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Endosomal vesicles as vehicles for viral genomes

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The endocytic pathway is the principal cell entry pathway for large cargos and pathogens. Among the wide variety of specialized lipid structures within endosomes, the intraluminal vesicles (ILVs) formed in early endosomes (EEs) and transferred to late endosomal compartments are emerging as critical effectors of viral infection and immune recognition. Various viruses deliver their genomes into these ILVs, which serve as vehicles to transport the genome to the nuclear periphery for replication. When secreted as exosomes, ILVs containing viral genomes can infect permissive cells or activate immune responses in myeloid cells. We therefore propose that endosomal ILVs and exosomes are key effectors of viral pathogenesis.

Virus entry through the endocytic pathway

Clathrin-medicated endocytosis is arguably the major entry pathway for large cargos and pathogens. As endocytosis proceeds, endocytic compartments acquire a wide variety of membranous structures including invaginations, tubules, and ILVs [1–4], each of which has specific functions in cargo selection, intracellular trafficking, cell signaling, or metabolism (Box 1). Many viruses utilize cellular receptors directly or indirectly associated with clathrin or clathrin adaptors to enter the host cell through the endocytic pathway. In enveloped viruses, the genome is enveloped in a lipid membrane, which must be fused to a cellular membrane to deliver the viral genome or nucleocapsid into the cytoplasm. The viral envelope glycoproteins catalyze this membrane fusion event with conformational changes triggered by environmental cues [5]. The set of physicochemical requirements required to trigger the fusogenic conformational change – such as pH, lipid composition, and post-translational modifications – determines where and when in the endocytic pathway viral membrane fusion occurs.

During endocytosis, it was assumed that enveloped RNA viruses and some enveloped DNA viruses fuse with limiting endosomal membranes, membranes that separate the cytoplasm from the endosomal lumen. This assumption was challenged when vesicular stomatitis virus (VSV) was found to fuse with ILVs in early/intermediate endosomes, releasing the nucleocapsid into the lumen of ILVs. These ILVs are then transported along microtubules to late endosomes (LEs) near the nucleus. VSV was the first virus reported to utilize ILVs as transport vehicles in this manner. In LEs, ILVs fuse with the limiting endosomal membrane to deliver the nucleocapsid into the cytosol [6], a process known as 'back-fusion'. Back-fusion of ILVs to the limiting membrane is dependent on bis(monoacylglycero)phosphate (BMP), phosphatidylinositol-3-phosphate [PI(3)P], and Endosomal Sorting Complexes Required for Transport (ESCRT) components in the limiting membrane. Back-fusion is regulated by Hrs, an ESCRT-0 component, and the ESCRT accessory protein Alix [6–9]. The availability of ILVs in the early endosomal compartment in which VSV undergoes its initial fusion event has been questioned [10]; however, other pathogens have been shown to utilize a similar ILV back-fusion pathway to enter the host cell. Lethal toxin from *Bacillus anthracis* utilizes ILV back-fusion to deliver its toxin subunit into the cytoplasm [11] and some members of the *Flavivirus* genus, which includes important human pathogens such as dengue, West Nile, and Japanese encephalitis viruses, were recently shown to utilize a similar pathway [7]. Moreover, recent studies revealed that ILVs containing genomic RNA from hepatitis C virus (HCV) can be secreted as exosomes, which proceed to infect human cells [12,13] or activate plasmacytoid dendritic cells [14]. The recent discovery that ILVs are used to transport the nucleocapsids of several different viruses, and perhaps shield them from degradation in the endosomal lumen, suggests that ILV-dependent cell entry pathways are more common and important for viral pathogenesis than previously appreciated. We review these and other recent advances and their implications for the prevention and treatment of viral diseases.

Physicochemical requirements of membrane fusion direct some viruses to ILVs

Membrane lipid composition determines the site of virus entry

Early and late endosomal membranes, and the wide variety of membranous structures they contain, have distinct lipid compositions (Figure 1) [1–4]. EEs, like the plasma membrane from which they originate, are rich in cholesterol, phosphatidylserine (PS), and phosphatidylinositols [15–17]. The lipid substructures that originate from EEs, including invaginations, tubules, and ILVs, initially have the same lipid composition (lipids can subsequently undergo sorting). As membranes progress along the endocytic pathway, their cholesterol content decreases. Cholesterol is replaced with ceramide in LEs and lysosomes, where it maintains membrane fluidity [18]. The anionic, acid-resistant lipid BMP

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[also known as lysobisphosphatidic acid (LBPA)] is enriched in the internal membranes of LEs and lysosomes, including the ILVs derived from them, but not in ILVs transported from EEs [15,16]. Hence ILVs derived from EEs or LEs form distinct pools. BMP increases the fusogenicity of vesicle membranes at pH <6 and induces internal vesiculation in liposomes, resembling multivesicular endosomes found *in vivo* [8,19]. Autoantibodies against BMP result in autoimmune disorders such as antiphospholipid syndrome and Niemann–Pick type C syndrome. Anti-BMP antibodies cause dysfunctional sorting and trafficking in LEs [20,21], suggesting a critical role for BMP in LE function and dynamics.

Recent evidence suggests that these changing lipid compositions in endosomes, combined with the pH threshold of fusion, may direct virus entry to specific points along the endocytic pathway. Indeed, certain enveloped RNA viruses require a specific lipid composition in their target cellular membrane for efficient fusion. For example, cholesterol and pH <6–6.5 are necessary for fusion of alphaviruses, such as Semliki Forest virus, with early endosomal membranes, where cholesterol is abundant [22]. In another example, flaviviruses, including dengue virus, require PS or other similar anionic phospholipids in the target membrane for efficient fusion [7,23]; cholesterol, although not strictly required, further enhances fusion [24,25].

