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Intracellular Trafficking of Bacterial Toxins

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Abstract

Bacterial toxins often translocate across a cellular membrane to gain access into the host cytosol, modifying cellular components in order to exert their toxic effects. To accomplish this feat, these toxins traffic to a membrane penetration site where they undergo conformational changes essential to eject the toxin's catalytic subunit into the cytosol. In this brief review, we highlight recent findings that elucidate both the trafficking pathways and membrane translocation mechanisms of toxins that cross the plasma, endosomal, or endoplasmic reticulum (ER) membrane. These findings not only illuminate the specific nature of the host-toxin interactions during entry, but should also provide additional therapeutic strategies to prevent or alleviate the bacterial toxin-induced diseases.

Introduction

To cause disease, many bacterial toxins must reach the cytosol of the target cell where they modify the activities of host components, leading to changes in cellular physiology that ultimately promote bacterial pathogenesis [1,2]. To do so, the toxins first bind to receptor(s) on the surface of the host cell. These engagements can initiate toxin translocation across the plasma membrane, enabling the toxin to gain access into the cytosol (Figure 1, pathway 1). Alternatively, the toxin-receptor interaction can stimulate endocytosis, bringing the toxin into endosomal compartments. Some toxins subsequently breach the endosomal membrane in order to reach the cytosol (Figure 1, pathway 2). Others traffic further along the retrograde pathway and are directed to the endoplasmic reticulum (ER). Here, translocation across the ER membrane allows the toxin to arrive into the cytosol (Figure 1, pathway 3). How a toxin selects a specific membrane translocation pathway depends partly on the nature of the toxin and the availability of host components that support all cellular events leading to cytosol translocation. In recent years, seminal reports have provided insights into the trafficking pathways and membrane translocation mechanisms of certain bacterial toxins that use the plasma, endosomal, or ER membrane for membrane penetration. In this review, our objective is to focus on one toxin for each membrane penetration site – these examples

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underscore the exquisite demand required to deliver an extracellular bacterial toxin into the interior of a host.

Translocation across the plasma membrane

The best case of a bacterial toxin that translocates across the plasma membrane is seen in the adenylate cyclase toxin (CyaA) (Figure 2A) secreted by *Bordetella pertussis*, the causative agent for pertussis or whooping cough [3]. Infection by this bacterium often compromises the host immune system. Structurally, CyaA contains an N-terminal adenylate cyclase (AC) domain followed by a C-terminal hydrophobic hemolysin domain important for pore formation and receptor-binding [4]. To cause disease, CyaA binds to the $\beta 2$ integrin complement receptor (CR3) on the cell surface of phagocytes [5]. While this step is not absolutely required for toxin entry, it enhances the process. Part of the hemolysin domain is then inserted into the plasma membrane, enabling the AC domain to subsequently cross the plasma membrane and reach the cytosol [6]. How the hemolysin domain assists in membrane translocation of AC is not well-understood, although there is evidence that an α -helical peptide located at the N-terminus of the hemolysin domain possesses a membrane-destabilizing activity [7] – this activity has been proposed to locally disrupt membrane bilayer integrity that in turn promotes AC translocation across the plasma membrane. Importantly, while cellular components involved in supporting this translocation event remained largely unclear [8,9,10], a recent report using an elegant *in vitro* translocation system was able to address this question [11**]. In this study, AC translocation across an artificial lipid bilayer (designed to mimic the plasma membrane) was clearly demonstrated to be dependent only on the presence of calcium ions and a negative membrane potential, without requiring additional host factors [11**]. Although calmodulin (CaM) was placed in the *trans* side of this *in vitro* membrane translocation system, it likely stimulates AC's catalytic activity [12] without necessarily playing any role in driving AC's membrane translocation [13]. Such an *in vitro* approach offers the advantage of evaluating a membrane translocation event under a highly controlled condition, a strategy that should be used to further evaluate membrane translocation of other bacterial toxins.

