Chapter 10

Pathogen and Toxin Entry – How Pathogens and Toxins Induce and Harness Endocytotic Mechanisms

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Additional information is available at the end of the chapter

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1. Introduction

Humans have been exposed to a plethora of pathogens (bacteria, viruses) ever since. Infectious diseases are among the leading causes of death worldwide. For example, in 2011, 1.34 million people died of tuberculosis, which is caused by an infection with *Mycobacterium tuberculosis*. Even more died of an infection by the human immunodeficiency virus (HIV; 1.78 million) or lower respiratory tract infection (3.46 million) [1]. In addition, recurring pandemic outbreaks of the influenza A virus, as in 2009, or an epidemic outbreak of enterohemorrhagic *E. coli* (EHEC) in Germany in 2011, show quite plainly that pathogens in the 21th century still are a severe health problem, not only in developing countries.

During evolution, defence mechanisms have been developed by the host to counter pathogens, which in turn needed to respond with new strategies to gain entry into host cells. As a consequence, a wide variety of invasion mechanisms have evolved, of which only a few have been characterised in molecular detail to date.

In this chapter, we describe the different invasion strategies of bacteria, viruses and toxins by illustrating the mechanisms using prominent examples. Rather than relying passively on cellular mechanisms of their hosts, diverse pathogens and toxins actively induce the first steps of their uptake into a wide range of target cells. In most cases, the pathogen plays a key role in subverting the cellular machinery to stimulate actin re-arrangements, which facilitates the invasion process. As an example, recent progress in our understanding of the molecular mechanism of lipid-mediated endocytosis of carbohydrate-binding viruses and toxins is presented. In particular, we highlight the critical role of lipid species underlying these processes.

Bacteria invade cells as a means to escape host immune responses. Once inside a hijacked cell, the pathogen is protected against active factors of the immune system (e. g. complement factors, antibodies) and is conveniently provided with nutrients. In addition, viruses critically depend on the cellular machinery of host cells for their replication.

The initial step in the cellular uptake process of diverse pathogens and toxins is characterised by the binding to carbohydrate moieties exposed by a lipid or a protein in the plasma membrane of target cells. For example: cholera toxin binds with its B-subunit to the ganglioside GM1 in intestinal cells, the opportunistic human pathogen *Pseudomonas aeruginosa* attaches to respiratory cells by binding to asialo-GM1 and asialo-GM2 through type IV pili [2, 3], and the influenza A virus initiates its uptake by binding to sialic acids in the host cell membrane [4]. Conventionally considered as adhesion receptors for toxins, viruses and bacteria, recent data indicate that glycosphingolipids are also crucial parameters for the self-induced endocytosis of toxins and viruses [5, 6].

Glycosphingolipids, such as Gb3 or GM1, are enriched in the external leaflet of the plasma membrane and comprise a glycan and a ceramide lipid moiety of sphingosine (a long-chain amino alcohol) linked to a fatty acid [7]. The structure of the ceramide moiety of glycosphingolipids is highly divers and varies in length, saturation degree and hydroxylation. However, glycosphingolipids are traditionally classified by the structure of their glycans.

A second important type of lipids, which is critically important for the uptake of pathogens, is phosphatidylinositol-phosphate (PIP). PIPs are essential components of cell membranes implicated in a variety of signalling events. They are glycero-phospholipids with a negatively charged myo-inositol head group, which can be phosphorylated at different OH-positions of the inositol ring (D1-D5) [8]. More than 50 enzymes have been identified to combinational phosphorylate and dephosphorylate the inositol ring [9].

PIPs are signalling molecules rather than structural components of the plasma membrane, considered to be involved in dynamic cellular processes like (plasma) membrane dynamics, vesicle trafficking and actin polymerisation [8]. Probably this is the reason why many invasive bacteria as well as viral pathogens hijack these lipids to manipulate the plasma membrane in order to ensure their proper uptake into host cells.

One strategy by which invasive bacteria manipulate the PIP metabolism is the translocation of effector proteins, which act as phosphatidylinositol phosphatases (e.g. IpgD of *Shigella* and SopB/SigD of *Salmonella*, discussed below). A second option to interfere with the PIP metabolism is the engagement of specific host cell receptors.

The invasion process of pathogens is highly complex because it involves a specific spatiotemporally regulated interplay of different subsets of host cell and pathogenic factors. In addition, the composition and architecture of biological membranes is extensive. To understand how individual factors contribute to the entry process, less complex and easy-to-manipulate synthetic systems are needed. Artificial membrane systems gain more and more in importance as simpler and controllable systems to reconstitute and study endocytotic processes (see “EXCURSUS” box).

EXCURSUS: Artificial Membrane systems – powerful tools to study endocytotic membrane processes

Liposomes (also called vesicles) are more and more used as simple synthetic models for biological membranes. They represent a sphere of a cytosol-free unilamellar lipid bilayer and consist of a defined lipid composition resembling e.g. that of the plasma membrane. Manipulations at the outside can be conducted with ease. Giant unilamellar liposomes (GUVs) with diameters between 1 and 50 μm can be obtained by swelling of a phospholipid bilayer in water within an electrical field [10] (Figure 1). Because of its simplicity compared to native cells, this type of liposomes has been used recently to identify the initial steps of the cellular uptake of Shiga toxin [5, 11].

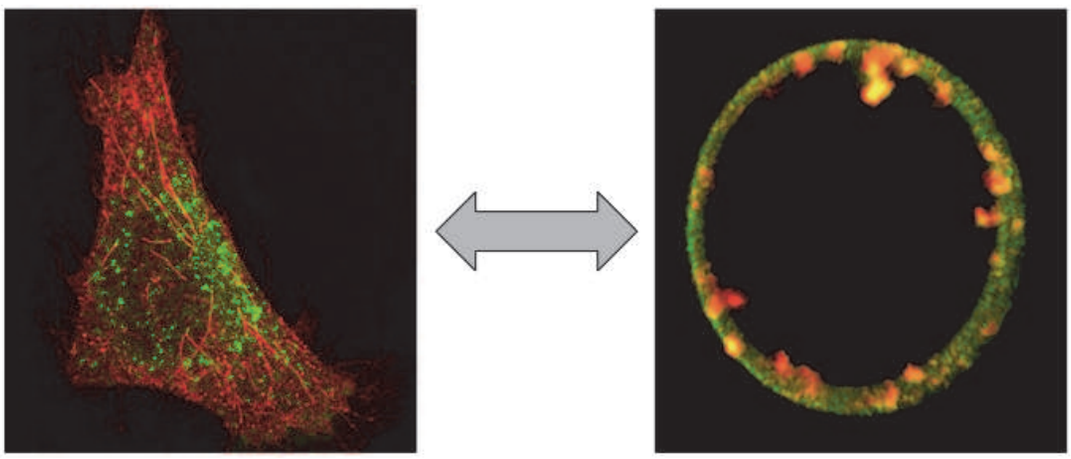

Figure 1. Giant unilamellar vesicles (GUVs) as a minimal membrane system to investigate endocytotic processes. Shiga toxin-induced tubular membrane invaginations on a HeLa cell (left image; in red colour) can be reconstituted on GUVs in the complete absence of cellular energy and cytosolic proteins (right image; red colour). Systems of different complexity are helpful and complementary to identify the molecular mechanisms of cellular processes.

