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ADP-Ribosylation of p53 Tumor Suppressor Protein: Mutant but not Wild-Type p53 Is Modified

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Abstract Poly(ADP-ribosyl)ation of mutant and wild-type p53 was studied in transformed and nontransformed rat cell lines constitutively expressing the temperature-sensitive $p53^{135val}$. It was found that in both cell types at 37.5°C, where overexpressed p53 exhibits mutant conformation and cytoplasmic localization, a considerable part of the protein was poly(ADP-ribosyl)ated. Using densitometric scanning, the molecular mass of the modified protein was estimated as 64 kD. Immunofluorescence studies with affinity purified anti-poly(ADP-ribose) transferase (pADPRT) antibodies revealed that, contrary to predictions, the active enzyme was located in the cytoplasm, while in nuclei chromatin was depleted of pADPRT. A distinct intracellular localization and action of pADPRT was found in the cell lines cultivated at 32.5°C, where p53 adopts wild-type form. Despite nuclear coexistence of both proteins no significant modification of p53 was found. Since the strikingly shared compartmentalization of p53 and pADPRT was indicative of possible complex formation between the two proteins, reciprocal immunoprecipitation and immunoblotting were performed with anti-p53 and anti-pADPRT antibodies. A poly(ADP-ribosyl)ated protein of 116 kD constantly precipitated at stringent conditions was identified as the automodified enzyme. It is concluded that mutant cytoplasmic p53 is tighly complexed to pADPRT and becomes modified. At 32.5°C binding to DNA of p53 or its temperature-dependent conformational alteration might prevent an analogous modification of the tumor suppressor protein.

Key words: p53 protein, ADP-ribosylation, rat cells, tumor suppressor protein

INTRODUCTION

The p53 tumor suppressor protein is a 53 kD nuclear protein that in normal cells is expressed in very low amounts due to extremely short half-life [Reich et al., 1984]. The protein p53 is activated in response to a variety of DNA-damaging agents, such as ultraviolet light, ionizing radiation or alkylating agents [Fritsche et al., 1993; Kastan et al., 1991]. p53 becomes stabilized and accumulates to high concentration. The accumulation of wild-type p53 results in inhibition of cell growth or induction of apoptosis thereby preventing the proliferation of damaged cells and allowing repair of damaged DNA [Eliyahu et al., 1994; Lin et al., 1992; Yeargin et al., 1995; Zhan et al., 1993]. Mutations of the p53 gene result in conformational changes in the protein and loss of wild-type p53 suppressor function [Legros et al., 1994; Zambetti, 1992]. In more than 50% of human tumors the gene is lost or mutated [Hollstein et al., 1991] [for review, see Cox et al., 1995b; Donehower et al., 1993].

Wild-type p53 protein levels may vary throughout the cell cycle and its activity as a growth suppressor may also be regulated by post-translational modifications. The phosphorylation is the most extensively studied modification of p53 protein. p53 is phosphorylated in vivo at multiple serine residues in the amino- and carboxy-terminal regions. It can be phosphorylated by several different protein kinases. Wild-type p53 has been shown to be hyperphosphorylated compared to mutant p53. There is evidence that changes in p53 phosphorylation may have distinct effects on the function of p53 [reviewed in Ullrich et al., 1992].

In response to DNA damage by genotoxic agents or by endogenous physiologic events another nuclear protein, poly(ADP-ribose)transferase (pADPRT) is strongly activated [reviewed in Berger, 1985; Boulikas, 1991; Shall, 1982; Ueda et al., 1985]. This enzyme utilizing NAD as substrate catalyzes transfer of the ADP-ribose moieties to covalent linkages with various acceptor proteins. Modification of proteins by ADP-ribosylation results in the alteration of their

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charge and also of their properties. The activity of several enzymes involved in breaking and rejoining of DNA strands such as topoisomerases I and II and ligase II is inhibited following their poly(ADP-ribosylation) [Darby et al., 1985; Ferro et al., 1983; Jongstra-Bilen et al., 1983; Yoshihara et al., 1985]. pADPRT activity mostly depends on the presence of DNA. The transferase molecule is inactive unless single- or double-strand breaks are present. Introduction of DNA strand breaks strongly induces the activity of pADPRT [Gradwohl et al., 1990]. The mechanism of stimulation by DNA free ends has not yet been fully understood, but it certainly involves the binding of pADPRT to DNA and creation of its active conformation [de Murcia et al., 1991]. Recently, a detailed analysis of nucleolar pADPRT [Leitinger et al., 1993] revealed the preferential association of the enzyme with nucleolar structures devoid of DNA [Mosgoeller et al., 1996]. Considering the involvement of pADPRT in the recognition of DNA strand breaks and the simultaneous activation of p53 following DNA injury we have addressed the question whether these two processes may be linked.

For biochemical studies of p53 it is desirable to dispose of a system allowing its expression at high level and with authentic post-translational modification. However, constitutive overexpression of the wild-type p53 in mammalian cells leads to growth arrest or even apoptosis. To overcome this problem, a temperature-sensitive mutant of p53 has been employed. A particular p53 mutant containing valine instead of alanine at position 135, one of the most extensively studied p53 mutants [Eliyahu et al., 1985; Michalovitz et al., 1990] exhibits a temperature-sensitive behaviour. Like other p53 mutants, it acts as an oncogene at 37.5°C. In contrast, at 32.5°C it acquires wild-type conformation and inhibits proliferation of transfected cells. We studied the ADP-ribosylation of mutant and wild-type p53 in primary rat embryo cells transfected with the mutant $p53^{135val}$ gene alone or in combination with an activated c-Ha-ras oncogene. This allowed in addition a comparative analysis of transformed versus nontransformed phenotype.

