Experimental report

15/09/2016

Proposal: 8-02-780 **Council:** 4/2016

Title: NR studies of clathrin-mediated endocytosis

Research area: Biology

This proposal is a new proposal

Main proposer: Nathan ZACCAI

Experimental team: Armando MAESTRO
Nathan ZACCAI
Ryan BRADY

Local contacts: Philipp GUTFREUND

Samples: Lipids
protein

Instrument	Requested days	Allocated days	From	To
FIGARO	2	2	03/09/2016	05/09/2016

Abstract:

Clathrin-mediated endocytosis is crucial for the internalization of most eukaryotic cell-surface proteins. Clathrin-coated vesicles (CCVs) assemble with their cargo at the plasma membrane then transport these to the early endosome inside the cell. A CCV consists of a clathrin scaffold coating a lipid vesicle, in which the cargo is bound, linked by adaptor proteins, like AP2, that are associated with effectors of CCV assembly, stability and disassembly.
In vivo, AP2 solely interacts with one leaflet of the cellular membrane. Therefore, we have been able to analyze the first stages of CCV assembly by using cargo embedded in a lipid monolayer and Langmuir trough methods. Importantly, addition of AP2, then of clathrin, results in distinct physical changes . Available structural information about CCV formation has however been indirect. EM has only been on mature CCVs, either lacking lipids and AP2, or with a mixture of proteins. Crystal structures of AP2 (without lipids) have also suggested that large structural changes occur on binding the cellular membrane. To clarify between these different scenarios, neutron reflectomery analysis of CCV formation will be very informative.

Neutron Reflectometry studies of clathrin-mediated endocytosis

Armando Maestro, David J Owen, Pietro Cicuta and Nathan Zaccai

***Abstract*—Clathrin-mediated endocytosis is crucial for the internalization of most eukaryotic cell-surface proteins. Clathrin-coated vesicles (CCVs) assemble with their cargo at the plasma membrane then transport these to the early endosome inside the cell. A CCV consists of a clathrin scaffold coating a lipid vesicle, in which the cargo is bound, linked by adaptor proteins, like AP2, that are associated with effectors of CCV assembly, stability and disassembly. In vivo, AP2 solely interacts with one leaflet of the cellular membrane. Therefore, we have been able to analyze the first stages of CCV assembly by using cargo embedded in a lipid monolayer and Langmuir trough methods. Importantly, addition of AP2, then of clathrin, results in distinct physical changes. Available structural information about CCV formation has however been indirect. EM has only been on mature CCVs, either lacking lipids and AP2, or with a mixture of proteins. Crystal structures of AP2 (without lipids) have also suggested that large structural changes occur on binding the cellular membrane. To clarify between these different scenarios, neutron reflectometry analysis of CCV formation has been very informative.**

I. Introduction

Clathrin-mediated endocytosis CME is the main mechanism by which eukaryotic cells internalize and recycle most membrane proteins: Clathrin adaptor proteins bound to the plasma membrane, recruit and polymerize clathrin to form clathrin-coated pits into which transmembrane protein cargoes are packaged to build clathrin-coated vesicles CCVs inside the cytoplasm. These vesicles are indeed responsible for the intracellular trafficking of proteins, protein ligands and lipids between internal membrane-bound organelles and the plasma membrane. A CCV consists of a clathrin scaffold coating a lipid vesicle, in which the cargo is bound, linked by adaptor proteins, like AP2, that are associated with effectors of CCV assembly, stability and disassembly.

We recently determined that a single adaptor protein AP2 is sufficient to initiate and drive clathrin-coated bud formation on appropriate membranes, enriched in PtdIns(4,5)P2 *(Kelly et al., Science, 2014)*. The resultant buds were spherical and uniform in size ($\sim$20 nm radius). This technical advance has now put us in a position where we can generate clathrin-coated vesicles with known protein and lipid composition. The opportunity to study simpler and more homogeneous CCVs has allowed us to revisit earlier solution scattering work, and subsequently extend it to the study of factors that influence CCV formation, size and disassembly. SAXS and SANS analysis of CCVs are however proving very challenging due to the resultant particles large size.

In vivo, AP2 solely interacts with one leaflet of the cellular membrane. Therefore, an alternative in vitro approach is the direct measurement of clathrin assembly on a flat lipid monolayer. We have been able to analyze the first stages of CCV assembly by using cargo embedded in a lipid monolayer. Importantly, addition of AP2, then of clathrin, results in distinct changes in lateral lipid pressure and compressibility. Besides, crystal structures of AP2 in complex with different cargos (but without lipids) have also suggested that large structural changes occur on binding the cellular membrane (see figure). For these reasons, neutron reflectomery analysis of CCV formation will be very informative offering structural information of AP2 binding to the lipid layer and clathrin polymerization.

