011]. The AnxA6 contribution to membrane repair mechanism has been further elaborated on the basis of *in vitro* observations that AnxA4 and AnxA6 involved in plasma membrane repair cause rapid closure of micron-size holes in membranes. It has been demonstrated that AnxA4 binds to membranes and generates curvature force, whereas AnxA6 induces constriction force. In cells, plasma membrane injury and concomitant Ca^{2+} influx result in AnxA4 recruitment to the vicinity of membrane wound edges. Then, homo-trimerization of AnxA4 leads to membrane curvature near the edges. Mediated by AnxA6 constriction force is responsible for pulling the wound edges together for membrane fusion and final repair [Boye *et al*. 2017]. In agreement are observations performed by means of whole genome sequencing and RNA sequencing which identified AnxA6 on the mouse model of muscular dystrophy associated with cardiomyopathy. Its truncated version called ANXA6N32 was found to be responsible for disrupting the whole AnxA6-rich cap and the associated (surrounding) repair zone at the site of sarcolemma disruption, resulting in a membrane leak, characteristic for muscular dystrophy [Swaggart *et al*. 2014].

VESICULAR TRANSPORT

AnxA6 features allowed many investigators to think about this protein as a potential modulator of vesicular transport events. It has been suggested that AnxA6 is implicated in endocytosis, especially at the stage of fusion of autophagosomes with endocytic compartment in hepatocytes [Tebar *et al*. 2014; Enrich *et al*. 2017]. Moreover, AnxA6 highly expressed in smooth muscles, hepatocytes, endothelial cells and cardiomyocytes, has been found to affect various stages of endocytotic route of cholesterol transport [Cubells *et al*. 2007; Enrich *et al*. 2011; Reverter *et al*. 2011; Rentero *et al*. 2018].

In addition, AnxA6 was found to participate in cholesterol storage and the control of late endosomal cholesterol levels, that modulate integrin recycling and cell migration [Garcia-Melero *et al*. 2018], as well as influenza A replication and propagation [Musiol *et al*. 2013]. AnxA6 has also been linked to triglyceride storage in adipocytes [Cairns *et al*. 2017].

Participation of annexins in exocytosis was first postulated almost 30 years ago [Creutz 1992]. Further studies has revealed, that the number of observations suggesting functioning of AnxA6 in exocytosis is limited. Investigators, however, agree that this multifunctional protein plays a regulatory role in membrane trafficking during exocytosis too [Enrich *et al*. 2017; Cairns *et al*. 2018].

CONCLUDING REMARKS

We have actively contributed to experiments, results of which show that AnxA6 is an exceptional member

of the annexin family of proteins resembling genuine cholesterol-interacting proteins. Our studies indicate particularly that AnxA6 intracellular localization and membrane binding at low pH is determined by cholesterol. Although, the overall picture of possible AnxA6 functions still requires further studies to identify/clarify/completely unveil physiological and/or pathological processes involving AnxA6 ability to change membrane permeability to ions or mechanisms of membrane repair. To sum up, step should be taken to elucidate the overall importance of AnxA6 for the whole organism as it may form specific target to identify and cure human diseases in which AnxA6 may play a significant role.

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Aneksyna A6, białko wiążące cholesterol i nukleotydy, uczestniczące w naprawie błon biologicznych i w transporcie pęcherzykowym

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Słowa kluczowe: aneksyna A6, cholesterol, naprawa błon biologicznych, transport pęcherzykowy

STRESZCZENIE

Aneksyny, rodzina białek wiążących jony wapnia i błony biologiczne, były badane w Instytucie Biologii Doświadczalnej im. Marcelego Nenckiego w Warszawie od wczesnych lat 90. XX wieku. Szczególną uwagę poświęcono strukturze aneksyn, potencjalnym ligandom tych białek oraz ich funkcji, np. w procesie biomineralizacji zachodzącym w normie i w stanach patologicznych. Wyniki badań prowadzonych w wielu laboratoriach na świecie wskazują, że aneksyny odgrywają bardzo ważną rolę w organizacji błon biologicznych. W tym artykule przeglądowym opisujemy jednego z największych pod względem masy cząsteczkowej przedstawiciela rodziny aneksyn, aneksynę A6 (AnxA6), białko wykazujące zdolność wiązania się z cholesterolem i nukleotydami oraz zmieniające przepuszczalność błony dla jonów w odpowiedzi na obniżenie wewnątrzkomórkowego pH. Dodatkowo, opisano funkcje AnxA6, takie jak udział w tworzeniu mikrodomen błonowych, w naprawie uszkodzeń błony plazmatycznej oraz w regulacji transportu pęcherzykowego.