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Visualizing membrane trafficking through the electron microscope: cryo-tomography of coat complexes

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Coat proteins mediate vesicular transport between intracellular compartments, which is essential for the distribution of molecules within the eukaryotic cell. The global arrangement of coat proteins on the membrane is key to their function, and cryo-electron tomography and subtomogram averaging have been used to study membrane-bound coat proteins, providing crucial structural insight. This review outlines a workflow for the structural elucidation of coat proteins, incorporating recent developments in the collection and processing of cryo-electron tomography data. Recent work on coat protein I, coat protein II and retromer performed on in vitro reconstitutions or in situ is summarized. These studies have answered long-standing questions regarding the mechanisms of membrane binding, polymerization and assembly regulation of coat proteins.

1. Introduction

Recent advances in cryo-electron microscopy (cryo-EM) have enabled numerous high-resolution studies which have addressed long-standing structural biology questions. The large body of cryo-EM work carried out for protein structure characterization has utilized single-particle electron microscopy (Cheng, 2018). Recently, developments in hardware, data collection and data processing have placed cryo-electron tomography (cryo-ET) and subtomogram averaging (STA) at the forefront of structural studies of repetitive assemblies, reaching resolutions comparable to those of single-particle EM (Schur et al., 2016; Wan et al., 2017; Hutchings et al., 2018; Dodonova et al., 2017; Himes & Zhang, 2018). This review discusses the application of cryo-ET to the study of intracellular coat proteins.

Eukaryotic cells are separated into membrane-bound compartments that constantly exchange molecules while retaining their own biochemical identity. This tightly controlled intercompartmental exchange is enabled by vesicular transport, in which coat proteins promote the formation of cargo-carrying vesicles from a donor compartment, and the budded vesicles travel towards a target compartment and fuse with it, delivering their cargo (Caro & Palade, 1964). Cargo molecules are selectively incorporated into vesicles by core coat proteins or additional interacting factors (Bonifacino & Glick, 2004). Different coat protein families are responsible for the transport between specific intracellular compartments, mediating local vesicular trafficking (Barlowe et al., 1994).

Four coat protein families have been described: coat protein II (COPII), coat protein I (COPI), clathrin and retromer (Fig. 1). The cytoplasmic coat protein complexes I and II (COPI and COPII) mediate retrograde and anterograde trafficking,
respectively, between the endoplasmic reticulum (ER) and the Golgi apparatus (Orci et al., 1986; Barlowe et al., 1994). COPI is also responsible for trafficking between Golgi cisternae (Ishii et al., 2016). Clathrin-coated vesicles mediate the transport of endocytic cargo from the plasma membrane, as well as trafficking from the trans-Golgi network to endosomal compartments (Roth & Porter, 1964; Robinson, 1994). Retromer mediates trafficking from the endosome to the trans-Golgi network and the plasma membrane (Seaman et al., 1997; Temkin et al., 2011). The common steps in vesicle formation shared by the known coat protein families are represented schematically in Fig. 2 (Bonifacino & Glick, 2004).

Questions that remain unanswered about coat proteins address the mechanism of their association with the membrane and their global assembly. Cryo-ET is well suited to address these questions as it can allow the high-resolution analysis of coat proteins in the process of modifying membranes.

2. A cryo-electron tomography workflow
We describe our workflow for the investigation of coat protein membrane assemblies by cryo-ET in Fig. 3.

2.1. Sample preparation
Structures of isolated coat components and subcomplexes have been elucidated by X-ray crystallography and single-particle electron microscopy (Fotin et al., 2004; Stagg et al., 2006, 2008; Lee & Goldberg, 2010; Bi et al., 2002, 2007; Mancias & Goldberg, 2008; Fath et al., 2007; Wilbur et al., 2010; Heldwein et al., 2004; Collins et al., 2002). However, isolated structures lack insight into the global interactions and arrangement defining the functional coats as assembled on membranes. This gap can be addressed by in vitro reconstitution studies with purified components assembled on artificial membranes (Dodonova et al., 2017; Hutchings et al., 2018; Kovtun et al., 2018; Faini et al., 2012; Bacia et al., 2011). Alternatively, coated vesicles can be purified from cells (Cheng et al., 2007; Malhotra et al., 1989; Heymann et al., 2013) or observed directly within cells (Bykov et al., 2017; Kovtun et al., 2018) for structural analysis. Cryo-ET and STA are then used to analyse the architecture of assembled coats, providing insights into the coat structure and its pleiomorphic assemblies (Zanetti et al., 2013; Hutchings et al., 2018; Kovtun et al., 2018; Dodonova et al., 2017, 2015; Faini et al., 2012).

