

# Functional Interactions of p53 with Poly(ADP-ribose) Polymerase (PARP) during Apoptosis following DNA Damage: Covalent Poly(ADP-ribosylation) of p53 by Exogenous PARP and Noncovalent Binding of p53 to the $M_r$ 85,000 Proteolytic Fragment<sup>1</sup>

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## Abstract

We have examined the domain-specific interactions between p53 and poly(ADP-ribose)polymerase (PARP) (E.C. 2.4.2.30) in apoptotic HeLa cells. Apoptosis was induced by exposing cells to 50  $\mu$ M *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) for increasing lengths of time and was confirmed by: (a) oligonucleosomal fragmentation of chromatin; (b) increase in p53 levels; and (c) degradation of PARP into the characteristic  $M_r$  85,000 (COOH-terminal catalytic domain) and  $M_r$  29,000 (DNA-binding domain) peptide fragments. We also immunodetected p53 in immunoprecipitates obtained with a PARP-specific antibody. However, intact PARP coimmunoprecipitated with a p53-specific antibody during the initial 30 min of MNNG treatment. After 60 min, only the COOH-terminal fragment coimmunoprecipitated with p53, indicating that PARP noncovalently binds p53 via its  $M_r$  85,000 catalytic domain. Therefore, we next examined p53 as a covalent target for poly(ADP-ribosylation). Although p53 was not endogenously poly(ADP-ribosylated) *in situ*, incubation of cell extracts with full-length PARP from calf thymus and [<sup>32</sup>P]βNAD<sup>+</sup> resulted in its time-dependent poly(ADP-ribosylation). In summary, our results are consistent with the conclusion that PARP and p53 are activated with nonoverlapping kinetics during apoptosis.

## Introduction

It has long been known that cells of multicellular organisms have the ability to invoke a suicide program leading to cell death or apoptosis. This program is activated in response to diverse signals during normal tissue development, cellular homeostasis, and disease pathogenesis (1–4). Some of the hallmarks of apoptosis are cell shrinkage, membrane blebbing, chromosome condensation, influx of Ca<sup>2+</sup>, and internucleosomal DNA cleavage. Conditions that induce cell commitment to apoptosis include DNA damage, hyperthermia, glucocorticoids, viral infections, and withdrawal of growth factors (5). Apoptosis due to genotoxicity may be defined as a mechanism for avoiding the proliferation of genetically aberrant cells that might have sustained a high level of DNA damage.

Over the last few years, several pathways of programmed cell death have been postulated. For example, the tumor suppressor gene *p53* has been shown to activate a DNA damage-responsive pathway that also involves many other genes (6, 7). Indeed, p53-mediated apoptosis is correlated with enhanced levels of p21 (8). Interestingly, the function of p53 as a transcriptional activator may not be required for apoptosis (9). Recently, it was shown that in a cell line harboring a thermocon-

ditionally active p53 protein, UV-induced apoptosis, as judged by internucleosomal DNA cleavage, was strictly dependent on a functional p53, *e.g.*, on incubation at the permissive temperature (9). However, p53-dependent apoptosis was also easily detected in the presence of transcription and translation inhibitors (9). Thus, a p53 function other than its role in transcriptional activation may be critical for apoptosis.

The execution of the apoptotic program in cells committed to die involves the controlled degradation of cellular macromolecules by hydrolytic enzymes. Indeed, highly specific proteases that biochemically degrade crucial polypeptides play a central role in this process (10–12). These proteases have collectively been identified as caspases (cysteine, aspartic acid-specific proteases; Ref. 10) and are presently classified into 10 distinct families on the basis of their phylogenetic properties, primary structure, and substrate preference. The intracellular distribution of caspases shows that they are active in both the cytoplasm and the nucleus of apoptotic cells (12). For example, the cleavage of PARP<sup>3</sup> (EC 2.4.2.30), an abundant DNA-binding protein of the nucleus, into an  $M_r$  85,000 COOH-terminal fragment (automodification and catalytic domains) and a  $M_r$  29,000 a NH<sub>2</sub>-terminal fragment (DNA-binding domain) has been widely used as a biochemical hallmark for the execution phase of apoptosis (13–15). Indeed, proteolysis of PARP is one of the earliest detectable protein degradation events after fragmentation of high molecular weight DNA in chromatin but before internucleosomal DNA cleavage (16, 17).

