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A novel pathway for exotoxin delivery by an intracellular pathogen

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Abstract

Fundamental to the biology of many bacterial pathogens are bacterial proteins with the capacity to modulate host cellular functions. These bacterial proteins are delivered to the host's molecular targets by a great diversity of mechanisms of varying complexity. The different delivery mechanisms are adapted to the specific biology of the pathogen. Here we focus our attention on a recently described delivery pathway adapted to the biology of an intracellular pathogen, in which an exotoxin is delivered from an intracellular location to its molecular target through autocrine and paracrine pathways.

Introduction

Pathogens that have evolved close associations with their hosts often utilize proteins that have the capacity to modulate cellular functions to shape the pathogen/host interface. In some cases these molecules inflict overt damage of the target cell, but in most cases they subtly regulate cellular functions in order to make the host a better environment for their survival and replication. These molecules are usually referred to as “exotoxins” or “effectors”, a distinction that at times could seem confusing. Although it is often considered that the activity of “exotoxins” is more destructive than that of “effectors”, from the pathogen biology standpoint, the distinction between these two types of bacterial virulence factors is blurred at best. Rather, the apparent different damaging-capacity of these molecules may simply reflect an intrinsic bias in the experimental approaches utilized in their study. The distinction between “exotoxins” and “effectors” can be arguably best defined by the mechanisms by which these bacterial molecules are delivered into host cells. Proteins secreted from the bacteria and subsequently delivered to cells at sites not necessarily in close association with the bacteria are usually referred to “exotoxins”. On the other hand, bacterial proteins delivered directly from the bacteria to the target cell in their immediate proximity by specialized multiprotein machines are usually referred to as “effectors”. These different mechanisms have evolved to accommodate not necessarily the biochemical activity of a given toxin/effector but the biology of the particular pathogen that delivers them or the biological context in which they are delivered. In this review, we will first briefly summarize the different strategies used by many toxins/effectors to reach their targets, and then examine in more detail a recently described pathway utilized by an intracellular bacterium to deliver a toxin to its target cells.

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Mechanisms for toxin/effector protein delivery

Bacterial products (i. e. toxins and effectors proteins) that have evolved to modulate cellular processes are targeted to host cells by a variety of mechanisms (Fig. 1). In the simplest form, some toxins contain in the same polypeptide all the necessary information for both regulating cellular functions and gaining access to their final destination. Examples of this class of toxins are the so called “large clostridial cytotoxins” such as the Rho-glucosylating *Clostridium difficile* toxins A and B [1]. These toxins possess a tripartite structure comprising a biologically active N-terminal domain, a middle domain with a small hydrophobic stretch presumably involved in toxin translocation, and a C-terminal receptor-binding domain. Additional examples in this category are arguably the so-called pore-forming toxins [2]. These toxins are single chain molecules that insert into the plasma membrane often after multimerization and formation of an amphipathic structure. By this mechanism they form a pore, which is largely responsible for their cellular effects. Although these toxins have traditionally been considered to exert their function by simply lysing cells (or lysing the phagosomal membrane in the case of Listeriolysin O), recent findings point toward more complex roles involving modulation of several cellular functions through the activation of signaling pathways [3,4].

A second mechanism for toxin delivery is the one exploited by the AB-type of toxins, such as cholera toxin or pertussis toxin [5]. These toxins are composed of an “A” (for “active”) subunit, which carries the enzymatic activity, and a “B” (for “binding”) subunit, which mediate the delivery of the A subunit to its place of action [6,7]. The B subunit, which can be formed by one or several components, usually targets a cellular receptor and exploits endocytic pathways in the target cell to deliver the A subunit. In some cases the B subunit is also required for intracellular endocytic transport of the complex and the translocation of the A subunit through the membrane. Most of these toxins (e. g., cholera toxin and shiga toxin) are retrogradely transported to the endoplasmic reticulum through the Golgi complex and their translocation to the cytosol is thought to be mediated by the translocation machinery of the endoplasmic reticulum [6]. Translocation of other AB toxins from endocytic compartments to the cytosol is mediated by the B subunit itself, which forms a pore in the membrane as a consequence of a conformational change induced by the acidic pH of the endosomal lumen [8].

