Mechanisms of carrier formation during Clathrin-independent endocytosis

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Abstract

Clathrin-independent endocytosis (CIE) mediates the cellular uptake of many extracellular ligands, receptors and pathogens, including several life-threatening bacterial toxins and viruses. So far, our understanding of CIE carrier formation has lagged behind that of clathrin-coated vesicles. Impediments have been the imprecise definition of some of the CIE pathways, the lack of specific cargoes being transported and that of exclusive cytosolic markers and regulators. Notwithstanding these limitations, three distinct molecular mechanisms by which clathrin-independent endocytic carriers form can be defined. Cargo capture by cytosolic proteins is the main mechanism used by FEME and IL-2R endocytosis. Acute signaling-induced membrane remodeling drives macropinocytosis. Finally, extracellular lipid or cargo clustering by the Glycolipid-Lectin (GL-Lect) hypothesis mediates the uptake of Shiga and cholera toxins and receptors by the CLIC/GEEC pathway. Here, we review these mechanisms and highlight current gaps in knowledge that will need to be addressed to complete our understanding of CIE.

Trends

- Clathrin-independent endocytosis (CIE) is involved in several key cellular processes, by mediating the uptake of many receptors and pathogens, although few cargoes use a single pathway exclusively.

- Clathrin-independent endocytic pathways remain poorly defined molecularly and their nomenclature is imprecise, with various routes named after carrier morphologies, cargoes, cytosolic markers or broad regulators.

- Some proposed CIE pathways have been reconsidered and their functions in endocytosis have been revised.

- CIE carriers form upon cargo capture by cytosolic proteins, acute signaling-induced membrane remodeling, or extracellular lipid or cargo clustering.

Evidence of clathrin-independent endocytosis

Endocytosis is the process of internalization of extracellular material and cell surface transmembrane proteins into cells through membrane-bound carriers. Clathrin-mediated endocytosis (hereafter, CME) is the best-characterized endocytic pathway and the dominant uptake mechanism to support housekeeping functions in cells [1,2]. Clathrin-independent endocytosis (CIE), also called non-clathrin endocytosis (NCE), exists in parallel to CME and has been reported in a wide variety of *in vitro* cell lines, as well as *in vivo* in mouse, worm, fly, plant and yeast [3-5]. Its proposed functions range from bulk extracellular protein and lipid uptake and removal of activated receptors from the plasma membrane, to the control of cell spreading, polarization and migration. Some clathrin-independent endocytic processes are faster than CME and function in physiological processes requiring quicker internalization from the cell surface, such as reaction to receptor hyper-stimulation, stress hormones ('fight-or-flight' response), chemotaxis or compensatory endocytosis following exocytosis of synaptic or hormone-containing vesicles [6]. CIE also mediates the cellular entry of over twenty viruses (including Ebola, HIV, Lassa, Herpes, Dengue and SV40 viruses), some bacteria, prions and bacterial toxins (including cholera and Shiga toxins) as well as Streptolysin O and VacA [7]. Finally, CIE may function in several diseases such as cancer, lysosomal storage disease or atherosclerosis.

The existence of clathrin-independent uptake by membrane structures with widely different size and morphology to that of clathrin-coated vesicles (which are 50-200 nm spheres) is unquestioned [3-5]. Membrane ruffles folding back onto the plasma membrane to form large (0.2 to >10 μm) endocytic vacuoles called macropinosomes were easily recognised as forming an independent portal of entry into cells. It is also clear that some cargoes (internalized receptors and/or ligands) can still enter cells when CME is inhibited [8]. However, the importance, and even the existence, of sub-micron CIE has been a topic of intense debate, with the contentions mostly focused on the activity of such pathways in non-perturbed cells [2]. The valid concern that inhibition of CME might activate pathways that would normally not contribute to uptake is supported by evidence that several cargoes are rerouted to other pathways upon perturbations [9-12]. Moreover, the capacity of clathrin-independent internalization fluxes has been reported from negligible [2] to as high as 85% of fluid uptake [13], likely due to differences in cell lines and experimental procedures (serum starvation or cold exposure), as well as in physiological states (cell confluency, nutriment status or oncogenic transformation) [12,14-17]. It is important to note that many clathrin-independent processes are not observed spontaneously in resting cells and are only activated by ligands (growth factors, cytokines, GPCR agonists), receptor cross-linking (by lectins or antibodies) or by bacterial toxins or viruses [18-21]. Temperature, membrane fluidity and tension also appear critical for some CIE pathways to operate, such as ultrafast endocytosis at synapses, fast Endophilin-mediated endocytosis (FEME) and Clathrin-independent carriers / Glycosylphosphatidylinositol (GPI)-anchored protein enriched endocytic compartments (CLIC/GEEC, hereafter CLIC) as these mechanisms do not function below physiological temperatures or upon increased membrane tension [21-24]. Part of the skepticism around CIE might also come from the revised functions of some of the proposed CIE pathways, such as caveolae and Flotillin-positive membrane nanodomains (see Text box 1).

