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ADP-Ribosylation of Wild-Type p53 *in Vitro:* Binding of p53 Protein to Specific p53 Consensus Sequence Prevents Its Modification

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We have recently reported that mutant but not wild-type (wt) p53 protein was ADP-ribosylated in primary rat cells overexpressing the temperature-sensitive murine $p53^{val135}$ gene. To examine whether the lack of susceptibility to modification is a specific feature of $p53^{val135}$ adopting wt conformation or rather a general property of this tumor suppressor protein, we have studied ADP-ribosylation of wt p53 of different origin in vitro using semi-purified poly(ADP-ribose) transferase (pADPRT). In vitro pADPRT modified human and mouse wt p53 and $p53^{val135}$. Under limiting substrate concentration, the molar mass of ADP-ribosylated p53 was only slightly altered. Chase experiments with high NAD concentration resulted in the formation of poly(ADP-ribosyl)ated p53 protein shifted to 64 kD. However, preincubation of wt p53 proteins with a p53 consensus sequence resulting in complex formation abolished the modification of wt p53. This indicates that in the cellular environment the specific DNA binding of wt p53 prevents its covalent modification by poly(ADP-ribose).

The p53 tumor suppressor gene is the most frequent target of genetic alterations in human and animal cancer (for review, see 1-3). Accumulating evidences indicate that loss of the normal wt function of the protein contributes to tumorigenesis. The wt p53, a nuclear protein with transcriptional activity, is expressed in normal cells in low amounts due to a very short half life (4). In response to DNA injury by genotoxic agents such as ionizing radiation, ultraviolet light or carcinogens (5-8) the rate of p53 protein synthesis increases and in addition p53 becomes stabilized resulting in its accumulation to high levels (5-8). Depending on the nature of damage and the affected cell type, respectively, the activated p53 protein is able either to inhibit cell proliferation (9, 10) or to induce apoptosis (11).

The activity of wt p53 protein is also regulated by post-translational modifications. p53 of mouse, rat and human origin is phosphorylated at multiple sites (12). At least five different kinases have been found to be involved in phosphorylation of p53 as deduced from several in vivo and in vitro studies (13). In general, wt p53 was shown to be hyperphosphorylated compared to several mutant forms. The role of p53 phosphorylation is not well understood. However, it has been suggested that the functional state of p53 might be modulated during the cell cycle by phosphorylation (12). It appears that not only changes in the phosphorylation level but also the sites of serine modification may have distinct effects on the growth suppressor activity of p53.

Since the complexity of DNA repair processes demands for a highly coordinated action of a pleithora of enzymes, we investigated recently whether pADPRT, a nuclear enzyme that is strongly stimulated upon DNA damage (14, 15), participates in the regulation of p53 activity. We have studied ADP-ribosylation reaction in primary rat cells overexpressing the temperature-sensitive mutant $p53^{val135}$. These cells cultivated at 37.5°C or 39.5°C express a mutant form of p53 that is localized in cytoplasm (16). After temperature shift to 32.5°C the p53 protein

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adopts wt conformation and translocates into the nucleus resulting in the arrest of the cells in G_1 phase of cell cycle. Using this cell system we found that only mutant but not wt p53 was ADP-ribosylated (17). These results were surprising for two reasons. How is the presumptively nuclear enzyme pADPRT able to modify a cytoplasmic protein and why is, on the other hand, colocalization of both proteins clearly not sufficient for modification. Immunostaining of cells grown at permissive temperature with anti-pADPRT antibodies revealed that the enzyme contrary to prediction was localized in the cytoplasm and moreover, was complexed to mutant p53. No modified wt p53 could be detected in cells maintained at 32.5°C despite spatial colocalization of both proteins. Thus, the question remained whether purified wt p53 protein can be at all ADP-ribosylated in vitro. For this purpose we have used not only $p53^{val135}$ exhibiting wt conformation but also genuine wt p53 of human and mouse origin overexpressed in a baculovirus system. We found that all tested wt p53 proteins were readily ADP-ribosylated in vitro. However, preincubation of wt p53 with the specific p53 consensus element (p53 CON) resulting in complex formation between DNA and p53, abolished very efficiently its modification. The protective effect of p53 CON was specific since its replacement by a non-specific oligonucleotide was not able to prevent ADP-ribosylation of wt p53 protein in vitro.

