An N-terminal Segment of the Active Component of the Bacterial Genotoxin Cytolethal Distending Toxin B (CDTB) Directs CDTB into the Nucleus*

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Cytolethal distending toxin (CDT), produced by Actinobacillus actinomycetemcomitans, is a putative virulence factor in the pathogenesis of periodontal diseases. It is a cell cycle specific inhibitor at the G2/M transition. CDTB, one of the subunits of the CDT holotoxin, is implicated in a genotoxic role after entering the target cells, whereby chromosomal damage induces checkpoint phosphorylation cascades. CDTB microinjected into the nucleus was shown to localize in the nucleus and induce chromatin collapse. To investigate the molecular mechanism involved in nuclear transport of CDTB, we used transient expression and microinjection of a CDTB-green fluorescent protein (GFP) fusion protein. After microinjection, His-tagged CDTB-GFP entered the nucleus in 3–4 h. Leptomycin B did not increase the speed of entry of the fusion protein, suggesting that the relatively slow entry of the fusion protein is not due to the CRM1-dependent nuclear export of the protein. Nuclear localization of the CDTB-GFP was temperature-dependent. An in vitro transport assay demonstrated that the nuclear localization of CDTB is mediated by active transport. An assay using transient expression of a series of truncated CDTB-GFP fusion proteins revealed that residues 48–124 constitute the minimum region involved in nuclear transport of CDTB. A domain swapping experiment of the target region involved in nuclear transport of CDTB with an SV40 T nuclear localization signal indicated that CDTB is composed of two domains, an N-terminal domain for nuclear transport and a C-terminal active domain. Our results strongly suggest that nuclear localization of CDTB is required for the holotoxin to induce cytidistension and cell cycle block. This is the first demonstration that a bacterial toxin possessing a unique domain for nuclear transport is transferred to the animal cell nucleus by active transport.

Cytolethal distending toxin (CDT) is a unique bacterial toxin that induces cell cycle arrest of cultured cells in the G2 phase. It has been identified in several pathogenic bacteria including Campylobacter spp., Escherichia coli, Shigella dysenteriae, Haemophilus ducreyi, Helicobacter hepiticus, and Actinobacillus actinomycetemcomitans. The CDT genes are tandemly located on the cdt locus (1, 5–7). The CDT-induced G2 arrest has been ascribed to the inactivation of the Cdc2-cyclin B complex, which is a key molecule for the progression of the cell cycle. In normal cells, dephosphorylation of the Thr14 and Tyr15 in Cdc2 triggers G2/M transition in the cell cycle. CDT-treated cells were found to maintain Cdc2 with these residues phosphorylated in the Cdc2-cyclin B complex (8). This is because of the recruitment of Cdc25C, a Cdc2-specific phosphatase, from the nucleus to cytoplasm, which prevents dephosphorylation of the Cdc2-cyclin B complex in the nucleus (8–12). Cdc25C is regulated by Checkpoint kinase 1 or 2, which are controlled by ATM or ATR (ataxia-telangiectasia mutated and Rad3-related) (13). Recently, two research groups indicated independently that CDT has structural homology to human DNase I and suggested that CDTB is an active component of the CDT complex acting as a DNase. In support of this, E. coli CDTB has been demonstrated to possess nicking activity toward purified plasmid in vitro (14). These findings have raised the possibility that CDTB directly damages chromosomal DNA, which results in the onset of phosphorylation of the checkpoint control cascade described above. Lara-Tajero and Galan (15) demonstrated that transiently expressed or microinjected Campylobacter jejuni CDTB in the cultured cell cytoplasm accumulates in the nucleus, consistent with a possible nuclear function of CDTB. However, no biochemical information is available for the mechanism of nuclear accumulation of CDTB component.

In eukaryotic cells, a number of cellular proteins that function in the nucleus are imported or exported through the nuclear pore complex (NPC), which is composed of about 100 subunits (Nup). Nucleoporins are involved in forming the NPC, and the NPC is an important site for protein transport between the cytoplasm and nucleus. The NPC is composed of a series of concentric rings that form a central channel for nuclear transport. The NPC is composed of two sets of proteins, the outer nuclear membranes and the inner nuclear membranes. The outer nuclear membranes are composed of a series of rings of proteins, which are arranged in a U-shaped structure. The inner nuclear membranes are composed of a series of rings of proteins, which are arranged in a Y-shaped structure. The NPC is a complex structure that is composed of a series of concentric rings of proteins. The NPC is composed of a series of concentric rings of proteins, which are arranged in a U-shaped structure. The NPC is composed of a series of concentric rings of proteins, which are arranged in a Y-shaped structure.
### TABLE I

