JCB Comment

To be helped or not helped, that is the question

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Diphtheria toxin (DT)* is the paradigm of the powerful A-B toxins. These bacterial poisons bind to cells, are endocytosed, and inject their catalytic domain into the cytosol causing the irreversible modification of a key component of the the host cellular machinery. The mechanism by which the hydrophilic enzymatic fragment of DT crosses the endosomal membrane and is released into the cytosol remains controversial. In this issue, Ratts et al. (2003) demonstrate that delivery of the DT catalytic domain from the lumen of purified early endosomes to the external medium requires the addition of a cytosolic translocation factor complex composed in part of Hsp90 and thioredoxin reductase.

Diphtheria was the first infectious disease to be studied at the molecular and cellular level (Pappenheimer, 1993). Roux and Yersin demonstrated in 1888 that a thermolabile and protease sensitive "poison" secreted by *Corynebacterium diphtheriae* was entirely responsible for the fatal disease. Upon the discovery of toxins, Paul Erlich put forward the idea that retargeting these proteins, molecules that he named "magic bullets," specifically to tumors cells might cure cancers. DT is the best studied A-B toxin to date, and was the first to be retargeted through genetic engineering, and DAB_{389}IL-2 (Ontak®) is the first fusion protein toxin approved for the treatment of human disease (vanderSpek and Murphy, 2000). However, the mechanism by which DT "injects" its enzymatic fragment through the membrane of early endosomes into the cytosol has indeed remained a puzzling and controversial problem. In this issue, the group of John Murphy (Ratts et al., 2003) provides evidence that the translocation of DT catalytic fragment (DT-C) out of purified early endosomes is not an autonomous mechanism performed by the toxin itself, as previously suggested (Oh et al., 1999), but requires host cellular factors.

DT consists of a catalytic A chain (21 kD) and a B chain (37 kD), which are linked by a disulfide bridge. The B chain encompasses both the transmembrane (DT-T) and receptor binding (DT-R). After binding to its cognate host cell receptor and endocytosis, the toxin reaches early endosomes, where it partially unfolds within the low pH of this compartment, unmasking the hydrophobic helices of DT-T. This allows membrane insertion of DT-T and translocation of DT-C into the cytosol. Within the cytosol, DT-C catalyzes the NAD^+-dependent ADP-ribosylation of elongation factor 2 (EF-2), thereby stopping protein synthesis and resulting in cell death (Collier, 2001). Studies performed with artificial planar lipid bilayers and unilammelar liposomes brought forward the idea that DT-T domain insertion into lipidic membranes at low pH is sufficient to allow DT-C to flip from the cis to the trans side of the leaflet (Oh et al., 1999). In this model, translocation is performed by the transient association–dissociation events between the T domain, playing the role of a transmembrane chaperone and the partially unfolded hydrophophic molten globule-like DT-C fragment (Ren et al., 1999). These results led to the notion that the DT-T domain contains the entire molecular machinery for mediating the transfer of DT-C across a membrane (Oh et al., 1999). In this model, DT uses an amazingly simple system for translocation of a large hydrophilic protein through lipid membranes.

In their studies, Ratts et al. (2003) took a different approach to study the DT-C translocation. They made use of an assay previously described for DT-C translocation across purified early endosomes (Lemichez et al., 1997), in a study which also demonstrated the necessity of crude cellular extracts for productive translocation of DT-C. This assay is based on the unique ability of DT, as compared with other toxins of this family, to selectively ADP-ribosylate EF2. By using radiolabeled NAD, concentrations as low as 10^{-14}–10^{-16} M DT-C can be detected. In their current study, Ratts et al. (2003) used DAB_{389}IL-2 instead of DT, a chimeric toxin constructed by substituting the native receptor-binding domain of DT with human interleukin-2 (hIL-2) (Ratts and vanderSpek, 2002). DAB_{389}IL-2 shares all the properties of a bona fide DT except that it binds to high affinity IL-2 receptors with a K_d similar to that of native IL-2. Using this approach, Ratts et al. (2003) now report that the in vitro translocation of DT-C from the lumen of acidified early endosomes into the cytosol requires a host cell cytosolic translocation factor (CTF) complex.

