Mechanisms and functions of endocytosis

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A recent EMBO-FEBS workshop entitled Endocytic Systems: Mechanism and Function, organized by Howard Riezman in Villars-sur-Ollon (Switzerland), showcased the multifaceted approaches and model systems used to study endocytosis. The meeting revealed how endocytosis controls multiple aspects of biology, ranging from development to immunity and neurotransmission.

Mechanisms of clathrin-dependent endocytosis

Although clathrin-mediated internalization has been investigated for many years, new technologies keep providing us with novel insights into its underlying mechanisms, with an unprecedented scale down to molecular details regarding the structure and dynamics of the proteins involved. The plasticity of clathrin-mediated internalization was well illustrated by the fact that this pathway is used not only to traffic extracellular molecules or cellular proteins, but also to mediate entry of certain toxins, viruses, and bacteria.

The issue of lifetimes of clathrin-coated pits (CCPs) and vesicles (CCVs) has remained controversial, as values reported in the literature range from seconds to minutes. Sandra Schmid (The Scripps Research Institute) described quantitative computational analyses to track the dynamics of CCP/CCV formation on the plasma membrane. In this way, three kinetically distinct populations of CCPs could be distinguished, two short-lived (early- and late-abortive with lifetimes in the range of seconds) and one long-lived productive population stable for over one minute. Interestingly, cargo appears to increase a number of productive, long-lived CCPs/CCVs without affecting their lifetimes, which can in turn be regulated by the activity of dynamin.

Morphological heterogeneity of CCVs was emphasized by Tomas Kirchhausen (Harvard Medical School). Cryo-electron tomography of individual CCVs revealed a broad range of patterns used to organize a clathrin lattice, with asymmetrically located membrane vesicles buried inside the shell (Cheng et al., 2007). Moreover, high-resolution imaging of live cells based on total internal reflection fluorescence technology indicates that AP-2 adaptor proteins are also localized nonsymmetrically within an individual CCV. This may result from an initially restricted localization of adaptors, as they are captured during the nucleation and early phases of coated pit assembly, while retaining the adaptors concentrated at the place of their original recruitment at the time of vesicle pinching and CCV formation.

Clathrin-mediated endocytosis serves some specialized functions in various tissues, including the nervous system. Knockout (KO) studies in mice, reported by Pietro De Camilli (Yale University School of Medicine), demonstrated that dynamin-1 appeared not to be essential for the biogenesis and endocytic recycling of synaptic vesicles (Ferguson et al., 2007), although studies of dynamin mutants in cultured cells would have predicted a crucial role for this protein in vivo. The role of dynamin-1 in synaptic vesicle endocytosis is activity dependent and becomes evident during strong stimulation of neurons. The morphology of KO nerve terminals was visualized by EM tomography followed by tridimensional reconstruction. Such synapses are filled with clusters of clathrin-coat components, forming tubular networks capped by clathrin-coated pits that open to the plasma membrane.

Role of actin in clathrin-dependent endocytosis

Because of the ease of genetic manipulations, the yeast *Saccharomyces cerevisiae* has been very useful for dissecting the molecular machineries of endocytosis. Genetic studies have revealed an essential role for actin in endocytosis in yeast, and a key question concerns how actin functions together with clathrin in endocytosis. Using real-time image analysis of yeast cells expressing fluorescently tagged versions of more than 40 endocytic proteins, David Drubin (University of California, Berkeley) has analyzed the dynamic appearance, movement, and disappearance of these proteins at endocytic sites. Drubin presented data indicating that these proteins can be grouped into four functional modules that mediate coat formation, membrane invagination, actin-meshwork assembly, and vesicle scission during clathrin/actin-mediated endocytosis. Maria-Isabel Geli (Instituto de Biología Molecular de Barcelona) described an in vitro assay to reconstitute the complex actin structures that participate in the formation of endocytic profiles and the use of immuno-electron microscopy to define the primary endocytic profiles in yeast and the localization of the actin machinery.

