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Review

Understanding Living Clathrin-Coated Pits

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Most knowledge of clathrin-mediated endocytosis has been gained by biochemical fractionation and *in vitro* assays. Recently, the study of endocytosis has extended into the living cell. The tracking of individual clathrin-coated pits and vesicles (CCPs and CCVs) has provided new insight into understanding the dynamic nature of CCPs. The use of total internal reflection fluorescence microscopy (TIR-FM), also termed evanescent field microscopy, has enabled the direct observation of events occurring within a restricted area of the cell adjacent to and including the adherent plasma membrane. TIR-FM is now actively being pursued in the study of endocytic processes. The direct observation of CCP-associated proteins including clathrin itself, dynamin and, most recently, AP-2 has considerably challenged old models, confirming some points but raising very interesting new questions.

Key words: AP-2, clathrin-coated pits and vesicles, clathrin, endocytosis, evanescent field fluorescence microscopy, time-lapse, total internal reflection (TIR)

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Since the original description of clathrin by Barbara Pearse (1), biochemical assays have characterized many of the steps and participating proteins in the processes of coat formation, pit invagination, vesicle fission and uncoating. Multiple accessory and adaptor proteins have been characterized, as have novel protein–protein and protein-lipid binding domains and interactions between the endocytic apparatus and cytoskeletal elements. Assays such as yeast-two-hybrid and coimmunoprecipitation have permitted the identification of binding partners and the domains through which interactions occur. Additionally, *in vitro* analysis of purified coat components and accessory proteins has provided insight into the processes regulating clathrin lattice polymerization and lipid deformation. More recently, the advent of *Aequorea victoria* green-fluorescent protein (GFP) has illuminated nearly all aspects of the living cell previously hidden to real-time analysis (2). The purpose of this review is to describe and evaluate data gathered in studies of clathrin-coated pit and vesicle (CCP and CCV, respectively) formation in living cells, and to place this new information in the context of what was observed using other methods. The study of clathrin-mediated endocytosis and analysis of the relevant molecular machinery are rapidly emerging as a paradigm for the benefits of real-time live-cell imaging.

A unifying model for CCP/CCV formation organized around the known functions of three historical actors, clathrin, the AP-2 complex and dynamin, has been detailed in several recent review articles (3–5). In this model, the clathrin adaptor complex AP-2 plays a central role in CCP formation and function, being responsible for the assembly of clathrin triskelia onto the cytosolic leaflet of the plasma membrane and selection of cargo receptors that will be internalized by the forming vesicles. The formed pits progressively invaginate and are finally released into the cytosol as a free CCV, a step proposed to require the GTPase activity of dynamin (Figure 1). New partners of these historical actors have been added to this initial framework that interact with cargo, clathrin, dynamin and/or AP-2. These can be broadly separated into three categories: those containing EH protein–protein interaction domains (Eps15 and Eps15R) and those containing either ENTH (epsin, Hip1 and Hip1R) or ANTH (AP180, CALM) phosphoinositide binding domains.

Current data suggests that Eps15, epsin and CALM are involved the recruitment of AP-2 onto the plasma membrane (6,7). Similarly, dynamin-interacting proteins including intersectin, amphiphysin and endophilin have been implicated in the targeting of dynamin to CCPs and as important cofactors in dynamin function in CCV formation. Amphiphysin and endophilin are believed to be involved in the invagination process and endophilin may play a direct role in the fission reaction through its lipid modifying activity (6,8). The ability of proteins to induce curvature in lipid bilayers *in vitro* has been demonstrated for epsin, amphiphysin, endophilin and dynamin, suggesting that the capacity for function as an adaptor and in the processes of invagination, and potentially fission, are not mutually exclusive. Several CCP-associated proteins (Intersectin, Hip1 and Hip1R, dynamin) appear to play a role in regulating actin polymerization, which may be required for the last step of vesicle fission and/or to push the nascent CCV into the cytosol (7,9). Following internalization, the uncoating reaction, which recycles clathrin coat components into the

