Journal of Structural Biology 168 (2009) 107–116

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Annexin-A6 presents two modes of association with phospholipid membranes. A combined QCM-D, AFM and cryo-TEM study

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ARTICLE INFO

Article history:
Received 22 December 2008
Received in revised form 3 March 2009
Accepted 13 March 2009
Available online 21 March 2009

Keywords:
Annexin A6
Annexin A5
Annexin A5 dimer
Dioleoylphospatidylserine
Calcium
Membrane binding
Membrane aggregation
Quartz crystal microbalance with dissipation monitoring (QCM-D)
Atomic force microscopy (AFM)
Transmission electron microscopy (TEM)
Supported phospholipid bilayer (SLB)

ABSTRACT

Annexins are soluble proteins that bind to biological membranes in a Ca^{2+}-dependent manner. Annexin-A6 (AnxA6) is unique in the annexin family as it consists of the repeat of two annexin core modules, while all other annexins consist of a single module. AnxA6 has been proposed to participate in various membrane-related processes, including endocytosis and exocytosis, yet the molecular mechanism of association of AnxA6 with biological membranes, especially its ability to aggregate membranes, is still unclear. To address this question, we studied the association of AnxA6 with model phospholipid membranes by combining the techniques of quartz crystal microbalance with dissipation monitoring (QCM-D), (cryo-) transmission electron microscopy (TEM) and atomic force microscopy (AFM). The properties of membrane binding and membrane aggregation of AnxA6 were compared to two reference systems, annexin A5 (AnxA5), which is the annexin prototype, and a chimerical AnxA5-dimer molecule, which is able to aggregate two membranes in a symmetrical manner. We show that AnxA6 presents two modes of association with lipid membranes depending on Ca^{2+}-concentration. At low Ca^{2+}-concentration (~60–150 μM), AnxA6 binds to membranes via its two coplanar annexin modules and is not able to associate two separate membranes. At high Ca^{2+}-concentration (~2 mM), AnxA6 molecules are able to bind two adjacent phospholipid membranes and present a conformation similar to the AnxA6 3D crystallographic structure. Possible biological implications of these novel membrane-binding properties of AnxA6 are discussed.

1. Introduction

Annexins are soluble proteins that exhibit the common property to bind membranes containing negatively charged phospholipids in a Ca^{2+}-dependent manner (Seaton, 1996). Annexins have been proposed to participate in many membrane-related processes, such as regulation of blood coagulation or inflammation, regulation of membrane organization and dynamics, participation in endocytosis or exocytosis and in membrane-cytoskeleton interactions (reviews in Raynal and Pollard, 1994; Gerke and Moss, 2002; Gerke et al., 2005). Annexins present a conserved core module made of the repeat of four homologous domains of about 70 amino-acid residues, which contain conserved sequences including calcium-binding sites. Annexins differ mainly by the length and sequence of their N-terminal segments, which harbor binding sites for diverse molecular partners. The annexin membrane-binding module presents a slightly bent shape with Ca^{2+}-binding loops exposed on the convex face (Huber et al., 1990; Concha et al., 1993; Luecke et al., 1995; Burger et al., 1996; Favier-Perron et al., 1996; Rosengarth et al., 2001). Several annexins, the prototype of which is Annexin A5 (AnxA5)[4], present the property to self-assemble into 2D ordered arrays upon binding to a membrane surface

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[4] *Abbreviations used:* AFM, atomic force microscopy; AnxA6, annexin A6; AnxA5, annexin A5; TEM, transmission electron microscopy; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine; QCM-D, quartz crystal microbalance with dissipation monitoring; SLB, supported lipid bilayer; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle.

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doi:10.1016/j.jsb.2009.03.007

(Brisson et al., 1991; Mosser et al., 1991; Andree et al., 1992; Voges et al., 1994; Reviakine et al., 1998, 2000; Oling et al., 2000, 2001). Some annexins have been shown in addition to aggregate membranes via 2D self-assembly. These properties have been proposed to regulate membrane organization and dynamics (Mosser et al., 1991; Andree et al., 1992; Rand et al., 1994; Reviakine et al., 1998; Oling et al., 2001; Kenis et al., 2004; Gerke et al., 2005).

Annexin A6 (AnxA6) is unique in the annexin family as it consists of two annexin core modules, connected via a 40 amino-acid long segment. In the high-resolution X-ray model of soluble AnxA6, the two annexin modules appear tilted with respect to each other in such a way that none of the two annexin modules can bind to a membrane with its convex face parallel to the membrane (Benz et al., 1996; Avila-Sakar et al., 1998, 2000) (Fig. 1A). Two independent transmission electron microscopy (TEM) studies of membrane-bound AnxA6 2D crystals have concluded that the two annexin modules reorient and become coplanar upon membrane binding (Benz et al., 1996; Avila-Sakar et al., 2000). However, two conflicting models of membrane-bound AnxA6 have been proposed, in which the two annexin modules present either the same orientation or opposite orientations.

In this study we re-investigated the structure of membrane-bound AnxA6 and addressed the question of its membrane-aggregation properties, which has not yet been studied at the structural level. This question is critical in the context of the proposed role of AnxA6 in endocytosis and exocytosis, in vesicular trafficking and in the organization of the interface between the cytoskeleton and the cytoplasmic membrane leaflet (Pons et al., 2001; Gerke and Moss, 2002; Grewal et al., 2005).

