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Differential Susceptibility of Normal and PARP Knock-Out Mouse Fibroblasts to Proteasome Inhibitors

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Abstract Recently we found a clearly reduced basal level of wt p53 protein in PARP-deficient cells. Interestingly, PARP deficiency affected only regularly spliced (RS) wt p53. No significant difference of the p53 transcription rate was observed between wt and PARP-lacking cells. To clarify whether the reduction of RS p53 protein is due to a lower translation rate or rather to its instability in the absence of functional PARP, we investigated the effect of the inhibition of proteasome activity and nuclear export on the p53 level. The p53 half-life was approximately eight-fold decreased in PARP-lacking cells. Surprisingly, treatment with three proteasome inhibitors increased RS p53 in normal but not in PARP-deficient cells. However, the inhibition of nuclear export resulted in a considerable accumulation of RS p53 in the latter. Therefore, we decided to increase concentrations of the inhibitors. Their higher concentrations strongly affected viability of normal, but not of PARP-deficient cells, about 70% of MEFs died. Interestingly, higher concentrations of proteasome inhibitors resulted in the appearance of RS p53 in PARP-lacking fibroblasts. Reconstitution of PARP-deficient cells with PARP restored the normal susceptibility to proteasome inhibitors thereby unequivocally demonstrating that the enhanced cytotoxicity of proteasome inhibitors and their action on p53 level depends on the presence of functional PARP. J. Cell. Biochem. 78:681–696, 2000.

Key words: PARP knock-out cells; p53 expression; cytotoxicity of proteasome inhibitors; resistance to proteasome inhibitors

The Wild-type (wt) p53 is a 53 kD nuclear phosphoprotein that is expressed in low amounts in normal cells due to a very short half-life [for review see Cox et al., 1995; Donehower et al., 1993; Ullrich et al., 1992; Velculescu et al., 1996; Vogelstein et al., 1992]. In response to cytotoxic or genotoxic stimuli, such as ultraviolet light, ionizing radiation, carcinogens, or hypoxia the level of p53 increases markedly [Reich et al., 1984]. The resulting accumulation of p53 leads to cell cycle arrest [Maltzman et al., 1984; Fritsche et al., 1993] or induction of apoptosis [Yonish-Rouach et al., 1991] thereby preventing the proliferation of injured cells and allowing repair of DNA lesions. The accumulation of wt p53 is mainly caused by its stabilization and is only rarely the result of enhanced transcription or translation [Chernov et al., 1998; Finlay et al., 1989]. The exact mechanism of the wt p53 stabilization has not been elucidated until now. It has been proposed that the posttranslational modifications, especially phosphorylation may affect the steady-state of p53 protein [Ullrich et al., 1992].

Poly(ADP-ribose) polymerase (PARP) is another nuclear protein involved in the DNA damage response in mammalian cells [D'Amours et al., 1999; Berger, 1985; de Murcia et al., 1994]. PARP is a 113 kD multifunctional enzyme catalyzing the modification of a few protein targets by oligo- or poly(ADP-ribose) chains covalently coupled to acceptor proteins. The analysis of structural organization of the PARP molecule revealed presence of three integral domains which closely correlate with

Abbreviations used: AS, alternatively spliced; hdm-2, human double minute 2; IF, immunofluorescence; KO, knock-out; L, lactacystin; LMB, leptomycin B; mdm-2, mouse double minute 2; MEFs, mouse embryo fibroblasts; MG-132, Cbz-Leu-Leu-Leucinal; NES, nuclear export signal; PARP, poly(ADP-ribose) polymerase; PSI, N-Benzyloxycarbonyl-Ile-Glu(OtBu)-Ala-Leucinal; RS, regularly spliced; ts, temperature-sensitive; wt, Wild-type.

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three distinct functions of the enzyme [Gradwohl et al., 1990; Schreiber et al., 1992]. PARP is ubiquitously distributed at about 1×10^5 molecules/cell, and in contrast to p53 its expression is not affected by DNA lesions. However, in response to DNA damage enzymatic activity of PARP is strongly stimulated resulting in transient modification of proteins. Interestingly, proteins directly involved in DNA metabolism and chromatin organization are major targets of poly(ADP-ribosyl)ation [D'Amours et al., 1999]. Recently, development of new molecular biological and genetic strategies substantially contributed to the elucidation of the biological function of PARP. It has been shown that PARP is an essential component of the base excision repair complex and its activity is necessary for the formation of functional complexes with XRCC1 protein [Masson et al., 1998]. The accessory role of PARP in DNA repair contributes to the increased sensitivity of PARP knock-out mice to alkylating agents and γ-irradiation [Masutani et al., 1999; Menissier-de Murcia et al., 1997; Wang et al., 1997]. PARP has also been shown to be involved in the execution of apoptosis in vitro [Lazebnik et al., 1995; He et al., 1998] and in vivo [Węsierska-Gądek et al., 1999a].

