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Membrane protein sequestering by ionic protein-lipid interactions

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Abstract

Neuronal exocytosis is catalyzed by the SNARE protein syntaxin-1A[1]. Syntaxin-1A is clustered in the plasma membrane at sites where synaptic vesicles undergo exocytosis[2,3]. However, how syntaxin-1A is sequestered is unknown. Here, we show that syntaxin clustering is mediated by electrostatic interactions with the strongly anionic lipid phosphatidylinositol-4,5-bisphosphate (PIP2). We found with super-resolution STED microscopy on the plasma membrane of PC12 cells that PIP2 is the dominant inner-leaflet lipid in ~73 nm-sized microdomains. This high accumulation of PIP2 was required for syntaxin-1A sequestering, as destruction of PIP2 by the phosphatase synaptojanin-1 reduced syntaxin-1A clustering. Furthermore, co-reconstitution of PIP2 and the *C*-terminal part of syntaxin-1A in artificial giant unilamellar vesicles resulted in segregation of PIP2 and syntaxin-1A into distinct domains even when cholesterol was absent. Our results demonstrate that electrostatic protein-lipid interactions can result in the formation of microdomains independent of cholesterol or lipid phases.

Phosphoinositides are lipids that contain an inositol headgroup conjugated to 1–3 phosphate groups. With ~1% of total lipids in the inner leaflet of the plasma membrane[4], PIP2 is the

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Author Contributions

G.v.d.B and R.J. designed the experiments and wrote the paper. K.M., B.E.H. and U.D. synthesized the peptides. J.H.R. and H.G. performed the simulations. H.A. performed the TIRF and K.I.W. and S.W.H the STED microscopy. M.D. contributed to the protein purification and immunofluorescence. G.v.d.B. performed all other experiments. All authors contributed to the manuscript.

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most abundant phosphoinositide. Earlier studies identified PIP2 as a second messenger in the phospholipase-C pathway. However, the list of cellular functions of PIP2 is rapidly growing, and PIP2 is also involved in membrane targeting, cytoskeletal attachment, endocytosis and exocytosis[4]. PIP2 interacts with many different proteins, either via unstructured basic residue-rich regions or via more structured domains[4,5].

Neuronal exocytosis requires plasma membrane PIP2[2,6-8]. PIP2 levels at the plasma membrane determine the rates of vesicle priming, the size of the readily releasable pool, and the rates of sustained exocytosis in stimulated cells[2,6,8]. This regulation is probably mediated by interactions of PIP2 with proteins involved in docking and fusion such as rabphilin, CAPS, synaptotagmin, SCAMP2 and Mints[6,9]. In docking, PIP2 clusters may act as molecular 'beacons' that target synaptic vesicles to the fusion sites. Indeed, PIP2 is locally enriched at the sites of docked vesicles and colocalizes with at least 5–10% of the microdomains of syntaxin-1A (Supp. Fig. 1)[2,3,9], the membrane-anchored t-SNARE of neuronal exocytosis[1].

The amount of PIP2 at the sites of membrane fusion in PC12 cells has been estimated at 3–6% PIP2 of surface area (Supp. Fig. 2)[9]. In these experiments, membrane sheets were specifically stained for PIP2 with the PH domain of protein lipase C delta fused to GFP[9] or citrine (a YFP analog[10]; $PH_{PLC\delta}$-citrine, Supp. Fig. 1–2), and the fluorescence from the punctuated PIP2-microdomains was quantified. However, as explained in reference[9], this approach underestimates the fraction of PIP2 if the size of the $PH_{PLC\delta}$-microdomains is smaller than the ~200 nm diffraction limited resolution of conventional fluorescence microscopy. To obtain a more accurate estimate, we re-analyzed PC12 membrane sheets labeled with $PH_{PLC\delta}$-citrine or an antibody raised against PIP2 using super resolution STED (stimulation emission depletion) microscopy[11] (Fig. 1a–c). These experiments revealed that the PIP2 stained clusters are much smaller than anticipated, with an average diameter of only 73 ± 42 nm (s.d.). Although this is still a higher estimate since it represents the microdomain size convoluted with the resolution of the STED microscope (~60 nm), it is in good agreement with the size of the syntaxin-1A microdomains[12]. Using this value, we re-calculated the surface density of PIP2 (Supp. Methods). For this calculation, we first estimated the total amount of PIP2 in a microdomain when sampled with the diffraction limited resolution of our epi-fluorescence microscope (Supp. Fig. 2; Fig. 1d, black curve). We then calculated the peak concentration when this PIP2 was concentrated into 73 nm microdomains (Fig. 1d, red curve). Here, we assumed a Gaussian distribution of PIP2 in the microdomains. A peak surface density of 82% PIP2 was obtained (Fig. 1d). It needs to be kept in mind that (*i*) at these high PIP2 concentrations, molecular crowding might hinder binding of $PH_{PLC\delta}$-citrine, (*ii*) relatively small errors in microdomain size and resolution of the microscopes result in substantial errors, and (*iii*) $PH_{PLC\delta}$-citrine and antibody binding may alter PIP2 localization and is only indicative of PIP2 microdomains. Nevertheless, our values are much higher than any previous estimate and it seems safe to conclude that PIP2 is the dominant inner-leaflet lipid in the microdomains. The question then arises by which molecular mechanism such high concentrations of PIP2 are achieved.

