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Bordetella Dermonecrotic Toxin Exerting Toxicity through Activation of the Small GTPase Rho

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***Bordetella* dermonecrotic toxin (DNT) is a virulence factor produced by bacteria belonging to the genus *Bordetella*. The toxin possesses novel transglutaminase activity that catalyzes polyamination or deamidation of the small GTPases of the Rho family. The modified GTPases loose their GTP hydrolyzing activity, function as a constitutive active molecule, and continuously transduce signals to downstream effectors, which mediate the consequent phenotypes of cells intoxicated by DNT. A dynamin-dependent endocytosis is required for the toxin to be internalized into cells although it is unlikely transported to deep organelles such as the Golgi apparatus or the ER. Several lines of evidence show that the toxin undergoes proteolytic cleavage by furin or furin-like protease probably in the early endosome, and then escapes into the cytoplasm to reach the GTPase.**

Key words: actin stress fiber, endocytosis, furin, Rho GTPase, transglutaminase.

Abbreviations: DNT, dermonecrotic toxin; ER, endoplasmic reticulum.

Many bacterial protein toxins exert their toxicity by modifying the small GTPases (*1*). It is well known that *Clostridium botulinum* C3 exoenzyme transfers the ADP-ribose moiety of NAD to the small GTPase Rho (*2*, *3*). Large clostridial cytotoxins including *Clostridium difficile* toxin A and B cause glycosylation of the small GTPases using UDP-glucose or UDP-*N*-acetylglucosamine as a cosubstrate (*4*–*7*). These modifications result in inactivation of the GTPases, which mediates their toxic actions. In contrast to these small GTPase-inactivating toxins, dermonecrotic toxin (DNT), which is produced by bacteria belonging to the genus *Bordetella*, has been recently found to activate the GTPases. DNT is essentially a transglutaminase that catalyzes deamidation or polyamination on members of the Rho GTPase family, such as Rho, Rac, and Cdc42. This article reviews the molecular nature of the modification of the GTPases by DNT and resultant phenotypes in intoxicated cells. The roles of DNT in infectious diseases caused by *Bordetella* spp. are also discussed.

Structure and biological activities of DNT as a toxin

DNT was first described in 1909 as a virulence factor of *B. pertussis*, which causes human whooping cough (*8*). It was subsequently found that *B. bronchiseptica* and *B. parapertussis* also produce almost identical toxins. The toxin is a single-chain polypeptide of 1,464 amino acids. Recent study revealed that the N-terminal 54 amino acids are responsible for binding to target cells and C-terminal 288 amino acids confer the enzymatic action (*9*–*11*); they are the designated B (binding) domain and A (active) domain, respectively (Fig. 1). Four transmembrane helices are predicted between the B domain and the A domain. The A domain has about 30% homology with the catalytic domain of cytotoxic necrotizing factor (CNF) from *Escherichia coli*, which is known to catalyze deamidation but not polyamination of the identical Gln of the Rho GTPases (*12*). Notably, the highly homologous sequences are in the vicinity of a Cys residue that is considered to be an active center of the molecules. On the other hand, the B domain is similar to no toxins.

DNT is highly lethal and causes splenoatrophy in mice and a characteristic dermonecrosis when injected intradermaly, from which the name "dermonecrotic toxin" is derived. At a cellular level, the toxin stimulates DNA and protein syntheses and reorganization of actin cytoskeletal systems, focal adhesions and stress fibers, leading to alterations in cell morphology (*13*). Moreover, the toxin inhibits the differentiation of osteoblastic MC3T3-E1

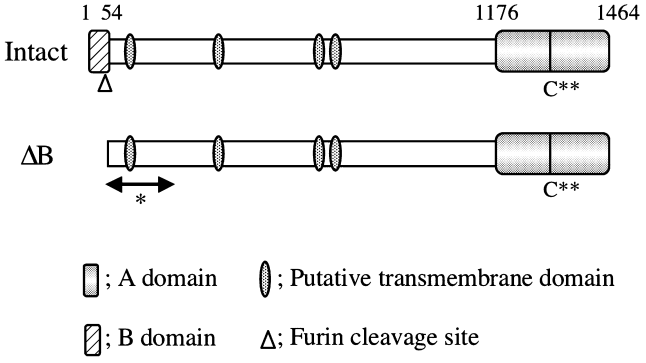

Fig. 1. **The organization of functional domains of DNT.** The numbers show the amino-acid positions at the boundaries of each domain. *The region which is considered to be responsible for the membrane translocation of the A domain. **The active Cys residue.