Obviously, p53 and PARP are involved in cellular response to DNA damage and contribute to the initiation or execution of apoptosis suggesting that both proteins can be functionally related. In fact, PARP is a p53-binding protein and form tightly complexes with wt as well as mutant form of p53 [Vaziri et al., 1997; Węsierska-Gądek et al., 1996a,b, 1998]. Interestingly, in rat cells constitutively expressing the temperature-sensitive (ts) mouse $p53^{135val}$ mutant, PARP was sequestered in the cytoplasm by p53 at the restrictive temperature and upon temperature shift to 32°C both proteins moved into the nucleus [Wesierska-Gadek et al., 1996a, 1998]. Moreover, in these cells mutant but not wt p53 was poly(ADP-ribosyl)ated [Węsierska-Gądek et al., 1996a]. The experiments performed in vitro with purified recombinant human and murine wt p53 revealed that wt p53 protein can be modified by poly(ADP-ribose) chains [Węsierska-Gądek et al., 1996b]. However, preincubation of p53 protein with the p53 consensus sequence, resulting in complex formation, abolished its modification [Węsierska-Gądek et al., 1996b] thereby implicating that under in vivo conditions functionally active wt p53 bound to DNA is not susceptible to covalent modification by poly(ADP-ribose). Recently, poly(ADP-ribosyl)ation of p53 was also reported by other groups [Simbulan-Rosenthal et al., 1999]. Furthermore, PARP regulates the stability of wt p53 protein. There are two different lines of evidence that PARP affects the steady-state of p53. We have recently reported that overexpressed PARP delays the release of rat cells from p53 mediated G_1 check point due to stabilization of wt p53 protein [Węsierska-Gądek et al., 1998]. Overexpressed PARP was able to protect wt p53 from degradation despite the presence of upregulated mdm-2 protein. Remarkably, the enzymatic activity of PARP was necessary for p53 stabilization since the treatment of G_1 arrested cells with PARP inhibitors abolished this effect [Węsierska-Gądek et al., 1998]. We have also found a clearly reduced constitutive expression of wt p53 protein in immortalized PARP -/- mouse embryo fibroblasts (MEFs) [Węsierska-Gądek et al., 1999b]. Correlation between PARP and expression of p53 protein was also reported by other groups [Agarwal et al., 1997; Menegazzi et al., 1999; Whitacre et al., 1995]. Interestingly, PARP deficiency affected only the regularly spliced (RS) form of wt p53 protein, whereas alternatively spliced (AS) p53 remained unchanged [Węsierska-Gądek et al., 1999b]. To prove whether the reduction of the RS form of p53 is a result of its extreme instability in the absence of functional PARP, we have investigated the effect of the inhibition of proteasome activity and of protein export on the level of wt p53.

Proteasome inhibitors markedly affected the phenotype and viability of normal mouse fibroblasts. The most dramatic changes were observed in response to MG-132. Immunoblotting analysis revealed the highest p53 increase in response to lactacystin, a highly specific and irreversible inhibitor of the cytoplasmic proteasome. Surprisingly, in vivo treatment of PARP -/- cells with three different proteasome inhibitors did not affect the staining pattern with PAb421, almost all nuclei remained p53 negative. However, the inhibition of nuclear export by leptomycin B resulted in a considerable accumulation of PAb421 immunoreactive wt p53 in nuclei of PARP-deficient cells, thereby showing that the nuclear retention protects RS p53 from proteolytic degradation. Pulse-chase analysis revealed approximately eight-fold de-

crease of the p53 half-life in PARP knock-out MEFs. Considering the lack of p53 accumulation upon inhibition of proteasome activity, we decided to increase concentrations of the inhibitors. PSI and MG-132 did not affect the viability of cells lacking PARP gene but were highly cytotoxic towards normal fibroblasts. Interestingly, the application of proteasome inhibitors at much higher concentrations resulted in accumulation of RS p53 in PARP deficient cells. The reconstitution of PARP -/- cells with human PARP restored the normal susceptibility of the cells to proteasome inhibitors. These data unequivocally show that the enhanced cytotoxicity of proteasome inhibitors depends on the presence of functional PARP.

MATERIALS AND METHODS

Cells

Primary cells and immortalized MEFs were obtained from PARP +/+ (EF-10, A-19) and from PARP -/- (EF-6, A-11, and A-12) mice [Wang et al., 1995, 1997]. Immortalized PARP -/- cells (clone A-11) were reconstituted with human PARP [Herceg et al., 1999]. An eukaryotic expression construct containing full-length human cDNA under the control of SV-40 promoter was used to generate stable cell lines expressing exogenous PARP. Cells were positively selected with hygromycin and resistant clones (A-11/wt2 and A-11/wt3) were isolated and characterized by Southern blot analysis. Cells were grown in DMEM supplemented with 10% FCS in an atmosphere of 7.5% CO_2. PARP reconstituted cells were cultivated with hygromycin.

Antibodies

Different anti-p53 antibodies recognizing distinct epitopes were used. Anti-p53 antibodies PAb421 (Ab-1), PAb240 (Ab-3), and PAb246 (Ab-4) were obtained from Oncogene Research Products (Cambridge, MA.). CM-1 was obtained from Novocastra Laboratories Ltd. (Newcastle upon Tyne, England). Anti-mdm-2 antibodies (SGM-14) were from DAKO A/S (Glostrup, Denmark). Appropriate secondary antibodies linked to horseradish peroxidase (HRP), Cy-2 or Cy-3 were from Amersham International (Little Chalfont, Buckinghamshire, England).

Cell Treatment

Cells were treated with 0.1–1 μM leptomycin B (LMB) [Wolff et al., 1997] and with proteasome inhibitors: Lactacystin, Cbz-Leu-Leu-Leucinal (MG-132) or N-Benzyloxycarbonyl-Ile-Glu(OtBu)-Ala-Leucinal (PSI; Calbiochem) [Fenteany et al., 1995; Griscavage et al., 1996; Traenckner et al., 1994] or with inhibitors of lysosomal proteases: Calpain inhibitor II (Calbiochem), E-64, and leupeptin (Roche) for indicated periods of time.

Cytotoxicity Assay

Sensitivity of cells to inhibitors of different proteolytic pathways was assessed using a microtiter plate colorimetric assay, which measures the ability of viable cells to cleave the tetrazolium salt (WTS-1) to a water-soluble purple formazan. Cells were plated at the appropriate density (10,000 cells per well) in a 96-well microtiter plate. After a 24 h incubation, cells were exposed to various concentrations of distinct protease inhibitors for an appropriate time. After treatment, the medium was removed and replaced with 200 μl of inhibitor-free medium and plates were incubated for further 72 h. At this time, 20 μl of WTS-1 was added to each well. After a 4 h incubation at 37°C plates were shaken for 5 min. Absorbance was measured at 460 nm on a Labsystem Multiscan microtiter plate reader. Each column represents the mean ± SD (bars) of three independent experiments, each performed in triplicate.

Incorporation of ^{3}H-Thymidine

To assess the effect of proteases inhibitors on cells progressing through S phase, incorporation of ^{3}H-thymidine was determined. Cells were pulse labeled 1 h before harvesting. Cells were washed with PBS, lysed and three aliquots were dropped onto cellulose filter discs. After precipitation with 10% trichloroacetic acid (TCA), discs were washed in 5% TCA, then in ethanol and dried. The radioactivity of the precipitated material was measured by scintillation counting. An additional aliquot of the cell lysate was used for measurement of DNA content. The DNA labeling (cpm/μg DNA) in treated cells was expressed as a percentage of values measured for corresponding untreated controls.

