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Rohana P. Dassanayake
University of Nebraska - Lincoln

Mark A. Griep
University of Nebraska-Lincoln, mgriep1@unl.edu

Gerald Duhamel
University of Nebraska - Lincoln, gduhamel1@unl.edu

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The cytolethal distending toxin B sub-unit of *Helicobacter hepaticus* is a Ca^{2+}- and Mg^{2+}-dependent neutral nuclease

Rohana P. Dassanayake[a], Mark A. Griep[b,c], Gerald E. Duhamel[a,c]

[a]*Department of Veterinary and Biomedical Sciences, University of Nebraska-Lincoln, Room 147, VBS Building, Lincoln, NE 68583-0905, USA*

[b]*Department of Chemistry, University of Nebraska-Lincoln, Lincoln, NE, USA*

[c]*Center for Biotechnology, University of Nebraska-Lincoln, Lincoln, NE, USA*

**Corresponding authors, email addresses: rohana1@gmail.com, gduhamel1@unl.edu*

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Abstract: The cytolethal distending toxin B (CdtB) of the mouse pathogen *Helicobacter hepaticus* has cation binding and DNA catalysis residues in common with members of the mammalian deoxyribonuclease I (DNase I) family. The purpose of the present study was to characterize CdtB nuclease. To establish optimal digestion conditions and to evaluate co-factor requirements, a novel and sensitive fluorometric assay that quantitatively determines double stranded DNA digestion was developed. Although the Ca^{2+}- and Mg^{2+}-dependence and neutral properties of CdtB were similar to DNase I, hydrolysis of DNA by CdtB was approximately 100-fold less active than DNase I and was considerably more resistant to inhibition by $ZnCl_2$ and G-actin.

1. Introduction

Enterohepatic *Helicobacter* species (EHS) colonize the lower intestine and hepatobiliary tract of a broad range of hosts including human [1]. Certain EHS cause enterocolitis and bacteremia in humans immunocompromised due to AIDS [2] and chronic colitis and hepatitis in laboratory animal models [3,4]. The prototype EHS, *Helicobacter hepaticus*, is a cause of typhlocolitis, hepatitis, and hepatocellular carcinoma in laboratory mice, and together with certain EHS produces a toxin, known as cytolethal distending toxin (Cdt) [5]. Homologues of EHS Cdt also are found in the enteric bacterial pathogens, *Campylobacter* species, *Shigella* species, and *Escherichia coli* as well as the periodontopathogenic bacterium, *Actinobacillus actinomycetemcomitans* and the venereal pathogen, *Haemophilus ducreyi* [6,7].

In concert with subunits A and C, Cdt subunit B (cdtB) causes progressive cytoplasmic and nuclear distension of target cells accompanied with growth arrest at the G_2/M transition phase of the eukaryotic cell cycle, and ultimately cell death [7–10]. More recent studies suggest that CdtB produced by *H. hepaticus* [5], *Campylobacter jejuni* [11], *E. coli* [12], and *Shigella dysenteriae* [13] might be an important virulence determinant in naturally-occurring and experimentally-induced disease.

The presence of conserved cation-binding and DNA catalysis residues between CdtB and members of the

