

Functional and structural characterization of chimeras of a bacterial genotoxin and human type I deoxyribonuclease

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Abstract

Chimeras composed of the *cdtB* gene of a novel bacterial genotoxin and the human *type I deoxyribonuclease* (DNase I) gene were constructed and their products characterized relative to the biochemical and enzymatic properties of the native proteins. The product of a *cdtB/DNase I* chimera formed a heterotrimer with the CdtA and CdtC subunits of the genotoxin and targeted mutations increased the specific activity of the hybrid protein. Expression of active chimeric gene products established that the CdtB protein is an atypical divalent cation-dependent endonuclease and demonstrated the potential for genetically engineering a new class of therapeutic agent for inhibiting the proliferation of cancer cells.

Keywords

Aggregatibacter actinomycetemcomitans; chimera; cytolethal distending toxin; epithelial cells; genotoxin; pathogen; type I deoxyribonuclease

Introduction

The human periodontal bacterium *Aggregatibacter actinomycetemcomitans* is among a small group of facultative or microaerophilic gram-negative pathogenic bacterial species that produce a genotoxin, known as the cytolethal distending toxin (Cdt), that inhibits the proliferation of certain types of eukaryotic cells. Oral cells of epithelial origin appear to be natural targets (Kang *et al.*, 2005; Kanno *et al.*, 2005). The Cdt is a heterotrimer that has a structural organization exemplified by A-B-type toxins (Spangler, 1992). Two heterogenous subunits, CdtA and CdtC, promote binding of the toxin to the cell surface by forming a lectin-like groove analogous to that found in homodimers of the B chain of ricin (Nešić & Stebbins, 2005). Deduced amino acid sequences of various CdtB subunits exhibit a weak phylogenetic relationship to the large superfamily represented by endonucleases, exonucleases, sphingomyelinases and inositol polyphosphate 5-phosphatases (Hofmann *et al.*, 2000). It has been reported that CdtB from *Escherichia coli* (Elwell & Dreyfus, 2000), *Helicobacter hepaticus* (Dassanayake *et al.*, 2005) and *A. actinomycetemcomitans* (Mao & DiRienzo, 2002) exhibits *in vitro* double-strand DNA nicking activity.

Cdt-susceptible cell types or lines only respond to the heterotrimer. The exact sequence of events and roles of the subunits during the cell intoxication process have not been fully elucidated. However, studies to date suggest that the Cdt binds to either a fucose-containing glycoprotein (McSweeney & Dreyfus, 2005) or ganglioside (Mise et al., 2005) on the cell surface and the CdtB subunit, either alone or with CdtC, enters the cell through the Golgi complex (Guerra et al., 2005) or by an endosomal pathway (Akifusa et al., 2005). The CdtB subunit is translocated to the nucleus and crosses the nuclear membrane with the aid of a nuclear localization sequence (Nishikubo et al., 2003; McSweeney & Dreyfus, 2004). In some sensitive cell types chromatin damage occurs as double-strand breaks (Frisan et al., 2003; Kanno et al., 2005). The unrepaired DNA damage is thought to signal the cell to arrest cell cycle progression due to the accumulation of checkpoint proteins (Escalas et al., 2000). The location of the cell cycle block is cell type or line dependent. It has been proposed in one study that human lymphocytes undergo cell cycle arrest and apoptosis in response to the *A. actinomycetemcomitans* CdtB protein through a signaling pathway involving phosphatidylinositol 3,4,5-triphosphate phosphatase activity (Shenker et al., 2007).

The ability of CdtB to (i) exhibit *in vitro* DNA nicking activity, (ii) induce DNA damage in some cell types or lines and (iii) display a tentative phylogenetic relationship to Mg^{2+} -dependent phosphohydrolases invites a more detailed comparison of the relationship between these prokaryotic and eukaryotic proteins. A well characterized example of a Mg^{2+} -dependent endonuclease is mammalian DNase I which functions as a digestive enzyme (Lacks, 1981). In this study we compared the biochemical properties of CdtB and DNase I and then used this information to characterize the products of chimeras composed of the *A. actinomycetemcomitans* *cdtB* and human *DNase I* genes. Analysis of hybrid proteins provides insight into the relationship between CdtB and DNase I beyond that obtained from the study of the native proteins alone. The implications of this relationship are discussed in view of the role of the CdtB subunit in cytotoxicity and in engineering inhibitory agents for certain types of cancer cells of epitheloid origin.

