Journal of Cellular Biochemistry 89:220–232 (2003)

Central and Carboxy-Terminal Regions of Human p53 Protein Are Essential for Interaction and Complex Formation With PARP-1

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

Institute of Cancer Research, University of Vienna, Vienna, Austria

Abstract It has been previously described by different groups that poly(ADP-ribose) polymerase-1 (PARP-1) and the product of the tumor suppressor gene p53 form tight complexes. We investigated which domains of human PARP-1 and of human wild-type p53 were involved in this protein–protein interaction. We generated baculoviral constructs encoding full length protein or distinct functional domains of both proteins. Baculovirally expressed wild-type p53 was post-translationally modified. 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. The amino-terminal part harboring the transactivation functional domain of p53 was dispensable. On the other hand, the amino-terminal and central fragments of PARP-1 were necessary for complex formation with p53 protein. Finally, we explored the functional significance of the interaction between both proteins. Inactivation of PARP-1 resulted in the reduction of p53 steady-state levels. Inhibition of nuclear export by leptomycin B prevented accelerated degradation of p53 in PARP-1 KO cells and led to accumulation of p53 protein. Considering the fact that the accelerated p53 nuclear export in the absence of PARP-1 contributes to enhanced p53 degradation, we conclude that PARP-1 may mask the NES of p53 through complex formation with its carboxy-terminal part, thereby preventing the export. J. Cell. Biochem. 89: 220–232, 2003.

Key words: p53 export; NES masking; p53 nucleocytoplasmic shuttling; p53 tetramerization

The most fundamental function of wild-type (wt) p53 protein is the regulation of the normal progression of cells through the cell cycle and the maintenance of genomic stability [reviewed by Bargonetti and Manfredi, 2002; Blagosklonny, 2002]. p53 is a short-lived protein that is expressed in low concentrations under physiological conditions and generally undetectable in unstressed cells. In response to several stressful conditions, the level and transcriptional activity of wt p53 protein rises resulting in activation of p53 target genes and downstream mediators of p53 [Maltzman and Czyzyk, 1984; Fritsche et al., 1993]. Increased levels of the p53 protein are primarily regulated through lengthening of its half-life. MDM2 protein, the product of a p53 inducible gene has been originally found to interact with and to inhibit p53-mediated transcriptional activity [Oliner et al., 1993]. It has also been shown to regulate the level of p53 protein by promoting its rapid degradation. MDM2 protein possessing intrinsic activity of E3 ubiquitin-ligase has been identified to efficiently target p53 for the ubiquitin-dependent proteolytic machinery [Haupt et al., 1997; Kubbutat et al., 1997]. The importance of MDM2 in the regulation of p53 levels is reflected by the fact that disruption of the *mdm2* gene was lethal in early embryos,

Abbreviations used: aa, aminoacid(s); ARF, alternative reading frame; ECL, enhanced chemiluminiscence; FCS, fetal calf serum; HPV, human papilloma virus; HRP, horseradish peroxidase; KO, knockout; LMB, leptomycin B; MEFs, mouse embryo fibroblasts; MDM2, mouse double minute; 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; ts, temperature-sensitive; WCL, whole cell lysate; wt, wild-type.

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

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

*Correspondence to: Dr. Józefa Węsierska-Gądek, Institute of Cancer Research, University of Vienna, Borschkegasse 8 a A-1090 VIENNA, Austria.

E-mail: Jozefa.Antonia.Gadek-Wesierski@univie.ac.at

Received 24 February 2003; Accepted 25 February 2003

DOI 10.1002/jcb.10521

whereas the concomitant inactivation of the *p53* gene rescued the animals from lethality [Jones et al., 1995; Montes de Oca Luna et al., 1995]. An autoregulatory feedback loop has been established between p53 and MDM2, where p53 activates expression of its own negative regulator. The precise mechanism by which p53 is stabilized is not yet entirely elucidated. An increase of p53 protein level can be achieved by inhibiting directly or indirectly its degradation as well as by upregulating the rate of translational initiation. Post-translational modifications of p53 including phosphorylation, acetylation or ADP-ribosylation contribute to its stabilization [Węsierska-Gądek et al., 1996a; Simbulan-Rosenthal et al., 1998; Appella and Anderson, 2001]. These modifications may abrogate the interaction between MDM2 and p53 preventing p53 from being targeted for degradation. It is becoming evident that a number of mechanisms exist to abrogate MDM2 mediated degradation of p53, thereby allowing the maintenance of a p53 response. One of the most effective ways to stabilize p53 is site-specific phosphorylation of p53 that minimizes the interaction between p53 and MDM2 and renders p53 resistant to MDM2 mediated degradation. Another mechanism allowing p53 to overcome the targeting by MDM2 is modification-independent and involves the upregulation of the human $p14^{ARF}$ (mouse $p19^{ARF}$) protein. The ARF protein is encoded by the INK4A-ARF locus that encodes two distinct polypeptides translated from two different mRNAs generated by alternative splicing. Alternative reading frame (ARF) is encoded by exons 1β, 2 and 3 [Mao et al., 1995; Quelle et al., 1995] and binds directly to MDM2 in a region distinct from the p53 binding region and prevents degradation of p53 [Pomerantz and Schreiber-Agus, 1998]. It is becoming evident that multiple mechanisms exert control over the stability of the p53 protein. Recently, the involvement of poly(ADP-ribose) polymerase-1 (PARP-1) in the regulation of p53 stability was demonstrated [Węsierska-Gądek et al., 1999, 2000; Węsierska-Gądek and Schmid, 2000, 2001]. This observation is of interest because both proteins are sensors of DNA lesions and can be induced in response to multiple stress stimuli. There are two independent lines of evidence for PARP-1 mediated stabilization of wild-type p53 protein. First, the inactivation of the *PARP-1* gene in mice resulted in a reduction in the constitutive expression of wild-type p53 to a barely detectable level due to the shortening of the half-life of p53 protein [Węsierska-Gądek et al., 1999, 2000]. The reconstitution of PARP-1 deficient cells with the human counterpart abrogated the reduced stability of p53 protein. A second line of evidence revealed that coexpression of PARP-1 with a temperature sensitive (ts) $p53^{135val}$ mutant increased the stability of wild-type p53 [Węsierska-Gądek et al., 2000; Węsierska-Gądek and Schmid, 2001]. This had functional consequences. Coexpression of PARP-1 markedly delayed the recovery of G_1 arrested cells in S-phase after temperature elevation. PARP-1 has also been shown to physically interact with p53 protein [Węsierska-Gądek et al., 1996a,c; Vaziri et al., 1998; Węsierska-Gądek and Schmid, 2000].

