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The *Haemophilus ducreyi* cytolethal distending toxin induces DNA double-strand breaks and promotes ATM-dependent activation of RhoA

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Summary

Among bacterial protein toxins, the cytolethal distending toxins (CDTs) are unique in their ability to activate the DNA damage checkpoint responses, causing cell cycle arrest or apoptosis in intoxicated cells. We provide direct evidence that natural intoxication of cells with the *Haemophilus ducreyi* CDT (HdCDT) holotoxin induces DNA double-strand breaks similarly to ionizing radiation. Upon DNA damage, epithelial cells and fibroblasts promote the formation of actin stress fibres via activation of the small GTPase RhoA. This phenomenon is not toxin specific, but is part of the ATM-induced cellular responses to genotoxic stresses, including ionizing radiation. Activation of RhoA is associated with prolonged cell survival, as HdCDT-treated epithelial cells expressing a dominant-negative form of RhoA detach and consequently die faster than cells expressing a functional RhoA. Our data highlight several novel aspects of CDT biology: (i) we show that a member of the CDT family causes DNA double-strand breaks in naturally intoxicated cells, acting as a true genotoxic agent; and (ii) we disclose the existence of a novel signalling pathway for intracellularly triggered activation of the RhoA GTPase via the ATM kinase in response to DNA damage, possibly required to prolong cell survival.

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Introduction

The chancroid bacterium *Haemophilus ducreyi* produces a potent toxin (HdCDT), which belongs to the recently described family of cytolethal distending toxins (CDTs). CDTs are produced by a variety of Gram-negative pathogenic bacteria including *Escherichia coli*, *Campylobacter* sp., *Actinobacillus actinomycetemcomitans*, *Shigella dysenteriae* and *Helicobacter hepaticus*. CDTs cause an irreversible cell cycle arrest and death of the target cells (reviewed by Cortes-Bratti *et al*., 2001a; Lara-Tejero and Galan, 2002).

The CDT activity is encoded by three linked genes, designated *cdtA*, *cdtB* and *cdtC*. Expression of all three genes is required to produce an active CDT (reviewed by Pickett and Whitehouse, 1999), which forms a tripartite protein complex (Lara-Tejero and Galan, 2001). In intoxicated cells, HdCDT induces similar responses to ionizing radiation, a well-characterized DNA-damaging agent, including activation of DNA damage checkpoints, phosphorylation of the histone H2AX and relocalization of the DNA repair complex Mre11 (Cortes-Bratti *et al*., 2001b; Li *et al*., 2002). These observations are consistent with the demonstration that the CdtB subunit of *E. coli*, *C. jejuni* and *H. ducreyi* CDTs possesses DNase I-like activity when tested *in vitro* or microinjected into cells (Elwell and Dreyfus, 2000; Lara-Tejero and Galan, 2000; Elwell *et al*., 2001). However, previous attempts to demonstrate directly CDT-induced DNA strand breaks in cells exposed to CDT holotoxins were not successful (Sert *et al*., 1999).

Cellular intoxication by CDTs promotes the formation of stress fibres in a cell type-dependent manner (Aragon *et al*., 1997; Cortes-Bratti *et al*., 1999). However, nothing has been reported on how this event is regulated or how it is associated with the cell cycle arrest induced by CDTs. Good candidates to control this process are the GTPases of the Rho subfamily, which are key regulators of the actin cytoskeleton (Hall, 1998). RhoA is involved in the formation of stress fibres and focal adhesions (Ridley and Hall, 1992). Cdc42 induces the formation of filopodia, protrusions from the membrane that are formed by polymerization of actin in parallel bundles (Kozma *et al*., 1995). Rac stimulates the polymerization of actin beneath the plasma membrane leading to the production of membrane ruffles (Ridley *et al*., 1992). Furthermore, the Rho proteins are

involved in several cellular processes such as membrane trafficking, transcriptional regulation, cell proliferation, transformation and apoptosis (reviewed by Bar-Sagi and Hall, 2000; Aznar and Lacal, 2001). The small GTPases regulate all these molecular events by cycling between two conformations: an inactive form (GDP-bound) and an active form (GTP-bound). Only the GTP-bound form can interact with its effector molecules and therefore exert its function. The process is negatively regulated by hydrolysis of the GTP to GDP, and consequent inactivation of the small GTPase. The nucleotide exchange is facilitated by nucleotide exchange factors (GEFs), and the intrinsic GTPase activity can be promoted by GTPase activating proteins (GAPs) (reviewed by Sander and Collard, 1999).

The ATM (ataxia-telangiectasia mutated) protein belongs to the phosphatidylinositol 3-kinase (PI3K) family and plays a central role in regulating the complex cellular responses to genotoxic agents. DNA double-strand breaks, caused by ionizing radiation, induce activation of ATM, which (i) blocks cell proliferation at different stages of the cell cycle; and (ii) activates DNA repair mechanisms, thus preventing genetic instability.

Cell cycle arrest at the G1/S border is caused by ATM-dependent phosphorylation and consequent stabilization of the tumour suppressor protein p53, whereas ATM-dependent phosphorylation of the chk2 kinase indirectly inhibits activation of the cyclinB/cdc2 complex, preventing G2 to M transition and inducing G2 arrest (reviewed by Shiloh, 2001). Recent data show that ATM-dependent activation of DNA repair processes is mediated via early phosphorylation of the histone H2AX, followed by the recruitment of the DNA repair complex Mre 11 at the site where DNA has been damaged (Paull *et al*., 2000; Burma *et al*., 2001). However, to date, there is no link between cellular responses to DNA damage and alteration of the actin cytoskeleton.

We demonstrate that HdCDT causes DNA double-strand breaks in intoxicated cells, similarly to ionizing radiation. We also demonstrate that the rearrangement of the actin cytoskeleton observed upon HdCDT intoxication is mediated by activation of the RhoA GTPase, and does not depend on Rac or Cdc42. This phenomenon is not toxin specific, but is part of the ATM-dependent response to genotoxic stresses. These data highlight a novel pathway, which enables cross-talk between damaged DNA in the nucleus and the actin cytoskeleton, possibly required to prolong cell survival.

Results

HdCDT induces DNA double-strand breaks (DSBs)

Despite the similarities of the cellular responses in irradiated and HdCDT-intoxicated cells, to date, there is no direct demonstration that CDTs can cause DNA strand breaks in cells naturally exposed to CDTs. We used pulsed field gel electrophoresis (PFGE) to separate high-molecular-weight DNA and showed that HdCDT intoxication induced DSBs in a time-dependent manner in HeLa cells. When ^{14}C-labelled DNA extracted from HdCDT-intoxicated cells was separated by PFGE and visualized by ethidium bromide, substantial fractions of double-stranded DNA were out in the gel 8 h after treatment (Fig. 1A). The pattern of DNA fragmentation was similar to that observed in HeLa cells exposed to a radiation dose of 20 Gy (Fig. 1B). Double-strand breaks were then quantified by measuring in a liquid scintillator the ^{14}C activity