Materials and methods

Construction of chimeric *cdtB* genes

Plasmids and primers used in this study are listed in Tables 1 and 2, respectively. All PCR and restriction digestion reactions were performed using standard techniques. Restriction endonucleases were purchased from New England Biolabs (Beverly, MA) and PCR oligonucleotide primers were obtained from Integrated DNA Technologies (IDT, Coralville, IA). Constructs were first selected in *E. coli* DH5 α [*supE44* Δ *lacU169* (ϕ 80*lacZ* Δ M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] and transformed into *E. coli* BL-21(DE3) for gene expression analysis and isolation of the gene product. Bacteria were grown in LB broth containing 75 μ g/ml ampicillin at 37°C with vigorous shaking. Plasmid DNA was isolated using either a QIAprep Miniprep (QIAGEN, Valencia, CA) or the Wizard Miniprep Kit (Promega Corporation, Fitchburg, WI). All constructs and mutations were confirmed by DNA sequencing. Automated cycle sequencing reactions were conducted by the DNA Sequencing Facility at the University of Pennsylvania Abramson Cancer Center using an Applied Biosystems 96-capillary 3730XL sequencer with BigDye Taq FS Terminator V 3.1.

The *cdtB* gene sequence used throughout these experiments was from construct pJDB7 originally made in Cao et al., (2005) and based on the sequence deposited in GenBank (accession no. AF006830). The full length DNase I sequence (GenBank accession no. AC005203) was amplified from plasmid GC-C0001 (GeneCopoeia, Inc., Germantown, MD) using the PCR primers N-DNase-F and X-DNase-R. This amplicon was cloned, in frame, to the His-tag in pETBlue2 (EMD Chemicals-Novagen, San Diego, CA) using *NcoI* and *XhoI* restriction endonuclease cleavage sites. Portions of the DNA sequence from the resulting

clone, pJD1, were used to construct the chimeras. To construct the *DNase I/cdtB* gene chimera the 5'-half of the *DNase I* gene sequence was obtained by digestion of pJD1 with *NcoI* and *SphI*, the 3'-half of the *cdtB* gene was obtained by digesting pJDB7 with *SphI* and *BamHI* and pET15b was cut with *NcoI* and *BamHI*. The three DNA fragments were ligated to obtain pETDB1 (Table 1). To construct the *cdtB/DNase I* gene chimera the PCR primers N-cdtB-F and S-cdtB-R were used to amplify the 5'-half of *cdtB* using pJDB7 as the template DNA. pJD1 was digested with *NcoI* and *SphI* and the large DNA fragment was ligated to the pJDB7 amplicon after digestion with the same enzymes (clone pBDN1). The insert DNA fragment from pBDN1 was amplified using the PCR primers N-cdtB-F and NdeI-His and the amplicon was cloned into the *NcoI* and *NdeI* sites of pET15b. The resulting plasmid, pETBDN2, contained the *cdtB/DNase I* chimeric gene, with codons for a His-tag at the 3'-end, in the same vector background as the *DNase I/cdtB* gene chimera.

Amino acid substitutions mutants

Amino acid substitutions were made in the CdtB/DNase I hybrid protein by site-directed mutagenesis as described previously (Cao *et al.*, 2006). Synthetic oligonucleotide primer pairs were used to change S99Y and R100E (both in same primer), L62E, G55R and V134R. Briefly, mutant DNA strands were made using PfuUltra DNA polymerase (Stratagene, La Jolla, CA) in PCR. Plasmid DNA from pETBDN2 was used as the first PCR template. Additional mutations were then made sequentially using plasmid DNA from pETBDN2-1 to pETBDN2-5. Methylated parental DNA strands were digested with *DpnI* and the intact mutated DNA strand was transformed into *E. coli* TOP10 [F⁻ *mcrAA*(*mrr-hsdRMS-mcrBC*) ϕ 80*lacZ* Δ M15*lacX*74 *recA1 araD139* Δ (*ara-leu*)7697 *galU galK rpsL*(str^R) *endA1 nupG*] chemically competent cells (Invitrogen, Carlsbad, CA). The mutations were confirmed by sequencing of the plasmid insert DNA from a single transformant.

The same strategy was used to change the active site H160 in CdtB-His₆ to Ala. DNA from pJDB7 was used as the PCR template for primers H160for and H160rev. The mutated protein from this clone was used as a negative activity control in the bioassays.

The large loop domain was added to CdtB/DNase I using an inverse PCR method (Cao *et al.*, 2004) with the Loop-F and Loop-R primers (Table 2) and the insert fragment from pETBDN2-5 (Table 1), containing the *cdtB/DNase I* nucleotide sequence, as the template DNA. Codons for LMLNQLRSQIT replaced NGLSDQLAQAI in pETBDN2-5LL.

