Cytolethal Distending Toxin Demonstrates Genotoxic Activity in a Yeast Model

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Cytolethal distending toxins (CDTs) are multisubunit proteins produced by a variety of bacterial pathogens that cause enlargement, cell cycle arrest, and apoptosis in mammalian cells. While their function remains uncertain, recent studies suggest that they can act as intracellular DNases in mammalian cells. Here we establish a novel yeast model for understanding CDT-associated disease. Expression of the CdtB subunit in yeast causes a G2/M arrest, as seen in mammalian cells. CdtB toxicity is not circumvented in yeast genetically altered to lack DNA damage checkpoint control or that constitutively promote cell cycle progression via mutant Cdk1, because CdtB causes a permanent type of damage that results in loss of viability. Finally, we establish that CDTs are likely to be potent genotoxins, as indicated by in vivo degradation of chromosomal DNA associated with expression of CdtB—suggesting that the varied distribution of CDT in bacteria implicates many human pathogens as possessors of genotoxic activity.

Cytolethal distending toxins (CDTs) are highly related, bacterially encoded proteins associated with gastrointestinal disease (34) and, perhaps, the unrelated diseases periodontitis (46) and chancroid (7). Intoxication with CDT causes cells to exhibit nuclear and cytoplasmic enlargement accompanied by a G2 arrest associated with invocation of the DNA damage checkpoint and eventual apoptotic cell death (9, 20, 42, 50). Campylobacter jejuni, a producer of CDT, is the most common bacterial cause of food-borne infectious illness in the United States and is responsible for approximately 2 million cases of gastrointestinal illness each year (47). Infections with this organism are also an antecedent to the paralytic disorder Guillain-Barre syndrome (26; B. Speed, J. Kaldor, and P. Cavanagh, Letter, J. Infect. 885–886, 1984).

Opportunities for CDT to intoxicate humans are abundant. By association with C. jejuni alone, CDT is prevalent in the majority of uncooked store-bought chicken carcasses (18). Moreover, CDT is present in other common human pathogens, including Campylobacter coli, Campylobacter fetus (23, 37), various Escherichia coli isolates (24, 36, 41), Haemophilus ducreyi (7, 8), enterohepatic Helicobacter spp. (5, 53, 54), Actinobacillus actinomycetemcomitans (31, 43, 46), and Shigella spp. (34).

The mechanism by which CDT acts is uncertain, but recent findings have suggested that it may act as an intracellular DNase (12, 17, 28). The toxin is encoded by three conserved genes: cdtA, cdtB, and cdtC (36). These gene products exhibit weak homologies to proteins outside the CDT family. CdtA bears homology to the ricin B chain, CdtC is most similar to CdtA (C. Pickett, unpublished data), and CdtB exhibits similarity to a broad class of enzymes sharing phosphoesterase activity, including nucleases, protein phosphatases, inositol polyphosphate phosphatases, and sphingomyelinases (12). The similarity of CdtB to DNase I, in particular, has attracted attention and led to the demonstration of in vitro DNase activity by CDT, but not by CDT containing CdtB subunits mutated in residues predicted to be critical for catalysis based on the DNase I mechanism (17). Furthermore, ectopic expression in mammalian cells of the CdtB subunit alone, but not the CdtA or CdtC subunits, recapitulates the toxic effects observed when cells are treated with CDT (28). These results readily explain the CDT-induced G2 cell cycle arrest that has been noted in multiple studies (6, 8, 35, 42, 44, 46, 50) as resulting from elicitation of the cell’s DNA damage checkpoint response. Contrary to this model, however, CDT-induced DNA damage in vivo was specifically rejected as a potential mechanism by Sert et al. (42) and the rather weak in vitro CdtB DNase activity (28) seems inconsistent with the potency of CDT as a toxin. Moreover, the critical residues of CdtB predicted to be important for its putative DNase activity are similarly predicted to be important for any potential phosphoesterase activity based on the similarities noted by Dlakic (12). Any of these other potential activities could conceivably result in the activation of a G2 checkpoint response.

In an effort to clarify some of these issues and gain further understanding of how CDT causes disease, we have explored the use of a more tractable model system to study the in vivo mechanism of CDT toxicity. In this study, we show that expression of CdtB—but not CdtA, CdtC, or a CdtB mutant in a residue predicted to be essential for phosphoesterase activity—in the yeast Saccharomyces cerevisiae induces an irreversible G2 cell cycle arrest accompanied by degradation of the chromosomal DNA.

