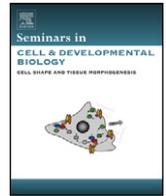




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Review

Modulation of membrane traffic between endoplasmic reticulum, ERGIC and Golgi to generate compartments for the replication of bacteria and viruses

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ABSTRACT

Several bacteria and viruses remodel cellular membranes to form compartments specialised for replication. Bacteria replicate within inclusions which recruit membrane vesicles from the secretory pathway to provide nutrients for microbial growth and division. Viruses generate densely packed membrane vesicles called viroplasm which provide a platform to recruit host and viral proteins necessary for replication. This review describes examples where both intracellular bacteria (*Salmonella*, *Chlamydia* and *Legionella*) and viruses (picornaviruses and hepatitis C) recruit membrane vesicles to sites of replication by modulating proteins that control the secretory pathway. In many cases this involves modulation of Rab and Arf GTPases.

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1. Introduction

Many intracellular pathogens replicate in specialised compartments in cells. For bacteria these are generally described as vacuoles or inclusions, and for viruses they are called virus factories or viroplasm (Fig. 1). The formation of replication sites can involve extensive modification of membrane compartments. Early changes probably reflect the mobilisation of cellular defences against infection such as phagocytosis and autophagy [1,2] while later stages indicate the steps taken by the pathogen to avoid cellular defences

and generate a compartment specialised for replication. Intracellular bacteria remain within membrane-bound vacuoles to avoid delivery to lysosomes and then recruit vesicles from the secretory pathway to provide nutrients necessary for microbial growth and cell division. Many viruses generate densely packed membrane vesicles to shield them from recognition by cellular defence pathways that recognise double-stranded RNA, and at the same time the membranes provide a platform to recruit viral and host proteins required for replication [3–5]. This review describes how recent work on intracellular bacteria such as *Salmonella*, *Chlamydia* and *Legionella* draws parallels with studies on (+) strand RNA viruses where microbial proteins recruit membranes by modulating proteins that control the secretory pathway. In many cases this involves modulation of Rab and Arf GTPases.

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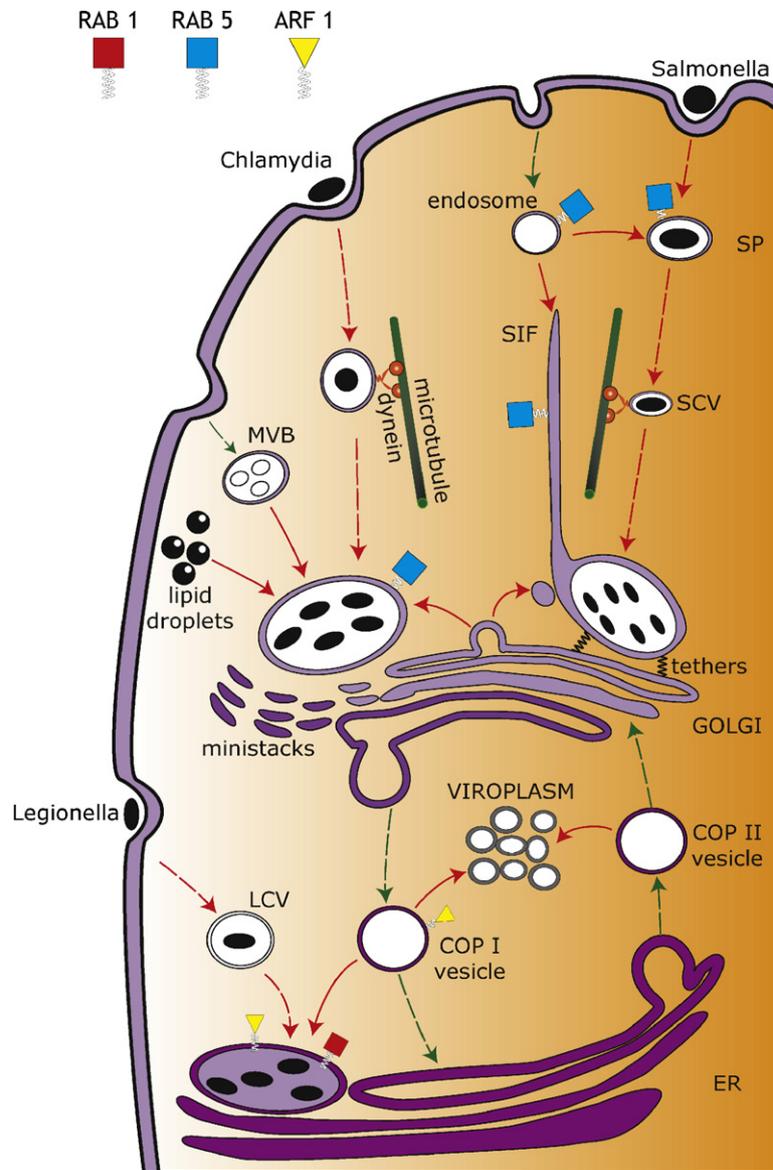


