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Identification of Functional Domains of *Bordetella* Dermonecrotizing Toxin

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***Bordetella* dermonecrotizing toxin (DNT) stimulates the assembly of actin stress fibers and focal adhesions by deamidating Gln63 of the small GTPase Rho. To clarify the functional and structural organization of DNT, we cloned and sequenced the DNT gene and examined the functions of various DNT mutants. Our analyses of the nucleotide and amino acid sequences revealed that the start codon of the DNT gene is a GTG triplet located 39 bp upstream of the reported putative initiation ATG codon; consequently, DNT contains an additional 13 amino acids at its N-terminal end. All of the N-terminally truncated mutants were found to modify Rho. The shortest fragment of DNT possessing the Rho modification activity consists of amino acids from Ile1176 to the C-terminal end. This fragment overlaps the region homologous to *Escherichia coli* cytotoxic necrotizing factors (CNFs), which show activity similar to that of DNT. The introduction of a mutation at Cys1305 located in the highly conserved region between CNFs and DNT eliminated the activity, indicating that this domain is the catalytic center of DNT. The N-terminal fragment (1 to 531) of DNT failed to modify Rho but reduced the DNT-induced polynucleation in MC3T3-E1 cells when simultaneously added with the holotoxin, suggesting competitive inhibition in the receptor-binding or internalizing step. Our finding that DNT consists of an N-terminal receptor-binding and/or internalizing domain and a C-terminal catalytically active domain may facilitate analysis of the overall action of the toxin on the mammalian target cells.**

Bordetella species such as *B. bronchiseptica*, *B. pertussis*, and *B. parapertussis* commonly produce dermonecrotizing toxins (DNTs) (2, 5, 33). The name DNT was given because of the toxin's ability to induce dermonecrotic lesions when injected into guinea pigs, mice, rabbits, and other laboratory animals (2, 10, 11, 17, 33). *B. bronchiseptica* DNT is considered to be responsible for turbinate atrophy in swine atrophic rhinitis (8, 12, 14, 19). At a cellular level, DNT is known to cause morphological changes in osteoblastic MC3T3-E1 cells and inhibit their differentiation into osteoblasts, indicating that the turbinate atrophy caused by DNT results from a deficiency of the osteoblastic differentiation in bone tissues (13). The morphological changes in DNT-treated cells resulted from the assembly of actin stress fibers and focal adhesions, which are caused by an anomalous activation of the small GTP-binding protein Rho (15, 16). It was recently demonstrated that DNT deamidates Gln63 of Rho and the corresponding Gln residues of the Rho family proteins Rac and Cdc42 (16). The deamidation results in the reduction of the GTPase activities of the Rho family proteins and renders them constitutively active. It was reported that cytotoxic necrotizing factor 1 (CNF1) produced by some strains of *Escherichia coli* also causes deamidation at the same amino acid residue of Rho (6, 29).

The DNT genes of *B. pertussis* and *B. bronchiseptica* were cloned and sequenced by two independent groups and found to be more than 99% identical (28, 32). Analyses of the sequence databases revealed that the amino acid sequence of DNT shows homology to CNF1 and the closely related CNF2 in the C-terminal regions (21, 32). CNF1 and -2 share 85% identical residues over the whole sequence of 1,014 amino acids. The N-terminal regions of CNF1 and -2, spanning more than 500 amino acids, show 27% homology to the N-terminal part of *Pasteurella multocida* toxin (PMT) (25), which is also considered to be the causative agent for turbinate atrophy in swine atrophic rhinitis (7). DNT and PMT show similar biological activities such as dermonecrotic and splenoatrophic activities and the stimulation of DNA synthesis, although their amino acid sequences are quite different (27, 28) and PMT, unlike DNT, does not cause the deamidation of RhoA (24). Based on their similarities in both structure and biological activity, DNT, PMT, CNF1, and CNF2 are considered to constitute a family of dermonecrosis-inducing toxins (32). Of these toxins, CNF1 was recently reported to consist of N-terminal receptor-binding, C-terminal catalytic, and deduced intermediate membrane-spanning domains (21). PMT was more recently reported to possess an intracellularly active domain in the N-terminal region (35). On the other hand, the organization of the functional domains of DNT is unknown.

Here we report that DNT has a domain organization similar to that of CNF; i.e., the receptor-binding domain is in the N-terminal region and the catalytic domain is in the C-terminal region. We identified Cys1305 in the catalytic domain as an essential amino acid for the enzyme activity of DNT. We also found that the N-terminal domain can block the DNT-induced polynucleation, presumably by inhibiting the cell surface binding or entry of DNT.

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MATERIALS AND METHODS

Bacterial toxins. DNTs from *B. bronchiseptica* S798 and *B. pertussis* Tohama were purified by a method described previously (11). C3 exoenzyme was kindly provided by S. Kozaki, University of Osaka Prefecture, Osaka, Japan.