mammalian deoxyribonuclease I (DNase I) family was independently reported by Elwell and Dreyfus for *E. coli* [8,10] and Lara-Tejero and Galán for *C. jejuni* [9]. DNase I is an endonuclease that digests single- and double-stranded DNA (dsDNA) by hydrolyzing phosphodiester bonds producing mono- and oligo-deoxyribonucleotides with 5'-phosphate and 3'-hydroxyl groups. Consistent with the structural similarities with DNase I, mutations of corresponding residues impaired the nuclease activity of *E. coli* CdtB in vitro [8]. However, demonstration of the nuclease activity of CdtB in vitro has only been shown by qualitative assessment of plasmid DNA digestion with CdtB of *E. coli* [8,10], and more recently *H. hepaticus* [14], and eukaryotic DNA double strand breaks with CdtB of *H. ducreyi* [7,15] by using gel-based assays. Digestion of plasmid DNA by CdtB under empiric conditions is estimated on the basis of disappearance of supercoiled form in agarose gels stained with ethidium bromide. Although this method is suitable for qualitative demonstration of nuclease activity, the detection limit is low, approximately 10 ng plasmid DNA, and does not allow precise biochemical characterization of nuclease activity, particularly determination of optimal assay conditions, co-factor requirements, and comparative digestion efficiency and inhibition. To address these limitations, hexahistidine (His_6)-tagged CdtB was overproduced and purified from the prototype pathogenic EHS, *H. hepaticus*, and a highly sensitive fluorometric assay that accurately quantitates ds DNA digestion was developed. The fluorometric assay uses the dye PicoGreen that selectively binds to dsDNA in solution and displays a linear correlation between dsDNA concentration and fluorescence with a detection range of 25 pg ml^{-1} –1 μg ml^{-1} dsDNA [16]. PicoGreen is a cationic bis-cyanine dye with very low fluorescence quantum yield in solution that changes to very high yield when it bis-intercalates into duplex DNA. The basis of the fluorometric nuclease assay is that a fractional decrease in fluorescence intensity of the PicoGreen–DNA complex is exactly proportional to the hydrolysis of a fractional equivalent of phosphodiester bonds in the dsDNA sample. As the duplex DNA is cleaved, termini are created to which the dye binds poorly. The fluorometric assay can not differentiate enzymatic cleavage of supercoiled plasmid DNA to linear and relaxed forms seen with the gel-based assay used in previous studies. However, it provides a highly sensitive and quantitative measure of nuclease activity unlike the subjective assessment of plasmid digestion in the gel-based assay. The data provide new insights into DNA hydrolysis by *H. hepaticus* CdtB, a representative member of a novel family of nuclease toxins produced by several important bacterial pathogens of humans and animals.

2. Materials and methods

2.1. Bacterial strains and growth conditions

H. hepaticus mouse strain 3B1/Hh-1 ATCC 51449^T [3] was propagated on trypticase soy agar supplemented with 5% (v/v) sheep blood incubated at 37 °C under microaerobic conditions as previously described [17]. Chemically competent *E. coli* (One Shot TOP10 and BL21 Star [DE3] strains; Invitrogen, Carlsbad, CA) were grown on Luria–Bertani (LB) agar plates at 37 °C under aerobic conditions. Transformants were selected by plating onto LB agar containing 30 μg ml^{-1} (w/v) kanamycin (Sigma, St. Louis, MO).

2.2. His_6-tagged H. hepaticus CdtB

The *cdtB* gene of *H. hepaticus* was amplified by using a PCR assay with forward 5'-GAATTC AATCTTGAAGATTATAGAG-3' and reverse 5'-CTCGAGCTAAAATCGTCCAAAATGC-3' oligonucleotide primers designed to amplify the full length gene without the first 48 nucleotides encoding the N-terminal 16-amino acid signal sequence (GenBank accession no. AF163667/AAF19158). One hundred ng of *H. hepaticus* genomic DNA was mixed with 48 μl containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM concentration each of dATP, dTTP, dGTP, and dCTP, 0.5 μM concentration of each primer, and 1.0 U of the high fidelity *Pfx* DNA polymerase (AccuPrime, Invitrogen) in sterile water. The cycling conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 25 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 1 min, and extension at 68 °C for 1 min. The 771 bp amplified product was visualized by electrophoresis in 0.8% agarose gel run at 7.0 V/cm after staining with ethidium bromide. For directional expression cloning into the His_6-tagged vector (Novagen, Madison, WI) 5'-*Eco* RI and 3'-*Xho* I overhangs were included in the respective primers (underlined sequence). After digestion of purified PCR products with appropriate restriction enzymes, the *cdtB* fragment was sub-cloned into pET-28a(+) (Novagen) and transformed into *E. coli* TOP10. The nucleotide sequences of both strands of plasmid DNA from selected recombinant clones were determined. The correct His_6-tagged CdtB construct was transformed into *E. coli* BL21 and induced with 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG, Invitrogen) at 25 °C for 5 h. At the end of the incubation, the cells were harvested by centrifugation and suspended in binding buffer (0.5 M NaCl, 20 mM Tris–HCl, 5 mM imidazole, pH 7.9; Novagen) containing 45–60 KU g^{-1} of lysozyme (Sigma). The cells were lysed by sonication and after centrifugation at 14,000*g* for 20 min at 4 °C, CdtB was pu-