| Strains, plasmids, and primers used in this study |
|--------------------------------------------------|
| **Actinobacillus equuli** expression vector       |
| CDTB is Transferred to HeLa Cell Nucleus by Active Transport |
| **Strain** | **Plasmid** | **Character** | **Used primer** | **Reference** |
| XL-I blue  | pTK3022       | cdh68C in pUC19 (Sim 1, EcR R1 site) | mu308 Oligo, L-2167 | 2 |
| H915/14 (DE3) | pET-3a (WT) | cdh68 (WT) in pET28a (Nov 4, EcR R1 site) | This study |
| XL-11     | pET-3a (WT) | cdh68 (WT) in pET28a (Nov 4, EcR R1 site) | This study |
| H915/14 (DE3) | pET-3a (pGEX-4T1) | cdh68 in pET28a (Nov 4, EcR R1 site) | This study |
| XL-11     | pET-3a (pGEX-4T1) | cdh68 in pET28a (Nov 4, EcR R1 site) | This study |
| H915/14 (DE3) | pET-3a (pGEX-4T1) | cdh68 in pET28a (Nov 4, EcR R1 site) | This study |
| M15       | pQE-cdH68C (WT) | cdh68C (WT) in pQE600 (Nov 4, EcR R1 site) | QIA-CDTA-U2, QIA-CDTC-L, L-mb C trm 11aa, L-mb C | This study |
| M15       | pQE-CDpH68C (A1630C) | cdh68C (A1630C) in pQE600 (Nov 4, EcR R1 site) | QIA-CDTA-U2, QIA-CDTC-L, L-mb C trm 11aa, L-mb C | This study |
| M15       | pQE-CDpH68C (A1630C) | cdh68C (A1630C) in pQE600 (Nov 4, EcR R1 site) | QIA-CDTA-U2, QIA-CDTC-L, L-mb C trm 11aa, L-mb C | This study |
| M15       | pQE-CDpH68C (A1630C) | cdh68C (A1630C) in pQE600 (Nov 4, EcR R1 site) | QIA-CDTA-U2, QIA-CDTC-L, L-mb C trm 11aa, L-mb C | This study |

**Mammalian expression vector**

| Strain | Plasmid | Character | Used primer | Reference |
|--------|---------|-----------|-------------|-----------|
| XL-11  | pEGFP-cDNA | cdh68 in pEGFP-C1 (Bgl II, EcR R1 site) | mu308 Oligo, L-2167 | This study |
| XL-11  | pEGFP-cDNA | cdh68 in pEGFP-C1 (Bgl II, EcR R1 site) | This study |
| XL-11  | pEGFP-cDNA | cdh68 in pEGFP-C1 (Bgl II, EcR R1 site) | This study |
| XL-11  | pEGFP-cDNA | cdh68 in pEGFP-C1 (Bgl II, EcR R1 site) | This study |
| XL-11  | pEGFP-cDNA | cdh68 in pEGFP-C1 (Bgl II, EcR R1 site) | This study |
| XL-11  | pEGFP-cDNA | cdh68 in pEGFP-C1 (Bgl II, EcR R1 site) | This study |
| XL-11  | pEGFP-cDNA | cdh68 in pEGFP-C1 (Bgl II, EcR R1 site) | This study |
| XL-11  | pEGFP-cDNA | cdh68 in pEGFP-C1 (Bgl II, EcR R1 site) | This study |
| XL-11  | pEGFP-cDNA | cdh68 in pEGFP-C1 (Bgl II, EcR R1 site) | This study |
| XL-11  | pEGFP-cDNA | cdh68 in pEGFP-C1 (Bgl II, EcR R1 site) | This study |

| Primer | Position | Sequence | Restriction enzyme site | Reference |
|--------|----------|----------|-------------------------|-----------|
| mu108  | 1414-1425 | 5'-GAACGTTCATCGATCGAAGAGG-3' | Bgl II | This study |
| Ae Sap | 1926-1949 | 5'-GAACGTTCATCGATCGAAGAGG-3' | Bgl II | This study |
| U-1523 | 1565-1556 | 5'-GAACGTTCATCGATCGAAGAGG-3' | Bgl II | This study |
| L-1527 | 1565-1556 | 5'-GAACGTTCATCGATCGAAGAGG-3' | Bgl II | This study |
| L-1527 | 1565-1556 | 5'-GAACGTTCATCGATCGAAGAGG-3' | Bgl II | This study |
| L-1527 | 1565-1556 | 5'-GAACGTTCATCGATCGAAGAGG-3' | Bgl II | This study |

### Note

- The table includes information on plasmids, characters, and primers used in the study, along with references for each entry.
clear pore complex (NPC), which forms a tunnel through the nuclear envelope (16–18). Although molecules smaller than 40–60 kDa pass by diffusion through the NPC, most of the macromolecules are generally carried by energy-dependent active transport. Such nuclear imported proteins have common features. For example they possess a conserved nuclear localization signal (NLS), which allows their rapid import via complex formation with carrier protein families in the cells. The classical monopartite or bipartite NLSs are characterized by lysine- or arginine-rich sequences, which bind to the carrier protein, importin-α. There are some variations of NLS that do not possess conserved basic amino acid residues, such as NLS in M9 (19) or the RPA protein (replication protein A) (20). They are called atypical NLS, and the number of reports on atypical NLS are increasing (21, 22).

