

DNA Damage Induces p53-dependent Down-regulation of hCHK1*

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The levels of the human checkpoint gene hCHK1 were measured in human cancer cells growing *in vitro* after treatment with the DNA damaging agent *cis*-dichlorodiammine platinum(II) (DDP). Treatment of human cancer cell lines with DDP induced a decrease in the hCHK1 protein levels starting 6 h after treatment, with a further decline at 24 and 48 h. A similar decrease in the levels of hCHK1 was found at the mRNA level by using Northern blot analysis. By using isogenic cell systems in which p53 was disrupted either by transfection with HPV-E6 or by targeted homologous recombination, we found that the DNA damage-induced down-regulation of hCHK1 was only observable in wild type p53-expressing cells, with only a minor decline in the hCHK1 levels observable 48 h after treatment in cells with disrupted p53. Similarly, treatment of mutant p53-expressing human cancer cell lines with DDP did not result in changes in the levels of hCHK1. The p53-dependent down-regulation of hCHK1 is likely to be at transcriptional levels, as suggested by the lack of down-regulation of the hCHK1 when transfected under the control of a heterologous viral promoter. In addition, p53 is able to down-regulate the luciferase activity under the control of the 5' flanking region of the hCHK1 gene. The data suggest a strict link between p53 and hCHK1 governing the activation and repression of the G₂ checkpoint in which both proteins participate.

The cellular response to DNA damage includes a transient arrest of the cell cycle either at the G₁ phase, before DNA replication (through the G₁ DNA damage checkpoint), or before mitosis (through the G₂ DNA damage checkpoint), presumably to allow time for DNA repair, minimizing the replication and segregation of damaged DNA. The G₁ checkpoint is in part dependent on the p53-regulated transcription of p21, a potent inhibitor of the cyclin-cdk complexes required for the G₁-S transition (1, 2). Cells lacking a functional p53 are defective in the G₁ checkpoint in response to DNA damage, still retaining checkpoint mechanisms by which cells are arrested in G₂ phase (3, 4). The G₂ DNA damage checkpoint prevents the activation of the cdc2-cyclin B1 complex, thereby inhibiting entry into mitosis in the presence of a damaged DNA (5, 6). This is thought to be determined by phosphorylation and inactivation of cdc25 phosphatase, which in the phosphorylated form binds

the proteins of the 14–3-3 family and is prevented from activating the cdc2 complex (7–9). The two main kinases described to phosphorylate cdc25 are hCHK1 and hCHK2 (8–10). The mechanism by which DNA damage activates hCHK2 and/or hCHK1, crucial enzymes in the cascade of reactions leading to G₂ arrest, is not fully elucidated yet, even if there is evidence of an involvement of ATM (11, 12).

p53 also is an important component of the G₂ checkpoint after DNA damage, possibly through the transactivation of the p21 and 14–3-3 σ genes (13, 14). 14–3-3 σ is required to sequester cdc2-cyclin B1 complexes in the cytoplasm and to prevent mitotic catastrophes, whereas p21 prevents any cdc2-cyclin B1 that enters the nucleus from becoming activated (4).

Moreover, a growing body of data is accumulating on a possible cross-connection between p53 and hCHK1 and hCHK2; in particular, there is evidence that hCHK1 and hCHK2 are able to phosphorylate and activate p53 in response to DNA damage (15–17). In the present study we investigated the consequences of the cellular response to the DNA-damaging agent *cis*-dichlorodiammine platinum(II) on hCHK1 and in particular on its regulation by p53.

MATERIALS AND METHODS

Cells and Drugs—The human col carcinoma cell line HCT-116 was maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum. The human endometrial HEC1A cells, the human ovarian cancer cell line SKOV3, and the human leukemic cell line Jurkat were grown in RPMI 1640 supplemented with 10% fetal calf serum. The human osteosarcoma cells U2OS, transfected with HA-hCHK1, were kindly supplied by Dr. C. Mercurio (IEO, Milan, Italy) and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 400 μ g/ml G418.

