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Regulation of the cell cycle by p53 after DNA damage in an amphibian cell line

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In mammalian cells, the p53 protein is a key regulator of the cell cycle following DNA damage. In the present study, we investigated the function of p53 in the A6 amphibian cell line. Using various specific *Xenopus* p53 monoclonal antibodies, we showed that *Xenopus* p53 accumulates after DNA damage, including gamma and UV irradiation or treatment with adriamycin. Such accumulation is accompanied by an increase in the apparent molecular weight of the protein. This change was shown to be the result of a phosphorylation event that occurs after DNA damage. Accumulation of *Xenopus* p53 is parallel to a drastic change in the cell cycle distribution. Brief exposure to adriamycin or gamma irradiation induces reversible growth arrest, whereas long-term exposure to adriamycin leads to apoptosis. Taken together, these results indicate that p53 has a similar behaviour in frog cells and mammalian cells, and that it conserves two activities, cell cycle arrest and apoptosis. *Oncogene* (2001) **20,** 3766–3775.

Keywords: p53; DNA damage; *Xenopus laevis*; cell cycle

Introduction

The p53 protein plays a crucial role in the cellular response to DNA damage by activating either an apoptotic or growth arrest pathway in proliferating cells (Levine, 1997). Among the various biochemical activities linked to the p53 protein, its main function seems to be its ability to activate transcription from genes containing two contiguous monomers of the sequence (Pu)3 × C(A/T)(A/T)G(Py)3 × (El-Deiry *et al*., 1992). The transcription domain of p53 is localized in the amino-terminal part of the protein (residues 1–42), whereas the DNA binding domain is localized in the central region of the protein (residues 90–290). The importance of this DNA binding region is emphasized by the observation that more than 13 000 described point mutations of the p53 gene are clustered in this region (Soussi *et al*., 2000). Most of these mutations are correlated with a loss of the wild-type function of the p53 (Ory *et al*., 1994). These p53 DNA binding sites are found in the promoter or the intron of many genes involved either in control of the cell cycle or apoptosis (Tokino and Nakamura, 2000).

All these studies were performed on human and murine p53 proteins. Starting with vertebrates, more than 25 p53 genes or cDNAs were isolated and sequenced, providing a basis for developing new animal models to study this gene (Soussi and May, 1996). Indeed, in the case of the cat, cattle and dog, mutations in the p53 gene have been detected in the central region where mutational hot spots for human cancer are located (Dequiedt *et al*., 1995; Mayr *et al*., 2000; Veldhoen *et al*., 1998). Even more interesting is the identification of the p53 gene in *Drosophila* (Brodsky *et al*., 2000; Jin *et al*., 2000). It should be noted that, although p53 has not been found in yeast, overexpression of human wild-type p53 inhibits cell division in *S. cerevisiae* and *S. pombe*, whereas mutant p53 does not induce a detectable phenotype (Nigro *et al*., 1992). The cloning of *Xenopus laevis* p53 (Xp53) in 1987 led to the identification of five highly conserved domains, with four of them corresponding to the key regions involved in the DNA binding activity of p53 (Soussi *et al*., 1987). Previous studies have demonstrated that Xp53 shares a number of biochemical properties with mammalian p53 (Ridgway *et al*., 1994; Wang *et al*., 1995). Xp53 can bind specifically to various human p53 DNA recognition sequences as long as p53 is activated either by a carboxy-terminus monoclonal antibody or a specific peptide (Hardy-Bessard *et al*., 1998; Soussi *et al*., 1989). Furthermore, Xp53 is able to transactivate human promoters such as WAF-1, Mdm2 or bax, which contain the p53 response element. Although no p53 target has been identified in the frog, we postulate that they contain a consensus sequence similar to those found in humans. The regulation of p53 via mdm2 protein is also conserved throughout evolution, and it has been shown that xdm2 is able to bind mammalian p53 (Kussie *et al*., 1996; Marechal *et al*., 1997). The binding of hdm2 to Xp53 led to inactivation of the transactivational activity of p53 (Hardy-Bessard *et al*., 1998).

All these studies were performed in mammalian cells using transfection experiments and exogenously expressed Xp53. Furthermore, due to the thermo-

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sensitivity of Xp53, which is inactive at 37°C, most experiments were performed at 30°C (Soussi *et al*., 1989). In the present study, we examined the biological function of endogenous p53 in *Xenopus* cells. Using one of the most widely used *Xenopus* cell lines, we demonstrated that it expresses wild-type p53 that is stabilized after DNA damage inducing either growth arrest or apoptosis. This accumulation is accompanied by the phosphorylation of Xp53 that activates its DNA binding activity.

Results

DNA damage induced the stabilization of Xp53 in A6 cell lines

Cell line A6 was developed by Raferty from *X. laevis* adult kidney (Rafferty, 1969). This non-tumoral epithelioma cell line has been extensively used for physiological studies, but genetic information is lacking. Sequencing of the endogenous p53 gene was not performed, as it can lead to ambiguous results. Previously studies from our laboratory have shown that *X. laevis* has two p53 genes with different polymorphisms in the various alleles (data not shown). This is due to the fact that genome of *X. laevis* is tetraploid and that the two p53 genes have slightly diverged (Bisbee *et al*., 1977). Furthermore, it appears that there are some polymorphisms between various *X. laevis* which are not inbred. Thus, we focused on the biological activity of the Xp53 in this cell line, including stabilization and cell cycle arrest after DNA damage. The stabilization of endogenous Xp53 was detected by Western blot using specific polyclonal and monoclonal antibodies. As shown in Figure 1a, adriamycin treatment led to the accumulation of a 46 kDa protein that is detected either with the monoclonal antibody X20 or with a rabbit polyclonal antibody. This molecular weight is similar to the size obtained when Xp53 is exogenously expressed in mammalian cells (data not shown, see also Soussi *et al*., 1989). Furthermore, the same protein is detected with various monoclonal antibodies (Figure 1 and data not shown). Both ionizing radiation and adriamycin are able to induce the stabilization of Xp53 (Figure 1b,c). Stabilization of Xp53 is also detected after UV radiation (data not shown). Adriamycin treatment led to a slow increase in Xp53 that peaked at 8 h, whereas ionizing radiation induced a rapid p53 induction that was detectable 30 min after irradiation (Figure 1b). When adriamycin was administered as a 1 h pulse, the kinetic of Xp53 induction was similar to that of ionizing radiation, with maximum induction 3 h after the pulse and a decrease thereafter. The level of stabilized Xp53 was higher with the pulse of adriamycin (data not shown) compared to ionizing radiation, but it did not reach the level observed with a long exposure to adriamycin. Induction of Xp53 was proportional to the amount of DNA damage induced either by adriamycin or irradiation (Figure 1c). Control experiments with the human HCT116 cell line, indicates that Xp53 behaves like Hp53 (Figure 1d).

