A Bacterial Toxin That Controls Cell Cycle Progression as a Deoxyribonuclease I–Like Protein

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Many bacterial pathogens encode a multisubunit toxin, termed cytolethal distending toxin (CDT), that induces cell cycle arrest, cytoplasm distention, and, eventually, chromatin fragmentation and cell death. In one such pathogen, *Campylobacter jejuni*, one of the subunits of this toxin, CdtB, was shown to exhibit features of type I deoxyribonucleases. Transient expression of this subunit in cultured cells caused marked chromatin disruption. Microinjection of low amounts of CdtB induced cytoplasmic distention and cell cycle arrest. CdtB mutants with substitutions in residues equivalent to those required for catalysis or magnesium binding in type I deoxyribonucleases did not cause chromatin disruption. CDT holotoxin containing these mutant forms of CdtB did not induce morphological changes or cell cycle arrest.

Campylobacter jejuni, the most common cause of food-borne infectious illnesses in the United States (*1*), encodes a toxin termed CDT, which is considered to be an important virulence factor (*2–4*). This toxin causes eukaryotic cells to arrest in the G_2/M transition phase of the cell cycle (*5*). Intoxicated cells show a characteristic accumulation of the phosphorylated form of the cell cycle regulator CDC2, as well as an increase in their DNA content (4N), consistent with a cell cycle blockage at the G_2/M boundary (*6–10*). After intoxication, cell division stops, but the cytoplasm continues to grow and distend, resulting in cells up to five times their normal size. Intoxicated cells maintain viability for extended periods of time, although they eventually show morphological changes in the chromatin such as condensation and/or fragmentation and ultimately die. In addition to *C. jejuni*, CDT-homologous toxins have been described in several other important bacterial pathogens (*11–17*), but little is known about their mechanism of action. CDTs are encoded by a cluster of three highly conserved genes of unknown function, *cdtA*, *cdtB*, and *cdtC* (*4*).

To investigate which of the Cdt subunits may have toxic activity within the host cell, we transfected COS-1 cells with plasmids expressing epitope-tagged CdtA, CdtB, or CdtC or vector control (*18*). Cells expressing CdtA or CdtC or transfected with the vector control displayed an apparently normal morphology and showed no signs of intoxication (Fig. 1). In contrast, cells transfected with a plasmid expressing CdtB exhibited striking alterations characterized by fragmented nuclei and often a total collapse of the chromatin (Fig. 1). Changes in the chromatin were apparent as early as 18 to 24 hours after transfection when nuclei of transfected cells began to exhibit a distinct smooth appearance (Fig. 1). Forty-eight hours after transfection, the nuclei of transfected cells appeared seriously compromised, exhibiting marked fragmentation or outright disappearance of the chromatin (Fig. 1). Thus, CdtB was responsible, at least in part, for the activity of the CDT toxin within the host cell.

We examined the predicted amino acid sequence of this toxin subunit in an effort to identify clues about its function. Amino acid sequence comparison revealed similarity to deoxyribonuclease (DNase) I–like proteins. Further alignment of CdtB with members of the DNase I protein family revealed a striking conservation of most residues that mutagenesis and structural analysis have shown to be essential for enzymatic activity (*19–21*) (Fig. 2A). These include residues that are part of the active site as well as residues that are important for Mg^{2+} binding, an essential requirement for the catalytic activity of this family of proteins (*21*). Further sequence comparison revealed that these residues are absolutely conserved in all CdtB proteins from other bacterial pathogens.

To confirm the putative DNase activity of CdtB, we constructed mutant derivatives of this toxin subunit carrying single amino acid substitutions in residues that have been shown to be critical for the catalytic activity of members of the type I DNase family of proteins (*22*). The residues that were mutated were equivalent to those shown in other type I DNases to be either components of the active site (i.e., His^{152}) or essential for magnesium binding (i.e., Asp^{185}) (*19–21*) (Fig.

