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A Novel Mode of Action for a Microbial-Derived Immunotoxin: The Cytolethal Distending Toxin Subunit B Exhibits Phosphatidylinositol 3,4,5-Triphosphate Phosphatase Activity

Bruce J. Shenker
University of Pennsylvania

Mensur Dlakic´,

Lisa Walker

Dave Besack
University of Pennsylvania

Eileen Jaffe

See next page for additional authors

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Abstract

The Actinobacillus actinomycetemcomitans cytolethal distending toxin (Cdt) is a potent immunotoxin that induces G2 arrest in human lymphocytes. We now show that the CdtB subunit exhibits phosphatidylinositol (PI)-3,4,5-triphosphate phosphatase activity. Breakdown product analysis indicates that CdtB hydrolyzes PI-3,4,5-P3 to PI-3,4-P2 and therefore functions in a manner similar to phosphatidylinositol 5-phosphatases. Conserved amino acids critical to catalysis in this family of enzymes were mutated in the cdtB gene. The mutant proteins exhibit reduced phosphatase activity along with decreased ability to induce G2 arrest. Consistent with this activity, Cdt induces time-dependent reduction of PI-3,4,5-P3 in Jurkat cells. Lymphoid cells with defects in SHIP1 and/or ptase and tensin homolog deleted on chromosome 10 (PTEN) (such as Jurkat, CEM, Molt) and, concomitantly, elevated PI-3,4,5-P3 levels were more sensitive to the toxin than HUT78 cells which contain functional levels of both enzymes and low levels of PI-3,4,5-P3. Finally, reduction of Jurkat cell PI-3,4,5-P3 synthesis using the PI3K inhibitors, wortmannin and LY290004, protects cells from toxin-induced cell cycle arrest. Collectively, these studies show that the CdtB not only exhibits PI-3,4,5-P3 phosphatase activity, but also that toxicity in lymphocytes is related to this activity. Copyright © 2007 by The American Association of Immunologists, Inc.

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Author(s)

Bruce J. Shenker; Mensur Dlakic´„; Lisa Walker; Dave Besack; Eileen Jaffe; and Ed LaBelle

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A Novel Mode of Action for a Microbial-Derived Immunotoxin: The Cytolethal Distending Toxin Subunit B Exhibits Phosphatidylinositol 3,4,5-Triphosphate Phosphatase Activity[1]

Bruce J. Shenker,[2]* Mensur Dlakić,[‡] Lisa P. Walker,* Dave Besack,* Eileen Jaffe,[¶] Ed LaBelle,[§] and Kathleen Boesze-Battaglia[†]

The *Actinobacillus actinomycetemcomitans* cytolethal distending toxin (Cdt) is a potent immunotoxin that induces G_2 arrest in human lymphocytes. We now show that the CdtB subunit exhibits phosphatidylinositol (PI)-3,4,5-triphosphate phosphatase activity. Breakdown product analysis indicates that CdtB hydrolyzes PI-3,4,5-P_3 to PI-3,4-P_2 and therefore functions in a manner similar to phosphatidylinositol 5-phosphatases. Conserved amino acids critical to catalysis in this family of enzymes were mutated in the *cdtB* gene. The mutant proteins exhibit reduced phosphatase activity along with decreased ability to induce G_2 arrest. Consistent with this activity, Cdt induces time-dependent reduction of PI-3,4,5-P_3 in Jurkat cells. Lymphoid cells with defects in SHIP1 and/or ptase and tensin homolog deleted on chromosome 10 (PTEN) (such as Jurkat, CEM, Molt) and, concomitantly, elevated PI-3,4,5-P_3 levels were more sensitive to the toxin than HUT78 cells which contain functional levels of both enzymes and low levels of PI-3,4,5-P_3. Finally, reduction of Jurkat cell PI-3,4,5-P_3 synthesis using the PI3K inhibitors, wortmannin and LY290004, protects cells from toxin-induced cell cycle arrest. Collectively, these studies show that the CdtB not only exhibits PI-3,4,5-P_3 phosphatase activity, but also that toxicity in lymphocytes is related to this activity. ***The Journal of Immunology,*** **2007, 178: 5099–5108.**

The cytolethal distending toxins (Cdts)[3] are a family of heat-labile protein cytotoxins produced by several different bacterial species including diarrheal disease-causing enteropathogens such as some *Escherichia coli* isolates, *Campylobacter jejuni*, *Shigella* species, *Haemophilus ducreyi*, and *Actinobacillus actinomycetemcomitans* (1–7). There is clear evidence that Cdts are encoded by three genes, designated *cdtA*, *cdtB*, and *cdtC*, which are arranged as an apparent operon (7–12). These three genes specify three polypeptides designated CdtA, CdtB, and CdtC with apparent molecular masses of 28, 32, and 20 kDa, respectively, that form a heterotrimeric holotoxin. Several cell lines and cell types have been shown to be sensitive to Cdt; these include human lymphoid cells, fibroblasts, human embryonic intestinal epithelial cells, a human colon carcinoma cell line, and human keratinocytes, among others (7, 11, 12). In response to Cdt, most of these cells exhibit G_2 arrest, cellular distension, and eventually cell death. However, the effects of Cdt on lymphocytes are different; Cdt-treated lymphocytes do not exhibit cellular distension and are nearly five orders of magnitude more sensitive to Cdt (10–50 pg/ml) relative to most other cells (1–5 μg/ml). For this reason, we believe that lymphocytes are a likely in vivo target of Cdt and propose that Cdt represents a novel immunotoxin.

The underlying basis for heightened lymphocyte sensitivity to Cdt remains unclear. However, there is considerable agreement that regardless of the microbial source of Cdt, the heterotrimeric holotoxin functions as an AB_2 toxin where CdtB is the active (A) unit and the complex of CdtA and CdtC comprise the binding (B) unit (13–15). In this regard, we have shown that CdtA and CdtC are required for the toxin to associate with lipid microdomains within lymphocyte membranes and that Cdt-mediated toxicity is dependent upon the integrity of these lipid domains (16). We have also demonstrated that CdtB is capable of inducing G_2 arrest and eventually apoptotic death in both mitogen-activated human T cells and T cell leukemia cell lines such as Jurkat (8, 9). There is compelling evidence that CdtB must be internalized to induce cell cycle arrest (17–19). Several investigators have also suggested that CdtB functions as a DNase-like moiety whereby it cleaves DNA and activates the G_2 cell cycle checkpoint (18, 20, 21). This mechanism of action, however, does not account for the huge difference in lymphocyte sensitivity to the toxin. Although it has been shown that Cdt-treated cells exhibit DNA degradation, we have shown that Cdt-induced DNA fragmentation in lymphocytes is not the result of direct effects of the toxin, but rather the irreversible effects of cell cycle arrest leading to activation of the apoptotic cascade (22).

The bias in recent literature toward rationalizing CdtB function is mostly based on its homology with DNase. This bias has obscured the fact that its protein fold, and most likely the reaction mechanism, are

*Department of Pathology and [†]Department of Biochemistry, University of Pennsylvania School of Dental Medicine, Philadelphia, PA 19104; [‡]Department of Microbiology, Montana State University, Bozeman, MT 59717; [§]University of Pennsylvania School of Veterinary Medicine, Philadelphia, PA 19104; and [¶]Fox Chase Cancer Center, Philadelphia, PA 19111

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[2] Address correspondence and reprint requests to Dr. Bruce J. Shenker, Department of Pathology, University of Pennsylvania School of Dental Medicine, 240 South 40th Street, Philadelphia, PA 19104-6030. E-mail address: shenker@pobox.upenn.edu

[3] Abbreviations used in this paper: Cdt, cytolethal distending toxin; PI, phosphatidylinositol; RT, room temperature; PTEN, ptase and tensin homolog deleted on chromosome 10.

