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Overexpressed Poly(ADP-Ribose) Polymerase Delays the Release of Rat Cells From p53-Mediated G_1 Checkpoint

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Abstract We have previously reported that in cells ectopically expressing temperature-sensitive $p53^{135val}$ mutant, p53 formed tight complexes with poly(ADP-ribose) polymerase (PARP). At elevated temperatures, $p53^{135val}$ protein, adopting the mutant phenotype, was localized in the cytoplasm and sequestered the endogenous PARP. To prove whether an excess of $p53^{135val}$ protein led to this unusual intracellular distribution of PARP, we have established cell lines overexpressing $p53^{135val}$ + c-Ha-ras alone or in combination with PARP. Interestingly, immunostaining revealed that PARP is sequestered in the cytoplasm by mutant p53 in cells overexpressing both proteins. Simultaneous overexpression of PARP had no effect on temperature-dependent cell proliferation and only negligibly affected the kinetics of p53-mediated G_1 arrest. However, if the cells were completely growth arrested at 32°C and then shifted up to 37°C, coexpressed PARP dramatically delayed the reentry of transformed cells into the cell cycle. Even after 72 h at 37°C the proportion of S-phase cells was reduced to 20% compared to those expressing only $p53^{135val}$ + c-Ha-ras. The coexpressed PARP stabilized wt p53 protein and its enzymatic activity was necessary for stabilization. J. Cell. Biochem. 80:85–103, 2000.

Key words: cell cycle; overexpressed PARP, ts p53, stabilization of wt p53, nuclear p53; mdm-2; G_1 arrest;

The highly conserved p53 gene encodes a tumor-suppressor protein essential for the maintenance of DNA replication fidelity and genome stability [for review, see Cox and Lane, 1995; Donehower and Bradley, 1993; Ullrich et al., 1992; Velculescu and El-Deiry, 1996]. The wild-type (wt) p53 protein is a 53-kDa nuclear phosphoprotein that is present in extremely low amounts in normal cells due to a very short half-life [Reich and Levine, 1984]. In response to DNA damage by genotoxic stimuli [Fritsche et al., 1993; Kastan et al., 1991; Maltzman and Czyzyk, 1984], p53 becomes stabilized and p53 protein level increases markedly. The resulting accumulation of p53 protein leads to inhibition of cell-cycle progression [Eliyahu et al., 1989; Finlay et al., 1989; Lin et al., 1992] or induction of apoptosis [Yonish-Rouach et al., 1991], thereby preventing the proliferation of damaged cells and allowing repair of damaged DNA. The exact molecular mechanism through which wt p53 exerts its activities and initializes a program leading to cell-cycle arrest or apoptosis has recently been the focus of intense research. The anti-proliferative function of wt p53 has been shown to be mediated by its sequence-specific binding to a variety of target genes [El-Deiry et al., 1992; Funk et al., 1992] including gadd-45, mouse double minute-2 (mdm-2), and $p21^{Waf1/Cip1/Sdi1}$ [El-Deiry et al., 1993; Momand et al., 1992; Smith et al., 1994] and regulation of their transcription. The product of the latter has been implicated in mediating the G_1 cell-cycle arrest because of its ability to inhibit cyclin-dependent protein kinases 4 and 6 [Harper et al., 1993] and to bind directly to proliferating cell nuclear antigen (PCNA) [Flores-Rozas et al., 1994; Waga et al., 1994]. Furthermore, it was found that p53 in-

Abbreviations used: 3-AB, 3-aminobenzamide; BA, benzamide; BS, benzoic acid; FTF, flow-through fraction; LMB, leptomycin B; mdm-2, mouse double minute-2; NAD, nicotinamide adenine dinucleotide; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; ts, temperature-sensitive; wt, wild-type.

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teracts with the transcription factors TAFII40 and TAFII60 [Sancar 1994; Thut et al., 1995].

In response to DNA damage, another nuclear protein, poly(ADP-ribose) polymerase (PARP), is strongly stimulated resulting in its binding to DNA strand breaks and concomitant synthesis of oligo- or poly(ADP-ribose) chains covalently coupled to various acceptor proteins [Berger 1985; D'Amours et al., 1999]. It has been shown recently that PARP is an essential component of the base excision repair complex [Masson et al., 1998] and its activity is necessary for the formation of a functional complex with the XRCC1 protein. PARP has also been shown to be involved in the execution of apoptosis [He et al., 1998; Kaufmann 1989; Lazebnik et al., 1995; Węsierska-Gądek et al., 1999a]. More recently, tankyrase, a telomere-associated protein possessing PARP-like activity, has been described implicating a role of ADP ribosylation in the regulation of cellular aging [Smith et al., 1998]. In the last few months, new PARP-related enzymes have been described [D'Amours et al., 1999; Kickhoefer et al., 1999]. They show a high degree of homology with the catalytic domain of PARP and possess poly(ADP-ribosyl)ating activity.

To better understand the physiologic function of PARP, mice lacking PARP have been generated by homologous recombination [Masutani et al., 1999; Menissier-de Murcia et al., 1997; Wang et al., 1995]. They show normal fetal and neonatal development but exhibit inherent genomic instability and are extremely sensitive to genotoxic agents, like γ-rays or monoalkylating substances [Menissier-de Murcia et al., 1997; Oliver et al., 1998; Trucco et al., 1998; Wang et al. 1997]. Interestingly, the inactivation of the PARP gene in mice affects the constitutive expression of wt p53 [Agarwal et al., 1997; Węsierska-Gądek et al., 1999b]. We have recently reported that in the absence of active PARP, the stability of the regularly spliced form of wt p53 was reduced to a barely detectable level [Węsierska-Gądek et al., 1999b] due to an eight-fold decrease in its half-life [Węsierska-Gądek et al., 2000]. These results are consistent with findings of other groups that showed a link between the expression of p53 and PARP [Simbulan-Rosenthal et al., 1999; Vaziri et al., 1997; Węsierska-Gądek et al., 1996a; Węsierska-Gądek et al., 1996b; Whitacre et al., 1995].

Moreover, PARP is a p53-binding protein and forms tight complexes with wt as well as mutant forms of the p53 protein [Węsierska-Gądek et al., 1996a; Węsierska-Gądek et al., 1996b] in cells constitutively overexpressing temperature-sensitive (ts) mouse $p53^{135val}$. The advantage of this ts $p53^{135val}$ mutant [Eliyahu et al., 1985] is its oncogenic phenotype at elevated temperatures (37°C and 39°C) connected with cytoplasmic localization of p53, and its tumor-suppressor activity at low temperature (32°C) accompanied by p53 translocation into the nucleus. In the cells maintained at high temperature, PARP exactly colocalized with p53 protein and was sequestered in the cytoplasm by mutant p53 protein [Węsierska-Gądek et al., 1996a].

However, in these cells the p53 protein was highly overexpressed and its level markedly exceeded that of endogenous PARP. Therefore, we have addressed the question whether the simultaneous overexpression of both proteins, p53 and PARP, in comparable concentration, would also result in the unusual PARP sequestration in the cytoplasm and how the simultaneously overexpressed proteins would affect the regulation of the cell cycle. Therefore, we have established c-Ha-ras transformed cells constitutively overexpressing ts $p53^{135val}$ alone or in combination with PARP. We have observed that the reentry of the G_1-arrested cells into S phase upon shift from 32°C to the basal temperature was significantly delayed in cells overexpressing PARP and $p53^{135val}$. In these cells, two different pools of p53 protein occurred concomitantly: significant amounts of wt p53 protein were still present in the nucleus even after a longer maintenance of cells at elevated temperature, and an increased level of mutant p53 appeared in the cytoplasm. The prolonged persistence of the nuclear pool of wt p53 was mediated by overexpressed PARP.

MATERIALS AND METHODS

Antibodies

Monoclonal anti-p53 antibodies recognizing mutant p53 (PAb240), wt p53 (PAb246), or both (PAb421), and anti-$p21^{waf-1}$ antibodies were from Oncogene Research Products (Cambridge, MA). Polyclonal anti-p53 antibodies CM-1 were obtained from Novocastra Laboratories Ltd. (Newcastle upon Tyne, England). Polyclonal anti-PARP antibodies were from

Roche (Roche Diagnostics GmbH, Wien, Austria), monoclonal anti-PARP antibodies C-2-10 were from Dr. G. Poirier. Anti-mdm-2 (SMG-14) and anti-human p53 (DO-7) antibodies were from DAKO A/S (Glostrup, Denmark). Anti-topoisomerase I antibodies were purified from human scl-70 serum by affinity chromatography [Mosgoeller et al., 1998]. Monoclonal anti-actin antibodies were from ICN Biomedicals. Monoclonal anti-BrdU antibodies and distinct secondary antibodies were from Amersham International (Little Chalfont, Buckinghamshire, England).

Plasmids

pLTRp53cGval135 comprising a chimera of mouse p53 cDNA and genomic DNA (generous gift of Dr. M. Oren) has been previously referred to as pLTRp53cG [Eliyahu et al., 1985]. It encodes a mutant protein exhibiting a substitution from alanine to valine at position 135. The plasmid encompassing the coding sequence for human PARP was a generous gift of Dr. G. de Murcia. Plasmids pVV2 bearing the neo selective marker and pVEJB coding for a mutated human c-Ha-ras gene cloned into pVVJ were used. Ewing sarcoma cell line overexpressing human ts mutant $p53^{138val}$ [Kovar et al., 2000] was a kind gift of Dr. H. Kovar.

Establishing Cell Clones

The establishment of transformed rat cell clones was performed according to the protocol previously described in detail [Węsierska-Gądek et al., 1996a]. Cells were cotransfected with pVV2 plasmid bearing the neo selective marker. After replating, cells were selected by cultivation in medium containing geneticin (200 μg/ml, G418, Gibco). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in an atmosphere of 7.5% CO_2. For experiments dealing with the change of the conformational state of p53 protein, cells grown at 37°C were shifted to 32°C or 39°C for varying periods of time. Cells maintained at 32°C for 48 h were shifted up to 37°C for varying periods of time.