Given the importance of actin and clathrin in endocytosis, proteins that link actin and clathrin functions are of special interest. Genetic studies in yeast have

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indicated that clathrin light chain may regulate the ability of Sla2 to control actin dynamics in endocytosis (Newpher et al., 2006). Frances Brodsky (University of California, San Francisco) described a study of Hip1 and Hip1R, the mammalian homologues of Sla2, which have overlapping but not identical functions in endocytosis. Brodsky presented evidence that Hip proteins interact sequentially with clathrin and actin rather than functioning as bridges between the two.

Clathrin-dependent endocytosis and pathogen entry

Certain toxins and pathogens harness clathrin-mediated internalization to enter cells. Endocytosis of anthrax toxin, described by Gisou van der Goot (Federal Polytechnic School of Lausanne), is clathrin- and dynamin-mediated but requires also the presence of lipid rafts, a classical hallmark of clathrin-independent entry routes. The protective antigen (PA) subunit of the toxin binds to cell surface receptors (TEM8 and CMG2) and induces their multiple post-translational modifications, such as palmitoylation, phosphorylation, and ubiquitination, which differentially regulate toxin internalization (Abrami et al., 2006). Yet another new player implicated in this process appears to be the Wnt coreceptor LRP6, which interacts with TEM8 and CMG2 and its depletion results in reduced toxin uptake. Anthrax toxin thus provides an interesting example of using complex intracellular endocytic and signaling mechanisms for precise regulation of its internalization in time and space.

Semliki forest virus (SFV) was one of the first viral pathogens identified to exploit clathrin-dependent internalization mode, as recalled by Ari Helenius (ETH Zurich) in his plenary lecture, along with a number of viruses using other pathways (see Fig. 1). Further viruses, such as species C adenovirus type 2 (Ad2) and Ad5 reported by Urs Greber (UZH Zurich), enter cells via clathrin- and dynamin-dependent mechanisms but escape the classical Rab5-Rab7-EEA1-Hrs pathway from early to late endosomes and are redirected to trans-Golgi compartments in an Arf1-dependent process.

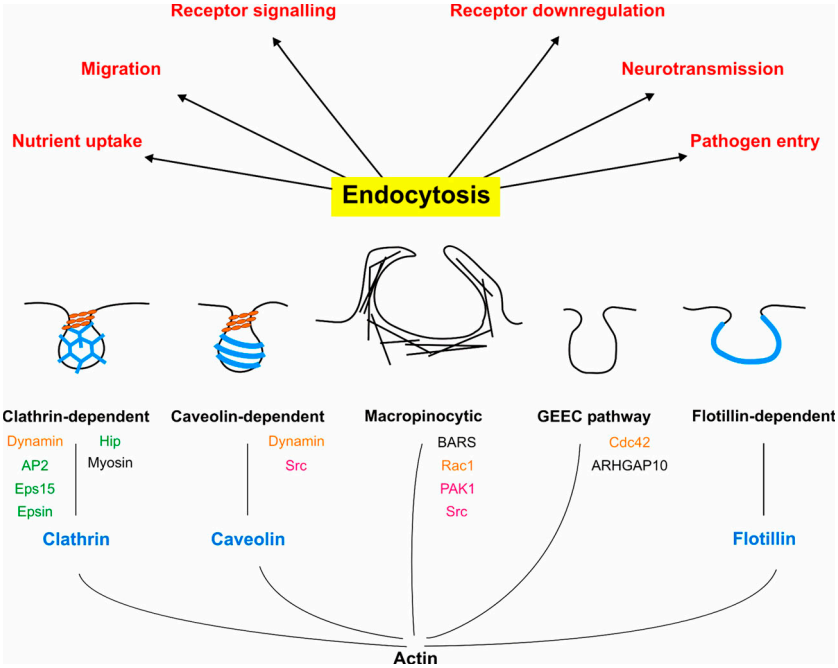

Figure 1. **Distinct endocytic internalization pathways discussed at the conference, with examples of key regulators.** Color code: blue, coat proteins; red, kinases; orange, GTPases; green, adaptors; black, other proteins. The various functions of endocytosis are indicated on top.

