Pyknotic cell death induced by *Clostridium difficile* TcdB: chromatin condensation and nuclear blister are induced independently of the glucosyltransferase activity

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**Summary**

TcdA and TcdB are the main pathogenicity factors of *Clostridium difficile*-associated diseases. Both toxins inhibit Rho GTPases, and consequently, apoptosis is induced in the affected cells. We found that TcdB at higher concentrations exhibits cytotoxic effects that are independent on Rho glucosylation. TcdB and the glucosyltransferase-deficient mutant TcdB D286/288N induced pyknotic cell death which was associated with chromatin condensation and reduced H3 phosphorylation. Affected cells showed ballooning of the nuclear envelope and loss of the integrity of the plasma membrane. Furthermore, pyknotic cells were positively stained with dihydroethidium indicating production of reactive oxygen species. In line with this, pyknosis was reduced by apocynin, an inhibitor of the NAPD oxidase. Bafilomycin A1 prevented cytotoxic effects showing that the newly observed pyknosis depends on intracellular action of TcdB rather than on a receptor-mediated effect. Blister formation and chromatin condensation was specifically induced by the glucosyltransferase domain of TcdB from strain VPI10473 since neither TcdBF from cdi1470 nor the chimera of TcdB harbouring the glucosyltransferase domain of TcdBF was able to induce these effects. In summary, TcdB induces two different and independent phenotypes: (i) cell rounding due to glucosylation of Rho GTPases and (ii) shrinkage of cells and nuclear blister induced by the high concentrations of TcdB independent of Rho glucosylation.

**Introduction**

*Clostridium difficile* toxins are causative for clinical symptoms of *C. difficile*-associated diseases. The toxins induce diarrhoea, inflammation, disturbance of the intestinal barrier function, lesions of the intestinal epithelium, and induce infiltration of granulocytes into the mucosa. Both toxins, TcdA and TcdB, monoglucosylate Rho GTPases (Rho, Rac, Cdc42) within the host cell but also glucosylate Rap and Ras GTPases as second-tier substrates (Zeiser et al., 2013). Glucosylation of substrate GTPases results in functional inactivation of these molecular switches with complete abrogation of downstream signalling. Glucosylation of Rho GTPases affects all actin cytoskeleton-dependent structures like tight junctions, focal adhesions, and membrane protrusions. In addition, actin cytoskeleton-dependent processes like phagocytosis, migration and cytokinesis are likewise affected (Huelsenbeck et al., 2009). Inactivation of Rho GTPases induces immediate to early pro-inflammatory responses such as upregulation of interleukin-8 or macrophage inflammatory protein MIP-1α (Kim et al., 2002; Meyer et al., 2007) and eventually triggers apoptosis of affected cells (Nottrott et al., 2007). Apoptosis that is based on Rho inactivation can be induced by TcdA (Brito et al., 2002; Matte et al., 2009) as well as by TcdB (Huelsenbeck et al., 2007; Matarrese et al., 2007). Although apoptosis is the predominant type of cell death associated with TcdA and TcdB, few descriptions of necrosis or non-apoptotic cell death suggest further effects of these toxins (Solomon et al., 2005; Lica et al., 2011). However, either the observed non-apoptotic cell death is related to very specific cell cycle phases, since only non-proliferating, confluent HT-29 cells show signs of this kind of cell fate (Lica et al., 2011). Or highly sensitive cells like monocytes are reported to exhibit signs of non-apoptotic cell death related with release of lactate dehydrogenase as surrogate for necrotic cell lysis (Solomon et al., 2005). A very recent report by Chumbler and co-workers also shows that high concentrations of TcdB induce necrosis of target cells that is independent on the autoprocessing function of TcdB and was also induced by mutated TcdB where the glucosyltransferase activity was affected (Chumbler et al., 2012). We here further characterized the new cytotoxic...
effects towards target cells. Cell death can be classified into four modes with typical morphological features, including apoptosis, autophagy, cornification, and necrosis (Kroemer et al., 2009). Especially apoptosis is the mode of cell death that includes pyknosis with reduction of the cellular and nuclear volume as key events. The present study describes new effects of the glucosyltransferase domain of TcdB that can be classified as pyknosis, associated with special forms of cell death. The rapid TcdB-induced cell death includes production of reactive oxygen species, and effects on the nuclear envelope and chromatin structure.