Mammalian cells functionally activate two pivotal DNA-binding proteins in the nucleus after exposure to DNA-damaging agents. These proteins correspond to PARP (18–20) and p53 (21), respectively. Early in this process, PARP becomes enzymatically active upon binding to DNA strand breaks and dimerization to catalyze an intermolecular (18, 19) autopoly(ADP-ribosylation) reaction (20) in a highly processive manner (22). The intranuclear concentration of p53 (see below), on the other hand, increases in a time-dependent fashion as a result of increased genetic expression (21). Coexistence of PARP and p53 in the vicinity of DNA strand breaks (23) suggests that the DNA-binding properties and function of p53 might be regulated by reversible poly(ADP-ribosylation). Therefore, p53 needs to be examined as a potential target for poly(ADP-ribosylation) in apoptosis. Indeed, Vaziri *et al.* (24) reported recently that the posttranslational activation of p53 involves poly(ADP-ribose) polymerase activity.

## Materials and Methods

**Cell Culture.** HeLa cells (#ccl2, American Type Culture Collection) were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in DMEM (Atlanta Biologicals, Norcross, GA) supplemented with 10% fetal bovine serum (Atlanta Biologicals).

<sup>3</sup> The abbreviations used are: PARP, poly(ADP-ribose) polymerase; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; LDS, lithium dodecyl sulfate.

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**Treatment of Cultures.** MNNG (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO and added to the culture medium at a concentration of 50  $\mu\text{M}$  (25, 26). The concentration of DMSO in the medium was 0.5% (v/v). Cells were incubated at 37°C for 0, 10, 30, 60, 90, and 120 min. Subsequently, cells were lysed in a buffer containing 5 mM iodoacetamide, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride for 1 h on ice, followed by centrifugation at  $10,000 \times g$  for 30 min.

**p53 Expression.** HeLa cell nuclear extracts were resolved by SDS-PAGE, electroblotted on nitrocellulose (Millipore, Bedford, MA), and immunoprobed with a p53-specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

**Immunoprecipitation Experiments.** Endogenous p53 and PARP were immunoprecipitated from HeLa cell extracts with either anti-p53 or anti-PARP antibodies. Samples were incubated with the appropriate antibody for 1 h with gentle agitation. Immune complexes were recovered with protein A-Sepharose. Beads were added for 60 min at 4°C with constant agitation. Next, the beads were washed twice with 0.1% Triton X-100 in Tris sodium azide, once with Tris sodium azide, and once with 50 mM Tris-HCl buffer (pH 6.8). Electrophoresis loading buffer was added to dissolve protein, and the samples were then incubated at 100°C for 5 min. Immunoprecipitates were resolved by SDS-PAGE and blotted on nitrocellulose.

**Western Blotting.** Nuclear pellets and immunoprecipitated protein samples from MNNG-treated cells were fractionated by SDS-PAGE (4–15% gradient gels) according to the method of Laemmli (27), and proteins from the gel were subsequently transferred to a nitrocellulose membrane (28) using an electroblotting apparatus (Bio-Rad, Hercules, CA). The membrane was blocked with 5% nonfat milk for 1 h and subsequently incubated with a primary antibody (0.1 mg/ml for p53 and a 1:1000 dilution for the PARP antibody) for 2 h at room temperature. Unbound antibody was washed with Tris buffered saline (pH 7.2) containing 0.5% Tween 20. The membrane was then incubated with the secondary antibody (alkaline phosphatase conjugate; Sigma) for 60 min at room temperature, washed, and the enzyme activity was detected upon addition of the substrate (BCIP/NBT tablets from Sigma Chemical Co).

