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Phosphorylation Regulates the Interaction and Complex Formation Between wt p53 Protein and PARP-1

Józefa Węsierska-Gądek,* Jacek Wojciechowski, and Gerald Schmid

Cell Cycle Regulation Unit, Institute of Cancer Research, University of Vienna, Vienna, Austria

Abstract We recently characterized the interaction between poly(ADP-ribose) polymerase-1 (PARP-1) and the product of the tumor suppressor gene *p53*. We investigated which domains of human PARP-1 and of human wild-type (wt) p53 were involved in this protein–protein interaction. We generated baculoviral constructs encoding full length or distinct functional domains of both proteins. Full length PARP-1 was simultaneously coexpressed in insect cells with full length wt p53 protein or its distinct truncated fragments and vice versa. Reciprocal immunoprecipitation of Sf9 cell lysates revealed that the central and carboxy-terminal fragments of p53 were sufficient to confer binding to PARP-1, whereas the amino-terminal part harboring the transactivation functional domain was dispensable. On the other hand, the amino-terminal and central fragments of PARP-1 were necessary for complex formation with p53 protein. As the most important features of p53 protein are regulated by phosphorylation, we addressed the question of whether its phosphorylation is essential for binding between the two proteins. Baculovirally expressed wt p53 was post-translationally modified. At least six distinct p53 isomeres were resolved by immunoblotting following two-dimensional separation of baculovirally expressed wt p53 protein. Using specific phospho-serine antibodies, we identified phosphorylation of baculovirally expressed p53 protein at five distinct sites. To define the role of p53 phosphorylation, pull-down assays using untreated and dephosphorylated p53 protein were performed. Dephosphorylated p53 failed to bind PARP-1 indicating that complex formation between both proteins is regulated by phosphorylation of p53. The marked phosphorylation of p53 at Ser392 observed in unstressed cells suggests that the phosphorylated carboxy-terminal part of p53 undergoes complex formation with PARP-1 resulting in masking of the NES and thereby preventing its export. The functional significance of the interaction between both proteins was investigated at two different conditions: inactivation of PARP-1 and overexpression of PARP-1. Our results unequivocally show that the presence of PARP-1 regulates the basal expression of wt p53 in unstressed cells. J. Cell. Biochem. 89: 1260–1284, 2003.

Key words: p53 phosphorylation; p53 stability; p53 nuclear export; NES; nucleocytoplasmic shuttling; cell cycle arrest, FACS analysis; 2D-PAGE; p53 isomers; p53 pull-down assay

p53, the product of a tumor suppressor gene, is a critical regulator of cell cycle progression and apoptosis (reviewed by Prives and Hall, 1999; Bargonetti and Manfredi, 2002; Blagosklonny, 2002a). It is a short-lived protein that is expressed in low concentrations in unstressed cells [Maltzman and Czyzyk, 1984; Fritsche et al., 1993]. The expression and intracellular localization of p53 varies throughout the cell cycle. p53 phosphoprotein

Abbreviations used: aa, aminoacid(s); ARF, alternative reading frame; AS, alternatively spliced; DAPI, 4′,6-diamidino-2-phenylindole; CRM1, chromosome maintenance region1; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; ECL, enhanced chemiluminiscence; FCS, fetal calf serum; HPV, human papilloma virus; HRP, horseradish peroxidase; KO, knock-out; LMB, leptomycin B; MEFs, mouse embryo fibroblasts; MDM2, mouse double minute2; MDR, multi-drug resistance; NES, nuclear export signal; NLS, nuclear localization signal; PARP-1, poly(ADP-ribose) polymerase-1; PBS, phosphate-buffered saline; PDs, Petri dishes; Rb, retinoblastoma; RS, regularly spliced; ts, temperature-sensitive; WCL, whole cell lysate; wt, wild-type.

Józefa Węsierska-Gądek and Gerald Schmid contributed equally to this work.

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Gerald Schmid's present address is KTB Tumorforschungs GmbH, Freiburg, Germany.

*Correspondence to: Dr. Józefa Wesierska-Gadek, Cell Cycle Regulation Unit, Institute of Cancer Research, University of Vienna, Borschkegasse 8 a, A-1090 Vienna, Austria.
E-mail: Jozefa.Antonia.Gadek-Wesierski@univie.ac.at

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in normal, unstressed cells is predominantly nuclear in G_1 and largely cytoplasmic during S and G_2 [Shaulsky et al., 1990; David-Pfeuty et al., 1996]. This distribution pattern is consistent with its role as a mediator of the G_1 restriction point of the cell cycle. However, in response to stress stimuli, p53 is stabilized and retained in the nucleus where it regulates the transcriptional activity of downstream genes responsible for cell cycle arrest and apoptosis [Maltzman and Czyzyk, 1984; Fritsche et al., 1993]. These observations show that the ability to retain p53 in the nucleus is essential for p53 activity as a transcription factor. The mechanisms involved in regulating p53 subcellular localization are not fully understood. The nuclear localization signal (NLS) and nuclear export signal (NES) are the essential elements determining the ability of proteins to shuttle between the nucleus and cytoplasm. p53 protein harbors three NLSs in the carboxy-terminus. The first NLS (aa 316–322) is the most active of the three in targeting p53 for nuclear import, whereas the two others are much weaker. The activated p53 protein induces the transcription of its own negative regulator, *mdm2* (human *hdm2*) gene [Momand et al., 1992]. The mdm2 protein inactivates p53 by two independent mechanisms. Firstly, it inhibits p53 activity as a transcription factor by preventing its interaction with the TBP-associated factors [Momand et al., 1992; Oliner et al., 1993]. Secondly, it targets p53 for polyubiquitination, which is followed by proteasome-mediated degradation [Haupt et al., 1997; Kubbutat et al., 1997]. Mdm2 possesses an intrinsic E3 ubiquitin ligase activity. The binding of mdm2 to p53 is essential for ubiquitination. Nuclear export seems to be required for mdm2 mediated degradation of p53 [Freedman and Levine, 1998]. Mdm2 harbors a NES and shuttles between the nucleus and the cytoplasm. Originally, there was good reason to believe that mdm2 binds p53 in the nucleus and shuttles p53 from the nucleus to the cytoplasm. However, the highly conserved leucine-rich NES that has been identified in the tetramerization domain of p53 [Stommel et al., 1999], indicates that p53 can be exported independently from mdm-2. The intrinsic NES determines the subcellular localization and mediates the nucleocytoplasmic shuttling of p53 through association with the export receptor CRM1 (exportin1) encoded by chromosome maintenance region 1 [Adachi and Yanagida, 1989]. Thus, the inhibition of nuclear export of p53 by leptomycin B (LMB) that blocks the interaction of the NES with CRM1 [Wolff et al., 1997; Kudo et al., 1998] results in the accumulation of p53 protein. The stability and nucleocytoplasmic translocation of p53 is regulated by multiple factors. Oligomerization, post-translational modifications as well as interaction with distinct proteins may regulate its nucleocytoplasmic shuttling by affecting the accessibility of the NLS and NES. The interaction of p53 with poly(ADP-ribose) polymerase-1 (PARP-1) represents one of several alternative pathways regulating the stability of p53 protein in unstressed cells. PARP-1 also seems to be a potential mediator of the intracellular distribution of p53. PARP-1 forms tight complexes with p53 and colocalizes with wt as well as with mutant p53 protein [Wesierska-Gadek et al., 1996a, 2000a]. It has previously been shown that PARP-1, upon binding to mutant $p53^{135val}$, was sequestered in the cytoplasm [Wesierska-Gadek et al., 1996a, 2000a]. After temperature shift to 32°C, the temperature at which p53 adopts wt conformation, both proteins were translocated into the nucleus.

Recently, we examined in detail the interaction between human wt p53 and PARP-1. We identified domains of p53 and of PARP-1 involved in the complex formation [Wesierska-Gadek et al., 2003]. We found that the central and the carboxy-terminal domains of p53 were capable of binding PARP-1, whereas the amino-terminal region failed to bind PARP-1. p53 is a phosphoprotein and harbors multiple serine residues that are modified by specific kinases in response to a variety of stress stimuli (reviewed by Appella and Anderson, 2001). The activation of upstream kinases seems to be stress specific and distinct kinases modify p53 at specific sites. Therefore, we decided to examine whether phosphorylation of p53 may affect its binding to PARP-1. In the first step, we determined whether baculovirally expressed wt p53 was post-translationally modified as claimed in previous reports [Fuchs et al., 1995]. For this purpose, we performed immunoblotting following two-dimensional separations of proteins. At least six distinct p53 isomers were detected by anti-p53 antibodies. Using antibodies directed against p53 forms phosphorylated at specific serine residues we showed that at least five serines of p53 expressed in insect cells were modified. To define the role of p53

phosphorylation, pull-down assays using untreated and dephosphorylated p53 protein were performed. Dephosphorylated p53 failed to bind PARP-1 indicating that complex formation between both proteins is regulated by phosphorylation of p53. The marked phosphorylation of p53 at Ser392 that occurs in unstressed cells suggests that the phosphorylated carboxy-terminal part of p53 undergoes complex formation with PARP-1 resulting in the masking of the NES and thereby preventing its export.

Finally, we explored the functional significance of the interaction between these two sensors of DNA damage. We showed that PARP-1 affected the stability of wt p53 protein. Inactivation of PARP-1 by gene disruption resulted in shortening of the p53 half-life. Remarkably, not only the endogenous but also transfected wt p53 protein showed reduced stability in the absence of functional PARP-1. The inhibition of protein export by LMB increased the stability of the endogenous as well as transfected wt p53 protein. This observation was additionally substantiated by a p53 gene reporter assay. It indicates that PARP-1, through binding to the central and carboxy-terminal domains of p53, prevents its nuclear export and accelerated degradation in the cytoplasmic proteasome. On the other hand, overexpression of PARP-1 led to stabilization of wt p53 under permissive temperature. PARP-1 mediated stabilization of wt p53 protein had serious consequences on the regulation of the cell cycle. High levels of nuclear p53 protein delayed the re-entry of G_1 arrested cells into S-phase. Our results suggest that wt p53 protein is still active as a transcription factor following complex formation with PARP-1 via the central and carboxy-terminal domains.

MATERIALS AND METHODS

Plasmids

pLTRp53cGwt, pLTRp53cGphe132, and pLTRp53cGval135 comprising a chimera of mouse p53 cDNA and genomic DNA including introns 2–9 (generous gift of Dr. M. Oren) have been previously characterized [Eliyahu et al., 1985]. The latter encodes a p53 mutant exhibiting at position 135 a substitution from alanine to valine. Plasmids encoding murine alternatively spliced (AS) wt p53 and mutant (a cys-132 → phe change) p53 were a generous gift of Dr. Varda Rotter. Plasmids encoding transcription reporters that contain the luciferase gene (expressed from a minimal thymidine kinase promoter, which is regulated by a p53 binding site, and derived from the cyclin G promoter pWT30tkluc or control ktluc) were a kind gift of Dr. K. Okamoto. The plasmid encompassing the coding sequence for human poly(ADP-ribose) polymerase-1 (PARP-1) was a generous gift of Dr. G. de Murcia. Plasmid pVV2 bearing the neo selective marker and pVEJB coding for a mutated human c-Ha-ras were used.

Cell Lines

Sf9 insect cells were used for expression of baculovirally encoded p53 and PARP-1 constructs. Mouse embryo fibroblasts (MEFs) were obtained from PARP-1 wt (A-19) and PARP-1 knock-out (KO) (A-12 and A-11) mice [Wang et al., 1995]. A-11 PARP-1 −/− cells were reconstituted with human PARP-1 [Herceg and Wang, 1999]. Cell clones expressing exogenous PARP-1 (A-11/wt2 and A-11/wt3) were positively selected with hygromycin. Primary rat embryo cells were transfected with c-Ha-ras, murine ts $p53^{135val}$, and human PARP-1. The establishment of transformed cell clones was performed according to a procedure previously described in detail [Wesierska-Gadek et al., 2000a]. Cells were cultivated at basal temperature (37°C) in DMEM supplemented with 10% FCS in an atmosphere of 7.5% CO_2. For experiments dealing with the change of the p53 phenotype, cells were shifted to 32°C for 48 h and thereafter were shifted up to 37°C for varying periods of time.

Cell Treatment

MEFs grown to 60–70% confluency were treated for 4 h with 2 μg/ml doxorubicin alone or pretreated for 2 h with 10 μM verapamil. To inhibit nuclear protein export, cells were treated for indicated periods of time with LMB (Sigma Chemical Co., St. Louis, MO) at a final concentration of 0.5 μM. In some experiments, primary rat cells overexpressing c-Ha-ras + $p53^{135val}$ + PARP-1 were treated with 3-aminobenzamide (3-AB) at a final concentration of 3 or 10 mM or with benzamide (BA) at a final concentration of 50 μM for indicated periods of time.