MATERIALS AND METHODS

Strains, medium, and cell culture methods. Yeast strains used in this study are outlined in Table 1. S. cerevisiae strains Y300 and Y610 were generous gifts from S. J. Elledge (Baylor University, Houston, Tex.). Y477 and Y510 were generous gifts from D. Lew (Duke University, Durham, N.C.). Where appropriate, yeast was grown in either standard YPD medium or SD medium at 30°C lacking the...
appropriate amino acid with either 2% glucose, sucrose, or galactose as a carbon source. Broth cultures were shaken at 250 rpm. Semisolid medium contained 1.5% agar (Difco, Detroit, Mich.).

**Plasmids.** The following plasmids were used: pTrc18CDT containing the wild-type cdtA, cdtB, and cdtC genes isolated from C. jejuni 81–176 (50); and pDCH-CdtA, pDCH-CdtB, and pDCH-CdtC, pDCH-CdtBΔD222A, which are yeast expression plasmids that express CdtA, CdtB, and CdtC, respectively. The yeast plasmids in this study are based on the low-copy-number-based yeast expression vector, pMDM00333, which contains a point mutation changing Asp-222 to Ala-222 by the ExSite protocol. 

**PCR isolation of cdt genes.** Using pTrc18CDT as a template, the three CDT subunits were individually PCR amplified with Vent DNA polymerase as per the manufacturer’s recommendations (New England Biolabs, Beverly, Mass.) with the mutagenic oligonucleotides 5’-CGCGTCTAGAAGCTTACATGAAATGTAAATGTTGGCGTGTTACCG-3’ and 5’-GGCGGTCGACATCTAGAGTTTGCGTTAG-3’, which was confirmed by sequence analysis.

**RT-PCR of yeast transcripts.** Using pTrc18CDT as a template, the three CDT subunits were individually PCR amplified with Vent DNA polymerase as per the manufacturer’s recommendations (New England Biolabs, Beverly, Mass.) with the mutagenic oligonucleotides 5’-CGCGTCTAGAAGCTTACATGAAATGTAAATGTTGGCGTGTTACCG-3’ and 5’-GGCGGTCGACATCTAGAGTTTGCGTTAG-3’, which was confirmed by sequence analysis.

**TABLE 1. Strains and plasmids used in this study**

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<th>Strain</th>
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**RESULTS**

Expression of CdtB unaccompanied by other CDT subunits is toxic in *S. cerevisiae*. We evaluated whether cdtA, cdtB, or cdtC possesses a toxic activity by ectopically expressing each CDT subunit under the control of the GAL1 promoter in the *S. cerevisiae* EY957 genetic background (Table 1). All strains grew normally on glucose when compared to the vector control strain (Fig. 1A). However, when expression of the CDT subunits was induced by restreaking colonies onto medium containing galactose, only CdtA, CdtC, and the vector control grew, requiring 2 to 3 days at 30°C to obtain colonies that were clearly visible. In contrast, growth was not detected when yeast expressed CdtB, even when plates were incubated for 5 days. To confirm the results of the subunits we expressed, we performed Western blots with protein derived from yeast grown in broth culture in the presence of galactose. Each of these subunit proteins was detected under the inducing conditions (not shown).

Mutations in phosphate-esterase-like residues of CdtB result in toxicity. To ascertain whether it was a possible phosphate-esterase-like activity that was responsible for the CdtB toxicity in yeast cells, we constructed a series of point mutations in residues of CdtB expected to be critical for its putative catalytic activity (12). Among these mutants was an aspartate-to-alanine mutation in position 222 of wild-type CdtB (CdtBΔD222A). CDT holotoxin in which CdtB was replaced with
the CdtBD222A allele was ineffective at intoxicating HeLa cells, while wild-type CDT caused intoxication (Pickett, unpublished). To determine whether CdtBD222A would also fail to intoxicate yeast, we expressed CdtBD222A in *S. cerevisiae* EY957 under control of the GAL1 promoter as before. Expression of CdtBD222A did not inhibit the growth of yeast on galactose-containing agar plates in contrast to control yeast that expressed wild-type CdtB (Fig. 1A). The presence of CdtBD222A in yeast growing in galactose-containing medium was confirmed by Western blot analysis (not shown). These results indicated that the toxicity was likely to be due to a specific enzymatic activity of CdtB and not just generally to its expression in a heterologous organism.

