Bordetella Dermonecrotic Toxin Undergoes Proteolytic Processing to Be Translocated from a Dynamin-related Endosome into the Cytoplasm in an Acidification-independent Manner*

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Takeshi Matsuzawa‡§, Aya Fukui‡, Takashige Kashimoto∥, Kaori Nagao, Kyomasa Oka, Masami Miyake, and Yasuhiko Horiguchi‡

From the Department of Bacterial Toxology, Research Institute for Microbial Diseases, Osaka University, Yamada-oka 3-1, Suita, Osaka 565-0871, Japan

Bordetella pertussis dermonecrotic toxin (DNT), which activates intracellular Rho GTPases, is a single chain polypeptide composed of an N-terminal receptor-binding domain and a C-terminal enzymatic domain. We found that DNT was cleaved by furin, a mammalian endoprotease, on the C-terminal side of Arg⁺⁴, which generates an N-terminal fragment almost corresponding to the receptor-binding domain and a C-terminal remainder (ΔB) containing the enzymatic domain. These two fragments remained associated even after the cleavage and made a nicked form. DNT mutants insensitive to furin had no cellular effect, whereas the nicked toxin was much more potent than the intact form, indicating that the nicking by furin was a prerequisite for action. ΔB, but not the nicked toxin, associated with artificial liposomes and activated Rho in cells resistant to DNT because of a lack of surface receptor. These results imply that ΔB, dissociated from the binding domain, fully possesses the ability to enter the cytoplasm across the lipid bilayer membrane. The translocation ability of ΔB was found to be attributable to the N-terminal region encompassing amino acids 45–166, including a putative transmembrane domain. Pharmacological analyses with various reagents disturbing vesicular trafficking revealed that the translocation requires neither the acidification of the endosomes nor retrograde vesicular transport to deeper organelles, although DNT appeared to be internalized via a dynamin-dependent endocytosis. We conclude that DNT binds to its receptor and is internalized into endosomes where the proteolytic processing occurs. ΔB, liberated from the binding domain after the processing, begins to translocate the enzymatic domain into the cytoplasm.

Bacterial protein toxins that enzymatically modify cytosolic substances of eukaryotic cells consist of functionally distinct domains. The designation A–B toxin refers to toxins composed of an A domain conducting enzymatic action and a B domain binding to a surface receptor on target cells. In addition, these toxins are equipped with transmembrane domains carrying a delivery system to transport the A domain across lipid bilayer membranes after the B domain binds to the receptor. The A–B toxins are classified into at least three groups on the basis of structure (1). In the first group, which includes diphtheria toxin, botulinum neurotoxin, and Bordetella pertussis adenylate cyclase toxin, both the A and B domains originally reside on a single polypeptide chain. The toxins of the second group possess the A and B domains on different subunits that are noncovalently associated with each other. Cholera toxin, pertussis toxin, and Shiga toxin belong to this group. The third group is composed of binary toxins, in which components carrying the A and B domains are produced as distinct peptides and assembled on the target cells. The toxins of this type are exemplified by botulinum C2 toxin, anthrax toxin, and Clos tridium perfringens ε-toxin. Regardless of molecular structure, many of the A–B toxins undergo proteolytic processing in the region between the A and B domain. After binding to the cell surface through the B domain, most of the A–B toxins are internalized into endocytosed vacuoles and then transported to different intracellular destinations, e.g. acidic endosomes and the endoplasmic reticulum (ER). ¹ from which the A domain escapes into the cytoplasm. Toxins such as diphtheria, botulinum, and anthrax undergo a conformational change in response to acidification of the endocytosed compartments, which triggers the translocation of the A domains into the cytoplasm (1–4). Other toxins, including cholera, pertussis, and Shiga, are considered to reach the ER and to pass across the membrane there (1, 5–7). Although how the toxins escape from the ER lumen remains to be clarified, at least in the case of cholera toxin, Sec61p, a luminal ER protein, was shown to be necessary for export of the catalytically active A1 subunit of the toxin (8). Thus, understanding the mechanisms by which bacterial toxins enter the cytoplasm has proven valuable to elucidating not only the actions of toxins but also the intracellular transport systems for macromolecules.