MATERIALS AND METHODS

Materials

[adenylate-^{32}P]-NAD-(19–50 Ci/mmol) was purchased from New England Nuclear (NEN), 3-aminobenzamide (3-AB) was from Sigma.

Antibodies

Monoclonal anti-p53 antibodies were obtained from Oncogenic Science Inc., Cambridge, MA (Ab-1, Ab-3, and Ab-4) and rabbit polyclonal anti-p53 antibody (CM-1) were obtained from Medac GmbH, Hamburg, Germany. PAb 421 [Ab-1] and CM-1 recognize both wild-type and mutated form of p53 protein. PAb 240 [Ab-3] and PAb 246 [Ab-4] react under native conditions with mutated or wild-type p53, respectively.

Monoclonal anti-lamin A/B/C antibodies (41CC4) established by Dr. B. Burke and characterized [Burke et al., 1983] were kindly provided by Dr. W.J. van Venrooij. Specific anti-pADPRT antibodies were isolated from a human serum. The serum was obtained from a patient, who following an allogeneic bone marrow transplantation (BMT) developed clinical complications defined as graft-versus-host disease. While sera obtained pre-BMT and 1 or 2 months after transplantation were negative for anti-nuclear antibodies, specimen obtained at day 100, and thereafter revealed a strong nuclear staining. In this serum, which displayed a homogenous nuclear and strong nucleolar staining pattern in indirect immunofluorescence, anti-pADPRT and anti-histone H1 autoantibodies were detected. To purify specific anti-pADPRT antibodies we performed affinity chromatography using human pADPRT [Giner et al., 1992] immobilized on agarose beads as described in detail previously [Mosgoeller et al., 1996]. Specific second antibodies and western blotting detection reagents (ECL kit) were from Amersham International, Little Chalfont, England.

Isolation and Culture of Rat Embryo Cells

The isolation of primary Fisher rat embryo cells (REC) has been described previously [Cerni et al., 1990]. Briefly, 15.5 gestation old embryos were dissociated into single cell suspension by fractionated trypsinization and plated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Flow) to obtain cell layers of 50% confluence the next day. Medium was changed, and after 4 hours, Ca^{++}-precipitated DNA was added (5 or 6 μg per 6 cm plate). Twenty hours later, cells were replated at 3×10^5 per Petri dish. Geneticin (G418; Gibco) was added at 200 μg/ml 3 days later when cells were still in growing condition. The selective medium was changed twice a week. G418 resistant colonies were isolated by means

of steel cylinders and further propagated in selection medium at 1:3 to 1:8 transfer schedule depending on the growth properties of the cell population. Clones were considered established after 25 population doublings of the founder cell. Cells were cultivated at 37.5°C. For some experiments cells were shifted to 32.5°C. 36 h after shift to 32.5°C 88% of the cell population was arrested at G_1 phase as determined by FACS analysis.

Plasmids

pLTRp53cGval135 comprising a chimera of mouse p53 cDNA and genomic DNA (generous gift of M. Oren) has been previously referred to as pLTRp53cG [Eliyahu et al., 1985]. It encodes a mutant protein with a substitution from alanine to valine at position 135. Plasmid pVV2, bearing the neo selective marker was previously described [Meneguzzi et al., 1984]. Plasmid pVEJB codes for a mutated c-Ha-ras gene [Land et al., 1983] cloned into pVV2 as described previously [Cerni et al., 1990].

Indirect Immunofluorescence Microscopy

For light microscopic investigation cells were grown on coverslips, rinsed with PBS, fixed in ice-cold methanol/acetone (3:7) for 5 min, air dried and extracted in 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 10 min at room temperature (RT), washed three times with PBS, and saturated with 1% bovine serum albumin (BSA) in PBS for 1 h. Then the cells were PBS washed, incubated with primary antibodies at appropriate dilution for 1 h at RT. After three washes in PBS and incubation with biotinylated second antibodies (Amersham International, Little Chalfont, England) at a dilution 1:500 for 1 h, the coverslips were washed again, and incubated with streptavidin-fluorescein conjugate diluted 1:100 for 30 min. Finally the coverslips were PBS washed and mounted with anti-fade medium. Immunofluorescence signal was detected by conventional fluorescence microscope. For visualization of nuclei, cells were additionally stained for 10 min with diamidinophenylindole (DAPI; 2 μg/ml in PBS).

Cell Lysis

The cells maintained at 32.5°C and at 37.5°C were harvested and washed with ice-cold PBS three times and lysed in RIPA buffer [50 mM Tris/HCl (pH 7.4), 500 mM NaCl, 1% NP-40, 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 analysis.

Determination of Protein Content

Protein concentration was determined by the DC Bio-Rad protein assay using bovine serum albumin as standard.

Labeling of ADP-Ribosylated Proteins

To avoid proteolytic degradation of proteins all steps were performed in the presence of protease inhibitors. Cells grown at 32.5°C and at 37.5°C were harvested and washed three times in PBS. The cells were suspended in reticulocyte suspension buffer (RSB) [10 mM Tris/HCl (pH 7.4), 10 mM NaCl, 1.5 mM $MgCl_2$] containing 0.15% sodium deoxycholate and 0.3% NP-40 and incubated until 90% of the cells became permeable as monitored by Trypan blue staining. For labeling, the permeabilized cells were incubated with 100 μM ^{32}P-NAD, 100 mM Tris/HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF at 25°C for 20 min. The reaction was stopped by transferring the tube to ice water. Then the permeabilized cells were pelleted and washed with the incubation buffer except that the ^{32}P-NAD was omitted and unlabeled NAD was added to a final concentration of 5 mM [Węsierska-Gądek et al., 1985]. The cells were subsequently lysed in RIPA buffer and submitted for immunoprecipitation.