Antibodies

Different anti-p53 antibodies recognizing distinct epitopes were used. Monoclonal anti-p53

antibodies recognizing an epitope in the amino-terminus (DO-1), in the core domain (PAb240), or at the carboxy-terminus (PAb421) were from Oncogene Research Products (Cambridge, MA). The polyclonal anti-p53 antibody CM-1 was obtained from Novocastra (Newcastle-upon-Tyne, UK). Additionally, monoclonal antibodies to phosphoserine (clone 4A9) were from Alexis Biochemicals (Laufelfingen, Switzerland). Polyclonal antibodies (N-20) specific for the amino-terminus of PARP-1 (aa 1–20) were from Santa Cruz (Santa Cruz, CA). Monoclonal anti-PARP-1 antibodies (C-2–10) and F-1, -2, -3 were from Dr. G. Poirier and F-2 was from Santa Cruz. Additionally, rabbit polyclonal anti-human PARP-1 antibodies from Boehringer (now Roche, Vienna, Austria) were used. Antibodies against MDM-2 protein (SMP14) were from DAKO A/S (Glostrup, Denmark). Monoclonal anti-MCM7 (clone DCS141.2) and anti-actin (clone C4) antibodies were from Oncogene Research Products and ICN (Aurora, OH), respectively.

Monoclonal antibodies X-press directed against a 15 amino acid motif present in recombinant proteins or against His-Tag sequence was obtained from Invitrogen Life Technologies (San Diego, CA) and from Roche, respectively. Immune complexes were detected using appropriate secondary antibodies linked to horseradish peroxidase (HRP) from Amersham International (Little Chalfont, Buckinghamshire, UK) or from Pierce (Rockford, IL).

Cloning of p53 and PARP-1

All cDNAs were cloned into a modified baculovirus recombination vector (pBlueBac-His2B, Invitrogen). One nucleotide in the vector was changed as follows: at position 166 a G was replaced by an A generating a Bgl II restriction site at position 167 without changing the amino acid sequence of the His-tag. The resulting vector was called pBlueBacHis2BGS and all mentioned cDNAs were cloned into this newly generated Bgl II site at their 5′ ends and into the Sal I or Hind III restriction site at their 3′ ends. To generate the appropriate restriction sites flanking the coding region, PCR reactions were performed using the appropriate primers as previously described [Wesierska-Gadek et al., 2003]. The annealing temperatures of the PCR reactions were 48–56°C. Resulting cDNAs were digested and cloned into pBlueBacHis2BGS. The DNAs were sequenced in order to verify the correct cloning.

Measuring DNA Content of Single Cells by Flow Cytometry

The measurement of DNA content was performed by flow cytometric analysis according to a slightly modified method of Vindelov et al. [1983] as previously described in detail [Wesierska-Gadek et al., 2000a].

Transactivation of the p53-Responsive Genes

To examine the transcriptional activity of p53 we used the dual-luciferase reporter assay system (Promega Corporation, Madison, WI). The use of dual reporters refers to the simultaneous expression and measurement of two individual reporter enzymes within a single system and improves experimental accuracy. Normalizing the activity of the experimental reporter (firefly luciferase) to the activity of the internal control (*Renilla* luciferase) minimizes experimental variability caused by differences in cell viability or transfection efficiency. MEFs were transfected in triplicate with transcription reporters containing the luciferase gene expressed from a minimal thymidine kinase promoter regulated by a p53-binding site derived from the cyclin G promoter. In additional assays a plasmid encoding murine wt regularly spliced (RS) p53, and wt AS p53 were cotransfected. Forty-eight hours after transfection, cells were lysed and the activities of firefly and *Renilla* luciferases were measured sequentially according to the manufacturer's protocol. The activity of firefly luciferase (mean of triplicate determinations) was normalized to *Renilla* luciferase activity. Activity was expressed as fold-induction relative to cells transfected only with dual reporter.

Determination of p53 Stability

Cells were grown in 10 cm PDs for 24 h (after plating) to about 90% confluency. Thereafter, protein synthesis was inhibited by emetine which was added to a final concentration of 10 μM. Emetine treated cells were maintained in culture for increasing periods of time, then harvested and lysed in SDS sample buffer. Cellular extracts (30 μg protein) from each time point were separated electrophoretically and subjected to immunoblotting using anti-p53 PAb421. Equal protein loading was confirmed by sequential incubation of the blot with

anti-actin antibodies. The intensity of p53 bands was determined by densitometric analysis of the immunoblots [Wesierska-Gadek et al., 2002].

In another series of experiments, cells were prestarved in DMEM medium lacking methionine and pulse-chase labeled with ^{35}S-methionine as previously described [Wesierska-Gadek et al., 2000b]. Whole cell lysates prepared from radioactively labeled cells were precipitated with anti-p53 antibody PAb421. Immunoprecipitates were visualized by autoradiography and quantification was performed using a phosphoimager.

Preparation of Cell Extracts

PBS washed cells were 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 phenylmethylsulfonylfluoride (PMSF)] for 20 min at $+4^{\circ}$C. For affinity purification of baculovirally expressed proteins, insect cells were lysed directly in native binding buffer provided in the X-press purification Kit (Invitrogen Life Technologies), and cell structures were destroyed by repeated freeze/thaw procedure. The cell suspension was spun off. Clear supernatant was used for further analysis. Protein concentration of cell extracts was determined by DC assay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as standard.

Affinity Chromatography on Ni-Agarose

Cell lysates prepared in native binding buffer were loaded on preequilibrated Ni-agarose beads (ProBond, Invitrogen Life Technologies) and purification of recombinant proteins was performed according to the manufacturer's protocol. Recombinant proteins were eluted from the Ni-beads with the native elution buffer at pH 4.0 and immediately neutralized.

Pull Down Assay

To examine the effect of p53 phosphorylation on its binding ability to PARP-1, a pull down assay was performed. Full-length His-tagged PARP-1 was immobilized on Ni-beads and then human native p53 lacking the His-tag-sequence was loaded. Before loading, p53 was incubated with calf intestine phosphatase or alternatively, left untreated. After thorough washing steps, bound p53 was stepwise eluted and then analyzed by immunoblotting. Sequential eluates were designated as eluate 1, eluate 2, etc.

Immunoprecipitation

Immunoprecipitation was performed as previously described in detail [Wesierska-Gadek et al., 1996a]. Equal protein amounts of each cell lysate (150 μg protein) were used for incubation with anti-p53 antibody CM-1 and for incubation with anti-PARP-1 antibodies in a final volume of 100 μl. Immune complexes were then purified by affinity chromatography on prewashed Gamma-Bind Sepharose beads (40 μl) (Pharmacia, Uppsala, Sweden) and eluted sequentially three times with SDS sample buffer. Sequential eluates were designated as eluate 1, eluate 2, etc. Each eluate was then divided and loaded on two gels. One gel was used for immunoblotting with anti-p53 antibodies and the second for incubation with anti-PARP-1 antibodies.

Electrophoretic Separation of Proteins

Proteins were separated by a one-dimensional PAGE on 10 or 15% SDS-gels as described by Laemmli 1970 or by a two-dimensional PAGE according to O'Farell [1975] with some modifications [Wesierska-Gadek et al., 1996b]. In the lysis buffer and gel mixture, NP-40 was replaced by 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Bio-Rad). To achieve good resolution of proteins within the pH range 4–8 of isoelectrofocusing (IEF) gels, carrier ampholytes with broad (3.5–10) and narrow (4–8) pH ranges (BDH, Poole, UK) were mixed in a ratio of 1:4. For calibration, BSA and two isoforms of carbonic anhydrase were used as marker. In the second dimension, samples were run on 10% SDS slab gels. After electrophoretic separation, proteins were detected by Coomassie-Blue R-250 or silver staining of gels, or were electrophoretically separated onto a polyvinylidene difluoride membrane (PVDF) (Amersham International).

Immunoblotting

Proteins dissolved in reduced SDS sample buffer were loaded on SDS polyacrylamide gels, electrophoretically separated, and transferred onto a PVDF membrane (Amersham International). Equal loading of proteins was confirmed by Ponceau S staining. Immunodetection of antigens was performed with specific antibodies [Wesierska-Gadek et al., 2002]. The

immune complexes were detected with appropriate peroxidase-conjugated secondary antibodies routinely diluted 1:20,000 and enhanced chemiluminescence detection reagent ECL-Plus (Amersham International).

RESULTS

Expression of PARP-1 and p53 Proteins in Sf9 Cells

We first verified the expression of full-length proteins and their truncated forms. Proteins solubilized during lysis of insect Sf9 cells were separated on SDS gels and electroblotted. The identity of both full-length and distinct truncated proteins was examined by immunoblotting using specific primary antibodies. The incubation with monoclonal antibody targeting the tagged X-press sequence revealed positive signals with all tested p53 recombinants. The reactivity pattern of various antibodies used with distinct p53 and PARP-1 fragments shown in our previous paper [Wesierska-Gadek et al., 2003] is summarized in Table I and II. Thus, the pre–dicted identity of the generated fragments of p53 and PARP-1 was unequivocally confirmed by the reactivity with specific antibodies.

Post-Translational Modification of wt p53 Protein

The p53 tumor suppressor protein is post-translationally modified under physiological conditions. To examine the modifications of baculovirally expressed p53 protein, we resolved lysates prepared from insect cells expressing human and mouse wt p53 proteins by two-dimensional electrophoresis and analyzed them by immunoblotting. A number of proteins were present in cell lysates of insect cells as visualized by silver staining (Fig. 1A). Immunoblotting of the twin blots with anti-p53 PAb421 revealed that both baculovirally expressed p53 proteins were resolved to a few isomers differing in their isoelectric points (Fig. 1B,C). Human p53 isomeres (Fig. 1B) were found within a more acidic range. To exclude the possibility that the position of the spots depends on the running conditions, we separated both lysates on the same 2D-gel. As shown in Figure 2, immunoblotting revealed that both proteins differ slightly not only in size but also in their charge. Faster migrating mouse wt p53 protein was resolved into six isomers whereas human p53 protein was separated into five isomers (Fig. 2B). Indeed, human p53 isomers were more acidic than the mouse counterparts. In an additional 2D blot, we examined the pattern of murine p53 overexpressed in mammalian cells. Lysate of primary rat cells constitutively overexpressing the murine ts $p53^{138Val}$ mutant, showed many additional protein spots after 2D-PAGE visualized by silver staining (Fig. 3A) when compared to Sf9 cell extracts. The comparison of the modification pattern of mouse p53 expressed in mammalian cells and insect cells did not reveal any significant differences. This observation is of great importance with regard to the relevance of the reactivity of p53 expressed in insect and mammalian cells.

Site-Specific Phosphorylation of wt Human p53 Protein

From at least five different known modifications, phosphorylation is the most essential for regulation of the biological activity of p53. Therefore, we addressed the question of whether the p53 protein generated in insect cells is phosphorylated. We used human p53 protein with and without His-tag sequence. To ensure that the phosporylation pattern did not depend on the cultivation conditions, we loaded cell lysates prepared from two different batches. All phosphospecific antibodies were diluted to the same final concentration. As shown in Figure 4, serine residues at the positions 15, 20, 37, 46, and 392 of p53 were phosphorylated. A comparison of the intensity

TABLE I. Reactivity of Anti-p53 Antibodies

	Size (kDa)	PAb421	DO-1	PAb240 (Ab-3)	CM-1	X-press
Epitope (aa)		373–382	20–25	213–217	n.d.	tag
Full length p53	53	+	+	+	+	+
aa 1–101	~20	–	+	–	+	+
aa 102–292	~28	–	–	+	+	+
aa 102–393	~40	+	–	+	+	+
aa 292–393	~20	+	–	–	+	+

n.d., not determined.

TABLE II. Reactivity of Anti-PARP-1 Antibodies

		N-20	Boe	C-2-10	F-1-2-3	F-2
	Size (kDa)	Polyclonal	Polyclonal	Monoclonal	Monoclonal	Monoclonal
Full length PARP-1	116	+	+	+	+	+
aa 1–232	~32	+	+	−	+	−
aa 1–524	~50	+	+	+	+	−
aa 232–372	~20	−	+	+	−	−
aa 232–524	~40	−	+	+	−	−
aa 524–1014	~45	−	+	−	−	+

of the phosphospecific bands showed the strongest signal for phosphoserine at 392. However, the resolution of the reactive bands was poor under the conditions used. Repeated experiments with ten-fold higher diluted primary antibody revealed well-resolved phosphospecific bands. On the other hand, the weakest signal was detected for Ser 37. No differences in the phosporylation pattern were observed between tagged and untagged p53 forms. These results are consistent with the previous observations [Fuchs et al., 1995] and show that p53 expressed in insect cells is modified at the same sites as observed in mammalian cells, and that the highest level of phosphorylation was detected for serine at the position 392.