**Poly(ADP-ribosyl)ation of p53.** HeLa cells treated with 50  $\mu\text{M}$  MNNG for different time intervals were lysed (see below), and the nuclear fraction was incubated with or without 200 ng of pure calf thymus PARP, 20  $\mu\text{g/ml}$  DNA, and 0.4  $\mu\text{M}$  [ $^{32}\text{P}$ ] $\beta\text{NAD}^+$  at 37°C. The reaction was quenched by the addition of 2 $\times$  electrophoresis buffer containing 1% LDS, and samples were boiled for 5 min and loaded onto the gel at pH 6.8 to stabilize the monoester bonds of the poly(ADP-ribose)-protein conjugates.

## Results and Discussion

Mammalian cells respond with a plethora of biochemical adjustments immediately after exposure to DNA-damaging agents. In this

highly coordinated effort to appropriately deal with genotoxicity, cells either: (a) proceed to repair the DNA lesions via highly sophisticated DNA repair mechanisms; or (b) alternatively, when the level of DNA damage is very high, proceed to the suicidal response known as programmed cell death or apoptosis.

Two of the immediate cellular responses to DNA damage include the activation of poly(ADP-ribose) polymerase (25) as well as the time-dependent increase of p53 expression (22). However, the temporal relationship between these two biochemical events has not been examined.

To evaluate the protein-protein interactions between p53 and PARP during apoptosis induced by alkylating agents, we first measured the expression levels of p53 in HeLa cells at different times after DNA damage with 50  $\mu\text{M}$  MNNG. Fig. 1A shows that the level of p53 increased with time after exposure of HeLa cells to 50  $\mu\text{M}$  MNNG. Interestingly, the levels of p53 continued to go up after 30 min of DNA damage, the time at which the [ $^{32}\text{P}$ ] $\beta\text{NAD}^+$  levels would be expected to almost completely be depleted (26). Indeed, it has been reported previously that the highest levels of PARP activity and protein-bound poly(ADP-ribose) accumulate during the first 30 min after MNNG treatment (25, 26), therefore suggesting that PARP is activated prior to the expression of p53 following DNA damage. This interpretation of our data agrees well with the results of Whitacre *et al.* (29), who showed that p53 expression does not take place in cells deficient in either  $\beta\text{NAD}^+$  levels, PARP activity, or both (29). In fact, these deficient cell lines fail to accumulate p53 after exposure to etoposide, a topoisomerase II inhibitor (29). It should also be mentioned that p53 was detectable in control HeLa cells as well (Fig. 1A, Lane 1), although at a very low concentration.

To properly document the schedule of apoptotic events in our system, we determined the kinetics of PARP proteolysis. Fig. 1B shows the time-dependent degradation of PARP into the  $M_r$  85,000 and the  $M_r$  29,000 signature peptide fragments of apoptosis. A residual fraction of intact PARP remained after 30 min of MNNG treatment (Fig. 1B, Lanes 1–3). However, no full-length PARP was detected after 60 min of DNA damage (Fig. 1, Lanes 4–6). These results coincide with the decrease reported previously in PARP activity after 1 h of MNNG treatment (25, 26). In fact, the decrease in PARP activity preceded the onset of oligonucleosomal DNA fragmentation (see above) and p53 accumulation. Therefore, we conclude

