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# Inhibition of mast cell degranulation by a chimeric toxin containing a novel phosphatidylinositol-3,4,5-triphosphate phosphatase

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ABSTRACT

It is well established that many cell functions are controlled by the PI-3K signaling pathway and the signaling lipid, phosphatidylinositol-3,4,5-triphosphate (PIP3). This is particularly true for mast cells which play a key regulatory role in allergy and inflammation through activation via high-affinity IgE receptors (FcεRI) leading to activation of signaling cascades and subsequent release of histamine and other pro-inflammatory mediators. A pivotal component of this cascade is the activation of PI-3K and a rise in intracellular levels of PIP3. In this study, we developed a novel chimeric toxin that selectively binds to mast cells and which functions as a PIP3 phosphatase. Specifically, the chimeric toxin was composed of the FcεRI binding region of IgE and the active subunit of the cytolethal distending toxin, CdtB, which we have recently demonstrated to function as a PIP3 phosphatase. We demonstrate that the chimeric toxin retains PIP3 phosphatase activity and selectively binds to mast cells. Moreover, the toxin is capable of altering intracellular levels of PIP3, block antigen-induced Akt phosphorylation and degranulation. These studies provide further evidence for the pivotal role of PIP3 in regulating mast cell activation and for this signaling lipid serving as a novel target for therapeutic intervention of mast cell-mediated disease. Moreover, these studies provide evidence for the utilization of CdtB as a novel therapeutic agent for targeting the PI-3K signaling pathway.



## 1. Introduction

Mast cells play a key regulatory role in allergy and inflammation through the activation of high-affinity IgE receptors (FcεRI) and subsequent release of histamine and other pro-inflammatory mediators (Kinet, 2007; Rivera and Olivera, 2008). Mast cell activation is dependent upon the binding of multivalent antigen to prebound IgE molecules leading to cross-linking of FcεRI. As a consequence of these events, a complex signaling cascade is activated leading to degranulation, synthesis of pro-inflammatory lipid mediators and cytokines. The high-affinity FcεRI is composed of an α-chain which is responsible for binding the Fc portion of IgE, a β-chain and two γ-chains. Upon antigen binding and FcεRI cross-linking, immunoreceptor tyrosine-based activation motifs (ITAMS) on the β- and γ-subunits become phosphorylated and then serve as docking sites for secondary signaling proteins such as the tyrosine kinases Lyn and Syk. These molecular interactions increase tyrosine phosphorylation events and increase enzymatic activity of signaling proteins at or near the receptor complex. Additionally, signaling pathways leading to mast cell activation are dependent upon protein-lipid interactions. In particular, the generation of the signaling lipid, phosphatidylinositol-3,4,5-triphosphate (PIP3), has been shown to be critical for mediating mast cell activation (reviewed by Nadler et al., 2001).

Phosphoinositides (PIs) are derivatives of phosphatidylinositol and while they represent minor components of membrane lipids, PIs regulate a wide range of biological processes including: cell proliferation, cell survival, differentiation, cytoskeleton organization and membrane trafficking (reviewed in Kraub and Haucke, 2007; Sasaki et al., 2007). PIs regulate these cellular processes by serving as site-specific membrane signals that mediate membrane recruitment and regulation of effector/signaling proteins (Lemmon, 2008). PIP3 is synthesized from PI(4,5)P2 following the activation of PI-3K and has received much attention for its critical role as a second messenger. In particular, FcεRI-mediated mast cell activation requires the recruitment and activation of PI-3K and concomitant synthesis of PIP3 (Rivera and Olivera, 2008; Abramson and Pecht, 2007; Nadler et al., 2001; Tkacyzk and Gilfillan, 2001). The generation of PIP3 is critical to recruiting pleckstrin homology domain

*Abbreviations:* PIP3, phosphatidylinositol-3,4,5-triphosphate; FcεRI, high-affinity IgE receptors; Cdt, cytolethal distending toxin; BMMC, bone marrow derived murine mast cells; MCF, mean channel fluorescence.

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containing proteins such as BTK, Akt and PDK1 to the plasma membrane where they become activated and coupled to upstream signals and, in turn, transduce those signals to downstream events ultimately leading to activation of the secretory and synthetic response. Thus, FcεRI-mediated mast cell degranulation, synthesis of lipid derived pro-inflammatory mediators and cytokine production is ultimately controlled by PIP3 thereby making this lipid messenger a potent target for pharmacologic intervention in treating mast cell-mediated allergic disorders.

Several investigators have demonstrated that a reduction in PI-3K activity resulting from mutations or the introduction of inhibitors results in decreased PIP3 synthesis with a concomitant inhibition of cell function (reviewed in Kim et al., 2008). Of particular relevance, we have recently demonstrated that the active subunit of the cytolethal distending toxin (Cdt), CdtB, exhibits potent PIP3 phosphatase activity making it a useful agent to modulate PIP3 signaling pathways and to advance our understanding of the role of PIP3 in regulating mast cell activation (Shenker et al., 2007). We now report on the effect of a novel chimeric toxin composed of CdtB to specifically deplete cells of PIP3 and the FcεRI binding region of IgE to confer mast cell specificity in order to inhibit the FcεRI/PI-3K/PIP3 signaling pathway. Our results clearly indicate that the chimeric toxin not only specifically binds to mast cells, but is capable of inhibiting antigen-IgE-mediated mast cell degranulation.