Fig. 1. Replication compartments formed by bacteria and viruses. *Salmonella*-containing vacuoles interact with the endocytic pathway while migrating along microtubules. After reaching a peri-Golgi position, the vacuoles generate long membrane filaments (Sifs) and initiate replication. *Legionella* and *Chlamydia* escape interaction with the endosomal/lysosomal pathway. *Chlamydia* inclusions migrate along microtubules and replicate next to a fragmented Golgi. *Legionella*-containing vacuoles attach to ER and Golgi-derived vesicles and replication begins following fusion of vacuoles with the ER. Picornaviruses and hepatitis C replicate within perinuclear arrays of densely packed membrane vesicles called viroplasm. ER: endoplasmic reticulum; MVB: multivesicular bodies; SCVs: *Salmonella*-containing vacuoles; SIF: *Salmonella* induced filaments; SP: spacious phagosome; LCV: *Legionella*-containing vacuole.

1.1. Small GTPases regulate formation of vesicles in the secretory pathway

The Rab and Arf families of small GTPases act as molecular switches which regulate the formation, docking and fusion of membrane vesicles that carry cargo from one membrane compartment to another (Fig. 2). Vectorial transport through the secretory pathway is maintained because different organelles recruit different GTPases and specific tethering proteins able to direct interactions between transport vesicles and acceptor membranes. Vesicle fusion is consolidated by pairing between SNARE proteins [6,7]. The Arf1 GTPase regulates the formation of COPI coated vesicles which form at Golgi membranes to carry cargo back to the ER. COPII coated vesicles assemble at the endoplasmic reticulum (ER) and carry proteins and lipids to the ERGIC and Golgi where docking and fusion are con-

trolled by Rab1 and Golgi tethering proteins such as p115, GM130 and Golgin-84. The functions of other Rab proteins are described when they appear in the text.

2. Bacteria replicate within membrane-bound inclusions and vacuoles

2.1. Role of Rab GTPases during Golgi positioning of *Salmonella*-containing vacuoles

Salmonella enterica enters the cell within a membrane compartment known as the spacious phagosome (SP), which then shrinks to enclose one or two bacteria within *Salmonella*-containing vacuoles (SCVs) [8–10]. Early SP and SCV maturation result in recruitment of Rab 5 and several Rab5 effector proteins which normally con-

