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Importance of Endocytic Pathways in Liver Function and Disease

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Abstract

Hepatocellular endocytosis is a highly dynamic process responsible for the internalization of a variety of different receptor ligand complexes, trophic factors, lipids, and, unfortunately, many different pathogens. The uptake of these external agents has profound effects on seminal cellular processes including signaling cascades, migration, growth, and proliferation. The hepatocyte, like other well-polarized epithelial cells, posses a host of different endocytic mechanisms and entry routes to ensure the selective internalization of cargo molecules. These pathways include receptor-mediated endocytosis, lipid raft associated endocytosis, caveolae, or fluid-phase uptake although there are likely many others. Understanding and defining the regulatory mechanisms underlying these distinct entry routes, sorting and vesicle formation, as well as the postendocytic trafficking pathways is of high importance especially in the liver, as their mis-regulation can contribute to aberrant liver pathology and liver diseases. Further, these processes can be “hijacked” by a variety of different infectious agents and viruses. This review provides an overview of common components of the endocytic and postendocytic trafficking pathways utilized by hepatocytes. It will also discuss in more detail how these general themes apply to liver-specific processes including iron homeostasis, HBV infection, and even hepatic steatosis.

Introduction

The liver, by way of hepatocytes, is responsible for a number of physiological processes that involve the uptake and subsequent metabolism or processing of various proteins, lipids, pathogens or toxins. In fact, one of the most prevalent processes conducted by the hepatocyte is vesicle trafficking. These endocytic- and postendocytic-based processes depend upon interactive, dynamic protein complexes to allow for tight spatial and temporal regulation of vesicle formation at different sites along the endocytic pathway. In general, this vesicle formation machinery comprises a coat protein, such as clathrin or caveolin, and a number of monomeric and multimeric accessory proteins with various protein- and/ or lipid-binding domains. The controlled endocytic entry route allows the hepatocyte to specifically sequester and internalize desired ligand/receptor complexes, such as growth factor/respective receptor tyrosine kinase and iron-bound transferrin (Tf)/transferrin receptor (TfR). It also aids in the maintenance of normal lipid serum levels through hepatocellular

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endocytosis of lipoproteins and lipoprotein receptors. Understanding the proteins and mechanisms underlying endocytosis and subsequent vesicle formation at different postendocytic sites along the overall endocytic pathway is of high importance, as their misregulation can contribute to aberrant liver pathology (e.g., steatosis) and liver diseases (e.g., hepatocellular carcinoma). Further, pathogens may “hijack” endocytic proteins, processes, and pathways to facilitate infection of hepatocytes. Therefore, this review will provide an overview of common components of the vesicle formation complexes assembled and utilized in hepatocytes, followed by more focused discussions on three examples of endocytic processes of particular relevance to liver function and disease: (i) TfR endocytosis and iron homeostasis, (ii) the biology of hepatocellular lipid droplet dynamics as it pertains to steatosis, and (iii) infection of the liver by Hepatitis B virus.

Components of the Endocytic Pathway

General aspects of endocytosis in nonpolarized cells

Endocytosis is defined as a process by which cells internalize fluids, proteins and lipids—whether extracellular or integral to the plasma membrane—through the formation and severing of membrane-bound vesicles. Endocytosis can be further defined based on the type of material that is internalized as follows: pinocytosis or fluid-phase endocytosis; phagocytosis, for example, in the case of bacteria; and receptor-mediated endocytosis (RME). Independent of the type of internalized cargo, the basic principle is that the plasma membrane invaginates into the cytoplasm, closes up, and allows a cargo-containing vesicle to enter the cytosol. Subsequently, these vesicles can be delivered to an early postendocytic organelle to begin the initial sorting and processing of the cargo, which might result in recycling of the cargo in whole or in part, or alternatively, its degradation. The endocytic process is highly regulated and requires a dynamic, integrated network of coat proteins and accessory proteins that control membrane dynamics, cargo selection and concentration, vesicle coating/uncoating and, finally, membrane scission and vesicle trafficking (28,85,86,122,133) (see Fig. 1).

Modes of entry into the cell: Coats, adaptors, and accessory proteins

The two general mechanisms by which extracellular material enters the cell are clathrin-mediated endocytosis (CME) and the clathrin-independent endocytic process, CIE (28,85). CME is well known for its role in the internalization of growth factor receptors and is regulated by the clathrin coat, its accessory proteins that recognize specific cytoplasmic sorting sequences. In contrast, CIE appears to be responsible for mediating the internalization of a variety of substrates, is less well defined and most importantly, lacks the requirement for Clathrin or specific internalization signals. Each of these two types of endocytosis and the associated proteins that mediate these processes are discussed in more detail below.

Clathrin dependent endocytosis

CME is also often referred to as RME, as it is initiated by the capture and subsequent concentration of ligand/receptor cargo into rounded shallow clathrin-coated pits (CCPs) at the plasma membrane. The hallmark of CME is a coat of clathrin triskelions—three clathrin

heavy chains (CHCs) and three clathrin light chains—in the form of a honeycomb lattice (Fig. 1A and B). Additionally, an inner shell of the adaptor protein (AP) complex AP-2 is generally present as part of the clathrin coat (105). As described below, AP-2 aids in selecting and concentrating cargo, as well as in recruiting clathrin and accessory proteins.

AP-2 is one of five [AP-1 through AP-5; (9, 53)] structurally related heterotetrameric protein complexes involved in cargo selection and assembly of an overall vesicle protein coat, although not necessarily a clathrin-containing coat in all cases (124). The different AP complexes display distinct subcellular localizations at organelles along the endocytic and secretory pathways (e.g., endosomes, lysosomes, and Golgi); however, AP-2 is the AP complex that localizes to the plasma membrane and contributes to endocytosis. Similar to the other four AP complexes, AP-2 consists of two large "adaptin" subunits (α and β2), one medium "μ-adaptin" subunit (μ2), and one small "σ-adaptin" subunit (σ2). The β2- and μ2-adaptin subunits are implicated in selection of cargo through recognition of sorting motifs contained in the cargo, including: NPXY motifs (one letter amino acid code), tyrosine-based sorting motifs and dileucine-based sorting motifs (10,123,124,154). The β2-adaptin subunit also enhances interactions of AP-2 with clathrin through the so-called "clathrin box" contained in this subunit (106). In addition to the association of AP-2 with proteins, the α- and μ2-adaptin subunits contain phosphatidylinositol-4,5-bisphosphate (PIP_2)-binding modules, which mediate AP-2's localization to the plasma membrane and cargo binding abilities (56, 159). The remaining subunit, the small σ2-adaptin, appears to serve largely as a structural component of the AP-2 complex, but it has also been found to bind endocytic dileucine-based sorting motifs (24,30,65).

A main function of AP-2 is the selection and concentration of cargo during the initial stages of CCP formation at the plasma membrane. However, AP-2 also functions in recruiting and interacting with accessory proteins involved in this endocytic process. Through interactions with DPF, DPW, and FXDXF motifs (one letter amino acid code), both the α- and β2-adaptin subunits mediate such recruitment of accessory proteins to the plasma membrane (14, 106, 133, 155). These accessory proteins in turn can also interact with other proteins via various interaction domains, further extending this dynamic protein network. For example, some accessory proteins contain their own "clathrin box" that mediates interactions with CHCs. Protein-protein interactions between accessory proteins might be mediated by NPF (one letter amino acid code)-Eps15 Homology (EH) domain interactions or Src Homology 3 (SH3) domain-Proline-rich Domain (PRD) interactions (86, 133). (See Table 1 for a list of interaction domains and examples of accessory proteins containing these domains.)