**CdtB expression is associated with an irreversible loss of viability.** Glucose is a potent inhibitor of GAL1-controlled expression (19). To determine whether CdtBD222A would also fail to intoxicate yeast, we expressed CdtB^{D222A} in *S. cerevisiae* EY957 under control of the GAL1 promoter as before. Expression of CdtB^{D222A} did not inhibit the growth of yeast on galactose-containing agar plates in contrast to control yeast that expressed wild-type CdtB (Fig. 1A). The presence of CdtB^{D222A} in yeast growing in galactose-containing medium was confirmed by Western blot analysis (not shown). These results indicated that the toxicity was likely to be due to a specific enzymatic activity of CdtB and not just generally to its expression in a heterologous organism.

**Expression of CdtB results in an accumulation of G2-arrested cells.** More detailed flow cytometric analysis was conducted to determine the DNA content of cells expressing a CDT subunit relative to vector control. Yeast cells were sampled at 0, 5, 7, 10, and 24 h postinduction to establish whether subunit-expressing yeast demonstrated an aberrant cell cycle.
distribution. At 7 h postinduction, we observed CdtB-expressing cells noticeably accumulating in G2 phase, while CdtBD222A-expressing yeast did not. At 10 h postinduction, this effect was even more prominent. The distribution of DNA content in cells expressing CdtA or CdtC (not shown) was similar to that of vector control cells at 10 h (Fig. 3A), suggesting that neither CdtA nor CdtC was able to affect cell cycle progression. These findings suggested that cells were arresting primarily at G2/M, as was seen previously in mammalian cells intoxicated with CDT (8, 10, 35, 42, 44, 46, 50, 52), and that this activity could be attributed directly to CdtB in an Asp-222-dependent fashion. This result provided an initial validation of the relevance of our yeast model to CDT-related disease, and we pursued it further.

The nuclear morphology of the arrested cells was examined by Hoechst 33342 staining (Fig. 3B). As expected for a G2/M arrest, the moderately sized cells possessing large buds accumulated with a single nucleus located at the mother-daughter neck. The larger cells, which had presumably sat at the arrest point for an extended period of time, often appeared to have an unequally fragmented nucleus, as if the cells had attempted to segregate chromosomes that were not fully attached to the spindle or that were unable to separate sister chromatids. This phenomenon in which cells bypass a checkpoint after extended arrest and which results in missegregation of chromosomal DNA has been observed previously (40, 48).

CdtB does not act directly at the level of Cdk1 in yeast. In animal cells, DNA damage results in the inhibitory phosphorylation of Cdk1 on Tyr-15 through the control of a network of protein kinases and phosphatases. Despite the existence of a
similar network of regulators controlling Cdk1 phosphorylation in *S. cerevisiae*, however, DNA damage does not result in inhibitory Cdk1 phosphorylation. These regulators are instead ultimately responsive to disruptions leading to loss of cell polarity, and the DNA damage cell cycle arrest response is accomplished by alternative means (11, 29, 32, 33, 49).

We took advantage of this divergence to determine whether CdtB acted on the pathway near Cdk1, possibly as a protein phosphatase, by expressing CdtB in yeast in which wild-type Cdk1 was replaced with the Cdk1-AF allele, which lacks both sites needed for inhibitory phosphorylation. If phosphorylation of Cdk1 were critical for CDT toxicity, this mutant would not become intoxicated when CdtB is expressed. If it acted through the DNA damage pathway, we expected this mutant to remain sensitive to CdtB. We found that expression of CdtB resulted in toxicity when expressed in yeast containing the Cdk1-AF allele as well as a syngenic control strain. This indicated that CdtB did not act upon Cdk1 or upon the proximal regulators in the pathway leading to Cdk1 inhibitory phosphorylation.