Local contact: Philipp Gutfreund and Richard Campbell

II. Experimental details

Specular neutron reflectivity (SNR) experiments were performed to study the interaction of AP2 with monolayers prepared from a mixtures of unsaturated lipids (DOPE, DOPS and DOPC), cholesterol and phosphatidyl inositol (PtdIns4,5P2)- to mimic mammalian cell membranes. In addition TGN peptide based lipids were also included in the sample mimicking transmembrane cargo proteins. The SNR measurements were made on the time of flight (TOF) reflectometer, FIGARO. In the experiments, neutrons of wavelengths ranging between 2 $\mathring{A}$ and 30 $\mathring{A}$ and at two incident angles, namely 0.62^o and 3.78^o, were used giving a Q-range from 0.002 $\mathring{A}^{-1}$ to $\sim$0.32 $\mathring{A}^{-1}$. Calibration of FIGARO was performed using a D2O sub-phase. Backgrounds were subtracted from the data by the simultaneous acquisition of off-specular data for each measurement on the area detector. No off-specular scattering was observed for the monolayers under the conditions of the experiment. All SNR experiments were performed at a temperature of 22 ^{o}C.

Lipid monolayers were prepared on a Langmuir trough with a total area of 97 cm^2 (Nima Technologies, Coventry, UK) by spreading from a chloroform solution (using a 50 μL Hamilton microsyringe) onto a pH 7.2 Hepes-Tris buffer solution. Two surface pressures Π (namely 18 mN/m and 30 mN/m) were studied. Care was taking to ensure Π was constant after lipids were spreading to ensure the formation of a stable monolayer. Then, SNR profiles of the lipid monolayers were recorder as shown Fig. 1. For each experimental condition explored, once the lipid monolayer was stable, AP2 in buffer solution was injected (100 - 200 μL) into the aqueous sub-phase beneath the lipid monolayer. SNR profiles were measured each five minutes to measure the kinetics of AP2 binding to the lipid monolayer (results not shown). The kinetics of AP2 binding was also recorded by monitoring Π versus time. When the interaction of AP2 with the lipid monolayer was

almost complete (i.e. no further change in surface pressure was recorded after at least one hour), the SNR of the monolayer was measured as shown Fig. 1. SNR data were obtained using two different H/D contrasts: namely lipid monolayer on D2O and lipid monolayer on air contrast matched water (ACMW; a mixture of composition 92% H2O and 8% D2O by volume).

III. Results

The main goal of this experiment was to use SNR to probe the interaction of AP2 with the lipid monolayer by obtaining the interfacial structure of the lipid monolayer bound to several AP2 molecules. In detail, *FIGARO* was used to identify whether the adsorbed amount of AP2 molecules pack tightly next to the membrane or even possibly embed themselves into the lipid outer leaflet. In Figure 1, we compiled the most representative SNR profiles obtained in this study. At a first glance (without analysing the results) it can be stated that at high surface pressures ($\Pi = 30mN/m$) there is a remarkable difference in the SNR profiles of both the lipid monolayer and when AP2 is bind to it in both D2O and ACMW experimental conditions (Fig. 1b,d). Nevertheless, at a low surface pressure ($\Pi = 18mN/m$), there is apparently no much difference in the SNR profiles measured in D2O for both the lipid monolayer and the lipid - AP2 monolayer (Fig. 1A). The SNR profiles of both in ACMW (Fig. 1C) are different. At this point we can only speculate about the AP2 positioning with respect to the monolayer that apparently depends on the surface pressure. In summary, we collected several complete NR datasets of AP2 interacting with cargo-containing lipid monolayer at different lateral pressures. They are currently being analyzed to gain further insight in the structure, although it is important to note that structural changes were directly observed in real time.

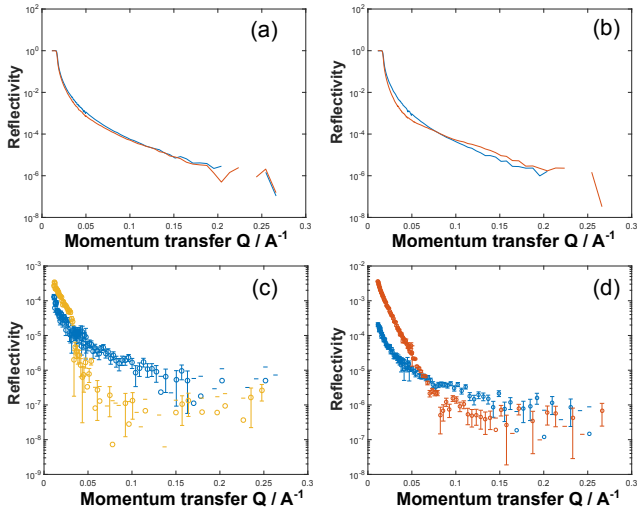

Fig. 1. Specular Neutron Reflectometry profiles of the lipid monolayer in D2O (a, b) and ACMW (c, d). Left panel: Results at $\Pi = 18mN/m$. Right panel: $30mN/m$. Top/bottom: blue lines always correspond to the lipid monolayer.

IV. Perspectives

We are now analysing the SNR profiles obtained to gain insight in the position of AP2 with respect to the lipids. This will position the lipids with respect to these proteins, providing new structural insights. In a future experiment, we want to address the binding of AP2 and clathrin interacting with a cargo-presenting lipid monolayer. An open question we want to address now is what influence does the clathrin scaffold have on the lipids?