Bordetella dermonecrotic toxin (DNT), consisting of 1,464 amino acids in a single chain, catalyzes polyamination or deamidation of the intracellular Rho GTPases (9–11) that regulate a variety of cell processes, including the reorganization of the actin cytoskeletons, cell motility, the transcription of certain genes, cell differentiation, and so on (12). As a result of the polyamination or deamidation, the GTPases become constitutively active and thereby cause the intoxicated cells to aber-

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‡ Present address: Kitasato Institute for Life Sciences, Kitasato University, Shirokane 5-9-1, Minato-ku, Tokyo 108-8641, Japan.
§ Both authors contributed equally to this work.
∥ Present address: School of Veterinary Medicine and Animal Sciences, Kitasato University, Higashi 23-35-1, Towada, Aomori 034-8628, Japan.
¶ To whom correspondence should be addressed. Tel.: 81-6-6879-8284; Fax: 81-6-6879-8283; E-mail: horiguti@biken.osaka-u.ac.jp.

1 The abbreviations used are: ER, endoplasmic reticulum; DNT, dermonecrotic toxin; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein.
rantly express the Rho-dependent phenotypes. Recently, we found that the binding and enzymatic domains of DNT were ascribed to the N-terminal 54 amino acids (13) and the C-terminal 300 amino acids (14), respectively. However, the internalization or translocation mechanism of DNT has not been dissected. Here, we show that DNT, after binding to cells, undergoes proteolytic cleavage by a mammalian protease such as furin. This cleavage is indispensable for the cellular action of DNT, and it seems to occur in endosomes derived from a dynamin-dependent endocytosis.

EXPERIMENTAL PROCEDURES

Materials—DNT was purified from Bordetella bronchiseptica S798 as described previously (15). In this study, the numbering of amino acids of DNT was based on the sequence available from the DDBJ/EMBL/GenBankTM databases under accession no. AB020025. C3 exoenzyme was provided by S. Kozaki, Osaka Prefecture University, Osaka, Japan. Furin was provided by K. Oda, Kyoto Institute of Technology, Kyoto, Japan. Liposomes were prepared with phosphatidyglycerol, phosphatidylcholine, and cholesterol at a molar ratio of 1:4:5 according to a method described elsewhere (16). pEGFP-C/Dyn2 and pEGFP-C/Dyn2K44A, expression vectors for human dynamin2 and its dominant negative form, were provided by Y. Nishimura, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan. pQEDNTwt, an expression vector for hexahistidine (His6)-tagged DNT, was constructed by insertion of the BamHI-NdeI fragment of pGEXDNT523 and the NdeI-HindIII fragment of pETDNTwt (14) into pQE40 (Qiagen, Hilden, Germany) digested with BamHI and HindIII. pQEDNT/R44G, pQEDNT/R44S, and pQEDNT/R44K were prepared by site-directed mutagenesis with a QuickChange kit (Stratagene, La Jolla, CA) and pQEEDNTwt as a template. All constructed DNAs were verified by nucleotide sequencing. The vectors were introduced into Escherichia coli M15[pRep4], and the recombinant proteins were produced and purified according to the manufacturer’s instructions (Qiagen). The mutant DNT523-1464 was prepared as described previously (14). ΔB was obtained from fractions passed through a His6-Bind Resin (Novagen, Darmstadt, Germany) column to which furin-treated His6-DNT was applied in the presence of 6 M urea. DNT573-C, DNT653-C, and DNT345-C were produced as glutathione S-transferase-tagged forms by E. coli DH5α harboring pGEX4T-3 (Amersham Biosciences) with each gene. The glutathione S-transferase-tagged proteins were absorbed by glutathione-Sepharose beads (Amersham Biosciences), and the tag-free proteins were eluted from the beads after treatment with thrombin.

