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# Upregulation of the Host SLC11A1 Gene by *Clostridium difficile* Toxin B Facilitates Glucosylation of Rho GTPases and Enhances Toxin Lethality

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**Pseudomembranous enterocolitis associated with *Clostridium difficile* infection is an important cause of morbidity and mortality in patients being treated with antibiotics. Two closely related large protein toxins produced by *C. difficile*, TcdA and TcdB, which act identically but at different efficiencies to glucosylate low-molecular-weight Rho GTPases, underlie the microbe's pathogenicity. Using antisense RNA encoded by a library of human expressed sequence tags (ESTs), we randomly inactivated host chromosomal genes in HeLa cells and isolated clones that survived exposure to ordinarily lethal doses of TcdB. This phenotypic screening and subsequent analysis identified solute carrier family 11 member 1 (SLC11A1; formerly NRAMP1), a divalent cation transporter crucial to host defense against certain microbes, as an enhancer of TcdB lethality. Whereas SLC11A1 normally is poorly expressed in human cells of nonmyeloid lineage, TcdB increased SLC11A1 mRNA abundance in such cells through the actions of the RNA-binding protein HuR. We show that short hairpin RNA (shRNA) directed against SLC11A1 reduced TcdB glucosylation of small Rho GTPases and, consequently, toxin lethality. Consistent with the previously known role of SLC11A1 in cation transport, these effects were enhanced by elevation of $Mn^{2+}$ in media; conversely, they were decreased by treatment with a chelator of divalent cations. Our findings reveal an unsuspected role for SLC11A1 in determining *C. difficile* pathogenicity, demonstrate the novel ability of a bacterial toxin to increase its cytotoxicity, establish a mechanistic basis for these effects, and suggest a therapeutic approach to mitigate cell killing by *C. difficile* toxins A and B.**

*Clostridium difficile*, which was first identified as a pathogen more than 50 years ago (1), is now recognized as the leading cause of antibiotic-associated diarrhea (AAD) and pseudomembranous colitis in the United States and Europe (2–4). During the past decade, the emergence of hypervirulent and antibiotic-resistant strains has resulted in a dramatic increase in mortality among infected patients (for reviews, see references 5, 6, and 7). *C. difficile* is an obligate anaerobe that produces two major virulence factors, known as toxin A and toxin B (TcdA and TcdB), which account largely for the clinical features of *C. difficile*-induced diseases. These toxins are similar in sequence, structure, and mechanism of action (8), although TcdB is 1,000 times more lethal to mammalian cells than TcdA (9). The N-terminal domains of TcdA and TcdB, which encode glucosyltransferase activity, are liberated from the intact toxins by autocatalytic cleavage in the cytosol of affected cells (10, 11), enabling transfer of glucose moieties from UDP glucose to specific threonine residues on low-molecular-weight GTPases of the Rho family (8, 12). Glucosylation of these residues inactivates the GTPases, disrupts multiple signaling cascades, and causes massive release of inflammatory cytokines and cell death (for reviews, see references 13, 14, and 15).

The outcome of exposure to pathogens and toxins is known to be affected by the actions of host genes (for reviews, see references 16 and 17). Whereas some host genes defend against detrimental effects of infections, others are exploited to assist pathogen entry, propagation, or release from infected cells or to promote the actions of toxic moieties produced by the pathogen (18–23). Pathogen-exploited host genes have been termed CGEPs (for cellular genes exploited by pathogens) (22, 24). Earlier work from our laboratory and others has established the usefulness of random gene inactivation and phenotype-based genetic screening to identify such genes (20, 22, 25–27).

Here, we report the discovery, using these strategies, of the role of solute carrier family 11 member 1 (SLC11A1; formerly known as natural resistance-associated macrophage protein 1 [NRAMP1]), a host cell transmembrane protein previously implicated in autoimmune disease and in host defense against infectious diseases (for reviews, see references 28 to 31), in promoting TcdB lethality. We show that SLC11A1 expression, which normally is detectable only in cells of myeloid lineage (32–35), is upregulated in intestinal epithelial and other nonmyeloid cells upon exposure to the toxin. We further demonstrate that manipulation of the extracellular divalent cation concentration can circumvent or mollify the actions of SLC11A1 in enhancing toxin lethality. Our findings reveal a novel mechanism of pathogen exploitation of host gene function and identify a previously unsuspected role of SLC11A1 as a facilitator of *C. difficile* pathogenicity.

## MATERIALS AND METHODS

**Cell culture, construction of EST library, and screening of EST libraries with TcdB.** HeLa cells, which were found to be the most sensitive to TcdB among multiple types of cell lines tested and consequently were used for screening of expressed sequence tag (EST) libraries, were cultured in Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Grand Island, NY) with 10% fetal bovine serum (FBS) (Atlanta Biological, Law-

Received 25 October 2012 Returned for modification 14 December 2012
Accepted 10 May 2013
Published ahead of print 20 May 2013
Editor: S. R. Blanke
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doi:10.1128/IAI.01177-12

renceville, GA). Briefly, a culture of HeLa cells expressing a tetracycline-repressed transactivator (HeLa-tTA) was infected with a collection of lentiviruses containing ~40,000 ESTs (pLEST) (20). Cells acquiring lentivirus were selected by growth in the presence of 600 μg/ml of G418 (Life Technologies) for 2 to 3 weeks (20). The EST library used in screening was constructed as previously described (20). To identify clones that are less sensitive to TcdB killing, cultures containing $2 \times 10^6$ HeLa-tTA EST library cells or a similar number of HeLa-tTA naive cells were plated in a 150-mm culture dish and treated with 2 ng/ml TcdB (List Biological Laboratories, Inc., Campbell, CA). At 3-day intervals, culture media were replaced with fresh media containing the same concentration of toxin until no surviving cells were observed on the plate of naive cells. Subsequent to the identification of SLC11A1 ESTs in HeLa cell clones showing reduced sensitivity to TcdB, HT-29, a human colon cancer cell line that is inherently less sensitive to the toxin but which more closely resembles the cells normally targeted by TcdB, was used for certain studies aimed at elucidating mechanisms of SLC11A1 action; HT-29 cells were cultured in McCoy's 5A (Life Technologies) with 10% FBS. Effects of SLC11A1 also were investigated in the human HL-60 promyelocytic leukemia cell line, which has been employed in earlier work to study SLC11A1 upregulation in response to environmental and cellular stresses (36, 37); these cells were cultured in RPMI (Life Technologies) containing 10% FBS.