Table I. *CdtB mutant constructs*

Plasmid	Primer	Sequence[a]
pGEMCdtB^{H160Q}	P1	GTATTTTTTACAGTGCA**G**GCTTTGGCCACA
	P2	TGTGGCCAAAGC**C**TGCACTGTAAAAAATAC
pGEMCdtB^{H274Q}	P1	CAAATTACATCCGATCA**G**TTTCCTGTTAGTTTTGT
	P2	ACAAAACTAACAGGAAA**C**TGATCGGATGTAATTTG
pGEMCdtB^{R117A}	P1	GATGTTGGGGCAAAC**GC**AGTGAACTTAGCTATCG
	P2	CGATAGCTAAGTTCACT**GC**GTTTGCCCCAACATC
pGEMCdtB^{D199S}	P1	GATGGTTGTTGGT**AG**TTTCAATCGTGCGCCGGT
	P2	ACCGGCGCACGATTGAAA**CT**ACCAACAACCATC

[a] Bold letters in the sequence indicate nucleotide substitutions.

also shared with many proteins found in a family of functionally unrelated signaling metalloenzymes that includes phosphatidylinositol (PI)-5-phosphatases (23). In this study we considered other possible modes of action for CdtB and explored the possibility that CdtB might function as a phosphatase (24). Indeed, we now report that CdtB exhibits PI-3,4,5-triphosphate (PI-3,4,5-P_3) phosphatase activity similar to that of the tumor suppressor phosphatases, ptase and tensin homolog deleted on chromosome 10 (PTEN) and SHIP1 (25, 26). Mutation analysis indicates that Cdt toxicity correlates with phosphatase activity; furthermore, lymphocytes treated with toxin exhibit reduced PI-3,4,5-P_3 levels. Finally, lymphocyte sensitivity to Cdt-induced G_2 arrest correlates with intracellular levels of PI-3,4,5-P_3.

Materials and Methods

Cell lines and analysis of cell cycle

The human leukemic T cell lines Jurkat, CEM, and Molt were maintained in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin. HUT78 cells were maintained in IMDM containing 4 mM L-glutamine and 20% FCS. Cells were harvested in mid-log growth phase and plated at 5×10^5 cells/ml, or as indicated, in 24-well tissue culture plates. The cells were exposed to medium, Cdt peptides, or CdtABC and incubated for 18 h. To measure Cdt-induced cell cycle arrest, Jurkat cells were washed and fixed for 60 min with cold 80% ethanol (27). After washing, the cells were stained with 10 μg/ml propidium iodide containing 1 mg/ml RNase (Sigma-Aldrich) for 30 min. Samples were analyzed on a BD Biosciences FACStar^{PLUS} flow cytometer (BD Biosciences). Propidium iodide fluorescence was excited by an argon laser operating at 488 nm and fluorescence measured with a 630/22-nm bandpass filter using linear amplification. A minimum of 15,000 events were collected on each sample; cell cycle analysis was performed using Modfit (Verity Software House).

Construction and expression of CdtB mutants

Amino acid substitutions were introduced into the *cdtB* gene by in vitro site-directed mutagenesis using oligonucleotide primer pairs containing appropriate base changes (Table I). Site-directed mutagenesis was performed using the QuikChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer's directions. Amplification of the mutant plasmid was conducted using PfuUltra HF DNA polymerase (Stratagene) and pGEMCdtB as a template; construction and characterization of this plasmid was previously described (27). All mutants were verified by DNA sequencing. Expression of the plasmids and purification of the mutant peptides is described below.

Expression and purification of Cdt peptides and Cdt holotoxin (CdtABC)

Construction and expression of the plasmid containing wild-type *cdtB* gene (pGEMCdtB) was previously described (27). In vitro expression of Cdt peptides and CdtB mutants was performed as previously described using the Rapid Translation System (RTS 500 ProteoMaster; Roche Applied Science). Reactions were run according to the manufacturer's specification (Roche Applied Science) using 10–15 μg of template DNA. After 20 h at 30°C, the reaction mix was removed and the expressed Cdt peptides were purified by nickel affinity chromatography as described (27).

Construction and expression of the plasmid containing the *cdt* genes for the holotoxin (pUCAacdtABC^{his}) has previously been reported (28). The plasmid was constructed so that the *cdt* genes were under control of the *lac* promotor and transformed into *E. coli* DH5α. Cultures of transformed *E. coli* were grown in 1 L Luria-Bertani broth and induced with 0.1 mM IPTG for 2 h; bacterial cells were harvested, washed, and resuspended in 50 mM Tris (pH 8.0). The cells were frozen overnight, thawed, and sonicated. The histidine-tagged peptide holotoxin was isolated by nickel affinity chromatography as previously described (9).

FIGURE 1. Structural alignment. Structural alignment of CdtB, inositol polyphosphate 5-phosphatase (IP5P), and DNase I was obtained by MUSTANG and slightly modified after visual inspection of superimposed structures. Protein Data Bank codes of corresponding structures are shown in parentheses next to protein names. The consensus line indicates the conservation of small (s), aliphatic (l), hydrophobic (h), charged (c), positive (+), and negative (−) residues, whereas identical residues are shown as capital letters. Numbers in parentheses within the alignment indicate the residues that were omitted either because of long insertions not shared by all proteins or for the lack of reliable alignment between the three structures. Amino acid residues mutated in this study are marked by red triangles. The remaining residues that are important for catalysis or Mg^{2+} coordination are identified by blue diamonds.

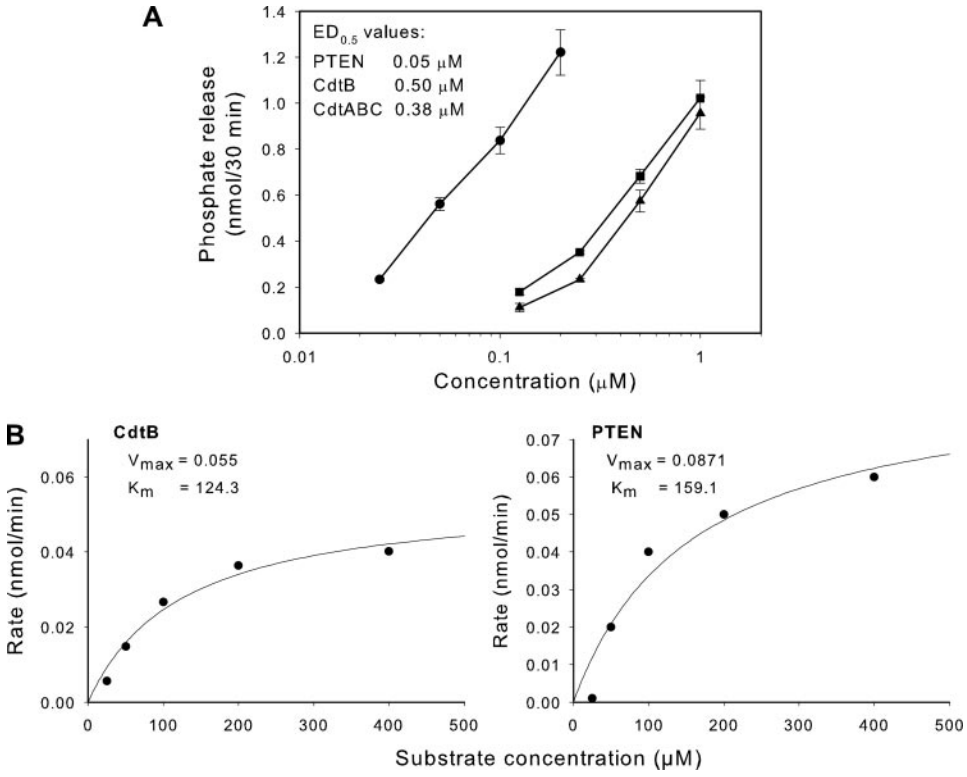

FIGURE 2. CdtB exhibits PI-3,4,5-P_3 phosphatase activity. As shown in *A*, varying amounts of CdtB (▲), CdtABC (■), and PTEN (●) were assessed for their ability to hydrolyze PI(3,4,5)-P_3 as described in *Materials and Methods*. The amount of phosphate release was measured using a malachite green-binding assay. Data are plotted as phosphate release (nM/30 min; mean ± SD) vs protein concentration; numbers shown are $ED_{0.5}$ values defined as the concentration required to induce ~50% (0.5 nM) phosphate release under these assay conditions. *B*, The rate of CdtB (0.5 μM) and PTEN (0.1 μM) mediated phosphate release in the presence of varying concentrations of PI-3,4,5-P_3 was assessed. Data were analyzed using Michaelis-Menten kinetics. The K_m values for CdtB and PTEN were 124.3 and 159.1 μM, respectively; V_{max} were 0.55 nM/min (CdtB) and 0.087 nM/min (PTEN).