Cell Cultures, Microinjection, and Staining for Actin Cytoskeletons—Cells used in this study were cultivated in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) or modified Eagle’s medium (α-MEM; Invitrogen) or α-modified Eagle’s medium (α-MEM), supplemented with 10% fetal calf serum in 5% CO2. For microinjection, cells were seeded on coverslips at an initial density of 520 cells/cm2, grown for 24 h, washed three times with DMEM or α-MEM, and further incubated in the same medium for 48 h at 37 °C. DNT preparations were injected into the cells together with 1 mg/ml rabbit IgG (ICN Pharmaceuticals, Inc., Aurora, OH) with an Eppendorf micromanipulator (Hamburg, Germany). After appropriate treatment, cells were fixed with 3% paraformaldehyde in PBS for 10 min, washed three times with PBS, and permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature. Alexa 568 phallolidin and Alexa 488 goat anti-rabbit IgG in PBS containing 10% fetal calf serum were overlaid on the cells at 2.5 units/ml and 4 μg/ml, respectively, and allowed to react for 1 h at room temperature. The cells were washed with PBS, mounted in PermaFluorTM aqueous mounting medium (Shandon/Lipshaw, Pittsburgh, PA), and observed under a fluorescence microscope. Images were taken with a SPOT color digital camera (Diagnostic Instruments, Sterling Height, MI) controlled by IP Lab software (Scalinlytics, Fairfax, VA).

Detection of the Polyaminated or Deamidated Rho—Cells appropriately treated with DNT preparations were disrupted by sonication, and the Rho in the lysates was labeled specifically with [32P]ADP-ribose by the C3 exoenzyme as described previously (17). The labeled Rho was detected by autoradiography after SDS-PAGE. Polyaminated and deamidated Rhos were identified on SDS-PAGE by their faster and slower mobilities, respectively (9, 10).

Gel Filtration Chromatography and Dot Blot Analysis—Gel filtration chromatography was performed using a SMART system (Amersham Biosciences) with a Superose 6 3.2/30 precision column. Twenty micro liters of sample containing about 50 μg protein was injected into the column, and 50-μl fractions of eluted samples were collected. A nitrocellulose membrane was blotted with each fraction and soaked in PBS containing 5% skim milk at 37 °C for 1 h. DNT and its fragments on the membrane were probed with anti-poly(His) (Sigma) or anti-DNT monoclonal antibody 1A3 (13) and horseradish peroxidase-conjugated goat anti-mouse IgG (Organon Teknika, Durham, NC). Proteins that specifically immunoreacted were visualized with the ECL system (Amersham Biosciences) and quantified from density evaluated using NIH image ver. 1.55 (rsb.info.nih.gov/nih-image). The results were expressed relative to the value of the densest blot in the same cycle of chromatography.

RESULTS

Proteolytic Cleavage of DNT at a C-terminal Site of the Binding Domain—DNT, when added extracellularly, causes an anomalous formation of actin stress fibers in susceptible cells through the constitutive activation of Rho resulting from polyamination or deamidation (9, 10, 17). However, when microinjected into cells, DNT did not have this effect (Fig. 1, A, B, G, and H). In contrast, microinjection of the catalytically active...
main and anti-DNT monoclonal antibody for AB. As shown in Fig. 2C, the binding domain and ΔB were concomitantly eluted at a position corresponding to that of intact DNT, whereas they were separately eluted in accordance with their molecular sizes under denaturing conditions. These results suggest that the binding domain and ΔB form a nicked molecule after the proteolytic cleavage, noncovalently associating with each other.

The DNT mutants in which the furin motif was destroyed by the substitution of Ser, Gly, or Lys for Arg44 (Fig. 3, C, E, and G), although they directly modified recombinant Rho in vitro (data not shown). These mutants showed competitive inhibition with wild type DNT in terms of the formation of stress fibers, implying that they retain the ability to bind cells (Fig. 3, D, F, and H). In contrast, the nicked form of DNT obtained by pretreatment with furin markedly potentiated the toxicity of intact DNT (Fig. 4). The magnitude of the activation of DNT by nicking with furin was further estimated on the basis of the extent, indicating that nicked toxin was more than 30 times as toxic as intact DNT. The furin motif is located on the C-terminal side of the binding domain of the sequence Arg41-Ala-Lys-Arg44 which corresponds to a recognition motif of a mammalian endoproteinase furin, Arg-X-Lys/Arg-Arg (Fig. 2B, underlined). In fact, treatment of DNT with furin yielded a fragment about 5 kDa smaller than the full-length toxin (Fig. 2B). The N-terminal amino acid sequence of the fragment corresponded to residues 45–56 of the toxin (Fig. 2A, boxed sequences), indicating that DNT was actually cleaved at the C-terminal peptide bond of Arg44 by furin. Unlike intact DNT, DNT pretreated with furin caused the cells to form actin cytoskeletons when microinjected (Fig. 1, I and J).