**Toxin treatment and cell viability assay.** For cell viability assays, 2,000 cells per well were seeded in 96-well plates, each containing 100 μl of culture medium, 16 h prior to addition of toxin. TcdB isolated from *C. difficile* VPI strain 10463 (Techlab, Blacksburg, VA) was added to each well to the indicated final concentration, and the cells and toxin were incubated at 37°C for 72 h. At the end of toxin treatment, the dye resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) (Sigma-Aldrich, St. Louis, MO), which was used to quantify viable cells, was added to each well at a final concentration of 44 μM, and plates were incubated for 4 h at 37°C. Cell viability was measured by reading the fluorescence resulting from resazurin reduction to resorufin using a microplate spectrofluorometer (Infinite 200; TECAN System Inc., San Jose, CA) at an excitation wavelength ($\lambda_{exc}$) of 560 nm and an emission wavelength ($\lambda_{em}$) of 590 nm. Cell viability was assessed by comparing readings against those obtained for wells that lacked TcdB and is shown as the percentage of surviving cells in the toxin-treated population relative to the number of cells in a similarly cultured cell population not exposed to the toxin (taken as 100%).

**RNA extraction, cDNA synthesis, quantitative real-time PCR, and calculation of relative mRNA level.** Total RNA was isolated using the RNeasy minikit (Qiagen, Valencia, CA). One μg of total RNA was used for cDNA synthesis with the QuantiTect reverse transcription kit (Qiagen). Quantitative real-time reverse transcription-PCRs (RT-PCRs) were performed using the Bio-Rad iCycler detection system and the IQ SYBR green supermix (Bio-Rad, Hercules, CA). The sequences of primer sets (forward and reverse) used for the real-time RT-PCR were the following: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5′ AAGGTCGG AGTCAACGGATTTGGT 3′ and 5′ AGCCTTGACGGTGCCATGGA ATTT 3′), SLC11A1 (5′ CTGGACGAATCCCACTCTGG 3′ and 5′ CGC GCCACCACATACTCAT 3′), and HuR (5′ GCTTGGGCTATGGCTTT GTGAACT 3′ and 5′ CGCTGATGTACAAGTTGGCGTCTT 3′). Relative mRNA abundance was calculated by Bio-Rad IQ5 software using the threshold cycle ($C_T$) value. Normalized gene expression levels were obtained with the $2^{\Delta\Delta CT}$ method, using the GAPDH gene as the reference, and are presented as the fold increase for the cell type and/or experimental condition indicated in each figure legend. All real-time PCR data represent the collective values obtained from three independent experiments, each of which included triplicate determinations. *P* values were calculated using REST 2009 software (Qiagen), which employed the Student's *t* test.

**shRNA and short interfering RNA (siRNA) methods.** Lentiviruses expressing short hairpin RNAs (shRNAs) directed against SLC11A1 mRNA were purchased from Open Biosystems (clones TRCN0000043268 to TRCN0000043272; Thermo Fisher Scientific, Waltham, MA); the shRNA sequences chosen showed 100% identity only to SLC11A1, as determined by a BLAST search. HT-29 cells were infected with lentivirus-shRNA constructs, and a pooled population of cells that stably express a puromycin resistance gene carried by the lentivirus was selected by culturing cells for 2 weeks in the presence of 1 μg/ml puromycin.

siRNAs directed against HuR (HuSi) and mutated HuSi (HuMC), which served as a negative control, were custom synthesized by Thermo Fisher Scientific using previously published sequences (37). The sequences chosen for these siRNAs are the following: HuSi (5′-AAGCCUG UUCAGCAGCAUUGG-3′) and HuMC (5′-AAGCCAAUUCAUCAGC AAUGG-3′). The underlined nucleotides indicate sites of mismatch between HuMC and HuSi.

**Immunoblotting.** Proteins in cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad) and transferred to nitrocellulose membranes using the iBlot transfer system (Life Technologies). Proteins interacting with rabbit polyclonal antibody directed against SLC11A1 or HuR were detected by fluorescence-labeled secondary antibody. Western blot images were detected by the Odyssey imaging system (LI-COR Biosciences, Lincoln, NE). Goat anti-SLC11A1 (SC16885) and mouse anti-HuR (SC56709) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse anti-α-tubulin antibody was purchased from Sigma-Aldrich. Mouse anti-TcdB antibody (GTX40413) was purchased from GeneTex (Irvine, CA). IRDye-conjugated secondary antibodies were purchased from LI-COR.

