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Single-molecule live-cell imaging of clathrin-based endocytosis

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Abstract

Clathrin-coated vesicles carry traffic from the plasma membrane to endosomes. We report here the first real-time visualization of cargo sorting and endocytosis by clathrin-coated pits in living cells. We have visualized the formation of coats by monitoring the incorporation of fluorescently tagged clathrin or its adaptor AP-2 (activator protein 2), and have followed clathrin-mediated uptake of transferrin, single LDL (low-density lipoprotein) and single reovirus particles. The intensity of a cargo-loaded clathrin cluster grows steadily during its lifetime, and the time required to complete assembly is proportional to the size of the cargo particle. These results are consistent with a nucleation-growth mechanism and an approximately constant growth rate. There are no preferred nucleation sites. A proportion of the nucleation events appear to be abortive. Cargo incorporation occurs primarily or exclusively in a newly formed coated pit, and loading appears to commit that pit to finish assembly. Our data led to a model in which coated pits initiate randomly, but collapse with high likelihood unless stabilized, presumably by cargo capture.

Background

Thirty years ago, while still a graduate student, I would browse cell physiology textbooks, and be fascinated by the number of ideas and models dwelling on the transport of water, ions and small molecules across cell membranes. What was notoriously lacking then, however, were molecular and mechanistic thoughts on how much larger molecules, such as protein hormones, lipoproteins and viruses in the cell milieu and membrane proteins bound to the cell periphery, the cargo, entered cells. The situation is vastly different today, as we

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now realize that this type of entry requires the regulated formation of membrane invaginations that, after sealing, form vesicular or tubular carriers that deliver the cargo typically to endosomes.

The clathrin-dependent pathway was the first system of membrane traffic to be recognized and studied in detail. This was due primarily to the distinctive morphological characteristics of budding coated pits, to the ease with which coated vesicles could be purified and to the widespread importance of clathrin-coated structures for receptor-mediated endocytosis. The clathrin pathway has thus acquired special status for analysing molecular mechanisms in membrane traffic.

The 'life-cycle' of a clathrin-coated vesicle, from coat assembly and cargo loading to coat disassembly and cargo delivery, involves a number of highly regulated events occurring on a rapid time scale (seconds). New tools of live-cell imaging, which provide the time resolution required to separate key steps, are starting to provide important new data of temporal sequences of protein associations and translocations. These results can now be integrated with static information, such as the identification of molecular partners and participating structures, derived largely from genetics and biochemistry, and the determination of three-dimensional structures of components, from X-ray crystallography and cryo-EM (cryo-electron microscopy). The goal now is to generate a mechanistic picture of clathrin-based membrane vesicular traffic — a 'molecular movie' of the molecular endocytic machinery.

High-resolution structure of clathrin and molecular anatomy of a coat

Clathrin was discovered 25 years ago [1]. It forms the striking lattices that surround coated pits and vesicles. It is a spider-like molecule, with three legs radiating from a central hub (a 'triskelion') [2]. Each leg contains a 190 kDa 'heavy chain' (the species discovered by Pearse [1]) and an associated ~25 kDa 'light chain' [2,3]. Antiparallel interactions of the legs from triskelions centred on adjacent vertices of the lattice allow assembly of a coat [3,4].

High-resolution structural studies of clathrin fragments by X-ray diffraction [5–7] and lower-resolution analyses of clathrin coats by cryo-EM to resolutions of 21 Å (1 Å=0.1 nm) [8,9] and 7.9 Å (Fotin et al., unpublished work) have led to a relatively detailed molecular picture of the framework of a coat and to a view of how clathrin assembly can be coupled to cargo incorporation.

Clathrin interacts directly with adaptor proteins, such as the heterotrimeric AP (activator proteins) complexes [10–13] and other, specialized, adaptors, such as β -arrestins [14] and GGAs (Golgi-localized, γ -ear-containing, ADP-ribosylation-factor-binding protein) [15]. These adaptors in turn link to the cytoplasmic segments of membrane-bound protein receptors (the cargo). The β -propeller structure of the clathrin N-terminal domain binds peptides such as the 'clathrin-box' motifs (LØ{D/E}Ø{D/E}) [5] and the W box motif (Pro-Trp-Xaa-Xaa-Trp) [16] present in disordered loops of various adaptor proteins and in other regulatory proteins. Combining the X-ray structure of the N-terminal 55 kDa N-terminal fragment of clathrin with the 21 Å resolution cryo-EM image reconstruction of a coat, we were able to show that

the clathrin terminal domain projects inward beneath a vertex, close to the presumed radial position of an adaptor complex [8]. The same work compared the conformation of the clathrin triskelion legs in each of the nine different packing environments within the coat, and suggested that the α -zigzag characteristic of most of the clathrin heavy chain provided a stiff, but slightly flexible, structure that could adapt to lattices of different size and design.

Coat assembly and disassembly

Despite this structural progress, we still do not fully understand the process of coat assembly and disassembly. Opinions are divided regarding the formation of coated pits. We believe that a curved lattice forms by sequential addition of individual cytosolic clathrin trimers to a growing shell [8]. Others have suggested that a flat hexagonal array of clathrin can restructure into the mixture of hexagons and pentagons in a curved assembly [17]. Such a rearrangement is physically impossible without substantial local dissociation, and we have proposed that flat arrays (observed in many electron micrographs) function as reservoirs for rapid assembly of curved coated pits at the edges of the arrays [8].