HCT-116/E6 cells were obtained and maintained as previously described (18). HCT-116 cells with p53 gene disrupted by targeted homologous recombination (clone 392.7, p53^{-/-}) and their relative controls (clone 40–16, p53^{+/+}) were kindly provided by Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD).

Cis-dichlorodiammine platinum(II) (DDP¹; Sigma) was dissolved in medium just before use. For each cell line, DDP treatment was performed for 24 h at the concentration approximately inhibiting the growth by 50% (IC₅₀). Nocodazole was purchased from Sigma.

Western Blotting Analysis—Cell extracts, obtained at the end of treatment and at 6, 24, or 48 h after recovery in drug-free medium, were prepared by lysing cells in 50 mM Tris-HCl (pH 7.4), 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 50 mM NaF in the presence of aprotinin, leupeptin, and phenylmethylsulfonyl fluoride as protease inhibitors, for 30 min on ice. Insoluble material was pelleted at 13,000 \times g for 10 min at 4 °C, and the protein concentration was determined using a Bio-Rad assay kit (Bio-Rad). Forty μ g of total cellular proteins were separated via SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose. Immunoblotting was carried out with polyclonal anti-hCHK1 antibody (8), p53 monoclonal antibody (DO-1, Santa Cruz Biotechnology, Heidelberg, Germany), anti-actin polyclonal antibody (Santa Cruz Biotechnology), and anti-HA monoclonal antibody (Roche

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¹ The abbreviations used are: DDP, *cis*-dichlorodiammine platinum(II); wt, wild type.

Molecular Biochemicals). Antibody binding was revealed by peroxidase secondary antibodies and visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Northern Blotting Analysis—Total RNA was isolated from cells growing in culture by the guanidine-thiocyanate method according to standard procedures (19), fractionated by electrophoresis on a formaldehyde-agarose gel, and transferred to nylon membranes. Filters were hybridized with cDNAs ³²P-labeled using a Rediprime kit (Amersham Pharmacia Biotech). Hybridizations were done in 50% formamide, 10% dextran sulfate, 1% SDS, 1 M NaCl at 42 °C for 16 h, followed by two 10-min washes at room temperature with 2× SSC (150 mM NaCl, 15 mM sodium citrate) and one 30-min wash at 65 °C in 2× SSC, 1% SDS.

Cell Cycle Analysis—Cells (2×10^6) were removed 24 h following treatment with DDP, washed twice in ice-cold phosphate-buffered saline, fixed in ice-cold 70% ethanol, washed in phosphate-buffered saline, resuspended in 2 ml of a solution containing 2.5 μg/ml propidium iodide and 25 μg/ml RNase, and stained overnight at 4 °C in the dark. Cell cycle analysis was done on at least 10,000 cells for each sample using the FACSORT system (Becton Dickinson). The percentage of cell cycle phase distribution was calculated as previously described (20).

Luciferase Assays—SKOV3 cells were cotransfected with 4 μg of pGL2-derived plasmids (Promega) containing the 5' flanking region of the hCHK1 gene isolated from a human P₁ artificial chromosome clone² or with 4 μg of p21-Luc construct (kindly provided by Dr. C. Prives, Columbia University, New York, NY) and with 4 μg of a plasmid encoding for human p53. 0.05 μg of untreated pRL-SV40 were used for internal normalization. Reporter gene activities were evaluated after 24 h using the Dual-Luciferase system (Promega). Results are expressed as the percentage of the control luciferase reported activity normalized by the renilla activity value. The mean ± S.D. of three independent experiments is shown.

RESULTS

Treatment of human HCT-116 cells with 25 μM DDP resulted in a decrease in the levels of hCHK1 starting 6 h after treatment, with a further strong decrease observable at 24 and 48 h (Fig. 1A), whereas no modification of the mobility of the protein could be observed. Treatment of the isogenic cell line (HCT-116/E6) in which p53 was inactivated (through HPV16/E6 transfection) with the same concentration of DDP (25 μM) did not result in such a decrease, although at 48 h after treatment a slight decrease in the hCHK1 levels could be found. The densitometric analysis of the results of three independent experiments (each performed in duplicate) is reported in Fig. 1B, where it is shown that after an initial decline of ~25% at 6 h, by 48 h the levels of hCHK1 in the HCT-116 cell line treated with DDP are less than 5% of the controls. At the same time point, in the HCT-116/E6 cell line treated with DDP the levels of hCHK1 are ~65% of the controls.