**Glucosylation assays.** HeLa cells were plated into 6-well plates ($4 \times 10^5$ cells/well) 1 day before addition of TcdB toxin. Two hours prior to toxin treatment, the medium was replaced with fresh DMEM or with DMEM containing either 1 mM EDTA or 100 μM $MnCl_2$. Cells were treated with 10 pg/ml of TcdB for 4 h and then detached from plates by addition of 200 μl of 1× trypsin (Life Technologies). Cell pellets were washed three times with ice-cold phosphate-buffered saline (PBS), resuspended in glucosylation reaction buffer (2 mM $MgCl_2$, 0.5 mM GDP, 150 mM KCl, 1 mM dithiothreitol [DTT], 50 mM triethanolamine HCl, pH 7.5), and sonicated five times for 20 s each time. After centrifugation (21,000 relative centrifugal force, 3 min), 6 μl of supernatant was removed for use in each glucosylation reaction, which was carried out as described previously (38). Eight-μl aliquots of reaction mixtures containing 30 μM UDP-[$^{14}$C]glucose and 20 μg/ml TcdB were incubated for 1 h at 37°C, and the reactions then were terminated by boiling in 1× SDS sample buffer (Bio-Rad). Proteins in reaction mixtures were separated by 4 to 12% SDS-PAGE and transferred onto a 0.22-μm nitrocellulose membrane. The radioactively labeled protein bands were detected by Typhoon (GE Healthcare Life Sciences, Piscataway, NJ).

**Detection of intracellular toxin.** HeLa cells were plated into 6-well plates at a density of $4 \times 10^5$ cells/well. After 16 h of incubation at 37°C, the culture medium was replaced with ice-cold, serum-free DMEM, or ice-cold DMEM containing either 1 mM EDTA or 100 μM $MnCl_2$, and placed on ice for 10 min. TcdB was added to a final concentration of 200 pg/ml, and the plates were transferred to a 37°C incubator for 30 min to allow toxin endocytosis. Cells were washed five times with ice-cold PBS and then lysed by addition of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate). The supernatant was subjected to Western blotting and image analysis as described above. The anti-TcdB antibody was purchased from GeneTex, Inc. (San Antonio, TX).

## RESULTS

**Isolation and characterization of human cell clones showing reduced sensitivity to TcdB.** The human cervical carcinoma HeLa cell line previously has been reported to be highly sensitive to TcdB and has been used extensively for studies of TcdB actions (9, 39). We found HeLa cells to be the most sensitive to TcdB among the multiple types of cell lines we tested and to show a minimal background of spontaneous resistance; thus, we used HeLa cells to screen for ESTs that alter TcdB lethality. Three plates, each con-

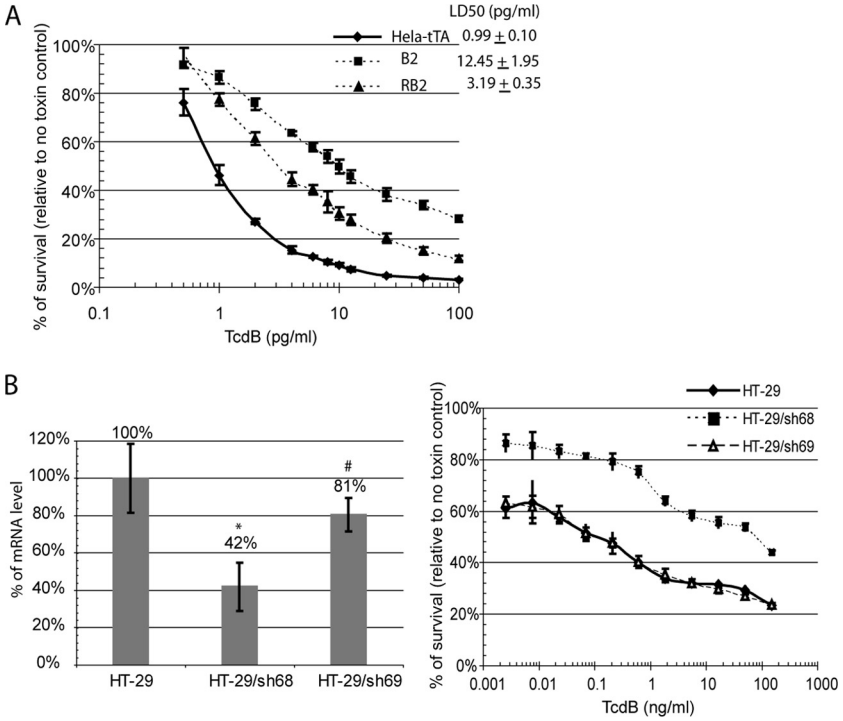


**FIG 1** Effects of genetic manipulations that alter SLC11A1 expression on cellular sensitivity to TcdB exposure, as measured by resazurin assay. (A) Cytotoxicity of TcdB was measured by cell viability assay in parental HeLa-tTA cells, in clone TcdBR-B2 (B2), which was isolated by screening as described in the text, and in reconstituted, pooled cells expressing the same SLC11A1 EST as that present in clone B2 (i.e., RB2). The $LD_{50}$ values shown at the top right were calculated from the graph, which presents data from one of three independent experiments that show similar results. Mean values and standard deviations from the means were calculated from seven determinations done at each TcdB concentration. (B, left) Relative SLC11A1 mRNA abundance determined by real-time RT-PCR in HT-29 cells subjected to knockdown of SLC11A1 expression by the indicated shRNAs. The bars indicate the percentages of SLC11A1 mRNA detected in HT-29 cells containing each shRNA relative to SLC11A1 mRNA detected in naive HT-29 cells that had not received shRNA (taken as 100%). The data shown are mean values from three independent experiments, each of which included triplicate determinations. The error bars indicate standard deviations. *, $P < 0.05$; #, $P > 0.09$. *P* values were calculated using REST 2009 software (Qiagen), which employed the Student *t* test. The error bars indicate the standard deviations from the means. (Right) Cytotoxicity of TcdB in the cells shown in the left panel. All cytotoxicity assays (A and B) were done 72 h after exposure of cells to toxin as indicated in Materials and Methods. Toxin concentrations are shown. Three independent experiments were carried out and gave similar results; the graph presents data from representative experiments and shows the mean values obtained from seven technical repeats. HT-29/sh68 is a pooled cell population of cells stably infected with a lentivirus shRNA construct corresponding to Open Biosystems number TRCN0000043268, and HT-29/sh69 contains lentivirus shRNA corresponding to Open Biosystems number TRCN0000043271.