The electroformation technique to produce liposomes is simple and rapid, but less suitable for embedding cytosolic proteins into the lumen of the lipid bilayer. For instance, the examination of the scission process of Shiga toxin-induced membrane invaginations requires the addition of the protein machinery at the internal (cytosolic) side of the membrane. For this, proteins could be microinjected into GUVs, or GUVs could be grown in the presence of protein machinery in the buffer solution. While the former method is very time-consuming and may easily provoke vesicle rupture, the latter is inefficient, not really well controlled, and proteins might be denatured (due to the application of an electric field during liposome formation). Moreover, in this setup, it is practically impossible to apply acute changes in buffer conditions (e.g. addition of ATP).

A model membrane system that better responds to the challenge of manipulating the protein machinery at both sides of the membrane are liposomes produced by the inverse emulsion technique [12] which allows for the use of two different buffers inside and outside. This technique has been used, for example, to reconstitute nucleation and

assembly of cortical actin at the inner side of a liposome. In principle, an inverted phospholipid micelle containing the protein(s) of interest is sedimented through a phospholipid monolayer at the phase boundary of oil and an aqueous buffer solution. By the addition of a pore forming α-hemolysin, ATP and ions can pass the liposome membrane to induce actin polymerization within the liposome [13]. In addition, this technique has already been successfully used to study the actin-driven scission of endocytotic membrane invaginations [11].

The inverse emulsion technique has several advantages compared to the now classical electroformation technique, apart from the preservation of protein functionality. First, it allows for the incorporation of proteins in very small volumes (a few microliters). Second, it allows for the use of two different buffers inside and outside of the vesicle (providing that the osmotic pressure is kept constant). Third, since the two membrane monolayers are prepared independently of each other, lipid asymmetry within the lipid bilayer can be taken into account.

However, the inverse emulsion technique still has some major limitations. Most notably, the inclusion of proteins within the liposomal lumen remains tedious as liposomes must be generated in the presence of the protein mix. Furthermore, the high diffusion mobility of small liposomes makes observation by microscopy cumbersome and prevents from following the same object over periods longer than a few seconds.

Already established for impedance spectroscopy measurements and single ion channel recordings [14, 15], pore-suspending membranes based on highly ordered pore arrays might represent marvellous tools to study endocytotic processes. This hybrid membrane system combines the advantages of freestanding and solid supported lipid membranes. While part of the lipid bilayer is anchored to the surface of the porous matrix and resembles a solid supported membrane, the pore suspending parts can be viewed as freestanding lipid membranes. Highly ordered alumina and silicon pore arrays with pore sizes in the nano- and micrometer range can be chosen as supports for lipid bilayer immobilization.

The porous support can either be covered by a synthetic lipid bilayer or native plasma membrane sheets, depending on the application. The advantage of this technique is that both sides of the membrane are freely accessible. This is particularly interesting, when native plasma membrane sheets are analyzed, considering their asymmetric composition. In general, two different methods exist to produce native plasma membrane sheets: the rip off and the lysis-squirting technique. In both cases adherent cells are first grown on the porous membranes. For the rip off technique, a chip with a nanostructured, porous surface is pressed on top of cultured, adherent cells to form direct molecular contacts with the cells. The chip is then removed, thereby peeling off the upper plasma membranes of the adhering cells, which are now located on the chip as supported membranes [16]. With the lysis-squirting technique, adherent cells are incubated in hypotonic buffer for a few minutes before being squirted with the same buffer. The basal part of the membrane remains attached to the support and can be studied further [17]. The pore-suspending membranes can be analyzed by e.g. atomic force microscopy, fluorescence microscopy or electrophysiological measurements.

Besides already established concepts of pathogen and toxin uptake, we will discuss the novel concept of glycosphingolipid-driven uptake starting at a low complexity level with toxins. With an increase in size and complexity of the objects of interest, we will continue our reflection about viruses and bacteria.

2. Toxins

The pathology of infections caused by different species of *Shigella*, enterohemorrhagic strains of *E. coli* (EHEC) and *Vibrio cholerae* is closely linked to the action of their glycosphingolipid-binding toxins: Shiga toxin (Stx), Shiga-like toxins (SLTs) and cholera toxin (Ctx), respectively. Commonly these toxins lead to severe diarrhoea, accompanied by hemorrhagic lesions in the intestine in the case of infections by EHEC and some strains of *Shigella*.

Because of their structural organisation with a monomeric A-subunit and a pentameric B-subunit, Stx, Ctx and SLTs belong to a group of toxins referred to as AB_5 toxins. The A-subunit consists of the enzymatic part of the toxin, which modifies intracellular targets: The RNA N-glycosidase activity of Stx, for example, targets cellular 28S rRNA, rendering ribosomes inactive for protein synthesis [18] and the ADP-ribosyltranferase activity of Ctx targets heterotrimeric G-proteins, thereby activating adenylate cyclases in mucosal epithelial cells of the small intestine [19]. The A-subunit is non-covalently linked to the B-subunit, which binds to host cell glycosphingolipids [20]. To exert their catalytic functions in the cytosol, the toxins have to be endocytosed and the A-subunits translocated into the cytosol. We will also introduce the plant toxin ricin, which gained notoriety due to its misuse as a bioterrorism weapon.

2.1. Shiga toxins and other bacterial toxins

Shigella dysenteriae and certain other *Shigella* strains secrete two types of enterotoxins: the so-called Shiga toxins I and II (Stx1 and Stx2; or verotoxins). These toxins are functionally and structurally related to the Shiga-like toxins I and II (SLTs), which are produced by enterohemorrhagic *Escherichia coli* (EHEC) strains [21]. In humans, these toxins cause serious complications in the gastrointestinal tract, including haemolytic colitis, which may (especially in children and elderly people) further progress to hemolytic-uremic syndrome (HUS) and severe complications of the central nervous system [22, 23]. Most recently, more than 50 patients in Germany died of EHEC infections [24-26].

While the sequence homology between Stx1 and Stx2 is only modest, *S. dysenteriae´*s Shiga toxin I and *E. coli´s* Shiga-like toxin I are 99% identical [27]. Shiga toxins are composed of a catalytic active A-subunit (StxA) of 32 kDa, which is non-covalently associated to the receptor-binding B-subunit pentamer (StxB; molecular mass of 5 x 7.7 kDa) [28, 29]. Despite the modest sequence homology (only 56% of the amino acid sequence), the B-subunits of Stx1 and Stx2 form a similarly structured homo-pentamer and bind to the same cellular receptor, the neutral glycosphingolipid globotriaosylceramide (Gb3, also known as CD77 or P^k blood group antigen) [30].

Other bacterial toxins, which use glycosphingolipids as their cellular receptors, include the GM1-binding cholera toxin (Ctx) of *Vibrio cholerae*, which is the causative agent of cholera [31], the respectively GM1 and GD1a-binding Heat-labile enterotoxins 1 and IIb of certain *E.coli* strains [32], Tetanus neurotoxin of *Clostridium tetani* and Botulinum toxin of *Clostridium botulinum*.