To test whether the difference in hCHK1 levels was related to a different DDP-induced cell cycle perturbation, a time course of cell cycle distribution induced by 25 μM DDP in HCT-116 and HCT-116/E6 cells was performed (Fig. 2A). As expected, a G₁ arrest was present only in HCT-116 wt cells at the end of treatment and at 6 h after drug washout, whereas in both cell lines DDP induced a G₂M block measurable at 24 and 48 h after treatment. At 48 h after drug washout the ratio between the percentage of cells in G₂M and G₁ phases was not so different between the two cell lines (3.1 and 2.7 for HCT-116 and HCT-116/E6 cells, respectively). Treatment of both HCT-116 and HCT-116/E6 cells with 0.4 μg/ml nocodazole, causing in both cell lines an accumulation of cells in the G₂ phase of cell cycle (Fig. 2B), did not result in any change in the levels of hCHK1 (Fig. 2C).

To check whether the DDP-induced decrease in the levels of hCHK1 was observable also at mRNA levels, a Northern blot analysis using the same experimental conditions reported for the Western blotting analysis was performed (Fig. 3A). A de-

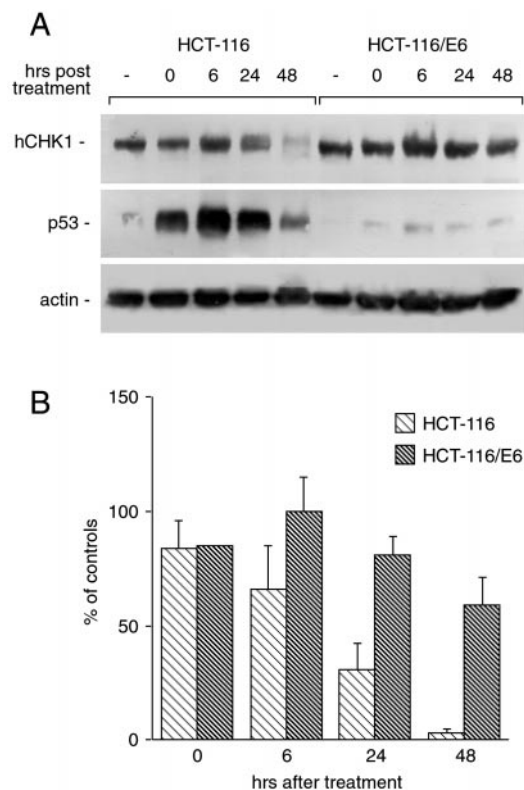


FIG. 1. A, Western blot analysis in HCT-116 and HCT-116/E6 cells treated with 25 μM DDP. Extracts were obtained at different time points after treatment. Blots were hybridized with antibodies recognizing hCHK1, p53, and actin. B, densitometric analysis of the levels of hCHK1 protein. Data are expressed as percents of untreated controls and represent the mean ± S.D. of the ratio between hCHK1 and actin levels of three different experiments, each performed in duplicate.

crease in the levels of both hCHK1 transcripts was clearly observable in HCT-116 cells but not in HCT-116/E6 cells. Densitometric scanning of three independent blots (performed on the lower transcript) (Fig. 3B), in fact, resulted in an ~20% decrease at 6 h, an 80% decrease at 24 h, and a 90% decrease at 48 h after treatment in HCT-116 cells, whereas no appreciable changes in HCT-116/E6 cells were found up to 24 h, with a marginal decrease in hCHK1 mRNA levels observable at 48 h after DDP treatment.