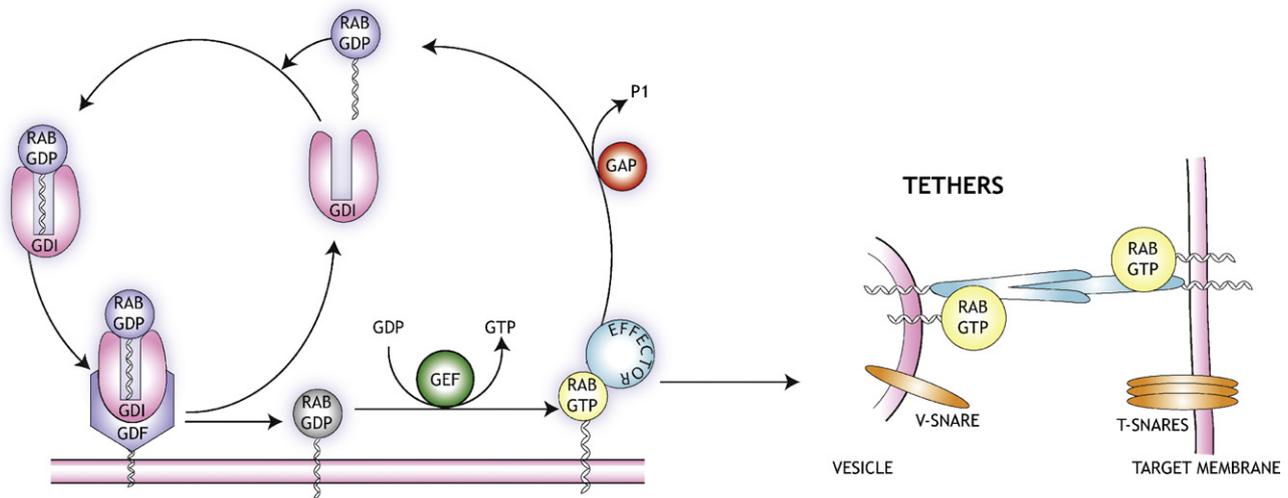


Fig. 2. Role of Rab and Arf proteins in directing vesicle transport. The small GTPases are held in the cytosol as inactive GDP-forms by GDI proteins. Translocation to membranes requires membrane-bound GDI displacement factors (GDFs) which release the GTPase and expose a C-terminal lipid group for membrane binding. Guanine nucleotide exchange factors (GEFs) exchange GDP for GTP and allow the activated GTPase to bind effector proteins which facilitate vesicle budding and fusion. The cycle is completed by GTP-hydrolysis, which requires GTPase activating proteins (GAPs), followed by extraction of the GDP-bound enzyme from the membrane by GDI.

tol endocytosis. Sorting nexins may facilitate shrinkage of the SP [11] during formation of the SCV, while Rab5 effectors such as early endosome antigen 1 prime the SCV for progress along the endocytic pathway. SCV lose Rab5 and accumulate Rab7, LAMP1 and acidify. Rab7 recruits Rab7-interacting lysosomal protein (RILP) which links the SCV to the dynein motor protein allowing movement to the Golgi and MTOC [12]. This, coupled with acidification of the vacuole, activates secretion of bacterial *Salmonella* pathogenicity island 2 (SPI-2) proteins which are needed for continued Golgi positioning and replication. The Pip2B and SifA proteins recruit host kinesin interacting protein SKIP to the SCV to displace kinesin and inhibit outward movement [13,14]. SifA may also impair the Rab7-RILP interaction to release dynein [12]. SseF and SseG locate to the surface of the vacuole where they may tether the vacuoles to the Golgi [15,16]. Golgi positioning may also involve SipA and SifA proteins, actin [17], and recruitment of myosin II [18].

Golgi positioning appears to be crucial for replication [19] and is thought to facilitate the supply of nutrients from Golgi-derived vesicles. The exact pathway followed by the vesicles remains to be determined. Vacuoles recruit 18 different RabGTPases during maturation [20] showing complex interaction with the secretory pathway. Vesicles leaving the trans-Golgi network (TGN) accumulate next to the SCV, but do not appear to fuse [21], making it unclear whether they deliver nutrients. Interestingly, long thin tubules of membrane called *Salmonella* induced filaments (Sifs) containing endosomal and lysosomal markers extend from the SCVs soon after positioning at the Golgi. Sifs require secretion of SPI-2 proteins, SseF and SseG, and extend towards the cell periphery along microtubules where they recruit Rab9 and Rab11 and take up fluid phase endocytic markers [20–24]. Sifs may provide nutrients to bacteria from outside the cell by fusing with vesicles trafficking between late endosomes and the Golgi.

