DNA AND CELL BIOLOGY
Volume 18, Number 4, 1999
Mary Ann Liebert, Inc.
Pp. 315–321

Function and Sequence Analyses of Tumor Suppressor Gene p53 of CHO.K1 Cells

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ABSTRACT

The tumor suppressor gene p53 plays an important role in guarding genomic integrity. When induced in response to environmental results, the gene product of p53 functions as a transcription factor to transactivate genes involved in arresting the cell cycle and as a facilitator of DNA repair. In contrast, the status of p53 in Chinese hamster ovary (CHO) cells, commonly used as a model system for various studies including those involving the cell cycle and transformation, remains an enigma. In this study, the function and sequence of p53 in CHO.K1 cells were investigated. The level of p53 proteins was elevated on ultraviolet (UV) irradiation of the cells, and the proteins formed specific complexes as probed with DNA containing p53-binding sequences. Its activities toward responsive promoters were inducible by UV in a dose-dependent manner. Although p53 in CHO.K1 contained a single missense mutation at codon 211, the mutation apparently had no effect on the functional properties of the protein. The CHO.K1 cells on X-ray irradiation failed to arrest at G_1 phase even when the cells were transfected with a wildtype human p53 gene, indicating that the failure probably was not caused by dysfunction of its p53, but by some other mechanism. This result is consistent with the finding that p21(Waf1/Cip1) is undetectable in UV-treated CHO.K1 cells, whereas Gadd45 is induced by UV light in the cells.

INTRODUCTION

THE TUMOR SUPPRESSOR GENE p53 plays critical roles at the checkpoints that control cell proliferation in higher eukaryotes (recently reviewed by Levine [1997]). As a guardian of genomic integrity, p53 protein is involved in the mechanisms of transient growth arrest, DNA repair, and apoptosis in response to environmental assaults (Canman *et al*., 1995; Levine, 1997). The p53 protein, normally unstable, accumulates concomitant with chromosomal damage (Levine, 1997). In some cells, p53-dependent stress responses are not activated, but p53-independent apoptosis is programmed in response to DNA damage (Aladjem *et al*., 1998).

The functions of the p53 gene are attributed largely to its activity as a transcription factor. It is responsible for the expression of many genes, including the human ribosomal gene cluster (Kern *et al*., 1991), mouse muscle creatine kinase (Zambetti *et al*., 1992), p21(Waf1/Cip1) (El-Diery *et al*., 1994), Gadd45 (Kastan *et al*., 1992), and mdm2 (Momand *et al*., 1992; Barak *et al*., 1993). In the promoter regions of these genes, a consensus p53 recognition sequence [PuPuPuC(A/T)(A/T)GPyPyPy-PuPuPuC(A/T)(A/T)GPyPyPy] has been identified (El-Diery *et al*., 1992; Zambetti and Levine, 1993). This sequence usually exists in a bent form (Nagaich *et al*., 1997).

The integrity of the p53 gene sequence also contributes to its functionality. The human p53 gene is frequently seen in mutated forms in malignancies, and mutational hot spots have been identified in its exons 5 to 8, between codons 130 and 290 (Zambetti and Levine, 1993). Three codons, 175, 248, and 273, stand out as extreme hot spots in colon and breast tumors. It is thus significant that many tumor cells are arrested when transfected with wildtype but not mutant p53 genes (Finlay *et al*., 1989; Michalovitz *et al*., 1990). This result suggests that p53 has a tumor suppression function. Furthermore, the efficacy of cancer radiotherapy or chemotherapy appears to depend on the status of p53 (Hawkins *et al*., 1996).