Crystal structures of Shiga toxins, cholera toxin, and Heat-labile enterotoxins revealed that the B-subunits of all these toxins fold into a doughnut-shaped pentamer and are of remarkable resemblance, although no amino acid sequence homology exists. Even more striking, the GM1-binding simian virus 40 capsid protein VP1, which we will present in more detail later, shares a structurally very closely related pentameric structure with each binding pocket arranged some 30 Å apart.

The binding affinity of the B-subunit of Shiga toxin (StxB) and cholera toxin (CtxB) to individual Gb3 and GM1 molecules, respectively, is very low (in the mM range) [33, 34], but the cooperative binding of multiple lipid molecules (up to 15 Gb3 molecules in the case of StxB) markedly increases the apparent affinity of the toxin to its receptor (in the nM range) [35-37]. Mutations of individual binding-pockets in the B-subunits of Stx and Ctx dramatically decrease the ability of the toxins to strongly associate to its receptor, and consequently to efficiently infect cells [38-40].

After receptor binding, Stx is internalized by clathrin-dependent as well as clathrin-independent endocytosis [41-44]. Even though Ctx has been found to be associated with caveolae, Ctx is efficiently endocytosed into cells devoid of caveolin-1 (a critical structural component of caveolae) [45], arguing that the caveolae-mediated endocytosis is not the major internalization pathway for Ctx in certain cells.

Studies on artificial membrane systems and energy-depleted cells (i.e. under conditions where the functionality of the cytosolic machinery is efficiently impaired) showed that StxB and CtxB are able to strongly cluster their glycosphingolipid receptors in the outer membrane leaflet, provoking the inward-bending of the plasma membrane and the generation of deep tubular membrane invaginations (Figure 1 and 2) [5, 6], suggesting that the toxin is able to trigger its own internalization into host cells, independently of host cell factors. These studies have uncovered a previously unknown mechanism for generating negative membrane curvature, and they have created a new paradigm that allows the conceptualization of why endocytotic coats are not detected at many sites of clathrin-independent endocytosis.

After binding of the toxins to glycosphingolipids, the invaginated membrane remains connected to the extracellular space as long as scission does not occur. The scission process requires cellular energy in contrast to the tubule formation. As a key factor in membrane scission, the GTPase dynamin has been described [46, 47]. However, dynamin-independent and cholesterol-dependent scission can also be observed, e.g. for the clathrin-independent endocytosis of clustered glycosylphosphatidylinositol (GPI)-anchored proteins when they laterally associate with proximal transmembrane proteins

[48]. Interestingly, the dynamin-independent scission involves glycosphingolipids. In line with this, it was shown that Stx-induced membrane tubules that undergo dynamin-independent and cholesterol-dependent scission by an Arp2-based reorganization of the cortical actin covering the tubule, ultimately leading to membrane constrictions [11]. How actin is linked to the plasma membrane is not clear, but binding of Stx to Gb3 leads to a redistribution of different proteins that are involved in the regulation of the cytoskeletal organisation including ezrin, CD44, vimentin, cytokeratin, paxillin, focal adhesion kinase (FAK), alpha- and gamma-tubulins beneath the plasma membrane [49]. Interestingly, among the redistributed proteins, ezrin (one of many proteins that links the actin cytoskeleton to the plasma membrane) [50] was shown to be phosphorylated in response to the binding of Stx to Gb3, in a process that is dependent on cholesterol, phosphoinositide 3-kinase (PI3K), Src family kinases and Rho associated kinase 1 [49]. These studies hint to ezrin as a possible linker between the plasma membrane and actin on Stx-induced membrane tubules. However, how the different identified kinases regulate the Stx-induced phosphorylation of ezrin and if ezrin phosphorylation is important for tubule scission has to be investigated.

On Hela cells and other toxin-sensitive cells, Stx- and Ctx-containing plasma membrane derived vesicles localise to early endosomes from which they are transported along the retrograde pathway via the Golgi apparatus to the ER. There, the A-subunit is retro-translocated into the cytosol to inhibit protein biosynthesis [51]. The escape of Stx from the early endocytotic pathway to enter the retrograde pathway critically depends on clathrin [42], the phosphatidylinositol (4)-phosphate-binding clathrin adaptor epsinR [44] and the curvature- and cargo-recognizing retromer complex [52]. Following a model proposed by Popoff et al. [53], clathrin, which is recruited to early endosomal membranes by the PI(4)P-binding protein epsinR [44], induces membrane curvatures on early endosomes to form retrograde tubules that are processed by retromer-dependent scission [21]. The subsequent transport from early endosomes to the Golgi complex is specifically regulated by the delta isoform of the protein kinase C (PKCδ), which is activated by binding of Stx to Gb3. Inhibiton of PKCδ results in the accumulation of Stx in early endosomes, which fails to reach the Golgi complex [54]. In addition, Stx also activates spleen tyrosine kinase (Syk) upon binding to Gb3, which causes a rapid phosphorylation of the clathrin heavy chain (CHC). Prevention of CHC-phosphorylation results in an ineffective transport of Stx from the early endosome to the Golgi complex. These findings again establish clathrin as a critical regulator of the endosome-to-Golgi transport of Stx. It needs to be addressed by additional studies how PKC and Syk interact on this part of the retrograde transport of Stx.

The retrograde transport is the main route for intoxication of Stx (and other AB_5-toxins), and highly specific, protective small-molecule inhibitors of intracellular toxin transport have recently been identified [55]. Indeed, human monocyte-derived macrophages and dendritic cells are resistant to Stx intoxication, probably because StxB fails to associate with membrane microdomains and does not detectably enter the retrograde route [56].

