Diphtheria toxin, diphtheria-related fusion protein toxins, and the molecular mechanism of their action against eukaryotic cells

Ryan Ratts and John R. Murphy

Abstract

Diphtheria toxin remains one of the most successfully studied of the bacterial protein toxins. A detailed understanding of the structure function relationships of the toxin and the role of each domain in the intoxication process is well understood. This understanding has led to the development of diphtheria toxin-related fusion protein toxins which are targeted toward specific cell surface receptors. The first of these targeted toxins are now approved for human clinical use in the treatment of refractory hematologic malignancies and graft-versus-host disease. In recent years, studies on the molecular mechanism by which the diphtheria toxin catalytic domain is translocated across the early endosomal membrane has revealed that a host cell cytosolic translocation factor complex facilitates the entry process. A detailed understanding of this process will further stimulate the development of new approaches toward the delivery of cargo, ranging from protein to nucleic acid and/or protein nucleic acids, to the eukaryotic cell cytosol.

1 Diphtheria toxin

Diphtheria toxin was first described by (Roux and Yersin 1888) in the culture medium of *Corynebacterium diphtheriae*. Of all the bacterial A/B toxins which exert their toxicity by the translocation of their enzymatically active domains into the cytosol of eukaryotic cells, diphtheria toxin (DT) and the DT-based fusion protein toxins have been among the most extensively and successfully studied. Diphtheria toxin is naturally expressed by strains of *C. diphtheriae* which are lysogenic for one of a family of closely related corynebacteriophages whose genome carries the structural gene encoding the toxin, *tox.* Diphtheria toxin is produced in precursor form with a 25 amino acid signal peptide and is co-translationally secreted as a single polypeptide chain. Upon cleavage of the signal sequence the toxin is released into the culture medium in its mature form as a 535 amino acid residue protein with a molecular weight of 58 kDa (Smith 1980; Smith et al. 1980; Kaczorek et al. 1983; Greenfield et al. 1983).

Early biochemical analysis of diphtheria toxin revealed that the protein is exquisitely sensitive to serine protease (e.g. trypsin) attack which results in 'nicking'

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in vitro and the formation of two fragments, A and B, which remain covalently attached by an interchain disulfide bond (Gill and Pappenheimer 1971; Drazin et al. 1971). Characterization of non-toxic serologically related mutants (Uchida et al. 1971) demonstrated that fragment A, the N-terminal 21.1 kDa peptide, contains the enzymatic activity (Collier and Kandel 1971; Gill and Dinius 1971); whereas, fragment B, the C-terminal 37.1 kDa peptide, mediates binding of the toxin to its cell surface receptors and facilitates the delivery of fragment A to the cytosol (Gill and Dinius 1971; Drazin et al. 1971; Uchida et al. 1971). X-ray crystallographic analysis, at a resolution of 2.5 Å, demonstrated that diphtheria toxin is composed of three structural domains: the amino terminal catalytic (C) domain corresponds to fragment A; whereas, the transmembrane (T) and carboxy-terminal receptor binding (R) domains comprise fragment B (Choe et al. 1992; Bennett et al. 1994).

A disulfide bond between Cys186 and Cys201 of the toxin 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 are prerequisites for both the DT and DT-related fusion protein toxins action against eukaryotic cells (Williams et al. 1990; Ariansen et al. 1993; Tsuneoka et al. 1993). While the mechanisms of enzymatic activity, receptor binding, and receptor mediated endocytosis are known in great detail, relatively little is known about the precise molecular mechanisms which mediate C domain translocation across the endosomal membrane and the release of an active appropriately folded enzyme into the cytosol.

2 Catalytic domain delivery into the eukaryotic cytosol results in an inhibition of protein synthesis

The intoxication of eukaryotic cells by either DT or the DT-related fusion protein toxins requires the productive delivery of the C domain from the lumen of an early endosomal compartment to the cytosol. It is well known that the C domain (amino acid residues 1 – 193, fragment A) catalyzes the NAD^+-dependent ADP-ribosylation of elongation factor 2 (EF-2). Elongation factor 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). The enzymatic transfer of the ADP-ribosyl (ADPR) moiety from NAD^+ to a modified histidine residue in EF-2 (diphthamide), as shown below, results in the irreversible arrest of polypeptide chain elongation, which ultimately leads to cell death (Collier et al. 1969).

$$\text{C domain}\quad \text{NAD}^+ + \text{EF-2} \rightleftharpoons \text{ADPR-EF-2} + \text{nicotinamide} + \text{H}^+$$

The DT C domain has been shown to ADP-ribosylate EF-2 from archea to man (Pappenheimer et al. 1972); whereas, the homologous elongation factors from both prokaryotic cells and mitochondria are not recognizable substrates (Richter et al. 1970). The crystal structure of DT indicates that the C domain is composed of

eight β-strands and seven α-helices. Binding of NAD^+ is mediated by residues His21 and Tyr65 (Papini et al. 1989, 1990, 1991), and Glu148 also plays a key role in catalysis (Carroll et al. 1984). The requirement for NAD^+ is highly specific and it cannot be replaced by $NADP^+$ or NADH (Goor et al. 1967).