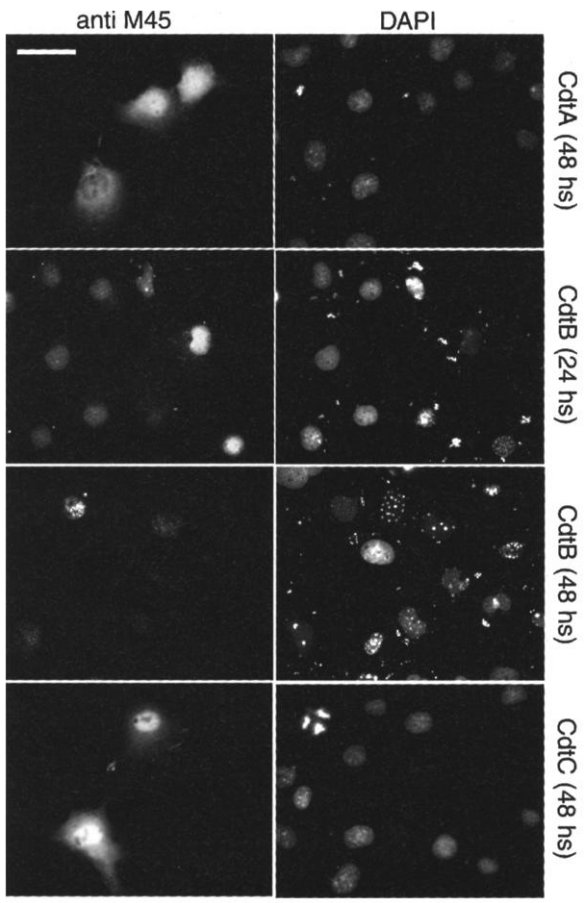

Fig. 1. Effect of the transient expression of CDT toxin components in cultured cells. COS-1 cells were transfected with vectors coding for M45 epitope–tagged *C. jejuni* CdtA, CdtB, or CdtC toxin subunits. Twenty-four or 48 hours after transfection, cells were stained with a monoclonal antibody directed to the M45 epitope tag to visualize cells expressing the individual toxin subunits and with DAPI to visualize the chromatin (*18*). Images were obtained with a Nikon Diaphot inverted fluorescence microscope fitted with a Princeton Instruments Micromax digital camera. Scale bar, 50 μm.

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2A). Plasmids encoding either epitope-tagged $CdtB^{H152Q}$ or $CdtB^{D185S}$ were transfected into COS-1 cells, and 48 hours after transfection, the cells were stained with an antibody directed to the epitope tag and with 4′,6′-diamidino-2-phenylindole (DAPI) to examine the structure of the chromatin. Cells transfected with either of these plasmids exhibited completely normal nuclear morphology despite the presence of high levels of mutant CdtB protein as judged by the bright fluorescence staining and Western blot analysis (Fig. 2B). This was in sharp contrast to cells transfected with wild-type CdtB, which showed a complete collapse of their chromatin (Fig. 2B). The mutant CdtB proteins exhibited exclusive nuclear localization, implicating the nucleus as the site of action for this toxin subunit and consistent with its potential role as a DNase (Fig. 2B). Because CdtB does not exhibit a detectable consensus nuclear localization signal (NLS), its mechanism of translocation to the nucleus may involve either an atypical NLS or a carrier protein (*23*).

To confirm the toxic activity of CdtB, we purified this protein to homogeneity and microinjected it into cells (*24*) (Fig. 3B). Microinjection into COS-1 or REF52 cells of a 1 mg/ml solution of purified CdtB resulted in marked changes in the chromatin as early as 4 hours after microinjection (Fig. 3A). In contrast, microinjection of equal amounts of $CdtB^{H152Q}$ purified in identical fashion did not cause any visible alteration to the chromatin of microinjected cells (Fig. 3A). Both wild-type CdtB and $CdtB^{H152Q}$ were exclusively localized to the nucleus (Fig. 3A).