To further evaluate the p53-dependent down-regulation of hCHK1, we used a clone derived from HCT-116 cells in which the p53 gene was disrupted by gene targeting (21). As reported in Fig. 4, 12.5 μM DDP induced hCHK1 decrease in HCT-116 p53^{+/+} cells, with minor changes induced only at 48 h in HCT-116 p53^{-/-} cells treated with the same DDP concentrations, in agreement with the results obtained with HCT-116 cells with HPV16-E6-inactivated p53. Similarly, when human cancer cell lines expressing mutated p53 (HEC1A, Fig. 5; and Jurkat, data not shown) were treated with cytotoxic concentrations of DDP (30 μM), no decrease in hCHK1 levels was observed, again strongly supporting the p53 dependence of hCHK1 decrease observed after DDP treatment.

Trying to define whether the p53-induced down-regulation of hCHK1 was due to a specific transcriptional repression of the hCHK1 gene, we used the p53 wild type U2OS cells transfected with the HA-CHK1 gene under the control of a CMV promoter. Treatment of these cells with 12.5 μM DDP induced down-regulation of endogenous hCHK1 (Fig. 6), whereas no effect (if not an increase) was found against the exogenous, HA-tagged hCHK1. The same filter was reprobbed with an anti-HA antibody, and a lack of CMV-driven, HA-tagged hCHK1 down-

² G. Damia, L. Carrassa, and M. Broggin, manuscript in preparation.

