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Diphtheria Toxin and Cytosolic Translocation Factors

Ryan C. Ratts[1] and John R. Murphy[2]
[1]*Dartmouth Medical School*
[2]*Boston University School of Medicine*
USA

1. Introduction

Diphtheria Toxin (DT) was the first investigated bacterial protein toxin. As one of the most extensively studied bacterial protein toxins, it has served as a model system for the analysis of other protein toxins (Pappenheimer, 1977). As reviewed by Pappenheimer, Loeffler identified *Corynebacterium diphtheriae* as the causative agent of diphtheria in 1884, and the toxin was first described in the culture medium of *C. diphtheriae* by Roux and Yersin in 1888. The gene for DT is encoded by a family of closely related corynebacteriophages (Uchida et al., 1971; Greenfield et al., 1983), and is expressed only under conditions of iron deprivation (Pappenheimer, 1977). Regulation of DT expression is under control of the iron-activated diphtheria toxin repressor, DTxR, which is encoded in the *C. diphtheriae* genome and inhibits transcription of DT in the presence of iron and other transition metal ions (Love and Murphy, 2000).

DT is translated with a 25 amino acid signal peptide and is co-translationally secreted as a single 535 amino acid residue polypeptide chain with a molecular weight of 58 kDa (Smith, 1980; Kaczorek et al., 1983). Biochemical analysis of DT demonstrated that proteolytic 'nicking' of the toxin *in vitro* results in two fragments, A and B, which remain covalently attached by an inter-chain disulfide bond (Gill and Pappenheimer, 1971). Fragment A contains the enzymatic activity (Collier and Kandel, 1971), whereas Fragment B mediates binding to cell surface receptors and facilitates the cytosolic entry of fragment A (Drazin et al., 1971). X-ray crystallographic analysis, at a resolution of 2.5 Å, demonstrated that DT is composed of three structural domains: the amino terminal catalytic (C) domain corresponds to fragment A (21 kDa), and the transmembrane (T) and carboxy terminal receptor binding (R) domains comprise fragment B (37 kDa) (Figure 1) (Choe et al., 1992). A disulfide bond between Cys186 and Cys201 subtends a 14 amino acid protease sensitive loop and connects fragment A with fragment B (Gill and Pappenheimer, 1971). Furin mediated cleavage within this loop and retention of the disulfide bond have been shown to be pre-requisites for intoxication of eukaryotic cells (Ariansen et al., 1993; Tsuneoka et al., 1993).

Once delivered into cytosol, the C-domain catalyzes the NAD+-dependent ADP-ribosylation of elongation factor 2 (EF-2). EF-2 is a soluble translocase involved in protein synthesis, and is the only known substrate for the DT C domain in eukaryotic cells (Pappenheimer, 1977). Transfer of the ADP-ribosyl moiety of NAD^+ to a modified histidine

residue in EF-2 (diphthamide) results in the irreversible arrest of chain elongation during protein synthesis (Collier and Cole, 1969), leading to cell death by apoptosis.

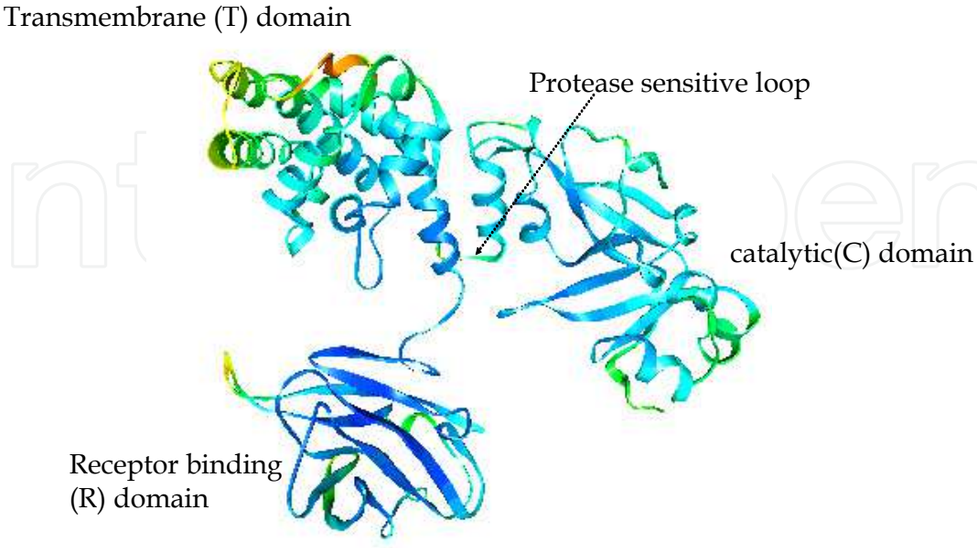

Fig. 1. X-Ray Crystallographic Structure of Diphtheria Toxin. PDB 1 MDT. Modified from Choe et al., 1992.

2. Receptor binding

Intoxication by DT involves an ordered sequence of events in which each structural domain of the toxin plays a precise and essential role and begins with toxin binding to cells expressing the heparin binding epidermal growth factor-like precursor (Figure 2) (Naglich et al., 1992). The sensitivity of targeted cells to intoxication by DT is roughly related to the number of receptors present on the cell surface, and is also enhanced by the diphtheria toxin receptor associated protein 27, DTRAP 27, which is the primate homologue of human CD9 (Mitamura et al., 1992). Human CD9 antigen, which is associated with the DT receptor but not DT itself, enhances sensitivity to DT through an unknown mechanism (Brown et al, 1993; Iwamoto et al., 1994),. In contrast to other AB toxins – such as abrin, ricin, and cholera – neither gangliosides nor galactosides have any effect on DT binding and intoxication (Pappenheimer, 1977).

In 1896, Paul Ehrlic coined the phrase "Zauberkugeln", or "magic bullet," for specifically targeting cells causing disease. This dream was realized almost a hundred years later by Murphy et al. (1986) with the design and synthesis of DT based fusion protein toxins that were targeted toward specific eukaryotic cell receptors. Substitution of the native R domain with a surrogate ligand results in the formation of a fusion protein toxin construct that targets cells expressing the appropriate cell surface receptor. The first genetically engineered fusion protein toxin, DAB_{486}MSH, consisted of DT fragment A and a portion of fragment B fused to α-melanocyte stimulating hormone (α-MSH) (Murphy et al., 1986). While this fusion protein toxin construct was prone to degradation, human interleukin 2 (IL-2) was selected as the next surrogate receptor binding domain and DAB_{486}IL-2 was next constructed (Williams et al., 1987). DAB_{486}IL-2 proved resistant to degradation, was