Indirect IF Microscopy

Cells grown on coverslips were rinsed with PBS, fixed in ice-cold methanol-acetone (3:2) for 20 min, and washed with PBS. The cells were incubated with anti-p53 antibodies at appropriate concentration, and the immune complexes were detected by incubation with corresponding secondary antibodies covalently coupled to Cy-2 or Cy-3 (Amersham International). To visualize nuclei, preparations were additionally stained with the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI), which is specific for DNA.

Pulse-Chase Analysis

Cells were prestarved in DMEM lacking methionine supplemented with 10% dialysed FCS for 30 min. Then ^{35}S-methionine (NEN Life Science Products) at 100 μCi/ml was added. After 1.5 h pulse labeling, the cells were rinsed three times with PBS and chased with medium containing 10% FCS and 2 mM unlabeled methionine. Cells were harvested at various times after chase and lysed in RIPA buffer. Equal numbers of counts were precipitated with anti-p53 PAb421 [Węsierska-Gądek et al., 1996a]. Immunoprecipitates were separated on 10% SDS gels and blotted onto polyvinylidene difluoride membrane (PVDF; Amersham International). Radioactively labeled proteins were visualized by autoradiography and quantification was performed by a phosphoimager.

Immunoblotting

Proteins dissolved in reduced SDS-sample buffer were separated on SDS-polyacrylamide gels, electrophoretically transferred onto PVDF. Equal protein loading was confirmed by Ponceau S staining. Blots were incubated with specific antibodies and the immune complexes were detected autoradiographically using appropriate peroxidase-conjugated secondary antibodies and enhanced chemiluminescent detection reagent ECL+ (Amersham International). In some cases, blots were stripped and used for sequential incubation. Equal protein loading was additionally proved by immunoblotting with anti-actin antibodies.

Determination of Proteasome Activity

Z-Leu-Leu-Leu-AMC degrading activity was determined [Tsubuki et al., 1993] by measuring the fluorescence of the generated aminomethylcoumarin. The reaction mixture contained 100 mM Tris-acetate (pH 7.0), 25 μM substrate dissolved in DMSO and an aliquot of cytosol fraction. Assays were performed either with or without the addition of 2 mM ATP [Ciechanover, 1994]. After incubation at 37°C for 15 min, the reaction was terminated by adding 10% SDS and 100 mM Tris-acetate (pH 9.0). Fluorescence of the reaction products was measured (excitation 380 nm/emission 440 nm). The activity was expressed as arbitrary units (1 unit = 1 absorbance unit/10 μg protein).

RESULTS

To elucidate the reason of reduced constitutive expression of RS p53 in PARP-deficient cells, we have focused our attention on cellular events downstream from protein synthesis. The level of wt p53 protein in normal cells is exactly controlled and one major pathway by which p53 concentration may be regulated, is mdm-2 mediated destabilization of p53 followed by its ubiquitination and degradation by the proteasome [Haupt et al., 1997; Kubbatat et al., 1997]. Moreover, it has been also reported that nuclear export is required for proteolytic cleavage of wt p53 protein [Freedman et al., 1998]. Therefore, we examined whether the feedback loop pathway involving mdm-2 activation is responsible for the accelerated degradation of RS p53 in PARP deficient cells and whether the inhibition p53 export from the nucleus could prevent the degradation cascade.

We have performed experiments with both: primary embryonal cells and immortalized fibroblasts from normal as well as PARP-deficient mice to eliminate eventual effects resulting from immortalization. The primary cells isolated from wt and from PARP -/- mice were very heterogeneous and no obvious phenotype difference was observed between them. In contrast, the phenotype of the established PARP -/- cells differed significantly from that of normal counterparts (Fig. 1A).

The inspection of cells in phase contrast microscopy revealed that the PARP -/- cells are considerably smaller than normal cells. At comparable cell density, a larger number of cells with PARP null genotype was present pro square unit. To rule out the possibility that the observed phenotypical difference was due to stronger bottom adherence of the wt MEFs or due to their more flat shape, we determined the

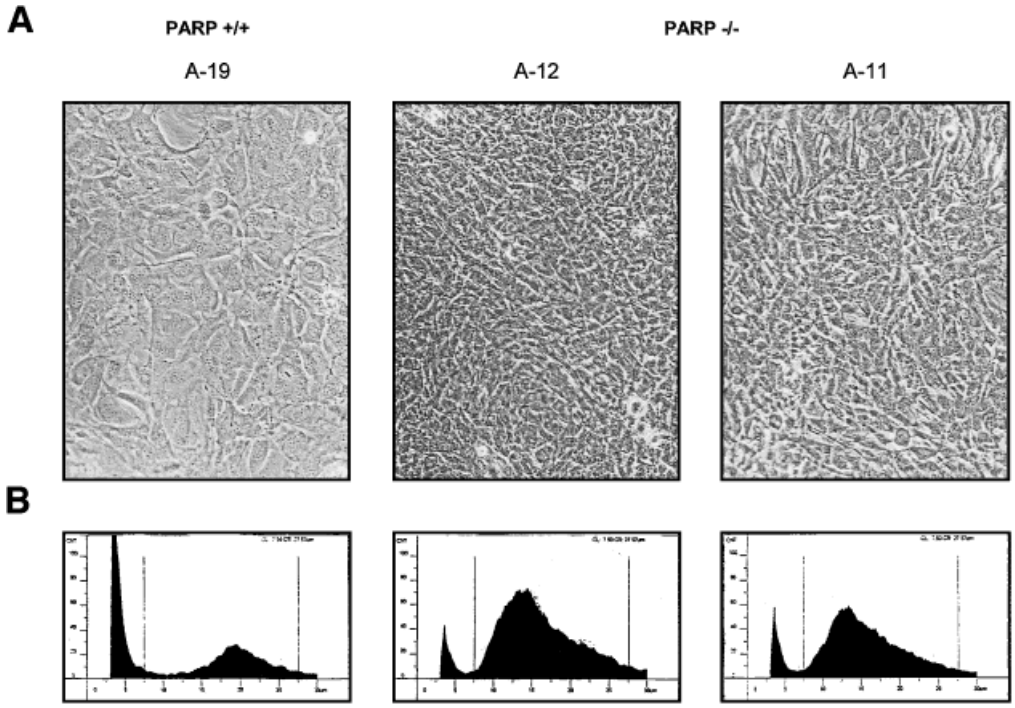

Fig. 1. Differential phenotype of PARP -/- cells. **A**: Phase contrast microscopy of MEFs. **B**: Cellular diameter distribution of suspended mouse fibroblasts.

volume of cells in suspension by CASY (Cell Counter + Analyser CASY1 Model TTC; Fig. 1B). Indeed, the size of both PARP deficient cell lines (medium diameter 15 μM) was clearly reduced as compared with wt cells (20 μM).