The CHO.K1 cell line, derived from Chinese hamster ovary tissue (Kao *et al*., 1979), is widely used in DNA transfection, toxicology, and tumorigenesis studies. However, although it is a transformed cell line, the status of its p53 gene remains largely

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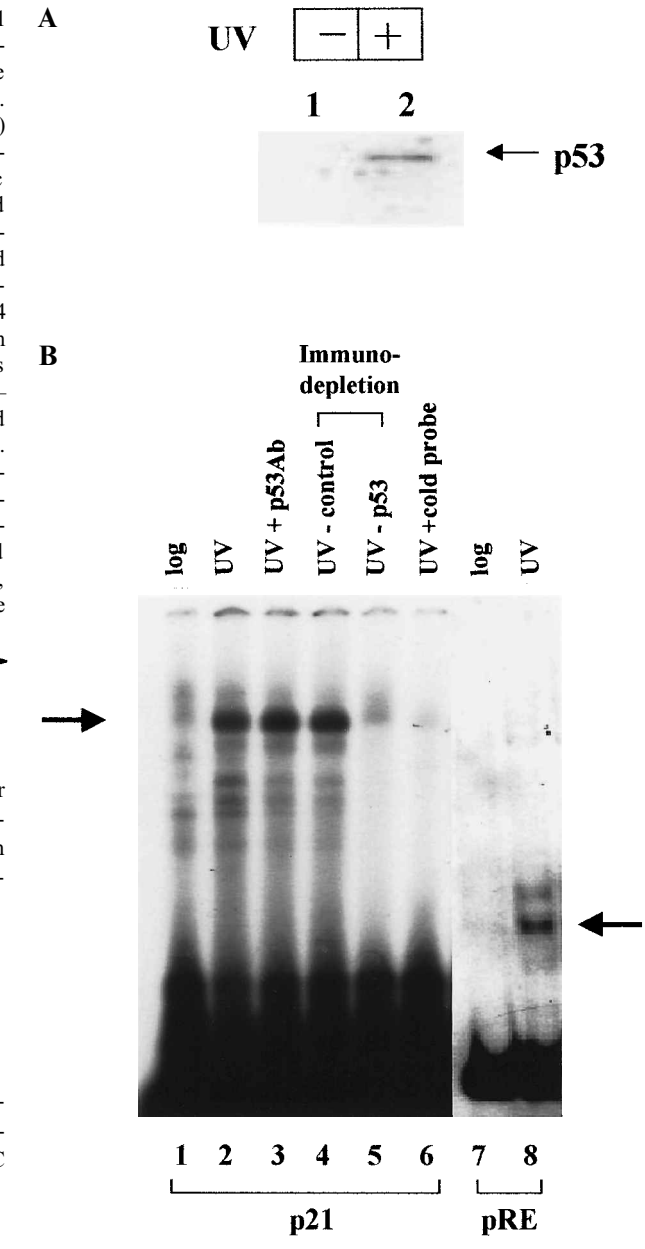

FIG. 1. Ultraviolet-inducible p53 expression in CHO.K1 cells. The cells were grown to 70%–80% confluence before being exposed to UV irradiation at dose of 40 J/m^2. Cell lysate was prepared after 24 h of recovery. **A.** Western blotting of p53. Monoclonal antibody to p53 (Pab240; Oncogene Science Co.) was used to detect p53 in the protein blot. P53 was greatly induced in CHO.K1 cells after UV irradiation. **B.** Electrophoretic mobility shift assay to monitor the expression of p53 induced by UV irradiation. The p53 response element, $(PRE)_3$, was isolated by cleaving the plasmid p3PRE-CAT with *Hin*dIII and *Sal*I and was ^{32}P-labeled by the Klenow enzyme fill-in reaction. Double-stranded p21 oligonucleotide was labeled by T4 polynucleotide kinase reaction. The EMSA was performed with control (log) or UV-treated (UV) cell extracts. Only the extracts prepared from cells treated with UV showed specific DNA–protein complexes (indicated by arrows), as the specific band was eliminated when excess cold probe was present (lane 6). To demonstrate the presence of p53 in the DNA–protein complexes, anti-p53 antibody was incubated with cell extract before probe was added (lane 3) for the antibody supershift assay. Alternatively, the p53 in cell extracts was immunodepleted with anti-p53 antibody (lane 5) or with an unrelated antibody, anti-PCNA (lane 4). The specific DNA–protein complexes were not detected in the p53-depleted cell extracts.

unknown. Recent studies indicated that the gene is mutated or dysfunctional (Lee *et al.*, 1997). In this study, we examined further its expression and function under environmental assault in an attempt to deduce the role of p53 and the cause of the dysfunction in terms of tumor suppression.