Herein, we demonstrate that the A. actinomycetemcomitans cdtB component is transferred to the nucleus by active transport. Furthermore, we have defined a unique and functional domain in the N-terminal segment of CDTB component that mediates nuclear import. We also demonstrated that nuclear entry of CDTB is required for the holotoxin to show cytopathic effect and cell cycle block. These studies strongly indicate that CDTB is a nuclear targeting genotoxin using the eukaryotic active transport system.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Plasmids—**HeLa cells (ATCC CCL2) and other mammalian cells were cultured in Dulbecco’s modified Eagle’s medium (Nissui) supplemented with 10% calf serum at 37 °C in a 5% CO2, 95% air atmosphere. Plasmids and bacteria used in this study are listed in Table I. All E. coli were laboratory strains and grown aerobically in Luria Bertani (LB) medium or on LB agar plates. A. actinomycetemcomitans was grown in Trypticase Soy Broth (BD Biosciences) supplemented with 1% (w/v) yeast extract in a 5% CO2 atmosphere. Ampicillin (50 μg/ml) or kanamycin (50 μg/ml) was added when necessary.

**Manipulation of DNA and Plasmid Construction—**The DNA region of A. actinomycetemcomitans cdtB gene, corresponding to the signal peptide-cleaved mature form of CDTB (23–233 aa), was isolated from A. actinomycetemcomitans Y4 genomic DNA by PCR amplification, using specific primers containing restriction enzyme sites, for subcloning into vectors. Primers used in this study are listed in Table I. The PCR-amplified cdtB gene was once cloned into pGEM-T Easy PCR cloning vector (Promega) and was subcloned into the pET28a (Novagen), the histidine-tagged expression vector, or pFla(+)M1 tag (Kodak), the FLAG-tagged expression vector. Various cdtB deletion mutants were generated by PCR using primers listed in Table I. The deletion mutants were also cloned into the pGEM-T Easy and subcloned into the pEGFP-C1, green fluorescence protein fusion vector (Clontech).

**Preparation of CDTB Subunit—**CDTB was highly expressed in recombinant E. coli carrying pET-cdtB in the presence of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at the point of OD600 = 0.5–0.6. After induction for 3 h at 30 °C, cells were harvested by centrifugation at 5000 × g for 5 min. Harvested bacterial cells were washed with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.3) twice. Cells resuspended in PBS with 1% Triton X-100 were broken by an ultrasonic disruptor (TOMY) and centrifuged at 5000 × g for 5 min to remove unbroken cell. The supernatant was further centrifuged at 100,000 × g to remove the membrane fraction. The resulting supernatant was incubated with nickel-chelated agarose (Ni-NTA, Qiagen). Ni-NTA metal affinity purification was carried out according to the instruction manual from Qiagen. The Ni-NTA-purified CDTB was further purified using high pressure liquid chromatography (Tosoh, Tokyo) equipped with a solvent delivery pump CCPM and UV/Vis01 absorbance detector. A Unos cation exchange chromatography column (Bio-Rad) was used for purification of CDTB protein. To obtain a better purification of CDTB protein, TSK gel G3000 PW (Tosoh) was used for gel permeation chromatography.

**Plasmid Transfection—**Plasmids were delivered into HeLa and other mammalian cells by calcium phosphate methods. Cells were prepared at 1 × 10⁶/ml in 5 ml on 60-mm tissue culture dish (Corning). Plasmid DNA (10 μg) was mixed with 500 μl of H₂O and 50 μl of 2.5 mM CaCl₂ and incubated at room temperature for 10 min. Then 500 μl of 2 × Hepes buffer (2.8 mM Na₂HPO₄, 280 mM NaCl, 50 mM Hepes, pH 7.1) was added to the DNA solution and kept at room temperature for 10 min. All of the solution was overlaid onto the cultured cells. After incubation at 37 °C for 2 h in a 5% CO2 incubator, cells were washed twice with PBS. 1.5 ml of glycerol-Hepes buffer (10% glycerol, 1 × Hepes buffer) was added at room temperature for 30 s. Cells were washed twice with PBS and cultured in Dulbecco’s modified Eagle medium with 10% calf serum in a 5% CO2, 95% air atmosphere.