In the next experiments we have tested the effect of three different proteasome inhibitors on the level of RS p53 in mouse fibroblasts. We have used lactacystin, an irreversible, highly specific inhibitor of proteasomes and additionally two hydrophobic peptide aldehydes (MG-132 and PSI) that are reversibly acting drugs. We have applicated them at concentrations of 5 μM. It has been previously shown that the degradation of p53 protein has been prevented by low concentrations of the inhibitors of proteasome activity [Maki et al., 1996]. Treatment with the inhibitors for 24 h affected strongly the morphology and viability of normal mouse fibroblasts, whereas no obvious effects have been observed in both PARP-deficient cell lines. The most dramatic changes occurred in response to MG-132. Wt mouse fibroblasts shrunk and lost their bottom adherence. The effects of the proteasome inhibitors were monitored by determination of cell viability and measurement of the cell size. As depicted in Figure 2 proteasome inhibitors resulted in cell shrinkage and induction of apoptosis of normal MEFs, whereas PARP -/- cells remained vastly unaffected.

The action of proteasome inhibitors on wt p53 protein was determined by immunofluorescence microscopy as well as by immunoblotting. The use of both methods gave us the opportunity to determine quantitative changes of p53 level, and to monitor its intracellular distribution. We have performed immunostaining with the monoclonal anti-p53 antibody PAb421, which is known to recognize an epitope within the COOH terminus of the RS form of p53 protein. The maintenance of normal mouse cells in the presence of proteasome inhibitors resulted in distinctive changes in the distribution of wt p53 protein. In response to lactacystin enhanced cytoplasmic accumulation of p53 as well as changes in the distribution of p53 immunoresponsive signals in nuclei were observed (Fig. 3). MG-132 treated cells, however, exhibited dramatic displacement of wt p53 within the nuclei. Nearly uniform staining of p53 in the control nuclei was converted upon MG-132 into a nuclear-body-like pattern. The

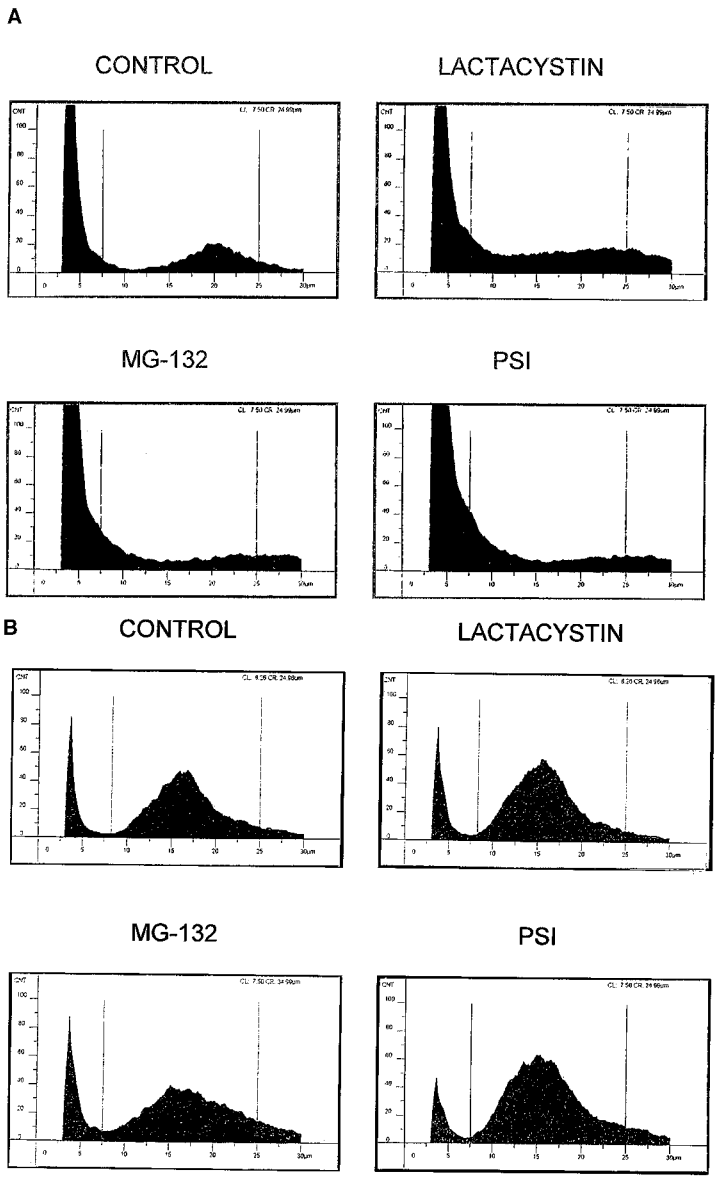

Fig. 2. Effect of proteasome inhibitors on the cellular diameter of suspended immortalized normal (A-19; **A**) and PARP -/- (A-11) MEFs (**B**). Twenty-four h after cell plating proteasome inhibitors dissolved in DMSO were added to the cell culture to a final concentration of 5 μM. Corresponding amounts of DMSO were added to the controls. After 18 h cells were harvested and the cell size was determined.

less apparent changes of p53 staining pattern resembling those after lactacystin occurred in response to PSI treatment.

