Study of the cytolethal distending toxin (CDT)-activated cell cycle checkpoint

Involvement of the CHK2 kinase

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Abstract The bacterial cytolethal distending toxin (CDT) triggers a G2/M cell cycle arrest in eukaryotic cells by inhibiting the CDC25C phosphatase-dependent CDK1 dephosphorylation and activation. We report that upon CDT treatment CDC25C is fully sequestered in the cytoplasmic compartment, an effect that is reminiscent of DNA damage-dependent checkpoint activation. We show that the checkpoint kinase CHK2, an upstream regulator of CDC25C, is phosphorylated and activated after CDT treatment. In contrast to what is observed with other DNA damaging agents, we demonstrate that the activation of CHK2 can only take place during S-phase. Use of wortmannin and caffeine suggests that this effect is not dependent on ATM but rather on another as yet unidentified PI3 kinase family member. These results confirm that the CDT is therefore responsible for specific genomic injuries that block cell proliferation by activating a cell cycle checkpoint.

Key words: Checkpoint 2 kinase; Cytolethal distending toxin; Cell cycle; CDC25C

1. Introduction

Bacterial cytotoxins are proteins whose ability to interfere with eukaryotic cell cycle opens new areas in our understanding of cellular proliferation. A family of cytolethal distending toxins (CDT) found in various bacterial species including *Escherichia coli*, *Shigella dysenteriae* and *Campylobacter jejuni* inhibit proliferation in a vast majority of vertebrate cell lines [1]. In HeLa cells, exposure to CDT induces a cell cycle block at the G2/M transition which results in the maintenance of CDK1, a key regulator of entry in mitosis, in a tyrosine 15 phosphorylated inactive form [2,3]. The CDC25 phosphatase is able to reverse this state to a fully active CDK1/cyclin B complex both in vitro and in vivo implying that phosphorylation on tyrosine 15 is responsible for the maintenance of an inactive CDK1 [3,4]. Although genomic injuries were not detected by comet assay upon CDT treatment of HeLa cells [3], this situation was reminiscent of a signalling cascade which activates a G2/M checkpoint following DNA damage [5]. In agreement with this hypothesis, it has recently been shown that DNase I homologous residues are present in cdtB and are critical for CDT-induced cell cycle arrest [6,7].

In eukaryotes, cell cycle checkpoints help to ensure the orderly progression and completion of critical events such as DNA replication and chromosome segregation [8]. By acting at different stages in the cell cycle, checkpoints delay G1/S or G2/M transitions or inhibit DNA synthesis depending on the type of injury [9]. Defects in the checkpoint pathway often result in enhanced sensitivity to damaging agents and could increase genomic instability. Two members of the phosphatidyl inositol kinase (PIK)-related kinase family are important components of this DNA damage response pathway ATM, which is mutated in ataxia telangectasia (AT) syndrome and a related protein called ATR (for ATM and Rad3-related). Cells from AT individuals display an increased rate of chromosomal recombination and are defective in ionizing radiation (IR)-inducible G1/S, S-phase and G2/M checkpoints. Once these PIK-related kinases have been activated, the next stage is activation/phosphorylation of downstream targets including tumor suppressor gene p53 [10–12], and checkpoint proteins 1 and 2 (CHK1 and CHK2). CHK2 is phosphorylated in an ATM-dependent manner in response to IRs which induce double-strand breaks and in an ATM-independent and possibly ATR-dependent manner in response to UV light and stalled replication [13–15]. In the case of CHK1 the available evidence suggests that in the case of UV irradiation its response to DNA damage is ATR-dependent [16,17].

Human CHK2 and CHK1 phosphorylate CDC25C on serine 216 [13,18–20] thus creating a binding site for 14.3.3 proteins that is responsible for the cytoplasmic sequestration of CDC25C and the subsequent impairment of nuclear CDK1 dephosphorylation [21]. Other identified substrates of CHK2 are the tumor suppressor protein p53 [22–24] and BRCA1 [25].

In this study, we have investigated the involvement of CHK2 in the cell cycle checkpoint response to CDT. We demonstrate that CHK2 is activated after CDT exposure during the S-phase. We also show that caffeine but not wortmannin is able to revert the CDT-dependent CHK2 activation. The CDT toxin is therefore able to target the G2/M DNA cell cycle checkpoint machinery through a mechanism that

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comprises CHK2 activation by an ATM-independent pathway.