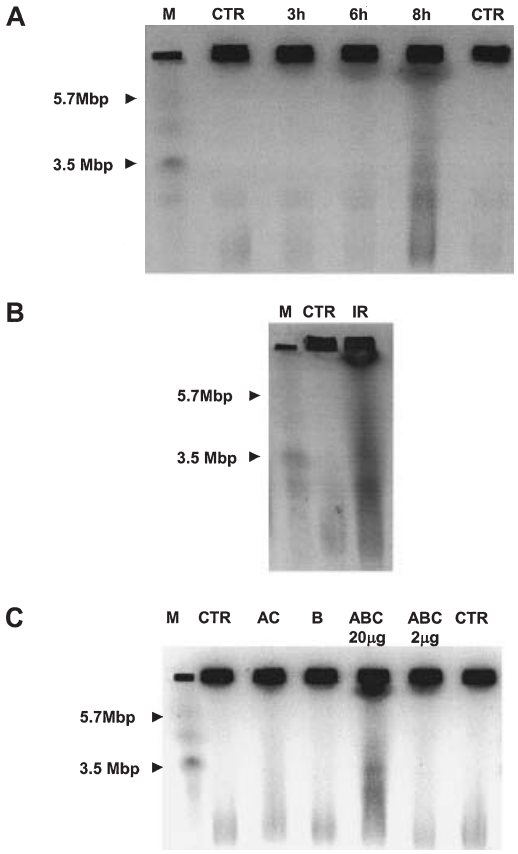

Fig. 1. HdCDT induces DNA double-strand breaks.
A. Fifty thousand HeLa cells per well were grown in 12-well plates in complete medium containing 4000 Bq [methyl-^{14}C]-thymidine for 72 h. The cells were then washed three times in PBS and chased for 2 h in complete medium. Cells were treated with HdCDT (20 μg ml^{-1}) for the indicated time periods or left untreated for 8 h (CTR), and processed for PFGE analysis as described in *Experimental procedures*. M, molecular weight marker.
B. HeLa cells were left untreated or irradiated (20 Gy) and immediately processed for PFGE analysis as described in *Experimental procedures*.
C. HeLa cells were left untreated or treated with: (i) CdtAC bacterial supernatant; (ii) purified *H. ducreyi* CdtB (20 μg ml^{-1}); (iii) CdtABC preparation described in *Experimental procedures*, for 8 h and then processed for PFGE analysis.

Table 1. HdCDT induces DNA double-strand breaks.

Treatment time	DSB/diploid cell	Equivalent radiation dose (Gy)
6 h[a]	133 ± 38	5.3 ± 1.5
8 h[a]	539 ± 16	21.6 ± 0.8
8 h[b]	490	19.5

a. Data and SEM from three to four gels from two separate experiments. Control values were subtracted, and DSBs were calculated as described in *Experimental procedures*.
b. Data relative to the DSBs induced by CdtABC (20 μg ml^{-1}) presented in Fig. 1C.

for DNA fragments <5.7 Mbp. We showed that 6 h and 8 h of treatment with 20 μg ml^{-1} HdCDT induced DSBs comparable to radiation doses of about 5 Gy and 22 Gy respectively (Table 1). To exclude the possibility that the DNA fragmentation observed in toxin-exposed cells resulted from contaminants present in the toxin preparation, we treated HeLa cells for 8 h with preparations containing only the CdtAC supernatant from recombinant *E. coli* expressing the relevant genes, purified CdtB alone (20 μg ml^{-1}) or the combination of CdtA, CdtB and CdtC (CdtABC). As shown in Fig. 1C, we did not detect any DNA DSBs in the presence of either CdtAC or CdtB alone. The combination of the three components was required to induce DNA damage at the concentration of 20 μg ml^{-1}. The extent of DSBs produced was comparable to an irradiation dose of 19.5 Gy (Table 1). Upon a 10-fold dilution of the CdtABC preparation, no DSBs above the control were observed (Fig. 1C).

DNA-damaging agents induce reorganization of the actin cytoskeleton via RhoA activation

Previous data have shown that cellular intoxication with *E. coli* and *H. ducreyi* CDTs is characterized by cell distension and induction of actin stress fibres in CHO and HEp-2 cells respectively (Aragon *et al*., 1997; Cortes-Bratti *et al*., 1999). We demonstrated that a similar pattern is detected in intoxicated or irradiated HeLa cells (Fig. 2A), suggesting that this is a default cellular response to DNA-damaging agents. Induction of stress fibres in intoxicated HeLa cells required the combination of the three CDT subunits, as shown in Fig. 2B, whereas CdtAC or CdtB alone did not have any effect, ruling out the possibility that stress fibre formation may result from contaminants present in the HdCDT preparation used in Fig. 2A.

To investigate this phenomenon further, we designed a set of experiments to clarify whether the small GTPases belonging to the Rho subfamily were involved in the cytoskeleton rearrangements observed in intoxicated and irradiated HeLa cells.

The first step was to transfect transiently HeLa cells with dominant-negative mutants for RhoA, Rac and Cdc42, and to assess whether any of them could prevent the formation of stress fibres 24 h after HdCDT intoxication. A fivefold reduction in the number of cells presenting stress fibres and a 50% decrease in phalloidin staining intensity per cell were observed in HeLa cells transiently transfected with dominant-negative RhoAN19, compared with the non-transfected HdCDT-treated cells (Fig. 3A–C). We did not detect differences in response to HdCDT in HeLa cells transiently transfected with dominant-negative RacN17 and Cdc42N17 mutants (Fig. 3B; data not shown). Interestingly, the cell distension induced by HdCDT treatment was not a consequence of stress fibre induction, as cells distended to an equal extent upon HdCDT intoxication independently of RhoAN19 expression (Fig. 3D).

Cell size increase was associated with activation of the PI3K kinase, as demonstrated by the use of a dominant-

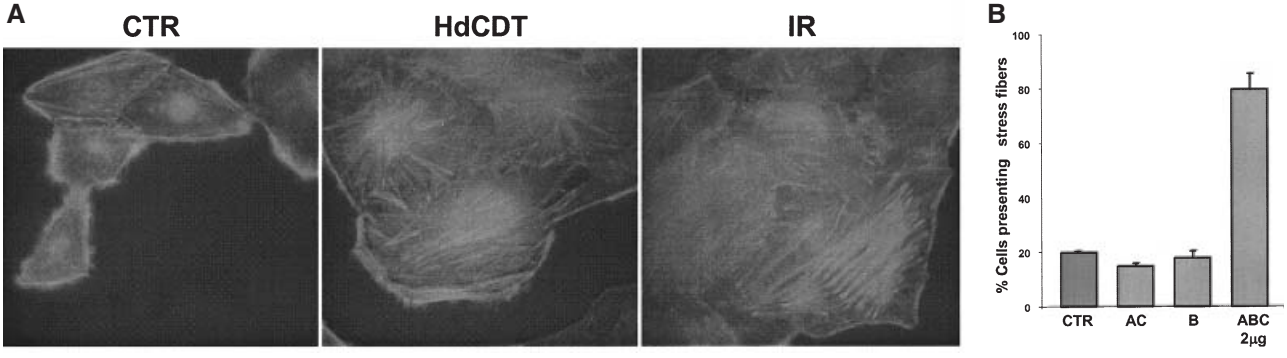

Fig. 2. DNA double-strand breaks induce rearrangements of the actin cytoskeleton.
A. HeLa cells were left untreated, treated with HdCDT (2 μg ml^{-1}) or irradiated (20 Gy), and incubated for 24 h in complete medium. F-actin was stained with FITC-phalloidin as described in *Experimental procedures*.
B. HeLa cells were treated with: (i) CdtAC filtered bacterial supernatant; (ii) purified *H. ducreyi* CdtB (20 μg ml^{-1}); (iii) CdtABC (2 μg ml^{-1}) for 24 h, and F-actin was stained with FITC-phalloidin as described in *Experimental procedures*. Approximately 150–200 cells were counted for each sample.