Surprisingly, the treatment of PARP deficient cells with the inhibitors at identical concentrations did not considerably affect their reactivity with PAb421. In response to lactacystin and MG-132 almost all cells remained p53 negative. Only a few p53 weakly stained nuclei were detected in the cell population fixed on the slides (Fig. 3). Upon treatment of PARP -/- cells with PSI a faint cytoplasmic staining appeared. These results called the question whether the PARP -/- cells are able at all to produce the RS form of wt p53 protein. Therefore, we have decided to check to what extent in vivo blocking of protein export would affect the level of RS p53 in this cells. Interestingly, the inhibition of protein export by LMB resulted in the appearance of PAb421 immunoresponsive signals in almost all nuclei of PARP -/- cells (Fig. 3) thereby demonstrating that these cells indeed synthesize RS p53 protein. In contrast, the LMB treatment of normal MEFs had no apparent effect on the PAb421 staining pattern: all control as well as LMB treated nuclei were stained with comparable intensity. The same results were obtained with primary cells (data not show) indicating that the observed reduction of the wt p53 protein level was the

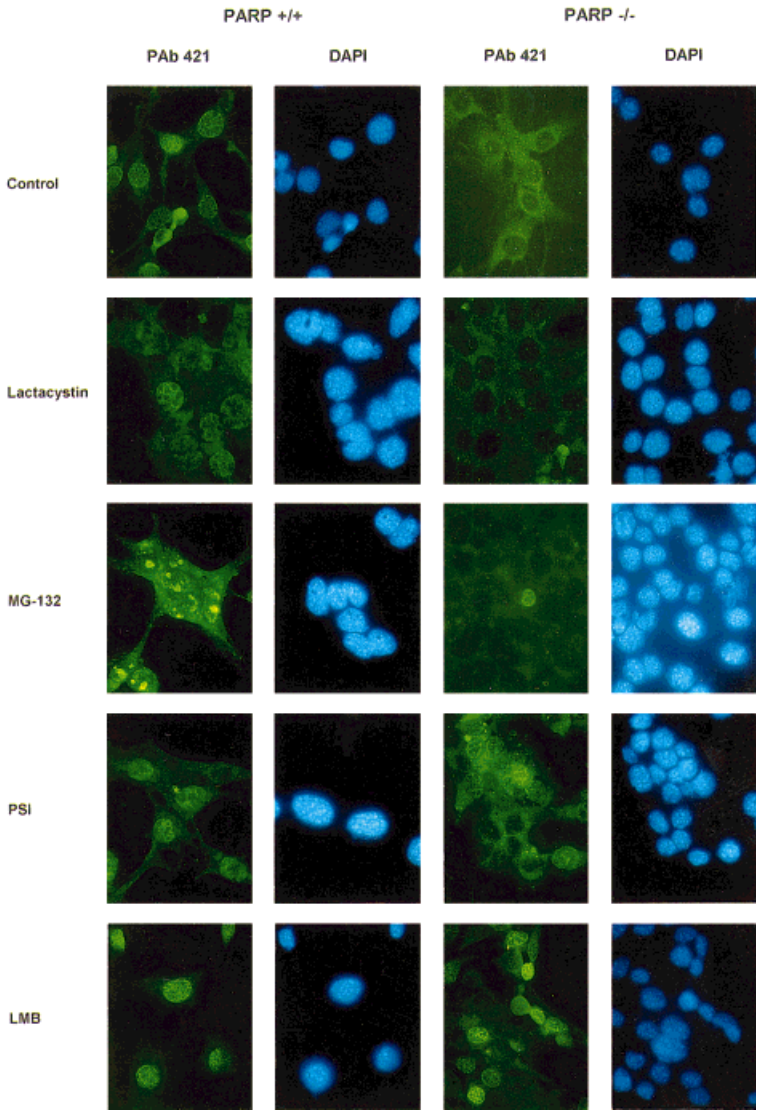

Fig. 3. Effect of inhibition of proteasome activity and nuclear export on the p53 staining. Nuclei were visualized with 4,6-diamidino-2-phenylindole (DAPI). Twenty-four h after cell plating proteasome inhibitors or LMB were added to a final concentration of 5 μM and 1 μM, respectively and cells were cultivated for further 24 h. Thereafter cells were fixed and immunostained with anti-p53 monoclonal antibody PAb421.

consequence of PARP inactivation but not the result of clonal changes during immortalization.

To examine whether the decrease of steady-state level of RS p53 in PARP-deficient cells was a result of diminished translation rate or enhanced p53 protein degradation, the half-life of the p53 protein was determined. Proteins were in vivo pulse-labeled with ^{35}S-methionine and then chased in medium containing an excess of unlabeled methionine. The pulse-labeling was performed in the presence and absence of LMB. Lysates were immunoprecipitated under stringent conditions with anti-p53 antibodies PAb421 and CM-1. Purified immune complexes were separated on 10% SDS gels, blotted onto the membrane and visualized by autoradiography (Fig. 4A). PAb421 and CM-1 precipitated labeled p53 with the comparable efficiency. Quantification revealed the half-life of p53 in normal immortalized MEFs of about 50 min confirming the previously published data [Freedman et al., 1998]. However, the pulse-chase analysis showed that the p53 half-life was substantially reduced in cells lacking PARP to approximately 6 min. The addition of LMB during ^{35}S-methionine pulse considerably increased p53 labeling in these cells showing that inhibition of protein export prevents p53 degradation in the cytoplasm.

The action of LMB and proteasome inhibitors on the level of RS p53 protein was additionally monitored by immunoblotting. The