The role that the C domain plays in its own delivery to the cytosol of targeted cells is, as yet, unknown. However, the complete unfolding of the C domain was postulated more than twenty years ago as a prerequisite for translocation (Donovan et al. 1981; Kagan et al. 1981). The necessity for complete unfolding of the C domain prior to translocation was then indirectly demonstrated by Wiedlocha et al. (1992) and by Falnes et al. (1994). Wiedlocha et al. (1992) genetically fused acidic fibroblast growth factor (aFGF) to the amino terminus of the C domain. The aFGF-DT fusion protein was found to be cytotoxic for eukaryotic cells, confirming the observation that polypeptides fused to fragment A may be delivered into the cytosol of targeted cells (Stenmark et al. 1991). In the presence of heparin, however, aFGF is known to retain a rigid tertiary structure and the aFGF-DT fusion protein was no longer cytotoxic. These results strongly implied that unfolding is a requirement for delivery through the nascent channel formed by the T domain.

Based upon the X-ray crystal structure of the toxin, Falsnes et al. (1994) constructed a double cysteine insertion mutant which formed an internal disulfide bond within the C domain. Under oxidizing conditions, with the disulfide bond intact, unfolding of the C domain is prevented. While this mutant toxin retained ADP-ribosyltransferase activity *in vitro*, it was not cytotoxic toward cells in culture. These results provided additional indirect evidence that complete unfolding of the C domain was prerequisite for intoxication of eukaryotic cells. In the case of the DT-related fusion protein toxins, tracking of internalized epitope tagged toxin has also been used to demonstrate that the C domain is denatured and loses its enzymatic activity in lumen of acidified early endosomes (Zeng 1998). Taken together, these studies indicate that the cytosolic delivery of the C domain occurs in at least a partially unfolded enzymatically inactive state.

3 Receptor binding and substitution of the native diphtheria toxin receptor binding domain with surrogate ligands

As with all of the A/B bacterial protein toxins, the intoxication of target cells by DT and DT-related fusion protein toxins begins with receptor binding, followed by receptor mediated endocytosis. The cell surface receptor for DT, DTR, has been shown to be the heparin binding epidermal growth factor-like precursor (Naglich et al. 1992a, 1992b). The relative sensitivity of a given cell line to intoxication by DT is related to the number of receptors present on the cell surface (Middlebrook et al. 1978; Dorland et al. 1979). Analysis of diphtheria toxin binding has also suggested that the sensitivity of targeted cells is also dependent upon the presence of DTR modulators, such as the human CD9 antigen (Mitamura et al.

1992). Human CD9 antigen, which is associated with the DTR (Mitamura et al. 1992; Iwamoto et al. 1994), enhances sensitivity to DT through an as yet unknown mechanism.

Analysis of the X-ray crystal structures indicates that the receptor binding (R) domain of native DT is composed of residues 386-535 (Choe et al. 1992; Bennett et al. 1992). Substitution of the native R domain with a surrogate cell surface receptor specific ligand has been shown to result in the formation of a fusion protein toxin whose action is targeted to only those cells which express that cell surface receptor. The first genetically engineered fusion protein toxin, $DAB_{486}\alpha MSH$, consisted of the diphtheria toxin fragment A and a portion of fragment B fused to α-melanocyte stimulating hormone (α-MSH) (Murphy et al. 1986). While $DAB_{486}\alpha MSH$ was selectively toxic toward cells that displayed the MSH receptor, it was prone to extensive proteolytic degradation when expressed in recombinant *E. coli*. Human interleukin-2 (hIL-2), 133 amino acid peptide versus the 13 amino acid aMSH, was used as the next surrogate receptor binding domain (Williams et al. 1987). Remarkably, DAB_{486}IL-2 was more stable when expressed in *E. coli* and was found to be extremely potent (IC_{50} of 2-4 x 10^{-11} M) against only those cells that displayed the high affinity receptor for IL-2 (Bacha et al. 1980; Williams et al. 1990a, 1990b). Deletion analysis of the carboxy-terminal residues in the diphtheria toxin portion of DAB_{486}IL-2 genetically mapped the junction between the transmembrane (T) domain of the toxin and the receptor binding domain (Williams et al. 1990a, 1990b). The internal in-frame deletion mutant, DAB_{389}IL-2, was shown to be at least a log more potent (*e.g.* IC_{50} = 2 -5 x 10^{-12} M) toward high affinity IL-2 receptor bearing cell *in vitro*.

