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Oligomeric structure of brain abundant proteins GAP-43 and BASP1

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ABSTRACT

Brain abundant proteins GAP-43 and BASP1 participate in the regulation of actin cytoskeleton dynamics in neuronal axon terminals. The proposed mechanism suggests that the proteins sequester phosphatidylinositol-4,5-diphosphate (PIP_2) in the inner leaflet of the plasma membrane. We found that model anionic phospholipid membranes in the form of liposomes induce rapid oligomerization of GAP-43 and BASP1 proteins. Multiply charged phosphoinositides produced the most potent effect. Anionic detergent sodium dodecyl sulfate (SDS) at submicellar concentration stimulated formation of similar oligomers in solution. BASP1, but not GAP-43, also formed oligomers at sufficiently high concentration in the absence of lipids and SDS. Electron microscopy study demonstrated that the oligomers have disk-shaped or annular structure of 10–30 nm in diameter. BASP1 also formed higher aggregates of linear rod-like structure, with average length of about 100 nm. In outward appearance, the oligomers and linear aggregates are reminiscent of oligomers and protofibrils of amyloid proteins. Both the synthetic N-terminal peptide GAP-43(1–40) and the brain-derived fragment GAP-43–3 preserved the ability to oligomerize under the action of acidic phospholipids and SDS. On the contrary, BASP1 fragment truncated by the short N-terminal myristoylated peptide was unable to form oligomers. GAP-43 and BASP1 oligomerization can be regulated by calmodulin, which disrupts the oligomers and displaces the proteins from the membrane. We suggest that *in vivo*, the role of membrane-bound GAP-43 and BASP1 oligomers consists in accumulation of PIP_2 in functional clusters, which become accessible for other PIP_2-binding proteins after dissociation of the oligomers.

1. Introduction

About one third of soluble and membrane-bound cellular proteins are estimated to be in oligomeric form (Goodsell and Olson, 2000). Oligomerization may provide the basis for some specific protein properties, including multivalent ligand binding and cooperative regulation. Symmetrical structure of homo-oligomeric proteins ensures their stability and finite control of assembly. Circular symmetry results in formation of ring-shaped (annular) oligomers. The examples include chaperonins (Ranson et al., 2001), Rad52 (Stasiak et al., 2000), AAA+ family proteins (Hanson and Whiteheart, 2005) and bacterial pore-forming toxins (Sekiya et al., 1993). Another case of annular oligomers is represented by prefibrillar oligomers of amyloid proteins involved in protein aggregation diseases (Quist et al., 2005; Lashuel and Lansbury, 2006). Oligomerization of some proteins strongly depends on environment and intracellular signaling pathways, while the transiently formed oligomers play an important role in many biological processes (Ali and Imperiali, 2005). Most oligomers are characterized by a definite (functionally important) number of subunits. In the present work, we have studied the oligomerization of brain proteins GAP-43 (also referred to as neuromodulin or B-50) and BASP1 (also referred to as CAP-23 or NAP-22), which is promoted by anionic phospholipids and results in formation of heterogeneously sized annular oligomers.

GAP-43 and BASP1 are abundant proteins at the inner surface of the plasma membrane of axon terminals. GAP-43 is well-known for its role in axon guidance, neuroregeneration and synaptic plasticity. The participation of GAP-43 in regulation of growth cone adhesion and motility and in the control of neurotransmitter release from presynaptic terminals of mature neurons has been well documented (Oestreicher et al., 1997; Denny, 2006). However, multiple functions of GAP-43 are poorly understood at the molecular level. BASP1 is much less studied than GAP-43 (Mosevitsky, 2005). These proteins are similar in a number of biochemical and functional properties, although their amino acid sequences share no homology. They are characterized by a molecular mass of 23–25 kDa, an acidic isoelectric point and a high content of charged

Abbreviations: CaM, calmodulin; ED, effector domain; GA, glutaraldehyde; PA, phosphatidic acid; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-diphosphate; PKC, protein kinase C; PS, phosphatidylserine; SEC, size-exclusion chromatography; SM, sphingomyelin; TEM, transmission electron microscopy.