The furin motif is located on the C-terminal side of the binding domain that we identified previously (Fig. 2A) (13). To elucidate whether the binding domain dissociates from the rest of the toxin molecule (ΔB) after the cleavage, we treated the N-terminal His6-tagged DNT with furin and compared the elution profile with that of intact DNT from gel filtration chromatography. Eluted samples fractionated and blotted on a nitrocellulose membrane were subjected to immunodetection with anti-poly(His) antibody for the N-terminal fragment and anti-DNT monoclonal antibody 1A3 for the C-terminal fragment. Open squares with broken lines and open circles with solid lines represent the N- and C-terminal fragments, respectively.

Fig. 2. Cleavage of DNT by furin. A, localization of the furin motif. B, SDS-PAGE of DNT treated with furin. DNT (1 μM) was incubated in vitro with furin at the indicated concentrations for 4 h at 37 °C. C, gel filtration chromatography of the furin-treated or intact DNT. His6-tagged DNT (12 μM) was treated with 10 units/ml furin at 37 °C overnight. The furin-treated (right panels) or intact (left panels) His6-tagged DNT was subjected to gel filtration chromatography in the presence (lower panels) or absence (upper panels) of 6 M urea. Each fraction from the column was subjected to dot blot analysis. Immunodetection was carried out with anti-poly(His) for the N-terminal fragment and anti-DNT monoclonal antibody 1A3 for the C-terminal fragment. Open squares with broken lines and open circles with solid lines represent the N- and C-terminal fragments, respectively.

mutant DNT523–1444 or trypsin-treated DNT, which are inactive when extracellularly added, caused the formation of stress fibers. These results imply that the toxin acts in the cytoplasm only after undergoing the intramolecular cleavage of a peptide bond, although the reason why remains unknown (Fig. 1, C, D, E, and F). Therefore, we searched for a consensus motif in DNT for cleavage by an endoproteinase and found at a C-terminal site of the binding domain the sequence Arg41-Ala-Lys-Arg44 which corresponds to a recognition motif of a mammalian endoproteinase furin, Arg-X-Lys/Arg-Arg (Fig. 2A, underlined). In fact, treatment of DNT with furin yielded a fragment about 5 kDa smaller than the full-length toxin (Fig. 2B). The N-terminal amino acid sequence of the fragment corresponded to residues 45–56 of the toxin (Fig. 2A, boxed sequences), indicating that DNT was actually cleaved at the C-terminal peptide bond of Arg44 by furin. Unlike intact DNT, DNT pretreated with furin caused the cells to form actin cytoskeletons when microinjected (Fig. 1, I and J).
active as intact toxin (Fig. 5, A and C). Nicked DNT began to modify intracellular Rho immediately, whereas intact DNT required a lag period of at least 30 min (Fig. 5, B and D). The time course of modification of intracellular Rho by nicked DNT apparently corresponded to that of binding of DNT1–54 (binding domain) to cells (13). These results indicate that proteolytic cleavage at the furin motif is a prerequisite and rate-limiting step for DNT to affect cells.

ΔB Is Translocated across the Membrane after Dissociating from the Binding Domain—Our previous findings demonstrated that DNT binds to target cells via the N-terminal domain and modifies intracellular Rho by the enzymatic action of its C-terminal domain (13, 14). The intervening region between the binding domain and the catalytic domain, therefore, should be involved in translocation of the catalytic domain into cytoplasm through the lipid membrane. The computer programs DAS, TMPred, and PHDhtm predicted two to four transmembrane helices in the intervening region (Fig. 6A). In some viral
Proteolytic Cleavage of Bordetella Dermonecrotic Toxin