2. Materials and methods

2.1. Cell culture and transfection

HeLa cells, obtained from the American Type Culture Collection, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (Eurobio), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified atmosphere containing 5% CO_2 at 37°C.

2.2. CDT, wortmannin and caffeine treatment

CDT was produced from *E. coli* DH5-α hosting recombinant plasmid pDS7-96, which contains the three open reading frames (*cdtA*, *cdtB* and *cdtC*) necessary to encode CDT activity. The toxic preparation consisted in the sterile supernatant of a 24 h trypticase soy broth aerated culture of this recombinant strain as previously described [2]. The toxic activity (CD100) was defined as the lower cytopathic dose that caused 100% of enlarged cells after 72 h of incubation and where more than 90% of the exposed cells are irreversibly blocked at stage G2/M after 24 h. Treatment with caffeine (2 mM) and wortmannin (20 μM) was started 1 h prior to CDT exposure. The cells were then washed and further treated with caffeine or wortmannin during 16 h before harvesting and biochemical analysis.

2.3. Immunofluorescence

Cells grown on glass coverslips were recovered, washed once with PBS, fixed in 3.7% formaldehyde in PBS at 4°C for 40 min then washed three times with PBS. Cells were then permeabilized with 0.25% Triton X-100 in PBS for 5 min at room temperature and with a further incubation in 100% cold methanol for 10 min at −20°C. Fixed and permeabilized cells were then saturated with 1% BSA in PBS for 15 min at room temperature. The cells were then incubated for 1 h at 37°C with polyclonal anti-CDC25C antibody (C20; Santa Cruz) diluted 1/250 in PBS/BSA 1%. After three washes with PBS/BSA 1%, the cells were incubated with Alexa 594-conjugated goat anti-rabbit antibodies (Molecular Probes) diluted 1/500 in PBS/BSA 1% for 45 min at room temperature, washed in PBS/BSA 1% and finally incubated with Hoechst 1 μg/ml for 10 min at room temperature. Coverslips were washed with PBS and mounted in Mowiol. Microscopic examinations were performed under a Leica photomicroscope.

2.4. Immunoblotting

Cells grown on 100 mm petri dishes were harvested by trypsinization and centrifuged at 200×*g* for 10 min. Cells were then lysed in 20 mM Tris–HCl buffer (pH 8.0) containing 250 mM NaCl, 0.5% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, aprotinin (2 μg/ml), leupeptin (2 μg/ml) and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 15 min at 4°C. The cell lysates were centrifuged for 10 min at 14 000 rpm. After centrifugation, proteins (10 μg) were separated by electrophoresis on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes using a semi-dry transfer apparatus. The membranes were then saturated with non-fat milk (5%) in TBST [26] for 30 min at room temperature then incubated with anti-CHK2 (gift from Elledge) diluted 1/1000 in TBST for 1 h. After three washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Biolabs) diluted 1/2500 in TBST for 30 min at room temperature, washed five times in TBST and finally detected by enhanced chemiluminescence.

2.5. Immunoprecipitation and kinase assay

Cell extracts were prepared as above described. 1 mg of protein was first pre-cleared by incubation with protein A-Sepharose beads for 45 min at 4°C. Cell extracts were centrifuged at 14 000 rpm for 10 min and the supernatant was then incubated with anti-CHK2 (1 μl) for 2 h at 4°C. Protein A-Sepharose beads were added for 1 h at 4°C and then the immunoprecipitated beads were washed four times with 20 mM Tris–HCl (pH 8.0), 250 mM NaCl, 0.5% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, aprotinin (2 μg/ml), leupeptin (2 μg/ml) and 1 mM PMSF. The CHK2 immunoprecipitates were then washed three times with 50 mM HEPES (pH 8.0), 10 mM $MgCl_2$, 2.5 mM EGTA, 1 mM DTT, 1 mM NaF, 0.1 mM Na_3VO_4 and 0.1 mM PMSF. The kinase assay was performed in 50 mM HEPES (pH 8.0), 10 mM $MgCl_2$, 2.5 mM EGTA, 1 mM DTT, 1 mM NaF, 0.1 mM Na_3VO_4, 0.1 mM PMSF, 20 μM adenosine triphosphate (ATP), and 5 μCi [γ-^{32}P]ATP for 30 min at 30°C in a 30 μl reaction volume. Proteins were then separated by SDS–PAGE and visualized by Coomassie blue staining and autoradiography.