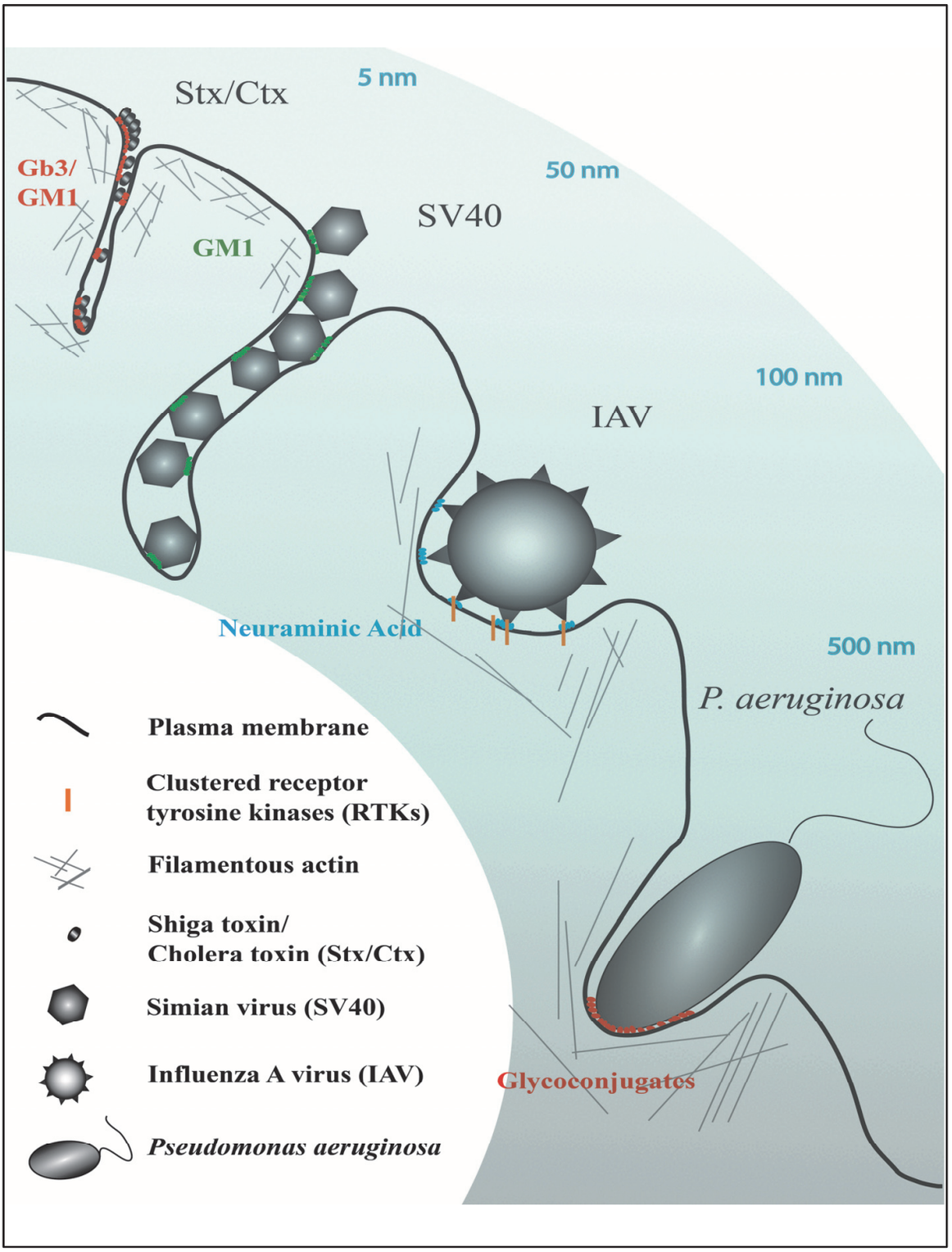

Figure 2. Schematic representation of entry strategies of some prominent pathogens and pathogenic products (toxins) and their (proposed) glycolipid receptors. As a common feature, clustering of glycolipids or glycosylated receptors has been proposed to trigger the endocytotic uptake of the toxin or the pathogen. The different elements in the scheme are not drawn to scale.

Lipid-mediated endocytosis represents a unique opportunity for several bacterial toxins to actively impose their invasion into host cells. This concept of a self-induced, lipid-mediated cellular uptake, seems not to be restricted to toxins alone. We will see in the section on viruses that a member of the *polyomaviridae* family, simian virus 40, follows this strategy in order to ensure efficient endocytosis into the host cell.

2.2. Ricin

Other lipid-binding toxins are the plant toxins ricin and the less-well characterized abrin, which are found in the seeds of castor beans (*Ricinus communis*) and of rosary pea (*Abrus precatorius*), respectively. These thermally stable proteins can be purified with ease in larger quantities, which prompted the Centers for Disease Control and Prevention (CDC) to categorise them as "Category B" agents (second highest priority) [57]. On the flipside, abrin and ricin were instrumental in Paul Ehrlich´s seminal work on the induction of immunity in mice, which have been fed with small amounts of ricin and later become immune against otherwise lethal toxin doses [58].

Despite being produced by unrelated plants and sharing only limited sequence homology, abrin and ricin exhibit a similar overall molecular structure [59, 60]: they are composed of a catalytic (toxic) A-subunit, which is covalently linked via a disulfide bond to a receptor-binding B-subunit.

The A-subunit possesses an N-glycosidase activity (identical to Shiga toxins and Shiga-like toxins) and strongly inhibits protein biosynthesis by removing a specific adenine residue from the 28S ribosomal RNA (A4324 in rat ribosomes), which prevents the binding of elongation factors [61]. These enzymes are highly efficient and a single toxin molecule suffices to kill a HeLa cell [62]. It is a remarkable observation that toxins produced by certain plants (ricin, abrin) and bacteria (Shiga toxins, Shiga-like toxins) exert their deleterious effect by an identical catalytic mechanism.

The B-subunit of ricin is a lectin with two functional binding pockets [63, 64] for ß1→4 linked galactose and N-acetylgalactoseamine residues of glycoproteins and glycolipids [65]. Whether these two binding sites operate independently from each other or cooperativity exists, remains a matter of debate. However, it is unlikely that an active lipid clustering effect, by which the toxin imposes its own uptake into cells as it has been proposed for Shiga toxin and simian virus 40, applies to ricin. Rather, ricin opportunistically binds to any glycoprotein or glycolipid with a terminal galactose at the cell surface and is passively taken up piggyback along with its binding partner. Due to its promiscuous binding, ricin enters cells by multiple endocytosis pathways (clathrin-dependent as well as clathrin-independent) [66] and its retrograde transport from the plasma membrane to the ER is highly inefficient: only an estimated 5% of cell-bound ricin is transported via the trans-Golgi network to the ER, from which it translocates into the cytosol, while the vast majority of toxin is either being recycled back to the cell surface or degraded in late endosomes and lysosomes [67]. This indiscriminate binding of ricin also explains why it is so difficult to identify distinct molecular players involved in the intracellular trafficking of ricin, while the retrograde

transport of the Shiga toxin, which specifically uses the glycosphingolipid Gb3 as cellular receptor, is currently much better characterised [21, 68]. Recently, a genome-wide RNAi screen shed some light on the molecular requirements of ricin intoxication [69]. This study corroborates earlier observations that only a subset of molecular factors that are required for ricin trafficking is also involved in the retrograde transport of other toxins, such as Shiga toxin or *Pseudomonas* exotoxin, and that several intertwined retrograde transport pathways exist in parallel. Ablating specifically the retrograde transport of ricin by small-molecule inhibitors in vivo protects mice against an otherwise lethal dosis of ricin [55].

3. Viruses

As opposed to bacteria (discussed in the next section), viruses require the cellular machinery of the host for their replication and therefore must deliver their genome into their eukaryotic target cells. In contrast to viruses that directly fuse with the plasma membrane, for example retroviruses, herpes viruses and HIV, most of the enveloped viruses, including Influenza A Virus, hijack endocytotic pathways for their cellular entry [70-72], thereby taking advantage of the endosomal sorting machinery to reach a defined cellular compartment for uncoating. In the following section, we exemplify the entry of non-enveloped as well as enveloped viruses through the simian virus 40 and the Influenza A Virus, respectively.