However, the clathrin-mediated pathway appears to be exploited not only by viruses, but also by much larger bacterial pathogens such as *Listeria monocytogenes*, as reported by Pascale Cossart (Pasteur Institute of Paris). In a process induced by the bacterial surface protein InlB, clathrin and auxilin are recruited around the entering bacteria, followed by actin polymerization. Nevertheless, despite certain similarities and common players involved (dynamin, Eps15, and the E3 ubiquitin ligase Cbl), this process appears mechanistically and kinetically different from the canonical clathrin-mediated internalization of macromolecules. This in turn argues that the networks of protein–protein and protein–lipid interactions involved in clathrin-mediated internalization can assemble in various combinations. This plasticity may be exploited not only by pathogens, but also under physiological conditions by different types of cells or tissues with particular needs for specialized forms of internalization.

Clathrin-independent endocytosis pathways

Once obscure, clathrin-independent internalization routes are a focus of intense research revealing the molecular players involved and cargos entering via these mechanisms. This allows us now to redefine these pathways more precisely and in positive terms, in contrast to their initial collective description as "non-clathrin endocytosis". Some classification schemes have been already proposed (Mayor and Pagano, 2007), based primarily on the dependence on dynamin and various small GTPases; however, the exact number of clathrin-independent pathways and their mutual relations remain unclear.

Satyajit Mayor (National Centre for Biological Sciences, Bangalore) reported progress toward further characterization of a clathrin-, dynamin-, and caveolae-independent internalization route that transports GPI-anchored proteins (GPI-APs) and involves GEECs (GPI-AP–enriched early endosomal compartments) as intermediates. This constitutive pinocytic pathway is initiated by a cholesterol-dependent recruitment and stabilization of active Cdc42 on the plasma membrane, which leads to localized actin polymerization (Chadda et al., 2007). Interestingly, the activity of Cdc42 is controlled by an Arf1-dependent recruitment of RhoGAP, such as ARHGAP10. This mechanism represents an interesting example of a cross-talk between different small GTPases of

the Ras superfamily in regulation of endocytosis.

Flotillins appear to be essential structural components of the internalization pathway independent of clathrin and caveolae, as reported by Ben Nichols (MRC Laboratory of Molecular Biology). Flotillin-1 and -2 coassemble on the plasma membrane into microdomains, which are laterally mobile, distinct from CCPs or caveolae but bearing certain features of lipid rafts (Frick et al., 2007). Electron microscopy and live-cell imaging demonstrated that these microdomains can induce membrane invaginations and eventually bud off from the plasma membrane in a process stimulated by overexpression of flotillins. The resulting primary endocytic structures contain cholera toxin B subunit, but not transferrin. They appear morphologically different to GEECs, although patched GPI-APs partly colocalize with flotillins.

Shiga toxin B-subunit (STxB) uses clathrin-dependent and -independent modes of internalization. Ludger Johannes (Institut Curie) reported that STxB can induce tubular invaginations, possibly acting as endocytic internalization intermediates. Their formation does not require clathrin, actin, dynamin, or caveolins, and is also observed on energy-depleted cells. Interestingly, the STxB-induced tubulation could be reproduced in vitro on giant unilamellar vesicles in the absence of cytosolic protein machinery. While the exact mechanisms underlying this phenomenon await further characterization, the findings indicate that some endocytic internalization events can be mediated by protein-induced rearrangements of a lipid bilayer as a driving force of membrane deformation.

