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Extensions of PSD-95/discs large/ZO-1 (PDZ) domains influence lipid binding and membrane targeting of syntenin-1

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ABSTRACT

Syntenin-1 is a PDZ protein involved in receptor recycling and clustering. Its two PDZ domains interact with various receptors and phosphoinositides, and are flanked by N- and C-terminal regions. Here, we report the identification of an autoinhibitory peptide stretch in the N-terminus that might be regulated by phosphorylation. We further establish that basic residues in the C-terminal region mediate electrostatic interactions with reconstituted liposomes and contribute to the plasma membrane targeting. Our study adds new components to the multi-dentate membrane targeting mechanism and highlights the role of N- and C-terminal PDZ extensions in the regulation of syntenin-1 plasma membrane localization.

Structured summary of protein interactions:
PDZ1-PDZ2 and **peptide** bind by fluorescence technology (View Interaction: 1, 2, 3, 4).

1. Introduction

Scaffolding proteins provide spatiotemporal organization of signaling complexes crucial for accurate flow of cellular information [1]. They are composed of modular domains linked and flanked by unstructured regions, and generally lack catalytic activity. Their proper distribution is commonly achieved by interactions with membrane receptors and/or membrane lipids [2]. The negative electrostatic potential of the plasma membrane plays a pivotal role in the recruitment of proteins containing poly-basic clusters [3–5]. The plasma membrane is enriched in phosphatidylinositol 4,5-bisphosphate (PIP2) which regulates, among other processes, actin polymerization and vesicular trafficking [6]. At physiological pH, PIP2 carries a net charge of ca. −4 and contributes thereby to the negative surface charge of the inner leaflet of the plasma membrane. Phosphatidylserine (PS) is the most abundant anionic phospholipid in the inner leaflet and carries one negative charge at pH 7 [7].

PDZ (postsynaptic density protein-95/discs large/zonula occludens-1) domains are among the most common protein–protein interaction modules in multi-cellular organisms. They are involved in a variety of cellular processes including establishment and maintenance of cell polarity [8]. PDZ domains are composed of 80–90 amino acids with a common fold comprising six β-strands and two α-helices. They commonly recognize the C-terminal peptides of their target proteins, but may also interact with internal peptide stretches, hetero- and homo-dimerize and/or interact with phosphoinositides [9,10]. Syntenin-1 is a scaffolding protein containing two PDZ domains. The PDZ domains can accommodate a wide range of C-terminal peptide ligands [11] and the plasticity of peptide binding specificity allows syntenin-1 to interact with a range of mainly transmembrane receptors including syndecans [12] and Frizzled-7 [13]. Syntenin-1 can also interact with plasma membrane PIP2, an interaction attributed to the PDZ-tandem. Indeed, syntenin-1 was the paradigm example of a PIP2-PDZ interaction [14]. The importance of the syntenin-1-PIP2 interaction was demonstrated in cellulo for the recycling of syndecans and cell spreading, and in vivo for directional movements in zebrafish [15,16].

While several functional studies have elucidated the functional importance of the PDZ-tandem, only few studies have been focused on the unstructured N- and C-terminal regions. The ca. 100 amino acid long N-terminal domain (NTD) has been proposed to hold an autoinhibitory region [11,17], recruit Sox4 [18] and to interact with ubiqitin [19,20]. The C-terminal extension (CTD) consisting of ca. 20 residues has in several cases been found to be a necessary extension of the PDZ tandem for high affinity interactions [21,22]. In this study we aimed to elucidate how, and to what extent, the NTD and the CTD of syntenin-1 regulate and contribute to its

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plasma membrane targeting. We identified a conserved autoinhibitory peptide stretch in the NTD that interacts with the PDZ-tandem; an interaction that may be regulated by a tyrosine phosphorylation switch. We discovered that basic residues in the CTD contribute to electrostatic interactions crucial for plasma membrane targeting and established that such interactions rely on the negative plasma membrane potential built up by PS. The results illustrate the importance of N- and C-terminal extensions in regulating and contributing to syntenin-1 function.

2. Materials and methods

2.1. Molecular biology

eGFP-Syntenin-1 and eYFP-PDZ1-PDZ2-CTD constructs were described previously [17]. The fragments encoding PDZ1-PDZ2-CTD (amino acids 103–298) or PDZ1-PDZ2 (103–275) were amplified by PCR using oligonucleotides carrying the restriction sites for NcoI and EcoRI (Roche) and subcloned into the pETM-11 plasmid (EMBL Heidelberg) for the expression of N-terminally His-tagged proteins. Site-directed mutagenesis was performed using QuikChange (Stratagene) on the eGFP-, eYFP- or pETM-11-templates. All constructs were verified by DNA sequencing.