MATERIALS AND METHODS

Materials. [α-adenylate]-^{32}P-NAD (30 Ci/mmol) was purchased from New England Nuclear (NEN). Monoclonal anti-p53 antibody Ab-1 (PAb 421) was obtained from Oncogenic Science Inc., Cambridge, MA. 24-mer representing the p53 consensus sequence (18,19) and non-specific oligonucleotide were synthesized and purified from shorter fragments on gels.

Cell cultivation. Freshly isolated primary Fisher rat embryo cells (REC) were transfected with plasmid pLTRp53cGval135 (16) comprising a chimera of mouse p53 cDNA and genomic DNA (gift of Dr. M. Oren) and with plasmid pVV2, bearing the neo selective marker (20). The cells were cultivated in selective medium as previously described in detail (21). The established clones were maintained at 37.5°C. For some experiments cells were shifted to 32.5°C or to 39.5°C. 36 h after shift to 32.5°C about 90 % of cell population was arrested at G_1 phase as determined by FACS analysis (not shown).

Insect Sf9 cells growing in TNM-FH medium (Sigma Chemical Co., St. Louis, MO) were transfected with recombinant baculovirus expressing human pADPRT (gift of Dr. G. de Murcia) (22), human wt p53 (gift of Dr. S. A. Maxwell) or mouse wt p53 (gift of Dr.D. Lane). 70 h post transfection cells were collected and used for lysis (23).

Cell lysis. The rat cells maintained at 32.5°C were harvested, washed with ice-cold phosphate-buffered saline and lysed in RIPA buffer [50 mM Tris/HCl (pH 7.4), 500 mM NaCl, 1 % Nonidet-P40, 0.5 % Na-deoxycholate, 0.1 % SDS, 0.05 % NaN_3, 1 mM PMSF] for 30 min at +4°C. The lysate was centrifuged at 5,000 rpm for 30 min and clear supernatant was used for further purification.

Purification of pADPRT. The human recombinant pADPRT overexpressed in insect Sf9 cells was purified according to the protocol of Giner et al (22).

Chromatographic purification of wt p53 proteins. Sf9 cell lysates containing the recombinant human or murine wt p53 were fractionated by affinity chromatography on Heparin-Sepharose as previously described (23) and wt $p53^{val135}$ protein was purified on DNA-Agarose (23).

ADP-ribosylation in vitro. Purified p53 protein and pADPRT were incubated in buffer containing 0.05 μM ^{32}P-NAD, 100 mM Tris/HCl (pH 7.8), 10 mM $MgCl_2$, 1mM DTT, 4 μg sonicated DNA for 30 min at 37°C. The reaction was terminated by transfer of the tubes to an ice-water bath and addition of 3-aminobenzamide (3-AB) to a final concentration of 5 mM. Then unlabeled NAD was added (C_E = 5 mM) and the samples were cold ethanol precipitated. In some experiments after incubation with radioactively labeled substrate, cold NAD was added (chase) and after 3 minutes reaction was stopped with 3-AB. The pelleted samples were dissolved in RIPA buffer and used directly for electrophoretic analysis or for immunoprecipitation that was performed according to a standard protocol (17) using the PAb 421.

RESULTS AND DISCUSSION

The aim of this study was to investigate whether wt p53 is susceptible to modification by ADP-ribosylation in vitro, and therefore it was important to test not only conformational wt $p53^{val\ 135}$ but also genuine wt p53. To obtain sufficient amounts of the proteins, we have overexpressed human and mouse wt p53 in insect cells and the recombinant proteins purified chromatographically on Heparin-Sepharose. Since $p53^{val\ 135}$ in wt conformation poorly binds to Heparin-