Initially, CIE was considered to be a single, non-selective, bulk entry process also called 'pinocytosis', with the 'macro' or 'micro' prefix depending on the size of the vacuole formed [3]. Subsequently, many parallel CIE pathways were proposed, but their nomenclature is imprecise and somewhat confusing. Pathways have been named after clathrin-independent cargoes (GPI-anchored proteins, IL-2 receptor), plasma membrane markers (Caveolin-1, Flotillin, FEME), carrier morphologies (macropinocytosis, caveolae, CLICs, ADBE) or the speed of the process (ultrafast endocytosis, FEME) [3,4,6]. GTPase regulators that perturb CIE when inhibited were also used, either to define pathways (e.g. Arf6 or RhoA), or to add broad descriptions, such as Rac-, Cdc42- or dynamin-dependent or -independent endocytosis [8,13]. As this review focuses on the molecular mechanisms driving CIE carrier formation, the reader is referred to recent excellent reviews describing the various CIE pathways and their characteristics [3,4,25,26].

The molecular mechanism of CME is better understood than that of any CIE pathway because its study has been accelerated by three main factors: i) characteristic electron-dense coats visible on electron micrographs; ii) specific cytosolic markers (*e.g.* AP-2, clathrin); and iii) cargoes relying primarily on the pathway to enter cells (mostly transferrin and LDL) [1]. Virtually all cell biology knowledge on CME derives from experiments using one or more of these readouts. Amongst known CIE pathways, none so far are known to have either a specific cargo, cytosolic marker or shape of carriers. Notwithstanding these limitations, to date we know of three main molecular mechanisms by which clathrin-independent endocytic carriers form: i) cargo capture and local membrane bending by cytosolic proteins; ii) acute signaling-induced membrane protrusions folding back onto the plasma membrane; and iii) extracellular lipid or cargo clustering according to the Glycolipid-Lectin (GL-Lect) hypothesis (**Table 1**). Our current understanding of each mechanism is described herein.

Cargo capture and local membrane bending by cytosolic proteins

The most common mechanism for transmembrane protein sorting into trafficking carriers is the recognition of motifs in their cytosolic tails by cytosolic adaptors. This drives cargo capture into COPI-, COPII- and AP2/clathrin-coated-vesicles, SNX3- and SNX-BAR-retromers, SNX17-retriever, as well as AP1/3/4/5-dependent carriers [27-30]. This leads to cargo enrichment into endocytic carriers of at least one order of magnitude (e.g. TfR and LDLR into CCPs) and the exclusion of proteins lacking sorting motifs. Carrier shaping and membrane bending around cargoes is mediated upon: i) the reversible insertion of hydrophobic protein motifs (e.g. amphipathic helices in Sar1, Arf1 or Epsin); ii) the local recruitment of membrane bending domains (e.g. BAR domains of Amphiphysin or SNX proteins); and iii) scaffolding by oligomerized hydrophilic protein coating vesicles (e.g. α to ζ-COP (COPI coat), Sec23/24 Sec13/31 (COPII coat) and clathrin) [31].

Many receptors that use CIE have large cytoplasmic tails but the understanding of their sorting motifs has lagged behind that of other trafficking routes. Amongst CIE pathways, FEME uses cargo capture by cytosolic proteins as the mechanism driving carrier formation. The SH3 domain of Endophilin binds to proline-rich motifs (PRMs) present in cytoplasmic parts of the G protein-coupled receptors (GPCRs), α2a-, β1-adrenergic, dopaminergic D3 and D4 receptors and muscarinic acetylcholine receptor 4 [21]. Endophilin dimers directly bridge the membrane (through their BAR domains), cytosolic effectors and cargoes (through their SH3) (**Figure 1a**). Furthermore, Endophilin can form higher order oligomers on membrane, creating a local network of multivalent interactions. Beside GPCRs, Endophilin binds indirectly to receptor-tyrosine kinases (RTKs) through adaptor protein recognizing motifs in their cytoplasmic tails. In the case of EGFR and HGFR, only activated (*i.e.* dimerized and transphosphorylated) receptors are recognised by Cbl through its SH2 domain, which in turn is bound by CIN85-Endophilin complexes [32,33]. Endophilin binds to neurotrophic Tropomyosin receptor kinase B (TrkB) through the cytosolic adaptor Retrolinkin and mediates its uptake [34].

Unlike CCPs, which nucleate at the same time as receptors are sorted into them [35], FEME initiation sites pre-exist receptor activation (**Figure 1a**). Endophilin is concentrated into distinct patches on the plasma membrane by $PI(3,4)P_2$-binding protein Lamellipodin, which possesses at least 10 Endophilin-binding motifs [36]. In the absence of receptor activation, these patches disassemble after 5-10 s and new ones form nearby, constantly probing the membrane. Upon stimulation by cognate ligands, activated cargoes are sorted into the FEME carriers [21]. This pre-enrichment of Endophilin on the plasma membrane might mediate the rapidity of FEME to form endocytic carriers following receptor activation. The precise mechanism for the coordination of receptor sorting and membrane bending is not understood. The N-BAR domain of Endophilin can induce extensive membrane tubulation and vesicle formation at high local concentration as it contains two amphipathic helices, thereby combining protein scaffolding with hydrophobic insertion [37,38]. However, Endophilin patches in resting cells do not corresponded to membrane invaginations [21], suggesting that cargo capture either increases local Endophilin levels over the critical concentration, inducing membrane curvature, or receptor clustering itself is contributing to bending, in synergy with Endophilin N-BAR domain (**Figure 1b**). BAR-domain-mediated membrane scaffolding, actin polymerization and Dynamin activity are all required for FEME carrier scission [39]. It is probable that BAR domain-mediated friction-driven scission [39,40], combined with the SH3-mediated local recruitment of N-WASP and Dynamin contribute to FEME carrier budding.