rified from the soluble (supernatant) and insoluble (pellet) fractions. Purification of CdtB from the soluble fraction was accomplished under native conditions with a 1.25 ml Ni^{2+}-charged His-Bind column (Novagen). The insoluble CdtB fraction was purified under denaturing conditions by adding 6 M urea. The eluted CdtB was diluted in dialysis buffer (25 mM Tris–HCl, 25 mM NaCl, 2 mM EDTA, pH 7.4) to 0.1 mg ml^{-1} and dialyzed (6 K MWCO Spectra, Spectrum, Medical Industries Inc., Los Angeles, CA) overnight at 4 °C. The urea was removed gradually during dialysis by sequential changes of dialysis buffer that contained 4, 2, and then 0 mM urea, to provide gradual re-folding of CdtB. The soluble CdtB and the re-solubilized CdtB were separately concentrated and desalted by filter centrifugation (Amicon Ultra-15 10K MWCO; Millipore, Bedford, MA) at 3000*g* for 30 min at 4 °C. The concentration of protein was determined by using a commercially available method (BCA protein assay kit; Pierce, Rockford, IL).

Purified CdtB and whole-cell lysate from induced and noninduced *E. coli* BL21 cells were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 4% stacking and 12% separation gel for 3 h at room temperature and staining with Coomassie brilliant blue R250 or electrotransferred onto nitrocellulose membrane for immunoblot analysis [18]. Identification of CdtB on the immunoblot was accomplished by incubation of the membrane with a mouse monoclonal anti-His_6 tag immunoglobulin (Ig) G_1 antibody (Novagen), followed by horseradish peroxidase conjugated goat anti-mouse IgG_1 (heavy and light chains) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and 4-chloro-1-naphtol substrate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) and washings. The amino acid sequence of purified His_6-tagged CdtB was confirmed by mass spectrometry [19].

2.3. Gel-based nuclease assay

The nuclease activity of the purified His_6-tagged CdtB was first confirmed by qualitative assessment of changes in band intensity after digestion of either purified plasmid DNA (pUC19 [Invitrogen], pBSIIKS+ [Stratagene, La Jolla, CA] and pTRE2hyg [Clontech]) or eukaryotic DNA from human epithelioid cervical carcinoma HeLa cells ATCC CCL-2, and separation of DNA fragments by electrophoresis in agarose gels stained with ethidium bromide, as previously described [8,14]. Briefly, 1 μg (1.5 μM final protein concentration) of CdtB and either 1 μg (150 μM final nucleotide concentration) of plasmid DNA or eukaryotic DNA in a volume of 20 μl of 50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 10 mM $CaCl_2$, and 0.4 μg ml^{-1} bovine serum albumin (BSA) were incubated for up to 6 h at 37 °C. At the end of the incubation, DNA digestion was stopped by adding sample loading buffer (50 mM EDTA, 0.25% bromphenol blue and 30% glycerol) and loading onto 0.8% agarose gel, and electrophoresis at 7.0 V/cm for 2 h. Disappearance of supercoiled plasmid DNA and digestion of eukaryotic DNA were visualized under UV light after staining with ethidium bromide.