**Microinjection—**Microinjection was performed using the Eppendorf Injectman N12. Purified proteins were adjusted to the concentration of 1 μg/ml and injected into the cytosol of cultured cells at a pressure of 50–120 hpa for a duration of 0.2 s. The cells injected with CDTB fused to green fluorescence protein (GFP) were observed directly by confocal microscopy (Carl Zeiss LSM 410). Cells without fluorophore were viewed by immunohistochemistry. Briefly cells were washed three times with PBS and fixed with 3.7% formaldehyde, 0.5% Triton X-100 in PBS for 10 min at room temperature. Cells were again washed three times with PBS and added with 1% bovine serum albumin in PBS (blocking buffer) at room temperature for 10 min. The primary antibody in blocking buffer was overlaid on cells and kept at room temperature for 40 min. Cells were washed three times with PBS, and the secondary antibody conjugated with fluorescein isothiocyanate or rhodamine in blocking buffer was added and incubated at room temperature for 30 min. After being washed with PBS three times, the cells were incubated with propidium iodide (PI) in PBS (5 μg/ml) for 5 min at 37 °C. After being washed again with PBS three times, the cells were observed by confocal microscopy.

**Cytosol Preparation—**Cytosol was prepared by the method described by Miyamoto et al. (23). HeLa cells were harvested by scraping in PBS and pelleted by centrifuge at 5000 × g for 5 min. Cells were washed in 5 ml of washing buffer (10 mM Hepes, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol), by centrifugation at 5000 × g for 5 min. Cells were then resuspended in lysis buffer (5 mM Hepes, pH 7.3, 10 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol, 20 mM cysteine, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin), kept on ice for 10 min, and homogenized for 5–10 strokes with a Dounce homogenizer. Lysed cells were centrifuged at 1,500 × g for 15 min. The supernatant was further centrifuged at 100,000 × g for 30 min, and the resultant supernatant was used as cytosol.

**In Vitro Transport Assay—**An in vitro transport assay was carried out by the method described by Nagoshi and Yoneda (24). HeLa cells (5 × 10⁵/ml) grown overnight were washed twice with transport buffer (20 mM Hepes, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, and 0.5 mM EDTA), and cytosol was added at a concentration of 40 μg/ml. Cells were permeabilized by being kept on ice for 5 min and washed with transport buffer containing 2 mM dithiothreitol, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. Permeabilized cells were overlaid with 1 μg/ml extracted cytosol, 0.1 mM ATP, 0.5 mM phosphocreatine, 2 units/ml creatine kinase, 0.05 mM GTP, and the protein of interest. The in vitro transport assay was carried out by incubating cells at 30 °C for an appropriate time. Cells were washed twice with TB containing 2 mM dithiothreitol, 1 μg/ml leupeptin, and 1 μg/ml pepstatin and then fixed with 3.7% formaldehyde in TB for 10 min.

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**TABLE I—continued**

| Primer | Position* | Sequence | Restriction enzyme site | Reference |
|--------|------------|----------|------------------------|-----------|
| L-1725-xyb | 1723-1738 | 5'-ATCGTTGGCAGCGGCGGTTCCTT-3' | | This study |
| U-175 | 1767-1698 | 1725-1735 | 5'-GTTTAGGATCTCGCTCAGG-3' | This study |
| L-175 | 1767-1698 | 1725-1735 | 5'-GTTAGCAGAACAACTCAAAGC-3' | This study |
| L-172 | 1754 | 1725-1735 | 5'-GTTAGCAGAACCCGGCGCACGG-3' | This study |
| L-171 | 1741 | 1725-1735 | 5'-GCGTCAGCGAGGGGAGTCT-3' | This study |

* Position corresponds to the DNA sequence published in GeneBank accession No. AB 011450.
FIG. 1. Microinjected CDTB enters into HeLa nucleus. A, purified His-CDTB was microinjected into HeLa cells, and His-CDTB was detected by immunostaining using rabbit anti-CDTB serum as primary antiserum followed by fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG goat serum as secondary antiserum. Wavelengths of 488 nm and 543 nm were used to excite FITC and PI, respectively. Emission spectra were collected with 510–525-nm band-pass filters and 570-nm long-pass filters. Computer-generated overlays of the fields in the fluorescence (green) and those of PI (red) are shown (Merge). The fluorescent signal began to be detected in the nucleus 30 min after microinjection. The chromatin stained with PI shows the decreased signal at 4 h. B, His-CDTB-GFP, which has a molecular mass of ~65.6 kDa, was purified by a nickel-affinity column and microinjected into HeLa cells. Localization of microinjected protein was monitored every hour after microinjection by laser confocal microscopy. GST-SV40 T NLS-GFP and GFP were used as positive and negative controls, respectively. C, leptomycin B, a specific inhibitor of CRM1/exportin 1, was added to the HeLa cells at 0, 10, and 50 ng/ml, 4 h before CDTB-GFP microinjection. Localization of microinjected protein was monitored every hour after microinjection by confocal microscopy.
**RESULTS**