Isolation of gene products and heterotoxin reconstitution

Escherichia coli BL-21(DE3) [F⁻ *ompT hsdS_B* (r_B⁻ m_B⁻) *gal dcm* (DE3)] (Novagen) containing pJDA9 (wild-type CdtA-His₆), pJDC2 (wild-type CdtC-His₆), pJDB7 (wild-type CdtB-His₆), pMUT160cdtB (active site mutant), pETBDN2 (*cdB-DNase I* chimera), pETDNB1 (*DNase I-cdB* chimera) and pETBDN2-1 to pETBDN2-5(*cdB-DNase I* mutated chimera) were used to isolate gene products by affinity chromatography on nickel-iminodiacetic acid columns (Novagen) as described previously (Cao *et al.*, 2005). All of the gene products have a His₆₋₇ tag at the carboxy-terminal end. The final protein preparations were dialyzed to remove urea, passed through 45 micron filters and quantified with the Micro BCA protein assay kit (Pierce, Rockville, IL). Purity was assessed by analysis on 10–20% polyacrylamide gels. The presence of the His-tag on all protein constructs was verified by western blotting using Anti-His•Tag Monoclonal Antibody (Novagen). Aliquots of the quantified protein samples were stored at –70°C in a buffer containing 10 mM Tris-HCl (pH 7), 100 mM NaCl, 5 mM MgCl₂ and 5 mM imidazole for use in the various bioassays. Wild-type heterotoxin and heterotoxin containing hybrid and mutated hybrid proteins were reconstituted as described previously (Mao & DiRienzo, 2002). Attempts to purify

recombinant human DNase I from either *E. coli* BL-21(DE3) (pJD1) or *E. coli* BL-21(DE3) (pETDN1) were unsuccessful most likely due to the poor expression of the human *DNase I* gene in *E. coli* as noted by Linardou *et al.* (2000).

***In vitro* DNase activity**

Supercoiled DNA nicking activity was determined, with minor modifications, as described previously (Elwell & Dreyfus, 2000; Mao & DiRienzo, 2000). In a typical assay 1 µg of supercoiled pBluescript II SK(+) DNA (Sigma-Aldrich, St. Louis, MO) was incubated with 1 µg of wild-type, mutant or hybrid CdtB protein in 25 mM HEPES (Sigma-Aldrich) (pH 7.0) containing 50 mM MgCl₂ at 37°C for 1 h. DNase I (0.1 ng), from bovine pancreas (Sigma-Aldrich), was used in place of human DNase I. Bovine DNase I is approximately 2.4-fold more active than the human homolog (Pan *et al.*, 1998). Each form of DNA (supercoiled, relaxed, linear) was quantified and compared using ImageJ version 1.34 (<http://rsb.info.nih.gov/ij/>) and digitized images of ethidium bromide-stained gels. Protein and divalent cation concentrations were varied in some kinetic and biochemical analyses.

Heat lability was compared by pretreating proteins at 100°C for up to 30 min prior to determining DNA nicking activity. Actin inhibition of DNA nicking activity was performed essentially as described by Ulmer *et al.* (1996). Rabbit skeletal muscle G-actin (Sigma-Aldrich) was de-polymerized at room temperature for 15 min in a buffer containing 25 mM HEPES (pH 7.5), 5 mM CaCl₂, 5 mM MgCl₂, 0.1% BSA, 0.05% Tween 20 and 0.5 mM 2-mecaptoethanol. Five micrograms of the depolymerized G-actin were preincubated with 1 µg of CdtB-His₆ or 0.1 ng of bovine DNase I for 60 min at 37°C. The treated proteins were then examined in the nicking assay. Subunit protection of DNA nicking activity was assessed as described previously (Nešić *et al.*, 2004) except that incubation was performed at room temperature for 1 hour.

***In vivo* CdtB activity**

Cell proliferation was measured with a colony-forming assay employing CHO cells [300 cells in 3 ml of medium per well (6-well plate) in triplicate] as described in Mao & DiRienzo (2002). The number of colonies per well was expressed as colony-forming units (CFU). A dose response curve for wild-type reconstituted heterotoxin has been published (Cao *et al.*, 2005). Cell cycle arrest was determined by flow cytometry of propidium iodide stained nuclei as described previously (Cao *et al.*, 2005).

Binding kinetics

Saturation kinetics were used to assess binding of the hybrid and mutated hybrid proteins to wild-type CdtA-His₆ and CdtC-His₆ in a thyroglobulin ELISA (Cao *et al.*, 2005). Wild-type CdtA-His₆ (4.0 µg) and CdtC-His₆ (3.5 µg) were added to thyroglobulin-coated wells. Hybrid or mutated hybrid protein (0–4 µg) was then added to triplicate wells. Bound protein was detected with a 1×10^{-6} dilution of anti-CdtB IgG rabbit antiserum and a 1×10^{-3} dilution of donkey anti-rabbit IgG-horseradish peroxidase conjugate (Amersham Pharmacia Biotech, Piscataway, NJ) (Cao *et al.*, 2008). The ability of the hybrid and mutated hybrid proteins to bind the other subunits was also determined by stoichiometric binding in the thyroglobulin ELISA as described previously (Cao *et al.*, 2005). Wild-type CdtA-His₆ was prebound to thyroglobulin-coated 96-well microtiter plates as described above. Hybrid and mutated hybrid proteins (4.5 µg) and wild-type CdtC-His₆ (3.5 µg) were added to triplicate wells. Total bound protein was detected with anti-His*Tag monoclonal antibody and anti-mouse IgG horseradish peroxidase conjugate both at a 1×10^{-7} dilution. An absorbance ratio of 3.0 is indicative of the binding of the hybrid or mutated hybrid protein to the other two subunits. CdtB-His₆ and DNase I do not bind to thyroglobulin. ELISA plates were washed in a BioTek Model EL405 HT microplate washer (BioTek Instruments, Inc., Winooski, VT).

