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Endocytosis and Signaling: A Relationship under Development

Meeting Review

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The ability to internalize macromolecules by endocytosis is a property of all eukaryotic cells. Frontline research on endocytosis has been presented in a successful series of biannual meetings in Europe. This year's meeting on "Membrane Dynamics in Endocytosis" was held September 13–18 in Acquafredda di Maratea, on the coast of southern Italy. Four key questions were addressed: What are the molecular mechanisms of endocytic membrane trafficking? How does endocytosis modulate receptor signaling and vice versa? What is the importance of endocytosis during development? How do endocytic organelles contribute to immunity or susceptibility to pathogens?

Mechanisms of Endocytosis

Endocytosis can occur by several mechanisms (Conner and Schmid, 2003b) (Figure 1). The best-understood mechanism involves formation of a cytoplasmic clathrin coat. Since clathrin itself does not capture cargo, clathrin-associated adaptor proteins are required for incorporating appropriate cargo proteins into clathrin-coated pits and vesicles. Our understanding of the mechanisms of cargo selection by adaptors is now the subject of rapid progress, leading to revision of previously accepted models. In addition, an emerging pattern is the high diversity of internalization routes into the cell: clathrin-dependent and -independent, dynamin-dependent and -independent, caveolar endocytosis, macropinocytosis, etc. (Figure 1). Another recurring theme is the importance of the lipids in the internalization process.

AP2 Cargo Selection

It has been widely assumed that AP-2 functions as a general adaptor for transmembrane proteins that are endocytosed via clathrin-coated pits. Margaret Robinson (University of Cambridge) argued that this view has to be modified. Using highly efficient RNAi knockdown of the AP-2 adaptor subunits α and μ2, she found that transferrin receptor endocytosis was blocked, as expected. Unexpectedly, however, endocytosis of both chimeric CD4/LDL receptors and EGF receptors proceeded largely unaffected in AP-2 depleted cells (although a poster from Alexander Sorkin, University of Colorado, Denver, reported a partial inhibition of EGF endocytosis by AP-2 depletion), whereas their endocytosis was blocked in clathrin-depleted cells (Motley et al., 2003) (Figure 1). AP-2 might thus function as a clathrin adaptor for only a subset of transmembrane proteins. Indeed, Robinson presented evidence that AP-2 is required for endocytosis of membrane proteins whose cytoplasmic tails contain the endocytosis signal YXXϕ (where X is any amino acid and ϕ is a bulky hydrophobic amino acid). This signal is found in a number of membrane proteins, including the transferrin receptor, TGN38, and the cation-independent mannose 6-phosphate receptor. Using the RNAi knockdown approach, Robinson showed that proteins with clathrin endocytosis signals different from YXXϕ, such as FXNPXY, are endocytosed by AP-2-independent pathways (Figure 1). Which, then, are the clathrin adaptors for these proteins? Robinson suggested that a group of clathrin- and cargo-associated proteins that have so far been regarded as accessory proteins for clathrin and AP-2 could be good candidates. These proteins include Epsin, Hip1, Dab2, AP180, β-arrestin, and ARH (Motley et al., 2003; Conner and Schmid, 2003a). This imposes a novel level of diversity on endocytic routes and mechanisms: even the mechanisms of cargo recruitment during the clathrin pathway involve different types of adaptors.

When AP-2 is involved, how is cargo recognition accomplished, and how is the cargo targeted specifically to the clathrin coat? It was established that the μ2 subunit of AP2 binds to the YXXϕ motif and thereby mediates cargo selection to the clathrin-coated pit (Owen and Evans, 1998). But David Owen (University of Cambridge) recently solved the crystal structure of the core of the AP2 adaptor and found that, surprisingly, the putative YXXϕ binding site in μ2 is blocked by the adjacent β2 subunit (Collins et al., 2002). Owen proposed that phosphorylation of μ2 and the presence of PI(4,5)P_2 in the plasma membrane could open up the conformation and release the block of the YXXϕ binding site to allow cargo binding. Indeed, Elizabeth Smythe (University of Sheffield) reported that phosphorylation of Thr156 in μ2 is required for cargo recruitment of transferrin receptors into clathrin-coated vesicles. Using a phospho-Thr156-specific antibody for μ2, Smythe found that phospho-μ2 is opened up and active for cargo recruitment only at the plasma membrane. This ensures that AP-2 interaction with the cargo occurs only if the binding is productive, i.e., when engaged in a clathrin coat at the plasma membrane. The AP2-associated kinase AAK1 is a good candidate for a μ2 kinase (Conner and Schmid, 2002), but how is its activity regulated so that phosphorylation is coupled to cargo sorting? Smythe presented evidence that clathrin itself activates AAK1 to phosphorylate μ2. Since only the phosphorylated adaptor can bind to the YXXϕ motif in the cargo, spurious cargo binding prior to coat formation can be avoided (Jackson et al., 2003).