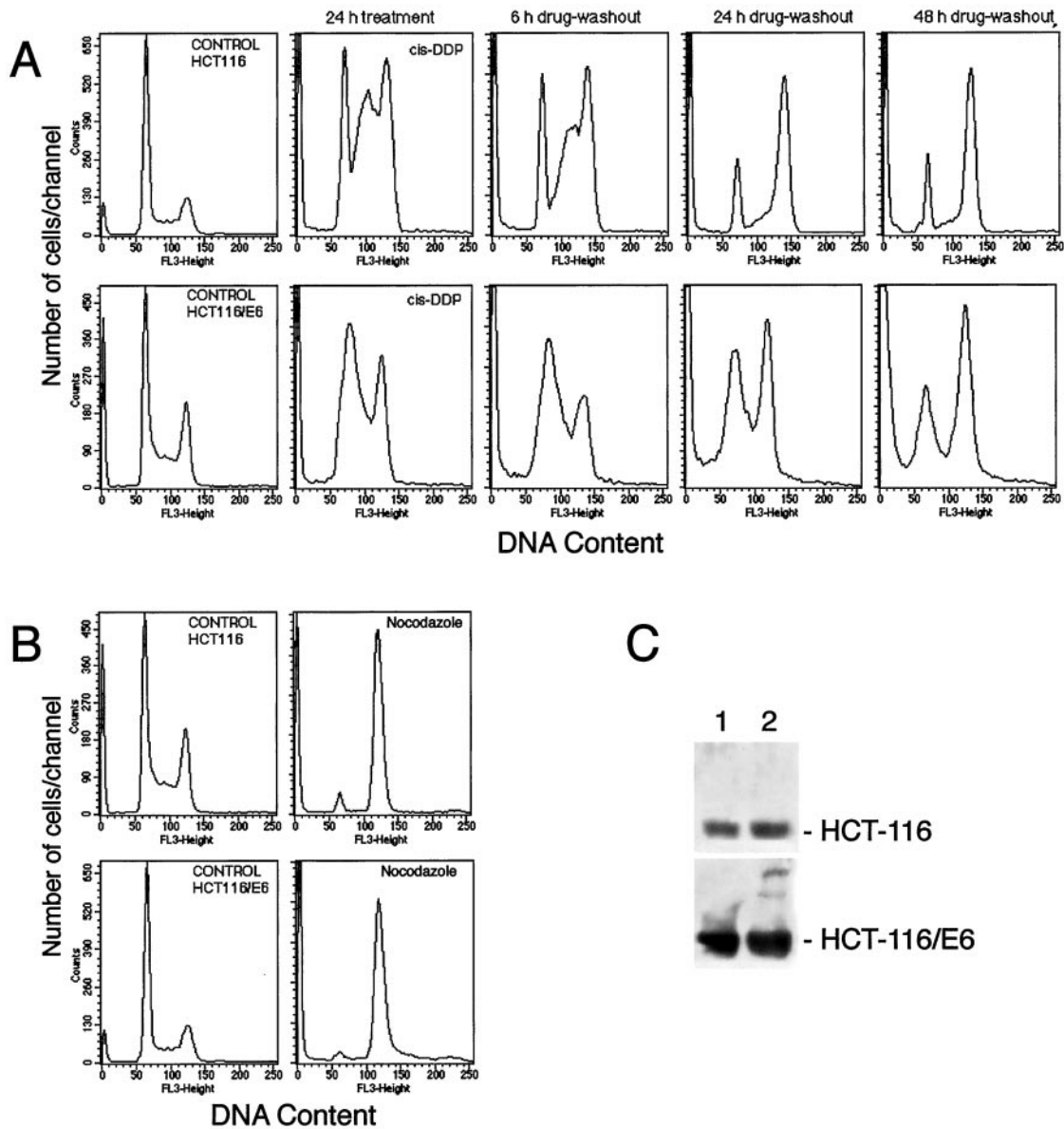


FIG. 2. *A* and *B*, flow cytometric analysis of the cell cycle phase distribution of HCT-116 and HCT-116/E6 cells treated with 25 μ M DDP (*A*) or 0.4 μ g/ml nocodazole (*B*). *C*, hCHK1 levels in HCT-116 and HCT-116/E6 cells treated with nocodazole.

regulation is clearly observed. Importantly, the ratio between endogenous and HA-tagged hCHK1 dramatically changed after DDP treatment. In fact, by densitometric analysis the levels of endogenous hCHK1 were 2–3-fold higher than exogenous hCHK1 before DDP treatment, whereas at 24 and 48 h after treatment, the ratio between endogenous and exogenous hCHK1 was 0.2.

Finally, we analyzed luciferase constructs containing in both orientations the 5' flanking region of the hCHK1 gene. Transfection of an 867-base pair fragment in sense, but not in anti-sense, orientation induced luciferase activity (Fig. 7) in p53 null SKOV3 cells. Cotransfection with a human p53 expression vector reduced this activity by \sim 50%. Similar results were obtained when other constructs (containing 1200 and 1600 base pairs of the 5' flanking region of the hCHK1 gene) were used (data not shown). The same p53-expressing vector was able to induce in the same experimental conditions the transcription of the p53-responsive promoter p21.

DISCUSSION

The product of the tumor suppressor gene p53 plays a central role in both G₁ and G₂ checkpoints (1, 2, 21). In different

cellular systems, after DNA damage the cells respond by inducing an increase in the levels of p53 and consequently a transcriptional activation of genes regulated by p53 (22, 23). In addition, p53 not only activates transcription but also induces repression (through a mechanism not yet established) of different genes (23). Posttranslational modifications of p53, such as phosphorylation and acetylation, are thought to play a key role in the mechanisms of activation of p53 (24, 25). In particular, phosphorylation at the N terminus of p53 can relieve the inhibitory effect of mdm2, resulting in an increase in the levels of p53 (26, 27). The kinases possibly involved in these phosphorylations are DNA-PK, ATM, ATR, hCHK1, and hCHK2, all shown to be able, at least *in vitro*, to phosphorylate p53 (15–17, 24). hCHK1 is the human homologue of the yeast chk1, a protein important for the G₂ DNA damage checkpoint that prevents mitosis when DNA is being repaired (8). Chk1 is phosphorylated after damage by a mechanism that required checkpoint Rad proteins, including Rad 3 and the *Saccharomyces cerevisiae* Mec1/Tel1 (28–30). It has been proposed that chk1 might regulate the activity of cdc2 by phosphorylating the tyrosine kinase Wee 1, which inactivates cdc2, or the protein