Xenopus *p53 DNA binding activity was induced by DNA damage*

One of the key functions of p53 is its specific DNA binding activity. Recombinant Xp53 is able to bind human p53 binding sites. We thus assessed the behaviour of endogenously expressed Xp53 toward a DNA binding site of human origin, as no *Xenopus* p53 response gene has been cloned thus far. No DNA binding activity could be detected in non-treated cells, whereas strong DNA binding activity could be detected in cells treated with adriamycin (Figure 2a). This complex was supershifted with a monoclonal antibody specific for *Xenopus* p53 (Figure 2a). Competition could be observed with an excess of unlabelled wild-type sequence, whereas a similar amount of mutant sequence did not affect the complex. Control experiments with recombinant Xp53 expressed in insect cells confirmed the specificity of these various complexes. The observation that recombinant Xp53 needs to be activated by a C-ter monoclonal antibody for efficient DNA binding has already been described (Hardy-Bessard *et al*., 1998). Similar activation of the DNA binding activity of Xp53 is also detected after ionizing radiation in a dose dependent manner (Figure 2b). In those experiments it was not possible to determine whether the DNA binding activity was due to an increase in the amount of Xp53 after DNA damage or whether it was linked to specific activation of the p53 protein, as suggested by several authors (Hupp *et al*., 1992; Woo *et al*., 1998).

DNA damage led to cell cycle arrest and apoptosis in A6 cells

It is of importance to associate such biochemical activity with the biological function of Xp53. Wild-type mammalian p53 induction is associated with a change in cell cycle, as it can lead to growth arrest or apoptosis. Using flow cytometry, we analysed the behaviour of A6 cells after DNA damage. Transient cell growth arrest was readily detectable 8 h after treatment with ionizing irradiation (5 Gy), as cells incorporating BrdU dropped from 40.3% in control cells to 19.4% in irradiated cells (Figure 3 and Table 1). This was also accompanied by an increase in cells in G1 (65 *versus* 51% in control) and an increase in G2 (13.5 *versus* 7.4% in control). After 24 h, the cell cycle resumed with cell distribution similar to that of a non-irradiated control. Such behaviour is totally identical to that described in mammalian cells.

Cells treated with adriamycin behaved differently. With a short pulse of the drug, the number of cells in S phase 8 h after treatment did not change (37 *versus* 40.3% in the control) but incorporation of BrdU dropped (MFI, 176 *versus* 652 in the control), indicating slower incorporation of BrdU. After 24 h, cell cycle arrest was readily detectable both at the S and G2 phase.

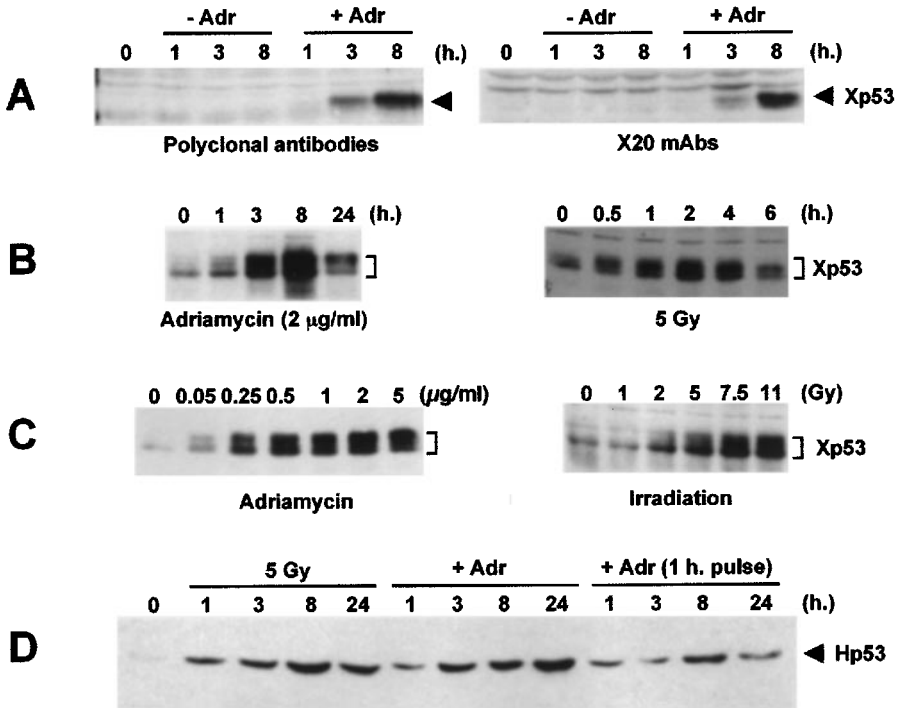

Figure 1 Stabilization of Xp53 is A6 cells after DNA damage. (**a**) A6 cells were treated with adriamycin (20 μg/ml) and protein extracts were prepared after various times of treatment. Western blots were performed either with a polyclonal antibody or a monoclonal antibody (X20). (**b**) Time course stabilization of Xp53 after adriamycin or gamma irradiation were analysed by Western blots with the X77 monoclonal antibody. (**c**) The effect of increasing the amount of ionizing radiation or adriamycin on the steady-state level of Xp53 was analysed by Western blot with the X77 monoclonal antibody. Autoradiography exposure for adriamycin was shorter than for γ irradiation. (**d**) Time course stabilization of Hp53 after adriamycin or gamma irradiation were analysed by Western blot with DO7 monoclonal antibody in HCT116 cells