MATERIALS AND METHODS

Cultured cells and cell synchronization

The CHO.K1 cells were originally obtained from the American Type Culture Collection and were maintained in 1× McCoy's 5A medium containing 10% fetal bovine serum at 37°C in 5% CO_2.

Plasmids and oligonucleotides

Plasmid p3PRE-CAT was obtained from Dr. Y.-S. Lin. This plasmid is a derivative of pE1BCAT containing three copies (in tandem) of the consensus p53-binding site 5′-AGCTAGGCATGTCTAGACATGCCT-3′. Plasmids pG13-CAT were provided by Dr. Bert Vogelstein. The oligonucleotides which were used in electrophoretic mobility shift assays (EMSA's) containing the p53 recognition site in the p21(Waf1/Cip1) promoter had the sequence 5′-CAACATGTTGGGACATGTTC-3′.

Transient transfection and UV treatment

A total of 5 × 10^5 CHO.K1 cells in a 60-mm dish were transfected with plasmid DNA (1 μg) by the liposome-mediated method using LipofectAMINE (GIBCO) according to the manufacturer's manual. Twenty-four hours after transfection, the cells were allowed to recover in serum-free medium for 48 h to reach the quiescent stage before UV treatment. The cells were rinsed with 1× PBS and were uncovered prior to UV exposure at a marked area in tissue culture hood previously determined to receive the required dose of UV light (254 nm) from the germicidal lamp with the aid of a UV radiometer.

CAT assay

The chloramphenicol acetyl transferase (CAT) activity in crude cell extracts was assayed as follows. The cell extracts were obtained by the freezing and thawing method. Prior to the CAT reaction, the extract was heated at 65°C for 15 min to inactivate endogenous deacetylase. The CAT assay was performed essentially as described in Gorman *et al.* (1982). Briefly, aliquots of cell lysate containing equal amounts of protein were

incubated with 5 μl of 4 mM acetyl CoA and 5 μl of ^{14}C-chloramphenicol (0.025 μCi/ml) at 37°C for a period of time (e.g., 4 h). The chloramphenicol and derivatives were extracted with 550 μl ethyl acetate followed by centrifugation, and the top phase was collected for analysis. After thin-layer chromatography, plates were exposed to a PhosphoImager. The CAT activity was quantified using a computing densitometer.

Western blotting analysis

The standard procedure of Western blotting as described in Sambrook *et al.* (1989) was followed for studying the protein level. In brief, 20 μg of CHO.K1 crude cell extract was mixed 3:1 (v/v) with sample buffer (5% SDS, 5 mM Tris HCl, pH 6.8; 200 mM dithiothreitol (DTT), 20% glycerol, 0.1% bromophenol blue) and boiled prior to SDS-PAGE. The proteins in the gel were transferred to the nitrocellulose membrane using a semidry electroblotter and probed with the antibodies. The antigen–antibody complexes were visualized by the enhanced chemiluminescence detection kit (Amersham Co.) as directed by the manufacturer's manual.

Preparation of nuclear extracts

Nuclear extracts were prepared according to the procedures described by Huang and Prystowsky (1996). Briefly, 0.5–1 × 10^6 cells were collected, washed with 1× phosphate-buffered saline, and pelleted by centrifugation. The cell pellet was resuspended in 400 μl of cold buffer A (10 mM HEPES, pH 7.9; 10 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride) by gentle pipetting. The cells were allowed to swell on ice for 15 min, after which 25 μl of a 10% solution of Nonidet P-40 was added, and the tube was vortexed vigorously for 10 sec. The homogenate was centrifuged for 30 sec in a microcentrifuge. The nuclear pellet was resuspended in 50 μl of ice-cold buffer C (20 mM HEPES, pH 7.9; 1 mM EDTA, 0.4 M NaCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride), and the tube was rocked vigorously at 4°C for 15 min on a shaking platform. The nuclear extract was centrifuged for 5 min in a microcentrifuge at maximum speed at 4°C, and the supernatant fluid was stored at −70°C until use.