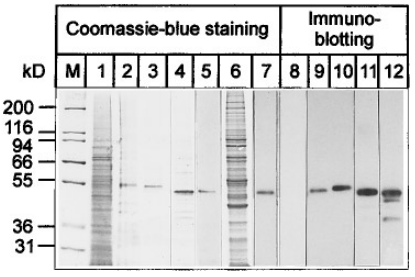

FIG. 1. Analysis of purified p53 proteins. Cell lysates and isolated proteins were separated on 10 % SDS slab gels. Proteins were Coomassie-blue stained or transferred onto nitrocellulose for immunoblotting using anti-p53 antibodies. Detection of immune complexes was performed with ECL system (Amersham) Lanes **1** and **8,** control lysat of insect cells; **2** and **9,** lysat of insect cells transfected with baculovirus expressing human wt p53; **3** and **10,** purified human wt p53; **4,** lysat of insect cells transfected with baculovirus expressing mouse wt p53; **5** and **11,** isolated mouse wt p53; **6,** lysat of rat cells expressing $p53^{val135}$; **7** and **12,** purified $p53^{val135}$.

Sepharose, separation on DNA-Agarose column was performed. As shown in Fig. 1 (lanes 2 and 4) cell lysates were highly enriched in p53 proteins migrating nearly to the position of 55 kD. Following chromatographic purification of p53 enriched lysates a single polypeptide at 53 kD was isolated. To ensure that chromatographically purified proteins represent p53 from different species, immunoblotting tests were performed using specific anti-p53 antibodies recognizing both human and murine p53. The immunostained bands exactly coincided with the proteins formerly visualized by Coomassie-blue staining. Human p53 protein migrated slightly slower than its murine counterparts. Moreover, comparison of the immunoreactivity between crude extract and isolated human recombinant p53 protein shows high enrichment of p53 (lanes 9 and 10). Control extract from non-transfected insect cells (lanes 1 and 8) did not react with anti-p53 antibodies. Then the purified wt p53 proteins were subjected to ADP-ribosylation reaction. The

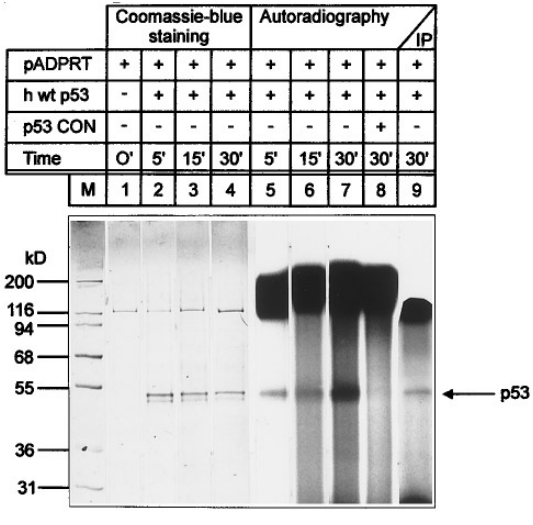

FIG. 2. ADP-ribosylation of human wt p53. 2 μg of isolated human pADPRT (lane **1**) was incubated with 2 μg h wt p53 in presence of ^{32}P-NAD for 5, 15 or 30 min. After ethanol precipitation samples were resolved on 10% SDS-gels, stained, dried and exposed to Kodak film. In some experiments samples after incubation with ^{32}P-NAD were used for immunoprecipitation using anti-p53 Ab and then analysed. The sample shown in lane **9** was from another gel run.

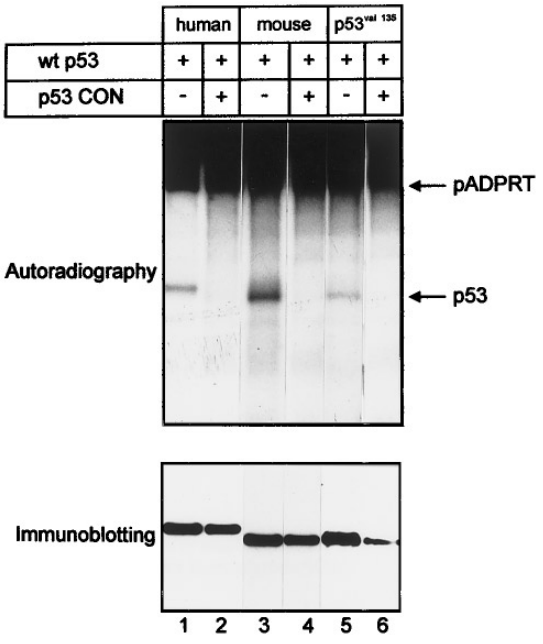

FIG. 3. Effect of preincubation of p53 proteins with p53 CON on their ADP-ribosylation. p53 of different origin (2-3 μg) was preincubated with p53 CON (2 μg) or with buffer alone for 30 min and then used for reaction with pADPRT (1 μg) for 20 min. The samples were electrophoretically separated, transferred onto nitrocellulose and exposed to Kodak X-Omat film. Then the immunoblotting with anti-p53 antibodies was performed.