taining $10^6$ HeLa cells expressing a tetracycline-dependent transactivator (i.e., HeLa-tTA cells), were infected with a library of human ESTs carried by lentivirus (20), resulting in the establishment of three independent HeLa-tTA EST populations after G418 selection. Before TcdB addition, $2 \times 10^6$ cells from each population were plated onto a 150-mm plate. Subsequent toxin treatment and maintenance during screening for clones that showed reduced toxin sensitivity was as described in Materials and Methods. A plate that contained $\sim 2 \times 10^6$ uninfected cells was tested concurrently. After three rounds of TcdB treatment, no surviving cells were detected on cultures of naive parental HeLa cells, suggesting a frequency of spontaneous resistance to TcdB of less than $2 \times 10^{-6}$ under the conditions tested.

Twenty to thirty surviving colonies were observed on each of the three HeLa-tTA EST plates, and these colonies were expanded by addition of fresh media until they reached a size suitable for isolation; the cells in each colony then were cloned for further analysis. Retesting of these survivors indicated that more than two-thirds of the clones had acquired heritably reduced toxin sensitivity; some, but not all, of these clones showed regulation of this phenotype by tetracycline, consistent with evidence that such regulation can be affected by the chromosomal site of lentivirus insertion (our unpublished data). Using primers complementary to vector sequences bracketing EST inserts, ESTs inserted into the chromosomes were PCR amplified and sequenced. Ten distinct ESTs were identified among the approximately 60 clones we picked. We reintroduced each of these ESTs back into naive HeLa-tTA cells by lentiviral infection and found that three of the 10 ESTs resulted in decreased TcdB lethality. The EST from clone B2, which had resulted in the greatest (11-fold) increase in 50% lethal dose ($LD_{50}$) in the original isolate, produced a 3-fold higher $LD_{50}$ in a reconstituted pool of cells infected with lentivirus expressing the EST (B2 versus RB2) (Fig. 1A). Both clone B2 and the pool of RB2 cells exhibited an increased $LD_{50}$ to *C. difficile* toxin A (TcdA) (data not shown), which was shown in previous studies to have the same UDP-glucosyltransferase activity as TcdB but to require different receptors for binding to cells (40), indicating that the effects of the B2 EST are not receptor specific. The role of the host gene represented by the B2 EST in TcdB toxicity is the subject of this report.

The sequence of the EST identified in clone B2 was found to correspond to an ~350-nucleotide image clone (image 2050382;