2.6. Flow cytometry

Cell cycle distribution was assessed essentially as described previously [3] using a FACScalibur flow cytometer (Becton Dickinson). Collection and analysis of the cell cycle were performed using the CellQuest and the ModFit software (Becton Dickinson).

3. Results

3.1. Effects of CDT on CDC25C phosphatase intracellular localization

We first examined the intracellular localization of CDC25C in HeLa cells treated or not for 24 h with CDT. As shown in Fig. 1A, while untreated cells displayed cytoplasmic or nuclear localization in agreement with previous publications [21], CDC25C was fully relocated in the cytoplasm of cells treated with CDT (Fig. 1B). This is a typical feature of the G2/M checkpoint activation, where the phosphorylation of CDC25C leads to its binding to 14.3.3 proteins and to its cytoplasmic accumulation and functional inactivation. This observation as well as our recent findings that CDC25C overexpression bypassed G2/M arrest in CDT-treated cells [4], led us to examine the involvement of an upstream regulator of CDC25C, the checkpoint kinase CHK2, in that regulation.

3.2. CHK2 is activated in CDT-treated HeLa cells

In order to investigate the effects of CDT on CHK2, HeLa cells were treated for 24 h with 1 CD100 CDT or for 1 h with 30 μM etoposide as positive control, then washed twice and harvested 23 h later. Protein extracts were prepared and subjected to Western blot analysis using CHK2 antibodies. It has been reported for genotoxic treatment or replication block that the activation of checkpoint 2 (CHK2) protein kinase leads to a reduction of its electrophoretic mobility [13,27]. As shown in Fig. 2A a slower mobility form of CHK2 was also detected after CDT or etoposide treatment. Similar results were also observed when normal diploid fibroblasts IMR90 were treated with CDT (data not shown). Exposure of immunoprecipitated CHK2 to lambda phosphatase led to disappearance of the slower electrophoretic mobility form indicating that CHK2 is indeed phosphorylated in CDT-treated HeLa cells (not shown).

It has been demonstrated that in response to gamma irradiation CHK2 phosphorylation correlated with an increase in its protein kinase activity [13,14]. CHK2 was therefore immunoprecipitated from treated and untreated cells and subjected to a kinase assay. As shown in Fig. 2B, CHK2 kinase activity was at least 3-fold higher in CDT-treated cells as compared to untreated cells strengthening the fact that CHK2 is activated in response to CDT.

In order to investigate the relation between CDT treatment and activation of CHK2, a kinetic analysis of its phosphorylation was also performed by monitoring CHK2 change in electrophoretic mobility (Fig. 2C). Surprisingly in asynchronous HeLa cells exposed to CDT, CHK2 phosphorylation was barely detectable after 8 h and required about 24 h to be at a maximum. This result contrasts with data obtained