Besides the "classic" heterotetrameric AP complexes, cells also employ a number of monomeric APs that serve a similar function [for reviews, see (86, 122, 133)]. Amongst the so called "CLASPs" (clathrin-associated sorting proteins) are ubiquitin adaptors such as epsin and Eps15; disabled homolog-2 (Dab2), which specifically regulates internalization of low density lipoprotein receptors; and members of the arrestin family, which are particularly important in G protein-coupled receptor trafficking (63,80,110,140). By way of these interactions, proteins associated with CME form a highly regulated network that contributes to, and aids in, regulating substrate recruitment, vesicle formation and severing of the vesicle from the plasma membrane (86).

Invagination and liberation of coated vesicles is mediated by the large GTPase dynamin 2 (Dyn2). The three members of the mammalian dynamin family share four conserved domains: a N-terminal, highly conserved GTP binding domain, followed by a pleckstrin homology domain (PH) that allows membrane binding, a coiled-coil domain (CC) and a PRD, that promotes association with a variety of effector proteins and is less conserved than the others (1,67,87,115,130,168).

Dyn2 has also been referred to as a “pinchase” that generates discrete vesicles from invaginated coated pits. Like clathrin, Dyn2 can self-assemble and it appears that GTP hydrolysis is the driving force for membrane fission (89,127). In addition to acting as a mechanoenzyme, Dyn2 can also promote vesicle scission in concert with other effectors, e.g., BAR domain containing proteins. Either way, Dyn2 action is indispensable for proper CME.

Another component that participates in the liberation of coated vesicles from the plasma membrane is the subcortical actin cytoskeleton. While much of the information about the contributions of actin to this process has come from the use of yeast models, there is also substantial evidence of the endocytic role of actin in mammalian cells. For example, the mammalian Hip1 (Huntingtin interacting protein 1) protein, a homologue of the yeast actin binding protein Sla2p that plays a role in endocytosis (55) has been demonstrated to colocalize with clathrin, AP-2, and endocytosed transferrin. These data suggest that Hip1 could link the actin cytoskeleton to endocytic processes (13, 14, 32). In addition, actin motors such as the minus-end motor Myosin VI and the plus-end motor Myosin 1E have been shown to participate in CME via an interaction with the adaptor Dab2 and PdtIns(4,5)P_2 or dynamin and synaptojanin 1, respectively (70,147). Other actin links to the endocytic machinery include actin-binding proteins, profilin, synapsin, syndapin, and cortactin, all of which, surprisingly, are also known binding partners of dynamin (51,66).

Vesicle formation and trafficking also involves a cooperative and dynamic interaction between lipids and proteins. The specific lipid composition of membranes affects membrane fluidity; the clustering of receptors; and the targeting, clustering and/or signaling of coat proteins and accessory proteins. To facilitate this lipid-protein interaction, a number of endocytic proteins, in particular those associated with CME, contain lipid-binding domains. As noted above, PIP_2 aids in targeting and localizing the α- and μ2-adaptin subunits of AP-2 to the plasma membrane. The lipid-binding domains of other endocytic proteins are also particularly sensitive to phosphoinositide-containing membrane domains, exhibiting a preference for PIP_2. (See Table 1 for examples.)

Lipid-protein interactions mediated by Epsin N-terminal Homology/ AP 180 N-terminal Homology (ENTH/ ANTH) domains and various forms of Bin-Amphiphysin-Rvs161/167p (BAR) domains, such as contained in epsin (ENTH domain) and amphiphysin and endophilin (N-BAR domain), mediate the bending, and tubulation of membranes during vesicle formation (62,78,92). These proteins also contain additional protein-protein interaction domains, which can facilitate cargo recruitment or the recruitment of other coat proteins. As an example, the SH3 domains of amphiphysin and endophilin interact with the PRD of the large GTPase Dynamin 2 (Dyn2; Fig. 1E). The interaction of Dyn2 with PIP_2,

which is mediated by its Pleckstrin Homology (PH) domain, combined with Dyn2's GTPase activity, adds a mechanochemical aspect to the tubulation process and, finally, vesicle scission (19,90,92,111,134).

Clathrin-independent endocytosis

Clathrin-independent endocytosis can be induced in several ways. It can be both Dyn2-dependent and independent (138,150) and account for a large amount of total fluid uptake in the cell (57). Various types of CIE have been described and the factors involved depend on the cargo and cell type examined. A central component of clathrin-independent uptake is small, flask-shaped invaginations named caveolae. Caveolae were previously thought to be dynamic endocytic structures. However, evidence accumulated over the years is convincing that this is probably not the case. While dispensable for endocytic processes, caveolae appear to be involved in transendothelial transport, organization of PM domains, and may also serve as signaling platforms that support a variety of different cascade networks (11,37,108,117).

A number of different types of membrane proteins are internalized via CIE due to the lack of specific internalization signals that target them for CME. These proteins are often important components of the PM (see below). These proteins are internalized by a "default" mechanism which ensures a necessary level of quality control (potentially in the endosomes) and helps maintain a proper and functional surface. Examples for proteins internalized via CIE include components of the immune response (e.g., MHCI and II), transporters (e.g., calcium and potassium channels, glucose, and amino acid transporter), some growth factor receptors (e.g., β-adrenergic receptor, and c-Met) or cell adhesion components (e.g., ICAM1, E-Cadherin, and integrins), just to name a few [for recent reviews see (82,132)].

Depending on the cargo protein, these processes may be dependent or independent of dynamin function and are regulated by small GTPases of the Rho family such as Cdc42 and Rac1 (fluid uptake), RhoA (IL-2Rβ) or Arf 6 (MHCI, GPI-anchored proteins). Interestingly, CIE cargo proteins merge with CME cargoes in Rab5 and EEA1 positive early endosomes. Moreover, other members of the Rab family of GTPases such as Rab11 and Rab22a further control the recycling of CIE cargo (97–99,161).

Many open questions in regard to CIE remain, e.g., what is the exact internalization machinery? How is the cargo selected? How is this process regulated in polarized cells? Future studies designed to answer these and other questions will provide us with novel insights into this important endocytic pathway.

Intracellular trafficking routes: Recycling versus degradation

Once cargo is internalized, the nascent vesicles are trafficked to various intracellular compartments for further sorting. In this way, the cell decides whether the cargo will be recycled back to the plasma membrane (e.g., TfR) or targeted for degradation (e.g., epidermal growth factor receptor (EGFR; Fig. 1A). Independent of the entry route, the initial sorting compartment along the endocytic intracellular trafficking pathway is often the sorting endosome or early endosome. Upon sorting at the early endosome, cargo to be recycled can be rapidly trafficked back to the plasma membrane. Alternatively, cargo

destined to be recycled may be trafficked through the perinuclear recycling endosome before being recycled to the plasma membrane. In contrast to recycling, some cargo protein and/or lipids are targeted for degradation. In this case, after sorting at the early endosome, these cargo molecules are trafficked to the late endosome or multivesicular body (MVB) and subsequently lysosome, where the cargo is ultimately degraded.

