



Portals and Pathways: Principles of Bacterial Toxin Entry into Host Cells

Many pathogenic bacteria generate potent toxins that breach the membrane barrier of host cells to access their intracellular targets

Steven R. Blanke

During infection, pathogenic microbes actively remodel host cells and tissues to create a more suitable niche for withstanding the rigors of the host environment. One of the most effective remodeling strategies is the generation of protein toxins that modulate important functions of both immune and nonimmune cells. The central importance of toxins to the virulence strategies of pathogens is most poignantly illustrated in diseases such as diphtheria or botulism, where essentially all of the symptoms can be attributed to toxins acting upon their host cells.

Many of the most potent bacterial toxins act inside host cells (see table). Their site of action is perhaps not surprising, if one considers that from the perspective of pathogenic microbes, the eukaryotic intracellular environment is a treasure trove of regulatory pathways and networks that are ripe for manipulation. The potency of intracellularly acting toxins is derived, in part, from their mode of action; most are enzymes that catalyze the covalent modification of specific molecular targets. To be successful, however, intracellularly acting toxins must access their substrates inside target cells. This is no small feat, as the eukaryotic plasma membrane is a formidable gatekeeper that effectively restricts macromolecules such as toxins from passing freely into cells. To overcome the membrane barrier, intracellularly acting toxins are either injected directly into host cells by pathogenic microbes or, alternatively, enter cells in a manner that is microbe-independent (Fig. 1).

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Overall Paradigms of Toxin Entry into Cells

Some gram-negative pathogens inject their toxins into the cytosol of host cells through bacterial transport machines that function as macromolecular syringes (Fig. 1). The syringes, which resemble either bacterial flagella or conjugative pili, facilitate the direct passage of toxin effectors from bacterial donor cells into eukaryotic cells by processes referred to as Type III or Type IV secretion mechanisms, respectively. The Type III and Type IV secretion machines do not allow the free exchange of proteins between prokaryotic and eukaryotic cells, but, instead, appear to selectively regulate which toxin effectors can pass through the syringe needle. While gram-positive bacteria do not appear to use

- Many bacterial pathogens generate toxins that act inside host cells, as a mechanism for remodeling the host environment.
- Pathogens directly inject toxins into host cells or, alternatively, release AB toxins, which must then enter host cells independent of the bacterium.
- AB toxins are “hard-wired” with properties that exploit cellular entry pathways, and these toxins co-opt host mechanisms for taking up molecules from their surfaces.
- Several questions surrounding entry of AB toxins into host cells remain unresolved, including how B fragments help to move A fragments of AB toxins across membranes and also how specific toxins are routed into cells by different mechanisms.

Type III and IV secretion mechanisms, some species apparently construct large pores within the plasma membrane of target cells that function as portals for direct effector delivery.

The direct delivery of toxin effectors into host cells has several implications for virulence. On one hand, direct injection is both efficient and selective, as the intimate interaction between microbes and host cells guarantees that most or all of the toxin effectors generated by a microbe can ultimately access their substrates. On the other hand, injection mechanisms limit the extent to which a pathogen can remodel the host, because a bacterium can directly affect only a single target cell at any given time. Pathogenic microbes exert a broader sphere of influence by releasing a bolus of toxin that can act upon many cells within a given tissue and/or diffuse away to modulate one or more types of cells at multiple locations within the host. For example, the lethal and edema toxins of *Bacillus anthracis* disseminate within the host bloodstream and act upon both immune and nonimmune cells. In this and similar cases, intracellularly acting, or AB, toxins are not injected into host cells by the bacterium, but must facilitate their own entry to access their targets (Fig. 1).

