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"Alternative" endocytic mechanisms exploited by pathogens: New avenues for therapeutic delivery?☆

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Abstract

Some pathogens utilize unique routes to enter cells that may evade the intracellular barriers encountered by the typical clathrin-mediated endocytic pathway. Retrograde transport and caveolar uptake are among the better characterized pathways, as alternatives to clathrin-mediated endocytosis, that are known to facilitate entry of pathogens and potential delivery agents. Recent characterization of the trafficking mechanisms of prion proteins and certain bacteria may present new paradigms for strategizing improvements in therapeutic spread and retention of therapy. This review will provide an overview of such endocytic pathways, and discuss current and future possibilities in using these routes as a means to improve therapeutic delivery.

Contents

1. Introduction

Studies in gene therapy and drug targeting have brought to light the importance of identifying cellular and intracellular barriers to efficient delivery. Accordingly, a broad audience has been made aware in recent years of the characteristics of a typical trafficking pathway for many targeted therapeutics. Such

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a pathway is characterized by: receptor binding followed by cell entry *via* receptor-mediated endocytosis into clathrin-coated pits and vesicles, delivery to early endosomes, and passage through late endosomes/lysosomes where cargo degradation otherwise takes place [1–3]. Ligand–receptor pairs, viruses and other pathogens, as well as non-viral gene delivery vectors are known to enter cells by such routes. If a targeted therapeutic, such as a gene delivery vector, is to impart therapeutic efficacy, however, the degradative pathway must somehow be avoided.

Endocytic pathways other than classical clathrin-mediated endocytosis targeted for the endosomal/lysosomal compartments have been better characterized in recent years. Such pathways may offer alternative uptake and trafficking routes for gene delivery vectors and targeted therapeutics that may avoid the barriers posed by the classical route. For example, the retrograde transport pathway, used by plant and bacterial toxins, facilitates endocytic trafficking from the cell surface to the Golgi, and from the Golgi to the endoplasmic reticulum (ER), in reverse of classical secretion [4,5] (Fig. 1). These toxins can then make use of the cell's own protein auditing system to become transported to the cytoplasm where the toxic activity can take place.

Endocytosis *via* caveolae has been well-studied, and the route by which SV40 and similar pathogens utilize caveolar uptake for infection has been characterized in recent years [6]. In this pathway, caveolar vesicles fuse with caveosomes, which facilitate prolonged survival of the pathogen in the cell before transit to the ER, from which nuclear entry can take place for viral replication (Fig. 3).

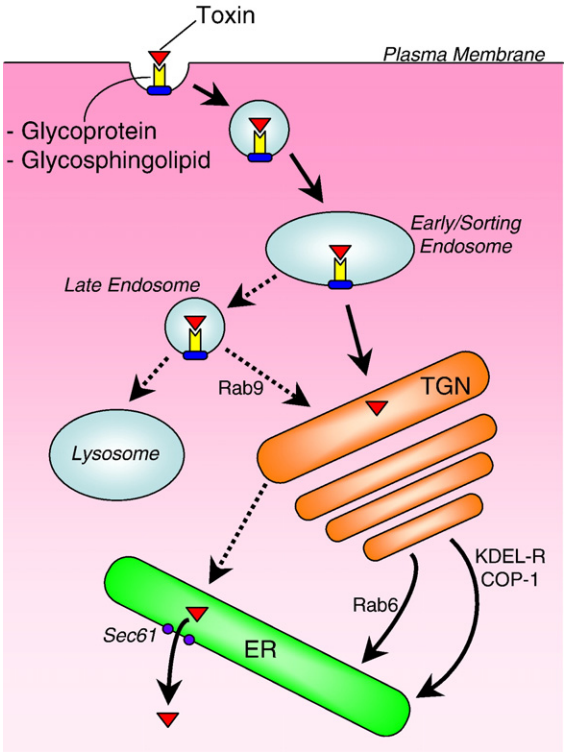

Fig. 1. Retrograde transport of plant and bacterial toxins. Initial binding to a cell-surface molecule triggers receptor-mediated endocytosis and delivery to early/sorting endosomes. Toxins may undergo delivery to the TGN from either early or late endosomes. From the TGN, toxins may transit through the Golgi cisternae and become transported to the ER through either a KDEL receptor-dependent or -independent pathway. Alternatively, transport may take place directly from the TGN to the ER. The toxin is displaced from the ER through a pore into the cytoplasm where the translation machinery can be accessed.

Formation of replication-competent vacuoles inside cells is a strategy used by *Brucella* and similar pathogens to gain long-term survival inside host cells [7]. Such a pathway is typified by endocytosis into pathogen-containing vacuoles, delivery to and interaction with the ER, followed by formation of an ER-derived replicative organelle (Fig. 4).

Endocytic pathways may also lead to effective delivery and spread to neighboring cells. The secretion of exosomes likely enables prion proteins to be transmitted from cell to cell [8,9] (Fig. 2).