Caveolae, Caveolins, and the Caveosome Pathway

The availability of caveolin-1 knockout mice (Drab et al., 2001) shed new light on the mechanisms of another internalization route, caveolar endocytosis, as discussed by Ari Helenius (Swiss Federal Institute of Technology,

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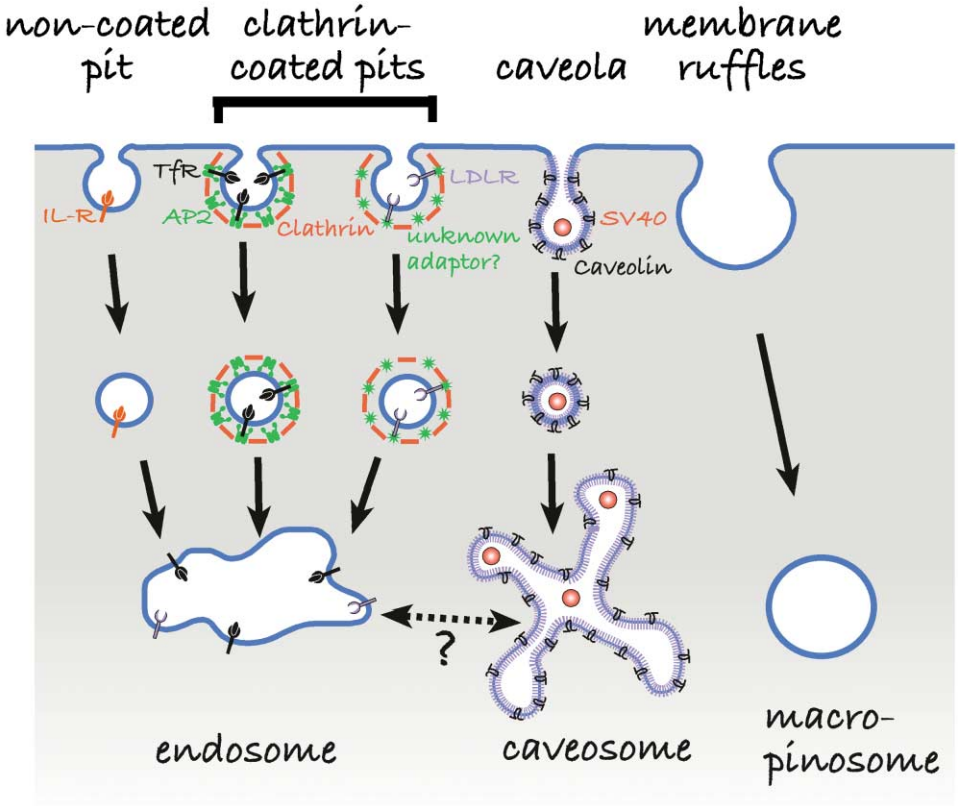

Figure 1. Multiple Pathways of Endocytosis

There are at least two distinct pathways of clathrin-dependent endocytosis, one involving AP-2 (used by, for instance, the transferrin receptor, TfR), and one involving unknown adaptors (used by, for instance, the low-density lipoprotein receptor, LDL-R) (Motley et al., 2003; Conner and Schmid, 2003a). Some membrane proteins, such as the interleukin-2 receptor (IL-R), are internalized from invaginations that are devoid of clathrin (Lamaze et al., 2001). Simian virus-40 (SV40) is internalized via caveolae, flask-shaped invaginations that are smaller than other types of endocytic invaginations (Pelkmans et al., 2001). Growth factors and certain bacteria can induce membrane ruffling, resulting in macropinocytosis (bulk uptake of fluid-phase) (Conner and Schmid, 2003b).

Zurich). The SV40 virus is endocytosed using the caveolar pathway and enters novel endosomal compartments termed caveosomes (Pelkmans et al., 2001). These are numerous, stationary compartments that are negative for standard endosomal antigens and are poorly reached by endocytic tracers (Figure 1). Caveolar endocytosis is characterized by sensitivity to cholesterol depletion (implying the involvement of lipid raft microdomains), requirement for dynamin, and independence from clathrin. SV40-containing caveosomes show a greatly increased motility, which may help the virus enter the endoplasmic reticulum from these organelles. Caveolin-1 is essential for the formation of caveolae (Fra et al., 1995), and Helenius addressed the interesting question of what happens to caveosomes and SV40 uptake when caveolin-1 is absent. Remarkably, cells from caveolin-1 knockout mice were found to be infected by SV40.

The Role of Lipids in Endocytosis

While most researchers who study the mechanisms of endocytosis focus on proteins, it is becoming increasingly evident that lipids play a very important role as well. For instance, it has been demonstrated that cholesterol depletion inhibits formation of clathrin-coated vesicles and flattens caveolae (Rodal et al., 1999). Another striking example was provided by Joost Holthuis (University of Utrecht). Aminophospholipids are distributed asymmetrically in the membrane bilayer: they are more abundant in the inner leaflet. Hoolthuis showed that this biased distribution requires two P type ATPases, Dnf1p and Dnf2p, which transport aminophospholipids from the exoplasmic to the cytoplasmic leaflet of the plasma membrane. Yeast cells devoid of these transporters showed delayed endocytosis of the membrane dye FM4-64, implying that the transbilayer lipid distribution is important for the ability of the plasma membrane to invaginate and form vesicles (Pomorski et al., 2003).