Absorbance values were obtained with a BioTek Synergy 2 Multi-Detection Microplate Reader.

Differential dialysis was performed as described previously using dialysis membrane with a molecular weight exclusion limit of 100 kDa (Spectrum Laboratories, Inc., Rancho Dominguez, CA) (Cao *et al.*, 2008). Immunopositive bands on western blots were quantified with ImageJ.

Computer modeling

The European Molecular Biology Open Software Suite (EMBOSS release 3.0; <http://emboss.sourceforge.net>) (Rice *et al.*, 2000) was used to obtain the deduced amino acid sequences for *cdtB-His₆* (from pJDB7), *cdtB/DNase I*, *DNase I/cdtB* and *human DNase I* (from pETDN1) and to create the alignment using ClustalX 1.83. The crystal structures of the *A. actinomycetemcomitans* Y4 Cdt (Yamada *et al.*, 2006) and bovine DNase I were modeled with UCSF Chimera 1.2197 (<http://www.cgl.ucsf.edu/chimera/>) (Pettersen *et al.*, 2004). Coordinates were obtained from the Protein Data Bank (accession nos. 2F2F and 3DNI, respectively). Human DNase I has not been crystalized. CdtB/DNase I and DNase I/CdtB structures were predicted using Modeller version 9v1 (<http://salilab.org/modeller/>) (Marti-Renom *et al.*, 2000).

Results

Kinetic analysis of CdtB-His₆ and DNase I

Purified recombinant *A. actinomycetemcomitans* Y4 CdtB from pJDB7 (Fig. 1a), converted supercoiled [superhelical (S) circular, Form I] plasmid DNA to relaxed [(R) nicked circular, Form II] and linear [(L) Form III] forms with dose dependent kinetics in the standard DNA nicking assay (Fig. 1b). This conversion was not affected by the location (amino- or carboxy-terminus) of the His₆-tag. One µg of recombinant CdtB-His₆ converted 95% of 1 µg of supercoiled plasmid DNA to relaxed or linear forms in 1 hour at 37°C. The substitution mutant CdtB^{H160A} (from pMUT160cdtB in Table 1) had no effect on supercoiled plasmid DNA confirming that recombinant CdtB-His₆ preparations were not contaminated with *E. coli* nucleases (data not shown). In comparison, 0.1 ng bovine DNase I converted 100% of 1 µg of supercoiled plasmid DNA to relaxed form in 1 hour at 37°C (Fig. 1c). A ten-fold higher concentration of DNase I converted 98% of 1 µg of supercoiled DNA to linear form and a 100-fold higher concentration of the nuclease completely digested the DNA into small fragments. Based on these results the specific activity of recombinant CdtB-His₆ was estimated to be as much as 10³-fold lower than that of bovine DNase I.

Maximum conversion of supercoiled DNA to relaxed and linear forms was obtained with either 50 mM MgCl₂, CaCl₂ or MnCl₂ (Fig. 1d). Reactions containing CaCl₂ consistently failed to go to completion. CdtB-His₆ nuclease activity was inhibited by MgCl₂, CaCl₂ and MnCl₂ when the cation concentrations were greater than 150–200 mM. No conversion of supercoiled DNA was observed at any divalent cation concentration in the absence of CdtB and various combinations of the cations (50 mM of each) did not affect digestion patterns relative to those obtained with the individual cations. A time course of digestion with 50 mM MgCl₂ in the reaction established that 1 µg of CdtB-His₆ completely converted 1 µg of supercoiled DNA to relaxed and linear forms in 1 hour at 37°C (data not shown). Incubation times of 2 hours or greater generated more linear form DNA. Thus, CdtB-His requires either Mg⁺⁺, Ca⁺⁺ or Mn⁺⁺ at an individual or combined concentration of 50 mM for optimum activity. This is a 10-fold higher concentration than that required for optimum DNase I activity (Price, 1975). The standard *in vitro* DNase activity assay used in all subsequent experiments contained 50 mM MgCl₂.

Cdt-B-His₆ retained greater than 90% of its DNA nicking activity following incubation for 5 min in a boiling water bath (data not shown). There was a loss of virtually all enzymatic activity after heating for 10 min. In contrast, bovine DNase I DNA nicking activity was less than 50% of the control after 5 min of heating. The DNA nicking activity of both enzymes was significantly more heat stable than the double-strand cleavage activity.