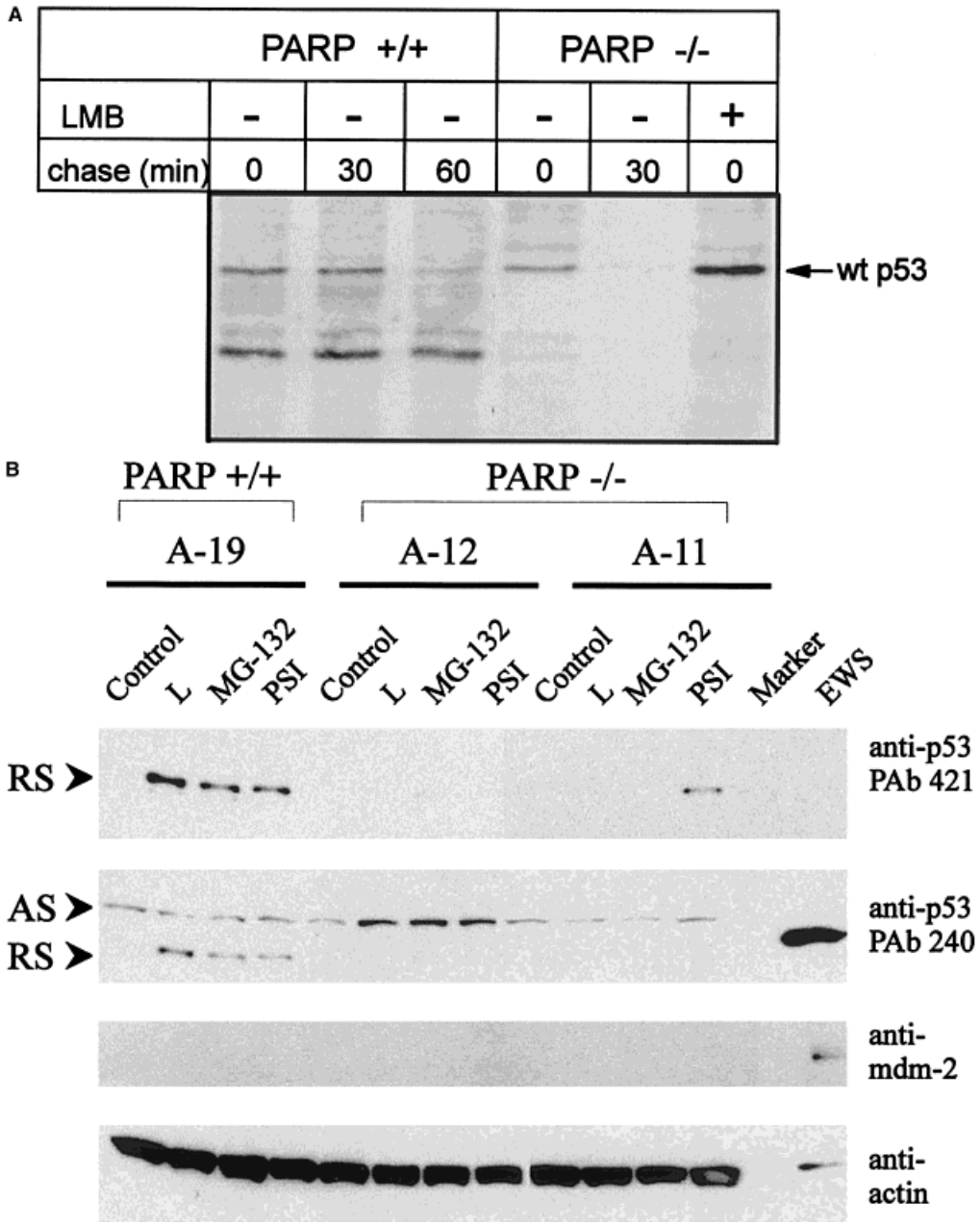

Fig. 4. **A**: Decrease of the p53 half-life in PARP -/- cells. Cells were in vivo labeled ^{35}S-methionine for 1.5 h in the presence or absence of 100 nM LMB, then chased in medium containing an excess of cold methionine for indicated time and lysed. Lysates were immunoprecipitated with anti-p53 PAb421 and separated on 10% SDS-gel. Precipitated proteins were visualized by autoradiography. **B**: Lack of the accumulation of RS p53 in PARP-deficient cells after treatment with proteasome inhibitors. Cells were treated with proteasome inhibitors for 24 h at a final concentration of 5 μM. Twenty μg of total cellular proteins was loaded on 10% SDS-slab gels. Immunoblotting was performed using anti-p53 antibody PAb421 and subsequently with anti-p53 PAb240, anti-mdm-2 and anti-actin antibodies. As p53 and mdm-2-positive controls, human cell line (EWS) expressing temperature-sensitive $p53^{138val}$ mutant was used. The cells were maintained at 32°C for 6 h to allow activation of hdm-2 protein by wt p53. Human $p53^{138val}$ protein is expressed in latent form and does not react with PAb421 but is recognized by PAb240 and DO-1.

treatment of normal MEFs with proteasome inhibitors resulted in significant increase of RS p53 concentration, as visualized by immunoreactivity with PAb421 (Fig. 4B). The strongest effect exhibited lactacystin. In contrast, no noticeable effect of the proteasome inhibitors on the RS p53 level could be detected in PARP null genotype cells.

