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AUTHOR MANUSCRIPT
Accepted for publication in a peer-reviewed journal

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Published as: *Curr Opin Microbiol*. 2014 February ; 17: 46–52.

Maintenance of Vacuole Integrity by Bacterial Pathogens

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Abstract

Many intracellular bacterial pathogens reside within a membrane-bound compartment. The biogenesis of these vacuolar compartments is complex, involving subversion of host cell secretory pathways by bacterial proteins. In recent years it has become clear that disruption of vacuole biogenesis may result in membrane rupture and escape of bacteria into the host cell cytosol. Correct modulation of the host cell cytoskeleton, signalling molecules such as small GTPases and the lipids of the vacuole membrane have all been shown to be critical in the maintenance of vacuole integrity. Increasing evidence suggests that vacuole rupture may result from aberrant mechanical forces exerted on the vacuole, possibly due to a defect in vacuole expansion.

Introduction

In order to survive, bacterial pathogens that replicate within the cells of their hosts must adopt lifestyles that both exploit and maintain intracellular niches. This is no simple task because eukaryotic organisms have evolved efficient strategies to destroy invading microbes. Intracellular bacteria enter the host cell via phagocytosis and the nascent phagosome is directed into the endocytic pathway. Phagosomes bearing nonpathogens will follow a path that fuses with the lysosomal compartment resulting in bacterial degradation. Pathogens have developed different strategies to avoid this fate. A few pathogenic bacteria including *Shigella, Listeria,* and *Francisella*, escape from the phagosome into the cytosol of the host cell. Although these pathogens avoid the endocytic pathway and the challenge of residing in a nutrient poor vacuolar compartment, they face other challenges in the cytosol notably the cytoplasmic innate immune system. Most of the characterized intracellular pathogens choose to remain within a membrane-bound compartment and modify this niche to facilitate their survival and replication. In recent years it has become apparent that in addition to establishing a replication vacuole, intravacuolar pathogens also actively promote

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the maintenance and integrity of these vacuoles. Here we review recent advances in the understanding of how pathogen-containing vacuoles are maintained.

Establishment of a replication vacuole

In order to survive and replicate within a vacuole, bacterial pathogens must direct the nature of this compartment to suit their needs. The specifics of this process vary from species to species but there are common strategies utilized by all vacuolar bacterial pathogens. Intracellular bacterial pathogens utilize specialized secretion systems to deliver bacterial effector proteins into the host cell. Many of these effector proteins modify the vacuolar niche directly or indirectly to allow the establishment of a replication vacuole. By targeting host cell proteins and lipids, effector proteins alter the identity of the nascent phagosome and promote selective interactions with endosomal membrane compartments. These selective interactions alter the trafficking of the pathogen-containing vacuole such that it avoids the toxic effects of fusing with lysosomes yet maintains constant interactions with host cell vesicles to allow vacuole expansion and nutrient acquisition [1].

Maintenance of a replication vacuole

The formation of a replication vacuole requires a complex interplay of host pathways and bacterial factors. Disruption of this process leads to the failure to establish a replication niche. Incorrect targeting of the vacuole may result in routing of the membrane bound compartment into the default endocytic pathway, resulting in destruction of the bacterial pathogen or the formation of a nonpermissive vacuole. However, it has become apparent that disruption of vacuole assembly can result in a compartment that has many features of permissive replication vacuole, but has compromised integrity, resulting in pathogen release into the cytoplasm (Fig. 1).

Role of the cytoskeleton in vacuole stability

Vacuole trafficking within eukaryotic cells is dependent upon the cell cytoskeleton and cytoskeletal motors. Therefore it is not surprising that pathogen-containing vacuoles (PCV) are tightly associated with cytoskeletal components, and that modulation of the cytoskeleton is a key strategy that facilitates vacuole formation. Host cell vesicles and organelles are transported on cytoskeletal networks, which can be manipulated to allow vesicle recruitment to the PCV. The cytoskeleton also determines the localization of the PCV within the host cell. Indeed many intracellular pathogens traffic to a perinuclear location after phagocytosis, which may facilitate interactions between the PCV and secretory vesicles necessary to maintain a functional replication vacuole. Correct modulation of the cytoskeleton has been shown to be required for expansion of both the *Salmonella* and *Chlamydia* vacuoles [2,3]. Finally, it has been shown that some PCVs are surrounded by a scaffold of F-actin and other cytoskeletal components that provide structural support for the vacuole [3,4].