Why is studying these pathways important for therapeutic delivery? Delivery to the lysosomal compartment poses one major barrier to gene and drug delivery. The appeal of using viruses or viral components in targeted therapeutics is partly due to the capacity of endosomal escape, and thus avoidance of lysosomal degradation, by penetrating the membrane of the maturing vesicle before cargo delivery to the lysosome. Peptides derived from several types of pathogens have been used to accomplish the same [10]. Such peptides are thought to change conformation in response to the acidifying environment of the endosomal lumen and as a result, interact with the endosomal membrane by forming pores or destabilizing the lipid bilayer, thus affording vesicle escape.

In the event that a gene delivery vector escapes the endocytic vesicle, cytosolic factors still pose additional barriers. The crowded cytosolic milieu can prevent rapid vector motility to the nucleus [11] while cytosolic nucleases can degrade the DNA cargo [12].

"Alternative" endocytic pathways such as those described above may contribute toward improvements in therapeutic delivery by facilitating: the avoidance of lysosomal delivery and degradation; enhanced delivery to a target organelle (such as the Golgi, ER, or nucleus) or compartment (such as the cytoplasm); and enhanced long-term therapy, such as the formation of an extranuclear replicating organelle.

It is clear that there is more than one route for entering a cell and studies on a variety of pathogens show that alternative endocytic pathways have been cleverly hijacked to avoid a degradative fate and evade the cell's defenses. Here we will examine some of these pathways, which may serve as possible routes for improving therapeutic delivery.

2. Retrograde trafficking of plant and bacterial toxins

Plant toxins, such as ricin and abrin, and bacterial toxins, such as Shiga toxin (STx), cholera toxin (CTx), and *Pseudomonas* exotoxin A (PEx), enter cells following a route in reverse of the classic secretory pathway [13]. Classical secretion is characterized by transport of newly synthesized proteins from the ER to the Golgi, followed by budding of vesiculated cargo from the *trans*-Golgi network, vesicle sorting in the cytoplasm, and fusion with the plasma membrane. While many types of viruses and similar pathogens can directly access the cytoplasm

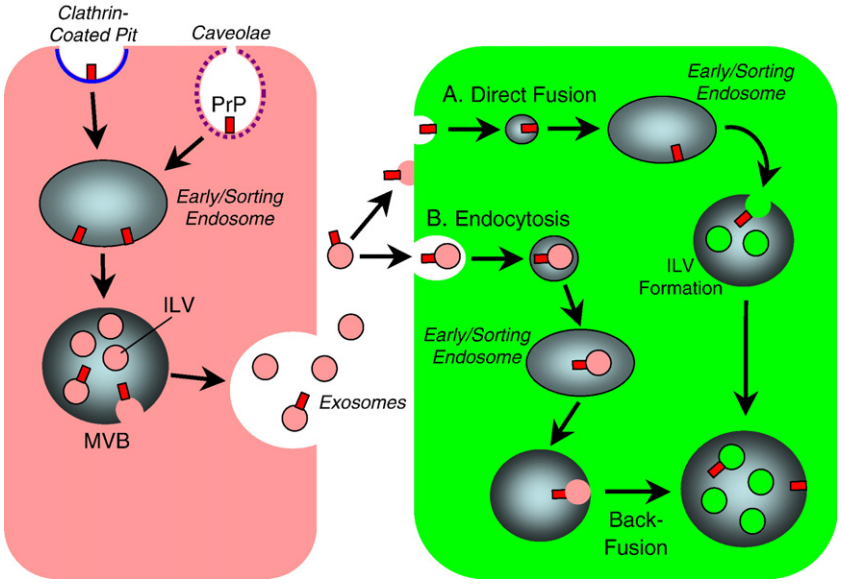

Fig. 2. Exosome-mediated spread of prion protein. Prion protein (PrP) may undergo either clathrin or caveolae-mediated endocytosis into early/sorting endosomes which can sort to multivesicular bodies (MVBs), into which intraluminal vesicles (ILVs) containing cytosolic material bud off into the endosomal lumen. ILV formation results in GPI-attached proteins remaining anchored at the outer surface of the ILV. MVBs can fuse with either lysosomes or with the plasma membrane, releasing the ILVs from the cell surface, which are now known as exosomes. Exosomes can either directly fuse with the plasma membrane of an uninfected cell (shown in green) or undergo endocytic uptake. Direct fusion may result in delivery of PrP to the membrane of endosomal vesicles, which may undergo ILV budding and result in the location of PrP on the ILV outer surface. Alternatively, endocytosed exosomes may undergo back-fusion with the endosomal limiting membrane, resulting in PrP localizing to the endosomal membrane.

from the endocytic vesicle after endocytosis, toxins follow a retrograde route of classical secretion to accomplish the same.