In vivo treatment of cells. In some experiments, cells maintained at 32°C were additionally incubated with the PARP inhibitors [Banasik et al., 1992; Sims et al., 1983] 3-aminobenzamide (3-AB; 3–10 mM), benzamide (BA; 50 μM), or the chemically related compound benzoid acid (BS; 1 mM). The treatment with these agents was started 24 h after temperature shift from 37°C to 32°C and was performed for an additional 24–72 h. Under these conditions, the PARP inhibitors did not impair the nucleotide metabolism.

The effect of leptomycin B (LMB), an inhibitor of protein export [Wolff et al., 1997], on nuclear retention of endogenous p53 in normal rat fibroblasts was tested within concentrations ranging from 20 nM to 100 nM. In further experiments, cells overexpressing ts p53 that were maintained at 32°C for 48 h were preincubated with 50 nM LMB for 1 h. Then the cells were shifted up to 37°C and the incubation was prolonged for 48 h.

Indirect Immunofluorescence Microscopy

For microscopic investigations, cells plated on slides were cultivated at 37°C for 24 h then shifted to the appropriate temperature for indicated time and stained as previously described in detail [Węsierska-Gądek et al., 1999b]. For detection of replicating cells, BrdU was added 1 h prior to the harvest.

Measuring DNA Content of Single Cells by Flow Cytometry

The measurement of DNA content was performed by flow cytometric analysis by modifying the method described by Vindelov et al. [1983]. The pelleted cells were washed with ice-cold phosphate-buffered saline (PBS) and aliquots of 5×10^5 cells were used for further analysis. The cells were spun down, resuspended in 200 μl PBS, and mixed with 300 μl of solution A [20.4 mM trisodium citrate, 3 mM Tris/HCl (pH 7.6), 0.6% Nonidet P-40, 9 mM spermin tetrahydrochloride] containing 54 μg trypsin. After 10 min incubation, 250 μl of solution B containing 750 μg chicken egg white and 150 μg RNase A were added and mixed carefully on a rotator for 10 min at room temperature. Finally, 250 μl of solution C containing 122 μg propidium iodide and 1.74 mg spermine tetrahydrochlorid were added; after at least 2 h, the stained cells were measured using the Becton Dickinson FACScan.

RNA isolation and Northern blotting. Total cellular RNA was prepared by the guanidium isothiocyanate, phenol, and chloroform extraction procedure. Total RNA samples (40 μg) were separated through a 1% agarose

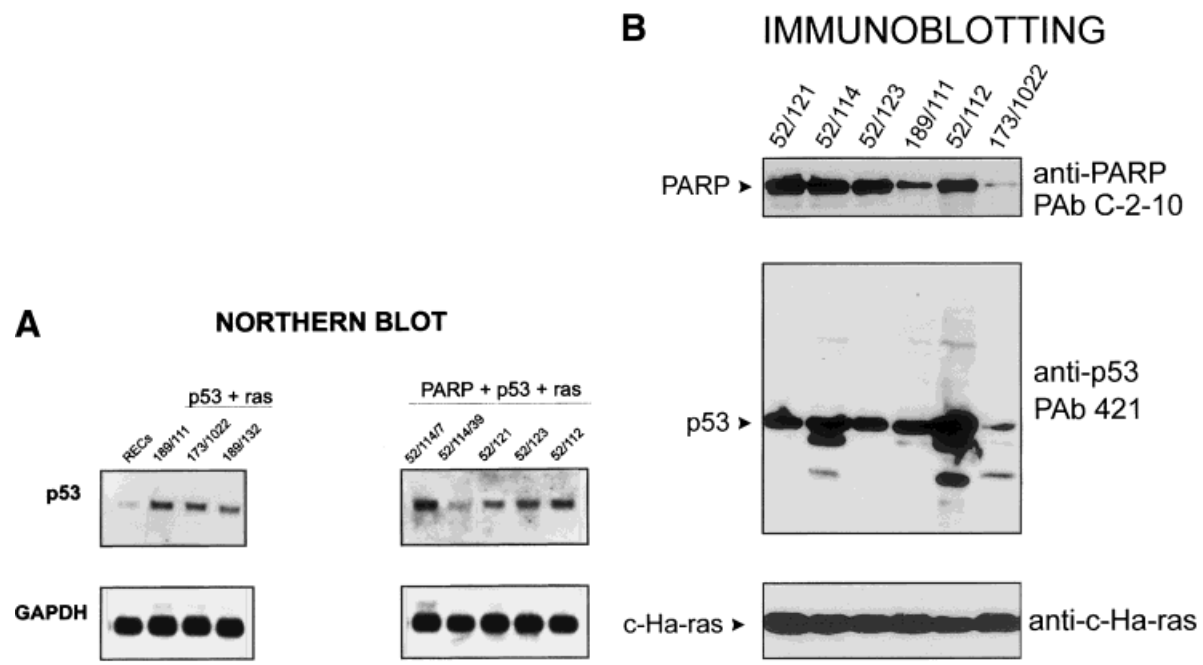
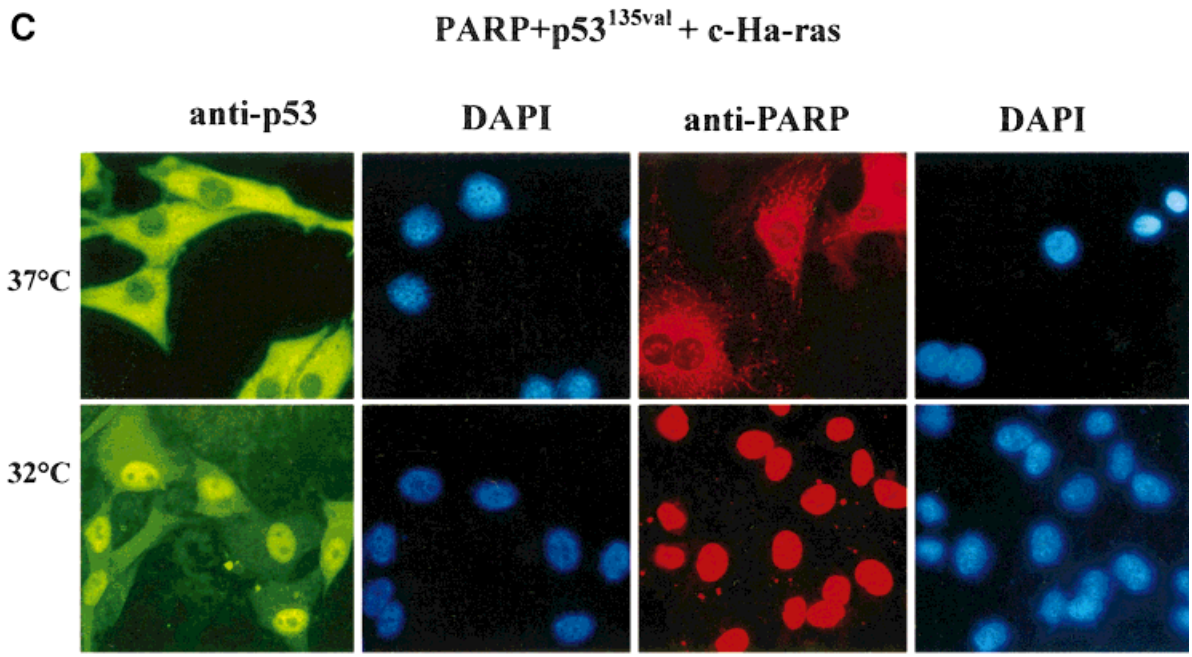


Fig. 1. Establishment of stable cell clones overexpressing $p53^{135val}$ and poly(ADP-ribose) polymerase (PARP). Freshly prepared primary rat cells were transfected with $p53^{135val}$ and c-Ha-ras alone or in combination with human PARP. **A:** Northern blotting analysis of different cell clones. Total RNA (40 μg/lane) was separated on agarose gel (1%) and transferred onto the nylon membrane. Fluorescein-labeled p53 probe or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used for hydridization. **B:** Expression of p53 and PARP in the established c-Ha-ras transformed cell clones. Total cell lysates (20 μg/lane) of corresponding cell clones were separated on 10% sodium dodecyl sulfate-slab gels. The blot was incubated sequentially with corresponding antibodies diluted to a final concentration of 1:2,000. Relatively weak p53 and PARP signal in the last lane (clone 173/1022) was due to pure protein transfer in the middle of the sandwich. However, repeated immunoblotting control revealed high expression of p53 protein. **C:** Immunolocalization of PARP and p53 proteins at restrictive and permissive temperature in PARP + $p53^{135val}$ + c-Ha-ras cells. Cells cultivated at 37°C or for 24 h at 32°C were fixed and stained with monoclonal anti-p53 PAb421 or with polyclonal anti-PARP antibodies. Antigens were visualized by appropriate Fluoro-link–labeled secondary antibodies. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). **D:** Nuclear localization of topoisomerase I and proliferating cell nuclear antigen (PCNA) in PARP + $p53^{135val}$ + c-Ha-ras cells maintained at 37°C − double immunostaining for p53/PARP and for PCNA/Topo I. Cells grown at 37°C were fixed 24 h after plating and sequentially stained with monoclonal (p53 or PCNA) and polyclonal (PARP or Topo I) primary antibodies. Antigens were detected after incubation with anti-mouse IgG coupled to Cy2 and with anti-rabbit IgG-Cy3 complex or anti-human IgG-Cy3. Nuclei were visualized by DAPI.

gel and transferred onto nylon membranes (Gene Screen Plus, DuPont). Membranes were hybridized with fluorescein-labeled probes (Gene Image, Amersham Internartional) and stringently washed. After a blocking step, the hybrids were detected by the incubation with anti-fluorescein-alkaline phosphatase conjugate. After the excess conjugate was washed off, probe-bound alkaline phosphatase was used to catalyze light emission in the presence of substrate. Chemiluminescence was detected by autoradiography.