3.1. Simian virus 40

Several non-enveloped viruses bind with their capsid to glycosphingolipids on the host cell and use them as viral receptors for efficient endocytosis and infection. The best characterized of these viruses are two members of the *polyomaviridae* family, the simian virus 40 (SV40) and the mouse polyoma virus (mPy), which bind to the gylcosphingolipids GM1, GD1a and GT1b, respectively. Other lipid-binding, non-enveloped viruses include the BK virus, Merkel cell polyomavirus and murine norovirus [73, 74].

Interestingly, the binding to glycosphingolipids at the plasma membrane pre-determines the uptake mechanism and intracellular trafficking route of viruses: instead of being degraded in the late endocytotic pathway or recycled back to the plasma membrane after endocytosis, these viruses are transported from the plasma membrane to the ER [75], from which they translocate into the cytosol [76]. Once they reach the nucleus, viruses subvert the cellular machinery and replicate.

Natural hosts for SV40 are Asian macaques, where it induces persistent infections in the kidneys. However, it was also shown that SV40 is significantly associated with human brain tumours and bone cancer [77], indicating its cell transforming properties.

The viral capsid is mainly composed of 72 VP1 protein pentamers in an icosahedral organisation [78]. The VP1 of SV40 folds into a doughnut conformation and bears five highly specific GM1-binding sites [79, 80]. Minor differences in the carbohydrate moiety of GM1, which is exposed into the extracellular space, strongly affect the binding affinity of the virus [80].

The multivalent binding of the VP1 pentamer to GM1 enables the tight association of the virus to the host cell despite the otherwise low affinity of individual binding sites of SV40 to GM1 [79]. In addition, a recent study on cellular and artificial membranes revealed that by virtue of this multivalent binding of GM1, SV40 induces the reorganization of membrane lipids and the segregation of specific lipids into membrane nanoscale domains, and thereby actively promotes its own uptake into the host cell [6]. This process critically depends on the lipid structure of GM1 and is essential for efficient infection by SV40 (Figure 2).

The precise physiological function of caveolae – uncoated, flask-like pits, enriched in cholesterol and glycosphingolipids (e.g. GM1, GM3) – still remains debated. A recent study supports the notion that caveolae act as a membrane reservoir to counter mechanical stress [81]. The role of caveolae in clathrin-independent endocytosis is equally a matter of much debate [82, 83]. FRAP (fluorescence recovery after photobleaching)-experiments on cells, which express GFP-tagged caveolin-1 (the major protein component of caveolae in epithelial cells) show that caveolae are rather immobile structures, a finding that argues against a major role of caveolae in constitutive endocytosis [84]. Though earlier studies suggest that the uptake of SV40 occurs via caveolae [85-88], recent work shows that the majority of SV40 does not partition into caveolae and that SV40 efficiently infects cells devoid of caveolin-1 [6, 89], corroborating the idea that the caveolin-independent, lipid-induced pathway represents the major route for efficient SV40-infection.

3.2. Influenza A Virus

Influenza A Virus (IAV) is the causative agent of flu, which is an infectious disease, primary affecting the deep respiratory tract. IAV is an enveloped virus, which possesses a single stranded RNA genome in a negative orientation. Infectious particles of influenza viruses are pleomorphic, filamentous or spherically shaped particles with a mean diameter of 120 nm [90]. IAV particles attach to their host cells by binding with their trimeric haemagglutinin (HA) to terminal α2,6 or α2,3 glycosidic-bound N-acetylneuraminic acids (sialic acids) on the surface of the host cells [4]. Following receptor binding, virions undergo endocytosis and become uncoated in a pH-dependent manner [91]. The low pH of late endosomes induces a conformational change in the HA, resulting in the fusion of HA with the endosomal membrane [92, 93] and the release of the RNA into the cytosol of the infected cell.

Electron microscopy-based studies revealed that plasma membrane-derived vesicles containing IAV are surrounded by clathrin, indicating that clathrin is involved in the uptake of IAV [93]. Since IAV particles have also been observed in smooth, non-coated vesicles, it was speculated that IAV also enter host cells by clathrin- and caveolin-independent endocytosis. This notion was supported by the observation that cells expressing dominant negative Eps15 (a clathrin adaptor) and caveolin-1 were still infected by IAV. Interestingly, subsequent studies showed that IAV actively induce the *de novo* formation of clathrin-coated pits by binding to the host cell surface [72]. The mechanism that triggers the recruitment of clathrin is unknown. It was speculated that IAV by binding to the host cell surface induces

negative membrane curvatures that are sensed by BAR-domain containing proteins, which in turn recruit clathrin. However, the membrane-bending properties of IAV have not yet been shown. In this regard it is interesting that IAV, SV40 VP1 and Shiga toxin all bind to glycosphingolipids to induce endocytotic processes. Although experimental data are missing to this end, it is conceivable that IAV is able to bend membranes through clustering of sialic acid receptors for entry (Figure 2). The process of membrane bending and receptor clustering would likely be more complex than for Shiga toxin and SV40 VP1, considering that two different membranes (i.e. plasma membrane of the host cell and the viral envelope) are involved in this process.

Although more specific receptors than sialic acids are not yet identified for IAV, the virus activates specific cellular kinases for its efficient uptake, for example PI3K. PI3K is activated during the first 60 min of infection and was demonstrated to be required for efficient uptake [94]. The precise function of PI3K for the entry of IAV is not completely understood. Interestingly, IAV-activated PI3K seems to regulate an entry step, which precedes endosomal sorting [94]. In the context of bacterial invasion, PI3K activation is often associated with dramatic actin re-arrangements leading to macropinocytosis of the bacterium. Interestingly, this pathway has been reported recently as an alternative entry pathway for IAV that is dependent on the kinases Rac1 and Src, but independent of dynamin [95]. It was speculated that the virus activates this pathway by interacting with receptor tyrosine kinases (RTK) in the plasma membrane of the host cell. A study published at the same time reports that EGF receptor, a RTK, is activated by sialic acid-dependent IAV binding to ensure the efficient uptake of IAV [96]. This study hints to RTK, such as the EGF receptor, as entry receptors that promote the efficient uptake of the virus in a sialic acid dependent manner. It was hypothesized that sialic acids containing signalling receptors and/or glycosphingolipids become clustered upon binding by viral HA, leading to the activation of these signalling receptors and subsequent induction of PI3K signalling required for the cellular uptake [96]. So far, PI3K activation has not been linked to the re-arrangements of the cytoskeleton by actin polymerisation, although it was shown for polarized epithelial cells; actin dynamics and the motor protein myosin IV are apparently indispensable for the internalisation of IAV [97].

Despite the vast number of reports analysing the entry processes of IAV at the plasma membrane, additional studies are required to understand the exact mechanistic role of sialic acids during the entry mechanism. In particular, less is known about the trans-bilayer signalling of sialic acids on the outer membrane leaflet towards the cytosolic machinery in the context of IAV entry.

So far, we have addressed the endocytotic mechanisms of toxins and viruses. In the following, we will review the internalization strategies of invasive bacteria. The most significant difference to toxin molecules and viruses regarding the initial entry steps, is that bacteria sense environmental changes (e.g. Ca^{2+} levels, temperatures, surfaces) and dynamically *respond* to them in a more complex manner than toxins and viruses can (e.g. by the expression of a secretion system for their uptake [98], as discussed below). By doing so, these pathogens can manipulate their local microenvironment to a certain level, which makes the invasion process more complex as compared to toxins and viruses.

