Kinases in clathrin-mediated endocytosis

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Abstract
The process of clathrin-coated vesicle (CCV) formation/disassembly has been studied intensively, and numerous proteins have been identified which aid this process. We have learnt a great deal about individual components of the CCV machinery, and now the ultimate aim is to elucidate the mechanisms regulating clathrin-mediated trafficking. One of the fundamental processes governing the complicated network of interactions is phosphorylation. It has been known for some time that several proteins associated with clathrin-coated vesicles are substrates for protein kinases. These proteins include clathrin, three of the four adaptor complex subunits, dynamin 1, synaptojanin 1 and the amphiphysins. However, the identities of the kinases involved in this process remained largely unknown until recently. This short review discusses advances in our knowledge of how CCV formation/disassembly is regulated by the phosphorylation/dephosphorylation cycle and the role played by specific protein kinases in that process.

Introduction to clathrin-mediated endocytosis (CME)
CME is a major vesicular transport mechanism in eukaryotic cells. It is designed to allow the internalization of nutrients, hormones and other molecules from the plasma membrane into intracellular compartments. The carrier vesicles of this pathway are designated clathrin-coated vesicles (CCVs), as they are encapsulated into the cage formed by the interaction of clathrin molecules. The clathrin molecule is called a triskelion, and consists of three heavy chains each of which is bound to one of two light chains (LCa and LCb). A central feature of clathrin triskelia is their ability to assemble spontaneously into polymers that play a pivotal structural role in CCVs (reviewed in [1,2]).

Receptors and their ligands are recruited into CCVs forming at the plasma membrane. This process is facilitated by specific adaptors that have the bivalent capacity to interact with both cytosolic domains (‘tails’) of receptors and clathrin. The main adaptor molecule of CME is the heterotetrameric assembly protein designated adaptor protein-2 (AP-2). AP-2 consists of two heavy (α and β2) subunits (which interact with clathrin), a medium β2 subunit (which binds to some sorting signals of internalized receptors) and a small σ subunit. AP-2 recognizes a variety of receptors that contain a tyrosine-based YXXΦ motif (where Φ denotes a bulky hydrophobic residue; e.g. transferrin receptor), a dileucine-based motif (e.g. CD3-γ) or an NPXY motif (e.g. low-density lipoprotein receptor) in their cytosolic tails (reviewed in [1,2]). In addition, other proteins such as monomeric β-arrestins can also act as adaptors. Arrestins are linker proteins for the sequestration of ligand-activated G-protein-coupled receptors (GPCRs) into clathrin-coated pits [3] (clathrin-coated pits form as a precursor to CCVs). Generally, endocytic CCVs should be viewed as a heterogeneous pool of vesicles that vary significantly in composition, but use a universal protein coat.

In addition to the main structural components, CCVs contain many accessory proteins that play various roles at different stages of vesicle formation and disassembly. Thus, for example, AP-180/CALM (adaptor protein-180/clathrin assembly lymphoid myeloid leukaemia protein), amphiphysin and synaptojanin are thought to regulate clathrin and adaptor assembly [4–6], epsin has recently been implicated in the process of membrane invagination [7], dynamin, amphiphysin and endophilin I participate in the fission reaction [8–11], while Hsc70, auxilin and synaptojanin play a role in CCV uncoating [12,13]. All of these, and the other proteins implicated in CME, are interconnected by a complicated network of interactions that ensure that CCV assembly and disassembly are well-orchestrated processes.

Regulatory role of kinases
Phosphorylation is one of the fundamental mechanisms governing interactions in cells. The CCV cycle does not seem to be an exception, since many of the core and accessory proteins of the CCV machinery are phosphorylated reversibly in vivo [14–17].