Phosphatase assay

Phosphatase activity was assessed by monitoring the dephosphorylation of PI-3,4,5-P_3 as described by Maehama et al. (29). Briefly, the reaction mixture (20 μl) consisted of 100 mM Tris-HCl (pH 8.0), 10 mM DTT, 0.5 mM diC16-phosphatidylserine (Avanti), 25 μM PI-3,4,5-P_3 (diC16; Echelon), and the indicated amount of CdtB, CdtABC, or PTEN (provided by G. Taylor, University of Nebraska Medical Center, Omaha, NE). Appropriate amounts of lipid solutions were deposited in 1.5-ml tubes, organic solvent was removed, the buffer was added, and a lipid suspension was formed by sonication. For experiments to determine substrate specificity, PI-3,4,5-P_3 was replaced by the indicated phosphatidylinositol phosphate (Echelon). Phosphatase assays were conducted at 37°C for 30 min; the reactions were terminated by the addition of 15 μl of 100 mM *N*-ethylmaleimide. Inorganic phosphate levels were then measured using a malachite green assay. Malachite green solution (Biomol Green; Biomol) was added to 100 μl of the enzyme reaction mixture and color was developed for 20 min at room temperature (RT). Absorbance at 650 nm was measured and phosphate release was quantified by comparison to inorganic phosphate standards.

Analysis of CdtB-mediated product formation

Inositol lipids were separated by thin layer chromatography. Silica gel G plates were dipped in boric acid (5% in methanol) and then dried. They were then spotted with the lipid samples dissolved in chloroform/methanol/water (1/2/0.1) together with solutions containing the standard inositol lipids phosphatidyl 4,5 bisphosphate, phosphatidyl 3,4 bisphosphate, phosphatidyl 3,5 bis phosphate, phosphatidyl 3,4,5 triphosphate, and PI. The plates were eluted with solutions containing propyl acetate/isopropanol/ethanol/ammonia/water (15/45/16/9/35), dried, and sprayed with solution containing $CuSO_4/H_3PO_4$ (8%/10%), and air dried again. Plates were then heated to 70°C then to 155°C to visualize the lipids.

DNase assay

CdtB peptides were assessed for DNase activity by monitoring changes in electrophoretic mobility of supercoiled plasmid DNA as described by Elwell and Dreyfus (20). Briefly, supercoiled pUC19 (1 μg/reaction) was incubated with CdtB, CdtB mutants, or bovine DNase I for 2 h at 37°C in a buffer containing 25 mM HEPES (pH 7.0), 10 mM $MgCl_2$, and 5 mM $CaCl_2$. The reaction was stopped by adding 10 mM EDTA (final concentration). The samples were then loaded onto 1% agarose gel and subjected to electrophoresis in Tris-Borate-EDTA buffer; gels were stained with ethidium bromide and analyzed by digital scanning densitometry.

Measurement of cellular PI-3,4,5-P_3 content

Jurkat cells (5×10^5/ml) were incubated in the presence of medium or Cdt holotoxin for 30–240 min. Replicate cultures (1×10^7 cells) were pooled and harvested. The cell pellet was treated with cold 0.5 TCA for 5 min, centrifuged, and the pellet was washed twice with 5% TCA containing 1 mM EDTA. Neutral lipids were extracted twice with methanol:chloroform (2:1) at RT. Acidic lipids were extracted with 2.25 ml of methanol:chloroform:12 M HCl (80:40:1) for 15 min at RT; the samples were centrifuged for 5 min and the supernatant was recovered. The supernatant was then treated with 0.75 ml of chloroform and 1.35 ml of 0.1 M HCl and centrifuged to separate organic and aqueous phases; the organic phase was collected and

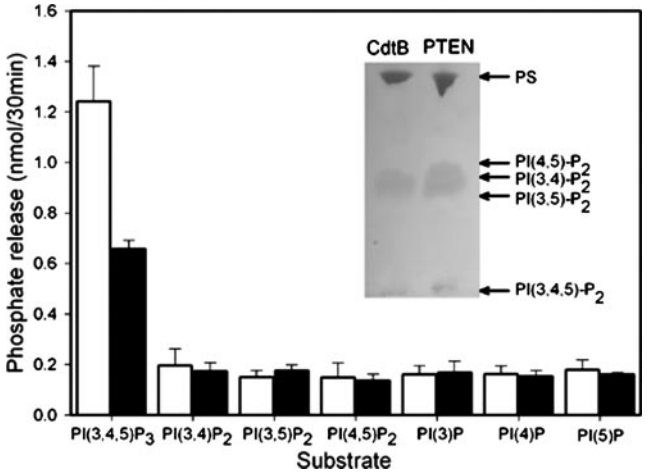

FIGURE 3. Analysis of substrate specificity and product formation for both CdtB and PTEN. CdtB (0.5 μM; ■) and PTEN (0.1 μM; □) were incubated for 30 min in presence of liposomes containing one of the PI phosphates shown. Data are plotted as phosphate release (nM/30 min; mean ± SD) for each substrate. It should be noted that no phosphate release was observed in presence of phosphatidylserine alone. *Inset*, Results from TLC analysis of product formation following PI-3,4,5-P_3 hydrolysis in the presence of 0.5 μM CdtB or 0.1 μM PTEN.

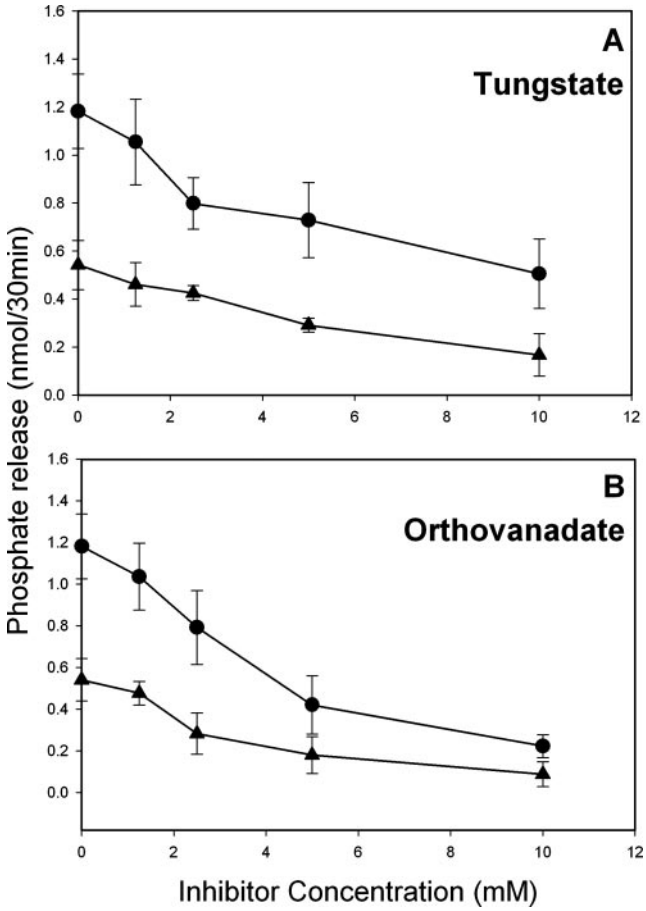

FIGURE 4. Effect of phosphatase inhibitors on CdtB-mediated hydrolysis of PI-3,4,5-P_3. CdtB (0.5 μM; ▲) and PTEN (0.1 μM; ●) were incubated with liposomes containing PI-3,4,5-P_3 in the presence of varying concentrations of tungstate (*A*) or orthovanadate (*B*) for 30 min. Phosphate release was measured using the malachite green-binding assay. Results are plotted as phosphate release (nM/30 min; mean ± SD) vs inhibitor concentration (millimoles).

dried. The dried lipids were resuspended in 120 μl of 50 mM HEPES buffer (pH 7.4) containing 150 mM NaCl and 1.5% sodium cholate, and left overnight at 4°C. PI-3,4,5-P_3 levels were then determined using a commercially available competitive ELISA according to the manufacturer's directions (PIP3 Mass ELISA kit; Echelon).