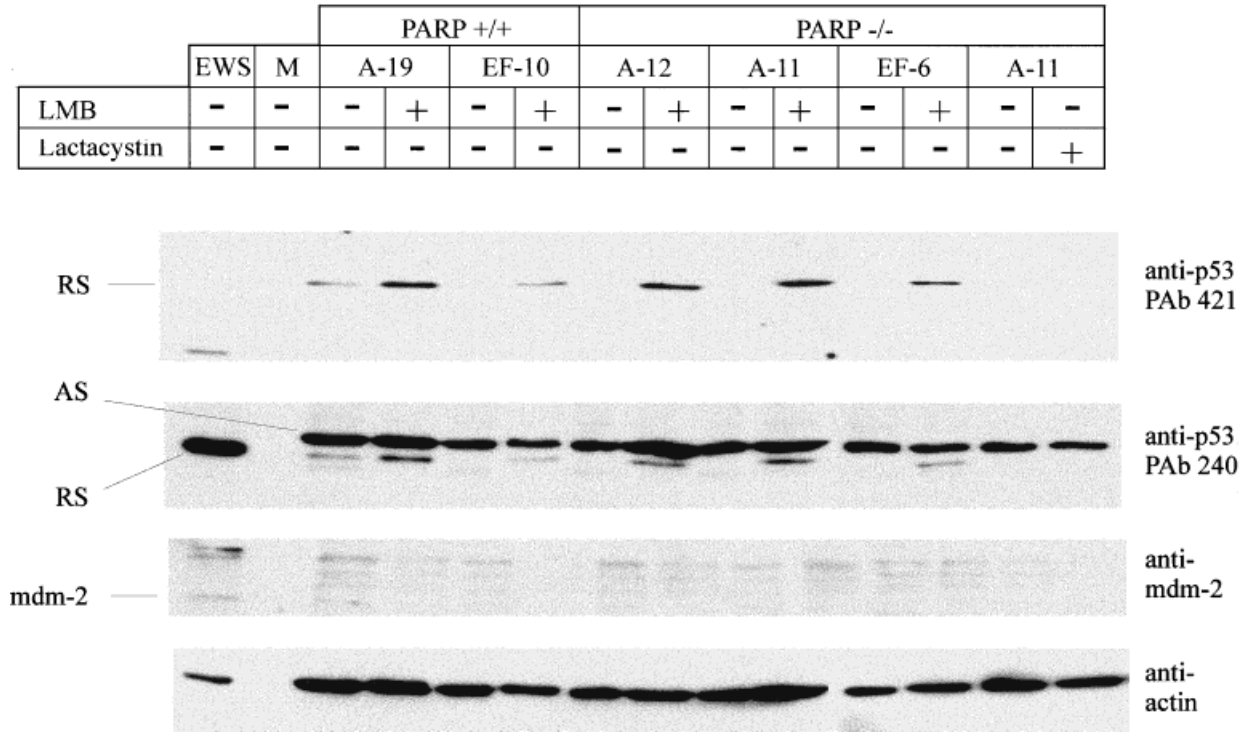

Fig. 5. Increase of the level of wt p53 protein in response to inhibition of protein export by LMB. Primary (EF-6, EF-10) and immortalized (A-19, A-12, A-11) fibroblasts were incubated in the presence of 1 μM LMB for 24 h. Total cell lysates were resolved on 10% SDS slab gels. Immunoblotting was performed as described in Figure 4. M, molecular weight markers; EWS, human cell line, for details see Figure 4.

After overexposing autoradiograms, a very weak, barely detectable RS p53 signal appeared in lysates of PARP -/- cells after treatment with PSI. Unfortunately, the weak reactive band was not satisfactorily resolved on photomicrograph. However, PARP-deficient cell lines clearly responded to LMB with an increase in p53 concentration: missing protein band of RS p53 in untreated controls became visible in response to LMB application (Fig. 5). The subsequent incubation of the blots with PAb246, recognizing both splice variants of p53 revealed also slight accumulation of AS p53 upon protease inhibitors and LMB. Despite the significant increase of p53 concentration in the PARP -/- cells after inhibition of nucleocytoplasmic trafficking, no activation of mdm-2 protein could be detected neither by the IF (not shown) nor by immunoblotting (Fig. 5). The reactivity of the anti-mdm-2 antibody was confirmed by a positive mdm-2 signal visualized in the lane loaded with a lysate of human cells expressing hdm-2 protein as a control. As illustrated in Figure 5 the concentration of RS p53 protein in wt MEFs changed only moderately upon treatment with LMB.

The lack of the accumulation of RS p53 protein in PARP KO cells after inhibition of proteasome activity was surprising and could be explained at least in two different ways. One could speculate that p53 protein in PARP deficient cells is processed by other proteolytic pathway or alternatively that these cells possess elevated activity of proteasomes and need much higher concentrations of corresponding inhibitors for effective protease inactivation. In the following experiments we examined both hypotheses. We have incubated the mouse fibroblasts with three specific inhibitors of lysosomal proteases: calpain inhibitor II, E-64, and leupeptin at two different concentrations. As shown in Figure 6 even the treatment with higher doses of lysosomal inhibitors did not affect the level of p53 protein in PARP null cells indicating that lysosomal degradation pathway is not involved in the accelerated degradation of RS p53 protein in PARP deficient cells. On the other hand, a moderate but

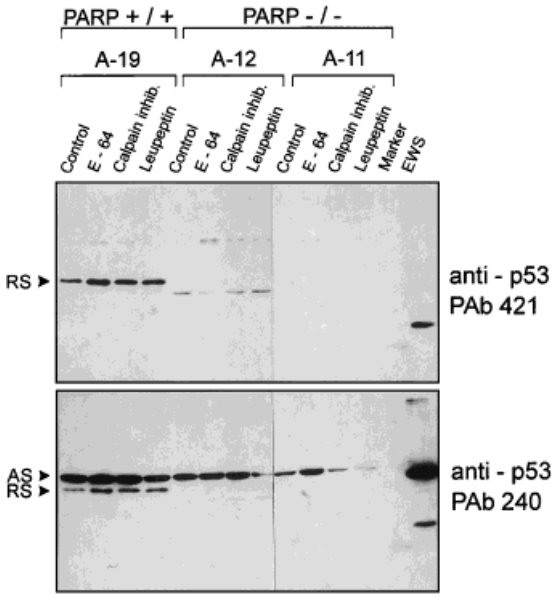

Fig. 6. Effect of lysosomal inhibitors on the level of wt p53 protein. Cells were treated with E-64 (20 μM), calpain inhibitor II (50 μM), or with leupeptin (20 μM) for 24 h. Then the cells were harvested and lysed. Total cell lysates were analysed by immunoblotting using anti-p53 antibodies PAb421 and PAb240.

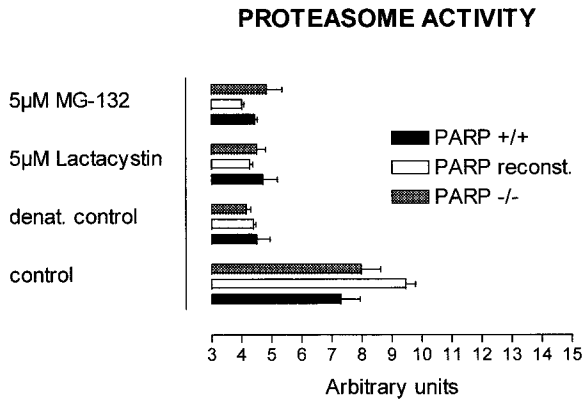

Fig. 7. A comparison of the proteasome activity between different cell lines depending on the PARP status. Cytosol aliquots in triplicate (10 μg/assay) were incubated with substrate in an appropriate buffer for 15 min at 37°C. After termination of the reaction fluorescence at 440 nm was measured. The effect of proteasome inhibitors and heat inactivation was additionally determined. Agents were added to the cytosol samples to a final concentration of 5 μM 5 min prior to the addition of the substrate.

clearly visible increase of both p53 splice variants was ascertained in wt cells.