The morphological changes induced by the overexpression of CdtB or the microinjection of a 1 mg/ml solution of purified CdtB do not completely resemble those changes induced by CDT holotoxin intoxication. We hypothesized that this difference could be the result of the vastly different intracellular levels of CdtB under these two different conditions. To test this hypothesis, we microinjected into COS-1 and REF52 cells increasingly lower amounts of purified CdtB protein and examined the effects on cellular and nuclear morphology (*25*). Microinjection of a solution containing purified CdtB (1 μg/ml) resulted in vast distention of the cell cytoplasm and severe enlargement of the nucleus 4 to 5 days after microinjection (Fig. 3B). These changes closely resemble those induced by the CDT holotoxin, indicating that, when microinjected into cells, CdtB by itself was capable of recapitulating the toxic effects observed by CDT holotoxin treatment. As expected, microinjection of similar amounts of $CdtB^{H152Q}$ did not result in any detectable cellular changes (*25*).

To investigate the importance of the DNase activity of CdtB on the toxicity of the CDT holotoxin, we constructed plasmids that encode the mutant proteins $CdtB^{H152Q}$ or $CdtB^{D185S}$ along with CdtA and CdtC in the same genetic organization as that of the wild-type locus (*26*). Extracts from *Escherichia coli* strains carrying these plasmids or the wild-type *cdtABC* locus were prepared, and their toxicity was examined by a variety of assays (*27*). The levels of the CdtB mutant proteins as well as those of the other components of the holotoxin were equivalent in all the extracts used in these studies (Fig. 4C). Extracts from *E. coli* expressing wild-type CdtB induced marked morphological changes in intoxicated cells characterized by a marked distention and enlargement of the cell cytoplasm and nucleus (Fig. 4A). In contrast, cells treated with extracts from *E. coli* expressing the catalytic ($CdtB^{H152Q}$) or the Mg^{2+}-binding ($CdtB^{D185S}$) mutants exhibited the same morphology as cells treated with extracts from *E. coli* carrying the vector alone (Fig. 4A). To examine the effect of the mutant toxins on cell cycle progression, we analyzed by flow cytometry the DNA content of cells treated with the same extracts of *E. coli* expressing wild-type or mutant forms of CdtB. Consistent with a G_2/M block, cells intoxicated with extracts from bacteria expressing wild-type holotoxin exhibited a 4N DNA content (Fig. 4B). In contrast, the flow cytometric profiles of cells infected with extracts from bacteria expressing either of the mutant forms of CdtB ($CdtB^{H152Q}$ or $CdtB^{D185S}$) were indistinguishable from the profile of untreated cells or cells treated with extracts from bacteria carrying the empty vector (Fig. 4B). Thus, CdtB DNase activity

A

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1 DHP-1    ----MGGPRALLAALWALEAAGTAALRIGAFNIQ-SFGDSKVSDPACGSIIAKILAGYDLALVCEVRDPDLSAVSALMEQIN--SVSEHE   83
2 DNase I  ---MRGMKLLGALLALAALLQGAVSLKIAAFNIQ-TFGETKMSNATLVSYIVQILSRYDIALVCEVRDSHLTAVGKLLDNLN--QDAPDT   84
3 DHP-2    -----MSRELAPLLLLLSIHSALAMRICSFNVR-SFGESKQEDKNAMDVIVKVIKRCDIILVMEIKDSNNRICPILMEKLNRNSRRGIT   84
4 DHP-3    MSLHPASPRLASLLLFILALHDTLALRLCSFNVR-SFGASKKENHEAMDIIVKIIKRCDLILLMEIKDSSNNICPLMEKLNGNSRRSTT   89
5 DNase-X  -------MHYPTALLFLILANGAQAFRICAFNAQ-RLTLAKVAREQVMDTLVRILARCDIMVLCEVVDSSGSAIPLLLRELN-RFDGSGP   81
6 CdtB     -----MKKIICLFLSFNLAFANLENFNVGTWNLQGSSAATESKWSVSVRQLVSGANPLDILMICEAGTLPRTATPTGRHVQQ---GGTPI   82