Characterization of cdtB/DNase I and DNase I/cdtB chimeric genes and gene products

Notable differences between the biochemical properties of CdtB and DNase I suggested that a genetic strategy based on the analysis of the products of chimeric genes could be used to further examine functional similarities and differences between the two proteins. This approach was facilitated by the presence of a unique *Sph*I restriction endonuclease cleavage site at approximately the mid-point of both wild-type gene sequences. Using each half of the CdtB-His₆ and human DNase I gene sequences two genetic constructs, pETBDN2 and pETDNB1 containing the chimeric ORFs cdtB/DNase I and DNase I/cdtB, respectively (Table 1) were made. Both ORFs were placed immediately downstream from a highly efficient, inducible promoter and contained six or seven histidine codons in frame at the 3'-end of each sequence.

Two active site catalytic histidines are conserved in CdtB from *A. actinomycetemcomitans* and bovine DNase I (Fig. 2a). A deduced amino acid sequence alignment showed that both hybrid gene products contain the two histidines [residues H160/157 and H278/271 in CdtB/DNase I and DNase I/CdtB, respectively] (Fig. 2b). Computer modeling indicated that only the CdtB/DNase I hybrid protein maintained a folded structure similar to those of native CdtB and DNase I (Fig. 2c). However, both hybrid proteins exhibited optimum *in vitro* supercoiled DNA nicking activity in the presence of 50 mM MgCl₂. The DNA nicking activity of CdtB/DNase I was not affected after incubation in a boiling water bath for 5 min but there was a significant reduction in the DNA nicking activity of DNase I/CdtB after the same treatment ((data not shown). In contrast to bovine DNase I, G-actin had no effect on the ability of CdtB-His₆ and CdtB/DNase I to convert supercoiled DNA to relaxed and linear forms. Conversely, actin inhibited the DNA nicking activity of DNase I/CdtB. The results of these assays demonstrated that the amino terminal portions of CdtB and DNase I carry the biochemical properties of thermostability and actin binding, respectively.

Subunit assembly of cdtB/DNase I and DNase I/cdtB hybrid proteins

A property specific to CdtB is the ability to bind the CdtA and CdtC subunits to form a biologically active heterotrimer. CdtB/DNase I but not DNase I/CdtB bound to CdtA-CdtC heterodimer at concentrations comparable to CdtB-His₆ (Fig. 3a). Both the CdtB-His₆ control and CdtB/DNase I hybrid proteins reached saturation binding at approximately 3–4 µg per 4.0 µg of CdtA-His₆ and 3.5 µg of CdtC-His₆. Consistent with these results, CdtB/DNase I but not DNase I/CdtB, formed a heterotrimer complex in the differential dialysis assay (Fig. 3a; inset). In addition, CdtB/DNase I but not DNase I/CdtB bound stoichiometrically to CdtA and CdtC (Fig. 3b) and reconstituted heterotrimer preparations containing the CdtB/DNase I hybrid protein failed to convert supercoiled DNA to linear and relaxed forms (Fig. 3b; inset). Although these four independent assays established that the CdtB/DNase I hybrid protein formed a heterotrimer, this complex failed to inhibit the proliferation and cell cycle progression of CHO cells.

Effect of targeted amino acid substitutions on the DNA nicking and subunit binding activities of CdtB/DNase I

Since the DNase I/CdtB hybrid protein failed to bind CdtA and CdtC (Fig. 3), lacked a key domain for nuclear localization (Fig. 2b) and appeared to have an unfolded conformation in computer models (data not shown) it was not studied further. Single amino acid substitutions

were sequentially made in the CdtB/DNase I hybrid protein based on the results from mutagenesis studies of the bovine and human forms of the *DNase I* gene (Pan *et al.*, 1998). Substitutions G55R, S99Y and V134R added DNA contact residues, L63E added a metal ion binding residue and R100E added a residue that hydrogen bonds to the catalytic H160 in DNase I (Fig. 2b and Table 1). Five hundred ng of the final mutated hybrid gene product, CdtB/DNase I^{mut5} (clone pETBDN2-5), converted 100% of 1 µg of supercoiled DNA to relaxed and linear forms under standard assay conditions resulting in an approximately 1.4-fold increase in specific activity relative to CdtB-His₆ and CdtB/DNase I (Fig. 4 and inset B). CdtB/DNase I^{mut5} exhibited classical first-order kinetics (inset A).

Two regions in CdtB considered to be important for subunit assembly are the α-helix H1 (residues N41-S54) and a large loop (residues L261-S272) which are predicted to bind to CdtC and CdtA, respectively (Hu *et al.*, 2006). Since the CdtB/DNase I hybrid contains the former but not the latter motif the large loop sequence LMLNQLRSQIT (residues 261–272 in Fig. 2b) was added to CdtB/DNase I^{mut5}. However, this modification failed to improve the ability of the hybrid protein to form an active heterotrimer with CdtA and CdtC.