The interaction of the *Salmonella*-containing vacuole (SCV) with the host cytoskeleton is highly complex and tightly controlled. Shortly after uptake, the SCV traffics to the microtubule organizing center (MTOC) in a manner dependent on both microtubules and actin [2,5,6]. A feature of the SCV is the formation of membrane filaments that extend from

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the SCV to the cell periphery. Transport to the MTOC, retention in this location and production of filaments require the coordinated efforts of several translocated effectors. SopB activates myosin II to promote retrograde movement of the SCV [6], whereas SifA, SseF, SseG, PipB2 and SopD regulate the binding of microtubule motors to the SCV. SseF and SseG promote retention of the SCV at the MTOC by recruiting dynein [7]. PipB2 and SopD2 recruit kinesin-1 to the SCV [8,9]; the presence of high levels of kinesin-1 on the SCV causes vacuole instability [10] but another bacterial effector, SifA, recruits kinesin-1-interacting protein SKIP to promote the transport of membrane filaments and vesicles toward the cell periphery [10,11]. This delicate balance of interactions with cytoskeletal motors is critical for maintenance of SCV integrity. Inhibition of myosin II, kinesin-1 or dynein leads to loss of SCV integrity as does the absence of *sifA* [5,6,12]. Schroeder et al. recently demonstrated that the accumulation of kinesin-1 on the SCV resulting from the absence of *sifA* can be prevented by the further deletion of *sopD2* and *pipB2* [9]. The authors observed a novel type of tubule extending from the SCV that is absent in the presence of SopD2 suggesting that SopD2 suppresses the formation of a novel type of tubule extending from the SCV that can compensate for the lack of SifA-induced filaments.

Both the *Salmonella* and *Chlamydia* vacuoles are surrounded by a network of F-actin [3,4]. This network is required for vacuole stability of both these pathogens as treatment with F-actin polymerisation inhibitors leads to the loss of integrity [3,4]. The role of the F-actin network has been studied most closely in the case of *Chlamydia* infection. It has been shown that F-actin and intermediate filaments combine to form a structural scaffold surrounding the *Chlamydia* inclusion that maintains the integrity of the inclusion [3]. The secreted chlamydial protease CPAF was shown to process intermediate filament proteins to form a filamentous structure that is able to support expansion of the inclusion [3]. More recently, it has been demonstrated that CPAF activity is required for inclusion integrity [13]. This may reflect its role in intermediate filament protein processing although it also targets many other host and bacterial proteins that may play a role in vacuole maintenance.

Role of small GTPases in vacuole stability

During phagocytosis of particles and formation of a membrane bound compartment, host cell proteins are acquired that regulate the trafficking of this compartment. With sequential membrane fusion and fission events, the repertoire of membrane-associated host proteins changes and directs the fate of the organelle. Foremost among these host cell proteins are the small GTPases. Small GTPases regulate host cell functions, including membrane trafficking, by cycling between inactive GDP-bound and active GTP-bound states. In their active state, small GTPases interact with downstream effectors that promote membrane transport. Not surprisingly, many vacuolar pathogens regulate the recruitment and activation state of host cell GTPases to alter the trafficking of their vacuole [1,14]. *Salmonella enterica* Typhimurium initially recruits Rab5, a marker of early endosomes to its vacuole [15,16]. Rab5 is subsequently replaced with Rab7 following fusion with late endosomes [17]. This process has been shown to be important in vacuole stability as expression of constitutively-active Rab5 or dominant-negative Rab7 cause loss of vacuole integrity and release of *S.* Typhimurium into the cytosol [18]. *Legionella pneumophila* recruits GTPases Rab1 and ARF1 which are typically present on early secretory vesicles and are required for formation

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of a replication vacuole [19,20]. *Legionella* utilizes at least 5 translocated effectors to modulate Rab1 function on the vacuolar surface [21]. Recently LidA, an effector that enhances Rab1 recruitment, has been implicated in vacuole stability. A *Legionella* strain lacking 2 translocated effectors, LidA and WipB, was shown to have decreased replication vacuole stability and a corresponding decrease in intracellular survival [22].

Role of lipids in vacuole stability

In addition to targeting the proteins found on the vacuole membrane, intravacuolar pathogens also target the lipids of the vacuolar membrane. Changes in membrane lipids alter the membrane identity of the organelle affecting trafficking. Recruiting or altering lipids is also important for metabolism, membrane dynamics and the tethering of effector proteins to the vacuole [23–25]. Several pathogens change the phosphoinositol signature found on their vacuoles. For instance, *Mycobacterium tuberculosis* depletes PI(3)P to prevent phagosome maturation, whereas *S. enterica* Typhimurium generates PI(3)P to promote vacuole fusion with late endosomes [23]. *L. pneumophila* utilizes phosphoinositides PI(4)P and PI(3)P to anchor bacterial effector proteins to the vacuole [26]. Although they play a key role in vacuole biogenesis, the involvement of phosphoinositides in vacuole stability has not been investigated.