2.2. Bacterial effector proteins recruit of Rab GTPases and SNARE proteins to *Chlamydia* inclusions

Chlamydia invades the cell as an infectious, but replication defective, elementary body (EB) and differentiates into a replicative form called the reticulate body (RB), within a membrane compartment called an “inclusion”. *Chlamydia* does not interact with the

endosomal/lysosomal pathway [25] but, as seen for *Salmonella*, the inclusions recruit dynein and migrate along microtubules to a peri-Golgi position [26], and again peri-Golgi positioning at the MTOC is required for replication. Inclusions receive sphingomyelin and cholesterol from exocytic vesicles that would normally deliver lipids from the TGN to the plasma membrane [27–30] and can also gain lipids directly from lipid storage organelles called lipid droplets. This involves the bacterial Lda3 protein which may link lipid droplets to the outer surface of the inclusion [31]. *Chlamydia* infection also results in proteolysis of tethering protein Golgin 84 by caspases leading to fragmentation of the Golgi into ministacks. Inhibition of Golgi fragmentation reduces replication and also prevents the delivery of lipids to the inclusion suggesting that fragmented Golgi ministacks provide vesicles able to fuse with the vacuole [32]. Non-lipid nutrients can also be delivered to *Chlamydia* inclusion by endocytosis and fusion with multivesicular bodies (MVB) [33].

Chlamydia effector proteins called ‘integral inclusion membrane’ (Inc) proteins recruit several RabGTPases to the inclusions [34]. Mature inclusions recruit Rab4 and Rab11 [35,36], which are generally present in recycling endosomes and could interact with incoming MVBs. Inclusions recruit Rab1, which may activate tethering and subsequent fusion with Golgi-derived vesicles or ministacks. Rab6 and Rab10, which are involved in Golgi trafficking, are associated with inclusions in a species-dependent manner [35]. Rab recruitment requires the GTP-bound form suggesting that the Inc proteins act as Rab effector proteins. Inc protein Cpn0585, has coiled coil domains that share sequence similarity with Rab effectors such as the tethering proteins Golgin 84 and Golgi matrix protein GM130, and interacts with Rab1, Rab10 and Rab11 [37]. Whether Cpn0585 plays a role in Golgi fragmentation seen during infection has not been tested. IncA proteins such as CT813 have SNARE motifs and interact directly with host SNAREs that facilitate vesicle fusion [38]. The glutamate residue in the SNARE-like motif of IncA suggests similarity with Q-SNAREs that would interact with R-SNAREs. This is consistent with co-precipitation of Vamp3, Vamp7 and VAMP8 with IncA, and location of Vamp8 in the inclusion membrane. The precise role the SNARE homologues play in inclusion formation is not known. They interact with SNAREs involved in endosome trafficking and may aid recruitment of MVBs to the inclusion.

2.3. Bacterial effector proteins modulate Rab1 and Arf1 during maturation and fusion of Legionella-containing vacuoles with endoplasmic reticulum

Legionella pneumophila is an intracellular bacterium of amoeba but can infect humans following inhalation of aerosols from contaminated water and air cooling systems. Inhaled bacteria infect alveolar macrophages and become surrounded by a membrane compartment called the *Legionella*-containing vacuole (LCV), which avoids interactions with the endosomal/lysosomal pathway [39]. The LCVs quickly recruit Arf1 and Rab1 GTPases, and associate with vesicles containing ER and ERGIC markers, suggesting recruitment of vesicles trafficking between ER and Golgi compartments. Membrane vesicles remain associated with the LCV for several hours during which time the LCVs lose Rab1 and Arf1 [40,41]. The vesicles tethered to the LCV [42] eventually fuse with the vacuole and deliver ER markers, and this is coincident with the onset of replication. Delivery is inhibited by Brefeldin-A, and a dominant negative Sar1[H79G] GTPase slows bacterial replication, suggesting involvement of COPI and COPII transport vesicles [42].

Rabs are recruited to the LCV by bacterial effector proteins DrrA (also called SidM) and LepB [43]. DrrA first acts as a Rab1-specific GDF recruiting Rab1 onto the cytosolic face of the LCV membrane [44,45]. DrrA then acts as a Rab-GEF protein promoting GDP-GTP exchange to preserve Rab1 in its active form [43,46]. The LepB protein binds the LCV at later times and is also present on mature LCVs that have acquired ER markers. LepB binds activated Rab1-GTP and acts as GAP [44] leading to release of Rab1 from the vacuole and this is coincident with fusion of the vacuole with ER-derived vesicles. DrrA and LepB show exclusive binding to Rab1, but Dot/Icm effector LidA binds Rab1, Rab6 and Rab8 [43]. LidA does not have GAP or GEF activity and cannot affect GTP hydrolysis, but works with DrrA/SidM to recruit Rab1 to vacuoles [43].

