Experimental report

09/09/2022

Proposal: 8-02-971 **Council:** 4/2021

Title: Role of molecular players in protein trafficking revealed by neutron scattering

Research area: Soft condensed matter

This proposal is a new proposal

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Samples: Lipid samples (DOPC, DOPE, PIP, PIP2)
Protein samples

Instrument	Requested days	Allocated days	From	To
D17	0	2	11/10/2021	13/10/2021
FIGARO	3	0		

Abstract:

Differently-phosphorylated phosphoinositides are distinctly distributed within the eukaryotic cell. For example, PI(4,5)P2 is predominantly enriched at the plasma membrane inner leaflet, while PI(4)P is located along Golgi-endosomal trafficking axis.

Acting as docking lipids, these phosphoinositides are directly involved in intra-cellular trafficking. PI(4,5)P2 binding Adaptor Proteins (such as epsin and AP2) control Clathrin-Mediated Endocytosis, which is the main mechanism by which eukaryotic cells internalize and recycle membrane proteins. Interacting with Golgi-enriched PI(4)P lipids, the oncogenic protein GOLPH-3 is essential for retrograde trafficking, including the correct localization of glycosyltransferases within the Golgi (where most glycosylation occurs in eukaryotic cells).

This proposal therefore focuses on the molecular interactions between phosphoinositide-enriched lipid bilayers and AP2, epsin and GOLPH-3. In conjunction with available crystal structures, the resultant NR data will clarify the lipid-bound proteins' orientation and position, and thus provide crucial insights into their biological function.

Experimental report of #8-02-971 on D17

Role of molecular players in protein trafficking revealed by neutron scattering

Scientific Background

Clathrin-mediated endocytosis (CME) is the main endocytic pathway used by cells to the internalize membrane proteins (termed cargoes). Molecular biology and proteomic approaches have been essential to understand the function and structure of the main proteins involved in such pathway, as CALM and AP2, which can bind the membrane and recognize the cargoes. However many questions remain unanswered concerning the interaction between proteins and lipids, which represents the very first steps of the CME. For example, although it is clear that the main interaction driving CALM and AP2 towards the inner leaflet of the plasma membrane is electrostatic, due to the presence of Phosphatidylinositol 4,5-bisphosphate (PIP_2), information is missing, regarding the orientation of these proteins with respect to the lipid interface, as well as their eventual insertion in the membrane.

The heterotetrameric Adaptor Protein complex AP2 is composed by four different subunits (α, β, μ, and σ) with different functions. It is characterized by a protein core made up of the trunk domains of the subunits[1], and flexible appendages. The former is provided with multiple PIP_2 binding sites[1]. Finally, the σ subunit contains the dileucine binding box[2] that can bind to the so called CD4 sorting signal peptide. The structure of the AP2 core was determined in complex with inositol(1,2,3,4,5,6) hexakisophosphate[1] that mimics PIP_2 headgroup, showing the molecular detail of the binding, and the influence of AP2 on PIP_2-containing membranes was investigated with EM tomography and fluorescence microscopy[3]. Recently, the interaction of AP2 with lipid monolayers was studied by NR and AFM[4], unravelling important pieces of information. However a detailed investigation of the binding of AP2 with PIP_2- and cargo-containing membranes in the form of lipid bilayers is still missing.

CALM (clathrin assembly lymphoid myeloid leukemia protein) is one of the most abundant clathrin adaptors[5]. It possesses a relatively small, compact, stacked-helical domain (ANTH domain), with large, natively unstructured, C-terminal tails[6]. The single PIP_2 binding site is located in the ANTH domain, Lysines 28, 38 and 40[6]. Moreover, CALM is characterized by a membrane-inserting amphipathic helix at the N-terminus (thus called amphipathic helix 0, AH0), whose 3D orientation is thought to change upon membrane binding. AH0 is able to drive pearlized liposomes tubulation[7]. Given this, a model for CALM binding and function was proposed, in which the protein is seen as curvature sensitive[8] but also actively driving further membrane curvature. However, the insertion of the helix was not experimentally proved, but only hypothesized on the basis of the obtained results, and the orientation of the ANTH domain upon membrane binding is unknown.

