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Clathrin-coated vesicles from brain have small payloads: A cryo-electron tomographic study

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ABSTRACT

Clathrin coats, which stabilize membrane curvature during endocytosis and vesicular trafficking, form highly polymorphic fullerene lattices. We used cryo-electron tomography to visualize coated particles in isolates from bovine brain. The particles range from ~66 to ~134 nm in diameter, and only 20% of them (all ⩾80 nm) contain vesicles. The remaining 80% are clathrin "baskets", presumably artifactual assembly products. Polyhedral models were built for 54 distinct coat geometries. In true coated vesicles (CVs), most vesicles are offset to one side, leaving a crescent of interstitial space between the coat and the membrane for adaptor proteins and other components. The latter densities are fewer on the membrane-proximal side, which may represent the last part of the vesicle to bud off. A small number of densities – presumably cargo proteins – are associated with the interior surface of the vesicles. The clathrin coat, adaptor proteins, and vesicle membrane contribute almost all of the mass of a CV, with most cargoes accounting for only a few percent. The assembly of a CV therefore represents a massive biosynthetic effort to internalize a relatively diminutive payload. Such a high investment may be needed to overcome the resistance of membranes to high curvature.

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1. Introduction

Clathrin-mediated endocytosis is responsible for cellular uptake in the context of receptor recycling (LDL) (Ehrlich et al., 2004), synaptic vesicle recycling (Augustine et al., 2006), virus infection (e.g. (Ehrlich et al., 2004; Matlin et al., 1981; Rust et al., 2004)), and import of the prion protein (Taylor et al., 2005), among other processes. Clathrin-coated vesicles are also involved in protein sorting at the trans-Golgi network (Traub, 2005), and the assembly of the Golgi apparatus itself requires clathrin (Radulescu et al., 2007). This functional diversity requires assembling polymorphic scaffolds that are able to accommodate large variations in the size, shape, and molecular nature of the cargoes.

The building-block – the clathrin triskelion – is a remarkable structure with three hinged 52 nm-long legs connected at a trimeric hub (Brodsky, 2012; Kocsis et al., 1991; Ungewickell and Branton, 1981). It is able to assemble into many different forms, including flat lattices (Heuser, 1989), clathrin baskets (CBs) which are proteinaceous particles devoid of lipid (Crowther and Pearse, 1981; Crowther et al., 1976; Pearse and Robinson, 1984; Vigers et al., 1986b), and clathrin-coated vesicles (CVs) (Crowther et al., 1976). The coats of CBs and CVs adopt a wide range of polyhedral shapes and sizes.

In CV assembly, the main role of clathrin is to impose curvature of the membrane or to stabilize curvature otherwise accomplished (Hinrichsen et al., 2006). This calls for an energetically unfavorable distortion of the lipid bilayer. The endocytic process starts with the formation of a coated pit, followed by deepening the invagination until it is pinched off from the membrane of origin as a CV through the action of the GTPase dynamin (Hinshaw, 2000). Once the CV detaches from the plasma membrane, it is rapidly uncoated by the ATPase, Hsc70, and the freed clathrin triskelions recycle back to the membrane. The lifetime of a CV is only a few seconds before it is uncoated (Taylor et al., 2011). In view of these kinetics, it is likely that biochemical isolates contain, in addition to *bona fide* CVs recently budded off, also CBs assembled in the homogenate and CVs completed from coated pits during the isolation procedure.

In CVs, the clathrin network is coupled to the vesicle membrane through various proteins, the major ones being the adaptor proteins (APs), AP-1 and AP-2, which also function in cargo selection

Abbreviations: CB, clathrin basket; CV, coated vesicle; AP, adaptor protein.

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(Edeling et al., 2006). Each AP is composed of a heterotetrameric complex, forming a four-chain core, with two chains extending from the core to form two appendages or "ears". Various parts of the APs have been put forward as the clathrin-binding parts, such as the AP-2 core (Matsui and Kirchhausen, 1990; Peeler et al., 1993), the α-appendage (Goodman and Keen, 1995), and the linker or hinge of the ß2-appendage (Shih et al., 1995) at a motif called the clathrin box (Dell'Angelica et al., 1998; ter Haar et al., 2000). It remains unsettled which potential interactions are important *in vivo*.

The polymorphism of clathrin-coated particles complicates analyses of their three-dimensional structures by "single particle" reconstructions from cryo-electron microscopy. Classification of images into homogeneous subsets represents one approach to overcoming this obstacle and most studies to date have focused on the D6, 36-triskelion, coat which is relatively abundant in preparations from brain (Fotin et al., 2004; Heymann et al., 2005; Smith et al., 1998, 2004; Xing et al., 2010). However, this structure is a CB lacking a cargo and *per se* it casts little direct light on the wide range of clathrin lattices that form nor on the interactions of the clathrin coat with other components. The more recently introduced technique of cryo-electron tomography (cryo-ET) (Baumeister and Steven, 2000; McEwen and Frank, 2001) has the advantage of rendering three-dimensional structures for individual particles and its potentiality for investigating clathrin-coated particles has been demonstrated (Cheng et al., 2007). Here we have followed a generally similar approach, working with a larger data set and focusing to a greater extent on coat polymorphisms, their complements of APs, the presence of vesicles, and, in particular, the quantitation of the mass contributions of the respective constituents.