The presence of Arf1 and Rab1 could facilitate fusion with both ER and Golgi-derived membranes. Rab1 may interact with effector proteins such as p115 or the GM130/Grasp65 complex would normally tether COPI and COPII vesicles to the cis-Golgi. Silencing experiments suggest that the TRAPP1 tethering complex, which facilitates docking of COPII vesicles to ERGIC and Golgi membranes, and Sec22b, which is a v-SNARE found in ER to Golgi transport vesicles are both required for replication [47–49]. These results suggest that COPII vesicles deliver the host factors required to sustain microbial growth and cell division within the ER-derived vacuole.

3. Viruses generate densely packed membranes for replication

The (+) strand RNA viruses produce replicase proteins with RNA-dependent RNA polymerase and helicase activity which are targeted to the cytoplasmic face of the ER by non-structural proteins with membrane binding motifs. This invariably results in the generation of densely packed membrane vesicles called viroplasm. Work in this area has concentrated on understanding how these vesicles are formed, and how their use as replication sites impacts on the secretory pathway. Multiple interactions between replicase proteins probably induce the membrane curvature required to generate vesicles in a way that parallels virus budding from membranes [4,50]. Virus replication can, however, slow secretion suggesting that the formation of viroplasm may divert vesicles normally involved transport between the ER and Golgi into the replication complex. As described above for bacteria, this can involve modulation of small GTPases.

3.1. Role of Arf1 and Arf-GEF proteins in modulating membrane traffic during picornavirus replication

A search for a link between virus replication and membrane trafficking proteins was stimulated by the observation that BFA blocks replication of enteroviruses such as poliovirus and coxsackieviruses [51]. BFA inhibits Arf-GTP exchange proteins (Arf-GEFs) and reduces the Arf1-GTP needed to generate COPI coats in the Golgi. Inhibition of replication by BFA has led to a model where viral activation of Arf-GEFs would recruit Arf1, and Arf effector proteins, needed to recruit membranes into replication compartments [52]. This is supported by genome-wide interference screens where COPI proteins are important for replication of both animal and plant picornaviruses [53,54], and is consistent with the increase in Arf-GTP levels seen in cells following poliovirus infection. Moreover, Arf-1 and Arf-GEF proteins GBF1 and BIG1/2 are recruited into replication complexes [55,56], and poliovirus replication is inhibited by siRNA for GBF1 [57]. Sequestration of Arf-1 into the replication complex would also explain the block in secretion seen in infected cells [58].

For poliovirus, secretion is blocked by the 3A protein which tethers the replicase to ER membranes. 3A does not have intrinsic Arf-GEF activity but the 3A protein recruits Arf-GEFs such as GBF1 and BIG1/2 to membranes, and 3A activates Arf-GEF activity 'in vitro' [56]. This provides an explanation for inhibition of poliovirus replication by BFA, and how the sequestration of Arf1 and Arf-GEFs into replication sites, could block secretion. Evidence that this generates densely packed vesicles is less clear. 3A alone cannot generate the vesicles, and recruitment of Arf-GEFs by 3A is not essential for replication [57]. Moreover, BFA does not prevent formation of densely packed vesicles by poliovirus, so vesicle formation does not require activated Arf-GEF proteins [57].

An alternative mechanism of action for 3A is provided from coxsackievirus, which is also a BFA-sensitive enterovirus. The coxsackievirus 3A protein inhibits Arf-GEF activity, and reduces Arf-GTP [59–61] and may inhibit ER to Golgi transport by stabilizing ARF1-GDP-GBF1 complexes which would in turn reduce the supply of Arf1-GTP for COPI coats. 3A may also slow secretion by inactivating LIS1, a component of the dynein-dynactin motor complex [62] and contribute to microtubule-dependent disruption of the Golgi apparatus seen during poliovirus infection [63]. These experiments explain how 3A would slow secretion, but again do not provide a mechanism for generation of the densely packed vesicles, or paradoxically, explain why the virus would be sensitive to BFA.