A particular class of lipids, the phosphoinositides (phosphorylated derivatives of phosphatidylinositol), play a key role in protein recruitment to cellular membranes (Simonsen et al., 2001). Pietro De Camilli (Yale University, New Haven) discussed the importance of phosphoinositides in synaptic endocytosis and regeneration of synaptic vesicles from endocytosed intermediates. In particular, $PI(4,5)P_2$ has an essential function by recruiting/activating AP-2 and a number of other possible adaptor proteins, and the enzymes that form and metabolize this lipid are therefore of special interest. De Camilli's group has previously found that the polyphosphoinositide phosphatase synaptojanin, which dephos-

phorylates PI(4,5)P_2 into PI(4)P, plays an important role in synaptic vesicle regeneration by promoting uncoating of clathrin-coated vesicles (Cremona et al., 1999). He now reported that the reverse enzyme, PI(4)P 5-kinase Iγ, also appears to be important for the normal function of the synaptic vesicle cycle. This kinase is recruited to the presynaptic plasma membrane through interactions with Talin and the small GTPase Arf6 (Di Paolo et al., 2002; Krauss et al., 2003). One possible mechanism of action for PI(4,5)P2 during endocytosis was proposed by Owen, who showed that binding of this phospholipid to the μ2 subunit of AP2 (together with its phosphorylation) could open up its conformation and release the block of the YXXϕ binding site by the β2 subunit to allow cargo binding.

Small GTPases as Regulators of Endocytic Membrane Trafficking

Rab GTPases function as membrane organizers and mediate directionality and specificity of vesicle delivery (Zerial and McBride, 2001). In the endocytic pathway, Rab5 has been implicated in endocytosis and homotypic fusion between early endosomes, Rab4 and Rab11 in endocytic recycling, and Rab7 and Rab9 in trafficking through late endosomes. Given the high degree of similarity between different Rab GTPases, their differential localization to distinct organelles has puzzled scientists for some time. There is good evidence that two related proteins, Rab escort protein (REP) and Rab GDP dissociation inhibitor (GDI), target GDP-bound and prenylated Rab GTPases to the correct membranes. Roger Goody (Max Planck Institute for Molecular Physiology, Dortmund) presented a structural model for monoprenylated Rab7 in complex with REP-1. Interestingly, the switch regions of Rab7 appear to undergo structural changes upon interaction with REP-1. It has not been clear how REP-1 and GDI bind to the highly hydrophobic geranylgeranyl group of Rab GTPases, but Goody and colleagues characterized the prenyl binding pocket of GDI complexed with yeast Ypt1p (homolog of mammalian Rab1) which had been chemically prenylated (Rak et al., 2003). The prenyl binding site appears to be exposed only upon ligand binding, which is likely to be important for the ability of GDI to deliver and retrieve Rab GTPases from membranes. But how is the GDI/Rab complex targeted to the correct membrane? Suzanne Pfeffer (Stanford University) described how a family of so-called "Yip" proteins could target Rabs to specific membranes by acting as Rab-specific GDI displacement factors (Sivars et al., 2003).

A Rab GTPase of special interest is Rab27A. Inherited mutations of this GTPase are associated with the Griscelli syndrome, a condition associated with partial albinism and impaired platelet and T cell functions. Rab27A has been shown to control the motility of melanosomes by recruiting melanophilin, which binds the actin motor protein myosin V (Wu et al., 2002). Miguel Seabra (Imperial College London) reported that melanophilin belongs to a family of effectors for Rab27 and Rab3, the latter protein previously associated mainly with neurosecretion (Strom et al., 2002). To study the expression of Rab27A in various tissues, Seabra generated transgenic mice expressing functional GFP-Rab27A under control of the Rab27A promoter. Analysis of these mice revealed that Rab27A is not only found on melanosomes but also on other lysosome-related organelles, such as melanosomes, T cell granules, basophilic granules, and Weibel-Palade bodies, as well as in exocrine and endocrine secretory vesicles (Tolmachova et al., 2003). The function of Rab27A on these organelles remains to be studied, as well as the functions of the related protein Rab27B, which has a more restricted expression pattern.

Besides the large Rab family, the 6 members of the Arf family of small GTPases are known to function as specific regulators of intracellular membrane traffic. While most Arfs function in the Golgi, the plasma membrane-localized Arf6 functions in phagocytosis, cell spreading, and endocytic trafficking (Niedergang et al., 2003). Philippe Chavrier (Curie Institute, Paris) reported from a novel two-hybrid screen for possible effectors of Arf6. Among the proteins he found to bind specifically to Arf6-GTP was mammalian Sec10, a subunit of the 8-membered exocyst complex. Electron microscopic and biochemical data were consistent with the possibility that Arf6 controls docking of endocytic recycling vesicles via the exocyst complex (Prigent et al., 2003). This suggests a novel function for the exocyst complex, which has previously been implicated in exocytosis through interactions with GTPases from the Rab and Rho families (Novick and Guo, 2002).