Actions of intracellularly acting toxins on target cells

Toxin	Pathogen	Activities
ExoS	<i>Pseudomonas aeruginosa</i>	ADP-ribosylation, GTPase activation
ExoT	<i>Pseudomonas aeruginosa</i>	ADP-ribosylation, GTPase activation
Adenylate cyclase	<i>Bordetella pertussis</i>	Adenylate cyclase, hemolysis
Cholera toxin	<i>Vibrio cholerae</i>	ADP-ribosylation, immunomodulatory effects
Pertussis toxin	<i>Bordetella pertussis</i>	ADP-ribosylation, T cell mitogen, hemagglutination, signal transduction through inositol phosphate
Shiga toxin	<i>Shigella dysenteriae</i>	Inhibition of protein synthesis, induction of cytokine expression
Toxin A	<i>Clostridium difficile</i>	Actin depolymerization, mitochondrial alterations and apoptosis
Lethal toxin	<i>Bacillus anthracis</i>	Alterations in dendritic cell function, apoptosis of various cell types, Hypoxic tissue injury, repression of glucocorticoid receptor transactivation, lysis of macrophages
PMT	<i>Pasteurella multocida</i>	Cytotoxicity of various cell types, mitogenesis of various cell types, immunomodulatory effects
Aerolysin	<i>Aeromonas hydrophila</i>	Hemolysis, cell vacuolation
Alpha toxin	<i>Staphylococcus aureus</i>	Hemolysis, apoptosis
Enterotoxin	<i>Clostridium perfringens</i>	Cytotoxicity, loosening of tight junctions
Pneumolysin	<i>Streptococcus pneumoniae</i>	Cytolysis, complement activation
RTX toxin	<i>Vibrio cholerae</i>	Cell rounding, actin cross-linking
VacA	<i>Helicobacter pylori</i>	Alterations in late endosomes, alterations in mitochondrial membrane permeability, inhibition of T-cell proliferation

Principles of AB Toxin Entry into Cells

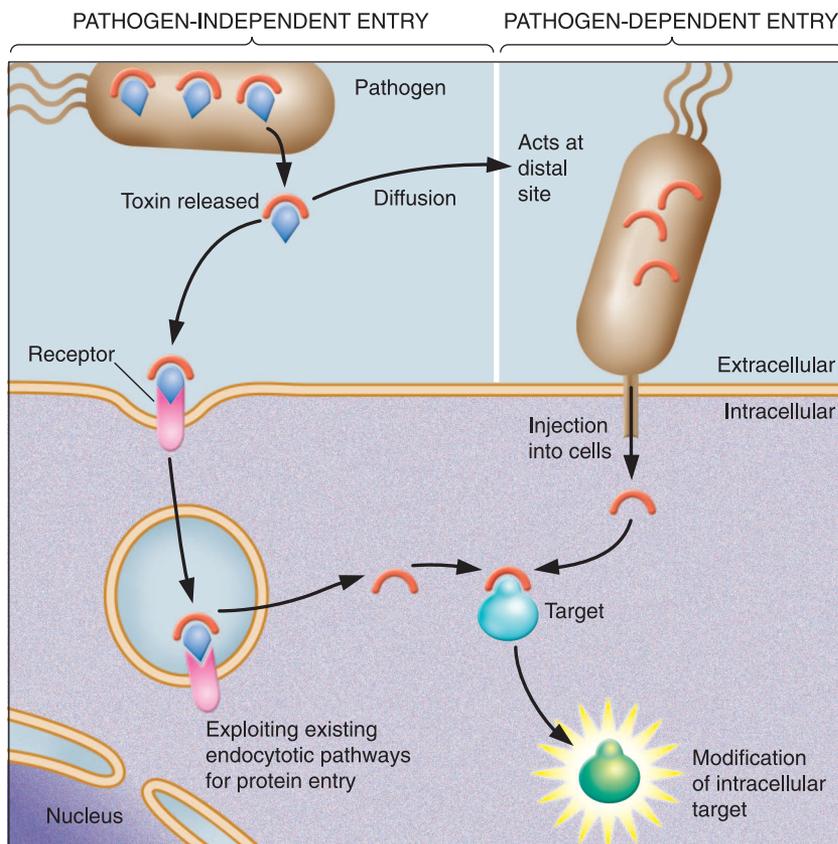
As the first step in cellular entry, AB toxins bind to one or more plasma membrane surface receptors (Fig. 1), which can be proteins, glycoproteins, or glycolipids. Host cells lacking such receptors are generally resistant to intoxication, underscoring the importance of toxin-receptor complexes. Some receptor-bound toxins (e.g., diphtheria toxin, *Pseudomonas* exotoxin A, and Shiga toxin) are targeted for clathrin-dependent endocytosis, which is how eukaryotic cells take up proteins and lipids from their surfaces. Other toxins, including cholera and tetanus toxins, enter cells by one of several alternative “clathrin-independent” endocytic pathways. Ultimately, AB toxins are trafficked to translocation portals, where they cross the membrane barrier into the cytosol. Collectively, these observations illustrate an important principle about AB toxins—namely, to access their intracellular substrates,

they coopt existing mechanisms for taking up molecular cargo from the host cell surface.