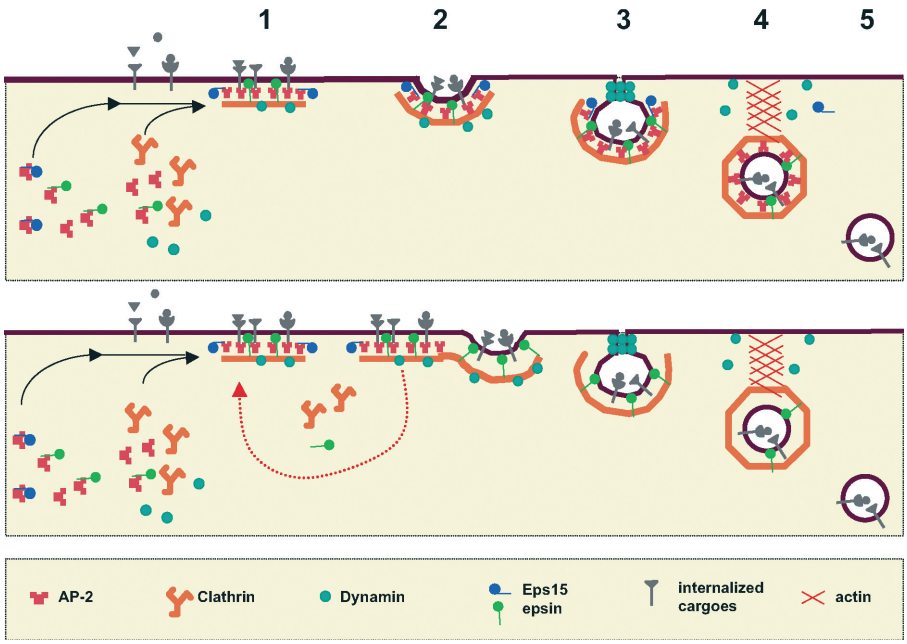

Figure 1: Models for CCP/CCV assembly. Previously proposed model for CCP/CCV formation (Top) assumed that AP-2 complexes are first recruited on the plasma-membrane, forming a nucleation site for further assembly of AP-2 and clathrin (1). Cargoes are then incorporated in these forming CCPs that progressively invaginate (2, 3) to form a free CCV (5) through the action of dynamin and actin polymerization (4). This linear model in which each CCP will be transformed in a vesicle was recently challenged by studies that have followed the dynamic of CCP-associated proteins in live cells by classical or TIR time-lapsed fluorescence microscopy. From these studies a new model could be proposed in which plasma membrane associated AP-2 complexes form stable platforms for clathrin assembly and cargo selection. From these platforms, clathrin lattices and cargoes are transferred onto and into forming vesicles that rapidly move laterally and then move in towards the cytosol through actin polymerization.

cytosol, is thought to proceed rapidly in a process catalyzed by Hsc70 and auxilin (4,5).

Some models for clathrin-mediated endocytosis predict that CCPs form stochastically at the plasma membrane, requiring only a local concentration of PIP2, which binds to AP-2, epsin and CALM, and a putative AP-2 anchoring protein likely to correspond to synaptotagmin in neuronal cells. An alternate view, which will be pursued further below, holds that certain areas of the plasma membrane serve as nucleation sites for the iterative formation and internalization of nascent CCVs.

This review covers the relatively recent use of techniques such as siRNA and fluorescence imaging that has allowed the study of clathrin-mediated endocytosis in living cells. In many cases the results are consistent with observations made from biochemical and *in vitro* assays. However, they have led to suggestions of alternative hypotheses to explain the prior observations. These results emphasize that our understanding of the process as a whole, as well as the particular role of each individual protein, still has many surprises in store for us.

Functional Studies Using siRNA

The respective roles of AP-2 and clathrin in CCP/CCV formation were recently revisited using small interfering RNA to knock-down expression of the relevant proteins. The data obtained by two different groups clearly confirm a central role for AP-2 in the assembly of CCPs at the plasma membrane (10,11). Indeed, the almost complete knock-down of AP-2 resulted in a drastic diminution of the number of CCPs (< 10% of control cells). These studies also confirmed that AP-2 complexes are likely recruited before clathrin on the plasma membrane because AP-2 shows similar punctate plasma membrane localization in the absence of clathrin. Other CCP markers such as epsin and CALM were similarly present on the membrane following AP-2 knock-down, suggesting that they are recruited prior to, or independently of, AP-2 complexes. Finally, these studies revealed some interesting new observations regarding the respective involvement of AP-2 in the internalization of different sets of receptors distinguished by their tyrosine-based endocytic signal subtype. The overall internalization process was greatly impaired in clathrin-deficient cells, suggesting that clathrin assembly on preformed AP-2 patches is required for vesicle formation. However, interesting differences were detected concerning the affects of AP-2 knock-down on the internalization of different types of receptor–ligand pairs. Internalization of the transferrin receptor (Tf-R) and of a low-density lipoprotein receptor (LDL-R) chimera were both completely blocked in clathrin knock-down cells. In contrast, internalization of the LDL-R chimera was not

perturbed at all in AP-2 depleted cells, whereas Tf-R was almost completely blocked.

The hypotheses suggested by the authors of these studies contained several interesting points. First, AP-2 is not required for the overall clathrin-dependent internalization pathway, suggesting the role of additional clathrin-dependent adaptors (recently discussed in (12)). Second, it seems that the ~10% remaining CCPs still present in the absence of AP-2 are able to form functional vesicles that are sufficient to support normal internalization of certain cargoes. This latter point suggests the existence of an AP-2-independent clathrin-dependent internalization pathway that would account for the internalization of receptors such as the LDL-R family and of others yet to be defined. The unexpected results of these siRNA studies suggest the existence of diverse, parallel, and possibly interconnected, clathrin-dependent internalization pathways. As discussed below, live cell imaging studies have begun to similarly suggest an apparent heterogeneity within the clathrin-decorated structures observable in the plasma membrane region under epifluorescence or TIR-FM microscopy.

Following the Dynamics of Clathrin

The first study reporting clathrin dynamics at the plasma membrane (13) challenged several points of the above-described model. In this study, a GFP-tagged clathrin-light chain was used to label clathrin triskelia (Figure 2). Initial control experiments demonstrated that the GFP fusion efficiently associated with endogenous clathrin heavy chain showed an intracellular staining pattern identical to that of endogenous clathrin and that expression did not perturb clathrin-dependent endocytosis of transferrin. Employing classical time-lapse fluorescence in areas of the cell devoid of significant out-of-focus fluorophore emission, Keen and colleagues were able to track plasma membrane-associated accumulation of the GFP chimera in real time. In these conditions, most of the CCPs were static and the dynamic ones (1/3 of total spots) showed a half-life of 20–80 s depending on the cell line, values compatible with previous estimations (see in (13) for details). The majority of the spots disappeared abruptly or moved laterally, arising from preexisting spots, and then disappeared. A minority of the spots was observed to move laterally on the plasma membrane without subsequent disappearance.