*mec1* mutants of *S. cerevisiae* are susceptible to intoxication with CdtB. The Mecl protein of *S. cerevisiae* is required for both the S-phase and G2 checkpoints responsive to DNA damage and unreplicated DNA (49). Mecl is a homologue of the mammalian ATM protein, which performs a similar function in mammalian cells (33). The G2/M accumulation of cells associated with CDT intoxication is abolished by caffeine (9, 42), an ATM inhibitor (4), and results from an ATM-dependent checkpoint (9). To determine whether the yeast arrest resulted from the activation of the corresponding checkpoint, CdtB was expressed in both a *mec1* null strain and its syngenic counterpart. We then analyzed these CdtB-expressing strains as before alongside vector controls. If CdtB were causing physical damage to *S. cerevisiae*, we expected that the *mec1* null yeast would still lose viability after expressing CdtB, but would not arrest at the G2 checkpoint. Alternatively, if CdtB were merely causing the perception of damage by manipulating the DNA damage sensory machinery at the level of Mec1 or upstream, the CdtB-expressing yeast should survive. All CdtB-expressing strains, including the *mec1* strain, failed to grow in galactose-containing media, suggesting physical damage and not manipulation of proteins involved in the checkpoint. Flow cytometric analysis of cellular DNA content indicated that cells continued to arrest with G2/M DNA content despite the absence of the G2 checkpoint (Fig. 3C). It is possible that the G2 arrest in the *mec1* background is due to the activation of an independent checkpoint, perhaps that involving spindle attachment to the kinetochore—a possibility currently undergoing investigation.

**RNR2 upregulation is associated with CdtB expression.** In light of the Asp-222-dependent toxicity of CdtB, we revisited the possibility of a CdtB-associated DNA damage response by examining RNR2 transcription. Upregulation of RNR2 occurs when yeast cells are exposed to DNA-damaging agents such as UV radiation or hydrogen peroxide (3, 14–16). Specific reporter systems have been described in yeast to identify genotoxins based on this upregulation (1, 2). We used RT-PCR to evaluate RNR2 expression semiquantitatively in yeast expressing either CdtB or CdtB*D222A* alongside positive and negative controls. CdtB expression resulted in an Asp-222-dependent increase in RNR2 transcription, which was also observed with hydrogen peroxide treatment, but absent in the vector control (Fig. 4). The RNR2 band is evident as early as 21 cycles of PCR in both CdtB-expressing cells and hydrogen peroxide-treated cells. Transcription of another gene unrelated to the DNA damage checkpoint, *GPD1*, was similar in both vector control yeast and CdtB*D222A*-expressing yeast. *GPD1* transcription was notably reduced by treatment with hydrogen peroxide and modestly reduced during expression of CdtB—possibly the result of nonspecific toxicity or stress associated with these treatments. The augmented RNR2 transcription suggested that CdtB possesses a genotoxic activity, consistent with its putative role as a DNase.

CdtB, but not CdtB*D222A*, causes degradation of chromosomal DNA in vivo. These results encouraged us to look at the integrity of yeast chromosomes by using PFGE to ascertain whether potential DNA damage resulting from CdtB expression could be directly observed. Chromosomes isolated from yeast expressing CdtB were compared to chromosomes from yeast expressing CdtA, CdtC, CdtB*D222A*, or the vector control at 0, 6, or 10 h postinduction. We found that chromosomes from all yeast obtained at time 0 were entirely intact relative to CdtB*D222A* expression (not shown). However, by 10 h postinduction, CdtB-associated

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**FIG. 4.** Heightened expression of RNR2 in CdtB-expressing yeast and hydrogen peroxide-treated yeast relative to CdtB*D222A* and vector controls. (A) The bar chart compares net intensities of bands shown on the agarose gel and is representative of three experiments (B) at 21, 25, and 32 PCR cycles. CdtB-expressing yeast cells are represented by solid bars (CdtB), CdtB*D222A*-expressing yeast cells are represented by hatched bars (D222A), vector control cells are represented by dotted bars (vector), and hydrogen peroxide-treated yeast cells are represented by open bars (H2O2). (B) The agarose gel shows bands arising from RT-PCR of RNA samples using either *GPD1*-specific or RNR2-specific primers. Intensity of RNR2 bands increases relative to controls in both CdtB-expressing and H2O2-treated yeast, while the intensity of *GPD1* bands is similar in all conditions except H2O2 treatment. CdtB-expressing yeast (CdtB), CdtB*D222A*-expressing yeast (D222A), vector control yeast (vector), and peroxide-treated yeast (H2O2).
with expression of CdtB. The white arrow indicates the position of an RNA product associated with the vector control strain (DCH6045; lanes 5 and 10) are compared.