Ubiquitination and Signaling

Beside the small linear internalization motifs in the backbone of the cytosolic tail of receptors, conjugated ubiquitin can also function as a signal for cargo recruitment during endocytosis (Hicke, 2001). This seems to play a key role for receptor signaling and hence during development. Pier Paolo Di Fiore (FIRC Institute for Moleceular Oncology, Milan) and Ivan Dikic (Goethe University, Frankfurt) reported that the EGF receptor becomes mono-ubiquitinated at multiple cytoplasmic lysine residues (multi-ubiquitinated) upon EGF binding (Haglund et al., 2003). Multi-ubiquitination may provide selectivity of interaction with different ubiquitin binding domains, depending on the particular array of ubiquitination sites, perhaps in a combinatorial manner. In addition, multi-ubiquitination may occur in a sequential manner, allowing the possibility of encoding different levels of activation by the different number of ubiquitin units in the modified protein. Epsin and its associated proteins Eps15 and Eps15R interact with ubiquitin, and these proteins are therefore candidates for serving as clathrin adaptors for activated EGF receptors (Di Fiore et al., 2003). To further investigate the role of ubiquitin in EGF receptor endocytosis, Di Fiore and Dikic studied the uptake of an EGF receptor whose cytoplasmic tail was replaced by ubiquitin. Unlike the wild-type EGF receptor, this receptor was endocytosed independently of EGF binding, supporting the view that ubiquitin may function as an internalization signal for this receptor (Haglund et al., 2003).

Ubiquitination as an MVB Sorting Signal

Mono-ubiquitination not only serves as an endocytosis signal, it may also function as a tag for targeting membrane proteins into MVBs (Hicke, 2001). The role of ubiquitination and MVB biogenesis during receptor down-

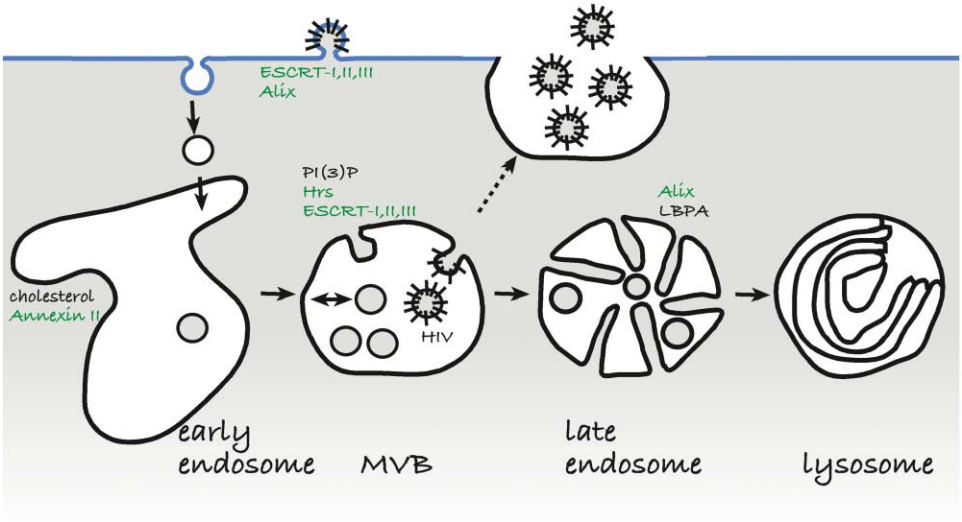

Figure 2. Regulators of MVB Biogenesis and Viral Budding

MVB biogenesis requires the cholesterol binding protein annexin II at an early stage and PI(3)P, Hrs, and ESCRT complexes at a later stage (Mayran et al., 2003; Petiot et al., 2003). Intraluminal vesiculation of late endosomes is promoted by LBPA and may involve Alix. HIV hijacks the ESCRTs and Alix to promote viral budding from the plasma membrane of lymphocytes (von Schwedler et al., 2003; Strack et al., 2003). In macrophages, HIV buds into MVBs and acquires properties of intraluminal vesicles (Pelchen-Matthews et al., 2003). Viral particles can possibly be released from macrophages in the same manner as exosomes, i.e., upon fusion of an MVB with the plasma membrane.

regulation is consistent with the idea that MVBs act as cisternal intermediates during lysosomal degradation. However, in recent years, it has become clear that protein sorting via MVBs plays a positive role during signaling events (Sorkin and Von Zastrow, 2002) as well as in the biogenesis of secretory lysosomes such as melanosomes, MHC-II compartments, and T cell granules (Blott and Griffiths, 2002). But how are MVBs formed? The protein and lipid machinery that mediates the formation of the budding of the limiting membrane to form the internal vesicle is starting to be elucidated (Figure 2).

The Protein Machinery of MVB Sorting

Scott Emr (University of California at San Diego) discussed the mechanisms of MVB biogenesis and sorting in yeast (Figure 2). Isolation of vacuolar protein mutants has yielded a subgroup of about 20 mutants with specific defects in MVB biogenesis and protein sorting into MVBs, the so-called class E *vps* mutants. Conjugated mono-ubiquitin serves as a tag for entry of transmembrane proteins into the MVB sorting pathway, and Emr's group has recently identified three subcomplexes consisting of Vps class E proteins which sort ubiquitinated cargo into MVBs. These *e*ndosomal *s*orting *c*omplexes *r*equired for *t*ransport (ESCRTs) contain at least two ubiquitin binding subunits (Vps23p in ESCRT-I and Vps36p in ESCRT-II) and probably interact directly with ubiquitinated cargo (Katzmann et al., 2002). They also appear to be required for MVB biogenesis, since *vps* class E mutants fail to accumulate intraluminal vesicles within the vacuole.