Immunoprecipitation

The samples diluted with buffer A [50 mM Tris/HCl (pH 7.4), 1 mM EDTA, 0.1% NaN_3, 1 mM PMSF] to a final concentration of 150 mM NaCl were incubated with corresponding antibodies at appropriate concentration for 1 h at room temperature. Then 100 μl of prewashed Protein-G-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) was added and incubation was carried on for 2 h at room temperature. Afterwards supernatant was discarded and Protein G-Sepharose was washed four times with 1% NP-40, 0.5% sodium deoxycholate in buffer A. Immune complexes were twice eluted with SDS sample buffer and were analysed.

Removal of Covalently Attached ADP-Ribose Chains

Cell lysates were incubated with snake venome phosphodiesterase (PDE I), immobilized on agarose beads (Pierce, Rockford, IL) in buffer containing 50 mM Tris/HCl (pH 8.9), 4 M urea,

2 mM NaCN, 2 mM PMSF at 37°C for 4 and 9 h. In second approach the cell lysates were incubated in the presence of 100 mM Tris/HCl (pH 10.5) and 10 mM $MgCl_2$ at 37°C for 2 h.

Electrophoretic Separation of Proteins

Proteins were separated by one-dimensional polyacrylamide gel electrophoresis (PAGE) on 10% SDS slab gels as described by Laemmli [1970]. Proteins dissolved in SDS sample buffer containing 50 mM DTT were boiled for 3 min and immediately cooled. Then 1 μl of 2.6 M DTT pro 100 μl sample was added. After electrophoretic separation, proteins were visualized by Coomassie Blue R-250 staining. Labeled ADP-ribosylated proteins were detected by autoradiography using X-Omat S film (Eastman Kodak Co., Rochester, NY).

Immunoblotting

Proteins separated on slab gels were electrophoretically transferred onto nitrocellulose (Bio-Rad Laboratories, Richmond, CA) and stained by Ponceau S. The blots were saturated with 5% nonfat milk powder in PBS, then probed with specific primary antibodies at appropriate concentration, extensively washed with 0.2% Tween-20 in PBS and incubated with second antibody covalently linked to horseradish peroxidase (HRP) at a dilution of 1:1,500. The reactive antigens were detected by HRP/hydrogen peroxide catalyzed oxidation of luminol under alkaline conditions using ECL western blotting detection reagents (Amersham International, Little Chalfont, England) and X-Omat S film.

RESULTS

A characteristic feature of the mutant $p53^{135val}$ protein is a temperature-dependent alteration of conformation accompanied by a distinct cellular compartmentalization. To confirm this temperature-sensitive behaviour and to examine the kinetic of protein translocation in our transfected nontransformed and transformed REC cell lines, we first performed immunofluorescence microscopy. Various monoclonal anti-p53 antibodies were used that allow to discriminate between mutant and wild-type conformation. PAb 421 (Ab-1) directed against an epitope located in the carboxy-terminus is known to react with either wild-type and mutant p53. Two other antibodies, PAb 240 (Ab-3) and PAb 246 (Ab-4), have been described to recognize under native conditions exclusively mutant or wild-type p53, respectively. As expected, at 32.5°C where $p53^{135val}$ assumed wild-type configuration, the protein was located in the nuclei of the growth arrested cells (Fig. 1a), whereas at 37.5°C the mutant form of p53 accumulated in the cytoplasm of the growing cells (Fig. 1b). Corresponding results were achieved with the wild-type or mutant-specific antibodies, respectively. The temperature-dependent translocation from the cytoplasm into the nucleus occured in our cell system about 24 h after temperature shift. 36 h after shift to 32.5°C about 90% of cellular p53 had wild-type form and only negligible amounts of mutant form were found as determined by immunoprecipitation (data not shown). Therefore, for temperature shift experiments cells were maintained for at least 36 h at the indicated temperatures.

In order to label ADP-ribosylated proteins, cell membranes were made permeable for the radioactive precursor since exogenous NAD can not enter the cell interior. Incubation of our cell lines in cold hypotonic buffer for 1 hour was not sufficient for their permeabilization. During this exposure only a small portion of cells became permeable. However, effective permeabilization was achieved upon addition of low concentration of detergents (to a final concentration of 0.3% NP-40 and 0.15% Na-deoxycholate). By this supplementation about 90% cells were permeabilized within 25 min as evidenced by Trypan blue staining. The cells were then labeled with ^{32}P-NAD for 20 min and free radioactive precursor was carefully removed. Cells were lysed and clarified lysates were submitted to immunoprecipitation using specific monoclonal anti-p53 antibodies. Analysis of immune complexes originating from transformed $p53^{135val}$ + ras cells grown at 37.5°C revealed the presence of two radioactively labeled protein bands of about 60 kD and 120 kD (Fig. 2, right panel). In control assays, in which the anti-p53 antibodies were omitted or preadsorbed with purified p53 no labeled proteins were precipitated. This suggested that the strong radioactive band at approximately 60 kD represents the ADP-ribosylated form of p53 protein. This finding was further evidenced by immunoblotting with anti-p53 antibodies (Fig. 3).