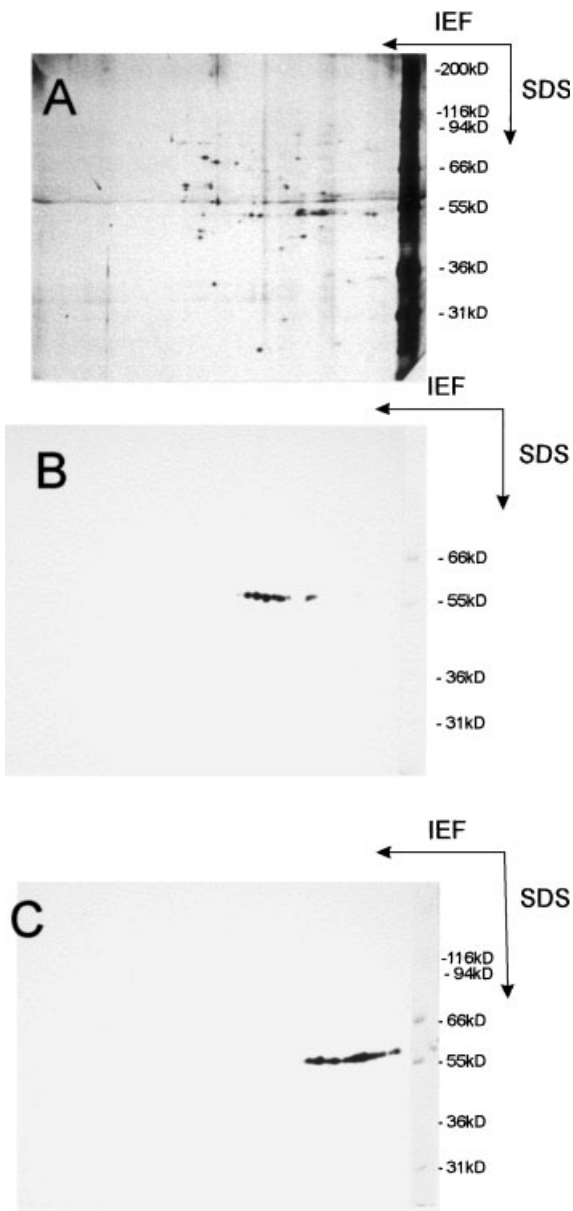

Fig. 1. Detection of multiple isomers of baculovirally expressed wt p53. Lysates prepared from Sf9 cells expressing human or murine wt p53 were separated by 2D-PAGE. Twin gels were prepared for each sample. One gel was silver stained to visualize the cellular proteins (**A**) and the second gel (**B** and **C**) was electroblotted. Proteins (20 μg) of Sf9 cells overexpressing human p53 protein (A, B) and murine p53 (C) were loaded on IEF gels. After electrofocusing, gels were run in the second dimension on 10% SDS-gel. p53 was detected after incubation with anti-p53 monoclonal antibody PAb421.

Reciprocal Co-Immunoprecipitation of p53 and PARP-1

To determine whether the generated constructs expressed in insect cells form complexes, we cotransfected full-length PARP-1 with full-length wt p53 protein or its distinct truncated fragments and vice versa. We then used equal amounts of cellular proteins for independent immunoprecipitations using anti-p53 and anti-PARP-1 antibodies. In the following experiments we used lysates of insect cells simultaneously coexpressing full-length PARP-1 with distinct p53 domains for immunoprecipitation.

Full-length p53 and two p53 fragments aa 102–292 and 293–393 were detected in the immune complexes precipitated by polyclonal anti-PARP-1 antibody (Fig. 5). However, the anti-PARP-1 antibody failed to precipitate the

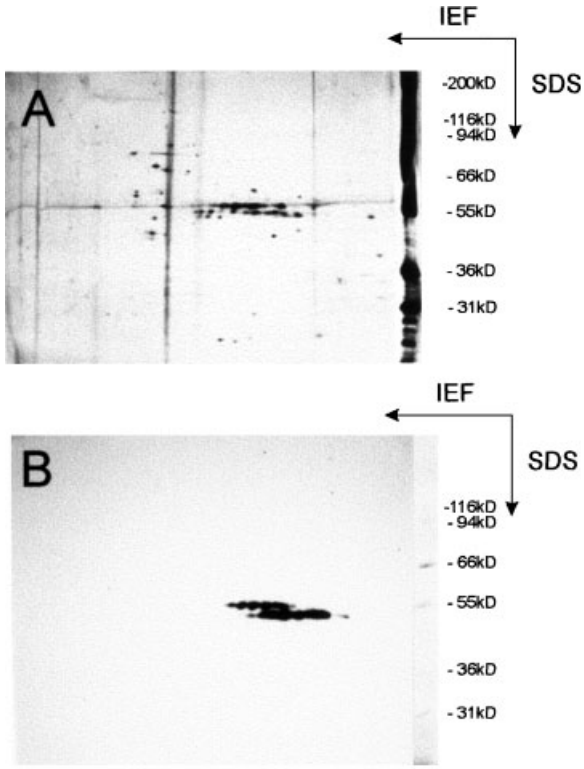

Fig. 2. Distinct pattern of human and murine p53 isomers. Lysates (10 μg protein) prepared from Sf9 cells expressing human or murine wt p53 were combined and separated by 2D-PAGE. Twin gels were prepared for each sample. Conditions of separation as in Figure 1. One gel was silver stained to visualize the cellular proteins (**A**) and the second gel was electroblotted (**B**). p53 was detected after incubation with anti-p53 monoclonal antibody PAb421 recognizing the antigen of human as well as of mouse origin.

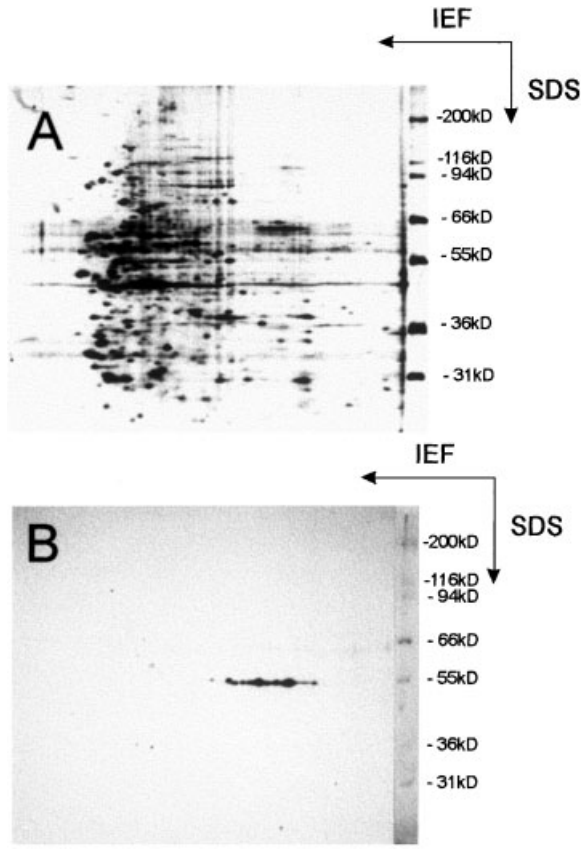

Fig. 3. Pattern of ts $p53^{135val}$ overexpressed in primary rat cells. The cell lysate (55 μg protein) was resolved by 2D-PAGE. Twin gels were prepared for the sample. One gel was silver stained to visualize the cellular proteins (**A**) and the second gel was electroblotted (**B**). Conditions of separation and immunoblotting as in Figure 1.

short aa 1–102 p53 fragment. Analysis of the non-bound fraction (not shown) or the other half of the samples precipitated by anti-p53 CM-1 antibody as well as sample used for precipitation (not shown), revealed the presence of a strong signal for the truncated aa 1–102 protein, thereby substantiating the evidence that the amino-terminus of p53 did not complex with PARP-1. The lack of the binding of this p53 fragment to PARP-1 was also confirmed by results of other experiments. The immunoblotting of complexes precipitated by anti-p53 CM-1 antibody revealed a very weak signal for full-length PARP-1 in samples coexpressing full-length PARP-1 with p53 aa 1–102, but a strong signal in samples coexpressing PARP-1 with aa 102–292 and aa 293–393. Remarkably, a comparison of the bands representing p53 fragments precipitated with anti-PARP-1 antibody shows the higher intensity of the band aa 293–393 indicating that the distal carboxy-terminal fragment possesses higher affinity to PARP-1.

Thus, the thorough analysis of the samples obtained by reciprocal immunoprecipitation shows that the central and carboxy-terminal p53 domains are involved in the complex formation with PARP-1, whereas the amino-terminus of p53 harboring the transactivation domain is dispensable. The carboxy-terminal region of p53 harbors motifs that are crucial for its intracellular localization as well as for its activity. The NLSs and NES are the essential elements for nucleocytoplasmic shuttling whereas the tetramerization domain is involved in tetramer formation. On the other hand, the carboxy-terminal part of PARP-1 encompassing the catalytic domain has very low affinity to human p53 protein and seems not to mediate binding to p53.

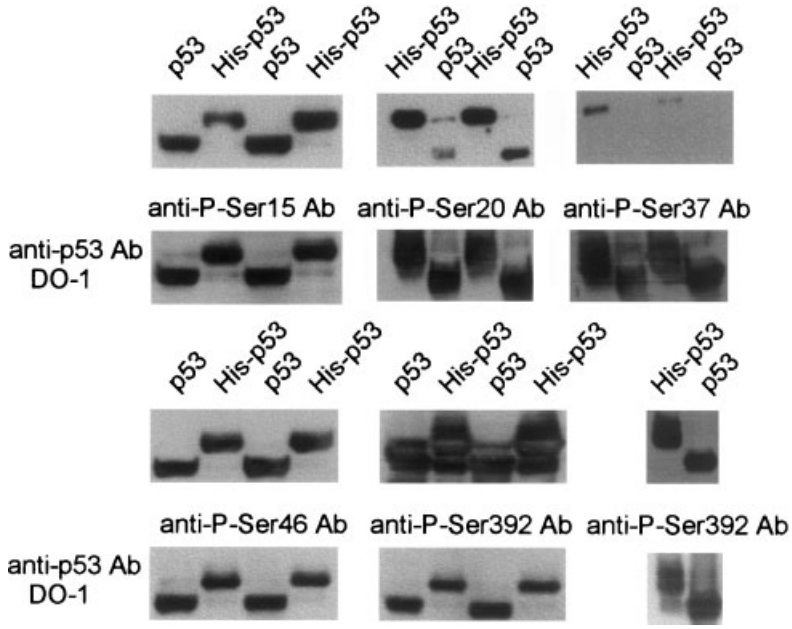

Fig. 4. Site-specific phosphorylation of human wt p53 expressed in insect cells. Sf9 cell lysates expressing human wt p53 protein without and with His-tag sequence prepared from two different batches were resolved on 10% SDS gels and transferred electorphoretically onto the PVDF membrane. Blots were incubated with antibodies directed against Phospho-p53 modified at distinct serine residues (Cell Signaling Technology) at a final concentrations of 1:5,000. Then the total p53 protein was visualized after incubation with anti-p53 antibody DO-1.

Pull Down Assay

To perform the pull down assay, we immobilized PARP-1 on Ni-beads and then loaded human p53 lacking the His-tag-sequence. The bound p53 was eluted and then analyzed by immunoblotting. To examine whether the phosphorylation of p53 was essential for its ability to complex with PARP-1, we compared the binding capacity of native and dephosphorylated full-length p53 protein. The phosphorylation state was checked by immunoblotting using antibodies specific for phosphoserine. As shown in Figure 6A, the phosphoserine signal observed in untreated p53 samples completely disappeared after treatment of p53 with alkaline phosphatase. To prove the specificity of the monoclonal antibody a phosphoserine positive control delivered by the manufacturer was loaded in the same blot.

As shown in Figure 6B, PARP-1 bound substantial amounts of native p53. Even in the forth eluate, a p53 signal was detected. However, dephosphorylation of p53 abolished its ability to form complexes with PARP-1. No p53 was eluted showing that dephosphorylated p53 was not retained on immobilized PARP-1. Thus, the lack of pull down of phosphatase-treated p53 is attributable to the loss of affinity between dephosphorylated p53 and PARP-1.