Results

The experiments performed during this beamtime with lipid bilayer were used to complete previous studies on both CALM and AP2 interacting with lipid monolayers. The results obtained were included in Andreas Santamaria's thesis, and are being readied for publication.

AP2

A lipid bilayer containing hydrogenous DOPC and DOPE, together with 10% in mol of PIP_2 and also 3.5% in mol of CD4 peptide was exploited, which mimics the intracellular domains of transmembrane cargo proteins recognized by AP2. The CD4 peptide covalently bound to a phosphatidylethanolamine lipid was kindly provided by Dr. Owen (Cambridge University). To the best of our knowledge, this is the first time that a bilayer containing a peptide moiety (to mimic a protein soluble domain) has been deposited onto a

silicon crystal surface for NR experiments. This novel *in vitro* lipid system has been characterized with NR, thus assessing the presence of the peptide in the headgroup layer. To characterize the out-of-plane structure of the bilayer, four different isotopic contrasts were employed: 100% D_2O *v/v*, 38% D_2O *v/v* (SiMW), 8.1% D_2O *v/v* (ACMW), and 100% H_2O *v/v*. This approach allowed us to determine the molar fraction of CD4 in each headgroup layer. Indeed, we experienced an asymmetry in the bilayer, leading to a 5% in mol of the peptide in the inner headgroup layer, closer to the support, and 2% in the outer layer, which might be due to CD4 overall positive charge, promoting interaction with the negatively charge support is favourable. **Figure 1 C** also shows the contribution of each moiety of lipid molecules to the bilayer, in term of volume fractions. The contribution of CD4 is also reported, shown as plain light magenta area. After the characterization of the lipid bilayer composition as well as out-of-plane structure, AP2 has been injected and its interaction with the model membrane investigated. The same isotopic contrasts were employed to shed light on the interaction of AP2, demonstrating an insertion of the protein in the outer headgroups layer. This is compatible with the interaction of AP2 with both the CD4 peptide and the phosphate groups of PIP_2 inositol ring, considered embedded in such layer. Besides, an extra layer has been taken into account to model the remaining protein structure present outside the bilayer. The total thickness of AP2 obtained here (69±2 Å) is compatible with the crystal structure of the protein[1], as well as with previous NR experiments that showed the orientation of AP2 with lipid monolayer containing PIP_2 and CD4, which reported a total thickness of ~80 Å[4].