The aim of this study was to precisely characterize the interaction between these proteins. p53 and PARP-1 consist of distinct functional domains. Therefore, we generated baculovirus constructs encoding full length human p53 and PARP-1 proteins as well as fragments corresponding to 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 to determine which domains of both proteins were involved in the complex formation. Our results show that the central and carboxy-terminal parts of p53 are indispensable for complex formation with PARP-1. On the other hand, the amino-terminal and central fragments of PARP-1 are necessary for p53 binding. We also studied the biological significance of the interaction between p53 and PARP-1. Inactivation of PARP-1 resulted in the reduction of p53 steady-state levels in mouse cells. However, the inhibition of nuclear export by leptomycin B prevented accelerated degradation of p53 in PARP-1 KO cells and led to accumulation of p53 protein. The fact that the accelerated p53 nuclear export in the absence of PARP-1 contributes to enhanced p53 degradation leads us to conclude that PARP-1 may mask the NES of p53 through complex formation with its carboxy-terminal part, thereby preventing export of p53.

MATERIALS AND METHODS

Plasmids

Plasmid encompassing the coding sequence for human poly(ADP-ribose) polymerase-1

(PARP-1) was a generous gift from Dr. G. de Murcia.

Cell Treatment

Mouse embryo fibroblasts (MEFs) grown to 60–70% confluency were treated with anticancer agents for 6 and 24 h. Etoposide (VP-16) (10 μg/ml), 5-fluorouracil (5-FU) (5 μg/ml), and cytarabine (Ara-C) (100 μg/ml) were used. To inhibit nuclear protein export, cells were treated for indicated periods of time with Leptomycin B (LMB) (Sigma Chemical Co, St. Louis, MO) at a final concentration of 300 nM.

Antibodies

Various 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). Polyclonal anti-p53 antibody CM-1 was obtained from Novocastra (Newcastle-upon Tyne, UK). 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.

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, England) or from Pierce (Rockford, IL).

Cloning of p53 and PARP-1

All cDNAs were cloned into a modified baculovirus recombination vector (pBlueBacHis2B, Invitrogen, CA). 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 with the following primers: PARP 1 Forward, 5′-AGGAATTCCCCGAGATCTGTACGACGATGACGATAAGATGGCGGAGTCTTCGGATAAG-3′; PARP 231 reverse, 5′-GGCTTTTTCAAGCTTTCAATCCTTGTCTTTTTCTTTTTTAGATTTCTT-3′; PARP 372 reverse, 5′-CACAGCAGCAGGAGCGTCGACTCAGGAGGGCGGAGGCGT-3′; PARP 524 reverse, 5′-GCTGCTCCTCCTTTGTCGACTCATTTCATTCTCTTTTCAGAT-3′; PARP 1014 reverse, 5′-GGCTACCTCGTCGACTTACCACAGGGAGGTCTTAAAATTGAA-3′; p53 1 forward, 5′-CTGGATTGGCAGAGATCTGTACGACGATGACGATAAGATGGAGGAGCCGCAGTCAGAT-3′; p53 102 forward, 5′-GCCCCCTCCTGGCCAGATCTGTACGACGATGACGATAAGATGACCTACCAGGGCAGCT-3′; p53 293 forward, 5′-GGGAGAGACCGGCGAGATCTGTACGACGATGACGATAAGATGGGGGAGCCTCACCACG-3′; p53 101 reverse, 5′-GAAACCGTAGCTGCCGTCGACTCATTTCTGGGAAGGGACAGAAG-3′; p53 292 reverse, 5′-GGGCAGCTCGTGGTGGTCGACTCATTTCTTGCGGAGATTCTC-3′; p53 393 reverse, 5′-CAAGAAGTGGGTCGACTCAGTCTGAGTCAGGCC-3′.

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.

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°C. Protein concentration of cell extracts was determined by DC assay (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as standards.

Immunoprecipitation

Immunoprecipitation was performed as previously described in detail [Węsierska-Gądek et al., 1996b]. 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. The eluates were then divided and loaded on two gels. One gel was used for immunoblotting with anti-p53 antibodies and the second was for incubation with anti-PARP-1 antibodies.