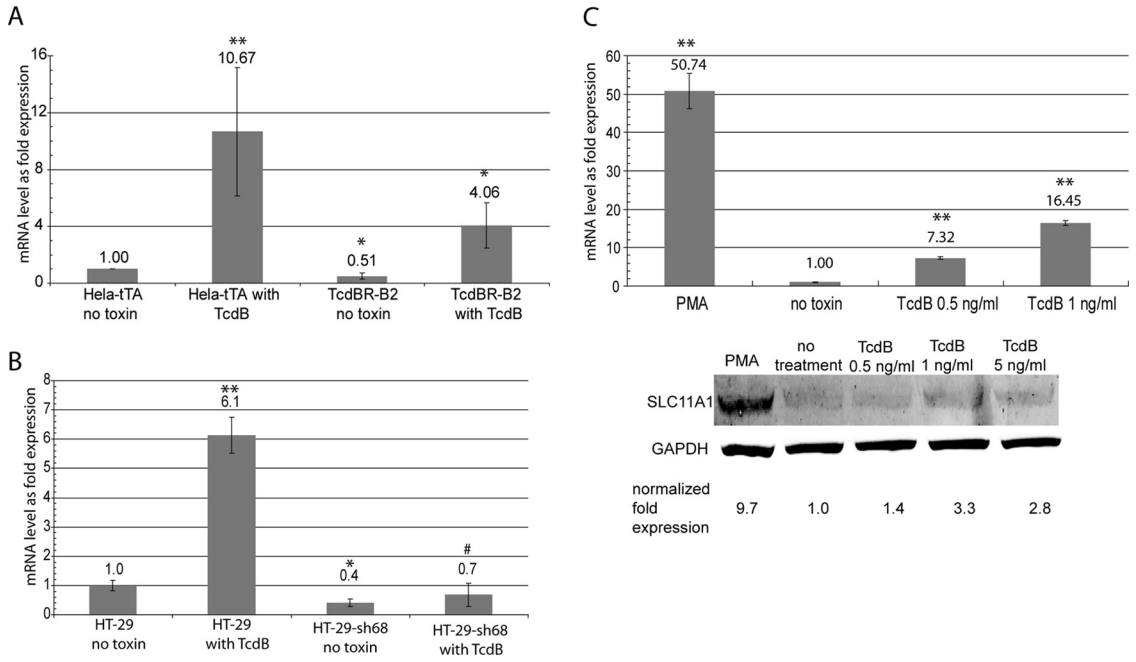


**FIG 2** Effect of altered SLC11A1 expression in response to TcdB treatment of mammalian cell lines. Cell lysates and total RNA were collected after 16 h of toxin exposure. SLC11A1 mRNA abundance was determined by real-time RT-PCR, and the data shown represent the calculated mean values from three independent experiments, each of which included triplicate determinations. *P* values were calculated using REST 2009 software (Qiagen), which employed the Student's *t* test. #, $P > 0.3$; *, $0.01 < P < 0.05$; **, $P < 0.01$. The error bars indicate the standard deviations from the means. (A) SLC11A1 mRNA abundance in HeLa-tTA and TcdBR-B2 cells. TcdB was added at a concentration of 2 pg/ml, and SLC11A1 mRNA abundance in mutant cells and/or cells treated with toxin is shown as the fold expression relative to that of SLC11A1 mRNA expression in HeLa-tTA cells that have not been exposed to toxin (fold expression of these control cells is assigned a value of 1). (B) SLC11A1 mRNA expression in HT-29 and HT-29/sh68. The TcdB concentration is 2 ng/ml, and the relative mRNA level of the mutant cells and/or cells treated with toxin was compared to the level in naive HT-29 cells that have not been exposed to toxin (fold expression in control cells is 1). (C) Expression of SLC11A1 in HL-60 and HL-60 cells treated with TcdB. The concentration of TcdB is indicated. HL-60 treated with 10 ng/ml of phorbol-12-myristate-13-acetate (PMA) served as a positive control for SLC11A1 induction (37). Upper panel, SLC11A1 mRNA expression level; lower panel, SLC11A1 protein abundance detected by Western blotting. The detected abundance of mRNA or protein in toxin-treated cells was compared to the abundance in untreated HL-60 cells (fold expression is 1).

GenBank accession number AI312979) inserted into the lentiviral vector in antisense orientation. A BLAT (UC Santa Cruz) search showed that the EST corresponds to a sequence extending from near the end of the coding region of the solute carrier family 11 member A1 (SLC11A1) gene, also known as Nramp1 (for natural resistance-associated macrophage protein 1), into the 3′-untranslated region. SLC11A1 has been widely studied previously because of its effects on the outcome of multiple types of bacterial infections, on macrophage activation, on autoimmune disease, and on anticancer drug treatment (for reviews, see references 41, 42, and 43). BLAST analysis showed that the sequence present in the cloned EST is specific to SLC11A1 and contains no homology with the SLC11A1 related gene, SLC11A2.

To confirm that the decrease in TcdB susceptibility observed in clone B2 and in RB2 cells results from decreased expression of the SLC11A1 gene and to learn whether the reduced toxin sensitivity observed in HeLa cells extends to intestinal cells, the cell type targeted by *C. difficile* infection in humans (44, 45), we tested the effects of shRNAs directed against several different regions of SLC11A1 in a pool of stably infected cells of the human intestinal colon adenocarcinoma cell line, HT-29 (see Materials and Methods). The effectiveness of shRNA in reducing SLC11A1 mRNA expression was determined by real-time RT-PCR. We found that the knockdown level of SLC11A1 correlates with the cell sensitivity to TcdB. sh69, an shRNA that only slightly reduced SLC11A1 mRNA levels (i.e., to 81% of the initial SLC11A abundance; $P = 0.09$; Fig. 1B, left), yielded a toxin sensitivity profile (Fig. 1B, right, open triangle) similar to the one observed for cells that received no shRNA (filled diamonds). In contrast, sh68, which produced the best SLC11A1 knockdown of the shRNAs we tested (i.e., to 42% of the initial SLC11A1 abundance), reduced TcdB sensitivity over a wide range of toxin doses (Fig. 1, right, filled squares), providing direct evidence of the effect of decreased SLC11A1 expression on TcdB toxicity.

**Effect of TcdB exposure on expression of SLC11A1.** As already noted, SLC11A1 is known to be expressed predominantly in monocyte/macrophage and dendritic cell lines, and expression of this gene has been reported to be almost undetectable in most other cell lineages (32, 46). Similarly, we also found only minimal amounts of SLC11A1 mRNA during real-time RT-PCR analysis of mRNA from the HeLa cell and HT-29 cell lines we tested. SLC11A1 protein was not detected in HeLa or HT-29 cells by Western blotting. The paradox that shRNA directed against SLC11A1 affected TcdB toxicity in cells where the baseline expression of the gene is only minimal led us to hypothesize that toxin exposure leads to an increase in SLC11A1 production, and that the shRNA acts by blocking this increase. We directly tested this possibility by determining the effects of the toxin on SLC11A1 expression using a dose of TcdB (i.e., 2 pg/ml) that was not lethal even to the parental cell line during 16 h of toxin exposure but which was