Native, IL-2 receptors, composed of IL-2Rα (also known as CD25), IL-2Rβ and common γ chains, dimerize upon IL-2 binding [41] and enter T cells within FEME carriers [21]. However, isolated IL-2Rβ or γc chains expressed ectopically in non-immune cells cannot bind IL-2 but are constitutively internalized into cells independently of clathrin [42,43]. Even though both FEME and IL-2Rβ uptake share several regulators (Rac1, PAK1 and 2, N-WASP, PI3K and dynamin), the mechanism of cargo sorting and endocytic vesicle formation appears to be different. Several receptors including IL-2Rβ, have a WIRS motif in their cytoplasmic tails that binds directly to a surface composed of Sra1 and Abi2 in the WAVE1 complex [44,45]. Clustering of IL-2Rβ chains during anti-IL-2R antibody feeding assays (used to trigger their uptake) might therefore locally gather enough copies of WAVE1, followed by N-WASP, to induce Arp2/3-mediated actin protrusions thus forming endocytic pits around them (**Figure 1b**). PI3K recruitment by clustered IL-2Rβ, local $Pi(3,4,5)P_3$ production and Rac1-mediated WAVE and PAK-1 and -2 activation are also contributing to actin polymerization and endocytic pit formation (**Figure 1b**). Although reminiscent of macropinocytosis (which is also WAVE- and N-WASP-dependent), membrane projections around cross-linked IL-2Rβ remain small (<0.5 μm) and confined, forming small and spherical carriers. The role of extracellular receptor clustering during IL-2Rβ uptake has not yet been tested but it might be an essential step for its endocytosis. Heteromerization of native IL-2R complexes is not expected given to the position of IL-2 at the middle of the trimer [41], suggesting that its sorting into the FEME pathway might rely on the recognition of activated receptors by cytosolic adaptors.

Acute signaling-induced membrane protrusions

Macropinocytosis is reminiscent of phagocytosis, which is activated by the engagement of opsonin receptor recognizing antibodies, complements or lectins around the surface of opsonized pathogens or apoptotic cells [46,47]. Instead of forming tight contacts between the membrane and the cargoes, macropinosomes form upon the extension and folding of large membrane ruffles back onto the cell surface, thereby mediating the bulk intake of extracellular fluids [46,47]. By doing so, receptors located on the plasma membrane patch used to form macropinosomes are indiscriminately internalized. As there are no sorting mechanisms at the cell surface, the fate of the internalized receptors (recycling back to the surface or degradation in lysosomes) is decided upon endosomal maturation instead [46,47].

Macropinocytosis is activated by specific signals but is not cargo-specific, as many other transmembrane proteins are internalized as 'collaterals' alongside the receptors emitting the signal. It is activated upon elevated and sustained signaling emanating from growth factor, chemokine or Toll-like receptors (TLRs) [46,47]. The signaling of such receptors mediates the process in the same way as oncogenic transformation of Ras and Src induces constitutive macropinocytosis, independently of active receptors [17]. Some viruses, bacteria and integrin substrates, as well as phosphatidylserine (PS)-containing apoptotic cell remnants, can also trigger macropinocytosis. At synapses, activity-dependent bulk endocytosis (ADBE) is the dominant endocytic mode and shares features with macropinocytosis [48]. Clustered synaptic vesicle cargoes, including VAMP4, induce membrane invaginations, retrieving non-specifically all molecules located on the patch of the plasma membrane being used to form large (up to 500 μm) bulk endosomes [49].

The signals required for macropinosomes induce membrane protrusions and ruffles powered by actin polymerization. Initially, activated receptors and Ras (Ras-GTP) both recruit and activate phosphoinositides 3-kinases (PI3Ks) that produce spots of $PI(3,4,5)P_3$ [50] (**Figure 2a**). In turn, the lipid recruits and activates Rac and Cdc42, as well as actin-nucleation-promoting and branching complexes SCAR/WAVE, WASP/N-WASP and Arp2/3 [46,47]. Cdc42 and N-WASP are also locally activated by SNX9 [51]. Active Rac-GTP and Cdc42-GTP also stimulate PAK1, which supports actin reorganization and membrane ruffling [46,47] (**Figure 2b**). Consistently, small compound inhibitors of PI3K, Rac, Cdc42 or PAK1 all inhibit macropinocytosis. Additionally, $PI(3,4,5)P_3$ mobilizes phospholipases (PLC) that hydrolyze $PI(4,5)P_2$ locally into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 and DAG then activate protein kinase C (PKC), thereby sustaining actin polymerization [46,47] (**Figure 2b**). The key role of PKC is apparent from the induction of macropinocytosis upon its activation by phorbol esters (such as PMA or TPA),

thus bypassing receptor and Ras activation. Electrostatic interaction between $PI(3,4,5)P_3$ and polybasic motifs in actin-nucleating factors is central to membrane ruffling, as accumulating cytosolic H^+ (which neutralizes negative charges of the inner leaflet) is an efficient way to block macropinocytosis. This is achieved by preventing Na^+/H^+ transfer across the plasma membrane using amiloride or its 5-(N-Ethyl-N-isopropyl)amiloride (EIPA) derivative [46,47]. Consistent with the central role of $PI(3,4,5)P_3$, phosphatases modulating its levels such as PTEN (which reverts it back to $PI(4,5)P_2$) and SHIP2 (which transforms it into $PI(3,4)P_2$) are keys in regulating macropinocytosis. It is intriguing that many inhibitors that block macropinocytosis also inhibit FEME and IL-2Rβ uptake, even though the latters produce much smaller endocytic carriers and function upon specific cargo capture. Future work should reveal the molecular basis for the difference in mechanisms.