Twenty-four hours after the pulse, 19.6% of the cells were found to have a DNA content between 2n and 4n, although they had not incorporated any BrdU during the labelling pulse, suggesting that these cells had arrested replication. However, the increase in MFI observed at 24 h (424) compared to 8 h (176) indicated that some cells had resumed normal replication. When the exposure to adriamycin was continuous, the phenotype was more pronounced, with a sharp drop in the MFI starting 3 h after the beginning of treatment (227 *versus* 730 in the control), culminating after 24 h (37 *versus* 503 in the control or 424 with the short pulse of adriamycin). This blocking of the cell cycle was undoubtedly due to the continuous induction of DNA damage that did not allow the cell cycle to resume. Furthermore, we observed that continuous treatment with adriamycin led to the release of the cells into the medium (50% after 24 h and more than 90% after 48 h). Such an event has not been observed either with γ-irradiation or after a short pulse of adriamycin (data not shown). Using TUNEL staining, we observed that a significant number of cells were apoptotic after treatment with adriamycin, 13% at 24 h and 45% at 48 h (Figure 4a). DAPI staining was also used to confirm these results (data not shown). Using FACS analysis, we also observed a specific increase in the sub-G1 population for cells treated with adriamycin. It is not detected either with γ-irradiation or after a short pulse of adriamycin (Figure 5b). For comparison, we analysed the human cell line HCT116 treated in a similar way (Figure 4 and Table 1). After irradiation or a short pulse of adriamycin, we observed a decrease of G1 and S phase and an increase of G2 phase as already described (Bunz *et al*., 1998). Long exposure to adriamycin led to a sharp drop in the MFI starting 3 h after the beginning of treatment (131 *versus* 721 in the control) as it was observed for A6 cells.

Xp53 was phosphorylated after DNA damage

During the course of these studies, we observed an increase in the apparent molecular weight of Xp53 after DNA damage (Figure 1b,c). This could be due to post-translational modification, including phosphorylation, known to occur in mammalian p53. Such a modification does not induce any change in migration of Hp53 in polyacrylamide gel, contrary to human Rb or BRCA1. Phosphorylation of mammalian p53 is believed to hamper the interaction with mdm2, leading to accumulation of p53. It has also been shown that mammalian p53 can be stabilized by using the calpain/proteasome inhibitor LLnL, but such stabilization is not linked to p53 phosphorylation (Shieh *et al*., 1997).

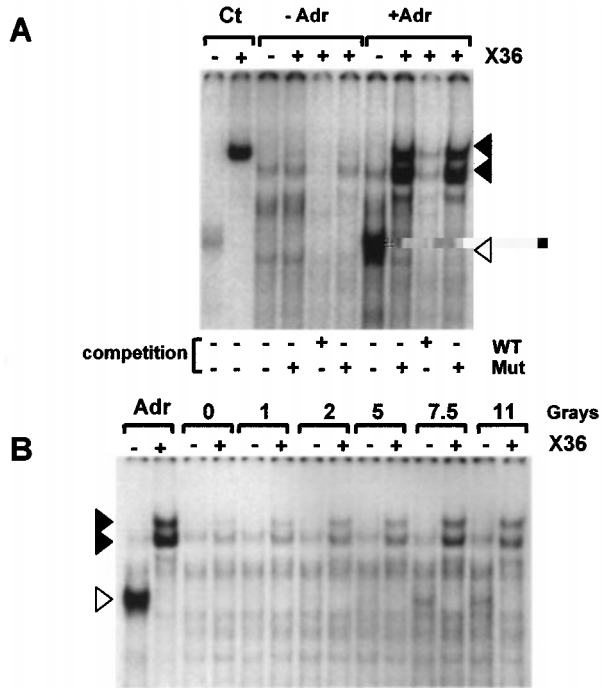

Figure 2 Specific activation of the DNA binding activity of Xp53 after DNA damage. (**a**) A6 cells were incubated in the absence (−Adr) or presence of 2 μg/ml of adriamycin for 8 h (+Adr). Nuclear extracts were used for gel mobility shift as described in Materials and methods. Competition experiments using a 50× molar excess of wild type or mutant DNA are indicated at the bottom of the figure. Supershift was performed with the monoclonal antibody X36 specific for the carboxy-terminus of Xp53. The control experiment (Ct) was done with recombinant p53 expressed in insect cells using a baculovirus expressing Xp53. (**b**) A6 cells were irradiated with an increasing amount of ionizing radiation and nuclear extracts were prepared 2 h after treatment. Gel mobility shifts were performed with (+) or without (−) the X36 p53 monoclonal antibody. A nuclear extract from A6 cell lines treated with adriamycin (Adr) was used as a control. Open and closed arrows mark the migration of the p53-DNA complex and the supershift complex respectively

As shown in Figure 5a, Xp53 accumulation induced by LLnL was not accompanied by a shift in molecular weight, whereas DNA damage induced by adriamycin or irradiation was associated with the appearance of more slowly migrating species recognized by Xp53 monoclonal antibodies (Figure 5a). Phosphatase treatment of Xp53 induced by adriamycin led to a decrease in the apparent molecular weight of Xp53, suggesting that phosphorylation was responsible for this change in migration (Figure 5b).

Several kinases have been shown to phosphorylate Hp53 after DNA damage. Using various purified kinases, we treated recombinant Human and *Xenopus* p53 in the presence of radiolabelled ATP. As shown in Figure 6a, each kinase, CKI, CKII and DNA-PK, is able to phosphorylate the two p53 proteins. An endogenous unidentified kinase led to a low level of phosphorylation, especially in the CKI and DNA-PK buffers. Phosphorylation of human p53 did not lead to any change in migration of the protein. Phosphorylation of Xp53 by CKI and DNA-PK led to a shift in the apparent molecular weight of the protein. In order to ensure that the target protein was really p53 and not a minor component of the extract, a Western blot linked to a kinase assay was performed (Figure 6b) (see Materials and methods). After the kinase reaction and electrophoresis, the gel was transferred to a membrane and blotted with a monoclonal antibody specific for Xp53. As shown in Figure 6b, increased activity of DNA-PK led to protein phosphorylation. Western blot experiments confirmed that the slower band was indeed Xp53. Treatment of ^{35}S-labelled Xp53 obtained by *in vitro* transcription–translation with DNA-PK and unlabelled ATP also led to an increase in the apparent molecular weight of Xp53 (Figure 6c). Taken together, these data demonstrate that phosphorylation of Xp53 by several, but not all, kinases led to an increase in the apparent molecular weight of the protein.