Although it is clear that viruses have requirements for different membrane types, the mechanisms underlying this specificity are only emerging. In the context of flaviviruses, virus particles from at least two species bind to PS with high affinity and specificity [7]. PS is normally restricted to the cytoplasmic leaflets of the plasma membrane and early endosomal membranes. However, when calcium is released into the cytoplasm – a common cellular distress signal during infection, apoptosis, or cancer – phospholipid transporters known as scramblases translocate PS to the extracellular and luminal leaflets [7,26–28]. PS in the extracellular leaflet usually serves as an 'eat-me' signal for phagocytosis of dying cells. However PS is also externalized on cancerous cells and during myeloid cell activation [29–31]. Notably, recent evidence suggests that the redistribution of PS toward extracellular or luminal leaflets following intracellular calcium release may be important for *Flavivirus* infectivity. Certain flaviviruses trigger cytoplasmic calcium release and activate PI(3)P signaling during cell entry [7] and chelation of intracellular calcium inhibits their infection [7]. The presence of PS on the outer plasma membrane leaflet of insect cells even in the absence of calcium release [32], in contrast to mammalian cells, provides an explanation for why dengue virus can fuse with the plasma membrane of insect but not mammalian cells in an acidic environment [23].

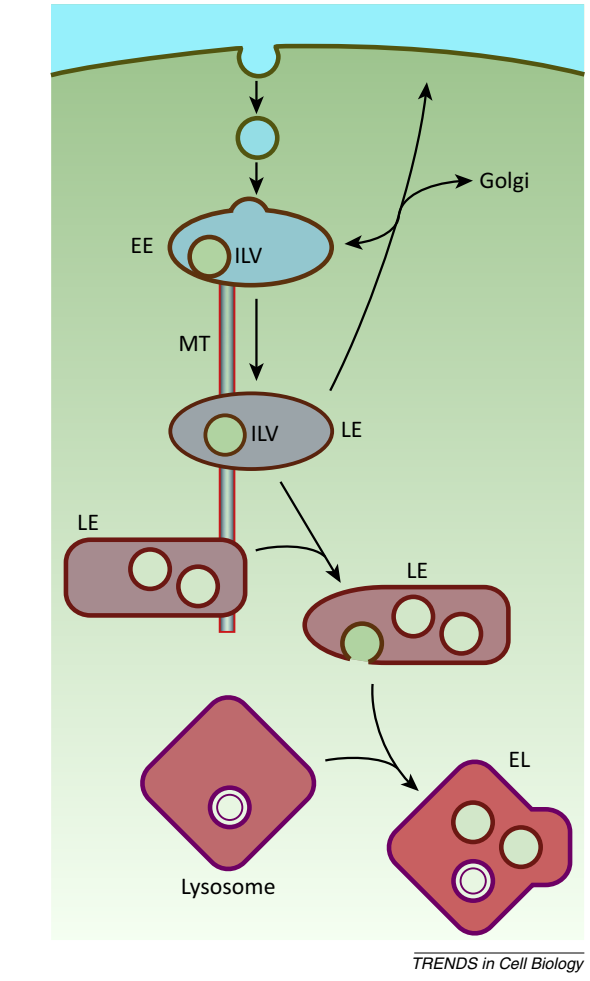

Figure I. Biogenesis and trafficking of ILVs in the endocytic pathway.

Box 1. The endocytic system

Endocytosis is the mechanism for internalization of molecules of all sizes, plasma membrane components, and nanoparticles by the invagination of the plasma membrane and the formation of vesicles through membrane fission (Figure I) (reviewed in [2]). Clathrin-mediated endocytosis is the major pathway for vesicles to bud from the plasma membrane, but several clathrin-independent pathways also contribute to endocytosis, including the caveolin-dependent pathway. Endocytic vesicles fuse with EEs in the peripheral cytoplasm. EEs are the main sorting station in the endocytic pathway. As they accumulate extracellular cargo from endocytic vesicles, some proteins and membrane components are recycled to the plasma, whereas others are transferred to the Golgi apparatus. However, most cargo is retained inside EEs. ILVs bud from the endosomal membrane, beginning in EEs, although the timing of when ILVs begin to form remains to be clarified. EEs and the ILVs they contain are transported toward the nucleus on microtubules (MTs). During this time, EEs mature into LEs. LE maturation involves changes in lipid and protein composition and in the pH of the lumen. Additionally, vesicles bud from the outer (limiting) membrane of the maturing endosome to form ILVs. As maturation proceeds, LEs undergo homotypic fusion, grow in size, acquire more ILVs, and receive newly synthesized components from the secretory pathway. Some ILVs can be secreted at the cell surface through scission of an LE fragment and subsequent fusion of the fragment with the plasma membrane. The fusion of an LE with a lysosome generates a transient hybrid organelle, the endolysosome (EL), in which active degradation takes place. ELs then mature to lysosomes, storage organelles for hydrolases and membrane components. Proteins from the Rab family of GTPases define many of the functional attributes of endosomes by regulating the biological activities of effector proteins, which include the ESCRT machinery responsible for the budding and sorting of endosomes and ILVs.

Endosomal ILVs deliver viral genomes into the cytosol via back-fusion

The lipid and pH requirements for fusion of flaviviruses to a host cell membrane usually result in fusion to early-to-intermediate endosomal compartments, which contain a multitude of ILVs [1] (except for insect cells under acidic