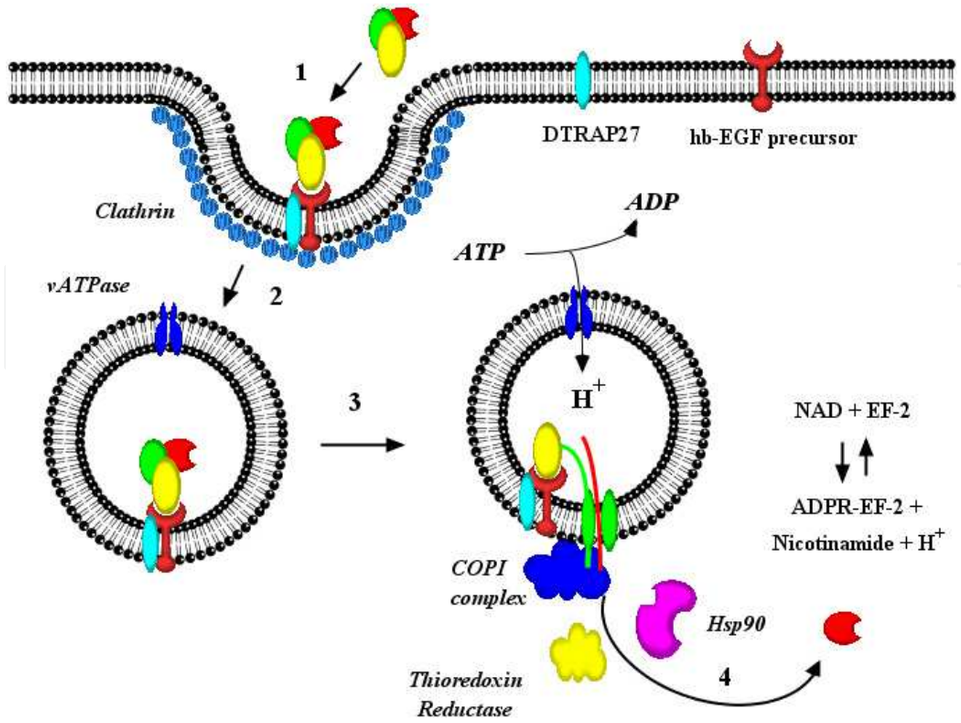

Fig. 2. Schematic Overiew of DT Intoxication. (1) DT binds to its cell surface receptor (2) Internalization of clathrin coated pits into early endosomal vesicles (3) Acidification of the endosomal lumen induces DT T domain insertion and pore formation (4) Translocation and cytosolic release of the DT C domain is facilitated by COPI complex, Thioredoxin Reductase and Hsp90. Refolded DT C domain catalyzes the ADP-ribosylation of EF-2. Diphtheria toxin: yellow = receptor binding (R) domain; green = transmembrane (T) domain; red = catalytic (C) domain. Reproduced from Murphy (2011).

remarkably potent (IC_{50} of 1 x 10^{-11} M), and was specifically targeted to cells only expressing high affinity IL-2 receptors (Bacha et al., 1980; Williams et al., 1990). Subsequent in-frame deletion analysis of the carboxy terminal residues in the DT portion of DAB_{486}IL-2 demonstrated that incorporation of a smaller portion of the diphtheria toxin fragment B, DAB_{389}IL-2, resulted in a chimeric toxin that was 10-fold more cytotoxic (Williams et al., 1990).

In the case of DAB_{389}IL-2, only the high affinity and intermediate affinity IL-2 receptor – toxin complexes are internalized (Waters et al., 1990). The specific expression of the high affinity IL-2 receptor on only activated and proliferating T-cells made DAB_{389}IL-2 a potential therapeutic agent for the treatment of both T-cell mediated malignancies and autoimmune diseases (Ratts and vanderSpek, 2002). In 1999, DAB_{389}IL-2 (ONTAK®) was the first fusion protein toxin construct approved by the U.S. Food and Drug Administration for clinical use in humans, and is currently used for the treatment of CD25 positive refractory cutaneous T cell lymphoma (Ratts and vanderSpek,2002). In designing fusion protein constructs, only those surrogate ligands that trigger clathrin dependent endocytosis, analogous to the mechanism of entry of endogenous DT, are functional. There are currently more than 20 different fusion protein toxins under clinical development. Since DAB_{389}IL-2 binds with

greater affinity to its receptor compared to native DT, this fusion protein toxin has proven to be an effective and novel probe for studying internalization of the C-domain by target cells.

3. Endocytosis

Receptor bound DT is concentrated in clathrin coated pits and internalized into clathrin coated vesicles (CCVs), which are then converted into early endosomes (Moya et al. 1985). Assembly of the clathrin coat is inhibited by depletion of intercellular potassium, and cells are protected against DT under such conditions (Moya et al., 1985; Sandvig et al., 1985). Sequestration of the coated pit from the plasma membrane requires additional proteins, including the GTPase dynamin (Simpson et al., 1998). Simpson et al. (1998) demonstrated that over expression of dominant negative dynamin blocks clathrin dependent endocytosis and protects cells against DT. Successful detachment of the coated pit results in CCVs which are subsequently released into the cytoplasm. After entering the cytoplasm, the CCVs are uncoated in an ATPase dependent manner and the subsequent homotypic fusion of uncoated vesicles results in the formation of early endosomes (Luzio et al., 2001). The clathrin triskelon is replaced with a new set of protein components on the vesicle membrane that includes Arf-1, COPI complex, Rab-5, early endosomal antigen (EEA1), and vesicular (v)-ATPase. Although the precise mechanism is unclear, the dynamic docking and release of these factors activates the process of membrane fusion through the formation of a fusion pore and activation of v-ATPase (Luzio et al, 2001).

The characteristic feature of endosomes is acidification of the lumen by v-ATPase. Acidification promotes protein sorting by dissociating ligand-receptor complexes and allowing some receptors to be recycled back to the plasma membrane. Acidification is also required for the formation of endosomal carrier vesicles (ECVs), which carry ligands and non-dissociated ligand-receptor complexes from early to late endosomes. Bafilomycin A1, a specific inhibitor of v-ATPase, blocks acidification and prevents the formation of ECVs, resulting in the accumulation of early endosomes (Bowman, et al.,1988). The formation of ECVs requires the binding of both β'COP (Sec 27) and ADP-ribosylation factor 1 (ARF1) to the cytoplasmic surface of the endosomal membrane, and binding of both factors is dependent upon a low pH within the endosomal lumen (Aniento et al., 1996). Inward invaginations of the endosomal membrane also occurs during ECV formation, resulting in the production of multi-vesicular bodies (Futter et al., 1996).

Several studies have confirmed the early endosomal compartment as the site fragment A translocation. Merion et al. (1983) showed that endosomes isolated from DT resistant mutants of chinese hamster ovary (CHO)-K1 cells were defective in acidification. In contrast, lysosomes isolated from the same mutants were not defective in acidification, suggesting that the endosomal compartment was the site of fragment A translocation (Merion et al., 1983). Umata et al. (1990) demonstrated that Bafilomycin A1, which prevents acidification of the endosomal lumen, protected cells against DT intoxication.

Cell fractionation experiments provided the best evidence that DT translocation occurs from early endosomes (Papini et al., 1993a; Papini et al. 1993b; Lemichez et al., 1997). While the DT C domain is most efficiently translocated from early endosomes, the majority of the toxin is actually sorted into ECVs and late endosomes where translocation of the C domain into the cytosol is marginal (Lemichez et al., 1997). Toxin trapped within ECVs and late

endosomes is ultimately targeted for lysosomes and degraded. Lemichez et al. (1997) provided two possible explanations for why toxin failed to translocate from ECVs or late endosomes: First, there might be cytosolic factors specific to early endosomes required for translocation. Second, translocation events might actually be occurring in the ECVs, but such events within the multi-vesicular body would result in the vectorial transfer of the DT C domain into the lumen of another intra-vaculolar vesicle rather than the cytosol.

4. Role for acidification

Ammonium salts (e.g., NH_4Cl), glutamine and other amines, and choloroquine were the first compounds found to inhibit the cytosolic entry of the DT (Kim et al., 1965; Sandvig et al., 1980). Although these compounds had no effect upon neither enzymatic activity nor receptor binding, these reagents did protect sensitive cells against DT intoxication. Choloroquine and ammonium salts are ionophores, and they raise the luminal pH of endosomes and lysosomes. These results led to the hypothesis that passage of DT through a low pH compartment was a required step for intoxication. Umata et al. (1990) confirmed this hypothesis by demonstrating that acidification of the endosomal lumen by membrane associated vesicular (v)-ATPase was a required step in DT intoxication.

In contrast to the endosomal route, low pH (5.5) exposure of toxin bound to the surface of cells results in decreased protein synthesis even in the presence of choloroquine and ammonium ions (Sandvig et al., 1980). This same study demonstrated that the entry of pre-nicked diphtheria toxin through the cell membrane in the low pH environment was time and temperature dependent. Using the same system, Sandvig and Olsnses (1981) also demonstrated that the cytosolic entry of the DT C domain could be blocked by the metabolic inhibitors 2-deoxyglucose and sodium azide, implying that a cellular ATPase was required for the membrane translocation of the DT C domain (Sandvig et al., 1981).