Phosphorylation of Xp53 led to its activation for DNA binding

Phosphorylation of p53 is essential for its stabilization and for the activation of its specific DNA binding activity. Phosphorylation of the amino-terminus of the protein is believed to be partially involved in interference in the interaction between p53 and mdm2 that leads to p53 stabilization after DNA damage. The carboxy-terminal of Hp53 (amino acids 368–383) represses the DNA binding activity of p53. *In vitro*, phosphorylation of this region by several kinases such as CKII or PKC is associated with increased DNA binding activity. We tested whether such activation could occur with Xp53. Using recombinant human or *Xenopus* p53, we performed gel shift assay in the presence of various amounts of PKC. As shown in Figure 7a and b, Xp53 is phosphorylated by PKC. This phosphorylation also leads to a slight decrease in the electrophoretic mobility of the protein. The upper band detected with PKC corresponds to auto phosphorylation of the kinase. For EMSA, incubation of Hp53 with PKC was performed at 30°C for 5 min. In the absence of PKC, such incubation led to irreversible denaturation of Hp53 with a loss of its DNA binding that could not be activated with the activation antibody (Figure 7c). In the presence of PKC, the phosphorylation of Hp53 led to substantial activation of the protein which bound very efficiently to DNA in the absence of activating antibody. Supershift experiments confirmed that this DNA binding was due to p53. The observation that incubation of Hp53 at 30°C leads to its inactivation has already been described (Hansen *et al*., 1996). The present data suggest that phosphorylation of the carboxy-terminus of Hp53 by PKC has a protective effect upon heat denaturation. For Xp53, phosphorylation was performed for 30 min at 20°C, as higher temperature led to total denaturation of the protein (data not shown). Phosphorylation of Xp53 by PKC also led to a moderate increase in the DNA binding activity of the protein in the absence of activating antibody (Figure 7d).

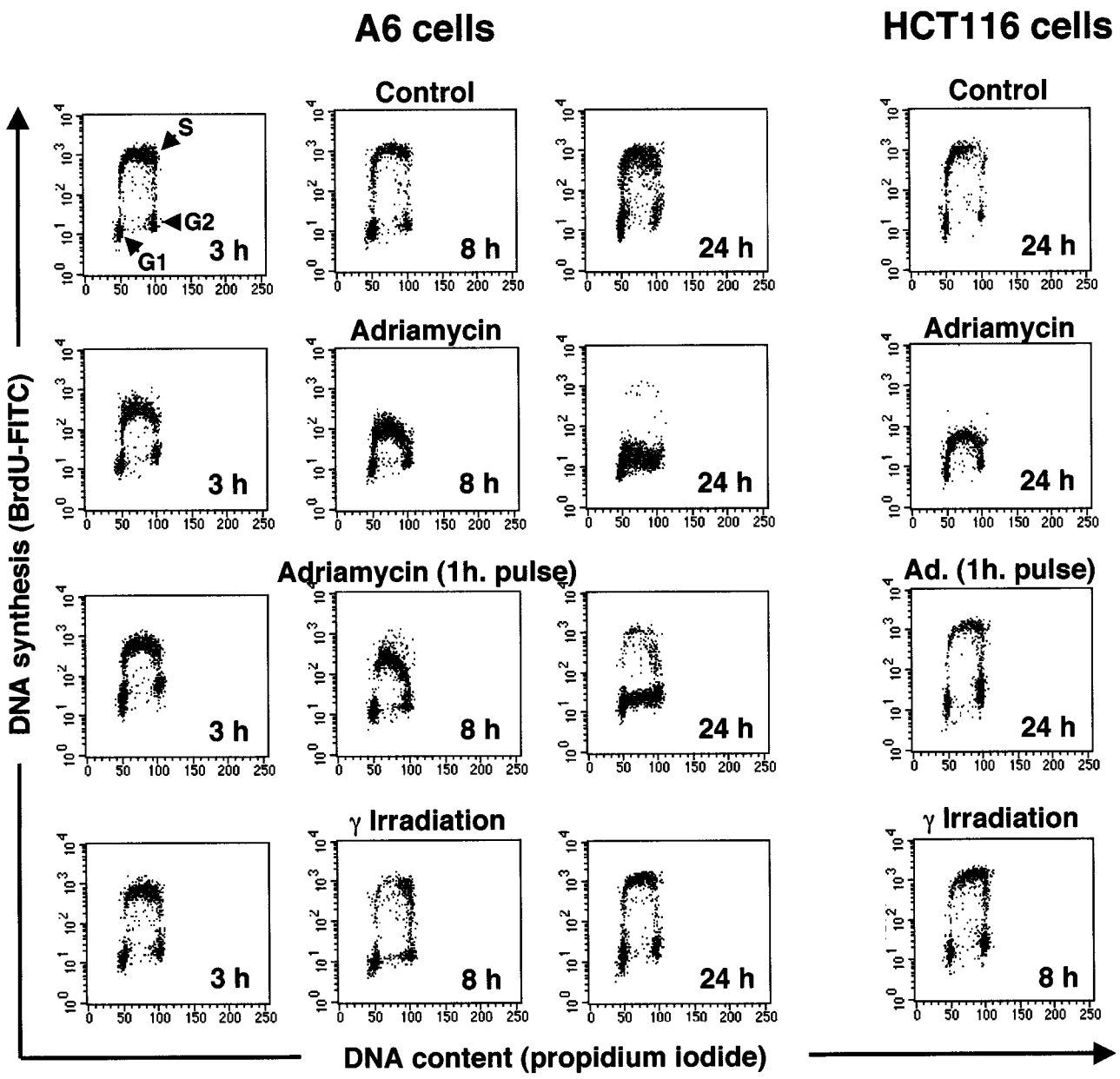

Figure 3 Flow cytometric analysis of A6 and HCT116 cells after DNA damage. Cells were pulsed with BrdU for 15 min before labelling with FITC-conjugated BrdU antibody and counterstaining with propidium iodide. Cell cycle distributions in control cells or cells at 3, 8 and 24 h after exposure to adriamycin (2 μg/ml), a 1 h pulse of adriamycin (2 μg/ml) or γ-irradiation (5 Gy) were analysed as described in Materials and methods