Structural comparisons

Multiple structural alignment of CdtB from *A. actinomycetemcomitans* with inositol polyphosphate 5-phosphatase and DNase I was made using MUSTANG (www.cs.mu.oz.aw/~arun/mustang/) (30–33). Positional sequence conservation of CdtB was derived from combined alignments of CdtB and inositol polyphosphate 5-phosphatase homologs (34) and residue conservation was determined (35). Conservation indices were converted to colors (red, most conserved; green, intermediate; blue, least conserved) and mapped onto the CdtB structure using Bobscript (36).

Results

It has been proposed, based on sequence comparisons, that CdtB shares catalytic residues and similar reaction mechanism with the large group of functionally diverse Mg^{2+}-dependent phosphoesterases (23). DNase I was the first structurally characterized member of this diverse enzyme superfamily (32); subsequent structural characterization of inositol polyphosphate 5-phosphatases and CdtB confirmed the initial prediction (13, 30, 31). An alignment of these three structures shows striking conservation of catalytic and divalent ion-chelating residues, despite low overall sequence identity (Fig. 1). As a general rule, all enzymes in this superfamily hydrolyze phosphate esters and their exact function depends on what substrate(s) can be accommodated in the active site. CdtB was originally characterized as a DNase-like enzyme and the putative PI phosphatase activity was never formally tested despite its weak nuclease activity (18).

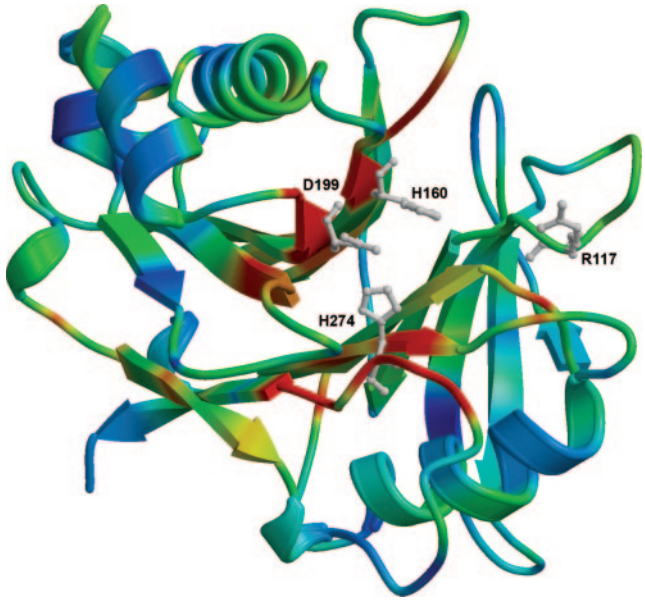

FIGURE 5. Residue conservation. A look from above into the active site of CdtB with mutated residues shown in ball-and-stick representation. Individual alignments of CdtB and IP5P homologs were combined and residue conservation was determined for each column of the alignment. Residues that are most conserved between these two groups of proteins are colored in red, with color shading changing toward green (for intermediate conservation) and blue (lack of conservation). Despite very low pairwise sequence identity between these proteins, the active site shows remarkable degree of conservation that reflects their similar reactions mechanisms.

In this study, CdtB was initially assessed for its ability to dephosphorylate PI-3,4,5-P_3. As shown in Fig. 2*A*, CdtB exhibits dose-dependent (0.2–1.0 μM) phosphate release which ranged from 0.1 to 0.9 nM in the presence of 0.2 and 1.0 μM CdtB, respectively. It should be noted that CdtA and CdtC failed to exhibit phosphatase activity under identical conditions (data not shown). The Cdt holotoxin (CdtABC), containing all three subunits, exhibited dose-dependent activity similar to that of CdtB. $ED_{0.5}$ values (concentration required to catalyze 0.5 nM phosphate release) were calculated; the $ED_{0.5}$ for CdtB and the holotoxin were 0.5 and 0.38 μM, respectively. For comparative purposes, PTEN was also assessed for activity. PTEN exhibited dose-dependent (0.02–0.2 μM) phosphate release; thus, PTEN was determined to be ~10 times more active then CdtB with an $ED_{0.5}$ of 0.05 μM. To further explore the propensity of CdtB to act as a PI phosphatase, we presumed a Michaelis-Menten relationship and determined K_m and V_{max} values with respect to cleavage of PI-3,4,5-P_3 and compared it to a similar analysis of PTEN (Fig. 2*B*). By this analysis, both CdtB and PTEN demonstrated similar K_m values of 124.3 and 159.1 μM, respectively. V_{max} values were 0.055 nM/min for 0.5 μM CdtB and 0.087 nM/min for 0.1 μM PTEN. Thus, CdtB exhibits PI-3,4,5-P_3 phosphatase activity that is comparable to that of PTEN.

CdtB substrate specificity was assessed using several different phosphoinositides. Preliminary experiments indicated that the lipid moiety was critical for CdtB phosphatase activity when PI-3,4,5-P_3 was used as a substrate; therefore, all PIs contained

