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Manipulation of host membrane machinery by bacterial pathogens

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Abstract

Subversion of host membrane machinery is important for the uptake, survival, and replication of bacterial pathogens. Understanding how pathogens manipulate host membrane transport pathways provides mechanistic insight into how infection occurs and is also revealing new information on biochemical processes involved in the functioning of eukaryotic cells. In this review we discuss several of the canonical host pathways targeted by bacterial pathogens and emerging areas of investigation in this exciting field.

Introduction

Pathogens have developed an amazingly wide variety of mechanisms to hijack the host cell membrane machinery to their own profit during infection. Conversely the cell also uses membrane trafficking to counteract the infection. The plasma membrane and more specifically its external face are evidently the first targets of pathogenic bacteria during infection but membranes of intracellular organelles such as endosomes, lysosomes and the intracellular face of the plasma membrane are also targeted by bacteria in various tissues and organs. Strikingly, not only the membrane proteins, but also phospholipids and various other components of the membrane, such as sugars, act as pathogen receptors/ligands/targets [1-3]. In most cases these interactions participate efficiently in the infection process, in other cases, they mostly act as danger signals and initiate cellular innate immune responses. In this review, we will focus on bacteria, for which detailed studies have been conducted, and thus appear as archetypes of cellular subversion for either extracellular colonization or intracellular parasitism. Among extracellular bacteria, we will discuss enteropathogenic *Escherichia coli* (EPEC), which adhere and colonize the mammalian intestine, and several streptococcal species (e.g. *S. pyogenes*, *S. pneumoniae*), which produce a variety of toxins, including pore-forming toxins critical for mucosal or deeper organs infections. Intracellular pathogens that will be covered include those that are passively engulfed by phagocytic cells but have developed various ways to survive in the aggressive environment of the phagosome (*Legionella pneumophila*, *Coxiella burnetii*, *Mycobacterium tuberculosis*, all three capable of replicating in alveolar macrophages), and

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bacteria generally named “invasive bacteria” that are able to induce their own phagocytosis in non-phagocytic cells, with *Salmonella*, *Shigella* and *Listeria* all representing paradigms of enteroinvasive pathogens (Figure 1 and Figure 2), and the last two being able to escape from the internalization vacuole and spread intra- and inter-cellularly using a now well characterized actin-based motility process (Figure 1).

Targeting the plasma membrane for adherence

Bacteria may adhere to cell membranes through protein-protein interactions mediated by bacterial adhesins present either on the bacterial surface, such the M protein of streptococci, or at the tip of bacterial appendages such as pili [3]. Interestingly, pili were up to recently considered to be uniquely present in gram negative bacteria (bacteria with an external membrane in addition to the internal membrane) such as *E. coli*, *Neisseria* species or *Helicobacter*. However, they have now been discovered in a variety of gram positive bacteria (bacteria with a single membrane and a thick cell wall), in particular streptococci and pneumococci, and thus appear as a common theme in bacterial adhesion [4]. The most sophisticated pili are the retractive pili, which after the initial contact between the bacteria and the host cell, retract to reduce the distance between the two partners and favor cross talk. In many cases, the bacterial adhesin interacts with a sugar moiety of a host cell surface protein.

