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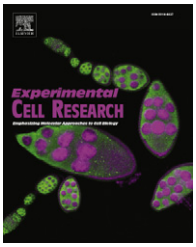

Research Article

Synaptotagmin 1 causes phosphatidyl inositol lipid-dependent actin remodeling in cultured non-neuronal and neuronal cells

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ABSTRACT

Here we demonstrate that a dramatic actin polymerizing activity caused by ectopic expression of the synaptic vesicle protein synaptotagmin 1 that results in extensive filopodia formation is due to the presence of a lysine rich sequence motif immediately at the cytoplasmic side of the transmembrane domain of the protein. This polybasic sequence interacts with anionic phospholipids in vitro, and, consequently, the actin remodeling caused by this sequence is interfered with by expression of a phosphatidyl inositol (4,5)-bisphosphate (PIP2)-targeted phosphatase, suggesting that it intervenes with the function of PIP2-binding actin control proteins. The activity drastically alters the behavior of a range of cultured cells including the neuroblastoma cell line SH-SY5Y and primary cortical mouse neurons, and, since the sequence is conserved also in synaptotagmin 2, it may reflect an important fine-tuning role for these two proteins during synaptic vesicle fusion and neurotransmitter release.

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Introduction

Actin polymerization is the principal mechanism behind formation of protruding filopodia and lamellipodia at advancing edges of motile cells. The filament-forming activity is a consequence of surface receptor activation and, orchestrated by numerous different actin binding proteins, it results in the plethora of dynamic arrangements typically characterizing the leading edge of migrating cells or the advancing growth cone of nerve axons.

The core of the finger-like filopodia is built by a tightly packed bundle of parallel actin filaments which grow through rapid addition of new actin subunits at their extending tips e.g. [1–2]. These projecting structures are used by cells to explore their surroundings for chemo-attractants and make initial contacts with the extracellular matrix and neighboring cells; thus steering migrating cells over their support surfaces in vitro or through the tissue in vivo [3–6]. This exploratory behavior often includes repeated protrusion and withdrawal of filopodia and lamellipodia, forming the ruffling phenomenon typically seen at cell edges during polarized growth and migration and known by cell biologists for more than four decades [7]. Although varying between cell types, filopodia formation is observed in a range of cells, including nerve cells [8–9]. For instance during early stages of synaptogenesis, both pre- and postsynaptic, filopodia contribute to axo-dendritic contacts within the neuronal system, [10]; reviewed in [11–13], and there appears to be extensive similarities between the signaling cascades involved in axon guidance and synapse formation [12].

Studies on the mechanisms behind remodeling of the actin microfilament system have led to a remarkable amount of data yet it is not entirely clear how this fundamental process is controlled. Actin nucleation and elongation promoting factors (NEPFs) such as the Arp2/3-dependent N-WASP and WAVE family of proteins,

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formins and Ena/VASP proteins are critical to the spatiotemporal control of actin filament formation in vivo. Their multimodular character allows for a fine-tuned activity by phosphatidyl-inositol lipids, RhoGTPases and other components in juxtaposition to the plasma membrane where they operate to incorporate actin from profilin: actin onto growing filaments. More recently discovered proteins such as Spire, Cobl and JMY, and numerous actin binding proteins influencing actin microfilament turn-over in response to different signals such as profilin, cofilin and capping protein further add to the complexity of this machinery, for reviews see [2,14–17].

Synaptotagmin 1 (Syt) is the archetype member of the 16 isoforms constituting the synaptotagmin protein family. It has a well established role in hippocampal Ca^{2+}-dependent synaptic vesicle (SV) fusion during neurotransmitter release [18–19] and contributes also to synaptic activity via interaction with components of the endocytotic machinery in the peri-active zone [20]. The protein is tethered to SVs via a single amino (N)-terminal transmembrane helix while its major part extends into the nerve cell cytoplasm, accessible for interactions with the fusion-mediating SNARE-complex and for binding Ca^{2+}-ions via its C_2A and C_2B-domains. Binding of Ca^{2+} to the C_2B domain enhances the interaction of the protein with phosphatidyl inositol-(4,5)-bisphosphate (PIP2) in the presynaptic membrane of the nerve terminal [21]. Upon action potentials and subsequent Ca^{2+}-influx, the fusion of pre-docked synaptic vesicles with the presynaptic membrane and subsequent neurotransmitter release occurs on a sub-millisecond time scale.

Early studies of the *Drosophila* motorneuron revealed a significantly decreased frequency of vesicle release after down-regulation of Syt [22–23], illustrating its importance for neurotransmission. Later, these observations were explained to be due to a combination of synaptotagmin's role as a Ca^{2+}-sensor and interaction partner with the SNARE-complex, [19,24]. Despite an extensive knowledge of Syt, its structure, and lipid and Ca^{2+}-binding properties, the exact mechanisms by which it triggers synaptic vesicle fusion are not fully understood. Nor has a possible involvement of Syt in the membrane flow directed toward the peri-active zone after vesicle fusion been addressed, although several of the vesicle proteins, including Syt, remain clustered at the presynaptic membrane after fusion [25–27].