Electrophoretic mobility shift assay

The DNA binding assays were performed in 1× EMSA buffer consisting of 12 mM HEPES, pH 7.6; 50 mM NaCl, 1 mM DTT, and 5% (v/v) glycerol. The DNA probes (~20,000 cpm) were added to 20-μl binding reactions containing 5 μg of nuclear extract proteins and 1.5 μg of poly(dI-dC)-poly (dI-dC). The incubations were conducted at 4°C for 15 min following a 20 min incubation at the same temperature in the absence of probe. The DNA–protein complexes were resolved on 0.4-mm thick 5% polyacrylamide (29:1 Bis)–0.5× TBE gels. Gels were run in the cold room at 10 V/cm without buffer recirculation. After separation, the gels were dried and exposed to X-ray films at −70°C. To show the presence of p53 in the DNA–protein complexes in EMSA, an antibody supershift assay, as described by Buratowski and Chodosh (1996), and the immunodepletion method described previously (Zhan *et al.*, 1998) were used. Briefly, in the supershift assay, 2 μl of anti-

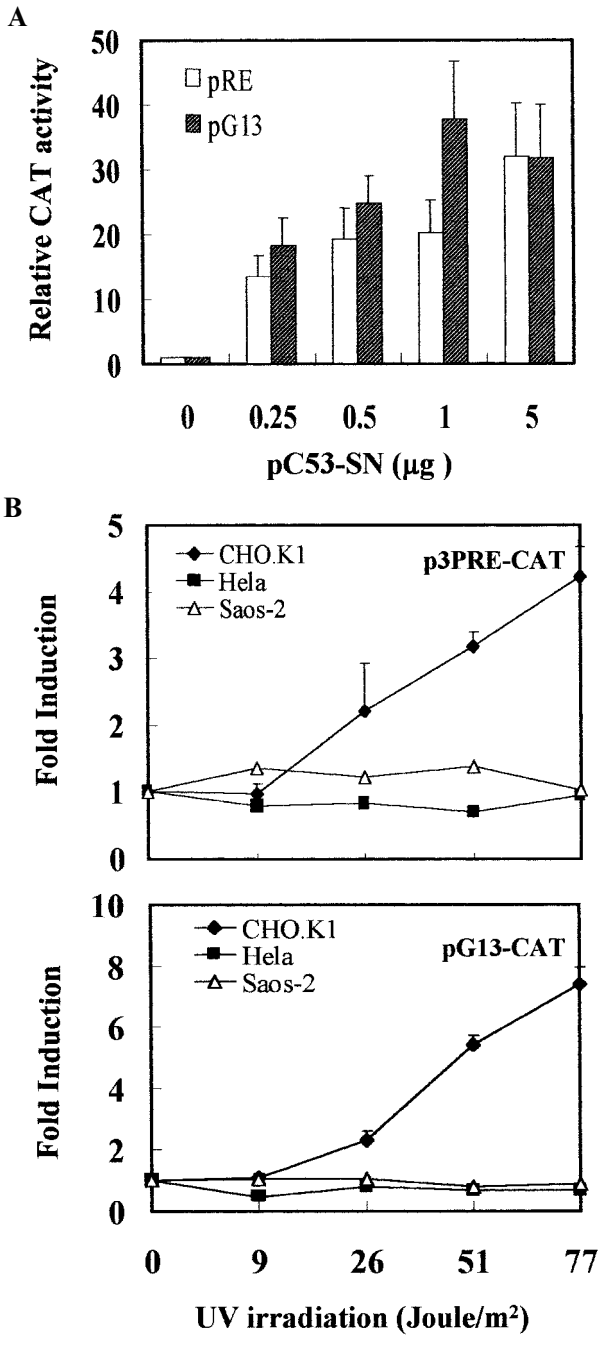

FIG. 2. Ultraviolet induction of chloramphenicol acetyl transferase (CAT) activity driven by p53 responsive promoters. **A.** P53 responsiveness of p3PRE-CAT and pG13-CAT promoters. A constant amount of p3PRE-CAT (or pG13-CAT) was cotransfected with the indicated amount of wildtype human p53 expression vector, pC53-SN, into CHO.K1 cells in a 60-mm dish. Both the PRE and pG13 promoters were activated by expression of p53. **B.** Dose dependence of UV induction of p3PRE-CAT and pG13-CAT promoters. The CHO.K1 cells in a 60-mm dish were transfected with 1 μg of p3PRE-CAT (top panel) or pG13-CAT (bottom panel) and then subjected to UV treatment at the indicated dose. The CAT activity was measured after cells had recovered for 24 h. Similar experiments were performed with HeLa and Saos-2 cells as controls. Each datum is the average of triplicate experiments. Standard errors are indicated as bars.

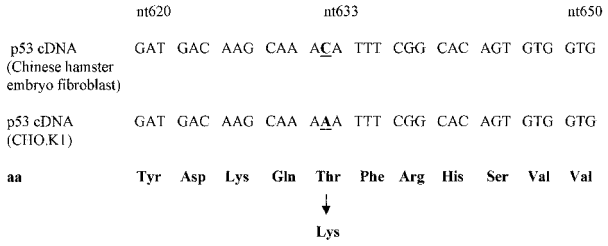