A unique adhesion system is that of EPEC. EPEC are able to inject into mammalian cells via a type three secretion system (TTSS), a transmembrane molecule Tir which then acts as a receptor for the bacterial surface protein intimin [5]. Tir has a cytoplasmic region that becomes tyrosine phosphorylated and recruits cytoskeleton elements that stabilize the interaction. Then, a well orchestrated signaling cascade takes place which leads to the formation of actin-rich pedestals that completely disrupt the intestine’s architecture and its specific properties [6-8]. Convergent results indicate that one tyrosine residue Tyr 474 is rapidly phosphorylated by the Src family kinase c-Fyn, in cholesterol rich detergent membrane microdomains (DRMs), which are rich in PIP2 early during infection [7]. This results in recruitment of Nck, N-WASP and Arp2/3. A second phosphorylated tyrosine residue, Y454, binds to PI3 Kinase and may generate PIP3 [7,8]. Interestingly, as shown recently, two other tyrosine residues, Y483 and Y511, are important for normal pedestal formation, but are not subject to host phosphorylation. These residues are critical for the recruitment of a inositol-5-phosphatase SHIP2 which is known to be able to dephosphorylate PIP3 into PIP2 [6]. Together this work highlights that both actin and membrane reorganization are necessary for pedestal formation and maturation. The capacity of the Tir cytoplasmic domain to interact with the cytoskeleton is in fact shared by a variety of cellular receptors that are used by bacteria for tight adherence to cells. Integrins, in particular, are used by many bacteria for host interactions either directly or through interaction of a bridging molecule such as fibronectin. Many bacteria have indeed virulence surface proteins that recruit fibronectin to adhere to host cells by binding to integrins [9].

Bacterial entry into cells

During bacterial entry, two morphologically distinct mechanisms characterized by two different types of membrane rearrangements are used: the zipper mechanism and the trigger mechanism.

In the first case, the membrane tightly enwraps the bacterium. This results from an initial interaction between a bacterial surface protein and its receptor on the host cell and triggers a cascade of signals including protein phosphorylations, ubiquitinations and phospholipid modifications that culminate in the formation of an internalization vacuole (Figure 1). A subset of bacterial pathogens can escape from the internalization vacuole to reach the cytosol and replicate therein. In this category, one finds the bacterium *Listeria monocytogenes*, which has evolved two main invasion proteins called internalin and InlB [10,11]. Internalin interacts with

the cell adhesion molecule E-cadherin and InlB mainly interacts with Met, the hepatocyte growth factor receptor. E-cadherin undergoes successive posttranslational modifications (phosphorylation, ubiquitination) during entry [12]. The same holds true for Met [13]. Quite interestingly, the presence of DRMs in the plasma membrane is critical for the entry process (Figure 1) [14]. However, while DRMs and components of DRMs (e.g. caveolin) are critical for the initial clustering of E-cadherin required for bacterial entry into cells that express E cadherin, the role of DRMs in the InlB-mediated entry occurs at a later stage, *i. e.* the Rac activation step downstream of PI3 kinase activation [15]. Indeed disruption of DRMs, with for example β methyl cyclodextrin, does not affect the normal activation of PI-3 kinase that follows Met activation by InlB. However, the Rac activation that also normally follows Met activation does not take place, probably due to an impaired distribution of the newly formed PIP3 molecules in the plasma membrane. The role of DRMs and lipid rafts in bacterial entry has been demonstrated for several other pathogens, including *R. conorii*, [16], *Brucella abortus* [17] and specific strains of *Chlamydia* [18].

The study of *Listeria* entry has recently led to the shift in paradigm that clathrin is not only used for internalization of small particles but also for that of large objects such as bacteria [13,19]. The clathrin-mediated endocytosis is indeed absolutely critical for the entry of *Listeria* and a series of experiments show that at least for the InlB-mediated entry, clathrin recruitment precedes actin rearrangements and is required for bacterial entry [19]. How clathrin is recruited is still a matter of debate. The classical AP2 adaptor protein is dispensable. In contrast, AP1 a ligand of PI4P at the Golgi has been detected, albeit not systematically, at the plasma membrane at the bacterial entry site and knock down of PI4 kinase dramatically impairs entry [20]. Together, a new mechanism of internalization is emerging but how clathrin is recruited and assembled at the entry site deserves further investigation. It is clear that the dynamics of clathrin recruitment in these cases is different from the normal clathrin-mediated endocytosis and probably results from the use of flat clathrin plaques that are present in the inner face of the plasma membranes. Clathrin would act as a scaffold to orchestrate the rearrangements of actin and other cytoskeleton elements necessary for entry. Indeed, septins which are small GTPases able to form filaments and interact with actin appear as important new players in bacterial entry [21]. The other well-studied bacterium that uses the zippering mechanism for entry is *Yersinia*, which uses a cell surface molecule called invasin to interact with $\beta 1$ integrins on host cell surfaces. As for *Listeria*, both clathrin and actin are critical for entry [19]. In a spectacular example of convergent evolution it was found that the critical residues important for invasin-mediated uptake are a structural mimic of the RGD peptide involved in the interaction between fibronectin and integrins [22].