Development of compartment-specific markers and fluorescent probes, together with improved imaging techniques, has allowed for more detailed tracking of cargo proteins through the endocytic pathways. As such, these technological advances have contributed to an increased understanding of endocytic trafficking routes. For example, the TfR first colocalizes with clathrin at the plasma membrane. It then enters and undergoes sorting at early endosomes, which are positive for the small GTPase Rab5 or its effector EEA1 (early endosomal antigen 1). The TfR is then sorted to the perinuclear Rab11-positive recycling endosome, from which the receptor is recycled back to the plasma membrane. In contrast, depending on the specific cellular conditions (see below), the EGFR may follow either trafficking pathway, being recycled in a manner similar to the TfR in some cases and undergoing degradation in others. If the EGFR is destined to be degraded, it also enters early endosomes after internalization. However, degradation-destined EGFR is then trafficked to MVBs/late endosomes, which are positive for CD63 and Rab7, and subsequently lamp1-positive lysosomes (44, 59, 60, 85, 128) (Fig. 1A).

How is the fate of internalized cargo determined? It appears cargo fate might depend on the cellular context. For example, ligand concentration may play a role in determining the fate of the receptor: low epidermal growth factor (EGF) concentrations favor recycling while high amounts of EGF target EGFRs for degradation (141, 143). Furthermore, over the past years it has been revealed that ubiquitination plays an important role in regulating the trafficking of growth factor receptors. While poly-ubiquitination through long chains of lysine 48-linked ubiquitin is well known to target proteins for degradation by the proteasome, modification of cargo proteins via mono-ubiquitination or short chain lysine 63-linked poly-ubiquitination, sometimes at multiple sites within the cargo, serves as a signaling module to target proteins for lysosomal degradation [for reviews see (22, 45)]. These mono- or short chain-ubiquitin modifications are recognized by the ESCRT (Endosomal Sorting Complex Required for Transport) machinery, a complex consisting of four multimeric subcomplexes (0, I, II, III) that recognizes ubiquitinated cargo and then facilitates simultaneous formation of, and cargo sorting into, intraluminal vesicles of early endosomes and MVBs/late endosomes [Fig. 1C and D; (5)]. MVBs/late endosomes then ultimately fuse with lysosomes to promote degradation of the target protein (Fig. 1A). In general, while ubiquitination can occur at the plasma membrane and may serve as an internalization signal, it is most important at the level of MVBs as described above. Therefore, it is not surprising that defective ESCRT function is linked to a variety of diseases, including cancer (e.g., hepatocellular carcinoma, pancreatic cancer, and breast cancer) and neurodegenerative diseases (e.g., Charcot-Marie-Tooth disease), and viral infections (e.g., Human Immunodeficiency Virus, Ebola; (60, 76, 129). Thus, the ESCRT machinery provides a set of proteins that may serve as targets for novel therapeutics and diagnostic tools.

Taken together, endocytosis is a highly regulated process, particularly when coupled with complex pathways for intracellular trafficking that determine the fate of the internalized cargo [for additional extensive and more detailed reading see (142)]. Intriguingly, endocytosis is often hijacked by pathogens to serve their own purposes. As the liver can be a site for clearing pathogens, examples of potential infecting agents might include bacteria (e.g., *Listeria monocytogenes*), protozoa (e.g., *Cryptosporidium parvum*), and various viruses (e.g., hepadnaviruses). Furthermore, misregulation of endocytic processes, in particular when associated with growth factors and their receptors, can contribute to the onset and/or exacerbation of malignancies. Examples in this case include aberrant trafficking of the ligand/ receptor complexes EGF/EGFR and hepatocyte growth factor/c-Met.

Endocytic trafficking in polarized epithelial cells

Epithelial cells line all organs that are in contact with the environment, providing selective barriers between the two systems. They display a unique organization with connecting and stabilizing junctions that promote an asymmetric architecture of the cell characterized by an apical and basolateral domain and specific associated proteins. Epithelial cells use a variety of sorting processes to guide specific proteins to a destination. MDCK (Madin-Darby canine kidney) cells for example, sort most apical and basolateral components in the TGN and then transport the cargo directly to their respective membrane (84).

Hepatocytes, the major epithelial cell in the liver, have a complex network that communicates with both the blood stream as well as adjacent cells in a very specialized way. Their apical domain is specialized in the transport of bile acids, release of cholesterol, phospholipids and pIgA while the basolateral domain transports nutrients, bile acids and amino acids to the blood stream. While cultured cell lines such as HepG2 employ the direct sorting pathway, it seems that hepatocytes *in vivo* prefer the indirect route (166).

It has been proposed that this indirect transcytotic pathway employs two endosomal sorting sites: (i) an early endosome at the basolateral periphery that resembles the early/sorting endosome in nonpolarized cells, and (ii) a subapical compartment (SAC) that receives transcytosing proteins destined for the apical surface, but also stains positive for a subset of lysosomal/degradative proteins such as endolyn-78 or LAMP1 (61). Functionally, apical endocytic trafficking specifically controls membrane retrieval from the apical domain (148), coordinates the delivery of apical PM proteins to lysosomes for degradation (137,155) and regulates canalicular bile secretion by recycling of ABC transporters (12,41,71,149).

The SAC appears to function as a recycling system; although, its physiological role remains poorly defined as efficient recycling from early endosomes from both membranes occurs also without the SAC (54). Structurally, this compartment appears as a tubulovesicular reticulum that is clustered in the apical region and extends to the cell periphery (3, 6, 68, 83, 118, 153). Its structural and functional integrity depends at least in part on MTs which is regulated by the activity of class III PI3 kinase (157,158).

Based on the cargo proteins found, one role of the SAC is to connect the transcytotic pathway (5′NT, pIgAR) with the apical to lysosomal pathways [endolyn-78; (61,118)]. As

for other endosomal compartments, the sorting process in this particular compartment also involves CCPs and CCVs.

Interestingly, no common structural information for directing cargo to the apical membrane has yet been identified, with the exemption of the pIgAR, that contains several targeting signals within the cytoplasmic tail. In contrast, most other apical cargo proteins do not display any apparent sorting signal and have unusually short tails, suggesting that the sorting information is buried somewhere else in the protein and/or in its interaction partners (61).

In addition, distinct members of the Rab GTPase family localize to specific regions in the SAC itself (Rab 11, 17, and 25) or the associated apical tubules (Rab 18 and 20) (17, 43, 49, 118, 146, 167). However, their exact role in the SAC-based sorting process is still unclear.

Taken together, because no basolateral cargo such as the asialoglycoprotein receptor (ASGPR), TfR, or other lysosomal proteins such as the M6PR are found in this particular apical compartment, it has been proposed that the SAC is not a common sorting station, but rather a one way route to the apical membrane and to some extent to the lysosome (61).

Endocytic Trafficking and Iron Homeostasis

Almost all cells of the body utilize iron as a cofactor for essential biochemical reactions such as energy metabolism or oxygen transport. However, absorption and cellular storage of excess iron can be toxic and iron overload is a characteristic of diseases such as hemochromatosis and various neurodegenerative diseases (35,88,113,135). Therefore, iron homeostasis, which is regulated in part by the liver, is an important process [for a review see (107)]. The liver employs two receptors, TfR1 and TfR2 (Fig. 2A and B), to internalize iron-bound Tf (holo-Tf), as well as to stimulate iron transporters in other cells and tissues.

While the TfR1 is viewed as an essential and ubiquitous receptor found in almost all cells, the TfR2 is largely specific to hepatocytes and differentiated erythroblasts. Comparison of the TfR1 versus the TfR2 amino acid sequence reveals that the ectodomains of the two receptors are ~45% identical whereas the small (80 amino acid) cytoplasmic domains are quite different (64) (Fig. 2A). Both receptors do contain an AP-2-binding motif, suggesting that the same mode of entry into cells may be used, namely CME. However, there are also reports linking TfR2 to lipid rafts and Cav-1-mediated endocytosis (15). Following internalization, the TfR1 follows an endocytic recycling route. In this endocytic pathway, the holo-Tf/TfR1 progresses from early endosomes to Rab11-positive recycling endosomes, where the iron is released from Tf. The apo-Tf/TfR1 complex is then transported back to the plasma membrane, allowing for the release of apo-Tf into the extracellular milieu for another round of iron absorption. Tf and its receptor (TfR) follow this pathway in most cell types, but can also enter the transcytotic pathway in hepatocytes and other polarized cells (18).