FIG. 3. Missense mutation at codon 211 in CHO.K1 p53 cDNA. The p53 cDNA clone from CHO.K1 was sequenced manually and by automatic sequencer. Partial nucleotide and deduced amino acid sequences of p53 cDNA clone derived from CHO.K1 mRNA are shown and compared to those of p53 from Chinese hamster embryonic fibroblast (GenBank Accession No. D86070). A transversion from C to A in CHO.K1 p53 at nucleotide 633 results in replacement of Thr by Lys at codon 211. The nucleotides were numbered starting from base A of the ATG initiation codon.

p53 antibody (Ab-3, Oncogene Co.) was added to nuclear extracts for 30 min before the DNA probe was added for EMSA. In the immunodepletion assay, nuclear extracts were incubated with 5 μl of anti-p53 or an unrelated antibody for 6 h, and the antigen–antibody complexes were immunoprecipitated with protein A agarose prior to EMSA.

Cloning of p53 cDNA

The mRNAs were isolated from CHO.K1 cells and converted to cDNA by reverse transcriptase AMV using poly(T) primer. The RNA templates were hydrolyzed with alkaline solution (0.1 M NaOH, 1% SDS). Double-stranded cDNAs were produced by polymerase chain reaction (PCR) using the primers with the sequences derived from the Syrian hamster p53 gene (forward primer: 5′TTCTGCCAGCTGGCGAA-3′; reverse primer: 5′-GTCAGTCCGAGTCAGGCCCC-3′). The PCR products were cloned directly into T-vector (Invitrogen).

FIG. 4. Wildtype human p53 cannot complement the deficiency of G_1 arrest in CHO.K1 cells following X-ray irradiation. Exponentially growing cells were pretreated with colcemid (0.5 μg/ml) 1 h prior to X-ray irradiation (6 Gy) with a TORREX 150D X-ray machine. Following the irradiation, cells were incubated in colcemid-containing medium for the indicated time before harvest for FACS analysis. The CHO.K1 cells (left panel), NIH3T3 cells (right panel), and CHO.K1 cells stably transfected with wildtype human p53 plasmid (pC53-SN) (middle panel) were treated or untreated with X-ray irradiation under the same conditions. The stable transfectants of pC53-SN were selected with the antibiotic G418 (400 μg/ml), as the plasmid confers resistance to this drug. The vertical and horizontal axes represent cell counts and DNA content, respectively.