incubation of human wt p53 with human pADPRT in the presence of low concentration of ^{32}P-labeled substrate resulted in the modification of p53 protein (Fig. 2). The reaction was rapid and time-dependent: longer incubation lead to higher incorporation of radioactively labeled substrate. Already after 5 min incubation a double p53 band appeared that was visualized by Coomassie-blue staining: one band migrating nearly to the position of unmodified p53 and a second slightly slower. The upper band exactly coincided in autoradiogram with the radioactive band. After 30 min of reaction the intensity of the lower band diminished indicating that larger amounts of p53 underwent the modification and were shifted in size. This correlated very well with increase of incorporation of radioactive ADP-ribose into p53 molecules. Interestingly, after longer incubation the size of modified p53 remained the same suggesting that under limiting substrate concentration p53 protein was modified by ADP-ribose chains of constant length. The molecular mass of modified p53 protein determined by densitometry was estimated as 55 kD implicating that about 4 ADP-ribose moieties were covalently coupled to target protein. To unequivocally prove the identity of the radioactively labeled protein, immunoprecipitation with anti-p53 antibodies was performed. Analysis of the immune complexes (Fig. 2, lane 9) revealed the radioactive band migrating to the same position as in the original sample. Furthermore, the heavily labeled band representing self-modified pADPRT was also immunoprecipitated due to complex formation between pADPRT and p53 protein. In the next experiments we have tested whether murine wt p53 proteins may also serve as targets for ADP-ribosylation. The incubation with the enzyme pADPRT under previously established conditions lead to the formation of ADP-ribosylated murine p53 as well as p53^{val135} derivatives (Fig. 3, lanes 1, 3 and 5). Regarding the modification of the latter in vitro, these results differed substantially from those obtained in vivo (17). Since p53^{val135} in cells maintained at non-permissive temperature adopts wt conformation and exerts its regulative function by specific DNA-binding, it was possible that under physiological condi-

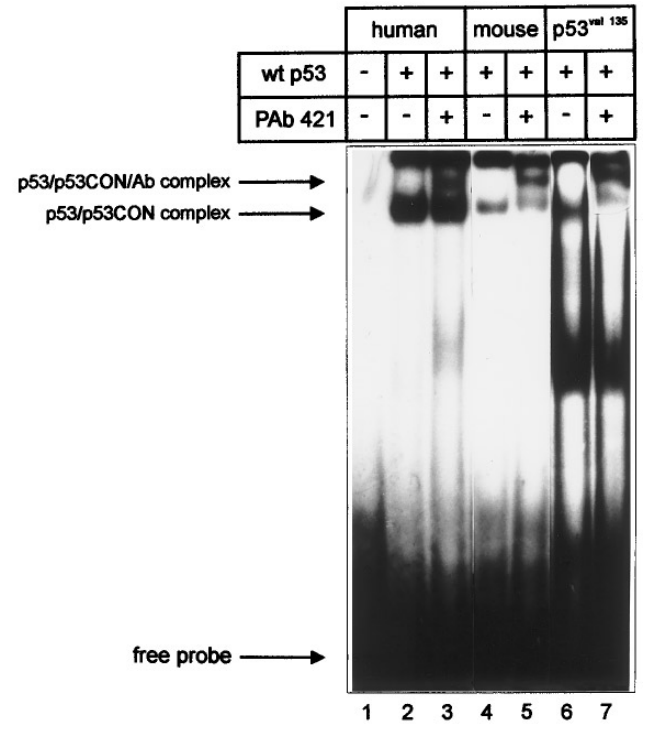

FIG. 4. The DNA-binding ability of isolated wt p53 of different origin. 1 μg of purified p53 protein was mixed with 2 ng of ^{32}P-end labeled CON p53 oligonucleotide. After a 30 min incubation, the mixture was run on a 7 % TBE polyacrylamide gel at 20 mA. When indicated, 100 ng of anti-p53 antibody PAb 421 was added. The gel was then dried and exposed to Kodak X-Omat film for 2 days.