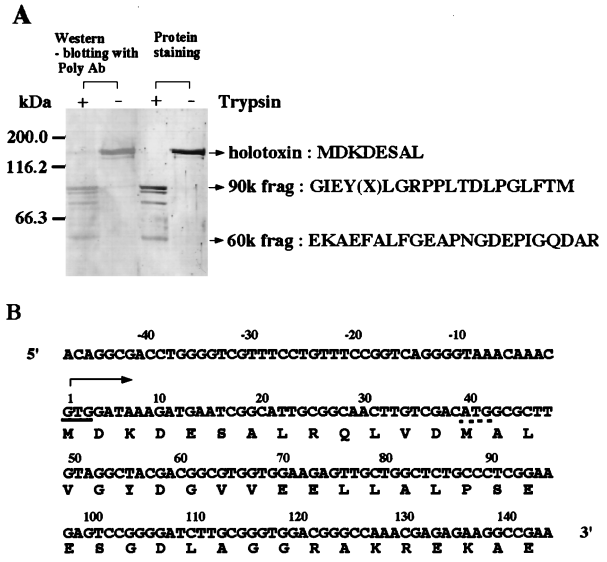


FIG. 1. Amino acid sequence analysis of the N-terminal region of DNT. (A) DNT and its tryptic fragment (frag) were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. One strip of the membranes was stained with 0.25% Coomassie brilliant blue R-250. Major protein bands indicated by arrowheads were cut out, and their N-terminal sequences were determined. Other strips were subjected to Western blot analysis with rabbit polyclonal antibody (Poly Ab) against DNT. Positions of molecular weight markers are indicated on the left. (B) Nucleotide and deduced amino acid sequences of the N-terminal region of the cloned DNT gene. The underline indicates the initiation GTG codon identified in this study; the dashed underline indicates the ATG codon reported previously as an initiation codon (28, 32).

Cell culture. MC3T3-E1 cells were cultured in alpha minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum at 37°C under 5% CO_2 in air. DNT-induced polynucleation of the cells was examined by a previously described method (13).

Cloning and sequencing of the *B. bronchiseptica* DNT gene. Genomic DNA was isolated from *B. bronchiseptica* S798 by the method of Murray and Thompson (23) and then purified by $CsCl_2$ density gradient centrifugation. The isolated DNA was digested with *Bam*HI and *Apa*I, and the DNA fragments migrating at 4.5 to 5.5 kbp were isolated and inserted into the *Bam*HI-*Apa*I site of pBluescript SK. *E. coli* DH5α was transformed with the ligated DNA, and the DNT gene was screened by hybridization with a ^{32}P-labeled oligonucleotide probe (5′-CATGG TGAAAAGACCCGGCAGATCG-3′) which was designed according to the N-terminal sequence of the 90k fragment (see Results and Fig. 1). One of the positive clones, designated pBSDNT, was then physically mapped, sequenced, and used for further constructions.

Construction of *E. coli* expression vectors for DNT mutants. The expression vectors for DNT and its mutants were constructed as follows. Sequences of the constructed DNAs were confirmed by nucleotide sequencing.

(i) pETDNTwt. pBSDNT was digested with *Apa*I, treated with T4 DNA polymerase, and then digested at the internal *Eco*RI site to isolate a 3′ portion of the DNT gene. The resultant 4.5-kbp fragment was subcloned into pUC13 digested with *Hin*cII and *Eco*RI. This plasmid was designated pUCDNT3′. The rest of the DNT gene in pBSDNT was amplified by PCR with the oligonucleotide primers 5′-GGGCCATGGATAAAGATGAATCGGCATTGC-3′ (the underline indicates an *Nco*I site) and 5′-GATTCAGTAGGTAGTAGATCTCG-3′ and subcloned into the *Nco*I-*Hin*dIII site of pET21d (Novagen, Inc., Madison, Wis.) together with the *Eco*RI-*Hin*dIII fragment of pUCDNT3′. The resulting plasmid was designated pETDNTwt and used for the expression of wild-type DNT.

(ii) pETDNT$_{1-531}$. pETDNT$_{1-531}$ was constructed by the treatment of pETDNTwt with *Not*I, followed by recircularization.

(iii) pETDNT$_{1-1162}$. First, to add in-frame stop codons to the 3′ end of the cloned fragment, we constructed a plasmid possessing a stop codon in every reading frame. pUC13 was digested with *Hin*dIII and *Xba*I and then ligated with the annealed oligonucleotides 5′-CTAGAGTCTGAGTGAGTGAGTGA-3′ (the underlines indicate stop codons) and 5′-AGCTTCACTCACTCACTCAG ACT-3′. The resultant plasmid was designated pUCSTOP. Second, we introduced an *Xba*I site into nucleotide position 3487 in the DNT gene by PCR with primers 5′-CCTTTAACGCAGTGGACCGGAAC-3′ and 5′-CCTCTAGAGGC AGGACCGAAGTT-3′ (the underline indicates an *Xba*I site). The *Eco*RV (nucleotide position 3301 in DNT)-*Xba*I fragment of the amplified DNA and the *Eco*RI-*Eco*RV (nucleotide position 3301 in DNT) fragment of pBSDNT were subcloned into the *Eco*RI-*Xba*I site of pUCSTOP. This plasmid was digested with *Eco*RI and *Hin*dIII, and the obtained fragment was ligated to pETDNTwt digested with *Eco*RI and *Hin*dIII.