The increase of molecular weight of radioactively labeled p53 was obviously due to the presence of the covalently attached ADP-ribose chain. Interestingly, the p53 was modified by ADP-ribose chains of defined length since only one radioactive derivate was detected that migrated slower than the unmodified p53 (Fig. 3). Using

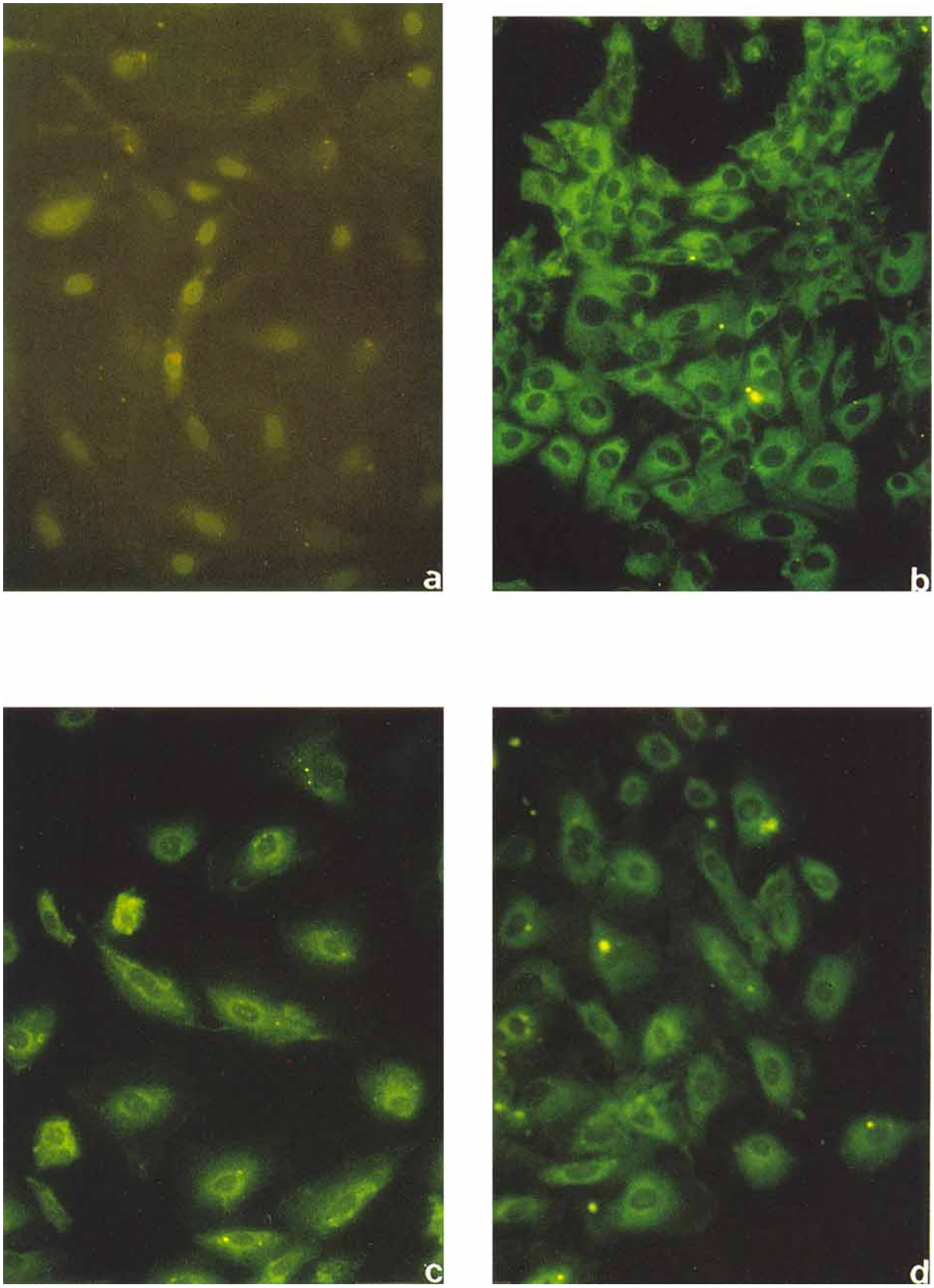

Fig. 1. Immunostaining of mutant p53 and pADPRT translocation in response to temperature-shift. Cells grown at 37.5°C (**b,d,f,h**) and shifted to 32°C for 36 h (**a,c,e,g**) were stained with anti-p53 Ab (Ab-1 at a dilution 1:100) (a and b) or with anti-pADPRT Ab (affinity purified anti-pADPRT Ab at a dilution 1:150) (c and d), with monoclonal anti-lamin A/B/C Ab (41CC4 at a dilution 1:100) (e, f), and with DAPI (g, h). Panels e, g and f, g represent the same cells that were double stained.