Immunoblotting

Proteins were separated by a one-dimensional PAGE on 10 or 15% SDS-gels. Proteins dissolved in reduced SDS sample buffer were loaded on SDS polyacrylamide gels, electrophoretically separated, and transferred onto a polyvinylidene difluoride membrane (PVDF) (Amersham International). Equal loading of proteins was confirmed by Ponceau S staining. Immunodetection of antigens was performed with specific antibodies. 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

In the first step we 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 (partially shown in Fig. 1A).

To further check the identity of the truncated p53 and PARP-1 proteins, we resolved the proteins on a number of identical blots and incubated with antibodies recognizing different epitopes localized within distinct functional domains. As shown in Figure 1A PAb421 recognized solely full length p53 protein as well as its carboxy-terminal fragments aa 102–393 and aa 293–393. PAb240 reacted with two truncated p53 forms: 102–292 and 102–393 as well as with full length p53 protein, but failed to recognize the amino-terminal domain aa 1–102 and the carboxy-terminal fragment 293–393. DO-1 reacted with full length human p53 and the amino-terminal part aa 1–102 and failed to react with the other fragments. The sequential immunoblotting of the same blot with DO-1 revealed a significant increase of the intensity of full length p53 band implicating that the affinity of antibody DO-1 towards full length wt human p53 protein is much higher than that of PAb421 (Fig. 1A).

For identification of expressed full length PARP-1 and its truncated forms, various antibodies were used (Fig. 1B). Polyclonal antibody N-20 from Santa Cruz (aa 1–20) reacted solely with full length enzyme and two amino-terminal fragments aa 1–232 and aa 1–524. The monoclonal antibody C-2–10 recognized full length protein and fragments aa 1–524, aa 232–372 and 232–524, and did not react with the DNA binding domain (DBD) aa 1–232 and carboxy-terminal fragment aa 524–1014. Monoclonal antibodies F-1-2-3 reacted with full length PARP-1 and fragments aa 1–524, the DBD aa 1–232, but did not react with aa 232–372 and 232–524 or with the carboxy-terminal fragment aa 524–1014. The monoclonal antibody F2 was specific solely for full length PARP-1 and the carboxy-terminal domain aa 525–1014. Rabbit polyclonal anti-PARP-1 antibody detected all PARP-1 fragments (Fig. 1B).

Thus, the predicted identity of the generated fragments of p53 and PARP-1 was unequivocally confirmed by the reactivity with specific antibodies.

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 first step we examined the ability of the rabbit polyclonal anti-p53 antibody CM-1 to precipitate all recombinant p53 proteins. As shown in Figure 2A, this antibody precipitated full length p53 and its three different truncated forms: aa 1–101, aa 102–292 and aa 293–393. It was therefore deemed suitable for our further studies.

As expected on the basis of previous observations, we could precipitate both proteins using anti-p53 and anti-PARP-1 antibodies (Fig. 2A). To exclude the possibility that the His-tag sequence had an effect on the complex formation,