In addition to phosphoinositides, pathogens also recruit or exclude other lipids from their vacuolar membranes. *Chlamydia trachomatis* recruits host cell sphingomyelin and cholesterol to its inclusion both of which are incorporated by the bacterium and are required for replication [27,28]. *C. trachomatis* has been shown to recruit sphingomyelin from both vesicular and non-vesicular sources for different purposes. Sphingomyelin from non-vesicular sources is required for bacterial replication whereas vesicular sphingomyelin is involved in inclusion expansion and is required for stability of the inclusion [29,30]. It has been suggested that a failure to acquire sphingomyelin and other host lipids prevents inclusion expansion to accommodate the replicating chlamydiae and results in inclusion fragmentation [29].

A final example of lipids affecting vacuole stability is the modulation of cholesterol levels by the *S. enterica* Typhimurium translocated effector SseJ. SseJ is a phospholipase that is activated by RhoA and exhibits glycerophospholipid:cholesterol acyltransferase (GCAT) activity leading to the production of cholesterol esters and the depletion of cholesterol from the SCV [31–33]. The activity of SseJ has been shown to destabilize the SCV membrane in the absence of correct interactions with cytoskeletal motors (described above). Additional deletion of *sseJ* inhibits the vacuole rupture phenotype of a *sifA* mutant [34] and the absence of *sseJ* reduces the vacuole destabilizating affect of myosin II inhibition [6]. Cholesterol affects the fluidity of membranes and it has been suggested that SseJ-dependent cholesterol depletion may contribute to tubulation of the SCV by increasing membrane fluidity while also increasing susceptibility to cytoskeleton motor-dependent forces that cause membrane rupture [32]. Interestingly, PlaA, a *Legionella* protein with homology to SseJ, has been shown to regulate stability of the LCV. PlaA is exported by the type 2 secretion system and has been shown to cleave phospholipids and lysophospholipids and to esterify cholesterol

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[35,36]. Similarly to SseJ, PlaA destabilizes the vacuole membrane in the absence of another *Legionella* effector, SdhA [37].

Vacuole disruption as a survival strategy

A few intracellular pathogens including *Listeria* and *Shigella*, escape a membrane-bound compartment shortly after uptake to avoid the restrictive environment of a phagosome targeted to the endocytic pathway. However it is becoming clear that pathogens thought to exclusively replicate within membrane-bound compartments may also exhibit a cytoplasmic stage of replication. It has been known for several years that a small percentage of *Salmonella* escape from the vacuole and reside in the cytosol of epithelial cells [38]. Recent work has shown that the bulk of *Salmonella* replication observed during growth in epithelial cells occurs in the cytosol; the significance of this during infection is unknown although it may play a role in bacterial dissemination [39,40]. Escape from the vacuole has also been observed for Mycobacterial species and *L. pneumophila* suggesting that an extra-vacuolar stage occurs in other bacterial infections [41–43]. The frequency and outcome of vacuole disruption during disease for both of these organisms is unknown. It is likely that the choice of intracellular niche reflects a complex balance between the need to hide from cytoplasmic immune responses and the restrictive nature of life in a vacuole [44].

It is also clear that pathogen-containing vacuoles exhibit some permeability during infection to allow the translocation of bacterial effector proteins into the host cytoplasm. This permeability is achieved through the function of specialized secretion systems forming pores in the vacuole membrane that effectors pass through. *M. tuberculosis* was recently shown to permeabilize its phagosome membrane during the early stages of infection via the ESX-1 secretion system [45]. This permeability results in the release of *M. tuberculosis* DNA into the host cytoplasm and the triggering of the cytosolic innate immune system. A similar immune response is seen during infection with other vacuolar pathogens that possess specialized secretion systems, suggesting that selective permeabilization of the vacuole membrane is a general strategy [46,47].