Fluorescence-activated cell sorter analysis

To study the cell-cycle populations with or without X-ray irradiation, cells (2×10^6) were harvested and fixed in alcohol (75%) for 12 to 16 h at 4°C followed by RNase (1 mg/ml) treatment at room temperature for 30 min. Cells were stained with propidium iodide 10 μg/ml for 15 min prior to analysis with a flow cytometer (FACScan, Becton Dickinson).

RESULTS

Expression of p53 and its activity in CHO.K1 cells

Because the p53 level is often elevated following UV irradiation of cells, to know if the expression of p53 in CHO.K1 is induced by UV, we performed Western blotting analysis. Compared with that in untreated cells, the p53 level in UV-treated cells was greatly increased (Fig. 1A).

The p53 expressed in CHO.K1 after UV irradiation was able to recognize specific DNA sequences, as is expected given that it is a transcription factor. This was demonstrated by EMSA. In this assay, two DNA probes were used: one, designated PRE, carried three copies of consensus p53 binding sites; the other, designated p21, consisted of the p53 binding site in the p21 (Waf1) promoter. The results, shown in Figure 1B, indicate that both the PRE and p21 probes were able to form DNA–protein complexes with the total extracts of UV-irradiated cells, which contained elevated levels of p53, but not with extracts from control cells, which contained little if any p53 (comparing lanes 1 and 2 and lanes 7 and 8, respectively).

To demonstrate the presence of p53 in the DNA–protein complexes, we tried two strategies; i.e., antibody supershift assay and immunodepletion. While the anti-p53 antibody was unable to cause a supershift in the EMSA experiment (comparing lanes 2 and 3), the nuclear extract after p53 depletion by the same antibody failed to form the specific DNA–protein complexes (comparing lanes 2 and lane 5) just as in the situation where the excess competitive cold probe was present (lane 6). Furthermore, as a control for the specificity of the immunodepletion experiment, an unrelated antibody did not alter the formation of specific DNA–protein complexes (lane 4). Hence, the data indicate that p53 was present in the specific DNA–protein complexes.

Moreover, the p53 induced UV-irradiated CHO.K1 cells was able to transactivate its target promoters. This conclusion was obtained from experiments with two p53-responsive promoters, p3PRE-CAT and pG13-CAT (Hsu *et al.*, 1995; Kern *et al.*, 1991; Fig. 2A). To study the transactivation activity of endogenous p53, CHO.K1 cells were transfected with a fixed amount of p3PRE-CAT or pG13-CAT plasmid, followed by UV treatment at various doses. Results, shown in Figure 2B (top and bottom for p3PRE-CAT and pG13-CAT, respectively), indicate that the promoter's activities were induced by UV light and the extent of induction was increased in a dose-dependent manner. As a control experiment, the same promoters were transfected into HeLa or Saos-2 for a similar study. The expression of p53 in HeLa cells is suppressed because of the presence of oncoprotein E6, which renders p53 more susceptible to degradation (Scheffner *et al.*, 1990). On the other hand, there was no p53 activity in Saos-2 because of a null genotype ($p53^{-/-}$). No UV induction was seen, irrespective of the dose, with HeLa and Saos-2 (Fig. 2B). These results are consistent with the notion that the p53 induced by UV in CHO.K1 cells is able to transactivate its cognate promoters such as p3PRE-CAT and pG13-CAT.