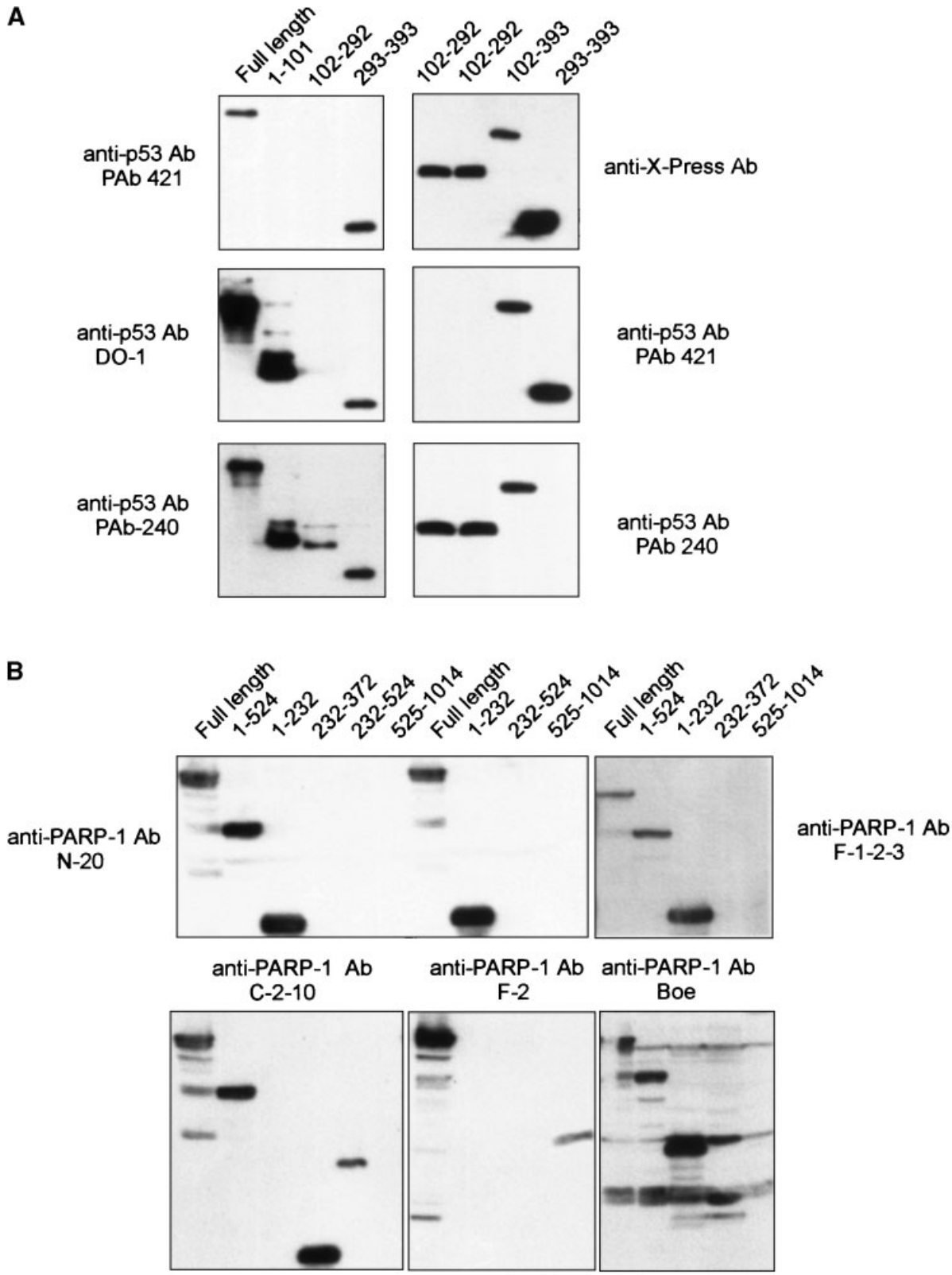

Fig. 1. Characterization of human wt p53 (**A**) and human PARP-1 (**B**) full length and truncated recombinant proteins expressed in Sf9 cells. A: Total cell lysates expressing p53 recombinant proteins (5 μg/lane) were separated on 15% SDS-slab gels. The blots were incubated with corresponding antibodies diluted to a final concentration of 1:5,000. The blot shown in the left panel was sequentially incubated with the antibodies. B: Total cell lysates expressing PARP-1 (5 μg/lane) were separated on 10% SDS-slab gels. The blots were sequentially incubated with corresponding antibodies diluted to a final concentration of 1:5,000.

we removed the tag sequence by enterokinase or used baculovirally expressed proteins without the tag sequence (not shown). No difference was observed in the affinity of p53 to PARP-1 indicating that the tag sequence did not affect the interaction between the proteins. 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 three p53 fragments aa 102–292, 102–393 and 293–393 were detected in the immune complexes precipitated by polyclonal anti-PARP-1 antibody (Fig. 2B). However, the anti-PARP-1 antibody failed to precipitate the short p53 fragment aa 1–102. The analysis of the non-bound fraction (not shown) and the other half of the samples precipitated by anti-p53 CM-1 antibody (Fig. 2B), as well as sample used for precipitation (designed as input), revealed the presence of a strong signal for the truncated aa 1–102 protein. This substantiated the evidence that the amino-terminus of p53 did not complex with PARP-1. The lack of 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