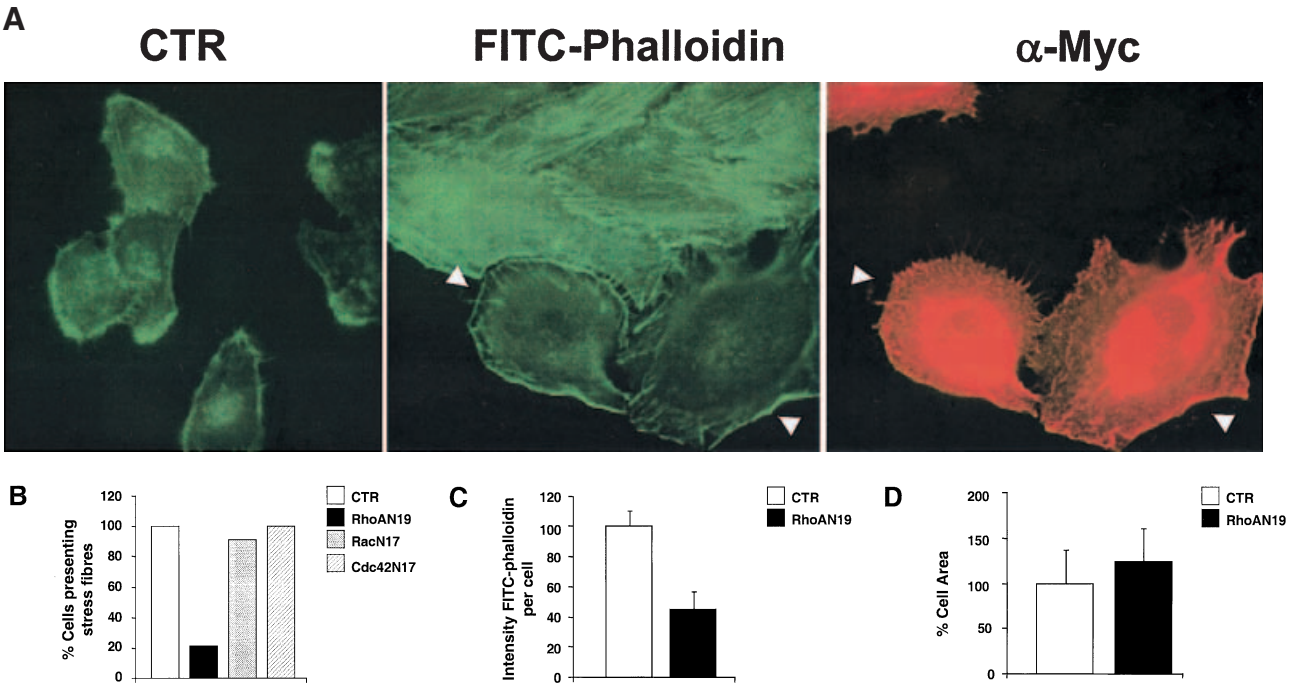

Fig. 3. Dominant-negative RhoAN19 inhibits promotion of actin stress fibres in HdCDT-intoxicated cells.
A. HeLa cells were transfected with dominant-negative RhoAN19 and, 24 h after transfection, cells were incubated with HdCDT (2 μg ml^{-1}) for an additional 24 h. Transfected cells were visualized by immunofluorescence using anti-Myc 9E10 antibody, and F-actin was stained with FITC-phalloidin as described in *Experimental procedures*. Arrows indicate RhoAN19-transfected cells.
B. Quantification of HdCDT-intoxicated cells presenting promotion of stress fibres upon transfection with the dominant-negative forms of RhoA, Rac and Cdc42. Data are presented as a percentage of the non-transfected HdCDT-treated cells.
C. The fluorescence intensity of each cell was quantified in HdCDT-treated cells expressing or not expressing the dominant-negative RhoAN19 protein using the NIH IMAGE software. Data are presented as a percentage of the intensity of non-transfected HdCDT-treated cells.
D. Quantification of the cell area of HdCDT-treated HeLa cells expressing or not expressing the dominant-negative RhoAN19 protein. Cell areas were quantified using the IMAGEQUANT (Molecular Dynamics) software, and data are presented as a percentage of non-transfected HdCDT-treated cells. One out of four independent experiments is shown, and 200 cells were counted for each experiment.

negative form of the p85 subunit (Fig. 4). A twofold increase in cell size was detected in non-transfected HeLa cells 24 h after intoxication; however, the p85αΔiSH2-N-expressing cells did not increase the cell size above the level of the non-intoxicated control. Expression of p85αΔiSH2-N did not alter the size or the viability of untreated HeLa cells (Fig. 4B). No clear effect of the p85 dominant-negative mutant was observed regarding induction of stress fibres (data not shown).

To elucidate further the role of RhoA, Rac and Cdc42, we used an affinity binding assay to detect exclusively the active form of the GTPases in lysates from intoxicated cells. The assay is based on the ability of the GTP-bound, but not the GDP-bound, form to bind a specific domain. GTP-Rac and GTP-Cdc42 bind the p21-binding domain (PBD) of p21-activated kinase (PAK), and GTP-Rho binds the Rho-binding domain (RBD) of the effector protein Rhotekin. The *E. coli* cytotoxic necrotizing factor (CNF), a toxin known to activate all three Rho subfamily GTPases, was used as positive control (Flatau *et al.*, 1997; Schmidt *et al.*, 1997). We performed time course experiments to examine the level of the GTP-bound forms of the Rho-GTPases after HdCDT intoxication. We did not detect any increase in the GTP-bound Rac and Cdc42 (Fig. 5A). In contrast, a time-dependent activation of RhoA was observed in HdCDT-intoxicated cells as early as 40 min, with maximum levels of the GTP-bound form of RhoA 6 h after intoxication, followed by a small decrease 24 h after treatment (Fig. 5A and B). To ensure that the total amount of small GTPases was similar in all samples, 20 μl of the total-cell lysate was probed with the relevant antibody.

The reorganization of the actin cytoskeleton observed upon irradiation was also induced by RhoA activation, as determined by the inhibition of stress fibre formation in irradiated HeLa cells transiently transfected with the dominant-negative mutant RhoAN19 (Fig. 6A), and by increased levels of the GTP-bound form of RhoA detected by affinity purification in time course experiments (Fig. 6B and C).

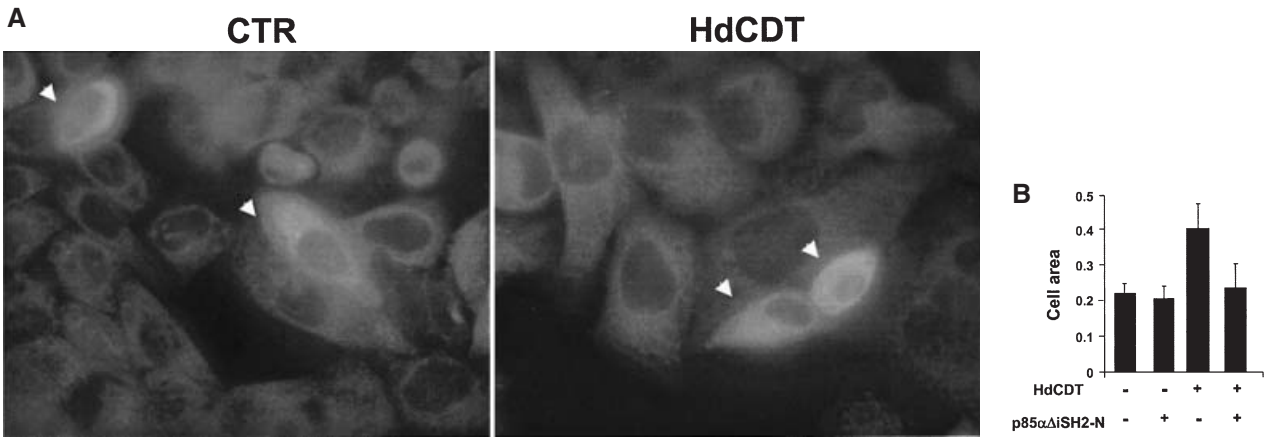

Fig. 4. PI3K controls cell distension in intoxicated cells.
A. HeLa cells were transfected with the dominant-negative p85αΔiSH2-N and, 24 h after transfection, cells were incubated with HdCDT (2 μg ml^{-1}) for an additional 24 h. Transfected cells were visualized by immunofluorescence using the anti-p85 specific antibody, and F-actin was stained with FITC-phalloidin as described in *Experimental procedures*. Arrows indicate p85αΔiSH2-N-transfected cells.
B. Quantification of the cell area in untreated or HdCDT-treated HeLa cells expressing or not expressing the dominant-negative p85αΔiSH-2 N protein. Cell area was quantified using the NIH IMAGE software. One out of three experiments is shown.

It is noteworthy that, in both HdCDT-treated and irradiated cells, the levels of RhoA activation were similar to those observed in cells intoxicated with the *E. coli* CNF, used as a positive control (Figs 5 and 6).