(iv) pETDNT$_{523-1464}$. The 0.9-kbp fragment of pETDNTwt was amplified by PCR with primers 5′-GATCCATGGGCATTGAATATTGCCTGGGG-3′ (the underline indicates an *Nco*I site) and 5′-CCTAAGCACTCGTCGTGGTGATG C-3′. The *Nco*I-*Bgl*II fragment of the amplified DNA and the *Bgl*II-*Hin*dIII fragment of pETDNTwt were inserted into the *Nco*I-*Hin*dIII site of pET21d.

(v) pETDNT$_{1163-1464}$. The 0.4-kbp fragment of pETDNTwt was amplified by PCR with primers 5′-TTTCTAGAGCCATCAGCCCGACGCGCCTGGATTA CGC-3′ (the underline indicates an *Xba*I site) and 5′-CAGGTAGCCCTCCTT GACCCCAACCATCGTCGTGCAC-3′ (the underline indicates an *Apa*LI site). The amplified fragment was digested with *Xba*I and *Apa*LI and subcloned with the *Apa*LI-*Hin*dIII fragment of pETDNTwt into pBluescript SK$^-$. The resultant plasmid was digested with *Xba*I, blunted with T4 DNA polymerase, and then ligated with a phosphorylated 8-mer *Nde*I linker. This plasmid was digested with *Nde*I and *Hin*dIII, and the obtained fragment was subcloned into pET3a (Novagen). The constructed vector, however, was not stably retained in *E. coli*; therefore, the *Xba*I-*Hin*dIII fragment of this plasmid was religated to the *Xba*I-*Hin*dIII site of pET21d.

(vi) pETDNT$_{1176-1464}$. A phosphorylated 12-mer *Nde*I linker was inserted into pETDNTwt digested with *Eco*RV. The *Nde*I-*Hin*dIII fragment of this plasmid was subcloned into pET3a. The *Xba*I-*Hin*dIII fragment of the resultant plasmid was subcloned into pET21d for the reason mentioned above.

(vii) pETDNT$_{1292-1464}$. pETDNT$_{1163-1464}$ was digested with *Nru*I and ligated with a phosphorylated 8-mer *Nde*I linker. The resultant plasmid was digested with *Nde*I and then circularized.

(viii) pETDNT$_{1176-1444}$ and pETDNT$_{1176-1364}$. PCR was carried out with primers 5′-TTTCTAGAGCCATCAGCCCGACGCGCCTGGATTACGC-3′ and 5′-TTTGTAAGCTTCAGCCTGCCCAGGCCAGATCCTCCGC-3′ or 5′-TTTGT AAGCTTCAGACCAAGTCGTCATTGCGCATCGG-3′ (the underlines indicate *Hin*dIII sites), with pETDNT$_{1163-1464}$ as a template DNA. The *Asc*I-*Hin*dIII region of pETDNT$_{1176-1464}$ was replaced with the amplified DNAs digested with *Asc*I-*Hin*dIII.

(ix) pETDNT$_{C1305A}$. pETDNT$_{C1305A}$ was prepared by site-directed mutagenesis with a Quick Change kit (Stratagene, La Jolla, Calif.), using pETDNT$_{1163-1464}$ as a template.

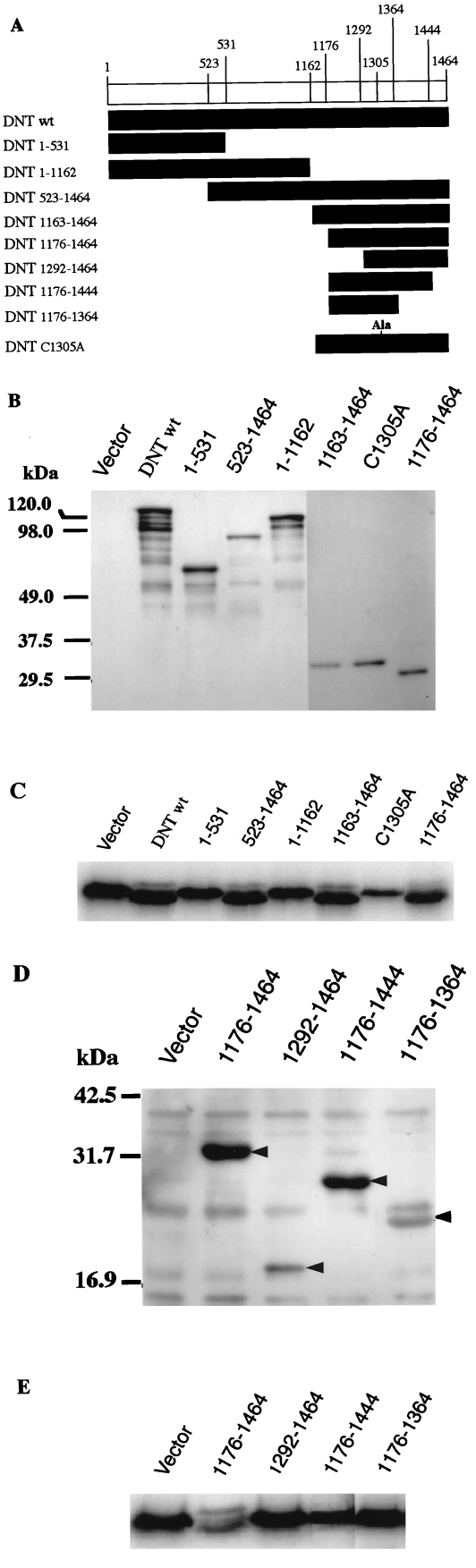
(x) pSTV29-FLAGRhoA. FLAG-tagged RhoA gene was obtained from pMEPyori FLAG-RhoA (16) digested with *Nco*I and *Bam*HI and inserted into the *Nco*I-*Bam*HI site of pET21d. This plasmid was digested with *Bgl*II, blunted with T4 DNA polymerase, and then digested with *Bam*HI. The resultant fragment was inserted into the *Sal*I (blunted)-*Bam*HI site of pSTV29 (Takara Biomedicals, Tokyo, Japan).