1 DHP-1    YSFVSSQPLGRDQYKEMYLFVYRKDAVSVVDTYLYPD----PEDVFSREPFVVKFSAPGTGERAPPLPSRRALTPPPLPAAAQNLVLIP  168
2 DNase I  YHYVVSEPLGRNSYKERYLFVYRPDQVSAVDSYYYDDGCEPCGNDTFNREPAIVRFFSR---------FT--EVR---------EFAIVP  154
3 DHP-2    YNYVISSRLGRNTYKEQYAFLYKEKLVSVKRSYHYHDYQDG-DADVFSREPFVVWFQSP----------H------T----AVKDFVIIP  153
4 DHP-3    YNYVISSRLGRNTYKEQYAFVYKEKLVSVKTKYHYHDYQDG-DTDVFSREPFVVWFHSP----------F------T----AVKDFVIVP  158
5 DNase-X  YSTLSSPQLGRSTYMETYVYFYRSHKTQVLSSYVYND----EDDVFARERPFVAQFSLPSN----------------------VLPSLVLVP  146
6 CdtB     DEYEWN--LGTLSRPDRVFIYYSRVDVGANRVNLATVSRMQAEEVIVLPPPTTVSRPIIG----------------------IRNGNDAFFN  150

1 DHP-1    LFAAPHQAVAEIDALYDVYLDVIDKWGTDDMLFLGDFNADCSYVRAQDWAAIRLRSSEVFKWLIPDSADTTVGN-SDCAYDRIVACGARL  257
2 DNase I  LFAAPGDAVAEIDALYDVYLDVQEKWGLEDVMLMGDFNAGCSYVRPSQWSSIRLWTSPTFQWLIPDSADTTATP-THCAYDRIVVAGMLL  243
3 DHP-2    LHTTPETSVKEIDELVEVYTDVKHRWKAENFIFMGDFNAGCSYVPKKAWKNIRLRTDPRFVWLIGDQEDTTVKKSTNCAYDRIVLRGQEI  243
4 DHP-3    LHTTPETSVKEIDELVDVYTDVRSQWKTENFIFMGDFNAGCSYVPKKAWQNIRLRTDPKFVWLIGDQEDTTVKKSTSCAYDRIVLCGQEI  248
5 DNase-X  LHTTPKAVEKELNALYDVFLEVSQHWQSKDVILLGDFNADCASLTKKRLDKLELRTEPGFHWVIADGEDTTVRASTHCTYDRVVLHGERC  236
6 CdtB     IHALANGGTDVGAIITAVDAHFANMPQVN-WMIAGDFNRDPSTITSTVDRELANRIRVVFP--------TSATQASGGTLDYAITGNSNR  230

1 DHP-1    RRSLKPQSATVHDFQEEFGLDQTQALAISDHFPVEVTLKFHR----------------------------  299
2 DNase I  RGAVVPDSALPFNFQAAYGLSDQLAQAISDHYPVEVMLK----------------------------  282
3 DHP-2    VSSVVPKSNSVFDFQKAYKLTEEEALDVSDHFPVEFKLQSSRAFTNSKKSVTLRKKTKSKRS------  305
4 DHP-3    VNSVVPRSSGVFDFQKAYDLSEEEALDVSDHFPVEFKLQSSRAFTNNRKSVSLKKRKKGNRS------  310
5 DNase-X  R--SLLHTAAAFDFPTSFQLTEEEALNISDHYPVEVELKLSQAHSVQPLSLTVLLLLSLLSPQLCPAA  302
6 CdtB     -----QQTYTPPLLAAILMLASLRSHIVSDHFPVNFRKF----------------------------  265
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B