Effect of PARP-1 on the Stability of wt p53 Protein In Vivo

To assess the effect of PARP-1 on the expression of wt p53, we examined the basal stability of p53 protein under two quite different biological conditions: a lack of PARP-1 protein or its overexpression, respectively. As shown in Figure 7, the concentration of wt p53 protein in mouse cells lacking PARP-1 was reduced to an undetectable level. It is known that steady-state levels of p53 protein are regulated primarily by the alteration of its stability and that after genotoxic stress stimuli p53 protein is induced by its stabilization. Remarkably, after the treatment of mouse cells with a high dose of doxorubicin p53 protein levels increased and a p53 signal was detected in PARP-1 deficient cells. Pretreatment of cells with verapamil enhanced the action of doxorubicin. However, doxorubicin strongly induced p53 protein in their wt counterparts. The combined treatment with verapamil did no visible effect on p53 concentration. These results are consistent with previous observations that PARP-1 deficient cells are resistant to some anti-cancer drugs due

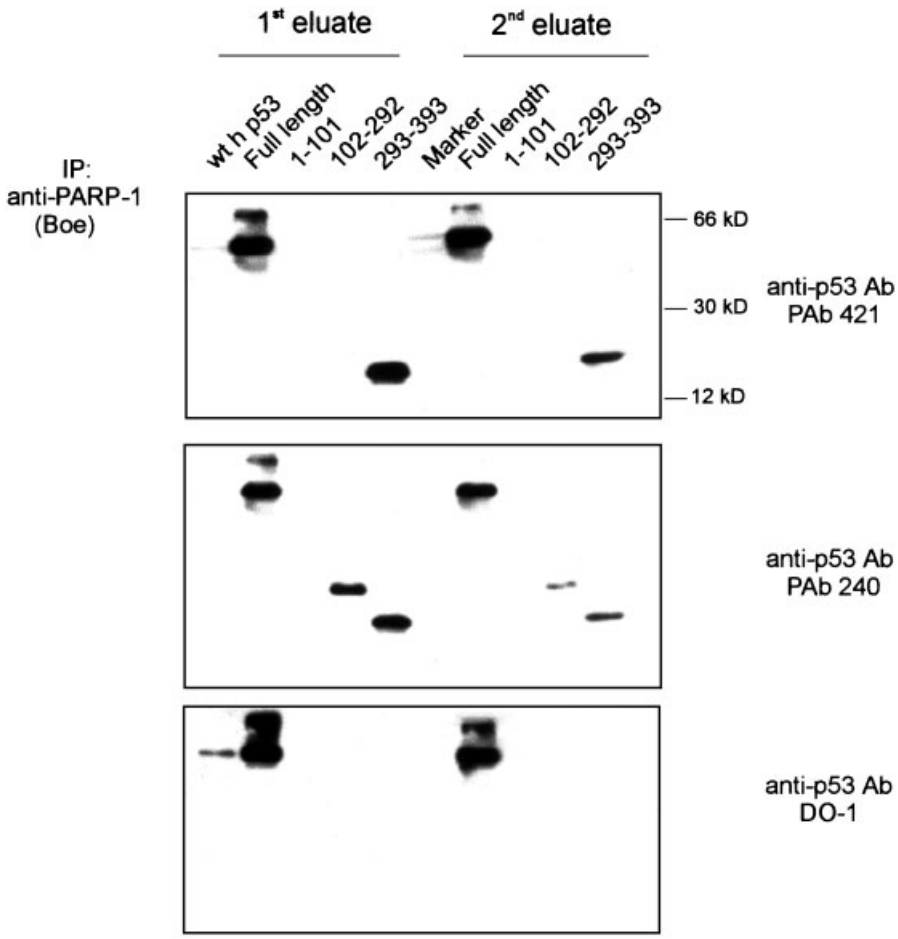

Fig. 5. Identification of p53 domains involved in the complex formation with PARP-1. Lysates prepared from Sf9 cells simultaneously expressing full-length human PARP-1 and distinct fragments of human p53 were divided and used for reciprocal immunoprecipitation. Affinity purified immune complexes were analyzed by immunoblotting. Two eluates were loaded on the gel. Conditions of separation and immunoblotting as in Figure 4.

to enhanced expression of P-gp protein [Wurzer et al., 2000]. Inhibition of P-gp activity by MDR modulator, verapamil increased action of doxorubicin in PARP-1 KO MEFs. Undetectable levels of p53 protein in individual cells lacking PARP-1 were also shown by immunofluorescence staining (Fig. 8B). Interestingly, inhibition of protein export by LMB resulted in the upregulation of p53 in a time-dependent manner and led to nuclear accumulation of p53 in PARP-1 KO cells (Fig. 8A,B). This result substantiates the assumption that the stability of p53 protein in cells lacking PARP-1 was significantly reduced.

To explore the mechanism contributing to the reduced expression of p53 in mouse cells lacking PARP-1, we determined the possible differences between wt and PARP KO cells in the kinetics of p53 degradation. Mouse cells at about 90% confluency were incubated in culture with emetine to inhibit protein synthesis. The relative amounts of p53 protein remaining at various points after emetine addition were then quantified by densitometric analysis of immunoblots. As illustrated in Figure 9A, steady-state levels of p53 protein in wt MEFs were relatively constant. The half-life determined after addition of emetine was approximately 7.5 h. However, a striking difference was found in cells lacking PARP-1. No p53 positive signals were detected (Fig. 9A). In the control and also after short chase time, p53 protein was not detectable. WCL obtained from human cervix carcinoma HTB-31 cells, which is known to overexpress mutant p53 protein, was loaded in the last lane as a positive control. This showed a

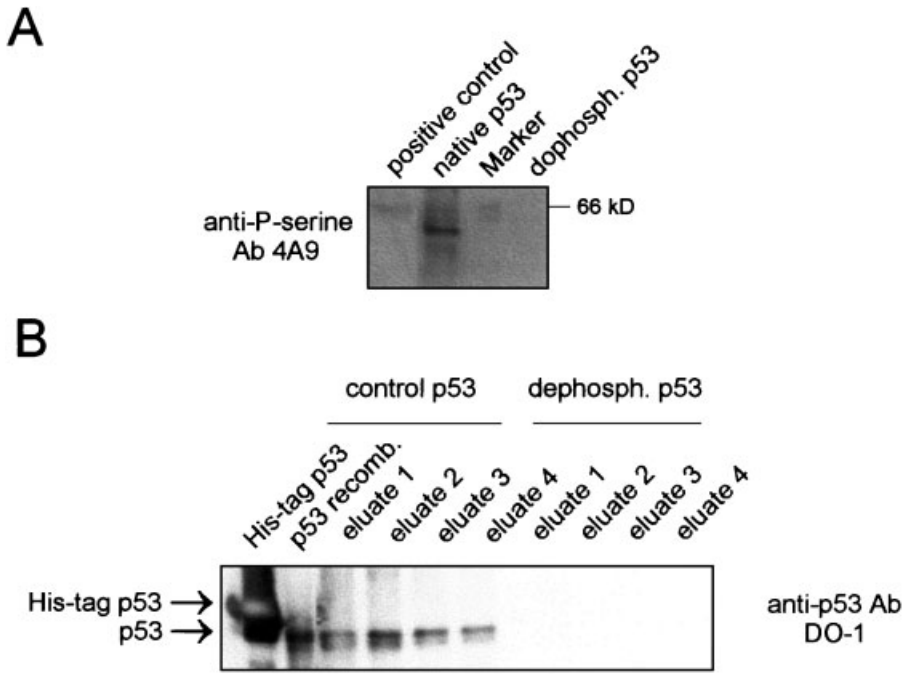

Fig. 6. Loss of the PARP-1 binding ability after dephosphorylation of p53 protein. **A**: Control and alkaline phosphatase treated p53 was resolved on 10% SDS gels and blots were probed with antibodies against phosphoserine 4A9. Phosphoproteins from rabbit muscle purified by affinity chromatography were loaded as a positive control. **B**: Pull down assay. PARP-1 was immobilized on ProBond (Invitrogen) beads. Equal amounts of untreated or dephosphorylated human wt p53 without the tag-sequence were loaded on the beads. After incubation and extensive washing, the bound fraction was pulled down and analyzed by immunoblotting. Human His-tag p53 and untagged p53 recombinant proteins were loaded as positive control. Both recombinant proteins differ slightly in size.

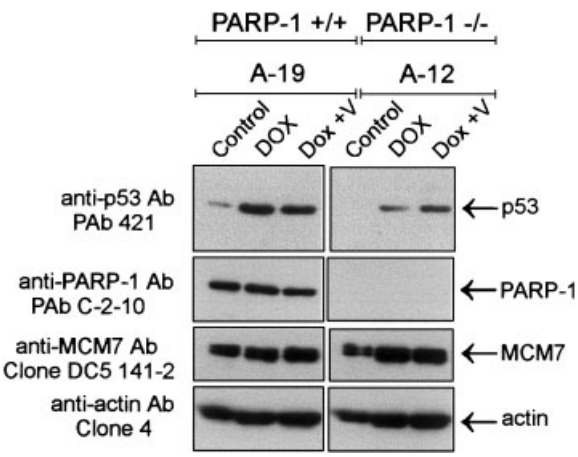

Fig. 7. Lack of basal expression of wt p53 protein in PARP-1 KO cells due to its reduced stability. **A**: No induction of p53 response in PARP-1 KO cells upon cellular stress. Normal (A-19) and PARP-1 KO (A-12) cells were treated with 2 μg/ml doxorubicin for 4 h or were pretreated with 10 μM verapamil. WCLs (30 μg protein/lane) were resolved on a 10% SDS gel. p53 protein was detected with the monoclonal anti-p53 antibody PAb421. The blot was sequentially incubated with anti-PARP-1, with anti-MCM-7, and finally with anti-actin antibodies.

strong p53 signal thereby evidencing that the immunoblotting worked and the lack of p53 detection in samples obtained from PARP-1 KO cells is specific. This was surprising because it is known that inhibition of transcription or translation by various agents immediately increases the level of p53 protein [Ljungman et al., 1999; Blagosklonny, 2002b]. To verify that the inactivation of PARP-1 had no effect on the protein stability per se, similar half-life studies were performed on MCM7. No substantial differences in the stability of this nuclear protein were found between PARP-1 KO cells and the normal counterparts.

It was, therefore, necessary to use another method to determine the half-life of p53 in PARP-1 deficient cells. We chose a short pulse-labeling followed by a chase. Samples obtained from wt and PARP-1 cells were resolved on the same gel allowing a direct comparison of the intensity of the p53 bands. Radiolabelled in vitro translated murine wt p53 was loaded as a positive control. Pulse-chase experiments combined with immunoprecipitation using anti-p53 antibody PAb421 (Fig. 9B, upper panel)

revealed a strong radioactively labeled p53 band in normal MEFs and its intensity remained almost unchanged after a chase for 30 min. Quantification of the radioactive band precipitated with the anti-p53 antibody CM-1 in a phosphoimager revealed a half-life of p53 of approximately 50 min confirming previously published data [Freedman and Levine, 1998; Wesierska-Gadek et al., 2000a]. However, the intensity of radioactive p53 protein precipitated by PAb421 from lysates obtained from cells lacking PARP-1 was significantly weaker than that in normal counterparts. After a chase for 30 min, the p53 band completely disappeared. In cells treated with LMB, the intensity of the p53 band slightly increased and the p53 signal was maintained after chase for 30 min. Therefore, to determine the half-life of p53 protein in PARP-1 KO cells, a pulse-labeling was performed using higher specific activity of the substrate. The labeling was chased in short intervals. As shown in Figure 9B (middle panel) the intensity of the p53 band was significantly reduced after chase for 5 min. The p53 half-life determined by measurement of the intensity of the radioactive p53 band in a phosphoimager was 7 min. Reconstitution of PARP-1 KO cells with human PARP-1 increased the stability of p53 protein (Fig. 9B, lower panel).

In the following experiments, we addressed the question whether the stability of murine p53 protein transiently expressed in wt and PARP-1 deficient MEFs would be comparable. We transfected mouse fibroblasts with plasmids encoding RS or AS p53, respectively, both in wt as well as mutant (phe132) form. It became evident that the PARP-1 status of mouse cells is essential for the stability of the transfected wt p53 protein but not for the mutant form (Fig. 10). To eliminate the possibility that the lack of detectable wt p53 protein in PARP-1 KO cells is due to a reduced uptake of the plasmids, we cotransfected with a sequence coding for GFP. The expression of GFP was comparable in all PARP-1 KO samples, irrespective of the type of plasmid used for transfection (not shown). Moreover, the inhibition of protein export after addition of LMB 20 h prior to cell harvesting resulted in the appearance of wt p53 protein in PARP-1 deficient cells (Fig. 10). This showed that PARP-1 regulates the stability not only of the endogenous but also of the transfected p53 protein, and only the steady-state of wt p53 protein depends on PARP-1 status. Taken together these results indicate that in cells lacking PARP-1 wt p53 protein is extremely unstable due to hyperactive nuclear export. In normal cells the binding of the carboxy-terminal domain of p53 that harbors the NES, to PARP-1 may mask the NES and may prevent the access to nuclear export machinery.