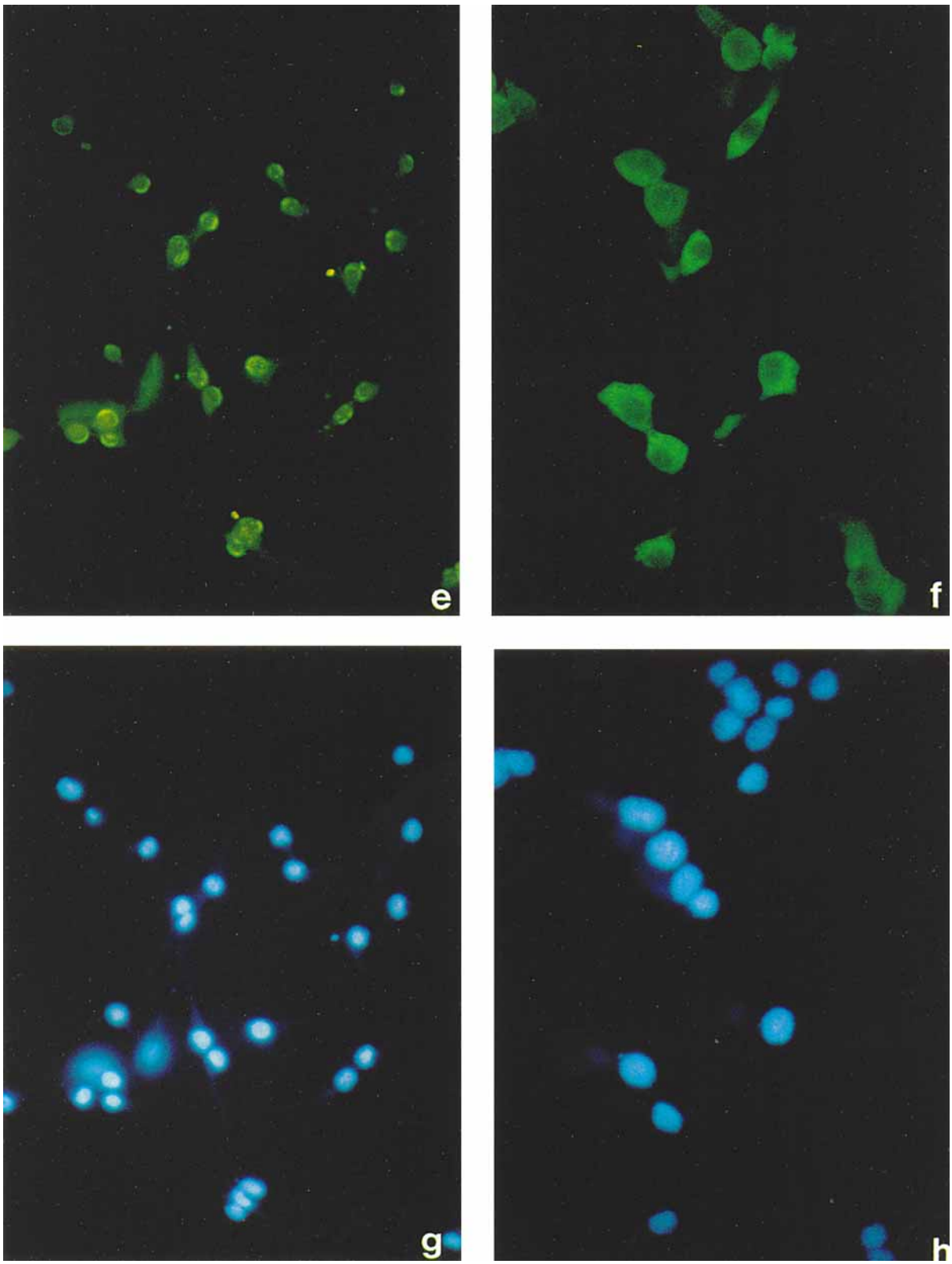

Figure 1. (Continued.)

densitometric scanning, the molecular mass of the ADP-ribosylated p53 form was calculated as 64 kD. From the increase of molecular mass by 11 kD it was deduced that the covalently attached chains consisted of about 20 ADP-ribose residues. Since the intensity of the immunostained protein band at 64 kD was lower than that corresponding to the unmodified p53 protein (Fig. 3), it was concluded that a part of the overexpressed p53 protein was modified.

The immunoprecipitation of lysates from nontransformed cells expressing only $p53^{135val}$ construct resulted in a very similar pattern of ADP-ribosylated proteins (Fig. 2, left panel).

However, a different pattern of ADP-ribosylated proteins was observed after precipitation of lysates from transformed and nontransformed cells maintained at 32.5°C. No modified p53 could be detected in cells transfected with $p53^{135val}$ construct alone and only trace amounts of ADP-ribosylated p53 were found after long exposure of samples from cells transfected with $p53^{135val}$ + ras. The only ADP-ribosylated protein precipitated with specific anti-p53 antibodies, was the approximately 120 kD protein band of unknown identity, which already had been detected in corresponding 37.5°C cultivated cells.

These results indicate that p53 protein was ADP-ribosylated in cells exclusively expressing mutant p53. However, in cells expressing predominantly p53 in wild-type configuration, no modification or very small amounts of ADP-ribosylated p53 protein were detected. The latter was found in transformed $p53^{135val}$ + ras cells and was presumably due to the presence of negligible amounts of mutant p53. Thus, these results clearly demonstrate that mutant but not wild-type p53 is modified by ADP-ribose chains. This enzymatic reaction did not depend on the temperature of cell cultivation because in cells maintained at 32.5°C small amounts of p53 probably representing mutant form were modified. Furthermore, in both cell lines maintained at permissive and restrictive temperature the enzyme catalyzing the ADP-ribosylation reaction was active since a variety of ADP-ribosylated proteins was observed in total cell lysates (Table I).

Considering the temperature-dependent compartmentalization of p53 protein on the one hand and the primarily nuclear localization of the pADPRT on the other hand, it was surprising that mutant p53 protein exclusively accumulated in the cytoplasm was ADP-ribosylated. This was in contrast to transformed and nontransformed cells maintained at 32.5°C where both the modifying enzyme and the putative target protein were assumed to colocalize in the nucleus. To approach the question how supposedly nuclear pADPRT was able to modify mutant p53 localized in the cytoplasm, we examined the intracellular distribution of pADPRT in proliferating and growth arrested cells. Surprisingly, in proliferating cells at 37.5°C affinity purified anti-pADPRT antibodies heavily stained the cytoplasm and nucleoli while the nucleoplasm remained negative. The cytoplasmic accumulation of pADPRT resembled that of mutant p53 (Fig. 1d). In contrast, in growth arrested cells maintained at 32.5°C the anti-pADPRT antibodies stained the nuclei with strong accentuation of the nuclear periphery (Fig. 1c) similarly to staining pattern observed with anti-lamin A/B/C antibodies (Fig. 1e). The nucleoli were only occasionally stained presumably due to their decreased activity in growth arrested cells. These results demonstrated a spatial colocalization of p53 protein and pADPRT and nicely explained how mutant p53 accumulated in the cytoplasm could act as target for pADPRT catalyzed modification.