**Results**

**Correlation of Rac1 glucosylation and cell viability**

The present study describes newly observed effects of TcdB NXN, of which the conserved DXD motif was changed to D286N, D288N. This mutant had no detectable glucosyltransferase activity in *in vitro* [14C]glucosylation assay and was estimated to be at least 5·10^4-fold less active in cell culture assay (Supplementary Fig. S1). A first systematic comparison of TcdB and TcdB NXN was done in concentration-dependent cytotoxicity assays to calculate EC_{50} values for Rac1 glucosylation and cell viability. Figure 1A shows Western blot analyses of non-glucosylated Rac1 of HEp2 cells treated with TcdB, TcdB NXN, and GTDBF-TcdB. GTDBF-TcdB is a chimera of the glucosyltransferase domain (amino acids 1–541) of variant TcdB from *C. difficile* strain 1470 (TcdBF) and the translocation domain of TcdB (amino acids 540–2366) from strain VPI10463. In contrast to TcdB, TcdBF has a different substrate spectrum and glucosylates Rac1 and Ras, but not RhoA or Cdc42 (Huelsenbeck et al., 2007). The identity and homology of the glucosyltransferase domain of both toxins is 79% and 87% respectively (Von Eichel-Streiber et al., 1995). We used the chimera of TcdB and TcdBF to warrant identical uptake, translocation, and cleavage kinetics for direct comparison of the effects of the respective GTDs. TcdB and GTDBF-TcdB both glucosylated Rac1 in a concentration-dependent manner with comparable kinetics. Incubation of cells with TcdB NXN led to a reduced signal of non-glucosylated Rac1 only at the highest applied concentration (3 nM). The double band of non-glucosylated Rac1 in immunoblots is typical for HEp2 cells when probed with antibody clone 23A8 (Genth et al., 2006). The upper band corresponds to the single band at 21 kDa detected with Rac1 antibody clone 23A8. Densitometrical evaluation of Western blots and data from WST assays for determination of cell viability were basis for the graph in Fig. 1B. Shown are data from Rac1 glucosylation (filled symbols) and WST assay (open symbols) from the linear phase of their effects on the respective end-points. The slopes were calculated from the linear regression and EC_{50} values were calculated from non-linear regression (sigmoidal curves) of data points including at least two values within maximum and minimum plateau phase, where applicable. Values are summarized in the table below. The graphs clearly show that Rac1 glucosylation and loss of cell viability induced by wild-type TcdB were detected in concentration ranges differing in three to four magnitudes. Furthermore, TcdB NXN does primarily reduce cell viability at comparable concentrations as TcdB (EC_{50}: 1.1 nM and 0.45 nM respectively) whereas specific Rac1 detection by glucosylation sensitive antibody was diminished only at highest concentration applied (EC_{50}: 0.1 pM for TcdB; a 50% effect could not be determined for TcdB NXN). Concentration dependency of GTDBF-TcdB-catalysed Rac1 was measured and dose–response relation was comparable to that of TcdB (EC_{50}: 0.1 nM and 0.11 nM respectively) whereas GTDBF-TcdB did not affect cell viability within the observed concentration range. These data clearly show that Rac1 glucosylation does not correlate with reduced cell viability. Under these experimental conditions, Rac1 glucosylation and cytotoxicity represent separate and independent end-points, depending on the toxin applied.

**TcdB NXN induces chromatin condensation and blister formation**

The morphological changes of HEp-2 cells induced by different concentrations of TcdB are shown in Fig. 2. TcdB induced the cytopathic effect (cell rounding) at 0.03 nM in >80% of cells after 4 h. At concentrations from 0.3 nM up to 3 nM the percentage of round cells was progressively reduced, whereas the number of shrunken and flat cells increased. At higher concentrations, increasing number of cells showed huge blister when TcdB was applied. These blisters were only observed in flat cells but not in rounded cells. The glucosyltransferase-deficient mutant TcdB NXN had no effect on the cell morphology at low concentration (up to 0.03 nM). From 0.3 nM on, TcdB NXN induced chromatin condensation and blister formation in a number of cells. At 3 nM, almost 100% of cells showed the blister phenotype with condensed chromatin. Figure 2A also illustrates the effect of TcdB on Hep-2 morphology. TcdBF only induced cell rounding from 0.03 nM up to the highest concentration applied. TcdBF did not induce blister formation or chromatin condensation, which is in good accordance with cell viability assay (compare Fig. 1B). Obviously, both phenotypes that were induced by TcdB and TcdB NXN, i.e. rounded cells or shrunken flat cells with blister, appeared in parallel. This is more evident in Fig. 2B where a wider concentration range of TcdB and
a lower magnification was chosen. Cells that were rounded up due to glucosylation of Rho GTPases did not show signs of chromatin condensation and blister formation. The effects of both toxins can be interpreted as follows: at high concentrations both, TcdB and TcdB NXN, induces shrinking of cells and blister formation. This phenomenon appears only in cells where Rho GTPases were not glucosylated to an extent that resulted in cell rounding. Interestingly, TcdB NXN induced condensation of the chromatin in association with blister formation. The condensed chromatin was not observed in all flat and shrunken cells after treatment with wild-type TcdB. We therefore characterized the effect of TcdB and TcdB NXN on the chromatin in more detail. We found that two phenotypes of the nucleus were observed in cells that were treated with TcdB and TcdB NXN. In contrast, TcdB NXN induces loss of viability, although no Rac1 glucosylation is detectable unless loss of viability interferes with analysis. The slopes were calculated from the linear regression, EC50 values were calculated from non-linear regression (sigmoidal curves) of data points including at least two values within maximum and minimum plateau phase, where possible. Values are summarized in the table below.
Fig. 2. Morphological changes induced by TcdB.
A. HEp-2 cells were incubated with different concentrations of wild-type (TcdB) or glucosyltransferase-deficient TcdB (TcdB NXN) for 4 h. At 0.03 nM TcdB induced almost 100% cell rounding whereas TcdB NXN had no effect. At high concentration (3 nM) TcdB led to rounding of less than 50% of cells and to crumpled or condensed chromatin (indicated by arrowheads) and necrotic blister (indicated by arrows) in > 50% of cells. TcdB NXN also induced necrotic blister and condensed chromatin in > 50% of cells at high concentration but had no effect at 0.03 nM. The variant TcdB (TcdBF) induced cell rounding but no chromatin condensation or blister formation.
B. The concentration-dependent biphasic effect of TcdB on HEp-2 cell rounding is more evident in lower magnification.