In the last series of experiments we compared the ts p53 expression in transformed cells possessing endogenous PARP-1 or expressing

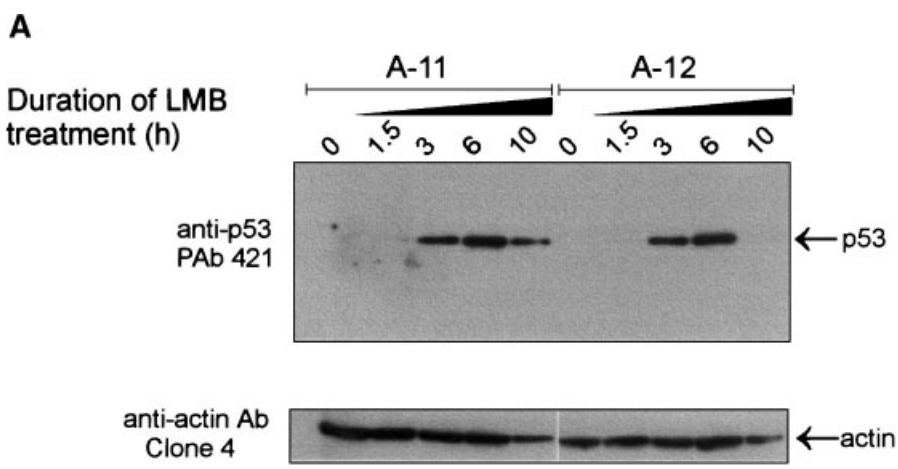

Fig. 8. Increase of p53 protein in PARP-1 KO cells after inhibition of protein export. **A**: Kinetics of the increase of cellular p53 protein in cells lacking PARP-1 after inhibition of protein export. PARP-1 KO (A-11 & A-12) cells were treated with 0.5 μM LMB for indicated periods of time. WCLs (20 μg protein/lane) were resolved on a 10% SDS gel. p53 protein was detected with the monoclonal anti-p53 antibody PAb421. Equal protein loading was confirmed by Ponceau S staining (not shown) and by incubation with anti-actin antibodies. **B**: Nuclear accumulation of p53 in PARP-1 KO cells after inhibition of protein export by LMB. Normal (A-19) and PARP-1 KO (A-11) cells were treated with 300 nM LMB for 6 h. The control and LMB treated cells were then acetone–methanol fixed and immunostained with anti-p53 antibody PAb421. Nuclei were visualized with DAPI.

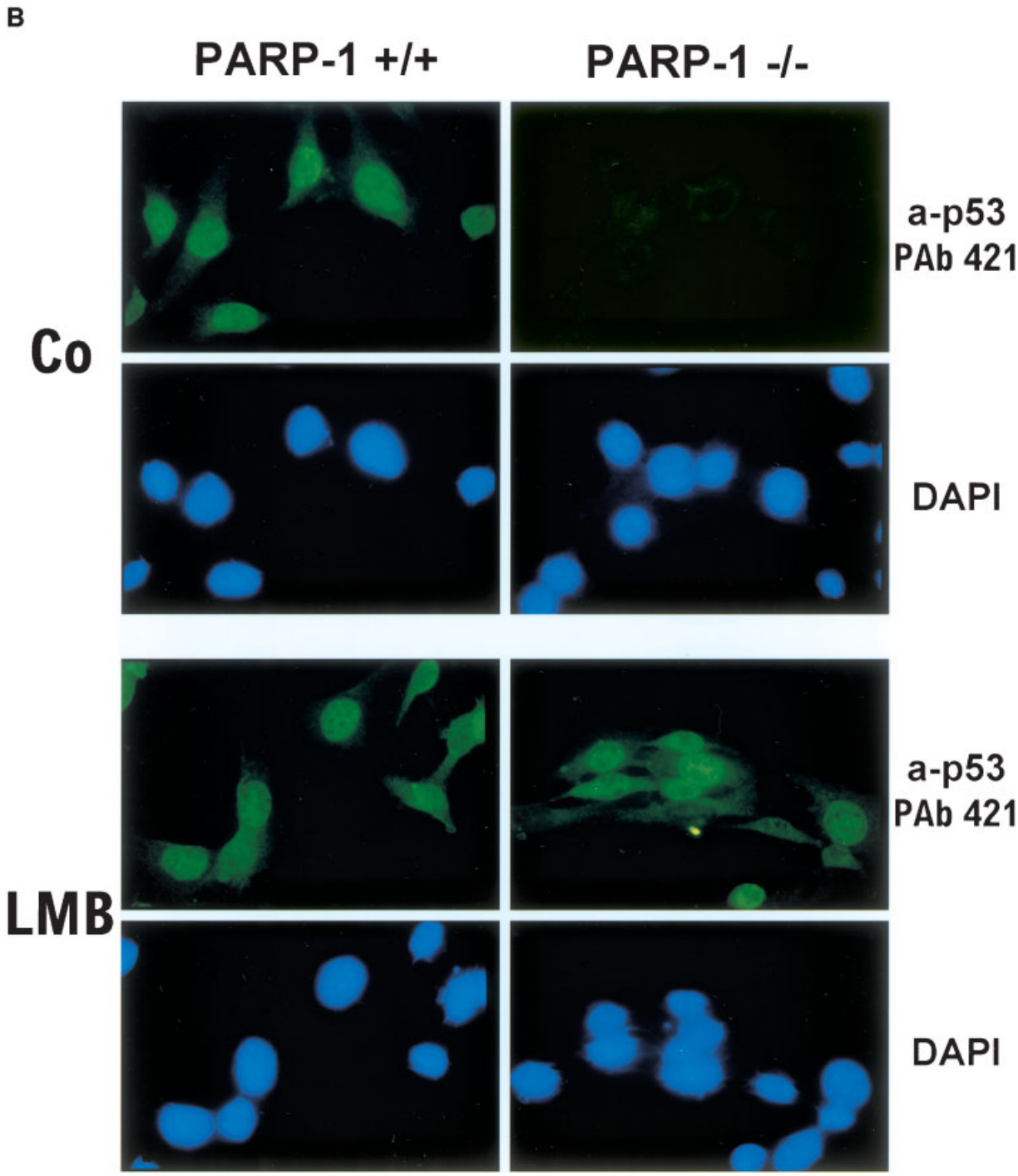

Fig. 8. (*Continued*)

human PARP-1 maintained for increasing periods of time at permissive temperature. The c-Ha-ras transformed cells overexpressing ts $p53^{135val}$ alone or in combination with human PARP-1 were previously established. The expression of c-Ha-ras, p53, and PARP-1 in different clones was examined by Northern blot and by immunoblotting. Due to features of the ts p53 mutant, at basal (37°C) and elevated (39°C) temperatures, p53 protein resides in the cytoplasm and exhibits mutant character [Eliyahu et al., 1985]. After temperature shift to 32°C, p53 protein moves into the nucleus within a few hours and simultaneously adopts

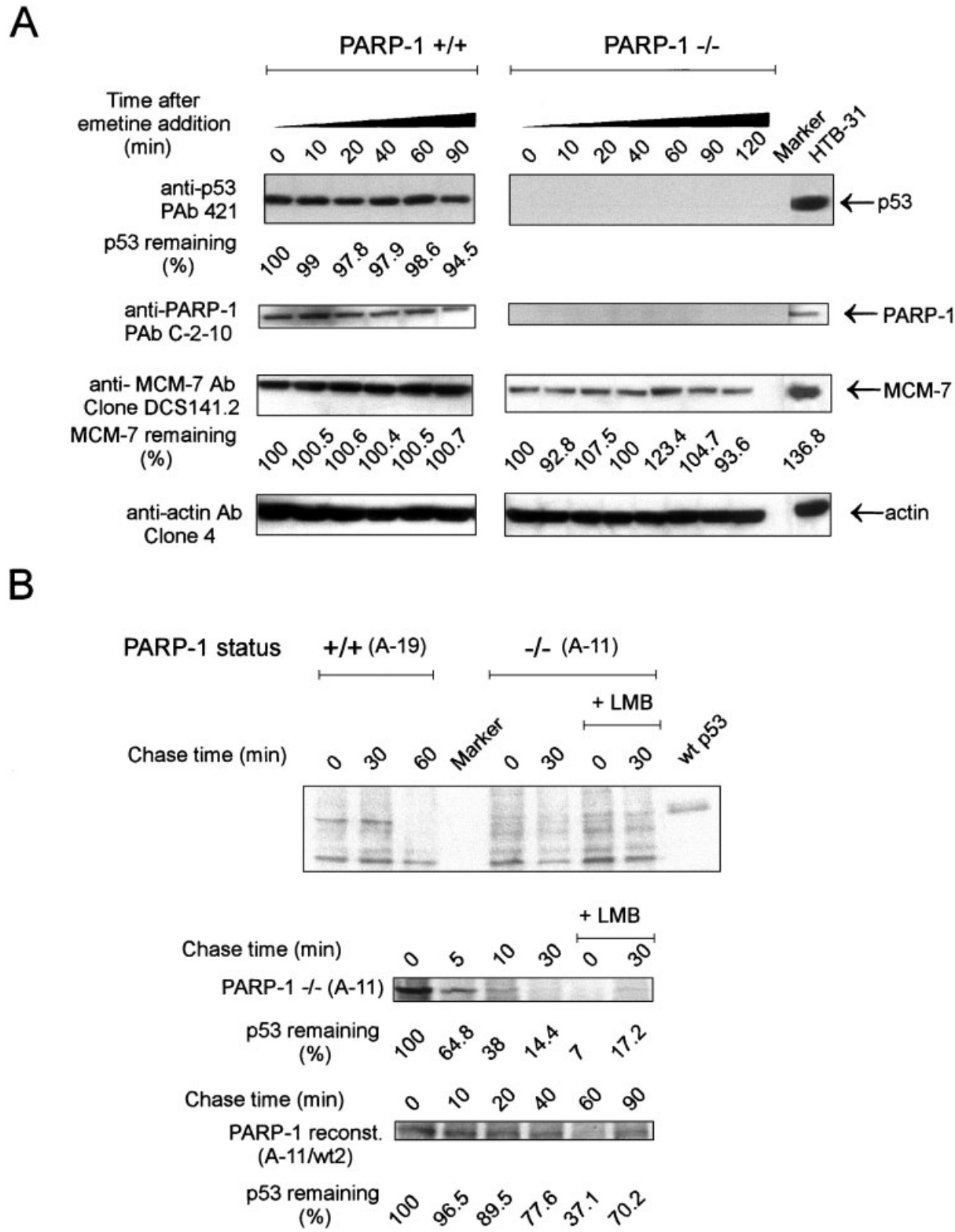
A
PARP-1 +/+
PARP-1 -/-
Time after emetine addition (min)
0 10 20 40 60 90
0 10 20 40 60 90 120
Marker
HTB-31
anti-p53 PAb 421
p53
p53 remaining (%)
100 99 97.8 97.9 98.6 94.5
anti-PARP-1 PAb C-2-10
PARP-1
anti- MCM-7 Ab Clone DCS141.2
MCM-7
MCM-7 remaining (%)
100 100.5 100.6 100.4 100.5 100.7
100 92.8 107.5 100 123.4 104.7 93.6 136.8
anti-actin Ab Clone 4
actin
B
PARP-1 status
+/+ (A-19)
-/- (A-11)
+ LMB
Chase time (min)
0 30 60 Marker 0 30 0 30 wt p53
+ LMB
Chase time (min)
0 5 10 30 0 30
PARP-1 -/- (A-11)
p53 remaining (%)
100 64.8 38 14.4 7 17.2
Chase time (min)
0 10 20 40 60 90
PARP-1 reconst. (A-11/wt2)
p53 remaining (%)
100 96.5 89.5 77.6 37.1 70.2

Fig. 9.