A third strategy of bacterial protein delivery to target eukaryotic cells involves complex multiprotein machines such as the type III and type IV secretion systems. [9–12]. These secretion systems are expressed by many pathogenic and symbiotic gram-negative bacteria and are designed to deliver molecules with high efficiency and extreme temporal precision, so that they can establish an elaborate cross-talk between the bacterium and its host during symbiosis or infection. These machines couple secretion through the bacterial envelope to translocation through the eukaryotic membrane and are therefore responsible for a direct transfer of molecules from the cytosol of bacteria to the cytosol of target cells. Discussion of these machines is beyond the scope of this article and therefore readers are referred to some recent reviews [9–12]. As discussed above, bacterial proteins delivered by these systems are most often referred to as “effector proteins” rather than “toxins”. From the standpoint of their actual biochemical activities or the functional consequences of their action, such a distinction is probably not warranted. However, unlike the delivery mechanisms for “exotoxins”, a distinctive feature of these machines is that they have evolved to deliver not just one or two proteins but many, which in many instances work in concert with one another.

Recently, a pathway was described for the delivery of a toxin expressed by an intracellular pathogen [13]. The distinctive feature of this pathway is that it has presumably evolved to deliver a toxin to target cells other than the cell in which it is produced. As it will be discussed below, this pathway, which delivers a multi-functional toxin from *Salmonella enterica* serovar

Typhi (*S. Typhi*) known as typhoid toxin, requires the export of the toxin from its site of production within host cells to target other cells via a paracrine pathway.

***Salmonella* Typhi: an intracellular pathogen with the capacity to persistently infect its human host**

Salmonella enterica is a facultative intracellular pathogen that can infect a variety of hosts. Some serovars of this pathogen, such as serovars Typhimurium or Enteritidis, can infect a broad range of animals and can cause acute gastroenteritis in the human host. In contrast, other serovars are host adapted and can cause systemic infection [14–16]. Central to the pathogenicity of all *S. enterica* serovars is their ability to induce their own uptake into cells that are normally non-phagocytic, such as those of the intestinal epithelium [17–19]. After entry, *S. enterica* also actively remodels the intracellular vacuole in which it resides in order to generate a niche suitable for its survival and replication. Both entry and remodeling of the vacuole are strictly regulated by bacterially encoded proteins, which are delivered through the two type-III secretion systems (TTSS) encoded by *Salmonella enterica* in its pathogenicity island 1 (SPI-1) and 2 (SPI-2). In particular, the SPI-1 TTSS promotes bacterial internalization and formation of an intracellular niche by modulating phosphoinositide metabolism and the activity of Rho-family GTPases. Later during infection, effector proteins delivered by the SPI-2 TTSS further modulate host intracellular membrane traffic to maintain a specialized compartment that allows its replication.

Salmonella enterica serovar Typhi (*S. Typhi*) is an exclusive human pathogen that causes typhoid fever, a systemic life threatening disease [20,21]. Unlike other *Salmonella enterica* serovars, which cause localized intestinal infection, *S. Typhi* establishes infection by breaching the intestinal epithelial barrier colonizing deeper tissues, such as the reticuloendothelial system, spleen and liver [21]. A unique property of *S. Typhi* is that, if untreated, it can cause persistent, often lifetime, infection by establishing residence in specific organs, most commonly the gall bladder. The molecular bases for *S. Typhi*'s unique virulence properties are not known but it is hypothesized that they may be the result of a combination of genome reduction and the acquisition of unique virulence genes [22,23]. Comparison of the genome of *S. Typhi* with those of other *S. enterica* serovars revealed the presence of a small number of putative virulence genes that are unique to this serovar or have a very limited distribution among other *S. enterica* serovars, and therefore may contribute to its distinctive virulence properties. Investigating these unique genes has shed light on the mechanisms leading to a persistent infection.

Exotoxin delivery by an intracellular pathogen

One of the unique features of the *S. Typhi* genome is the presence of a small genomic islet containing 5 open-reading frames. One of these genes encodes a homologue of CdtB, the active or “A” subunit of the cytolethal distending toxin (CDT) [24,25]. This toxin, which is a tripartite AB-type toxin expressed by several bacterial pathogens, interferes with eukaryotic cell cycle progression [24,26]. In epithelial cells CDT induces a dramatic cellular distension, as a consequence of G2-phase cell cycle arrest [27–29], while in lymphocytes and monocytes it causes apoptosis [30–34]. CDT induces activation of the ATM-dependent DNA damage response and the formation of DNA repair complexes [35,36]. The CDT active subunit CdtB shares limited amino acid sequence similarity with DNase I and is able to disrupt cell chromatin when microinjected into cells at very low concentrations [37,38]. Therefore, by generating double strand breaks in the chromosomal DNA, CdtB initiates a DNA damage response in the intoxicated cell that finally results in cell cycle arrest or apoptosis [24,26]. However, to be delivered into target cells, CdtB requires the assistance of a B subunit, which is composed of CdtA and CdtC, two ricin-like lectin domain proteins [39,40]. Similarly to other AB-type

toxins, the B subunit is responsible for the interaction of the complex with a surface receptor and the internalization and endocytic traffic of the active subunit CdtB [41].