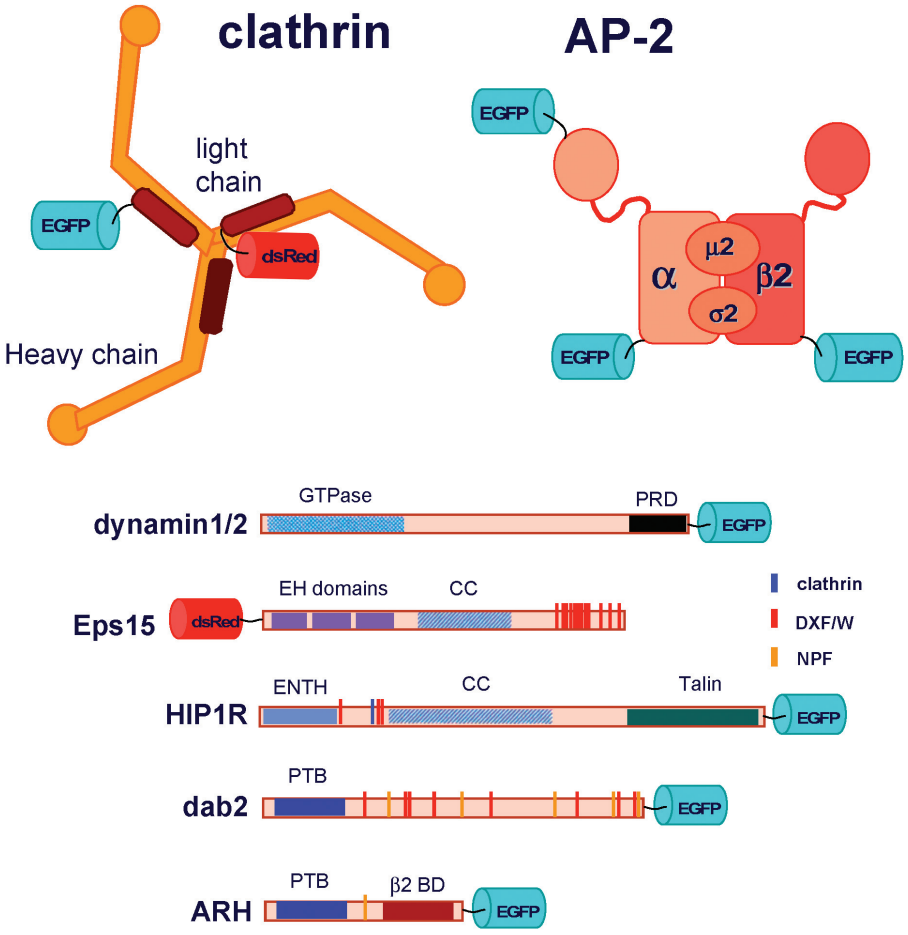

Figure 2: Fluorescent-tagged CCP associated proteins. β2 BD: β2-adaptin ear binding domain; CC: coiled-coil; EH: Eps15 homology; ENTH: Epsin *N*-Terminal domain Homology; PRD: Proline Rich Domain; PTB: Phospho-Tyrosine Binding domain.

The rapid vanishing of plasma membrane associated clathrin spots (CS) was interpreted as the direct observation of CCV formation and transport deeper in the cytosol, leaving the focal plane, and/or loss of their clathrin coat (uncoating reaction). The most striking result was that CCPs seemed to form in restricted areas of the plasma membrane since CS seemed to reappear preferentially in regions from which spots disappeared few seconds before. Similar observations were obtained following clathrin dynamics in dendrites, suggesting that CCV formation in these compartments may be similar to that in non-neuronal cells (14). These results were really not expected from some of the different models for clathrin-mediated endocytosis and suggest that only some restricted regions of the plasma membrane are competent to form CCPs. To date, no convincing molecular explanation for this phenomenon has been provided, even if a role for actin cytoskeleton is invoked.

Improving *in Vivo* Analysis Using TIR-FM

There were two experimental limitations to this otherwise significant, seminal work. First, it was necessary to assume that clathrin spots that disappeared corresponded to events of endocytosis, but direct proof of this assertion is still lacking (see below). Second, it could not resolve whether the fluorescent spots were actually associated with the plasma membrane or in the cytosol. It was not possible to distinguish between CCPs, CCVs, clathrin-coated regions of endosomes (or endosomal-derived vesicles) or TGN-derived CCVs (Figure 3). This specific problem has recently been reduced using the technique of TIR-FM. This microscopic methodology makes use of laser-based illumination that is restricted to the most basal volume of the sample (typically cited as 50–150 nm) including the extracellular milieu between the coverslip, the adherent plasma membrane of the cell and a thin layer of adjacent cytosol (15–17). Since the size of a CCV is ~100 nm, it should be possible to observe forming CCPs and CCVs. Indeed, as soon as the vesicles begin to move into the cell, their fluorescence should 'blink out' as they exit the evanescent field.