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**Fig. 3.** TcdB induces shrivelling of the nucleus or chromatin condensation.

A. Rhodamin-phalloidin staining of the actin cytoskeleton and DAPI staining of the chromatin of HEp-2 cells after treatment with high concentrations (3 nM) of TcdB or TcdB NXN. DAPI staining reveals different shapes of nuclei (white arrows): n, normal; c, condensed; s, shrivelled.

B. 3D images of nuclei were calculated from stacks of 80–90 images (0.12 μm thickness) by Imaris software. Shown are representative pictures of six per nuclei.

C. Dimensions (length and width, see DAPI-stained control) of all nuclei of randomly chosen micrographs were measured and displayed in a bar chart (length = black bars; width = grey bars). Significant changes compared to controls were indicated by asterisks (mean ± SD, \( n = 200–350 \), \( P < 0.01 \)).
glucosylated Rho GTPases exhibited shrivelled and uneven nuclei. The 3D images of representative nuclei calculated from stacks of single micrographs in z-dimension illustrate the characteristic 'normal', 'condensed' and 'shrivelled' morphologies that were observed (Fig. 3B). To discriminate between the effects on the nucleus due to glucosylation of Rho GTPases and other reasons, we measured dimensions of DAPI-stained nuclei from pictures randomly chosen after treatment of HEp-2 cells with TcdB or TcdB NXN (Fig. 3C). The length and width of all nuclei were measured and mean values are displayed in a bar chart. At low concentration (0.03 nM), TcdB induced significant shrinking of nuclei, whereas TcdB NXN had no effect compared to controls. At high concentration (3 nM) both toxins induced significant reduction in width and length. TcdB NXN significantly reduced the volume to 69.6 ± 22.8% compared to controls (mean ± SD, n = 6, P < 0.05) suggesting that TcdB has glucosyltransferase-dependent and -independent effects on the nucleus.

In relation with chromatin condensation we observed that high concentrations of TcdB arrested cells within the 2n chromatin status. Figure 4 shows flow cytometry of propidium iodide stained Hep-2 cells treated with 3 pM or 3 nM TcdB for 4 h, 8 h and 24 h. TcdB at low concentration (3 pM) progressively reduced the peak of 2N cells in G1/G0 phase to 9 ± 0.5% after 24 h. Simultaneously, the percentage of 4N cells (diploid cells in G2-M phase and tetraploid cells in G1 phase) increased to 67 ± 1%. Control values were 47 ± 2% (2N) and 19 ± 0.6% (4N), respectively, after 24 h. The high number of cells within the 4n peak resulted from delayed G2-M transition (Ando et al., 2007) and inhibition of cytokinesis with resulting in binucleated cells (Huelsenbeck et al., 2009). At high concentration (3 nM), TcdB-treated cells showed a 2N population of 22 ± 1% and a 4N population of 58 ± 2%, indicating reduction in cell cycling due to a delayed G1/S transition or arrest within the G1/G0 phase. The reduced G1/S transition was also observed in cells treated with 3 nM TcdB NXN where 55 ± 1.5% of 2N cells in G1/G0 phase and 23 ± 0.6% of 4N cells (diploid cells in G2-M phase and tetraploid cells in G1) were counted. The 2N and 4N cell populations are summarized in a bar chart (Fig. 4B). The pronounced effect on the chromatin does also involve histone H3. Treatment of cells with TcdB and TcdB NXN at high concentration leads to Ser-10 dephosphorylation of histone H3 after 8 h and to a compensatory upregulation of histone H3 after 24 h. This was however not observed after treatment with low concentration of either toxin. The reduced histone phosphorylation did not correlate with Rac1 glucosylation (Fig. 4C). Reduced histone H3 phosphorylation was semiquantified by densitometrical analysis of immunoblots of 4 separate experiments and is shown in a bar chart (Fig. 4D).