Although it shares morphological and functional features with macropinocytosis, ADBE is activated by a different mechanism. High neuronal activity-induced Ca^{2+} elevation in synaptic terminals activates the phosphatase Calcineurin [52], which dephosphorylates and activates many endocytic proteins (including the membrane scission GTPase Dynamin-1 on Ser-774). This is further supported by the AKT-mediated inactivation of Glycogen Synthase Kinase 3 (GSK3) [53], which is the main kinase of Ser-774 Dynamin-1 and the main inhibitor of ADBE [54]. Interestingly, a rise in Ca^{2+} also induces the clathrin-independent uptake of EGFR at high EGF concentration [55]. Reticulon-3-dependent ER-plasma membrane contact sites are required for IP_3-mediated localized Ca^{2+} signaling [55]. However, the size and shape of the EGFR-containing endocytic carriers are not consistent with macropinocytosis, even though at least one receptor (CD147) is being internalized alongside EGFR, potentially as collateral. Thus, acute intracellular signaling produced upon the strong (high ligand dose) or sustained stimulation of many receptors induces actin cytoskeleton and plasma membrane remodeling to generate several types of clathrin-independent carriers.

Extracellular cargo clustering and GL-Lect hypothesis

Several CIE cargoes do not contain known sorting motifs or do not have any cytoplasmic tails: one such example is GPI-anchored proteins, which are bound to the exoplasmic leaflet of the plasma membrane through a short lipid anchor [18]. Initially, cell surface proteins devoid of sorting motifs were assumed to enter cells passively, up until molecular events other than recognition by cytoplasmic machinery were identified [56]. One such mechanism, named the Glycolipid-Lectin (GL-Lect) hypothesis, occurs through the extracellular clustering of glycosphingolipids and/or glycosylated proteins into nanodomains that bend into the cytosol [56]. Glycosphingolipids such as GM1 or Gb3 are exoplasmic lipids that cluster upon binding to secreted lectins (galectin-1/3/4/7-9) or to pathogenic factors such as SV40 and other polyomaviruses, botulinus, tetanus, cholera or Shiga toxins [56]. An initial hypothesis proposed that glycosphingolipids would group laterally with cholesterol into 'lipid rafts', trapping transmembrane or GPI-anchored proteins [57]. This hypothesis likely gained popularity because simple cholesterol sequestration or extraction (using nystatin or filipin and MβCD, respectively) was considered sufficient for determining whether the uptake of a given receptor or pathogen occurs through 'lipid raft endocytosis'. However, molecular details on how lipid rafts would bend membrane inward to form endocytic carriers were missing. As most cholesterol is located at the inner leaflet of the plasma membrane [58,59] and only polyvalent extracellular proteins (galectin-3 or pentameric cholera and Shiga toxins) induce uptake, protein-mediated lipid clustering is likely the main driver of inward membrane bending [56]. In addition to glycolipids, lectins also bind to the N- and O-glycosylated extracellular domains of cell surface proteins, inducing their dimerization and, in the case of galectin-3 on CD44 and β1 integrin, their oligomerization and internalization [20].

The extracellular clustering of glycolipids into nanodomains by cholera or Shiga toxins or galectin-3 is sufficient to induce inward membrane bending in GUVs prepared with plasma membrane-like lipid composition [19,20]. The glycolipids *per se* do not appear to confer endocytosis specificity, as cholera toxin-binding GM1 lipid is internalized through multiple pathways, including CCPs. Instead, the GL-Lect mechanism overcomes the steric stress induced by the asymmetric crowding of proteins clustered on the exoplasmic leaflet, which induces outward buckling of the membrane [60]. For Shiga and cholera toxins, the tilt of glycosphingolipid headgroups induced by the location of the binding sites and the pentameric assembly of the toxins induce the bending of the membrane [61,62] (**Figure 3a**). However, it is not yet clear how

galectin-3 binding to protein glycosylation sites located on flexible domains (and often many nanometres away from the cell surface) could transmit the force to the membrane.

Similarly, even though it is a slow process (half-time in the order of multiple minutes to hours), it is not yet fully understood how the extracellular clustering of GPI-anchored proteins induces their uptake into cells. Clustering of their large bulky extracellular domains is required but the composition of their GPI tails has little influence [63]. Clustering is likely mediated by lectins, which could bind to the glycan core, as well as to glycosylations on the protein part. Such cross-linking of their bulky extracellular domains might impose a steric force, tilting away the lipid tails (**Figure 3a**). Steric bulk and lipid tail tilting would be propagated into local inward membrane bending upon trans-bilayer coupling [64].