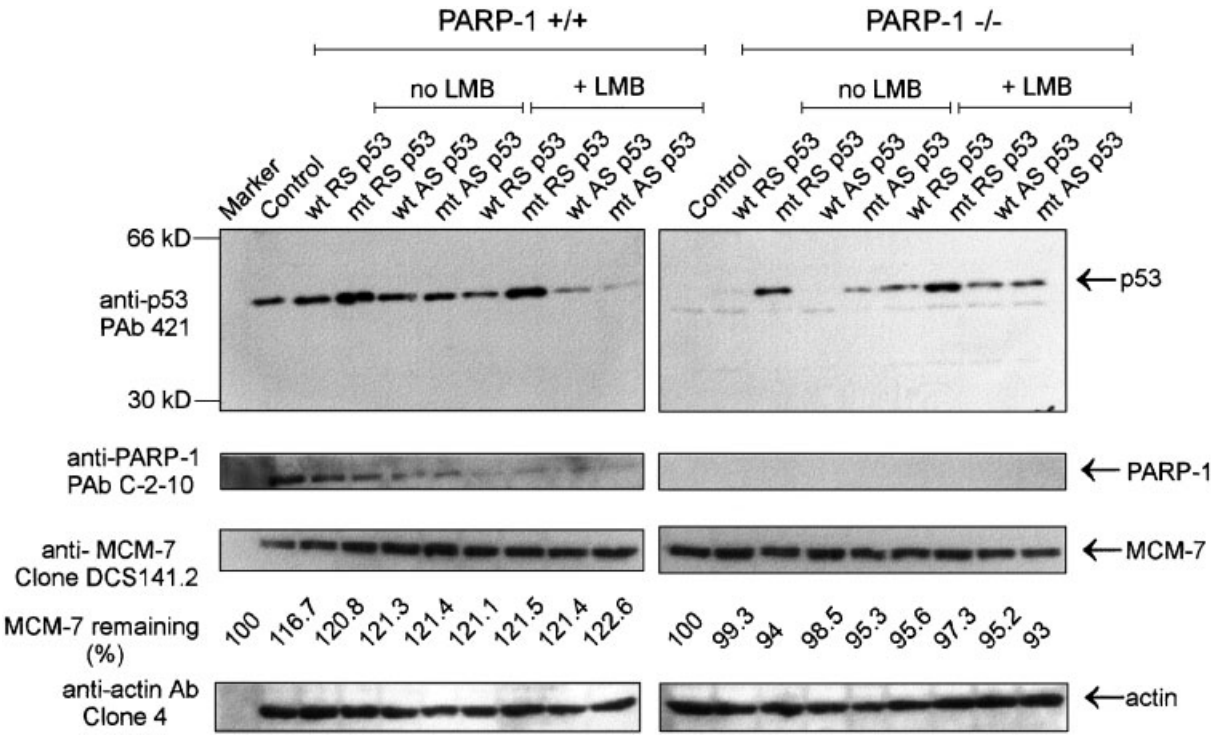

Fig. 10. Reduced stability of transiently expressed wt p53 protein in PARP-1 KO cells. Normal (A-19) and PARP-1 KO (A-11) cells were transfected with equal amounts of plasmids (2 μg) encoding murine wt and mutant p53 in both splicing forms: RS and AS. In an additional assay, GFP was cotransfected to assess the transfection efficiency for each combination. Medium was changed 24 h post transfection. Four hours after medium change LMB was added to a final concentration of 300 nM to a subset of PDs. Forty eight hours post-transfection cells were collected and lysed. WCLs (20 μg/lane) were resolved on 10% SDS gels. Immunoblotting conditions were as described in Figure 7.

wt conformation. Cells grown at basal temperature to 60–70% confluency were shifted for indicated periods of time to 32°C. As shown in Figure 11A simultaneous coexpression of $p53^{135val}$ and PARP-1 increased the steady state of wt $p53^{135val}$ protein. Even after the maintenance of cells for 4 days at permissive temperature, the level of p53 was not reduced (Fig. 11A). In contrast, in cells overexpressing $p53^{135val}$ alone, the concentration of p53 decreased gradually during the cultivation of cells at permissive temperature. The decrease of wt p53 protein in cells possessing endogenous PARP-1 levels coincided with the diminution of the expression of mdm-2 protein, which is the main p53 downstream target. The PARP-1 mediated stabilization of wt p53 at permissive temperature depended on PARP-1 enzymatic activity. Treatment of cells with 3-AB, a competitive inhibitor of PARP-1, affected the level of p53 protein in a dose-dependent manner. After longer treatment of cells (for 72 and 96 h) wt p53 became undetectable even at a lower concentration of 3-AB (Fig. 11B). On the other hand, application of BA at a concentration that did not impair PARP-1 activity in vivo, did not

Fig. 9. (Overleap) Decreased stability of wt p53 protein in PARP-1 deficient mouse fibroblasts. **A**: Determination of the p53 stability in MEFs. Normal (A-19) and PARP-1 KO (A-11) MEFs were exposed to emetine to inhibit protein synthesis for the indicated periods of time. WCLs (30 μg/lane) were separated on 10% gels. Immunoblotting was performed with the anti-p53 antibody PAb421. Cell lysate obtained from human cervix carcinoma HTB-31 cells expressing mutant p53 was loaded as a p53 positive control. Human p53 was visualized after incubation with anti-p53 antibody DO-1. The blots were sequentially incubated with anti-PARP-1, with anti-MCM-7 and with anti-actin antibodies. **B**: Decrease of the p53 half-life in PARP-1 −/− cells. Proteins were labeled in vivo with ^{35}S-methionine for 1.5 h in the presence or absence of 100 nM LMB, respectively, and then chased in medium containing an excess of cold methionine for the indicated periods of time and thereafter lysed. Lysates were immunoprecipitated with anti-p53 antibody PAb421 and separated on 10% SDS-gels. Radiolabelled in vitro translated murine wt p53 was loaded as a positive control. Precipitated radioactively labeled proteins were quantified by scintillation counting and by measurement of the intensity of the p53 band in a phosphoimager.

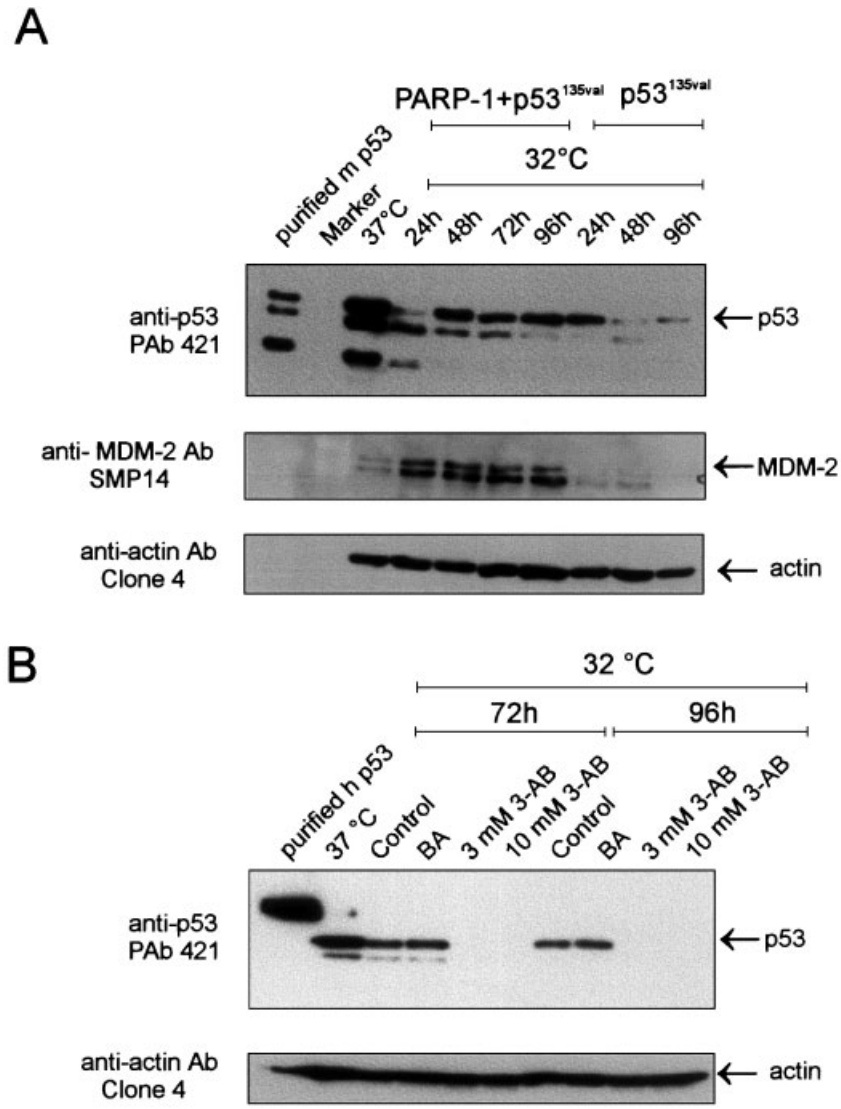

Fig. 11. Increased steady-state of wt $p53^{135val}$ in cells coexpressing human PARP-1. **A**: Cell clones expressing c-Ha-ras + wt $p53^{135val}$ alone or in combination with PARP-1 cultivated at 37°C were shifted to 32°C for the indicated periods of time. Cells were lysed and analyzed on 10% SDS gels. Immunoblotting conditions as described in Figure 7. The blot was sequentially incubated with anti-MDM2 antibodies. **B**: Enzymatic activity of PARP-1 is necessary for stabilization of wt p53 protein. Cells expressing c-Ha-ras + wt $p53^{135val}$ + PARP-1 maintained at 32°C for indicated periods of time were treated with the PARP-1 inhibitors 3-AB and BA. Cells were lysed and analyzed on 10% SDS gels. Immunoblotting conditions as described in Figure 7.

reduce the level of wt p53 protein (Fig. 11B). These data show that elevation of PARP-1 expression contributes to the stabilization of wt p53 protein, and that the enzymatic activity of PARP-1 is essential for this action.

Inhibition of Protein Export Induces p53 Phosphorylation at Serine 392

The inhibition of protein export by LMB resulted in a time-dependent accumulation of p53 in two PARP-1 KO cell lines (Fig. 8A,B). Monitoring of the total cellular level of p53 by immunblotting and its nuclear accumulation detected by immunofluorescence revealed an increase of p53 levels after 3 h of LMB treatment. p53 protein reached the highest concentration after 6 h. LMB is known to block the interaction of the export receptor with CRM1 (exportin1), thereby inhibiting the export [Kudo et al., 1998, 1999]. The p53 NES consisting of nine predominantly hydrophobic amino acid residues is embedded within the tetramerization domain. We examined whether the p53 which accumulated in PARP-1 KO cells after

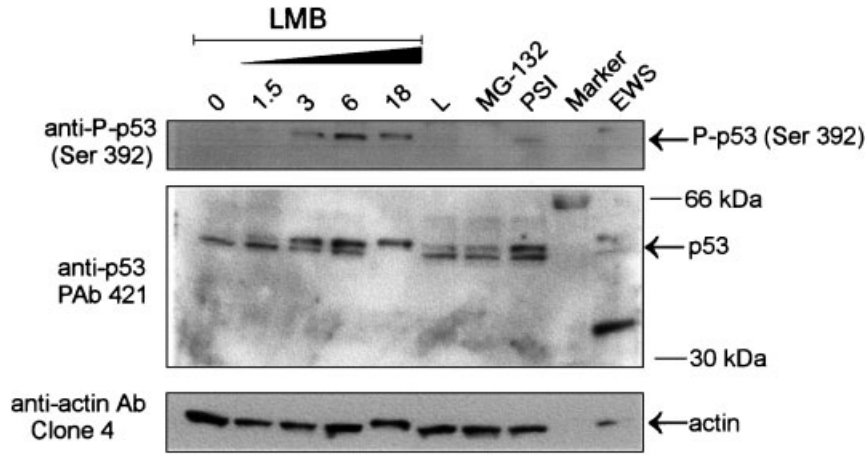

Fig. 12. Induction of p53 phosphorylation in PARP-1 KO cells after inhibition of protein export. PARP-1 KO (A-11) cells were treated with 0.5 μM LMB for indicated periods of time or with proteasome inhibitors (lactacystin (L), MG-132, and proteasome inhibitor I (PSI)) for 24 h at a final concentration of 5 μM. WCLs (20 μg protein/lane) were resolved on a 10% SDS gel. The blot was incubated with antibody against Phospho-p53 (Ser392) and sequentially with the monoclonal anti-p53 antibody PAb421. Equal protein loading was confirmed by incubation with the monoclonal anti-actin antibody. Cell lysate prepared from Ewing sarcoma cell line overexpressing ts p53 $p53^{138val}$ (EWS) was loaded as a p53 positive control. Human ts p53 $p53^{138val}$ protein was visualized after incubation with anti-p53 DO-1 antibody.

LMB treatment was phosphorylated within the carboxy-terminus. As shown in Figure 12 LMB induced time-dependent phosphorylation of p53 at Ser 392. The site specific phosphorylation coincided with the onset of p53 accumulation. It seems to be specific because proteasome inhibitors did not induce this kind of modification (Fig. 12).

Reduced Transactivation of the p53-Target Genes in PARP-1 KO Cells

To examine the functional consequences of the reduced stability of p53 protein in the absence of functional PARP-1, we performed a gene reporter analysis. We used the dual reporter assay system that allows the minimization of experimental variability caused by differences in cell viability or transfection efficiency. We normalized the activity of the experimental reporter (firefly luciferase) to the activity of the internal control (*Renilla* luciferase). As shown in Figure 13, cotransfected wt p53 transactivated p53 responsive genes in normal, but not in PARP-1 KO MEFs. The extent of induction of luciferase activity in wt MEFs correlated with the amounts of introduced wt p53. RS p53 activated the p53-responsive genes stronger than AS p53 protein. The lack of activation of luciferase in PARP-1 KO cells is attributable to extreme instability of exogenous p53 protein as illustrated in Figure 10.