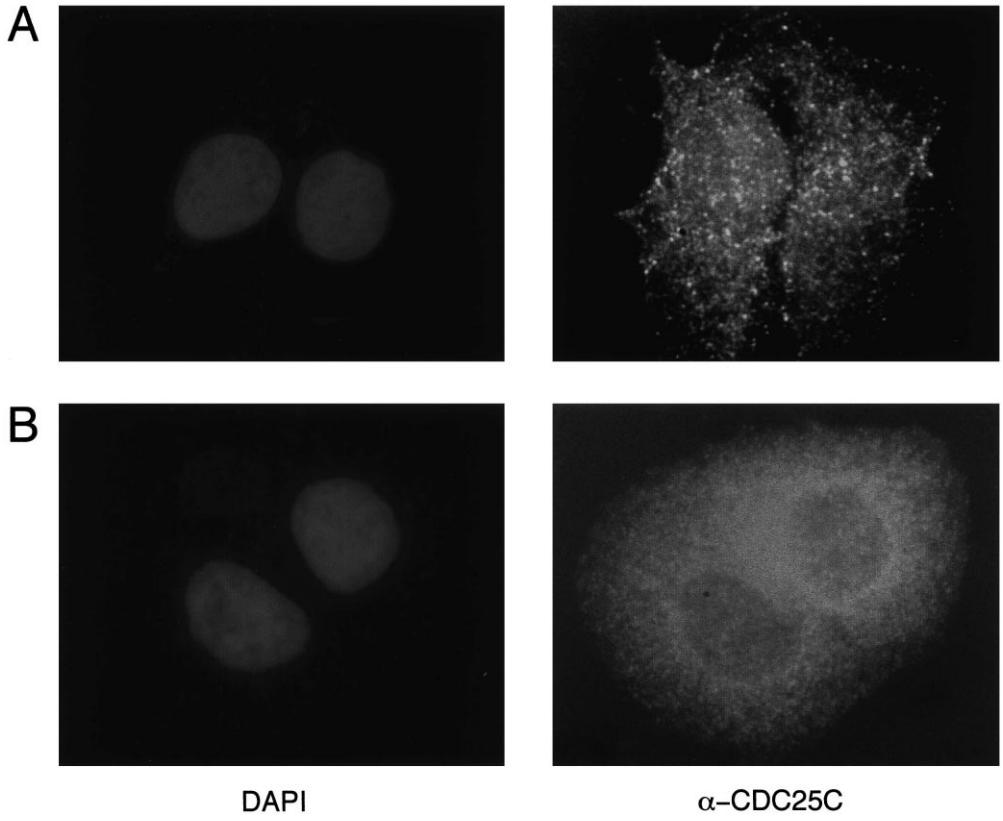

Fig. 1. Intracellular localization of CDC25C in CDT-treated cells. Exponentially growing HeLa cells were treated (B) or not (A) for 24 h with CDT. After 24 h the cells were fixed and processed for immunofluorescence detection of CDC25C using C20 antibodies.

after gamma irradiation of HeLa cells where CHK2 was fully phosphorylated in less than 30 min [13].

3.3. *CHK2 is rapidly phosphorylated in S-phase but not in G2-phase in HeLa cells*

The slow kinetics of accumulation of the CHK2 phosphorylated form together with the fact that passage of the cells through S-phase has been shown to be required for CDT activity [3], prompted us to investigate whether they should proceed to a specific stage of the cell cycle in order to allow CHK2 activation by CDT. To address that issue, HeLa cells were synchronized at the G1/S boundary using a double thymidine block then released and CDT was subsequently added at different stages of the cell cycle. Progression in the cell cycle was monitored in these experiments by flow cytometry in treated and untreated cells. In the case of CDT added just after release from thymidine block (i.e. at G1/S), the CHK2 phosphorylation was completed after 8 h and remains at this level up to 24 h (Fig. 3A). This phosphorylation of CHK2 fully paralleled the accumulation of the cells in G2. When the cells were treated by CDT 4 h after release from G1/S (i.e. in S-phase), complete CHK2 phosphorylation was achieved in about 4 h (Fig. 3B) and was also coincident with G2 accumulation. In contrast, when the cells were treated by CDT 8 h after release from thymidine block, i.e. when they were already mostly in G2, at least a 16 h delay was necessary to observe 50% of CHK2 phosphorylated form and nearly 24 h for a complete phosphorylation (Fig. 3C). That delay was also necessary to observe the accumulation of the cells with a G2 DNA content. This set of results indicates that in contrast to what has been reported with IRs, progression of the cells

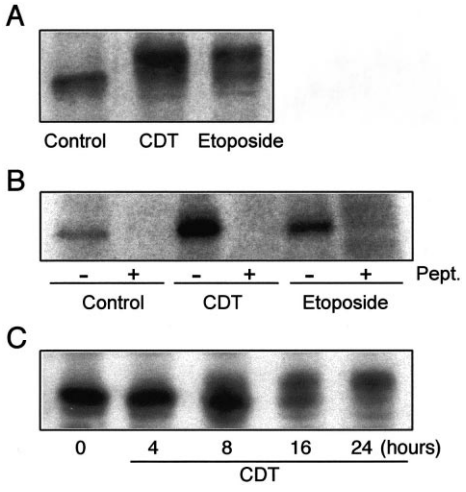

Fig. 2. Phosphorylation and activation of CHK2 in HeLa cells exposed to CDT. A: Asynchronous HeLa cells were treated or not with CDT or etoposide. For CDT the cells were treated for 24 h then harvested. For etoposide, cells were treated for 1 h then washed twice and collected 23 h later. Proteins were fractionated on SDS–PAGE and CHK2 was detected by Western blotting using anti-CHK2 antibodies. B: Immunoprecipitation of CHK2 from untreated, CDT-treated or etoposide-treated cells was performed in the presence or in the absence of immunizing peptide (pept.) then incubated with [γ-^{32}P]ATP for 30 min at 30°C then subjected to SDS–PAGE and autoradiography. C: Asynchronous HeLa cells were treated with CDT then collected at various times and analyzed as in A.