Macropinocytosis represents clathrin-independent internalization of vast areas of plasma membrane and large volumes of extracellular fluid that are enclosed in macropinosomes, big (>1 μm) vacuolar structures. Such voluminous membrane rearrangements involve actin ruffles and are driven by Rac1 and Src kinases. Fission of nascent macropinosomes does not involve dynamin, but no alternative mechanisms have been proposed. As reported by Prisca Liberali (Consorzio Mario Negri Sud), CtBP1/BARS, a protein controlling membrane fission in other transport steps, assumes this role also in macropinocytosis. CtBP1/BARS is locally recruited to the closure site of a macropinocytic cup where it is phosphorylated by p21-activated kinase-1 (Pak1). This is a key event in the fission process in which CtBP1/BARS acts as a scaffold in a larger complex coupling membrane rearrangements with cytoskeletal machinery.

The morphological features and the molecular players involved in the entry of vaccinia virus make it a new addition to the list of cargo internalized via macropinocytosis, as presented by Ari Helenius (ETH Zurich). Besides exploiting features of regular macropinocytosis (dependence on actin, Rho GTPases, cholesterol, and Pak1), the virus uses additional specialized mechanisms, involving formation of transient blebs on the plasma membrane induced upon virus binding and preceding its internalization. This surprising effect was tracked down to the presence of phosphatidylserine (PS) on the viral particle, as a key factor for successful infection. PS externalization is a known hallmark of apoptosis, resulting in extensive plasma membrane blebbing. Thus, this "apoptotic mimicry" used by the virus causes a transient stimulation of membrane rearrangements to ensure an efficient entry.

Even within one family of viruses, different species can use various internalization routes. In contrast to its relatives entering via clathrin-dependent mechanisms (see above), the species B adenovirus type 3 (Ad3) described by Urs Greber (UZH Zurich) induces macropinocytosis to mediate its own entry. This process requires cell surface receptor CD46 and αv integrins, in addition to other proteins regulating macropinocytosis, including CtBP1/BARS.

Although phagocytosis represents a very distinct and specialized class of endocytic internalization, it shares certain mechanisms and machinery with other pathways. Sergio Grinstein (Hospital for Sick Children) reported that lipid remodeling and the resulting changes in membrane surface charge can regulate protein recruitment in phagocytosis, a mechanism that likely could be extended to endocytosis in general. Novel genetic probes revealed a decrease of surface potential upon sealing of the phagocytic cup, due mainly to hydrolysis of phosphoinositides (Yeung et al., 2006). This affects binding of several signaling and regulatory molecules (e.g., K-Ras, Rac1, c-Src) recruited to the plasma membrane via electrostatic interactions. Internal endocytic compartments have lower negative surface charge than the plasma membrane, so depending on the strength of the cationic targeting sequences, proteins can be differentially recruited to the plasma membrane or endocytic compartments, whereas proteins lacking such motifs localize increasingly to the endoplasmic reticulum.

Systems biology approaches to study endocytosis

Current technologies enable global analyses of physiological pathways, and a few systems biology approaches to study endocytosis in various organisms were presented. Genome-wide analysis of endocytic recycling in *S. cerevisiae* was reported by Liz Conibear (University of British Columbia). The screen involved measuring an increased or decreased presence of the v-SNARE Snc1p on the plasma membrane, thus identifying both exocytic and endocytic defects among the mutant collection encompassing deletions of all nonessential genes. Genetic interaction analysis of the top hits established a network of gene clusters involved in various intracellular processes contributing to Snc1p trafficking, containing both known and novel components.

Endocytic internalization of transferrin (destined for recycling) and epidermal growth factor (EGF; directed for degradation) in mammalian cells has been a target process for a multiparameter, genome-wide RNAi screen undertaken by Marino Zerial (Max Planck Institute of Molecular Cell Biology and Genetics) and colleagues. Given the high frequency of off-target effects by commercially available siRNA libraries, the screen has been performed using at least seven independent siRNA oligonucleotides and a mixture of endoribonuclease-prepared siRNAs (esiRNA) per gene, making this an effort of unprecedented scale. The initial analysis of the screen

results included over 60 highly quantitative parameters describing the morphology, intracellular distribution, and cargo content of transferrin- and EGF-bearing endosomes. In addition to the identification of novel genes regulating endocytosis, the study revealed interesting correlations and general principles pertaining to the organization of the endocytic pathway in mammalian cells.