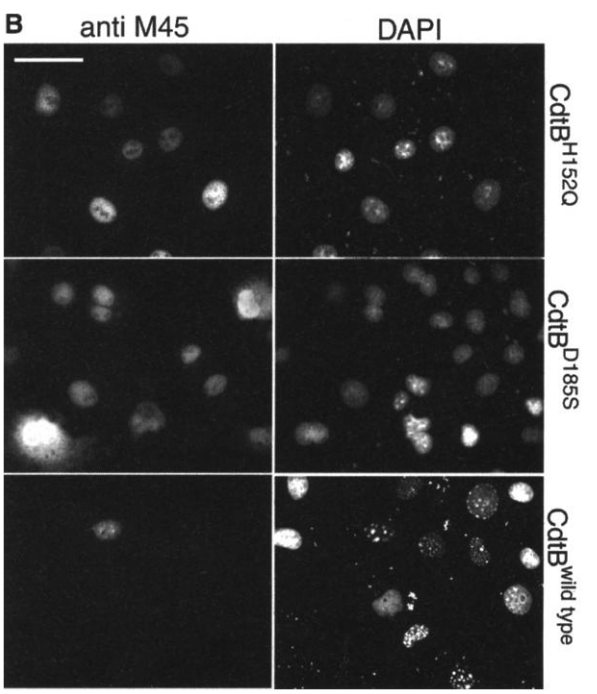


Fig. 2. (**A**) Amino acid sequence comparison of *C. jejuni* CdtB toxin subunit with members of the DNase-I family of related proteins (*33*). Residues that have been shown to be essential for catalysis are boxed. Arrows indicate the CdtB residues that were mutated for these studies. The alignment was done with the ClustalW program (*34*). The DHP-1, DHP-2, DHP-3, DNase I, and DNase X sequences correspond to human DNases. (**B**) Effect of the transient expression of CdtB mutants in cultured cells. COS-1 cells were transfected with vectors coding for different M45 epitope–tagged CdtB mutants as indicated (*18*). Forty-eight hours after transfection, cells were stained with a monoclonal antibody directed to the M45 epitope tag to visualize cells expressing the different CdtB proteins and with DAPI to visualize the chromatin. Images were obtained with a Nikon Diaphot inverted fluorescence microscope fitted with a Princeton Instruments Micromax digital camera. Scale bar, 50 μm.