Certain plant and bacterial toxins share a common structure comprised of two major domains or chains, termed A and B. The A chain forms the catalytic or toxic domain, whereas the B chain is responsible for cell binding [14]. The pro-form of the protein is non-toxic, whereas proteolytic cleavage inside the cell activates the toxin after cell entry. In PEx and ricin, this cleavage releases the A subunit, whereas in CTx and STx, the A subunit is separated into A1 and A2 chains.

The targets of toxic activity are specific components of the protein synthesis machinery. The A chains of Stx and ricin are RNA *N*-glycosidases that remove a conserved adenine residue from 28S rRNA that is the site of interaction with elongation factor (EF)-2 ternary complex [15,16]. The effect is inhibition of protein synthesis, leading to cell death. The CTx A chain is an ADP-ribosyltransferase, that modifies the heterotrimeric G protein Gs-α to activate adenylyl cyclase [17], inducing intestinal chloride secretion [18]. The *Pseudomonas* exotoxin A chain ADP-ribosylates EF-2, preventing protein synthesis and leading to cell death [19]. As toxic activity requires interaction with protein synthesis molecules, these toxins depend on delivery to the cytoplasm to access this machinery.

2.1. Mechanism of toxin entry and trafficking

Consistent with most pathogen infection mechanisms, toxin cell entry is enabled by binding to a cell-surface molecule, thus triggering endocytic uptake. Infection by STx and CTx is initiated by B chain binding to host cell membrane glycolipids. STx binds the trisaccharide domain of globotriaosylceramide (Gb_3/CD77) [20–22], whereas CTx binds the ganglioside, GM1 [23,24]. The PEx B chain binds α_2-macroglobulin receptor/low-density lipoprotein receptor-related protein [25]. The ricin B chain is a lectin that binds β1-4 linked galactosides, which are displayed on a wide range of cell-surface glycoproteins and glycolipids, thus owing to the promiscuity of ricin [26].

Cell-surface binding triggers internalization of the toxins into endocytic vesicles, which undergo lumen acidification as the vesicles mature to late endosomes (Fig. 1). Typically, cargo of late endosomes would become degraded due to vesicle fusion with lysosomes, which contain proteolytic enzymes. Vesiculated toxins evade this fate by transit to the TGN from either early or late endosomes (Fig. 1). These pathways are dependent, at least in part, on lipid association, certain Rabs, and specific vesicle and TGN receptors known as SNAREs (soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptors).

Association with lipid rich membrane domains is a common mode of cell uptake, and accordingly, STx and CTx associate with detergent resistant membrane microdomains (DRMs) [27] and undergo transport from early endosomes. PEx, on the other hand, undergoes lipid-independent transport from late endosomes to the TGN [28]. While the late endosome pathway used by PEx is dependent upon the small GTPase, Rab9 [29], transport from early endosomes to the TGN is a Rab9-independent route used by CTx, STx, and ricin [30].

Transport of cargo from endocytic vesicles to target organelles entails the fusion of vesicle and target membranes, which requires interaction of specific vesicle and target membrane SNAREs (or v-SNAREs and t-SNAREs, respectively).

Correspondingly, early endosome to TGN, and late endosome to TGN pathways depend on the contributing SNAREs, as each route is characterized by its own separate v-SNAREs and t-SNAREs [31,32].

Once vesiculated toxins reach the TGN, the cargo is transported to the endoplasmic reticulum (ER) *via* several routes (Fig. 1). One is through the Golgi cisternae by interaction with the KDEL receptor, which cycles between the TGN and ER in a coatamer protein complex (COP-1)-dependent manner [33–35]. The COP1 protein complex, which coats vesicles budding from the Golgi apparatus, can sort vesiculated cargo based on interactions with the cytoplasmic domains of membrane proteins. The KDEL receptor recognizes and binds KDEL motifs on cargo proteins, and is responsible for retrieving escaped ER proteins from the Golgi. PEx contains a KDEL-like sequence that is exposed after A chain release by furin cleavage in early/recycling endosomes [28,36–39]. The lipid-sorted pathway, used by STx, is both KDEL receptor and COP1-independent, and controlled by Rab6 [40–43]. PEx may also use this pathway [28]. A third poorly characterized pathway bypasses the Golgi cisternae and instead transports cargo directly from the TGN to the ER in a KDEL receptor and COP1-independent manner. CTx uses this pathway, despite the existence of a KDEL motif in its peptide sequence [44]. It is possible that this motif functions to retain CTx in the ER after delivery, and prevent possible anterograde transport to the Golgi.

Ricin may utilize all 3 pathways. While ricin lacks a KDEL sequence, evidence shows that it can bind the chaperone, calreticulin, which has a KDEL motif, and undergoes COP1-dependent trafficking to ER [45]. Ricin can elicit cell death when both the classical COP1-dependent and Rab6-dependent pathways are inhibited, suggesting that ricin can bypass Golgi stacks along a similar pathway as CTx [46]. As ricin can bind glycolipids containing a terminal galactose, it may also follow a lipid-sorting pathway.