Since the original *vps* screen only identified nonessential proteins, Emr and colleagues set up a new replica plate-based screen for lethal temperature-sensitive MVB sorting mutants. The first protein to be identified by this new screen was a ubiquitin ligase, Rsp5p. Similar to *vps* class E mutants, *rsp5* mutants are defective in MVB sorting. However, unlike Vps class E proteins, Rsp5p is not required for MVB biogenesis. Rsp5p is localized to punctate intracellular structures (possibly endosomes or Golgi elements) and is required for ubiquitination of carboxypeptidase S, a biosynthetic cargo known to be sorted into intraluminal vesicles of MVBs. These results implicate Rsp5p as a crucial component of the machinery that recognizes and labels cargo that is destined for MVBs.

If the evolutionarily conserved ESCRTs represent the machinery that sorts ubiquitinated proteins into MVBs, how are these complexes recruited to membranes? Harald Stenmark (Norwegian Radium Hospital, Oslo) presented evidence that the ubiquitin binding protein Hrs (the mammalian homolog of yeast Vps27p) serves to recruit ESCRT-I to endosomal membranes via interactions with the subunit Tsg101 (homolog of Vps23p). RNAi-mediated depletion of Hrs inhibits MVB biogenesis, probably because ESCRT recruitment is inhibited. Hrs could serve a dual function by first recognizing ubiquitinated cargo and then recruiting ESCRT complexes to membrane domains in which cargo is enriched (Bache et al., 2003).

Hrs and ubiquitinated proteins are localized to an intriguing bilayered clathrin coat on endosomes (Sachse et al., 2002), which was characterized by Judith Klumperman (University Medical Centre, Utrecht). Using electron tomography (Murk et al., 2003), she provided spectacular three-dimensional reconstitutions of MVBs with inward budding profiles. Interestingly, these invaginations were always found adjacent to the bilayered clathrin coat. Klumperman proposed a model for endosomal sorting by retention of ubiquitinated cargo in the Hrs-containing clathrin coat. Graca Raposo (Curie Institute, Paris) showed that this coat was particularly abundant in maturing MVBs of melanocytes and proposed

that it might serve an important function in early melanosome biogenesis by mediating intraluminal sorting of melanosome-specific cargo proteins such as Pmel17 (Raposo and Marks, 2002).

The Lipid Machinery of MVB Biogenesis

Like endocytosis, MVB biogenesis is likely to depend on lipids as well as on proteins (Figure 2). Jean Gruenberg (University of Geneva) showed that PI(3)P is required for sorting of EGF receptors into MVBs (probably by mediating membrane recruitment of Hrs), but not for biogenesis of endosomal carrier vesicles that bud from early endosomes (Petiot et al., 2003). The latter requires instead annexin II, a cholesterol binding protein localized to early endosomes (Mayran et al., 2003) (Figure 2). While intraluminal vesicles of MVBs are enriched for PI(3)P, intraluminal vesicles of late endosomes contain large amounts of the hydrolase-resistant lipid lyso-bisphosphatidic acid (LBPA) (Figure 2). Gruenberg has previously shown that antibodies against LBPA inhibit the biogenesis of late endosomes (Kobayashi et al., 1998), and he now showed that liposomes that contain LBPA spontaneously form intraluminal vesicles when the liposomes are acidic, similar to natural endosomes. Gruenberg pointed out that in vivo, this process is very likely to rely on an interplay between lipids and proteins, and he presented evidence that Alix may be part of such a regulatory mechanism. Alix has recently been proposed to act as a link between ESCRT-I and ESCRT-III (von Schwedler et al., 2003; Strack et al., 2003), and the finding that it also regulates LBPA functions provides a potential connection between ESCRTs and LBPA in MVB biogenesis.

MVB Dynamics in Health and Disease

Lysosome-related organelles such as melanosomes and T cell granules are thought to be derived from MVBs, and their biogenesis requires a functional endocytic pathway. Using T cells from patients with diseases such as Hermansky-Pudlak syndrome, Chediak-Higashi syndrome, and Griscelli syndrome, Gillian Griffiths (Oxford University) has been studying the molecular machinery responsible for the regulated fusion of T cell granules with the plasma membrane (Blott and Griffiths, 2002). She discussed the involvement of a Rab GTPase (Rab27A), a Rab geranylgeranyl transferase, an adaptor complex (AP-3), and a large BEACH domain-containing protein (Lyst) in this process. Curiously, loss of the β subunit of AP3 prevents microtubule-mediated movements of lysosomes along microtubules in cytotoxic T lymphocytes. This suggests a novel function for adaptor proteins (Clark et al., 2003).