Labeling of ADP-ribosylated proteins. Cells were suspended in cold hypotonic medium and after permeabilization they were incubated with 100 μM ^{32}P-NAD in 100 mM Tris/HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride at 25°C for 20 min. Then the cells were pelleted, washed with incubation buffer containing 5 mM cold NAD, and lyzed as described previously in detail [Węsierska-Gądek et al., 1996a].

Immunoprecipitation. Cell lysates (100 μg) were precleared with 1 μg of mouse or rabbit IgG bound to GammaBindG-Sepharose. Then they were incubated with antibodies generated against residues 88–109 of murine wt p53 detecting only wt p53 in native conformation (PAb246) or with anti-p53 antibodies recognizing only mutant form of p53 (PAb240) under nondenaturing conditions or with poly-



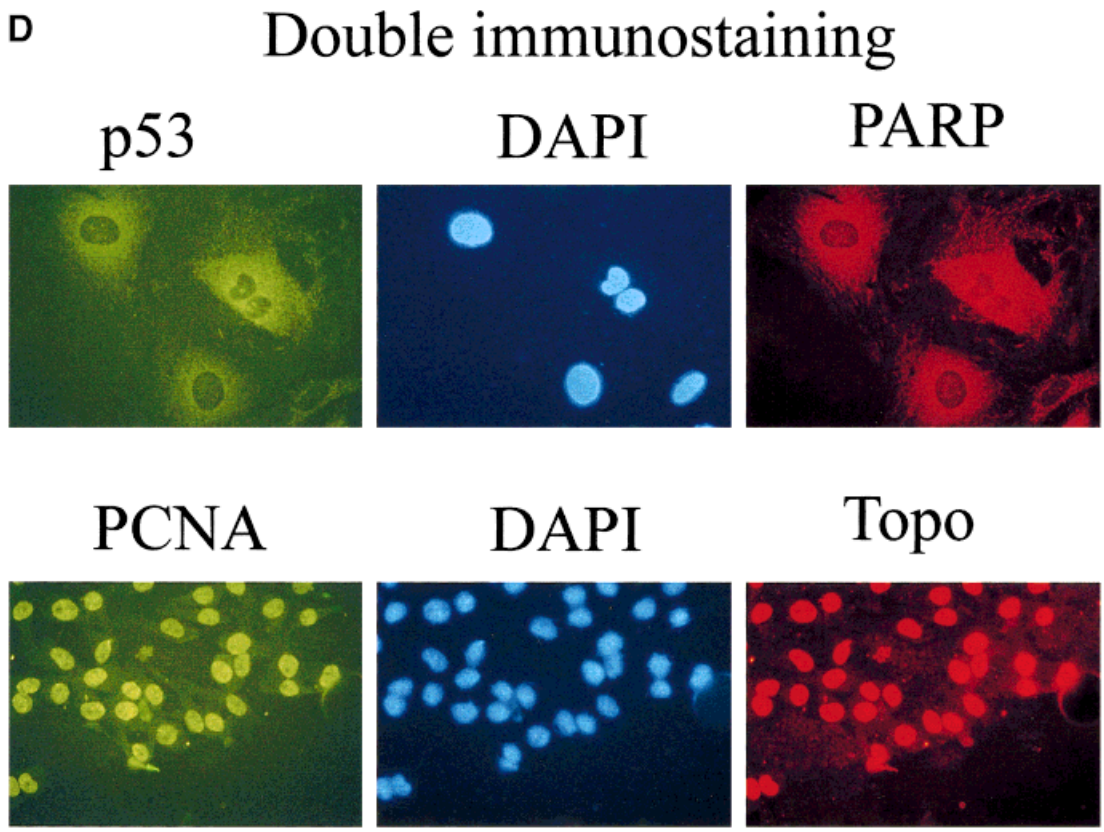


Figure 1. (Continued.)

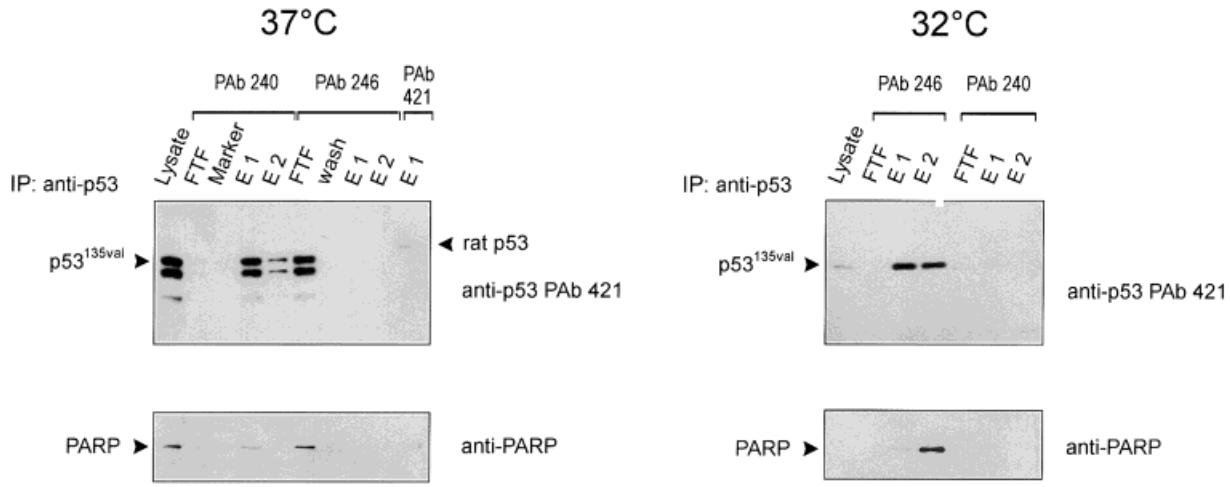


Fig. 2. Determination of the phenotype of p53 protein in cells maintained at 37°C and 32°C. Discrimination between wild type and mutant phenotype was based on the specificity of two distinct anti-p53 monoclonal antibodies: PAb246 recognizes selectively murine wt p53 in native conformation and PAb240 detects solely the mutant form of p53 under nondenaturing conditions. Lysates of cells expressing poly(ADP-ribose) polymerase (PARP) + $p53^{135val}$ + c-Ha-ras (clone 52/112) that were maintained at 37°C or at 32°C were used for separate immunoprecipitations with PAb240 and PAb246. Immune complexes bound to GammaBindG-Sepharose beads were eluted stepwise with a small amounts of sodium dodecyl sulfate (SDS)-sample buffer. Original samples, nonbound proteins (flow-through fraction; FTF), and purified immune complexes eluted stepwise (E1–E2) were electrophoretically separated on 10% SDS-slab gels and analyzed by immunoblotting using anti-p53 PAb421 recognizing both wild type and mutant forms of p53 proteins. Sequential immunoblotting was performed with anti-PARP antibodies.

clonal anti-PARP antibodies, respectively. Affinity-purified immune complexes were analyzed by immunoblotting with anti-p53 PAb421, anti-p53 DO-7, or anti-PARP-C-2-10 [Węsierska-Gądek et al., 1995].

Immunoblotting. Proteins were separated on sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride membrane. Immunodetection of antigens was performed with specific antibodies and enhanced chemiluminescent detection reagent (Amersham International). Equal loading of proteins was confirmed by Ponceau S staining and additionally by sequential incubation of blots with anti-actin antibodies. For detection of p53 protein on blots, monoclonal anti-p53 PAb421 antibody was routinely used. It is a general feature of distinct anti-p53 antibodies recognizing wt or mutant p53 form that they react specifically only with native antigens. However, if used for immunoblotting, they are not able to discriminate between wt and mutant conformation.

RESULTS

Colocalization of p53 and PARP

We have established transformed cell clones overexpressing ts $p53^{135val}$ + activated c-Ha-ras alone or in combination with PARP using primary rat cells. The overexpression of the corresponding genes was confirmed by Northern blotting (Fig. 1A) and immunoblotting (Fig. 1B). Several transformed cell clones exhibiting the highest level of p53 (189/111, 173/1022) and of both PARP and p53 (52/112, 52/114) as proved by serial immunoblotting experiments were chosen for further experiments. All established cell clones were transformed and exhibited transformed phenotype as monitored by phase contrast microscopy (not shown) or by the high expression of activated c-Ha-ras protein (Fig. 1B). We regularly monitored the $p53^{135val}$ expression level during the course of continuous cell propagation in culture. We observed a gradual decline of ectopically overexpressed p53 beyond passage 37 (Fig. 1A, clone 52/114; compare passage #7 and #39). Therefore, all experiments were performed with cells that did not exceed the passage number 30. The advantage of the ts $p53^{135val}$ mutant is its oncogenic character at elevated temperature connected with cytoplasmic localization of p53 protein, and its wild-type phenotype at 32°C accompanied by p53 translocation into the nucleus. To prove whether mutant p53 is also able to sequestrate PARP in the cytoplasm when