TcdB and TcdB NXN induce ballooning of the nuclear envelope and dissociation of nucleoplasmin from the chromatin

Blistering formation in response to high concentrations of TcdB is a so far unreported phenomenon. The question arose whether the huge blister induced by TcdB were due to ballooning of the plasma membrane or of the nuclear envelope. We took advantage of a stably transfected A375 cell line that expresses a GFP-fusion protein of nucleoplasmin (GFP-Np) (Andrade et al., 2009). Nucleoplasmin associates with histones and is necessary for chromatin organization. We first applied TcdB NXN at high (3 nM) concentration (Fig. 5A). Control cells showed a specific localization of GFP-Np within the nucleus of most cells. Treatment with TcdB NXN (3 nM) led to condensation of the chromatin and a more intense staining of the nucleus in most cells. Some cells showed localization of GFP-Np within the TcdB NXN-induced blister, indicating dissociation of nucleoplasmin from the chromatin and ballooning of the nuclear envelope instead of solely the plasma membrane. This phenomenon was also observed when TcdB was applied at high concentration. At low concentration (3 pM) TcdB induced cell rounding and increased appearance of binucleated cells. We also investigated the effect of TcdB and TcdB NXN on the integrity of the plasma membrane. Therefore, DAPI was added directly to the culture medium of cells after 4 h of toxin treatment. Control cells that were not exposed to toxins excluded DAPI and did not allow incorporation into the chromatin (Fig. 5B). Subsequent to DAPI-exclusion assay cells were fixed and the actin cytoskeleton was additionally stained with rhodamin-phalloidin. TcdB (3 pM) induced cell rounding but did not disturb membrane integrity at low concentration as can be seen by lack of DAPI-positive cells. In contrast, cells treated with high concentration of TcdB (3 nM) showed incorporation of DAPI. The same was true for TcdB NXN. At low toxin concentration, cells appeared like controls whereas at high concentration, TcdB NXN allowed incorporation of DAPI into the chromatin indicating disturbance of the plasma membrane integrity.

Pyknotic cell death is an intracellular effect of the glucosyltransferase domain of TcdB

The GTD deletion mutant of TcdB (ΔGTD-TcdB) and the chimera of TcdB1–541 and TcdB 540–2366 were generated to address the question of intracellular action that underlies all the observed effects. The DAPI exclusion assay was again used as end-point to investigate the domain that is responsible for disturbance of plasma membrane integrity and chromatin condensation. Figure 6 shows that DAPI incorporation into about 80% of...
Fig. 4. Chromatin condensation is associated with reduced G1-S transition and reduced histone H3 phosphorylation.
A. Cell cycle analyses by flow cytometry after propidium iodide staining shows cells with diploid chromosomes (2n, G1/G0 phase) or tetraploid chromosomes (4n, G2/M transition or binuclear cells) after treatment of HEp-2 cells with 0.003 nM or 3 nM TcdB or TcdB NXN after indicated times. Treatment with 3 nM TcdB resulted in an increased number of cells with 2n chromatin. TcdB at 0.003 nM allowed G1-S transition and accumulation of cells with 4n chromatin.
B. Bar graphs show the percentage of cells 2n or 4n DNA content after treatment for 24 h (mean values ± SD, n = 3).
C. Western blot analyses of Rac1 and histone phosphorylation (pSer-10 H3) are representative for four separate experiments. Note the reduced histone H3 phosphorylation at Ser-10 and simultaneous compensatory upregulation of histone H3 in cells treated with high concentration of TcdB or TcdB NXN, which was independent on Rac1 glucosylation.
D. Graphs show densitometrical evaluation of H3 phosphorylation of immunoblots from four separate experiments after 24 h treatment (mean ± SD).
cells was specifically induced by TcdB or TcdB NXN. Cells that were incubated with ΔGTD-TcdB or GTD_{BF}-TcdB showed no DAPI incorporation. All toxins were applied at a concentration of 3 nM or, in case of ΔGTD-TcdB, in an equimolar concentration. The magnifications in Fig. 6A show the merged pictures of DIC and fluorescence micrographs. As indicated by arrows, some cells were not affected by TcdB or TcdB NXN with respect to pyknosis. These cells were sensitive to cell rounding (TcdB) or left unaffected with respect to morphological changes. The intracellular effect of GTD_{BF}-TcdB can easily be detected by cell rounding due to glucosylation of Rac1. Rounding