Discussion

p53 is a tightly regulated transcription factor that induces cell cycle arrest or apoptosis in response to cellular stress such as DNA damage or oncogene activation (Levine, 1997). Most of these studies were performed with either human or mouse p53. Recently, p53 from *Drosophila* (Dmp53) has been identified (Brodsky *et al*., 2000; Jin *et al*., 2000). Dmp53 overexpression induces apoptosis, but in contrast to mammalian p53, it does not induce G1 cell cycle arrest, and inhibition of Dmp53 activity does not affect X-ray-induced cell cycle arrest. It has been suggested that this ancestral p53 may have been restricted to eliminating damaged cells by apoptosis. Extensive analysis of frog p53 has revealed several important biochemical feature similar to Hp53, such as DNA binding activity, negative regulation of specific DNA binding activity by the carboxy-terminus of the protein, or fixation to mdm2 (Hardy-Bessard *et al*., 1998; Kussie *et al*., 1996; Ridgway *et al*., 1994; Wang *et al*., 1995). Thus far, all these studies have been performed either *in vitro* or in mammalian cells transfected with an expression vector. In order to gain a clear view of the biological function of wild-type *Xenopus* p53 in its natural environment, we undertook functional studies in frog cells. Using the

Table 1 Cell cycle parameters after stress. Fractions of cells in the G0/G1, S and G2/M phases of the cell cycle

		Percentage of cells						*Percentage of cells*			
Treatment	*Time*	*G0/G1*	*S* (BrdU ±)	*G2/M*	*MFI*	*Treatment*	*Time*	*G0/G1*	*S* (BrdU ±)	*G2/M*	*MFI*
A6 cells						HCT116 cells					
Control	3 h	48.4	43.2/0.9	7.5	730	Control	3 h	27.6	55/0.5	16.9	721
	8 h	51.2	40.3/1.1	7.4	652		8 h	30.2	52/0.6	17.2	861
	24 h	54.9	36.5/0.7	7.9	503		24 h	32.5	52/0.3	15.2	602
Adriamycin	3 h	49.4	41.5/0.9	8.2	227	Adriamycin	3 h	32.6	50.4/0.7	16.3	131
	8 h	50.2	40.5/0.5	8.9	77		8 h	28.9	54.2/0.1	16.9	94
	24 h	56.4	10.4/15	18.2	37		24 h	22.1	59.4/2	16.6	37
Adriamycin	3 h	48.1	42.2/0.8	8.9	496	Adriamycin	3 h	24.8	58.5/0.5	16.1	401
(1 h pulse)	8 h	52.9	37/0.9	9.2	176	(1 h pulse)	8 h	21	55.1/0.5	23.4	574
	24 h	51.6	8.3/19.6	20.5	424		24 h	15.5	16.3/0.9	67.4	667
γ-irradiation	3 h	49.9	38/1.1	11	519	γ-irradiation	3 h	27.6	54.7/0.6	17.2	730
	8 h	65.5	19.4/1.6	13.5	434		8 h	17.6	41.4/1.1	39.9	1049
	24 h	60.6	32.2/0.9	6.3	822		24 h	30.6	33.5/1	35	599

MFI: Mean Fluorescence Intensity of BrdU labeled cells

common cell line A6 from *X laevis*, we analysed its behaviour after DNA damage. Under normal growth conditions, Xp53 is barely detectable in this cell line, whereas it is heavily stabilized after DNA damage such as ionizing radiation, UV light or adriamycin. Such stabilization is accompanied by a change in the cell cycle. All these features indicate that this non-tumoral cell line expresses wild-type p53.

Ionizing radiation induced reversible G1/S arrest similar to that described in mammalian cells (Dileonardo *et al*., 1994; Kuerbitz *et al*., 1992). Prolonged treatment of these cells with adriamycin led to cell cycle arrest followed by irreversible apoptosis. These differences in behaviour after irradiation or adriamycin could be due to the greater extent of DNA damage resulting from continuous exposure to adriamycin. Extensive accumulation of DNA damage could lead to apoptosis due to a failure to repair. It has been suggested that the level of p53 induced after DNA damage could determine cell death or growth arrest (Chen *et al*., 1996). In the present study, we consistently observed that the level of Xp53 induction was always higher during adriamycin treatment, supporting the hypothesis that the high amount of DNA damage induced by this treatment overcomes growth arrest and leads to apoptosis. This is supported by the observation that treatment of the cells by a short pulse of adriamycin is similar to that observed with irradiation, ruling out the possibility that both agents could lead to different types of DNA damage associated with a different cellular response. These data indicate that Xp53 is closer to its mammalian counterparts than Dmp53, as it can induce both cell cycle arrest and apoptosis.

Phosphorylation of p53 is a post translational modification essential for its activation (Meek, 1998). It has been demonstrated that several kinases specifically phosphorylate the amino and carboxy-terminus of mammalian p53 depending on the type of damage. Modification of the amino-terminus of mammalian p53 is believed to hamper the binding of mdm2, leading to p53 accumulation. In the present study, we demonstrate that Xp53 is also phosphorylated after DNA damage. This was demonstrated by a change in the apparent molecular weight of the protein that could be reversed by treatment with phosphatase. *In vitro* treatment of recombinant Xp53 with various human kinases demonstrates that Xp53 can be phosphorylated, suggesting an evolutionary conservation of several phosphorylation sites. Except for CKII, such phosphorylation leads to a change in the apparent molecular weight of Xp53. This feature does not allow us to distinguish the kinases involved in phosphorylation of Xp53 *in vivo*. Phosphorylation of mammalian p53 is also believed to be involved in the induction of a latent non-DNA binding protein to an efficient DNA binding transcription factor (Hupp *et al*., 1992). We previously demonstrated that recombinant Xp53 also needs to be activated in order to bind efficiently to DNA. In the present study, we show that the DNA binding activity of endogenous Xp53 from A6 cell is activated after DNA damage. *In vitro* phosphorylation of recombinant Xp53 by PKC is able to activate such specific DNA binding. The observation that phosphorylation leads to a distinguishable p53 form could be useful for the study of specific phosphorylation inhibitors.