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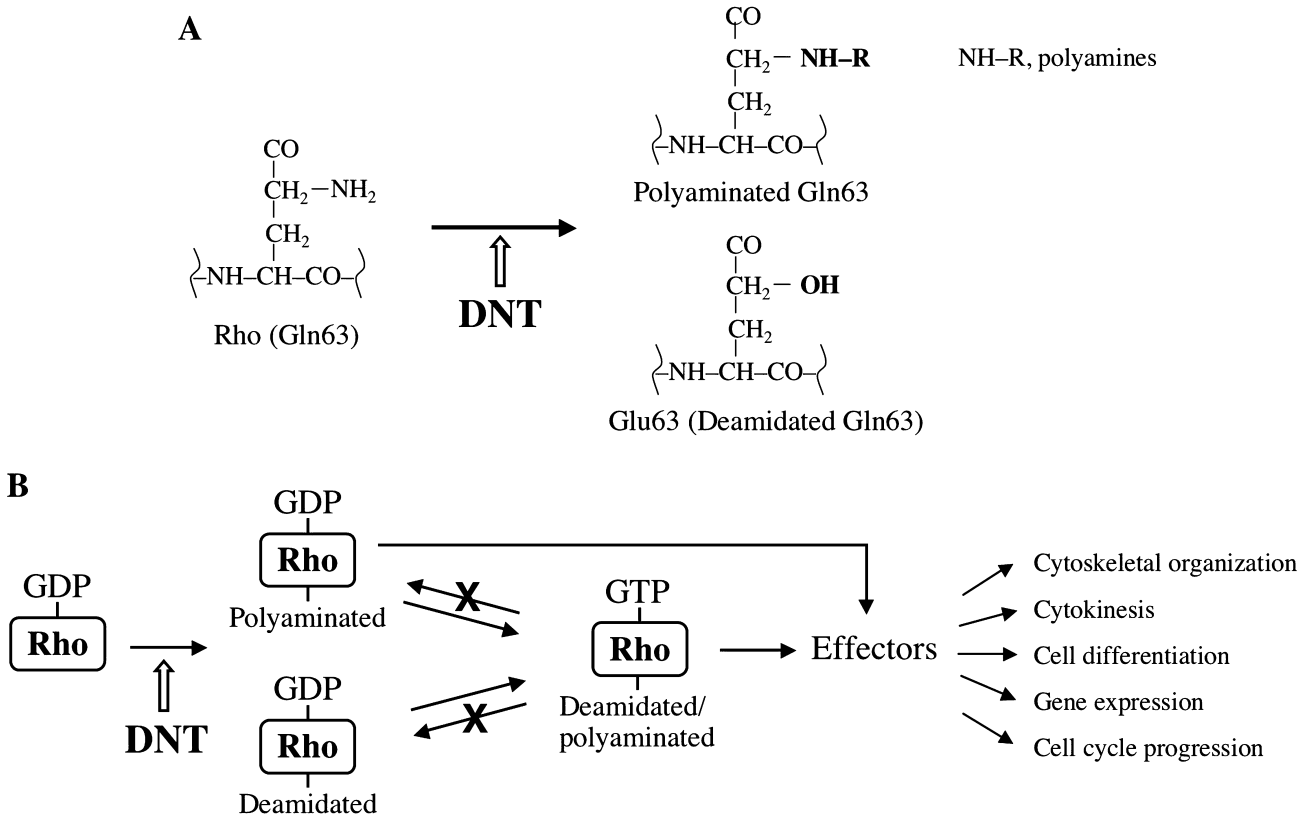

Fig. 2. **Modification of Rho GTPase by DNT.** (A) Transglutaminase activity of DNT. DNT catalyzes polyamination or deamidation at Gln63 of RhoA. See the text for details. (B) The modifications abrogate the GTP-hydrolyzing activity of the GTPases. Furthermore, in the case of the polyamination, the GTPases gain the ability to interact with downstream effectors (especially ROCK) in a GTP-independent manner. As a result, the modified GTPases function as constitutive analogues and induce anomalous-cellular events.

cells (*14*). Multinucleated cells appear in the culture of DNT-treated cells, which probably result from interference with cytokinesis (*15*). The cultured cell strains sensitive to DNT are limited; in our experience, the toxin affects MC3T3-E1, Swiss3T3, and C3H10T1/2 cells and a few other strains, suggesting that a distinctive receptor is present on the susceptible cells (*11*).