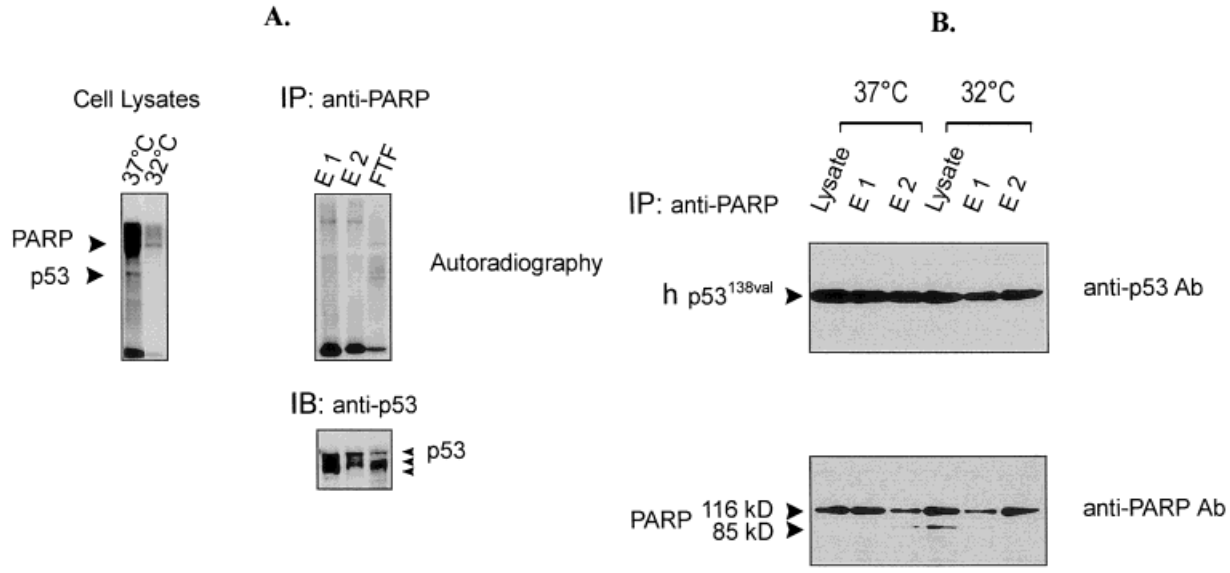


Fig. 3. Complex formation between poly(ADP-ribose) polymerase (PARP) and p53 protein. **A:** Rat cells overexpressing PARP + temperature-sensitive (ts) $p53^{135val}$ + c-Ha-ras were permeablized and labeled with ^{32}P-NAD. Cell lysate obtained from cells cultivated at 37°C was used for immunoprecipitation with anti-PARP antibodies. ^{32}P-labeled ADP-ribosylated proteins were visualized by autoradiography and the blot was incubated with anti-p53 antibodies PAb421. **B:** Human Ewing sarcoma cell line constitutively expressing human ts $p53^{138val}$ was maintained at restrictive temperature and then shifted down to 32°C for 6 h. Cells were lysed and subjected to immunoprecipitation using anti-p53 CM-1 antibodies. Immune complexes were separated on 10% sodium dodecyl sulfate (SDS)-gels. Immunoblotting was performed with antibodies against human p53 (DO-7) and then with anti-PARP antibodies C-210.

PARP is overexpressed, we performed immunostaining experiments. As shown in Figure 1C, strong immunoresponsive PARP signals corresponding to the p53 localization were detected in the cytoplasm at 37°C. Interestingly, in a number of cells anti-PARP antibodies additionally stained the nucleoli [Mosgoeller et al., 1996]. Upon temperature shift to 32°C, both antigens were detected in the nucleus. To substantiate this observation, we sequentially stained exactly the same cells for p53 and then for PARP. p53-responsive signals were detected by a secondary antibody coupled to Cy2, whereas PARP was visualized by a secondary antibody linked to Cy3. Using different filters, the signals from the same cells were sequentially photographed. To exclude unspecific reactions, highly specific secondary antibodies that did not cross-react with immunoglobulins from other species were used. The double immunostaining (Fig. 1D) corroborated the colocalization of p53 and PARP at both temperatures. Furthermore, we proved whether any nonhistone DNA-binding proteins also exit from the nucleus under these conditions. Staining of topoisomerase I and PCNA protein (Fig. 1D) revealed that the cytoplasmic expression of mutant p53 at 37°C does not affect their nuclear localization. These results demonstrate that PARP is selectively sequestered in the cytoplasm by mutant p53 and that it occurs also under conditions in which both proteins are overexpressed.

Correlation Between Phenotype of ts $p53^{135val}$ and Its Localization

The strict correlation between temperature-dependent p53 phenotype and intracellular localization has been previously shown [Eliyahu et al., 1985]. To additionally prove that the $p53^{135val}$ overexpressed in rat cells possesses mutant character at elevated temperatures and adopts wt conformation at 32°C, we have performed immunoprecipitation using specific antibodies detecting selectively native murine wt p53 (PAb246) or mutant p53 protein (PAb240), respectively. Equal protein amounts prepared from cells cultivated at 37°C and 32°C were used for independent incubations with two anti-p53 monoclonal antibodies. The immune complexes were purified by affinity chromatography. Then the original sample, an

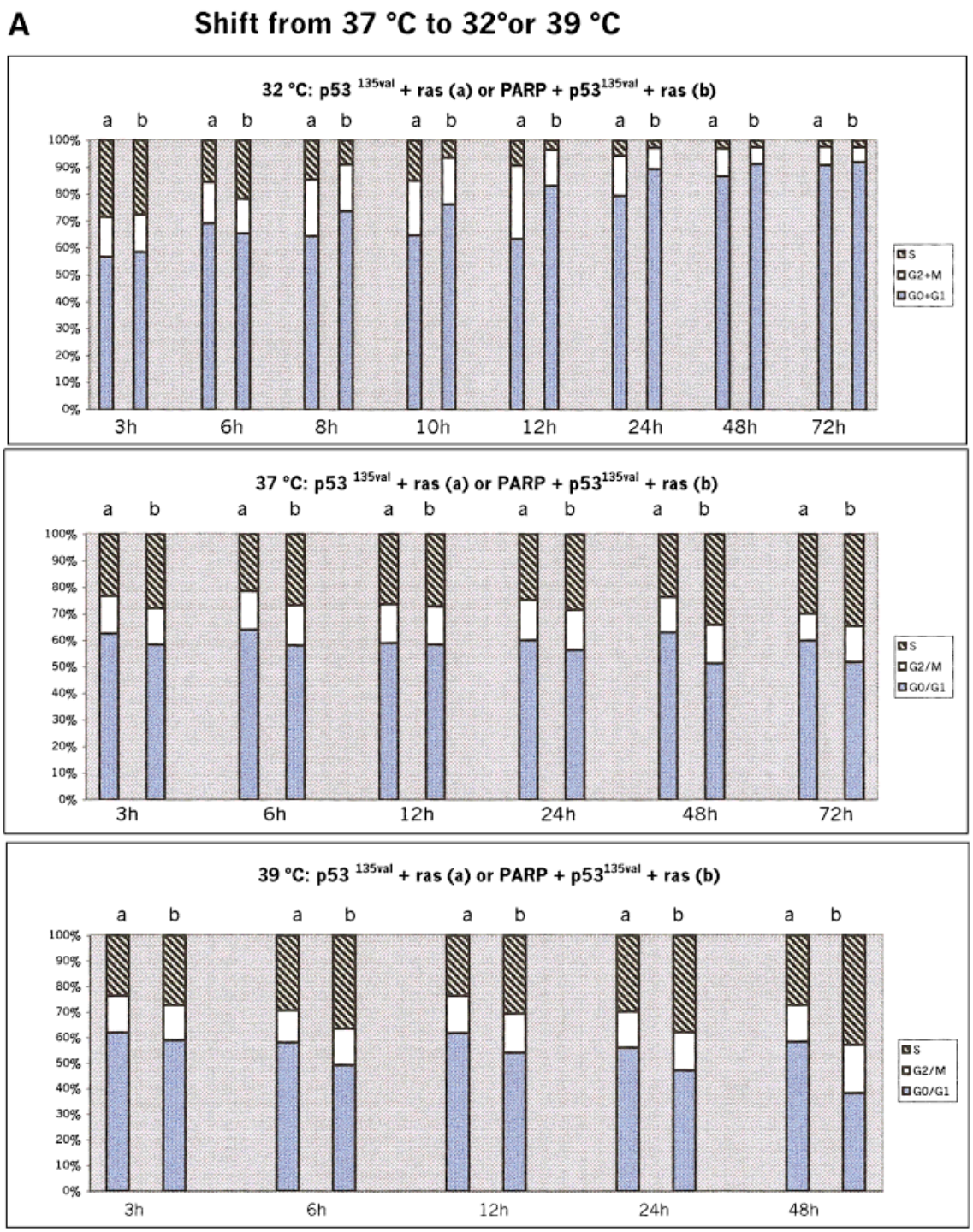


Fig. 4. Cell cycle analysis for representative cell clones **A:** $p53^{135val}$ + c-Ha-ras (**a**) and poly(ADP-ribose) polymerase (PARP) + $p53^{135val}$ + c-Ha-ras (**b**) expressing cells were maintained at basal temperature (37°C) and shifted to 32°C and 39°C. The time indications in the figure on X-axis refer to the duration of the cell maintenance at corresponding temperature. Four separate analyses of the cell cycle were performed using at least three different cell clones, each determination in duplicate. The deviations between distinct cell clones were 1–3% at 32°C and 4–6% at 37°C and 39°C. The variations in these ranges occurred also within the same cell clone during cultivation at elevated temperatures.

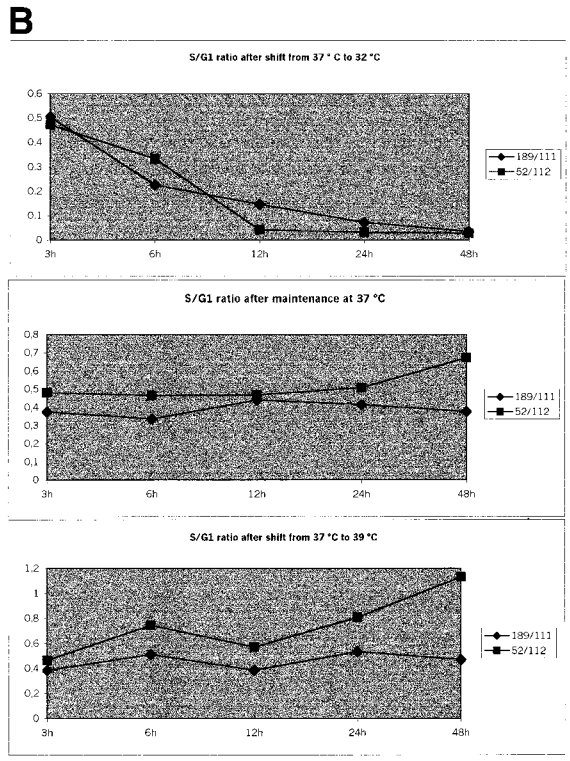


Fig. 4. **B:** A comparison of the S/G_1 ratio between transformed cells expressing $p53^{135val}$ alone (clone 189/111) or in combination with PARP (clone 52/112).

aliquot of flow-through fraction (FTF), and isolated immune complexes were analyzed by immunoblotting. For detection we used PAb421, which recognizes both mutant and wt form of p53. As illustrated in Figure 2, PAb240 almost completely precipitated the p53 protein present in the lysates from cells maintained at 37°C, and no p53 signal could be detected in the FTF. The concentrations of the original sample and FTF were normalized to ensure that comparable protein amounts were loaded. After parallel incubation of the lysate with PAb246, no detectable p53 protein could be precipitated. However, a strong p53 signal was detected in FTF, indicating that the bulk of p53 protein did not bind to PAb246 and remained in the supernatant. Interestingly, sequential precipitation of post-PAb240 supernatant with PAb421 revealed a very weak p53 band. This band migrated slightly slower than ts $p53^{135val}$ and represented the endogenous rat wt p53 protein. Complementary results were achieved after immunoprecipitation of extracts from cells maintained at 32°C. p53 protein was precipitated solely with PAb246, but not with PAb240, thereby showing that p53 changed its conformation and localization in a temperature-dependent manner.

