Antisense inhibition of Chk2/hCds1 expression attenuates DNA damage-induced S and G2 checkpoints and enhances apoptotic activity in HEK-293 cells

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Abstract The cellular response to DNA damage involves checkpoint controls that delay cell cycle progression in order to provide time for repair of damaged DNA. Chk2/hCds1 is a recently identified homolog of the yeast Cds1 kinase that is involved in cell cycle checkpoint response to DNA damage. To investigate the functions of Chk2/hCds1 in response to DNA damage in mammalian cells, we established a stable human kidney embryonic cell line (HEK-293) that expresses antisense Chk2/hCds1 (Chk2AS) under the control of an inducible promoter. Cells that express Chk2AS display defective S-phase delay in response to DNA replication-mediated DNA damage induced by the topoisomerase I inhibitor camptothecin. The defective G2 checkpoint was also observed in Chk2AS cells exposed to the DNA damaging agent VP-16 or γ-radiation. Enhanced apoptosis was observed in Chk2AS cells after exposure to γ-radiation or camptothecin. No p53 activation was observed after DNA damage in HEK-293 or Chk2AS cells. Our results indicate that perturbation of Chk2/hCds1 expression adversely affects the S- and G2-phase checkpoints following DNA damage or DNA replication block, and suggest that reduced expression of Chk2/hCds1 might promote a p53-independent apoptotic response. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Cell cycle checkpoint; Chk2; hCds1; Apoptosis; 293 cells; p53

1. Introduction

Cell cycle checkpoints are signal transduction pathways that ensure the time, sequence, and fidelity of critical cell cycle events and orchestrate cellular responses to environmentally induced genotoxic stress. DNA damage induced by ionizing radiation or chemotherapeutic agents trigger checkpoint activation and delay cell cycle progression [1]. Such a delay in cell cycle would presumably allow time to repair damaged DNA or to complete DNA replication before entry into mitosis [2]. Disruption of cell cycle checkpoint control leads to increased genomic instability, and enhances the predisposition to cancer [3]. A majority of human cancers have a defect in the G1 checkpoint because of mutations of the p53 tumor suppressor gene. Compared to the G1 checkpoint, less is known about the genes that contribute to G2- and S-phase checkpoint regulation.

Chk2/hCds1, a mammalian homolog of the *Saccharomyces cerevisiae Rad53* and *Schizosaccharomyces pombe cds1* has been recently identified by several groups [4–7]. Chk2/hCds1 is implicated as an important component in DNA damage response pathways. In human cells, Chk2/hCds1 has been shown to be phosphorylated and activated in response to ionizing radiation in an ataxia telangiectasia mutated (ATM)-dependent manner, as well as in response to UV and replication block in an ATM-independent manner [4–7]. Chk2/hCds1, like its yeast counterpart *cds1*, can phosphorylate Cdc25C on the inhibitory phosphorylation site S216 in vitro, and thus may activate the G2 checkpoint by inactivation of Cdc2 through Cdc25C [4–7]. Although Chk2/hCds1 has been shown to be activated by DNA damage, its role in the checkpoint control remains to be elucidated in human cells. While the preparation of this manuscript was in progress, Hirao et al. have shown that Chk2/hCds1 is required for cell cycle G2 checkpoint because Chk2/hCds1-deficient mouse embryonic stem (ES) cells fail to maintain a G2 arrest after DNA damage [8]. In the study that follows, we generated a cell line that conditionally expresses antisense Chk2/hCds1 (Chk2AS) and investigated the role of Chk2/hCds1 in regulating cell cycle checkpoints and apoptotic response in human cells. Our results indicate that Chk2/hCds1 plays a role in S- and G2-phase checkpoints in response to DNA damage or replication block. We demonstrate that p53 is inactive in HEK-293 cells and that reduced expression of Chk2/hCds1 in these cells promotes apoptosis, suggesting that Chk2/hCds1 deficiency might lead to enhanced apoptosis in p53-defective cells.