Once the toxins have reached the ER, it is thought that the cytoplasm is accessed by taking advantage of the protein auditing system in the ER known as ERAD (ER-associated protein degradation). This mechanism eliminates misfolded proteins from the ER by discard into the cytoplasm through a pore known as the Sec61 translocon (now termed a dislocon) [47,48] (Fig. 1). Typically, such proteins are ubiquitinated and targeted to the proteasome for degradation. However, toxins contain abnormally low lysine content, thus are poor substrates for ubiquitination [49], and hence are spared from degradation but survive in the cytoplasm to access the protein synthesis machinery.

2.2. Retrograde trafficking for cytosolic delivery of therapeutics?

Similar types of bacterial toxins, such as diphtheria toxin, can directly penetrate from the endosomal membrane into the cytosol, thus avoiding the retrograde route altogether [4]. This feature has been utilized for the delivery of exogenous genes [50] and peptides [51,52] into the cytoplasm. Gene delivery conjugates have also been produced and tested that make use of components from toxins, like PEx, that undergo retrograde trafficking. For example, multidomain fusion proteins containing the translocation domain of PEx have been used to deliver genes into the cytosol [53,54]. Whether these conjugates actually trafficked in similar fashion to wild-type PEx is unknown, though it appears that the intention of the molecular design was to breach the endosomal membrane. Shiga toxin and cholera toxin, which also enter the cytoplasm from the ER, have been tested as gene transfer agents [55–58], though, like PEx derived vectors, it is not clear whether these agents traffic as the wild-type toxins when used for gene delivery.

Protein toxins have also been used to generate specific cytotoxic T lymphocyte (CTL) activation against certain epitopes [59,60]. Delivery of antigenic peptides by fusion to modified toxins has facilitated cytosolic entry of antigens, which can then be degraded in the proteasome and displayed on MHC class I molecules to prime a CTL response. This approach to generating a new type of vaccine has been used to deliver epitopes *via* PEx [61], STx [62], and other types of toxins [14]. As the molecular players of the toxin/retrograde trafficking pathway are better characterized, it may be possible to utilize this route as a means to target compounds to the other specific organelles contributing to this pathway. One could envisage the design of new molecules that can interact with target cells similarly to the toxins discussed here, and mimic the toxin trafficking pathway to deliver peptides specifically to the Golgi or ER to, perhaps, correct a defect, or elicit toxicity for the treatment of cancer. Moreover, given its avoidance of the lysosome, perhaps this route could be of better use for gene delivery and enable greater survival of gene therapy vectors after target cell entry.

3. Prion protein trafficking and intercellular delivery

The cellular prion protein (PrP^c) is a glycosylphosphatidylinositol (GPI)-anchored protein that is ubiquitously expressed, though found at higher levels in neurons, some non-neuronal tissue, and immune cells [63,64]. The function of PrP^c remains unclear, but is thought to contribute to: copper and/or zinc ion transport or metabolism, protection from oxidative stress, cellular signaling, membrane excitability and synaptic transmission, apoptosis, and neurite outgrowth. The diseases associated with prions occur when PrP^c undergoes conversion to a scrapie form (PrP^{sc}) [65,66], resulting from the transconformation of an α-helix to β-sheet-rich structure. PrP^{sc} can seed further conversion reactions, thus greatly increasing the rate of transconformation [67]. The resulting molecules can oligomerize into an amyloid fibril, and acquire the tendency to form amyloid deposits in brain tissue.

In humans, 15% of prion diseases are inherited, due to mutation in the prion protein gene. The infectious form, which causes Kuru and Creutzfelt–Jakob disease in humans, scrapie in sheep, and bovine spongiform encephalopathy in cattle, is thought to enter the host through the gastrointestinal tract and become acquired by peripheral nerves and lymphoid tissue where replication takes place [68,69]. Invasion of the central nervous system is likely due to transfer *via* phagocytic mononuclear cells [70,71]. The transfer of the infectious agent from

cell to cell is hypothesized to occur *via* exosomes [72], which are vesicles that are secreted from the cell surface.

3.1. Mechanism of intracellular and intercellular transport

After synthesis, PrP^c is secreted to the cell surface where its GPI anchor is inserted at plasma membrane lipid rafts [73,74], which are domains within the lipid bilayer exhibiting a more ordered assembly of specific lipids (usually glycosphingolipids and cholesterol) compared to surrounding plasma membrane [75]. PrP^c can constitutively endocytose *via* either clathrin-coated vesicles or caveolae (Fig. 2), likely depending on cell type or lipid microenvironment [74]. Once internalized, PrP^c traffics through late endosomes/lysosomes, with a steady state fraction localized to multivesicular bodies (MVBs) in neurons, brain, and non-neuronal cells [76–78]. MVBs are formed by the pinching off of cytosol-filled, or intraluminal vesicles (ILVs) into the endosomal lumen [79,80] (Fig. 2). The sorting of cargo into ILVs involves at least 18 proteins and is a tightly regulated process [81]. Among the cellular factors contributing to this process is the Endosomal Sorting Complex Required for Transport (ESCRT) machinery, which are cytosolic proteins that selectively sort cargo to ILVs [81]. In this process, ubiquitinated cargo proteins are recognized by Hrs complex (or ESCRT-0) proteins, which then recruit Tsg101 and the ESCRT-I complex, that also recognize ubiquitinated cargo. Tsg101 then recruits ESCRT-III *via* ESCRT-II or Alix, which together sequester cargo proteins into the inward-budding ILVs.