Advances in detecting vacuole disruption

Most studies examining the integrity of pathogen-containing vacuoles utilize transmission electron microscopy to directly visualize membranes or immunofluorescent microscopy to detect a marker of the vacuole membrane such as LAMP1 or Chlamydial Inc proteins [e.g. 3,12]. An alternate approach is to selectively permeabilize infected cells such that antibodies can pass the plasma membrane and detect cytoplasmic bacteria but are excluded from membrane-bound compartments [12,37]. There are significant limitations with these techniques including the possibility of artefacts resulting from sample processing and the inability to visualize vacuole integrity in real time using live cells. To address these limitations, Enninga and colleagues have developed two novel approaches that allow live visualization of membrane integrity and as such are suitable for high throughput and kinetic assays (Fig. 2). The first assay takes advantage of the observation that cytosolic galectins are specifically recruited to the remnants of ruptured vacuoles [48,49]. Cells expressing GFP-Galectin 3 have been successfully used to follow the escape of *Shigella* into the cytosol in

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real time [50,51]. The second technique also utilizes fluorescent microscopy to detect vacuole rupture and takes advantage of the ability of Gram negative bacteria to display β-lactamases on their surface. Infected cells can be preloaded with the fluorescent β-lactamase substrate CCF4. If the bacteria have access to the cytosol, CCF4 is cleaved and there is a change in fluorescence due to a loss of FRET. This technique has been used to study vacuolar escape of *Shigella* and *M. tuberculosis* [42,50] and is suitable for detection by flow cytometry making it appropriate for high throughput studies [52]. A third live-cell imaging approach was developed by Steele-Mortimer and colleagues [39]. The authors utilized fluorescent-tagged dextran and mCherry expressing *Salmonella*. The dextran labels the endocytic compartment of infected cells and accumulates within the SCV. SCV integrity was tracked using live fluorescent microscopy to detect colocalization of Alexa488-Dextran and mCherry *Salmonella* (Fig. 2).

Conclusion and Perspectives

With our increasing understanding of PCVs, it is clear that a frequent outcome of aberrant vacuole biogenesis and expansion is the loss of vacuole integrity and the release of pathogenic bacteria into the cytoplasm. The formation of a replication vacuole is a tightly regulated process involving both bacterial effector proteins and host pathways. Many of these effector proteins and host pathways have been shown to be required for intracellular replication of bacterial pathogens but their role in vacuole maintenance has not be investigated. As we move forward dissecting the biology of PCVs it is important to revisit these old studies and investigate the effect of disrupting these systems on vacuolar integrity.

The mechanisms of vacuole disruption are largely unclear. They may involve biochemical disruption by host or bacterial enzymes or mechanical disruption caused by aberrant vacuole expansion to accommodate bacterial replication or inappropriate forces exerted by cytoskeletal motors. Much of the evidence to date supports a mechanical model of vacuole rupture and the biophysics involved in vacuole biology is an important consideration.

Finally, given that vacuole disruption often results in triggering of the cytosolic immune system and destruction of the pathogen, it is likely that pathogen-containing vacuoles are themselves targeted by the host. Indeed interferon-inducible GTPases target pathogen-containing vacuoles to inhibit pathogen replication via several mechanisms including fusion with lysosomes or autophagosomes [53]. An outcome of recruitment of interferon-inducible GTPases by the *Toxoplasma gondii* parasitophorous vacuole is membrane disruption [54]. It remains to be seen whether this is also occurs during infection with bacterial pathogens.

Acknowledgments

We thank Dr. Sina Mohammadi for reviewing the manuscript. EAC was supported by HHMI, and RRI is an Investigator of HHMI.

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Highlights

- Many pathogens direct the formation of a specialized vacuolar niche
- Vacuole assembly and trafficking involve modulation of host proteins and lipids
- Disruption of vacuole assembly often leads to loss of vacuole integrity
- Aberrant mechanical forces may cause vacuole rupture

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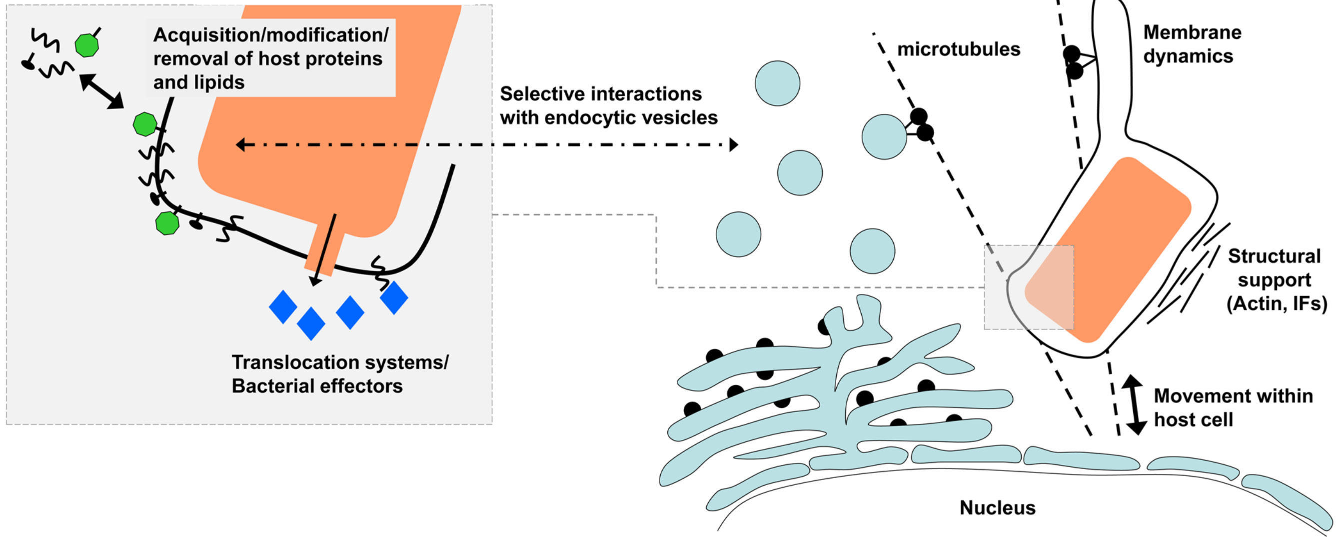