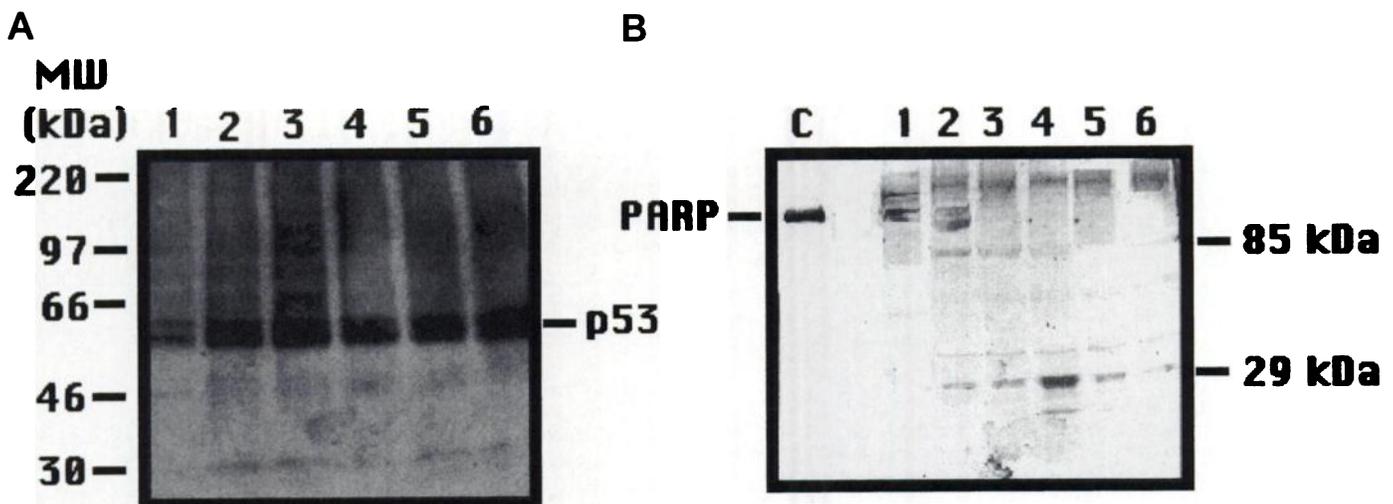


Fig. 1. Time-dependent expression of p53 (A) and proteolytic degradation of PARP (B) in HeLa cells after DNA damage with 50  $\mu\text{M}$  MNNG. In A, Lanes 1–6 show the immunodetection of p53 by Western blotting after 0, 10, 30, 60, 90, and 120 min of exposure of HeLa cells to 50  $\mu\text{M}$  MNNG. Left, the relative mobility of the molecular weight (MW; in thousands) markers. In B, Lane C shows the immunodetection of pure PARP (control). Lanes 1–6, the polypeptides immunodetected with a polyclonal antibody specific for PARP. Right, the relative electrophoretic mobility of the signature peptides of apoptosis (85 kDa,  $M_r$  85,000; 29 kDa,  $M_r$  29,000).