Pronase protection assays were used to examine which portions of DT inserted into the plasma membrane when toxin bound cells were exposed to low pH (Moskaug et al., 1991). Moskaug et al. (1991) found a translocated fragment A (20 kD) in the cytosol and a plasma membrane associated 25 kDa peptide derived from fragment B. Furthermore, an inwardly directed proton gradient was required for the translocation of fragment A, but not for membrane insertion of fragment B (Sandvig et al., 1988). Analogously, it has also been shown that translocation of fragment A requires a lower pH as compared to the membrane insertion of fragment B (Falnes et al., 1992).

5. Pore formation

Exposure of DT transmembrane (T) domain to artificial lipid bilayers at low pH results in spontaneous membrane insertion and the formation of voltage dependent and cation selective channels (Boquet et al., 1976; Donovan et al., 1981). Kagan et al. (1981) observed a channel diameter of approximately 18-22 Å, which is theoretically large enough to accommodate the passage of a fully denatured fragment A. The crystal structure of DT shows that the T domain is composed of nine α-helices (TH1-9) and their connecting loops, and that the helices are arranged in three layers (Figure 1, Choe et al., 1992). The first three helices (TH1-3) comprise the first layer and are amphipathic in nature. Helices TH5, 6, and 7

compose a second hydrophobic layer. The third, central core layer is composed of the hydrophobic helices TH8 and 9, connected by transmembrane loop 5 (TL5).

Insertion of this third α-helical layer (Th8-9) is required for pore formation, which is then stabilized by the second α-helical layer (TH5, 6, and 7). Assays used to measure the formation and conductance of membrane pores, such as patch clamp experiments, molecular marker exclusion studies, and pH sensitive dyes have been used in conjunction with diphtheria toxin mutants to demonstrate the importance of specific residues in pore formation and support this model of helix insertion (Figure 3). Upon acidifcation of the endosomal lumen, residues Glu 349 and Asp 352 located at the tip of loop (TL5) connecting TH8 and TH9 are protonated, and the third helical layer spontaneously inserts into the membrane and forms a cation selective channel (O'Keefe et al., 1992; Mindell et al., 1994). Deletion or disruption of these helices by introducing proline residues ablates channel formation and results in non-cytotoxic mutants (vanderSpek et al., 1994a; Hu et al., 1998), suggesting that the full length helices arranged in a specific conformation is required for channel formation. While helices TH8 and 9 alone can create pores (Silverman et al., 1994), pore formation by TH8 and 9 alone is not sufficient for effective delivery of the C domain (vanderSpek et al., 1994a).

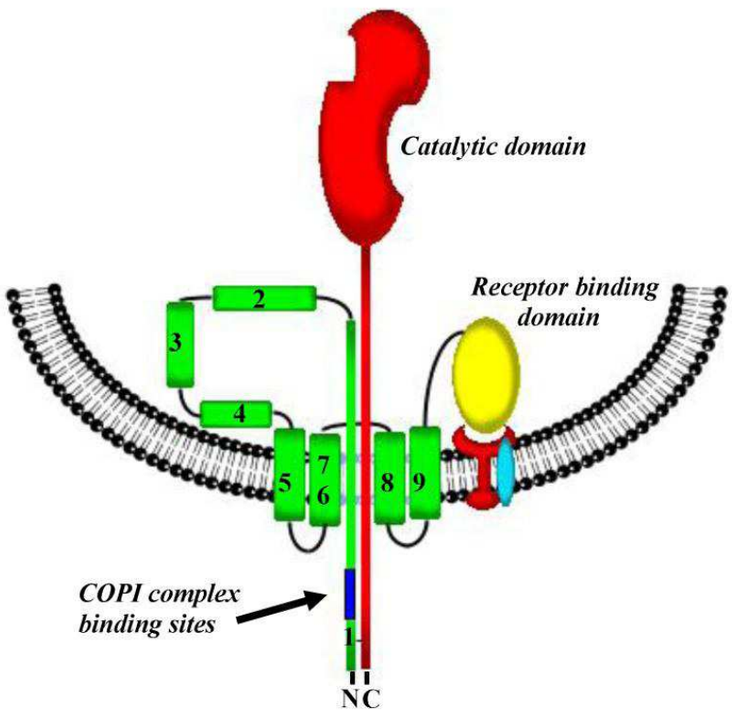

Fig. 3. Schematic of DT transmembrane (T) domain membrane insertion and pore formation. Following furin mediated nicking at Arg194 and denaturation of the catalytic (C) domain, the *N*-terminal portion of the T domain with the disulfide bond linked *C*-terminal end of the C domain is threaded into the pore. Emergence of one or more of the KXKXX motifs in first alpha helix (TH1) of the T domain on the cytosolic side of the vesicle membrane allows for binding of the COPI complex required for translocation of the catalytic domain. Reproduced from Murphy (2011).

The role that the Pro 345 residue, located at the end of TH8, plays in channel formation remains unclear. Mutation of Pro 345 to either a Glu residue, an α-helix former, or to a Gln residue, an α-helix breaker, resulted in a marked decrease in DT toxicity (Johnson et al., 1993). The *cis-trans* isomerization of proline by membrane associated peptidylprolyl *cis-trans* isomerases (PPIases), or cyclophilins, is important in the gating mechanisms of other cation selective channels, and a role for PPIases in DT channel formation or translocation of the DT C domain have been proposed (Johnson et al., 1993).

Following membrane insertion of the third helical layer, the second helical layer is subsequently inserted next and is thought to stabilize the channel formed by helices TH8 and 9 (Cabiaux et al., 1993; Cabiaux et al. 1994). Insertion of proline residues into the second helical layer of DAB_{389}IL-2 resulted in non-cytotoxic mutants with abnormal channel formation (Hu et al., 1998). Although this layer is not required for channel formation, it appears that the second helical layer is required for the formation of productive channels capable of supporting C domain translocation across the early endosomal membrane.

Deletion of the first three helices of DAB_{389}IL-2 resulted in a non-cytotoxic mutant that still formed characteristic channels and retained enzymatic activity (vanderSpek et al., 1993). The amino terminal residues of TH1 are translocated across the membrane and presented to the cytosol (Madshus et al., 1994a). Replacement of the charged residues in TH1 with uncharged residues strongly inhibits translocation of TH1 (Madshus et al., 1994a, vanderSpek et al., 1994b). The insertion of proline residues into the first helical layer also resulted in non-cytotoxic mutants that formed characteristic channels and retained enzymatic activity (Hu et al., 1998).

Taken together, these results suggested that the first helical layer facilitates the orientation and insertion of the C domain through the nascent channel formed by the T domain. Assuming that the disulfide bond connecting fragments A and fragment B remains intact, translocation of amino terminal residues of TH1 across the endosomal membrane would be anticipated to effectively thread the carboxy terminal residues of the C domain through the nascent channel and present them to the cytosol (vanderSpek et al., 1994b), and this work implied possible interactions with an unidentified translocation apparatus. These early findings were prescient of the recent identification of the T1 motif in TH1 by Ratts et al (2005) and role of the di-lysine motif KXKXX in recruiting components COPI complex required for toxin entry that is discussed below (Trujillo et al., 2010).