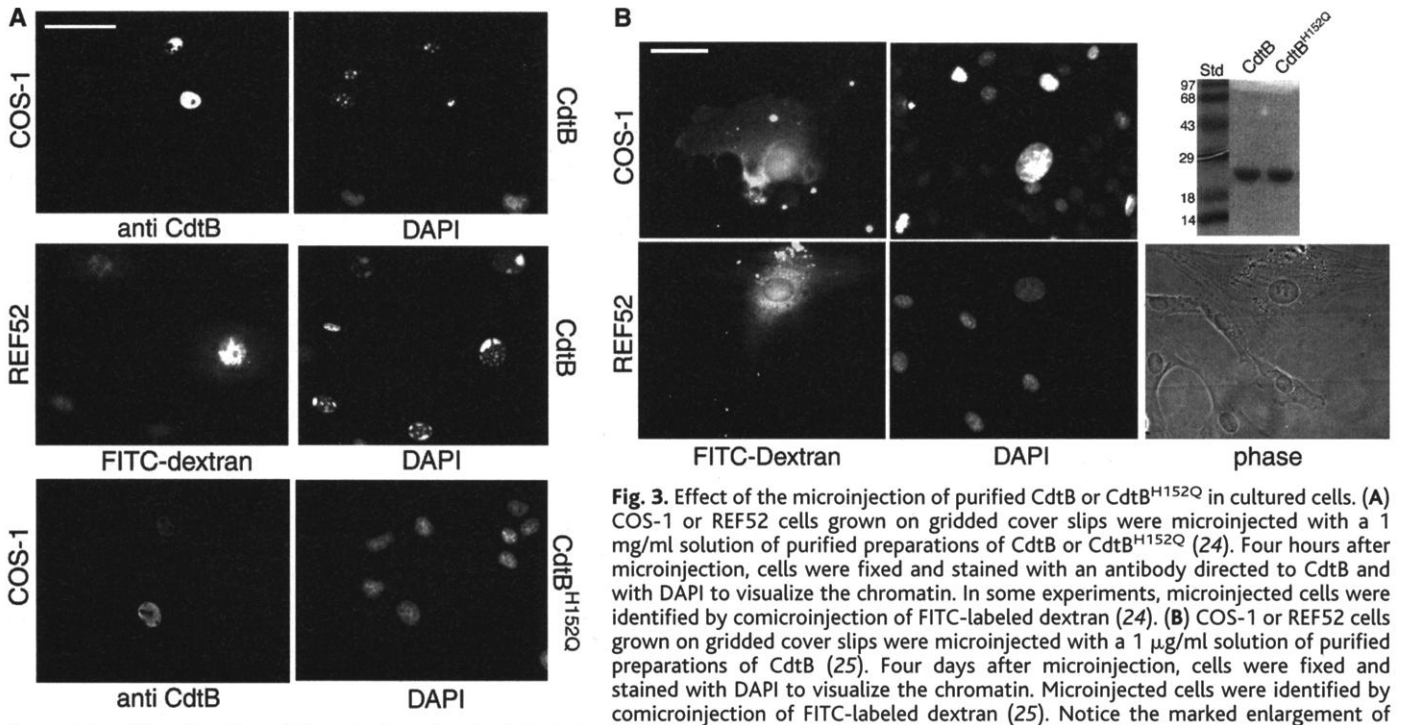
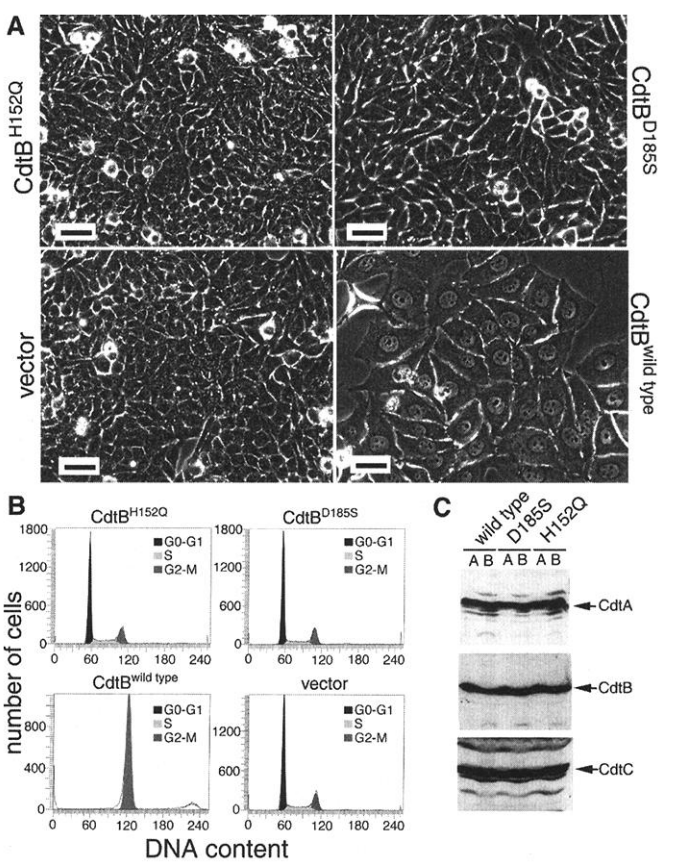


Fig. 3. Effect of the microinjection of purified CdtB or CdtB^{H152Q} in cultured cells. (**A**) COS-1 or REF52 cells grown on gridded cover slips were microinjected with a 1 mg/ml solution of purified preparations of CdtB or CdtB^{H152Q} (*24*). Four hours after microinjection, cells were fixed and stained with an antibody directed to CdtB and with DAPI to visualize the chromatin. In some experiments, microinjected cells were identified by comicroinjection of FITC-labeled dextran (*24*). (**B**) COS-1 or REF52 cells grown on gridded cover slips were microinjected with a 1 μg/ml solution of purified preparations of CdtB (*25*). Four days after microinjection, cells were fixed and stained with DAPI to visualize the chromatin. Microinjected cells were identified by comicroinjection of FITC-labeled dextran (*25*). Notice the marked enlargement of the nuclei and the distention of the cytoplasm in microinjected cells. Similar results were obtained in several repetitions of this experiment (*25*). Images were obtained with a Nikon Diaphot inverted fluorescence microscope fitted with a Princeton Instruments Micromax digital camera. Scale bar, 50 μm. A coomassie blue–stained SDS-polyacrylamide gel of the purified CdtB protein preparations used in the microinjection studies is shown.