**Fig. 5.** TcdB and TcdB NXN induce dissociation of GFP-NP from the chromatin.
A. Treatment of GFP-Np transfected A375 cells with TcdB and TcdB NXN. Black arrows indicate nuclear blister containing GFP-Np, white arrows indicate binuclear cells resulting from inhibition of cytokinesis due to Rho-inactivation.
B. DAPI incorporation induced by high concentrations of TcdB or TcdB NXN correlates with dissociation of GFP-Np from chromatin in A375 melanoma cells.
Fig. 6. Pyknotic cell death is ROS mediated and specifically induced by the glucosyltransferase domain of TcdB.
A. DAPI exclusion assay was performed with HEp-2 cells treated with TcdB, TcdB D286/288N (TcdB NXN), TcdBF1–539-TcdB540–2366 Chimera (GTDΔ-GTD-TcdB), or TcdB540–2366 (ΔGTD-GTcdB) each 3 nM for 4 h. The Cartoon illustrates 2D structure of TcdB and toxin constructs used for experiments. Dihromate and fluorescence micrographs are shown. Arrows in merged detail figure indicate round cells (TcdB) or flat cells (TcdB NXN) that did not respond in terms of the new cytotoxic effect. DAPI-positive nuclei were quantified and displayed in a bar chart (right panel).
B. Pyknosis as measured by DAPI incorporation can be inhibited by bafilomycin A1 (left panel), as proof for intracellular effect of toxins. Quantification of DAPI-positive nuclei from four separate experiments (mean ± SD).
C. Dihydroethidium (DHE) staining of cells treated with 3 nM TcdB or TcdB NXN for 3 h indicated production of reactive oxygen species in the affected cells. Inhibition of NADPH oxidase by apocynin (1 mM) reduced cytotoxic effect of TcdB and TcdB NXN, each 3 nM for 4 h. TcdB-induced cell rounding was not affected by apocynin.

Discussion

We here report on a new aspect of TcdB-induced cytotoxicity, the pyknotic cell death with characteristic cell shrinkage and chromatin condensation. We provide evidence that the newly observed cytotoxic effect is induced by the glucosyltransferase domain of TcdB, albeit it is independent of the glucosyltransferase activity.

We chose the term pyknotic cell death for the newly observed cytotoxic effect because of the prominent chromatin condensation, the nuclear blister, shrinking of cells and the rapid loss of viability. In general, chromatin condensation and pyknosis are specifications for programmed cell death (PCD) (Reed, 1995). On the other hand, typical signs for apoptosis such as blebbing of the plasma membrane or chromatin fragmentation were not observed. In addition, flow cytometry data (Fig. 6A) show that the DNA was not fragmented (no abundance in sub-G1 cells), consistent with a previous report on TcdA (Nottrott et al., 2007). Although few observations like rapid loss of cell viability or huge blister formation might argue in favour of necrosis, we do suggest a special form of PCD that is induced by high concentrations of TcdB or TcdB NXN respectively. This assumption is supported by the immunoblot analyses in Fig. 6C, where an upregulation of histone H3 is shown, indicating cell activity typical for PCD/apoptosis.

The mechanism leading to pyknosis is completely unknown. High concentrations of TcdB induce a moderate G1 arrest or at least a delay in the G1-S transition. Changes in histone H3 phosphorylation at Ser-10 are critical for chromatin condensation within the prometaphase prior to subsequent chromosome

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TcdB-induced pyknosis

A

DIC  DAPI

control

TcdB

TcdB NXN

ΔGTD-TcdB

merge

DAPI-positive cells (%)

0 20 40 60 80 100 120

B

-bafilomycin  + bafilomycin

control

TcdB NXN

DAPI-positive cells

0 50 100 150 200

C

DIC  DHE  merge

TcdB NXN

TcdB

Apocynin

cytotoxic effect (%)

(relative number DAPI-positive cells)