2. Materials and methods

2.1. The ecdysone-inducible expression system for Chk2AS expression

To develop a cell line that conditionally expresses Chk2AS, we used the ecdysone-inducible mammalian expression system (Invitrogen, Carlsbad, CA, USA). The expression system is based on the heterodimeric ecdysone receptor of *Drosophila*. On binding of ecdysone, the receptor activates an ecdysone-responsive promoter, which promotes high-level expression of the gene. A human Chk2/hCds1 cDNA was ligated into the pIND expression vector in the antisense orientation. The resulting pIND-Chk2/hCds1AS vector was transfected into a

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Abbreviations: UCN-01, 7-hydroxystaurosporine; PARP, poly(ADP-ribose)polymerase

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human embryonic kidney cell line that constitutively expresses the ecdysone receptor (EcR-293) (Invitrogen, Carlsbad, CA, USA) by means of Fugene (Boehringer Mannheim, Indianapolis, IN, USA). EcR-293 cells transfected with pIND empty vector plasmid DNA were used as a control. Stable clones were selected by limiting dilution in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 600 μg/ml G418 and 400 μg/ml zeocin. To determine the expression of Chk2/hCds1 in transfected cells, the ecdysone analog ponasteron A (PonA; Invitrogen) was added to the cultures to a final concentration of 5 μM, and cells were collected after 24 h for Western blot analysis. The resulting cell line that expresses reduced Chk2/hCds1 was herein named Chk2AS.

2.2. Cell cycle analysis

Cell cycle assays were performed as described previously [9]. Briefly, cells were harvested and fixed in 70% ethanol. The fixed cells were then stained with propidium iodide (50 μg/ml) after treatment with RNase (5 μg/ml). The stained cells were analyzed for DNA content by fluorescence-activated cell sorting (FACS) in a FACScan (SOBR model, Becton Dickinson Instrument, San Jose, CA, USA). Cell cycle fractions were quantified with CellQuest (Becton Dickinson).

2.3. Protein analysis

Cell lysates were prepared as reported previously [9]. Briefly, cells were lysed with cell lysis buffer (0.3% NP-40, 1 mM EDTA, 50 mM Tris–HCl, pH 7.4, 2 mM EGTA, 10% Triton X-100, 150 mM NaCl, 25 mM NaF, 1 mM Na_3VO_4, 2 mM AEBSF, 5 μg/ml aprotinin, 1 μg/ml leupeptin) for 30 min on ice, and the lysates were clarified by centrifugation at 12 000×*g* for 15 min at 4°C. Protein concentration was quantified by the protein assay (Bio-Rad, Hercules, CA, USA) and protein samples (100 μg) were separated by SDS–PAGE (12% polyacrylamide) and transferred onto Immobilon membranes (Millipore, Bedford, MA, USA). Chk2/hCds1 or p53 proteins were identified using anti-Chk2/hCds1 or anti-p53 primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and reactive bands were visualized using the enhanced chemiluminescence detection system (NEN Life Science Products, Boston, MA, USA).

2.4. Apoptosis detection

Apoptosis was assessed by flow cytometry using a FITC-conjugated anti-PARP (poly(ADP-ribose)polymerase) cleavage site-specific antibody (Biosource, Camarillo, CA, USA) according to the manufacturer's instructions. Briefly, cells were treated with the indicated stimuli to induce apoptosis. The cells were washed with phosphate-buffered saline (PBS) twice and lifted with 2 mM EDTA. Once detached, the cells were washed once with PBS and fixed with PBS containing 4% paraformaldehyde for 20 min at 4°C, and permeabilized in PBS containing 0.1% saponin for 10 min. The cells were then incubated with 10 μl of FITC–PARP antibody in 100 μl PBS for 30 min at room temperature. After washing, the cells were resuspended in PBS and analyzed by FACS.

3. Results and discussion

3.1. Inhibition of Chk2/hCds1 protein expression by antisense transfection

To generate a cell line that expresses reduced Chk2/hCds1 protein conditionally, we stably transfected an ecdysone-inducible Chk2AS expression vector into EcR-293 cells. The resulting clones were treated with the ecdysone analogue PonA to induce Chk2AS expression. Shown in Fig. 1 is the Western blot analysis of Chk2/hCds1 protein expression in stably transfected EcR-293 cells after 24 and 48 h treatment with PonA (5 μM). Chk2/hCds1 expression was reduced to 50% after 24 h of PonA treatment and no further decrease was observed after 48 h treatment. PonA treatment of mock-transfected cells did not result in a decrease in Chk2/hCds1 expression (data not shown). The EcR-293 cells that are induced to express antisense Chk2/hCds1 are named Chk2AS throughout this report.

