Watching real-time endocytosis in living cells

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The precise sequence of events promoting clathrin-coated vesicle assembly is still debated. In this issue, Kadlecova et al. (2017. *J. Cell Biol.* https://doi.org/10.1083/jcb.201608071) test structural models using quantitative microscopy in living cells to investigate the hierarchy and temporal importance of molecular events required for clathrin-coated pit initiation.

In eukaryotic cells, the majority of internalized transmembrane protein receptors and ligands enter by clathrin-mediated endocytosis (CME). Clathrin-coated vesicles (CCVs) assemble at the plasma membrane by forming clathrin-coated pits (CCPs) that mature into fully formed coated vesicles and undergo scission via the large GTPase, dynamin. The clathrin adaptor complex AP2 (a heterotetramer made up of α, β2, μ2, and σ2 subunits) is a well-studied key regulator of CME, important for the recruitment of both cargo into CCVs and clathrin itself. AP2 directly engages short amino acid motifs found in protein cargoes: the μ2 subunit binds YXXΦ motifs (Φ is a bulky hydrophobic residue; Owen and Evans, 1998), and σ2 binds acidic dileucine ([E/D]xxxL[L/I]) motifs (Kelly et al., 2008). AP2 also binds clathrin via a clathrin box motif in the β2 hinge (ter Haar et al., 2000) and a variety of accessory proteins through C-terminal domains of α and β2 subunits (Owen et al., 1999).

Models based on x-ray crystal structures (Collins et al., 2002; Jackson et al., 2010) revealed how AP2 assumes at least two conformations. In the cytoplasm, AP2 adopts an autoinhibited closed state with obstructed cargo binding sites that prevents futile clathrin binding (Fig. 1 A; Collins et al., 2002). At the plasma membrane, AP2 undergoes a conformational change to an open state that is able to bind cargo (Jackson et al., 2010). Structure-based mutagenesis combined with surface plasmon resonance experiments (Höning et al., 2005; Jackson et al., 2010) suggest the conformational change is triggered when AP2 binds phosphatidylinositol-4,5-bisphosphate (PIP2) head groups in the membrane. Further structural work (Kelly et al., 2014) revealed how the conformational change expels a short motif in the unstructured β2 hinge that recruits clathrin, which then polymerizes to form a scaffold around vesicles. The coplanar arrangement of the PIP2 and cargo binding sites on AP2 directs it, and thus clathrin polymers, to cargo-enriched sites on the membrane. This model suggests AP2 allostery is sufficient to drive CCP initiation and growth. Other data indicate that CCP initiation requires nucleation sites for AP2 formed by complexes of Eps15 with FCHo (Ma et al., 2016), which is itself hypothesized to serve as an AP2 allosteric activator (Hollopeter et al., 2014).

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Although these structural models are appealing, they have not been directly tested in real time in living cells. In this issue, Kadlecova et al. have now conducted a technically excellent and important study that demonstrates functional hierarchy in binding events. The authors first generated stable cell lines with highly specific structure-based point mutations. This approach allows cells to be defective in binding phosphoinositide or cargo without affecting other functions. They used total internal reflection fluorescence microscopy to quantify in real time the extent to which PIP2 and cargo binding sites on AP2 affect the dynamics of CCP initiation, formation, and stabilization. Their methods allow quantitative detection of very transient clathrin-labeled structures, as well as both abortive and productive CCPs. This allowed them to verify structural predictions and furthermore provided key additional insights into the role of AP2 in coated pit initiation and stabilization.