However, there is a second important principle, which is that AB toxins are not simply delivered into cells as passive cargo, but, instead, must be “hard-wired” to engage and exploit cellular entry pathways. AB toxins incorporate two discrete and essential functional components that vary considerably in their physical arrangement but are generally conserved in terms of function (Fig. 2). Thus, toxin “A fragments” are the active moiety that can modify intracellular target molecules by one of several identified enzymatic activities, including ADP-ribosylation, UDP-glucosylation, or proteolysis. Meanwhile, the “B fragments” serve as delivery vehicles for their A components by binding to plasma membrane surface receptors and facilitating translocation of the A components into the cytosol through available portals.



FIGURE 1



Intracellularly acting bacterial toxins access their substrates within host cells by one of several mechanisms. Some gram-negative pathogens directly inject toxin effectors through flagellar- or pilus-adapted transport machines into eukaryotic cells by Type III or IV secretion systems, respectively. Alternatively, bacteria release intracellular-acting toxins, also called AB toxins, into the host environment where they act locally or diffuse to act distally to the site of colonization. AB toxins commonly exploit endocytic pathways that eukaryotic cells use for importing proteins. Finally, *Bordetella* adenylate cyclase toxins directly enter the cytosol from the plasma membrane.

Toxins Exploit the Acidic Environment of Endosomal Compartments

To transport their A fragments into the cytosol, some AB toxins, including diphtheria, anthrax, and the botulinum neurotoxins, exploit the drop in pH to between 5.0 and 6.0 as endocytic vesicles are trafficked from the plasma membrane into the cell (Fig. 3). Endosome acidification triggers profound structural changes in these toxins, resulting in the insertion of B fragments into the membrane and the formation of ion-conducting channels. Partially unfolded A fragments are generally thought to use these B fragment-derived channels as conduits into the

cytosol, although it has been challenging to prove this model.

While toxin B fragments sense and respond to the relatively low pH of host endosomes, toxin A fragments also embody properties that promote translocation. For instance, they must be able to reversibly unfold at pH 5–6, engage the translocation machinery, pass through the endosomal membrane, and then functionally refold within the cytosol. Recently, host factors have been implicated in this process, as suggested by the discovery that *in vitro* translocation from endosomes requires both a source of energy (ATP) and a host cell cytosolic translocation factor (CTF) complex consisting of chaperonin heat shock protein (Hsp) 90 and thioredoxin reductase. Thus, bacterial toxins engage host-cell components that are ordinarily involved in protein folding.

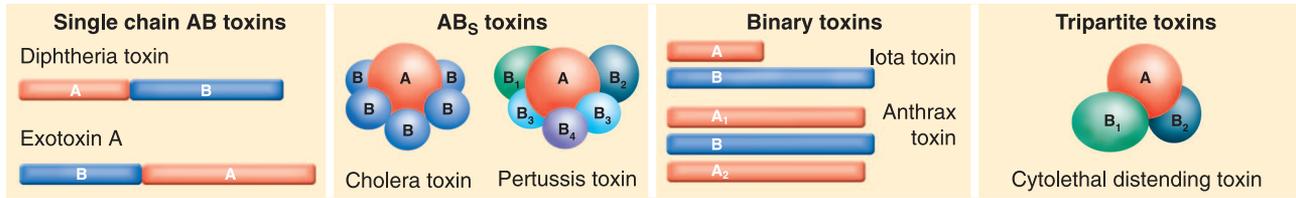
Toxins Exploit the Sec61 Retro-Translocon in the Endoplasmic Reticulum

Members of a second group of AB toxins access cytosolic substrates by exploiting membrane transport complexes located in the endoplasmic reticulum (ER) (Fig. 3). Upon entering their target cells, these toxins exploit one of several “retrograde” trafficking pathways destined for the ER. Within the lumen of the ER, the A fragments are transported through an existing

membrane complex whose primary player is a protein called Sec61. The Sec61 complex ordinarily functions as a “retrotranslocon”—sending improperly folded proteins back into the cytosol to be degraded in a proteasome-dependent manner.