## 2. Materials and methods

### 2.1. Cell culture

RBL-2H3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml) (Venkatesha et al., 2004). Murine bone marrow derived mast cells were (BMMC) derived from bone marrow cells obtained from the femurs of C57/BL6mice (Zaidi et al., 2006). Cells were cultured for 4–6 weeks in Iscove's medium supplemented with 10% fetal bovine serum, glutamine (2 mM) penicillin (100 units/ml), streptomycin (100 μg/ml), and rmIL-3 (10 ng/ml). The homogeneity of the mast cells was confirmed by acid toluidine blue staining; the cell population used for these studies was >95% BMMC.

### 2.2. Assay of degranulation

RBL-2H3 and BMMC were incubated overnight in the presence of 1 μg/ml anti-dinitrophenyl (DNP) mouse IgE (Sigma Chemical, St. Louis, MO). Cells ($5 \times 10^4$) were seeded into 96-well plates in a total volume of 50 μl of HEPES-buffered saline containing 0.1% BSA and exposed to DNP-BSA (Bioresearch Technologies; Novato, CA) for 30 min. For total β-hexosaminidase release, control cells were lysed in 50 μl of 0.1% Triton X-100. Aliquots of supernatants or cell lysates were incubated with 1 mM p-nitrophenyl-N-acetyl-β-D-glucosamine for 1.5 h at 37 °C. The reaction was stopped by adding 250 μl of a 0.1 M $Na_2CO_3$/0.1 M $NaHCO_3$ buffer and absorbance was measured at 405 nm (Ali et al., 1993).

### 2.3. Construction and expression of chimeric toxin

Construction of the plasmid containing wildtype *cdtB* gene (pGEMCdtB) was previously described (Shenker et al., 2005). In order to construct the plasmid containing the *cdtB* gene fused to the coding sequence for the Fc binding region of IgE, designated pIVEXCdtBFcε, we inserted NcoI and NotI restriction sites by PCR using pGEMCdtB as a template with the following primers:

CAT GCC ATG GCA AAC TTG AGT GAT TTC AAA GTA GCA
ATA GTT TAG CGG CCG CGA TCA CGA ACA AAA CTA ACA GGA

A new plasmid (pIVEXCdtB) containing *cdtB* was generated from the PCR product which was ligated into pIVEX2.3d (Roche Applied Science; Indianapolis, IN). The Fcε fragment was generated by PCR using cDNA obtained from LPS activated murine B-cells as described (Belostotsky and Lorberboum-Galski, 2003); the following primers were used to incorporate NotI and XmaI restriction sites into the PCR product:

ATA AGA ATG CGG CCG CCA GCA ATG GAT GTC TGA AAG CA
CCC CCC GGG TGG GGT CTT GGT GAT GGA ACG CA

The PCR product contains 411 residues encoding 135 amino acids corresponding to residues 137–271 of the mouse IgE heavy chain (Genbank accession J00476); this sequence corresponds to the C terminus of domain 2 and domain 3. The PCR product was ligated into pIVEXCdtB to yield a new plasmid designated, pIVEXCdtBFcε. The chimeric protein was expressed using an *in vitro* coupled translation/transcription system (RTS 500 ProteoMaster; Roche Applied Science) that we routinely employ and the protein purified by nickel affinity chromatography as we incorporate a His tag into the construct (Shenker et al., 2004, 2005).

### 2.4. Phosphatase assay

Phosphatase activity was assessed by monitoring the dephosphorylation of $PIP_3$ as described by Maehama et al. (2000) and Shenker et al. (2007). Briefly, the reaction mixture (20 μl) consisted of 100 mM Tris–HCl (pH 8.0), 10 mM dithiothreitol, 0.5 mM diC16-phosphatidylserine (Avanti Polar Lipids, Alabaster, AL), 25 μM $PIP_3$ (diC16, Echelon Biosciences, Salt Lake City, UT) and the indicated amount of toxin. Lipid solutions were deposited in 1.5 ml tubes, organic solvent removed, the buffer added and a lipid suspension formed by sonication. Phosphatase assays were carried out 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, Plymouth Meeting, PA)] was added to 100 μl of the enzyme reaction mixture and color was developed for 20 min at RT. Absorbance at 650 nm was measured and phosphate release quantified by comparison to inorganic phosphate standards.

### 2.5. Measurement of cellular $PIP_3$ content

BMMC ($5 \times 10^5$ ml$^{-1}$) were incubated in the presence of medium or toxin for the time indicated. Replicate cultures ($1 \times 10^7$ cells) were pooled and harvested. The cell pellet was treated with cold 0.5 M TCA for 5 min, centrifuged and the pellet 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 methanol:chloroform:12 M HCl (80:40:1) for 15 min at RT; the samples were centrifuged for 5 min and the supernatant recovered. The supernatant was then treated with 0.75 ml chloroform and 1.35 ml 0.1 M HCl and centrifuged to separate organic and aqueous phases; the organic phase was collected and dried. The dried lipids were resuspended in 50 mM Hepes buffer (pH 7.4) containing 150 mM NaCl and 1.5% sodium cholate, and left overnight at 4 °C. $PIP_3$ levels were then determined using a commercially available competitive ELISA according to the manufacturers directions (PIP3 Mass ELISA Kit and PIP2 Mass ELISA Kit; Echelon Biochemicals).

### 2.6. Immunoassays

Samples were separated on 12% SDS-PAGE gels and then transferred to nitrocellulose. The membrane was blocked with BLOTTO and then incubated with anti-CdtB mAb (CdtB194), anti-Akt (Cell Signaling Technology, Danvers, MA) or anti-pAkt antibody (pAkt 473; Cell Signaling Technology) for 18 h at 4 °C (Shenker et al., 1999). Membranes were washed, incubated with goat anti-mouse immunoglobulin (Southern Biotech, Birmingham, AL) conjugated to horseradish peroxidase. The Western blots were developed using chemiluminescence [SuperSignal West Pico (Thermo Scientific, Rockford, IL)] and analyzed by digital densitometry (Kodak Scientific Imaging Systems, Rochester, NY).