RhoA activation is cell type dependent

To test whether the HdCDT-induced RhoA activation is a general phenomenon, we used a broader panel of human cell lines, including primary foreskin (HFS) fibroblasts, the SV40 transformed fibroblasts GM0847 and the B-cell line SN-B1. Each of these cell lines responded differently to the HdCDT-induced DNA damage. We showed previously that primary HFS arrest in both G1 and G2 phases of the cell cycle, whereas B cells die rapidly upon toxin exposure (Cortes-Bratti *et al*., 2001b). Here, we demonstrated that the SV40 transformed fibroblasts are arrested exclusively in the G2 phase of the cell cycle (Fig. 7). Cells of epithelial and fibroblast origin showed a time-dependent activation of RhoA, detected by a twofold increase in the GTP-bound form of the small GTPase 3 h after intoxication, independently of the type of cell cycle arrest. However, we did not observe increased levels of activated RhoA in the B-cell line even upon longer toxin exposure (Fig. 7; data not shown).

Activation of RhoA is ATM dependent

The data acquired here indicate that the RhoA-dependent reorganization of the actin cytoskeleton is not exclusively associated with CDT intoxication, but is a default response to DNA damage. However, it was not known whether under these circumstances RhoA activation is directly regulated by the same set of molecules that senses DNA damage and activates checkpoint responses, or is induced by an independent pathway.

The ATM kinase is a key protein responsible for detecting DNA damage and for activating cellular responses to avoid genetic instability (reviewed by Shiloh, 2001). We tested whether caffeine, an inhibitor of ATM (Zhou *et al*., 2000), could prevent RhoA activation upon irradiation or intoxication. To select for the concentration of caffeine required to block the ATM kinase, we incubated HeLa cells with increasing amounts of caffeine for 1 h; the cells were then irradiated and incubated further for 40 min. Activation of ATM was assessed indirectly by monitoring the ATM-dependent phosphorylation of the histone H2AX (Paull *et al*., 2000). A large decrease in H2AX phosphorylation was achieved at a caffeine concentration of 20 mM, whereas lower concentrations did not exert any effect (Fig. 8A). Subsequently, RhoA activation was measured by affinity purification of the GTP-bound form from cytosolic extracts of untreated or irradiated cells. A threefold inhibition of H2AX phosphorylation was detected in caffeine-treated cells (Fig. 8B). As shown previously in Fig. 6B, irradiation induced a time-dependent accumulation of the GTP-bound form of RhoA, which was decreased in caffeine-treated cells by 1.6-fold to 2.8-fold after 30 min and 1 h of treatment respectively (Fig. 8B). Caffeine alone did not affect the levels of GTP-bound RhoA (data not shown).

To confirm further the ATM dependency of RhoA acti-

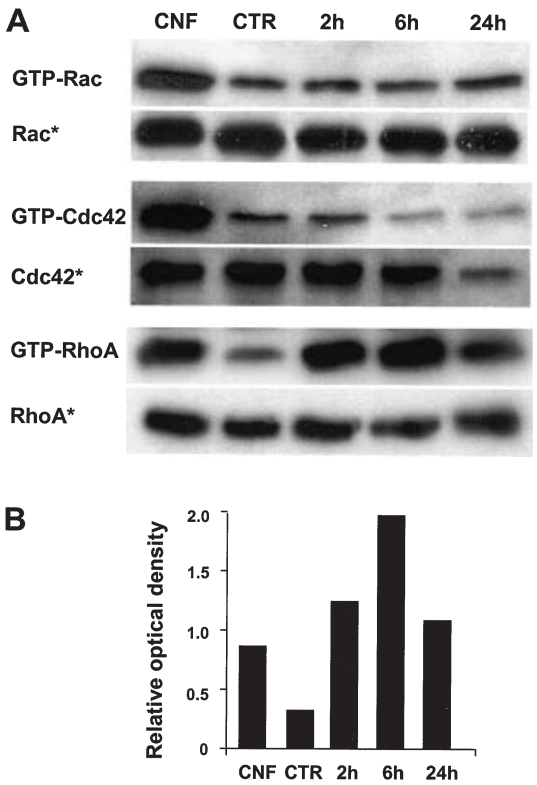

Fig. 5. Affinity precipitation of GTP-Rac, GTP-Cdc42 and GTP-Rho in HdCDT-intoxicated cells. HeLa cells were left untreated (CTR) or treated with HdCDT for the indicated times. Affinity purification of the GTP-bound GTPases was performed as described in *Experimental procedures*. CNF was used as a positive control.
A. GTP-Rac and GTP-Cdc42 were precipitated using GST-PBD coupled to glutathione sepharose. The GTP-RhoA was precipitated using GST-TRBD coupled to glutathione sepharose. Twenty microlitres of cytosolic lysate was kept as a control of the total amount of Rac, Cdc42 and RhoA present in the cytosolic extract (indicated as Rac*, Cdc42* and Rho*). Samples were processed for Western blot and developed with anti-Rac (1:200), anti-Cdc42 (1:200) or anti-RhoA (1:500) antibodies.
B. Quantification of the GTP-bound RhoA. Data are presented as a ratio between the optical density of the GTP-bound RhoA and the optical density of RhoA present in the cytosolic extract. One out of three experiments is shown.

vation, we performed similar experiments using the ATM wild-type GM00847 and the ATM-deficient GM05849 SV40 transformed human fibroblasts. A twofold increase in the GTP-bound form of RhoA was detected in intoxicated ATM wild-type cells 2 h after treatment, whereas no change in the level of activated RhoA was observed in the ATM-deficient cells (Fig. 9). In both cases, treatment with the *E. coli* CNF was used as a positive control.

Inhibition of RhoA activation enhances cell detachment

In order to test whether RhoA activation induced by DNA damage had any effect on cell survival, we used as a model the canine cell line T23 MDCK stably transfected with the c-Myc-tagged tetracycline-regulated negative form of RhoA (RhoAN19). RhoAN19 expression was induced upon withdrawal of tetracycline from the culture medium for 40 h (Fig. 10A). In the conditions used for the induction, the percentage of RhoAN19-positive cells varied from 30% to 40% in four independent experiments, and we could not achieve higher levels of positive cells, despite several rounds of subcloning.

T23 MDCK RhoAN19 cells were grown for 40 h in the presence (RhoAN19 off) or absence (RhoN19 on) of 1 μg ml^{-1} tetracycline. The cells were left untreated or treated with HdCDT (2 μg ml^{-1}) for 40 h, and then cell survival was assessed indirectly by counting the number of detached cells present in the culture supernatant. A twofold increase in detached cells was observed in the HdCDT-treated cells expressing the RhoAN19 dominant-negative mutant compared with the HdCDT-treated cells grown in the presence of tetracycline and thus not expressing RhoAN19 (Table 2). Expression of RhoAN19 did not affect cell viability in non-intoxicated cells as shown by: (i) intact chromatin structure (Fig. 10B); and (ii) similar amounts of detached cells in induced and non-induced samples (Table 2).

Discussion

In the field of bacterial protein toxins, CDTs are quite unique as cellular intoxication resembles the effects of DNA-damaging agents such as ionizing radiation (Cortes-Bratti *et al*., 2001b; Li *et al*., 2002). The aim of this study was to assess whether CDTs induce DNA DSBs in naturally intoxicated cells, and whether the activation of the actin cytoskeleton observed as a consequence of the intoxication was mediated by the small GTPases of the Rho subfamily as a default response to DNA damage.