2. Materials and methods

2.1. Preparation of clathrin-coated particles

Material was isolated from fresh bovine brains essentially following the procedure of Nandi et al. (1982) and using a 12% sucrose-D_2O ultracentrifugation step (SW28 rotor, 100,000×*g* for 3 h) for purification. The preparation was stored at a protein concentration of about 5 mg/ml at 4 °C in the homogenization buffer (0.1 M MES, 0.1 mM EGTA, 0.5 mM $MgCl_2$, and 3 mM NaN_3, pH 6.5). Multiple preparations were done and each was used within a week (longer storage resulted in precipitation and freezing with cryo-protectants also failed).

2.2. Data acquisition

The specimen was mixed with an equal volume of 10 nm colloidal gold particles to serve as fiducial markers (BBInternational, Ltd), giving $\sim 3 \times 10^{12}$ particles/ml, applied to a glow-discharged lacy carbon grid and plunge-frozen (Reichert Jung KF80 Cryofixation System). The SerialEM package (Mastronarde, 2005) was used to record tilt series on a Tecnai T12 electron microscope (FEI) operating at 120 kV, using a post-column energy filter (in zero-loss mode with a 20 eV energy slit width) and a CCD camera of 2048 × 2048 pixels (GIF2002, Gatan, Inc.). Tilt series consisted of up to 141 micrographs taken from −70° to 70° in 1° steps. The total dose for a series was approximately 60 e^-/nm^2, and the target defocus was 4 μm under focus, putting the first zero of the CTF at $\sim(3.7\ nm)^{-1}$. They were recorded at magnifications of 54,000 (3 tomograms), 38,500 (11 tomograms) and 26,000 (9 tomograms), giving pixel sizes of 0.55 nm, 0.78 nm and 1.14 nm, respectively. The intermediate magnification yielded the best tomograms (obtained from two sample preparations), providing a sufficient number of fiducial markers and particles within the field-of-view. The lower magnification appeared to decrease the quality of the clathrin spars in the tomograms. All tomograms further discussed are at the intermediate magnification.

2.3. Tomogram reconstruction

Tilt series were aligned and tomograms reconstructed using the package Bsoft (Heymann and Belnap, 2007; Heymann et al., 2008a). The tomograms were denoised by a non-linear anisotropic diffusion algorithm (Frangakis and Hegerl, 2001). Individual clathrin particles were selected and extracted. The resolution of each individual micrograph was estimated by Fourier ring correlation using a cutoff of 0.3 ($FSC_{0.3}$) (Cardone et al., 2005; Heymann et al., 2008a). The zero-tilt micrographs showed resolutions of 5.4–6.5 nm.

2.4. Modeling the clathrin lattice

Polyhedral models of the clathrin network were built into the tomographic subvolumes using the program Chimera (Pettersen et al., 2004). Symmetry was determined by visual inspection. The coordinates of the polyhedral vertices were regularized (Heymann et al., 2008b). The reference inter-spar angles were set to the canonical angle for the associated polygon (90° for a tetragon, 108° for a pentagon, 120° for a hexagon and 128.6° for a heptagon).

2.5. Modeling the vesicle membrane

The vesicles in CVs are approximately spherical. Accordingly, for each vesicle, a set of closely spaced points was generated, evenly distributed at a radius approximately equal to that of the vesicle. The model was generated and positioned relative to the tomogram by cross-correlation. The positions of points were refined by cross-correlation to a reference membrane patch. The offset of the vesicle within each CV was calculated as the difference between the geometric centers of the coat polyhedron and the vesicle model. To represent the volume of the membrane, a shell mask with a thickness of 5 nm was generated (in a eukaryotic bilayer the P–P distance is ∼4 nm (Mitra et al., 2004) and typically phospholipid bilayers are ∼5 nm (Woodka et al., 2012)).

2.6. Segmenting the coated vesicle

For a given CV, a synthetic clathrin coat map was constructed using the polyhedron and averaged spar density from a single particle reconstruction of the 36-vertex D_6 barrel (Heymann et al., 2005). The use of this map avoids including spurious densities that might be present in a tomogram. This map was binarized by thresholding at a level that gave a good representation of the N-termini. This mask and a mask covering the region outside the polyhedral model were used to isolate the interior of the CV, excluding the coat.

The membrane model (see above) was refined within this interior mask, and a membrane mask produced as a shell with a thickness of 5 nm. The membrane mask and a mask outside the membrane model were used to isolate the vesicle lumen. The membrane mask and a mask of the vesicle lumen were used to produce a mask of the interstitial volume.

With these four masks (coat, interstitial, membrane and lumen), each region was isolated as a separate map.

2.7. Counting adaptor protein (AP) densities

The AP core-sized densities in the interstitial space of each CV were counted manually. In addition, potential APs in the interior of CBs and interstitial spaces of CVs were located as follows: The