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amino acids as well as alanine and proline. Like GAP-43, BASP1 participates in regulation of subplasmalemmal actin dynamics both in non-neuronal cells (Wiederkehr et al., 1997; Laux et al., 2000) and in axonal growth cones (Frey et al., 2000). Enhanced expression of BASP1 in mature neurons is also strongly correlated with increased neuron's capacity for axonal regeneration and sprouting, as in the case of GAP-43 (Caroni et al., 1997; Bomze et al., 2001). Knocking-out a gene of any of these two proteins in mice resulted in severe abnormalities in nervous system development and high postnatal lethality (Frey et al., 2000; Shen et al., 2002). On the other hand, knock-in mice produced by insertion of GAP-43 cDNA instead of BASP1 gene coding sequence were viable and indistinguishable from wild-type mice in many respects (Frey et al., 2000). This fact indicates that GAP-43 can functionally substitute for BASP1 at least partially. However, in contrast to GAP-43, BASP1 is widely distributed in various non-nervous tissues and cells (Mosevitsky et al., 1997; Zakharov et al., 2003).

The probable mechanism of action of GAP-43 and BASP1 proteins in growth cones is based on the regulation of local actin dynamics, which in its turn is under the control of protein kinase C (PKC) and calmodulin (CaM). There is evidence that phosphorylation of Ser41 residue by PKC plays a central role in GAP-43 function. The phosphorylated GAP-43 accumulates in filopodia and lamellipodia at the leading edge of growth cones, whereas the quiescent and collapsing regions of growth cones contain dephosphorylated GAP-43 (He et al., 1997; Dent and Meiri, 1998). Phosphorylation sites Ser41 of GAP-43 and Ser5 of BASP1 reside within positively charged effector domains (EDs) (39–55 in GAP-43, 1–9 in BASP1) involved in CaM binding (Chapman et al., 1991; Takasaki et al., 1999). N-terminal glycine of BASP1 ED is bound to the myristic acid residue, which participates in binding to the membrane, PKC and CaM (Mosevitsky et al., 1997; Takasaki et al., 1999). BASP1 binds CaM only in the presence of calcium (Maekawa et al., 1993), whereas GAP-43 has stronger affinity for apo-CaM (Alexander et al., 1987).

At present, two probable models have been suggested to account for the role of GAP-43 and BASP1 in regulation of actin cytoskeleton dynamics (Denny, 2006). According to the first model, this regulation may be accomplished by their direct interaction with actin or actin-binding proteins (He et al., 1997; Odagaki et al., 2009). The second model presumes that the proteins can bind electrostatically (by means of their basic EDs) and thereby sequester anionic phosphatidylinositol-4,5-diphosphate (PIP_2) in the inner leaflet of the plasma membrane (Laux et al., 2000; Wang et al., 2002; Epand et al., 2004; Tong et al., 2008). Phosphorylation by PKC or, alternatively, CaM binding disrupts the interaction of GAP-43 and BASP1 with phospholipids. This leads to uncovering PIP_2 clusters, which influence the activity of a number of actin-associated proteins that directly regulate the architecture of actin skeleton (Laux et al., 2000; Caroni, 2001). The molecular details of PIP_2 sequestration and accumulation by GAP-43 and BASP1 proteins still remain unclear. The electrostatic mechanism of protein-PIP_2 interaction suggests that the single molecule of the protein is unable to form the PIP_2-enriched microdomain. GAP-43 and BASP1 EDs have a net charge of +7 and +5, respectively, while PIP_2 has a charge of –4. Consequently, the single protein chain can bind only one or two PIP_2 molecules. This predicts that effective PIP_2 accumulation may be performed only by oligomeric protein form capable for multivalent PIP_2 binding. Formation of GAP-43 oligomers was suggested by several early studies on the basis of size-exclusion chromatography (SEC) data, which provided different estimates of apparent molecular mass of GAP-43 aggregates, from 120 kDa up to higher than 300 kDa (Benowitz et al., 1987; Schotman et al., 1989; McIntosh et al., 1989). In the case of BASP1, sedimentation experiments showed that the protein forms highly aggregated complexes at concentrations above 0.2 mg/ml (Epand et al., 2003).