PIP2 has a net negative charge of -3–5[4] and interacts with polybasic stretches of amino acids[4,5,13,14]. Proteins with such stretches can sequester PIP2 even in excess of monovalent

anionic lipids, such as MARCKS, spermine and even pentalysine (Lys_5)[5,14]. Similar to these proteins, syntaxin-1A also possesses a stretch of basic amino acids. These residues are adjacent to the transmembrane domain and are in contact with the head-groups of the phospholipids (Supp. Fig. 3a)[15,16]. Indeed, it is well established that this conserved stretch with 5 positive residues (260-KARRKK) interacts with PIP2[9,15-17]. Removal of charge diminishes this interaction (Supp. Fig. 3b–c), but syntaxin-1A remains capable of fusing membranes even upon removal of all 5 charges[9,16]. Because PIP2 colocalizes with at least a fraction of syntaxin-1A microdomains (Supp. Fig. 1)[2], we speculated that their interaction might drive domain formation similar to various soluble lipid binding proteins[5,14]. Two independent approaches were used to test this hypothesis: (*i*) reconstitution in giant unilamellar vesicles (GUVs)[18], and (*ii*) hydrolysis of PIP2 in PC12 cells using a membrane-targeted variant of the PIP2 phosphatase synaptojanin-1[7].

Murray and Tamm[17] showed syntaxin-1A clustered in a non-raft way in neutral cholesterol:phosphatidylcholine (PC) membranes. Here, cholesterol clusters syntaxin-1A by competing for solvation by PC. Indeed, a synthetic *C*-terminal peptide of syntaxin-1A (residues 257–288; 3 mol%; Fig. 2, Supp. Fig. 4a) clustered in domains in > 50% of GUVs composed of DOPC (1,2-dioleoyl-*sn*-glycero-3-PC) with 20 mol% cholesterol. This peptide contained both the polybasic juxtamembrane linker and transmembrane region and was *N*-terminally labeled with either rhodamine red or Atto647N. Analysis of fluorescence showed a 1.6 ± 0.2 (s.d.; $n = 18$) fold enrichment of syntaxin-$1A_{257–288}$ in these clusters, but this lower estimate is limited by the optics. Negatively charged PIP2 or DOPS (1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine) dispersed these clusters (Fig. 2)[17]. Thus, while cholesterol competition might explain syntaxin-1A clusters that are not enriched in PIP2 (Supp. Fig. 1), they cannot explain the high accumulation of PIP2 at the sites of docked vesicles. However, 1.5 mol% (total lipids) PIP2 also clustered syntaxin-1A in 1–10 μm domains in 1–5% of the GUVs (Fig. 2, Supp. Fig. 4b–c). These domains did not depend on cholesterol or DOPS. In these domains, PIP2 was 1.9 ± 0.2 (s.d.; $n = 13$; Supp. Fig. 5) and syntaxin-$1A_{257–288}$ 5.5 ± 1.4 (s.d.; $n = 27$) fold enriched based on fluorescence. Importantly, no domains were observed without peptide or when the PIP2 concentration exceeded 5 mol%. Divalent cations can act as bridges between two adjacent lipids and induce aggregation of PIP2 into clusters[19-21], but even 1 mM Ca^{2+} was not sufficient to compete syntaxin-1A away from the microdomains. Domains were present with both synthetic dioleoyl-PIP2 and with PIP2 extracted from pig brain (Supp. Fig. 4b). Thus, syntaxin-1A can be clustered in the membrane both by cholesterol and PIP2.