Fig. 4. Effect of mutations in the predicted catalytic and Mg^{2+}-binding sites of CdtB on CDT toxicity. (**A**) Morphology of Henle-407 intestinal epithelial cells after treatment with CDT holotoxin containing either mutant or wild-type CdtB. Henle-407 cells were treated with different extracts of *E. coli* expressing the *C. jejuni* CDT toxin containing either wild-type CdtB or its mutant derivatives CdtB^{H152Q} or CdtB^{D185S}. As a control, cells were treated with an extract of *E. coli* carrying the empty vector (vector). Images were captured 48 hours after treatment with a Nikon Diaphot inverted microscope fitted with a Princeton Instruments Micromax digital camera. Scale bar, 50 μm. (**B**) Cell cycle progression of Henle-407 intestinal epithelial cells after treatment with CDT toxin containing different CdtB mutants. Henle-407 cells were treated in an identical manner and with the same preparations described in (A). Seventy-two hours after treatment, cells were fixed, stained, and examined for DNA content by flow cytometry (*27*). The peaks corresponding to cells in G_0-G_1, S, or G_2 are indicated. A minimum of 10,000 nuclei per sample were analyzed. (**C**) Expression of CdtA, CdtC, and wild-type CdtB or its mutant derivatives CdtB^{H152Q} or CdtB^{D185S} in the extracts used in experiments described in (A) and (B). Whole-cell lysates (labeled A) and sonicated extracts (labeled B) were separated by SDS-polyacrylamide gel electrophoresis, and the levels of the CdtA, CdtB, and CdtC proteins were evaluated by Western immunoblot with specific antibodies.



is essential for CDT toxicity.

DNA damage triggers a series of carefully controlled processes that stop cell cycle progression to ensure that cell division will not proceed to the next phase until the DNA damage has been repaired (*28*). The G_2/M cell cycle arrest induced by CDT toxins is therefore most likely the consequence of DNA damage inflicted by the DNase activity

of its CdtB subunit upon delivery into the cell. Although DNA damage can lead to cell cycle arrest in either G_1/S or G_2/M, CDT-intoxicated cells invariably arrest in G_2/M. A plausible explanation for this observation may be that CdtB DNase activity is directed to single-stranded DNA only present during the S phase of the cell cycle, which immediately precedes G_2. Alternatively, CdtB may have access to the chromatin only in G_2 or may require a cofactor for its activity that is only available in this phase of the cell cycle. Consistent with either hypothesis, purified CdtB exhibited only very poor DNase activity in vitro, which was only detectable on single-stranded DNA templates.

Bacterial infections are increasingly considered a potential predisposing factor for the development of cancer. The presence of a bacterial toxin capable of causing DNA damage in a commonly occurring intestinal pathogen such as *C. jejuni* may not only aid its pathogenicity but may constitute a predisposing factor for intestinal cancer.