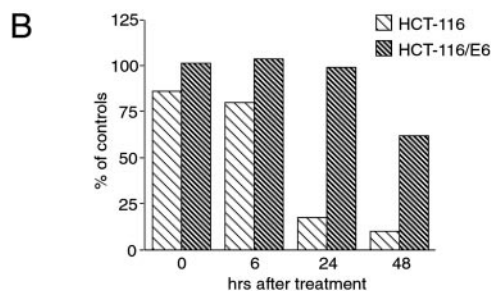
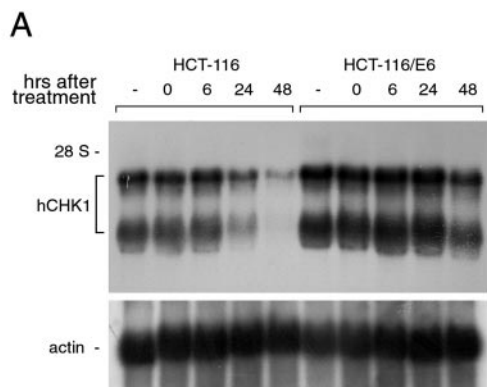


FIG. 3. *A*, Northern blot analysis of hCHK1 mRNA levels in HCT-116 and HCT-116/E6 cells. RNAs were obtained at different time points after treatment with 25 μ M DDP and processed as described under "Materials and Methods." The filter was hybridized with hCHK1 cDNA and subsequently with actin cDNA. The two hCHK1 mRNA transcripts are shown. *B*, densitometric analysis of the levels of hCHK1 mRNA. Data are expressed as percents of untreated controls and represent the ratio between hCHK1 and actin mRNA levels.

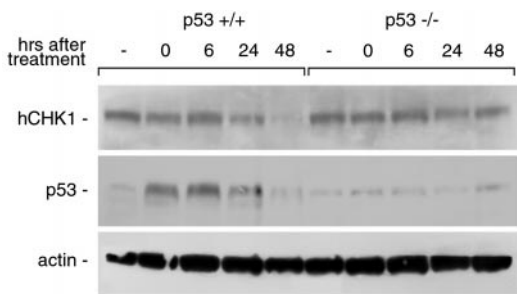


FIG. 4. Western blot analysis in HCT-116 p53^{+/+} and p53^{-/-} cells treated with 12.5 μ M DDP and processed as described in the legend to Fig. 1.

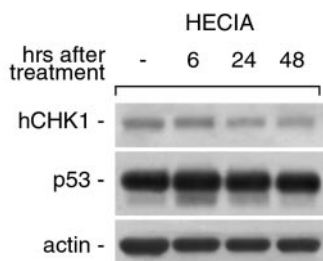


FIG. 5. Western blot analysis in p53 mutant-expressing HEC1A cells treated with 30 μ M DDP. Treatment conditions and blot hybridizations were as described in the legend to Fig. 1.

phosphatase cdc25, which activates cdc2. Recently it has also been shown that in *S. cerevisiae* chk1 is required for the function of the DNA damage checkpoint maintaining the abundance of Psd1, an anaphase inhibitor (31). It seems likely that hCHK1 also operates downstream from ATM and, once activated, phosphorylates cdc25C, creating a consensus site for the

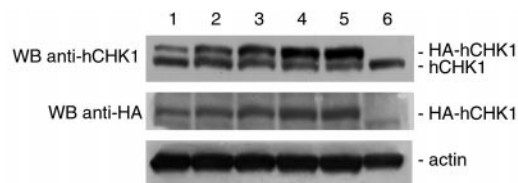


FIG. 6. Western blot analysis in U2OS cells transfected with the HA-CHK1 gene under the control of the CMV promoter (lanes 1–5) and in untreated HCT-116 cells used as internal negative controls for HA-hCHK1 (lane 6). Extracts obtained at different time points were separated via SDS-polyacrylamide gel electrophoresis and filter-hybridized to antibodies recognizing hCHK1, HA, and actin. Lanes 1–5 are extracts taken from untreated cells and after 0, 6, 24, and 48 h following 12.5 μ M DDP, respectively. WB, Western blot.