2.4. Fluorometric nuclease assay

Further quantitative determinations of the nuclease activity of CdtB were accomplished by using the newly developed fluorometric assay. Standard assay conditions for digestion consisted of 1 μg (1.5 μM final protein concentration) CdtB mixed with 1 μg (150 μM final nucleotide concentration) DNA in a volume of 20 μl of 50 mM HEPES, pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$, and 0.4 μg ml^{-1} BSA reaction buffer. After incubation for up to 6 h at 37 °C, the reaction was quenched by transferring 4 μl of the reaction mixture to 96 μl of 10 mM Tris, pH 7.4, 1 mM EDTA buffer (TE), in a flat-bottom 96-well plate (Nalge Nunc Intl., Naperville, IL). After diluting the DNA quantitation reagent (PicoGreen dsDNA Quantitation Reagent and Kits; Molecular Probes, Eugene, OR) to 1:200 into TE buffer, 100 μl was added to each well and incubated at room temperature for 3 to 5 min in the dark. After incubation, the fluorescence was measured using a fluorescence microplate reader (FLUOstar OPTIMA BMG LABTECH, Durham, NC) with excitation and emission wavelengths of 485 and 520 nm, respectively. Data were expressed as mean percent digested ng ml^{-1} of ds DNA by determining the ratio of digested to control undigested dsDNA in ng $ml^{-1}\times$ 100 from at least three independent experiments, and using a standard curve with known DNA concentrations from 0 to 1 μg ml^{-1} (0 to 150 μM). Bovine pancreatic DNase I (0.1 U or 3.7 μM; Fermentas, Hanover, MD) was included as a positive control in all of the assays. Negative control consisted of dsDNA without nuclease under the same conditions.

2.5. Multiplex reaction buffer and pH optimization and cation requirement

First, different concentrations of the reaction buffer HEPES pH 7.4 (0–100 mM) containing 0–50 mM $CaCl_2$, 0–50 mM $MgCl_2$, and 0–100 μg ml^{-1} BSA were examined for digestion of 1 μg (150 μM nucleotide) plasmid DNA by 1 μg (1.5 μM) CdtB in a final volume of 20 μl for 1 h at 37 °C.

Once the optimal DNA digestion conditions were determined, the pH dependence of CdtB nuclease activity was determined using a range of pH from 3 to 9 in 0.5 unit increments by using APHTC buffer containing 40 mM each of acetic acid (p*K* 4.8 at 25 °C), PIPES (p*K* 6.8), HEPES (p*K* 7.6), Tris (p*K* 8.3), and CHES (p*K* 9.3) (Sigma). Then, HCl was added to half of the APHTC mixture to create solutions with pHs from 3 to 6.5. Similarly, NaOH was added

to the other half of the mixture to create solutions with pHs from 7 to 9.

The cation requirement for nuclease activity of CdtB was determined by pre-incubating the reaction buffer containing 1 μg (1.5 μM) CdtB with either 4 to 20 mM EDTA or 50 to 250 mM NaCl for 10 min at 37 °C. Then 1 μg (150 μM nucleotide) plasmid DNA was added, and after adjusting the final volume to 20 μl with distilled water, the reaction mixture was incubated at 37 °C before determination of the mean percent DNA digestion by using the fluorometric assay. In order to assess the synergistic effect of Ca^{2+} and Mg^{2+}, 0 to 50 mM of Mg^{2+} were assayed in the presence of 10 mM Ca^{2+} or 0 to 50 mM Ca^{2+} were assayed in the presence of 10 mM Mg^{2+}.

Subsequent experiments examined the kinetics of plasmid DNA and eukaryotic DNA digestion for up to 6 h, by using varying concentrations of CdtB from 0.2 to 2 μg (0.3 to 3 μM) and constant 1 μg (150 μM nucleotide) dsDNA or keeping the concentration of CdtB constant at 1 μg (1.5 μM) and varying the concentration of dsDNA from 0.1 to 2 μg (15 to 300 μM nucleotide) under optimal buffer conditions.