As an example, we briefly describe the basic steps for COPII budding reconstitution in vitro (Fig. 4). The sequential assembly of the COPII coat components is well understood in yeast and is conserved in mammals. In the vesicle formation process, COPII proteins detect and induce membrane curvature, recognize and concentrate cargo, and bud the membrane into cargo carriers. COPII assembly is initiated by the recruitment of the Sar1 GTPase to ER exit sites (ERES). Sar1 associates with the membrane upon GTP binding, facilitated by its cognate guanine nucleotide-exchange factor (GEF), Sec12. GTP binding triggers a conformational change in Sar1, which inserts an amphipathic N-terminal helix into the
membrane. Sar1 then recruits Sec23/24, cytoplasmic heterodimers that form the COPII inner coat, which in turn recruit Sec13/31 heterodimers that assemble into the outer coat. This detaches the COPII vesicle, completing the COPII coat assembly, which is short-lived owing to an intrinsic propensity for disassembly. GTP hydrolysis by Sar1 is stimulated by the specific GAP activity of Sec23/24, and in turn accelerated by Sec13/31, resulting in dissociation of the COPII coat (Zanetti et al., 2012; Bi et al., 2007; Antonny et al., 2001).

COPII budding has been reconstituted in vitro by incubating purified yeast COPII proteins with giant unilamellar vesicles (GUVs; Bacia et al., 2011). The GUV composition and protein concentrations have been optimized to mimic physiological conditions and maximize budding efficiency (Matsuoka et al., 1998; Bacia et al., 2011). To stabilize the intrinsically unstable COPII complexes on the membrane, GMP-PNP, a nonhydrolysable GTP analogue, must be used (Bi et al., 2007; Bacia et al., 2011). Gold fiducial markers are added to reconstituted reactions prior to plunge-freezing to facilitate cryo-tomography tilt series alignment. Reconstituted COPII ‘budding’ reactions produce a variety of COPII morphologies, including budded and straight membrane tubules coated with COPII (Fig. 4).

2.2. Data collection

For subnanometre-resolution analysis of coats by STA, imaging should be performed on a high-end cryo-microscope with a stable stage, equipped with a direct detector and an energy filter (Dodonova et al., 2017; Hutchings et al., 2018; Schur et al., 2016). Tilt series are typically collected at defoci ranging from −1.5 to −3.5 μm. The transmission of high-frequency signal degrades with dose accumulation, limiting the total electron dose used as distributed across the tilt series. Signal is best transmitted at lower tilts, which have a lower apparent thickness and thereby suffer fewer inelastic scattering events. An efficient, dose-symmetric tilt scheme which maximizes the amount of electron dose spent on lower tilt images has been established: the first image is collected at zero tilt, and then the sample is tilted, alternating both directions at incremental angles (Hagen et al., 2017). A tilt angle range of ±60° with 3° increments and exposures of up to 150 e− Å⁻² is typically used (Hagen et al., 2017; Wan et al., 2017; Schur et al., 2016; Bharat et al., 2017). Multiple frames can be collected per tilt and aligned using the MotionCor2 software (Zheng et al., 2017).

To achieve subnanometre resolutions, a high number of subtomograms must be available, which necessitates high-throughput automated data collection. For example, to analyse the COPII budding reconstitution described above, SerialEM software was used to automatically collect 90 tomograms (Mastronarde, 2005).