**CDTB Microinjected into Cytosol Translocates to the Nucleus**—To know the final destination of the functional CDTB subunit, we first attempted to microinject the His-CDTB protein into the cytoplasm of cultured cells and trace its moiety. Microinjection of His-CDTB and subsequent immunostaining of the cells with anti-CDTB serum showed the nuclear localization of His-CDTB and confirmed the observation made on *Campylobacter* CDT (15) (Fig. 1A). Time course measurement of His-CDTB localization after microinjection revealed that His-CDTB entered into the nucleus 30 min after microinjection and remained in situ as late as 4 h (Fig. 1B). We simultaneously stained the cells with PI to observe the chromatin. PI staining showed chromatin disintegration at 4 h, but not at 1 h, suggesting that microinjected CDTB acts as a DNase or indirectly induces DNA injury. To exclude the possibility that His₆ worked as a nuclear localization signal, we constructed the plasmid pET-cdtb-gfp, that produces His-tagged CDTB-green fluorescence protein fusion (His-CDTB-GFP), which has a predicted molecular mass of 65.6 kDa. His-CDTB-GFP can be monitored directly by confocal microscope without fixation or antibody reaction. After purification by affinity chromatography, His-CDTB-GFP was microinjected into the cytoplasm of HeLa cells. GST-SV40 T NLS-GFP, which is imported into the nucleus by a classical monopartite NLS (PPKKRKVEDP), was used as a positive control. As shown in Fig. 1B, microinjected GST-SV40 T NLS-GFP was imported into the nucleus in 30 min. Microinjected His-CDTB-GFP was also transported into the nucleus with relatively slow rate. After microinjection, His-CDTB-GFP was detected in the nucleus from as early as 2 h and apparently as late as 3 h, whereas GFP alone did not accumulate in the nucleus even after 4 h. It should be noted that even after 4 h the microinjected cells exhibited a fairly weak cytoplasmic staining besides the strong nuclear staining. These phenomena of His-CDTB-GFP localization could be due to nuclear export of the protein. To address this possibility, cells were treated with an inhibitor of the CRM1-exportin pathway, leptomycin B (25), and the subcellular localization of microinjected His-CDTB-GFP was assessed. Leptomycin B was added to the cells prior to microinjection and localization of His-CDTB-GFP was monitored up to 4 h. As shown in Fig. 1C, there was no difference in the import rate and the staining of the nucleus and cytoplasm irrespective of the presence or absence of leptomycin B. These data suggest that these phenomena do not result from CRM1-dependent nuclear export of His-CDTB-GFP. We next assessed the temperature dependence of His-CDTB-GFP nuclear transport. Incubation of cells on ice suddenly after microinjection prevented the transport of GST-SV40 T NLS-GFP into the nucleus as expected (Fig. 1B). Similarly, the nuclear transport of His-CDTB-GFP was prevented when the microinjected cells were placed on ice (Fig. 1B). The temperature dependence of His-CDTB-GFP nuclear localization prompted us to investigate...
Identification of region involved in nuclear transport of CDTB. A series of cdtB deletions was cloned into pEGFP in-frame to express various EGFP-deleted CDTB mutants. Each recombinant plasmid was transfected into HeLa cells, and localization of green fluorescence was monitored by a laser confocal microscope. A, schematic representation of deletion constructs. B, green fluorescence of deletion clone transiently expressed in COS7 cells. C, immunoblotting analysis of homogenate of COS7 cells transiently expressed EGFP-deleted CDTB mutants. Anti-EGFP rabbit antibody was used as the primary antibody. Immunodetection was performed as described elsewhere (2). D, alignment of CDTB sequences from a variety of bacteria. Amino acid sequences of CDTB from various bacteria and mouse DNase I are aligned using the Clustal W program. The identical amino acids are boxed in gray. The domain for nuclear transport is indicated by a bold underline. The conserved catalytic amino acids are boxed with a red line. The conserved metal binding sites are boxed with a blue line with an asterisk underneath. Actinobacillus actinomyce-
the energy requirement for the nuclear import. We used the in vitro transport assay to identify the molecules necessary for CDTB-nuclear import. Digitonin-permeabilized HeLa cells were overlaid with a solution containing His-CDTB-GFP, HeLa cell cytosol, energy regeneration components (ATP, GTP, phosphocreatine, and creatine kinase), and incubated at 30°C for 4 h. As a control, GST-SV40 T NLS-GFP was used. The nuclear import of GST-SV40 T NLS-GFP has been shown previously to be energy-dependent, and we were able confirm these results (Fig. 2). Similarly, His-CDTB-GFP was able to enter into the nucleus in the presence of the cytosol extract and energy resources. The temperature dependence of the nuclear transport of His-CDTB-GFP was again confirmed using the in vitro transport system. In the absence of GTP/ATP, His-CDTB-GFP failed to enter into the nucleus. Treatment with apyrase, one of the ATP hydrolases, also abolished the entry of both His-CDTB-GFP and GST-SV40 T NLS-GFP into the nucleus.