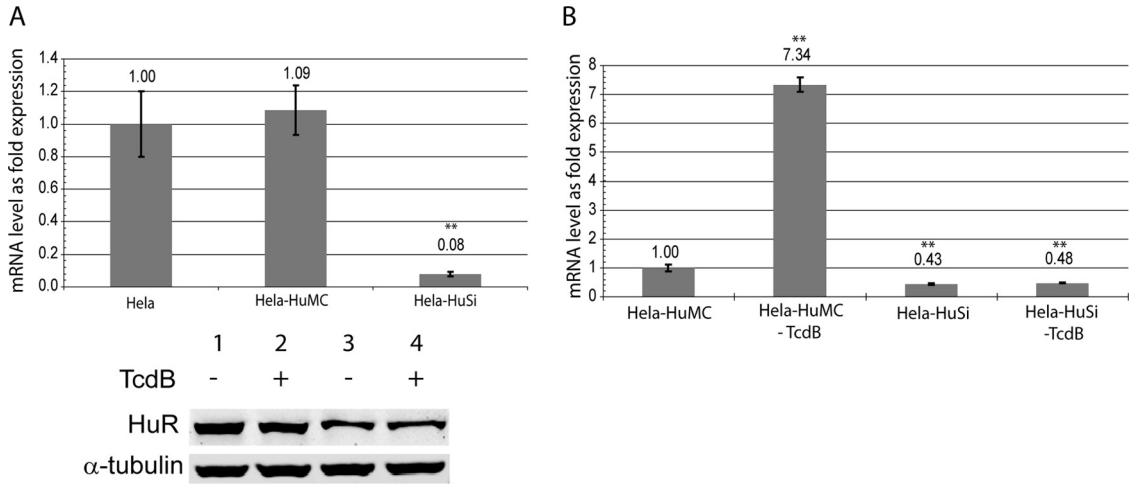


**FIG 3** HuR protein is required for the SLC11A1 mRNA upregulation induced by TcdB. (A) Expression level of HuR. HeLa cells were transiently transfected with siRNA. Sixteen h after transfection, each sample of transfected cells was split into two wells, and TcdB was added at a final concentration of 2 pg/ml in one of the wells. Sixteen h after toxin treatment, the total RNA and protein were collected for further analysis. (Top) HuR mRNA abundance in HeLa cells with no transfection, transfected with HuR mutation control siRNA (HuMc), or transfected with HuR-specific siRNA (HuSi), as determined by real-time PCR. (Bottom) Western blotting showing the protein level of HuR. Lanes 1 and 2, HeLa cells transfected with HuMC; lanes 3 and 4, HeLa cells transfected with HuSi. Cell lysates from lanes 2 and 4 were collected after TcdB treatment. (B) Real-time PCR analysis showing SLC11A1 mRNA level. As mentioned in Materials and Methods, all real-time PCR data represent the calculated mean values from three independent experiments, each of which included triplicate determinations. The *P* values were calculated using REST 2009 software (Qiagen), which employed Student's *t* test. **, $P < 0.01$.

sufficient to yield the cell-rounding phenotype characteristic of inactivation of small Rho GTPases by TcdB (12, 47) in nearly all of the population. As reported previously (39), cells incubated with toxin that had been pretreated at 65°C for 30 min to inactivate their ability to carry out glucosylation of Rho GTPase did not show such rounding. The effect of TcdB exposure on SLC11A1 mRNA abundance was quantified using real-time RT-PCR analysis (Fig. 2A); mRNA of the housekeeping gene GAPDH, which was unaffected by sublethal concentrations of TcdB in high-throughput RNA sequencing (RNA-Seq) analyses (our unpublished data), was used as an internal control. This revealed a 5- to 10-fold increase in SLC11A1 mRNA after exposure of parental HeLa-tTA cells to TcdB (Fig. 2A). An increase in SLC11A1 expression was not detected in cells exposed to heat-inactivated TcdB (data not shown). The level of SLC11A1 expression after exposure of cells to TcdB was sharply diminished in TcdBR-B2 cells (Fig. 2), which, as described above, express the SLC11A1 EST in antisense direction. Elevation of SLC11A1 expression by TcdB also was observed in HT-29 cells (Fig. 2B), and this increase was reversed by cells transfected with shRNA68, which limits SLC11A1 expression, as seen in Fig. 1. In HL-60, a human promyelocytic leukemic cell line that previously has been found to have increased SLC11A1 expression when the cells were induced toward monocyte/macrophage differentiation (34), also showed elevation of SLC11A1 expression when the cells were exposed to TcdB (Fig. 2C). As has been observed during induction of expression of other genes by TcdB (48, 49), the relationship between TcdB concentration and SLC11A1 abundance was not linear.

**TcdB-induced increased abundance of SLC11A1 mRNA requires the RNA binding protein HuR.** Collectively, the results described above establish that exposure of cells to TcdB leads to upregulation of the steady-state level of SLC11A1 mRNA and further indicate that such upregulation is associated with increased TcdB lethality. Earlier work has shown that upregulation of SLC11A1 expression by a variety of agents requires the HuR protein, which binds to an AU-rich element in the 3′-untranslated region of SLC11A1 and stabilizes SLC11A1 transcripts (37). To learn whether HuR is also required for the observed TcdB-mediated increase in SLC11A mRNA, we transfected cells with a small interfering RNA (siRNA) that has been shown previously to specifically reduce HuR expression (37); a mutant siRNA (i.e., HuMC) that lacks the ability to reduce HuR expression (37) was used as a negative control (Fig. 3A). Quantitative real-time RT-PCR analysis and Western blotting (Fig. 3A) demonstrated the effectiveness and specificity of HuSi versus HuMC in reducing HuR expression. Additional quantitative real-time RT-PCR analysis of the effects of such HuR knockdown on SLC11A1 mRNA abundance (Fig. 3B) showed that the increase in SLC11A1 mRNA normally observed following exposure of HeLa cells to TcdB did not occur in cells in which HuR expression was inhibited by HuSi. In contrast, cells transfected with an siRNA that has been mutated to prevent its actions on HuR mRNA expression (37) (i.e., HuMC) continued to show a dramatic elevation of SLC11A1 mRNA expression in response to TcdB exposure (Fig. 3B). These findings indicate that HuR actions are necessary for TcdB-mediated elevation of SLC11A1.

Exposure of cells to phorbol ester treatment leads to translocation of HuR from the nucleus to cytoplasm in HL-60 cells (37), enabling its mRNA-stabilizing actions to occur (50, 51). However, we observed no alteration in the nuclear/cytoplasmic ratio of HuR following treatment of HL-60 cells with TcdB (data not shown). Similarly, we detected no evidence by real-time RT-PCR or Western blotting of activation of HuR mRNA synthesis or protein synthesis by TcdB. Thus, while our results establish that siRNA-mediated reduction of HuR protein interferes with TcdB stimulation of SLC11A1 production, the mechanism underlying the requirement for HuR has not been elucidated.