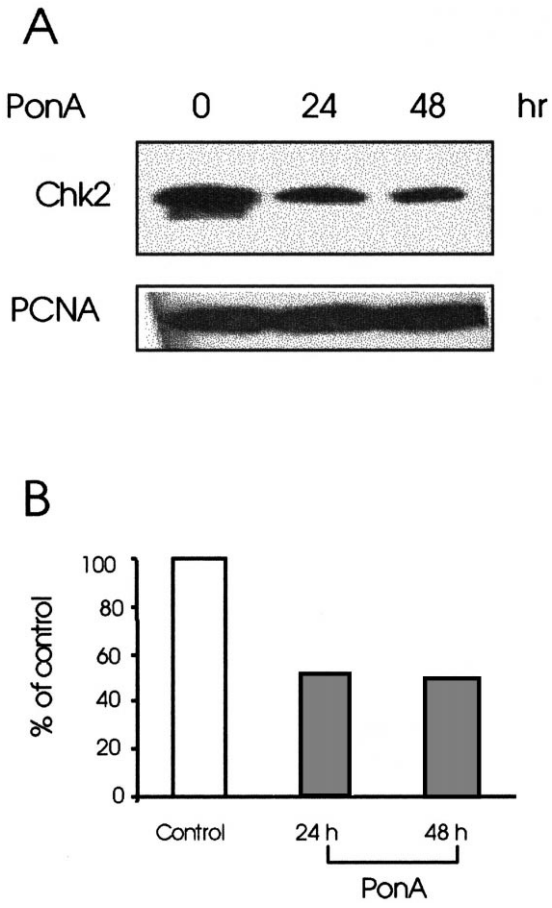
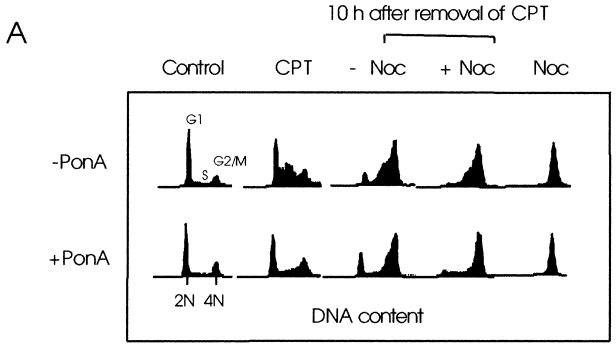


Fig. 1. Inhibition of Chk2/hCds1 protein expression after induction of Chk2AS. The EcR-293 cells that were transfected with a pIND plasmid containing Chk2AS cDNA were cultured in DMEM containing 800 μg/ml G418 and 400 μg/ml zeocin. A: Cells were treated with or without 5 μM PonA for 24 and 48 h, and cell extracts were separated by SDS gel electrophoresis, transferred to a membrane, and blotted with anti-Chk2/hCds1 antibody. PCNA was used as a loading control. B: Quantitation of the data presented in (A).

3.2. Inhibition of Chk2/hCds1 expression leads to impaired S and G2 checkpoints

Chk2/hCds1 and its yeast homolog *cds1* have been suggested to be important in both DNA damage and replication checkpoints [5,10–13]. To determine the requirement of Chk2/hCds1 for cell cycle checkpoint regulation, we first examined whether reduced expression of Chk2/hCds1 affects S-phase arrest caused by the topoisomerase I inhibitor camptothecin (CPT). CPT is know to form topoisomerase I cleavage complexes which are converted into DNA double-strand breaks by DNA replication [14]. Thus, CPT was used to activate a replication checkpoint in the S phase [15]. A study by Chaturvedi et al. [6] indicated that topoisomerase I inhibitor topotecan, an analog of CPT, can activate Chk2 in human cells. To study the role of Chk2/hCds1 on the S-phase checkpoint, cells were treated with PonA for 24 h to express Chk2AS before further exposure to 100 nM CPT. FACS analysis showed a similar cell cycle profile 24 h after treatment of control cells with PonA, indicating that this compound did not perturb the cell cycle distribution in the absence of DNA damage. 8 h after CPT treatment, approximately 55% of control cells were accumulated in S phase, as measured by FACS analysis. By contrast, the S-phase population was reduced to