The CLIC pathway is the main CIE route that utilizes the GL-Lect hypothesis to build endocytic carriers. It is a constitutive pathway internalizing large volumes of fluid and extracellular material that do not have surface receptors (probed by dextran or HRP uptake), in addition to GPI-anchored proteins, CD44 and some integrins [13,18,20,65,66]. The constant formation of CLICs is consistent with their reliance on galectin-3 [20], which is secreted at high concentration into the extracellular space (up to low μg/mL range). Membrane tension and bilayer fluidity plays an important role in CLIC formation, consistent with its drastic reduction upon cell stretching, cell confluency or cholesterol lowering [15,24] and its increase at the leading edge of migrating cells [13]. It is still unclear how membrane protrusions formed from extracellular cargo and extending inside the cytoplasm are recognized by cytoplasmic components involved in CLIC.

GRAF1 marks CLICs and controls their formation [66] (**Figure 3b**). Its BAR domain senses and induces membrane curvature but so do those of many other BAR domain-containing proteins. Other GL-Lect-driven endocytic carriers such as the one produced by Shiga and cholera toxins are recognised by Endophilin, a step required for their scission [39]. However, GRAF1 also functions in non-endocytic trafficking events and Endophilin is recruited to budding FEME carriers as well as a subset of clathrin-coated pits. Thus, cues other than simple membrane curvature might be present during early CLIC bulging. Precise spatio-temporal N-WASP/Arp2/3-mediated actin polymerization is required during CLIC formation, and Cdc42 must be activated and switched off in a timely manner (**Figure 3a-b**). Cdc42 inactivation is mediated on the plasma membrane by Arf1-mediated ARHGAP10 and after CLIC budding by the GAP domain of GRAF1 [66,67]. Even though GRAF1 binds to Dynamin, the budding of CLICs is insensitive to dynamin inhibition and relies instead on forces generated by actin polymerization [13,18,66] and potentially on friction-driven scission generated by BAR proteins [39,40]. Dynamin-independent scission events drive the formation of several carriers such as: the Sar1-mediated scission of COPII vesicles [68], the BAR protein Arfaptin1/2-mediated budding of AP1/clathrin-coated vesicles at the Golgi [69] and the SNX-retromer carriers budding at endosomes [29]. However, the precise molecular events resulting in CLIC severing from the plasma membrane are not yet known.

The detachment of Shiga and cholera toxin-containing carriers is much better understood. Their budding occurs upon synergy between the pulling forces generated by actin polymerization and dynein-mediated traction on microtubules, membrane scission by dynamin and friction-driven scission generated by Endophilin [39,40]. The precise stoichiometry, spatial location and timing of each of the components will be important to elucidate as imbalances between the different components perturb membrane scission [39, 70]. The scission mechanism of toxin-containing CIE carriers is reminiscent to that of FEME, even though they are formed by different mechanisms upstream. How Endophilin and the cytoskeleton machinery distinguish between toxin-induced and featureless membrane protrusions of similar diameter to that of other endocytic pathways will be important to understand. One cannot exclude the possibility that co-receptor clustering mediates their recruitments, as transmembrane proteins such as VAMP2, 3 and 8 SNAREs are efficiently sorted into toxin-induced invaginations to mediate their intracellular trafficking to the retrograde pathway [71].

Concluding remarks

CIE is comprised of several parallel routes, which use different mechanisms to form endocytic carriers. So far, all known CIE routes deliver their cargoes into early endosomes; thus the differences concern the initial steps at the plasma membrane. We reported here our current knowledge of CIE carrier formation by three distinct mechanisms. However, it is highly probable that some pathways use hybrid

versions of those. For example, a role for extracellular cargo clustering during IL2-Rβ uptake or FEME was not tested and therefore a potential synergy with cytosolic cargo capture cannot be ruled out (see Outstanding Questions). Interestingly, galectin-3 interacts with EGFR [72], which enter cells through CIE, including FEME [21, 55]. Likewise, inter-molecular interactions between the extracellular domains of two receptors could bring receptors that have cytoplasmic motifs into CLICs (see Outstanding Questions). Cytoplasmic tail-less GPI-anchored proteins are co-sorted into multivesicular bodies (MVBs) by tetraspanins [73] and thus might be co-sorted into CLICs as well. The canonical CLIC cargoes CD44 and CD90 (Thy-1) interact with EGF, FGF and TGFβ receptors, as well as some integrins and metalloproteases [74]. Such co-receptor sorting may occur upon extracellular inter-molecular bridging by lectins. Indeed, galectin-3 is known to crosslink integrin β1 with LRP1 or PDGF receptors [75]. CD44 may bind to cytoplasmic BAR domain-containing SNX18 and SNX33 [74], which would suggest a potential contribution from the cytosolic cargo sorting mechanism.

Some BAR domain-containing proteins may emerge as major CIE regulators as they are ideally equipped to sense, stabilize and/or induce membrane curvature, locally regulate actin cytoskeleton dynamic, gather cytosolic regulators (*e.g.* dynamin, cargo adaptors) and, in some cases, directly recruit receptors. So far, SNX5 and 9 are known to be involved in macropinocytosis, Endophilin in FEME and IL-2R, Shiga and cholera toxin uptake and GRAF1 in CLIC. Ideally, some BAR domain proteins will be found to be reliable and specific markers for CIE pathways, which would help the study of their molecular mechanisms. The addition of pathway-specific cargoes, if they exist, will also greatly assist in deepening our understanding of each of the CIE routes.