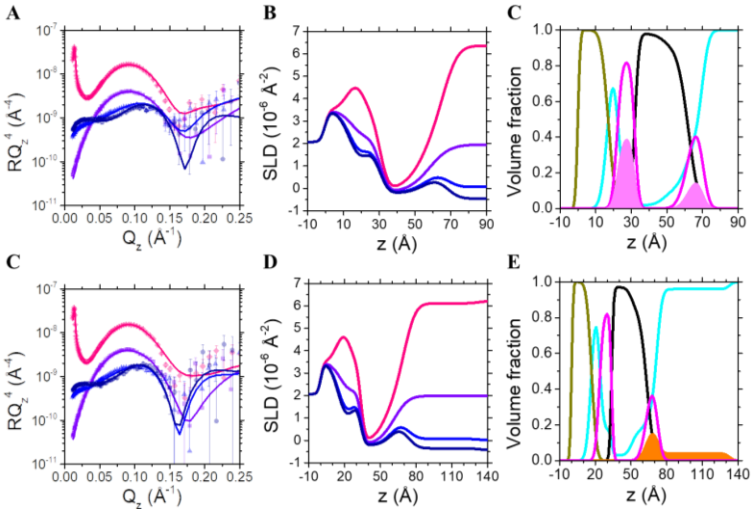

Figure 1 Experimental (symbols) and simulated (lines) neutron reflectivity profiles of lipid bilayer **A** before and **C** after AP2 addition. Data at four isotopic contrasts have been measured: 100% D_2O (pink diamonds), SiMW (violet squares), ACMW (blue triangles) and 100% H_2O (dark blue circles). Figures are displayed on an RQ_z^4 scale to show the quality of the fits at high Q_z values. SLD profiles corresponding to fits are plotted in **B** and **D**. Pink, violet, blue and dark blue continuous lines indicate the SLD profiles in 100% D_2O, SiMW, ACMW and 100% H_2O isotopic contrast, respectively. **C**, **E**, Volume fraction profiles derived from the fit highlighting the distribution of tails (black), headgroups with CD4 (magenta), water (cyan). Panel **C** also shows the contribution of CD4 (light magenta area), while panel **E** shows the contribution of AP2 (orange area).

CALM

A lipid mixture made of hydrogenous DOPC, DOPE and 10% in mol of PIP_2 was employed. To characterize the out-of-plane structure of the bilayer, three to four different isotopic contrasts were employed: 100% D_2O *v/v*, 38% D_2O *v/v* (SiMW), 8.1% D_2O *v/v* (ACMW), and 100% H_2O *v/v*. After bilayer characterization, the protein was injected. We used both hydrogenous CALM and fully deuterated CALM, provided by the D-Lab. The same multilayer-slab model was employed to fit the reflectivity data, showing the insertion of the protein in the headgroup layer. Such results were in agreement with previous results obtained with lipid monolayer. Indeed the same total thickness for CALM has been obtained.

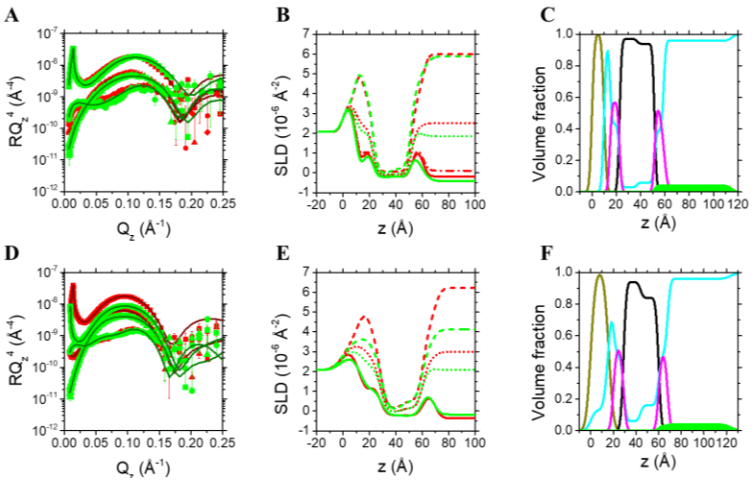

Figure 2 Experimental (symbols) and simulated (lines) neutron reflectivity profiles of solid-supported bilayers in the absence (red) and presence (green) of **A** hydrogenous CALM and **D** deuterated CALM. Data at three isotopic contrasts have been measured for hydrogenous lipid bilayers: D2O (red squares), H2O (red circles) and SiMW (red triangles). An additional contrast was measured for the bilayer prior to the injection of hydrogenous CALM (ACMW), shown as red diamonds. Data at three isotopic contrasts have been measured for bilayers+CALM: D2O for hydrogenous CALM and 62% D2O for deuterated CALM (green squares), H2O (green circles) and SiMW (red triangles). Figures are displayed on an RQ_z^4 scale to show the quality of the fits at high Q_z values. SLD profiles corresponding to fits are plotted in **B** and **E**. Red continuous, short dotted, short dashed lines indicate the bilayer SLD profile in H2O, SiMW and D2O isotopic contrast, respectively, and the dotted-dashed line indicated the bilayer SLD in ACMW. Green continuous, short dotted, short dashed lines indicate the bilayer+CALM SLD profile in H2O, SiMW and D2O (62% D2O for deuterated CALM) isotopic contrast, respectively. Volume fraction profiles derived from the fits highlight the distribution of silicon oxide (dark yellow), tails (black), heads (magenta), water (cyan) and **C** hydrogenous CALM (green), **F** deuterated CALM (green).

References

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