MVBs can either fuse with the lysosome or with the plasma membrane. Plasma membrane fusion enables the release of ILVs (now known as exosomes) extracellularly (Fig. 2). Exosomes may serve as a mode of intercellular communication, a means to discard proteins, and may be exploited by pathogens for disease transmission [82]. Both PrP^{sc} and PrP^c have been detected in cell culture supernatants, associated with secreted exosomes [9]. These exosomes elicited conversion of endogenous PrP^c to PrP^{sc} when incubated with naïve host cells [72], and caused acute typical neuropathology when inoculated into mice [9]. These findings suggest that exosomes may facilitate transfer of infectious prion agent.

Once released, exosomes may transport associated cargo to remote as well as neighboring cells, which likely explains how prions may be delivered to sites distant from the site of introduction. Transfer of the protein to recipient cells is thought to take place by interaction of exosomes with recipient cell membranes through two possible types of mechanisms (Fig. 2). One mechanism is the direct fusion of exosomes with the plasma membrane, thus transferring contents of exosomal membranes to the plasma membrane. This has been suggested by the observation that recipient cells receive incoming PrP^{sc} into recipient raft domains, and exchange of membrane components can take place between exosomes and recipient cells [83]. A more likely mechanism, however, is that exosomes undergo endocytic uptake into recipient cells, then back-fuse with the limiting membrane of the endocytic vesicle, thus transferring exosomal membrane contents. Exosomal delivery of transconformation activity is supported by this mechanism, as the conversion process is thought to occur at low pH [84,85].

3.2. Exosomes for therapeutic spread?

Gene therapy treatment of solid tumors relies on efficient delivery to as many cells as possible to obtain the most potent level of gene expression and potential tumor ablation. However, delivery of a gene to 100% of cells in a tumor may be an improbable task. Expression of a gene product that can somehow spread a therapeutic effect to neighboring cells is a more feasible approach. Thus, the use of the herpes simplex virus thymidine kinase (HSV-TK) gene as a therapeutic transgene has sustained great appeal in gene therapy due to the 'bystander effect' caused by the toxic enzyme product [86]. This effect describes the ability of the HSV-TK enzyme product, triphosphorylated ganciclovir, to spread to neighboring cells *via* gap junctions within the solid tumor, whereas the substrate has no such effect on cells. More recently, attempts have been made to enhance this effect by producing TK as a recombinant fusion to the VP22 protein [87], which has an ability to become secreted from the expressing cell and taken up by neighboring cells through a mechanism that remains unclear [88,89]. The capacity of secreted exosomes from a 'producer' cell to be taken up by neighboring as well as remote cell targets could be a potent means of delivering the most efficient levels of a therapeutic to cell targets. One could envisage the ability to modify such exosomes with a membrane bound targeting ligand to perhaps limit the spread of a therapeutic to surrounding tumor tissue while at the same time target remote, metastatic tumors. Such modified exosomes containing a therapeutic protein or compound could efficiently deliver the therapy into recipient cells by fusion of the exosomal membrane with recipient cell membranes, thus releasing the product directly into the target cells. Further characterization of the pathogenic and cellular factors contributing to this process would determine whether this approach to therapeutic dissemination could be a feasible option in the future.

4. SV40 trafficking *via* caveolae and caveosomes

Caveolae are flask-shaped invaginations at the plasma membrane [90] characterized by a coat comprised mostly of caveolin-1 (Cav1) [91,92], which is a palmitylated, cholesterol-binding protein [93,94]. Caveolae regulate several different signaling cascades, thus caveolin defects can contribute to a broad range of diseases, including cancer, cardiovascular disease, diabetes, atherosclerosis, pulmonary fibrosis, and muscular dystrophies [95]. Certain mammalian viruses, such as polyomaviruses, influenza viruses, coronaviruses, and echovirus, use caveolae-mediated transport from the cell surface to enter cells [96–100]. The trafficking of simian virus 40 (SV40), a non-enveloped DNA virus of the papovavirus family, has been most extensively studied, and follows a pathway of caveolae-mediated transport that facilitates nuclear delivery of the virus while avoiding the endosomal/lysosomal degradation route [101–104].