The authors first investigated the role of PIP2 binding by the α and β2 subunits (Fig. 1 B). Previous studies probed the role of PIP2 binding in CCV formation through methods like bulk depletion of PIP2; this study takes a less disruptive approach by specifically targeting AP2-dependent CME and allows the authors to dissect the role of each PIP2 binding site. Previous data suggested that both the α and β2 PIP2 binding sites were paramount for initiating the conformational change; both in vitro and cell-based data supported the importance of the α binding event in particular (Collins et al., 2002; Höning et al., 2005). Kadlecova et al. (2017) confirmed that the α-PIP2 binding site is required for both nucleation and downstream events; cells with AP2 lacking the α binding site also yielded many fewer productive CCPs and a slower rate of clathrin polymerization. The PIP2 binding site on β2 was tested directly here for the first time in cells. The AP2 mutant lacking the β2 binding site produced the same phenotype as the α mutant, suggesting both sites are equally important. The authors used electron microscopy to show that these mutants have an increased number of small, flat CCPs. These data suggest that PIP2 binding by α and β2 is required beyond the earliest stages of CCP formation, in contrast to recent work that suggests clathrin assembly is sufficient to drive CCP formation after nucleation (Dannhauser and Ungewickell, 2012). Full allosteric activation of AP2 is necessary for rapid clathrin polymerization and efficient CCP production, and AP2 appears to require both α and β2 phosphoinositide binding sites.

The authors next tested the μ2 PIP2 binding site (Fig. 1 C). Based on structural work, this site was hypothesized to be accessible after the conformational change induced by membrane

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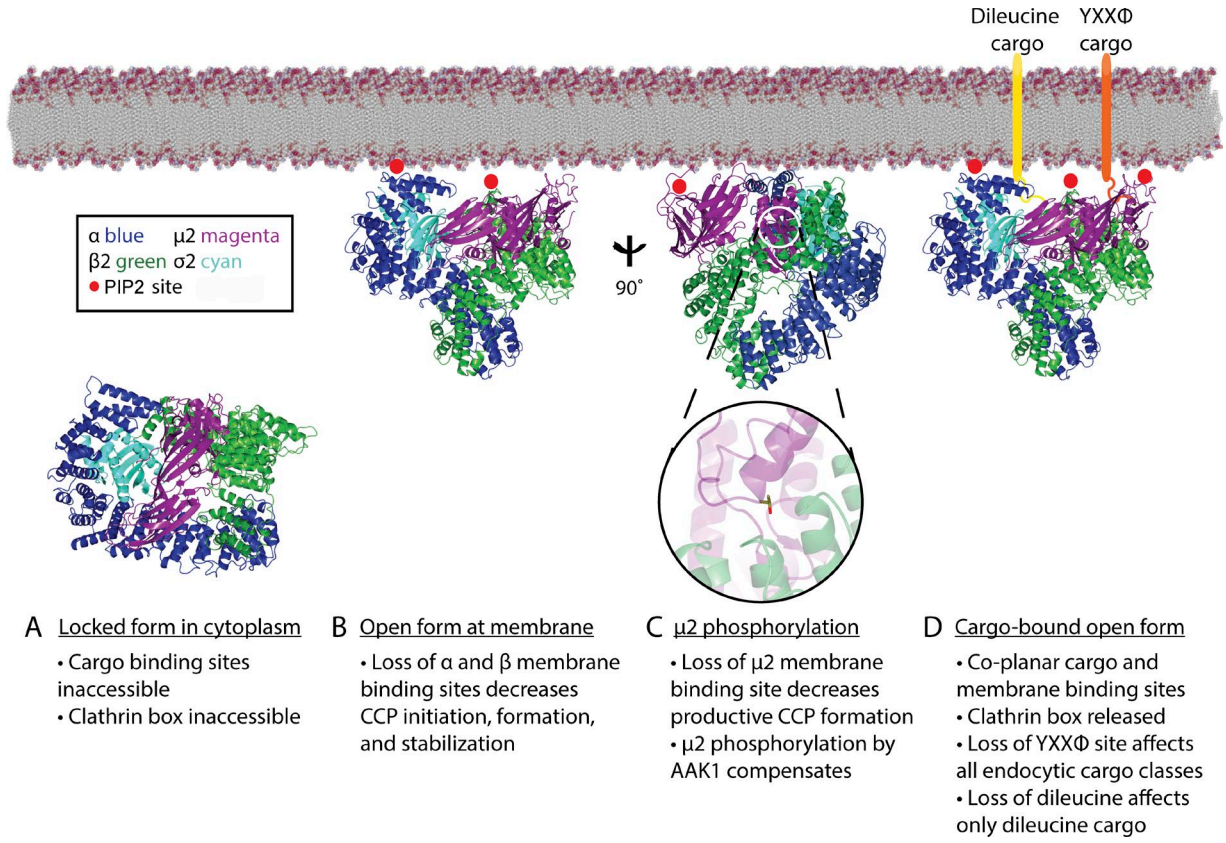