Taken together, our results clearly demonstrate that the p53 pathway is intact in the *X. laevis* A6 cell line. Treatment of this cell line with various DNA damaging agents led to phenotypes indistinguishable from those observed in mammalian cells, including both cell cycle arrest and apoptosis. It has been previously shown that Xp53 is stored in the cytoplasm of oocytes and migrates to the nucleus after fertilization (Amariglio *et al*., 1997; Tchang and Mechali, 1999). Such delocalization could act by supplying the p53 necessary for the protection of the embryo during the development. Although $p53^{-/-}$ mouse is viable, several works suggest that p53 could act as a teratologic suppressor during stress occurring in development (Nicol *et al*., 1995; Norimura *et al*., 1996). Our present study indicates that we have sufficient specific tools to

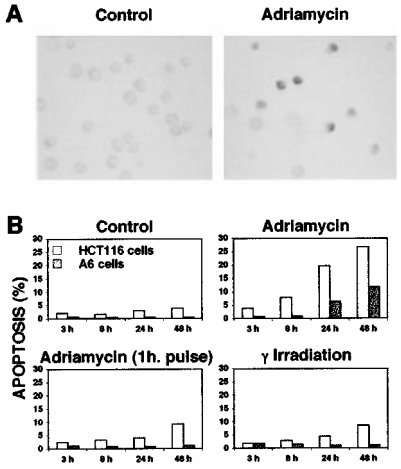

Figure 4 Apoptotic response of A6 and HCT116 cells after DNA damage (**a**) Cells were subjected to adriamycin (2 μg/ml) for 48 h and stained with a TUNEL assay. (**b**) Apoptosis was determined by the content of cells in the sub G1 population measured by flow cytometry after staining with propidium iodide. Cells were treated by adriamycin (2 μg/ml), a 1 h pulse of adriamycin (2 μg/ml) or γ-irradiation (5 Gy). For analysis of A6 cells, adherent and floating cells were pooled and analysed. For HCT116 cells, no increase of floating cells were detected after any treatment

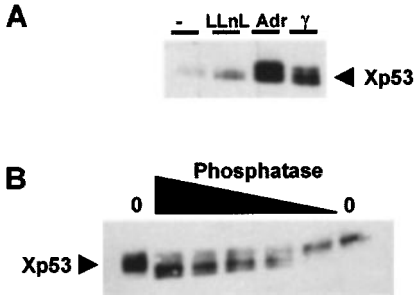

Figure 5 Dephosphorylation of *Xenopus* p53 from A6 cells. (**a**) A6 cells were treated with LLnL (200 μM), adriamycin (2 μg/ml) or ionizing radiation (5 Gy). Protein extracts were performed respectively 6, 8 or 3 h after treatment. Similar amounts of protein were used for Western blot using X77 monoclonal antibody. (**b**) A6 cell extract treated for 8 h with adriamycin (2 μg/ml) was incubated with 0, 10, 50, 100, 500 or 1000 units of lambda phosphatase as described in Materials and methods. Extracts were then subjected to Western blot with X77 monoclonal antibody

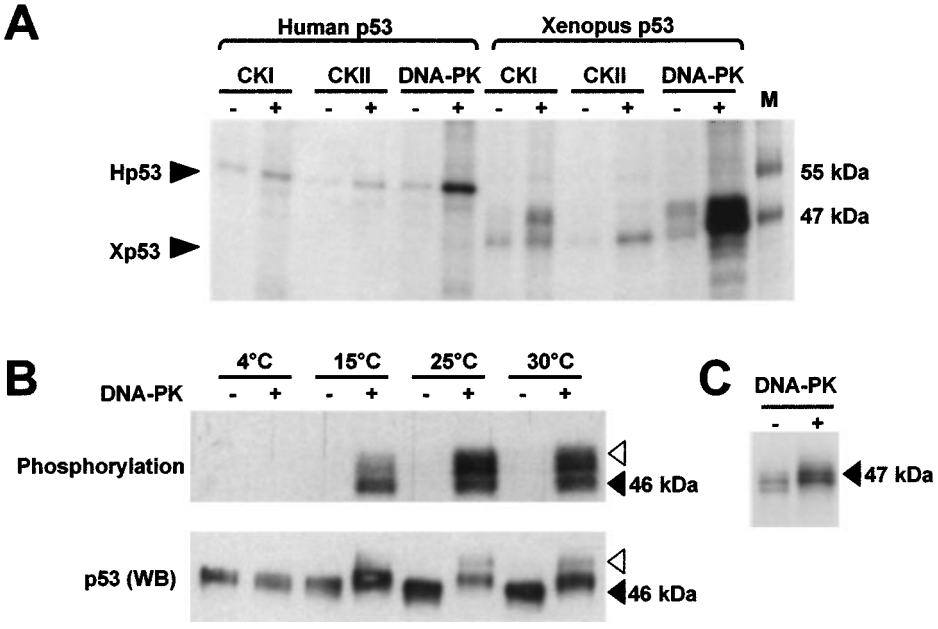

Figure 6 Phosphorylation of p53. (**a**) Recombinant human or *Xenopus* p53 (20 ng) were incubated with various kinases in their respective reaction buffers (+) in the presence of [γ^{32}]ATP (CKI, 15 U; CKII, 15 U; DNA-PK, 60 U). Control reactions without kinase were also included (−). (**b**) Xp53 (20 ng) was incubated with DNA-PK 60 U) at various temperatures to modulate the activity of the enzyme. Reactions were performed in the presence of [γ^{32}]ATP. After a 30 min incubation, the reactions were split into two samples. The first series of sample was run on a gel that was directly dried and subjected to autoradiography for monitoring the extent of phosphorylation. After electrophoresis of the second series of sample, gels were transferred to a nitrocellulose filter and processed for Western blot using X77 monoclonal antibody (p53(WB)). (**c**) ^{35}S labelled Xp53 (30 000 c.p.m.) was obtained by *in vitro* transcription-translation and incubated with DNA-PK (60 U) in the presence of unlabelled ATP (+) or only in the reaction buffer without enzyme (−). After the reaction (10 min, 30°C), samples were immunoprecipitated with the X36 monoclonal antibody and subjected to SDS–PAGE and fluorography. For human p53, phosphorylation by CKI and CKII lead to a specific increase of signal of 1.9 ± 0.6 and 2.1 ± 0.9 respectively