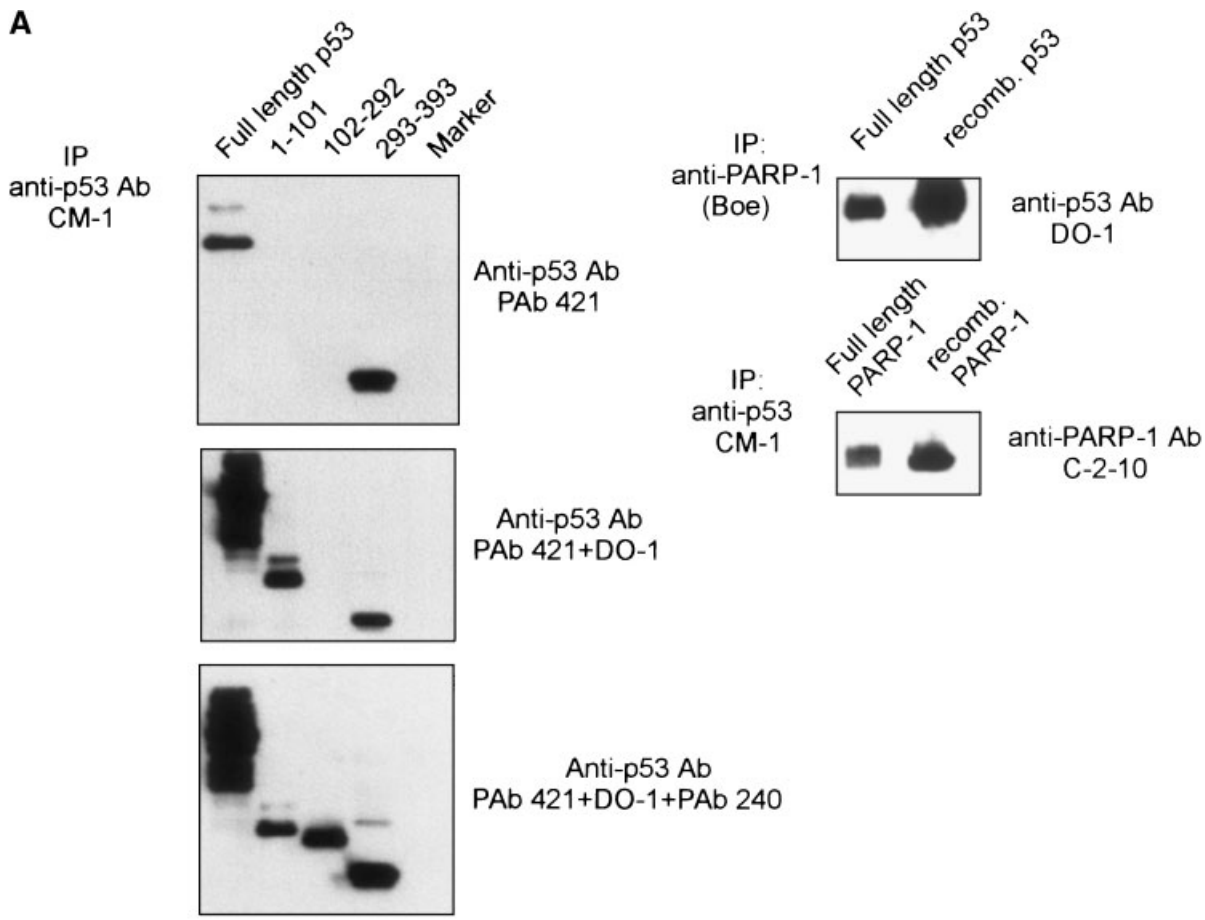

Fig. 2. Identification of p53 domains involved in the complex formation with PARP-1. **A**: Reactivity of the polyclonal anti-p53 antibody CM to distinct p53 recombinant proteins. Different p53 fragments were precipitated by CM-1 antibody. The identity of precipitated proteins was proved by immunoblotting using specific antibodies. Lysates prepared from Sf9 cells simultaneously expressing full length human p53 and full length human PARP-1 were divided and precipitated with anti-p53 or anti-PARP-1 antibodies, respectively. Affinity purified immune complexes were analyzed by immunoblotting. Corresponding recombinant proteins were loaded as a positive control. **B**: 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 from two independent experiments were analyzed by immunoblotting. Immune complexes bound to Gamma-Bind Sepharose were eluted sequentially three times with SDS-sample buffer. We showed results from two independent experiments. Two eluates from the first experiment were loaded on the gel. Samples used for second immunoprecipitation (input) were additionally analyzed. Wt human p53 was loaded as a positive control.