The *S. Typhi* genome does not encode any homologue of CdtA or CdtC. Surprisingly, however, intestinal epithelial cells infected with *S. Typhi* undergo G2-phase cell cycle arrest and cellular distension in a CdtB-dependent manner [25]. This observation raised the issue of how the CdtB protein is delivered into host cells. The first clue to answer this question came from the observation that CdtB expression is induced after bacteria are internalized into host cells and, consequently, intoxication is strictly dependent on bacterial internalization [25]. These findings indicated that the delivery of CdtB to the target cells occurs from bacteria that already reside within a phagocytic vacuole.

Therefore, before delivery, CdtB must first somehow cross the vacuolar membrane. Since neither of the TTSSs are involved in the delivery of CdtB [25], it follows that this toxin must utilize specific mechanisms to reach the nucleus, its site of action. Quite unexpectedly, further studies showed that in order to be delivered to its site of action, CdtB has to be transported outside of the infected cell, where through autocrine and paracrine pathways, it can intoxicate both infected and uninfected cells [13]. This finding originated from the initial observation that during bacterial infection of tissue culture cells, not only cells containing bacteria but also those that have not internalized any bacteria, undergo cell cycle arrest and cellular distension. Final proof for the autocrine and paracrine pathway of CdtB delivery came from experiments showing that: 1) toxic activity can be recovered from the extracellular medium; and 2) addition of a toxin-neutralizing antibody to the medium of infected cells can prevent intoxication. Interestingly, the neutralizing antibody not only blocks the intoxication of uninfected cells but also prevents the intoxication of all the cells in the tissue culture dish. These results indicate that even the intoxication of infected cells occurs preferentially through a pathway requiring extracellular delivery of the toxin [13].

The finding that CdtB-dependent intoxication requires its transport to the extracellular medium raised the question of how this may occur. Immunofluorescence experiments showed that ~5 hours post-infection CdtB can be seen in association with intracellular bacteria. However, later in infection (<12 hours post infection), CdtB can be seen associated with small intracellular puncta radiating from the bacteria and 24 hours after infection CdtB-stained puncta can be seen throughout the entire cell (Fig. 2). Subcellular fractionation of infected cells showed that the CdtB fraction that is not directly associated with bacteria is contained in a membrane-bound compartment [13]. These observations suggest that CdtB is transported out of the *Salmonella*-containing vacuole in transport carriers. It is possible that these carriers, once formed, move to the periphery fusing to the plasma membrane to release the toxin to the extracellular space. Understanding the molecular mechanism of this transport pathway will certainly require further investigation. At this point it is unknown whether this transport mechanism utilizes cellular machinery known to be involved in well-described budding or exocytic pathways. However, the fact that the *Salmonella*-containing vacuole is a unique compartment suggests that the machinery involved in the generation and traffic of the transport carriers may also be unique. It is possible that the characterization of this machinery may reveal yet undescribed vesicle traffic or exocytic pathways. In any event, these observations clearly indicate that the *Salmonella*-containing vacuole is a dynamic compartment capable of exchanging cargo with other cellular compartments. Interestingly, a dynamic behavior of a bacteria-containing vacuole has been previously reported for *Mycobacterium tuberculosis*, although in this case it was shown that bacterial lipids are transported outside of the bacteria-containing phagosome [42]. Taken together these observations have important implications not only for toxin delivery but also for the interaction of intracellular pathogens with the immune system.