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18. The genes encoding the CdtA, CdtB, and CdtC proteins without the predicted secretion signal sequence were individually amplified from *C. jejuni* strain 81-176 and cloned into the M45 epitope–tagging vector pSB616 (*29*). The M45 epitope–tagged genes were then cloned into the mammalian expression vector pSG5 (Stratagene) and expressed in COS-1 cells. All constructions were verified by nucleotide sequencing. The M45 epitope–tagged versions of the three Cdt proteins were shown to function in a manner indistinguishable from that of wild type. Transfections were carried out with 0.2 μg of plasmid DNA with the FuGENE6 (Roche) reagent following the recommendations of the manufacturer. Twenty-four or 48 hours after transfection, cells were fixed and stained with monoclonal antibody to M45 and DAPI as previously described (*30*).
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22. Site-directed mutagenesis was carried out with the QuickChange site-directed mutagenesis system (Stratagene) following the recommendations of the manufacturer.
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24. Protein fusions between glutathione *S*-transferase (GST) and the different CdtB proteins without their signal sequences were constructed by standard recombinant DNA methods. Purification of the different GST fusions and the subsequent release of the CdtB moiety by thrombin cleavage were carried out as described elsewhere (*31*). Microinjection of CdtB proteins into the cytoplasm of COS-1 and REF-52 cells grown on gridded cover slips was carried out as previously described (*32*). Microinjected cells were identified by the comicroinjection of fluorescein isothiocyanate (FITC)–labeled dextran or by staining with an antibody to CdtB.
25. Different dilutions of purified CdtB or $CdtB^{H152Q}$ were microinjected into the cytoplasm of COS-1 and REF52 cells, and the morphological changes were followed over time by standard microscopy. These preliminary experiments involved the microinjection of at least 50 cells for each protein dilution. Once the CdtB dilutions that led to cytoplasmic distention were identified, a more detailed examination of microinjected cells was carried out involving the staining of the nucleus with DAPI and subsequent examination under a fluorescence microscope. Microinjected cells were identified by comicroinjection of FITC-labeled dextran. In this case, a minimum of 300 cells in different experiments were examined. When a concentration of 1 μg/ml was used, 4 days after microinjection, ~50% of the cells exhibited cytoplasmic distention, ~20% showed no changes, and ~30% showed compromised chromatin. The difference in response is most likely due to differences in the actual amount of protein microinjected into the cells, which may vary from cell to cell.
26. A wild-type operon encoding a COOH-terminal M45 epitope–tagged CdtB was constructed by amplifying a fragment containing *cdtAB* by polymerase chain reaction (PCR) and cloning it into the epitope-tagging vector pSB616 (*29*). A DNA fragment containing *cdtC* and its ribosome-binding site was amplified by PCR and cloned into a site located immediately downstream of the epitope tag. The resulting plasmid, pSB1735, encodes an operon composed of *cdtA cdtB-M45 cdtC*. DNA fragments encoding either the epitope-tagged $CdtB^{H152Q}$ or $CdtB^{D185S}$ proteins were swapped with the wild-type copy of *cdtB-M45* present in pSB1735 with unique restriction sites. All constructs were verified by nucleotide sequence analysis.
27. Henle-407 intestinal epithelial cells were treated with filter-sterilized cell extracts from sonicated cultures of *E. coli* strains expressing the wild-type *cdtABC* locus, the mutant derivatives $cdtAB^{H152Q}C$ or $cdtAB^{D185S}C$, or extracts from *E. coli* carrying the empty vector. In all cases, expression of these genes was under the control of the strong $para_{ABC}$ promoter. For cell cycle analysis, Henle-407 intestinal epithelial cells were seeded on 60-mm tissue culture dishes and treated with the different filter-sterilized lysates of *E. coli* strains as indicated above with a dose sufficient to intoxicate 100% of the cells present on the dish. Forty-eight hours after intoxication, cells were processed for flow cytometry as follows. Cells were collected from dishes by trypsinization, and trypsin was neutralized with serum containing media. The cell suspensions were centrifuged for 5 min at 1500 rpm at room temperature, the supernatants were removed, and the cell pellets were resuspended in 0.5 ml of phosphate-buffered saline (PBS) at room temperature. The cell suspensions were slowly added to tubes containing 4 ml of cold (−20°C) 90% ethanol solution under continuous mixing. Cells were kept in fixative for at least 2 hours on ice. The fixed cells were collected by centrifugation, and the fixative was decanted thoroughly. The pellet was washed once with 5 ml of PBS, and the cell pellet was resuspended in 1 ml of a solution containing 0.1% Triton X-100, DNase-free ribonuclease A (20 mg/ml), and propidium iodide (20 μg/ml; Molecular Probes) in PBS. The stained cells were analyzed by flow cytometry with a FACStar Plus flow cytometer (Beckton Dickinson).
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33. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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35. We thank C. Peña and J. Wilson for critical reading of the manuscript and M. Blazer for *C. jejuni* strain 81-176. M.L.-T. was supported by a fellowship from "la Caixa," Barcelona, Spain.

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