Modifications of the Rho GTPases by DNT

The alterations in the actin cytoskeletal systems caused by DNT implied that the toxin directly or indirectly influenced the small Rho GTPases. (*16–18*). Thus, analyzing the DNT action in relation to the Rho GTPases, our research group found that the toxin catalyzes deamidation or polyamination at Gln63 of RhoA and the corresponding Gln61 of other Rho family member, Rac and Cdc42 (*19*, *20*) (Fig. 2). The deamidation and the polyamination are essentially the same acyl-transfer reaction, in which the γ-carboxamide group of the Gln is an acyl donor. In the presence of polyamines such as putrescine, spermidine, and spermine, DNT utilizes primary amino groups as acyl acceptors. In the absence or at less than threshold concentrations of polyamines, water acts as an acyl acceptor to accomplish deamidation (Fig. 2A). The polyamination preferentially occurs rather than the deamidation. Rho and Rac are equally sensitive to DNT while Cdc42 is less sensitive than the others. The GDP-bound Rho but not the GTP-bound Rho serves as a substrate for DNT. Furthermore, the GDP-bound Rho in the presence of GDP dissociation inhibitor (GDI) is not modified by DNT, indicating that Rho in the complex with GDI is not sensitive to the toxin. The Gln to be modified by DNT resides in the common switch II region of the small GTPases, which plays a key role in the GTP hydrolyzing activity of the GTPases. In fact, upon the modification by DNT, GTPases lose both the intrinsic and GTPase activating protein (GAP)-stimulated GTPase activities but retain the GTP-binding activity (*19*). In addition, the polyaminated Rho acquires the ability to associate in a GTP-independent manner with its effector ROCK, which mediates the signal leading to the stress fiber formation (*21*). Therefore, the modified GTPases both in the GDP-bound form and in the GTP-bound form function as constitutively active analogues and upregulate the downstream signal transductions, leading to the reorganization of the actin cytoskeletons and other cellular responses (Fig. 2B). In fact, stress fibers, lamellipodia, and filopodia, which are regulated by Rho, Rac, and Cdc42, respectively, obviously form in cells in response to DNT (*20*). Other cellular responses including DNA synthesis, multinucleation, and interference of the osteoblastic differentiation could be explained by the constitutive activation of the Rho GTPases although experimental evidence has yet to be fully obtained, because they have been known to regulate cytokinesis (*22–24*), cell differen-

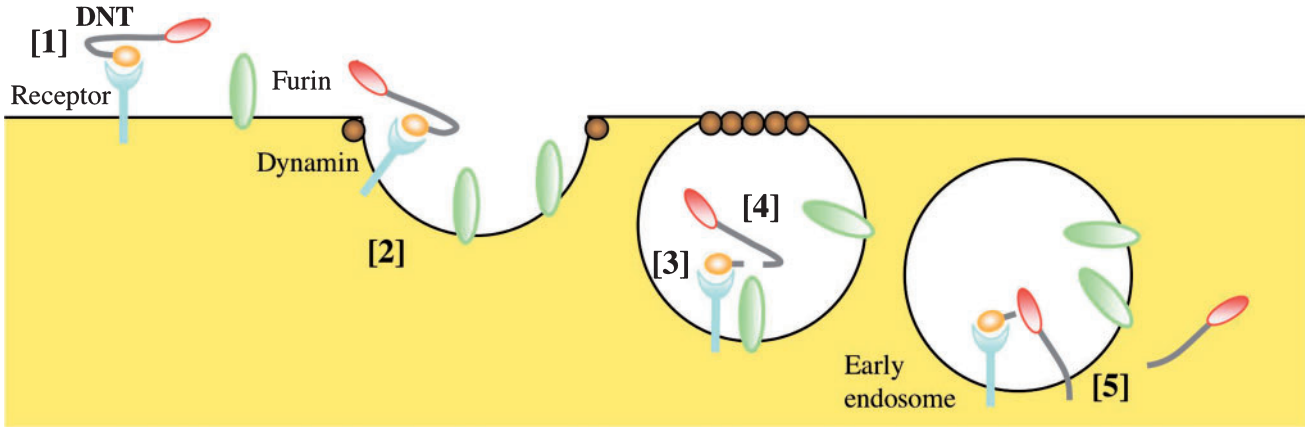

Fig. 3. **The mode of DNT entry into cells.** [1] DNT binds to a specific receptor on the cell surface, [2] is internalized by a dynamin-dependent endocytosis, and [3] is cleaved at the C-terminal side of the B domain by furin or furin-like endoprotease. [4] The B domain liberates ΔB, the rest of DNT. [5] ΔB penetrates the endosome membrane *via* the region containing a transmembrane helice (Fig. 1) and translocates the A domain into the cytoplasm. Because furin is internalized from the cell surface through clathrin-coated pits and is delivered to the early endosome and then retained there (*40*, *47*), it may be convenient for DNT to be internalized into the early endosome where furin is condensed by the clathrin-dependent endocytosis. The triggers for liberating ΔB from the B domain and the dynamic alterations in the toxin molecule to translocate the A domain into the cytoplasm remain to be elucidated.

tiation (*25*, *26*), gene expression (*27*, *28*) and cell cycle progression (*29*) besides the cytoskeletal organization.