**Effect of $Mn^{2+}$ on alteration of TcdB sensitivity by SLC11A1 deficiency.** As noted above, the cytotoxicity of *C. difficile* toxins A

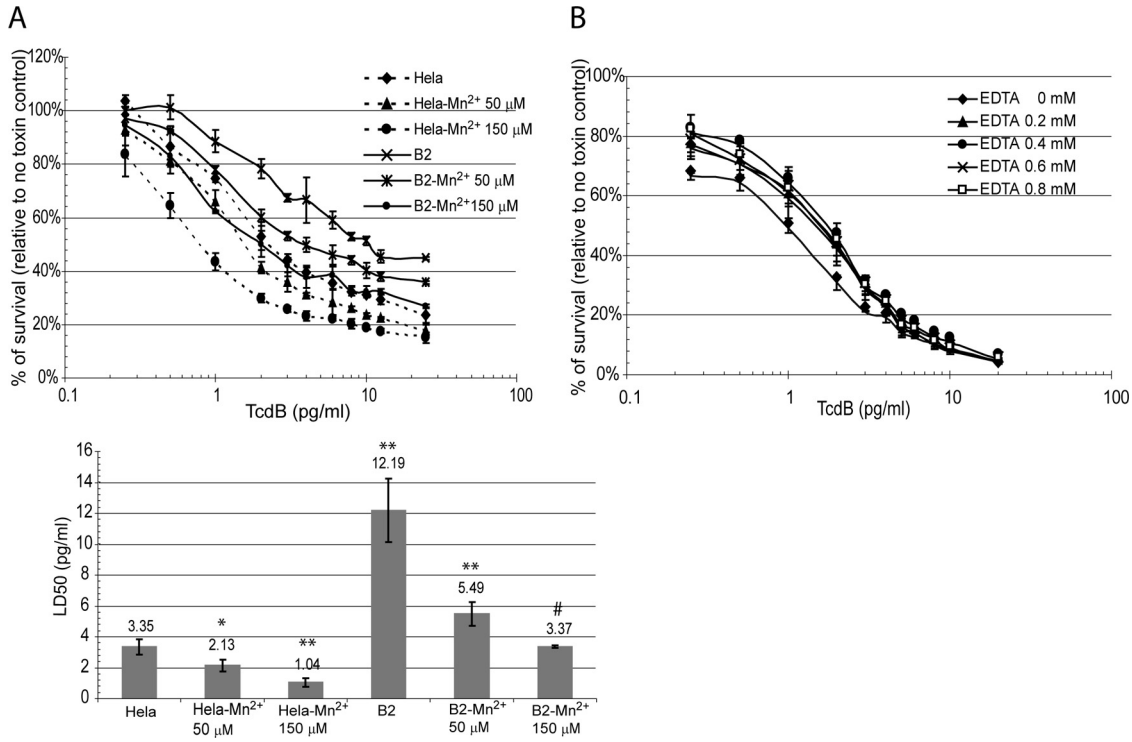


**FIG 4** Effect of divalent cation concentration in cell culture media on cell sensitivity to TcdB. Culture media containing the indicated concentration of $MnCl_2$ were added 30 min before toxin treatment. Cell viability assays were done 72 h after toxin treatment. (A, top) Survival ratio of HeLa-tTa and TcdBR-B2 cells after TcdB treatment in various concentrations of $MnCl_2$. The graph is a representative of three independent experiments. The values shown in the graph represent the means from four technical repeats, and error bars indicate the standard deviations of the means. (Bottom) Calculated $LD_{50}$ (means ± standard deviations) from data shown in the upper panel. We compared the $LD_{50}$ of every sample to that of wild-type HeLa cells ($LD_{50}$, 3.35 pg/ml) and calculated the *P* values using the two-tailed Student's *t* test. **, $P < 0.01$; *, $0.01 < P < 0.05$; #, $P > 0.05$. (B) Effect of EDTA on cell survival after TcdB treatment. Culture media containing the indicated concentration of EDTA were added 30 min before toxin treatment. Cell viability assays were done 72 h after toxin treatment. The graph is a representative of three independent experiments. The values shown in the graph represent the means from four technical repeats, and error bars indicate the standard deviations. For both A and B, the survival ratio of each toxin concentration was the normalized value relative to 100% survival when no TcdB had been added at the indicated $MnCl_2$ or EDTA concentration.

and B results from their activity as cation-dependent UDP-glucosyltransferases (52), which glucosylate the bulk of low-molecular-weight Rho GTPases within 2 to 4 h after toxin exposure. This event activates signaling cascades that disable biological and cellular biochemical functions in the exposed cells and result in cell death approximately 68 to 72 h later (13, 14, 39). Thus, the endpoints for measurement of TcdB-induced glycosylation and TcdB-induced cytotoxicity do not temporally coincide. The glucosyltransferase activity of *C. difficile* toxins is stimulated by multiple divalent cations and is especially sensitive to $Mn^{2+}$ (52). SLC11A1 is known to increase the cytosolic concentration of such cations (53, 54), leading us to hypothesize that the observed effects of altered SLC11A1 expression on both TcdB-induced glucosylation of Rho GTPases and consequent cytotoxicity can be circumvented by increasing the concentration of $Mn^{2+}$ in the culture media. Indeed, we found that elevating the extracellular concentration of $Mn^{2+}$ increased Rho GTPase glucosylation and toxin sensitivity of both TcdBR-B2 cells and parental HeLa-tTA cells (Fig. 4A). These findings suggest that the effects of SLC11A1 on TcdB lethality result at least in part from the known actions of SLC11A1 on $Mn^{2+}$ transport. Consistent with this notion, we found that addition of the cation-chelating agent EDTA, which is routinely used to reduce the concentration of free divalent cations, to culture media reduced TcdB cytotoxicity (Fig. 4B). Consistent with the known role of $Mn^{2+}$ in glucosylation of Rho GTPase by TcdB, addition of EDTA to culture media also reduced glycosylation of the substrate, whereas elevating the $Mn^{2+}$ concentration increased it (Fig. 5A). The amount of toxin detected in cells, which reflects events that affect binding and/or endocytosis, was not changed by the addition of either $Mn^{2+}$ or EDTA (Fig. 5B), suggesting that these agents do not affect events that occur prior to the inactivation of small Rho GTPase by toxin.