Discussion

A number of studies have shown that the CdtB subunit of the Cdt promotes inhibition of cell proliferation of epithelial cells through a DNA-damaging mechanism. However, questions remain about the mechanism of action of CdtB based on its phylogenetic (Hofmann *et al.*, 2000) and structural (Dlakic, 2000) connection to a wide range of eukaryotic phosphohydrolases. Theoretically, each of these classes of enzyme are capable of inducing cell cycle arrest through a variety of mechanisms all requiring Mg²⁺-dependent hydrolysis of phosphate (Mock & Ullmann, 1993). The largest body of empirical evidence supports an *in vivo* endonuclease type mechanism of DNA damage (Oswald *et al.*, 2005). Indeed, it has been reported that the *cdtB* gene product from *E. coli* (Elwell & Dreyfus, 2000) and *Helicobacter hepaticus* (Dassanayake *et al.*, 2005) have *in vitro* biochemical properties consistent with a Mg²⁺-dependent nuclease. We found that although both the *A. actinomycetemcomitans* CdtB and mammalian DNase I exhibit *in vitro* DNA nicking activity there were significant differences such that CdtB requires a 10-fold increase in divalent cation concentration to optimally convert supercoiled to relaxed form DNA, is more heat stable than DNase I, is not inactivated by actin and has a relatively lower specific activity. These differences are not surprising since the *cdtB* gene has evolved as part of a prokaryotic cytotoxin and, as such, would not be expected to be identical to a typical endonuclease.

Obtaining functional chimeras of the *cdtB* and human *DNase I* genes strongly supports the close relationship between the two gene products. The chimeric *cdtB-DNase I* genes were expressed in *E. coli* and the gene products exhibited nuclease activity, *in vitro*, comparable to that of CdtB. The fact that these artificial prokaryotic/eukaryotic gene constructs were expressed by *E. coli* and satisfied very stringent requirements to maintain a specific enzymatic activity provides compelling and novel evidence that the *cdtB* gene has evolved as an atypical divalent cation-dependent nuclease with remarkable similarities to mammalian DNase I. There appears to be a significantly weaker relationship between other phosphohydrolases and CdtB. For example, multiple large gaps have to be inserted to force an alignment between the deduced amino acid sequences of phosphatidylinositol 3,4,5-triphosphate phosphatase and CdtB (Shenker *et al.*, 2007). A typical CdtB and human DNase I alignment requires only a few relatively small gaps (see Fig. 2b).

Even though the CdtB/DNase I hybrid protein exhibited *in vitro* DNA nicking/cutting activity and appeared to form a heterotrimer with CdtA and CdtC, as determined by four

independent assays, it failed to form a biologically active heterotoxin. The hybrid protein may be defective in either the ability to enter cells or to migrate to the cell nucleus because it has a non-native conformation. Additional details about the cell intoxication process will be required to design experiments to examine these possibilities. However, the successful construction of the chimeric genes indicates that there may be significant potential to exploit the functional relationship between CdtB and human DNase I to genetically engineer novel therapeutic reagents that take advantage of the most desirable features of each native protein. DNase I has therapeutic applications including the treatment of wounds and ulcers, bronchitis, inflammatory conditions, herpes infection and cystic fibrosis (Lloyd, 1968; Shak *et al.*, 1990). Most notably, DNase I has a significantly more potent DNA cutting/nicking activity than CdtB. In contrast, CdtB can enter cells and translocate to the nucleus. We found that immortalized epitheloid cell lines, including HeLa, KB, HEP-2 (DiRienzo *et al.*, 2002) and GSM-K (Kanno *et al.*, 2005), as well as cells from oral squamous cell carcinomas (manuscript in preparation), are particularly sensitive to the cytotoxic effects of the Cdt. Cells of mesenchymal and ectomesenchymal origin, such as human periodontal ligament fibroblasts, cementoblasts and osteoblasts are resistant (Kang *et al.*, 2005; Kanno *et al.*, 2005; unpublished data). Since the effects of the Cdt are most pronounced on rapidly proliferating epitheloid cells the toxin has potential anti-cancer applications. Our approach, initiated in this study, is to develop a genetically engineered CdtB/DNase I chimera to attempt to increase the DNA-damaging activity of the toxin while maintaining a native mechanism for delivery to the cell nucleus.

Acknowledgments

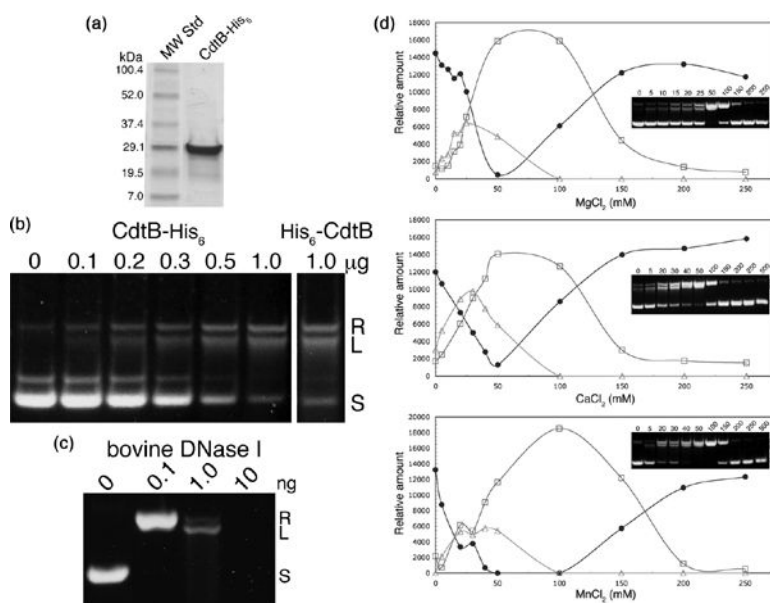
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**Fig. 1.**