address such questions during the development of *X. laevis*, as this amphibian's development is rapid and autonomous, and the embryo is large enough to allow experimental manipulation.

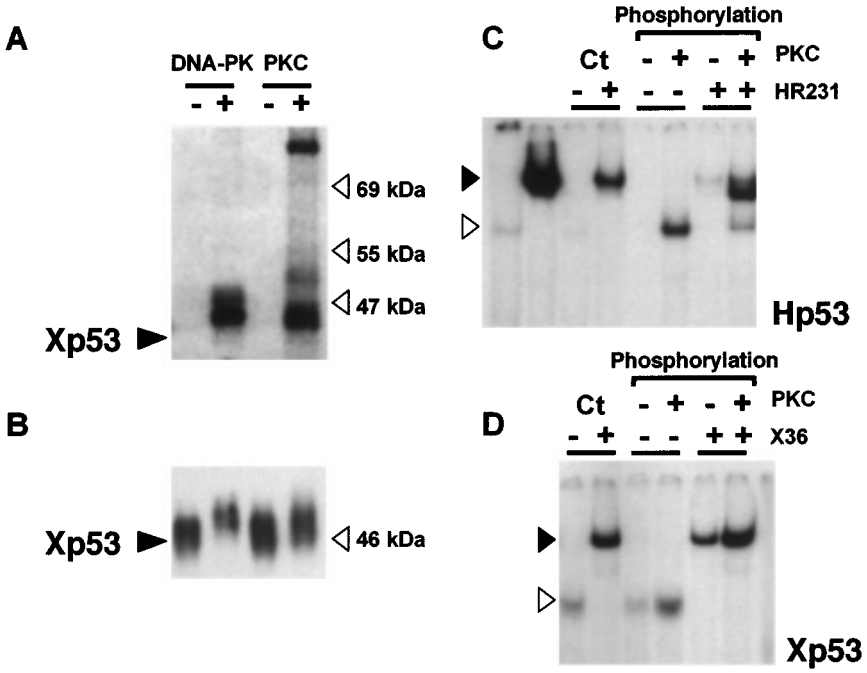

Figure 7 Activation of the specific DNA binding activity of human and *Xenopus* p53 by protein kinase C. (**a**) Recombinant Xp53 (20 ng) was incubated with DNA-PK (30 U) or PKC (55 mU) in their respective reaction buffers in the presence of (γ^{32}]ATP (+). Control reactions without kinase were also included (−). After electrophoresis, the gel was dried and subjected to autoradiography. (**b**) Same as in **a** except that unlabelled ATP was used. The gel was transferred to nitrocellulose filter and processed for Western blot using X77 monoclonal antibody. (**c**) Recombinant Hp53 (20 ng) either treated or untreated with PKC (PKC + or −) for 5 min at 30°C was subjected to EMSA in the presence or absence of the activating monoclonal antibody HR231. Control experiments were performed with p53 that had not been processed for phosphorylation. (**d**) Same as **b** with Xp53, except that phosphorylation was performed for 20 min at 20°C to avoid denaturation of the p53. The activating antibody was X36

Materials and methods

Cell lines and culture conditions

X. laevis A6 epithelial cells were provided by G Almouzni (Institut Curie), and were grown in modified Leibowitz L-15 medium (Leibowitz L-15 medium containing 20% sterile distilled water, 10% foetal bovine serum and 100 U/ml penicillin/streptomycin), at 23°C. The HCT116 cell lines (human wild-type p53) was obtained from the American Type Culture Collection. Cells were maintained in McCoy medium supplemented with 10% heat-inactivated foetal calf serum and antibiotics. Cells were grown at 37°C in a humidified 5% CO_2 atmosphere. Irradiation was performed at the indicated doses with a ^{137}Cs Source (IBL 637, CisBio International, Gif sur Yvette). Adriamycin, a topoisomerase II inhibitor that induces double-strand breaks, was purchased from Sigma.

Western blotting and antibodies

To prepare proteins for immunoblotting, cells were lysed in RIPA buffer (10 mM Tris hydrochloride, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.5% sodium deoxycholic acid) with protease inhibitors (1 mM PMSF, 1 μg/ml leupeptine and pepstatine) and phosphatase inhibitors (50 mM NaF, 2 mM sodium orthovanadate) for 30 min at 4°C. After centrifugation at 15 000 *g*, extracts were stored at −80°C until use. Protein concentrations were determined using the BCA protein assay reagent kit (Pierce). Next, equal amounts of sample lysate were separated by SDS–PAGE and electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in PBST (PBS with 0.2% Tween 20) at room temperature for at least 2 h. The membrane was then incubated in the same buffer with the primary antibody against p53 for 2 h at 20°C. After five washes in PBST, the membrane was incubated with appropriate secondary antibody (horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG). Revelation was performed using the chemoluminescence kits (Pierce). *Xenopus* p53 monoclonal antibodies have been previously described. X77 recognized the amino-terminus of the protein, whereas X20 and X36 had non-overlapping epitopes in the carboxy-terminus (Hardy-Bessard *et al*., 1998). Polyclonal antibodies have been raised by immunization of a rabbit with a recombinant *Xenopus* p53 (Soussi *et al*., 1989).