Another effort based also on siRNA high-throughput screening technology was described by Lucas Pelkmans (ETH Zurich) to determine infectomes, and reveal detailed infection pathways for 15 mammalian viruses. This approach allows for identification of critical host genes and grouping together viruses using similar intracellular components for establishing infection. However, the efficiency of viral infection depends strongly on several parameters such as local cell density, cell and colony size, or number of cells. These factors, describing cell population properties of infection, are also quantitatively assessed as a part of a tri-dimensional dataset along with the corresponding viruses and siRNA phenotypes. Secondary screens for endocytosis of various cargo types will complement the infection screens in order to pinpoint the common machinery involved.

Presenting a genome-wide RNAi screen for endocytic regulators in *Caenorhabditis elegans*, Barth Grant (Rutgers University) identified 168 candidate genes. Surprisingly, a group of proteins known for their role in embryonic and epithelial polarity, PAR-3, PAR-6, PKC-3, and CDC-42, were identified in this screen. A closer analysis revealed that ablation of these proteins disrupted the morphology and function of recycling endosomes. Consistent with these data, CDC-42 and its mammalian homologue Cdc42 were found on recycling endosomes in *C. elegans* and mammalian cells. One possible function of the polarity proteins may be, together with actin, to mediate scission of vesicles or tubular elements from the recycling endosome. These findings are consistent with the view that membrane trafficking, and in particular endocytic recycling, may contribute to epithelial polarity (Balklava et al., 2007).

Trafficking to lysosomes

Many endocytosed membrane proteins, including receptors for growth factors, cytokines and hormones, are transported to lysosomes for degradation. Conjugation with ubiquitin serves as a signal for this pathway, and endosomal ubiquitin-binding proteins have consequently been sought as components of the sorting machinery. Through genetic and biochemical studies of yeast mutants defective in vacuolar protein sorting, Scott Emr (Cornell Institute for Cell and Molecular Biology) has identified three endosomal sorting complexes required for transport, ESCRTs, that mediate degradative sorting of ubiquitinated membrane proteins (Saksena et al., 2007). Emr showed evidence that ESCRT-II may nucleate the formation of ESCRT-III multimers and suggested that some ESCRT-III subunits may function as capping proteins that prevent chain elongation. This controlled multimerization, reversed by Vps4, may drive the membrane rearrangements that underlie MVB biogenesis. In support of this hypothesis, Phyllis Hanson (Washington University, St. Louis) presented a poster showing that overexpressed ESCRT-III subunits assemble on membranes to form curved filaments that associate into circular arrays. The ESCRTs are evolutionarily conserved, and consistent with their role in lysosomal receptor down-regulation they function as tumor suppressors in *Drosophila* (Hariharan and Bilder, 2006). Harald Stenmark (University of Oslo) presented evidence that these complexes may also protect against neurotoxicity by facilitating autophagic degradation of toxic protein aggregates (Rusten et al., 2007).

Jean Gruenberg (University of Geneva) showed that back-fusion of intralumenal vesicles (ILVs) with the limiting membrane of the MVB is exploited by vesicular stomatitis virus (VSV), which enters MVBs through endocytosis and subsequent trafficking and releases its nucleocapsid to the cytosol when VSV-containing ILVs fuse with the limiting MVB membrane. This appears to happen in late MVBs and depends on the late endosome–specific lipid LBPA and its effector protein Alix. Gruenberg presented an in vitro assay to study back-fusion of ILVs and showed evidence that ESCRT-I is required for back-fusion, and Alix depletion stimulates this process. He proposed that Alix stabilizes a post-fusion, pre-fission intermediate between ILVs and the limiting MVB membrane, whereas ESCRT-I controls ILV biogenesis directly.