In order to determine if CdtB$^{Fc\varepsilon}$ influenced mast cell sensitization with IgE, BMMC were incubated with IgE as described above. Cells were then treated with CdtB$^{Fc\varepsilon}$ (20 μg/ml) for 2 h min. The cells were then stained with anti-IgE conjugated to FITC (Southern Biotech) and analyzed by flow cytometry.

## 3. Results

The plasmid expressing the CdtB-IgE Fc chimeric protein (pIVEXCdtBFcε) was constructed in pIVEX (Roche) as described in Materials and Methods section; the insert contains 1207 bp that encodes a fusion toxin protein consisting of 135 aa encoding the FcεRI binding region from the CH2/CH3 region of IgE and the entire sequence for CdtB (32 kDa; Fig. 1A). Expression of the chimeric protein was achieved using an *in vitro* coupled translation/transcription system and the protein purified by nickel affinity chromatography as we incorporated a His tag into the construct (Shenker et al., 2004, 2005). Fig. 1B and C shows SDS-PAGE and Western blot analysis of samples containing both crude and purified CdtB$^{Fc\varepsilon}$ that was expressed from pIVEXCdtBFcε using the *in vitro* coupled translation/transcription system. The chimeric toxin (CdtB$^{Fc\varepsilon}$) exhibits a mw of approximately 49 kDa compared to 32 kDa for CdtB$^{WT}$.

CdtB$^{Fc\varepsilon}$ was assessed for its ability to dephosphorylate PIP3 using malachite green to measure inorganic phosphate release. As shown in Fig. 2A, CdtB$^{Fc\varepsilon}$ exhibits dose-dependent phosphate release which ranged from 0.5 ± 0.03 to 1.5 ± 0.17 nmol/30 min in the presence of 0.125 and 1.0 μM CdtB$^{Fc\varepsilon}$, respectively. For comparative purposes, CdtB$^{WT}$ was also assessed. CdtB$^{WT}$ also exhibits dose-dependent phosphate release which was lower than the chimeric toxin; phosphate release ranged from 0.1 ± 0.02 to 0.96 ± 0.07 nmol/30 min in the presence of 0.125 and 1.0 μM CdtB$^{Fc\varepsilon}$. We have previously demonstrated that CdtB$^{WT}$ exhibits Michaelis–Menten kinetics (Shenker et al., 2007). Therefore, to further assess the phosphatidylinositol phosphatase activity of CdtB$^{Fc\varepsilon}$ we compared the Michaelis–Menten kinetics to that of CdtB$^{WT}$ and determined $K_m$ and $V_{max}$ values with respect to cleavage of PIP3 (Fig. 2B and C). By this analysis, both CdtB$^{Fc\varepsilon}$ and CdtB$^{WT}$ demonstrated $K_m$ values of 593.7 and 124.3 μM, respectively. $V_{max}$ values were 0.117 nmol/min (CdtB$^{Fc\varepsilon}$) and 0.055 nmol/min (CdtB$^{WT}$) for 0.5 μM toxin. Thus, the chimeric toxin exhibits approximately twice the PIP3 phosphatase activity along with reduced substrate affinity compared to that observed with wildtype CdtB.

We next determined whether CdtB$^{Fc\varepsilon}$ was capable of selectively interacting with mast cells by binding to FcεRI. BMMC and Jurkat cells were exposed to varying amounts (0–10 μg/ml) of CdtB$^{Fc\varepsilon}$ for 60 min. The cells were then extensively washed, solubilized and analyzed by Western blot with anti-CdtB mAb (Fig. 3A). CdtB$^{Fc\varepsilon}$ was detected in extracts prepared from both BMMC and RBL cells (data not shown). The specificity of this interaction is demonstrated by the fact that Jurkat cells, which do not contain FcεRI, failed to bind the chimeric toxin. CdtB$^{WT}$ failed to associate with cells (data not shown); this observation is consistent with our earlier studies indicating that CdtB requires the binding units, CdtA and CdtC, in order for it to bind to lymphocytes (Shenker et al., 2005). These studies were extended to demonstrate specificity of CdtB$^{Fc\varepsilon}$ binding to the FcεRI. As shown in Fig. 3B, pre-exposure of BMMC to varying amounts of IgE (1–10 μg/ml) blocked CdtB$^{Fc\varepsilon}$ binding.