The association of AnxA6 with lipid membranes was compared with two reference annexin systems: AnxA5 and a chimerical AnxA5-dimer molecule. AnxA5 is the smallest member of the annexins, consisting only of the membrane-binding module (Huber et al., 1990) (Fig. 1B). The binding mechanism of AnxA5 to model lipid membranes is well described at the physico-chemical and structural levels (Reutelingsperger et al., 1985; Tait et al., 1988, 2004; Meers and Mealy, 1993; Oling et al., 2001; Richter et al., 2005). In particular, it is well established that, upon Ca^{2+}-dependent membrane binding, AnxA5 molecules self-assemble into large 2D ordered arrays of trimers. AnxA5 is also the prototype of annexin that does not aggregate membranes (Blackwood and Ernst, 1990; Lambert et al., 1997). The chimerical AnxA5-dimer is made of two AnxA5 modules arranged symmetrically with their concave face facing each other around a disulfide bond (Brisson, 2005; Bérat et al., 2007; Granier et al., in preparation) (Fig. 1C). As shown below, the AnxA5-dimer is a prototype of annexin that binds two membranes in a symmetrical manner.

In this study, we combined the physico-chemical method of quartz crystal microbalance with dissipation monitoring (QCM-D) with the structural methods of (cryo)-TEM and atomic force microscopy (AFM) in order to obtain complementary information on the membrane-association and membrane-aggregation properties of AnxA6. The QCM-D method is well suited for analyzing protein-membrane-association processes (Govorukhina et al., 2003; Janshoff and Steinem, 2005; Richter et al., 2005) because it allows separating the succession of events occurring in membrane-aggregation processes (Kastl et al., 2002; Ross et al., 2003) using supported lipid bilayers deposited on silica-coated quartz crystals as membrane models (Keller and Kasemo, 1998; Richter et al., 2003, 2006). (Cryo)-TEM and AFM are proven methods for the structural analysis of membrane-bound proteins and their assemblies, as shown previously in the annexin field (Mosser et al., 1991; Voges et al., 1994; Lambert et al., 1997; Reviakine et al., 1998; Kaetzel et al., 2001; Ross et al., 2003; Dabitz et al., 2005; Richter et al., 2005).

This study reveals that AnxA6 presents two possible modes of association with phospholipid membranes, one at low and one at high Ca^{2+} concentration, respectively. Possible biological implications of this Ca^{2+}-sensing property of AnxA6 are discussed.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-Dioleoyl-*sn*-glycero-3-[phospho-L-serine] (DOPS) were purchased from Avanti Polar Lipids (AL, USA). All other chemicals were of ultrapure grade. Water was purified with a RiOs system (Millipore, France).

Silica-coated QCM-D sensor crystals (5 MHz) were purchased from Q-SENSE (Gothenburg, Sweden). Muscovite mica discs of 12-mm diameter were from Metafix (Montdidier, France). Most experiments were performed with a buffer containing 150 mM NaCl, 10 mM Hepes, pH 7.4 and 2 mM NaN_3 in ultrapure water (buffer A), supplemented with either 2 mM $CaCl_2$ (buffer B) or 2 mM EGTA (buffer C), unless otherwise indicated.

2.2. Annexin protein preparation

Human recombinant AnxA6 (isoform 1) was expressed in *Escherichia coli* strain BL21 (DE3) and purified to homogeneity as described previously (Kirilenko et al., 2002). Rat recombinant rat AnxA5 and AnxA5-dimer were produced and purified as described in (Richter et al., 2005) and (Bérat et al., 2007), respectively. The AnxA5 moiety of AnxA5-dimers is a double mutant (C314S, T163C) in which the only cysteine in wt-Anx5 has been replaced by a serine (C314S) and a single cysteine has been introduced at position 163 (T163C) in a highly exposed region on the concave face of the protein.

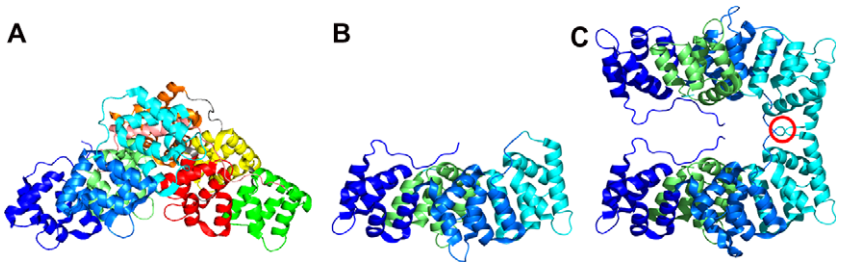

Fig. 1. X-ray models of AnxA6 (A), AnxA5 (B) and AnxA5-dimer (C). X-ray models of AnxA6 (Avila-Sakar et al., 2000), AnxA5 (Concha et al., 1993) and AnxA5-dimer (Granier et al., in preparation). The AnxA6 molecule is observed with the pseudo 2-fold axis relating the two annexin modules oriented vertically. The AnxA5 molecule is observed with its convex membrane-binding face pointing downwards. The AnxA5-dimer molecule is presented with the lower AnxA5 module oriented as AnxA5 in (B) while the upper AnxA5 module was rotated by 120° along a vertical axis passing through the centre of gravity of the molecule. In (C), the location of the disulfide bond is marked with a red circle. In the three models, domain I is located on the left side. Scale bar: 2 nm.