Fig 1. Maintenance of vacuole stability

Vacuolar pathogens translocate effector proteins into the host cell to modify the vacuolar niche and subvert host cell pathways. Effectors direct the acquisition, modification and removal of host proteins and lipids of the vacuole membrane. Changes in lipid and protein composition alter the identity of the vacuole leading to selective interactions with endocytic vesicles. Interactions with the host cytoskeleton determine the subcellular localization of the vacuole, provide structural support and may facilitate vesicle recruitment. The dynamics of the vacuole membrane are affected by changes in the membrane lipids and interactions with microtubules and microtubule motors. Vacuole integrity is closely linked to vacuole biogenesis and subversion of these events frequently leads to vacuole rupture. In addition, the vacuole membrane may be compromised by the specialized secretion systems used to translocate effector proteins into the host cell.

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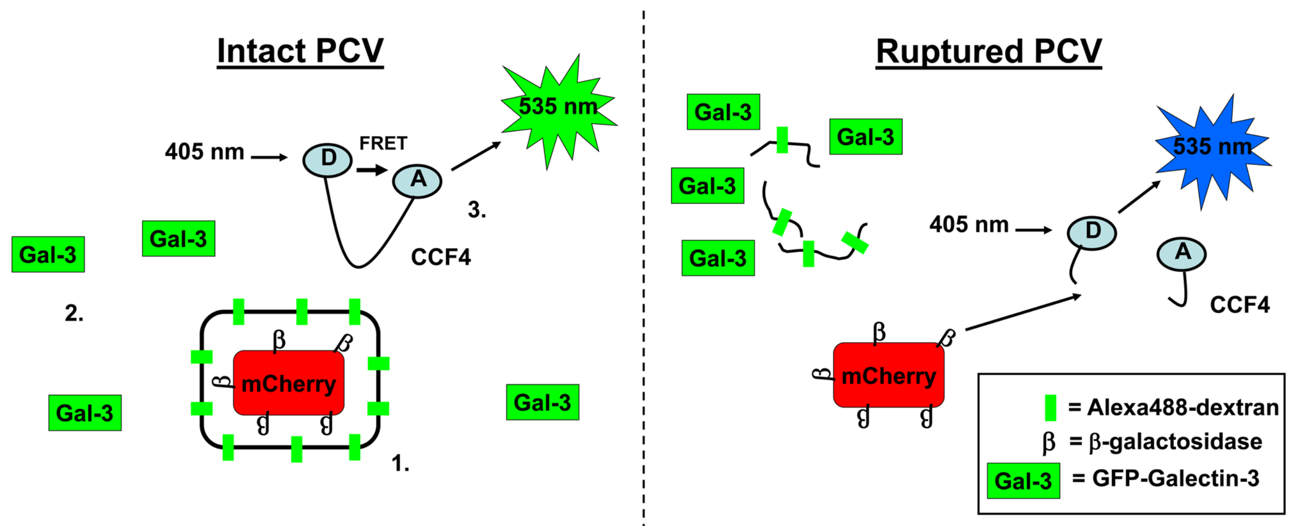

Fig 2. Detection of vacuole rupture

Three recent techniques allow the detection of vacuole rupture in real time on a single cell basis. (1) Alexa488-Dextran marks the vacuole membrane and colocalization with bacteria expressing mCherry can be detected by fluorescence microscopy. (2) GFP-tagged Galectin-3 is specifically recruited to the remnants of ruptured vacuoles. (3) Following vacuole disruption, surface-expressed β-lactamases can cleave cytosolic CCF4. Cleavage of CCF4 relieves the intramolecular FRET and causes a change in fluorescence emission.