Complex Formation Between p53 and PARP

The unusual cytoplasmic sequestration of PARP seems to be a consequence of complex formation between PARP and the p53 protein. To prove this assumption we performed reciprocal immunoprecipitations followed by immunoblotting. As shown in Figure 2 (lower panel), PARP was coprecipitated with p53 protein by specific anti-p53 antibodies. At elevated temperature, PARP was coprecipitated solely by antibodies reacting with the mutant form of p53 (PAb240) but not by antibodies reacting with the wt p53 under native conditions. Precipitation of cell lysates obtained from cells maintained at 32°C revealed the presence of PARP in complexes with wt p53 protein. Further experiments were performed with permeabilized cells incubated with ^{32}P-NAD (Fig. 3A). The analysis of radioactively labeled lysates from cells at 37°C revealed the presence of a heavily ADP-ribosylated band at approximately 120 kDa (representing automodified PARP) and a second band at approximately 60 kDa [Węsierska-Gądek et al., 1996a]. In lysates of cells maintained at 32°C, only one radioactive at approximately 120 kDa was detected. Immunoprecipitation of lysates obtained from cells at 37°C showed that p53 protein was coprecipitated by anti-PARP antibodies (Fig. 3A). Under these conditions, p53 protein has a mutant phenotype and is localized in the cytoplasm. Careful analysis of autoradiograms revealed that two labeled protein bands were precipitated by anti-PARP antibodies: the upper band at approximately 120 kDa represented automodified PARP and the lower band at approximately 60 kDa was p53 protein. Anti-p53 antibodies stained the protein doublet corresponding to unmodified and ADP-ribosylated p53. To further substantiate this observation, we performed reciprocal immunoprecipitation using lysates of the human Ewing sarcoma cell line expressing human ts $p53^{138val}$ [Kovar et al., 2000]. It is known that in Ewing sarcoma cells PARP is amplified [Prasad et al., 1990]. As shown in Figure 3B, wt as well as mutant p53 was coprecipitated by anti-PARP antibodies. These results demonstrate that PARP forms complexes with wt as well as mutant p53 protein in both rat and human cells. Moreover, the origin of the protein was not relevant for complex formation because hu-

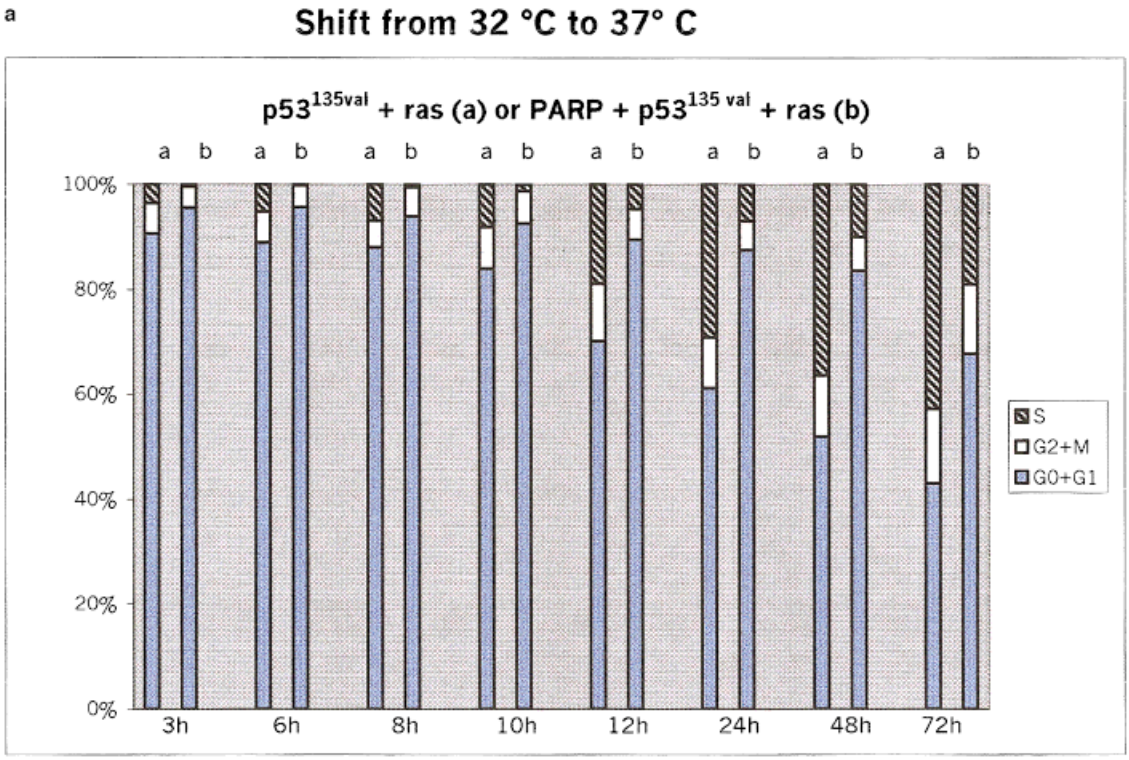


Fig. 5. **A:** Differing kinetics of the recovery of $p53^{135val}$ + c-Ha-ras cells (**a**) and poly(ADP-ribose) polymerase (PARP) + $p53^{135val}$ + c-Ha-ras cells (**b**) from G_1 arrest. Cells were maintained for 48 h at 32°C and then shifted up to 37°C. At indicated times after temperature shift, cell-cycle analysis was performed. The determinations were repeated at least three times for three distinct cell clones. The diagram depicts representative results. The time indications in the figure on X-axis refer to the duration of the cell maintenance at 37°C after temperature shift.

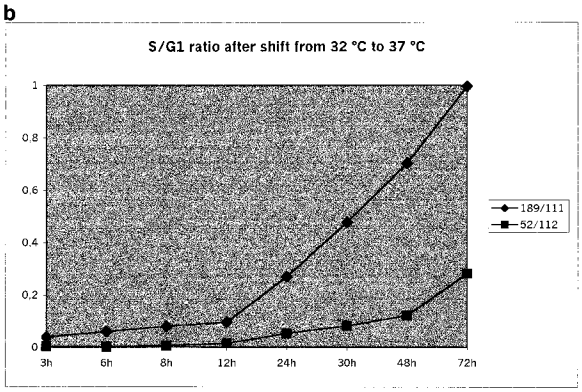


Fig. 5. **B:** A comparison of the S/G_1 ratio between $p53^{135val}$ + c-Ha-ras (clone 189/111) and PARP + $p53^{135val}$ + c-Ha-ras cells (clone 52/112).

man PARP formed complexes with human and mouse p53 protein. Moreover, the PARP binding to p53 explains the unusual cytoplasmic localization of PARP at restrictive temperature.

Effect of PARP Coexpression on Cell Proliferation and Cell-Cycle Regulation

First, we have compared the kinetics of cell proliferation at 32°C, 37°C, and 39°C for distinct cell lines. According to the characteristics of the ts $p53^{135val}$ mutant, cells grew rapidly at elevated temperatures, but 24 h after being shifted down to 32°C they ceased to proliferate (not shown). No remarkable difference in the proliferation profiles at corresponding temperatures was observed between cells overexpressing $p53^{135val}$ with or without PARP. In contrast, cultivation of primary rat cells at 32°C did not retard their growth, thereby indicating that the inhibition of proliferation of cells expressing ts p53 was due to wt p53-mediated downstream signals.

In the next step we studied the effects of the temperature-dependent p53 phenotype on the cell-cycle distribution. At basal temperature (37°C), about 30% of cells were in

S-phase (Fig. 4A). After temperature shift to 32°C, a successive accumulation of G_1 arrested cells was observed in both cell lines within the first 24 h. Notably, cells overexpressing $p53^{135val}$ and PARP accumulated more rapidly in G_1 phase than those overexpressing $p53^{135val}$ alone, as shown by the comparison of the S/G_1 ratio (Fig. 4B). At 48 h, the number of cells in S-phase was reduced to 1% and 2% , respectively, reflecting the cessation of cell proliferation. This strong reduction of the proportion of S-phase cells was additionally confirmed by determination of in vivo BrdU labeling (not shown). The concomitantly performed immunostaining of p53 provided information about the kinetics of p53 translocation from the cytoplasm into the nucleus. In both cell lines, p53 protein moved rapidly from the cytoplasm into the nucleus; and 24 h upon shift to 32°C, the p53 staining was restricted to the nucleus in almost all cells.