Wheat germ agglutinin is a lectin that presumably inhibits nuclear protein transport by interacting with the nuclear pore complex protein with O-linked N-acetylglucosamine moieties (26, 27). When the permeabilized HeLa cells were preincubated with wheat germ agglutinin, nuclear entry of both His-CDTB-GFP and GST-SV40 T NLS-GFP was inhibited. These results strongly suggested that nuclear import of CDTB required energy and that the His-CDTB-GFP passes through the nuclear pore complex just like other mammalian nuclear proteins.

N-terminal Stretch of CDTB (48–124 aa) Is Responsible for Nuclear Localization—Because the amino acid sequence of CDTB does not contain a typical nuclear localization signal, it was of interest whether CDTB possesses functional region(s) involved in nuclear transport in the molecule. A preliminary trial to purify a series of truncated CDTB proteins using an E. coli-vector system was unsuccessful because of the instability of the recombinant products. Therefore, we switched to using direct expression of the truncated CDTB-GFP fusion protein in COS7 cells, and expression of truncated CDTB was verified by immunoblot analysis of the transfected cells (Fig. 3B). The cdtB gene was cloned in-frame into pEGFP-C1 and transiently transfected into COS7 cells to generate GFP fused to a mature form of CDTB (CDTB-GFP). As shown in Fig. 3C, GFP-CDTB was detected in the nucleus 4 h after plasmid transfection. In order to know the region involved in nuclear transport of temcomitans, locus cloned from A. actinomycetemcomitans Y4 (GenBankTM accession no. AB011405); Haemophilus ducreyi, locus cloned from H. ducreyi 55000 (GenBankTM accession no. U53215); Campylobacter jejuni, locus cloned from C. jejuni 81–176 (GenBankTM accession no. U51121); Helicobacter hepaticus, locus cloned from H. hepaticus (GenBankTM accession no. AAF19158); E. coli CDT I, locus cloned from E. coli E6468/62 (GenBankTM accession no. U03293); E. coli CDT II, locus cloned from E. coli E6468/62 (GenBankTM accession no. U04208); E. coli CDT III, locus cloned from E. coli 1404 (GenBankTM accession no. U89305); DNase I, locus cloned from Mus musculus (GenBankTM accession no. AAH30394).
CDTB, we first divided CDTB into two parts, the N-terminal (23–200 aa) and C-terminal (167–283 aa) domains, and constructed plasmids to express each fragment as GFP-fused protein in the cells. After plasmid transfection, localization of the GFP-fused protein was assessed. As shown in Fig. 3C, nuclear localization was observed in the cells expressing the N-terminal portion of CDTB fused to GFP but not in the cells expressing the C-terminal half fused to GFP. Therefore, we created a series of deletion constructs of the N-terminal portion of CDTB and searched for the region with which the fusion protein clearly localized into the nucleus. In some cases, the intensity of fluorescence appeared equal in both the nucleus and the cytoplasm. Finally, a region corresponding to 48–124 aa was found to be the minimum region responsible for nuclear localization of the CDTB molecule. Further deletion of either the N-terminal or C-terminal ends of this segment resulted in loss of function of the apparent nuclear localization. Similar results were obtained when HeLa cells were used to transiently express truncated CDTB-GFP fusion protein (not shown). We also tried to express GFP fused to CDTB 48–124 aa (GFP-CDTB-(48–124)) in other cell lines such as Chinese hamster ovary cells (CHO) and mouse keratinocyte (Pam). The accumulation of GFP-CDTB-(48–124) was also observed in the nucleus of these cells (not shown).

CDTB Is Composed of an N-terminal Domain for Nuclear Transport and a C-terminal Activity Domain—Comparison of amino acid sequences of CDTB component from a variety of bacteria with members of the DNase-I family revealed that there are five conserved regions implicated as essential amino acid residues for DNase activity, as shown in Fig. 3D (boxed). Among them, all but Glu66 are located in the C-terminal half of the CDTB molecule. Previously, Elwell and Dreyfus demonstrated that His154, Asp229, Asp260, and His261 of E. coli CDTB are essential for the activity, but Glu66 is not (14). Taken together with the notion that N-terminal 48–124 aa plays a role as a domain for nuclear transport, we hypothesized that CDTB forms a two-domain structure composed of the N-terminal domain and C-terminal genotoxic activity domain.