(xi) pETDNT$_{1-531}$ · glutathione S-transferase (GST). The *Ssp*I-*Hin*dIII fragment of pUCSTOP was inserted into the *Eco*RV and *Hin*dIII site of pBluescript SK$^-$. The resultant plasmid was designated pBSSTOP. The GST gene was amplified by PCR with primers 5′-AATATGCGGCCGCTCATGTCCCCTATA CTAGG-3′ (the underline indicates an *Not*I site) and 5′-GGCAGATCGTCAG TCAGTCACG-3′, with pGEX4T3 (Amersham Pharmacia Biotech Ltd., Uppsala, Sweden) as a template DNA. The amplified DNA was digested with *Not*I and *Eco*RI and inserted into the *Not*I-*Eco*RI site of pBSSTOP. The *Not*I-*Hin*dIII fragment of this plasmid was ligated with the *Nco*I-*Nde*I and *Nde*I-*Not*I fragments of pETDNT$_{1-531}$ and pET21d digested with *Nco*I and *Hin*dIII.

(xii) pGEXGST · DNT$_{523-1464}$. A *Bam*HI site was generated upstream of the gene encoding DNT$_{523-1464}$ by PCR with primers 5′-CCGGATCCGGCATTG AATATTGCCTGG-3′ (the underline indicates a *Bam*HI site) and 5′-CCTAA GCACTCGTGGTGATGC-3′, with pBSDNT as a template. The *Bam*HI and *Bgl*II fragment of the amplified DNA was inserted into pBSDNT digested with *Bam*HI and *Bgl*II. This plasmid was digested with *Apa*I, blunted with T4 DNA polymerase, and then digested with *Bam*HI. The resultant fragment was subcloned into pGEX4T3 digested with *Bam*HI and *Sma*I.

Expression and purification of GST fusion proteins. pETDNT$_{1-531}$ · GST and pGEXGST · DNT$_{523-1464}$ were introduced into *E. coli* JM109(DE3) (Promega Co., Madison, Wis.) and DH5α, respectively. The bacteria were cultivated in Luria-Bertani (LB) broth containing 50 μg of ampicillin per ml and induced to produce the GST fusion proteins by 1 to 100 μM of isopropyl-β-D-thiogalactopyranoside. The GST fusion proteins were purified with a glutathione-Sepharose 4B (Amersham Pharmacia Biotech) column according to the manufacturer's instructions.

ADP ribosylation. One of the DNT mutant genes was introduced into *E. coli* BL21(DE3) carrying pSTV29 FLAG-RhoA. The bacteria were grown on LB agar containing ampicillin (25 μg/ml) and chloramphenicol (12.5 μg/ml) at 37°C overnight. The bacterial cells of two or three colonies were picked up and incubated for 12 h at 37°C in the same medium. The cells were suspended in 500 μl of 10 mM sodium phosphate buffer (pH 8.5) and disrupted by sonication. After centrifugation, 30 μg of protein of the supernatants was incubated with 300 ng of C3 exoenzyme for 60 min at 37°C in 150 μl of reaction buffer (Tris-HCl [pH 7.6], 10 mM thymidine, 10 mM dithiothreitol [DTT], 10 mM nicotinamide, 5 mM $MgCl_2$) and 10 μM [^{32}P]NAD (800 Ci/mmol; Dupont NEN, Wilmington, Del.). Fifteen microliters of 100% trichloroacetic acid was added to the reaction mixture. The precipitates obtained after centrifugation were washed with ice-cold ethyl ether, solubilized in 67.5 mM Tris-HCl (pH 6.8) containing 1% sodium



dodecyl sulfate (SDS), 25 mM DTT and 20% glycerol, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (20). Radioactive bands were detected with a Fuji BAS 1500 image analyzer (Fuji Film Co., Tokyo, Japan).

Other methods. Protein concentration was determined by the method of Lowry et al. (22) or Bradford (1). The N-terminal amino acid sequences were determined with an Applied Biosystems 492 sequencer (PE Applied Biosystems, Foster City, Calif.). Rabbit anti-DNT serum was obtained as reported previously (11). Anti-DNT immunoglobulin G was purified with an Affi-Gel protein A MAPS II kit (Bio-Rad, Richmond, Calif.). Expression of the DNT mutants was confirmed by Western blot analysis using the anti-DNT immunoglobulin G. Specific immunoreactivity was detected with a substrate mixture of 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (Promega Co., Madison, Wis.) or an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech).

Nucleotide sequence accession number. The nucleotide sequence of the DNT gene is available from the DDBJ/EMBL/GenBank databases under accession no. AB020025.