HdCDT causes DNA double-strand breaks

Sert *et al*. (1999) have reported previously that the *E. coli* CDT did not induce DNA strand breaks in HeLa cells 24 h after treatment, as assessed by the gel electrophoresis-based comet assay. In the literature, this is the only study in which CDT-induced DNA fragmentation was studied in naturally intoxicated cells. DNA fragmentation was observed in *Saccharomyces cerevisiae* expressing the *C. jejuni* CdtB (CjCdtB) subunit under the *GAL1* promoter (Hassane *et al*., 2001), and chromatin collapse, but no DNA strand breaks, was observed in cells upon microinjection of 1 mg ml^{-1} purified CjCdtB (Lara-Tejero and Galan, 2000). However, direct DNA damage has never been demonstrated in cells exogenously exposed to the CDT holotoxin. To clarify whether natural intoxication, without ectopic expression of CdtB, is associated with DNA damage, we used PFGE analysis. Cells exposed to

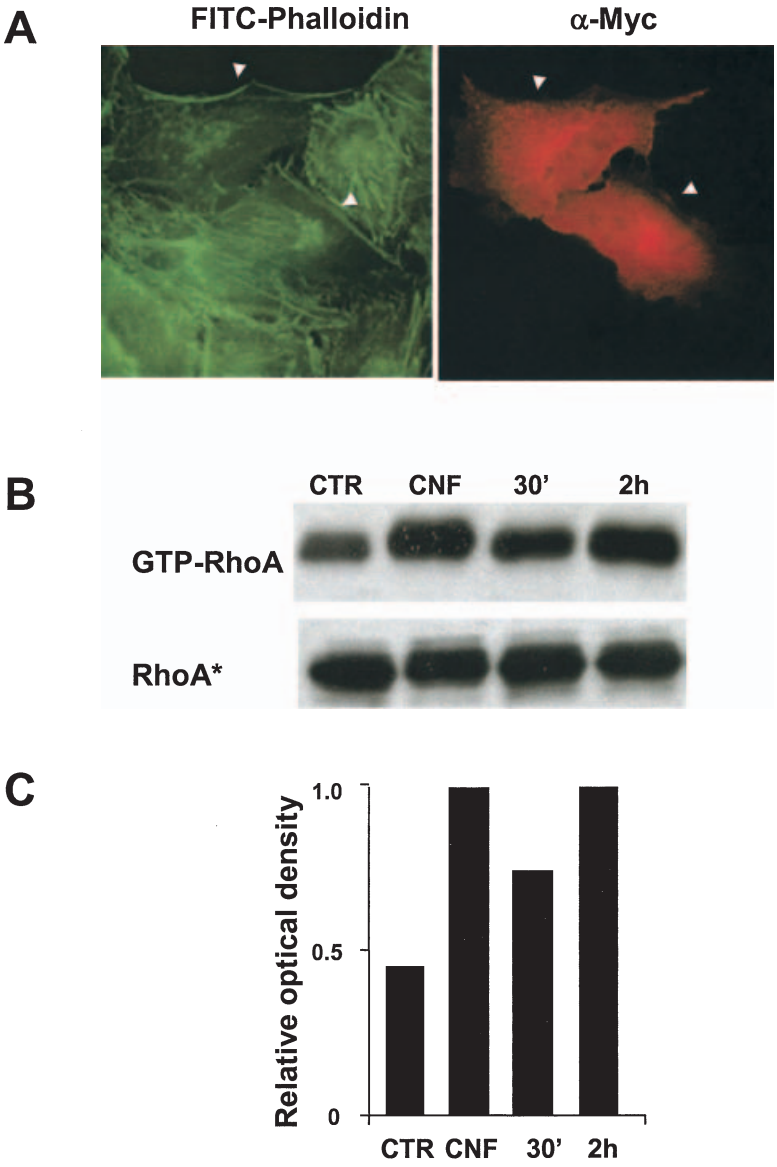

Fig. 6. RhoA is activated in irradiated cells.
A. HeLa cells were transfected with dominant-negative RhoAN19 and, 24 h after transfection, cells were irradiated (20 Gy) and incubated for an additional 24 h in complete medium. Transfected cells were visualized by immunofluorescence using anti-Myc 9E10 antibody, and F-actin was stained with FITC-phalloidin as described in *Experimental procedures*. Arrows indicate RhoAN19-transfected cells.
B. HeLa cells were irradiated (20 Gy) and GTP-bound Rho A was precipitated using GST-TRBD coupled to glutathione sepharose at the indicated time periods. Western blot analysis was performed as described in the legend to Fig. 3A.
C. Quantification of the GTP-bound RhoA was performed as described in the legend to Fig. 3B. One out of three experiments is shown.

20 μg ml^{-1} HdCDT for 8 h showed a number and a distribution of DNA fragments similar to that observed in irradiated cells and corresponding to an irradiation dose of 22 Gy (Fig. 1 and Table 1). The discrepancy between the comet and PFGE assays probably reflects a different sensitivity of the two methods used. The choice of PFGE was based on previous results demonstrating that HdCDT intoxication induced all the molecular effects observed in

Table 2. Effect of RhoAN19 expression on cell survival.

	RhoAN19 OFF			RhoAN19 ON			
	CTR[a]	HdCDT[a]		CTR[a]	HdCDT[a]		% RhoAN19-positive cells
Expt 1	5	10	2[b]	5	20	4[b]	38%
Expt 2	5	14	2.8	3	23	7.6	ND
Expt 3	1.75	5.8	3.3	1.75	10.3	5.9	36%

a. Number of suspension cells (×10^4) 40 h after intoxication. Forty thousand T23 MDCK RhoAN19 cells per well were grown in 12-well plates for 40 h in the presence (CTR) or absence (N19) of 1 μg ml^{-1} tetracycline. The cells were further left untreated or treated with HdCDT (2 μg ml^{-1}) for 40 h; at this time point, detached cells present in the culture supernatant were counted.
b. Ratio between the number of intoxicated and untreated cells.
ND, not done.

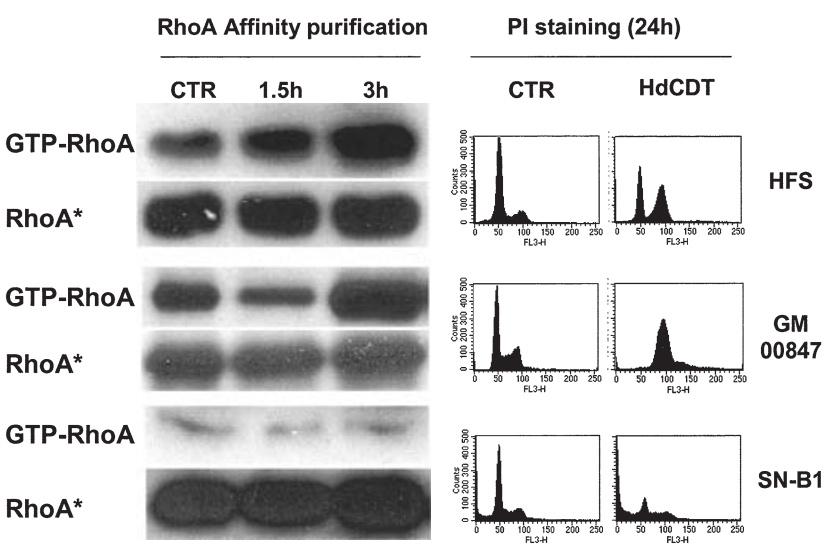

Fig. 7. The effect of HdCDT on RhoA activation is cell type dependent. GTP-bound RhoA was purified from cell lysates derived from HdCDT-treated or untreated primary foreskin fibroblasts (HFS), SV40 transformed GM00847 fibroblasts and the B-cell line SN-B1 at the indicated time periods as described in *Experimental procedures*. Samples were processed for Western blot as described in the legend to Fig. 3A. RhoA* indicates the total amount of protein present in the cytosolic extract. Cell cycle distribution was assessed by DNA staining with PI and flow cytometry analysis 24 h after intoxication. The G1 peak was arbitrarily set at the mean fluorescence intensity value of 50.

cells exposed to an irradiation dose of 20 Gy, including the ATM-dependent phosphorylation of the histone H2AX and the relocalization of the Mre11 DNA repair complex (Li *et al*., 2002). It is noteworthy that induction of DNA DSBs upon HdCDT treatment was a time-dependent phenomenon (Fig. 1 and Table 1). DNA fragments were clearly seen 6 h after intoxication, and this pattern corresponded to a radiation dose of 5 Gy. The time-dependent induction of DNA fragments is consistent with the changing distribution of phospho-H2AX foci reported previously and interpreted as an increasing number of DNA lesions produced during the course of intoxication (Li *et al*., 2002). There was a 10-fold difference between the toxin concentration required to detect DNA DSBs by PFGE analysis (20 μg ml^{-1}, Fig. 1C) and that sufficient to induce complete cell cycle arrest and H2AX phosphorylation (2 μg ml^{-1}, data not shown), probably due to the different level of sensitivity of these methods.