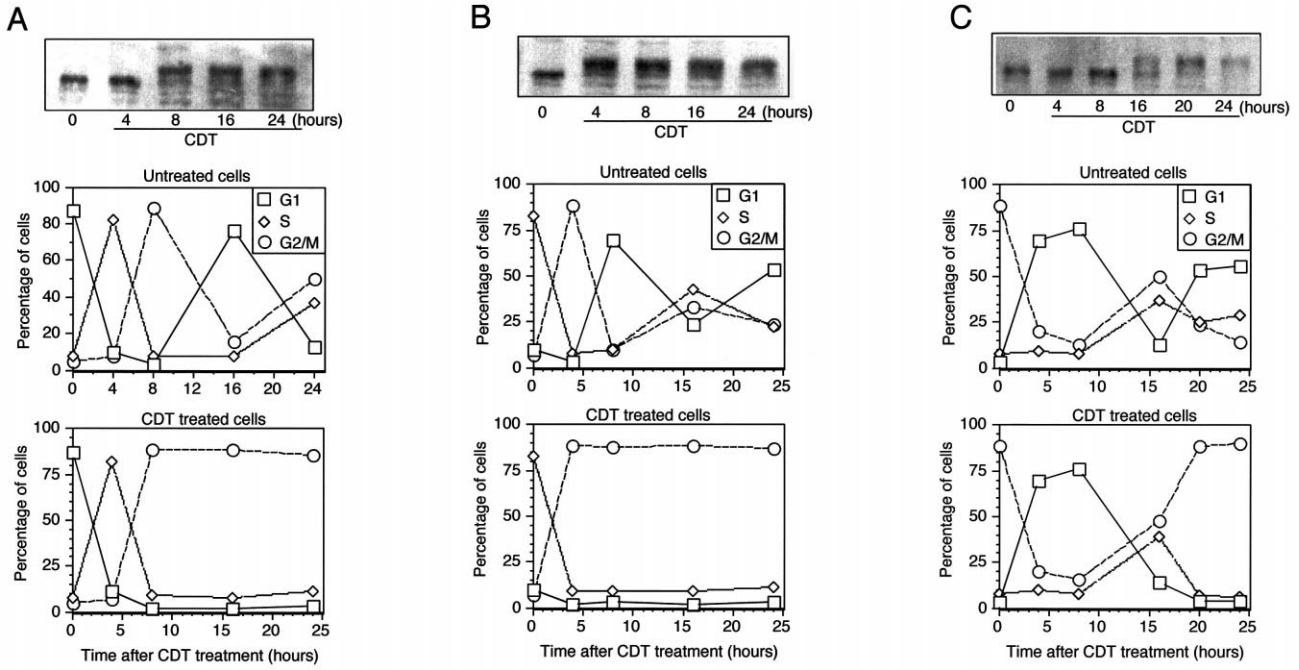

Fig. 3. Cell cycle regulated activation of CHK2 following exposure to CDT. HeLa cells were synchronized by a double thymidine block as described [3]. Cells were then released from G1/S block by double washing with PBS. A: Cells were treated immediately with CDT. B: Cells were released for 4 h before exposure to CDT. C: Cells were released for 8 h before exposure to CDT. A, B and C: Cells were harvested at the indicated times after treatment by CDT and subjected to Western blot analysis of CHK2 phosphorylation (top panel) and to cell cycle distribution analysis by flow cytometry after propidium iodide staining. Both untreated and CDT-treated cells were analyzed by flow cytometry. Note that in all panels the experiment started when CDT was added, that is to say immediately after thymidine block release (A), 4 h later (B) and 8 h later (C).

through S-phase is necessary to achieve the activation of CHK2 after treatment by the CDT toxin.

3.4. *ATM independence of CHK2 activation in cells exposed to CDT*

As we have already shown previously that caffeine is able to revert CDT-dependent cell cycle arrest in G2 [3] and given the fact that caffeine appears to inhibits ATM and ATR, we examined whether these PI3-related kinases were involved in CDT-dependent CHK2 activation. To examine that question, asynchronous HeLa cells were incubated with 2 mM caffeine or 20 μM wortmannin prior to CDT treatment. After 16 h, cells were lysed and protein extracts were analyzed by Western blot with anti-CHK2 antibody. As shown in Fig. 4, caffeine led to an important decrease in the phosphorylated CHK2 form. In contrast, wortmannin had little or no effect on the phosphorylation status of CHK2.