Coexpressed PARP Delays the Recovery of Cells From G_1 Arrest

The next experiments were designed to test the recovery of G_1-arrested cells after shift up to the basal temperature. To ensure that the G_1 arrest was complete, cells were maintained at 32°C for 48 h. A comparison of the results for both cell lines shows dramatic differences in the kinetics of the recovery from G_1 arrest (Fig. 5A). While cells expressing $p53^{135val}$ alone rapidly reached a characteristic S-phase level upon back shift to 37°C, cells expressing both $p53^{135val}$ and PARP were retarded in the reentry into S-phase. Twenty-four hours after shift up from 32°C to 37°C, about 20% of cells expressing $p53^{135val}$ were in S-phase compared to 5% of those expressing $p53^{135val}$ and PARP. Even after 48 h and 72 h at 37°C, the proportion of S-phase cells was significantly reduced in the latter (Fig. 5A and 5B).

Persistence of wt p53 in the Nuclei of Cells Coexpressing PARP

During recovery from G_1 arrest, the cells were monitored for p53 distribution. Immunostaining revealed substantial differences in the p53 localization between both cell lines after elevation of temperature (Fig. 6A). In approximately 95% of $p53^{135val}$ cells, p53 protein was exclusively detected in the cytoplasm 3 h after temperature elevation; in all cells overexpressing $p53^{135val}$ and PARP, p53 protein was found in the nuclei. The nuclear staining was very intense. Interestingly, in about 40% of the cells, weak p53 signals were additionally detected in the cytoplasm. Twenty-four hours after temperature shift, anti-p53 antibody still stained the nuclei stronger than the cytoplasm. As shown in Fig. 6A , a substantial number of cells had p53 reactivity still restricted to the nucleus. This unusual p53 distribution in cells overexpressing $p53^{135val}$ plus PARP persisted for an additional 72 h, despite their maintenance at 37°C. The nuclear persistence of p53 closely correlated with the observed retardation of reentry into S-phase. To ensure that nuclear p53 protein found in PARP plus $p53^{135val}$ cells after temperature shift up to 37°C represents the wild-type phenotype, we performed immunoprecipitation (Fig. 6B). Indeed, the precipitation of extracts from cells harvested 24 h after shift up to 37°C revealed presence of two subpopulations of $p53^{135val}$: one representing wt phenotype was reactive with PAb246 and another possessing mutant character was precipitated by PAb240. These results revealed that cells expressing $p53^{135val}$ and PARP exhibited simultaneously two different pools of p53: wt protein located in the nucleus, and mutant form present in the cytoplasm.

Increased Level of wt p53 Protein in Cells Coexpressing PARP

The above-mentioned results clearly indicated that the stability of nuclear wt p53 protein significantly differed between cell lines and suggested that the prolonged stability might depend on coexpressed PARP. Therefore, using immunoblotting, we compared the levels of wt p53 protein in both cell types maintained for longer time at 32°C. As illustrated in Figure 7, the amounts of wt p53 expressed in $p53^{135val}$ cells decreased during the period of observation falling to a very low level by 96 h. In contrast, cultivation of cells overexpressing PARP plus $p53^{135val}$ for 96 h at the permissive temperature did not affect the level of wt p53. These cells exhibited markedly higher concentrations of wt p53 at the corresponding time points accompanied by an increased level of mdm-2 protein. On the other hand mdm-2 concentration in $p53^{135val}$ cells was clearly lower, and decreased to a barely detectable level after 96 h. Sequential incubation of the

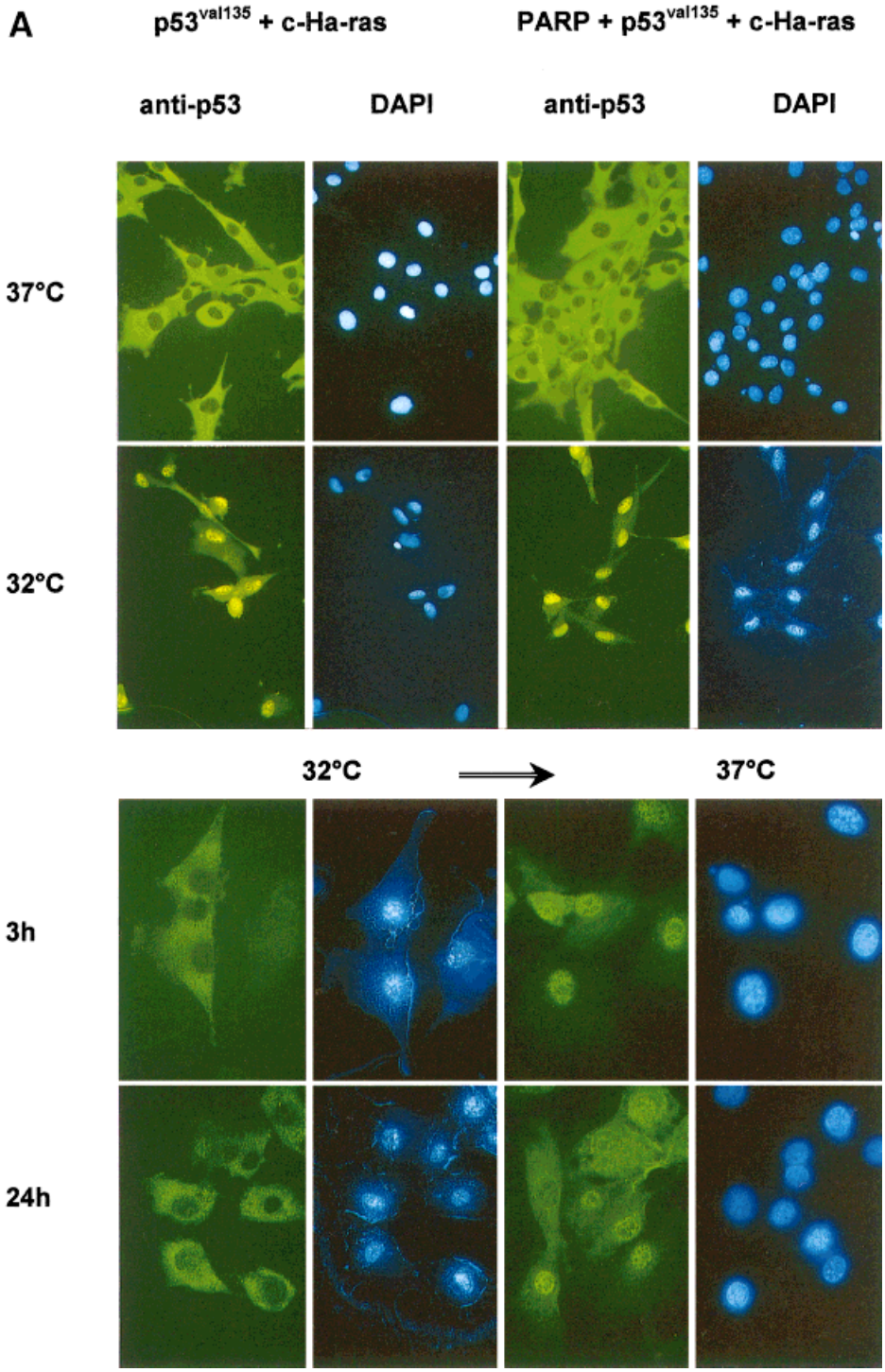


Fig. 6. **A:** Kinetics of p53 redistribution upon temperature shift up from 32°C to 37°C. Cells maintained 24 h after plating at 37°C (first panel) were shifted down to 32°C for 24 h (second panel). After an additional 24 h at 32°C, cells were shifted up to 37°C for 3 h (third panel) or for 24 h (fourth panel). Cells were stained with anti-p53 PAb421 antibodies. Nuclei were visualized by 4,6-diamidino-2-phenylindole (DAPI).

blot with anti-actin antibodies confirmed equal protein loading. These results indicate that the coexpressed PARP resulted in prolonged nuclear accumulation of wt p53 protein that was functionally active as reflected by upregulation of its target gene mdm-2.

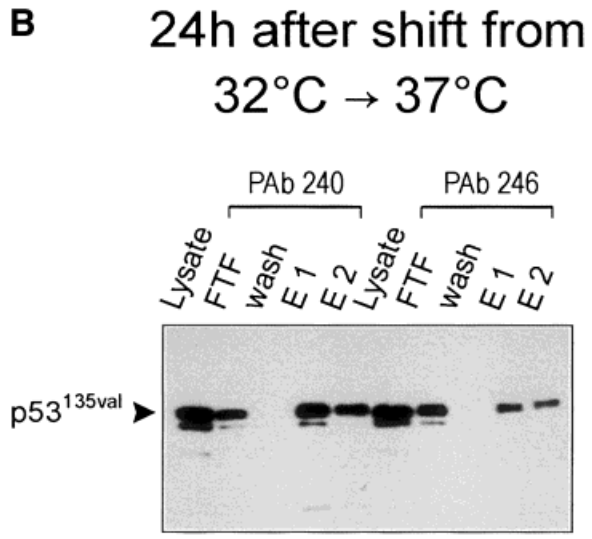


Fig. 6. **B:** Presence of two p53 forms in lysates of cells expressing poly(ADP-ribose) polymerase (PARP) + $p53^{135val}$ + c-Ha-ras prepared 24 h after temperature shift from 32°C to 37°C. Lysates were used for separate immunoprecipitations with two anti-p53 antibodies discriminating between wild type and mutant phenotype. Immunoblotting was performed with anti-p53 PAb421 recognizing both wild type and mutant p53 proteins.