DNA sequence analysis indicates that p53 of CHO.K1 has a single mutation at codon 211

The p53 cDNA of CHO.K1 cells was cloned by RT-PCR using primers designed on the basis of the sequences derived from the Syrian hamster p53 gene. The sequence was then determined. It was found to be virtually identical to that of p53 cDNA derived from Chinese hamster embryonic fibroblast (EMBL/GenBank Accession No. D86070), except for a C to A trans-

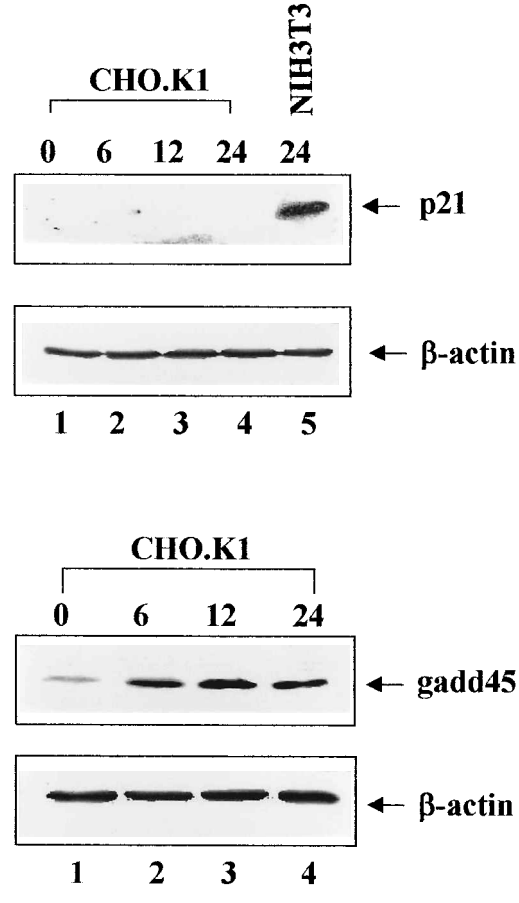

FIG. 5. Gadd45 but not p21(Waf1/Cip1) is induced by UV irradiation in CHO.K1 cells. The cells were irradiated at dose of 25 J/m^2 and allowed to recover for 0, 6, 12, or 24 h before harvest for analysis. Western analysis of p21(Waf1/Cip1) and Gadd 45 was done using anti-p21(Waf1/Cip1) monoclonal antibody (Santa Cruz Co.; sc-6246) and anti-Gadd45 polyclonal antibody (Santa Cruz Co.; sc-797). The NIH3T3 cells used as positive control for p21(Waf1/Cip1) were treated with UV (25 J/m^2) as K1 cells and allowed to recover for 24 h. Each lane was loaded with an identical amount of protein as indicated by the equality of their β-actin levels. In K1 cells, Gadd45 was induced by UV treatment, whereas p21(Waf1/Cip1) was undetectable even with UV treatment.

version at position 633 (codon 211) (Fig. 3). This base difference corresponds to an amino acid change from Thr (ACA) to Lys (AAA) in the DNA-binding domain (Cho *et al*., 1994). This mutation was also identified recently by Lee *et al*. (1997).

Transfection of wildtype human p53 was unable to complement the deficiency of G_1 arrest in CHO.K1 cells following X-ray irradiation

Previous study has observed that CHO cells lack G_1 arrest after irradiation (Larner *et al*., 1994). The deficiency of G_1 arrest was also seen by us using CHO.K1 cells and a protocol including colcemid in the culture medium 1 h prior to X-ray irradiation. Colcemid is an inhibitor of mitotic spindle formation. The inclusion of colcemid in the culture medium was designed to arrest cells in metaphase and thereby prevent the mitotic cells from entering G_1. Therefore, cells would not be at G_1 unless they were arrested by G_1 checkpoint control. Compared with the untreated CHO.K1 cells, the irradiated cells did not show any significant G_1 population at 12 to 24 h after irradiation, as indicated by the results of the FACS analysis, shown in Figure 4 (left panel). This result is in contrast to the findings in NIH3T3 cells, which showed a significant G_1 population after the same treatment (Fig. 4, right panel), suggesting that CHO.K1 cells failed to arrest at G_1 after the treatment. Furthermore, when a similar experiment was done with cells stably transfected with wildtype human p53 (pC53-SN), the cells remained unable to arrest at G_1 following the same treatment (Fig. 4, middle panel). Therefore, because the wildtype human p53 did not complement the defect in CHO.K1 cells, and because the wildtype human p53 was functional in this cell line, as indicated in Figure 2A, the lack of G_1 arrest in CHO.K1 is unlikely to be attributable to the dysfunction of its p53, as suspected by previous investigators (Lee *et al*., 1997).