ATM-dependent RhoA activation: novel signalling pathway

One of the effects of CDT intoxication in fibroblasts and epithelial cells is cell distension and induction of actin stress fibres. Several bacterial toxins are known to modify Rho proteins directly, causing either inactivation (Just *et al*., 1995a,b) or activation (Horiguchi *et al*., 1995; Flatau *et al*., 1997; Schmidt *et al*., 1997). The *E. coli* CNF activates Rho by deamidation of glutamine 63 (Flatau *et al*., 1997; Schmidt *et al*., 1997) and induces a similar pattern of stress fibre promotion to that observed in CDT-treated cells. However, previous work performed in our laboratory failed to detect any differences in the molecular size of either Rho or Ras proteins in toxin-treated cells, suggesting that no covalent modification had taken place, and possibly excluding a direct effect of CDTs on these proteins (Cortes-Bratti *et al*., 1999). Here, we demonstrate that promotion of actin stress fibres in HdCDT cells is mediated by activation of the RhoA GTPase as part of the complex response to the cellular intoxication. Requirement for RhoA, but not Rac or Cdc42, was demonstrated by (i) inhibition of the promotion of actin stress fibres in

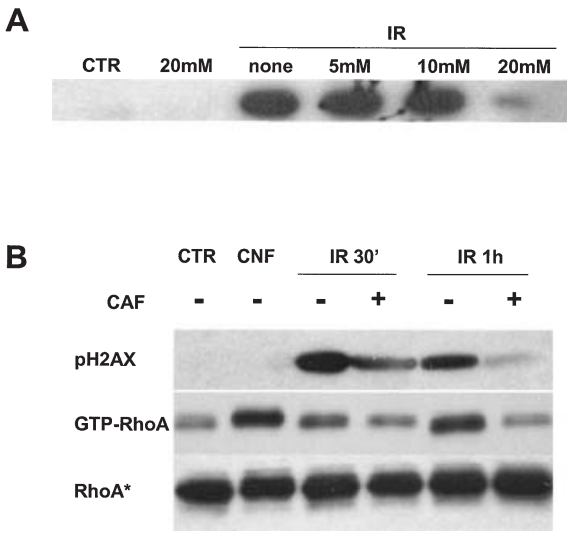

Fig. 8. Caffeine inhibits RhoA activation.
A. HeLa cells were incubated without or with increasing amounts of caffeine for 1 h. Cells were further left untreated (CTR) or irradiated (20 Gy). After a 40 min incubation at 37°C in complete medium, samples were prepared for Western blot analysis with the phospho-specific H2AX antibody as described in *Experimental procedures*.
B. HeLa cells were incubated with or without caffeine (20 mM) for 1 h. Cells were further left untreated (CTR) or irradiated (20 Gy), and affinity purification of the GTP-bound RhoA was performed at the indicated time periods as described in *Experimental procedures*. Western blot analysis was performed as described in the legend to Fig. 3A. To detect H2AX phosphorylation, 25 μg of nuclear extract was fractionated in a 12% SDS-polyacrylamide gel.

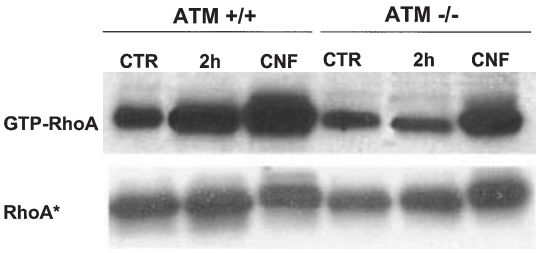

Fig. 9. RhoA activation is prevented in ATM-deficient cells. The GM00847 (ATM+/+) and the GM05849 (ATM–/–) cell lines were left untreated (CTR) or treated with HdCDT (2 μg ml^{-1}). Affinity purification of the GTP-bound RhoA was performed 2 h after intoxication as described in *Experimental procedures*. Western blot analysis was performed as described in the legend to Fig. 3A. One out of two experiments is shown.

HeLa cells transfected with the dominant-negative RhoAN19; and (ii) affinity precipitation of increasing amounts of active (GTP-bound) RhoA in intoxicated cells while the total RhoA remained constant (Figs 3 and 5). Activation of the actin cytoskeleton occurred with similar kinetics in HeLa cells and human fibroblasts exposed to ionizing radiation (Figs 2A and 6; data not shown).

Activation of the Rho GTPases via cell surface receptors has been studied extensively, and many details of these pathways have been elucidated (reviewed by Hall, 1998; Sander and Collard, 1999; Bar-Sagi and Hall, 2000). However, little is known about activation of these proteins via intracellularly triggered signals. We asked whether the same molecules that sense DNA damage and activate cell cycle checkpoint responses also control RhoA activation. Cross-talk between DNA damage-induced pathways and the actin cytoskeleton was suggested by Althaus *et al*. (1999), who showed that polyADP ribosylation mediated by the poly(ADP-ribose) polymerase (PARP) may work as a DNA break signal mechanism and activate the MARCKS protein, involved in the regulation of the actin cytoskeleton. Here, we assessed whether the ATM kinase is required for RhoA activation in irradiated or intoxicated cells. ATM was chosen, because it plays a key role in the cellular response to genotoxic agents, and many details of the ATM-dependent pathways activated by ionizing radiation are well characterized (reviewed by Shiloh, 2001). Blocking ATM with caffeine (Fig. 8) or lack of functional ATM (Fig. 9) was sufficient to inhibit early RhoA activation. These data highlight a novel signalling pathway, linking nuclear events to the actin cytoskeleton, with ATM acting as a key effector molecule.

Members of the PI3K family, which also includes ATM, ATR and DNA-PK, play an important role in the regulation of cell size. In particular, the PI3K-activated mTOR has been implicated directly in these pathways (Fingar *et al*., 2002; reviewed by Schmelzle and Hall, 2000). We observed that both intoxication and irradiation induce a remarkable increase in cell size. This distension was not dependent on RhoA activation, as toxin-treated cells expressing the dominant-negative mutant RhoAN19 show similar distension to non-transfected cells (Fig. 3D).

As expected, activation of PI3K played an important role in controlling cell distension upon intoxication, whereas no clear effect of the dominant-negative p85 mutant was observed regarding induction of stress fibres (Fig. 4; data not shown).

Conceivably, actin cytoskeleton reorganization and cell distension could be controlled by members of the PI3K family via activation of two independent pathways.

RhoA activation and cell survival

We investigated whether the DNA damage-induced RhoA activation played any role in cell survival. Forty hours after HdCDT treatment, the T23 MDCK cells overexpressing the RhoAN19 mutant protein detached and died faster than cells expressing a functional RhoA (Fig. 10 and Table 2). The importance of Rho for cell survival has been demonstrated previously in several systems. RhoA activation mediated by the *E. coli* CNF protected HEp-2 cells from UVB-induced apoptosis via upregulation of the Bcl-2 and Bcl-X_L proteins (Fiorentini *et al*., 1998). Mice transgenic for the C3 transferase of *Clostridium botulinum*, which inactivates Rho by ADP ribosylation, showed a striking proliferative and cell survival defect during T-cell development (Henning *et al*., 1997). Inhibition of Rho in primary and transformed cell lines by the C3 transferase disrupted cellular attachment and induced apoptosis (Bobak *et al*., 1997).

RhoA activation was observed at early stages upon intoxication in cells of epithelial and fibroblast origin (Figs 3 and 7), including HeLa cells, primary foreskin and SV40 transformed fibroblasts. The effect on RhoA did not depend on the type of cell cycle arrest or the status of the tumour suppressor gene *p53*, as it was similarly activated in cell lines carrying both functional and non-functional p53. However, RhoA activation was not detected in the SN-B1 B-cell line growing in suspension. This kind of response may take place only in cells growing as adherent monolayers, as B cells and dendritic cells, which grow in suspension, died upon intoxication without a clear arrest in any phase of the cell cycle (Fig. 7; Cortes-Bratti *et al*., 2001b; Li *et al*., 2002), supporting the hypothesis that RhoA may be essential for extending the survival time. We did not detect any change in the levels of expression of the Bcl-2 and Bcl-X_L proteins upon intoxication or irradiation (data not shown). Thus, RhoA may activate alternative survival signals upon induction of DNA DSBs.