One can classify phosphorylation events in CCVs into two functionally opposite classes: (i) those that are inhibitory for CCV formation (‘negative phosphorylation’) and (ii) those that facilitate the assembly of CCVs (‘positive phosphorylation’). Probably the best characterized example to date of negative regulation by phosphorylation is phosphorylation in the hinge region of the α and β2 subunits of the AP-2 complex. Phosphorylated AP-2 has a reduced ability to interact with clathrin and requires dephosphorylation for efficient interaction [14,19]. Clathrin LCs (primarily LCb) are also subject to phosphorylation both in vitro and in vivo.

Key words: adaptor protein-2 (AP-2), clathrin-coated vesicle, clathrin-mediated endocytosis, kinase, phosphorylation
Abbreviations used: CCV, clathrin-coated vesicle; CME, clathrin-mediated endocytosis; LC, clathrin light chain; AP, adaptor protein; GPCR, G-protein-coupled receptor; GAK, cyclin G-associated protein kinase; AAK, adaptor-associated kinase; EGF, epidermal growth factor

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and are thought to serve as regulatory subunits [14,18]. The very limited data on this suggest that the phosphorylation of LCs could be inhibitory for coat assembly [15,19]. Phosphorylation also appears to be important in modulating the function of several other proteins implicated in endocytosis; these include dynamin 1, amphiphysins 1 and 2, AP-180, synaptotagmin, epsin and Eps15 (collectively called dephosphophins). These proteins are found in the phosphorylated state in resting nerve terminals, and are dephosphorylated when stimulation invokes a burst of CCV formation [17]. It was shown that phosphorylation of dynamin 1 and synaptotagmin inhibits their binding to amphiphysin, while phosphorylated amphiphysin has an impaired affinity for AP-2 and clathrin [20].

In contrast with the bulk of CCV-associated proteins (where phosphorylation appears to correlate with disassociation of the coat from the vesicle), phosphorylation of the μ2 subunit is an example of positive regulation in the CCV cycle. When phosphorylated on its medium subunit, AP-2 has a 25-fold higher affinity for tyrosine-containing sorting motifs in the cytosolic tails of receptors and an increased affinity for lipid membranes [21,22]. Recent structural data on AP-2 suggest a possible molecular explanation for these observations. Apparently, the binding site in μ2 for the tyrosine-containing motif is buried in the AP-2 complex; thus a conformational change is required for its exposure. This could be triggered by phosphorylation of μ2 [23].

Tyrosine phosphorylation of the clathrin heavy chain in its hub domain by Src-family kinases is another example of positive phosphorylation. It was shown that ligand binding to the epidermal growth factor receptor leads, via activation of protein kinase activity, to clathrin phosphorylation. This phosphorylation enhances clathrin recruitment to the plasma membrane [24]. Enhanced interaction with CCV components is also observed during internalization of ligand-activated GPCRs. In this case, ligand binding leads to phosphorylation of the GPCR, which is then recognized by β-arrestins [3].

### Identification of CCV-associated kinases

It has been known for many years that kinase activities could be found in purified preparations of CCVs. Several groups attempted to identify the kinases responsible for the phosphorylation events mentioned above; this information is presented in Table 1. Thus it was shown that CK2 (formerly known as casein kinase 2) plays an essential role in endocytosis, as inhibition of CK2 leads to a significant decrease in transferrin uptake [25]. CK2 is highly enriched in CCV preparations and phosphorylates LCb in vitro [18]. As mentioned above, LCb is also found to be phosphorylated in vivo, but the functional role of this phosphorylation event in endocytosis is not entirely clear at present. It was also found that CK2 phosphorylates the α and β subunits of the AP-2 adaptor complex in vitro [26]. It is possible that CK2 plays the same role in vivo. In vitro phosphorylation of AP-2 by CK2 prevents AP-2 from binding to clathrin cages, thus mimicking the effect observed in vivo [14].

There is also evidence that a kinase other than CK2 may phosphorylate AP-2 in vivo. This comes from the observation that β2-adaptin was phosphorylated even in the presence of a CK2 inhibitor [27]. It is therefore possible that more than one kinase is responsible for phosphorylating β2-adaptin. Additional studies are required to ascertain the identity of the kinase(s) phosphorylating the α and β subunits of AP-2 in vivo.