These cholesterol- and PIP2-mediated clusters both differ from ‘rafts’. They also differ from each other. First, PIP2-domains are always round and only 1–2 per vesicle, whereas cholesterol generally (but not always) induces many small domains (Supp. Fig. 4). Second, fluorescence recovery after photobleaching showed that syntaxin-1A remained mobile in the PIP2-domains while syntaxin-1A was essentially immobile in the cholesterol-dependent clusters (Supp. Fig. 6). Syntaxin-1A thus diffuses in the PIP2-domains and forms large circular domains for minimizing boundary energy[21]. Third, 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan)[22] showed a high hydration of the PIP2-domains, whereas the cholesterol domains were much denser packed (Supp. Fig. 7). Fourth, phase

contrast microscopy showed a thickening of the cholesterol-dependent clusters, but not of the PIP2-domains (Supp. Fig. 8). Thus, even though no saturated lipids are present, the cholesterol-dependent domains show behavior that essentially resembles the Lo phase. In contrast, the PIP2-domains seem much more disordered and resemble the Ld phase. Ca^{2+} demixing of polyanionic amphiphiles showed that electrostatic interactions can indeed lead to liquid-like domains[21].

The transmembrane helix of syntaxin-1A has been reported to homodimerize. However, introducing the M267A C271A I279A mutations that prevent homodimerization of the syntaxin-1A peptides[23] did not prevent cholesterol or PIP2 mediated clustering (Supp. Fig. 9). In contrast, no PIP2-domains were observed when two charges (K264A K265A) from the polybasic linker were removed, but cholesterol-dependent clusters were still observed (Supp. Fig. 5). Overexpression of the *C*-terminal part of syntaxin-1A fused to GFP[24] in PC12 cells also showed 4–8-fold loss of clustering of the K264A K265A mutant (Supp. Fig. 10–11). These data show that electrostatic interactions between PIP2 and the juxtamembrane helix of syntaxin-1A are *sufficient* for domain formation.

We then set out to investigate to what extent PIP2 is *required* for syntaxin-1A clustering in PC12 cells. For this purpose, we expressed a RFP-tagged construct containing the phosphatase domain of synaptojanin-1 fused to a CAAX-box, resulting in its targeting to the plasma membrane[7]. Synaptojanin-1 is a polyphosphoinositide 5-phosphatase, and the expression of the construct completely removed PIP2 from the plasma membrane (Supp. Fig. 12)[6,7]. Importantly, synaptojanin-1 expression 3.7-fold reduced the punctuate distribution of endogenous syntaxin-1A (Fig. 3; Supp. Fig. 13). Thus, this provides evidence that PIP2 is indeed required for at least part of syntaxin-1A microdomain formation.

We performed molecular dynamics simulations to gain insight in the precise conformation of the PIP2-syntaxin-1A microdomains. In these coarse-grained simulations, several atoms are represented by one simulation bead[25,26] (Supp. Fig. 14). This allowed for simulations of relatively large lipid bilayers of ~2,500 copies of a 4:1 molar ratio of DOPC:DOPS and 40–64 copies of syntaxin-$1A_{257–288}$ and PIP2. Within 10 μs simulation time, up to 10 copies of syntaxin-$1A_{257–288}$ clustered with PIP2 into microdomains (Supp. Fig. 15). Equal amounts of PIP2 and syntaxin-1A were present in the bulk-phase of those domains, while more PIP2 and DOPS associated transiently to the periphery. We used this information to construct a domain with 64 copies of syntaxin-1A (Fig. 4, Supp. Movie 1), which is comparable to the syntaxin-1A content in the microdomains in PC12 cells[12]. These domains were stable over 6 μs simulation time and contained <10% residual DOPC or DOPS. Together, we conclude that syntaxin-1A and PIP2 can form dynamic, amorphous networks with PIP2 acting as a ‘charge-bridge’ and spanning the distance between the various syntaxin-1A molecules (Fig. 4c).