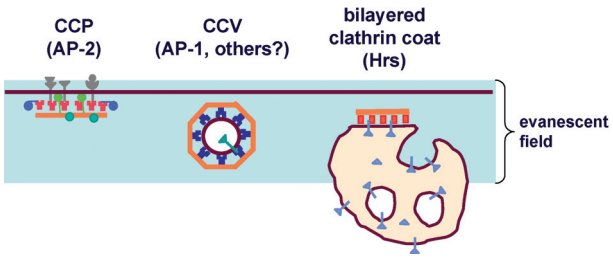

Figure 3: Clathrin coats potentially 'visible' by TIR-FM. The evanescent field allows the excitation of a 100–150 nm region adjacent to the coverslip that includes the plasma membrane and a thin layer of cytosol. Within this region could be theoretically observed CCPs, invaginated CCPs and plasma membrane-derived CCPs but also other organelle-derived CCVs (AP-1 positive) and clathrin-coated areas on sorting endosomes (Hrs positive).

However, given that the decay of the evanescent field is exponential, and not absolute, brighter objects deeper than the commonly cited *d* (depth at which the field falls to 1/e (e is defined as the inverse natural log of 1: ln(e) = (1))) can in fact be observed by TIR-FM. Interestingly, several studies have used TIR-FM to evaluate the behaviors associated with other endocytic structures within the evanescent field, including endosomes (early, recycling and late) and lysosomes (18–20). That endosomes could be clearly observed by TIR-FM suggested that they could be responsible for some of the clathrin spots (see below).

Observing clathrin light-chain derived constructs (labeled with either GFP and dsRed) by TIR-FM has greatly improved the analysis of the behaviors of plasma membrane-associated clathrin by eliminating nearly all the intracellular (e.g. *trans*-Golgi network) clathrin-labeled structures. These studies have confirmed the results obtained by classical FM and the existence of three populations of clathrin spots, the static (70–80%), the disappearing (10–20%) and the ones that move laterally parallel to the plane of the plasma membrane (2–8%) (20–22). Furthermore, the use of combined classical and TIR-FM can allow the monitoring of CCVs as they disengage from the plasma membrane and move deeper into the cytosol, providing the opportunity to monitor endocytosis as well as uncoating (23).

Are Disappearing Spots Truly CCVs?

Formal proof that a CS that disappears is a nascent cargo-containing CCV is still lacking. One critical piece of evidence would be the identification of endocytic cargo inside disappearing clathrin puncta. Unfortunately, it is not as yet possible to gain a great deal of insight into the process of endocytosis through the use of fluorescent ligands such as LDL or Tf in TIR-FM studies. This is because imaging while a ligand is in the extracellular milieu between the coverslip and the plasma membrane causes a very strong 'background haze' due to its location near the origin of the evanescent field. In addition, the use of fluorescent-tagged receptors has remained unsuccessful due to the presence of labeled protein both in post-Golgi exocytic vesicles in the TIR field, as well as in diffuse fluorescence in the plasma membrane.

The ability to track fluorescent ligands and receptors would additionally allow the analysis of which receptors are targeted to preformed CCPs and which induce clathrin polymerization *de novo*. This would help address the issue of heterogeneity within the process of clathrin-mediated endocytosis. Newer technologies such as photo-activatable GFP (24) may help surmount these obstacles. In addition, the imaging of fluorescent-tagged viruses by TIR-FM should be a useful tool in both the study of viral entry and in the elucidation of endocytic processes (25). Finally, recent work by our lab and others has demonstrated the benefits of quantum dot labeling of proteins for use in live

cell imaging studies, although the direct application of this technology to the study of endocytosis is really just beginning ((26) and reviewed in (27)).

Static colocalization of tagged clathrin in puncta with receptors (e.g. Tf-R), ligands (e.g. Alexa488-Tf) and other endocytic proteins (e.g. AP-2) provides circumstantial evidence that clathrin concentrations present in the TIR field represent stages in the life of a clathrin-coated endocytic vesicle. Simultaneous observation of clathrin together with other GFP-tagged markers (dynamins, actin, and clathrin adaptor proteins, see below) has provided better evidence that disappearing spots are CCVs. It was found that a dsRed-tagged version of clathrin-light chain was useful for these kinds of studies. This is of great benefit as in many cases dsRed induces inappropriate aggregation of tagged proteins. The ability to successfully express and image a red-tagged clathrin has permitted the simultaneous imaging of other proteins tagged with GFP without fluorophore emission bleed-through becoming a major concern (as it is with the simultaneous imaging of CFP and YFP).

Whereas previous cellular analyses were limited to fixed cells imaged either by immunofluorescence or immunogold transmission electron microscopy, live cell imaging studies have allowed questions to be addressed on a molecular level, in real-time. The results of these dynamic colocalization studies have provided several interesting new observations regarding the spatial–temporal regulation of the machinery of endocytosis. For example, while the fluorescence associated with dynamin1, the neuronal specific isoform, was shown to increase dramatically at the sites of clathrin spots just before they disappear (21, 23), the ubiquitously expressed isoform dynamin2 was shown to exactly follow clathrin (21). Although the studies conducted to date on living cells cotransfected with dynamin1 and clathrin have involved heterologous expression in non-neuronal cell lines, similar observations were made when dynamin1-EGFP was imaged in stimulated PC12 neuro-endocrine cells (28). The presence of dynamin at the sites of endocytosis was in agreement with the proposed models for dynamin function in CCV formation and the difference observed between the two isoforms may reflect differences in CCV formation in the brain vs. the rest of the body. In addition, actin accumulation was observed at clathrin spots while they were disappearing (23), evidence in favor of models in which actin polymerization provides a force pushing the forming vesicle away from the plasma membrane. This hypothesis has been supported by the observation that in cells treated with Latrunculin, a drug that depolymerizes actin filaments, the number of disappearing CS decreases (14,29). However, both the microscopy and separate biochemical analyses suggest that the Latrunculin effect is not complete, rather only an ~40% decrease in the number of disappearing spots was observed (30). Thus, as further discussed below, the potential direct and/or indirect roles for cytoskeletal elements in clathrin-mediated endocytosis have yet to be fully elucidated.