2.6. Inhibition of CdtB nuclease activity by $ZnCl_2$ and G-actin

The requirement for Ca^{2+} and Mg^{2+} for CdtB nuclease activity was further examined by pre-incubating 1 μg (1.5 μM) CdtB in reaction buffer containing 0.03 to 1.0 mM $ZnCl_2$ for 15 min at 37 °C, then 1 μg (150 μM nucleotide) plasmid DNA was added and further incubated for up to 6 h at 37 °C. G-actin has been shown to specifically inhibit mammalian DNase I [20–22]. Following incubation of 1 μg (1.5 μM) CdtB in reaction buffer containing between 1 and 400 μg ml^{-1} rabbit skeletal muscle G-actin (Molecular Probes) for 20 min at 37 °C, 1 μg (150 μM nucleotide) plasmid DNA was added and further incubated for up to 6 h at 37 °C. In control experiments, 0.1 U (3.7 μM) bovine pancreatic DNase Ipre-incubated with either $ZnCl_2$ or G-actin, was allowed to digest 1 μg (150 μM nucleotide) plasmid DNA for 20 min at 37 °C. In all assays, the mean percent DNA digestion was quantified using the fluorometric assay.

3. Results and discussion

The single band with a molecular mass of approximately 32 kDa was present on the Coomassie blue stained SDS-PAGE of purified protein (Fig. 1A). This mass was consistent with the predicted molecular mass of the recombinant *H. hepaticus* His_6-tagged CdtB. The identity of the purified recombinant CdtB protein was confirmed by immunoblot analysis with anti-His_6 antibody (Fig. 1B) and amino acid sequence determination by mass spectrometry.

Both the soluble and re-solubilized proteins were active as determined in the gel-based assay. Incubation of either plasmid or eukaryotic DNA with the purified enzymes showed disappearance of supercoiled plasmid DNA over 6 h incubation (Fig. 2) or digestion of eukaryotic DNA indicative of nuclease activity.

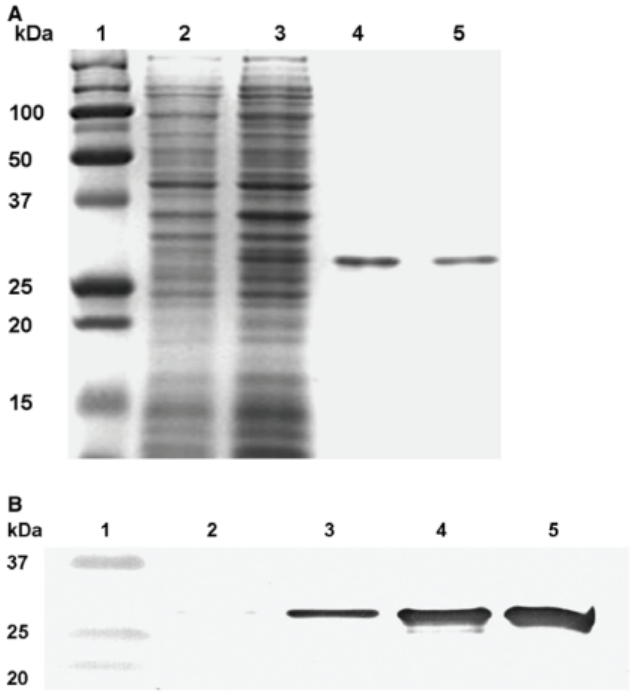

Figure 1. SDS-PAGE and immunoblot analyses of recombinant *H. hepaticus* His_6-tagged CdtB. SDS-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue R250 (A), and immunoblot analysis using a mouse monoclonal anti His-tagged IgG_1 antibody followed by horseradish peroxidase-conjugated goat anti-mouse IgG_1 antibody (B). Lanes: 1, molecular weight marker; 2, whole-cell lysate of *E. coli* BL21 before IPTG induction; 3, whole-cell lysate of *E. coli* BL21 5 h after IPTG induction; 4, purified CdtB from the soluble fraction (approx. 1 μg); and 5, purified re-solubilized CdtB from the insoluble fraction (approx. 1 μg).