RESULTS

Cloning and sequencing of the DNT. Two different groups have reported the nucleotide sequences of the DNT genes from *B. bronchiseptica* and *B. pertussis* (28, 32). We also independently cloned and sequenced the DNT gene from the *B. bronchiseptica* S798 strain from which we routinely isolate DNT. The N-terminal sequences of the purified toxin and its tryptic fragments were also determined (Fig. 1A). Our analysis of the N-terminal amino acid sequence of the holotoxin indicated that the coding sequence of DNT started from the GTG codon located 39 bp upstream from the putative initiator ATG codon (Fig. 1B). This is not peculiar to our bacterial strain, because DNT from *B. pertussis* Tohama has an identical N-terminal sequence (data not shown), and the corresponding GTG codon is conserved in all DNT genes so far reported (28, 32). According to our observations, the open reading frame of the DNT gene should consist of 4,395 bp coding for 1,464 amino acids with a calculated molecular mass of 160,602; therefore, in this report we adopt position numbers of amino acids in DNT 13 greater than those reported to date (28, 32). The nucleotide sequence of the DNT open reading frame was 99.3 and 99.8% identical to those reported by Walker and Weiss (32) and Pullinger et al. (28), respectively. The N-terminal sequences of the two major tryptic fragments designated 60k and 90k corresponded to the deduced amino acid sequences from Glu45 to Arg67 and from Gly523 to Met542, respectively (Fig. 1A). These results confirm that the clone indeed encodes DNT.

Functional domains of DNT. We attempted to localize the catalytic domain of DNT by using the coexpression method of Oswald et al. (25). One of the DNT mutant genes was introduced into *E. coli* BL21(DE3) carrying pSTV29 FLAG-RhoA. The expressed RhoA and DNT mutants were allowed to react in the bacterial cells, and the cell lysates were examined for the

FIG. 2. Localization of the catalytic domain of DNT. (A) A schematic representation of wild-type DNT (DNT wt) and deletion mutants of DNT. Numbers in mutant names indicate positions of the N-terminal and C-terminal amino acids. (B and D) Expression of the DNT mutant proteins in *E. coli*. *E. coli* BL21(DE3) coexpressing FLAG peptide-tagged RhoA along with various mutants of DNT was solubilized in 67.5 mM Tris-HCl (pH 6.8) containing 1% SDS, 25 mM DTT, and 20% glycerol and subjected to SDS-PAGE. After transfer onto polyvinylidene difluoride membranes, the expression of each mutant was examined in a Western blot analysis using the anti-DNT polyclonal antibody. In panel D, arrowheads indicate mutant proteins. (C and E) Mobility shifts of RhoA in *E. coli* BL21(DE3). *E. coli* BL21(DE3) harboring pSTV29 FLAG-RhoA and the indicated mutant DNT expression plasmids were cultured at 37°C overnight in LB broth supplemented with ampicillin (25 μg/ml) and chloramphenicol (12.5 μg/ml). The cells were disrupted by sonic treatment, and the soluble fractions were subjected to [^{32}P]ADP-ribosylation followed by SDS-PAGE. Radiolabeled RhoAs were detected with a Fuji BAS 1500 image analyzer.

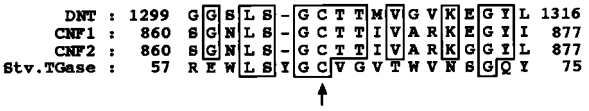


FIG. 3. Sequence alignment showing the homology in the putative active sites of DNT, CNF1, CNF2, and *Streptoverticilium* sp. transglutaminases (Stv. TGase). Groups of identical amino acids are boxed. Cys1305 of DNT exists in the consensus sequence LSGCTT (arrow).

modifications of RhoA that are detected by the mobility shifts in SDS-PAGE. The band moving more slowly than the intact RhoA was found to be the deamidated RhoA (16), whereas the nature of the faster-moving band is unknown. As shown in Fig. 2C, $DNT_{523-1464}$, $DNT_{1163-1464}$, and $DNT_{1176-1464}$ modified RhoA as effectively as the wild type did, whereas DNT_{1-531} and DNT_{1-1162} did not. Expression of the mutant proteins was confirmed by Western blotting with the anti-DNT polyclonal antibody (Fig. 2B). These results suggest that the catalytic domain of DNT is located in the C-terminal region spanning from Ile1176 to the C-terminal Val (Fig. 2C). To determine the minimal region responsible for the catalytic action of DNT, we prepared further the smaller fragments and examined them for the Rho modification activity. However, neither C-terminally nor N-terminally truncated forms of $DNT_{1176-1464}$ modified RhoA (Fig. 2D and E). A 20-amino-acid deletion of the C terminus was enough to eliminate the catalytic activity of $DNT_{1176-1464}$ (Fig. 2E, $DNT_{1176-1444}$). We recently found that DNT is a transglutaminase catalyzing the polyamination of Rho (unpublished data). The active core regions of transglutaminases include a Cys residue of which the thiol group is considered essential for their enzyme activities (26). In the catalytic domain of DNT, there is one Cys residue at position 1305. The region including this cysteine residue shows slight homology to the putative active site of another bacterial transglutaminase from *Streptoverticillium* sp. (18) and is very well conserved among DNT and CNFs (Fig. 3). Therefore, to determine whether Cys1305 is involved in the activity of DNT, we examined the activity of DNT_{C1305A} in which the Cys residue was exchanged with Ala. As shown in Fig. 2C, this mutant failed to modify RhoA (Fig. 2C).