TfR1 endocytosis was thought to be constitutive, meaning independent of binding to iron-bound Tf; however, TfR1 was recently reported to undergo regulated endocytosis (16). In this study, ligand binding stimulated a Src-dependent signaling cascade that led to

phosphorylation, and regulation, of Dyn2 and Dyn2's actin-associated binding partner cortactin.

In comparison to the TfR1, internalization and endocytic trafficking of the TfR2 is less clear. In contrast to the recycling route followed by the TfR1, TfR2 has been reported to enter a degradative pathway. Although the TfR2 itself was not reported to be ubiquitinated, receptors appear to follow a MVB-dependent sorting pathway in being targeted for degradation in the lysosome (20). Despite the recent advances in understanding TfR2 endocytosis and trafficking, additional studies will be needed to determine the exact molecular machinery that regulates the different endocytic/trafficking pathways used by the two TfR types. For example, do TfR1 and TfR2 use the same entry route and employ the same initial APs? How does the internalization machinery distinguish between the two receptors and determine their fate? How is TfR2 targeted into a MVB pathway independent of an ubiquitin signal?

The TfR2 also exhibits functions that are related, but distinct, from the TfR1. As part of an iron-regulated iron regulatory pathway mediated by the liver peptide hormone hepcidin, TfR2 plays a role in maintaining iron homeostasis throughout the entire body. Under normal conditions, blood plasma iron concentrations in humans remain stable at 10 to 30 μmol/L. This relatively stable concentration must be maintained in the context of: (i) fluctuations in dietary iron intake and cellular iron stores, and (ii) intermittent iron losses through bleeding and desquamation of epithelial cells, (3) diverse immune responses and (4) macrophage-mediated degradation and recycling of erythrocytes. Multiple signals from these activities converge at the level of hepatocytes, including formation of a holo-Tf/TfR2/HFE (hemochromatosis protein) signaling complex, to allow for iron-regulated production of hepcidin. Namely, transcription and synthesis of hepcidin by hepatocytes is increased when iron is abundant, whereas little or no hepcidin is produced under conditions of iron demand (e.g., erythropoiesis). In the body, hepcidin limits absorption of dietary iron in the intestine, as well as release of iron from absorptive enterocytes. Additionally, hepcidin downregulates export of iron from cellular stores, mainly from macrophages of the liver and spleen and from hepatocytes. These inhibitory actions result from hepcidin binding to, and inducing endocytosis and degradation of, ferroportin, a multipass transmembrane protein that mediates the export of cellular iron. When hepcidin levels are low, intestinal iron absorption is active. Also, ferroportin can remain at the plasma membrane of iron-storing and -exporting cells, thus allowing for cytoplasmic iron to be released into the blood plasma. Insufficient production of hepcidin by hepatocytes, resulting from, for example, mutations in the hepcidin gene or in genes encoding for proteins involved in regulating hepcidin production (e.g., TfR2, HFE, and hemojuvelin), is a main cause of hereditary hemochromatosis (40,88,107).

Endocytic Proteins Associated with Lipid Droplets and Autophagy

A major function of the liver is to control lipid homeostasis, including cholesterol synthesis and very low density lipoprotein secretion. Additionally, the liver is responsible for converting consumed fats into stored energy—a process that, when taken to excess, can lead to hepatic steatosis (e.g., nonalcoholic fatty liver disease, NAFLD; nonalcoholic

steatohepatitis, NASH) (2, 96, 152). For a variety of reasons, this disease process has become increasingly more prevalent in the United States. Indeed, the incidence of these diseases has increased dramatically over the past 20 years and at present is observed in greater than 60% of obese patients and, remarkably, more than 35% of the nonobese (77). Importantly, alcohol consumption greatly increases fat storage by the hepatocyte through unknown mechanisms.

The central fat storage organelles, namely, lipid droplets (LDs; Fig. 3A–E), are found in all cell types but are most predominant in adipose, muscle and the liver [for reviews, see (31, 94)]. LDs contain a hydrophobic core of esterifies neutral lipids such as triacylglycerides and cholesterol ester surrounded by a phospholipid monolayer with scores of associated proteins (Fig. 3A). The accumulation of LDs in hepatocytes is obvious in steatosis (Fig. 3B–B″); however, surprisingly little is known about LD origin, formation/maturation, and breakdown (39,104,112,131).

It has been known for some time that LDs are coated by lipases and different members of the PAT adaptor family [perilipin, adipophilin/ADRP (adipose differentiation-related protein), TIP47 (tail-interacting protein of 47 kDa)] (Fig. 3C). More recently, proteomic studies have shown that LDs are also coated with several proteins best known for their roles in endocytic trafficking [e.g., caveolins, Rab5, Rab7 (8,38,95)] (Fig. 3D). This suggests that lipids and/or proteins internalized at the plasma membrane and trafficked along an endocytic pathway might also influence LD formation and function.

The discovery of Cav-1 and -2 on LDs in nonmuscle cells and adipocytes was one of the first links between an endocytic protein and LDs (7, 23, 38). In addition to observations made using cultured cells (Fig. 3E), the caveolin-LD association also occurs in primary cells and under physiological conditions such as liver regeneration after partial hepatectomy (34, 75, 114). The association of caveolins with LDs occurs very rapidly after challenging the cells with exogenous cholesterol or oleate. Furthermore, this lipid-induced trafficking is also dependent on the membrane-severing protein Dyn2 and the nonreceptor tyrosine kinase Src (75). Despite recent findings that Cav-1 deficiency alters the composition of surface phospholipids on the LD and affects LD size (7), details regarding the manner in which Cav-1, Dyn2, and Src might regulate LD dynamics are poorly defined. However, a recent paper by Schulze and colleagues demonstrates that Dyn2 regulates autophagic LD breakdown by controlling autolysosomal reformation. As this process ensures regeneration of functional lysosomes that are essential for lipophagy to occur, Dyn2 appears to be a central player in starvation-induced LD breakdown (136).

The functional link between endocytic Rab GTPases and LD formation, and/or possibly lipolysis, is also undefined. More than 15 different Rab GTPases have been copurified with LDs, but in most cases, detailed studies aimed at determining a potential role for a specific Rab protein with respect to LD formation, function or dynamics have not been conducted. One study did, however, find that Rab5 can promote the contact between LDs and early endosomes via recruitment of the Rab5 effector EEA1 (early endosomal antigen 1) to LDs (79). The authors speculated that, in general, LDs contain “docking sites” for various small GTPases allowing contacts between LDs and different endosomal compartments. These

contacts are transient in nature and might allow for an efficient bidirectional exchange of cholesterol between the cell surface and cytosolic compartments. Although this is an attractive model, it still lacks experimental evidence and needs to be verified on a cellular level.

A recent very interesting hint on how and why LDs might interact with components and compartments of the endocytic trafficking pathway is based on the discovery that breakdown of LDs is mediated by autophagic mechanisms [for a recent review see (29)]. Autophagy, literally meaning “self-eating,” refers to a process that regulates important cellular functions such as differentiation, immunity, and aging (Fig. 4A–H). This process enables cells to produce energy under conditions of nutrient deprivation, but it also helps degrade excessive or damaged intracellular organelles. Three different forms of autophagy are defined, depending on the cargo and mode of entry into lysosomes: macroautophagy, microautophagy, and chaperone-mediated autophagy.