The ability of Syt to cause actin polymerization and dramatic filopodia formation when expressed in non-neuronal cells [28] has led to little attention with respect to its activity in neuronal cells. This may perhaps be because the presence of actin at presynaptic terminals has mainly been interpreted to have a non-propulsive role, organizing and separating the synaptic vesicle cluster from the rest of the nerve cytoplasm [29]. Here we have focused on the ability of Syt to cause actin polymerization with subsequent formation of filopodia [28], and identified a polybasic sequence motif immediately on the C-terminal side of the transmembrane domain as being responsible for this activity. Our observations suggest that this sequence in Syt is an important target for physiological studies and that Syt may be a useful tool for further analysis of the mechanisms involved in filopodia formation.

Material and methods

DNA constructs

Rat synaptotagmin1 cDNA was kindly provided by Dr Kasim Diril, Freie Universität, Berlin, as a BamHI/EcoRI insert in pcDNA-FLAG (pcFLAG-Syt1); N-terminally HA-tagged Syt1 was generated from pcFLAG-Syt1 by subcloning of the BamHI/EcoRI full-length Syt1 cDNA into the XhoI/EcoRI sites of pEGFP-C2. The HA-tag was amplified from the pCGN vector [30], using primers fwd 5′-GTTGCTAGCATGGCTTCTAGCTATCCTTA-3′ and rev 5′-ATCTGTACATTCTAGAAGGTCCTCCCAG-3′ and further subcloned into the Nhe1/BsrG1 sites of pEGFP-C2 thereby replacing the GFP-sequence for the HA-tag. This construct was used to generate a full length Syt with C-terminal GFP by inserting GFP into the Pst I/BamHI-site (used in Supplementary Video 1). For truncation and mutagenesis analyses, Syt(159–421) and Syt(1–169) were generated using restriction enzyme digestion and ligation, and Syt(1–96) was generated by PCR amplification and subsequent subcloning of the fragments into the XbaI/BamHI sites of the pCGN-HA vector. The Syt(1–96)GFP construct was generated by PCR from the pEGFP-C2 vector and the fragment cloned into the BamHI site of Syt 1–96, thus this construct referred to in text as Syt_{1-96} still contained the HA-tag. Mutations of the Syt(1–96) GFP were introduced using oligonucleotide-based site-directed mutagenesis to generate the following mutations: $2K/NQ_1$: (K85N/K86Q); $2K/NQ_2$: (K89N/K90Q); 4K/NQ: (K85N/K86Q/K89N/K90Q); 4K/R: (K85R/K86R/K89R/K90R); 8K/NQM: (K85N/K86Q/K87M/K89N/K90Q/K91M/K93Q/K95M); C82S; and, finally, Syt1–81 with residues 82–421 deleted. For the generation of SUMO-fusion constructs for bacterial expression, DNA-oligonucleotides corresponding to Syt(80–96) and Syt(80–96) 8K/NQM, respectively, were annealed with overhangs matching cleaved BamHI/Eco31I sites and then subcloned into the corresponding sites of $pHis_6$-SUMO [31]. The plasmids for depletion of cellular PIP2, mRFP-FKBP12, mRFP-FKBP12-5-ptase-domain, PM-FRB-RFP and PM-FRB-CFP were generously provided by Tamas Balla, NIH [32] and used together with 100 nM rapamycin (Sigma). To construct FKBP12-Syt(80–96), annealed oligonucleotides corresponding to Syt(80–96) were subcloned into the PvuI/KpnI sites in mRFP-FKBP12. All constructs produced were sequenced to ensure correctness. The VASP targeting constructs GFP-FP4-Mito and GFP-AP4-Mito were kindly provided by Frank Gertler, MIT [33].

Membrane preparation

The procedure for preparation of inside-out microsomal membranes was essentially as described in [34]. Briefly, six 10 cm plates each of B16 cells untransfected (control sample) or transfected with Syt(1–96)GFP were washed in ice cold PBS, scraped off in 800 µl 10% sucrose 10 mM Tris–HCl pH 7.5 containing EDTA free protease inhibitor (PI; Roche) and homogenized in a glass douncer by 35 strokes. The cell lysates were cleared from debris by centrifugation at 14,300×*g* for 20 min, the resulting supernatants were centrifuged at 105,000×*g* for 30 min and the microsomes collected in the pellets were then resuspended in 375 µl 10 mM Tris pH 7.5 containing 0.6 M KCl and PI, dounced and incubated for 2 h on ice. Subsequently the samples were loaded onto a sucrose gradient (starting with 25% sucrose in 10 mM Tris–HCl pH 7.5 and then serially increasing the sucrose concentration in steps of 2.5% until reaching 50% with a bottom cushion of 70%, using a layer volume 375 µl) and centrifuged at 72,000×*g* for 15 h in a swing out rotor. Fractions of 375 µl each were collected from the top of the gradient, diluted in an equal volume of 10 mM Tris–HCl pH 7.5 and centrifuged at 142,000×*g*

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