2.3. Pre-processing and reconstruction

Accurate tilt series alignment is central to retaining high-resolution information in the reconstructed tomogram. The low electron doses per tilt result in an inherently low

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**Figure 3**
A cryo-electron tomography workflow used for the structural determination of COPII assembled on membranes.
signal-to-noise ratio per tilt (SNR), which can affect tilt series alignment by cross-correlation. High-contrast gold fiducial markers are typically added to in vitro reconstitution reactions and are used to manually minimize the alignment residual error between tilts, which can be performed using the Etomo IMOD interface.

The incremental electron damage suffered by the sample results in the progressive loss of higher frequency information. The contribution of this low-SNR information can be reduced by dose compensation, which attunes progressively lower frequency components with the accumulation of electron dose (Grant & Grigorieff, 2015). Current tomography software that implements dose compensation includes emClarity and IMOD (Himes & Zhang, 2018; Mastronarde & Held, 2017).

Contrast transfer function (CTF) correction is essential for the recovery of higher resolution information. Appropriate CTF correction relies on accurate defocus determination. Modern CTF detection software such as CTFIND4 (Rohou & Grigorieff, 2015) and Gctf (Zhang, 2016) is usually able to detect CTF oscillations seen in Thon rings for all direct detector images across a tilt series. However, the accuracy can significantly decrease for the higher tilts, where less signal is available to guide CTF estimation, owing to increased thickness and the defocus gradient. The consequences of this are partially attenuated in the process of dose compensation.

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of STA is maximized if CTF correction is performed using defocus values adjusted across the thickness of the reconstruction (Jensen & Kornberg, 2000). This has recently been implemented in the NovaCTF 3D CTF correction software, in which CTF correction is performed during the tomogram reconstruction process (Turoňová et al., 2017; Himes & Zhang, 2018; Kunz & Frangakis, 2017).

To reconstruct the original 3D density from its 2D projections (i.e. the tilt series), the finely aligned and pre-processed tilt series are back-projected from their assigned orientation angles. To gain contrast for better visualization in the initial phases of processing, filters can be applied during the reconstruction process. For initial tomogram visualization, weighted back-projection (WBP) with iterations of simultaneous iterative reconstruction technique (SIRT)-like filtering can be performed using the Etomo IMOD interface (Mastronarde & Held, 2017).

2.4. Subtomogram extraction and averaging

Subtomogram particle picking of repetitive coat subunits assembled on membranes is facilitated by a priori information about the expected localization and orientation of coat subunits and their distribution. Typically, subtomograms are extracted using centre coordinates generated on the surface of the coated membranes. These can be defined upon manual membrane segmentation (Castaño-Díez, 2017) or generated automatically using tubular or spherical geometrical parameters (Zanetti et al., 2013; Faini et al., 2012). For repetitive assemblies, coat lattices are typically oversampled, and the convergent alignment of subtomograms containing redundant information is used to define the centre of unique particles (Zanetti et al., 2013). Initial rough assignment of two Euler angles is possible for coats, as subunits are all expected to be oriented normally to the membrane. In the case of tubules, in-plane rotations can also be assigned relative to the direction of the tube axis. This both helps to obtain an initial reference for alignments, as discussed below, and limits the angular search to small ranges, significantly decreasing the computational time. Initial alignments are often performed using binned 8× data, and low-resolution signal can be further enhanced by applying SIRT-like filters. Subtomogram averaging can be performed using a number of software packages, including Dynamo, emClarity, TOMAV3, PEET, Subtomo, MLTOMO, RELION, PyTom and EMAN2 (Castaño-Díez, 2017; Himes &
Initial references are typically generated by averaging extracted subtomograms and smoothening (i.e. low-pass filtering) the result to obtain a featureless membrane density. This is used for initial alignment of the subtomograms, which helps to define particle centres and can often constitute a first cleaning step. The subtomogram dataset is typically split in half and processed separately during further alignment, following a ‘gold-standard’ approach (Scheres & Chen, 2012).

Starting with subtomograms that are binned 8/C2 and heavily low-pass filtered, binning factors and low-pass filtering can be gradually decreased while restricting the angular search in order to reveal higher resolution information without falling into local minima (Hutchings et al., 2018; Zanetti et al., 2013; Faini et al., 2012; Dodonova et al., 2015, 2017). It is important that conservative low-pass filters are applied at each iteration in order to avoid bias from the high-resolution noise contained in the reference. Resolution can be estimated using Fourier shell correlation (FSC) between the two half datasets (Harauz & van Heel, 1986). The two half datasets are combined for the final reconstruction and low-pass filtered to the resolution indicated by the FSC at the 0.143 threshold (Rosenthal & Henderson, 2003).