The second well-studied entry mechanism is the trigger mechanism that is characterized by the apparition of large membrane ruffles at the bacterial entry site. These structures finally engulf the bacterium in a sort of macropinocytosis (Figure 1). The paradigms of the trigger mechanism are *Salmonella* and *Shigella* [2,3]. This mechanism is bacterially induced *i. e.* bacteria inject directly in the host cell cytosol via a type three secretion system, a series of effectors which trigger a variety of different intracellular signaling cascades necessary for entry. Some of these effectors activate or control cytoskeletal regulators such as small G proteins. For example, the *Salmonella* effectors SopE and SopE2 mimic endogenous GEFs. Other act as GAPs *e.g.* the Spt effector of *Salmonella*, which has in addition a tyrosine phosphatase domain. Interestingly, other bacterial effectors, such as *Salmonella* SopB or *Shigella* IpgD, are phospholipid-modifying enzymes, resulting in membrane composition modification necessary for bacterial engulfment. The delivery of effectors is stringently controlled [23,24] and the half-lives of translocated effectors in the host cytosol are also regulated [25], both of which are mechanisms designed to temporally regulate the activities of these bacterial protein during infection.

Intravacuolar life

A pathogenic microbe that has been internalized by a eukaryotic host cell must either avoid delivery to a degradative lysosomal compartment or develop strategies for survival within this degradative organelle. Although somewhat simplistic, pathogens that establish residence in a non-degradative vacuole can be divided into two different categories. There are pathogens that reside in vacuoles that maintain properties of either early-endosomal or late-endosomal organelles, and pathogens that divert from the endocytic pathway to establish a vacuole that has unique properties (Figure 2).

Examining the association of Rab family GTPases on vacuoles containing bacterial pathogens has facilitated the determination of which host membrane transport pathways are utilized during infection [26]. Representative pathogens residing in the endocytic pathway are often defined by the association of Rab5 (*M. tuberculosis*) or Rab7 (*Salmonella enterica*), whereas, those that interact with the secretory pathway are typically defined by the appearance of Rab1 (*L. pneumophila*), Rab2 (*Brucella abortus*) [27], and Rab6 (*Chlamydia trachomatis*) [28] on the vacuole membrane (Figure 2). Although localization of signature Rab proteins is useful for the characterization of host pathways that provide membranes to the pathogen-occupied vacuole, the ability of pathogens to subvert host functions results in atypical combinations of Rab proteins on the vacuole membrane, which make these organelles unique. This is illustrated by a “Rabomic” analysis in which the association of individual fluorescently-tagged Rab proteins revealed a novel pattern of Rab association on vacuoles containing virulent *S. enterica* over time compared to vacuoles containing an avirulent mutant of *S. enterica* [29].

Coxiella burnetii provides an interesting example of a pathogen that has evolved to survive in a lysosome-derived vacuole [30]. This bacterium requires an acidic lysosomal environment for intracellular replication (Figure 2). Remarkably, cultivation of *C. burnetii* in defined medium required both acidic pH conditions and low levels of oxygen [31], suggesting that the lysosome is a hypoxic environment. *C. burnetii* is not a passive occupant of the lysosome. Once *C. burnetii* initiates replication, the fusion properties of the lysosome in which it resides are altered and this vacuole fuses with other lysosomes in the cell creating a large vacuole that occupies much of the cytosol. The host GTPase Rab7 and the autophagy pathway has been implicated in the biogenesis of the vacuole containing *C. burnetii* [32], and proteins delivered into the host cell cytoplasmic compartment by the Dot/Icm-related type IV secretion system have been suggested to regulate the fusogenic properties of the vacuole [33,34]. Identification and biochemical characterization of these effectors should provide mechanistic insight into the interesting phenomenon of hijacking of the host lysosome by *C. burnetii*.