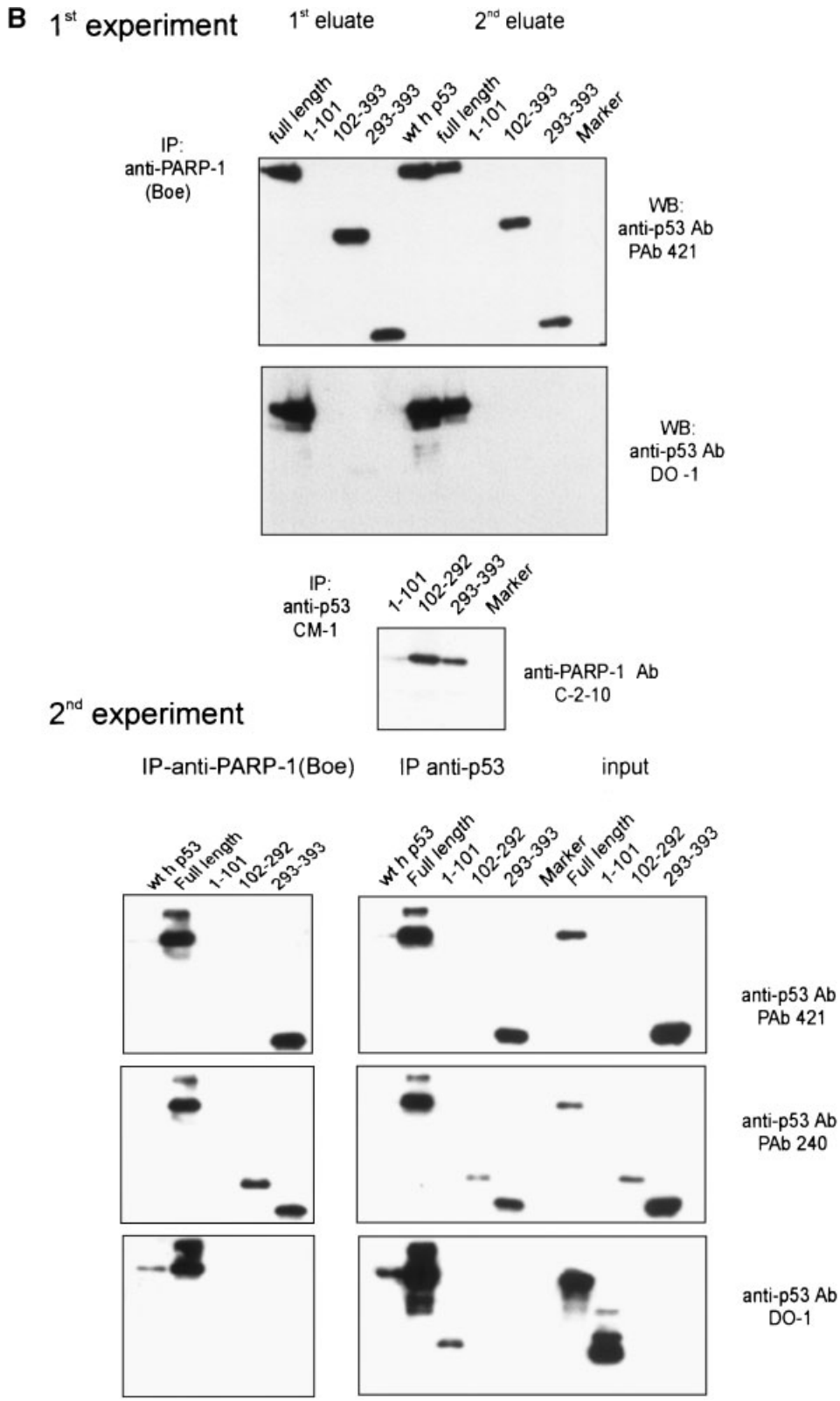

Fig. 2. (*Continued*)

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 (Fig. 2B). The latter was slightly weaker.

The experiments performed with lysates prepared from insect cells simultaneously expressing full length p53 and different fragments of PARP-1 showed the strong binding of its amino-terminal and central domain to full length p53, whereas the carboxy-terminal part showed only a very weak interaction (Fig. 3). The latter was detected in the immune complexes only after longer exposure of the blot.

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. Comparison of the intensity of immunoprecipitated p53 fragments aa 102–293, aa 102–393 and 293–393 led to the conclusion that carboxy-terminal domain of p53 possesses higher affinity to PARP-1 than its central region. 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 oligomerization 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.

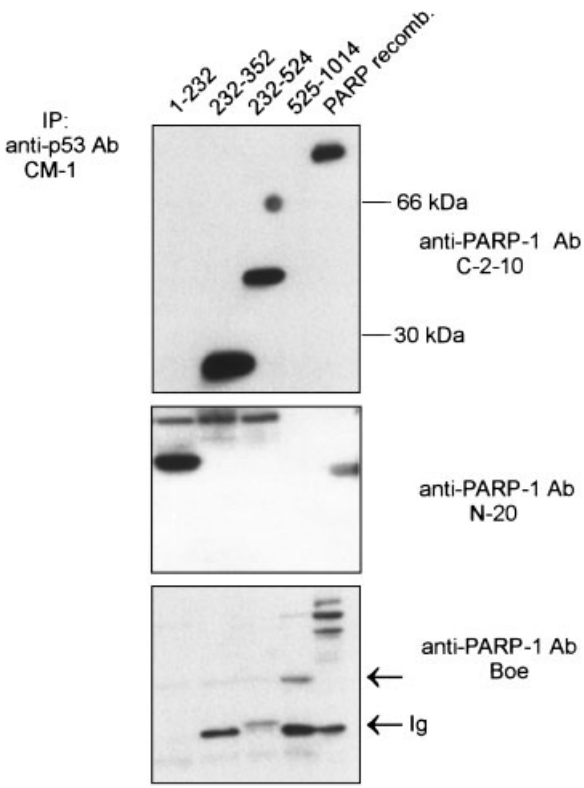

Fig. 3. Identification of PARP-1 domains involved in the complex formation with p53. Lysates prepared from Sf9 cells simultaneously expressing full length human p53 and distinct fragments of human PARP-1 were divided and precipitated with anti-p53 or anti-PARP-1 antibodies, respectively. Affinity purified immune complexes were analyzed by immunoblotting. Recombinant PARP-1 protein was loaded as a positive control.

Reduced Stability of wt p53 Protein in the Absence of PARP-1 In Vivo

To assess the effect of PARP-1 on the expression of wt p53, we examined the basal expression of p53 protein in mouse cells lacking PARP-1 protein and compared it with that in PARP-1 competent cells. As shown in Figure 4A, 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, the treatment of mouse cells with three distinct anti-cancer drugs (VP-16, 5-FU, and AraC) for 6 h (not shown) and for 24 h (Fig. 4A) did not elevate p53 levels in PARP-1 deficient cells. However, p53 protein was induced in their wt counterparts. The highest increase of p53 in normal MEFs was observed in response to VP-16. Undetectable levels of p53 protein in individual cells lacking PARP-1 were also shown by immunofluorescence staining (not shown). 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. 4B). Sequential incubation of the blot with antibodies against MCM-7 protein revealed that the accumulation of p53 after LMB was specific because the level of another nuclear protein, MCM-7 was not changed.