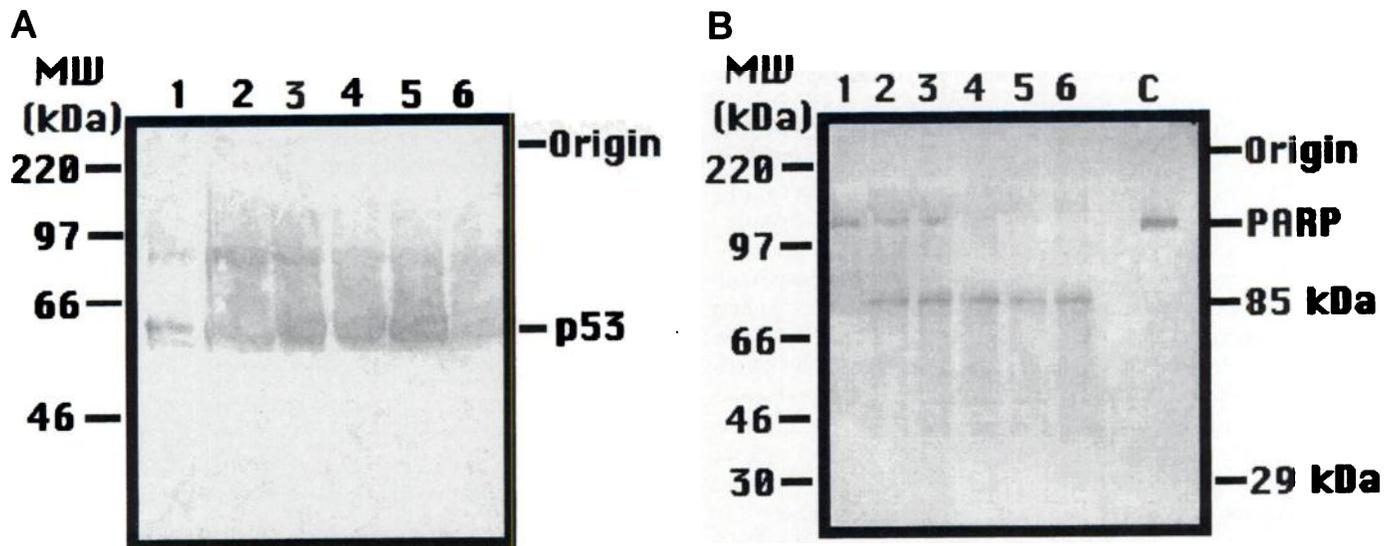


Fig. 2. Coimmunoprecipitation of p53 with a PARP-specific antibody (A) and of PARP with a p53-specific antibody (B) as a function of time after DNA damage. In A, Lanes 1–6 show the immunodetection of p53 in immunoprecipitates of HeLa cells obtained with a PARP-specific antibody after 0, 10, 30, 60, 90, and 120 min of treatment with 50  $\mu$ M MNNG. In B, Lanes 1–6 show the immunodetection of PARP and its proteolytic fragments in immunoprecipitates of HeLa cells obtained with a p53-specific antibody after 0, 10, 30, 60, 90, and 120 min of treatment with 50  $\mu$ M MNNG. Lane C, immunodetection of pure PARP (control). A and B: MW, molecular weight in thousands.

that PARP is stable during the time that the cell is attempting to repair alkylation damage and that it is proteolytically degraded when p53 triggers the execution phase of programmed cell death.

We also evaluated the integrity of chromatin DNA after MNNG treatment. After 1 or 2 h of treatment with MNNG, we observed the typical internucleosomal fragmentation of chromatin DNA (data not shown). Not surprisingly, nucleosomal chromatin degradation coincides with the highest levels of p53 observed (Fig. 1A).

In light of recent reports (23, 24) that suggest the colocalization of PARP and p53 in living cells as well as the reciprocal coprecipitation of PARP-p53 complexes by antibodies raised against either of the two partners, we proceeded to examine the domain specificity of interaction between p53 and PARP in HeLa cells as a function of time after MNNG treatment.

Immunoprecipitation studies were carried out using both anti-PARP (Fig. 2A) and anti-p53 (Fig. 2B) antibodies. HeLa cell extracts were immunoprecipitated with a polyclonal anti-PARP antibody, and samples were subsequently immunoblotted with an anti-p53 antibody. Fig. 2A shows that intact p53 was efficiently coimmunoprecipitated by an antibody specific for PARP after DNA damage at all times examined. Fig. 2B, Lane 1, shows that PARP coimmunoprecipitated with p53 when immunoprecipitation was carried out with a p53-specific antibody. Interestingly, however, after proteolytic degradation of PARP (Fig. 2B, Lanes 2–6), only the  $M_r$  85,000 COOH-terminal fragment coimmunoprecipitated with p53. These results indicated that PARP possesses a highly specific p53-binding site(s) in either the centrally located automodification domain (30, 31) or the COOH-terminal fragment (31).

The physical interaction of p53 with the catalytic domain of PARP, even in the absence of DNA damage (Fig. 2A, Lane 1, and Fig. 2B, Lane 1), strongly suggests that p53 might be a covalent target for poly(ADP-ribosylation). Indeed, it has been shown previously that a mutant of p53 can be poly(ADP-ribosylated) by pure PARP *in vitro* (23).

Therefore, we next proceeded to examine whether DNA damage-inducible p53 from HeLa cells was a target for endogenous poly(ADP-ribosylation). Unfortunately, incubation of extracts from HeLa cells, exposed to 50  $\mu$ M MNNG, with a low micromolar concentration of high specific radioactivity [ $^{32}$ P] $\beta$ NAD $^{+}$  did not result in the