Other studies that inhibited clathrin spot internalization have provided further evidence that disappearing CS represent endocytosis. Depletion of cholesterol using methyl-β-cyclodextrin inhibited CCV formation and increased the proportion of flat CCPs (31,32). The fact that the proportion of disappearing clathrin spots was drastically reduced in the same conditions (32) further suggest that they correspond to forming CCVs. Finally, a recent report where the FK506 binding domain was used in conjunction with dimeric FK506 (FK1012-A) to functionally inhibit GFP-tagged clathrin light chain has demonstrated by TIR-FM and more conventional measures of endocytosis (radioiodinated ligand uptake) that these methodologies represent suitable means to study endocytosis in living cells on a microscopic basis (29). Thus, the use of live-cell imaging by TIR-FM has permitted a new perspective on the study of clathrin-mediated endocytosis.

Live Cell TIR-FM of AP-2

One benefit of live-cell imaging is the ability to identify and then track individual puncta throughout their lifetimes in order to characterize differences among various populations. In the proposed linear models of CCV formation, AP-2 is present all along the CCV formation process. These models suggest that AP-2 plays its adaptor function beginning with the early steps of CCP assembly and then is incorporated in the budding vesicle. The presence of AP-2 in CCVs was mainly assumed on the basis that AP-2 was always found coenriched with clathrin in CCV preparation from brain homogenates. However, recent studies have challenged this point. First, AP-2 complexes were not strictly required for CCV formation in an *in vitro* assay following CCV formation from purified plasma membrane fragments (33). Furthermore, siRNA experiments indicated that following reduction of AP-2, a proportion of CCPs (~10%) are still present on the plasma membrane and efficient endocytosis of LDL still occurs (see above).

These results have been complemented by observations obtained through real-time imaging of clathrin-mediated endocytosis. We recently generated a GFP-tagged α-adaptin construct (34) and used a previously characterized GFP-tagged β2-adaptin (35) to investigate the dynamics of AP-2 in live cells. The α-adaptin fusion was correctly targeted to CCPs upon transient transfection, colocalizing with endogenous AP-2 and clathrin at the plasma membrane. Its expression did not perturb the localization of α-adaptin ear domain binding proteins such as Eps15, epsin and CALM, which were all found to colocalize with GFP-α-adaptin in plasma membrane-associated puncta. Expression of the construct did not perturb overall bulk clathrin-dependent endocytosis of Tf or LDL, as directly followed by uptake

experiments, or GFP-α-adaptin colocalized with endogenous cell surface Tf-Rs, suggesting that CCPs containing this fusion protein are still able to concentrate cargoes. Similar conclusions were obtained independently by Lois Greene and colleagues, who generated an α-adaptin construct with GFP appended to the carboxy terminal ear domain (36). Additionally, Juergen Knoblich has reported that α-adaptin with GFP on the amino terminus localizes to *Drosophila* CCPs and is able to rescue α-adaptin-deficient flies (personal communication). Although these results indicated that tagging α-adaptin with GFP is not deleterious to the function of the protein, as discussed below, the potential for effects of overexpression of exogenous proteins on endocytic processes is a continual concern to studies analyzing GFP-tagged proteins.

Simultaneous dual color TIR-FM imaging performed following dsRed-clathrin and GFP-α-adaptin showed that GFP-α-adaptin (and therefore presumably the AP-2 complex) was present in most, but not all, plasma membrane-associated clathrin puncta (Figure 4). However, somewhat surprisingly, GFP-α-adaptin was clearly excluded from both disappearing and laterally mobile CS, while being present in nearly all static CS. Furthermore, these observations were verified in experiments analyzing cells expressing dsRed-clathrin and β2-adaptin-GFP (34) and most recently in our lab using the α-adaptin-GFP construct generated by L. Greene and colleagues (unpublished results, Figure 5). The absence of GFP-tagged α-adaptin markers for AP-2 from laterally moving CS was also confirmed following a currently uncharacterized α-adaptin construct with GFP appended to the ear domain (20). However, in this study it was claimed that α-adaptin-GFP is present in disappearing CS. The reasons for this apparent discrepancy remain to be found (e.g. cell lines, constructs, expression parameters) (see Remaining Controls below).