4.1. Trafficking via caveolae and caveosomes

SV40 binds two receptors. One is ganglioside GM1, located in lipid rafts [105], and the other is major histocompatibility (MHC) class I [106]. Virus binding induces receptor clustering, and sequestration of lipid rafts and associated receptor/virus complexes into caveolae [107,108] (Fig. 3). Binding also induces a cascade of signaling events, including tyrosine kinase phosphorylation and protein kinase C activation [101,109], which contribute to caveolar formation and endocytosis. One downstream effect of signaling is actin depolymerization, which facilitates caveolar internalization, and recruitment of dynamin-2 to pinch off the caveolar neck and release caveolar vesicles [104], which some have termed 'cavicles' [110]. MHC class I molecule is not endocytosed with the virus [107].

After release from the cell surface, caveolar vesicles require intact microtubules to traffic within the cell [110], suggesting that dynein motors may contribute to motility. These vesicles may fuse with endosomes from the clathrin-mediated pathway [111], or with caveosomes [101], which are Cav1-positive, pH neutral compartments rich in cholesterol and glycosphingolipids [101] (Fig. 3). In non-infected cells, these compartments are likely to serve as intermediate depots for transport of sphingolipids and GPI-linked proteins from the plasma membrane to the Golgi apparatus [112,113]. After delivery to caveosomes, SV40 can be retained in these compartments for several hours. This contrasts with findings from uninfected cells showing that caveosomes may mediate receptor turnover [114,115], and suggests that delivery to and function of caveosomes depends on the protein cargo.

SV40-containing caveosomes are sorted by Cav1-negative carriers that traffic along microtubules to the ER [101,116] (Fig. 3). COPI and COPII-coated carrier vesicles may contribute to this process. It is thought that SV40 somehow penetrates into the cytosol after delivery to the ER, from which nuclear entry is gained *via* the nuclear pore complex [117] (Fig. 3). In an alternative and more rapid endocytic pathway, SV40 may be directly internalized *via* lipid rafts in a dynamin-2-independent manner and vesiculate into Cav1-negative organelles [102]. This pathway was identified because SV40 could infect caveolin-1 knock-out mouse cells.

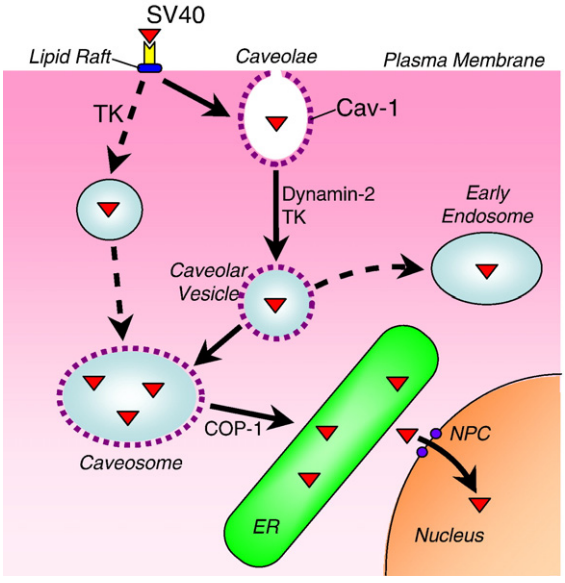

Fig. 3. Trafficking *via* caveolae and caveosomes. SV40 bound to the cell surface can distribute to caveolae, which can pinch off to form a vesicle that is released from the cell surface and transported to Cav1-positive caveosomes. Alternatively, SV40 may directly internalize *via* lipid rafts and fuse with caveosomes. COP1-mediated transport facilitates delivery from caveosomes to the ER, from which SV40 possibly exits into the cytosol and enters the nucleus *via* nuclear pores.

The molecular mechanisms contributing to caveolar-mediated trafficking remain to be fully characterized. Even the role of caveolin is unclear. Expression of Cav1 in cells lacking caveolae is sufficient to generate caveolar formation [118], whereas overexpression of Cav1 can inhibit this [119]. It has been proposed that Cav1 stabilizes caveolar invaginations whereas Cav1-negative intermediates can rapidly bud from the cell membrane and endocytose [115]. This may partly explain findings using green fluorescent protein (GFP)-tagged Cav1 showing that a large pool of immobile caveolin exists in cultured cells [120]. Such reports in addition to the SV40 trafficking pathway have suggested that caveolae mediate slow (up to 1–2h) endocytosis that requires sequestration of cargo at the cell surface before internalization [101]. This contrasts with the rapid endothelial transcytosis mediated by caveolae *in vivo* that has been observed using specific targeted nanoparticles [121]. These studies demonstrated that *in vivo* circulating particles targeted to caveolae rapidly crossed the endothelium and underlying basement membrane, and accumulated into the interstitial space at lung tissues within minutes. The contrasting findings of the latter and former studies may depend on whether the studies are being performed *in vivo* or in cultured cells, as well as the type of probe being used.