B

% of cells in S and G2 after CPT treatment

	CPT 8h			10hr after removal of CPT					
				-Noc			+ Noc		
	G1	S	G2	G1	S	G2	G1	S	G2
-PonA	27	55	18	7	43	50	2	40	58
+PonA	32	32	36	16	20	64	3	22	75

Fig. 2. Attenuated S-phase checkpoint in cells that express Chk2AS. The EcR-293 cells that were transfected with a pIND plasmid containing Chk2AS cDNA were grown in the presence or absence of PonA (5 μM) for 24 h before exposure to 100 nM of CPT. 8 h after CPT treatment, CPT was removed, and cells were grown for an additional 10 h in the presence or absence of nocodazole (Noc). A: Flow cytometry analyses (propidium iodide staining of DNA). The positions of the G1, S, and G2 populations have been highlighted in the control sample, and the DNA content of G1 and G2 cells are labeled as 2N and 4N, respectively. B: Quantitation of the flow histograms presented as percentages of cells in G1, S and G phases.

←

32% in cells expressing Chk2AS (Fig. 2). 10 h after CPT removal, cells were arrested in S and G2. Decreased S-phase arrest was observed in Chk2AS cells both in the presence and absence of nocodazole, indicating that Chk2/hCds1 down-regulation enabled the cells to progress through the S phase. These results suggest that Chk2AS cells have defective S-phase arrest in response to CPT treatment, implying a role of Chk2/hCds1 in the replication checkpoint. Very recently, an independent study also demonstrated a role of Chk2 in S-phase checkpoint regulation via Cdc25A phosphorylation [32].