Upon reaching the cytosol, the AC domain converts cytosolic ATP to the crucial second messenger cyclic AMP (cAMP) [14]. cAMP in turn stimulates a signaling cascade that disrupts the bactericidal functions of the phagocytes, and impairs other host cell innate immune responses against the bacteria [15]. Curiously, when a natural ligand binds to CR3, this interaction normally stimulates signal transduction events that lead to cellular responses important for innate immune defense [16], including CR3-dependent phagocytosis of bacteria [17]. How then does binding of CyaA to CR3 avoid activating this normal cascade? A partial clue to this enigma was revealed in a new study demonstrating that CyaA binds to an atypical binding site in the so-called “bent and closed” CR3 conformation, in contrast to natural ligands that typically interact within a well-defined canonical binding site in the “extended and open” CR3 conformation [18**]. While this atypical binding still allows translocation of CyaA's AC domain across the plasma membrane, it fails to stimulate the normal ligand-activated CR3 signaling pathway. In fact, the AC-generated rise in cAMP can counter the usual CR3-dependent signaling induced by a natural ligand. In this manner, CyaA binding to its host receptor is thought to thwart the ability of a host to properly mount

an immune response. This example emphasizes how subtle differences in receptor engagement can lead to profound differences in cellular physiology.

Translocation across the endosomal membrane

In contrast to CyaA, other toxins undergo receptor-mediated endocytosis to reach endosomal compartments where they translocate across the endosomal membrane to access the cytosol. In addition to anthrax and tetanus toxins that use this entry pathway to reach the cytosol [19], another well-characterized toxin that also uses this route is the botulinum neurotoxin (BoNT) (Figure 2B). BoNTs are secreted by various *Clostridia* species and are classified into seven serotypes (A-G) [20]. These toxins cause severe flaccid paralysis of muscles observed in botulism [21] and remain the most potent toxins known to date. Structurally, a BoNT harbors a metalloprotease light (L) chain linked via a disulfide bond to a heavy (H) chain that is further divided into three subdomains: one that supports L chain membrane translocation (HN) into the cytosol, followed by a domain likely involved in lipid binding (HC-N) [22], and then a domain that mediates receptor-binding (HC-C) [23]. To intoxicate cells, BoNT binds to receptors, including polysialoganglioside lipid and synaptic membrane (SV) protein receptors on the surface of the presynaptic nerve terminals via the HC-C domain. A recent X-ray structure of a long sought-after complex formed between the HC-C domain of a BoNT (BoNT/A) and the toxin-binding domain of a host protein receptor (called synaptic vesicle glycoprotein 2, SV2) revealed a striking backbone-to-backbone mode of interaction [24**]. Importantly, this finding led to the development of a peptide that successfully blocked toxin entry, suggesting that such structural information is likely to provide a reasonable platform for generating effective therapeutic agents against a toxin.

After receptor binding, BoNT is endocytosed and traffics to endosomes within the nerve terminals called synaptic vesicles [25]. The low pH in the endosomal environment triggers structural rearrangements that enable the HN chain to insert into the synaptic vesicle membrane where it acts as a channel, translocating the L chain into the cytosol [23]. The L chain, however, remains tethered to the H chain via the interchain disulfide bond. The cytosolic thioredoxin reductase-thioredoxin (TrxR-Trx) system is thought to be responsible for reducing this disulfide linkage [26], thereby releasing the L chain into the cytosol. To further characterize the TrxR-Trx system, the same group recently uncovered the presence of this system on the cytosolic side of the synaptic vesicles [27**]. This strategically localizes the redox system at the interface between the synaptic vesicle membrane and cytosol where it can reduce BoNT's disulfide bond. The ensuing release of the L chain allows it to cleave and inactivate the SNARE proteins, which are membrane proteins that normally promote fusion of vesicles with their proper target membranes. As a consequence, vesicles harboring the neurotransmitter acetylcholine cannot be released from the cell, thereby leading to muscle paralysis which characterizes botulism. In fact, in this same study [27**], inhibitors against the TrxR-Trx system were demonstrated to be effective at alleviating BoNT-induced disease in a mouse model. Hence, the principle of identifying host components that execute critical roles during toxin entry remains a sound approach in combating toxin-induced diseases, as it provides an opportunity to generate specific inhibitors against the host factor.