tions wt p53 was complexed with DNA and therefore was not capable to serve as an acceptor of ADP-ribosylation. To prove this possibility, we preincubated wt p53 proteins with specific p53 CON followed by ADP-ribosylation reaction. We used mobility shift assay to examine whether the chromatographically purified wt p53 proteins bind to the specific p53 binding DNA elements. The data in Fig. 4 show that the purification did not impair their binding capability to p53 CON. The specific complexes between human, mouse and p53^{val135} protein were super-shifted upon addition of anti-p53 antibodies PAb 421 (lanes 3, 5 and 7). Interestingly, the preincubation of wt p53 proteins with p53 CON completely abolished their ADP-ribosylation (Fig. 2, lane 8; Fig. 3, lanes 2, 4 and 6). On the other hand, the replacement of the latter by the non-specific oligonucleotide did not interfere with the modification of p53. The presence of p53 CON did not affect the enzymatic activity of pADPRT, since the intensity of radioactive band representing self-modified pADPRT was not altered. To ensure that lack of p53 modification was due to complex formation with p53 CON and not to lower amounts of p53 protein in the assays, the samples immobilized on membrane were submitted for immunoblotting with anti-p53 antibodies. The data show that comparable amounts of p53 were applied in both assays, thereby confirming the blocking effect of p53 CON. Finally, we have addressed the question as to what extent substrate concentration may affect the length of ADP-ribose chains attached to p53 protein. For this purpose we carried out the standard reaction and thereafter added high concentration of cold NAD for 3 min. The following analysis revealed presence of the additional ADP-ribosylated form of p53 migrating to the position of 64 kD (Fig. 5).

Our experiments show that wt p53 proteins from different species are readily ADP-ribosy-

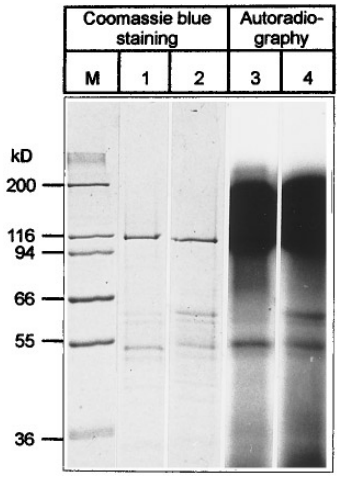

FIG. 5. Chase experiment. Human wt p53 protein was incubated with low concentration of ^{32}P-NAD in the presence of pADPRT for 10 min (lanes **1** and **3**). Then cold NAD was added to a final concentration of 5 mM and after 3 min reaction was stopped with 3-AB (lanes **2** and **4**). The samples were analysed by SDS-PAGE on a 10 % slab gel followed by autoradiography.

lated in vitro. Furthermore, our results clearly demonstrate that the preincubation of p53 proteins with the p53 consensus element resulting in the complex formation between p53 and DNA completely abolished their modification thereby indicating that under physiological conditions the state of p53 would determine its capability for ADP-ribosylation.

It is open for speculation and further experiments, how this novel modification of wt p53 protein affects its stability and/or biological activity. The crystal structure analysis of DNA-binding and tetramerization domain of p53 revealed a highly dynamic conformational structure extremely prone to alterations of the folding state (24). Not only a multitude of individual amino acids substitutions but also temperature or presence of metal chelating agents can exert a destabilizing influence. Moreover, it was shown that the nascent form of normal p53 adopts transiently ''mutant'' character (25). Therefore it is conceivable that ADP-ribosylation could modulate the balance between folded and unfolded species. It was reported recently that pADPRT-deficient cell lines showing reduced baseline levels of p53 failed to accumulate p53 in response to etoposide VP-16 treatment and showed subsequent reduction of VP-16 induced apoptosis (26).

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