2.2. Cell culture, transfections and microscopic analysis

MCF-7 cells obtained from American Type Culture Collection (Manassas, VA) were cultured in DMEM/F-12 medium (Life Technologies) supplemented with 10% fetal bovine serum (Gibco). For microscopy experiments cells were plated on eight-well chamber slides (Nagle Nunc International, Roche) and transfected after 4 h with FuGENE (Roche). Confocal images were obtained 10 h after transfection with an Olympus Fluoview 1000 microscope (Olympus) using appropriate filter sets. The enrichment of fluorescently tagged constructs at the plasma membrane was scored in living cells by wide-field microscopy (Leica AS-MDW) in at least 30 cells for each condition in three independent experiments. Cells with comparable fluorescence were selected for quantifications. Mean grey values were typically between 700 and 1600 (see also Supplementary Table 1). Myc-tagged PtdInsP 5 kinase was visualized using anti-Myc antibody (9E10, Sigma–Aldrich) and Alexa 647-conjugated goat anti-mouse secondary antibody (Invitrogen) and the staining was performed as described previously [17]. For dibucaine treatment, cells were incubated in a synthetic medium containing 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 100 μM EGTA, 2 mM $CaCl_2$, and 20 mM HEPES, pH 7.4 and dibuciane (Sigma–Aldrich) was added to the final concentration of 1 mM for 30 min at 37 °C.

2.3. Protein purifications

N-terminally his-tagged syntenin-1 variants were expressed in *Escherichia coli* ER2566 and purified by nickel affinity chromatography as described previously [23].

Table 1
Apparent affinities for 5% PIP2 in the background of composite liposomes (30% PC/20% PS/40% PE/5% PI and 5% PIP2) as determined by SPR binding experiments.

	Apparent K_D^{PIP2} (μM)
PDZ1-PDZ2	44 ± 12
PDZ1-PDZ2-CTD	11 ± 2
PDZ1-PDZ2-CTD/K280A/R281A	50 ± 9
PDZ1-PDZ2-CTD/K280A/R281A/M282R/A283R	8 ± 1
PDZ1-PDZ2-CTD/M282R/A283R	10 ± 2
PDZ1-PDZ2-CTD/S285D/S289D	28 ± 4

2.4. Peptide binding experiments

Peptide binding experiments were performed in 150 mM NaCl, 25 mM HEPES, 1 mM DTT, pH 7.2 using a Cary Eclipse Spectrofluorimeter (Varian) at 25 °C, essentially as previously described [23]. Synthetic peptides (ELSQYMGLSL-NH2 and ELSQEMGLSL-NH2) were from GeneCust and of high purity (>95%). PDZ1-PDZ2, PDZ1-PDZ2/G126E and PDZ1-PDZ2/G210D (3 μM) were titrated with increasing peptide concentrations (10–800 μM), and the change in intrinsic tryptophan fluorescence was recorded (excitation at 280 nm, emission at 320–380 nm). The observed signal was corrected for background by subtraction of the signal recorded when performing the same titration in the absence of proteins. The observed transitions were fitted by simple binding isotherms using Prism (GraphPad). The experiments were repeated independently three times.

2.5. SPR experiments

SPR experiments were performed with a Biacore T100 (GE Healthcare). Bulk phospholipids (phosphatidyl cholin (PC), PS, phosphatidyl ethanolamine (PE) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)) were from Sigma–Aldrich. PI and PIP2 were from Echelon Biosciences. Composite liposomes (30% PC/20% PS/40% PE/5% PI and 5% PIP2 or 10% PI for background reference) or DOPC liposomes (95% DOPC/5% PIP2 or 5% PI for background reference, or 80% DOPC/20% PS with 100% DOPC for background reference) were prepared and immobilized as described previously [24] in 150 mM NaCl, 25 mM HEPES, 1 mM DTT, pH 7.2. Liposomes were coated on L1 chips, with immobilization levels of 5000–6000 RU. Proteins were freshly prepared, dialyzed against the running buffer and injected at seven different concentrations. The surface was regenerated between runs by two 30 s pulses of 50 mM NaOH. The flow was 30 μL/min, the temperature 25 °C, and the injection time 240 s. Sensorgrams were double reference subtracted (e.g. PI-liposomes and buffer effect) and the responses observed 5 s before injection stop were plotted as a function of protein concentrations. Apparent K_D values were determined by fitting the data to a 1:1 Langmuir binding isotherm (GraphPad Prism). The experiments were repeated using different batches of liposomes and proteins.

3. Results

3.1. Identification of the autoinhibitory element in the NTD

The syntenin-1 NTD has been proposed to work as an autoinhibitory region because fluorescently tagged full length syntenin-1 (eGFP-syntenin-1) is mainly diffused upon over-expression in MCF-7 cells, on the contrary to syntenin-1 with the NTD deleted, which is highly enriched at the plasma membrane [17]. Indeed, discrete plasma membrane enrichment of full length syntenin-1 is found in only 17% of transfected cells (Fig. 1A, B and G;) and deletion of the first 102 amino acids confers a strong plasma membrane enrichment of the protein in all cells (eYFP-PDZ1-PDZ2-CTD) (Fig. 1C and G). To identify the autoinhibitory element, we made truncation variants of the fluorescently tagged protein (Fig. 1) and quantified the plasma membrane enrichment of the constructs. Truncation of the NTD from amino acid 1–51 did not improve the membrane enrichment (Fig. 1D and G). However, when truncated at amino acid 57, or further, the protein became strongly enriched at the plasma membrane (Fig. 1E and G). The region between amino acids 56 and 59 is highly conserved among syntenin-1 proteins (Fig. 1H). We hypothesized that the peptide stretch could interact with the PDZ-tandem and probed for a direct