The nature of a clathrin coat is such that triskelions can assemble and disassemble by rotations centred on any vertex (for a computer animation of our model for clathrin assembly consult <http://www.cbrinstitute.org/kirchhausen/research.html>). In fact, it has been discovered that such exchange occurs in living cells [18]. A large fraction of the clathrin structures bound to membranes underwent constant exchange with the cytosolic pool. The *in vivo* exchange was a surprise, however, as the prevailing model for coat formation predicted a simple sequential recruitment of coat components culminating with the formation of a coated vesicle. It is possible that Hsc70 (heat-shock cognate 70) and auxilin, proteins that drive the ATP-dependent disassembly of the clathrin lattice [19], are involved in the exchange reaction as well, but, at present, this is only a speculation. Although many mechanistic aspects of the Hsc70/auxilin-uncoating reaction are known, including the domains of these proteins required in the uncoating reaction [20], essentially nothing is known about where these proteins bind on the lattice, what type of contacts they establish with each other and with clathrin, and whether a rigid rotation of the triskelion driven by a ratchet mechanism combined with slight changes in leg conformation, rather than a very large conformational change, explains the process.

Coat formation *in vivo*

We can distinguish the following steps in coated-vesicle formation: nucleation or initiation of a coated pit; propagation of the clathrin lattice, accompanied by bilayer invagination and cargo recruitment; completion of the clathrin lattice and pinching off of the vesicle (budding); transport or diffusion of the vesicle away from the membrane; removal of the clathrin coat (uncoating); fusion of the uncoated vesicle with a target membrane [21,22]. Each of the

steps is very fast, and it is estimated that the whole cycle occurs in 1 min or less [18,23]. Many of these steps can now be resolved with the aid of live-cell imaging tools [23–26].

Visualization of clathrin-coated pits and vesicles

Keen's group was the first to tag clathrin coats with an EGFP (enhanced green fluorescent protein)–LCa (light chain A) fusion protein and visualize clathrin clusters at the plasma membrane of living cells [23]. They found that clathrin tends to assemble at defined sites or 'hot spots', and that they exhibit a characteristic 'blinking' in time-lapse imaging. They suggested that each such on–off cycle might represent clathrin recruitment (increase in fluorescence intensity), budding and uncoating (abrupt disappearance of signal). A closer look at their results, at those of other groups [18], and at new data we have obtained [27] suggests a more complex and richer set of phenomena.

To visualize clathrin structures in living cells, we created a number of cell lines derived from monkey BSC1 epithelial cells stably expressing chimaeras of EGFP (and variants) attached to clathrin light chains as originally described by Keen's group [23] and of σ 2-adaptin of the clathrin adaptor AP-2. We also detected the incorporation of EGFP-tagged dynamin to the clathrin structures and followed the incorporation to the forming coats of fluorescently tagged transferrin, single LDL (low-density lipoprotein) or reovirus particles. We examined the formation of coats with the aid of a spinning-disk confocal microscope, and found conditions that allow long-term imaging for more than 150 images before noticeable photo-quenching and undertook an unbiased analysis of clathrin dynamics.

One can detect four classes of fluorescent clathrin clusters: (i) those that do not fluctuate in intensity; (ii) those that 'blink' at a fixed position, tend to be short-lived and do not reach the fluorescence intensity associated with a fully formed coated vesicle; (iii) those that appear and do reach the fluorescence intensity associated with a coated vesicle, and typically show abrupt changes in lateral position before disappearing; and (iv) those that tend to be larger, brighter and highly mobile. In general, new spots do not appear at the location at which these last sorts of clusters formed. We suggest that many of the stationary spots (class i) might represent reservoirs for coat components. We propose that the clusters that disappear before becoming a coated vesicle (class ii) correspond to abortive events that fail to endocytose. We believe that the clusters that acquired rapid lateral mobility before disappearing (class iii) represent true endocytic budding events. The highly mobile clusters (class iv) correspond to clathrin associated with endosomes.

We noticed that cargo incorporation occurs primarily in a newly formed coated pit, and, in general, we could not detect incorporation of cargo into the abortive coats. The fluorescence intensity of a cargo-loaded clathrin cluster grows steadily during its lifetime, and the time required to complete coat assembly is proportional to the size of the cargo particle. These characteristics are consistent with the requirements of a system that must entrap cargo of highly variable size and shape.

Correlated dynamics of clathrin and AP-2 assemblies

By automatically tracking the fluorescent signals of clathrin and AP-2 along the time series, we discovered that every spot contains signals for both, and, regardless of their age, their fluorescent signals always appeared and disappeared together with similar rates during the life of a cluster. Thus clathrin and adaptor complexes co-associate in coated pits, and clathrin uncoating and adaptor dissociation also occur together.

Cargo capture and commitment to completion

To investigate the relationship between cargo capture and formation of a coated pit, we used fluorescently tagged transferrin, LDL and reovirus, and monitored their fate. It became apparent that these cargo molecules failed to associate with abortive clathrin clusters (class ii), and that the only successful interactions occurred with young clathrin clusters, which then proceed to grow, bud and uncoat (class iv). Budding followed by uncoating (e.g. endocytosis) was established with certainty for these events, because both fluorescent signals (cargo and clathrin or AP-2) were monitored as part of the same cluster before uncoating. Our results indicate a causal relationship between cargo capture and completion of the coat. It shows that assembly aborts unless cargo is captured. The molecular basis for the abortive step remains to be determined, however, and, at present, we cannot distinguish between an active dissolution process and a passive reaction where a molecular clamp fails to stabilize the nascent coat.

Exploratory model

Our observations suggest that the clathrin-based endocytic machinery has an exploratory character. The coated pits appear to dissolve abruptly unless some intervening event, perhaps cargo loading, determines that they continue. Our model separates the mechanism of initiation from the process of cargo recognition. It allows a variety of different cargo adaptors to participate equally, and it avoids the need to postulate differentiated budding sites on the membrane. Important tests of this description of endocytosis as an exploratory process will be to establish the mechanism by which abortive coated pits collapse and finding the signalling process that allows assembly to proceed.

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