Figure 1. **Effects of AP2 structure-based mutagenesis in CME of living cells.** (A) Structural models suggest AP2 exists in a closed form in the cytoplasm, preventing futile interactions with clathrin in the absence of membrane-embedded cargo. (B) The authors demonstrate that AP2 recruitment to the plasma membrane is dependent on the PIP2 binding sites on the α and β2 subunits (shown as red filled circles), as predicted by x-ray crystal structures and biophysical data. When these sites are absent, coated pit initiation and stabilization is significantly decreased, as are productive vesicles. (C) Loss of the PIP2 binding site on μ2 (red filled circle) increases coated pit initiation events but decreases the number of productive structures. The authors suggest that phosphorylation of μ2 Thr156 by AAK1 compensates for loss of the PIP2 binding site. (D) At the plasma membrane, all phosphoinositide and cargo binding sites are coplanar on the same surface of AP2, and the clathrin box motif is released. The authors show that loss of the YxxΦ binding site (orange cargo) results in decreased uptake of all classes of endocytic cargo, whereas loss of the dileucine site (yellow cargo) affects only dileucine cargo.

binding, which suggests that it functions downstream of the α and β2 sites. In contrast to their findings for the other PIP2 sites, Kadlecova et al. (2017) observed increased CCP initiation rates and increased rates of clathrin recruitment in the μ2 mutant but did not see significant changes in early nucleation markers. However, these increased rates did not correspond to an increase in productive CCVs; the structures were short lived and flat. The authors postulate that a compensatory mechanism accounts for these results. μ2 is phosphorylated on Thr156 by the kinase AAK1, which is stimulated by clathrin assembly (Jackson et al., 2003). μ2 phosphorylation enhances its affinity for cargoes (Ricotta et al., 2002) and is proposed to stabilize the open conformation (Jackson et al., 2010), but the structural basis for this remains unclear. Although the authors could not fully eliminate phosphorylation on μ2 Thr156, they demonstrated that μ2 mutants lacking PIP2 binding sites exhibit increased phosphorylation levels. They propose that increased rates of CCP initiation and clathrin recruitment likely result from increased phosphorylation of μ2 by AAK1 (Fig. 1 C).

Finally, the authors addressed the importance of cargo binding by AP2 for the first time in living cells. Structural models of AP2 suggest that binding both PIP2 and cargo motifs in the membrane is vital for promoting continued CCP growth and productive vesicle formation. Conceptually, it makes sense that cargo binding should constitute an early event in vesicle formation. The primary purpose of trafficking is to move cargo between membrane-bound organelles, and cells surely must decide early (i.e., before expending significant energy) whether correct cargoes have been incorporated into vesicles. Although there is consensus that cargo binding stabilizes AP2 at the membrane, whether or not cargo binding is necessary for CCP nucleation has been debated (Godlee and Kaksonen, 2013). To address this question, the authors specifically tested the importance of YxxΦ and dileucine binding sites (Fig. 1 D) and provide some of the most interesting data in the paper.