Salmonella enterica serovar Typhimurium-containing vacuoles have properties of early and late endosomes, and studies on biogenesis of these organelles has revealed a variety of strategies for bacterial manipulation of membrane transport processes (Figure 2). The *S. enterica* effector protein SopB is a phosphoinositide phosphatase that promotes high phosphatidylinositol-three-phosphate (PI3P) levels on the vacuole containing this pathogen, which maintains Rab5 localization to arrest vacuole maturation [35]. Effector proteins delivered by *S. enterica* assist in vacuole maintenance through interactions with host factors that affect the dynamics of the motor protein kinesin [36,37] and tether the vacuole to membranes of the Golgi apparatus [38]. Disrupting the balance of *S. enterica* interactions with the host trafficking machinery by elimination of a single effector protein, such as the SifA protein, has the unintended consequence of vacuole disruption. These *S. enterica* mutant bacteria are recognized by the host ubiquitination machinery in the host cytosol and targeted for destruction by the autophagy pathway [39,40].

Interestingly, studies examining the vacuole containing *Chlamydia* have implicated elements of the cytoskeleton as also playing an important role in vacuole stabilization [41]. Thus, maintaining a functional vacuole that supports bacterial replication requires a balanced interaction with multiple host pathways that control the dynamics of vesicular transport.

Studies on a model intracellular pathogen, *Legionella pneumophila*, are revealing novel mechanisms by which bacteria can subvert host factors involved in the transport of secretory vesicles to generate a vacuole derived from the host endoplasmic reticulum. Using a type IV secretion system called Dot/Icm [42,43] this intracellular pathogen prevents fusion of the vacuole in which it resides with endosomal compartments and recruits vesicles derived from the ER [44]. Fusion of the plasma membrane-derived vacuole in which *L. pneumophila* resides initially with ER-derived vesicles is promoted in part by the non-canonical pairing of a plasma membrane t-SNARE complex consisting of a syntaxin molecule and SNAP23 with the ER-localized v-SNARE protein Sec22b [45]. Surprisingly, this pathogen-driven event could represent a tightly regulated process that is also used by phagocytic cells [46] and could explain studies that have demonstrated the delivery of resident ER proteins to phagosomes [47]. Recent data suggests that in addition to utilizing host SNAREs, *L. pneumophila*, and other vacuolar pathogens such as *Chlamydia*, may encode SNARE mimics that directly modulate membrane transport [48,49].

Host GTPases that regulate membrane transport have emerged as direct targets for several *L. pneumophila* effector proteins translocated into host cells by the Dot/Icm system [44]. The effector protein RalF is a bacterial Sec7 domain-containing protein that stimulates the activation the ARF1 family of GTPases on the vacuole containing *L. pneumophila*. Similar to studies on eukaryotic Sec7 protein family members [50], structural and biochemical analysis suggest that RalF has a capping domain that makes contacts with residues in the Sec7 domain critical for ARF recognition [51]. These structural studies indicate that this bacterial ARF GEF is autoinhibited and robust enzymatic activity would require a conformational change in the capping domain. Determining the *in vivo* signals that mediate RalF activation should provide novel insight into the cellular processes by which ARF GEF activities are regulated.