The physiologically most relevant form of autophagy with respect to LD breakdown is, however, macroautophagy—or more correctly, a special subtype thereof, now termed “lipophagy” (29,144).

Autophagosome formation is a very complex and highly controlled process that is regulated by proteins encoded by the *atg* (autophagy-related) genes. Different combinations of these atg proteins and effectors regulate each step of the autophagosome formation. Initially, a so-called isolation membrane or phagophore forms around the cytosolic targets, which elongates and finally closes to a complete autophagosome, a double-membrane vesicle. The origin of the autophagosome membrane is still unclear, as contributions from the PM (120, 121), ER (4, 47, 48, 50, 165), or the mitochondria (46) have been reported. In macroautophagy, the autophagosome encloses cellular organelles and protein complexes. Autophagosomes fuse with lysosomes in a Rab7-dependent manner to generate autolysosomes. Degradation in the autolysosomes is mediated by the digestive enzymes derived from the lysosomes and the hydrolytic products are released into the cytoplasm for re-use (Fig. 4A–E).

Evidence supporting that hepatic LDs may undergo autophagic degradation has been reported [(145); Fig. 4G)]. In this study, breakdown of LDs in the context of either pharmacological inhibition of autophagy or small interfering RNA (siRNA)-mediated reduction in the levels of a protein that plays a key role in the formation of autophagosomes resulted in an increase in the number and size of LDs in comparison to control cells. Interestingly, the increase in number and size of LDs also occurred in cells treated with the autophagy inhibitors absent a lipid stimulus, suggesting that basal levels of LD lypolysis might also be regulated by autophagy. Furthermore, in cases of abnormal increases in intracellular lipid, autophagic clearance of LDs was decreased. If such a process were to occur physiologically over the long term, this could result in a harmful cycle that promotes lipid accumulation and retention in hepatocytes.

Autophagic pathways in the liver are not only of interest based on their link to LDs. These “self-eating,” degradative pathways also contribute to the regulation of liver physiology in

general and are often negatively associated with hepatic diseases such as NASH and potentially Hepatitis virus infections [for a recent review see (26)]. Thus, this is certainly an area of hepatocellular research that warrants additional attention and further study.

Endocytic Processes and Hepatitis B Virus Infection

Chronic hepatitis B virus (HBV) infections occur in more than 350 million people worldwide, with many infected individuals developing severe conditions such as liver cirrhosis, liver failure, and/or hepatocellular carcinoma (33,73,74). The infectious 45 nm HBV virion consists of an inner nucleocapsid, which surrounds the HBV genome consisting of partially double-stranded DNA (dsDNA), and an outer surrounding envelope of lipids and surface proteins (Fig. 5A). While the specific hepatocyte surface receptor utilized by the HBV for endocytic internalization has remained elusive for some time, recent studies have implicated the bile salt transporter NTCP (sodium-taurochlorate cotransporter peptide) (102, 162–164). How this transporter is recognized by the clathrin-AP2 machinery remains unclear.

Following infection, the HBV genome is delivered to the nucleus by an unknown mechanism, where it is converted into covalently closed, circular DNA (42,116). Transcripts of pregenomic-(pg)RNA are produced, exported into the cytoplasm and translated into the capsid-forming C protein and the viral reverse transcriptase polymerase (RT-Pol). Within the cytoplasm, the pgRNA and a single copy of the polymerase are encapsidated into viral nucleocapsids. Within these capsids, a single-stranded (ss)DNA copy of the HBV genome is produced by reverse transcription, which then serves as the template for second strand DNA synthesis. Upon completion of the synthesis of the partially dsDNA HBV genome, the capsid gains the capacity to bud. It is suggested that, initially, the capsids bind to the envelope proteins at the endoplasmic reticulum (ER), with cytoplasmic components of the large HBV surface antigen protein (LHB) facilitating interactions with capsid C proteins and LHB playing an essential role in the envelopment process. Cellular exit of infectious virions might then occur through an MVB pathway (Fig. 5B–D).

As part of its infection cycle, HBV hijacks cellular processes, in particular the endocytic machinery. The LHB protein, especially the N-terminal, pre-S1 domain, plays an essential role in the viral entry process. Neurath and colleagues were the first to demonstrate that a synthetic peptide consisting of amino acids 21–47 of pre-S1 or antibodies against that region can block entry of viroids into HepG2 hepatocytes (100). While a role for the LHB and the pre-S1 is well accepted, it is still unclear which host cell components might serve as receptors for HBV viroids and mediate their entry. Several candidates that bind to the LHB pre-S1 have been identified, including TfR, interleukin-6 (IL-6) receptor, and ASGPR (27,36,42,52,101,156). Thus, HBV may use one or more receptors present at the hepatocyte plasma membrane to mediate entry. Regarding internalization of HBV, it has been observed that inhibition of CME via expression of a dominant-negative form of either the clathrin adaptor Eps15 or Rab5 interferes with viroid uptake (25). Also, coimmunoprecipitation experiments indicated that LHB, likely through the pre-S1 domain, can complex with clathrin and AP-2, and short hairpin RNA (shRNA) knockdown of clathrin or AP-2 protein levels significantly reduced the susceptibility of cells to HBV infection (58). Nonetheless,

HBV may also use a clathrin-independent pathway, such as caveolae, to gain entry to cells (81,151).

Moreover, HBV also hijacks cellular machinery to mediate the budding/release of mature virions from host cells such that neighboring cells can then be infected. Mature capsids are thought to bud into the ER in an LHB-mediated manner to gain access to a secretory pathway. Studies have also detected viral core particles in a CD63-positive late endosomal compartment (Fig. 5B), suggesting a potential role of late endosomal components in viroid assembly and release (125). Indeed, Watanabe and colleagues showed that HBV colocalizes with components of the ESCRT machinery—such as Alix, an ESCRT-associated protein (Fig. 5C), and Vps4, an AAA-ATPase that is required for ESCRT disassembly and recycling. Furthermore, they demonstrated that a functional ESCRT complex is important for HBV production and release (21, 72, 116, 160). In addition, the HBV C protein contains a proline-based sequence similar to that contained in retrovirus proteins that have been implicated in mediating viroid release via an MVB pathway. Taken together, these findings strongly support the importance and essential roles of endocytic and postendocytic proteins in a productive HBV infection cycle (Fig. 5D). However, the exact mechanisms by which HBV entry, assembly, and release are regulated remain to be determined.

Concluding Remarks and Future Directions

Over the past decade, improved imaging and biochemical techniques in concert with new genetic models have allowed us to gain more detailed insights into the spatiotemporal regulation of endocytic processes in the hepatocyte. Nevertheless, the list of new players in these processes, including protein and lipid components, is still expanding. Also, their specific roles in endocytosis and endocytic trafficking pathways—whether structural, regulatory, enzymatic or a combination thereof—remain to be established. As such, an ongoing challenge for the future will be to understand how the hepatocyte coordinates the complex network of proteins and lipids that mediate and regulate cargo sorting and vesicle formation at endocytic and postendocytic sites. As examples of future areas of study, we will need to gain additional insights into how different cargo proteins are sequestered from each other in the same endosomal compartment, how subdomains within a compartment are established and maintained and how higher order signaling networks control sequential trafficking events. Expanding our understanding of these processes will provide us with insights toward the development of novel therapies to more effectively treat liver diseases.