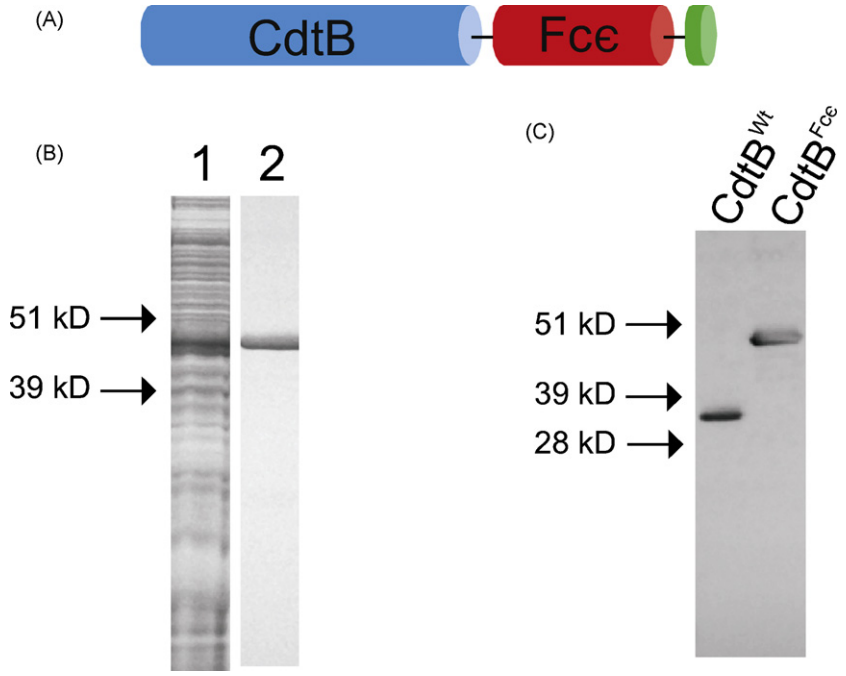


**Fig. 1.** Expression of CdtB$^{Fc\varepsilon}$ chimeric toxin. Panel A is a schematic representation of the protein encoded by pIVEXCdtBFcε showing the CdtB subunit on the N-terminus of the chimera (blue), the Fcε binding region downstream (red) and the histidine tag (green) on the C terminus. CdtB$^{Fc\varepsilon}$ was expressed from pIVEXCdtBFcε using *in vitro* coupled translation/transcription system and purified as described in Section 2; panel B shows a Coomassie stained SDS-PAGE gel of the unpurified expressed protein (lane 1) and the purified chimeric toxin (lane 2). Panel C shows a Western blot analysis of the expressed chimeric toxin which exhibits a mw of approximately 47 kDa; this compares to 32 kDa for CdtB$^{wt}$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

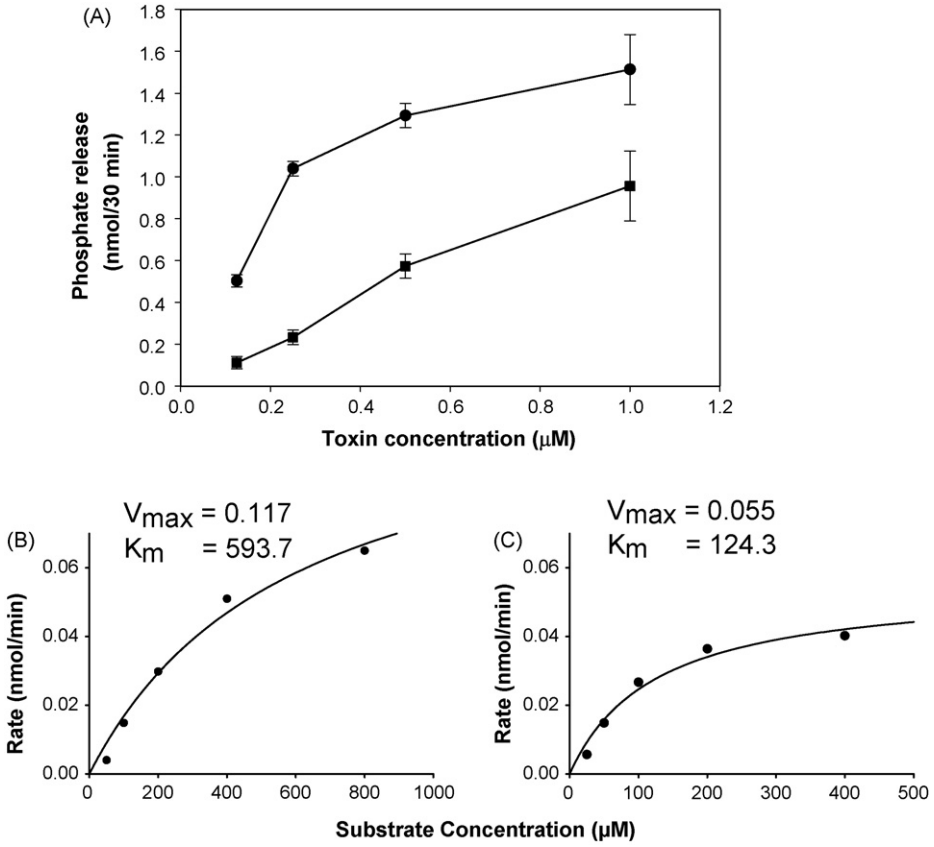


**Fig. 2.** Analysis of CdtB$^{Fc\varepsilon}$ for PIP3 phosphatase activity. Varying amounts of CdtB$^{WT}$ (squares) and CdtB$^{Fc\varepsilon}$ (circles) were assessed for their ability to hydrolyze $PIP_3$ *in vitro* as described in Section 2 (panel A). The amount of phosphate release was measured using a malachite green binding assay. Data are plotted as phosphate release (nmol/30 min; mean ± S.D.) versus protein concentration. CdtB$^{Fc\varepsilon}$ exhibited dose-dependent phosphatase activity; results are the mean ± S.D. of three experiments. The rate of CdtB$^{Fc\varepsilon}$ (0.5 μM; panel B) and CdtB$^{WT}$ (0.5 μM; panel C) mediated phosphate release in the presence of varying concentrations of $PIP_3$ was assessed. Data were analyzed using Michaelis–Menten kinetics; $K_m$ values for CdtB$^{wt}$ and CdtB$^{Fc\varepsilon}$ were 124.3 and 593.7 μM, respectively and $V_{max}$ were 0.055 nmol/min (CdtB$^{wt}$) and 0.117 nmol/min (CdtB$^{Fc\varepsilon}$).