Overexpressed PARP-1 Mediates a Delay of the Cell Cycle Re-Entry of G_1 Arrested cells

To explore the functional consequences of the persistence of wt $p53^{135Val}$ in G_1 arrested cells maintained for a few days at 32°C, we examined the recovery of G_1 arrested cells in the active cell cycle after their shift up to the basal temperature (37°C). To ensure that the G_1 arrest was complete, cells were maintained at 32°C for 48 h and then shifted up. A comparison of the profiles

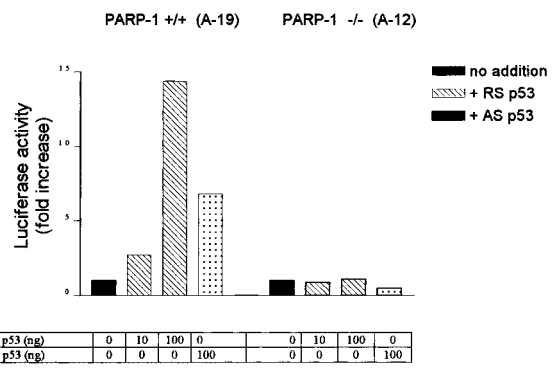

Fig. 13. Lack of p53 mediated transactivation in PARP-1 deficient cells. Normal (A-19) and PARP-1 KO (A-11) cells were transfected with a transcription reporter containing the luciferase gene expressed from a minimal thymidine kinase promoter regulated by a p53-binding site derived from the cyclin G promoter and control reporter alone or in combination with plasmids encoding both murine splice variants of wt p53. Luciferase activity was normalized as described in Materials and Methods. The activity was expressed as fold induction relative to cells transfected only with dual experimental reporters.

of propidium iodide stained cells (Fig. 14A and Table III) revealed that c-Ha-ras transformed cells expressing solely $p53^{135val}$ rapidly entered active cell cycle. After 12 and 24 h, about 8 and 20%, respectively, of the total cell population was in S-phase. After 72 h, S- and G_1-phase reached the same level. In contrast, in cells simultaneously overexpressing PARP-1 and $p53^{135val}$ the re-entry into the active cell cycle proceeded very slowly. Only 5% of cells reached S-phase after 24 h at 37°C. A comparison of the S/G_1 ratio between both cell types shows approximately a fivefold difference 72 h following temperature elevation (Fig. 14B). The significant retardation of the re-entry into active cell cycle of G_1 arrested cells was caused by PARP-1 stabilized wt p53 protein that was present in the nuclei, whereas newly synthesized mutant p53 resided in the cytosol (not shown).

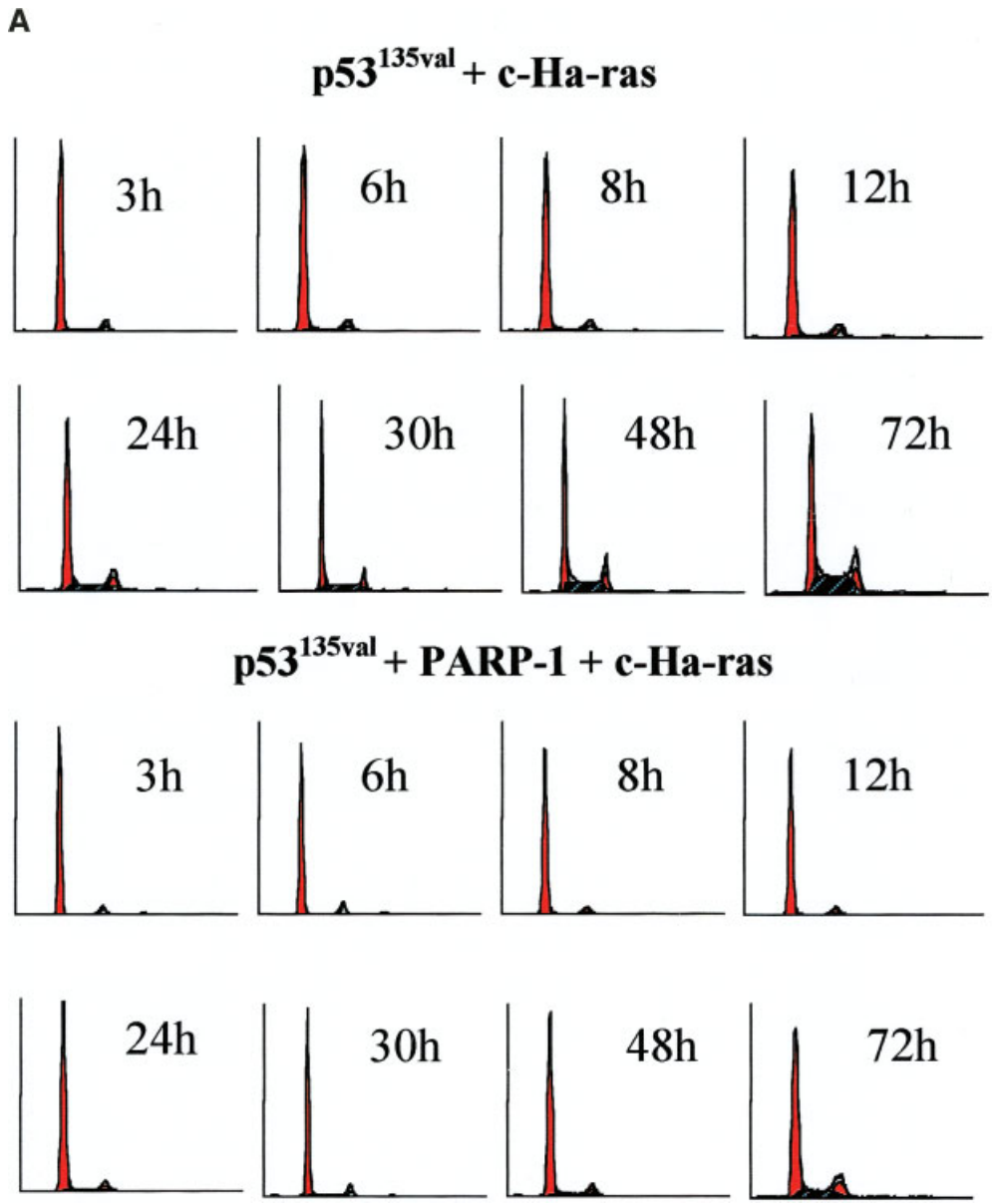

Fig. 14. PARP-1 stabilized wt p53 delays the cell cycle recovery of G_1 arrested cells. **A**: Different kinetics of cell cycle re-entry between cells expressing c-Ha-ras + wt $p53^{135val}$ alone or in combination with PARP-1. Cell clones expressing c-Ha-ras + wt $p53^{135val}$ alone or in combination with PARP-1 were maintained at 32°C for 48 h to achieve G_1 arrest. The cells were then shifted up to 37°C for the indicated periods of time. Cells were collected, stained with propidium iodide and the DNA content of single cells was measured by FACS (Beckton Dickinson). **B**: Comparison of S/G_1 for both cell types. Ratio determined for each profile.

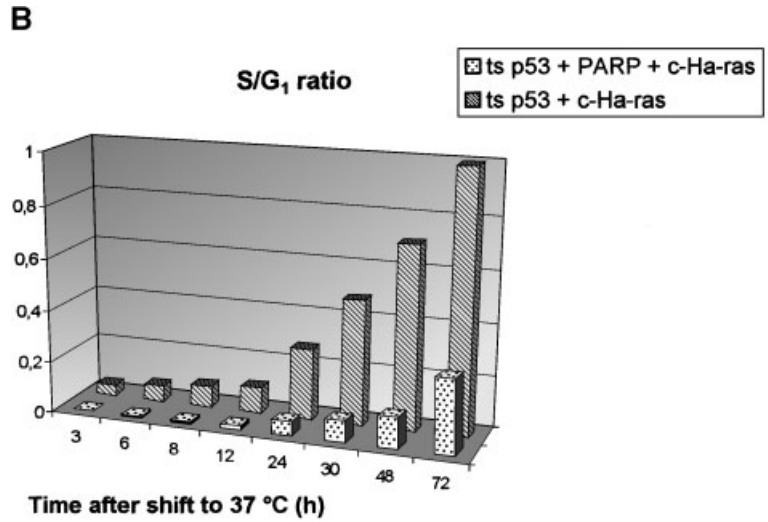

Fig. 14. (*Continued*)

DISCUSSION

Wild-type (wt) p53 has received a great deal of attention for its essential role in the prevention of malignant transformation. The biological function of p53 protein can be modulated by its activity and stability as well as by its intracellular localization. Nuclear import and export of p53 protein is controlled by an active nuclear transport pathway. The NLSs and NES of p53 are recognized by the transport proteins importin alpha and exportin (CMR1), respectively. Binding of the specific transporters to corresponding signal motifs is essential for protein shuttling. The nuclear exclusion of wt p53 protein represents one of the inactivating lesions observed in diverse neoplasms [Moll et al., 1992, 1995]. Recently, the mechanism of the aberrant cytoplasmic localization of p53 occurring in about 40% of the breast cancer tissues was studied [Kim et al., 2000]. Mutations in the importin alpha gene resulting in the production of a truncated form of importin alpha were identified. Truncated importin alpha deleted in the region encoding the putative NLS binding domain of p53 is unable to bind p53 [Kim et al., 2000]. Thus, the mutation of the transporter led to the cytoplasmic retention of p53 protein. The precise mechanism of the export of p53 protein is still enigmatic. Different and partially contradictory observations have been made for the mechanisms of p53 export from the nucleus. On the one hand, MDM2 functions as a mediator of p53 export [Freedman and Levine, 1998; Tao and Levine, 1999]. On the other hand, p53 has its own NES localized in the oligomerization domain that has been shown to be fully capable of mediating nuclear export independently of MDM2 [Stommel et al., 1999]. However, it seems that the NES is in an active p53 tetramer form inaccessible to transport proteins.

TABLE III. Cell Cycle Distribution of Cells Overexpressing $p53^{135val}$ + c-Ha-ras Alone or in Combination With PARP-1 After Shift to 37°C

Time after shift to 37°C (h)	$p53^{135val}$ + c-Ha-ras			$p53^{135val}$ + PARP-1 + c-Ha-ras		
	G_1 (%)	S (%)	G_2 (%)	G_1 (%)	S (%)	G_2 (%)
3	90.5	3.6	5.9	95.5	0.4	4.1
6	88.9	5.3	5.8	95.4	0.3	4.3
8	87.9	6.9	5.2	93.6	0.7	5.6
12	84.0	8.1	7.9	92.5	1.3	6.1
24	70.1	18.8	11.0	89.5	4.7	5.8
30	61.1	29.2	9.7	87.5	7.1	5.4
48	51.9	36.3	11.7	83.4	10.1	6.5
72	43.0	42.7	14.3	67.7	20.0	13.2

Therefore, it has been suggested that p53 tetramer has to be disassembled in order to render NES accessible for exportin [Stommel et al., 1999]. Recent data suggest that MDM2 mediated sequential ubiquitination of p53 is sufficient for the unmasking of NES in a p53 tetramer [Gu et al., 2001]. However, it remains unclear whether ubiquitinated p53 is exported via its own NES or in a complex with MDM2. The binding of MDM2 to p53 is essential for ubiquitination, but p53's tertiary structure may also be important for this reaction. Moreover, MDM2 binding to p53 and its ubiquitination can be abrogated by alternative reading frame p14 protein ($p14^{ARF}$) [Mao et al., 1995; Quelle et al., 1995; Kamijo et al., 1997]. $p14^{ARF}$ (murine $p19^{ARF}$) binds to MDM2 in a region distinct from the p53 binding site and acts by attenuating MDM2 mediated p53 degradation [Bothner et al., 2001]. $p14^{ARF}$ inhibits ubiquitin ligase activity [Pomerantz and Schreiber-Agus, 1998; Honda and Yasuda, 1999] and sequesters MDM2 in the nucleoli thus physically separating MDM2 and p53 in different subcellular compartments [Weber et al., 1999; Lorum et al., 2000; Lianos et al., 2001]. Recently, CARF, a novel protein of the $p14^{ARF}$–p53 pathway was discovered thereby extending the family of p53 interplayers [Hasant et al., 2002]. Furthermore, post-translational modifications of p53 may abrogate the interaction between MDM2 and p53 preventing p53 from being targeted for degradation. Human p53 has been reported to be modified on at least 18 sites [Appella and Anderson, 2001]. p53 protein is phosphorylated (reviewed by Appella and Anderson, 2001), acetylated [Gu et al., 1997], poly(ADP-ribosyl)ated [Wesierska-Gadek et al., 1996a,c; Simbulan-Rosenthal et al., 1998], and sumoylated [Gostissa et al., 1999; Rodriguez et al., 1999]. The amino-terminal and carboxy-terminal domains seem to be the primary targets for these modifications. The amino-terminus is heavily phosphorylated while the carboxy-terminus harbors phosphorylated, acetylated, and sumoylated residues. Although post-translational modifications at most sites occur in response to stress stimuli, clear differences in the kinetics of modifications and in susceptibility at individual sites to different agents have been observed. For example, exposure to ionizing radiation increased phosphorylation at Ser 6, 9, and 15, detectable as early as 30 min post treatment. However, in response to UV light a less rapid but more long-lived increase in the phosphorylation of these sites was observed [Appella and Anderson, 2001].