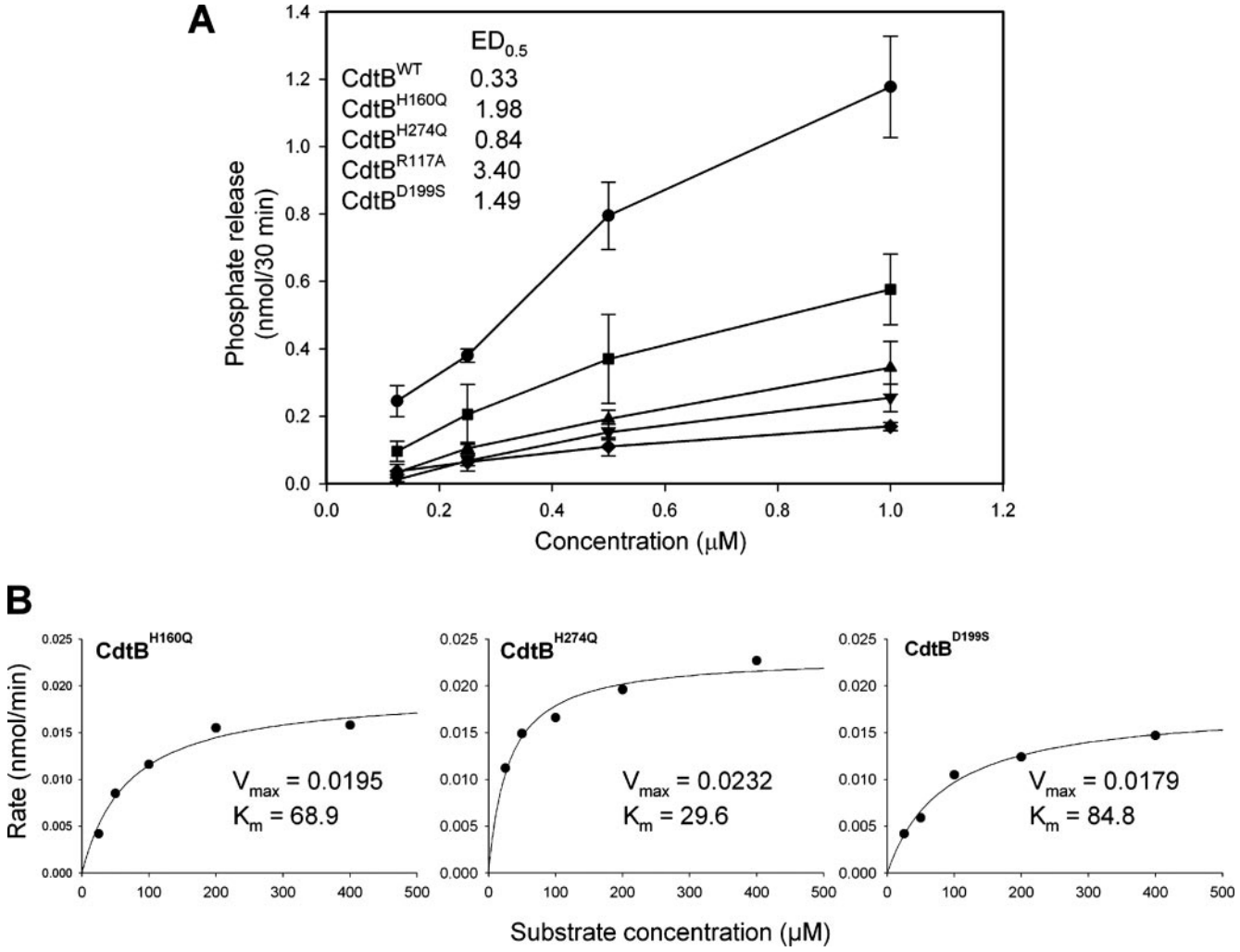

FIGURE 6. Analysis of CdtB mutants for PI-3,4,5-P_3 phosphatase activity. Plasmids were constructed containing mutations at residues critical to the putative active site: $CdtB^{H160Q}$, $CdtB^{H274Q}$, $CdtB^{R117A}$, and $CdtB^{D199S}$. The expressed proteins were purified and varying amounts of each were assessed for their ability to hydrolyze PI-3,4,5-P_3 (*A*): $CdtB^{WT}$ (●), $CdtB^{H160Q}$ (▼), $CdtB^{H274Q}$ (■), $CdtB^{R117A}$ (⬣), and $CdtB^{D199S}$ (▲). Data are plotted as phosphate release (nM/30 min; mean ± SD) vs protein concentration. *B*, The rate of phosphate release from PI-3,4,5-P_3 was assessed for mutants involving the catalytic site: $CdtB^{H160Q}$, $CdtB^{H274Q}$, and $CdtB^{D199S}$. No analysis was performed on the $CdtB^{R117A}$ mutants because of the low level of activity. Data were analyzed using Michaelis-Menten kinetics; both K_m and V_{max} values are indicated in the respective panels.

di-C16 acyl side chains. The PIs were incorporated into a lipid bilayer with phosphatidylserine as the carrier lipid. As shown in Fig. 3, CdtB-catalyzed phosphate release only occurred in the presence of PI-3,4,5-P_3; neither phosphatidylinositol diphosphates nor monophosphates were able to serve as a substrate. PTEN exhibited substrate specificity for PI-(3,4,5)-P_3 identical with CdtB. In other experiments, we also determined that CdtB was unable to dephosphorylate inositol triphosphate or inositol tetrakisphosphate (data not shown). It should also be noted that CdtB did not exhibit detectable protein phosphatase activity when the synthetic substrate, p-nitrophenyl phosphate, was used (data not shown). Additional similarities between PTEN and CdtB were observed with respect to susceptibility to the phosphatase inhibitors, tungstate and orthovanadate. As shown in Fig. 4, tungstate reduced CdtB phosphatase activity in a dose-dependent fashion; phosphatase activity was reduced 15% at 1 mM and 70% at 10 mM tungstate. Likewise, orthovanadate inhibited CdtB by 10% at 1 mM and 85% at 10 mM. PTEN exhibited similar dose-dependent sensitivity to these inhibitors. It should be noted that molybdate failed to inhibit CdtB or PTEN (data not shown). To determine whether CdtB is a 3- or 5-phosphatase, product formation was assessed using thin-layer chromatography. Although both CdtB and PTEN share many properties, they differ with respect to product produced. Whereas PTEN is specific for dephosphorylation at the D3 position of the inositol ring yielding PI-(4,5)-P_2, CdtB appears to be specific for the D5 position producing PI-(3,4)-P_2 (Fig. 3, *inset*) suggesting that CdtB activity may more accurately reflect that of an inositol 5-phosphatase similar to SHIP.

To further explore the relationship between CdtB and inositol polyphosphate 5-phosphatase, we generated several CdtB mutants that involve amino acid substitutions in residues that correspond to the inositol polyphosphate 5-phosphatase active site and which have previously been shown to be crucial for CdtB toxin activity. Fig. 5 shows a view into the active site of CdtB, with red color corresponding to the best residue conservation estimated from the combined alignment of CdtB and inositol polyphosphate 5-phosphatase homologs. The active site mutations included: $CdtB^{H160Q}$, $CdtB^{H274Q}$, $CdtB^{R117A}$, and $CdtB^{D199S}$; positions of these residues within the active site are shown in Fig. 5. The mutants were first assessed for their ability to release phosphate from PI-(3,4,5)-P_3. As shown in Fig. 6*A*, each of the mutants was observed to catalyze dose-dependent phosphate release; however, all of the mutants exhibited reduced activity relative to wild-type CdtB ($CdtB^{WT}$);

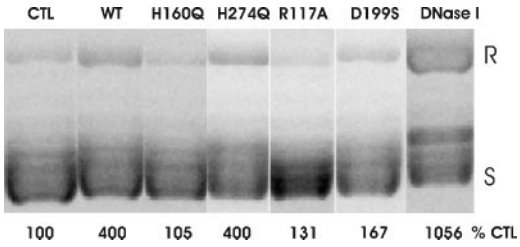

FIGURE 7. Analysis of CdtB mutants for DNase activity. $CdtB^{WT}$ and each of the CdtB mutants (2.5 μg) were incubated with supercoiled pUC19 DNA for 2 h at 37°C as described in *Materials and Methods*; bovine DNase (1 pg) was used as a positive control. The samples were then subjected to electrophoresis in agarose gels; after staining with ethidium bromide the gels were analyzed by digital scanning densitometry. S and R refer to supercoiled and relaxed form of plasmid DNA, respectively. Numbers reflect the relative density of the R band expressed as a percentage of the control.

$ED_{0.5}$ values increased from 0.3 μM for $CdtB^{WT}$ to 1.9 ($CdtB^{H160Q}$) 0.8 μM ($CdtB^{H274Q}$), 3.4 $CdtB^{R117A}$ and 1.5 μM ($CdtB^{D199S}$). The Michaelis-Menten kinetic parameters were also assessed for these mutations; as shown in Fig. 6*B*, the K_m values for the mutants was reduced to 68.9 ($CdtB^{H160Q}$), 29.6 ($CdtB^{H274Q}$), and 84.8 ($CdtB^{D199S}$) while the V_{max} was also reduced to 0.019, 0.023 and 0.018 nM/min, respectively. Phosphatase activity expressed by $CdtB^{R117A}$ was not sufficient to allow for kinetic analysis. Fig. 7 compares the ability of $CdtB^{WT}$ along with the CdtB mutants to exhibit DNase I-like activity using supercoiled plasmid DNA as a substrate. $CdtB^{WT}$ exhibited detectable nuclease activity although it was less than five orders of magnitude of that observed with bovine DNase I. All of the mutants, with the exception of $CdtB^{H274Q}$, exhibited a reduction in DNase activity; in contrast, $CdtB^{H274Q}$ nuclease activity was comparable to that of $CdtB^{WT}$.