RhoA activation has been implicated in controlling progression from the G1 to the S phase of the cell cycle, via repression of the G1 cyclin-dependent kinase inhibitor p21

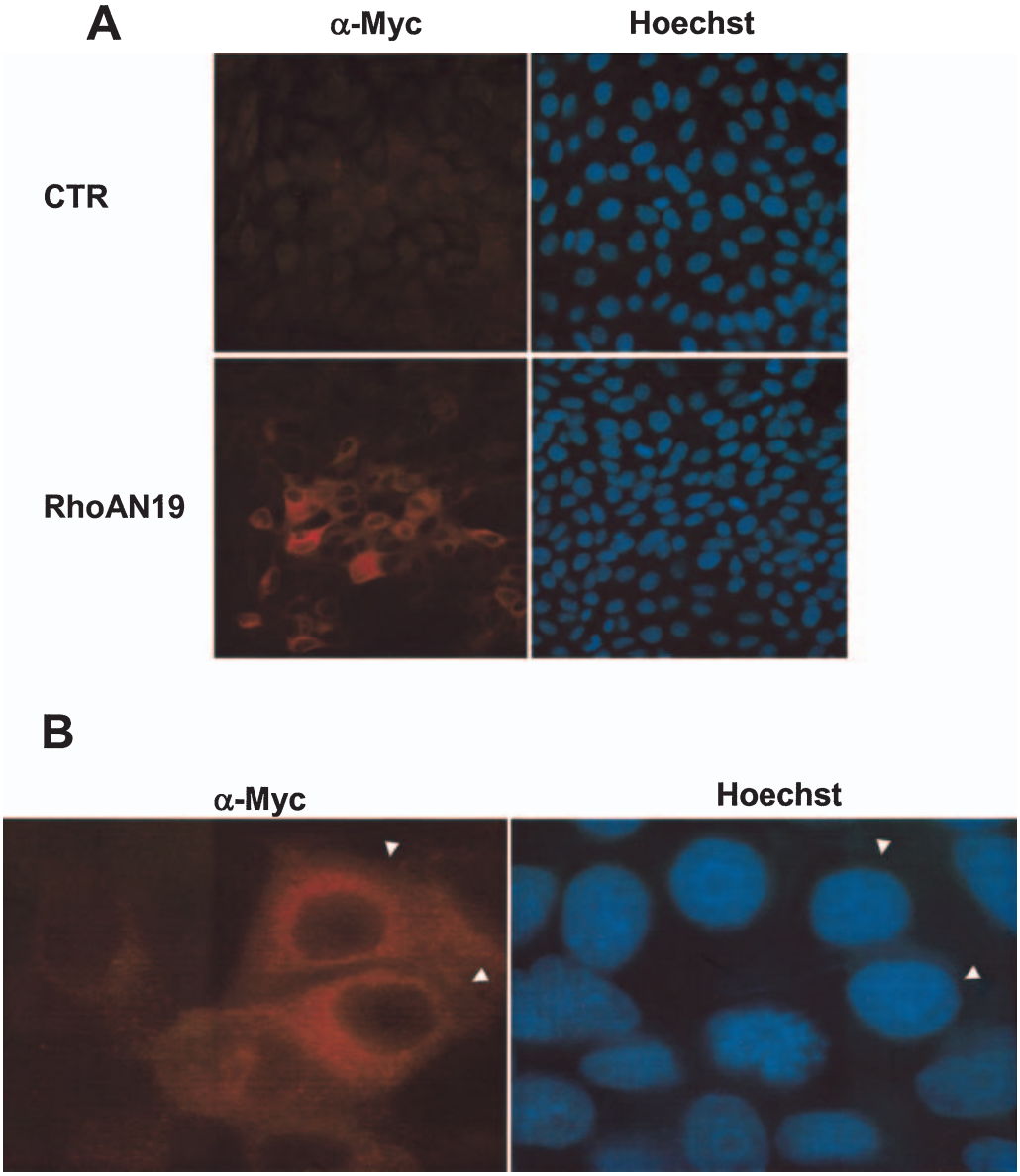

Fig. 10. Expression of tetracycline-regulated RhoAN19 and effect on chromatin structure. Twenty thousand T23 MDCK RhoAN19 cells per well were grown in 12-well plates on 13 mm slides for 40 h in the presence (CTR) or absence (RhoAN19) of 1 μg ml^{-1} tetracycline. Expression of RhoAN19 was assessed by indirect immunofluorescence using the anti-Myc 9E10 monoclonal antibody as described in *Experimental procedures*.

(Olson *et al*., 1998). Our data show that cell cycle arrest caused by DNA damage is also associated with RhoA activation. Upon induction of DNA damage, the checkpoint responses activated via the ATM/ATR kinases apparently over-ride the ability of RhoA to induce progression into the S phase, and instead favour its antiapoptotic effects.

In conclusion, we have demonstrated that one of the early effects of CDT intoxication is induction of DNA DSBs, with consequent promotion of actin stress fibres via an ATM-dependent activation of the small GTPase RhoA. Our results disclose a novel signalling pathway that connects damaged DNA in the nucleus with the actin cytoskeleton, possibly involved in regulating cell survival under stress conditions. Our data further underline the importance of bacterial toxins as tools to dissect unknown intracellular signalling pathways.

Experimental procedures

Cell lines

The following cell lines were used: HeLa (ATCC CCL-2), SV40 transformed GM00847 (ATM wild type) and GM05849 (ATM-deficient) human fibroblasts (Coriell Cell Repositories), the SN-B1 lymphoblastoid cell line (LCL), obtained by *in vitro* infection

of B lymphocytes from healthy donors with the B95.8 strain of Epstein–Barr virus as described previously (Miller and Lipman, 1973), T23 MDCK expressing RhoAN19 under the control of the tetracycline-repressible transactivator (a kind gift from Dr J. W. Nelson; described by Jou and Nelson, 1998). All cell lines were cultivated in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum (FBS), 5 mM L-glutamine, penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}) (complete medium) in a humid atmosphere containing 5% CO_2. The T23 MDCK RhoAN19 cells were grown in complete medium containing 1 μg ml^{-1} tetracycline.

Materials

HdCDT was purified from culture medium of recombinant *E. coli* DHα expressing the *H. ducreyi cdtABC* genes as described previously (Svensson *et al.*, 2001), and it was kindly supplied by T. Lagergård, University of Gothenburg, Sweden. The protein concentration of the stock solution was 2 mg ml^{-1}.

To test the specific effect of HdCDT and to rule out artifacts resulting from potential contaminants in the toxin preparation, we used the following material: (i) filtered bacterial supernatants containing the *H. ducreyi* CdtA and CdtC subunits (Frisk *et al.*, 2001), named CdtAC; (ii) *H. ducreyi* CdtB subunit purified from the pGEX-CdtB expression, at a final concentration of 20 μg ml^{-1}; (iii) the CdtAC supernatant and the purified CdtB (final CdtB concentration 200 μg ml^{-1}) were incubated for 30 min at 37°C and then used as holotoxin stock (named CdtABC). This preparation could induce complete cell cycle arrest 24 h after intoxication, and H2AX phosphorylation in 80% of HeLa cells 4 h after intoxication when diluted 100-fold (CdtB final concentration 2 μg ml^{-1}; data not shown). Further dilution resulted in partial G2 arrest and induction of H2AX phosphorylation in 35% of the cells. The limiting factor for this effect was possibly the low concentration of CdtA and CdtC present in the bacterial supernatant.