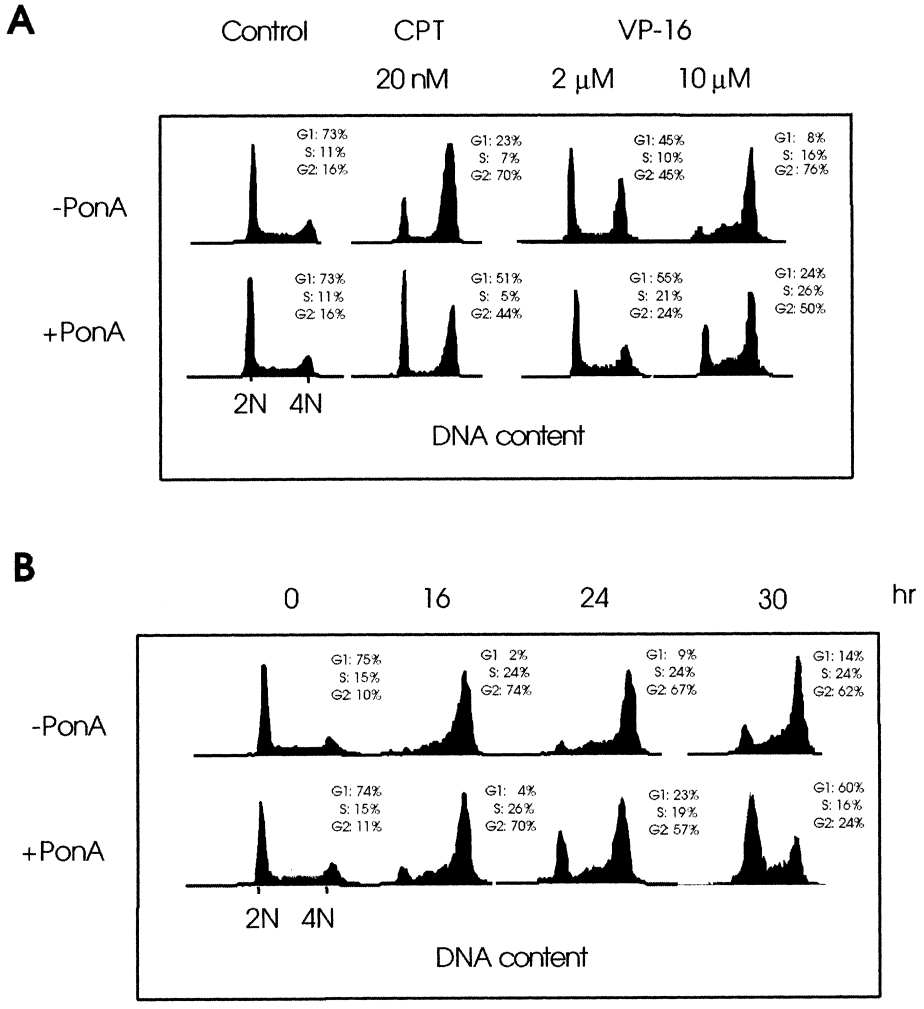


Fig. 3. Cells that express Chk2AS are defective for G2 checkpoint after exposure to CPT or VP-16. Cells grown in the presence or absence of PonA were harvested at the indicated times after drug treatment and analyzed by flow cytometry. A: Cells were treated with CPT (20 nM) or VP-16 (2 and 10 μM) for 24 h. B: Time-course analysis of cell cycle progression for cells treated with 6 μM VP-16.

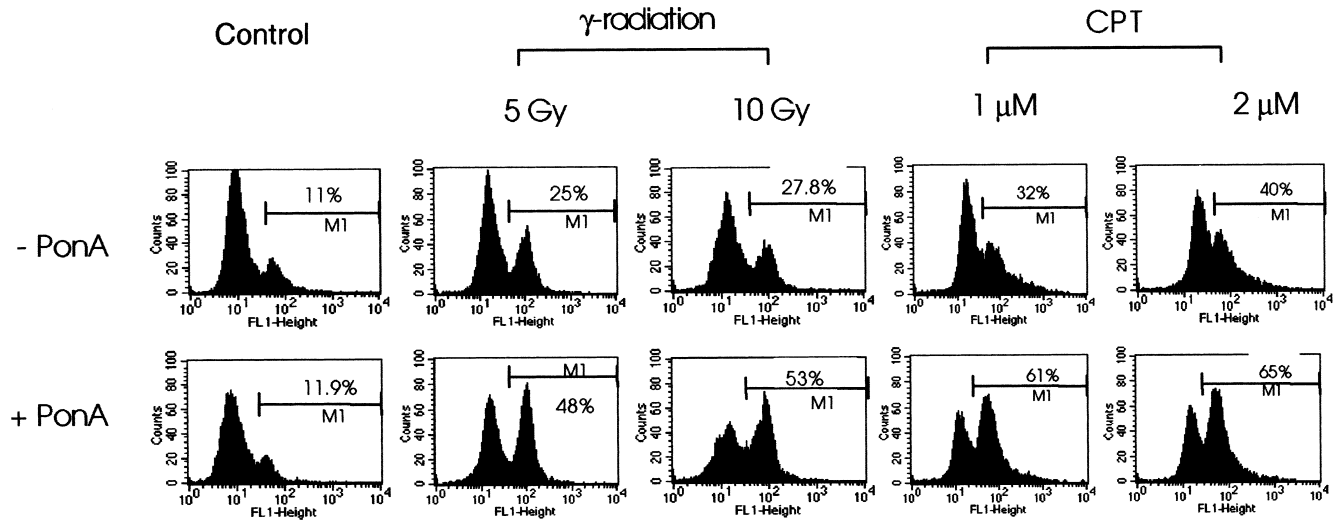


Fig. 4. Inhibition of Chk2/hCds1 expression results in enhanced apoptosis induced by IR and CPT. Chk2AS cells were cultured in the presence or absence of 5 μM PonA for 24 h before exposure to IR (5 and 10 Gy) or CPT (1 and 2 μM). 24 h after treatment, cells were harvested and stained with FITC-conjugated PARP antibody for flow cytometry analysis. The percentages of positive staining cells are indicated.