We next assessed whether the chimeric toxin was also capable of entering cells and exhibit PIP3 phosphatase activity by assessing the intracellular levels of both $PIP_3$ and the degradative product, PI-3,4-P2. RBL cells were treated with CdtB$^{Fc\varepsilon}$ (10 μg/ml) for varying periods of time and the levels of $PIP_3$ determined (Fig. 4A). Toxin-treated cells exhibited a time-dependent reduction in PIP3; baseline levels were reduced from 10.9 ± 0.14 pmol/$10^7$ cells to 9.6 ± 0.88 and 7.0 ± 0.47 pmol/$10^7$ cells at 30 and 60 min, respectively. Reductions in $PIP_3$ levels were also dependent upon the dose of chimeric toxin employed (data not shown). A concomitant increase in the levels of PI-3,4-P2 (Fig. 4B) was observed from a baseline of 0.33 ± 0.03 pmol/$10^7$ cells to 0.67 ± 0.13 and 1.3 ± 0.03 at 30 and 60 min, respectively. Similar results were observed with BMMC (data not shown). It should be noted that in the absence of CdtA and CdtC, CdtB$^{wt}$ was not able to alter phosphatidylinositol levels.

The next series of experiments focused on whether CdtB$^{Fc\varepsilon}$ was capable of inhibiting antigen-IgE-FcεRI mediated activation of mast cells. For these studies we employed both RBL cells and murine BMMC and assessed the ability of the toxin to inhibit

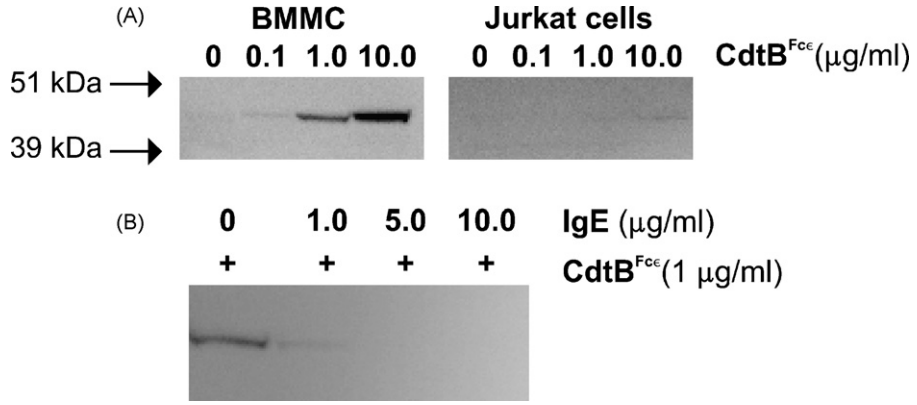


**Fig. 3.** Selective binding of CdtB$^{Fc\varepsilon}$ to mast cells. BMMC and Jurkat cells were exposed to varying amounts of CdtB$^{Fc\varepsilon}$ for 60 min at 4 °C (panel A). The cells were washed, solubilized and analyzed by SDS-PAGE and Western Blot using anti-CdtB mAb. CdtB$^{Fc\varepsilon}$ was observed in BMMC samples, but not with Jurkat cells. Similar results were observed with RBL cells (data not shown). It should be noted that CdtB$^{wt}$ does not bind to either Jurkat cells or BMMC (data not shown). Panel B is a Western blot demonstrating that the binding of CdtB$^{Fc\varepsilon}$ to BMMC can be blocked by pre-exposing cells (30 min) to varying amounts of IgE prior to the addition of chimeric toxin.

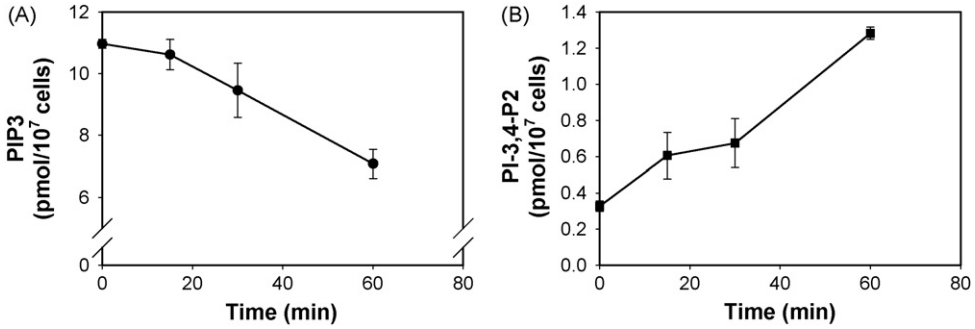


**Fig. 4.** Analysis of CdtB$^{Fc\varepsilon}$-induced changes in PIP3 and PI-3,4-P2 levels in RBL cells. RBL cells were incubated with CdtB$^{Fc\varepsilon}$ (10 μg/ml) for 15–60 min. Lipids were then extracted; PIP3 (panel A) and PI-3,4-P2 (panel B) levels were determined by ELISA. Results are the mean ± S.D. of three experiments. The chimeric toxin induced a time dependent reduction in PIP3 and increase in PI-3,4-P2.