Induction of Gadd45 but not p21(Waf1/Cip1) in UV-irradiated CHO.K1 cells

We then analyzed the protein levels of two p53-inducible genes, Gadd45 and p21(Waf1/Cip1). With NIH3T3 cells as a positive control for p21(Waf1/Cip1), 21(Waf1/Cip1) was undetectable in UV-irradiated (or nonirradiated) CHO.K1 cells, whereas Gadd45 was induced in UV-treated CHO.K1 cells (Fig. 5). In contrast, the level of β-actin in each sample as a control of the Western blotting analyses appears equal. As Gadd45 is UV inducible in the cells, suggesting that the endogenous p53 is able to induce Gadd45, the absence of p21(Waf1/Cip1) is unlikely to be attributable to the dysfunction of p53. Rather, it is probably because of the defect of the p21 gene or of other mechanism as yet not known. This result is consistent with the conclusion derived from the previous experiments that p53 is functional in CHO.K1 cells. In addition, the data suggest that deficiency of p21(Waf1/Cip1) expression may be responsible for the cell's failure to arrest at G_1 following DNA damage treatments.

DISCUSSION

We show in this study that the p53 gene in CHO.K1 cells is induced by UV irradiation, as expected for a normal stress response to this environmental assault. This normal activity of p53 is further supported by our observations that its product binds cognate response sequence elements and transactivates the respective promoters. Sequence analysis indicated that codon 211 of CHO.K1 p53 encodes Lys instead of Thr, as in Chinese hamster embryos (EMBL/GenBank Accession No. D86070) or in young Chinese hamster liver (Lee *et al*., 1997). Although one may be tempted to attribute this change to functional mutation and correlate it with the transformed state of CHO cells, the capability of this p53 protein to bind DNA and its effectiveness in transactivation are comparable to the properties of the wildtype. Apparently, the presence of such a replacement in the DNA-binding domain is inconsequential to the function of p53. This finding is in contrast to the effects of many other mutations in human p53, which render the gene product ineffective. Previous studies also observed that the presence of a mutation does not strictly correlate with p53 protein inactivation (Ory *et al*., 1994; Tsutsumi-Ishii *et al*., 1995), as certain p53 mutants display wildtype activity. It is interesting to note that a missense mutation is rarely seen in codon 211, compared with other sites such as in Arg-175, Arg-248, and Arg-273.

Unlike other cell lines, CHO.K1 cells are characterized by arrest at G_2 and not G_1 after X-ray irradiation. This is so even with ectopic overexpression of wildtype human p53. Our study proffers the possibility that CHO.K1 p53 protein is functional as a transcription activator and that the failure of G_1 arrest is secondary to its interacting component(s). In accordance with the hypothesis, we have found that p21(Waf1/Cip1) is undetectable in the UV-treated (or untreated) cells, whereas Gadd45 is UV inducible, as expected (see Fig. 5). As mentioned earlier, expression of these two genes is UV inducible and is mediated by p53. Thus, the specific mechanism that involves the failure of endogenous p21(Waf1/Cip1) to be expressed lends credence to our speculation and should be further investigated.

ACKNOWLEDGMENTS

The authors are grateful for Drs. B. Vogelstein and Y.-S. Lin for providing their plasmids. We thank Dr. Peter C. Keng for his valuable suggestion on FACS analysis and Dr. R. Rodewald for his critical review. This study was supported by the National Science Council of Taiwan with Grant NSC87-2311-B-007-029.

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Received for publication October 1, 1998; accepted in revised form January 15, 1999.