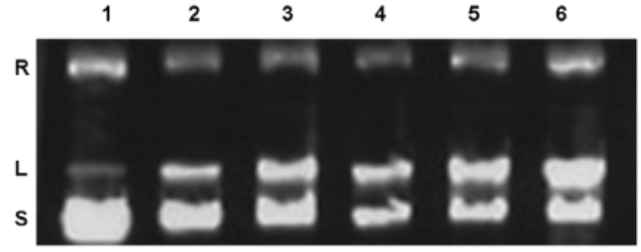

Figure 2. Nuclease activity of *H. hepaticus* CdtB with plasmid DNA. Purified His6-tagged CdtB was incubated with plasmid DNA (pTRE2hyg, Clontech) in the presence of Ca^{2+} and Mg^{2+} for up to 6 h and supercoiled (S), linear (L), and relaxed (R) forms of plasmid DNA were stained with ethidium bromide after separation by electrophoresis in a 0.8% agarose gel. Lanes: 1, control undigested plasmid DNA; 2, 1 h; 3, 2 h; 4, 3 h; 5, 4 h; 6, 5 h incubation. The data are representative of three independent experiments.

A rapid and quantitative fluorometric assay was used for optimization of the enzyme, including co-factor requirements, and then to characterized the inhibitory effects of Zn^{2+} and G-actin. Quantitative assessment of the nuclease activity of purified CdtB by using the fluorometric assay revealed that although 25 mM HEPES, 5 mM $CaCl_2$, 5 mM $MgCl_2$, and 100 μg ml^{-1} BSA had been empirically used in a previous study [10], a final concentration of 50 mM HEPES, 10 mM $CaCl_2$, 10 mM $MgCl_2$, and 0.4 μg ml^{-1} BSA provided optimal conditions for DNA digestion. Because the nuclease activities of CdtB obtained from the soluble fraction and the re-solubilized fraction were comparatively similar, CdtB obtained from the soluble fraction was used in all subsequent experiments. Although DNA digestion was observed between pH 5 and 9, maximum DNA digestion was seen at neutral pH between 7 and 8.

Next, we evaluated the efficiency of DNA digestion by varying the concentrations of CdtB and DNA, respectively. Because the digestion of 1 μg (150 μM nucleotide) plasmid DNA increased from 15% with 0.2 μg of CdtB to 40% with 1 to 2 μg of CdtB, a concentration of 1 μg (1.5 μM) of CdtB was used in all subsequent experiments. When increasing concentrations of plasmid DNA from 0.1 to 2 μg (15 to 300 μM nucleotide) were digested with a constant concentration of 1 μg (1.5 μM) of CdtB, the maximum nuclease activity (over 70% digestion) was observed when the DNA concentration was below 0.2 μg (30 μM nucleotide). At concentrations of DNA above 0.4 μg (60 μM nucleotide), the efficiency of digestion by CdtB nuclease was reduced and remained around 40% during the 6 h incubation period. Incubation of 1 μg (150 μM nucleotide) eukaryotic DNA with 1 μg (1.5 μM) of CdtB yielded similar results; 53% digestion during the 6 h incubation. Digestion with 1 μg (1.5 μM) CdtB and 1 μg (150 μM nucleotide) plasmid DNA revealed a linear relationship and an average digestion of 6.1 ± 1.2% of DNA/h (1.5 μM nucleotides/h; a specific activity of 1 M nucleotides/h/M enzyme). By contrast, 0.1 U (3.7 μM) of bovine pancreatic DNase I hydrolyzed 80% of 1 μg (150 μM nucleotide) of either plasmid or eukaryotic DNA within 20 min (specific activity of 97 M nucleotides/h/M enzyme). Therefore, CdtB nuclease was 97-fold less active than DNase I.

Comparative analysis of the predicted amino acid sequence of *H. hepaticus* CdtB with members of the mammalian DNase I family indicates that although the DNA hydrolysis residues are highly conserved [9,23], only five of 15 DNA binding residues, namely E37, S93, R139, N190, and T224 are present in CdtB (personal observation). The absence of several essential residues in CdtB may account for reduced efficiency of plasmid and eukaryotic DNA digestion compared with DNase I.