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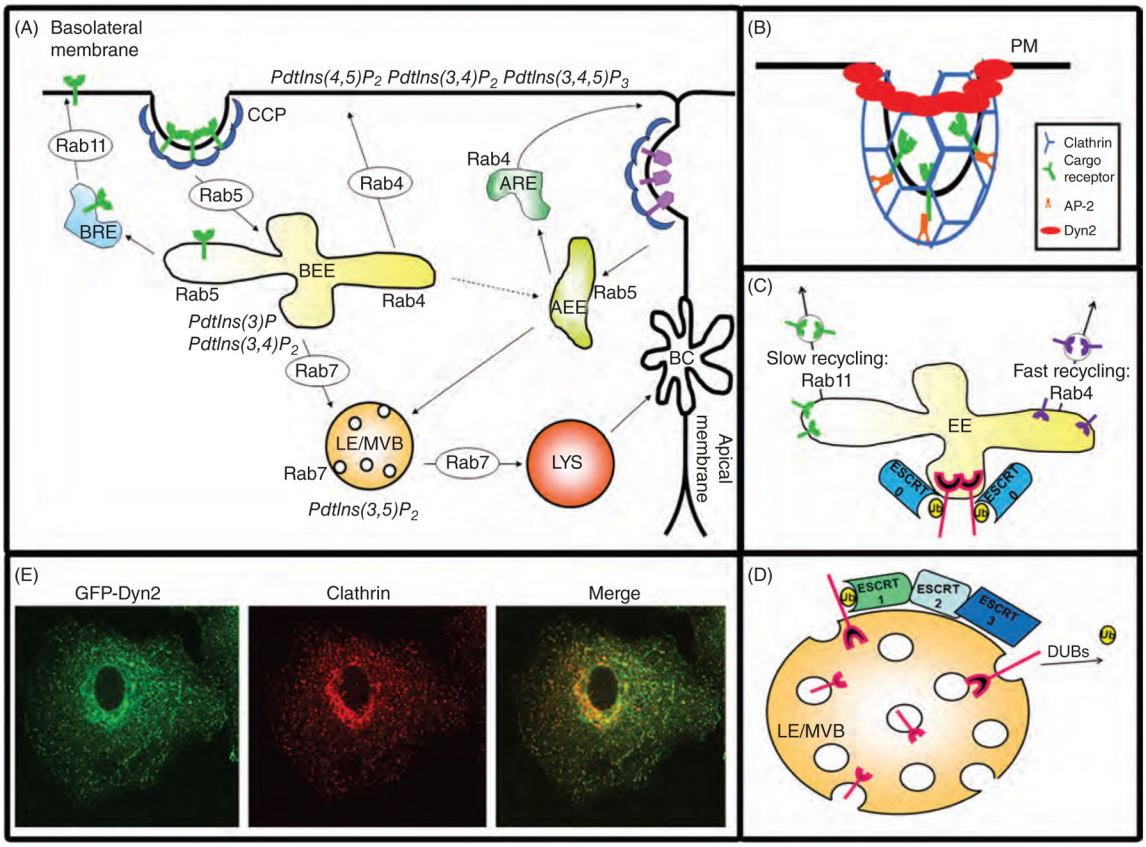

Figure 1.

Endocytic trafficking routes in hepatocytes. (A) Overview of degradative and recycling pathways that are controlled by RabGTPases and marked by specific phosphoinositides. (B) Assembly of the clathrin coat at the plasma membrane. Clathrin forms a triskelion (blue) that is connected to the cargo (green) via adaptor proteins (orange). The large GTPase Dynamin 2 (Dyn2, red) controls the scission of the nascent vesicle which is subsequently trafficked to endosomes for further sorting. (C) Early endosomes are the sorting stations in the cell. Different cargo molecules are concentrated at distinct subdomains of the sorting endosomes: cargo destined for degradation is marked by ubiquitination, which is recognized by the ESCRT machinery while nonubiquitinated cargo is accumulates at distinct subdomains of the early endosome and is recylcled back to the plasma membrane via a Rab11- or Rab4-dependent pathway. (D) ESCRT complexes do recognize and sort ubiquitinated cargo into intraluminal vesicles (ILVs) of late endosomes to form multivesicular bodies (MVBs). Subsequent fusion with lysosomes leads to the degradation of the cargo molecule. (D) Immunofluerescence image showing the colocalization of Dynamin 2 (Dyn2, green) and Clathrin (red) in Clone 9 cells. The image was kindly provided by Dr. H. Cao, Mayo Clinic, Rochester. PM: plasma membrane; EE: early endosome; LE: late endosome; MVB: multivesicular body; lys: lysosome; DUB: deubiquitinating enzymes.

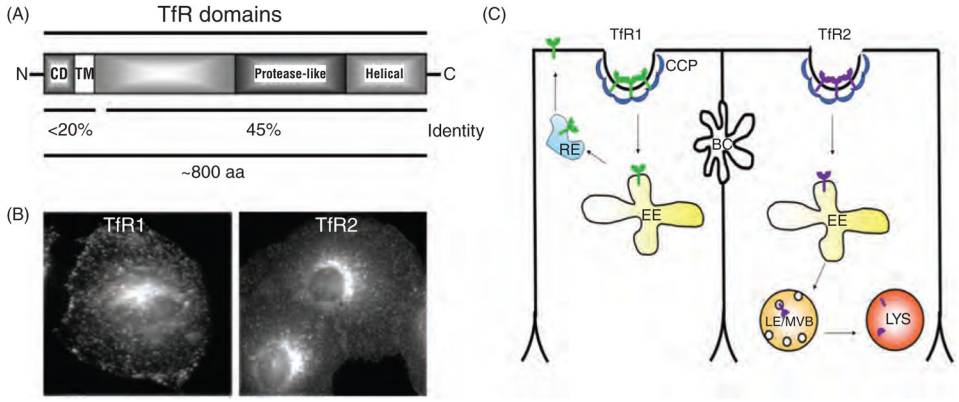

Figure 2.

Trafficking pathways of the Transferrin Receptor 1 and 2 essential for iron homeostasis. (A) Schematic representation of the domain structure of transferrin receptor 1 and 2 (TfR1 and 2) illustrating a cytoplasmic domain (CD), transmembrane domain (TM), protease-like domain and a C-terminal helical domain. While the CD is quite distinct between the two receptors, the ectodomains share about 45% identity. (B) Localization of TfR1 and TfR2 in Clone 9 cells. Both receptors were stained with specific antibodies. Due to the loss of TfR2 in cultured primary hepatocytes, this receptor was stably expressed in Clone 9 cells and then stained. The images were kindly provided by Dr. H. Cao, Mayo Clinic, Rochester. (C) Overview of the different trafficking pathways used by TfR1 and TfR2. While TfR1 is recycled back to the plasma membrane in a Rab11-dependent manner, TfR2 has been reported to enter the degradative pathway; however, the exact trafficking routes of TfR2 remain to be determined. PM: plasma membrane; CCP: clathrin-coated pit; EE: early endosome; LE: late endosome; MVB: multivesicular body; lys: lysosome.