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The action of the C. jejuni Cdt subunits. The action of CDT on mammalian cells has been shown to cause G2 arrest, likely due to invoking of the DNA damage-incomplete replication check points. Expression of CdtB alone was sufficient to cause a G2 block in S. cerevisiae, and use of S. cerevisiae mutants allowed us to clearly show that CdtB does not directly act on cell cycle machinery, but instead appears to involve direct damage and subsequent activation of the checkpoint pathway. In addition to use of the mutants for examining possible invocation of the checkpoint pathway, we showed that activation of RNR2, a gene known to be upregulated in response to direct DNA damage, occurred as early as 4 h after induction of CdtB expression. At 4 h, this observation occurred several hours before loss of chromosomal integrity could be detected by PFGE and before cells accumulated in G2/M. There is current evidence supporting this hypothesis: an unspecified amount of purified CdtB demonstrates DNase activity (17), albeit weak (28), in vitro, and when CdtB is microinjected into mammalian cells, these cells become distended at low doses, while chromatin collapse visualized by nuclear staining is observed at relatively higher doses (28). The results reported here add further support to the idea that CDT is indeed a nuclease and to the finding that CDT invokes the DNA damage checkpoint reported previously by Cortes-Bratti et al. (9) and Sert et al. (43). Finally, since yeast have relatively small and separable chromosomes compared to mammalian cells, we were able to use PFGE to analyze whether CdtB caused noticeable DNA damage in our system. This is the first time this type of analysis has been done with CDT-affected cells and provides visual confirmation of the action of CdtB in vivo in a way not previously demonstrated. Thus, this study indicates the likelihood that CdtB does indeed have DNase I-like activity.

FIG. 5. Chromosome degradation is dependent on a DNase-related residue of CdtB. Yeast chromosomes, ranging in size from 220 to 1,600 kb, are resolved by PFGE. Chromosomes isolated at time 0 (lanes 1 to 5) are compared to chromosomes isolated after 10 h of growth in galactose-containing medium (lanes 6 to 10). The CdtA-expressing strain (DCH6041; lanes 1 and 6), CdtB-expressing strain (DCH6042; lanes 2 and 7), CdtC-expressing strain (DCH6043; lanes 3 and 8), CdtB<sup>D222A</sup>-expressing strain (DCH6044; lanes 4 and 9), and the vector control strain (DCH6045; lanes 5 and 10) are compared. The white arrow indicates the position of an RNA product associated with expression of CdtB.

degradation was very apparent and consisted of a smear of high-molecular-weight DNA (Fig. 5, lane 7). CdtB expression was also accompanied by production of a relatively low-molecular-weight RNA fragment (Fig. 5, lane 7, white arrow) as determined by sensitivity to RNase A, presumably arising from continued biosynthesis in the absence of cell division. We are further characterizing this observation. In contrast, yeast expressing CdtA, CdtC, or CdtB<sup>D222A</sup> did not undergo detectable damage (Fig. 5, lanes 6 and 8 to 10). Thus, the degradation was only associated with CdtB and depended absolutely on the DNase-related residue, Asp-222.

DISCUSSION

We have shown here that a yeast model system can be used to analyze the action of the C. jejuni Cdt subunits. The action of CDT on mammalian cells has been shown to cause G2 arrest, likely due to invoking of the DNA damage-incomplete replication check points. Expression of CdtB alone was sufficient to cause a G2 block in S. cerevisiae, and use of S. cerevisiae mutants allowed us to clearly show that CdtB does not directly act on cell cycle machinery, but instead appears to involve direct damage and subsequent activation of the checkpoint pathway. In addition to use of the mutants for examining possible invocation of the checkpoint pathway, we showed that our findings contradict a report by Sert et al. (42) in which it was suggested that cells intoxicated with CDT did not undergo DNA damage. Possibly, the extent of DNA damage associated with closer-to-physiological CDT doses is not detectable in the assays used by those authors. In our study, amounts of CdtB per cell (and per unit genome size) are likely to be considerably higher than during intoxication of mammalian cells with the CDT holotoxin. Consequently, the extent of DNA degradation is likely enhanced in our system, making the biological role of CdtB more readily apparent in vivo. This would appear to be another advantage of using this novel yeast model system for the study of a bacterial protein that brings about a cell cycle block apparently through very modest DNA damage. We consider it possible that CdtB preferentially targets a particular DNA site or structure, the damage to which cannot be detected by standard assays at lower doses of CdtB.
The ability of CdtB to recapitulate in yeast the major effects observed with CDT-treated mammalian cells (38) suggests that yeast can serve as an excellent model organism that can be exploited to further understand how CDTs cause disease in vivo. Moreover, the CdtB-expressing yeast developed in this work have potential application as a drug screening system in which CDT-inhibiting compounds are identified by selection for yeast that can grow during expression of CdtB.

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