To determine whether Chk2/hCds1 is required for G2 checkpoint, cells were subjected to continuous treatment with low concentration of CPT, which produces primarily G2 delay with minimal S-phase effect. 24 h after CPT treatment (20 nM), ~70% of control cells were arrested at the G2 phase with a 4N DNA content, as shown by FACS analysis (Fig. 3A). By contrast, the percentage of cells in G2 was reduced to 44% when cells were induced by PonA to express Chk2AS (Fig. 3A). To expand our analysis to other agents that cause DNA damage by different mechanisms, cells were subjected to VP-16, a known topoisomerase II inhibitor that arrests cells in G2 by causing DNA double-strand breaks. As anticipated, 24 h after VP-16 treatment, the control cells were arrested in G2 in a dose-dependent manner with 76% of cells in G2 at 10 μM VP-16. By contrast, cells that express Chk2AS had a marked decrease in their G2 accumulation (50%), and a corresponding increase of cells in G1 from 8 to 24%. A time-course analysis of cell cycle response to VP-16 showed that both control cells and Chk2AS cells appeared to initiate a G2 arrest similarly, with 74 and 70% of cells in G2 by 16 h after DNA damage, respectively (Fig. 3B). However, at later times, Chk2AS cells prematurely escaped from the G2 checkpoint, as seen by the decrease of cells in G2 to 62% (at 24 h) and 24% (at 30 h), and a corresponding increase of cells in G1. In contrast, the control cells remained arrested at G2 (Fig. 3B). Similar results were observed in γ-radiation-treated cells (data not shown). These results suggest that initiation of the G2 checkpoint in response to DNA damage was independent of Chk2/hCds1, as both control and Chk2AS cells arrested in G2 with similar kinetics. However, sustaining of the G2 checkpoint appears to require Chk2/hCds1, as the Chk2AS cells failed to maintain the G2 arrest. While our experiments were in progress, a study by Hirao et al. reported that Chk2-deficient mouse ES cells failed to maintain G2 arrest after DNA damage [8], which is consistent with our observations. Furthermore, a more recent study by the same group using a similar approach has suggested that another checkpoint protein kinase Chk1 is required for initiating the G2 checkpoint following DNA damage [16]. Taken together, these data suggest a model in which both Chk1 and Chk2/hCds1 are required for the DNA damage-induced G2 checkpoint. While Chk1 would be responsible for the initiation of the G2 checkpoint in response to DNA damage, Chk2/hCds1 would be required to sustain it. Our results provide the first support for this hypothesis in human cells.

We currently do not know the molecular basis by which reduced expression of Chk2/hCds1 contributes to the inability to maintain the S and G2 arrest. Chk2/hCds1 has been shown to phosphorylate Cdc25C on Ser216 in vitro [4,5], suggesting (but not proving) that Chk2/hCds1 may regulate the DNA damage-induced G2 checkpoint by inactivating Cdc2 kinase through Cdc25C. Our preliminary data, however, showed no significant difference in Cdc2 kinase activity after DNA damage between control cells and Chk2AS cells (data not shown). We do not currently exclude the possibility that the experimental conditions in our system are not optimized. However, it is likely that Chk2/hCds1 may target separate pathways to cause cell cycle arrest. Indeed, recent evidence shows that considerable redundancy exists in the regulation of DNA damage-induced G2 checkpoint [17]. A recent study reported that Chk2/hCds1 activity is not necessarily correlated with Cdc2 activity, suggesting that Chk2/hCds1 may not target Cdc25C for G2 regulation [18]. This implies that either the deregulation of Chk2/hCds1 in our system is not sufficient to enhance the tightly regulated Cdc2 activity or that Chk2/hCds1 is not only targeting Cdc25C physiologically. Furthermore, Cdc25C phosphorylation in vivo can be completely abolished by the Chk1 inhibitor UCN-01 (7-hydroxystaurosporine) [19,20], implying that Chk2/hCds1 may not be the sole kinase for Cdc25C in vivo. Taken together, it is tempting to speculate that Chk2/hCds1 kinase may regulate the G2 checkpoint through substrate(s) other than the Cdc25C–Cdc2 regulatory pathway. This possibility is currently being investigated. Regardless of the mechanism, our data demonstrate the importance of Chk2/hCds1 in the G2 checkpoint control, as loss of this checkpoint in cancer cells might contribute to the tumorigenesis [21].