Since the unidentified protein of about 120 kD was constantly ADP-ribosylated in all our conditions tested and since its molecular mass coincided with that of the pADPRT, it appeared possible that it could represent the selfmodified enzyme. To prove this possibility, reciprocal immunoprecipitation experiments were performed. Coimmunoprecipitation of two proteins can be due to either shared epitopes or a physical association of the proteins. Radioactively labeled extracts prepared from cells either maintained at 32.5°C and 37.5°C were subjected to immunoprecipitation with either anti-p53 or anti-pADPRT antibodies. Proteins precipitated by both antibodies were electrophoretically separated and transferred onto nitrocellulose. The blots were Ponceau S stained and the ADP-ribosylated proteins were detected autoradiographically. As shown in Figure 4 the immunoprecipitation with either anti-p53 or anti-pADPRT antibodies revealed the same pattern of ADP-ribosylated proteins (panel autoradiography) thereby confirming the presumption that pADPRT was indeed complexed to p53 protein. The analysis of ^{32}P-ADP-ribosylated proteins is summarized in Table I. Both antibodies immunopre-

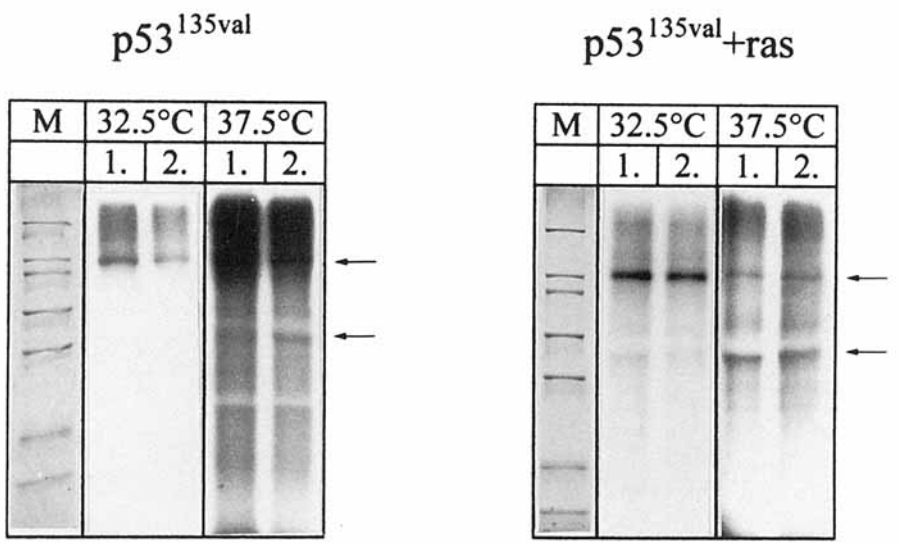

Fig. 2. Immunoprecipitation of ^{32}P-ADP-ribosylated proteins with anti-p53 antibodies. Cell lysates were incubated with 1 μg of anti-p53 antibodies PAb421. Immune complexes were eluted twice with SDS sample buffer and both eluates were loaded on 10% SDS-slab gel. Gels were Coomassie blue stained, dried, and exposed to a film for 3 days. M-protein markers from the top: Myosin-200 kD; β-galactosidase-116 kD; phosphorylase b-94 kD, BSA-66-kD; Dehydrogenase 55 kD, Lactate dehydrogenase 36.5 kD; Carbonic anhydrase-31 kD. *1.* First eluate, *2.* second eluate. To avoid overexposure, the 37.5°C samples were exposed for 20 h.

TABLE I. Analysis of ^{32}P-ADP-Ribosylated Proteins Immunoprecipitated by Anti-p53 and Anti-pADPRT Antibodies*

Cell lines	Temperature	^{32}P-ADP-ribosylated proteins in cell lysates	*Immunoprecipitates*	
			anti-p53 Ab	anti-pADPRT Ab
		cpm	cpm	cpm
p53	32.5°C	568.200	22.440	15.800
p53	37.5°C	1.042.900	93.920	88.400
p53 + ras	32.5°C	294.300	18.040	17.200
p53 + ras	37.5°C	2.377.600	306.200	266.600

*One-half of each lysate was submitted for immunoprecipitation with anti-p53 antibodies (PAb-421) and one-half with anti-pADPRT antibodies.

cipitated comparable amounts of ^{32}P-ADP-ribosylated proteins.

Thereafter, the blots were incubated with appropriate reciprocal antibodies. Proteins precipitated with anti-pADPRT antibodies were submitted for incubation with anti-p53 antibodies and vice versa. The immunoblots showed that an additional band immunoprecipitated with anti-pADPRT antibodies was recognized by anti-p53 antibodies. On the other hand, higher molecular weight protein at 116 kD precipitated by anti-p53 antibodies turned out to be pADPRT. These results unequivocally demonstrate that p53 protein forms complexes with pADPRT. Moreover, it is obvious that this complex formation depends neither on the conformation of p53 protein nor on its ADP-ribosylation since the complexes occur in growing and growth arrested cells as well.