The observation that AP-2 is absent from disappearing CS has led to a new hypothesis for the overall process of CCV formation. These data suggest that, contrary to predictions, AP-2 complexes may be absent from CCVs and may actually form stable platforms at the plasma membrane that serve to coordinate the assembly of clathrin lattices and accumulation of cargoes into the budding vesicles (Figure 1). The observation that AP-2 can be released from clathrin coats independently of clathrin itself (37) similarly suggests that clathrin and AP-2 are not inexorably linked spatially and temporally. The model

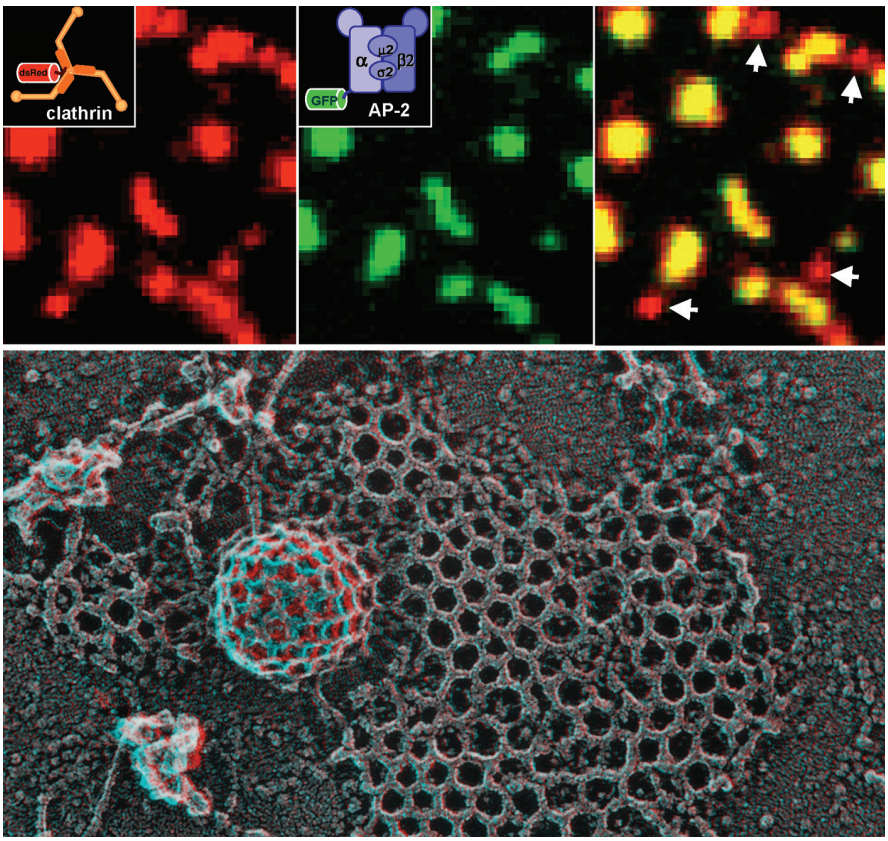

Figure 4: Imaging clathrin and AP-2 in living cells. HeLa cells were transfected with dsRed-clathrin and GFP-α-adaptin and observed by TIR-FM. Arrows point to clathrin spots that are devoid of AP-2. Stereoview of a clathrin-coated area of the plasma membrane of unroofed fibroblasts showing partial budding of flat clathrin lattice (kind gift of J. Heuser, see [38] for details). The bottom image can be viewed in 3-D using red/blue anaglyph glasses.