4.2. Caveolar uptake to avoid lysosomal degradation of therapeutics?

Trafficking studies on non-viral gene delivery vectors have shown that such vectors may enter the same cells using multiple cell entry routes [122,123]. Some of these routes may support delivery to the degradative pathway, while the caveolar route used by SV40 may not only spare cargo from degradation but enable prolonged residence in the cell for a period of time before routing to the ER. Given that cellular proteins, such as transforming growth factor β receptor (TGFβ-R), elicit receptor signaling when internalized *via* clathrin-coated pits whereas caveosome uptake promotes receptor turnover [114], it appears that using caveosomes as a means to evade degradation would depend on modulation by the vesicle cargo itself. In this regard, further studies characterizing the molecular interactions between pathogens like SV40 and the host cell that enable pathogen survival in caveosomes would be useful.

It has been shown that particles resembling non-viral gene delivery vectors can utilize either clathrin or caveolar-mediated uptake into cells, depending on particle size [124]. While the same vector or protein may enter the same cells *via* different routes, some routes appear to support gene transfer while others do not. Accumulating studies on vector trafficking do not show, however,

that one route consistently supports gene transfer. For example, recent studies have shown that certain polyplexes entered cultured cells by both clathrin and caveolar-mediated pathways, yet the latter pathway preferentially led to gene expression whereas inhibition of the former pathway had no effect [125]. The reverse was observed for histidinylated polyplexes, whereby clathrin-mediated endocytosis appeared to preferentially support gene expression even though the vector entered cells by both clathrin and non-clathrin-mediated pathways [126].

Recent studies showing that caveolae mediate the rapid transcytosis of targeted particles across the endothelium, thus enabling access to deep tissue cells *in vivo* [121], demonstrate that it is possible to overcome the endothelial barrier and target tissue from the circulation. While it has been suggested that tumor vasculature can be 'leaky', and thus facilitate accumulation of therapeutics into a solid tumor, normal tissue, containing different types of endothelium, may not be as easily accessible. Thus, targeting to endothelial cell-surface proteins for the transport of molecules across the vessel wall would be an important strategy for drug and gene delivery.

5. Vacuole and replicative organelle formation by *Brucella*

Some pathogens acquire prolonged survival in a host cell by forming a vacuole in which the pathogen resides and evades the host defenses. Among the bacterial pathogens that share a similar vacuole trafficking mechanism are: *Shigella*, *Listeria*, *Mycobacterium*, *Salmonella*, *Legionella*, *Francisella*, and *Brucella* [127,128]. The trafficking of *Brucella* is among the most extensively studied, and characterized by endocytosis into a special vacuole that interacts with the ER and forms a replicative organelle in which the bacterium establishes long-term survival. *Brucella* replicates inside infected host cells, such as macrophages [129], and causes brucellosis, which can affect a broad range of mammals, including livestock and humans [130]. Human brucellosis can be a chronic and debilitating disease, and the long-term survival of *Brucella* in host cells contributes to disease chronicity. *Chlamydia* also forms pathogen-containing vacuoles after cell entry. *C. pneunomiae* (Cpn) is associated with respiratory tract infections, including pneumonia, asthma, bronchitis, sinusitis, and sarcoidosis [131].

5.1. *Brucella* trafficking

Brucella infection is initiated by binding of bacterial surface-exposed Hsp60 to the cellular prion protein, PrP^c [132]. While PrP^c may typically undergo clathrin-mediated endocytosis, *Brucella* is somehow able to modulate its own endocytic uptake and avoid targeting to the lysosomal degradation pathway [133]. Instead, it is thought that uptake occurs *via* lipid raft endocytosis, as lipid raft disruption affects cell entry, short-term survival and replication of *Brucella suis* and *Brucella abortus* [134].

Endocytic uptake results in the formation of a *Brucella*-containing vacuole (BCV) (Fig. 4), which can interact with early endosomes but avoids fusion with late endosomes/lysosomes, as observed by the absence of GTPase Rab7 and other late endosomal markers from the vacuole [135,136]. While BCV can acquire LAMP-1, it is argued that other compartments in addition to late endosomes/lysosomes can harbor the LAMP-1 marker [137]. As the BCV vacuole matures, it undergoes acidification [138], and comes in close contact with the ER [129] (Fig. 4). The BCV interacts with the ER and forms an ER-derived replicative organelle, establishing long-term survival (Fig. 4). In epithelial cells, the replicative organelle is ER-derived, whereas in phagocytic cells, BCVs dock at the ER and acquire ER-specific markers *via* limited fusion events [129]. At