6. Unfolding of the catalytic domain

Unfolding of the DT C domain occurs *in vitro* in acidic conditions similar to those found inside the lumen of the endosome, and unfolding is required for delivery. Given the limited size of the pore, unfolding of the DT C domain was postulated as a pre-requisite for translocation (Donovan et al., 1981; Kagan et al., 1981). The necessity for complete denaturation of the DT C domain prior to translocation was then indirectly demonstrated by (Wiedlocha et al., 1992) and by (Falnes et al., 1994). Wiedlocha et al. (1992) fused acidic fibroblast growth factor (aFGF) to the amino terminus of fragment A. This aFGF-DT fusion protein construct was cytotoxic, confirming the observation that polypeptides fused to fragment A are delivered into the cytosol of targeted cells (Stenmark et al., 1991). In the presence of heparin, however, aFGF retains a rigid tertiary structure and the aFGF-DT

fusion protein was no longer cytotoxic, implying that unfolding is a requirement for delivery through the nascent channel formed by the T domain. Falsnes et al. (1994) also created a double cysteine mutant which formed a disulfide bond within fragment A. With the disulfide bond intact, unfolding of fragment A does not occur. This mutant retained ADP-ribosyltransferase activity, but it was not cytotoxic. Taken together, these studies indicated that the cytosolic delivery of the C domain occurs in at least a partially unfolded state and that once delivered into the cytosol, the C domain must be refolded into an active conformation.

7. Reduction of the interchain disulfide bond

The C domain is separated from the T and R domains by a protease sensitive loop that is sub-tended by the disulfide bond between residues Cys 186 and Cys 201. Upon binding and internalization of the toxin-receptor complex, this loop is nicked by the enzyme furin (Tsuneoka et al., 1993). Retention of the interchain disulfide bond following nicking is a pre-requisite for intoxication (Falsnes et al., 1992), and it presumably mediates threading the DT C domain through the channel formed by the T domain. The pivotal role of reducing the inter-chain disulfide bond is underscored by the observation that reduction and release of the C domain appears to be the rate limiting step for the entire intoxication process (Papini et al., 1993b).

The precise location where reduction of the DT inter-chain dilsulfide bond occurs remains somewhat controversial. Moskaug et al. (1987) showed that only membrane permeate sulfhydryl blockers were able to prevent the release of the C-domain into cytosol. Papini et al. (1993b) reported that reduction of the DT interchain disulfide bond occurs after the low pH induced membrane insertion of the T domain within the early endosome. Since unreduced DT C domain and membrane inserted DT fragment B are both targeted for proteolytic degradation (Madshus et al. 1994b), these results suggested that reduction of the interchain disulfide bond occurs during or post-translocation. In contrast, Ryser et al. (1991) found that membrane impermeate sulfhydryl blockers prevented DT intoxication, and proposed that reduction occurs prior to translocation, presumably on the cell surface or within the endosomal lumen. *In vitro* studies have shown that thioredoxin 1 (Trx-1) (12 kDa) reduces the DT inter-chain disulfide bond under acidic conditions (Moskaug et al., 1987). This result is consistent with observations that exposure of the DT interchain disulfide bond on the protein surface occurs upon denaturation (Blewitt et al., 1985). Trx-1 is predominately cytosolic, but a shorter form (10 kDa) is actively secreted by a non-classical ER-Golgi independent pathway and is present on the luminal side of the endosomal membrane (Rosen et al., 1995). It is not known whether or not Trx-1 interacts directly with DT *in vivo*.

8. Model for autonomous delivery

Initial studies of T domain insertion and pore formation in artificial lipid bilayers in the presence of a pH gradient led to the hypothesis of autonomous delivery of the C domain, and Deleers et al (1983) first suggested that a pH gradient was required to facilitate C-domain delivery. Shiver and Donovan (1987), using asolectin vesicles, demonstrated that diphtheria toxin could deliver its own C-domain across the artificial bilayer in a pH dependent fashion, independent of added proteins or factors. These studies demonstrated a

requirement for a pH gradient, in which the endocytic vesicle luminal pH is optimally between 4.7 and 5.5 and the cytosolic pH is at or near 7.4. The topography of DT inserted into both cell plasma membranes and artificial bilayers has been studied using protease digestion and analyzing the enzymatic cleavage products (Moskaug et al, 1991, Cabiaux et al, 1994). Although insertion of transmembrane helices 8 and 9 is a common finding of these studies, discrepancies arise in interpreting cleavage products that include the C-domain and whether or not they represent translocation intermediates (Madhus, 1994). Taken together, the apparent ability of DT to transfer its C-domain across synthetic lipid bilayers, in the absence of other proteins, led to a model of autonomous C-domain delivery.

The model of autonomous C-domain delivery was advanced by Oh et al. (1999) using planar lipid membranes and DT labeled with an *N*-terminal histidine tag (6× His). Since addition of Ni2+ to the *trans* compartment prevented the rapid closure of pores, these investigators concluded that the N- terminal end of the C-domain containing the His tag was translocated from the *cis* to the *trans* side of the lipid bilayer upon channel formation by the T-domain. These investigators also used biotin to label cysteine site-directed mutants at either position 58 or 148. The addition of streptavidin to the *trans* side of the planar lipid membrane also interfered with the channel closure. Again, these results suggested that Cys_{58} and Cys_{148} were on the *trans* side of the membrane following channel formation.

Also using artificial lipid bilayers, Ren et al. (1999) demonstrated that in a low pH environment, the presence of proteins in a partly unfolded molten globule-like conformation (e.g. unfolded C-domain) were able to convert the T domain from a shallow membrane inserted form to a fully *trans*- membrane inserted form. Hammond et al (2002) confirmed that the DT T-domain has chaperonin-like properties, but also observed that the T domain had a significantly greater affinity for other molten globule-like polypeptides compared to its own C-domain. The chaperonin-like property of membrane inserted T domain towards unfolded substrates under acidic conditions has been confirmed (Hayashibara *et al*, 2005; Chassaing *et al*., 2011).

In the autonomous translocation model, delivery of the C-domain is thought to be achieved through the chaperonin-like activity of the T-domain. Although these studies clearly demonstrate ability for DT to utilize a pH gradient, in conjunction with a relatively high membrane potential, to mediate autonomous translocation *in vitro*, it is not at all clear that these conditions occur *in vivo*. Many proteins are imbedded in the endosomal membrane and decorate both the luminal and cytosolic face of endocytic vesicles, and the impact these proteins may have on toxin delivery across the endosomal membrane are not included in artificial membrane bilayer systems. Since protease digestion patterns of DT inserted into planar lipid bilayers differ from those of DT inserted into the plasma membrane (Moskaug et al., 1991; Cabiaux et al., 1994), it seems likely that interaction(s) between the toxin and proteins associated with the endosomal membrane influence the orientation and/or stoichiometry of T-domain membrane insertion and translocation of the C-domain.

9. Model for facilitated delivery

A role for cellular factors in facilitating DT membrane translocation was first suggested by Sandvig and Olsnses (1981) with their observation that the successful delivery of toxin artificially inserted into the plasma membrane under acidic conditions required the function

of a cellular ATPase. This hypothesis was expanded by Kaneda et al (1984), who established hybrid cell lines resistant to DT and demonstrated that the resistance of these cell lines appeared to be independent of receptor binding, receptor trafficking, and susceptibility of EF2 to ADP-ribosylation. They concluded that the resistance of these hybrid lines was due to cellular factors required for toxin entry.

The rationale for cellular factors facilitating the delivery of the DT C-domain across the endosomal membrane is congruent with other known and similar mechanisms of protein translocation across membranes within eukaryotic cells such as mitochondrial import, ER synthesis, and the retrograde translocation of toxins from the ER. During mitochondrial import, an electrochemical membrane potential is initially required for insertion of the proteins synthesized in the cytosol into the translocation complex and its subsequent transfer to the translocation complex present on the mitochondrial inner membrane (Bauer et al., 2000). The inward movement of the protein, however, requires unfolding and its translocation into the mitochondrial matrix is mediated by an ATP dependent import motor consisting of at least three components, including mitochondrial heat shock protein 70 (Hsp 70) (Bauer et al., 2000). The translocation of newly synthesized proteins into the ER occurs co-translationally through channels formed by the Sec 61 translocon complex (Tsai et al., 2002). Bip, an ER luminal resident homologue of Hsp 70, functions analogously to mitochondrial Hsp 70 in mediating protein translocation into the ER lumen (Baker et al., 1996). In contrast, the ERAD pathway involves the retro-translocation of misfolded proteins, as well as several toxins (*e.g.* cholera, pseudomonas exotoxin A, ricin), from the ER lumen into the cytosol through the same Sec 61 channel (Tsai et al., 2002). In ERAD, as reviewed by Tsai et al (2002), misfolded luminal proteins are recognized and unfolded by chaperones prior to retro-translocation through the Sec 61 channel. Cellular factors that are conserved from yeast to humans, are then required for the extraction of from the ER membrane and their subsequent release into the cytosol. In all of these systems, translocation is facilitated by the sequential binding and refolding of denatured proteins by chaperonins as they emerge through the membrane.