4. Non-invasive bacteria and the role of phagocytosis

A limited number of immune cells, such as macrophages, monocytes, dendritic cells, and neutrophils, are able to incorporate large particles in an actin-dependent process called phagocytosis and thus eliminate cellular debris, apoptotic bodies and pathogens [99]. During phagocytosis, the tight interaction between the particle and cell surface receptors of the host cell (e.g. Fc or complement receptors) induces a transient reorganization of the actin cytoskeleton and the generation of local membrane protrusions that engulf the particle.

Several pathogens, such as *Mycobacteria* (including *M. tuberculosis* and *M. leprae*) and *Brucella,* have exploited this mechanism for their uptake into host cells. During the invasion by means of phagocytosis, the pathogen is passively taken up into the cell together with extracellular fluid. After internalization, pathogens alter the cellular machinery (e.g. prevention of the fusion of phagosomes and lysosomes) or are equipped to counter the phagocytic attacks (e.g. certain components in the outer bacterial membrane protect the pathogen against lysosomal enzymes; secreted enzymes neutralize toxic oxygen species) in order to survive inside the phagocytic cell, where they can replicate. The uptake of *M. tuberculosis* nicely illustrates the role for host cell PIPs in the invasion of pathogenic bacteria. Under normal conditions $PI(4,5)P_2$ and $PI(3,4,5)P_3$ are mainly localized and formed at the plasma membrane and recruit proteins important for phagocytosis. During the maturation of the phagosomes the small GTPase Rab5, the most abundant protein on pre-mature phagocytic vacuoles, recruits the PI3K hVps34 to generate PI(3)P. PI(3)P is now the dominating PIP species on the phagosomal membrane and attracts PI(3)P-binding proteins. These include the early endosomal antigen 1 (EEA1), which is critical for the further maturation of pre-mature phagocytic vesicles into phagolysosomes [100]. *M. tuberculosis* has evolved a mechanism to prevent the fusion of the bacterium-containing phagosome with early endosomes. By secreting the phosphatidylinositol analogon lipoarabinomannan (LAM), *M. tuberculosis* inhibits an increase of the cytosolic calcium in infected cells, thereby blocking Ca^{2+}/calmodulin kinase II, which is required for the activation of the PI3K hVps34 and the generation of PI(3)P [101]. Phosphatidylinositol mannoside (PIM), which is another mycobacterium-secreted phosphoinositide, stimulates early endosome fusion and consequently blocks phagosomal maturation [102]. PI(3)P is also directly dephosphorylated by the mycobacterium-secreted PI phosphatase SapM, which additionally contributes to the arrest of phagosomal maturation. [103]. These examples illustrate the powerful defence of *M. tuberculosis* to prevent its digestion in lysosomes by interfering with the host cell PIP metabolism.

5. Invasive bacteria: trigger versus zipper mechanism

As only a subset of cells phagocytose, so-called "invasive bacteria" have developed strategies to actively induce their own uptake into non-phagocytic cells (e.g. intestinal epithelial cells). These invasive bacteria are categorized by their entry mechanism into two groups: "triggering" and "zippering" bacteria. Swanson and Baer were the first who proposed these mechanisms for particle phagocytosis in 1995 [99].

Principally, "triggering" bacteria secret effector proteins into their target cells. These cells respond with a re-arrangement of the cytoskeleton that promotes the entry of the bacterium. In contrast, bacteria that enter cells via the "zipper" mechanism engage specific surface receptors of the target cell, leading to just moderate actin re-modelling concomitant by less dramatic alterations of the host cell surface. As a result of both strategies, these bacteria are tightly engulfed by the host cell plasma membrane [104] (Figure 3). In the following, we discuss these invasion strategies by using the examples of the best-characterised "triggering" and "zippering" bacteria.

5.1. "Triggering" bacteria

Many Gram-negative bacteria, such as *Salmonella enterica*, *Shigella flexneri*, and *Pseudomonas aeruginosa*, invade cells through a "trigger" mechanism.

Salmonella, as well as *Shigella*, (described in the toxin section above), *Listeria* and *Yersinia* (described below), are foodborne pathogens, which cause gastritic infections by ingestion of contaminated food or water. Typical symptoms of an infection by *Salmonella* species, e.g. *Salmonella enterica,* are diarrhoea, abdominal cramps and fever.

By virtue of a type III secretion system (T3SS), which serves as a translocation pore, *Salmonella*, *Shigella* and *P. aeruginosa* inject virulence factors directly into the host cell cytosol during infection. The T3SS spans the bacterial membrane and is then inserted into the host cell membrane. It is assembled in the different species by SipB and SipC in *Salmonella*; IpaB and IpaC in *Shigella*; and EspB and EspD in enteropathogenic *Escherichia coli* [105, 106].

For an efficient invasion, a specific membrane microenvironment is critical. Indeed, it has been shown for *Salmonella, Shigella,* FimH-expressing *E. coli* and *P. aeruginosa* that specialized lipid membrane microdomains, which are enriched in cholesterol and sphingolipids, are required for efficient binding of bacteria to target cells, the activation of their T3SS, the translocation of effectors into the host cell cytosol and for the activation of cellular signalling pathways essential for bacterial invasion [107-110].

The translocated bacterial virulence factors subvert various cellular activities of the host cell, which leads to a massive polymerization of actin and enables the internalization of the pathogen into the target cell [111, 112]. In the case of *Shigella*, VirA binds to $\alpha\beta$-tubulin heterooligomers and induces a local destabilization and depolymerisation of microtubules, which triggers the activation of the kinase Rac1 and promotes membrane ruffling [113]. Other virulence factors, such as SipC, SipA, SopE and SopE2 in *Salmonella* and IpaB and IpaC in *Shigella*, induce the polymerization of actin directly (SipC) or in a Rac1- and Cdc42-dependent manner via activation of the Arp2/3 complex (IpaC and SopE/SopE2) [114-116]. A further stimulus of the actin polymerization is mediated by phosphatidylinositol phosphatases (IpgD in *Shigella* and SopB/SigD in *Salmonella*), which hydrolyse $PI(4,5)P_2$ into PI(5)P, causing the disconnection of cortical actin from the plasma membrane and enhancing actin dynamics at the bacterial entry site [117, 118]. The resulting membrane protrusions engulf the pathogen, which is then (i.e. upon actin depolymerisation) internalized into the host cell.

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen leading to acute infections of the respiratory tract, the urinary tract and the skin. Like other Gram-negative bacteria mentioned above, *P. aeruginosa* also injects effector proteins into host cells by virtue of a T3SS. Among the different strains presented so far, those that produce the T3SS effector proteins ExoT and ExoS are more efficiently internalized than those that do not produce these proteins [119]. Both, ExoS and ExoT are bifunctional proteins containing a C-terminal ADP-ribosylase and a N-terminal GTPase activating protein (GAP)-activity. Both functions redundantly disrupt the actin cytoskeleton [120]. Surprisingly, ExoS and ExoT act as anti-internalization factors [121], probably by interfering with components of the Abl pathway [122]: Rac1, Cdc42 and Crk were demonstrated to be activated by, and necessary for, the cellular uptake of *P. aeruginosa* [123]. Interestingly, additional experiments performed with ΔExoS and ΔExoT deletion mutants of *P. aeruginosa* revealed that ExoT abrogated Rac1 and Cdc42 activation, whereas ExoS activates these GTPases in order to promote efficient uptake [123]. However, the role of ExoS/T in the *P. aeruginosa* entry seems to be complex, in particular considering that ExoS/T production is characteristic for invasive strains.