In the case of DAB_{389}IL-2, only the high affinity and intermediate affinity IL-2 receptor:: fusion protein toxin complexes are internalized (Re et al. 1996). The IL-2 receptor is composed of three subunits α, β, and γ_c (Takeshita et al. 1992). The α subunit alone forms the low affinity receptor (Kd ~10 nM), while the β and γ_c subunits form a heterodimeric intermediate affinity receptor (Kd ~5 nM). All three subunits together form a heterotrimeric high affinity receptor (Kd ~60 pM). IL-2 is a hematopoietic cytokine, and binding to the high affinity IL-2 receptor stimulates the activation of genes promoting DNA synthesis and the proliferation of IL-2 dependent cells (Rayhel et al. 1988). Signaling pathways mediated by the IL-2 receptor are a requirement for T-cell activation and proliferation, and IL-2 receptor expression is frequently upregulated in leukemia and lymphoma tumors of T-cell origin (Waldmann 1989). Expression of the high affinity IL-2 receptor is also an obligatory event in the development of T-cell mediated immune response, and upregulation of the receptor on auto-aggressive T-cells marks an early common event in the pathogenesis of essentially all autoimmune diseases (Ratts and vanderSpek 2002). The specific expression of the high affinity IL-2 receptor on only activated and proliferating T-cells suggested that intervention with DAB_{389}IL-2 could result in a beneficial outcome for patients presenting with CD25 positive (α-chain of the IL-2 receptor) malignancies and autoimmune diseases. Indeed in 1998, DAB_{389}IL-2 (Ontak®) was approved by the U.S. Food and Drug Administration for the treatment of refractory cutaneous T cell lymphoma,

and current studies have shown this fusion protein toxin to be an effective therapeutic in the treatment of steroid resistant graft vs. host disease (Ho et al. 2004). Based on the success of $DAB_{389}IL\text{-}2$ in the clinic, a growing number of diphtheria toxin based fusion protein toxins have been constructed as potential therapeutics for a variety of malignant oncological disorders (Ratts and vanderSpek 2002).

4 Intoxication of target cells requires the toxin to pass through a low pH endosomal compartment

Ammonium salts (e.g. NH_4Cl), glutamine, certain other amines, and choloroquine were the first compounds found to block the action of diphtheria toxin by inhibiting the cytosolic delivery of the C domain (Kim et al. 1965). It is well known that choloroquine and ammonium salts are ionophores, and raise the luminal pH of endocytidic vesicles and lysosomes (Wibo et al. 1974). These early results led to the hypothesis that passage of diphtheria toxin through an acidic compartment was an integral step in the intoxication process. Umata et al. (1990) confirmed the hypothesis by demonstrating that acidification of the endosomal lumen by membrane associated vesicular (v)-ATPase was, in fact, a required step in DT intoxication. The v-ATPase inhibitor bafilomycin A1 (Bowman et al. 1988) blocked acidification of the endosomal lumen and protected cells against DT intoxication (Umata et al. 1990).

In contrast to the internalization and endosomal route of entry, brief exposure of toxin bound to the surface of cells to low pH (*e.g.* 5.0) results in the delivery of the C domain to the cytosol and a decrease protein synthesis even in the presence of choloroquine and ammonium ions (Sandvig et al. 1980). This study also 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 (1981b) also demonstrated that the cytosolic entry of the C domain could be blocked by the metabolic inhibitors 2-deoxyglucose and sodium azide, implying that ATP was required for the membrane translocation (Sandvig et al. 1981b). Proteinase protection assays were used to examine which portions of diphtheria toxin inserted into the plasma membrane when toxin bound cells were exposed to low pH (Moskaug et al. 1988). These investigators described the translocation of fragment A (20 kD) into 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). In addition, it has been shown that translocation of fragment A into the cytosol requires a lower pH then the membrane insertion of fragment B (Stenmark et al. 1989; Falnes et al. 1992).