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Fig. 7. Effect of TcdB and TcdB NXN on primary mouse lung fibroblast.
A. MRC-5 cells were treated with low (0.003 nM) or high (3 nM) concentrations of TcdB and TcdB NXN. Morphological changes are shown in micrographs.
B. Higher magnification of MRC-5 cells treated with 3 nM of TcdB and TcdB NXN with typical shrinkage of cells and blister formation (indicated by arrows).
C. WST-test and DAPI incorporation measured in MRC-5 cells after treatment with TcdB and TcdB NXN. Barcharts show mean values ± standard deviation (n = 9, **P-value < 0.001).
separation (Crosio et al., 2002; Goto et al., 2002). Obviously, TcdB and TcdB NXN induce prominent changes in the chromatin structure. This is also evident by the GFP-Np data of A375 cells which show that nucleoplasmin is homogenously distributed within TcdB-induced blister of the nuclear envelope. Nucleoplasmin in its phosphorylated state associates with the chromatin and causes decondensation. Accordingly, the toxin-induced dissociation of nucleoplasmin from the chromatin is in good accordance with apoptotic chromatin condensation. It was reported by Lu and co-workers that dephosphorylation of nucleoplasmin at Tyr-124 abrogates chromatin decondensation activity and leads to exclusion from the chromatin (Lu et al., 2005). Interestingly, the authors showed that chromatin condensation and DNA fragmentation are independent processes, which also is in good accordance with our findings. In contrast to nucleoplasmin, the role of histone H3 dephosphorylation observed in our study is not in line with known functions: Phosphorylation of H3 was reported to be positively involved in apoptosis and mitotic chromatin condensation (Goto et al., 1999; 2002; Park and Kim, 2012). Phosphorylation of H3 is also critical for specific gene expression and neoplastic transformation (Dong and Bode, 2006; Sawicka and Seiser, 2012). This is contradictory to our findings where PCD and chromatin condensation correlated with dephosphorylation of histone H3. Further studies are necessary to discriminate between the reason for and the consequence of chromatin condensation.

We do not exactly know the molecular mechanism underlying PCD and whether the newly observed effect involves signalling of Rho GTPases. Our impression is that glucosylation of Rho GTPases masks or even prevents chromatin condensation, since TcdB primarily causes shrivelled nuclei and only TcdB NXN is able to induce quantitative chromatin condensation. A more detailed investigation of kinetics and sequential application of TcdB and TcdB NXN will elucidate the role of Rho GTPases in pyknotic cell death induced by TcdB. Son and co-workers reported that H$_2$O$_2$ stimulates pyknotic and caspase-independent cell death (Son et al., 2009). We provided evidence that TcdB and TcdB NXN induced ROS production by DHE staining of the nuclei and by reduction of cytotoxic effect via apocynin, an inhibitor of the NADPH oxidase. It is noteworthy that Rac1 is upstream involved in activation of the NADPH oxidase. These observations together nicely fit into our hypothetical model, where the kinetic of Rac1 glucosylation is decisive for whether pyknosis can be triggered by TcdB or not. Rac1 dependency of the cytotoxic effect was also shown in transfection experiments by Farrow et al. (2013). Our assumption is supported by the fact that HUVEC’s did not show the newly described phenotype. HUVEC’s are rather insensitive towards TcdB (Supplementary Fig. S2). Accordingly, flush or accumulation of toxin to a critical intracellular concentration where at the same time Rac1 function is yet not seriously hampered cannot be achieved. The kinetic of ROS production and Rac1 inhibition might also be critical for pyknotic cell death associated with PCD or necrosis. We cannot exclude that in case of massive ROS production lipid peroxidation induces necrosis before PCD is executed.

Although the molecular mechanism leading to TcdB-induced PCD is not completely known, we nevertheless could specify the domain of TcdB that is responsible for all the observed effects. The glucosyltransferase-truncated mutant of TcdB (ΔGTD-TcdB) had no effect at all which revealed that the GTD is the causative domain. Since only TcdB, but not the chimera GTD$_{AF}$-TcdB harbouring the GTD from variant TcdB (TcdBF), was able to induce pyknosis effects were unique for the TcdB GTD. Comparable EC50 values for Rac1 glucosylation proved full functionality of the GTD from TcdB in our system. By applying the chimera GTD$_{AF}$-TcdB we furthermore excluded that the mere translocation process, which includes pore formation, triggers PCD. By additional bafilomycin A1-induced inhibition of the translocation process we moreover dissected between intracellular and extracellular effects and precluded a ligand effect of the GTD. We suppose that a subdomain within the GTD from TcdB triggers activation of the NADPH oxidase upstream of Rac1. This subdomain is not present in the GTD from TcdBF due to different amino acid sequence.

In conclusion, our study describes the newly observed cytotoxic effect as an intracellular effect specific for the GTD of TcdB. Comparatively high concentrations of TcdB, i.e. 5000-fold higher in terms of potent Rho glucosylation, induce pyknosis that is associated with chromatin condensation and dysregulation of the cell cycle. PCD is visible not only by chromatin condensation, but also by cell shrinking and blister formation of the nuclear envelope.

**Experimental procedures**

**Antibodies used**

Monoclonal anti-Rac1 (clone 102), BD Transduction Laboratories; monoclonal anti-Rac1 (clone 23A8), Merck (Upstate); monoclonal anti-β-actin (clone AC-15), Sigma; monoclonal anti-GAPDH, Zytomed; rhodamin-phalloidin, Invitrogen (Molecular Probes); Polyclonal anti-histone H3 was from Cell Signalling; monoclonal anti-pH3(S10) (clone 9H10), was from Millipore.