Fig. 7. Non-selective toxicity of ΔB in cells originally resistant to the full-length DNT. A, control Balb3T3 cells (1). Cells were incubated with 620 nM (100 μg/ml) of intact DNT (2) or the furin-treated DNT (3), or 62 nM (10 μg/ml) of ΔB (4) at 37 °C for 24 h. The cells were stained for actin cytoskeletons. B, modifications of Rho in various cells exposed to the toxin preparations for 24 h at 37 °C at the concentrations mentioned in panel A. I-DNT, intact DNT; N-DNT, nicked DNT; C, schematic representation of intact DNT; nicked DNT, and N-terminal truncated DNTs. The toxins were extracellularly added (Ext.) to or microinjected (Ml.) into MC3T3-E1 (E1) or Balb3T3 (Balb) cells. Biological activities were estimated based on stress fiber formation in the treated cells.

Fusion glycoproteins, proteolytic cleavage is known to expose internal hydrophobic domains and confer the ability to fuse with the host cell membrane (18, 19). The transmembrane domains of DNT may also contribute to its hydrophobic interaction with the lipid bilayer membrane. To test this point, we applied ΔB by removing the binding domain from the full-length DNT after treatment with furin, mixed it with liposomes, and subjected the mixture to gel filtration (Fig. 6B). The elution profiles of furin-treated DNT from the gel filtration were unchanged irrespective of the presence or absence of liposomes. In contrast, ΔB, when mixed with liposomes, was partly eluted at a void volume of the column, indicating interaction with liposomes. These results prompted us to examine whether ΔB interacts not only with artificial liposomes but also with the cell membrane. For this purpose, we applied ΔB to Balb3T3 cells, which were resistant to the full-length DNT because of a lack of the cell surface receptor (13). If ΔB interacts with the cell membrane as well as liposomes and translocates the catalytic domain into the cytosol, it should exert toxicity in the cells independently of the cell surface receptors. As shown in Fig. 7A, Balb3T3 cells showed massive stress fiber formation in response to ΔB but not intact or furin-treated DNT. The action of ΔB was confirmed on the basis of the Rho modification in Balb3T3, PAE, and K562, all of which were resistant to the full-length DNT (Fig. 7B). The minimum effective dose of ΔB was appreciably higher than that of the full-length DNT. In MC3T3-E1 cells sensitive to the holotoxin, at least 10 μg/ml of ΔB was required to modify intracellular Rho, whereas 5 ng/ml of intact DNT was enough to exert the same effect (9, 10, 17). In contrast, both intact DNT and nicked DNT at 100 μg/ml showed no activity against Balb3T3 cells, whereas ΔB at 10 μg/ml was as effective as in MC3T3-E1 cells. Unlike ΔB, DNT167-C, DNT243-C, and DNT345-C, whose N termini start with Leu167, Tyr243, and Ala345, respectively, had no ability to affect cells when added extracellularly (Fig. 7C). Microinjections with all these mutants induced stress fiber formation. These results imply that although it does not bind to cells efficiently, ΔB retains the ability to translocate the catalytic domain across the membrane, which is attributable to the region encompassing amino acids 45–166.

Fig. 8. Inability of bafilomycin, brefeldin, cytochalasin, and nocodazole to block the DNT action on cells. MC3T3-E1 cells were incubated with 0.5 and 2 μg/ml bafilomycin A1 for 30 min (A), 0.5 and 2 μg/ml brefeldin A for 30 min (B), 1 and 10 μg/ml cytochalasin D for 30 min (C), or 10 and 100 μM nocodazole for 6 h (D) at 37 °C. The cells were further treated with 31 μg/ml brefeldin A for 5 h. Photographs on the right show that the reagents were effective against the cells at the concentrations adopted. A, cellular acidic compartments stained by acridine orange disappeared in response to 0.5 μg/ml bafilomycin A1. B, the Golgi apparatuses stained by 12-(N-methyl-N(-7-nitrobenz-2-oxa-1,3-diazol-4-yl)) C6- ceramide were diffused in the cells treated with 0.5 μg/ml brefeldin A. C, stress fibers visualized with Alexa 568 phalloidin were disrupted in cells treated with 10 μg/ml cytochalasin D. D, microtubules stained with mouse anti-β-tubulin and Alexa 488 anti-mouse IgG were disrupted in cells treated with 100 μM nocodazole.
MC3T3-E1 cells were transfected with pEGFP-C/Dyn2 (panels 1, 2, 5, and 6) or pEGFP-C/Dyn2K44A (panels 3, 4, 7, and 8), endowing expression of EGFP-tagged human dynamin2 (Dyn2WT) and its dominant negative form (Dyn2DN), respectively, and incubated at 37°C for 24 h in α-modified Eagle’s medium supplemented with 10% fetal calf serum. The medium was changed to fresh medium without serum and cells were further incubated for 12 h.