3. Studying coat protein assemblies by tomography

Coat complexes assembled on their cognate membranes form complex and unique 3D objects. Since their global assembly underlies their function, it is informative to study their structure within the context of the coat in the act of remodelling membranes. Cryo-ET is suited for investigating interactions between coat protein subunits in their assembled state and between coat proteins and the membrane. Within the 3D volume of the tomogram, coat proteins at different heights along the microscope axis, which overlap in 2D projection images, are resolved, allowing their isolation and analysis in silico. Averaging multiple copies of identical building blocks within an object of interest through STA leads to higher resolution views of coat subunits. Available structural information from X-ray crystallography and single-particle studies can be fitted into densities determined by STA. Together with the ability to map the relative positions and orientations of the aligned coat components within each tomogram, this provides information about the structure of subunits, their assembled architecture, and in some cases their physiological context, providing a pseudo-atomic model of unique pleomorphic coat assemblies where the quality of the fits permits.

High-resolution structures of coat proteins have elucidated essential protein–protein and protein–membrane interactions that underpin coat assembly on membranes (Hutchings et al., 2018; Kovtun et al., 2018; Dodonova et al., 2017). These recent studies shed light on the structural basis of the flexibility that allows the formation of carriers of different shapes and sizes to accommodate the needs of the cell. We outline the insights gained in recent studies of COP II, COPI and retromer (Fig. 5).

3.1. The structure of COP II assembled in vitro

Our recent cryo-ET and STA studies of in vitro reconstituted COP II budding yielded a structure of the COP II inner coat, composed of Sar1 and Sec23/24, at an average resolution...
of 4.9 Å, providing important new insights into the mechanisms of coat assembly and membrane remodelling (Fig. 5a). Firstly, it revealed the association of the coat and the Sar1 GTPase with the membrane. We observed for the first time how the N-terminal amphipathic helix of Sar1 is associated with the membrane: a helical hook is bent by 90° and inserts horizontally into the outer leaflet, running roughly parallel to the tube axis (Fig. 6). Also, the membrane-proximal surface of the Sec23/24 dimer is tightly associated with the bilayer, in particular a negatively charged patch on the surface of Sec24, which corresponds to its zinc-finger domain. Together, amphipathic helix insertion and the scaffolding action of the concave inner surface of Sec23/24 provide the force necessary to remodel membranes into tubules (Hutchings et al., 2018).

Moreover, our structure unveiled how the inner and outer coat are associated: through the binding of motifs embedded within the outer coat disordered C-terminal tails to two distinct sites on Sec23. Together with previous biochemical and X-ray crystallographic studies (Antonny et al., 2001; Bi et al., 2007; Ma & Goldberg, 2016), our work revealed how the outer coat affects the stability of COPII assemblies and the formation of the inner coat lattice and suggested how this process might be regulated during COPII-mediated budding.

3.2. The structures of COPI and retromer in vitro and in situ

Two other coat protein assemblies, COPI and retromer, were analysed by cryo-ET and STA of in vitro reconstituted assemblies and within the cell. Three consecutive studies have investigated the structure of COPII assembled in vitro from purified coatamer components on GUVs (Faini et al., 2012; Dodonova et al., 2015, 2017). The most recent study determined the structure of the COPII leaf, composed of a coatamer complex bound to two Arf1 GTPases, to 9.2 Å resolution (Fig. 5b; Dodonova et al., 2017). This showed an asymmetric subunit of a coatamer component bound to two membrane-interacting Arf1 molecules and elucidated interactions with ArfGAP2, which is essential for GTP hydrolysis by the membrane-bound Arf1 GTPase. Recent developments in cellular preparation with focused ion beam (FIB) milling allowed the study of protein assemblies in their native intracellular context by cryo-ET. This enabled the identification of novel interactions, the proteomic context and intracellular localization. Since coat proteins interact with diverse cargoes in a native intracellular context, in situ structural studies can reveal the interactions of coat proteins with cargo molecules. The power of in situ studies has been demonstrated in a recent study of COPI as a way of identifying protein–protein interactions that are relevant in an intracellular context (Bykov et al., 2017). Here, the authors demonstrated that coat architecture in vitro mimics that seen in physiological contexts. Moreover, they identified a novel density that is not present in in vitro reconstitutions, which was attributed to cargo or cargo receptors as informed by previous biochemical knowledge regarding the cargo-binding site of β-COP.