The large heterogeneity of sizes and shapes of carriers produced by CIE implies that the coupling between membrane invagination and scission is less strict than during CME. The absence of a rigid coat dictating the spatial positioning of scission proteins at the neck of the budding carriers is an obvious difference. A more stochastic order of events might happen during CIE carrier budding, compromising reproducibility in the coupling to gain rapidity or to accommodate a greater range of cargo shapes or copy number per carrier. Consistent with this proposal, core molecular machineries involved in CIE (actin-polymerization factors, BAR domain proteins, Dynamin) might be used in a modular manner, depending on the cellular and/or physiological contexts (see Outstanding Questions). This is exemplified by the differential reliance on Dynamin for Shiga and Cholera toxin uptake in different studies [39,65,76]. If such modularity were to be proven, it would blur the lines between current CIE pathways (proposed so far as well-defined order of events) and would open the possibility that the same fundamental mechanisms are used during all Clathrin-independent endocytic events.

Outstanding questions

- Are there additional mechanisms for clathrin-independent endocytic carrier formation?
- Does the extracellular cargo clustering mechanism contribute to FEME and IL-2R endocytosis?
- Are co-receptor sorting and cytoplasmic motif recognition involved in CLIC and GL-Lect-driven endocytosis?
- Are core molecular machineries used in a modular manner during all CIE events?

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Text Box 1. Roles of caveolae and Flotillins in Clathrin-independent endocytosis

Caveolae are cholesterol- and sphingolipid-rich nanodomains on the plasma membrane, which form characteristic 50-100 nm cave-like invaginations that look like endocytic carriers before scission [77,78]. Cholesterol-binding proteins Caveolin-1 to 3 associate with cytosolic proteins Cavin 1-4 to form a coat that shapes caveolae and traffics between early endosomes and the plasma membrane, albeit at low frequency [77,78]. The involvement of the endocytic GTPase dynamin and the localization of EGF or Insulin receptors, SV40 virus or cholera toxin within caveolae have solidified the belief that they constitute CIE carriers. However, no receptors, ligands or pathogens have been found so far to enter cells specifically through caveolae [79]. While many publications have reported the internalization of various cargoes by caveolae, in the vast majority of cases, colocalization with Caveolin-1 or cholesterol depletion/sequestration (MβCD, filipin or nystatin treatment) was the only evidence. Indeed, perturbation of cholesterol affects other CIE pathways such as FEME and CLIC [15,21] and disturbs plasma membrane lipid organization, receptor lateral diffusion and availability, thus impacting their internalization indirectly [57]. Moreover, caveolae, Caveolin and Cavins inhibit the CLIC pathway by regulating of the local availability and activity of the main CLIC regulator Cdc42 [80].

Recently, caveolae were proposed to function as mechano-sensing membrane domains [81]. Membrane stretching induces their flattening and lateral redistribution of Caveolin-1 and sphingolipids on the plasma membrane together cause the release of Cavins into the cytosol and activation of signal transduction [81,82]. Flattening of caveolae provides mechanoprotection of cells with abundant caveolae, such as adipocytes, skeletal muscle and zebrafish notochord [82-85]. As such, in response to membrane stretch, inhibited receptors localized in caveolae nanodomains are released, diffuse laterally on the plasma membrane and start signaling. Caveolin binding partners Cavins are released into the cytoplasm and might induce lasting cell changes by regulating transcription [86]. The phosphorylation of Caveolin-1 by Src family kinases regulates its assembly into caveolae and cell signaling without stretching also controls the clustering of receptors into nanodomains such as integrins or B-cell receptors [87]. Instead of mediating the uptake of specific cargoes, the main function for the shuttling of caveolae between the plasma membrane and early endosomes might be to regulate the cellular location and abundance of caveolae and caveolar proteins, as exemplified during cell division [88]. Thus, in absence of evidence that caveolae are a portal of entry for specific ligands, we did not discuss their mechanisms of carrier formation in this review.

Flotillin-1 and 2 were proposed to mark a CIE pathway as they concentrate in spots that blink at the plasma membrane, without colocalizing either with Clathrin or Caveolin [89]. However, further work suggested that their role in endocytosis was more likely indirect, by controlling clustering and lateral diffusion and availability of receptors for uptake by other pathways, such as Dopamine transporter (DAT), EGF receptor (which enters cells through many pathways), or the GPI-anchored protein CD59 (which is a CLIC cargo) [13,89-91].