The GTPase dynamin is known to regulate membrane trafficking events at the cell surface (24). We examined the effects of wild type or dominant negative dynamin expressed in cells on intoxication by DNT. The expression of dominant negative dynamin markedly reduced sensitivity to intact DNT but only moderately reduced sensitivity to nicked DNT (Fig. 9). In both cases, the extent of resistance correlated well with the expression level of dominant negative dynamin. In contrast, the expression of wild type dynamin did not influence cellular sensitivity to intact and nicked toxins.

**DISCUSSION**

We previously demonstrated that the binding domain of DNT could be delimited within the N-terminal 54 amino acids and bound to sensitive MC3T3-E1, C3H10T1/2, Swiss3T3, and NIH3T3 cells but not to resistant COS7, Balb3T3, L929, PAE, and K562 cells, indicating that a specific receptor confers sensitivity to DNT (13). In the present study, we revealed that DNT, probably after binding to cells, is cleaved by furin or a furin-like protease on the C-terminal side of the binding domain. Many protein toxins are known to undergo proteolytic processing by furin or furin-like protease (25–28), which is necessary for the subsequent translocation step (29–32). This was also the case for DNT. The cleavage step was essential and rate-limiting for DNT intoxication. The results presented here indicate that ΔB yielded by the proteolytic cleavage translocates its active domain into the cytoplasm. Unlike ΔB, whose N terminus is Glu^45, DNT^167-C and the shorter fragments did not show the ability to associate with artificial liposomes and to affect the DNT-resistant cells. Therefore, we consider that the region encompassing amino acids 45–166 of ΔB is involved in the translocation of the active domain across the membrane. Particularly, a putative transmembrane domain in this region may contribute to hydrophobic interaction with the lipid membrane. Because nicked DNT in which the binding domain and ΔB remain associated did not show such activities as ΔB did, it seems that ΔB must be dissociated from the binding domain so as to initiate the translocation. The binding domain may prevent ΔB from interacting with the lipid membrane, probably by sterically covering the 45–166 region. On the other hand, the binding domain should play a role in potentiating the efficiency of the toxin by binding to a specific receptor, because ΔB was over 1,000 times less potent than the full-length toxin against the DNT-sensitive cells. The receptor for DNT has yet to be identified but is unlikely to be furin. Furin is too ubiquitous to serve as the receptor for DNT, whose range of sensitive cells is quite narrow (17, 33). In fact, Balb3T3 cells to which the binding domain of DNT did not bind were found to express furin to a similar extent to DNT-sensitive MC3T3-E1 cells, as judged by immunoblotting (data not shown).

We next examined the pathway through which DNT is internalized and finally enters the cytoplasm. Bafilomycin A1, a pH-elevating agent (4, 20, 21), and brefeldin A, the Golgi apparatus-disrupting agent (22, 23), block the former and the latter mechanisms, respectively. Neither bafilomycin A1 nor brefeldin A blocked the DNT action (Fig. 8, A and B). Ammonium chloride, another pH-elevating agent, was also ineffective against the intoxication of cells by DNT (data not shown). Moreover, DNT was still active against cells in which actin filaments or microtubules were disrupted by pretreatment with cytochalasin D or nocodazole, respectively, indicating that the internalization of DNT is not mediated by actin-dependent endocytosis or vesicular trafficking along microtubules (Fig. 8, C and D).