Early studies demonstrate that *P. aeruginosa* binds to asialo-gangliosides [2]. Among these, asialo-GM1 seems to be important for the attachment of the bacteria to target cells in the respiratory tract by type IV pili [3]. Surprisingly, studies applying the small-molecule inhibitor PPMP, which inhibits the glucosylceramide synthase and consequently the biosynthesis of glycosphingolipids [124], revealed that glycosphingolipids are rather important for the internalization instead of the adhesion to target cells [125]. Based on these observations, one can speculate that those bacterial lectins, which bind to glycosphingolipids, might also promote the cellular invasion of *P. aeruginosa* into host cells. In this scenario, such lectins bend the plasma membrane by multivalent binding to their glycosphingolipid receptors in a similar manner as StxB (see section: Toxins), which might at least facilitate the initial steps of bacterial uptake. However, additional studies are required to proof this concept.

On the cytoplasmic side of the plasma membrane, PIPs were found to be important for the entry of *P. aeruginosa*. It was shown that invasive *P. aeruginosa* activate, and depend on, PI3K activity for efficient internalization [126]. Interestingly, $PI(3,4,5)P_3$ was found to accumulate at the bacteria entry site. However, it is not clear, which bacterial factor activates PI3K signalling and how PI3K leads to the internalization of *P. aeruginosa*. It was speculated that PI3K-regulated actin dynamics in the context of macropinocytosis leads to the internalization of the bacterium. This idea is supported by the fact that actin is required for the internalization of *P. aeruginosa* into host cells [127]. In subsequent studies using polarized epithelial cells, *P. aeruginosa* activates and recruits PI3K to the bacterial attachment site at the apical membrane of the cells. These processes were accompanied by the induction of membrane protrusions, enriched in filamentous actin and PI(3)P [128]. It needs to be determined how PI(3)P induces actin dynamics at the plasma membrane in order to promote the internalization of *P. aeruginosa*.

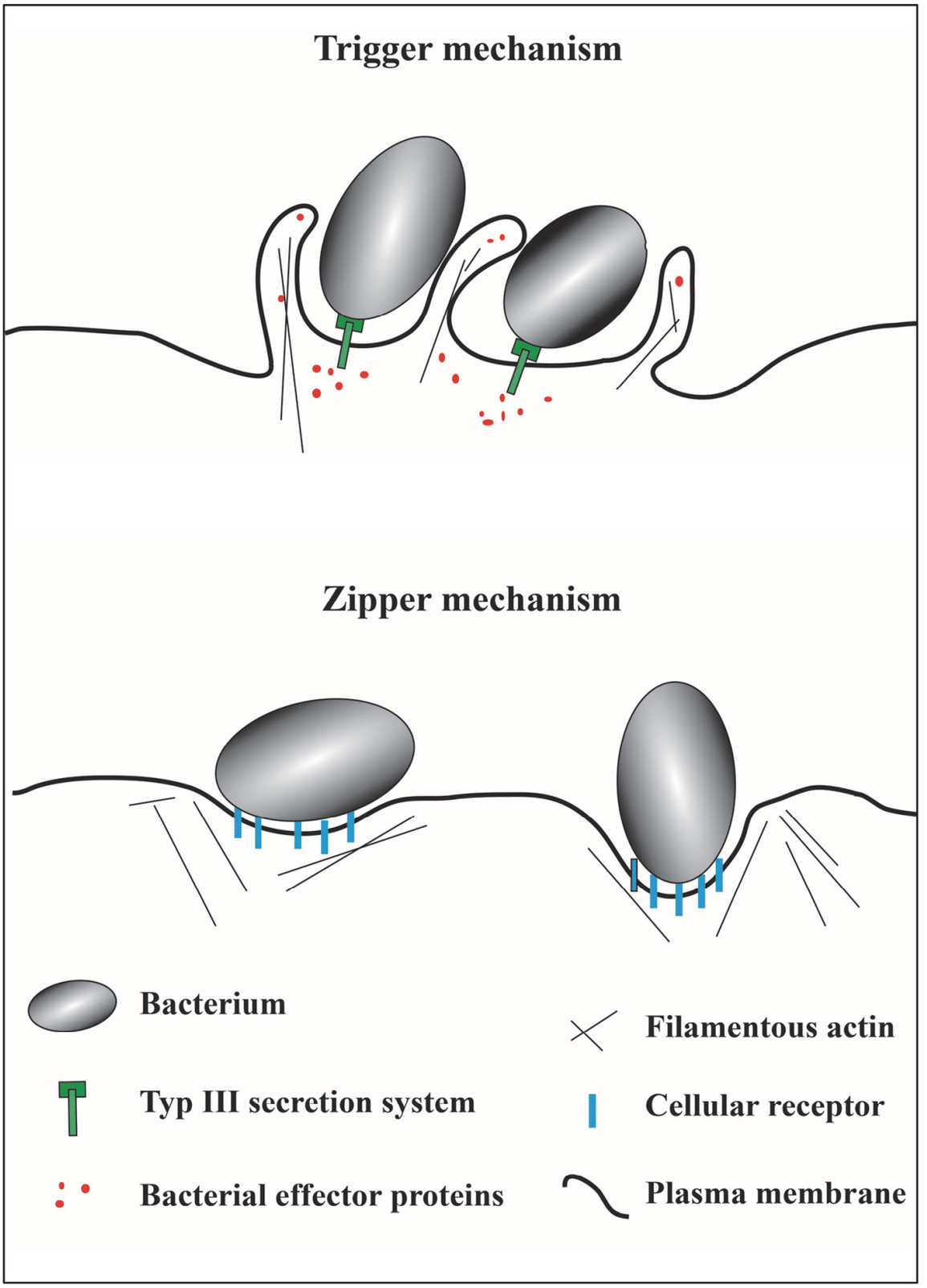

Figure 3. The two principle invasion mechanisms for invasive bacteria: the trigger and the zipper mechanism.

In general, less is known about the PIP metabolism in the context of *P. aeruginosa* uptake. It would be interesting to analyse whether *P. aeruginosa* also affects other PIPs and whether the T3SS effectors ExoS and ExoT also interfere with the PIP metabolism to induce the cellular uptake of the bacterium. These open questions necessitate additional studies to obtain more detailed insights into the molecular invasion mechanism of *P. aeruginosa.*

5.2. "Zippering" bacteria

The zipper mechanism is best characterized for the cellular invasion of *Listeria monocytogenes* and *Yersinia pseudotuberculosis*. By interacting with host cell receptors, these pathogens induce signalling events in the host cell to stimulate modest cytoskeletal rearrangements and membrane extensions for efficient invasion. *L. monocytogenes* is a foodborne pathogen, which crosses several host barriers leading to listeriosis, gastroenteritis and central nervous system infections [129]. The foodborne *Y. pseudotuberculosis* causes gastrointestinal and extra-intestinal infections, which can be accompanied by an abscess-forming mesenteric lymphadenitis [130].