The structure of retromer as assembled in vitro from purified retromer Vps5 components on liposomes was recently determined to subnanometre resolution (Fig. 5c; Kovtun et al., 2018). This revealed a membrane-coating array of the bar-domain protein Vps5, from which arches formed by retromer components Vps26, Vps35 and Vps29 protruded. Vps26 acted as an adaptor, while Vps29 was exposed at the tip of the arches, allowing modulation of the assembly by cytosolic factors. When subtomograms were positioned into the original tomogram density with their respective orientations, a semi-regulated arch array was observed. Similarly to COPI, in situ validation of the structure of retromer obtained from in vitro reconstitutions confirmed that in vitro reconstituted assemblies mirrored native retromer arrangements within the cell (Kovtun et al., 2018).

4. Challenges and future perspectives

Cryo-ET and STA have been applied to the study of units that do not form arrays, such as bacterial secretion systems (Chang et al., 2016; Nans et al., 2015; Hu et al., 2015, 2017; Wang et al., 2017; Chang et al., 2018), ribosomes (Pfeffer, Burbaum et al., 2015; Khoshouei et al., 2017; Englmeier et al., 2017; Pfeffer, Woellhaf et al., 2015; Pfeffer et al., 2012; Ortiz et al., 2010), the proteasome (Asano et al., 2015; Albert et al., 2017) and transmembrane proteins (Kudryashev et al., 2016; Davies et al., 2012).

While some studies have demonstrated that near-atomic resolution is possible with STA (Schur et al., 2016), technical and practical challenges limit tomographic analysis of many targets. Practically, data collection for tomography is low-throughput, as a tomogram takes significant time to be collected, and often the number of particles to average per tomogram is low. Coat protein assemblies are good systems as they form orderly arrays of repeating subunits and often contain hundreds of subunits per tomogram (Zanetti et al., 2013; Dodonova et al., 2017; Kovtun et al., 2018), but for many other samples, especially when targeted within cellular environments, achieving large datasets remains a challenge (Asano et al., 2016; Nans et al., 2015).
et al., 2015; Albert et al., 2017; Chang et al., 2016). To minimize the time needed for tomogram collection, a fast-incremental tilt method has recently been proposed, which could hold the potential to optimize data collection for high-resolution analysis (Chreifi et al., 2019).

Data processing also requires significant user expertise and manual intervention. The ongoing developments in automatic tilt series alignment procedures and the establishment of user-friendly software for STA will greatly benefit the speed of processing (Mastronarde & Held, 2017; Chen et al., 2017; Castaño-Díez, 2017; Himes & Zhang, 2018; Noble & Stagg, 2015).

In vitro reconstitutions provide control over sample composition and enable studies of the effect of varying the reaction environment in a controlled manner. Additionally, investigating the effect of mutations, which is central to the validation of structural information, is technically straightforward and is isolated from possible off-target effects. However, isolation from potential interacting partners, especially transient interactors that have not been biochemically established, limits discovery and the collection of exhaustive structural data. Information is also lost regarding the cellular context of the complex of interest. In situ studies can bridge the gap between in vitro analysis with purified proteins and the cell, allowing the identification of novel interacting surfaces, validating in vitro structural information, and providing global spatiotemporal insight (Kovtun et al., 2018; Bykov et al., 2017; Mahamid et al., 2016; Engelmeyer et al., 2017). While the challenges faced by in situ structural determination are beyond the scope of this review, we note that automated and sophisticated technologies for identifying assemblies of interest through template-matching may allow higher throughput and lower subjectivity (Lucić et al., 2013; Asano et al., 2015).

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