CK2 may phosphorylate a number of other targets implicated in endocytosis; these include AP-180, dynamin and synaptotagmin [4,16,28]. These phosphorylation events can

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**Table 1** | Proteins involved in CME and kinases regulating their function

<table>
<thead>
<tr>
<th>Protein</th>
<th>Kinase(s) functional in vitro</th>
<th>Kinase(s) functional in vivo</th>
<th>Possible role of phosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clathrin LC</td>
<td>CK2 [18]</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>α-Adaptin</td>
<td>CK2 [26]</td>
<td>α2-adaptin</td>
<td>Inhibition of clathrin binding [14,19]</td>
</tr>
<tr>
<td>β2-Adaptin</td>
<td>CK2 [26]</td>
<td>β2-adaptin</td>
<td>Inhibition of clathrin binding [14,19]</td>
</tr>
<tr>
<td>μ2-Adaptin</td>
<td>AAK1 [31,21], GAK [29,26]</td>
<td>Unknown</td>
<td>Facilitates receptor and membrane binding [21,22]</td>
</tr>
<tr>
<td>Amphiphysin</td>
<td>CK2 [4], cdk5, cdk2 [32]</td>
<td>Different from PKC [17], cdk5 and cdk2 [32]</td>
<td>CK2 inhibits interaction with AP-2 and clathrin [4,20]</td>
</tr>
<tr>
<td>Dynamin</td>
<td>PKC, CK2 [16], MAPK [33], cdk2 [34]</td>
<td>PKC [16,17]</td>
<td>Inhibits interaction with binding partners [20]; PKC stimulates GTPase activity [16]</td>
</tr>
<tr>
<td>Epsin</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Inhibits interaction with binding partners [13]</td>
</tr>
<tr>
<td>Eps15</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Inhibits interaction with AP-2 [13]</td>
</tr>
<tr>
<td>Synaptotagmin</td>
<td>CaMKII, PKC, CK2 [28]</td>
<td>CaMKII, PKC [28]</td>
<td>Probably inhibitory [28]</td>
</tr>
</tbody>
</table>

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probably be classified as ‘negative’ (ones that inhibit CCV formation), so the presence of an active kinase on a CCV would be harmful to the integrity of that CCV. Therefore it is significant that we have found CK2 to be inactive on intact CCVs, but active after uncoating of CCVs [26]. The inactivity of CK2 when associated with CCVs is due to inhibition by an unknown component of lipid membranes. Such inhibition might be a regulatory mechanism for preventing unwanted phosphorylation of CCV components [26]. The molecular mechanisms regulating the activity of CK2 remain to be elucidated.

Another kinase activity found in purified CCVs is directed towards the medium (µ2) subunit of the AP-2 adaptor complex. The identity of the kinase that phosphorylates µ2 remained obscure for a long time. Recently, two candidate kinases have been described in the literature [21, 26, 29–32]. One of them, termed GAK (for cyclin G-associated kinase), was shown to phosphorylate both µ1 (the medium-chain subunit of the AP-1 adaptor complex that functions in clathrin-coated vesicular transport between the trans-Golgi network and endosomes) and µ2 recombinant proteins in vitro [29]. It was also demonstrated that GAK co-purifies with CCVs, and that immunoprecipitated GAK can phosphorylate the endogenous µ2 subunit in vitro [26].

GAK is a complex protein; in addition to an N-terminal kinase domain, it contains a central tensin homology domain and a C-terminal J domain. This C-terminal domain shows similarity to auxilin, a neuronal protein that facilitates Hsc70 in the uncoating of CCVs. Hence an alternative (and possibly more appropriate) name for GAK is auxilin 2 [30]. GAK was shown to bind directly to both clathrin and the appendage domain of α-adaptin [29]. GAK/auxilin 2 is localized mostly to the trans-Golgi network (in HeLa cells at least). However, its overexpression completely prevents transferrin uptake [29, 30], implying a role for GAK/auxilin 2 in endocytosis. Given the multifunctional nature of GAK/auxilin 2, it is presently difficult to conclude whether the effect of GAK/auxilin 2 overexpression on endocytosis is due to its role in clathrin uncoating or µ2 phosphorylation.