In addition to targeting proteins for lysosomes or exosomes (Stoorvogel et al., 2002), intraluminal vesicles of MVBs may also serve as a temporary storage for MHC class II molecules and their associated invariant chain (Ii). Willem Stoorvogel (University Medical Centre, Utrecht) presented evidence that the packaging of MHCII-Ii complexes into MVBs of dendritic cells (which are critical for the initiation of primary immune responses) protects Ii from processing. Upon stimulation of the dendritic cell by lipopolysaccharides, intraluminal vesicles fuse back with the limiting membrane, thus triggering Ii processing and peptide loading onto the MHCII molecule. In this way, the dendritic cell may ensure that peptide loading of MHCII occurs efficiently only when contacted by pathogens. After peptide loading, the MHCII-peptide complex is finally transferred from the limiting membrane of the MHCII compartment to the plasma membrane. But how can MHCII-Ii complexes exist within MVBs of immature dendritic cells without being degraded by lysosomal enzymes? Ira Mellman (Yale University, New Haven) reported that lysosomes from immature dendritic cells fail to acidify, and that lysosomal acidification occurs upon maturation, apparently by a regulated assembly of the vacuolar proton ATPase (Figure 2). Moreover, the lysosomes also appear to have remarkably low levels of proteolytic enzymes (Trombetta et al., 2003). Future work should reveal the signaling mechanisms that regulate this lysosomal acidification pathway.

Beside their physiological roles, MVBs are also exploited by pathogens. An increasing number of viruses have been found to usurp this machinery for viral assembly. For example, HIV hijacks this pathway during infection of macrophages (Mark Marsh MRC, University College London) (Figure 2). Macrophages are thought to be one of the first sets of cells in the body to be infected by HIV, and to pass on large amounts of virus to T helper cells. Recent studies have revealed that the membrane-associated structural proteins of HIV and several other viruses recruit Alix and the ESCRT machinery in order to facilitate viral budding (von Schwedler et al., 2003; Strack et al., 2003). While HIV budding appears to occur from the plasma membrane of lymphocytes, Marsh presented evidence that HIV buds into MVBs of macrophages (Pelchen-Matthews et al., 2003) (Figure 2). The viral membrane even incorporates several endosomal proteins such as LAMP-1 and CD63. The HIV particles within MVBs thus resemble intraluminal vesicles. Marsh pointed out that interactions between the infected macrophage and a T helper cell may elicit a signaling response that triggers fusion of MVBs with the plasma membrane and thus facilitates viral transfer to the T cell. It remains a challenge to identify the molecular mechanisms of signaling and MVB fusion.

Endocytosis and Cell Asymmetry

Recent work from a number of laboratories has uncovered key roles of endocytosis in the establishing of cell polarity. In particular, asymmetric cell division of the sensory organ precursors (SOPs) during development of the peripheral nervous system in *Drosophila* seem to involve a number of polarized endocytic events (Jürgen Knoblich, IMP, Vienna) (Figures 3A–3C). In the SOPs, Numb protein concentrates in the area of the cell cortex overlying one of the two centrosomes (Knoblich et al., 1995) and is thus segregated into one of the two daughter cells upon cytokinesis (Rhyu et al., 1994). The fate of the daughter cells is then determined by a directed signaling event from one daughter cell to the other, which involves Notch and its ligands Delta and Serrate (Figures 3A–3C). Indeed, the asymmetric distribution of Numb controls the fate of the daughter cells by repressing Notch signaling. Numb binds to α-adaptin, a component of the AP2 complex, both in *Drosophila* and in vertebrates (Santolini et al., 2000; Berdnik et al., 2002). In the asymmetrically dividing SOPs, α-adaptin, like Numb, is concentrated at one side of the cell cortex