In summary, our findings show that electrostatic interactions between the membrane lipid PIP2 and the SNARE syntaxin-1A suffice to induce membrane sequestering and microdomain formation without the need for high local PIP2 production or a (complex) ‘molecular fence’ restricting PIP2 and protein diffusion[27]. This does not exclude an additional role for protein-protein interactions between either transmembrane helices or

soluble domains. In fact, these seem essential for segregation of proteins in similar structure and size, such as syntaxin-1A and syntaxin-4 (both have polybasic regions and cluster separately)[12,24]. The mutual enrichment of syntaxin-1A and PIP2 at the fusion sites by electrostatic interactions has clear advantages. First, accumulation of syntaxin-1A may facilitate SNARE interactions and thereby increase the membrane fusion efficiency[3,28]. Second, the lipid environment modulates the energetic requirements for fusion[13,16]. Third, both PIP2 and syntaxin-1A function as molecular docking sites and facilitate assembly of the complete fusion machinery[1,2,6-9]. Our findings that electrostatic protein-lipid interactions are sufficient for membrane sequestering constitute a novel mechanism for the formation of protein microdomains in the membrane that is clearly distinct from the well-established lipid phases[18,29].

Methods Summary

$PH_{PLC\delta}$-citrine was expressed in *Escherichia coli* and purified with his-tag affinity purification. PC12 cells were maintained and propagated as described[3,24]. PC12 cells were transfected using Lipofectamine LTX (Invitrogen). Membrane sheets were prepared by rupturing the cells with probe sonication as described[24]. Immunostaining[24] and microscopy[11,24] were performed as described. The peptides were synthesized via microwave-assisted Fmoc solid phase synthesis. Peptides were mixed with lipids in organic solvent and GUVs were formed by the drying rehydration procedure. The molecular dynamics simulations were performed with the GROMACS simulation package and the MARTINI coarse-grained model[25,26]. See Supp. Methods for details.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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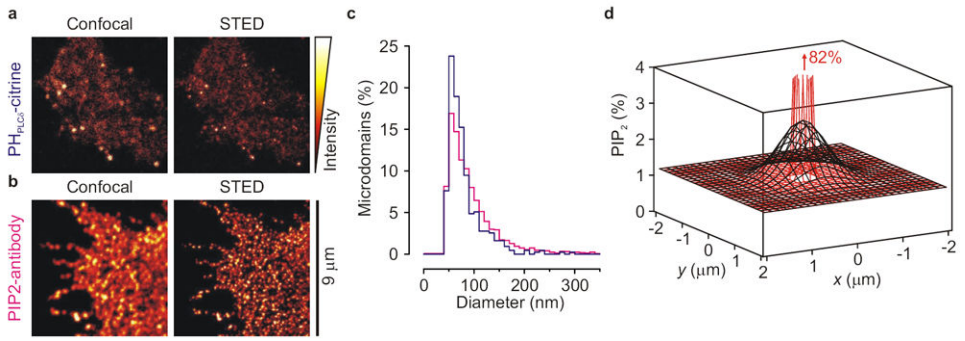

Figure 1.

PIP2 is the predominant inner-leaflet lipid in roughly 73 nm-sized microdomains. (**a**) Confocal and corresponding nanoscale-resolution STED image of a $PH_{PLC\delta}$-citrine stained membrane sheet of PC12 cells. Note the increase in resolution. (**b**) Same as **a**, but now immunostained with a monoclonal PIP2-antibody and a secondary antibody labeled with Alexa Fluor 488. (**c**) Size distribution of microdomains with $PH_{PLC\delta}$-citrine (blue; n = 433, 24 sheets, 2 independent preparations) and PIP2-antibody (pink; n = 2,959, 22 sheets, 2 independent preparations). The average diameter (full width at half maximum) was 73 ± 42 nm (s.d.) of $PH_{PLC\delta}$-citrine and 87 ± 62 nm (s.d.) for PIP2-antibody. (**d**) Spatial distribution of PIP2. Black: the PIP2 distribution when sampled at too low (377 nm) diffraction limited resolution (from Supp. Fig. 2). Red: approximation of the PIP2 distribution in the ~73 nm microdomains. PIP2 was accumulated at ~82% of total surface area. See Supp. Methods for details.