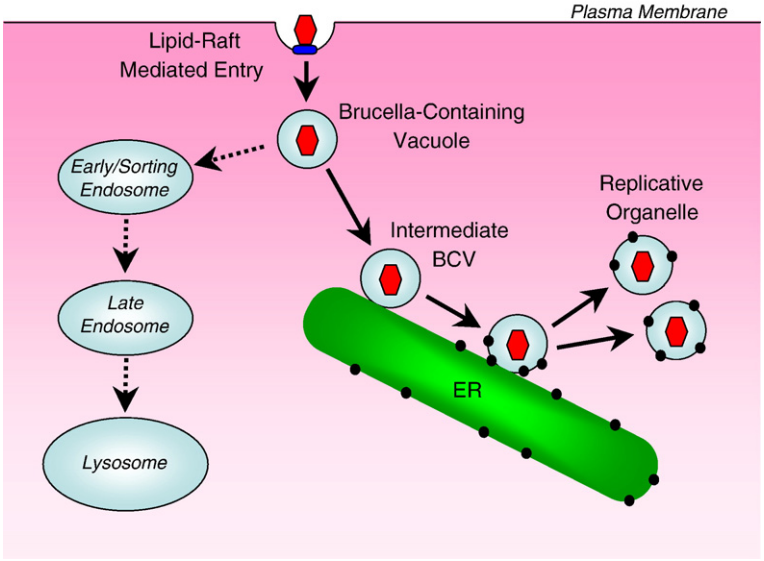

Fig. 4. Vacuole and replicating organelle formation. Membrane-bound *Brucella* is internalized into a *Brucella*-containing vacuole (BCV), which can either fuse with early/sorting endosomes, or undergo maturation, which entails transit to the ER. Interactions with the ER can result in acquisition of ER markers, while the vacuole undergoes maturation to a replicative organelle.

this stage, the organelle becomes permissive for bacterial replication. Requirement for additional membrane output for replication is likely provided by further fusion interactions with the ER membrane. Studies on *L. pneumophila* show that biogenesis of the ER-derived organelle requires interception of COPI vesicular trafficking from the ER [139]. This is not true for *Brucella*, however, as blocking such trafficking has no effect on BCV maturation or replication [129].

The molecular mechanism of entry and survival is still unclear, though VirB protein has received much attention. The screening of *Brucella* survival mutants generated by transposon insertion identified the VirB operon [140], which encodes a type IV-related secretion system that typically secretes/exports nucleoprotein complexes or proteins [129,141]. In contrast to wild-type *Brucella*, the VirB mutants undergo lipid raft-independent endocytosis, remain in immature vacuoles, can dock with the ER but do not sustain fusion with the ER, and are ultimately targeted to the degradative pathway (thus failing to reach a replicative niche). The relevance of VirB in endocytic entry is unclear, as a minority of ingested wild-type bacteria survive in the host cell, suggesting that the majority of internalized bacteria do not escape the degradative pathway [129,142,143]. Furthermore, studies in *B. suis* show that VirB expression takes place after cell entry [144]. VirB is likely involved in late events corresponding with ER fusion [129]. It is possible that VirB mediates the transport of bacterial effector molecules into the host cell that may modulate BCV maturation.

5.2. Vacuologenesis for extranuclear replication and retention of therapeutics?

Gene therapy may be administered as a temporary, short-term treatment for a disease, such as cancer, or as a long-term or permanent solution to replace or correct a defective gene. Long-term approaches have typically entailed the use of a viral vector with the capacity to integrate a gene of interest into the host genome, which can pose serious concerns, including the possibility that gene insertion can activate an oncogenic effect [145–147]. Alternatively, DNA vectors containing elements for extrachromosomal retention in host cells may support a long-term, though not permanent, solution for transgene delivery [148]. In the same vein, perhaps an extranuclear organelle, such as the type of vacuole providing a haven for pathogens like *Brucella*, may enable the long-term residence and retention of a gene therapy vector or drug in a target cell. Bacterial-like vectors have been considered for cancer therapy and gene therapy [149–151]. While the mechanism for the formation of pathogen-containing vacuoles and replicative organelles needs further characterization, studies so far appear to suggest that these intracellular bodies occur as a result of interaction between pathogen gene products and host cell factors. Further studies identifying the players in these interactions and associated functions may direct future efforts to design molecules that can mimic these dynamics and induce the formation of similar intracellular organelles. One could envisage such organelles as being useful in the long-term intracellular retention of therapeutic compounds or genes, whose release over time could be of more benefit in comparison to large, potentially toxic, bolus doses of therapeutic that may require numerous repeat doses to be effective.

6. Conclusion

In the development of drug and gene delivery, directing the entry and trafficking of therapeutics to specific intracellular pathways is a worthwhile consideration. While the typical clathrin-mediated endocytic pathway used by many gene therapy vectors and targeted therapeutics introduces common cellular barriers, alternative pathways such as those discussed here may provide a means to evade such barriers. Moreover, such pathways may facilitate delivery to specific organelles and cellular compartments. Future studies on the molecular mechanisms mediating pathogen trafficking through these pathways may enable design of new vectors and delivery agents with improved ability to direct therapy to desired intracellular targets.

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