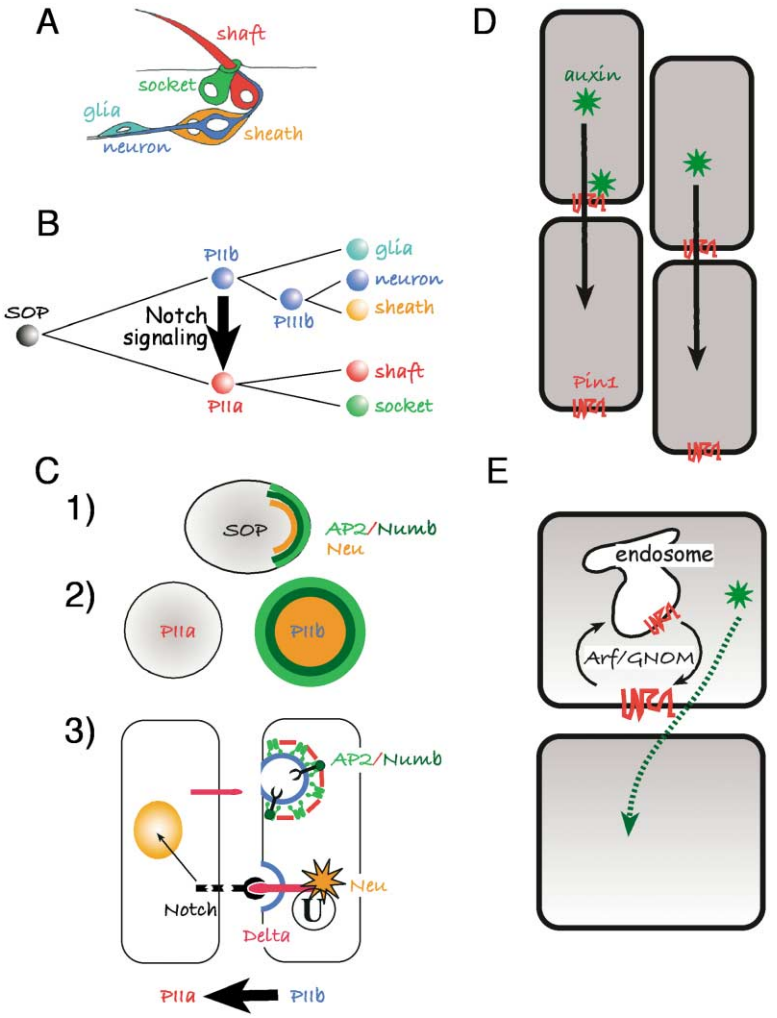

Figure 3. Endocytosis and Cell Asymmetry

(A–C) Endocytosis during asymmetric cell division of the *Drosophila* SOPs. The external sensory organ of the fly is formed by four cells (A) which together with a glial cell are generated by two or three consecutive asymmetric cell divisions controlled by directed Notch signaling events (B). Two endocytic mechanisms control the directionality of the signaling event underlying asymmetric cell division (C). Asymmetric localization of Numb, α-adaptin (Berdnik et al., 2002), and the E3 ubiquitin ligase Neuralized (Neu) in the cortex of the SOP (C1) (Le Borgne and Schweisguth, 2003) leads to different accumulation of these proteins in the two daughter cells (PIIa and b; C2). In the PIIb, accumulation of the receptor Notch (and thereby Notch signaling) is downregulated by AP2/Numb endocytosis. In addition, ubiquitin-mediated endocytosis of Dl in PIIb triggers the cleavage/nuclear import of the cytosolic tail of Notch (and thereby elicits Notch signaling) in the PIIa cell.

(D and E) Endocytosis and polarized auxin transport in *Arabidopsis*. Polarized auxin transport is mediated by the polarized distribution of the auxin efflux carrier Pin1 (D). Polarized localization of Pin1 is accomplished by its trafficking from an endosomal compartment, a process mediated by an Arf GTPase and its exchange factor GNOM (E) (Steinmann et al., 1999; Geldner et al., 2003).

(Berdnik et al., 2002) (Figure 3C). Since Numb can also directly interact with Notch, Numb may antagonize Notch activity by recruiting α-adaptin, causing Notch internalization and signal downregulation preferentially in the daughter cell receiving the higher levels of Numb/α-adaptin. In addition, it has been recently shown that the E3 ubiquitin ligase Neuralized also localizes asymmetrically in the dividing SOP and cosegregates with Numb (Le Borgne and Schweisguth, 2003) (Figure 3C). Neuralized upregulates endocytosis of the Notch ligand Delta, and it has been proposed that endocytosis of the ligand when bound to Notch receptor in the sibling cell triggers receptor cleavage and signaling (Figure 3).

In plants, polarized endocytic trafficking within single cells mediates the asymmetric flow of a key hormone, Auxin, during development (Niko Geldner, University of Tübingen) (Geldner et al., 2003) (Figure 3). Polar transport of the phytohormone auxin mediates various processes in plant growth and development, such as apical dominance, tropisms, vascular patterning, and axis formation. The polar auxin flux is caused by the asymmetric distribution of efflux carriers, such as PIN1, acting at the plasma membrane (Figure 3). The coordinated PIN1 localization to the region of the plasma membrane distal to the flow of Auxin is abolished by mutations in the *Arabidopsis* GNOM gene, one of the 8 Arf-GEF homologs in the plant (Steinmann et al., 1999). BFA treatment causes auxin-transport-related phenotypes by specifically impairing Gnom function, as indicated by the total rescue of the BFA effect by using a BFA-insensitive mutant GNOM rescue construct (Geldner et al., 2003). Upon BFA treatment, GNOM and PIN1 colocalize to the same compartment and GNOM-GFP colocalizes with FM4-64 positive structures. In addition, Rab5-positive compartments are structurally altered in gnom mutants. This suggests that polarized endocytic trafficking mediated by the Arf-GEF GNOM targets PIN1 and thus regulates auxin flux.

Beyond Receptor Downregulation: Endosomal Compartmentalization of the Signal Transduction Event

A well-established role of endocytosis is to internalize surface receptor, thereby downregulating the signaling event. In addition, accumulating evidence suggests that receptor signaling can take place from endosomes, and compartmentalized signaling could be physiologically important (Sorkin and Von Zastrow, 2002; Gonzalez-Gaitan, 2003). Both signaling downregulation and endosomal signaling were discussed at length at the Maratea meeting. It has been previously shown that, in the embryo, Wingless (Wg) emanating from a single source

signals at different ranges toward anterior and posterior, and that enhanced endocytosis and degradation of Wingless restricts the signaling range (reviewed in Piddini and Vincent, 2003). Using Hrs mutants in *Drosophila*, Hugo Bellen (Baylor College of Medicine, Houston) and Eugenia Piddini (National Institute for Medical Research, London) now showed evidence that Wg signaling is attenuated by Hrs, thereby suggesting a role of MVB biogenesis.