**Cell culture and cell viability and ROS assays**

The human epithelial cell line HEP-2 was cultured in MEM Eagles medium supplemented with 10% fetal bovine serum, 100 μM penicillin, and 100 μg ml$^{-1}$ streptomycin. The eGFP
C1-nucleoplasmin transfected human melanoma cell line A375 (kindly provided by Juan Aréchaga, University of the Basque Country) was cultured in DMEM supplemented with 10% fetal bovine serum, 100 μM penicillin, 100 μg ml⁻¹ streptomycin and 1.5 mg ml⁻¹ G418. Both cell lines were subcultured twice a week at subconfluent status. For experiments cells were seeded at a density of about 10⁵ cells cm⁻² and cultured for 16 h to achieve confluency of less than 50%.

MRC-5 lung fibroblasts were from ATCC and kept in DMEM supplemented with 10% fetal bovine serum, 100 μM penicillin, 100 μg ml⁻¹ streptomycin. Viability of cells was measured by WST-test (Roche, Germany) according to the protocol supplied by the manufacturer. WST assay was performed with cells at 50% confluency grown in multicthie plates.

DAPI exclusion assay was also performed at subconfluent status of cells. DAPI was added to a final concentration of 200 nM after toxin treatment and cells were incubated for further 5 min. Cells were directly subjected to fluorescence microscopy to document DAPI incorporation into nuclei. DAPI-positive cells were considered as non-viable.

Production of reactive oxygen species was investigated by dihydoroethidium (DHE) staining. Cells were treated as indicated and afterwards DHE (Sigma-Aldrich) was added to a final concentration of 10 μM. Cells were further incubated for 10–30 min to allow oxidation of DHE by ROS and to allow binding of oxidized DHE to the chromatin. DHE staining of nuclei was analysed by fluorescence microscopy. Inhibition of ROS production by NADPH oxidase was achieved by apocynin (Calbiochem). To this end, cells were pre-incubated for 15 min with indicated concentrations of apocynin.

Molecular cloning and recombinant expression of C. difficile toxins and toxin fragments

All toxins and toxin fragments were cloned in pWH1522 expression vector with a C-terminal 6×His tag as described by Olling et al. (2011). Mutation of the DXD motif of TcdB was done by Quick change XL site-directed mutagenesis Kit (Stratagene). The D268N/D288N mutant of TcdB (TcdB-NNX) was generated via site-directed mutagenesis Quickchange of the pHis1522 TcdB construct using 5′-GGTGGTATGTATTTAAAAATGGTTAACAGGAATACAACCAG-3′ as sense and 5′-CTGGTTGATATTCTGGTAAATACATATAACCATACCCACCC-3′ as antisense primer. A toxin chimera of TcdB (GTDT₃₋₅-TcdB) where the glucosyltransferase domain has been exchanged with the glucosyltransferase domain (amino acids 1–541) of variant TcdB from C. difficile serotype F strain 1470 was generated as follows: coding sequence 1–1623 of the TcdBF gene (Acc-No. AF217292) was amplified by PCR using 5′-ACTGGATTGATATTCAAGAACAGTTAG-3′ as sense and 5′-ACTGGATCCCTTCAAAATTTTCTTGTATTCTTC-3′ as antisense primer flanked by recognition enzyme BsrGI (5′ end) and BamHI (3′ end) recognition sequences. This fragment was ligated into the expression vector pHIS1522 TcdB, where base pair 1621–1626 were mutated from GGTTCT to GGATCC. This ligated into the expression vector pHIS1522 TcdB, where base pair 1621–1626 were mutated from GGTTCT to GGATCC. This resulting coding sequence was ligated via BsrGI and BamHI into pHIs1522.

All constructs were sequenced. The toxins were expressed in Bacillus megaterium (Mobitec, Germany). All proteins were purified by Ni²⁺ affinity chromatography using Ni²⁺-TED columns (Macherey-Nagel, Germany) according to the protocol supplied by the manufacturer.

Fluorescence staining and confocal laser scanning microscopy

Nuclei of permeabilized and formaldehyde fixed cells was done by adding 200 nM DAPI for 15 min followed by two times washing with PBS. The actin cytoskeleton was stained for 30 min with rhodamin-phalloidin (3 U per ml PBS) and subsequent washing with PBS. Micrographs were made with a Leica Inverted-2 confocal laser microscope. 3D images of cell nuclei surface were calculated from stacks of approximately 80 pictures (each 0.12 μm thickness) in z-dimension of DAPI-stained nuclei. Pictures were generated and volumes were calculated by Imaris software.