The ability of the mutants to induce G_2 arrest in Jurkat cells was also assessed. It should be noted that, as previously reported, the ability of CdtB to induce G_2 arrest requires the presence of CdtA and CdtC; the combination of individual Cdt subunits produces an active toxin that is not quite as potent (requiring nanograms of each subunit) as when the three Cdt genes are coexpressed to form a holotoxin (27). Thus, these experiments were done in the presence of CdtA and CdtC under conditions that we previously demonstrated results in an active toxin complex. As shown in Fig. 8, 13.9% G_2 cells were observed in control cultures and 14.7% G_2 cells in cultures exposed to only CdtA and CdtC; when 4 ng of $CdtB^{WT}$ was added to CdtA and CdtC, the G_2 population increased to 54.8%. In comparison, all of the CdtB mutants lost the ability to induce G_2 arrest; the percentage of G_2 in these cultures were 15.5% ($CdtB^{H160Q}$), 15.8% ($CdtB^{H274Q}$), 13.1% ($CdtB^{R117A}$), and 15.3% ($CdtB^{D199S}$). It should be noted that the mutants retained residual toxicity if used at higher concentrations (micrograms per milliliter). These experiments demonstrate a correlation between decreased lipid phosphatase activity and loss of the toxins ability to induce cell cycle arrest in lymphocytes.

To further define the relationship between lipid phosphatase activity and Cdt intoxication of lymphocytes, we used two lines of investigation. We first demonstrated that exposure of Jurkat cells to Cdt results in both a dose- and time-dependent reduction of the intracellular levels of PI-3,4,5-P_3. As shown in Fig. 9*A*, treatment of Jurkat cells with a toxic dose of CdtABC (50 pg/ml) results in a time-dependent reduction in PI-3,4,5-P_3 levels from 8.3 pM/10^7 cells to 6.5 and 4.7 pM/10^7 cells within 15 and 30 min, respectively; levels were further reduced to 3.3 pM/10^7 cells at 120 min and to 1.5 pM/10^7 cells at 240 min. Cdt treatment also induced a dose-dependent reduction in PI-3,4,5-P_3 levels when Jurkat cells

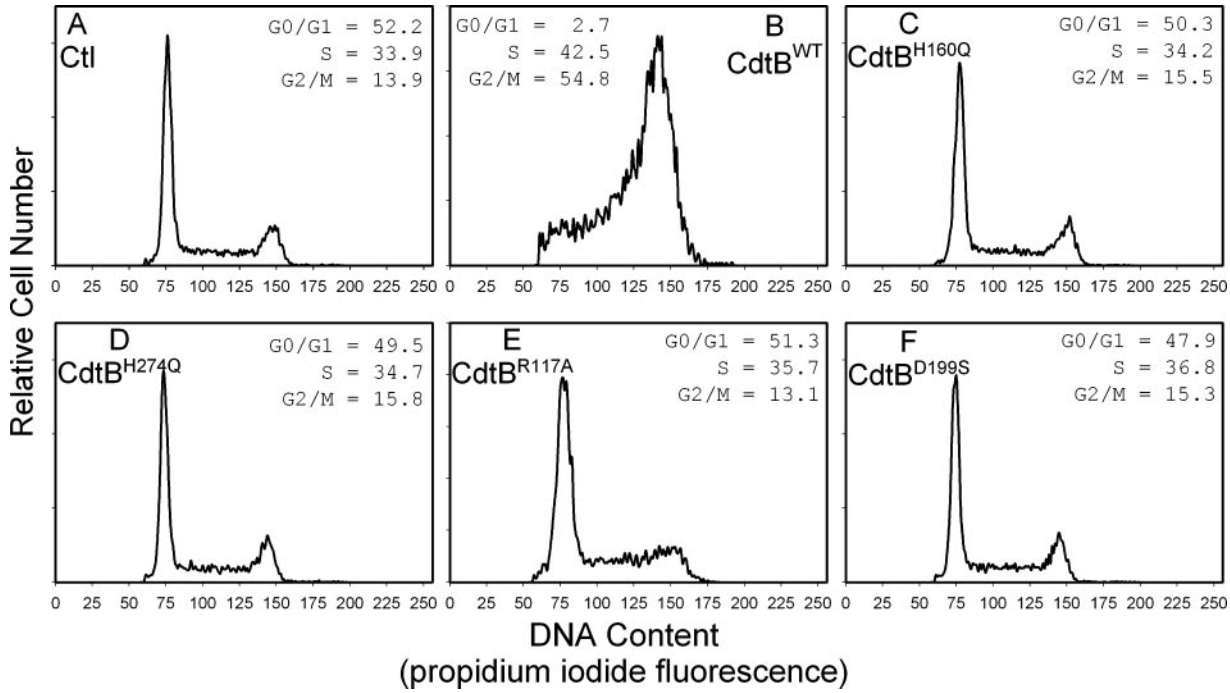

FIGURE 8. Assessment of CdtB mutants for their ability to induce G_2 arrest in Jurkat cells. Jurkat cells were exposed to medium alone (*A*) or 10 ng/ml each of CdtA and CdtC in the presence of 4 ng/ml $CdtB^{WT}$ (*B*) or $CdtB^{H160Q}$ (*C*), $CdtBH^{274Q}$ (*D*), $CdtB^{R117A}$ (*E*), or $CdtB^{D199S}$ (*F*). Cells were analyzed for cell cycle distribution 18 h after exposure to toxin subunits using flow cytometric analysis of propidium iodide fluorescence (27). The numbers in each panel represent the percentages of cells in G_0/G_1, S, and G_2/M. Cells exposed to only 10 ng/ml each of CdtA and CdtC exhibited 14.7% G_2 cells (data not shown). Results are representative of three experiments.

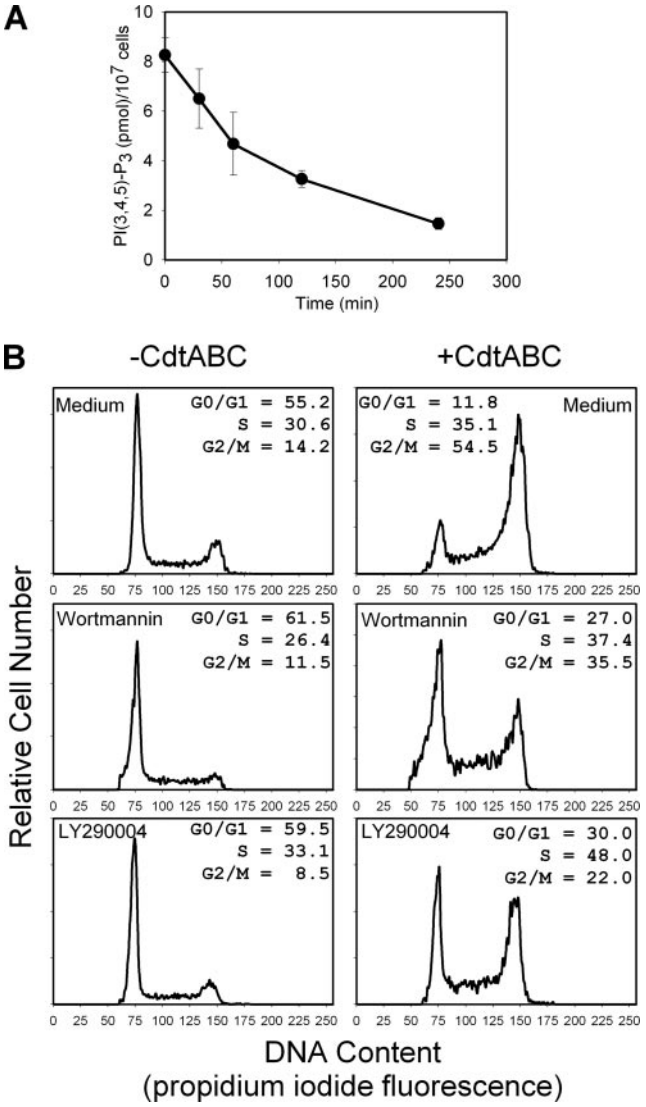

FIGURE 9. Relationship between exposure to Cdt, toxicity, and cellular content of PI-3,4,5-P_3. *A*, Jurkat cells were incubated in the presence of 50 pg/ml CdtABC for varying periods of time. Cells were then harvested, phospholipids were extracted, and PI-3,4,5-P_3 levels determined by ELISA. Data are plotted as PI-3,4,5-P_3 content (pM/10^7 cells; mean ± SD) vs time. *B*, PI3K inhibitors were used to lower Jurkat cell PI-3,4,5-P_3 and thereby alter susceptibility to CdtABC. Jurkat cells were preincubated in medium, 250 nM wortmannin, or 40 μM LY290004. The cells were then treated with medium or 40 pg/ml CdtABC and assessed for cell cycle distribution by flow cytometry. The numbers in each panel represent the percentages of cells in G_0/G_1, S, and G_2/M.