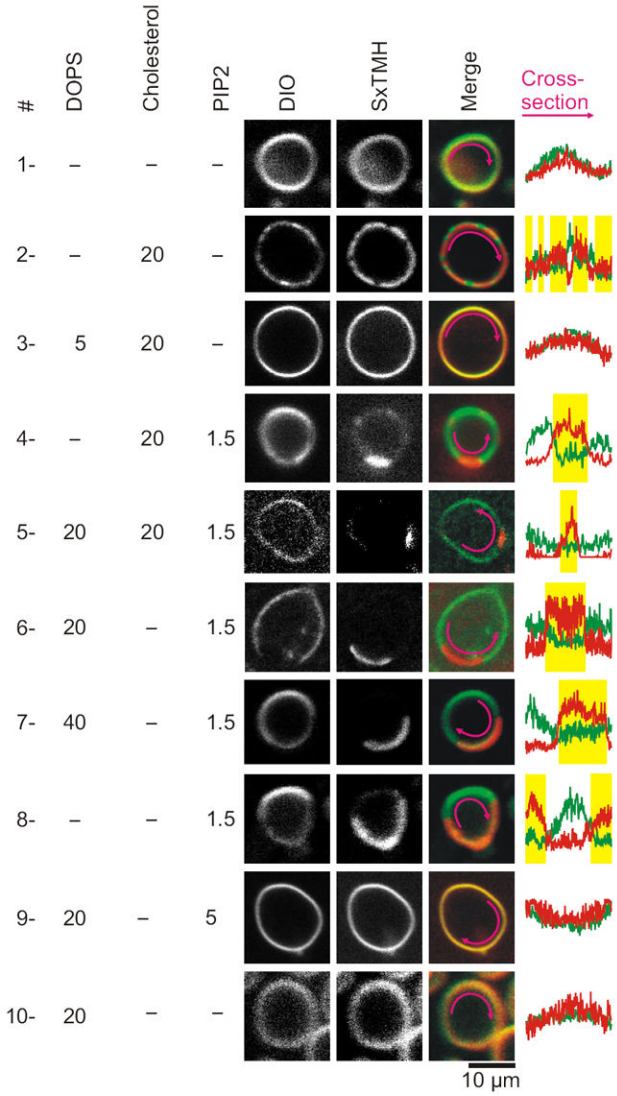

Figure 2.

Confocal microscopy of syntaxin-1A domains in artificial membranes. Syntaxin-1A$_{257–288}$ labeled with Atto647N (SxTMH; red) was reconstituted in GUVs. The membranes were composed of DOPC with 1.5 mol% of the fluorescent lipid analog DiO (3,3'-dioctadecyloxacarbocyanine; green) and the percentages DOPS, cholesterol and PIP2 indicated in the figure. In absence of anionic phospholipids, 20% cholesterol clustered syntaxin-1A in many small clusters (condition #2), as predicted by Murray and Tamm[17]. Inclusion of > 5% anionic DOPS dispersed these clusters (#3). 1.5% PIP2 partitioned SxTMH in 1–10 µm-sized domains regardless of cholesterol or DOPS (#4–8). These clusters were no longer observed with 5% PIP2 (#9). The pink arrows show the part of the membrane used for cross-sections. Yellow bars indicate the position of the domains. More data is presented in Supp. Fig. 4–9.