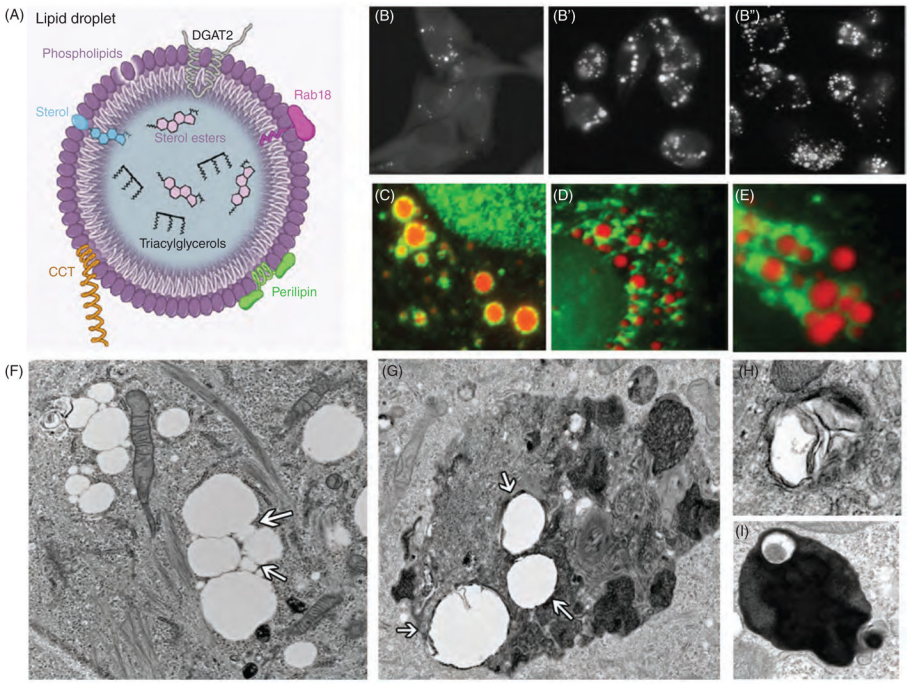

Figure 3.

Hepatocytes accumulate lipid droplets under steatotic conditions. (A) Cartoon depicting the composition of a lipid droplet (LD) and its associated proteins. The cartoon was reprinted from Krahmer *et al*. with permission (69). (B-B″) Accumulation of lipid droplets in VA-13 cells under nonfed conditions (B), upon oleate exposure (B′), or after oleate + EtOH treatment (B″). LDs were visualized using Oil Red O stain. Note that the oleate-induced accumulation of LDs is further enhanced by cotreatment with EtOH mimicking fatty liver disease. (C) Association of ARDP (green) on LDs (red) in Hep3B human hepatoma cells after o/n feeding with 150 μmol/L oleate. (D) Close association of GFP-Rab7 (green) with LDs (red) in Hep3B cells after o/n feeding with 150 μmol/L oleate. (E) Localization of GFP-Cav1 (green) on LDs (red) in HuH7 human hepatoma cells after o/n feeding with 150 μmol/L oleate. (F–I) TEM images from Hep3B cells loaded with 150 μmol/L oelate on/ showing LDs under resting (F) and starved (G–I) conditions. Arrows point to sites of potential fusion events (F) or LDs engulfed by a gigantic autophagosome (G). Starvation was induced by incubation in medium with 0.1% FBS for 24 h. (G–I) show three different examples of autophagic breakdown of LDs which are engulfed by lysosomes (electron dense compartments surrounding LDs). All TEM images were provided by Eugene Krueger, Mayo Clinic, Rochester.

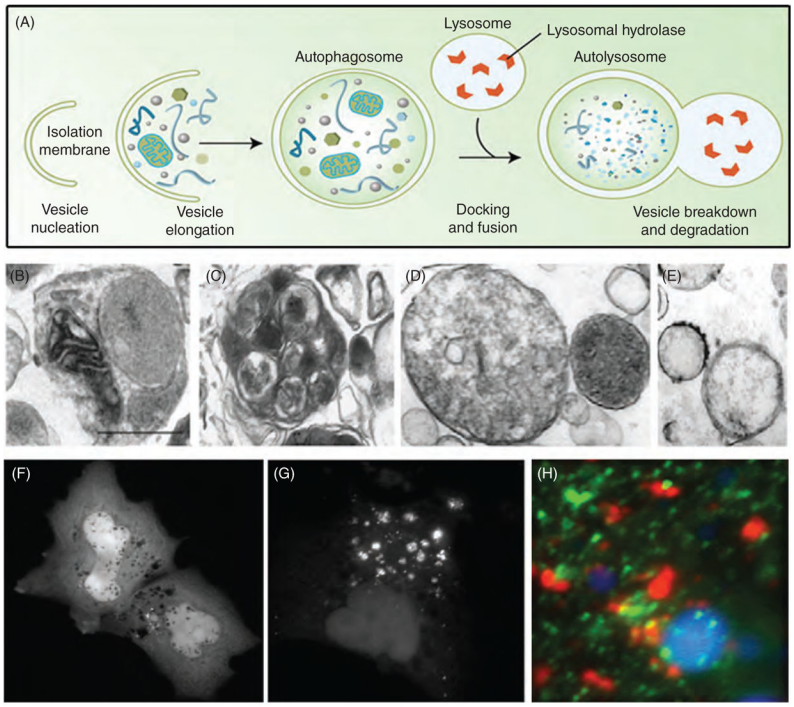

Figure 4.

Autophagy contributes to lipid droplet breakdown. (A) Cartoon showing the different stages of autophagosome formation. During the nucleation phase, an isolation membrane forms which then extends around the cargo destined for autophagic degradation to form an autophagosome. The autophagosome eventually fuses with lysosomes to a hybrid compartment, named the autolysosome, in which cargo proteins are degraded. Cartoon reprinted from Melendez and Levine with permission (91). (B–E) EM gallery depicting the different stages of autophagosome and autolysosome formation as described in (A). The gallery shows the entrance of cargo into endosomes (B), maturing autophagosomes containing still recognizable, but partially degraded material (C), heterogeneous intraluminal material (D) and lysosomes with fully degraded material leading to a less dense appearance. EM images reprinted from Nixon with permission (103) (F, G) Visualization of autophagosome formation in Hep3B hepatoma cells. Hep3B cells expressing GFP-LC3, an autophagic marker, were compared under resting (F) and starved conditions (24 h in medium containing 0.1% FBS). Note that starvation results in a redistribution of LC3 from the cytosol and nucleus to distinct vesicular structures. (H) Colocalization of autophagosomes (green) and lysosomes (red) on lipid droplets (LDs, blue). Hep3B cells expressing mCherry-lamp1 were starved for 24 h in medium containing 0.1% serum and the autophagosomes were stained using a LC3 antibody (green). LDs were visualized using MDH (blue). The image was kindly provided by Shaun Weller, Mayo Clinic, Rochester.