Even though the canonical function of lysosomes is in degradation of endocytosed and autophagocytosed material, these organelles do have additional functions. Norma Andrews (Yale University School of Medicine) explained that lysosomes are the major vesicle type responsible for calcium-dependent exocytosis in nonsecretory cells to re-seal the plasma membrane after injury. Other cell types contain specialized lysosomes that fuse with the plasma membrane independently of cell damage. Such secretory lysosomes are exemplified by granules in cytotoxic T lymphocytes (CTLs), which contain enzymes that kill target cells, as presented by Gillian Griffiths (University of Cambridge).

Endocytosis in signaling and development

In addition to its well-described roles in nutrient uptake and receptor down-regulation, endocytosis plays a direct role in the modulation of cell signaling responses (Miaczynska et al., 2004). Marcos Gonzalez-Gaitán (University of Geneva) presented studies on Smad anchor for receptor activation, SARA, which links Dpp (TGFβ) receptors with Smad signaling adaptor proteins and is a central component of the Dpp signaling pathway in *Drosophila*. SARA is located to a subset of endosomes that mediate cellular memory of Dpp signaling in wing imaginal discs by distributing evenly between the two daughter cells during cell division (Bokel et al., 2006). Using development of the sensory organ precursor (SOP) as a model for asymmetrical cell division, Gonzalez-Gaitán showed that SARA-containing endosomes accumulate at the central spindle during cell division and then partition asymmetrically into the signal-receiving daughter cell. Even though SARA is a mediator of Dpp signaling, the SARA-containing endosomes could well function as vehicles for other signaling pathways, and Gonzalez-Gaitán is currently investigating the possibility

that asymmetrical Notch-Delta signaling, strongly implicated in binary fate decisions, could be mediated via SARA-containing endosomes. Another evidence that endocytosis regulates SOP development was provided by Roland Le Borgne (Université de Rennes), who proposed that transcytosis of Delta may be required for Notch-Delta signaling in the SOP.

Recent studies have shown that recycling endosomes contribute membrane to the advancing cleavage furrow during the cytokinesis phase of cell division (Strickland and Burgess, 2004). How endosomes are targeted specifically to the cleavage furrow and the midbody is still an open question. Philippe Chavrier (Institut Curie) presented evidence that a novel family of effectors of the endocytic small GTPase Arf6 are involved in the completion of cytokinesis via control of membrane delivery at the midbody through interactions with microtubule motors.

One interesting function of endocytosis in developing tissues is its involvement in cell migration, and transparent zebrafish embryos have been one of the favorite models for such studies. Carl-Philipp Heisenberg (Max Planck Institute of Molecular Cell Biology and Genetics) showed that mesoderm polarization and directed migration strongly depend on Rab5-dependent trafficking and cellubrevin-mediated recycling, indicating that the endocytic cycle is required for proper mesoderm cell migration. Endocytosis and recycling may be important for membrane rearrangements during cell migration, but could also play a role in signal sensing during migration. An interesting example of the latter was discussed by Erez Raz (University of Münster), who has been studying the involvement of the chemokine SDF-1a and its receptor CXCR4b in germ cell migration in zebrafish. The receptor is expressed on the surface of the primordial germ cells, which receive directional cues from somatic tissues that secrete SDF-1a. While wild-type germ cells put on the brakes as they approach their target tissue where the gonad develops, cells expressing a non-internalizable CXCR4b mutant fail to down-regulate signaling, which causes longer runs and less precise targeting (Minina et al., 2007).

Among clathrin-independent endocytic mechanisms, an internalization route involving caveolae has been investigated in much detail. Unlike constitutive clathrin-dependent internalization, caveolar endocytosis is inducible upon signaling triggers. As presented by Miguel Del Pozo (Universidad Complutense de Madrid), cell adhesion and integrin signaling play a crucial role in this process, with global implications for cell polarity, motility, and invasiveness.

Conclusion

In conclusion, significant progress has now been made regarding the mechanisms of endocytosis and post-endocytic trafficking, and their roles in cell signaling, development, and host–pathogen interactions. The recent introduction of systems biology approaches promises to provide a deeper understanding of how endocytosis works, and how it serves to orchestrate biological processes.

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