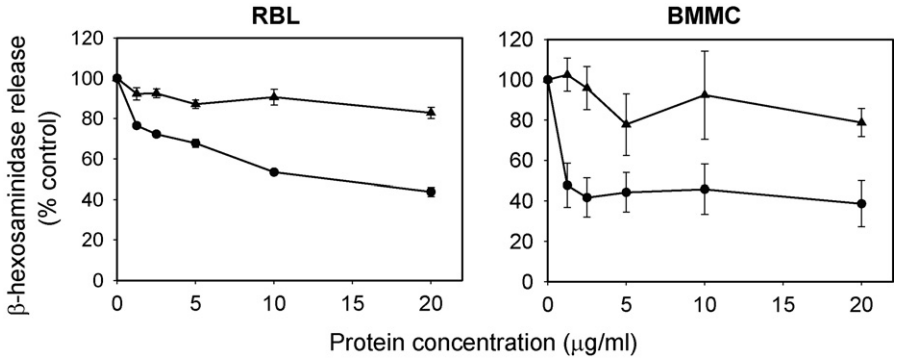


**Fig. 5.** Effect of CdtB$^{Fc\varepsilon}$ on mast cell degranulation. RBL cells (left panel) and BMMC (right panel) were sensitized to anti-DNP IgE. Varying concentrations of CdtB$^{Fc\varepsilon}$ (circles) or CdtB$^{wt}$ (triangles) were added to cells 60 min prior to exposure to antigen (DNP-BSA). Degranulation was measured 30 min after the addition of antigen as a function of the release of β-hexosaminidase. Data are expressed as percent control (sensitized cells treated with antigen in the absence of toxin) and are the mean ± S.D. of three experiments. CdtB$^{Fc\varepsilon}$ inhibited degranulation in both RBL and BMMC. All data points are statistically significant differences ($p < 0.05$) between CdtB$^{Fc\varepsilon}$ and CdtB$^{wt}$ except for the 10 μg/ml values for BMMC ($p = 0.138$).

antigen (DNP)-induced degranulation in anti-DNP IgE sensitized cells. As shown in Fig. 5, CdtB$^{Fc\varepsilon}$-treated RBL cells exhibit a dose-dependent inhibition of DNP-induced degranulation measured as a function of β-hexosaminidase release. Degranulation was reduced from 100% in control cells (exposed to antigen in the absence of of toxin) to 76.5%, 67% and 43% in the presence of 1.25, 5.0 and 20.0 μg/ml CdtB$^{Fc\varepsilon}$, respectively. In contrast, degranulation was minimally impaired in cells similarly treated with CdtB$^{WT}$; β-hexosaminidase release was 81.8% ± 2.7 in cells treated with 20 μg/ml CdtB$^{Wt}$. In a parallel series of experiments, the chimeric toxin was also assessed for its ability to inhibit BMMC degranulation. CdtB$^{Fc\varepsilon}$ reduced β-hexosaminidase release to 47.7% ± 10.9 in the presence of 1.25 μg/ml CdtB$^{Fc\varepsilon}$ and to 38.7% ± 11.4 in the presence of 20 μg/ml chimeric toxin. CdtB$^{WT}$ had a minimal effect on degranulation as exposure to 20 μg/ml only reduced β-hexosaminidase release to 79%. In addition to degranulation, targets downstream of $PIP_3$ were also assessed. Fig. 6 demonstrates the effect of CdtB$^{Fc\varepsilon}$ on IgE-FcεRI-mediated activation of mast cells as a function of phosphorylation of Akt (pAkt), a $PIP_3$-dependent event. Antigen activation of mast cells resulted in increased pAkt within 2 and 5 min in sensitized BMMC. By contrast, BMMC treated with CdtB$^{Fc\varepsilon}$ exhibited reduced pAkt at both 2 and 5 min.

Finally, we wanted to eliminate the possibility that CdtB$^{Fc\varepsilon}$ impairment of mast cell function was the result of reduced IgE binding to BMMC as opposed to direct effects of reduced $PIP_3$ levels. The chimeric toxin was assessed for its ability to reduce IgE sensitization of BMMC. As shown in Fig. 7, BMMC were incubated in medium alone or containing CdtB$^{Fc\varepsilon}$ (controls); additionally, cells were sensitized to IgE (panels C and D) as described in Section 2 and then incubated in medium (panel C) or with CdtB$^{Fc\varepsilon}$ (panel D) for 2 h. Surface IgE binding was assessed by immunofluorescence following staining of cells with anti-IgE conjugated to FITC and fluorescence measured by flow cytometry. CdtB$^{Fc\varepsilon}$ did not reduce IgE binding to mast cells as evident by the level of immunofluorescence; mean channel fluorescence (MCF) was 12.7 in control cells sensitized with IgE and 13.4 in cells pre-exposed to CdtB$^{Fc\varepsilon}$. Background MCF for control cells that were not treated with chimeric toxin or IgE was5.6. It should be noted that the immunofluores-

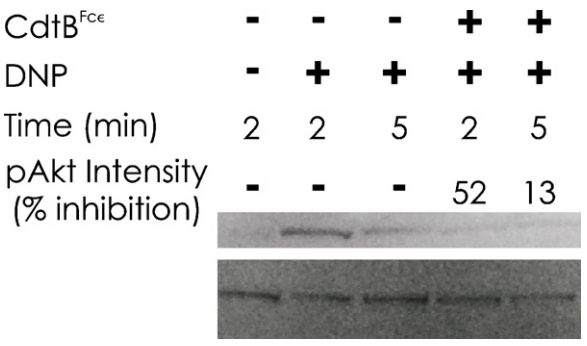


**Fig. 6.** Western blot analysis of pAkt during BMMC activation. BMMC were sensitized with IgE as described in Section 2. Cells were then treated with medium (control) or CdtB$^{Fc\varepsilon}$ (10 μg/ml) for 60 min and challenged with DNP-BSA for the time indicated. Replicate samples were pooled, the cells solubilized, fractionated by SDS-PAGE and analyzed for Akt and pAkt by Western blot. DNP-BSA induced phosphorylation of Akt within 2–5 min. Akt phosphorylation was reduced in the presence of CdtB$^{Fc\varepsilon}$. The pAkt intensity is indicated and expressed as inhibition of the relative control for both 2 and 5 min. Results are representative of three experiments.