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**Fig. 9. Effects of expression of dynamin on sensitivities of cells to DNT.** MC3T3-E1 cells were transfected with pEGFP-C/Dyn2 (panels 1, 2, 5, and 6) or pEGFP-C/Dyn2K44A (panels 3, 4, 7, and 8), endowing expression of EGFP-tagged human dynamin2 (Dyn2WT) and its dominant negative form (Dyn2DN), respectively, and incubated at 37°C for 24 h in α-modified Eagle’s medium supplemented with 10% fetal calf serum. The medium was changed to fresh medium without the serum, and cells were further incubated for 12 h. A, intact DNT (panels 1–4) or nicked DNT (panels 5–8) obtained by treatment with furin at 10 units/ml overnight was added to the culture at a final concentration of 5 ng/ml. After 16 h, cells were stained for actin cytoskeletons with Alexa 568 phalloidin (panels 1, 3, 5, and 7). The expression of dynamin was detected as fluorescence of EGFP (panels 2, 4, 6, and 8). A, representative images are shown. B, the expression level of dynamin in a cell was evaluated based on fluorescence intensity/pixel with IP Lab software from images taken at a constant exposure time. Cells were classified into three groups by the expression level as follows. Low (L), <100 intensity/pixel; medium (M), 100–400 intensity/pixel; high (H), >400 intensity/pixel. The number of cells with intensive stress fiber formation caused by DNT was counted and represented as a percentage of the total number of cells in the field.

transported via the Golgi apparatus to the ER where they escape into the cytoplasm. Bafilomycin A1, a pH-elevating agent (4, 20, 21), and brefeldin A, the Golgi apparatus-disrupting agent (22, 23), block the former and the latter mechanisms, respectively. Neither bafilomycin A1 nor brefeldin A blocked the DNT action (Fig. 8, A and B). Ammonium chloride, another pH-elevating agent, was also ineffective against the intoxication of cells by DNT (data not shown). Moreover, DNT was still active against cells in which actin filaments or microtubules were disrupted by pretreatment with cytochalasin D or nocodazole, respectively, indicating that the internalization of DNT is not mediated by actin-dependent endocytosis or vesicular trafficking along microtubules (Fig. 8, C and D).

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bacterial toxins which are endocytosed into cells need to be transported to either acidic endosomes or Golgi/ER. To clarify this point, we attempted to visualize the toxin in cells by fluorescence microscopy. However, definitive evidence was not obtained because of much nonspecific fluorescence caused by nonspecific binding of DNT to some extracellular matrices. Next, we examined whether the action of the toxin on cells is influenced by the expression of dynamin, which is considered to universally regulate endocytotic events at the cell surface. The dominant negative dynamin markedly blocked the effects of intact DNT but only moderately blocked those of nicked DNT. Therefore, we consider that intact DNT needs to be internalized into cells by a dynamin-dependent endocytosis, whereas nicked DNT is partly able to bypass this process. Furin is known to be localized in endosomes cycling between the trans-Golgi network and cell surface (37). DNT should undergo proteolytic cleavage in a dynamin-related endosome that is rich in furin family proteases. This step is likely time-consuming because nicked DNT modified intracellular Rho immediately after addition to the culture, whereas intact DNT required a lag of at least 30 min. The dynamin-dependent endocytoses can be further classified into clathrin-dependent, caveolae-dependent, and clathrin- and caveolae-independent (4, 38). DNT does not seem to utilize caveolae-dependent endocytosis because cholesterol-sequestering reagents such as filipin and methyl-β-cyclo-dextrin did not inhibit the cellular action of the toxin (data not shown). The clathrin-dependent endocytosis is more likely because it also involves the recycling of furin from the cell surface, although further study is needed to prove this. The endosomes are the most probable sites where DNT enters the cytoplasm.

In conclusion, DNT, after binding to its cell surface receptor, is internalized by a dynamin-dependent endocytosis into endosomes where it undergoes proteolytic processing and conformational change to liberate ΔB. Finally, ΔB translocates its catalytic domain into the cytoplasm through the region encompassing amino acids 45–166, which does not require the acidic environment. It may be worth examining what triggers the liberation of ΔB in the endosome and how ΔB allows the catalytic domain to pass through the lipid membrane.

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