Figure Legends

Figure 1. Cargo capture and local membrane bending by cytosolic proteins.
a, (left) Initiation of IL2Rβ uptake is triggered by binding to anti-IL-2R antibodies. (right) Fast Endophilin-mediated endocytosis (FEME) priming occurs upon the local pre-enrichment of Endophilin by $PI(3,4)P_2$-binding protein Lamellipodin (Lpd) (right). **b**, (left) antibody-induced IL2Rβ clustering stimulates WAVE recruitment to WIRS motifs present in the cytoplasmic tails of IL2Rβ. This activates local actin polymerization and membrane protrusions. PI3K produces locally $PI(3,4,5)P_3$, which recruits Rac1, thereby activating WAVE and PAK1, thus stimulating actin polymerization. N-WASP, Cortactin and Dynamin are then mediating IL2Rβ endocytic pit closure and detachment from the cell surface. (right) Receptor (in this case, a GPCR) activation reveals binding motifs in the cytoplasmic tails of FEME cargoes, which are bound by the SH3 domain of Endophilin. Cargoes such as receptor-tyrosine kinases (*not depicted here*) are captured into FEME carriers through the binding of adaptor proteins to Endophilin. Concomitant BAR-domain-mediated membrane bending, inter-molecular oligomerization and SH3-mediated adaptor and cargo recruitment by Endophilin mediates the formation of FEME carriers. The synergistic action of, actin polymerization, dynamin activity and BAR domain scaffolding-induced membrane friction mediates the scission of IL2Rβ and FEME carriers.

Figure 2. Acute signaling-induced membrane protrusions.
a, Local receptor activation (in this case, receptor tyrosine kinases, RTKs) recruits and activate Ras and PI3K, thereby producing $PI(3,4,5)P_3$. **b**, Sustained and/or elevated receptor signaling trigger intense local Ras and PI3K signaling, which recruits and activate Cdc42-N-WAS and Rac1-WAVE complexes as well as PAK1, thereby promoting actin polymerization-induced membrane ruffling and protrusions. $PI(3,4,5)P_3$ activates and recruits PLC, which hydrolyzes PI(4,5)P2 into IP_3 and DAG, which in turn activate PKC, thus stimulating further actin polymerization. Non-activated receptors located on the patch of plasma membrane used in the ruffles forming the macropinosomes are internalized indiscriminately as 'collaterals'. Soluble material (amino acids, soluble proteins) and fluid are captured into the forming macropinosomes. Macropinosomes scission is mediated by CtBP1 and/or Dynamin.

Figure 3. Extracellular cargo clustering and GL-Lect hypothesis.
a, (left) The extracellular domains of glycosylated cargoes (*e.g.* CD44) are clustered by galectin-3. The mechanism by which glycosylphosphatidylinositol (GPI)-anchored proteins (*e.g.* CD59, CD90) are clustering is not yet fully understood but is supported by Cholesterol nanodomains. (right) Glycosphingolipids (*e.g.* Gb3 or GM1) are bound and clustered by pentameric Shiga or cholera toxins. **b**, (left) Clustered glycosylated receptors or GPI-anchored proteins induce inward membrane bending into the cytosol. CLIC formation is then supported by Cdc42/N-WASP-induced actin polymerization and GRAF1 binding. Local and timely Cdc42 inactivation is mediated by ARHGAP10 recruited by Arf1 to the plasma membrane (likely at initial stages) and then by Cdc42-GAP activity of GRAF1 (at later stages). (right) Clustered glycosphingolipids induce inward membrane bending into the cytosol. Endophilin is recruited to nascent endocytic carriers. The synergistic action of BAR domain scaffolding-induced membrane friction, actin polymerization and/or Dynamin activity mediates the scission of CLICs and toxin-containing carriers.