Instead of manipulating individual cargoes, the authors globally eliminated either the YxxΦ or dileucine cargo binding sites by introducing structure-based point mutations in μ2 or σ2, respectively, and looked at the effects on model CD8 reporter cargoes. Surprisingly, loss of the μ2 binding site strongly inhibits endocytosis of both YxxΦ- and dileucine-based cargoes, as well as other classes of endocytic cargoes (FxNPxY motif–containing cargoes like LDL receptor or EGF receptor). In contrast, loss of the dileucine binding site on σ2 does not affect internalization of other cargoes. The μ2 cargo-defective mutant also mimics the α and β PIP2-defective mutants in terms of decreased CCP initiation rates and clathrin recruitment. The σ2 cargo-defective mutant does not influence initiation rates or clathrin recruitment, and overexpressing dileucine model cargoes cannot rescue defects in μ2 cargo-binding mutants. These

data demonstrate that AP2 broadly requires multiple PIP2 binding sites, as well as the μ2-YxxΦ binding site, to nucleate CCPs efficiently at the membrane.

This study raises several interesting and important questions. First, what is the significance of the proposed "functional hierarchy" of cargo binding events? Observed differences between μ2 and σ2 cargo-defective mutants could be explained by structural considerations. The dileucine binding site on σ2 becomes accessible in a partially open conformation of AP2 called the unlatched conformation (Kelly et al., 2008) so dileucine cargo could bind a partially open, though not fully activated, AP2. But how would cells effectively endocytose dileucine cargoes if AP2 does not transition to a fully open form that can recruit clathrin? Nearby phosphorylation events have been shown to enhance the interaction between AP2 and dileucine motifs or to decrease the affinity of AP2 for YxxΦ motifs. Perhaps AP2 can adopt a range of "cargo-competent" conformations based on cellular needs. The authors find that YxxΦ motifs are much more commonly found in human plasma membrane receptors than are dileucine motifs. In vertebrates, this may imply that binding and recruiting YxxΦ cargoes play key roles in initiating CCP formation and recruiting other classes of cargo at the plasma membrane. The situation is different in yeast: AP2 is dispensable for endocytosis, and there are few YxxΦ cargoes. Although dileucine-based cargoes have been identified for related AP complexes in yeast (AP1 and AP3), this motif does not seem to be important for internalizing most endocytic cargoes. Have these two cargo binding sites, especially the YxxΦ site, evolved in vertebrates to promote a more active or stable AP2 that can mediate rapid endocytosis at the cell surface, as required for key physiological processes like neurotransmission? The role and relevance of cargo binding sites for AP2 function may partly explain some of the differences observed between yeast and mammalian CME.

A second outstanding question is the molecular mechanism by which μ2 phosphorylation affects clathrin coat assembly. μ2 phosphorylation on Thr156 increases the rate of transferrin uptake, and the authors show here that loss of PIP2 binding by μ2 leads to increased phosphorylation events. How does AP2 sense that its open form has been destabilized by losing a membrane binding site? One possibility is that AAK1 binds preferentially to open-form AP2 at the membrane to stabilize the complex. This could be especially important in the absence of a membrane binding site on μ2, as if the regulatory kinase were trying to stabilize the open form to promote cargo binding and productive vesicle formation. Structural data on this key event will aid our understanding of the molecular basis of regulation by phosphorylation.

Finally, this work provides key insights into how multiple low-affinity interactions contribute in a hierarchical manner to promote efficient vesicle formation in living cells. It raises questions about how related AP complex family members function at different membranes. Although AP2 is recruited to the plasma membrane primarily by binding PIP2, other members of the AP family are recruited by the small GTPase Arf1 in its GTP-bound state. AP1 is a clathrin adapter that forms CCVs at the Golgi. Would loss of Arf1 binding sites on AP1 alter CCP initiation and assembly rates in the same way as AP2 mutants that cannot bind PIP2? How different might the steps of nucleation and growth look in vesicles that are coated by non-clathrin AP coats (e.g., AP4 and AP5)? Recent advances in high-resolution microscopy should make it feasible in the future to probe a variety of these interesting and critical events that take place further away from the cell periphery.

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