Finally, we digested the cell lysates with snake venom phosphodiesterase (PDE I) for 4 h and for 9 h at 37°C or incubated in the presence of 100 mM Tris-buffer at pH 10.5 for 2 h at 37°C and submitted them for immunoprecipitation with anti-p53 antibodies. As shown in Figure 5 enzymatic treatment of the samples for 4 h resulted in the disappearance of radioactively labeled protein band at 64 kD in the immunprecipitates due to the removal of poly(ADP-ribose) residues. The intensity of the labeled band at 116 kD was significantly diminished. After longer PDE I treatment (data not shown) or incubation under alkaline conditions both labeled band disappeared (Fig. 5). This experiment confirms that

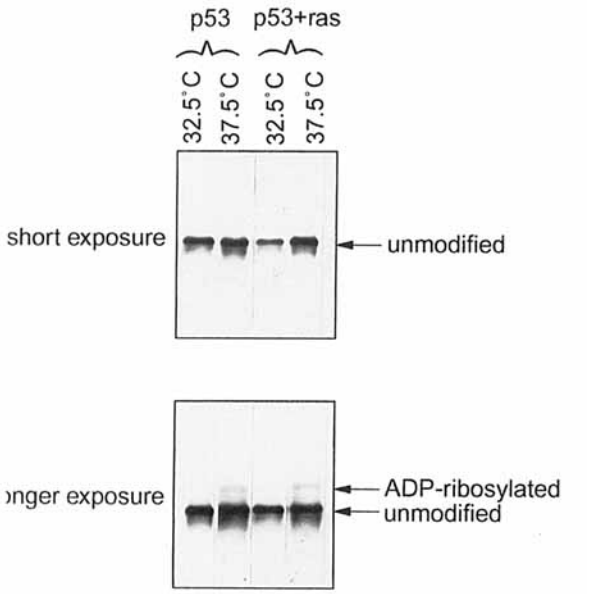

Fig. 3. Immunoblotting of ^{32}P-ADP-ribosylated proteins immunoprecipitated by anti-p53 antibodies PAb 421. Proteins immobilized on nitrocellulose were incubated with polyclonal anti-p53 antibodies CM-1 at a dilution 1:1,000 and with anti-rabbit antibodies horseradish peroxidase coupled at a concentration of 1:2,000. The immune complexes were detected using ECL kit. After short exposure overexpressed p53 was visible. After longer exposure an additional band at 64 kD representing the ADP-ribosylated p53 protein was visualized.

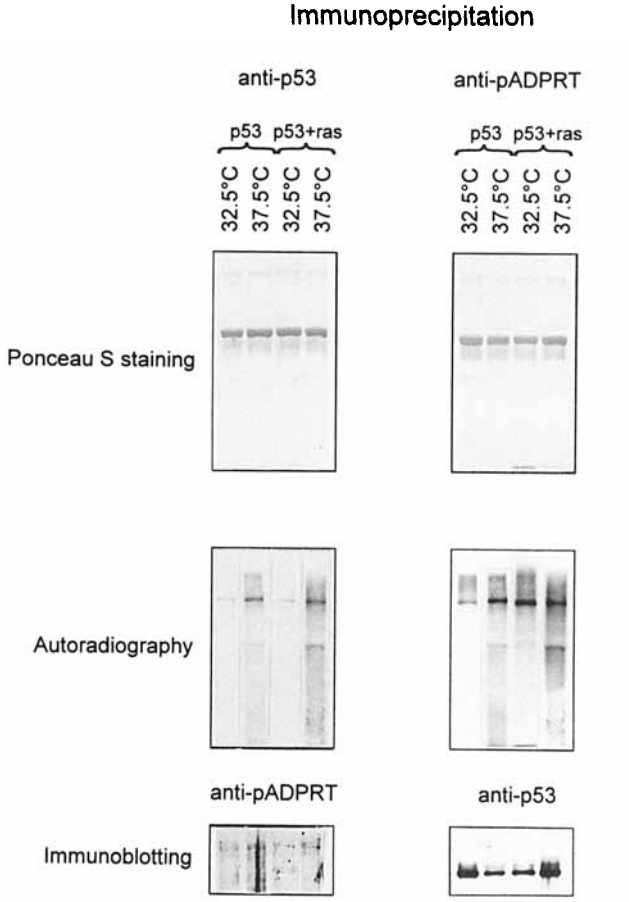

Fig. 4. Reciprocal immunoprecipitation and immunoblotting experiments. Cell lysates containing ^{32}P-ADP-ribosylated proteins were submitted for immunoprecipitation using either anti-p53 antibodies PAb 421 or anti-pADPRT antibodies. Precipitated proteins were electrophoretically separated and transferred onto nitrocellulose. The immobilized proteins were Ponceau S stained and ^{32}P-ADP-ribosylated proteins were detected autoradiographically. Then the proteins were incubated with appropriate antibodies: proteins immunoprecipitated with anti-p53 antibodies were submitted to immunoblotting using anti-pADPRT antibodies and vice versa. The results shown in Figures 2–5 were obtained from the same experiment. About one-third of the samples immunoprecipitated by anti-p53 antibodies was used in this figure.

the labeled 64 kD protein specifically precipitated by anti-p53 antibodies represents ADP-ribosylated form of p53.

DISCUSSION

The established mutant p53^{135val} offers an appropriate system for studying the biological activities of p53 protein. This mutant, constitutively overexpressing p53 protein, has been shown to exhibit wild-type activity at low temperature and to adopt mutant conformation at elevated temperatures [Eliyahu et al., 1985]. Since the time course of effective p53 translocation after temperature shift obviously depends on the cells used [Michalovitz et al., 1990; Gannon et al., 1991], we first examined the intracellular distribution of p53 protein and the kinetic of its translocation in our cell lines to ensure optimal conditions. As expected, in both nontransformed and transformed cells maintained at 32.5°C p53 protein was located in the nuclei. The majority of p53 was translocated from the cytoplasm into the nuclei within 24 h after temperature shift.