Another kinase, called AAK1 (for adaptor-associated kinase 1), is also capable of phosphorylating the µ2 subunit of AP-2. AAK1 was identified using a phage display library screening strategy [31]. Just like GAK/auxilin 2, AAK1 is enriched in purified CCV preparations and phosphorylates the µ2 subunit of AP-2 in vitro [31]. Importantly, the phosphorylation site for AAK1 in µ2 (Thr-156) is identical to the one used by the endogenous kinase [21]. AAK1 is composed of three domains: an N-terminal serine/threonine kinase domain that is 40% identical to the kinase domain of GAK/auxilin 2, a QPA-rich domain and a C-terminal α-adaptin-interacting domain that contains both adaptin- and clathrin-interacting motifs. In cultured cells AAK1 co-localizes with AP-2 in sites that show active endocytosis, and overexpression of AAK1 in HeLa cells selectively inhibits transferrin uptake [31].

An intriguing question concerns the relationship between GAK/auxilin 2 and AAK1 kinase activities in endocytosis. It is possible that the kinases act simultaneously, or that their functions might be temporally and/or spatially separated. Co-localization and direct interaction of GAK/auxilin 2 with the AP-1 adaptor complex [29] also hint at a possible role for GAK/auxilin 2 in transport from the trans-Golgi network.

**Other kinases in CCVs**

Various kinases have been suggested to be present in CCVs, and several of the peripheral membrane proteins associated with CCVs have been shown to be substrates for a range of different kinases (e.g. see Table 1). However, it is beyond the scope of this short review to go beyond the major kinases that appear to be involved, i.e. CK2, GAK/auxilin 2 and AAK-1.

**A model for the roles of CK2, GAK/auxilin 2 and AAK-1 in CME**

Figure 1 illustrates the possible role(s) of GAK/auxilin 2 and AAK-1 in the CCV assembly/disassembly cycle. Although very little is known about the precise timing of the action of the different kinases, it is possible to speculate that the µ2 subunit of AP-2 is phosphorylated immediately prior to the adaptor complex interacting with a tyrosine-based motif in a receptor protein, thus facilitating this interaction. µ2 is predicted to remain phosphorylated during the life of the CCV. Dissociation of clathrin then triggers release of GAK/auxilin 2 and/or AAK-1 from the CCV. This leads to dephosphorylation of µ2 and the subsequent release of AP-2 from the CCV membrane. Newly formed CCVs would also incorporate CK2 in an inactive, membrane-bound form that prevents premature phosphorylation of several components of the CCV machinery and subsequent disassembly. Uncoating of clathrin would immediately release CK2 from the inhibition imposed by the CCV membrane and allow CK2 to phosphorylate its diverse CCV peripheral membrane protein substrates. This phosphorylation would render the relevant CCV peripheral membrane proteins incompetent for assembly, and thus preclude re-assembly of the clathrin coat components on the membrane of the vesicle from which the coat has just been removed. Thus phosphorylation of LC8 might prevent its re-association, and phosphorylation of the heavy chains of AP-2 would lead to their release from vesicles. Subsequent dephosphorylation of these coat components in the cytosol would make them available for incorporation into newly forming CCVs at the cell surface.

**What is next?**

As is evident from Table 1, we currently have only a few pieces of the puzzle available. Many collaborative efforts are required in order to obtain more detailed insight into how the process of endocytosis is regulated at the molecular level. The identity of many kinases and phosphatases, their location and the timing of their action remain to be determined. The
mechanisms by which their activity is in turn regulated remain even more obscure.

References


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