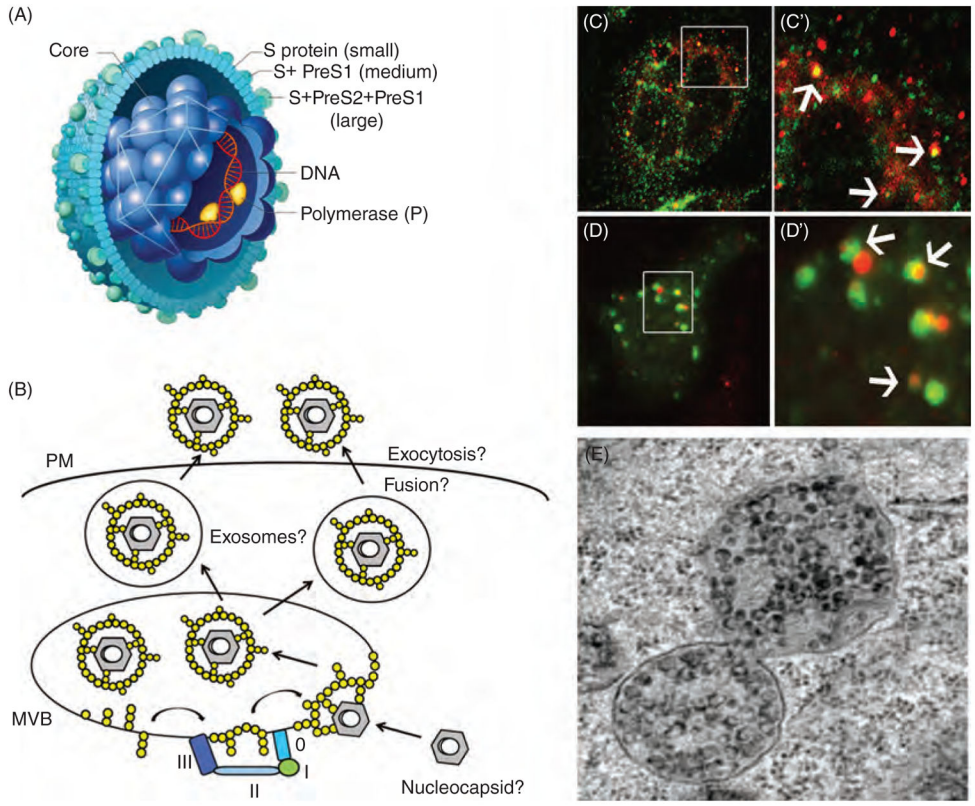

Figure 5.

Hepatitis B virus hijacks the endocytic system to ensure proper reproduction. (A) Cartoon (© James A. Perkins; used by permission) depicting the hepatitis B virion (HBV). HBV is comprised of a core, embedding the virus DNA and polymerase, and a protein-rich capsid consisting of large, small and medium surface proteins as indicated. (B) Cartoon showing the current model for HBV/MVB (multivesicular body) association and their involvement in virus reproduction. Cartoon modified after Patient with permission (109). HBV assembly potentially starts on the limiting membrane of MVBs and may eventually be delivered to intraluminal vesicles (ILVs). The fully assembled virus may then be separated from the MVB in the form of exosomes and delivered to the plasma membrane (PM), where it is released by fusion with the PM or by exocytosis to reinfect neighboring cells. (C and D) HBV associates with components of the ESCRT machinery in late endosomes. Confocal images show colocalization of the large HBs (LHBs, red) with Hrs (green), a component of the ESCRT-0 complex (C), or with the late endosomal/MVB marker GFP-Rab7wt (green; D). Boxes depict the regions that are enlarged in (C′ and D′), respectively. Arrows point to the sites of colocalization with the marker as indicated. The immune-fluorescence images were kindly provided by Dr. Jun Inoue, Mayo Clinic, Rochester. (E) TEM image showing virus particles (dense regions) residing in MVBs in HepG2.2.15 cells. Image provided by Eugene Krueger, Mayo Clinic, Rochester.

Table 1

Domain	Function	Endocytic proteins containing domain	Endocytic proteins or lipids interacting with domain
Protein-binding			
Clathrin box	Binding to clathrin heavy chain	AP-2 β 2-adaptin hinge, Amphiphysin, β-arrestins, CALM, Dab2, Epsin, HIP1/HIP1R	Clathrin heavy chain
DPF, DPW, and/or FXDXF motifs	Binding to AP-2 α-adaptin and β 2-adaptin appendages	Amphiphysin, CALM, Dab2, Eps15, Eps15R, Epsin, HIP1	AP-2 α-adaptin and β 2-adaptin appendages
NPF motifs	Binding to EH domains	CALM, Dab2, Epsin, Stonin	Eps15, Eps15R, Intersectin
EH	Binding to NPF motifs	Eps15, Eps15R, Intersectin	CALM, Dab2, Epsin, Stonin
PXY motifs	Binding to WW domains	α-arrestins, certain cargo proteins (e.g., ENaC), Nedd4	Nedd4 (intra- and intermolecular interactions)
WW	Binding to PXY motifs	Nedd4	α-arrestins, certain cargo proteins (e.g., ENaC), Nedd4 (intramolecular interaction)
SH2	Binding to phosphotyrosine residues	Grb2, Src	Growth factor receptors and accessory proteins with phosphorylated tyrosine residues
SH3	Binding to proline-rich domains	Amphiphysin, CIN85, Cortactin, Endophilin, Grb2, Intersectin, SNX9, Src, Syndapin	Dab2, Dyn2
PRD	Binding to Src Homology 3 domains	Dab2, Dyn2	Amphiphysin, CIN85, Cortactin, Endophilin, Grb2, Intersectin, SNX9, Src, Syndapin
UIM	Binding to ubiquitin moieties of ubiquitin-modified proteins	Eps15, Eps15R, Epsin	Ubiquitinated cargo molecules; ubiquitinated accessory proteins, including intramolecular interactions; ubiquitinated ubiquitin ligases or ubiquitin ligases with a ubiquitin-like domain
Lipid-binding			
ENTH/ANTH	Membrane binding; membrane bending	CALM, Epsin, HIP1/HIP1R	Phosphoinositide-containing membranes, with a preference for phosphatidylinositol-4,5-bisphosphate
BAR, F-BAR, N-BAR	Membrane binding; membrane bending; sensing of membrane curvature	Amphiphysin, Endophilin, FCHo, SNX9, Syndapin	Phosphoinositide-containing membranes, with a preference for phosphatidylinositol-4,5-bisphosphate
PH	Phosphoinositide lipid binding	Dyn2, Intersectin	Phosphatidylinositol-4,5-bisphosphate

Domains: DPF, aspartic acid-proline-phenylalanine; **DPW,** aspartic acid-proline-tryptophan; **FXDXF,** phenylalanine-X-aspartic acid-X-phenylalanine; **NPF,** asparagine-proline-phenylalanine; **EH,** Eps15 homology; **PXY,** proline-X-tyrosine; **WW,** protein domain of approximately 40 amino acids containing two highly conserved tryptophan residues spaced 20–22 residues apart; **SH2,** Src homology 2; **SH3,** Src homology 3; **PRD,** proline-rich domain; **UIM,** ubiquitin-interacting motif; **ENTH/ANTH,** Epsin N-terminal homology/adaptor protein (AP) 180 N-terminal homology; **BAR,** Bin-amphiphysin-Rvs161/167p; **F-BAR,** Fer/CIP4 homology (FCH) domain adjacent to a region that shares some homology with the C-terminal half of the BAR domain, also termed EFC for “extended FC”; **N-BAR,** BAR domain with an N-terminal unstructured amphipathic helix; **PH,** pleckstrin homology.

(Proteins) AP-2, adaptor protein complex 2; **CALM,** clathrin assembly lymphoid myeloid leukemia; **CIN85,** c-Cbl-interacting protein of 85 kDa; **Dab2,** disabled homolog-2; **Dyn2,** dynamin 2; **Eps15,** epidermal growth factor receptor pathway substrate 15; **Eps15R,** epidermal growth factor receptor pathway substrate 15 related; **ENaC,** epithelial Na^+ channel; **FCHo,** Fer/CIP4 homology domain-only; **Grb2,** growth factor receptor-bound protein 2; **HIP1,** huntingtin interacting protein 1; **HIP1R,** huntingtin interacting protein 1 related; **Nedd4,** neuronal precursor cell expressed, developmentally downregulated 4; **SNX9,** sorting Nexin 9.

The table is based on information from the following articles, mostly reviews in lieu of original papers (78, 93, 119, 122, 126, 133, 139, 169).