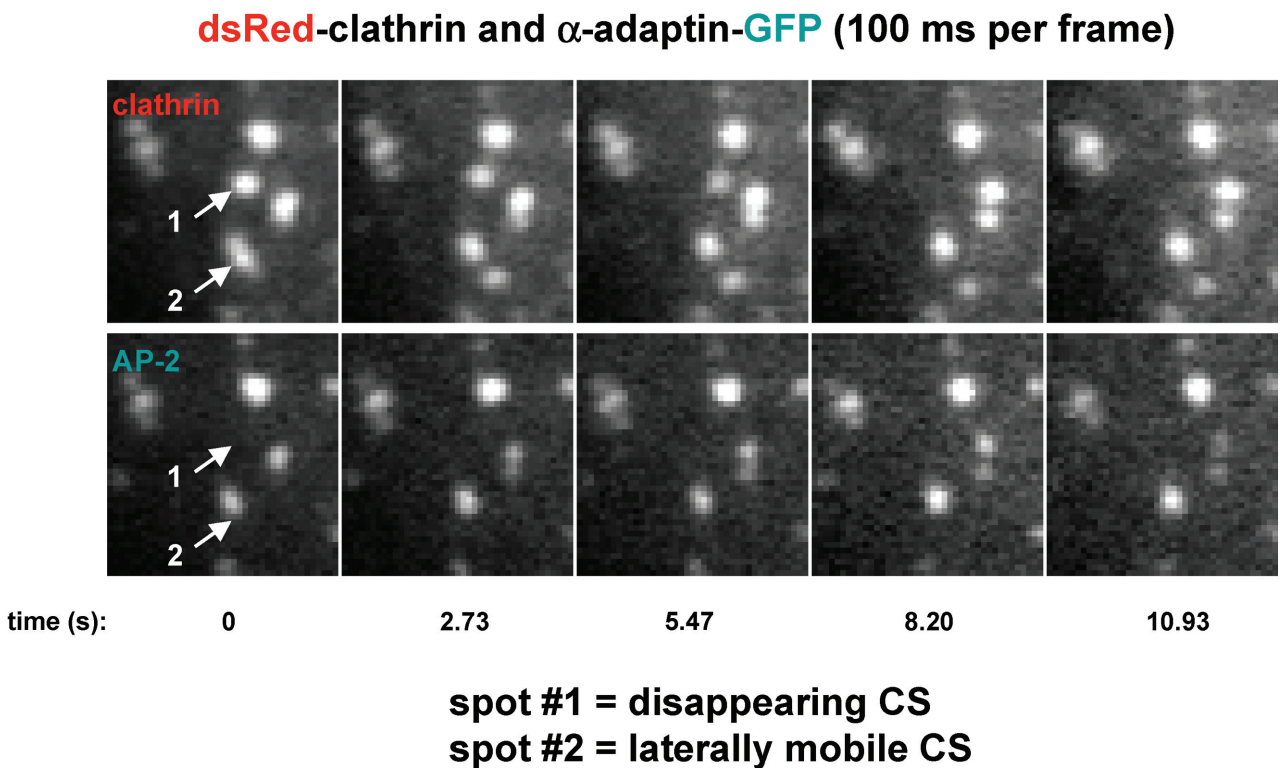

Figure 5: AP-2 is absent from disappearing and LM-CS. HeLa cells were transiently transfected with dsRed-clathrin and α-adaptin-GFP constructs and observed by TIR-FM 40 h later. Spots #1 and 2 denote disappearing and laterally mobile CS, respectively.

presented in Figure 1 fits with data showing that disappearing clathrin spots may emerge from static clathrin/AP-2 puncta at some point before internalization (13). Additionally, this model is consistent with the classical electron microscopy images obtained by J. Heuser (Figure 4) showing that budding vesicles often emerge from the periphery of flat clathrin lattices ((38), and personal communication). A conclusion that the disappearing population of clathrin spots does not contain AP-2 immediately prior to internalization implies the active and static clathrin populations may represent separate entities. Finally, in the case of cargo, the fact that activated GPCR/β-arrestin complexes are recruited to static preexisting CS (39,40) suggests that at least in this case they can correspond to preexisting platforms for cargo selection.

Although these latter steps would proceed by an unknown mechanism, they bear some resemblance to sorting events in endosomes mediated by Hrs-dependent endosomal flat clathrin-coats, also called bilayered clathrin-coats (reviewed in (41)). It has been shown that activated growth factor receptors are specifically accumulated within the bilayered clathrin coats before being transferred into vesicles that bud inward from the endosomal lumen (Figure 3). The mechanisms responsible for the transfer of the cargo to the nascent vesicle could be similar in these two very different events, topologically speaking. The parallel between them is also striking, concerning the formation of the vesicles; it has been observed that vesicles budding inside the endosomal lumen emerge at the periphery of the flat clathrin coat (42–44).

How can these data be reconciled with the enrichment of AP-2 in CCV preparation? Most of the AP-2 found in CCV preparation could be due to broken CCPs and/or artificially assembled clathrin cages. Finally, the forming of stable platforms at the plasma membrane by AP-2 complexes could provide a plausible molecular clue to how clathrin spots appear to form in restricted areas of the plasma membrane (13). However, the term 'stable' only accounts for the overall structure, i.e. a clathrin-coated region of the plasma membrane, not for individual molecules since FRAP (Fluorescence Recovery After Photobleaching) studies showed that plasma membrane-associated AP-2 and clathrin both rapidly exchange with cytosolic pools (36, 45). These latter very interesting results suggest that CCPs are stable overall structures undergoing constant remodeling. They also have interesting consequences for the mechanism of the uncoating reaction (see discussion in (45) for details). Alternatively, it is possible that only the GFP-fusion proteins of α-adaptin are excluded from the nascent clathrin-coated vesicle.

If AP-2 is excluded, are there alternative adaptor(s) for active clathrin spots? What links clathrin lattices to the receptors and cell adhesion molecules present in the vesicle

membrane? A number of the recently identified accessory proteins are potential candidates since they are able to bind both phospholipids and clathrin, a prerequisite for adaptor function. This list includes epsin, CALM, amphiphysin, Hip1R, ARH and Dab2. Hip1R was already shown to follow clathrin and disappear in the same time (46). The presence of Hip1R, as well as dab2 and ARH, in disappearing CS has been recently confirmed using TIR-FM (20). In that context, it will also be interesting to follow Eps15 and Eps15R that only bind AP-2 and not clathrin (47–49), as, according to our hypotheses, these two should be only present in static spots.

Laterally Moving Spots?

Another advantage of sensitive techniques such as TIR-FM that permit the analysis of individual events, structures and potentially molecules is the ability to focus on minority populations. The benefits of this were recently demonstrated by the characterization of laterally motile clathrin spots (LM-CS). Although the population of spots observed to move laterally parallel to the plasma membrane represents the minority (2–8% depending on the cell type) of CS observed by TIR-FM, three independent analyses have confirmed their existence and frequency (13,20,22). These spots often seem to originate from preexisting static CS, where they form as relatively dimmer concentrations that move quite fast in linear trajectories often toward another preexisting static CS. The linear trajectories (~2.5 μm) and the speed (0.5–1 μm/s) of these motile spots are compatible with their movement along microtubules. Both simultaneous imaging with microtubule markers and the complete elimination of this population of CS by nocodazole treatment confirmed this hypothesis (22).

What do these LM-CS correspond to? These structures could correspond to the microtubule-dependent transport of preformed flat clathrin lattices from one CCP to another and/or nascent CCVs moving along microtubules prior to uncoating. But plasma membrane-associated clathrin adaptors such as AP-2, ARH, dab2 and Hip1R were not found in the LM-CS, suggesting that they are not derived from plasma membrane CCPs (20,34). Finally, they could correspond to clathrin-coated buds on endosomes localized very close to the plasma membrane (Figure 3). Indeed, the movement of LM-CS along microtubules (22) is in agreement with their endosomal origin since microtubules are involved in the movement and fusion of these organelles *in vivo* (50). Several different types of clathrin coats have been characterized on both early and late endosomes with AP-1 (51), AP-3 (52) and Hrs as adaptors (53). Since both early and late endosomes could be observed by TIR-FM (18–20), each of these coats could then account for a proportion or for all of the observed LM-CS.