Combining two toxic activities: the typhoid toxin

While the host-cell factors involved in the transport of CdtB to the extracellular medium are still unknown, two bacterial proteins involved in this process were serendipitously identified. These two proteins, PltA and PltB, are encoded by an operon contained in the same genomic islet that encodes CdtB. PltA and PltB show significant amino acid sequence similarity to the pertussis toxin ADP-ribosyltransferase A subunit, and to just one of the 5 components of its heteropentameric “B” subunit, respectively [43]. There is no other gene in the *S. Typhi* genome encoding homologues of the other components of the pertussis “B” subunit, establishing an intriguing parallel with CdtB, namely the presence of homologues for A subunits of well characterized toxins but in the absence of homologues for critical components of the respective B subunits. A rather unexpected finding was that *pltA* or *pltB*-defective *S. Typhi* strains lacked CdtB-dependent toxicity [13]. Further studies determined that such a toxicity defect is due to the failure of CdtB to exit the *Salmonella*-containing vacuole in the absence of PltA or PltB. In addition, PltA and PltB can form a complex with CdtB, and the three proteins co-localize in infected cells. The ADP-ribosyltransferase activity of PltA is not required for CdtB export to the extracellular medium and cytolethal distending toxicity [13]. This finding suggests that formation of the CdtB/PltA/PltB complex is required for its recognition by the cellular machinery that transports it from the *Salmonella*-containing vacuole to the extracellular medium. Furthermore, the *in vitro*-assembled complex formed by recombinant CdtB, PltA and PltB is able to intoxicate tissue culture cells when added to the extracellular medium, while CdtB alone or in combination with either PltA or PltB is not [13]. This observation indicates that formation of the tripartite complex is also required for the delivery of CdtB after its release to the extracellular medium. Therefore, CdtB/PltA/PltB constitutes the typhoid toxin, a multipartite exotoxin with two distinct biochemical activities, a DNase activity associated with CdtB, and an ADP-ribosylating activity associated with PltA. It is likely that evolution has reshaped what may have originally been two independent toxins to combine them into one single toxin that is suited for its delivery from an intracellular location, and hence adapted to the biology of an intracellular pathogen.

Biological significance of the paracrine intoxication pathway in *S. Typhi* pathogenicity

What advantages might this unusual toxin delivery pathway confer to *S. Typhi*? Unlike other *S. enterica* serovars, *S. Typhi* is known to persistently infect humans for extended periods of time and, if untreated, potentially for life. It is thought that *S. Typhi* establishes residence within an intracellular compartment (presumably in the gall bladder) although the specific cell type (s) in which it may reside has not been defined [14,21]. It is possible that *S. Typhi* exploits typhoid toxin and its unique delivery pathway to target cells other than those infected by these bacteria. A necessary assumption for this hypothesis is that the cells infected by *S. Typhi* must lack the toxin receptor and therefore would be refractory to the autocrine delivery of the toxin. Therefore, according to the model described in Fig. 3, infected cells lacking receptors for the toxin could represent a safe haven for the bacteria and at the same time serve as a source of toxin that could act on other cells, even at systemic sites, if the toxin can access the lymph or the bloodstream. CDTs produced by other bacterial pathogens are known to hamper the activity of immune cells through the action of their active subunit CdtB [24,34,44]. Thus, it is possible that the CdtB component of the typhoid toxin and its paracrine delivery pathway may be critical for *S. Typhi* to modulate the host immune response to establish a persistent infection.

Conclusions

Bacterial pathogens have developed a variety of different strategies to deliver proteins to the host. One of these strategies, which is discussed in this article, is adapted to the biology of an

intracellular pathogen able to persistently infect its host. The distinctive feature of this strategy is that it can deliver a multifunctional toxin to target cells other than the cell in which it is produced. The understanding of this pathway may help to understand not only the biology of intracellular pathogens such as *S. Typhi* but also it may reveal novel vesicle trafficking pathways. Furthermore, this pathway may reveal important aspects of the interaction of intracellular pathogens with the immune system.

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Figure 1. Different strategies for the delivery of bacterial toxins or effectors

In the simplest strategy, represented by the large clostridial cytotoxins, all the necessary information for toxin delivery and enzymatic activity is encoded in a single polypeptide (A). Another strategy, represented by the AB toxins, utilizes the B subunit to deliver the enzymatically active A subunit (B). The most complex strategy, represented by the type III and type IV secretion systems, involves multi-protein machines to deliver multiple effectors (C) (see text for details).

Figure 2. Localization of the CdtB subunit of the typhoid toxin in infected cells

Immunofluorescence staining of cells infected with a strain of *S. Typhi* that encodes a 3x-FLAG epitope-tagged CdtB. Cells were infected and stained at different times post-infection (as indicated) with a mouse monoclonal antibody specific for the FLAG epitope (green), and a rabbit antibody specific for *S. Typhi* (red), and DAPI (blue). Scale bar, 5 μ m.

Fig. 3. Model for typhoid toxin delivery

After internalization (I) *S. Typhi* reaches a compartment where expression of *cdtB*, *pltA*, and *pltB* can take place (II). The CdtB/PltA/PltB complex is secreted from the bacteria into the lumen of the *S. Typhi* containing vacuole and it is recognized and packaged into transport carriers (III). The complex is then transported to the plasma membrane and secreted to the extracellular medium (IV) from where it can target susceptible non-infected cells (e. g. cells of the immune system) and induce DNA damage (V). Infected cells that do not express a receptor for the toxin would be resistant to the toxin and provide a safe haven for the bacteria (IV and V) (adapted from [13])