(a) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of affinity purified recombinant *A. actinomycetemcomitans* CdtB-His₆. The gel is stained with Coomassie Brilliant Blue. (b) Dose response kinetics of the conversion of supercoiled (S) plasmid DNA to relaxed (R) and linear (L) forms by CdtB-His₆. Agarose gel stained with ethidium bromide. The activity of CdtB labeled with a histidine tag at the amino terminal end of the protein is compared at the highest concentration (1 μ g/1 μ g of DNA) of CdtB-His₆ tested. (c) Dose response kinetics of the activity of bovine DNase I. (d) DNA nicking assay containing increasing concentrations of either MgCl₂ (top panel), CaCl₂ (middle panel) or MnCl₂ (bottom panel) in the standard reaction buffer. Supercoiled (S), relaxed (R) and linear (L) forms of DNA were quantified by densitometry of ethidium bromide stained agarose gels (insets). The data shown is representative of several repeat experiments.

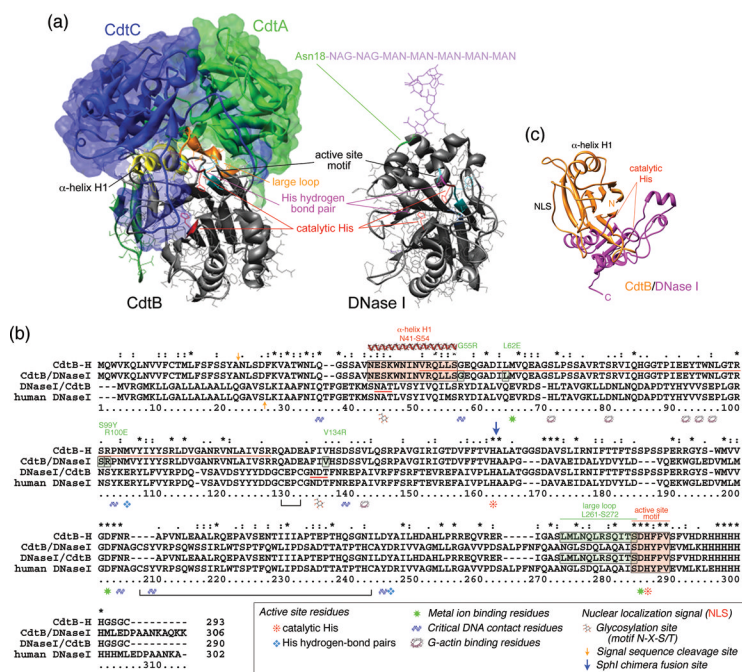
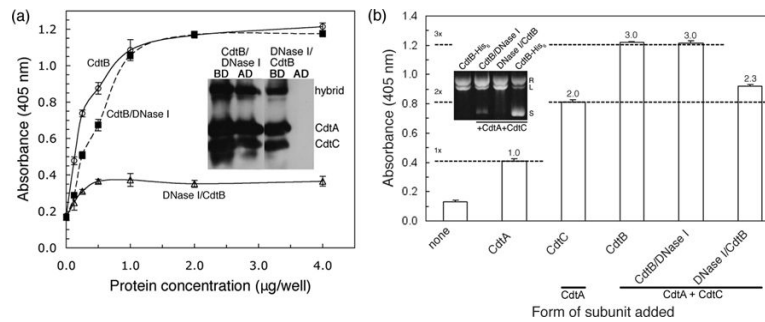


Fig. 2.

(a) Computer models of the *A. actinomycetemcomitans* CdtB (as part of the Cdt heterotrimer) and bovine DNase I. (b) Sequence alignment of the four His-tagged constructs. Conserved and identical residues are marked by dots and asterisks, respectively. The blue and orange arrows mark the gene fusion position and predicted signal sequence cleavage sites, respectively. Amino acid substitutions are marked in green and predicted functional domains are in red. Location of the nuclear localization signal (NLS) and information about the location of functional residues in DNase I was from Nishikubo et al. (2003) and Pan et al. (1998), respectively. Disulfides in DNase I are designated by brackets. (c) Theoretical model of the CdtB/DNase I hybrid protein construct. The CdtB (amino-terminal half) and DNase I (carboxy-terminal half) portions of the hybrid protein are shown in orange and magenta, respectively. The amino- (N) and carboxy (C)-termini are labeled.