Lemichez et al (1997) provided the first direct evidence supporting the hypothesis that delivery of the C-domain across the endosomal membrane requires both ATP and cytosolic factors. This pivotal study demonstrated that DT translocation occurs from within early endosomes, and that Bafilomycin A1 resulted in the accumulation of DT within the lumen of arrested early endosomes. These investigators established an *in vitro* translocation assay system using purified early endosomes pre-loaded with DT from cells treated with bafilomycin A1. The *in vitro* translocation of the C-domain across the endosomal membrane required the addition of both ATP and cytosolic factors. Lemichez et al (1997) also observed that DT co-localizes with β'COP in tubular structures, and that antibodies to β'COP inhibited the *in vitro* translocation of the DT C domain across the endosomal membrane.

10. Defining the DT Cytosolic Translocation Factor (CTF) complex

Using the *in vitro* translocation assay developed by Lemichez *et al.* (1997) as a purification assay, Ratts *et al.* (2003) confirmed and extended the observations that cytosolic translocation factors (CTFs) are essential for the translocation and release of the DT C-domain from the lumen of early endosomes pre-loaded with the fusion protein toxin DAB_{389}IL-2. Control endosomes loaded with horse radish peroxidase and a pH sensitive dye

(OG514) demonstrated that endosomal lysis did not occur and that the cytosolic factors were not required for endosomal acidification, respectively, under assay conditions. Protein complexes mediating toxin translocation were then partially purified from both human and yeast cytosolic extracts, and individual proteins were identified using mass spectrometry sequencing. The potential role of individual proteins as putative CTFs were then examined using specific inhibitors and/or neutralizing antibodies. Ratts et al (2003) showed that both heat shock protein 90 (Hsp 90) and thioredoxin reductase 1 (TrR-1), and their yeast homologues Hsp 82 and TrR, respectively, are components of the CTF complex required for DT entry. Importantly, CTF activity was limited to the translocation step and neither factor inhibited the enzymatic ADP-ribosylation of EF2 by the DT C-domain. Finally, a physiologic role for Hsp 90 and TrR-1 was confirmed using specific inhibitors in cytotoxicity assays to protect cells against toxin. Although Hsp 90, TrR-1, β-COP have all been confirmed as CTFs for the entry of diphtheria toxin, these factors alone are not sufficient for translocation (Ratts et al., 2003; Ratts et al 2005). The additional required components of the CTF remain to be identified. The *in vitro* translocation assay utilized by Lemichez et al (1997) and Ratts et al (2003) cannot distinguish between direct translocation of the DT C domain across the endosomal membrane and the release of the DT C domain from the cytosolic surface of the endosomal membrane. In either scenario, the assay system does accurately assess the physiological delivery of C-domain from lumen of early endosomes into the cytosol and the *in vitro* translocation assay has rapidly become the gold standard for studying toxin translocation across the endosomal membrane.

Protein complexes of similar composition to the diphtheria toxin CTF complex have been described in the protein-trapping proteomic analysis of yeast by Ho et al. (2002). Cyclophilin (Cpr6) trapped complexes from yeast contain Hsp 82, TrR-1 and Sec 27 (β-COP) (Ho et al, 2002). There are two considerations for interpreting the data obtained from protein-trapping proteomic analysis: First, the protein complexes are most likely of heterologous nature. Second, only the proteins that were readily detectable are included. Cyclophilin is functionally active in Hsp 90 chaperonin complexes, and a role for cyclophilin as a putative CTF will be discussed below. Surprisingly, EF-2 is present in several yeast complexes containing the diphtheria toxin CTFs (Ho et al., 2002), and Hsp 90 has previously been shown to directly interact with elongation factor-2 kinase (Palmquist et al., 1994). Recently, Bektas et al. (2011) provided evidence that EF-2 itself may augment the *in vitro* translocation of the DT C domain across endosomal membranes in the presence of actin filaments .

10.1 Hsp 90 functions as a CTF

Hsp 90 is ubiquitously expressed and comprises the core of several multi-molecular chaperonin complexes that are highly conserved in eukaryotes (Schulte et al., 1998). These complexes also contain additional chaperones, co-chaperones, and adapter proteins. Interaction of these proteins with Hsp 90 is mediated through a tetracopeptide repeat acceptor site (TPR domain) found in Hsp 90, and the formation of discrete subcomplexes with distinct co-chaperones mediates Hsp 90 substrate recognition (Caplan, 1999). Although Hsp 90 does not usually directly bind nor refold nascent polypeptides, it is known to refold a growing list of proteins including membrane associated protein kinases (Bijlmakers et al., 2000). In addition to its refolding activity, Hsp 90 complexes are also known to regulate the trafficking of membrane associated proteins through interactions with cytoskeletal motors (Pratt et al., 1999).

Ratts et al (2003) established a functional role for human Hsp 90, and the yeast homologue Hsp 82, as a component of the CTF complex by immunoprecipitation and the use of specific inhibitors in both the *in vitro* translocation assay and cytotoxicity assays. Using the Hsp 90 specific inhibitors geldanamycin and radicicol, Ratts et al (2003) demonstrated that Hsp 90 ATPase activity is capable of refolding *in vitro* denatured DT C domain into a biologically active conformation. Geldanamycin binds to the Hsp 90 active site, blocks the binding of ATP, and consequently inhibits substrate dissociation from the Hsp 90 refolding complex (Grenert et al., 1997). Radicicol, different in structure from geldanamycin, binds to a different location within the ATP binding pocket of Hsp 90 but also blocks the binding of ATP, and consequently inhibits substrate dissociation from the Hsp 90 refolding complex (Schulte et al., 1998).

Surprisingly, neither the addition of geldanamycin nor radicicol alone inhibited the *in vitro* translocation of the DT C-domain (Ratts et al., 2003). There are several reports demonstrating the synergistic inhibitory effects of geldanamycin and radicicol on Hsp 90, and inhibition is thought to result from either the disruption of substrate binding or the interaction with co-chaperonins (Schulte et al., 1998). When both inhibitors were used together, the *in vitro* translocation of the DT C-domain was inhibited (Ratts et al., 2003). The synergistic inhibition of C domain translocation was specific to Hsp 90, and protected cells against toxin. These results indicated that refolding of the denatured C-domain into an active conformation and translocation of the C domain across the early endosomal membrane were mutually exclusive events, and that redundant mechanisms exist for refolding any unfolded DT C domain following translocation. Dmochewitz et al (2011) confirmed and extended the observations made by Ratts et al (2003) using the anthrax pore to deliver the DT C domain across endosomal membranes *in vitro.* In this system, the *in vitro* translocation of the DT C domain across endosomal membranes was dependent on Hsp 90 ATPase activity. This study also demonstrated that the *in vitro* translocation of the DT C domain through the anthrax pore required the activity of cyclophilin, a known Hsp-90 co-chaperone. Dmochewitz et al. (2011) also provided the first evidence for interaction between the DT C domain and Hsp 90, either directly or in the presence of an adaptor protein.