were exposed to 50–5000 pg/ml toxin for 2 h (data not shown). In a second series of experiments, we explored the relationship between lymphocyte susceptibility to toxin and PI-3,4,5-P_3 levels. We first lowered PI-3,4,5-P_3 levels in Jurkat cells by using inhibitors of PI3K. Jurkat cells were pretreated for 30 min with 250 nM wortmannin or 40 μM LY290004; Cdt holotoxin was added and, 16 h later, the cells were assessed for cell cycle distribution. As shown in Fig. 9*B*, control cells exhibited 14.2% G_2 cells while the addition of 40 pg/ml CdtABC increased the percentage of G_2 cells to 54.5%. Pretreatment with either wortmannin or LY290004 reduced Cdt-induced accumulation of G_2 cells to 35 and 22.0%, respectively. Under these conditions, the drugs alone had minimal affect on cell cycle progression. Thus, reducing PI-3,4,5-P_3 synthesis protects cells from Cdt-induced G_2 arrest.

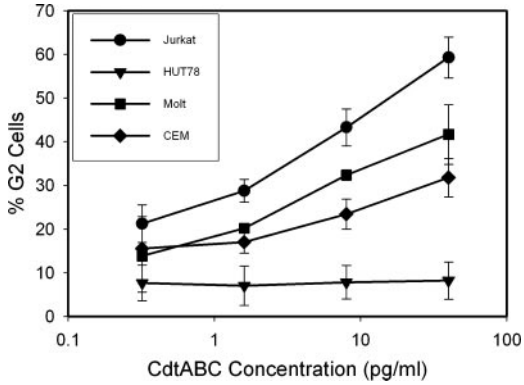

FIGURE 10. Comparison of lymphoid cell line susceptibility to Cdt-induced G_2 arrest. Jurkat cells (●), Hut78 cells (▼), Molt cells (■), and CCRF-CEM cells (♦) were treated with varying amounts of CdtABC and incubated for 18 h. The cells were then harvested, stained with propidium iodide, and analyzed by flow cytometry for cell cycle distribution. Data are plotted as percent G_2 cells vs CdtABC concentration. Results represent the mean ± SD of three experiments.

PTEN and SHIP1, both PI-3,4,5-P_3 phosphatases, regulate cell proliferation and survival by opposing the action of PI3K, thereby, maintaining low levels of PI-3,4,5-P_3 and blocking activation of Akt (37–39). As a consequence of this mode of action, PTEN and SHIP function as tumor suppressors (25, 26). Indeed, somatic deletions or mutations have been identified in a variety of cancers making them, along with p53, the most commonly mutated genes in human cancers (40). Of particular relevance, cell lines derived from leukemia and lymphoma patients often exhibit defects in either one or both of these lipid phosphatases (25, 26). In the final series of experiments, we took advantage of such naturally occurring defects of these phosphatases in lymphoid cell lines and assessed their relative sensitivity to Cdt. Jurkat cells have been shown to be deficient in both PTEN and SHIP resulting in relatively high levels of PI-3,4,5-P_3 (25, 26). As shown in Fig. 10, Jurkat cells exhibit the highest sensitivity to Cdt; the percentage of G_2 cells increased from 12.5% in untreated cultures to 21 and 59% in the presence of 0.32 and 40 pg/ml Cdt. In contrast, the cutaneous T cell lymphoma cell line, Hut78, which contains functional levels of SHIP1 and PTEN as well as low levels of PI-3,4,5-P_3, was resistant to the toxin at all concentrations tested. Molt and CEM cells are deficient in PTEN, but have functional SHIP1 activity; these cells exhibit susceptibility to Cdt holotoxin that was less than that observed with Jurkat cells. The percentage of G_2 cells increased to 13 (Molt) and 15% (CEM) in the presence of 0.32 pg/ml Cdt and to 41 (Molt) and 32% (CEM) in the presence of 40 pg/ml toxin. Thus, it appears that lymphoid cell susceptibility to Cdt-induced G_2 arrest is dependent upon the endogenous levels of PI-3,4,5-P_3 and possibly the cells dependence on this lipid for proliferation and survival.

Discussion

Over the past several years, lipids in general, and PI-3,4,5-P_3 in particular, have become recognized for their central role in regulating an array of biological responses which include cell growth, proliferation, and survival. PI-3,4,5-P_3 is normally maintained at low levels and increases rapidly in response to a variety of signals that involve plasma membrane recruitment and activation of PI3K. Normal cell function requires that PI-3,4,5-P_3 levels be tightly regulated; three enzymes, PTEN, SHIP1, and SHIP2, have been shown to play critical roles in this capacity (37–39). The tumor

suppressor phosphatase, PTEN, was originally identified as a dual-specificity protein phosphatase. However, PTEN appears to be a poor protein phosphatase and its biologically relevant substrates are 3-phospholipids, specifically PI-3,4,5-P_3 which is hydrolyzed to PI-4,5-P_2. SHIP1 and SHIP2 are inositol 5-phosphatases; whereas SHIP2 is ubiquitously expressed, SHIP1 appears to be found in a limited subset of cells including most immune cells. Both SHIP enzymes hydrolyze PI-3,4,5-P_3 to PI-3,4-P_2 and inositol 1,3,4,5-tetrakisphosphate to inositol 1,3,4 triphosphate. Therefore, it is noteworthy that our observations clearly demonstrate that the active Cdt subunit, CdtB, is capable of hydrolyzing PI-3,4,5-P_3 and appears to function as an inositol 5-polyphosphate phosphatase similar to the SHIP enzymes. However, unlike the SHIPs, CdtB does not hydrolyze inositol 1,3,4,5-tetrakisphosphate.

The relationship between CdtB and SHIP is not surprising since the initial observations of Dlakić (24) indicated sequence and structural homology between CdtB and a larger group of divergent enzymes that includes inositol polyphosphate 5-phosphatases. These enzymes have been predicted, based on sequence analysis, to be similar in fold and mechanism to DNase I and related metal-dependent phosphohydrolases (31, 41–44). Recently, the Cdt holotoxin was crystallized and analyzed by x-ray crystallography (13, 30). Of particular relevance to this study, the analysis reveals structural similarity with not just DNase I, but equal structural homology with inositol polyphosphate 5-phosphatase (Fig. 1). Despite the lack of significant pairwise sequence identity, structural similarity was predicted on the basis of sensitive multiple sequence alignments as an extension of an earlier discovery of homology between the nucleases and bacterial sphingomyelinase (31). Analysis of CdtB indicates that its structure can be superimposed on inositol polyphosphate 5-phosphatase with an RMSD of 3.5 Å over 183 Cα atoms; this compares to structural overlap of CdtB with DNase I with a root-mean-square deviation of 3.1 Å over 207 Cα atoms. Moreover, additional analysis of Cdt sequence homology with inositol polyphosphate 5-phosphatases demonstrate conserved regions corresponding to the active site (Fig. 5). To further explore this relationship, we generated several CdtB mutants that involve amino acid substitutions in the putative catalytic site ($CdtB^{H160Q}$, $CdtB^{H274Q}$, $CdtB^{D199S}$, and $CdtB^{R117A}$). In all instances, the mutants exhibited reduced phosphatase activity when compared with $CdtB^{WT}$. Furthermore, each of the mutants lost the ability to induce Jurkat cells to undergo G_2 arrest in the presence of the CdtA and CdtC subunits. These mutants also exhibited reduced DNase I-like activity when they were assessed for their ability to relax supercoiled plasmid DNA. It is noteworthy, however, that the DNase activity of one mutant, $CdtB^{H274Q}$, was not altered. Thus, it appears that there is a good correlation between the ability of CdtB to function as a PI-3,4,5-P_3 phosphatase and its toxicity in lymphocytes.