Different complexes seem to act as scaffolds that recruit factors that mediate alternative pathways downstream EGFR signaling either to the plasma membrane or the late endosomes. Lukas Huber (University of Innsbruck) reported that, upon EGF stimulation, a P14/MP1 complex binds to Mek1/Erk1 in the late endosome, where it is associated to DRMs (Teis et al., 2002). P14 itself seems to be essential for the targeting of the complex to the late endosome, but its depletion does not affect the morphology of the compartment, suggesting that P14 is involved in targeting the EGF-receptor transduction machinery rather than being involved in endosomal dynamics. At the endosome, the P14-containing complex mediates the phosphorylation of Erk1/2, but not P38, in an alternative EGFR pathway. In addition, activation of Erk1/2 at the plasma membrane is P14-independent. The emerging picture is that two different scaffold complexes mediate EGFR signaling through two alternative pathways: a plasma membrane scaffold containing KSR, and an endosomal scaffold containing P14/MP1. If true, this opens the possibility that different scaffolds target the transduction event to different intracellular compartments, thereby conferring different "signaling outputs," such as proliferation, cell fate specification, and survival. It also implies a new strategy to design specific drugs that will affect differentially alternative signaling outputs from the same receptor.

An endosomal compartment important for signaling was uncovered while searching for Rab5 effectors in Marino Zerial's lab. Using a GTP-bound Rab5 column, this group has found around 40 factors interacting (directly or indirectly) with activated Rab5, including EEA1, Rabaptin-5, Rabenosyn-5, Rabankyrin-5, and PI 3-kinases (Zerial and McBride, 2001). Marta Miaczynska (Max Planck Institute for Molecular Cell Biology and Genetics, Dresden) reported that two homologous proteins interact specifically with GTP-Rab5 at the endosome. The endosomes containing these Rab5 effectors are characterized by the absence of PI(3)P and the PI(3)P binding effector, EEA1, and do not correspond to clathrin-coated vesicles or caveosomes. These membrane structures may therefore represent a novel subcompartment of the early endosome. Interestingly, EGF internalized into these structures initiates a signaling event mediated by the two Rab5 effectors themselves. Again, a subcompartmentalization of the transduction event leads to a specific molecular response mediated by factors which, like the novel Rab5 effectors, are restricted to a defined compartment.

Beyond Signal Transduction: Endocytosis and Morphogens

Recent work on a number of signaling pathways has uncovered a novel signaling role for endocytosis: it controls the spreading of morphogens. Morphogens, like Wingless, Hedgehog, and the TGFβ superfamily homolog Decapentaplegic (Dpp), are signaling molecules which are secreted from a restricted group of cells and are thereby distributed in the target tissues with graded concentration profiles. This graded distribution of morphogens provides the target cells with positional information: cells know their position by reading the morphogen concentration around them. Endocytosis seems to play different roles during the spreading of different morphogens. During Wingless signaling, it seems to play a restricting role: the ligand seems to be cleared up in the lysosomes upon receptor-mediated endocytosis (Piddini and Vincent, 2003). In the case of Dpp, endocytosis seems to power the spreading of the ligand (Marcos Gonzalez-Gaitan, MPI Dresden). It has been proposed that Dpp traffics through the target tissue by passing through cells, with only a limited movement through the extracellular space (Entchev et al., 2000). Endosomal trafficking of the ligand, recycling, and degradation mediated by the endocytic Rab proteins Rab5, Rab4, Rab11, and Rab7 control in each cell the degradation of a percentage of the internalized ligand, while the rest is resecreted to be passed on to the next cells. This ensures the formation of stable gradients.

How do the morphogens travel from cell to cell? It was recently proposed that lipid-linked morphogens like Wingless and Hedgehog might travel on lipid-containing particles called argosomes (Greco et al., 2001). Movement of morphogens on particles rather than as free proteins might allow more precise control of trafficking, depending on which other proteins might also be present on the same particle. Evidence presented by the Eaton lab (Suzanne Eaton, MPI Dresden) suggests that the gpi-linked HSPG Dally could be a candidate for such a regulatory protein.

Conclusion and Future Perspectives

Endocytosis research is an exciting and very fast-moving field, with elements of molecular and structural cell biology as well as neurobiology, developmental biology, immunology, and microbial pathogenesis. It was evident from this meeting that important progress has been made concerning the molecular mechanisms of endocytosis and MVB sorting, and an increasing body of structural insight is accumulating. However, it still remains a challenge to characterize the various mechanisms of endocytosis, and to better define the membrane flow within the endocytic pathway. Although some of the major molecular players in endocytosis and endosomal sorting have been identified, it will take much further work to understand the mechanisms that orchestrate the amazing diversity of pathways and specificity of transport in endocytic membrane traffic.

A major emphasis of this meeting was the importance of endocytosis during development. A functional endocytic pathway is essential for maintaining gradients and long-range distribution of key morphogens, for controlling asymmetric cell divisions, and for restricting signaling outputs—all crucial factors in the process that starts with simple cells and ends with complex tissues. It is therefore quite possible that major components of the endocytic pathway may have coevolved with signaling pathways. Intriguing connections between signaling molecules and endosomes were revealed, and the in-

creasing efforts to merge model systems from developmental and cell biology have already proven rewarding.

Endocytosis is critical for immunity but also serves as a gateway for pathogens. It is interesting to observe that endosomes not only serve as compartments of antigen presentation—they may also function as hatcheries for viruses. Novel insight into the molecular mechanisms of how viruses subdue the immune system and reprogram the endocytic machinery can provide us with much-needed armor to combat infections.

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