Translocation across the ER membrane

A different strategy is implemented for certain bacterial toxins that do not harbor a domain that can be used as a channel to support membrane translocation. These toxins, including cholera toxin (CT) [28], shiga toxin [29], and the cytolethal distending toxin [30], take a rather unusual path, trafficking from the cell surface to the ER where they hijack a pre-existing channel to gain entrance into the cytosol. This ER-to-cytosol membrane transport pathway is normally geared to retro-translocate misfolded ER proteins to the cytosol where the misfolded proteins are ubiquitinated and degraded by the proteasome in a process called ER-associated degradation (ERAD) [31]. Here we highlight retro-translocation of CT, given the recent advances in our understanding of this pathway (Figure 2C).

CT, produced by *Vibrio cholera*, is responsible for cholera, a secretory diarrheal disease that remains a global health concern [32]. The CT holotoxin is composed of the toxic A (CTA) subunit inserted into the pore formed by five receptor-binding B (CTB) subunits. CTA cleavage produces CTA1 and CTA2, which remain associated with each other through a disulfide bond. During entry into intestinal epithelial cells, CTB interacts with the ganglioside GM1 receptor on the plasma membrane, triggering endocytosis and retrograde trafficking to the ER [33,34]. GM1's ceramide structure [35], in conjunction with the C-terminal KDEL ER retention sequence in CTA [36], appear to be critical determinants in guiding the toxin to the ER. While it is possible that the plasma membrane-to-ER transport pathway is generated due to toxin-binding, there is also evidence that such a route represents a constitutive pathway in which endogenous ligands are transported in a retrograde manner from the extracellular milieu to the ER [37].

Regardless, in the ER, CTA is thought to disguise as a misfolded protein, exploiting elements of the ERAD machinery to gain entry into the cytosol [38]. To accomplish this, the ER-resident BiP chaperone, acting in concert with its co-chaperones, was recently found to recognize and target CT to the ERAD machinery [39,40**]. The most striking observation in these studies is that the BiP co-chaperone ERdj5 is physically localized to the ERAD membrane machinery, thereby providing a mechanism to target CTA to the retro-translocation site. CTA is then reduced (by an unknown reductase) to generate CTA1, which is in turn unfolded by protein disulfide isomerase [41,42]. Unfolded CTA1 retro-translocates across the ERAD channel - postulated to be the transmembrane E3 ubiquitin ligase Hrd1 [43,44] - into the cytosol. How CTA1 avoids degradation by the proteasome in the cytosol remains unclear, but the lack of ubiquitination [45,46] due to the paucity of lysines in this toxin fragment may partially explain how it averts the degradative fate [38]. Interestingly, despite the lack of ubiquitination, CTA1 retrotranslocation requires an intact ubiquitination-deubiquitination cycle mediated by Hrd1's E3 ligase [47] and the YOD1 deubiquitinase [46] activities, raising the possibility that ubiquitination and deubiquitination of *trans* cellular factors control CTA1's ER membrane translocation. There is currently no consensus on the precise nature of the cytosolic force used to eject CTA1 into the cytosol [48]. In the cytosol, CTA1 triggers a signaling cascade that results in pathologic water loss, leading to secretory diarrhea that characterizes cholera. It is interesting to note that while the ER represents CT's membrane penetration site crucial for inducing toxicity, an intriguing study suggests that the ER also possesses a sensor (IRE1) that can be activated by the toxin to trigger an immune

response [49], suggesting that trafficking to the ER may have a deleterious consequence for the toxin.