Several picornaviruses families are resistant to BFA, and their 3A proteins do not slow ER to Golgi transport [60,64,65]. For these viruses COPII coated vesicles may provide membranes for replication [66–69] while other studies implicate a role for autophagosomes [70]. In the case of Foot-and-Mouth Disease virus, secretion is blocked by the ER-tethered 2BC protein [65,71], rather than 3A, and although less well studied, the 2B and 2BC of enteroviruses also slow secretion when expressed alone in cells [58,72]. These proteins can therefore affect membrane traffic and for 2C this may involve binding to ER membrane protein reticulon 3, which is involved in ER to Golgi trafficking and is required for replication [73].

3.2. Recruitment of membrane trafficking proteins to membrane webs formed from the endoplasmic reticulum during replication of hepatitis C virus

Hepatitis C virus replication occurs within a web of ER membranes. The NS5A protein is anchored to the cytoplasmic face of the web and recruits the RNA-dependent RNA polymerase, NS5B. Both NS5A and NS5B bind VAMP associated proteins (VAPs) [74,75]. NS5B binds to a coiled coil domain (CCD) in VAP that resembles

the CCD of SNARE proteins. Silencing VAP-B reduces the stability of NS5B, and depletion of VAP-B from cell free assays reduces RNA replication. Current models suggest that a VAPA/B heterodimer forms a scaffold that supports the replication complex. VAP proteins also bind to SNAREs involved in ER to Golgi transport, making it is possible that the VAP scaffold also facilitates membrane fusion to generate the membrane web from the ER. This may be true for other (+) strand RNA viruses such as Norwalk virus where membrane-bound nsp48 also binds VAP-A [76].

NS5A also binds TBC1D20, a Rab1-GAP protein, and both TBC1D20 and NS5A co-localise with ER markers. TBC1D20 activates Rab1 GTP hydrolysis, and since depletion of TBC1D20 and Rab1 greatly reduce HepC replication [77,78] it is likely that activation of Rab1-GTP is in some way involved in virus infection. Local blockade of Rab1 could, for example direct transport vesicles to make membrane webs. Rab5 is also recruited to the replication complex [79] by binding to NS4B, and virus-induced membranes recruit Rab5 effectors EEA1, rabaptin 5 and Rab4 suggesting the web may fuse with endosomes. A functional role for Rab5 in viral genome replication is suggested because dominant negative Rab5 reduces replication of replicons, and partial silencing of Rab5 reduces synthesis of viral RNA.

4. Conclusions

The studies described above set the stage or further work where it will be important to analyse interactions between host and microbial proteins at a structural level, and see how these interactions contribute to virulence and/or immune evasion. The work also provides a precedent for studies on other pathogens that use membrane compartments for replication. Brucella for example, traffic through lysosomes before fusing with the ER [80], and Coxiella replicate in a spaceous parasitophorous vacuole formed from secondary lysosomes [81]. Recent work on coronaviruses draws parallels with poliovirus where SARS-CoV replicates within densely packed membranes [82], and mouse hepatitis virus shows sensitivity to BFA and requires GBF1 mediated Arf1 activation [83,84]. Disruption or use of the Golgi is not restricted to the (+) strand RNA viruses. The negative strand RNA Bunyaviruses replicate in the Golgi apparatus where they form tubes that contain replicase proteins [85]. The large DNA viruses such as Fowlpox encode homologues of SNAP proteins involved in SNARE pairing [86], while African Swine Fever virus replicates at the MTOC, slows secretion, and causes Golgi fragmentation and microtubule-dependent loss of the TGN [87]. ASFV also encodes a multigene family of proteins with C-terminal KDEL sequences able to interfere with KDEL-receptor mediated ER to Golgi trafficking and redistribute ER proteins to the ERGIC [88]. In all these cases the consequences of modulating the early secretory pathway in the context of virus survival and/or pathogenesis remain to be discovered.

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