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-

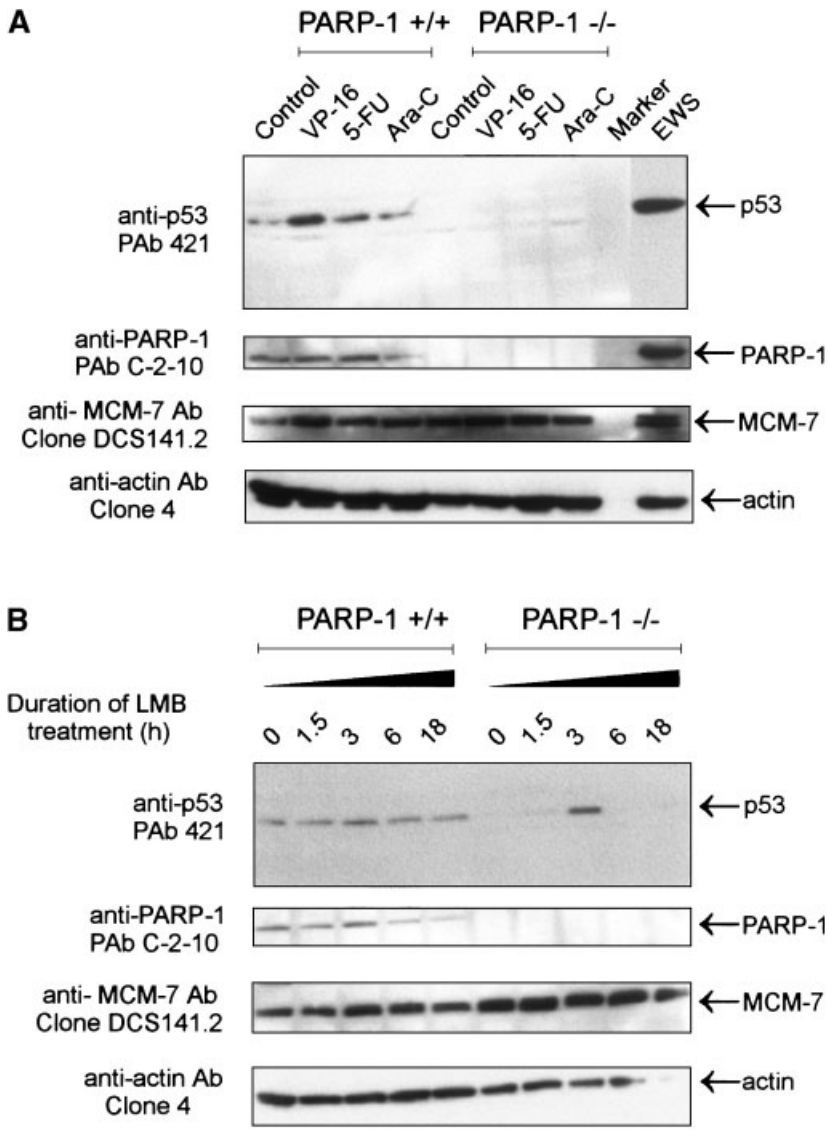

Fig. 4. Lack of basal and induced expression of wt p53 protein in PARP-1 KO cells. Normal (A-19) and PARP-1 KO (A-11) cells were treated with anti-cancer drugs (VP-16, 5-FU and Ara-C) for 24 h (**A**) or with 300 nM LMB for the indicated periods of time (**B**). WCLs (20 μg protein/lane) were resolved on 10% SDS gel. p53 protein was detected with monoclonal anti-p53 antibody PAb421. Cell lysate prepared from Ewing sarcoma cell line (EWS) overexpressing ts $p53^{138val}$ was loaded as a p53 positive control. Human ts $p53^{138val}$ was visualized after incubation with anti-p53 antibody PAb240. The blots were sequentially incubated with anti-PARP-1 and with anti-MCM-7 antibodies. Equal protein loading was confirmed by Ponceau S staining (not shown) and by incubation with anti-actin antibodies.

terminal domain of p53, which harbors the NES to PARP-1, may mask the NES and may prevent access to the nuclear export machinery.

DISCUSSION

In unstressed cells, the tumor suppressor protein p53 is maintained at low levels due to targeted degradation. The rapid p53 turnover in normal cells is mediated by MDM2, a negative regulator of p53, possessing intrinsic E3 ubiquitin ligase activity. In human papilloma virus (HPV) 16/18 positive cells, p53 turnover is mediated by HPV encoded E6 oncoprotein recruiting the cellular ubiquitin-protein ligase E6-AP [Munger et al., 1989; Scheffner et al., 1990, 1993]. The polyubiquitination of p53 protein catalyzed by these factors results in shortening of the p53 half-life by its enhanced degradation in the proteasome. However, a

variety of stress stimuli initiate signaling pathways that transiently stabilize the p53 protein causing its accumulation in the nucleus and its activation as a transcriptional regulator. Multiple post-translational modifications are essential in mediating these events.

The steady-state of wild-type p53 protein is also regulated by PARP-1. We showed in the present paper, and previously reported, that 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 [Węsierska-Gądek et al., 1999, 2000]. Interestingly, PARP-1 deficiency affected only the regularly spliced form of wt p53 protein, whereas the alternatively spliced p53 remained unchanged [Węsierska-Gądek et al., 1999]. The half-life of regularly spliced p53 protein was remarkably decreased in cells lacking PARP-1. 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 [Węsierska-Gądek et al., 2000, Wurzer et al., 2000]. 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. Regarding the reduced stability of the wt p53 in PARP-1 knock-out 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 ts p53 mutant confirmed this assumption [Węsierska-Gądek and Schmid, 2000].

The use of a temperature-sensitive (ts) p53 mutant 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 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 no remarkable effect on the cellular processes regulated by mutant p53. However, after temperature-dependent switching of p53 from mutant to wt phenotype, the strong effect of the cellular level of PARP-1 became evident. Co-expressed PARP-1 remarkably increased stability of wt p53 protein resulting in a significant retardation of the reentry of G_1 arrested cells into the cell cycle [Węsierska-Gądek and Schmid, 2000].