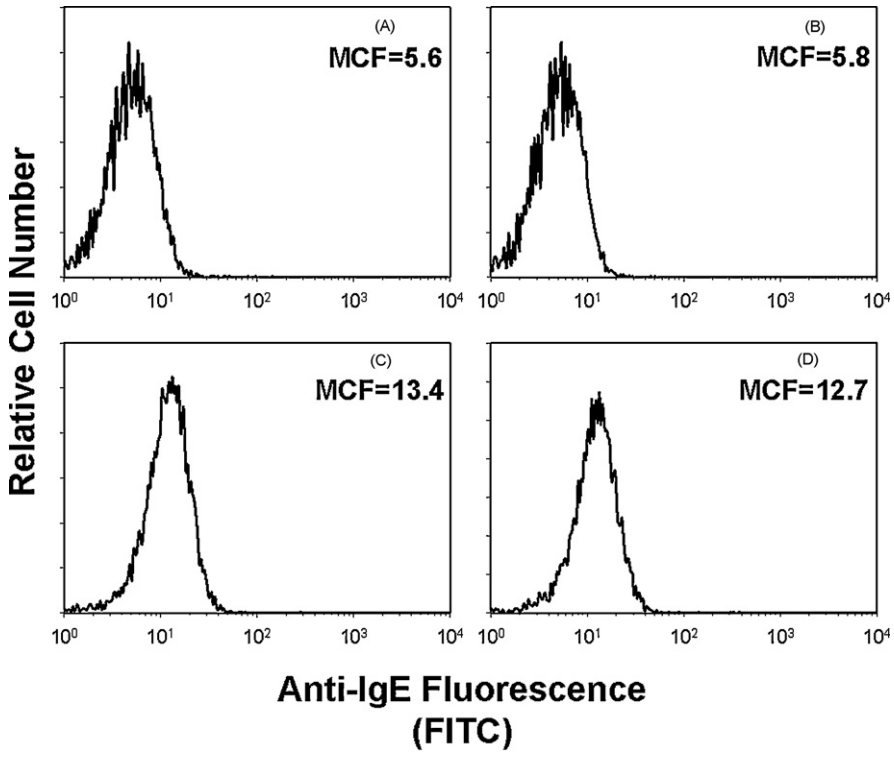


**Fig. 7.** Assessment of CdtB$^{Fc\varepsilon}$ for effects on IgE binding to mast cells. BMMC were incubated in medium alone (panel A), medium containing CdtB$^{Fc\varepsilon}$ (panel B; 10 μg/ml) or cells were sensitized with IgE (panels C and D) as described in Section 2. The cells were then exposed to medium only (panels A–C) or CdtB$^{Fc\varepsilon}$ (panel D; 10 μg/ml) for 2 h. The level of IgE bound to BMMC was determined by flow cytometry following staining with anti-IgE antibody conjugated to FITC. Mean channel fluorescence (MCF) is shown and indicates that similar amounts of IgE were present on BMMC in the presence or absence of chimeric toxin. It should also be noted that the anti-IgE antibody does not recognize CdtB$^{Fc\varepsilon}$ (panel B). Results are representative of three experiments.

cence analysis assesses surface IgE as the anti-IgE antibody did not recognize CdtB$^{Fc\varepsilon}$ (panel B; MCF = 5.8).

## 4. Discussion

The Cdts are a family of heat-labile protein cytotoxins produced by several different bacterial species including diarrheal disease-causing enteropathogens such as *Escherichia coli*, *Campylobacter jejuni*, Shigella species, *Haemophilus ducreyi* and *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* (Comayras et al., 1997; Okuda et al., 1997, 1995; Scott and Kaper, 1994; Pickett et al., 1994; Mayer et al., 1999; Pickett and Whitehouse, 1999). Cdts are encoded by three genes, designated *cdtA*, *cdtB*, and *cdtC*, which are arranged as an apparent operon encoding three polypeptides designated CdtA, CdtB and CdtC with apparent molecular masses of 28, 32 and 20 kDa, respectively, that form a heterotrimeric holotoxin (Shenker et al., 1999, 2000, 2001; Pickett and Whitehouse, 1999; De Rycke and Oswald, 2001; Thelastam and Frisan, 2004). There is considerable agreement that 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 (Nesic et al., 2004; Elwell et al., 2001; Lara-Tejero and Galan, 2001). In this regard, we have shown that CdtA and CdtC are required for the holotoxin to associate with lipid microdomains within lymphocyte membranes and that the CdtC subunit specifically binds to cholesterol (Boesze-Battaglia et al., 2006, 2009). Moreover, we recently determined that the active subunit, CdtB, functions as a lipid phosphatase (Dlakic, 2001). Specifically, CdtB exhibits $PIP_3$ phosphatase activity similar to that of the tumor suppressor phosphatases, PTEN and SHIP1 (Horn et al., 2004; Seminario et al., 2003). Mutation analysis indicates that CdtB-mediated toxicity correlates with phosphatase activity; furthermore, lymphocytes treated with Cdt exhibit reduced $PIP_3$ levels. Moreover, lymphoid cell line sensitivity to CdtB-induced cell cycle arrest correlates with PTEN deficiencies, elevated basal levels of $PIP_3$ and dependence on the PI-3K signaling pathway for survival and proliferation (Shenker et al., 2007).