The cation-dependence of the DNase I family of nucleases is well-established [22–24], and although cation-binding sites are present in CdtB, the requirements for specific cation(s) for nuclease activity have not been investigated. To determine the cation requirement for nuclease activity of CdtB, recombinant CdtB was incubated with plasmid DNA in the presence of increasing concentrations of $CaCl_2$, $MgCl_2$, and NaCl. CdtB had no activity when incubated in the absence of either Ca^{2+} or Mg^{2+}, whereas maximum activity was seen at concentrations of 20 and 15 mM of the respective divalent cation (Fig. 3). Moreover, CdtB nuclease activity showed a synergistic effect when the concentration of one cation was reduced while the other cation was kept unchanged (10 mM). The nuclease activity of CdtB was impaired at concentrations of Ca^{2+} and Mg^{2+} above 20 and 15 mM, respectively (Fig. 3). Complete inhibition was present by chelation of divalent cations with a concentration of EDTA greater or equal to 20 mM (data not shown). Based on these observations, we concluded that CdtB is a Ca^{2+} and Mg^{2+}-dependent nuclease similar to DNase I [22–24].

Monovalent ions, including Na^+, K^+, and NH_4^+ are known inhibitors of mammalian DNase I activity [24]. The nuclease activity of CdtB was impaired at a concentration of 50 mM NaCl, and complete inhibition was found at a concentration of 250 mM NaCl (Fig. 3). This effect might be attributable to an increase in the ionic strength of the solution rather than a direct binding of the Na^+ ions to the cation-binding site of CdtB.

The nuclease activity of members of the DNase I family is completely inhibited by Zn^{2+} ion [22,24], therefore we determined the 50% inhibition concentration (IC_{50}) of Zn^{2+}

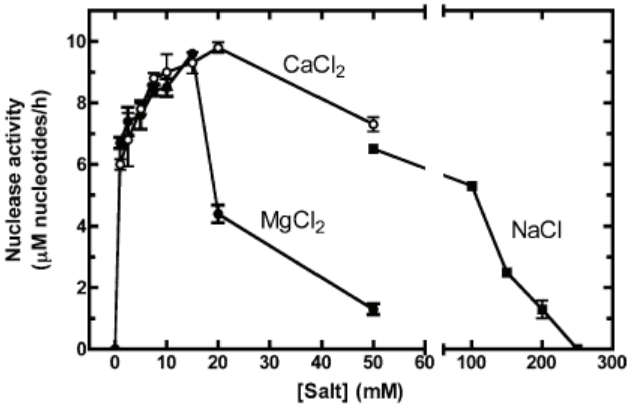

Figure 3. Nuclease activity of *H. hepaticus* CdtB in the presence of Ca^{2+}, Mg^{2+}, and Na^+ salts. His_6-tagged CdtB and plasmid DNA (pTRE2-hyg, Clontech) were incubated with increasing concentrations of $CaCl_2$, $MgCl_2$, and NaCl for 6 h and the remaining undigested double stranded DNA concentrations were determined by fluorometric assay. The nuclease activity of CdtB was impaired at concentrations of $CaCl_2$, $MgCl_2$, and NaCl above 20, 15, and 50 mM, respectively. The data are representative of three independent experiments.

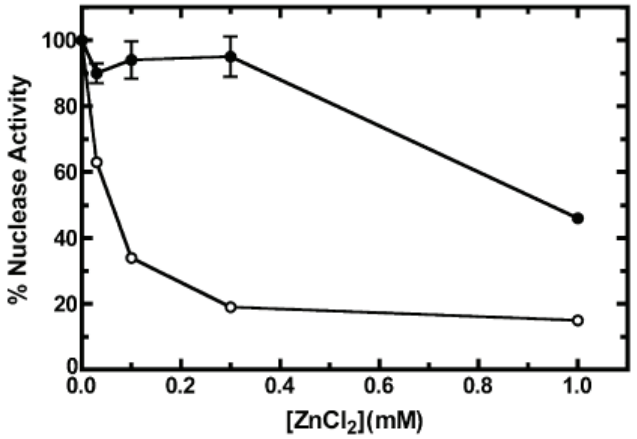

Figure 4. Inhibition of *H. hepaticus* CdtB nuclease activity by $ZnCl_2$. His_6-tagged CdtB (closed circles) or bovine pancreatic DNase I (opened circles) were pre-incubated with increasing concentrations of $ZnCl_2$ for 15 min followed by the addition of plasmid DNA (pTRE2hyg, Clontech) and further incubation for 6 h for CdtB and 20 min for DNase I. The concentration of undigested double stranded DNA was determined by using the fluorometric assay. Approximately 1.0 mM of $ZnCl_2$ was required to inhibit 50% (IC_{50}) of CdtB nuclease activity compared with approximately 0.06 mM for DNase I. The data are representative of three independent experiments.