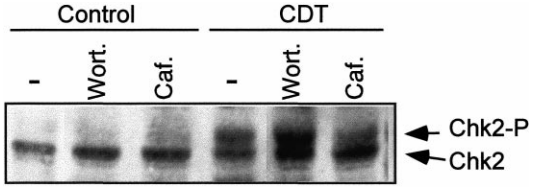

Fig. 4. ATM independence of CDT-induced CHK2 phosphorylation. Asynchronous HeLa cells were pre-treated with caffeine or wortmannin for 1 h before addition of CDT. Cells were washed and inhibitors were added again. Cells were collected after 16 h and proteins were fractionated on SDS–PAGE and visualized by immunoblotting with anti-CHK2 antibodies. CHK2 and its phosphorylated form are indicated with arrows.

4. Discussion

Previous studies have shown that the checkpoint 2 protein kinase (CHK2) is phosphorylated/activated in response to various stimuli as replication blocks by hydroxyurea or genotoxic treatment with gamma irradiation or agent causing DNA breaks such as etoposide. Here, we report that CHK2 is activated when HeLa cells are treated with *E. coli* CDT. This activation was in the first place surprising because no DNA damages were detected using a comet assay in our previous work [3]. However, two recent publications strongly suggest that CDT toxins are able to target directly the DNA of the host cells through an intrinsic DNase I-like activity [6,7]. Our results presented here demonstrate that in response to DNA damages that are either beyond the detection level or not detectable by comet assay, CDT activates a CHK2-dependent pathway that leads to cell cycle arrest in G2. However, there are a number of significant differences with the CHK2 activation that has been reported after other genomic injuries.

Studies in fission yeast *Schizosaccharomyces pombe* have shown that CHK2/cds1 could be activated only in S-phase following DNA damage [28]. When cds1 vertebrate homologous CHK2 was identified, similar studies could not have been reproduced suggesting that the activation of CHK2 in animals was not cell cycle-dependent. In particular Elledge's group has studied CHK2 activation following gamma irradiation and has found that it occurs in less than 2 h independently of cell cycle phase [13]. In this work, we took advantage of the fact that CDT could not trigger an immediate G2/M block when cells were exposed in G2-phase [2,3]. When HeLa

cells were synchronized by double thymidine block, we found that exposure to CDT in the middle of S-phase leads to CHK2 phosphorylation in less than 4 h which was compatible with a specific time window in S-phase.

The discrepancy between timing of CHK2 activation following gamma irradiation and CDT treatment could be explained by toxin processing requirements. Each of the steps of this process could be unavailable during a specific stage of the cell cycle and therefore could delay signalling. In contrast DNA breakage provoked by radiations is immediately sensored and therefore not subjected to cell cycle regulation. Alternately, as proposed by others [6] the DNase I-like activity of the cdtB subunit may only be active in S-phase because it is specifically directed against single-strand DNA or it is dependent on co-factors that are only present in S-phase.

In the aim to identify the signalling cascade involved in the cellular response to CDT, we investigated if we could inhibit CHK2 activation by pre-treating HeLa cells with caffeine or wortmannin. Caffeine is well known to overcome G2 checkpoint control related to DNA damage or replication blocks [29]. Recently it has been shown that caffeine is able to directly inhibit ATM and ATR in vitro with an IC_{50} of 0.2 mM [30]. We have found in this work that caffeine was able to interfere with CDT-dependent CHK2 activation and as reported in a previous study, this effect was able to prevent CDT-dependent cell cycle arrest [3]. In contrast, wortmannin had no effects on CHK2 phosphorylation at a concentration that is known to inhibit ATM but not ATR [31]. Altogether, these data suggest that an ATM-related kinase, as a caffeine sensitive and wortmannin insensitive protein (at the concentration used in this study) is an upstream regulator of CHK2 in CDT-treated cells. Whether this kinase is ATR remains to be fully demonstrated. In agreement with this proposal, ATR has been involved in replication checkpoint and we have shown in this paper that CHK2 activation takes place during S-phase. One can reasonably conclude that the bacterial toxin CDT can activate CHK2, a major key regulator of G2/M checkpoint, probably by creating DNA damages in S-phase that are signalled through an ATM-related kinase-dependent pathway.

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