In addition to cell proliferation and survival, it is well documented that PIP3 plays a central regulatory role in a diverse array of cell functions involving a wide range of cell types. Thus PIP3 represents a potent target for modulating cell function, in general, and mast cell-mediated inflammatory disorders in particular. We propose that the novel lipid phosphatase activity exhibited by CdtB makes this toxin a potentially useful agent for manipulating cellular responses for both pharmacologic purposes and for advancing our understanding of the regulatory role of PIP3. However, CdtB by itself has minimal capacity to associate with cells and the holotoxin lacks cell specificity as it is capable of binding to a wide range of cell types. In order to harness the pharmacologic potential of CdtB, we chose to develop a chimeric toxin in which we confer cell binding specificity by fusing the toxin's active subunit to the CH2/CH3 region of IgE thereby targeting the fusion toxin to FcεRI-bearing cells. Indeed, we have demonstrated that the chimeric toxin not only retains PIP3 phosphatase activity, but exhibits increased enzymatic activity relative to wildtype toxin. The explanation for this elevation in phosphatase activity is not clear but most likely reflects increased stability of the complex. Additionally, the chimeric toxin selectively associates with cells expressing FcεR1, RBL and BMMC, as opposed to Jurkat cells which do not express this receptor. Evidence that the chimeric toxin binding to cells involves FcεRI is further supported by our observation that IgE can block toxin binding.

It is well established that in order for the Cdt holotoxin to induce toxicity, the active subunit, CdtB, must be internalized following binding. This requirement is consistent with its functioning as a lipid phosphatase requiring that it gains access to intracellular pools of PIP3. Our observations clearly indicate that RBL cells treated with CdtB$^{Fc\varepsilon}$ exhibit reduced intracellular levels of CdtB

substrate (PIP3) and increased levels of enzymatic product (PI-3,4-P2) providing indirect evidence that the chimera is indeed able to gain access to intracellular compartments within these cells. It should be noted that the kinetics of these changes are dependent upon toxin concentration. The ultimate test, however, was whether the toxin was capable of preventing mast cell activation. To this end, we assessed the chimeric toxin for its ability to prevent antigen (DNP-BSA)-IgE-mediated mast cell degranulation. Our results clearly demonstrate that pre-exposure to $CdtB^{Fc\varepsilon}$ results in up to 60% inhibition of antigen-induced degranulation of both RBL and BMMC. Furthermore, the increase in pAkt, normally observed following antigen-induced activation was inhibited in $CdtB^{Fc\varepsilon}$-treated BMMC; control cells treated with $CdtB^{WT}$ were unaffected. Moreover these affects were not the result of the chimeric toxin blocking IgE binding to FcεRI since both control and toxin treated mast cells bound similar amounts of IgE.

The observations reported in this study are the first demonstration that direct depletion of PIP3 via increased degradation results in impaired mast cell function. In this context, it is important to recognize that our extensive knowledge of the regulatory role for PIP3 in cellular processes in general, and mast cells, in particular, has been derived primarily from indirect evidence in which the synthesis of PIP3 has been altered or degradation blocked. As a key regulatory molecule, it is essential that $PIP_3$ be maintained at low levels until its synthesis is stimulated by a variety of signals involving activation of PI-3K which utilizes PI(4,5)P2 to generate PIP3 (Nadler et al., 2001; Bachelet et al., 2006; Furumoto et al., 2006; Huber et al., 2002, 1998). There are essentially three classes of PI-3K (I, II, and III); class I is subdivided into two subclasses, IA and IB. It is generally accepted that class IA PI-3K isoforms are involved in FcεRI signaling (Kim et al., 2008). Inhibition of PI-3K activity via mutation or inhibitor results in defective mast cell degranulation and cytokine production as well as anaphylactic reactions *in vivo* (Rivera and Olivera, 2008, 2007; Kim et al., 2008; Marone et al., 2008). It should be noted that similar results were not noted for all isoforms suggesting a high level of redundancy of regulatory PI-3K isoforms in mast cell function. As is often the case with enzyme inhibitors, the specificity of PI-3K inhibitors is questionable; for example, wortmannin and LY294002 target a broad range of PI-3K related enzymes (Marone et al., 2008). The importance of PIP3 in mast cell responsiveness has also been demonstrated in studies that involve reduced expression of the degradative enzymes, PTEN, SHIP 1 and 2, resulting in sustained increases in intracellular PIP3; these increases represent a "gain of function" which is manifest in mast cells as a state of hyperreactivity (Seminario and Wange, 2002; Kyrstal, 2000; March and Ravichandran, 2002).

In summary, the observations reported in this paper provide further evidence that CdtB represents a highly potent lipid phosphatase. These observations are consistent with our previous demonstration that the action of the Cdt holotoxin is dependent upon CdtB-mediated depletion of intracellular levels of PIP3. It should be noted that our previous studies were based upon the toxins ability to induce cell cycle arrest and subsequent cell death. We now demonstrate that this bacterial-derived phosphatase is also capable of inhibiting a PIP3-dependent functions that do not involve proliferation. Moreover, these studies further establish the central regulatory role of this signaling lipid in regulating mast cell function and provides further evidence that PIP3 is a potent target for pharmacologic intervention to modify mast cell activity and associated pathologic processes.

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