Internalin (InlA) of *L. monocytogenes* binds to the cellular adhesion glycoprotein E-cadherin [131, 132], which is critically involved in the formation and integrity of adherens junctions in epithelial cells. Actin reorganization required for the entry of *L. monocytogenes* is triggered by the cytoplasmic domain of E-cadherin, which binds transiently to the actin cytoskeleton via interactions with α- and β-catenins and in concert with ARHGAP10 (the guanosine-activating protein (GAP) for RhoA and Cdc42), and the GTPase Arf6 [133, 134]. E-cadherin-mediated actin remodelling is further stimulated by the phosphorylation of cortactin [135] and the activation of the Arp2/3 complex in a Rac-dependent manner [136]. The efficient entry of *L. monocytogenes* also requires caveolin for the clustering of E-cadherin. In this scenario, caveolin is a prerequisite for the InlA-induced clustering of E-cadherin, which is localized around the bacterium. The clustered E-cadherin becomes tyrosine phosphorylated by Src, recruiting the ubiquitin ligase Hakai, which mediates the ubiquitination of E-cadherin. Finally, the ubiquitinated E-cadherin triggers the recruitment of clathrin to the entry site, which leads to the internalization of *Listeria* [137]. Furthermore, myosin VIIA and its ligand vezatin have been implicated in the endocytosis of *L. monocytogenes* [138].

The second invasion protein of *L. monocytogenes*, Internalin B (InlB), interacts with the ubiquitously expressed HGF (hepatocyte growth factor)-receptor Met [139]. Upon binding, the tyrosine-kinase Met dimerizes and autophosphorylates, which leads to the recruitment of the adaptor proteins Cbl, Gab1, Shc and Crk2 [140]. The subsequent activation of PI3K, which phosphorylates $PI(4,5)P_2$ into $PI(3,4,5)P_3$, promotes actin polymerization through the stimulation of the Arp2/3 complex in a Rac1-dependent manner [141]. Interestingly, depletion of membrane cholesterol with methyl-ß-cyclodextrin (MβCD) diminishes the activation of Rac1, but not of PI3K, indicating a possible need for the repartitioning of $PI(3,4,5)P_3$ into cholesterol-enriched membrane microdomains [142]. Surprisingly, clathrin, dynamin and several other components of the endocytotic machinery co-localize with the bacterial entry site and are essential for invasion of *L. monocytogenes* and *Y. pseudotuberculosis* [143].

While clathrin is crucial for the internalization of these "zippering" bacteria, it is not required for the entry of "triggering" bacteria (*Salmonella*, *Shigella*) [144]. More studies are needed to elucidate the functional interplay between clathrin and cholesterol-enriched membrane domains.

Recently, another class of GTPases – Septins - have been identified to be required for the efficient, InlB-dependent entry of *L. monocytogenes* [145]. Septins regulate actin organisation [146] and phagosome formation in macrophages [147]. The mechanism of septins during the uptake of *L. monocytogenes* is not yet clear. Interestingly, septin and actin colocalize at the same bacterial entry site. However, based on the ring-like assembly of septins, which is different from the actin architecture at the bacterial entry site, it is suggested that actin and septins fulfil distinct or complementary roles during the internalization process [145].

Upon internalisation, *L. monocytogenes* is located within a vacuole, from which it eventually escapes into the cytosol by the synergistic action of the pore forming toxin listeriolysin O and the bacterial encoded, PIP-specific PLC [140].

Similar to *L. monocytogenes*, enteropathogenic *Yersinia* species activate PI kinases by interaction of the *Yersinia* outer membrane protein invasine to the heterodimeric β1 integrin receptor [148] and subsequent activation of the Rac1 pathway. The activation of the Rac1 pathway leads to the local enrichment of $PI(4,5)P_2$ through the recruitment and activation of PI5K [148], which is a lipid kinase that selectively phosphorylates the inositol ring at D5 position of PIs. However, the final activation of PI5K seems to be induced by Arf6, a GTP binding protein that normally regulates the production of $PI(4,5)P_2$ [149]. It is assumed that Arf6-driven actin dynamics mediate the formation of the phagocytic cup surrounding *Yersinia* [150]. At this stage of entry, *Yersinia* is located in an intermediate compartment, termed prevacuole, which is still connected to the plasma membrane. It could be shown that $PI(4,5)P_2$ needs to be hydrolyzed in order to proceed the maturation of the prevacuole into a separate, sealed compartment. This step is mediated by the inositol-5-phosphatases OCRL and Inpp5b, which are recruited to the *Yersinia*-containing prevacuole. As a prerequisite for this recruitment, the GTPase Rab5 must associate with the prevacuole and it could indeed be shown that PI3K seems to mediate this step [151].

This is an intriguing example that illustrates how invasive pathogens dynamically regulate host cell PIPs to complete their internalization.

6. Conclusion

The example of SV40 shows how pathogens are able to initiate their uptake by engaging glycosphingolipids on the surface of host cells. It is intriguing to see that this concept of endocytotic uptake resembles those found for Stx uptake. This suggests that glycosphingolipid-driven endocytosis is not restricted to specific pathogens or toxins, but rather seems to be a general concept for initiating endocytotic processes at biological membranes. Considering that invasive bacteria bind carbohydrate receptors in the plasma

membrane for proper attachment to host cells, an interesting question is whether such adhesion receptors are also engaged as internalization receptors allowing invasive pathogens to gain access to host cells. In such a scenario, the invasive bacterium binds via some of its lectins to host cell receptors, which subsequently become clustered, thereby creating asymmetrical stress in the lipid bilayer and leading to membrane invaginations that facilitate the bacterial uptake. In particular, it would be interesting to know if the putative lectin-induced membrane invagination modulates host cell PIP metabolism at the cytoplasmic side of the plasma membrane for the efficient entry of the pathogen. Additionally, it needs be clarified whether the lectin-bended plasma membrane is sensed by cellular effector proteins, for example by BAR domains, that could stabilise and/or further assist in the invagination of the plasma membrane to accomplish the endocytosis process.

As most pathogens, in particular invasive bacteria, depend on the rearrangement of actin for their cellular uptake, it remains to be determined to which extent actin dynamics, as observed for Stx-induced membrane tubules, also contribute to the scission of the bacteria-containing vacuoles.

Glycolipids are often found co-clustered with protein receptors in the plasma membrane. It remains to be identified, if specific glycolipids, which are used as pathogen or toxin receptors, preferentially interact with other proteins in the plasma membrane to specify the endocytotic route of a toxin or an invasive pathogen.

As yet, less is known about these specific issues of the initial steps of pathogen internalization, and many more studies need to be carried out to examine the exact functional and mechanistic role of glycosphingolipids and phosphoinositides in pathogen invasion.

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