Therefore we tried to see whether a holotoxin containing SV40 T NLS was used as a positive control.

**FIG. 4. Chimera protein of CDTB 48–124-SV40 T NLS acts as a genotoxin in the nucleus.** A, schematic representation of wild type CDTB (WT) and CDTB with its amino acid sequence (48–124 aa) exchanged with SV40 T NLS (CDTB Δ48–124-SV40 T NLS). B, wild type CDTB and CDTB with its amino acid sequence (48–124 aa) exchanged with SV40 T NLS were purified using Ni-NTA. Purified protein was analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining. C, purified chimeric protein was microinjected into the cytosol of HeLa cells. The protein was localized by immunostaining using anti-CDTB serum. Chromatin was stained simultaneously with propidium iodide. GST-SV40 T NLS-GFP was used as a positive control.
FIG. 5. Activity of holotoxin containing mutant CDTB with the 11-amino acid truncation in the NLS. A, schematic representation of wild type CDTB (WT), CDTB with truncation of 11 amino acids in position 114–124 (CDTBΔ11aa (Δ11aa)), and CDTBΔ11aa in which truncation was replaced with SV40 T NLS (Δ11aa + SV40 T NLS). B, transient expression of mutant CDTB and its localization. Wild type CDTB, CDTBΔ11aa, and CDTBΔ11aa-SV40 T NLS were expressed transiently by pEGFP-constructs in HeLa cells as GFP fusion protein. The GFP fusion protein was monitored by confocal microscopy using the FITC channel signal (Fluorescence). Computer-generated overlays of the fields in the fluorescence (green) and those of phase contrast microscopy (red) are shown (Merge). C, Western blotting analysis of purified CDT holotoxin of wild type, holotoxin containing CDTBΔ11aa, and holotoxin containing CDTBΔ11aa-SV40 T NLS. Holotoxin was purified by His tag sequence fused to the C terminus of CDTC using a Ni-NTA column. Western blotting analysis was performed using anti-CDTA, anti-CDTB, or anti-CDTC as the primary antibody. D, cytodistending activity of CDT holotoxin of wild type, holotoxin containing CDTBΔ11aa, and holotoxin containing CDTBΔ11aa-SV40 T NLS. One microgram of the purified holotoxin was incubated with a 10-ml culture of HeLa cells for 24 h. CDT activity was estimated as 50% cytotoxic dose, which was titrated as the endpoint of the highest 2-fold dilution of the sample showing 50% cytodistending cells after 72 h of incubation; CDT activity was defined as the reciprocal of the dilution. E, flow cytometry analysis of HeLa cells treated with CDT holotoxin of wild type, holotoxin containing CDTBΔ11aa, and holotoxin containing CDTBΔ11aa-SV40 T NLS. One microgram of the purified holotoxin was incubated with a 10-ml culture of HeLa cells for 24 h. Control, cells without treatment.
CDT Is Transferred to HeLa Cell Nucleus by Active Transport

CDT is the first bacterial protein toxin found to be seemingly active as a DNase in target mammalian cells (14, 15). Previous studies suggest that CDTB is a toxic component and that CDTA and -C are carriers of CDTB to target cells (15, 28). Cortes-Bratti et al. (29) demonstrated that H. ducreyi CDT was internalized and transported to the Golgi using clathrin-coated pits (29). The final destination of CDTB is suggested to be the nucleus (15). As demonstrated in previous work (15) and in this study, transiently expressed CDTB or microinjected CDTB are transported into the nucleus. We have demonstrated that CDTB is transferred to the HeLa cell nucleus by active transport and that CDTB possesses a unique functional domain for nuclear transport. This is the first demonstration that a bacterial toxin is transferred to the animal cell nucleus by active transport. This is the first demonstration that a bacterial toxin is transferred to the animal cell nucleus by active transport and that CDTB has lost its ability to localize in the nucleus (Fig. 5A). On the other hand, GFP-CDTB11aa-SV40 T NLS expressed in HeLa cells was found to localize in the nucleus. CDT holotoxins containing the respective chimeric CDTB were successfully purified from the periplasmic fraction of recombinant E. coli using His tag in the C terminus of CDTB (Fig. 5C). Wild type holotoxin (WT) revealed strong cytostatidesting activity and successfully arrested the HeLa cells at G2/M (Fig. 5, D and E). On the other hand, CDT holotoxin containing CDTB11aa (Δ11aa) completely lost its activity for cytostidension and cell cycle block, whereas CDT holotoxin containing CDTB11aa-SV40 T NLS (Δ11aa-SV40 T NLS) restored those activities. Taken together, these results strongly suggest that entry of CDTB into the nucleus is necessary for the induction of cytotoxic effect by CDT holotoxin.