The finding that the C-terminal region possesses the catalytic activity raised the possibility that the receptor-binding domain resides in the other part of the DNT molecule. To examine this, we prepared GST fusion proteins of DNT_{1-531} and $DNT_{523-1464}$ and tested their inhibitory effects on the DNT-induced polynucleation. As shown in Fig. 4, the DNT-induced polynucleation was blocked in the presence of DNT_{1-531} in a dose-dependent manner, whereas $DNT_{523-1464}$ had no effect at any dose. Given that the catalytically active domain does not reside in DNT_{1-531}, it is likely that DNT_{1-531} retains the receptor-binding or internalizing property and thereby competitively inhibited the action of the holotoxin.

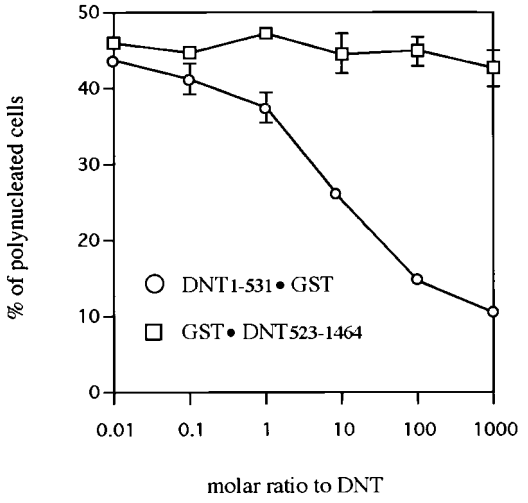


FIG. 4. Inhibition of the DNT-induced polynucleation by the DNT mutant proteins. MC3T3-E1 cells were plated on 24-well plates at an initial density of 2.5×10^4 cells per cm^2. The purified GST-DNT_{1-531} (○) or GST-$DNT_{523-1464}$ (□) fusion was added to the cell culture together with 5 ng/ml of DNT. After incubation for 72 h at 37°C, the mono- and polynucleated cells were enumerated and the percentage of polynucleated cells in at least 400 cells was calculated as described elsewhere (13). Each point represents the mean ± standard deviation of four determinations.

DISCUSSION

Many bacterial protein toxins exert their toxic effects on target cells by efficiently binding and entering into the cells. In the cells, the toxins modify the intracellular targets and alter their functions, eventually inducing various toxic effects. To accomplish these steps, the toxins have several functional domains that play specific roles in the toxin action. The so-called A-B toxin refers to protein toxins composed of two functionally different domains, i.e., an A domain that carries an enzyme activity and a B domain that is responsible for the binding to receptors on target cells (4). Diphtheria toxin, *Pseudomonas* exotoxin A, and botulinum neurotoxins are classified as this type (3, 34). Large clostridial cytotoxins such as *Clostridium difficile* toxins A and B possess more than one receptor-binding unit in a single molecule, and therefore the designation $A\text{-}B^x$ toxin, of which the superscript "x" indicates multivalent binding sites in a single chain, is proposed for them (4). Cholera toxin, *E. coli* heat-labile toxin, and Shiga toxin are designated $A\text{-}B_5$ toxins, because they consist of an A subunit and five B subunits, which are noncovalently associated (4, 31).

Based on our analysis of the structure-function relationship of DNT, we conclude that the toxin is composed of an N-terminal receptor-binding and/or internalizing domain and a C-terminal enzymatically catalytic domain. DNT has no repetitive oligopeptides in the receptor-binding domain, unlike large clostridial cytotoxins (4), and therefore should be classified into the A-B toxin group, not the $A\text{-}B^x$ group. The typical A-B toxins such as diphtheria toxin and botulinum neurotoxins undergo limited proteolysis after being synthesized as a single polypeptide chain, and in their active form they exist as heterodimers of an A fragment and B fragment linked by a single disulfide bond (3). This proteolytic cleavage has been reported to be requisite for exerting the toxic activities (4). Although DNT was also cleaved by trypsin in vitro into the N-terminal 60k and the C-terminal 90k fragments nearly corresponding to the receptor-binding DNT_{1-531} (an A fragment) and the catalytically active $DNT_{523-1464}$ (a B fragment), respectively, it remains unknown whether such protelolytic cleavage is essential for the DNT action in vivo.

The catalytically active mutants $DNT_{523-1464}$, $DNT_{1163-1464}$ and $DNT_{1176-1464}$ share the region homologous to CNFs. For example, $DNT_{1176-1464}$ shows 27.1% homology to $CNF1_{721\text{-C terminus}}$ and 31.6% homology to $CNF2_{721\text{-C terminus}}$. The similarity in activity between DNT and CNFs may reflect the homologous catalytic domains in the C-terminal regions. In fact, the enzyme activity of CNF1 has also been localized to the C-terminal homologous region (21). In this study, we show that a Cys residue in this homologous region is essential for the