Oligonucleotides and electrophoretic mobility shift assay (EMSA)

The following oligonucleotide was used in this study (only the sequence of the upper strand is given): BB9 5′-TGTCCGGGCATGTCCGGGCATGTCCGGGCATGT-3′ (Halazonetis *et al*., 1993). This corresponds to DNA binding consensus sequence specific for human p53 (Hp53). Complementary oligonucleotides were hybridized and end-labelled using the Ready to Go kinase kit (Pharmacia) and ^{32}P-γ-ATP. Probes were stored at 4°C until use.

Recombinant p53 (*Xenopus* or human) was expressed in insect cells using recombinant baculovirus. Nuclear extracts containing 50% pure p53 were used for the DNA binding assay (Hardy-Bessard *et al*., 1998).

For EMSA with A6 cells, nuclear extracts were prepared as described by Andrews and Faller (1991). Cell pellets were resuspended in buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF). Cells were allowed to swell for 15 min, then NP40 was added to 0.6% and cells were vortexed to 10 min. Samples were centrifuged for 10 s. The pellet was resuspended in buffer C (20 mM HEPES-KOH pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) and incubated on ice for 20 min for high-salt extraction. After centrifugation, the supernatant fraction containing DNA binding proteins was stored at $-80°C$ until used.

EMSA was performed in a two-step procedure. In the first step, the p53 was activated with monoclonal antibody in the DNA binding buffer (10 mM HEPES, pH 8.0, 0.1 mM EDTA, 50 mM NaCl, 50 mM DTT, 4 mM spermidine, 18% glycerol, 0.05% NP40, 11 μg/ml of poly dldC). This activation reaction was performed in a volume of 20 μl for 30 min at 20°C with 1 μg of a carboxy-terminus monoclonal antibody, HR231 for human p53 and X36 for *Xenopus* p53. In the second step, 0.2 ng of labelled DNA probe was added and a second incubation for 30 min at 20°C was performed. Reaction products were loaded onto a 4% polyacrylamide gel containing 0.5 × TBE. Electrophoresis was performed for 2 h. Gels were dried and exposed to X-ray film.

Flow cytometry

Cells were pulse-labelled with 30 μM BrdU for 15 min, washed in PBS and collected by centrifugation following trypsinization. For cell cycle analysis, adherent and floating cells were resuspended in PBS and fixed with ethanol (75%) at $-20°C$. BrdU-labelled cells were detected as described by Wilson *et al*. (1985). Briefly, nuclei were isolated following treatment with pepsin (0.5% in 30 mM HCI for 20 min) and cellular DNA was partially denatured with 2N HCI or 20 min at 37°C. After extensive washing, the cells were incubated successively with rat anti-BrdU antibodies for 1 h at room temperature (RT) and with FITC-conjugated goat anti-rat IgG secondary antibody for 30 min at RT. Then they were washed again twice in PBS and stained with 25 μg/ml propidium iodide (PI) for 20 min at RT. Data were collected using a FACsort flow cytometer (Becton Dickinson & Co., San Jose, CA, USA). For apoptosis, the cell suspension (adherent and floating cells) was washed in a balanced salt solution resuspended in 70% ethanol and stored at $-20°C$ until analysis. One hour before flow cytometry analysis, the fixed cells were washed twice and incubated for 30 min at room temperature in Hank's balanced salt solution in order to allow the release of low molecular weight DNA characteristic of apoptotic cells. Cells were resuspended in PBS solution at a final concentration of 10^6 cells/ml and incubated in the presence of propidium iodide (PI) and DNase-free RNase A for 20 min at RT. The samples were analysed using a Facsort (Becton-Dickinson, San Jose, CA, USA) equipped with an argon laser working at 15 mW. Cellquest was run for data acquisition for DNA ploidy, delineation of cell cycle compartments and calculation of the mean fluorescence intensity (MFI). Tunel staining was performed using the 'DeadEnd Colorimetric Apoptosis Detection System' (Promega) according to the manufacturer's instructions.

Phosphorylation of p53

Purified DNA-PK, casein kinase 1 (CKI), casein kinase II (CKII) and protein kinase C (PKC) were purchased from Promega. The DNA-PK reaction (20 μl) was performed in a buffer containing 12.5 mM HEPES, pH 7.5, 7 mM $MgCI_2$, 10% glycerol, 0.05% NP40, 0.5 mM DTT, 25 mM KCI, 1.3 mM spermidine, 0.2 mM ATP, 200 ng of double-strand linear plasmid, 5 μCi of [γ-^{32}P]ATP and 60 U or DNA-PK for 15 min at 30°C. The CKI reaction (20 μl) was performed in a buffer containing 25 mM Tris hydrochloride, pH 7.4, 10 mM $MgCI_2$, 0.2 mM ATP with 1 μCi of [γ-^{32}P]ATP and 15 U of CKI for 15 min at 37°C. The CKII reaction (20 μl) was performed in a buffer containing 25 mM Tris, Ph 7.4, 10 mM $MgCl_2$, 200 mM NaCl, 0.2 mM ATP with 1 μCi of [γ-^{32}P]ATP and 15 U of CKII for 15 min at 37°C. The PKC reaction (20 μl) was performed in a buffer containing 20 mM Tris hydrochloride, pH 7.4, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.1 mg/ml phosphatidylserine, 1.0 μg/ml diolein, 0.2 mM ATP with 1 μCi of [γ-^{32}P]ATP and 55 mU of PKC for 30 min at 30°C.

For dephosphorylation, cellular extracts were treated with lambda phosphatase (New England Biolabs) in 50 mM Tris hydrochloride pH 7.5, 0.1 mM EDTA, 5 mM DTT, 0.01% Brij 35, 2 mM $MnCl_2$ for 30 min at 30°C.

For phosphorylation of p53 before EMSA, the protocols were adjusted in order to avoid the thermal denaturation of p53 during prolonged incubation at 30 or 37°C. Hp53 was phosphorylated for 5 min at 30°C with 20 mU of PKC and Xp53 was phosphorylated for 30 min at 20°C with 20 mU of PKC. Only unlabelled ATP was used for this assay.

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