Hsp 90 mediates the entry of other bacterial toxins from the lumen of endosomes including the *C. botulinum* C2 toxins (Haug et al., 2003), iota toxin (Haug et al., 2004), and *C. perfingens* toxin (Haug et al. 2004). Like diphtheria toxin, passage through a low pH compartment and unfolding of the *C. botulinum* C2 toxin catalytic domain are pre-requisites for entry (Barth et al., 2011). In the case of the *C. botulinum* C2 toxins, Haug et al (2003) clearly demonstrated that Hsp 90 ATPase activity was not acting as an allosteric regulator of v-ATPase, and ruled out the possibility that Hsp 90 inhibition resulted in the enhanced proteosomal degradation of toxin. In addition to bacterial protein toxins, Hsp 90 has also been show to to mediate the endosomal membrane translocation of the HIV viral TAT protein (Vendeville et al, 2004), the endogenous protein fibroblast growth factor (Wesche, et al. 2006). Hsp 90 mediated translocation is not limited to the endosomal membrane, and Hsp 90 function is also required cytosolic entry of cholera toxin, another ADP-ribosylating toxin, from the ER via the ERAD pathway (Taylor et al, 2010).

Subtle differences between these toxins and their interaction with CTFs may reveal insight into the precise molecular role of Hsp 90 within the CTF complex. All of the bacterial protein toxins requiring Hsp 90 for cytosolic entry that have been identified to date are ADP-

ribosyltransferases, and Barth (2011) has hypothesized that there is some conserved component of the ADP-ribosyltransferase domain that mediates interaction with Hsp 90, either directly or indirectly through an adaptor protein or co-chaperone.

10.2 TrR-1 functions as a CTF

TrR-1 is an ubiquitously expressed homodimeric NADPH-dependent flavin adenine dinucleotide containing reductase, and is the only protein known to date to reduce thioredoxin (Trx-1) (Mustacich et al., 2000). Trx-1 reduces *in vitro* the DT inter-chain disulfide bond under acidic conditions (Moskaug et al., 1987), and reduced Trx-1 has also been shown to bind a variety of misfolded cytosolic proteins and directly facilitate refolding (Hawkins et al., 1991). Ratts et al (2003) established a functional role for TrR-1 and the yeast homologue as a component of the CTF complex by both immunoprecipitation, affinity depletion, and the use of the TrR-1 specific inhibitor *cis*-13-retinoic acid in both *in vitro* translocation assays and cytotoxicity assays. Under reducing conditions, TrR-1 was an essential component of the CTF complex indicating that it is structurally present or directly interacting with other CTFs that are required for DT C domain translocation, and this role is independent of its enzymatic activity. Ratts et al. (2003) demonstrated that TrR-1 function *in vitro* is required for translocation and/or release of the C-domain from early endosome under non-reducing conditions.

TrR-1 may be important for the entry of other bacterial protein toxins. TrR-1 reduces *in vitro* the inter-chain disulfide bond in both the botulinum neurotoxins and tetanus neurotoxins (Kistner et al., 1992; Kistner et al., 1993). These toxins are organized in a similar fashion to DT, and their mechanism across endosomal membranes parallels that of DT (Montecucco et al., 1996). An *in vivo* role for TrR-1 in mediating the entry of these neurotoxins, however, has not been shown. A role for TrR-1 in the intoxication of ricin has recently been reported by Bellisola et al. (2004), who showed that Trx and PDI mediated *in vitro* reduction of the ricin inter-chain disulfide bond depends upon TrR-1 activity under non-reducing conditions. When cytosolic extracts were depleted of TrR-1, effective reduction of ricin into two fragments still occurred, but protein(s) or protein fragment(s) of 15 kDa were associated with the ricin catalytic domain. Bellisola et al. (2004) hypothesized that this factor(s) associated with the ricin catalytic domain were chaperones required for toxin entry.

10.3 Cyclophilin may function as a CTF

Cyclophilin is a peptidylprolyl *cis-trans* isomerase and co-chaperone of Hsp 90, and mammalian cyclophilin – Hsp 90 complexes are conserved in yeast (Dolinski et al., 1998). A potential role for proyl isomerases in DT T domain membrane insertion and channel formation has been proposed (See Pore Formation above). Cyclophilin does facilitate the cytosolic entry of the *C. botulinum* C2 toxin, *C. perfingens* toxin, and the *C difficile* actin-ADP ribosylating CDT toxin (Kaiser et al., 2011). Dmochewitz et al (2011) demonstrated that *in vitro* translocation of the DT C domain across endosomal membranes using the anthrax pore was inhibited by cyclosporin, a specific inhibitor of cyclophilin. It has not yet been demonstrated, however, if translocation of the DT C domain using the DT T domain requires cyclophilin. Cyclophilin has not yet been identified in the purified diphtheria CTF complex, but cyclophilin trapped complexes from yeast do contain the other known CTFs required for diphtheria toxin entry. Although additional analysis is required to confirm that

cyclophilin plays a role in the diphtheria toxin CTF complex, cyclophilin does play a role in the entry of other toxins.

11. Identification of the T1 motif

Since a highly conserved CTF complex is required for DT entry, we reasoned that a sequence specific binding site mediating interaction between toxin and the CTF complex exists. Given the common route of entry of diphtheria toxin, the anthrax lethal and edema factors, and the botulinum neurotoxins across endosomal membranes, we performed *in silico* sequence analysis of these toxins. Initial analysis was limited to portions of the DT C domain (residues 140-193) and T domain (residues 194-272) which were hypothesized to be the first portions of the toxin threaded through the nascent pore and presented to cytosol, using position-specific-iterated (PSI)-BLAST (Basic Local Alignment Search Tool) analysis (Karlin and Altschul, 1990). This initial analysis elucidated a 12 amino acid motif corresponding to DT residues 212-223 in transmembrane helix 1, and was therefore named the T1 motif (Figure 4) (Ratts dissertation 2004). Next, *in silico* analysis of the entire primary amino acid sequence of DT that employed PSI-BLAST, Clustal W Alignment (Thompson et al, 1994), and MEME (Multiple Expectation maximization for Motif Elucidation) (Bailey et al, 1994) using overlapping 12 amino acid sequences from DT to probe the data base revealed a conserved 10 amino acid motif corresponding to the same region within diphtheria toxin, anthrax lethal factor, anthrax edema factor and botulinum neurotoxins serotype A, C, and D (Figure 4) (Ratts et al., 2005). Although these two methods essentially defined the same motif, the two algorithm derived consensus sequences contain subtle differences highlighting the import of functional analysis to confirm any physiological relevance such motifs may have in mediating protein-protein interactions.

Anthrax			p-value
Edema Factor	50-65	EKNKTEKEKFKDSINN	2.4 x 10-7
	404-420	KLDHLRIEELKENGII	1.8 x 10-6
Lethal Factor	27- 42	ERNKTQEEHLKEIMKH	5.5 x 10-8
Botulinum neurotoxin			
Serotype A	719-734	AKVNTQIDLIRKKMKE	5.4 x 10-6
	828-843	GTLIGQVDRLKDKVNN	2.1 x 10-7
Serotype Cl	755-770	ENIKSQVENLKNSLDV	2.4 x 10-9
Serotype D	751-766	ENIKSQVENLKNSLDV	2.4 x 10-9
Diphtheria toxin			
	212-227	DKTKTKIESLKEHGPI	9.0 x 10-8
MEME Consensus		TQIENLKEKGX	
Blast Consensus		EKXKTXXEXLKE	

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DT       198-SSLSCINLDWDVIRDKTKTKIESLKEHGPIKNKMSESPNKTVSEEKAKQYLEE-250
LF        10-KEKEKNKDENKRDEERNKTQEEHLKEIMKHIVKIEKGEEAVKKEAAEKLLEKV-65
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Fig. 4. BLAST and MEME analysis of anthrax edema and lethal factor (LF), botulinum neurotoxins, and diphtheria toxin (DT). P-values are for toxins compared to MEME consensus sequence. Longer sequence for LF and DT are shown indicating the flanking di-lysine motifs as underlined and described in the text.