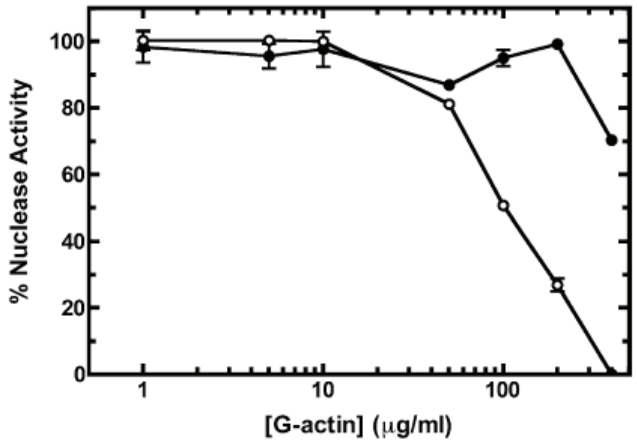

Figure 5. Inhibition of *H. hepaticus* CdtB nuclease activity by G-actin. His_6-tagged CdtB (closed circles) or bovine pancreatic DNase I (opened circles) were pre-incubated with increasing concentrations of G-actin for 20 min followed by the addition of plasmid DNA (pTRE2hyg, Clontech) and further incubation for 6 h for CdtB and 20 min for DNase I. The concentration of undigested double stranded DNA was determined by using the fluorometric assay. Greater than 400 μg ml^{-1} of G-actin was required to inhibit 50% (IC_{50}) of CdtB nuclease activity compared with approximately 100 μg ml^{-1} for DNase I. The data are representative of three independent experiments.

for CdtB. In the fluorometric assay, the IC_{50} of Zn^{2+} for bovine pancreatic DNase I and CdtB were 0.06 and 1.0 mM, respectively (Fig. 4). This data further suggested that CdtB is a nuclease similar to members of the mammalian DNase I family, however, CdtB was 17-fold more resistant to inhibition by Zn^{2+} than DNase I.

Monomeric actin, G-actin, binds to bovine and human DNase I and inhibits their nuclease activities [22,25]. The nuclease activity of CdtB was partially resistant to inhibition by G-actin; the IC_{50} of CdtB was greater than 400 μg ml^{-1} (the highest concentration that could be tested) compared with a IC_{50} of approximately 100 μg ml^{-1} for bovine pancreatic DNase I (Fig. 5). The inability of G-actin to inhibit CdtB correlates with the nonconservation of the DNase I actin-binding residues: Glu13, His44, Asp53, Tyr65, Val67, and Glu69 in this protein. The resistance of CdtB to inhibition by G-actin, a precursor cytoskeletal protein, might allow evasion of the nuclease from inhibition during translocation to the eukaryotic cell nucleus.

Although CdtB produced by *H. hepaticus* has structural characteristics homologous to members of the mammalian DNase I family, hydrolysis of DNA by CdtB was less active and also more resistant to inhibition by Zn^{2+} and G-actin than DNase I. Because CdtB is broadly distributed among bacterial pathogens and has nuclease activity in vitro that correlates with eukaryotic cell toxicity [8,9] and pathogenicity in animal models [5,11–13], it is unique among bacterial toxins. The novel and sensitive fluorometric assay provides the first detailed characterization of the biochemical properties of CdtB nuclease for bacterial pathogens in general and *H. hepaticus* in particular. These observations provide a framework for comparative biochemical analysis of this novel family of bacterial toxins.

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