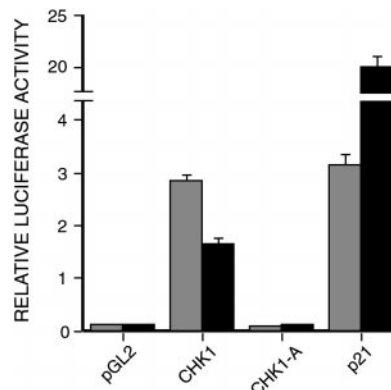


FIG. 7. p53 null-SKOV3 cells were transfected with pGL2 empty vector, CHK1, CHK1-A (containing 867 base pairs of the 5' flanking region of the hCHK1 gene in sense and antisense orientations, respectively), and p21 in the absence (gray bars) or presence (black bars) of 4 μ g of a human p53 expression plasmid. Results are expressed as relative luciferase activity, normalized for the renilla activity.

binding to 14–3-3 family members and preventing the entry of cells in mitosis (5, 8, 9). As already reported by others (32), we did not observe in our experimental condition the reduction in the electrophoretic mobility of hCHK1 described in HeLa cells after UV treatment (8) and possibly related to an activation of hCHK1 through phosphorylation. These apparently contrasting results might be due to the different cellular systems used and do not exclude the possibility that other forms of hCHK1 activation do indeed occur. On the contrary, a clear-cut decrease in hCHK1 protein level was observed, and this event was found to be p53-dependent. Here we show that p53, which has recently been shown to be phosphorylated and activated *in vitro* and *in vivo* by hCHK1 (15), is then able to induce a down-regulation of hCHK1. The evidence reported here, obtained in isogenic cell systems (in which p53 was inactivated by either viral transfection or targeted homologous disruption), suggests a possible regulatory loop between p53 and hCHK1 in which, after DNA damage, both p53 and hCHK1 are activated; once activated, p53 represses the transcription of the hCHK1 gene, resulting in the inactivation of this checkpoint protein at later times. This regulatory loop between hCHK1 and p53 is reminiscent of other regulatory strategies that have been described either in prokaryotes or in eucaryotic systems and that underlie the fact that a specific DNA damage response (*i.e.* the maintenance of a sustained G₂M block) might be deleterious and need to be quickly down-regulated once the cells have repaired the DNA damage (33, 34). Another negative feedback loop exists for p53 that, once activated, transcriptionally up-regulates mdm2, which is in turn a negative regulator of its transcriptional activity and stability (35).

As for the molecular mechanisms responsible for the p53-de-

pendent hCHK1 down-regulation, the experiments performed in cells transfected with HA-tagged hCHK1 clearly show that the hCHK1 gene under the control of a viral promoter is not a target of p53-induced repression, and the initial analysis of the 5' flanking region of the hCHK1 gene strongly suggests that the action of p53 occurs, at least partially, at the level of the hCHK1 promoter. Furthermore, the use of proteasome inhibitors did not modify the DDP-induced hCHK1 down-regulation in HCT-116 cells (data not shown). The difference in cell cycle perturbations induced by the DNA damaging agent DDP in cells expressing a wt p53 compared with cells with inactivated p53 is not the reason for the drug-induced difference in the levels of hCHK1 between these cell types. In fact, in both cell systems DDP induces an accumulation of cells in G₂M phases. At early times the G₁ block was present, as expected, only in cells with a wt p53. Furthermore, nocodazole treatment induces a superimposable G₂M arrest in both wt p53 and p53-inactivated cells without inducing changes in the levels of hCHK1 in both cell lines.

The strong p53-dependent decrease of hCHK1 protein levels at late time points after drug treatment, together with the recent observation that cell lines with a wt p53 express lower levels of hCHK2 compared with mutant p53-expressing cells (36), might be the way that cells tend to resume the G₂ block. This phenomenon could also partially describe the stronger and persistent G₂ block induced by anticancer drug treatment in cancer cells not expressing p53 and the effect of caffeine and other inhibitors that was reported to occur mainly in cells without p53 (37–39).

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