The GTPase Rab1 is targeted by multiple *L. pneumophila* effector proteins (Figure 2). DrrA (also called SidM) is a *L. pneumophila* effector that activates Rab1 by functioning as a specific GEF that can also displace Rab1-GDP bound to the chaperone protein Rab GDP dissociation inhibitor (GDI). Recent structural studies demonstrate that DrrA interacts specifically with the Rab1 switch regions to remove GDP and promote GTP binding [52-54]. The affinity of DrrA for GDP-bound Rab1 is higher than the interaction between Rab1-GDP and the GDI protein, a property that facilitates the displacement of GDI during the exchange reaction [52-54]. Unlike RalF, which is similar in sequence and structure to eukaryotic Sec7 proteins, the DrrA protein does not share sequence or structural homology with eukaryotic Rab GEFs, providing evidence that divergent evolutionary processes led to the emergence of this bacterial activator of Rab1. There is evidence that the *L. pneumophila* effector protein LidA interacts with Rab1 on the vacuole membrane to facilitate the tethering of ER-derived vesicles that function in remodeling of this organelle [55]. Lastly, the effector protein LepB has been shown to function as a Rab1 GTPase activating protein, which would presumably function in the down-regulation of Rab1 activity on the vacuole after successful maturation [56]. These data reveal that the effector repertoire of *L. pneumophila* includes a complete array of proteins that can control Rab1 activity on the vacuole.

Some interesting mechanisms have been uncovered in studies on the spatial and temporal control of *L. pneumophila* effector proteins on the vacuole membrane. Ubiquitination by the host machinery has been proposed to play an important role in modulation of host proteins and effector turnover during *L. pneumophila* infection [57-61]. This coupled with differential

expression of effector protein during infection facilitates the temporal patterns of effectors displayed on this organelle [62]. In addition, several *L. pneumophila* effectors sense phosphoinositide signatures on the vacuole membrane [63]. Biochemical studies have shown that the effectors SidC and DrrA interact with phosphatidylinositol-four phosphate (PI4P) [63,64]. A predicted PI4P-binding region near the C-terminus of DrrA was recently revealed by structural analysis [52]. Interestingly, when a truncation derivative containing only this C-terminal region in DrrA is expressed in mammalian cells it localizes to the plasma membrane [65], where the concentration of lipids displaying PI4P is low, and not to the PI4P-rich Golgi apparatus, suggesting that determinants in addition to PI4P are important for spatial localization of DrrA. Thus, these bacterial effectors have evolved a variety of biochemical mechanisms to sense changes to the vacuole membrane over time and these cues are important for regulating their activities.

Escape from the vacuole and evasion from autophagic recognition

Some bacteria succeed to rapidly escape from the phagosome and establish their replication niche in the cytosol (Figure 1). This is the case for *Listeria*, *Shigella*, *Rickettsia*, and also *Francisella*. Replication in the cytosol implies escape from autophagy, a highly conserved process during which a double layered isolation membrane wraps around undesirable cytoplasmic constituents and degrades them by fusion with a lysosome. Autophagy is thus a key innate defense for the possible elimination of invading microbes [66].

The best characterized bacterial factor allowing escape from the phagosome is undoubtedly listeriolysin O (LLO), the cholesterol dependent pore-forming toxin produced by *Listeria*, whose precise mechanism of action is unknown [67]. Listeriolysin O oligomerizes in cholesterol-containing membranes and creates large pores which allow calcium influx. Interestingly, LLO is mostly active at acidic pH, the pH of the vacuole. Recent data indicate that LLO full activity requires activation by a host factor named GILT, a thiol reductase [68]. It is generally accepted that internalized bacteria then either escape from the vacuole or are killed following fusion of the vacuole with lysosomes. However, as reported bacteria may *in vivo* in SCID mice and also *in vitro* reside in large vacuoles called SLAPs for spacious *Listeria*-containing phagosomes. These vacuoles are non acidic and bacteria replicate therein, albeit slowly. A LLO mutant was impaired in phagosome escape but replicated even more slowly suggesting that LLO may promote replication in SLAPS [69]. Once the bacterium is released in the cytosol, LLO is rapidly inactivated by denaturation, ubiquitination and degradation. This degradation prevents LLO from acting as a general cytotoxin that kills the host cells, which would limit intracellular infection. Together, LLO appears as a major player in the intracellular life of *Listeria*. In particular, it also contributes to the early escape from the autophagy machinery, the major actor however being the surface protein ActA [70].