**DISCUSSION**

The nuclear transport of bacterial component was first demonstrated by Agrobacterium tumefaciens virulence-related proteins (30). A. tumefaciens interacts with plant cells and is able to transfer DNA to the plant cell (31). The bacterial DNA, called T-DNA, which is a portion of the tumor-inducing plasmid of Agrobacterium, travels from Agrobacterium into the plant cells where the T-DNA integrates into the plant cell nucleus genome. For this purpose, the T-DNA must traverse the cytoplasm into the nucleus. This movement is carried out by two virulence-related proteins bound to T-DNA, called VirD2 and VirE2 (32, 33). These proteins have been demonstrated to possess a bipartite NLS (34, 35). Another example is the protein AvrBs3 produced by Xanthomonas campestris pv. Vesicatoria (36). In pepper plants, AvrBs3 is targeted to host plant cells by the bacterial Hrp type III secretion system and is transported into the nucleus by a monopartite NLS in the C terminus of the protein (37). It induces a rapid, localized cell death of the target plant. In the case of CDTB, a homology search or domain matching of CDTB amino acid sequences using available data bases did not hit any of the NLS candidate sequences. To investigate the region involved in the nuclear transport in the CDTB amino acid sequences, a series of deletion mutants of CDTB fused to GFP were constructed, and localization of the GFP fusion protein was monitored. In some cases, the intensity of fluorescence appeared equal in both the nucleus and the cytoplasm. This applied to constructs 28–93, 23–59, and 23–102. These constructs appeared to retain partial activity to transport the GFP fusion protein into the nucleus. Finally the domain from the 48th to 124th aa was found to be the shortest stretch. Alignment of the CDTB amino acid sequences of a variety of bacteria clearly indicated that the amino acid sequences in residues 48–124 were highly conserved among CDTBs (Fig. 3C). However, there are no basic amino acid clusters, which are often observed in classical NLSs of nuclear proteins, in this region.

In the case of eukaryotic or viral nuclear proteins, classical NLSs require binding proteins that act as receptors to carry the NLS to the NPC and to pass through the pore (38–40). In *Saccharomyces cerevisiae*, 14 receptors have so far been found by sequence homology, and mammalian cells are supposed to possess more receptors, including importins (21, 41). Our preliminary attempt to see the interaction between CDTB and importin-α or -β failed to show any direct association. The *in vitro* transport assay demonstrated that nuclear transport of His-CDTB-GFP required the HeLa cell cytosol and an energy source, suggesting that CDTB might use unknown factor(s) to enter into the nucleus.

The chromatin collapse induced by microinjection of His-CDTB or FLAG-CDTB could not be observed in CDT holotoxin-intoxicated cultured cells. Lara-Tejero and Galan (15) suggested that this difference was because of the difference of the intracellular concentration of CDTB molecule, and they demonstrated cytostidension by microinjecting a very low concentration of *C. jejuni* CDTB into the cytosol. Elwell and Dreyfus (14), and Lara-Tejero and Galan (15) independently identified that CDTB has significant similarity to the DNase I family. The essential amino acids for metal binding and catalytic domains of the DNase I family are conserved in the CDTB family, and all of them are located at the C terminus of CDTB (14). Site-directed mutations of these amino acids in CDTB resulted in inactivation of chromatin-disintegrating activity when microinjected. In this study, we have hypothesized that CDTB forms a modular structure composed of an N-terminal domain for nuclear transport and a C-terminal DNase-like domain. We could also reproduce chromatin collapse by microinjecting CDTB with its N-terminal domain for nuclear transport exchanged with SV40 T NLS. These results further support the hypothesis that CDTB acts as a DNase-like molecule and that the N-terminal domain may not be important for genotoxic activity. Furthermore, we demonstrated that nuclear entry of CDTB is necessary for cytostidension and the cell cycle block by CDT holotoxin. Recently, two groups reported that CDT induced ATM-dependent early response, which is observed by DNA strand breaks induced by irradiation. Li et al. (42) demonstrated that CDT induces phosphorylation of H2AX and Mre11 relocalization in HeLa cells. Another study demonstrated that CDT induces the formation of Rad50 foci in primary human fibroblast (43). These results further support that CDT directly induces DNA damage. However, the discrepancies between the reported specific nucleuse activites of CDTB and those of DNase I remain an open question. The precise molecular mechanism by which CDTB induces DNA damage remains to be elucidated.

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