How is it then that some bacterial toxins can successfully exploit a cellular system whose primary function is to send misfolded proteins to their destruction? The answer to this question may again derive from several intriguing properties hardwired into the cargo itself. First, the crystal structures of several of these A fragments reveal regions of poorly defined electron densities, suggesting that they may engage the Sec61

FIGURE 2



The diverse architectures of AB intracellular acting toxins. AB intracellular-interacting toxins are classified according to their architectures. In general, the B fragments are responsible for transporting the catalytic A fragments into cells. For single chain AB toxins, including diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A, the A and B fragments are contiguous, until separated by a host cell protease (furin) and reduction of a disulfide linkage. Among the AB₅ toxins, including cholera toxin, the *E. coli* heat-labile toxins, pertussis toxin, and the Shiga toxins, discrete genes encode the A and B fragments, which assemble as non-covalent complexes in which the A fragment rests upon a donut-shaped pentamer of B fragments. The A fragment may be cleaved into two polypeptides, with the catalytic A₁ fragment released from the shorter A₂ fragment that extends down into the hole of the B-fragment-derived pentamer. For the binary toxins, including anthrax toxins and the *Clostridium perfringens* iota toxin, discrete genes encode the A and B fragments, which are released as separate polypeptides that interact at the surface of the host cell following activation of the B fragment. The tripartite AB toxins, represented by the cytolethal distending toxins, are encoded as three separate fragments, with two of them combining as the B fragment to deliver the single A fragment into cells.

retrotranslocon by masquerading as partially misfolded proteins.

How do transported toxin fragments escape proteasome-mediated degradation? Proteins destined for degradation typically are posttranslationally decorated with ubiquitin on their lysine residues. However, toxin catalytic fragments transported to the cytosol through the Sec61 retrotranslocon contain remarkably few lysine residues, thereby escaping ubiquitination-mediated targeting to host proteasomes. These toxins must have idiosyncratic properties allowing them to enter the cytosol through the ER retrotranslocon.

Applying the Lessons Learned from Intracellularly Acting Bacterial Toxins

There has been an enormous outlay of intellectual and financial capital in the development of systems and vectors for the delivery of macromolecular-based therapeutics into cells. However, the pharmaceutical industry has been largely unable to emulate the success of pathogenic microbes in sending proteins into mammalian cells.

One of the most important lessons learned from the study of AB toxins is that the intended protein cargo must not only possess desired intracellular modulating activities, but must also be hard-wired with innate properties for delivery. For example, toxin A fragments partially unfold to pass through either toxin-medi-

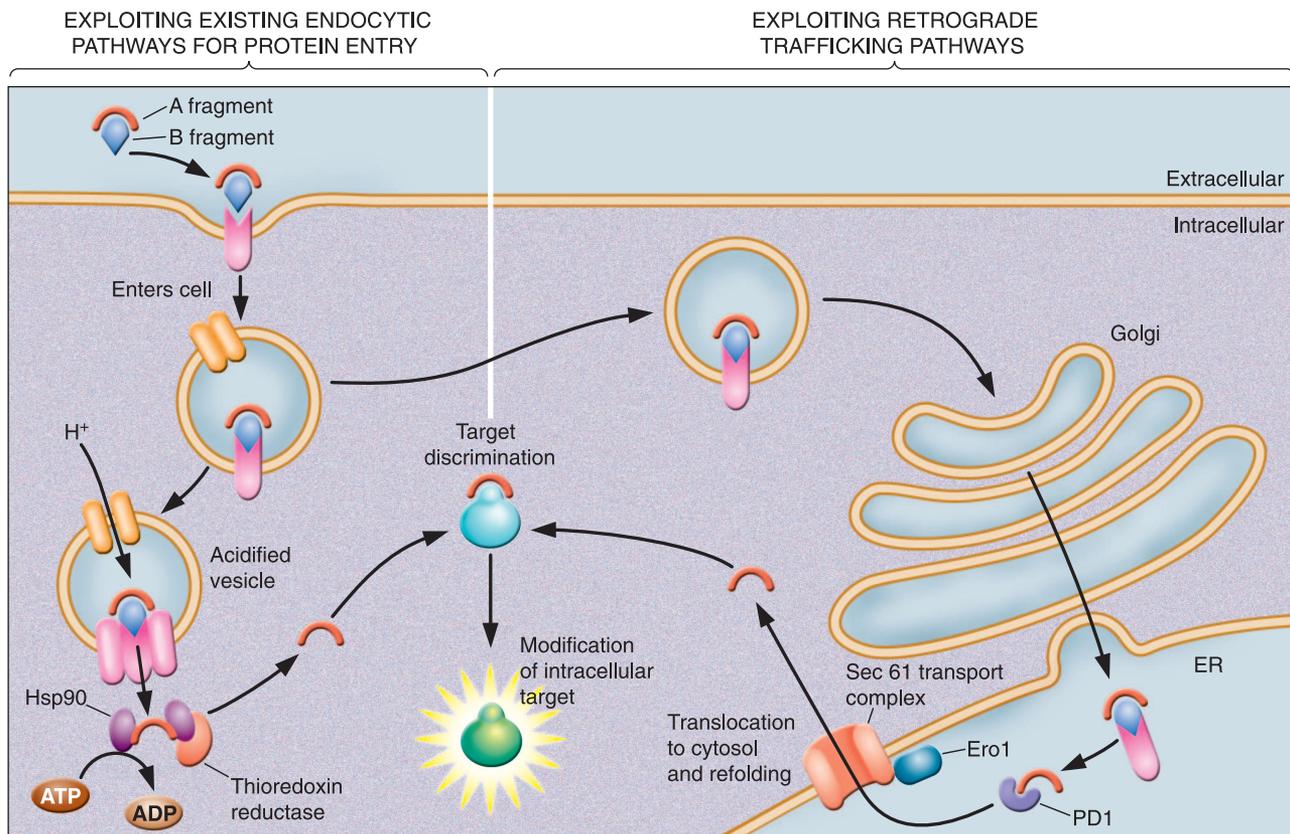
ated or Sec61 retrotranslocon-mediated transport channels. However, many potential protein cargos, including those that are stabilized with extensive disulfide linkages, may not be capable of being readily unfolded (and refolded) during translocation. In addition, toxin A fragments that pass through the Sec61 retrotranslocon have additional primary sequence requirements (e.g., lysine paucity within the primary structure) that, again, may be too restrictive for most proteins. Finally, despite the apparent success of AB toxins in modulating host cells, there is considerable evidence that the translocation process is relatively inefficient, implying that protein delivery into cells may be restricted to enzymes, so that by catalytic turnover, the effects of even a few successfully transported molecules may be amplified throughout the cell.