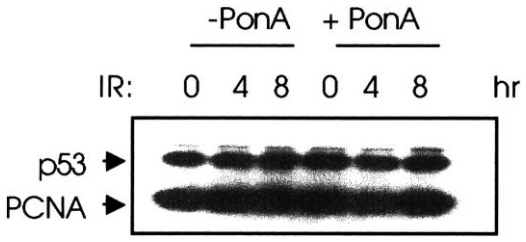


Fig. 5. Lack of p53 induction in response to γ-radiation in EcR-293 cells. Cells were treated with 20 Gy IR and harvested at the indicated times. Protein lysates were prepared and analyzed by Western blot for p53 protein levels. PCNA analysis was included to assess protein loading and transfer.

3.3. Inhibition of Chk2/hCds1 expression results in enhanced apoptosis in p53-inactive HEK-293 cells

During apoptosis, the activation of a group of caspases, such as caspase-3, and subsequent cleavage of cellular substrates, such as PARP, are crucial components of the cell death pathways [22]. Several recent reports indicate that Chk2/hCds1 can activate p53 [8,23,24], and thus promote p53-mediated apoptosis [8]. To examine the ability of Chk2AS cells to undergo apoptosis, cells were treated with γ-irradiation or CPT. 24 h after treatment, cells were harvested, fixed and stained for PARP cleavage using FITC-conjugated PARP antibody. Cells were then analyzed by flow cytometry. The appearance of the FITC–PARP Ab-positive cells was used to measure levels of apoptosis. Compared to control cells, Chk2AS cells exhibited a high level of fluorescence intensity after treatment with both ionizing radiation and CPT (Fig. 4). These data indicate that cells expressing reduced levels of Chk2/hCds1 are more susceptible to DNA damage-induced apoptosis.

It has been reported by Hirao et al. that Chk2/hCds1 can promote apoptosis by activating p53 as a result of p53 phosphorylation on Ser20, and that Chk2-null ES cells exhibit resistance to apoptosis in response to DNA damage due to loss of p53 activation [8]. This observation appears to be contradictory to our results. However, p53 in adenovirus-transformed HEK-293 cells has been demonstrated not to be functional [25–27]. Consistently, with observed high levels of p53 in HEK-293 cells, there was no elevation of p53 protein levels following γ-irradiation (Fig. 5). Furthermore, no detectable increase of p53 downstream targets, such as p21 and Bax, were observed in irradiated HEK-293 cells (data not shown). In Chk2-null ES cells, loss of p53 activation by Chk2 leads to a decreased susceptibility to apoptosis when compared to the wild-type ES cells that hold functional p53 [8] – a similar phenotype when comparing p53 wild-type to p53 mutant cells. However, in HEK-293 cells that are p53 inactive already, deregulation of Chk2/hCds1 will result in deficient S and G2 checkpoints, thus enhancing apoptosis in a p53-independent manner. Our data provide the first evidence that deregulation of Chk2/hCds1 in p53-inactive cells contributes to enhanced apoptosis. Thus, our data, combined with the report by Hirao et al. [8], further suggest a central role of p53 in determining the sensitivity to apoptosis when Chk2/hCds1 is deficient. Down-regulation of Chk2/hCds1 in cells with functional p53 would prevent the induction of p53-mediated apoptosis. However, down-regulation of Chk2/hCds1 in cells with defective p53 function would cause primarily deficient G2 checkpoint, and thus promote apoptosis in a p53-independent manner. It is well known that G2 checkpoint abrogators, such as caffeine or UCN-01, selectively sensitize p53-deficient cells to radiation or chemotherapeutic agents [9,20,28–31]. It is generally believed that p53-deficient cells, which lack G1, arrest enter G2 with more damaged DNA compared to the p53 wild-type cells. Thus, these cells would be more dependent on an intact G2 checkpoint to repair DNA damage. Accordingly, abrogation of S and G2 checkpoints would result in the greater radiosensitization of p53-deficient cells. The current findings reported here support this view and further suggest that a therapeutic gain might be achieved in the treatment of tumors harboring p53 mutations by targeting Chk2/hCds1. Therefore, it seems rational to develop Chk2/hCds1 inhibitors that can be used to pharmacologically abrogate the S and G2 checkpoints and enhance the sensitivity to chemotherapeutic agents.

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