**Fig. 3.**

(a) Saturation binding kinetics of the two hybrid proteins were compared to that of CdtB-His₆. The same proteins were incubated with wild-type CdtA and CdtC in refolding buffer and subjected to differential dialysis. The reconstituted samples were examined before (BD) and after (AD) dialysis on a western blot (inset). (b) The stoichiometric binding of the two hybrid proteins to wild-type CdtA and CdtC immobilized on thyroglobulin was examined by ELISA. Numbers above the columns are absorbance ratios calculated as described in Methods. The hybrid proteins, and CdtB-His₆ as a control, were mixed with wild-type CdtA and CdtC, as in the differential dialysis experiment in the inset in panel A, and the reconstituted samples assayed for DNA nicking activity (inset). The reaction examined in the first lane contained non-complexed CdtB-His₆. Abbreviations are the same as in the legend for Fig. 1. Experiments were performed a minimum of three times. Mean values and standard deviations were plotted where appropriate.

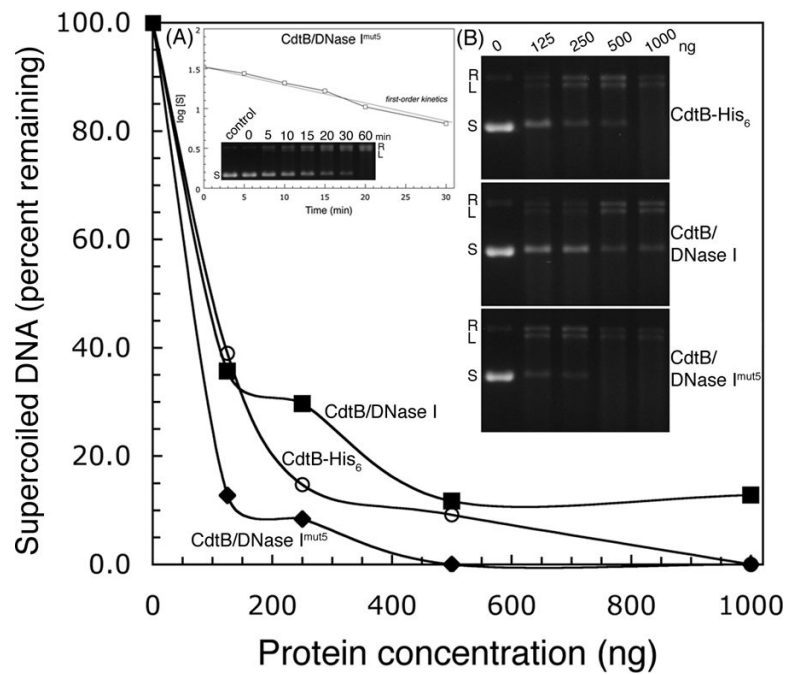


Fig. 4.

Nuclease activity of CdtB/DNase I^{mut5} was compared to that of CdtB-His₆ and the original CdtB/DNase I construct. The data is expressed as a percentage of the supercoiled (S) form DNA remaining after the reaction. These values were obtained by densitometry of ethidium bromide stained agarose gels (inset A). Semilogarithmic plot of the log of the substrate concentration [S] versus time of the reaction (expressed in minutes) for CdtB/DNase I^{mut5} (inset B). [S] represents the amount of S-form DNA obtained by densitometry of the agarose gel in the inset). The control lacked enzyme. The data shown is representative of several repeat experiments.

Table 1

Plasmids used in this study

Plasmid	Features	Source or reference
GC-C0001	human <i>DNase I</i> gene in pReceiver-B02	GeneCopoeia
pJD1	<i>DNase I</i> gene containing 6 His codons at 3'-end in pETBlue2	This study
pJDB7	<i>cdtB</i> gene containing 6 His codons at 3'-end in pET15b	Cao et al., 2005
pMUT160cdtB	pJDB7 with substitution H160A	This study
pETDN1	human <i>DNase I</i> gene containing 7 His codons at 3'-end in pET15b	This study
pBDN1	<i>cdtB/DNase I</i> chimeric gene containing 6 His codons at 3'-end in pETBlue2	This study
pETBDN2	<i>cdtB/DNase I</i> chimeric gene containing 7 His codons at 3'-end in pET15b	This study
pETDNB1	<i>DNase I/cdtB</i> chimeric gene containing 6 His codons at 3'-end in pET15b	This study
pETBDN2-1	pETBDN2 with substitutions S99Y and R100E	This study
pETBDN2-2	pETBDN2-1 with substitution L62E	This study
pETBDN2-4	pETBDN2-3 with substitution G55R	This study
pETBDN2-5	pETBDN2-4 with substitution V134R	This study
pETBDN2-5LL	pETBDN2-5 with substitution N265-I275 to L265-T275	This study
pETBlue2	Cloning vector	Novagen
pET15b	Cloning vector	Novagen

Primers used in this study

* Underlined bases show the NcoI (CCATGG), *XhoI* (CTCGAG), *SphI* (GCATGC), *NdeI* (CATATG) and *BamHI* (GGATCC) restriction endonuclease cleavage sites. Bold type marks the changed codon(s).