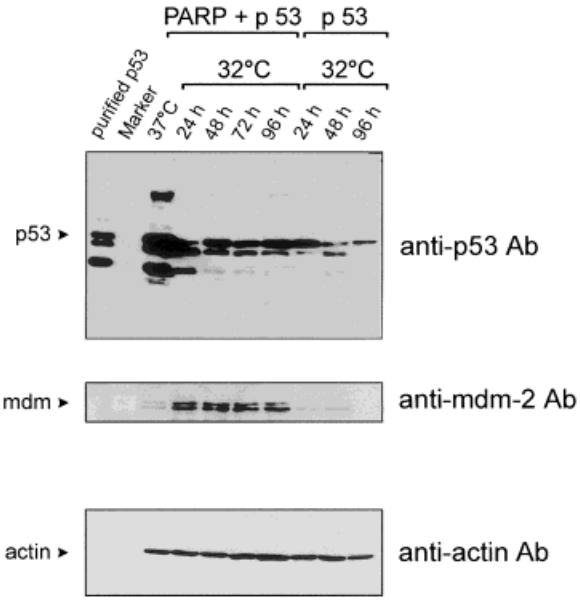


Fig. 7. Long persistence of wild type (wt) p53 protein in cells expressing poly(ADP-ribose) polymerase (PARP) + $p53^{135val}$ + c-Ha-ras. Total cell lysates (20 μg/lane) were separated on 10% sodium dodecyl sulfate gel and transferred onto the membrane. Immunoblotting was performed with anti-p53 antibody PAb421 and then with anti-mouse double minute (mdm)-2 antibodies. In total cell lysate obtained from cells at 37°C, only low levels of mdm-2 protein (presumably representing p53 independent expression) was detected. Low expression of mdm-2 in $p53^{135val}$ + c-Ha-ras cells seems to reflect the decreasing level of wt p53 protein. Equal protein loading was confirmed by immunoblotting with anti-actin antibodies.

Inhibition of PARP Abolished its Effect on Nuclear Accumulation of wt p53 Protein

After we could show the obvious dependence of the wt p53 level on coexpressed PARP, we have addressed the next question: Is the overexpressed PARP protein sufficient to mediate the stabilization of wt p53, or is its enzymatic activity required for p53 stabilization? To prove the latter possibility, we have cultivated cells at 32°C in the presence of two different PARP inhibitors [Banasik et al., 1992; Sims et al., 1983]. As a control, a chemically related compound (BS) was used, which is known not to affect the activity of PARP. As assumed, cultivation of PARP + $p53^{135val}$ cells in the presence of 3-AB strongly affected the level of wt p53 protein (Fig. 8). After 48 h, the intense p53 double band present in untreated controls declined in response to 3-AB in a dose-dependent manner. After longer treatment of cells, wt p53 became undetectable even at a lower concentration of 3-AB (3 mM). Sequential incubation of the same blot with anti-mdm-2 antibodies revealed a delayed decrease of the mdm-2 level that reflected the changes of wt p53 concentrations. Higher concentrations of 3-AB resulted in reduced expression of mdm-2. On the other hand, application of BA (at a low concentration that did not impair PARP activity in vivo) or application of BS did not reduce the stability of either wt p53 or mdm-2 in cells expressing PARP and $p53^{135val}$ (Fig. 9). These results unequivocally showed that the enzymatic activity of PARP is necessary for the nuclear maintenance of wt p53.

No Effect of LMB on p53 Cellular Distribution

We performed our next experiments to examine whether reentry of the G_1-arrested cells into the active cell cycle depends on p53 translocation from the nucleus into the cytoplasm or rather on its new synthesis. Therefore, we treated cells with LMB, a known inhibitor of protein export from the nucleus [Wolff et al., 1997], or cycloheximide. Unfortunately, the application of cycloheximide was not suitable to resolve this problem because even its low concentrations resulted in nuclear accumulation of p53. This was presumably due to the inhibition

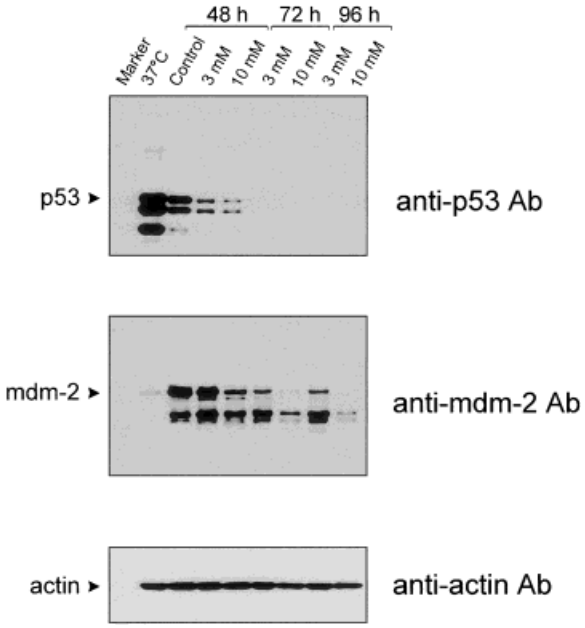


Fig. 8. Decrease of wild type (wt) p53 level in response to the inhibition of poly(ADP-ribose) polymerase (PARP) activity in PARP + $p53^{135val}$ + c-Ha-ras cells by 3-aminobenzamide (3-AB). Cells were maintained for 24 h at 32°C and then 3-AB was added to a final concentration of 3 or 10 mM, respectively. Cells were maintained at 32°C for an additional 24 h, 48 h, or 72 h. The time indications in the figure refer to the duration of the cell maintenance at 32°C. The level of wt p53 protein and its ability to transactivate targets genes, e.g., mouse double minute-2 (mdm-2) was proved by sequential immunoblotting. Equal protein loading was confirmed by immunoblotting with anti-actin antibodies.

of synthesis of a short-living protein sequestrating p53 in the cytoplasm [Gannon and Lane, 1991]. The specific effect of LMB on the p53 export was previously described [Freedmann and Levine 1998; Stommel et al., 1999]. To find out the optimal inhibition of p53 protein export in rat fibroblasts, we have tested different LMB concentrations. The treatment of rat cells with 50 nM LMB resulted in a marked increase of wt p53 protein (Fig. 10). No further p53 elevation could be observed in response to 100 nM LMB. Therefore, in further experiments, 50 nM LMB was applied. As shown in Figure 11, the inhibition of protein export by LMB did not affect the p53 compartmentalization in both cell lines. This indicated that a strong cytoplasmic staining appearing in cells expressing $p53^{135val}$ a few hours upon the temperature elevation was due to newly synthesized p53 protein.

DISCUSSION

The tumor-suppressor proteins p53 and PARP are potent sensors of DNA damage and are important for the maintenance of genome stability. It appears interesting that both proteins are involved in such cellular processes as DNA repair and apoptosis. The fact that p53 and PARP contribute to the capability of cells to rescue from DNA damage, or to commit to apoptosis, leads to the assumption that both proteins may be functionally related. Indeed, there are two lines of evidence for such a link. First, PARP interacts directly with p53 and forms tight complexes [Węsierska-Gądek et al., 1996a, 1996b]. Secondly, the functional status of PARP seems to directly affect the level of p53 protein by regulating its stability. It has been previously reported that PARP-deficient human cell lines exhibit reduced baseline levels of wt p53 and fail to accumulate p53 in response to etoposide VP-16 treatment, resulting in a lowered rate of apoptosis [Whitacre et al., 1995]. A low p53 level was also observed in PARP knock-out mouse fibroblasts [Agarwal et al., 1997; Węsierska-Gądek et al., 1999b]. Interestingly, PARP deficiency affected only the regularly spliced form of wt p53 protein, whereas alternatively spliced p53 remained unchanged [Węsierska-Gądek et al., 1999b]. The considerable reduction of the concentration of regularly spliced p53 protein had a functional consequence: PARP −/− cells failed to transactivate p53-responsive genes even after treatment with genotoxic agents [Węsierska-Gądek et al., 1999b].

In light of these results it was especially exciting to investigate the effect of PARP overexpression on the cellular functions mediated by p53 protein. The use of the ts p53 mutant offered the possibility to investigate the interaction between PARP and p53 in the same cellular background, but under two quite different conditions that depended on the phenotype of the p53 protein: one mimicking clonal expansion of neoplastic cells and the other resembling the response of normal cells to stress stimuli. Therefore, we established transformed cell clones expressing $p53^{135val}$ mutant + activated c-H-ras alone or in combination with PARP. The establishment of cell clones overexpressing simultaneously PARP and $p53^{135val}$ mutant was very difficult. In the first set of experiments using primary cells isolated from

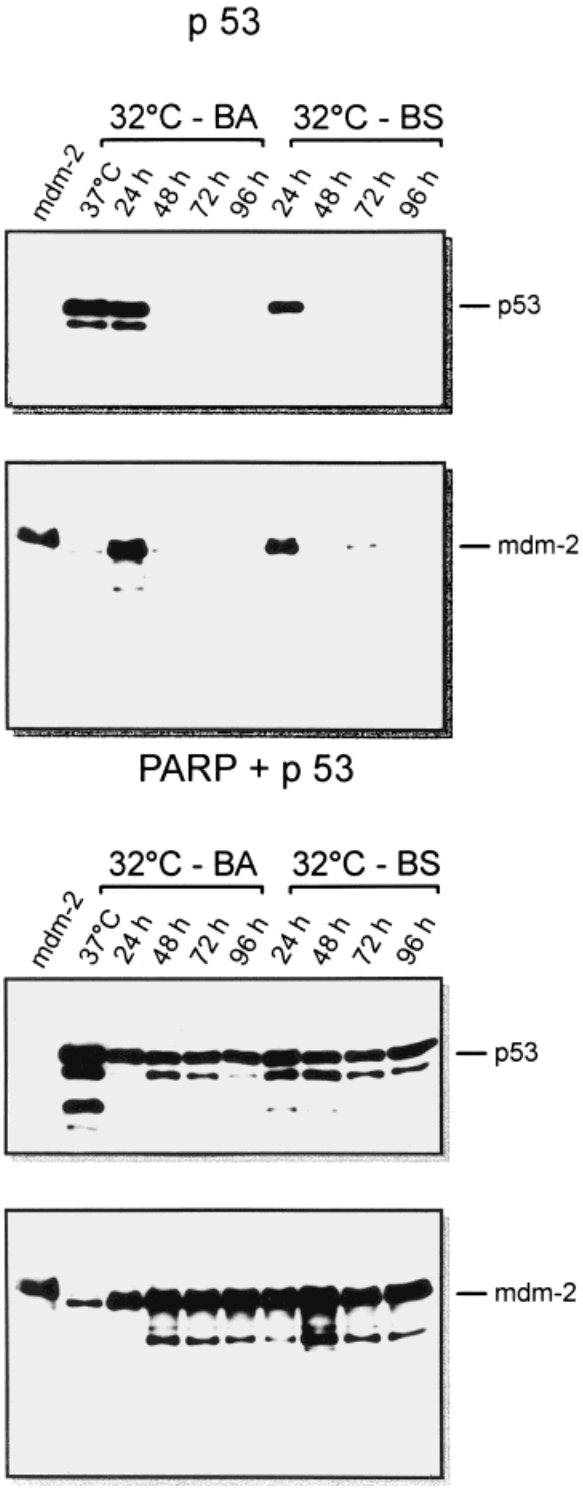


Fig. 9. Effect of benzamide (BA) and benzoic acid (BS) on the level of wild type (wt) p53 protein. Cells were maintained for 24 h at 32°C and then BA or BS was added to a final concentration of 50 μM and 1 mM, respectively. Cells were maintained at 32°C for an additional 24 h, 48 h, or 72 h. The time indications in the figure refer to the duration of the cell maintenance at 32°C. Immunoblotting was performed with monoclonal anti-p53 PAb421 antibody.