The relationship between lipid phosphatase activity and the ability of Cdt to intoxicate lymphocytes was further explored using two lines of investigation. First, we demonstrate that exposure of Jurkat cells to Cdt results in both a dose- and time-dependent reduction of the intracellular levels of PI-3,4,5-P_3. It should be noted that these effects occur at Cdt concentrations (50 pg/ml) equivalent to those that also induce maximal G_2 arrest in lymphocytes. A second series of experiments demonstrate that lymphocyte susceptibility to Cdt is dependent upon the cellular content of PI-3,4,5-P_3. In this regard, we first used two PI3K inhibitors, wortmannin and LY290004, to reduce Jurkat cell PI-3,4,5-P_3 levels; cells were then assessed for susceptibility to the toxin. Both PI3K inhibitors protected Jurkat cells from Cdt-induced G_2 arrest. We also compared Cdt sensitivity of several lymphoid cell lines that have been shown to contain different levels of PI-3,4,5-P_3 resulting from inherent defects in lipid phosphatase activity. It is of particular relevance to this study that Jurkat cells have been shown to be deficient in both functional PTEN and SHIP1 and as a result these cells have high basal levels of PI-3,4,5-P_3 (25, 26). It should be noted that elevated levels of PI-3,4,5-P_3 have been shown to be critical for the survival of Jurkat and many other leukemic cell lines. Thus, of the cells examined, Jurkat cells were the most sensitive to Cdt-induced G_2 arrest; this is consistent with their dependence on elevated levels of PI-3,4,5-P_3 for survival. In contrast, HUT78 cells, a cutaneous T cell lymphoma cell line that contains functional levels of both PTEN and SHIP1 and concomitant lower intracellular levels of PI-3,4,5-P_3, were resistant to the effects of Cdt at the concentrations used in this study. Finally, CEM and Molt cells contain normal SHIP1 expression and activity, but lack PTEN. These cells were responsive to Cdt, albeit, they exhibited reduced sensitivity than Jurkat cells.

It should be noted that current dogma is that CdtB toxicity is the result of DNase I-like activity whereby the toxin cleaves DNA and in turn activates cell cycle checkpoints (18, 21). As demonstrated in this study and by others, CdtB exhibits $<0.01\%$ of the activity of bovine DNase I (13, 20); in contrast, CdtB is approximately one-tenth as active as PTEN. In addition to partial sequence and structural homology, the link between CdtB and DNase I was initially based upon two lines of investigation. One approach used mutation of the *cdtB* gene at loci that are believed to be critical to DNase activity (13, 18, 20). We now demonstrate that many of these mutations also result in a decline in PI-3,4,5-P_3 phosphatase activity. Other investigators have demonstrated that Cdt exhibits DNase activity by its ability to denature or relax supercoiled plasmid DNA (13, 14, 20, 45). Most of these studies used large quantities of Cdt holotoxin or the CdtB subunit and even under these conditions relatively low levels of DNA fragmentation was observed. In another study, HeLa cells were treated with Cdt and assessed for both cell cycle arrest and the presence of DNA strand breaks (46). In addition to using relatively large doses of toxin, these studies do not discriminate between direct DNase activity associated with Cdt and the possibility of indirect effects whereby toxin treatment leads to activation of DNase endogenous to the cell. Indeed, our own studies support the notion that the vast majority of DNA fragmentation that we and others have reported in association with exposure to Cdt result from activation of the apoptotic cascade (10, 12, 47, 48). Nonetheless, our studies do not eliminate the possibility that Cdt may indeed exhibit dual functions whereby DNase activity is important for toxicity in some cell types while lipid phosphatase activity is critical for toxicity in other cells such as lymphocytes. It should be noted that in most cases, enzymes exhibit fairly strict specificity for one substrate, or at the most a few related substrates, as dictated by the active site constraints. In this particular case, one would not expect that an enzyme could evolve to have very strong DNase and lipid phosphatase activities, simply because the corresponding substrates are too different. However, a putative lipid phosphatase could potentially evolve into low-activity nuclease (if this was ultimately beneficial to its overall function) while giving up only part of its original phosphatase activity.

Collectively, our observations demonstrate that the active Cdt subunit, CdtB, is capable of functioning as a PI-3,4,5-P_3 phosphatase and that this activity appears to be critical to toxin-induced G_2 arrest in lymphocytes. Two lines of evidence support the candidacy of PI-3,4,5-P_3 as the major cellular target of CdtB. First, as noted earlier, the in vitro phosphatase activity of CdtB is much more robust than its nuclease activity, at least in relative terms when compared with other similar enzymes. Second, the results of

mutagenesis experiments done in this study and by Nesic et al. (13) are fully compatible with the phosphatase function. For example, the $CdtB^{R117A}$ mutation, either alone or in the context of the triple DNA-binding mutant, eradicates the G_2 arrest normally induced by $CdtB^{WT}$ without significantly affecting the DNase activity (13); we demonstrate that this mutation severely affects the PI-3,4,5-P_3 phosphatase activity of CdtB. Two arginine residues in the active site of inositol phosphate 5-phosphatases interact with either phosphate or hydroxyl groups of inositol and a similar function of R117 could explain the failure of our kinetic measurements to detect any product with the $CdtB^{R117A}$ mutant (31). It is worth noting that the mutation of the flanking valine, V118E, also eliminates the G_2 arrest normally induced by $CdtB^{WT}$, even though this particular mutation was expected to boost the DNase activity of CdtB (13). Overall, it appears that the ability of CdtB and its mutants to cause cell cycle arrest always correlates with their phosphatase activities and only sometimes with their DNase activities.

In summary, we propose that lymphocytes represent one of the primary in vivo targets of Cdt; this is based in part upon their exquisite sensitivity to toxin-induced cell cycle arrest. We further propose that the underlying basis for this heightened sensitivity to the toxin is related to a combination of CdtB-associated lipid phosphatase activity and lymphocyte dependence upon PI-3,4,5-P_3. Our observations suggest that CdtB, like SHIP1 and PTEN, mediates its regulatory effects by dephosphorylating PI-3,4,5-P_3 and thereby modulating the activity of pleckstrin homology containing proteins such as Akt. In this regard, it is well-established that antigenic and mitogenic activation leading to clonal expansion of lymphocytes is dependent upon increases in PI-3,4,5-P_3 and subsequent activation of the Akt pathway. This mechanism of action also accounts for the heightened sensitivity of leukemic cells to Cdt because mutations of PTEN and/or SHIP1 appears to be a common feature of cells such as Jurkat. Therefore, we propose that the mechanism of action for Cdt in lymphocytes, and other cell types, involves the depletion of PI-3,4,5-P_3 and a concomitant inactivation of the Akt pathway. Indeed, it has been shown that inactivation of Akt can lead to both cell cycle arrest and the activation of the apoptotic cascade, events that are also associated with the action of Cdt. In conclusion, it is likely that as a result of CdtB phosphatase activity, lymphocytes undergo cell cycle arrest resulting in impaired host immunity. In vivo, these events most likely create a situation that favors chronic infection and thereby contribute to the pathogenesis of disease associated with Cdt-producing organisms such as *A. actinomycetemcomitans.*

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Disclosures

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