One striking observation is that when β2-adaptin GFP construct is expressed at very high levels, it can localize to LM-CS ((20) and our unpublished observations). These results were quite surprising since GFP-tagged α-adaptin constructs were clearly excluded from this population of CS (20,34). However, as overexpressed β2-adaptin was shown to be misincorporated into AP-1 complexes (54, 55), it was suggested that LM-CS do correspond to AP-1-containing endosomal clathrin-coated buds (20). However, direct evidence to confirm this hypothesis is still lacking. Arf inhibition with brefeldin A treatment does not abolish LM-CS (manuscript in preparation), which suggests that even if some do correspond to AP-1 clathrin coats on endosomes, they do not behave as their TGN counterparts. It may be that the LM-CS are of heterogeneous origins. Indeed, our preliminary results using a well-characterized GFP-tagged Hrs construct (43) indicate that only ~1/3 of LM-CS correspond to bilayered clathrin-coated endosomes (manuscript in preparation).

Are all LM-CS of endosomal origin? Can some be ascribed to other compartments such as TGN-derived transport intermediates? At present, a systematic analysis of the identity of LM-CS is lacking. Even if all of the sources of LM-CS in the plasma membrane region are inventoried, there is no guarantee we will understand their function(s). If some of these structures do correspond to endosomes, this would still not necessarily explain the apparent fusion and separation of mobile and static spots. An alternate hypothesis is that microtubules immediately adjacent to the plasma membrane connect CCPs and therefore forming CCVs to clathrin-coated early endosomes. Although this hypothesis is hard to envisage and it will be even harder to directly test.

Remaining Controls

One major limitation to this type of analysis is the potential effects of GFP tagging and/or overexpression. It has been shown that overexpression of some endocytic proteins actually reduces endocytosis rates (56,57). Additionally, it has recently been observed that cells expressing very high amounts of β2-adaptin demonstrate mislocalization and misincorporation in AP-1 complexes (see above).

In the future, it will be useful to demonstrate that all the fusion proteins employed in these studies fully retain wild type functionality. This is a critical point for the α-adaptin chimeras. They are part of a multimeric protein complex, and different constructs have seemed to generate different results. In this precise case, the generation of stable cell lines would be required to check very important points such as the impact of fusing the GFP at the *N*-terminus on PIP2 binding (58) and the impact of fusing the GFP on the ear domain on the binding of all the accessory proteins such as Eps15, epsin or CALM (59). The use of stable cell lines would also allow more detailed analysis of the resulting AP-2 complexes. This may allow the determination of

whether GFP-tagged α-adaptin chimeras effectively incorporate into complexes, replacing a proportion or all of the endogenous protein.

The use of well-characterized stable cell lines would definitively eliminate the possibility that upon transient overexpression, monomers of GFP-tagged adaptin constructs could exist, even temporarily, outside of AP-2 complexes, therefore being responsible for some of the observed results. Indeed, in the case of greatly overexpressed GFP-β2, this could explain its colocalization with all clathrin spots, since β2-adaptin does contain an efficient clathrin-binding site (60) that could account for its general incorporation in all clathrin coats (static, disappearing, laterally moving CS). In addition, stable cell lines could be useful for complementation experiments after knock-down of endogenous α-adaptin using siRNA. However, the high degree of conservation among mammalian α-adaptins will make it difficult. Another possibility will be to complement *Drosophila* and/or *Dictyostelium* strains containing mutations or deletion in the corresponding genes.

Finally, it is important to mention a few caveats regarding the acquisition and analysis of live cell microscopy data. As in any case where multiple fluorophores are simultaneously visualized, one must take great care to ensure that bleed-through from the emission of one fluorophore into the recording channel for the second fluorophore does not produce misleading results. Each of the organic fluorophores emits over a broad region of the spectra and it is difficult to resolve them clearly. There is the further complication from fluorophores, such as dsRed, which mature over time, changing their emission spectra. For example, early in the expression of a dsRed tagged protein, much of the emission will be observed in the GFP channel. Additionally, the intertwined issues of exposure time, frame rate and illumination are of great concern to all who would image apparent acts of endocytosis. On one hand, one must make sure that enough excitation and emission is employed so that streams of data are temporally robust enough for analysis of the dynamic behaviors of individual puncta (in our experience 100–300 ms per frame in streaming mode). Most importantly, though, photobleaching will occur and it is imperative that the investigator remain constantly vigilant to the potential pitfall of misscoring events of internalization.

Conclusions

The dynamic analysis of CCP-associated proteins will be a very useful tool to develop for better understanding the rules of CCP and CCV formation, as it has been the case for many complex cellular processes (reviewed in (61)). The initial studies have already confirmed some points of existing models (e.g. the involvement of dynamin in vesicle formation). They have also brought some definitive answers to old questions, such as the demonstration that activated receptors are recruited to preexisting CCPs, and have raised some very interesting new ones, such as the origins of the apparent heterogeneity within the populations of clathrin-positive structures in close proximity to the plasma membrane. These recent results demonstrate the power of direct observation and confirm that experiments performed on live cells can provide unexpected clues to better understand how things work *in vivo*.

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