Conclusion

Here we highlighted recent advances in our understanding of how three different bacterial toxins penetrate the plasma, endosomal, or ER membrane to reach the cytosol from the extracellular environment. Depending on the toxin, this is accomplished by hijacking host receptors that promote toxin binding, transport routes that facilitate toxin trafficking, and/or cellular cues that impart conformational changes essential for membrane translocation. These insights are gained through the use of sophisticated structural, biochemical, and cell-based approaches, strategies that will continue to be invaluable in deciphering the trafficking pathways of other bacterial toxins. The advent of powerful technologies, including the CRISPR-dependent regulation of gene activation and suppression approach [50] and the haploid genetic screening method [51] should provide researchers with additional, exciting strategies to further unveil how bacterial toxins engage their host cells to cause disease.

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Figure 1. Intracellular transport of bacterial toxins

Bacterial toxins can enter host cells by at least three different pathways. The toxins can bind to the host cell surface, initiating translocation across the plasma membrane to reach the cytosol (pathway 1). Alternatively, the toxins can undergo endocytosis, targeting to endosomal compartments where they penetrate the endosomal membrane to reach the cytosol (pathway 2). Other toxins traffic further along the retrograde pathway to reach the ER - here they translocate across the ER membrane and arrive in the cytosol (pathway 3).

Figure 2. Host cell entry of CyaA, BoNT, and cholera toxin

A. CyaA binds to the bent form of the CR3 receptor. A portion of the C-terminal hemolysin domain of CyaA then inserts into the plasma membrane, enabling translocation of the N-terminal catalytic adenylate cyclase (AC) domain. This transport event appears to require only calcium and a negative membrane potential. In the cytosol, AC converts ATP to cAMP in a reaction that depends on calmodulin (CaM). cAMP triggers a signaling cascade that inhibits CR3's normal signal transduction pathway, which is activated when CR3's natural ligand binds to CR3 in the extended conformation. Because normal CR3 signaling promotes phagocytosis and innate immunity, CyaA is thought to cripple the host defense response in this manner. B. Through its C-terminal receptor-binding HC-C domain, BoNT interacts with its host receptors, including the lipid receptor ganglioside and protein receptor SV2. Receptor-mediated endocytosis targets BoNT into endosomes (synaptic vesicles) in presynaptic nerve terminals. The low pH in this environment induces a toxin conformational change such that the HN domain is inserted into the synaptic vesicle membrane. Membrane-inserted HC acts as a channel, translocating the metalloprotease L chain into the cytosol, which remains associated with the H chain via a disulfide bond. The thioredoxin reductase-thioredoxin (TrxR-Trx) system present on the cytosolic side of the synaptic membrane reduces the BoNT disulfide bond, releasing the catalytic L chain into the cytosol. L chain cleaves cytosolic SNARE proteins to impair release of acetylcholine from the nerve terminals, leading to paralysis that characterizes botulism. C. Via its receptor-binding B (CTB) subunit, cholera toxin binds to ganglioside receptor on the surface of intestinal epithelial cells. This triggers endocytosis, driving the holotoxin to the ER. In the ER, the holotoxin targets to the ERAD membrane machinery (whose central component includes Hrd1, one of a few ER-resident E3 ubiquitin ligases) by the actions of the BiP chaperone and co-chaperone (ERdj5 and Grp170/Sil1) complex. An unidentified reductase reduces the disulfide bond in the catalytic A (CTA) subunit generating CTA1, which is in turn unfolded

by PDI. Unfolded CTA1 retro-translocates into the cytosol by crossing the ERAD membrane channel postulated to be Hrd1. Although CTA1 is not ubiquitinated, an intact ubiquitination-deubiquitination cycle catalyzed by Hrd1 and the YOD1 deubiquitinase is required. In the cytosol, CTA1 activates a signaling cascade, leading to water secretion across epithelial cells that results in diarrhea.

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