The CTF complex was purified from either human T cells or yeast extracts and found to contain multiple protein bands ranging in apparent molecular mass from 12 to 100 kD. Identifying individual components of this complex by mass spectrometry and the use of specific inhibitors, Ratts et

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*Abbreviations used in this paper: DT, diphtheria toxin; DT-C, DT catalytic fragment; DT-T, DT transmembrane; EF-2, elongation factor 2.

al. (2003) found that Hsp90 (or its orthologue Hsp82 from yeast extracts) as well as thioredoxin reductase (TrR-1) were essential but not sufficient for DT-C translocation. To establish the functional role of Hsp90 and TrR-1 for DT-C translocation, the authors conducted a series of immunoprecipitation experiments and demonstrated that the removal of these proteins from the CTF complex prevented DT-C translocation across endosomal membranes. A role for Hsp90 as merely a "cofactor" required for DT-C activity seems to have been excluded since adding Hsp90 back to the immunodepleted CTF complex did not restore ADP ribosylation activity to the external medium. In addition, using two inhibitors of Hsp90 geldanamycin and radicicol, and cis-13-retinoic acid, an inhibitor of TrR-1, Ratts et al. (2003) were able to show that both factors participate in DT-C translocation both in vitro and in vivo. These observations favor a role forHsp90 and/or TrR1 in DT-C membrane translocation. However, to firmly establish that these proteins are integral components of the CTF complex and not indirectly acting to maintain the folded conformation of true components of the CTF, will require the complete reconstitution of the CTF complex in vitro.

These exciting results have initiated the process of purifying and identifying components of this CTF complex. Once highly purified, it will be interesting to determine whether the CTF complex acts directly on the DT-C domain by "traction" or facilitates its translocation in another manner. The need of cellular factors for the membrane translocation of toxin A domains might be a general requirement. For example, it is known that the membrane translocation of the cholera toxin catalytic domain needs the endoplasmic reticulum sec61 pore and chaperones (Tsai et al., 2002).

The earlier observations made through the use of artificial lipid bilayers led to the conclusion that DT-C domain translocation was likely to occur autonomously (Oh et al., 1999); whereas, using purified early endosomes a host cell CTF complex appears to be required for this process. These conflicting results seem to raise a paradox. However, as indicated by Ratts et al. (2003), both hypotheses might be reconciled in view of earlier observations showing that the structure of DT upon insertion into lipid bilayers differs depending on whether it is embedded in either cellular or artificial membranes. It is also important to point out that the assay used by Ratts et al. does not directly monitor the DT-C movement across the lipid membrane. However, Ratts et al., by following the decrease in DT-C enzymatic activity in the lumen of endosomes and its increase in the external medium, show that a productive membrane translocation of this molecule has likely occurred.

The possible involvement of Hsp90 in the mechanism of DT-C membrane translocation might also be an interesting finding since this protein is different from other chaperones in that most of its known substrates to date are signal transduction proteins such as steroid hormone receptors and signaling kinases (Young et al., 2001). Very recently, it has been shown that binding of Hsp90 to certain mitochondrial preproteins is required for their docking to the Tom70 import receptor and their translocation into the inner membrane/matrix (Young et al., 2003). This indicates a new role for Hsp90 in membrane protein translocation, and may be related to its detection in the CTF complex.

Following the observations of Ratts et al. (2003), which provide evidence that cellular factors are required for DT-C translocation, one is tempted to speculate that this mechanism may have broader implications. For example, the Botulinum neurotoxins and *Bacillus anthracis* toxins are deadly poisons, which might be used for bioterrorism. These toxins, like DT, are A-B toxins that require passage through an acidic endosomal compartment to inject their catalytic fragment into the cytosol. The assay reported by Ratts et al. (2003) may also allow screening for drugs that may impair the translocation of DT-C and the catalytic domain of other toxins into the cytosol. Such an assay may also allow the screening for pharmaceutical drugs that enhance the activity of chimeric toxins by boosting the efficiency of the membrane translocation of their catalytic domain resulting in a larger family of targeted "magic bullets," thereby more completely fulfilling the dream of Paul Ehrlich some 100 year ago.

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