## DISCUSSION

We applied a previously developed gene inactivation method (20) and phenotypic screening to identify cells that survived ordinarily lethal doses of *C. difficile* toxin B. This approach revealed that the gene encoding solute carrier family 11 member 1 (SLC11A1) in host cells modulates cellular sensitivity to this toxin, and that exposure of cells to TcdB increases expression of SLC11A1. The role of SLC11A1 as a determinant of *C. difficile* toxin sensitivity was confirmed by reconstituting the phenotype in naive cells as well as by shRNA experiments.

In macrophages, the SLC11A1 protein is localized to acidic endosomes and lysosomes (55, 56), where it can restrict replication of intercellular pathogens by acting as a metal ion efflux pump to transfer divalent cations, principally $Mn^{2+}$ and $Fe^{2+}$, from the phagolysosome to the cytosol (53, 54, 57). This action

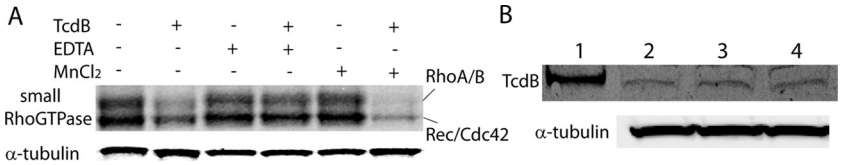


**FIG 5** Effect of alteration of divalent cation concentration on the glucosyltransferase activity of TcdB and on the toxin absorption in HeLa cells. (A) The level of glucosylated small Rho GTPases by TcdB. (Top) Autoradiograph showing small Rho GTPases labeled *in vitro* by UDP-[$^{14}$C]glucose. The signals of two bands reflect the fraction of the small Rho GTPases that are not glucosylated *in vivo* by TcdB but that are substrates for TcdB substrates, such as RhoA, RhoB, Rac1, and cdc42 (38). (Bottom) Western blot analysis of the same membrane showing the amount of α-tubulin as a loading control. As has already been mentioned in Materials and Methods, the final concentrations of EDTA and $MnCl_2$ are 1 mM and 100 μM, respectively, TcdB is at 10 pg/ml, and the cell lysates were collected 4 h after toxin treatment. The figure is the representative of two independent experiments with similar results. (B) Western blot analysis showing TcdB absorbed by cells. Lane 1, 0.5 ng of purified TcdB; lane 2, HeLa cells in regular media; lane 3, HeLa cells in medium containing additional 1.0 mM EDTA; lane 4, HeLa cells in media containing additional 100 μM $MnCl_2$. As has already been mentioned in Materials and Methods, the TcdB concentration for lanes 2 to 4 is 200 pg/ml, and the cell lysates were collected 30 min after toxin treatment.

aids cellular defense against at least some pathogens, and mutations that result in the loss of SLC11A1 function have been shown to increase host susceptibility to *Mycobacterium*, *Salmonella* species, and *Leishmania* species (58–60). In humans, SLC11A1 is expressed constitutively in the myeloid lineage, including monocytes, macrophages, polymorphonuclear neutrophils, and dendritic cells (34–36). It has been shown previously that expression of SLC11A1 is normally minimal, but that it can be upregulated by bacterial lipopolysaccharide, by interferon, and by granulocyte/macrophage colony-stimulating factor in such cells (46, 61). Consistent with earlier evidence that the production of SLC11A1 is minimal in most cell lineages (62), we observed that expression of SLC11A1 was barely detectable in the absence of TcdB exposure in the HeLa-derived cell line we used for EST library screening and also in the colon cancer cell line HT-29, but that it was upregulated by TcdB.

The observed upregulation of SLC11A1 expression by *C. difficile* toxin B was shown in our experiments to be associated with an increase in cation-dependent UDP glucosyltransferase activity on host Rho GTPases. We further found that upregulation of SLC11A1 expression requires the function of a second host gene, HuR, which is known from earlier work also to stabilize SLC11A1 mRNA during mycobacterial infection (36, 63). HuR reduces mRNA degradation by interacting with AU-rich elements near their 3′ ends (51, 64–66; for a review, see reference 67). Whereas the cellular location of HuR can be affected by a variety of stresses (68, 69), we found no evidence that TcdB alters HuR stabilization of SLC11A1 mRNA by this mechanism. We have not investigated whether experimentally induced overexpression of HuR is sufficient to alter SLC11A1 mRNA degradation.

A wide variety of host genes are known to be upregulated upon bacterial infection, and such upregulation commonly assists the host in defending itself against pathogens (for reviews, see references 70 and 71). Additionally, pathogens are known to exploit normal host gene functions to enter, reproduce in, or exit from cells (72–74). In the events reported here, the functions of a gene that aids host defense against other bacterial infections are exploited by TcdB to increase toxin activity. Our finding that upregulation of SLC11A1, a known host defense gene, enhances the action of TcdB instead of defending the host against these actions raises the prospect that other host genes upregulated by pathogens may also assist pathogenicity. Additionally, the role we have discovered for SLC11A1 in TcdB toxicity suggests a clinical approach to mitigation of the effects of *C. difficile* toxins. Orally administered chelating agents, which were initially developed to treat heavy metal poisoning (75, 76) and which have been used for other medical purposes as well (77, 78), may prove to be of therapeutic value in reducing the gastrointestinal toxicity of *C. difficile* infection.

## ACKNOWLEDGMENTS

These studies were supported by an award (HDTRA1-06-C-0039-P00011) from the Defense Threat Reduction Agency, by NIH grant AI08619, and by funds granted to S.N.C. by the Friends of Stanford Foundation in Taiwan.

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