For each toxin, the T1 motif is positioned on the surface of the protein within an amphipathic alpha helix that is located in a region of the toxin consistent with potential

function in the translocation process. For diphtheria, the T1 motif is present within the first amphipathic helix of the DT T domain - TH1 – which is responsible for threading the DT C domain into the nascent pore formed by the remainder of the T domain. Deletion of TH1, proline disruption of TH1, or change in the charge distribution within this region all result in the loss of toxicity (vanderSpek et al., 1993; vanderSpek et al., 1994b). Furthermore, these mutations had no effect upon receptor binding, channel conductance in artificial lipid bilayers, nor the ADP-ribosyltransferase activity of the C domain.

The proposed 'entry' motif is also consistent with the known mechanism of entry for anthrax lethal factor. Anthrax toxin is a binary complex assembled from three distinct protein chains: protective antigen (PA), lethal factor (LF), and edema factor (EF) (for review see Mourez et al., 2002). Protective antigen (PA83) binds to a universal cell surface receptor and a 20 kDa fragment is removed by furin digestion (Molloy et al., 1992). The remaining 63 kDa fragment (PA63) remains on the cell surface and spontaneously oligomerizes into a heptamer. The heptameric complex is then capable of binding either LF or EF (Pimental et al., 2004). The overall route of entry closely follows that of diphtheria. PA bound with either LF or EF, is internalized into an endosomal compartment, where acidification induces a conformational change in PA, driving membrane insertion and formation of a cation selective channel (Abrami et al 2003; Blaustein et al., 1989). Wesche et al. (1998) showed that the acid-induced translocation of LF, like diphtheria, must undergo complete unfolding for passage through the channel formed by PA, and is then refolded into an active conformation in the cytosol (Wesche et al., 1998). In contrast, EF remains associated with the vesicle compartment (Guidi-Rontani et al., 2000). In the case of anthrax LF, the putative entry motif is located between amino acid residues 27 – 39 in the mature protein, a region N-terminal to the PA binding domain. Analysis of anthrax LF N-terminal deletion mutagenesis (Arora and Leppla, 1993) demonstrated that the deletion of amino acids 1 – 40 in lethal factor results in a complete loss of toxicity for macrophages. More recently, Lacy et al. (2002) confirmed these results, and also showed that deletion of the N-terminal 27 amino acids had no effect. Although results by Lacy et al. (2002) suggested that the deletion of amino acid residues 1-40 may abrogate LF binding to PA, it is clear that the region is required for toxicity.

12. β COP Functions as a CTF

To demonstrate that the T1 motif mediates physiologically relevant interaction with CTFs, Ratts et al. (2005) engineered toxin resistant cells by transfecting a mini-gene encoding the T1 motif (amino acids 210-229 of DT). Cells expressing the T1 peptide were resistant to both DAB_{389}IL-2 and wild type DT, but were not protected against pseudomonas exotoxin A which enters cells through the ER and once delivered to the cytosol inhibits protein synthesis via an identical NAD+ dependent ribosylation of EF2. These results suggested that the T1 motif was not interfering with receptor binding, receptor trafficking, nor inhibiting the ability of toxin to ADP-ribosylate EF-2. Ratts et al (2005) then showed that knockdown of the T1 motif mini-gene using siRNA restored sensitivity to toxin, and we reasoned the T1 peptide was inhibiting an essential protein-protein interaction with CTFs.

In order to confirm such an interaction, a fusion protein was constructed between GST and DT amino residues 140-271 (Ratts et al., 2005). Because other regions outside the T1 motif

might also be important in the entry process we used a longer segment of DT, corresponding to regions of T domain and C domain that are first threaded through the pore and presented to the cytosol. While the T1 motif alone might be sufficient in blocking protein-protein interactions, additional regions of the toxin may be required for actually binding CTFs. In pull down experiments, Ratts et al (2005) affinity purified several proteins that specifically bound to DT140-271, and identified them by mass spectrometry sequencing. One of these identified proteins was β-COP. Using labeled [35S]-β-COP that was synthesized *in vitro* using a rabbit reticulocyte transcription and translation reaction mixture, we found that GST-DT140-271 specifically bound β-COP and that bind was inhibited by synthetic T1-motif peptide. Confirming and extending Lemichez et al. (1997), there results suggested direct interaction between toxin and β-COP via the T1 motif.

A role for the DT T1 Motif in mediating the cytosolic entry of anthrax lethal factor (LF) was demonstrated by Tamayo et al. (2008) using an *in vitro* translocation assay consisting of early endosomes pre-loaded with anthrax protective antigen (PA) and the anthrax LFn-DTa fusion protein construct. The LFn-DTa is a fusion protein consisting of the LF binding domain for PA and the C domain of diphtheria. Tamayo et al. (2008) clearly demonstrated that the anthrax LFn-DTa fusion protein construct was translocated across the endosomal membrane in an ATP and cytosol dependent fashion, and this observation was confirmed by Dmochewitz et al (2011). Tamayo et al. (2008) also demonstrated using GST-LFn pull downs that the T1 motif in anthrax lethal factor directly binds β-COP, as well as zeta (ζ)-COP, and that a synthetic peptide containing the DT T1 motif blocked this interaction.

13. Lysines adjacent to the T1 motif region bind COPI

COPI is a heptameric structure that is composed of α-, β-, β'-, γ-, ε-, δ-, δ-subunits, and this complex functions to facilitate endosomal vesicular trafficking, the retrograde transport of vesicles between Golgi compartments, and between the Golgi apparatus and the endoplasmic reticulum (Serafini et al, 1991; Waters et al., 1991; Whitney et al., 1995). As previously mentioned, both β'COP (Sec 27) and ADP-ribosylation factor 1 (ARF1) bind to the cytoplasmic surface of the endosomal membrane to promote ECV formation, and the binding of both factors is dependent upon a low endosomal lumen pH (Aniento et al., 1996). COPI complexes have also been shown to be recruited to the cytosolic surface of vesicle membranes *en bloc* by Arf-GTP (Donaldson et al, 1992; Palmer et al., 1993). The recognition of di-lysine motifs (KXKXX, KKXX) by coatomer in the cytoplasmic tails of cargo proteins is well established (Cosson et al., 1994; Eugster et al., 2004). Interactions between COPI and the p23/24 adaptor is also mediated by a di-lysine motif, and is thought stabilize coatomer binding to the membrane surface (Harter et al., 1998).

Trujillo et al (2010) hypothesized that the multiple di-lysine motifs adjacent to the T1 motif in DT are the specific amino acid residues that interact with coatomer. This hypothesis was then confirmed by site-directed mutagenesis of the specific lysine residues K213, K215, K217, and K222 demonstrating that at least three of the four lysine residues in the region of the T1 motif are required for both COPI binding and for the cytotoxic activity of DAB389IL-2 (Trujillo et al., 2010). Using a similar *in vitro* COPI precipitation assay as described by Hudson and Draper (1997), Trujillo et al (2010) demonstrated that synthetic peptides of the DT T domain transmembrane helix 1 would cross-link and induce precipitation of COPI complexes *in vitro.* Synthetic peptides containing lysine to alanine mutations at either the N-

terminal or C-terminal end of the peptide, or all five positions failed to precipitate COPI *in vitro.* The addition of monoamine 1,3-cyclohexanebis (methylamine), CBM, to the reaction mixture also blocked peptide binding to COPI complex. Trujillo et al. (2010) also demonstrated that DT directly interacts with only the β-COP and γ_1-COP components.

These observations suggested that the ε-amino moieties of the lysine residues immediately adjacent to the T1 motif specifically bind to COPI, and Trujillo et al (2010) reasoned that this region within DT was functioning as a mimetic of the cytoplasmic tail regions of either the cargo or p23/24 adaptor proteins that are normally recognized by COPI. This theory was validated by domain swapping the 13 amino acid COPI binding sequence from the cytoplasmic tail region of the p23 adaptor protein with native T1 motif and adjacent upstream lysine residues in DAB_{389}IL-2. The COPI domain swap fusion toxin mutant $DAB_{(212p23)389}$IL-2 retained full cytotoxic potency relative to the wild type-fusion protein toxin (Trujillo et al., 2010). Regardless of sequence, a major role for the DT transmembrane helix 1 is COPI complex binding and this interaction is essential for toxin entry.