Immunoblot analyses

Cells were grown in Petri dishes (5 cm diameter) and incubated as indicated. Detached cells were harvested, washed once with ice-cold PBS by centrifugation and pooled with attached cells, that were also washed once with PBS, in ice-cold lysis buffer [50 mM NaCl, 20 mM Tris-HCL pH 7.4, 1% (w/v) Triton X-100, supplemented with protease/phosphatase inhibitors]. Protein concentration of cell lysates was measured by Bradford assay and adjusted with milk powder and probed with specific primary antibody overnight at 4°C. Detection was performed using corresponding HRP-conjugated secondary antibody and ECL detection reagent Super signal West-femto (Pierce, Germany). Documentation and densitometrical evaluation of specific signals was done using Kodak-Image station and Kodak 1D 3.5 software.

Flow cytometry

Estimation of cell population in different cell cycle phases was done by flow cytometry (FACScan flow cytometer, Becton Dickinson). Detached cells and adherent cells which were suspended by trypsinization were pooled before FACS analysis. For analyses, 5 × 10⁵ cells per sample were fixed in ice-cold ethanol (70%) for 30 min and washed afterwards with 1% bovine serum albumin in PBS. Total DNA content was stained with 150 μg ml⁻¹ propidium iodide in Tris/HCl, pH 7.4, containing 1% BSA and 1% Triton X-100. The RNA was removed by incubating cells with 0.5% RNase for 30 min. Subsequently, cells were subjected to FACS analysis. A fluorescence area (FL2) of 200 was set to correlate with a 2n-set of chromosomes within the G1 phase. Cells in the fluorescence area 400 were considered as tetraploid.
Statistics

All statistical evaluations were performed with GraphPad Prism 5 applying Student’s t-test, two-tailed and paired. A P-value of less than 0.05 was considered as significant.

Gene IDs

TcdB from C. difficile strain VPI10463: GenBank X53138 and variant TcdB from C. difficile, serotype F strain 1470 (TcdBF): GenBank AF217292.

Conflict of interest

The authors declare that there is no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. The residual glucosyltransferase activity of TcdB D286/288N was estimated in a UDP-[14C]glucosylation assay. [14C]glucosylation of recombinant Cdc42 (2 μg) was performed in 50 μl glucosylation buffer (100 mM KCl, 50 mM Hepes pH 7.4, 1 mM MnCl₂, 2.5 mM MgCl₂) for 45 min at 37°C. TcdB-catalysed [14C]glucosylation showed strong signals in the filmless autoradiography when applied at a concentration of 60 pM (corresponding to 1 ng per 50 μl sample) and even more at 6 nM (Supplementary Fig. S1A, left panel) There was no detectable signal of [14C]glucosylated Cdc42 when TcdB D286/288N was applied at 1000-fold higher concentration (60 nM). We additionally tested the glucosyltransferase domain of TcdB NXN, since we could achieve higher concentrations with the isolated domain. Even at a concentration of 3 μM (corresponding to 15 μg per 50 μl sample) no [14C]glucosylated Cdc42 was detectable. Thus, the residual glucosyltransferase activity of TcdB NXN was estimated to be at least 5·10⁴-fold reduced compared to wild-type TcdB. The Coomassie-stained gel used for autoradiography is shown in the right panel. The glucosyltransferase activity of TcdB and TcdB NXN was also tested in a cytotoxicity assay using HEp-2 cells. Therefore, cells were incubated with the indicated concentrations of toxins for 24 h. Cells were harvested in Lämmli buffer, sonicated and the cell lysates were probed for glucosylated Rac1 in a Western blot assay using glucosylation-sensitive antibody (clone 102) that does not recognize glucosylated Rac1 in comparison with an antibody (clone 23A8) that is not glucosylation sensitive. TcdB (3 fM) reduced the ratio of glucosylated Rac1 and total Rac1 to 0.5 with respect to controls (ratio: 1.0) after 24 h incubation (Fig. 1B). 30 fM reduced non-glucosylated Rac1 to a ratio of about 0.2. In contrast, TcdB NXN at 30 nM (≈10 μg ml⁻¹) reduced the ratio to 0.5, which indicates a 10⁷-fold reduced glucosyltransferase activity in cell culture assay. Together these experiments show that the maximum residual glucosyltransferase activity of TcdB NXN is at least 5·10⁴-fold less than wild-type TcdB.

Fig. S2. Effect of TcdB and TcdB NXN on cell viability of human umbilical vein endothelial cells (HUVEC’s). HUVEC’s were obtained from PromoCell, Germany, and kept in ready-to-use Endothelial Medium (PromoCell, Germany). Cells were passaged twice a week and only cells of passage 2–5 were used for experiments. Cells were treated with toxin at indicated concentrations for 6 h. Only TcdB at high concentration induced morphological changes of cells but not complete cell rounding (Supplementary Fig. S2A). In contrast to MRC-5 primary mouse lung fibroblasts, cells are quite insensitive towards TcdB. Cell viability of cells after treatment as mentioned above was not significantly reduced as measured by WST assay in either of the experiments (mean values ± standard deviation, n = 6) (Supplementary Fig. S2B).