Analysis of ADP-ribosylated proteins revealed that mutant but not wild-type p53 was modified and no substantial difference was observed between its modification in nontransformed and transformed cells. At 37.5°C, in both cell lines the molecular mass of ADP-ribosylated p53 was shifted to 64 kD indicating that poly(ADP-ribose) chains of constant length were covalently attached to p53 molecules. Usually acceptor proteins for poly(ADP-ribosyl)ation are modified with chains of varying size resulting in proteins of different molecular masses. However, covalently attachment of poly(ADP-ribose) chains of exactly defined length has been also

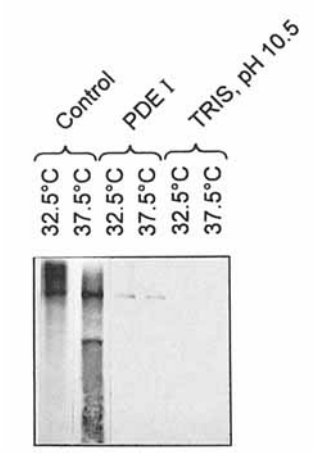

Fig. 5. Effect of removal of poly(ADP-ribose) on the pattern of labeled proteins immunoprecipitated by anti-p53 antibodies. Cell lysates of the transformed line treated with PDE I for 4 h or incubated with 100 mM Tris buffer (pH 10.5) for 2 h were immunoprecipitated with anti-p53 antibodies PAb421. Precipitated proteins were electrophoretically separated and transferred onto nitrocellulose. ^{32}P-ADP-ribosylated proteins were detected autoradiographically.

reported [Wong et al., 1982]. Our finding raised two questions: how supposedly nuclear pADPRT was able to modify mutant p53 accumulated in cytoplasm and why mutant but not wild-type p53 was ADP-ribosylated.

Considering the latter, wild-type p53 protein possesses sequence-specific DNA binding activity and binds to a consensus-binding site present in regulatory sequences of p53-dependent genes [Bayle et al., 1995; Cox et al., 1995a; Hsu et al., 1995]. Mutational inactivation of p53 is primarily due to the induction of an overall change in the conformation of the p53 protein leading to the loss of its DNA-binding ability. Therefore, mutant cytoplasmic p53 is obviously accessible for ADP-ribosylation, whereas wild-type p53 is complexed to DNA and does not act as target of ADP-ribosylation. It is also possible that in p53 protein adopting wild-type conformation some epitopes may be cryptic and not accessible for modification. Interestingly, it was reported that phosphorylation of mutant p53 molecules was found to be altered compared to wild-type p53 [Ullrich et al., 1993]. This observation combined with our findings suggests that modifications considerably depend on conformational p53 status. With regard to possible biological consequences of presently described novel modification of mutant p53, preliminary results of our current experiments suggest that poly(ADP-ribosyl)ation may affect the stability of p53 protein.

Attempting to elucidate how primarily nuclear pADPRT could modify mutant p53 accumulated in cytoplasm we examined the intracellular localization of the enzyme. Surprisingly, we detected spatial colocalization of p53 and pADPRT. In both nontransformed and transformed cells maintained at 37.5°C pADPRT was located in the cytoplasm and in the nucleoli whereas chromatin was depleted of pADPRT. It seems that activity of cytoplasmic pADPRT does not depend on the presence of DNA [Niedergang et al., 1979]. This is in accordance with other reports supporting the idea that pADPRT is a multifunctional enzyme playing a role in various cellular processes with or without direct involvement of DNA [Fakan et al., 1988; Leitinger et al., 1993; Mosgoeller et al., 1996].

Interestingly, nucleoli associated enzyme appeared unaffected and its reactivity with the affinity purified antibodies was comparable to that recently observed in HeLa cells [Mosgoeller et al., 1996]. It is not clear why pADPRT did undergo partially cytoplasmic sequestration in cells constitutively overexpressing mutant p53. Comparative experiments with other appropriate p53 mutants may help us to clarify this question. Reciprocal immunoprecipitation experiments combined with immunoblotting revealed that pADPRT binds to p53 protein and the formed complexes are very tightly bound since even the use of high concentration of nonionic and ionic detergents during experimental procedures did not affect the complex formation. A high affinity to p53 was previously described for cellular proteins hsp70 and mdm-2 [Hinds et al., 1987; Momand et al., 1992; Oliner et al., 1992]. Complex formation with hsp 70 is confined to mutant form of p53 [Hinds et al., 1987; Michalovitz et al., 1990; Pinhasi-Kimhi et al., 1986]. This is in contrast to pADPRT, which like mdm-2 has the capability to bind both mutant and wild-type p53.

The complex formation between pADPRT and p53 protein could be of functional importance. It is known that the p53 protein is strongly activated in response to DNA damage. However, the mechanisms mediating the recognition of DNA strand breaks are still unknown. On the other hand, the pADPRT is able to recognize sites on DNA where free ends occur. pADPRT contains the DNA binding domain localized in the amino-terminus that is characterized by the presence of two zinc-finger motifs. Studies with recombinant fragments encompassing DNA binding do-

main revealed that zinc is essential for its binding to DNA. DNase I protection experiments showed that pADPRT specifically recognizes and binds to single strand breaks in DNA and is able to protect symmetrically 7 ± 1 nucleotides on either side of a free DNA end [Gradwohl et al., 1990; de Murcia et al., 1991]. Considering the finding that pADPRT forms complexes with p53 protein we postulate that it could be of functional significance in mediating recognition of DNA damage.

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