Despite inherent difficulties in delivering exogenous proteins into cells, there are success stories. By swapping the normal toxin receptor-binding domain with antibodies or portions of antibodies targeting surface receptors, immunotoxins have been selectively sent into malignant cells to kill them (Fig. 4). Moreover, the anthrax toxin has been engineered to deliver T-cytotoxic lymphocyte-stimulating epitopes into cells for processing and MHC class I presentation that stimulates a T-cell response with the capacity to be protective.

Exciting new ideas are also emerging from many areas of chemistry and bioengineering that may circumvent restrictions on protein de-



FIGURE 3



Entry of AB toxins into target cells. Some toxins use host endocytic pathways that cells use ordinarily for degrading exogenous proteins within lysosomes. However, several AB toxins, including diphtheria, anthrax, and the botulinum neurotoxins, translocate their A fragments into the cytosol by exploiting the acidic environment of early endosomes. Endosome acidification triggers conformational changes, with toxins inserting their B fragments into the membrane and forming ion-conducting channels. Partially unfolded A fragments use these toxin-derived channels as conduits into the cytosol. Host factors are also involved. Other AB toxins exploit the degradation pathway for misfolded proteins. Nascent proteins destined for secretion are transported from the endoplasmic reticulum (ER), via the Golgi stack and the trans-Golgi network (TGN) to secretory vesicles that fuse with the plasma membrane to release the proteins. The secretion pathway is at least partially reversible, enabling several bacterial toxins to travel this “retrograde” pathway from the plasma membrane to the ER lumen through pores to the cytosol. Several AB toxins, including cholera toxin, shiga toxin, and *Pseudomonas aeruginosa* exotoxin A, enter the cytosol through the ER complex whose primary protein is Sec61. The B fragments of these toxins bind to receptors to facilitate the trafficking of catalytic A fragments to the ER. Toxin A fragments pass through the Sec61 retrotranslocon, but escape ubiquitination and proteasome-mediated degradation.