modification of RhoA. It has also been demonstrated that the substitution of Ser for the corresponding Cys resulted in the elimination of the CNF1 activity (30). In addition to the Cys residue, Schmidt et al. claimed that His is necessary for the CNF1 activity, forming a catalytic dyad with Cys (30). This might also be the case for DNT, because His is also conserved at the corresponding position in DNT (His1320) (21). Although DNT and CNFs possess similar enzyme activities, their substrate specificities are different: Rho $\approx$ Rac $>$ Cdc42 for DNT and Rho $>$ Cdc42 $\gg$ Rac for CNF1. Furthermore, DNT causes an unknown modification, which shifts the RhoA band downward in SDS-PAGE, in addition to the deamidation (16), whereas CNF causes only the deamidation under physiological conditions (29). To localize the pivotal domain(s) responsible for the differences in the substrate specificities and catalytic activities, we constructed DNT-CNF2 and CNF2-DNT chimeric toxins, in which the N- and C-terminal portions are connected via the consensus sequence LSGCTT (Fig. 3), and examined their catalytic actions on Rho family proteins. We expected that these chimeric toxins might help us to localize the regions involved in recognizing the substrates or effecting the unknown modification to portions either upstream or downstream of the consensus LSGCTT region. These chimeric toxins, however, did not catalyze any modification on RhoA (18a), probably because of gross alterations in the overall structure. Further investigation is necessary to address this matter. DNT was reported to possess the nucleotide-binding motif (^{1317}**AFYHTGKS**^{1324}; boldface indicates the consensus residues in the catalytic domain) (28). Pullinger et al. reported that the toxic activity of DNT was abolished by the nonconservative mutation of this motif and pointed out the possibility of DNT as a nucleotide-binding protein (28). However, this idea may have to be reconsidered because the enzyme action of DNT is obviously ATP independent and the binding of GTP was not detected by the filter assay method (data not shown). Furthermore, this motif does not exist in CNFs.

In this study, we localized the receptor-binding or internalizing and catalytic domains of DNT. These fragments with different functions should enable us to analyze the molecular mechanisms of DNT action in various respects such as binding to the cell membrane, internalization into the cytoplasmic environment, and translocation to the target molecule.

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REFERENCES

1. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
2. **Bruckner, I. E., and D. G. Evans.** 1939. The toxin of *Br. parapertussis* and the relationship of this organism to *H. pertussis* and *Br. bronchiseptica*. J. Pathol. Bacteriol. **48:**67–78.
3. **Collier, R. J., and J. Kandel.** 1970. Structure and activity of diphtheria toxin. J. Biol. Chem. **246:**1496–1503.
4. **Eichel-Streiber, C. V., P. Boquet, M. Sauerborn, and M. Thelestam.** 1996. Large clostridial cytotoxins-a family of glycosyltransferase modifying small GTP-binding proteins. Trends Microbiol. **4:**375–382.
5. **Evans, D. G.** 1940. The production of *pertussis* antitoxin in rabbits and the neutralization of *pertussis*, *parapertussis* and *bronchiseptica* toxins. J. Pathol. Bacteriol. **51:**49–58.
6. **Flatau, G., E. Lemichez, M. Gauthler, P. Chardin, S. Paris, C. Fiorentini, and P. Boquet.** 1997. Toxin-induced activation of the G protein p21 Rho by deamidation of glutamine. Nature **387:**729–733.
7. **Foged, N. T.** 1992. *Pasteurella multocida* toxin. The characterisation of the toxin and its significance in the diagnosis and prevention of progressive atrophic rhinitis in pigs. APMIS Suppl. **25:**5–56.
8. **Hanada, M., K. Shimoda, S. Tomita, Y. Nakase, and Y. Nishiyama.** 1979. Production of lesions similar to naturally occurring swine atrophic rhinitis by cell-free sonicated extract of *Bordetella bronchiseptica*. Jpn. J. Vet. Sci. **41:**1–8.
9. **Hettasch, J. M., and C. S. Greenberg.** 1994. Analysis of the catalytic activity of human factor XIIIa by site-directed mutagenesis. J. Biol. Chem. **269:**28309–28313.
10. **Horiguchi, Y., T. Nakai, and K. Kume.** 1989. Purification and characterization of *Bordetella bronchiseptica* dermonecrotic toxin. Microb. Pathog. **6:**361–368.
11. **Horiguchi, Y., T. Nakai, and K. Kume.** 1990. Simplified procedure for purification of *Bordetella bronchiseptica* dermonecrotic toxin. FEMS Microbiol. Lett. **66:**39–43.
12. **Horiguchi, Y., T. Nakai, and K. Kume.** 1991. Effects of *Bordetella bronchiseptica* dermonecrotic toxin on the structure and function of osteoblastic clone MC3T3-E1 cells. Infect. Immun. **59:**1112–1116.
13. **Horiguchi, Y., N. Sugimoto, and M. Matsuda.** 1993. Stimulation of DNA synthesis in osteoblast-like MC3T3-E1 cells by *Bordetella bronchiseptica* dermonecrotic toxin. Infect. Immun. **61:**3611–3615.
14. **Horiguchi, Y., T. Okada, N. Sugimoto, Y. Morikawa, J. Katahira, and M. Matsuda.** 1995. Effect of *Bordetella bronchiseptica* dermonecrotizing toxin on bone formation in calvaria of neonatal rats. FEMS Immunol. Med. Microbiol. **12:**29–32.
15. **Horiguchi, Y., T. Senda, N. Sugimoto, J. Katahira, and M. Matsuda.** 1995. *Bordetella bronchiseptica* dermonecrotizing toxin stimulates assembly of actin stress fiber and focal adhesions by modifying the small GTP-binding protein rho. J. Cell Sci. **108:**3234–3251.
16. **Horiguchi, Y., N. Inoue, M. Masuda, T. Kashimoto, J. Katahira, N. Sugimoto, and M. Matsuda.** 1997. *Bordetella bronchiseptica* dermonecrotizing toxin induces reorganization of actin stress fibers through deamidation of Gln-63 of the GTP-binding protein Rho. Proc. Natl. Acad. Sci. USA **94:**11623–11626.
17. **Iida, T., and T. Okonogi.** 1971. Lienotoxicity of *Bordetella pertussis* in mice. J. Med. Microbiol. **4:**51–60.
18. **Kanaji, T., H. Ozaki, T. Takao, H. Kawajiri, H. Ide, M. Motoki, and Y. Shimonishi.** 1993. Primary structure of microbial transglutaminase from *Streptoverticillium* sp. strain s-8112. J. Biol. Chem. **268:**11565–11572.