The pGEX-CdtB plasmid was constructed by polymerase chain reaction (PCR) amplification of the *H. ducreyi cdtB* gene from the pAF-tac1cdtB plasmid (Frisk *et al.*, 2001) using the following primers: 5′-TACCGAATTCTTAGCGATCACGAACAAAACT-3′ and 5′-ATTCGGATCCAGTCATGCAGAATCAAATCCTGA-3′. The PCR fragment was cloned into the *Bam*HI and *Eco*RI restriction sites of the pGEX4T3 expression vector (Amersham Biosciences). Purification and thrombin cleavage of the fusion protein from the *E. coli* BL21 DE3 strain was performed according to the manufacturer's instructions (Amersham Biosciences).

The plasmids expressing the c-Myc epitope-tagged RhoAV14, RhoAN19, Rac1V12, Rac1N17 derived from the pEXV vector (Qiu *et al.*, 1995a,b) and the plasmids encoding the c-Myc epitope-tagged Cdc42V12 and Cdc42N17 derived from the pMT90 vector were kindly provided by G. Flatau and P. Boquet from Institut National de la Santé et de la Recherche Médicale, Nice, France. The pGEX-2T-TRBD plasmid expressing the GST-tagged TRBD (Rhotekin Rho-binding domain) was kindly provided by X.-D. Ren and M. A. Schwartz from The Scripps Research Institute, California, USA. The p21-binding domain (PBD) of p21-activated kinase (PAK) is expressed as a GST fusion protein from a derivative pGEX-2T plasmid, and was a generous gift from G. M. Bokoch of The Scripps Research Institute, California, USA. The pSG5 p85αΔiSH2-N plasmid has been described previously (Rodriguez-Viciana *et al.*, 1997) and was a kind gift from Dr J. Downward (Signal Transduction Laboratory, London Research Institute, London, UK). The *E. coli* CNF was prepared as described previously (Flatau *et al.*, 1997).

Treatments

HdCDT intoxication. Cells were incubated for the indicated time periods with HdCDT (2 μg ml^{-1}) in complete medium.

CNF intoxication. Cells were incubated for 3 h with CNF (3 ng ml^{-1}) in complete medium.

Ionizing radiation. Cells were irradiated (20 Gy), washed once in phosphate-buffered saline (PBS) and incubated for the indicated time periods in complete medium.

Detection of DNA DSBs by PFGE

Fifty thousand HeLa cells per well were grown in 12-well plates in complete medium containing 4000 Bq [methyl-^{14}C]-thymidine for 72 h. The cells were then washed three times in PBS and chased for 2 h in complete medium. Cells were treated with HdCDT (20 μg ml^{-1}) for the indicated time periods or left untreated for 8 h. Irradiated cells (20 Gy) were used as a positive control for detection of DSBs. Cells were washed once in PBS and trypsinized in a 150 μl volume. The cell suspension was mixed with low gelling point agarose (InCert; BMA) at 37°C to a final agarose concentration of 0.6% and 1.0×10^6 cells ml^{-1} and pipetted into plastic moulds for 100 μl plugs. Moulds were incubated at 4°C for 20 min to solidify the plugs. The solid plugs were transferred into 1 ml of ice-cold lysis buffer (2% sarkosyl, 1 mg ml^{-1} proteinase K, in 0.5 M Na_3-EDTA, pH 8.0) overnight at 4°C, followed by a second lysis step in high-salt solution (1.85 M NaCl, 0.15 M KCl, 5 mM $MgCl_2$, 2 mM EDTA, 4 mM Tris, 0.5% Triton X-100, pH 7.5) overnight at 4°C (Stenerlöw *et al.*, 2003). Plugs were washed three times for 1 h in 0.1 M EDTA, and double-stranded DNA was separated on an agarose gel (0.8% SeaKem Gold; BMA) in a PFGE unit (Gene Navigator; Amersham Bioscience) in 0.5× TB as described previously (Stenerlöw *et al.*, 2003). After electrophoresis, the gels were sliced at the position of the 5.7 Mbp chromosome from *Schizosaccharomyces pombe* (BMA), and ^{14}C in the gel segments was measured by liquid scintillation. The fraction of radioactivity corresponding to DNA <5.7 Mbp was used to quantify the number of DSBs (Stenerlöw *et al.*, 1999) assuming an induction of 25 DSBs per diploid cell per Gy (Stenerlöw *et al.*, 2003).

Transfection and immunofluorescence

Fifty-thousand HeLa cells were grown on 13 mm slides. Transfection was performed with 1 μg of the relevant plasmid with Lipofectamine 2000 (Life Technologies), according to the manufacturer's instructions. Twenty-four hours after transfection, cells were either intoxicated or irradiated and incubated further for 24 h, when cells were prepared for immunofluorescence analysis.

Cells were washed three times in PBS, fixed with 3.7% paraformaldehyde for 15 min at 22°C and permeabilized with 0.5% Triton X-100 in PBS for 30 min at 22°C. After permeabilization, slides were blocked in PBS, 3% bovine serum albumin (BSA) for 30 min at 22°C and incubated further for 1 h at 22°C with anti-Myc 9E10 (Santa Cruz) or PI3K-p85 (BD Transduction Laboratories) monoclonal antibodies diluted 1:50 in PBS. Slides were washed three times for 5 min in PBS, and then incubated

with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated rabbit anti-mouse antibody (Dako; diluted 1:100 in PBS) for 30 min at 22°C. Finally, cells were stained with 1 μg ml^{-1} fluorescein isothiocyanate (FITC)-phalloidin (1 h at 37°C). Nuclei were counterstained with Hoechst 33258 (Sigma; 0.5 μg ml^{-1} for 1 min at 20°C), and slides were mounted and viewed via fluorescence microscopy.

Affinity precipitation of GTP-Rho, GTP-Rac and GTP-Cdc42

The GST-TRDB and the GST-PDB proteins were purified as described previously (Benard *et al*., 1999; Ren *et al*., 1999). Approximately 400 000 cells per well were grown in six-well plates and irradiated or treated with HdCDT for the indicated times. Lysates were prepared in ice-cold buffer containing 1% Triton X-100, 0.1% SDS, 0.3% Nonidet P-40, 50 mM Tris-HCl, pH 7.2, 500 mM NaCl, 10 mM $MgCl_2$ and 1 mM phenylmethylsulphonyl fluoride (PMSF). Cell lysates were clarified by centrifugation at 13 000 r.p.m. at 4°C for 3 min (defined as cytosolic lysate). The nuclei, resuspended in 150 μl of SDS electrophoresis sample buffer (Laemmli, 1970) were used to assess the level of H2AX phosphorylation by Western blot analysis. As a control for total GTPase content, 20 μl of the cytosolic lysate was taken for Western blot analysis. Equal volumes of cytosolic lysates were incubated with GST-TRBD or GST-PBD coupled to glutathione sepharose and incubated at 4°C for 45 min. Proteins bound to the glutathione sepharose were washed three times with cold buffer and resuspended in 30 μl of SDS-PAGE sample buffer (Laemmli, 1970).

Western blot analysis

Proteins were fractionated by 12% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) and probed with the respective antibodies. The following antibodies were used: anti-RhoA, anti-Cdc42 (Santa Cruz), anti-Rac (Transduction Laboratories), antiphospho-H2AX (Ser-139; Upstate Biotechnology), anti-Bcl-2 (Zymed Laboratories), anti-Bcl-X_L (PharMingen) and anti-phospho Akt (Cell Signalling). Blots were developed with enhanced chemiluminescence (ECL; Amersham Biosciences), using the appropriate horseradish peroxidase-labelled secondary antibody, according to the manufacturer's instructions (Amersham Biosciences).

Cell cycle analysis

Cells were trypsinized, centrifuged and washed once with PBS. The cell pellet was resuspended and fixed on ice for 15 min with 1 ml of cold 70% ethanol. The cells were subsequently centrifuged and resuspended in 1 ml of propidium iodide (PI) solution (0.05 mg ml^{-1} PI, 0.02 mg ml^{-1} RNase, 0.3% NP40, 1 mg ml^{-1} sodium citrate) for 1 h at 4°C. Flow cytometry analysis was performed using a FACSort flow cytometer (Becton Dickinson). Data from 10^4 cells were collected and analysed using the CELLQUEST software (Becton Dickinson).

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