The mechanism by which DNT enters cells

To reach the cytoplasm where the Rho GTPases are located, DNT must be internalized into target cells and translocated across the membrane after binding to the cell surface receptor via the B domain. Most bacterial enzymatic toxins are internalized into cells through endocytosis (*30*). For example, diphtheria toxin and tetanus toxin enter the cytoplasm from endosomes upon their acidification (*31*, *32*), whereas others like cholera toxin or shiga toxin are retrogradely transported via the Golgi apparatus to the ER where they escape into the cytoplasm (*33*, *34*). Actions of the former and latter toxins are blocked by bafilomycin A1, a pH-elevating agent and by brefeldin A, a Golgi apparatus-disrupting agent, respectively. However, neither reagent blocks DNT action (*35*). Nocodazole, which disrupts microtubles and thereby impairs vesicular trafficking, is also ineffective against the intoxication of cells by DNT. Therefore, the acidic late endosomes or the Golgi/ER compartments are unlikely involved in the internalization of the toxin. However, the toxin does not affect cells expressing a dominant negative form of dynamin (*35*), which is prerequisite for endocytosis to pinch off the necks of invaginated pits at the cell surface and make vesicles (*36*, *37*), indicating it needs to be endocytosed. Taken together, it is likely that DNT is internalized into the cells by dynamin-dependent endocytosis and is translocated from the early endosomes into the cytoplasm.

The mechanism by which DNT is translocated across the endosome membrane has been partly elucidated. DNT has the sequence Arg^{41}-Ala-Lys-Arg^{44}, corresponding to a consensus motif recognized by furin, a mammalian endoprotease (*38–40*), and is actually cleaved at the C-terminal peptide bond of Arg^{44}. This cleavage yields two fragments, the N-terminal chain almost corresponding to the B domain and the rest of the toxin designated ΔB, which remains associated, being in a nicked form. DNT mutants in which the furin motif was destroyed, bound to but did not affect cells. In contrast, the nicked DNT is much more potent against cells than the intact toxin. Therefore, the cleavage by furin should be indispensable for DNT action after the binding to the cells. ΔB but not the nicked toxin has the ability to translocate the A domain across the cell membrane independently of the specific receptor to the toxin, indicating that the furin cleavage and subsequent dissociation between the B domain and ΔB trigger the translocation of the A domain. The N-terminal 122 amino acid region of ΔB, which contains one of the putative transmembrane helices, seems to be involved in the translocation (Fig. 1). An overview of the internalization and translocation procedures of the toxin is depicted in Fig. 3. Presumably, the internalized DNT undergoes proteolytic cleavage by the endosome-resident furin and liberates ΔB to initiate the translocation of the A domain across the endosome membrane (*35*).

Roles in *Bordetella* infectious diseases

B. pertussis and *B. bronchiseptica* producing DNT are known to cause human whooping cough and swine atrophic rhinitis, respectively. *B. bronchiseptica* DNT has been particularly studied as to its involvement in the disease. Turbinate atrophy is often seen in juvenile pigs infected with swine atrophic rhinitis. The cell extract of bacteria producing much DNT caused severe turbinate atrophy indistinguishable from that seen in naturally infected pigs (*41*). Histological analyses of the atrophied turbinate revealed that the degenerative changes are most severe in osteoblasts (*42*). Purified DNT caused a similar bone lesion when injected into the subcutaneous tissue overlying the calvaria in neonatal rats (*43*). In addition, DNT inhibits the osteoblastic differentiation of MC3T3-E1 cells as discussed above. From these observa-

tions, it is considered that DNT impairs the osteoblastic differentiation in turbinate which eventually leads to its atrophy. On the other hand, the role of DNT in *B. pertussis* infection is obscure. The ability of bacteria to produce DNT did not correlate with their lethality after an intranasal challenge in infant mice (*44*). It also remains to be answered whether DNT contributes to the characteristic symptoms seen in whooping cough, such as the paroxysmal cough. Lack of an appropriate animal model that reproduces the cough has been an obstacle to addressing this issue.

Concluding remarks

The function of DNT has long remained to be elucidated because of its extreme instability and the property of aggregating with other bacterial components (*45*, *46*). Since it has become possible to keep the toxin active for a long period, research has progressed remarkably. At present, DNT is found to be the only bacterial toxin whose toxicity derives from transglutaminase activity. DNT as a transglutaminase is also novel in that it has a strict specificity for substrates, the Rho GTPases. The current understanding of the molecular action of DNT and the future identification of the DNT receptor should help to dissect the roles of this toxin in *Bordetella* infections, especially whooping cough, at a molecular level. In addition, we hope that DNT, like other bacterial toxins with unique biological activities, will serve as a useful tool for studies in the fields of molecular and cellular biology and biochemistry concerning the small GTPases.

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