18a. **Kashimoto, T., et al.** Unpublished data.

19. **Kimman, T. G., C. W. Lowik, L. J. A. V. D. Wee-Pals, C. W. Thesingh, P. Defize, E. M. Kamp, and O. L. M. Bijvoet.** 1987. Stimulation of bone resorption by inflamed nasal mucosa, dermonecrotic toxin-containing conditioned medium from *Pasteurella multocida*, and purified dermonecrotic toxin from *P. multocida*. Infect. Immun. **55:**2110–2116.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
21. **Lemichez, E., G. Flatau, M. Bruzzone, P. Boquet, and M. Gauthier.** 1997. Molecular localization of the *Escherichia coli* cytotoxic necrotizing factor CNF1 cell-binding and catalytic domains. Mol. Microbiol. **24:**1061–1070.
22. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
23. **Murray, M. G., and W. F. Thompson.** 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. **24:**1428–1434.
24. **Ohnishi, T., Y. Horiguchi, M. Masuda, N. Sugimoto, and M. Matsuda.** 1998. *Pasteurella multocida* toxin and *Bordetella bronchiseptica* dermonecrotizing toxin elicit similar effects on cultured cells by different mechanisms. J. Vet. Med. Sci. **60:**301–305.
25. **Oswald, E., M. Sugai, A. Labigne, H. C. Wu, C. Fiorentini, P. Boquet, and A. D. O'Brien.** 1994. Cytotoxic necrotizing factor type 2 produced by virulent *Escherichia coli* modifies the small GTP-binding proteins Rho involved in assembly of actin stress fibers. Proc. Natl. Acad. Sci. USA **91:**3814–3818.
26. **Pedersen, L. C., V. C. Yee, P. D. Bishop, I. L. Trong, D. C. Teller, and R. E. Stenkamp.** 1994. Transglutaminase factor XIII uses proteinase-like catalytic triad to crosslink macromolecules. Protein Sci. **3:**1131–1135.
27. **Petersen, S. K.** 1990. The complete nucleotide sequence of the *Pasturella multocida* toxin gene and evidence for a transcription repressor, TxaR. Mol. Microbiol. **4:**821–830.
28. **Pullinger, G. D., T. E. Adams, P. B. Mullan, T. I. Garrod, and A. J. Lax.** 1996. Cloning, expression, and molecular characterization of the dermonecrotic toxin gene of *Bordetella* spp. Infect. Immun. **64:**4163–4171.
29. **Schmidt, G., P. Sehr, M. Wilm, J. Selzer, M. Mann, and K. Aktories.** 1997. Gln 63 of Rho is deamidated by *Escherichia coli* cytotoxic necrotizing factor-1. Nature **387:**725–729.
30. **Schmidt, G., J. Selzer, M. Lerm, and K. Aktories.** 1998. The Rho-deamidating cytotoxic necrotizing factor 1 from *Escherichia coli* possesses transglutaminase activity. J. Biol. Chem. **273:**13669–13674.
31. **Sixma, T. K., S. E. Pronk, K. H. Kalk, E. S. Wartna, B. A. M. van Zanten, B. Witholt, and W. G. J. Hol.** 1991. Crystal structure of a cholera toxin-

related heat-labile enterotoxin from *E. coli*. Nature **351:**371–377.
32. **Walker, K. E., and A. A. Weiss.** 1994. Characterization of the dermonecrotic toxin in members of the genus *Bordetella*. Infect. Immun. **62:**3817–3828.
33. **Wardlaw, A. C., and R. Parton.** 1983. *Bordetella pertussis* toxins, p. 327–371. *In* F. Dorner and J. Drews (ed.), Pharmacology of bacterial toxins. Pergamon Press Ltd., Oxford, England.
34. **Wick, M. J., A. N. Hamood, and B. H. Iglewski.** 1990. Analysis of the structure-function relationship of *Pseudomonas aeruginosa* exotoxin A. Mol. Microbiol. **4:**527–535.
35. **Wilson, B. A., V. G. Ponferrada, J. E. Vallance, and M. Ho.** 1999. Localization of the intracellular activity domain of *Pasteurella multocida* toxin to the N terminus. Infect. Immun. **67:**80–87.

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