radiolabeling of p53 (data not shown). By contrast, the addition of 200 ng of pure calf thymus PARP and [ $^{32}$ P] $\beta$ NAD $^{+}$  to the poly(ADP-ribosylation) mixture resulted in the time-dependent covalent poly(ADP-ribosylation) of p53 (Fig. 3, Lanes 2–6). Interestingly, the automodification of PARP was only observed in control cells (Fig. 3, Lane 1) and extracts from DNA-damaged cells after 90 and 120 min of MNNG treatment (Fig. 3, Lanes 5 and 6). Under these conditions, the spectrum of caspases that leads to PARP degradation during apoptosis is apparently not fully functional anymore. Therefore, in extracts where PARP is specifically proteolyzed by endogenous proteases, e.g., after 10, 30, and 60 min of MNNG treatment (Fig. 1B), exogenous PARP is also degraded. This is obviously not the case in control cells (Fig. 3, Lane 1) as well as 2 h after DNA damage (Fig. 3, Lane 6). Our results also indicate that constitutive p53 (Fig. 1A, Lane 1) is not poly(ADP-ribosylated) by exogenously added PARP (Fig. 3, Lane 1), presumably due to the relative absence of breaks on DNA. An alternative explanation is that endogenous PARP may not modify p53 early after DNA damage because of its highly automodi-

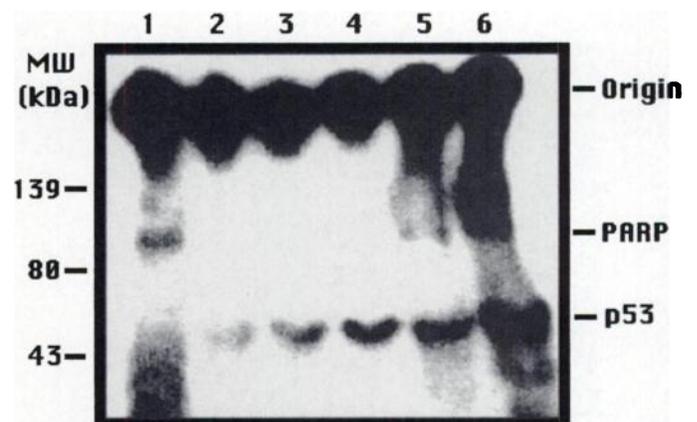


Fig. 3. Autoradiography of poly(ADP-ribosylated)-p53 from DNA-damaged HeLa cells after LDS-PAGE. Lanes 1–6, samples after 0, 10, 30, 60, 90, and 120 min of MNNG treatment incubated with 200 ng of pure PARP and 0.4  $\mu$ M [ $^{32}$ P] $\beta$ NAD $^{+}$  for 10 min at 37°C. Left, the electrophoretic mobility of the molecular weight (MW; in thousands) markers.

fied status (20) and as a result of preferential homodimerization over heterodimerization (32).

Recently, it also has been shown that p53 contains amino acid consensus sequences or "poly(ADP-ribose)" motif(s) that noncovalently bind to PARP-bound ADP-ribose polymers (33) in a highly specific manner. Therefore, it is likely that polymers covalently bound to the  $M_r$  85,000 fragment of PARP are responsible for specifically pulling down p53 (Fig. 2B) via ionic interactions and hydrogen bonding when immunoprecipitating extracts with the PARP antibody. On the other hand, it should be pointed out that nonpoly(ADP-ribosyl)ated PARP from control cells also displayed a strong binding affinity for constitutive p53 (Fig. 2A, Lane 1). Therefore, it would be safe to assume that the primary structure of the  $M_r$  85,000 COOH-terminal apoptotic fragment of PARP also contains the consensus sequence(s) that facilitates specific protein-protein interactions with p53. In fact, the p53 binding motif of PARP may overlap with the protein-protein dimerization motif located just upstream from the automodification domain (30) of this highly versatile enzyme.

It also appears logical that p53 reaches maximum intracellular levels after 60 min of MNNG treatment (Fig. 1A) when PARP has been quantitatively proteolyzed to the  $M_r$  85,000 (DNA-independent) catalytic fragment (Ref. 31; Fig. 1B). In summary, our results are consistent with the conclusion that p53-PARP interactions are highly coordinated during programmed cell death and that PARP activation and p53 expression do not overlap to avoid the poly(ADP-ribosyl)ation of p53 that might potentially interfere with the DNA-binding ability and apoptotic function of this tumor suppressor protein after MNNG treatment.

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