The steady-state of wt p53 protein is also regulated by PARP-1. As shown in this work, and by previous reports, the basal expression of wt p53 protein was reduced to a barely detectable level in mouse cells and tissues, in which the *PARP-1* gene was disrupted [Wesierska-Gadek et al., 1999]. Interestingly, PARP-1 deficiency affected only the regularly spliced form of wt p53 protein, whereas the AS p53 remained unchanged [Wesierska-Gadek et al., 1999]. The half-life of wt p53 protein was extremely decreased in cells lacking PARP-1 [Wesierska-Gadek et al., 2000b]. Therefore, we undertook two different approaches to assess the stability of p53 protein. In the first approach, we cultivated cells until they became confluent to increase the number of cells resting in G_1 phase of the cell cycle. It is known that the p53 level changes during the cell cycle reaching the highest level in G_1. Then we inhibited protein synthesis and prepared cell lysates at different time points after addition of the inhibitors. In the second approach, we performed pulse labeling of exponentially growing cells to get the maximal incorporation of the radioactively labeled methionine and chased the label for increasing time. The attempts to determine the p53 stability using a conventional method based on the estimation of the kinetics of the protein loss after inhibition of protein synthesis were not successful. No p53 band could be detected in PARP-1 −/− cells after inhibition of protein synthesis by emetine or cycloheximide. It has been shown previously that inhibition of transcription by various agents results in transient accumulation of p53 [Ljungman et al., 1999; Blagosklonny, 2002b]. This is a surprising result because the use of this method was successful for the determination of the p53 half-life even in cells known to express an extremely low level of p53 protein such as human cervix carcinoma HeLa cells [Wesierska-Gadek et al., 2002]. The reduced stability of wt p53 protein in HeLa cells is a consequence of its enhanced ubiquitination by HPV encoded E6 protein resulting in an accelerated targeting for degradation by the proteasome [Scheffner et al., 1990, 1993; Kessis et al., 1993]. We recently showed that treatment of HeLa cells with cisplatin increased the cellular level of p53 protein as a result of elongation

of the p53 half-life [Wesierska-Gadek et al., 2002]. We observed that after addition of emetine or cycloheximide to HeLa cell culture immediately before harvesting the cells, p53 protein was detectable even in untreated control. However, after addition of both inhibitors of protein synthesis to PARP-1 deficient mouse cells, p53 remained undetectable. In contrast, in normal counterparts, p53 levels increased after inhibition of protein synthesis allowing the determination of its half-life. The half-life of p53 protein in normal confluent MEFs was 7.5 h. Interestingly, this value greatly differed from the results assessed by pulse-chase experiments. The p53 half life assessed after precipitation of labeled cellular proteins was 50 min and was in concordance with previous reports [Freedman and Levine, 1998; Wesierska-Gadek et al., 2000b]. The half-life of p53 protein in PARP-1 deficient cells determined by pulse-chase experiments combined with immunoprecipitation was 7 min. It became evident that in cells lacking PARP-1 the p53 half-life was significantly reduced. The reduction of p53 stability was even more dramatic in resting cells. Whereas in normal cells growing to confluency, the p53 half-life was extended, no p53 could be detected in PARP-1 deficient cells. Remarkably, PARP-1 regulates the stability not only of the endogenous but also of the transfected p53 protein, and only the steady-state of wt p53 protein depends on PARP-1 status. We showed that the transfected mutant p53 was also stable in cells lacking PARP-1. It is noteworthy that the steady-state of p53 in PARP-1 cells was significantly changed after inhibition of protein export by LMB. The p53 protein accumulated in the nuclei of PARP-1 −/− cells after LMB exposure, thereby suggesting that p53 degradation primarily occurs in the cytoplasmic proteasome. Interestingly, LMB affected not only endogenous but also transfected wt p53 in PARP-1 deficient cells. Moreover, LMB induced site specific phosphorylation of p53 protein. In contrast, in normal MEFs LMB had no significant effect on p53 expression. Nuclear export of p53 is likely to be achieved through its direct interaction with the export receptor CRM1, and may occur only when the NES is unmasked. LMB, which covalently modifies CRM1 at cysteine 529 [Kudo et al., 1999], thereby impairing the interaction between the NES and CRM1, very efficiently blocks p53 export.

A large accumulation of wt p53 protein in the nuclei of PARP-1 −/− cells after inhibition of protein export by LMB suggests that the reduced p53 stability in the absence of PARP-1 is attributable to hyperactive p53 export. We showed in this study that PARP-1 binds to the central and to the carboxy-terminal domains of p53 protein. Interestingly, the carboxy-terminal fragment seems to possess the highest capacity to bind to PARP-1. This conclusion may be drawn from the comparison of the intensity of the precipitated fragments 102–292, 102–393, and 293–393. Moreover, we showed that the phosphorylation of p53 regulates its binding to PARP-1. Remarkably, dephosphorylated p53 failed to bind PARP-1 as evidenced by results of the pull-down assay. The comparison of the wt p53 proteins expressed in insect and mammalian cells shows very similar patterns of isomers detected by immunoblotting after 2D-PAGE. Detailed analysis of the phosphorylation of baculovirally expressed human p53 protein revealed that serine 392 was modified to the highest degree in a comparison of the five phosphorylated serine residues. On the basis of the phosphorylation pattern, and due to the fact that the carboxy-terminal p53 fragment possesses the strongest PARP-1 binding capacity, it seems that phosphorylation of Ser392 is essential for the interaction. This observation raises the question of how the binding of PARP-1 to the carboxy-terminal domain of p53 may affect its nuclear export. One could speculate that PARP-1 complexed with p53 may mask the NES, thereby resulting in the nuclear retention of p53 protein through preventing its export and subsequent degradation. It would also be possible that binding to PARP-1 prevents MDM2 ubiquitination that seems to be necessary to render NES accessible for transporter proteins [Geyer et al., 2000]. The assumption that PARP-1 binding to the carboxy-terminal domain of p53 may regulate its export is consistent with the previous observation that the inactivation of PARP-1 in mouse cells reduced only the stability of the regularly spliced form of p53, whereas the AS p53 was not affected. Both splice variants of murine p53 differ solely in the distal carboxy-terminus. Furthermore, exposure of PARP-1 cells to LMB induced phosphorylation of p53 at Ser392 which coincided with the onset of accumulation of the p53 protein. One might speculate that the phosphorylation of p53 at Ser 392 may regulate p53 export. This concept is

consistent with the recent results of Zhang and Xiong [2001]. The authors characterized the new NES within the amino-terminus of p53 that seems to collaborate with the carboxy-terminal NES and observed that DNA damage-induced phosphorylation of p53 at Ser15 inhibited p53 nuclear export.

The considerable reduction in the level of regularly spliced p53 protein had a functional consequence: PARP-1 −/− cells failed to transactivate p53-responsive genes even after treatment with genotoxic agents [Wesierska-Gadek et al., 1999]. These results are in accordance with previous observations that hamster cell lines selected for PARP-1 deficiency exhibit a reduction in baseline levels of wt p53 and fail to accumulate p53 in response to etoposide treatment [Whitacre et al., 1995]. This resulted in a lower rate of apoptosis. Considering the reduced stability of the wt p53 in PARP-1 KO cells, one could speculate that the overexpression of PARP-1 would exert the opposite effect. Indeed, the experiments performed with transformed rat cells overexpressing simultaneously PARP-1 and a ts p53 mutant confirmed this assumption [Wesierska-Gadek et al., 2000a].

The use of a temperature-sensitive (ts) p53 mutant [Eliyahu et al., 1985] offered the possibility to investigate the interaction between PARP-1 and p53 in the same cellular background but under two quite different conditions depending 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.

Our results show that the elevated expression of PARP-1 had little effect on the cellular processes regulated by mutant p53. There were no differences in the kinetics of cell proliferation, the distribution in the cell cycle, or p53 localization between cells expressing $p53^{135val}$ alone or in combination with PARP-1 [Wesierska-Gadek et al., 2000a]. However, after temperature-dependent switching of p53 from mutant to wt phenotype, the strong effect of the cellular level of PARP-1 became evident. Under permissive conditions, the prolonged expression of nuclear wt p53 attributable to increased protein stability persisted for at least 4 days, and was observed solely in cells coexpressing PARP-1. Increased p53 stability in cells possessing a high level of PARP-1 resulted in a significant retardation of the reentry of G_1 arrested cells into the cell cycle. This was reflected by a fivefold reduced S/G_1 ratio after 72 h. The enzymatic activity of PARP-1 was necessary for the stabilizing effect since treatment of cells with 3-AB, an inhibitor of PARP-1, abolished this effect in a dose dependent manner.

Considering the obvious involvement of PARP-1 in the regulation of the stability of wt p53 protein, the elucidation and characterization of the interaction between PARP-1 and p53 is of importance. PARP-1 binds directly to p53 and forms tight complexes. The first observation that p53 and PARP-1 undergo strong binding was accomplished using transformed rat cells overexpressing ts $p53^{135val}$ [Wesierska-Gadek et al., 1996a,c, 2000; Wesierska-Gadek et al., 2001]. Both proteins were co-precipitated. The complex formation did not depend on the p53 status: both wt and mutant p53 bound to PARP-1. This observation was later confirmed in other cell systems by various groups [Simbulan-Rosenthal et al., 1998; Vaziri et al., 1998]. However, no systematic studies were made to identify which parts of both proteins were involved in the interaction. In a study by Kumari et al. [1998], binding between the 89 kDa apoptotic fragment of PARP-1 and p53 protein was suggested. However, this report seems to be questionable due to serious methodological problems and general obstacles. Moreover, the results reported by Kumari et al. are contradictory to other reports [Roser et al., 2001] and our results (unpublished data). Thus, our results provide evidence for the first time that the protein–protein interaction between p53 and PARP-1 is very specific and is restricted to specific regions. Remarkably, the amino-terminal domain of p53 that harbors the transactivation domain is dispensable for the complex formation. On the other hand, the carboxy-terminal part of PARP-1 encompassing the catalytic domain of the enzyme shows a very weak, if any, affinity for p53. These results unequivocally implicate that both proteins undergo complex formation without involvement of their functional domains. This suggests that wt p53 if complexed with PARP-1 may retain its activity as a transcription factor. This assumption is supported by the observation that in primary rat cells simultaneously overexpressing p53 and PARP-1, p53 functioned as a transcription factor at the permissive temperature and activated its downstream genes such as *mdm-2* and $p21^{waf\text{-}1}$ [Wesierska-Gadek et al., 2000a]. However, no direct evidence exists as to

whether or not p53 transcriptional function takes place in a complex or requires dissociation from PARP-1. Furthermore, we present evidence in this study that the physical interaction between p53 and PARP-1 depends on the phosphorylation state of p53. This observation implicates the possible role of PARP-1 in the regulation of p53 mediated cellular response to stress stimuli. Whereas PARP-1 is a long-lived protein constitutively expressed at about 1×10 copies per cell, wt p53 protein is short-lived and its expression at steady-state is very low. Unlike PARP-1, p53 level is strongly inducible in response to stress stimuli. The activation of p53 protein is mediated primarily by its stabilization and to a lesser extent by an increase in transcription rate. However, not only the in-duction of p53 protein but also the duration of p53 response is of physiological importance. Therefore, it seems reasonable that another protein being a sensitive detector of even negligible DNA lesions could cooperate with p53 in this regard. PARP-1 is a good candidate as mediator of the p53 response. First, this nuclear enzyme is constantly expressed irrespective of cellular stress. Secondly, it is immediately activated upon DNA damage and is able to detect DNA lesions even at very low levels. Furthermore, PARP-1 self regulates its affinity and binding to DNA. PARP-1 may mask the NES through complex formation with p53 and may thereby prevent its export. However, considering the complexity of the nuclear protein export process we can not exclude that PARP-1 might regulate additional factor(s).

Since acetylation of p53 protein is also involved in the fine regulation of its activities, it would be of interest in future studies to examine whether site-specific acetylation of p53 may affect its binding capacity to PARP-1.

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