Considering the clear involvement of PARP-1 in the regulation of the stability of wild-type 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 seen using transformed rat cells overexpressing ts $p53^{135val}$ [Węsierska-Gądek et al., 1996a]. Both proteins were co-precipitated. The complex formation did not depend on p53 status: both wild-type and mutant p53 bound to PARP-1. This observation was later confirmed in other cell systems by other groups [Vaziri et al., 1998]. However, no systematic studies were made to identify which parts of both proteins were involved in the interaction. In an effort 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. First, the authors did not present any evidence that MNNG treated HeLa cells underwent apoptotic changes within the observation period. In response to genotoxic stimuli cells can initiate two different pathways of cell death: necrosis and apoptosis. Therefore, discrimination between both processes based on at least a few independent lines of evidence has to be presented. The conscientious determination of the type of induced cell death is especially important regarding the cleavage pattern for PARP-1 [Shah et al., 1996]. Secondly, the quality of the immunodetection of PARP-1 was poor and it is unlikely that the weak band at 80 kDa present only 10 min following onset of treatment could represent the apoptotic fragment of PARP-1. There is good evidence that apoptosis induced in HeLa cells by distinct cytostatic drugs such as cisplatin [Horky et al., 2001; Węsierska-Gądek et al., 2002], doxorubicin and etoposide (unpublished data) is a slow biphasic process executed within 24 h. On the basis of former reports one could expect that after longer treatment with strong genotoxic agents degradation of PARP-1 should proceed resulting in an increase in the intensity of the 89 kDa fragment. However, the intensity of the 80 kDa band shown by Kumari et al. decreased

during the course of MNNG treatment suggesting that the 80 kDa band was caused by unspecific binding [Budihardjo et al., 1998]. This suspicion was further strengthened by our observation that the onset of PARP-1 degradation occurred not until 6 h after MNNG treatment (unpublished data). Also, the upregulation of p53 protein upon a short MNNG treatment of HeLa cells, as well as detection of p53 positive signals in untreated control cells suggested by Kumari et al. is doubtful. We did not detect any accumulation of p53 after addition of MNNG (unpublished data). Even after longer MNNG treatment for 6 and 15 h, p53 protein remained undetectable in HeLa cells (unpublished data). It is known that p53 protein in HeLa cells is reduced to an undetectable level as a consequence of its targeting for enhanced polyubiquitination by HPV encoded E6 oncoprotein and accelerated degradation in the proteasome. It has been recently shown that even inhibition of proteasome activity or protein export is not sufficient to accumulate p53 in HeLa cells [Węsierska-Gądek et al., 2002]. It seems plausible that p53 protein can be reactivated primarily by its nucleolar targeting accompanied by repression of HPV encoded E6 protein induced during treatment by distinct anti-cancer drugs [Węsierska-Gądek et al., 2002].

Thus, we show for the first time that the protein–protein interaction between p53 and PARP-1 is very specific and restricted to specific regions of both proteins. Remarkably, the amino-terminal domain of p53 which 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. It would suggest that wt p53 if complexed with PARP-1 retains its activity as a transcription factor. This assumption is supported by the observation that in primary rat cells overexpressing simultaneously p53 and PARP-1, p53 at the permissive temperature functioned as a transcription factor and activated its downstream genes such as *mdm2 and p21^{waf-1}* [Węsierska-Gądek and Schmid, 2000].

It is known that fine regulation of the activity and stability of p53 can be regulated not only by its post-translational modifications but also by alteration of its intracellular distribution. The NLS and NES are the essential elements for proteins subjected to nucleocytoplasmic shuttling. Three NLS were identified in the carboxy-terminus of p53. The first NLS (aa 316–322) is the most effective of the three in targeting p53 nuclear import, whereas the two other NLS are much weaker. The highly conserved leucine-rich NES has been identified in the tetramerization domain of p53, indicating that p53 can be exported independently from MDM2 [Stommel et al., 1999]. 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 impairs the interaction between NES and CRM1, blocks very efficiently p53 export. In normal unstressed cells p53 protein is predominantly nuclear in G_1 and is largely cytoplasmic during S and G_2 [Shaulsky et al., 1991; David-Pfeuty et al., 1996]. This intracellular distribution is consistent with its role as a mediator of G_1 checkpoints. In response to stress, however, p53 is stabilized and retained in the nucleus where it induces the expression of genes involved in the induction of cell cycle arrest or apoptosis.

A strong 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 a hyperactive p53 export. We showed in this study that PARP-1 binds to the central domain and to the carboxy-terminal region of p53 harboring the NES. The latter seems to possess higher capacity to bind to PARP-1. The assumption that PARP-1 may regulate the nuclear export of p53 protein via interaction with its carboxy-terminus is consistent with the observation that inactivation of PARP-1 in mouse cells reduced only the basal expression of regularly spliced form of p53 whereas alternatively spliced form remained unaffected. Both splice variants differ in their distal carboxy-terminal region. Therefore, it seems 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.

Since the activity and stability of p53 protein strongly depend on its post-translational modifications, we examined in the following paper the role of p53 phosphorylation for its binding capacity to PARP-1.

ACKNOWLEDGMENTS

We thank Dr. Gilbert de Murcia for the baculovirus encoding full length human PARP-1. We also thank Christian Balcarek for technical assistance. The authors are grateful to Dr. Mike Roberts for careful reading of the manuscript and helpful suggestions.

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