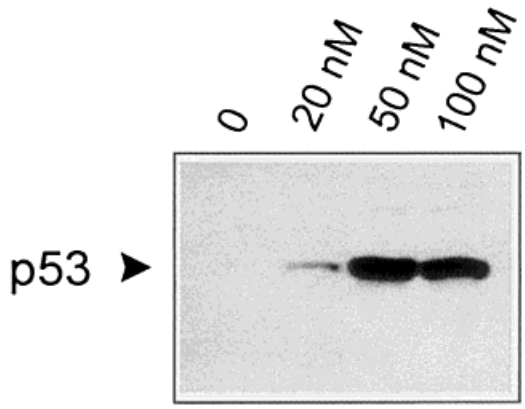


Fig. 10. Accumulation of endogenous p53 protein in rat fibroblasts in response to increasing concentrations of leptomycin B (LMB). Twenty-four hours after cell plating, LMB was added to the culture medium to a final concentrations of 20 nM, 50 nM, or 100 nM and the incubation was performed for further 24 h. Thereafter cells were harvested, phosphate-buffered saline (PBS)-washed and lysed in RIPA buffer. Total cell lysates (25 μg/lane) from untreated control and LMB-treated cells were resolved on 10% sodium dodecyl sulfate-slab gel and electroblotted onto the membrane. Immunoblotting was performed with anti-p53 antibodies PAb421.

rat embryo at gestation day 15.5, no PARP + $p53^{135val}$-positive clones were obtained. Similar observations have been previously made in murine fibroblasts [Bhatia et al., 1990]. However, in further experiments, primary rat cells at gestation day 13.5 were transfected yielding a number of cell clones overexpressing both genes after selection. This was consistent with recent reports about functional overexpression of human poly(ADP-ribose) polymerase in hamster and rat tumor cells [Bernges et al., 1997; van Gool et al., 1997].

Interestingly, in rat cells constitutively overexpressing both $p53^{135val}$ mutant + PARP, we have reproduced the previous observation that both proteins form tight complexes [Węsierska-Gądek et al., 1996a]. Moreover, in these cells maintained at restrictive temperature, the complex formation with mutant p53 led again to an unusual cytoplasmic sequestration of PARP. The presence of PARP in the cytoplasm depended on the phenotype of p53: Upon adopting wt conformation, p53 and PARP translocated into the nucleus with similar kinetics. Because these cells produce both proteins at comparable concentrations, we can exclude previous speculation that abundant amounts of mutant p53 restrain endogenous PARP in the cytoplasm unspecifically.

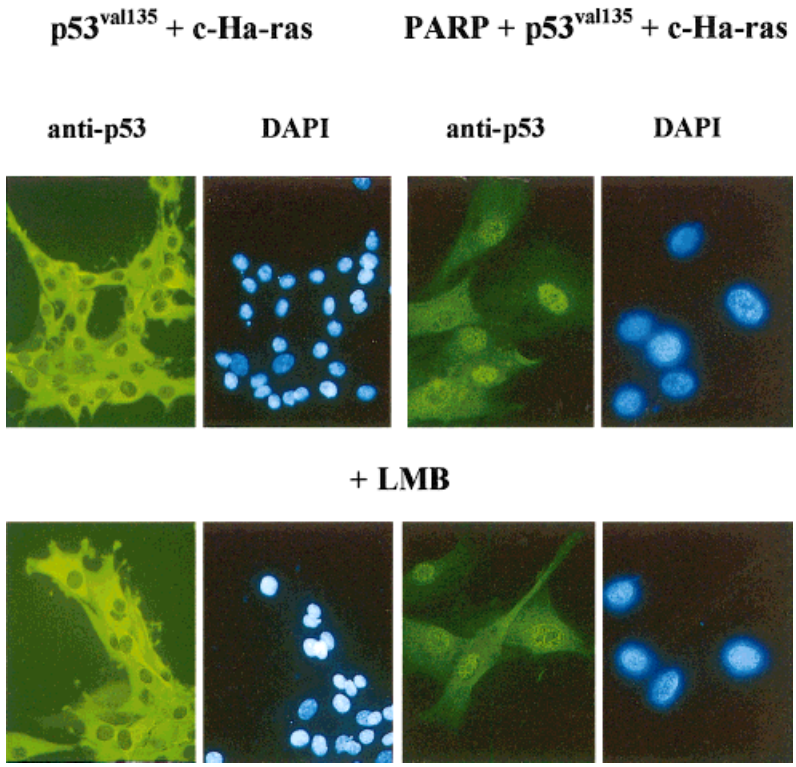


Fig. 11. Effect of inhibition of protein export by leptomycin B (LMB) on p53 localization. LMB was added to G_1-arrested cells 1 h before cells were shifted up to 37°C. p53 localization was monitored every 3 h within the first 24 h after elevation of temperature. Figure depicts immunostaining performed 24 h after elevation of temperature. Cells showing strong p53 signals in the cytoplasm were photographed at lower magnification (20× objective). On the other hand, cells exhibiting primarily nuclear p53 staining showed lower fluorescence intensity and were photographed at higher magnification (40× objective) to obtain satisfying resolution.

Our results show that elevated expression of PARP had no remarkable effect on cellular processes regulated by mutant p53. There are no differences in the kinetics of cell proliferation, distribution in the cell cycle, or p53 localization between cells expressing $p53^{135val}$ alone or in combination with PARP. However, after temperature-dependent switching of p53 from mutant to wt phenotype, the strong stabilizing effect of catalytically active PARP became evident. The reentry of G_1-arrested cells into the cell cycle was significantly retarded in cells possessing a higher level of PARP. Notably, in cells shifted up to 37°C, two different p53 pools were concomitantly present: wt p53 in the nucleus and mutant form in the cytoplasm. The simultaneous existence of two functionally antagonistic p53 forms led to a conflicting situation: Newly synthesized mutant p53 triggering G_1-arrested cells into S-phase was barely effective because of the presence of wt p53. The high level of wt p53 in the nucleus was obviously sufficient to induce p53 responsive genes and to mediate specific downstream signals. The prolonged expression of wt p53 in the nuclei under permissive conditions persisted for at least four days and was observed solely in cells coexpressing PARP. The immunoblotting experiments revealed that in the presence of overexpressed PARP, wt p53 protein exhibited a markedly increased level after longer maintenance of cells at permissive temperature, implicating overexpressed PARP is stabilizing the wt p53 protein. This assumption seems to be correct considering the fact that inactivation of the PARP gene reduced the half-life of wt p53 in mouse fibroblasts about eight-fold [Węsierska-Gądek et al., 2000]. The enzymatic activity seems to be necessary for the stabilizing effect of PARP because treatment of cells with the PARP inhibitor 3-AB abolished this effect in a dose-dependent manner. 3-AB is known to exert side effects, especially in proliferating cells, by impairing nucleotide metabo-

lism [Cleaver et al., 1983]. However, under our experimental conditions this putative interference was not relevant, because 3-AB treatment was performed on quiescent cells. Moreover, the application of BA at low concentration or a chemically related compound that did not inactivate PARP, did not affect the stability of wt p53. This was additionally confirmed by strong expression of mdm-2 that is known to be upregulated by wt p53 protein.

The monitoring of the mdm-2 level was important for two reasons. First, among several p53-responsive genes, mdm-2–P2 transcript [Bull et al., 1998] seems to be exclusively induced by p53, whereas other genes, e.g., $p21^{waf\text{-}1}$, are also regulated by alternative, p53-independend pathways [Gartenhaus et al., 1996]. Therefore, the expression of mdm-2 may serve as an indicator for the functional activity of wt p53. Secondly, mdm-2 plays a key role in the negative regulation of wt p53 expression [Haupt et al., 1997; Kubbatat et al., 1997]. Tight regulation of p53 function is critical for normal cell growth and development, and one major mechanism by which p53 function is controlled is through interaction with the mdm-2 protein. The interaction with mdm-2 can abrogate p53-mediated cell-cycle arrest and apoptosis, and can also result in a considerable reduction in the p53 level through enhanced proteasome-dependent degradation [Haupt et al., 1997; Kubbatat et al., 1997]. In this context it is noteworthy that in $p53^{135val}$ and PARP cells, wt p53 protein remained stable despite an elevated mdm-2 level. One can speculate that in these cells the physical interaction of wt p53 with mdm-2 was impaired or that other cellular components directly interfered with the negative regulation by mdm-2. One pathway protecting wt p53 from its mdm-2–regulated degradation has been reported recently. It was demonstrated that upregulated $p19^{ARF}$ is able to bind mdm-2 even if it is complexed with p53, thereby preventing p53 degradation [Pomerantz et al., 1998; Quelle et al., 1997].

These results are consistent with our previous findings and clearly show that PARP is involved in the stabilization of wt p53 protein. PARP-mediated stabilization of wt p53 offers an attractive model for control of the duration of the p53 response.

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