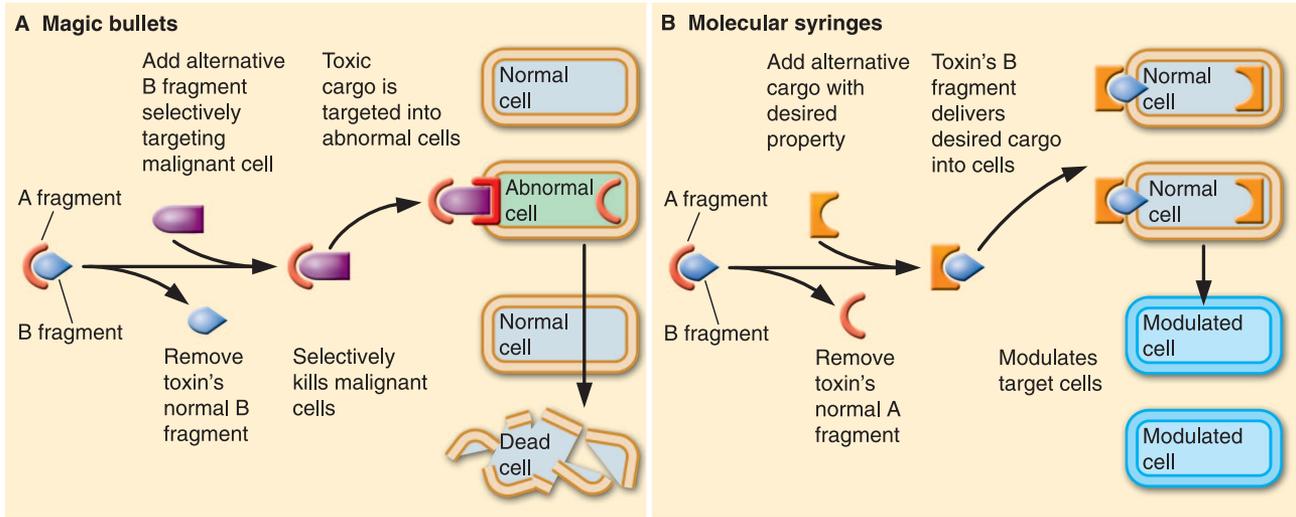
livery. For example, “smart polymers” are being targeted to endosomes that, upon sensing the lowering of pH, interact with and destabilize endosomal membranes to promote the controlled release of endosomal contents into the cytosol. Such a system would potentially increase the repertoire of molecules that could be delivered into the cytosol by removing the constraints of having to be translocated through the membrane. A striking feature is that many systems under development exploit some of the same innate properties of target cells used by AB

toxins, such as the capacity to target proteins for internalization through existing endocytic mechanisms.

Future Prospects

Despite intensive study, many exciting issues surrounding the entry of AB toxins into host cells remain unresolved. The mechanisms underlying the “routing” of toxins into cells, including the extent to which receptor binding determines both the trafficking patterns and ultimate destination, may not be as clear-cut as

FIGURE 4



Efforts to develop macromolecule-based therapeutics based on attributes of bacterial toxins. Some investigators are seeking to exploit attributes of toxins in protein-based therapeutics. (A) Magic bullets—by linking toxin catalytic A fragments to the receptor binding domains of eukaryotic proteins, the goal is to selectively deliver the catalytic domains of some toxins into malignant cells. (B) Molecular syringes—the goal is to use toxin B fragments to deliver heterologous cargo into target cells.

previously thought. Indeed, several toxins, including cholera toxin, have the capacity to enter cells by several different mechanisms mediated by the same receptor. The issue is further confounded by the demonstration that some toxins, such as the vacuolating cytotoxin (VacA) from *Helicobacter pylori*, exert differential effects upon multiple cell types. Because VacA binds to multiple components on the plasma membrane surface of a single cell type, an exciting possibility is that it might engage alternative entry pathways to access multiple targets within the same cell.

The fate of toxin A fragments subsequent to their translocation into the cytosol is also poorly understood. While some catalytic domains may simply diffuse to their intracellular substrates, signals for intracellular targeting may also be hard-wired in some toxins, as recently shown for the *Pseudomonas aeruginosa* Type III effector ExoS and the catalytic fragment of the *Clo-*

tridium botulinum neurotoxin B. The details of how these targeting signals function, as well as whether other toxins require additional mechanisms to locate their targets within the highly compartmentalized intracellular milieu, are not understood.

Probably the least understood step in the entry process of AB toxins remains membrane translocation, especially the exact mechanism(s) by which B fragments facilitate the movement of the A fragment across the membrane.

Finally, it remains to be seen whether all the portals and pathways used by bacterial toxins to enter the cell have been identified. The discovery of additional cellular entry pathways and mechanisms would not only provide novel insights into eukaryotic cell biology, but also benefit ongoing efforts to design protein-based therapeutics that act upon intracellular molecular targets.

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