14. A new model for translocation

The autonomous model for entry explains the initial steps in toxin translocation across the endosomal membrane, while the completion of translocation and release of the DT C domain into cytosol requires cytosolic factors. Acidification within the endosomal lumen promotes unfolding of the C domain and membrane insertion of the T domain in a mutually augmented process. It is not known if any host cell proteins help facilitate unfolding. The chaperonin-like qualities of the T domain then appear to thread the *C*-terminal end of the C domain, connected by its disulfide bond to the *N*-terminal end of the T domain, into the nascent pore. Presentation of the di-lysine motifs (KXKXX) in the N-terminal end of the T domain to the cytosolic side of the endosomal membrane then allows for targeting of the toxin by the COPI complex. Coatomer recognition of the toxin as cargo or a p23 mimetic essentially designates the N-terminal end of the T domain for "retrieval" into endogenous membrane sorting pathways. The effect of coatomer is to normally retrieve, or pull, membrane bound proteins into carrier vesicles. The effect of retrieving, or pulling, the mobile region of the N-terminal T domain actually results in the "retrieval" of the disulfide linked C domain through the pore and facilitating its complete translocation.

During this translocation process, Hsp 90 and TrR-1 perform critical steps that are required for toxin entry. Coatomer binding is a dynamic process consisting of many factors, and whether or not Hsp 90 and TrR-1 directly interact with the toxin or are rather involved in regulating or stabilizing coatomer remains to be elucidated. It is interesting that coatomer stabilization and release from the membrane is regulated by an endogenous ADP-ribosylation factor, and that the role of Hsp 90 in mediating toxin entry appears to be limited to toxins whose catalytic domains, like diphtheria, are ADP-ribosyltransferases. Likewise, the potential role for cyclophilin within the CTF complex also needs clarification.

Potential models for Hsp 90 function in the CTF complex include (but are not limited to): power stoke, regulation of other CTFs, Brownian ratchet, or architectural adaptor. The power stroke model implies that the Hsp 90 ATPase functions as a motor that directly drives the translocation, and is unlikely to apply to diphtheria entry since Ratts et al. (2003) demonstrated that translocation of the DT C domain across the endosomal membrane did

not require processive cycles of Hsp 90 ATPase function. Dmochewitz (et al. 2011) has demonstrated interaction between the C domain and Hsp 90, implying that Hsp 90's role is direct rather than indirect, i.e. regulatory, within the CTF complex. In the Brownian ratchet model, Hsp 90 would bind to progressive nascent regions of translocating C domain, preventing retrograde translocation back into the endosome. In other words, Hsp 90 binding and stabilization of exposed hydrophobic residues in the DT C domain as they emerge from the endosome effectively facilitates translocation. The Brownian ratchet model would be consistent with the observed synergistic inhibition by geldanamycin and radical – only when both inhibitors are used concomitantly, the emerging diphtheria toxin residues are no longer recognized by Hsp 90 and translocation is inhibited. Alternatively, Hsp 90 may function merely as an architectural adaptor, i.e. scaffold, within the CTF complex mediating a purely structural interaction between the toxin C domain and other CTFs.

Whether or not TrR-1 directly reduces the DT inter-chain disulfide bond *in vivo* remains unknown. It is possible that TrR-1 first reduces another reductase, which then directly reduces the DT inter-chain disulfide bond. Potential candidates include (Trx-1), presumably present on the endosomal membrane. Thioredoxin peroxidases, such as the alkyl hydroperoxide reductase-1 (Ahp1) identified in the yeast partially purified CTF complex; or a hitherto unidentified reductase, such as the novel protein YOR011C identified in the yeast partially purified CTF complex that bears homology to known NADPH oxidoreductases (Ratts et al. 2003). Conversely, another reductase might first reduces the DT inter-chain disulfide bond, and TrR-1 then subsequently mediates the release of the DT C domain by reducing the newly formed intermediate disulfide bond. A third possibility is that TrR-1 plays no role in reduction of the DT inter-chain disulfide bond during intoxication. Rather, TrR-1 may be responsible for the reduction of a key component of the CTF machinery required for translocation. For example, the reactivity of the free cysteines in Hsp 90 have been implicated in mediating chaperonin activity (Nardai et al., 2000).

As we learn more about toxin entry, our models will continue to need refinement. In the case of anthrax lethal factor, for example, Tamayo et al. (2011) recently reported that the chaperone Grp78 is required for intoxication and it unfolds the LF catalytic domain within the endosomal lumen. This finding appears to contradict long held beliefs that unfolding of the anthrax LF catalytic domain naturally occurs solely under acidic conditions *in vitro* and that the pore formed by protective antigen has chaperonin-like properties that facilitates autonomous delivery – both of which parallel and are analogous to models of diphtheria toxin entry. The report by Tamayo et al. (2011) reiterates the importance of studying toxin entry in biologically relevant systems containing the heterogeneous population of proteins naturally encountered by the toxin during intoxication. While the chaperones facilitating translocation of anthrax LF and DT have so far been found on opposite sides of the endosomal membrane, the entry of both toxins requires COPI binding in the cytosol. The identification of any other additional CTFs will further refine our models of toxin entry.

15. Conclusion

Diphtheria was the first investigated bacterial protein toxin, and more than a century later remains a paradigm for toxin entry. While there is no longer any question that cytosolic translocation factors (CTFs) facilitate the entry of diphtheria toxin, much work remains. The remaining components of the CTF complex required for the cytosolic entry of diphtheria

toxin need to be identified, and the precise role each cellular factor performs during translocation requires definition. Current methods for the purification of CTF complexes remain limited, and novel techniques that are more cost effective and readily available are desperately needed.

It is now apparent that a divergent group of toxins have convergently evolved to exploit similar mechanisms of entry to that of diphtheria toxin, and comparing and contrasting the differences in the CTF complexes for each toxin will serve as a valuable probe into the endogenous functions of coatomer assembly, cyclophilin and Hsp 90 function, thioredoxin redox pathways, and any other yet unidentified factors. Defining the precise molecular interaction between toxins and the CTF complex will allow for the design of novel therapeutics targeted towards virulence factors. Indeed, geldanamycin has already been shown to protect rat ileal gut from cholera toxin (Taylor et al., 2010) and clinical trials will likely soon follow. In light of the apparent evolutionary pressures, it is tempting to hypothesize that the CTF complex described for diphtheria toxin entry endogenously participates in discrete inter-intracellular signaling mechanisms that are highly conserved in eukaryotes.

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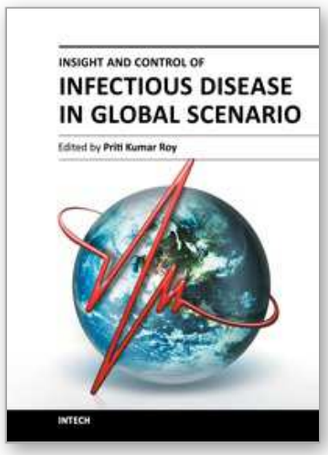

Insight and Control of Infectious Disease in Global Scenario
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This book is projected as a preliminary manuscript in Infectious Disease. It is undertaken to cover the foremost basic features of the articles. Infectious Disease and analogous phenomenon have been one of the main imperative postwar accomplishments in the world. The book expects to provide its reader, who does not make believe to be a proficient mathematician, an extensive preamble to the field of infectious disease. It may immeasurably assist the Scientists and Research Scholars for continuing their investigate workings on this discipline. Numerous productive and precise illustrated descriptions with a number of analyses have been included. The book offers a smooth and continuing evolution from the principally disease oriented lessons to a logical advance, providing the researchers with a compact groundwork for upcoming studies in this subject.

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University Campus STeP Ri
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Phone: +385 (51) 770 447
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