In this work, we have shown for the first time that GAP-43 and BASP1 are able to form regular high-order oligomers of similar structure. Our results are consistent with the notion that formation of the oligomers is a structural prerequisite for GAP-43 and BASP1 functional activity.

2. Materials and methods

2.1. Protein isolation and purification

BASP1, GAP-43 and GAP-43–3 proteins were isolated from bovine brain by successive chloroform and 3% perchloric acid extractions as described previously (Zakharov and Mosevitsky, 2007). The proteins were purified by preparative electrophoresis in 12% polyacrylamide gel cross-linked with 0.15% *N,N'*-diallyltartardiamide in acetic acid/urea/Triton X-100 system (Zakharov et al., 2003). Further protein purification was achieved by reversed-phase HPLC (Agilent 1200 Series chromatographic system) on C8 column, using 10–50% acetonitrile gradient in 25 mM sodium phosphate pH 2.1. Purified proteins were dialysed overnight at 4 °C against 10 mM sodium phosphate pH 7.3 and then against pure water and stored at −20 °C in aliquots. Protein concentrations were determined by the microplate Bradford assay (Sigma–Aldrich, St. Louis, MO, USA) using BSA as a standard.

2.2. Liposome binding assay

Small unilamellar vesicles were prepared from dioleoyl forms of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA), porcine brain phosphatidylinositol-4-phosphate (PIP), PIP_2, sphingomyelin (SM), bovine brain PS (in some experiments) and cholesterol (Avanti Polar Lipids, Alabaster, AL, USA) according to the following procedure. The lipids were solubilized in chloroform/methanol (2:1) at 2 mM concentration and mixed in a total volume of 20 μl to obtain the required lipid composition. Lipid mixtures were dried in chloroform-resistant 1.5 ml microtubes in vacuum centrifugal evaporator for 2 h at 22 °C. The dry lipid films were hydrated in 19 μl of PBS solution (20 mM sodium phosphate pH 7.3, 0.15 M NaCl, 0.2 mM EGTA, 5 mM $MgCl_2$, 0.5 mM dithiothreitol) at 22 °C for 1 h in a centrifuge/vortex mixer and then sonicated in a cup horn sonicator for 2 min at 0–10 °C. The resulted SUV samples were mixed with 1 μl of 40 μM (about 1 mg/ml) protein (GAP-43 or BASP1) solution. The final lipid and protein concentrations were 2 mM and 2 μM, respectively. The protein/liposome mixture was frozen in liquid nitrogen for 20 s, left to thaw at 22 °C for 15 min and then sonicated for 10 s as above. After incubation at 37 °C for 30 min, the liposomes with bound proteins were pelleted by centrifugation at 100,000*g* for either 1 or 6 h at 5 °C. The supernatants and pellets were carefully separated and analyzed by denaturing SDS PAGE. In some experiments, we used multilamellar vesicles prepared similarly except that the sonication steps were replaced by three cycles of freezing-thawing. The efficiency of liposome sedimentation was determined by TLC. Lipids present in the pellets and supernatants were extracted with chlorophorm/methanol (2:1), resolved on silica gel plates in chlorophorm/methanol/water (2:1:0.1) and stained with phosphomolybdic acid.

2.3. Oligomer cross-linking assay

Protein cross-linking was performed in the presence of 0.06% glutaraldehyde (GA) prepared from 1.2% stock solution (Sigma–Aldrich, St. Louis, MO, USA). The proteins (2–5 μg) at various concentrations (0.1–1.0 mg/ml) were incubated with GA in 20 mM sodium phosphate pH 7.3, 0.5 mM dithiothreitol at 37 °C for 30 min. The reaction was quenched with denaturing SDS PAGE