In the case of *Shigella*, the main factor involved in vacuolar escape is a type three effector named IpaB (Figure 1), which is also involved in entry. Other factors involved in vacuolar escape include IpaC and IpaH7.8. As recently shown, upon vacuolar membrane lysis, proteins associated with membrane remnants are polyubiquitinated, recruit the autophagy marker LC3 and adaptor p62, and are targeted to autophagic degradation [40,71]. Further, inflammasome components and caspase-1 are localized to these membranes. Thus the host membranes remnants after vacuole lysis act as danger signals for the host cell, an unsuspected and new concept in the field.

Once in the cytosol, some bacteria e.g. *Shigella*, *L. monocytogenes* and some *Rickettsia* succeed to circumvent autophagy (Figure 1), replicate efficiently and in addition use actin polymerization to promote their intra- and inter-cellular dissemination [72]. Strikingly, convergent results point to a close link between actin-based motility and autophagy. The

proteins that allow actin-based motility are now well known [2]. These are IcsA/VirG in the case of *Shigella* and ActA in the case of *Listeria*. IcsA/VirG recruits N-WASP which then recruits and activates the Arp2/3 complex and produces the actin comet tails. ActA directly recruits and activates the Arp2/3 complex. How *Shigella* and *Listeria* bacteria escape autophagy has been recently well characterized. Interestingly, *Shigella* escapes autophagy by secreting a type three secretion effector protein called IcsB, which interacts with IcsA and prevents the IcsA protein to interact with the autophagy protein Atg5 [73]. An IcsB mutant is much more efficiently targeted to autophagy than wild type bacteria. In the case of *Listeria*, escape from autophagy is mediated by ActA, which disguises and protects the bacterium from ubiquitylation, p62 and LC3 recruitment and subsequent autophagy [74]. The ability of ActA to prevent autophagic recognition is independent of the ability to mediate bacterial motility. It is thus quite striking that proteins originally considered as involved uniquely in actin-based motility are now both connected with autophagy recognition. Nevertheless, how autophagic membranes are recruited around bacteria remain elusive although as said above, an initial recruitment of ubiquitinated proteins now appears as an essential initial step for destruction by the autophagy machinery [39,40].

Concluding remarks

Co-evolution of prokaryotic organisms with eukaryotic cells has provided the selective pressure for the emergence of microbial factors that manipulate host functions. As a result, these simple microbes have developed sophisticated strategies to manipulate host membrane transport. The identification of microbial proteins with biochemical activities directed against components of the host membrane transport machinery not only reveal infection strategies, but analysis of these microbial factors provides unique insight into how host targets function in specific membrane transport pathways and how these pathways are regulated in the cell. Thus, studies aimed at elucidating the molecular mechanisms of microbial pathogenesis are critical for understanding processes of fundamental importance in cell biology, human disease, and host immunity.

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Figure 1. Interactions with membranes and strategies used by the intracytosolic pathogens *Listeria monocytogenes*, *Shigella flexneri*, and *Rickettsia conorii*

Depicted are the different stages involved in delivering the indicated pathogens into the host cytosol. They include entry, internalization within a vacuole, escape from the vacuole, actin-based motility and escape from autophagy. The essential bacterial products involved in each step and the cellular components or organelles are indicated (see also text).

Figure 2. Membrane transport pathways used by intravacuolar pathogens

Depicted are several bacterial pathogens that replicate inside specialized vacuoles inside of eukaryotic host cells. Indicated are the Rab proteins and other host proteins that are found on these unique pathogen-occupied organelles. Interactions between the bacterial vacuoles and host early endosomes (EE), late endosomes (LE), lysosomes, and the endoplasmic reticulum (ER) are indicated. The vacuolar association of host proteins including glucose 6-phosphatase (G6-Phase), early endosomal antigen-1 (EEA1), protein disulfide isomerase (PDI), are indicated.