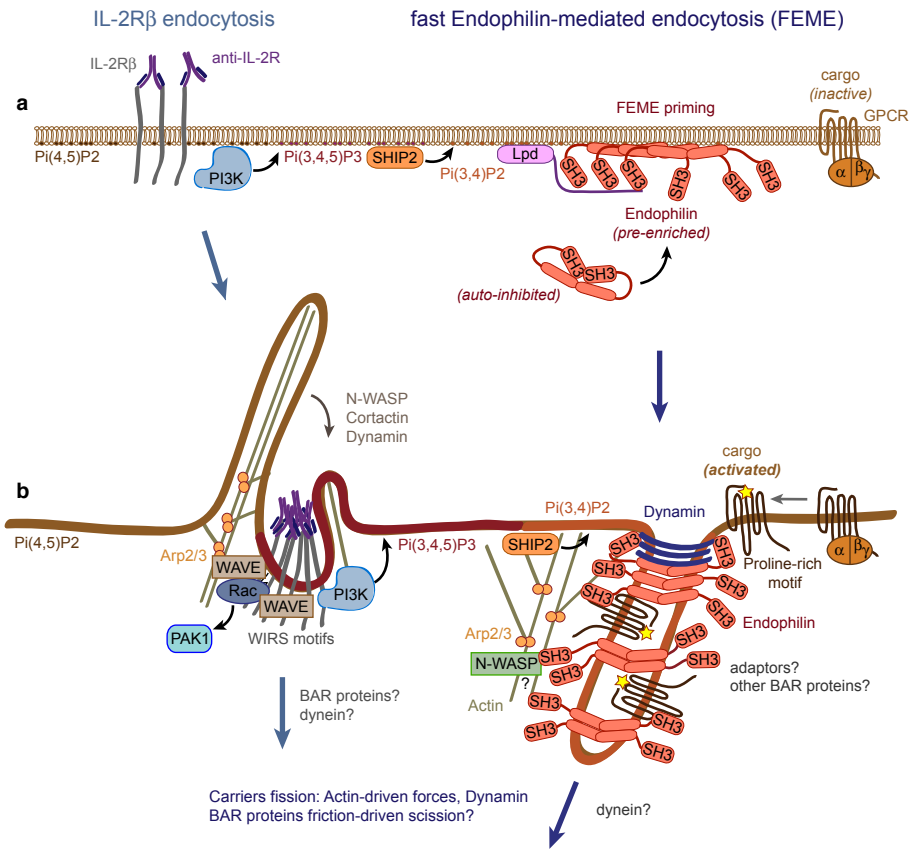

Figure 1. Cargo capture and local membrane bending by cytosolic proteins

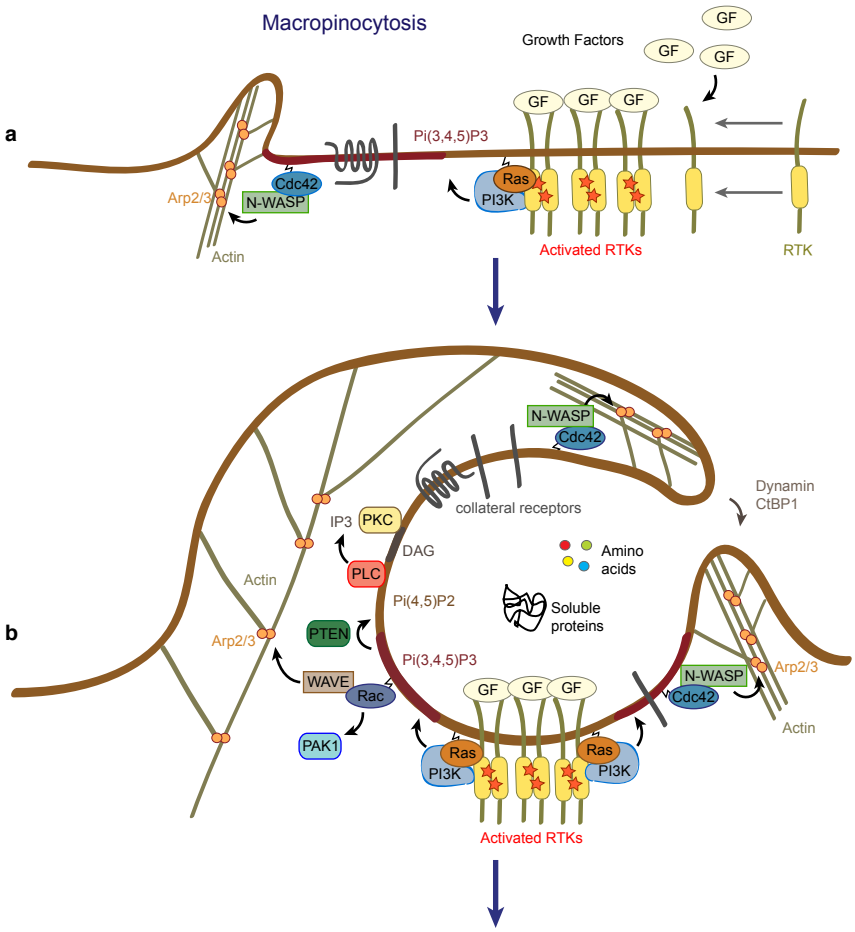

Figure 2. Acute signaling-induced membrane protrusions

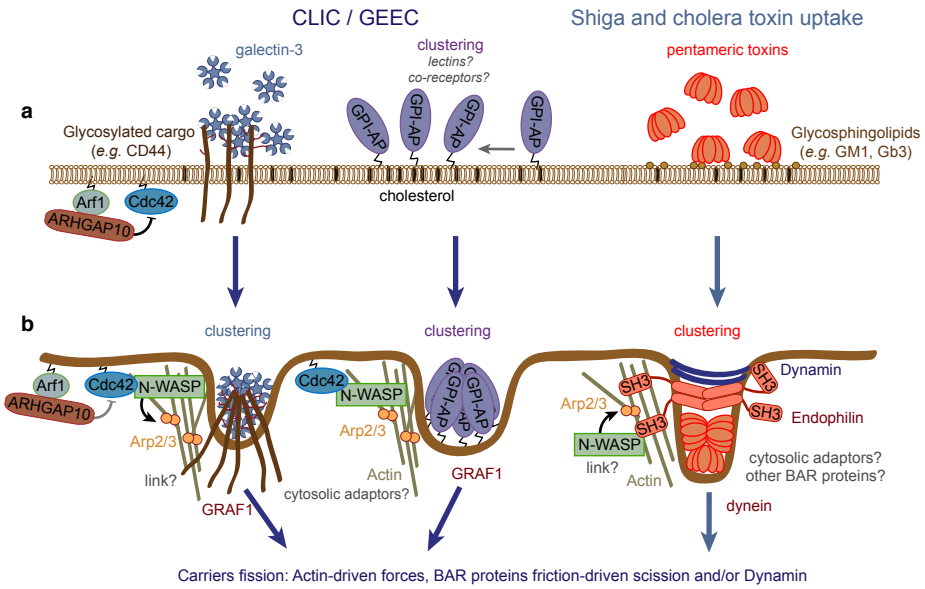

Figure 3. Extracellular cargo clustering - GL-Lect hypothesis