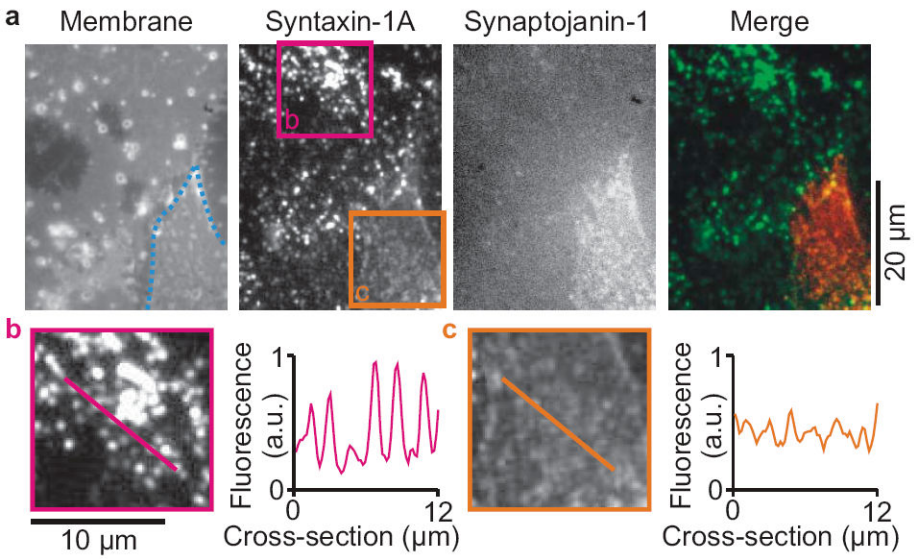

Figure 3.

Removal of PIP2 reduces syntaxin-1A clustering in PC12 cells (**a**) Membrane sheets of PC12 cells stained with TMA-DPH (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene)[12,24]. Immunostaining with a monoclonal antibody raised against syntaxin-1A and a secondary antibody labeled with DyLight649 showed that endogenous syntaxin-1A clustered in microdomains (region b; pink)[2,3,9,12,24]. Overexpressing the RFP-tagged and membrane-targeted catalytic region of synaptojanin-1 (residues 498–901; cell outlined in blue)[7] reduced this syntaxin-1A clustering 3.7-fold (region c; orange; see Supp. Fig. 13). Synaptojanin-1 is the 5-phosphatase of PIP2 and overexpression of the construct completely removes PIP2 from the membrane (Supp. Fig. 12). (**b–c**) Magnification of the regions of interest from **a** and cross-sections to indicate the clustering.

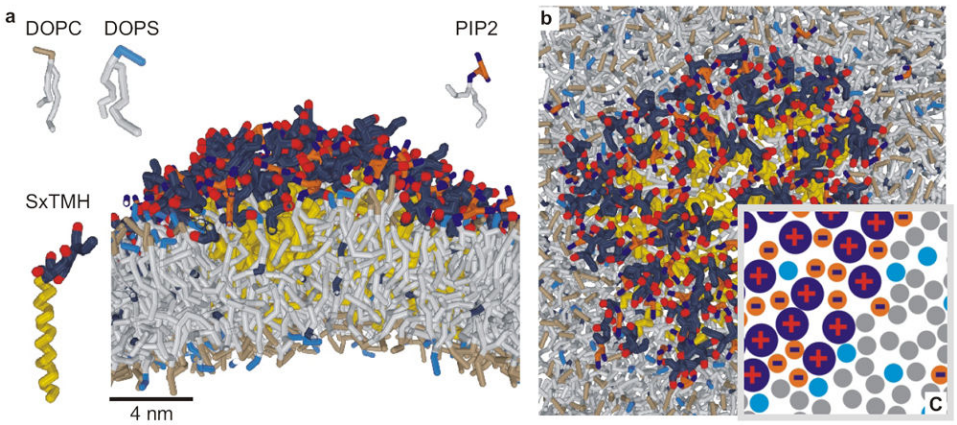

Figure 4.

Simulations of the dynamic and amorphous PIP2-syntaxin-1A microdomains (**a**) Side and (**b**) top-view of a coarse-grained molecular dynamics simulation. 64 copies of syntaxin-1A$_{257–288}$ (SxTMH) and 64 copies of PIP2 were incorporated in a bilayer composed of a 4:1 molar ratio DOPC:DOPS. PIP2 was only present in the membrane leaflet facing the *N*-terminus of syntaxin-1A$_{257–288}$. Simulations were performed with 150 mM NaCl. See Supp. Methods for details. White: alkyl chains of the lipids. Cyan: DOPS headgroup. Grey: DOPC headgroup. Yellow: transmembrane region of syntaxin-1A$_{257–288}$ (residues 266–288). Blue-red: polybasic linker region (residues 257–265, charges in red). Orange-blue: anionic PIP2 headgroup (charges in blue). The domains were stable over 6 μs simulation time; see supplementary movie 1. (**c**) Simplified scheme of the cluster.