In the next step we compared the proteasome activity between the distinct cell lines. We determined the Z-Leu-Leu-Leu-AMC degrading activity in cytosolic fraction using the equal amounts of protein. As depicted in Figure 7, the normal MEFs as well as PARP -/- cells exhibited comparable levels of the Z-Leu-Leu-Leu-AMC degrading activity. The addition of lactacystin, MG-132 or preincubation of cytosolic fraction at 100°C for 3 min strongly repressed the proteasome activity in all tested cells lines. However, calpain inhibitor did not inhibit the degrading activity (not shown). Addition of ATP stimulated the degradation by 10–15% (not shown). These results demonstrate that the proteasome activity in PARP deficient cells barely differs from that of normal counterparts and that the proteasomes possess in vitro similar sensitivity to the specific inhibitors.

Despite this observation, we decided to test the action of higher concentrations of proteasome inhibitors on the level of RS p53. In view of their cytotoxic effects initially observed after treatment of normal MEFs, we examined first their influence on the cell cycle progression and cell viability. The inhibitors decreased markedly the incorporation of ^{3}H-labeled thymidine in DNA in normal and PARP deficient cells (Fig. 8). The kinetics of inhibition of DNA replication was very similar in both cell types and the most dramatic reduction of the S-phase cells arose within first 6 h of treatment with all three agents. LMB also affected the DNA synthesis in both cell lines but the kinetics of the inhibition slightly differed. The repression of the nucleotide incorporation after LMB proceeded slower and more continuous.

The determination of the cell viability revealed that the individual proteasome inhibitors exert quite distinct cytotoxic effects on normal mouse fibroblasts. Whereas lactacystin even at high concentrations was low cytotoxic (Fig. 9), MG-132 already affected at low concentrations the viability of cells. In response to higher concentrations (20 μM) of PSI about 60% and in case of MG-132 about 70% of cells died. Contrary to highly cytotoxic action of proteasome inhibitors on normal MEFs, PARP -/- cells were completely resistant and did not die after treatment with PSI and MG-132 even at high concentrations despite of remarkable inhibition of DNA replication. Gaining by the lack of cytotoxicity of the proteasome inhibitors toward PARP KO cells, we tested their action at high concentrations on the level of RS p53.

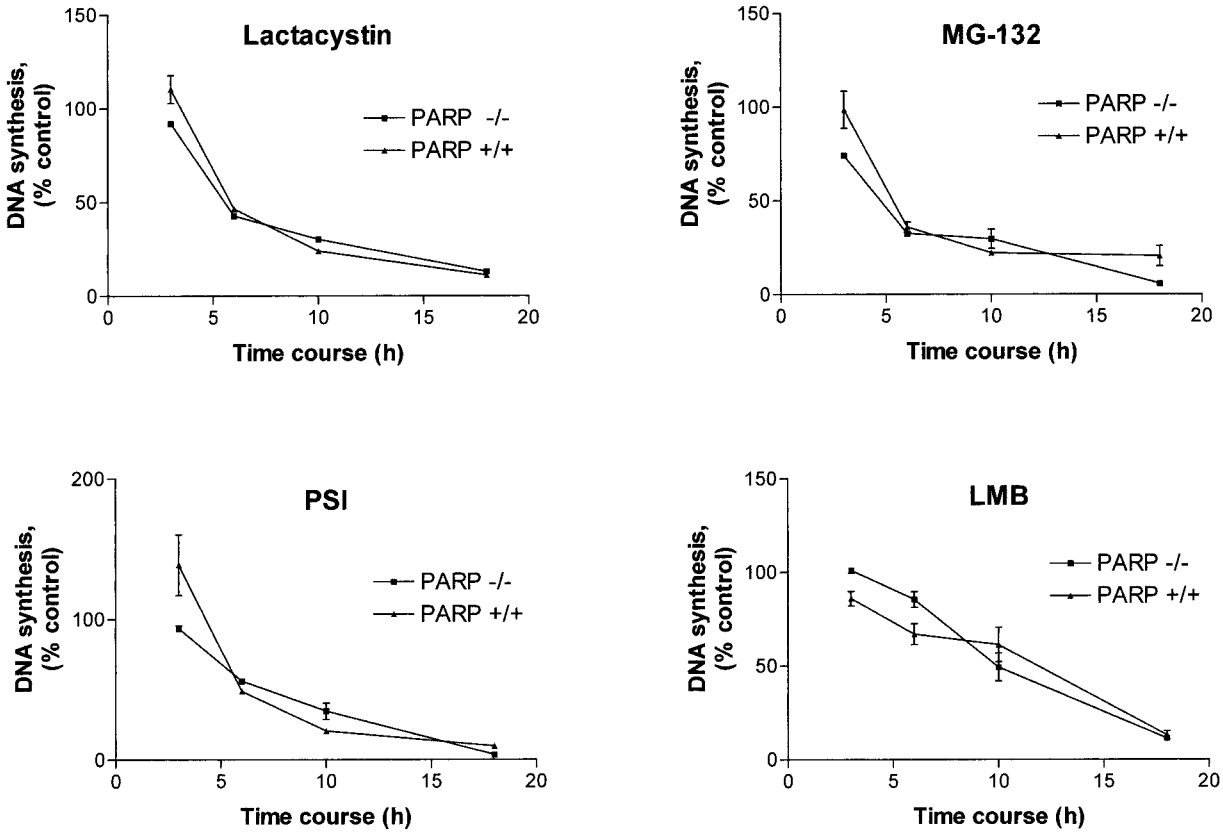

Fig. 8. Effect of proteasome inhibitors and LMB on the DNA replication. Twenty-four h after cell plating proteasome inhibitors and LMB dissolved in DMSO were added to a final concentration of 5 μM and 1 μM, respectively. Corresponding amounts of DMSO were added to the controls. Incubation was performed for indicated periods of time. One h prior to the cell harvesting ^{3}H-thymidine was added. Cells were washed and lysed. The incorporated radioactivity and DNA content were determined. The DNA labeling (cpm/μg DNA) in treated cells was expressed as a percentage of values measured for corresponding untreated controls.

As shown in Figure 10, some differences in the kinetics of p53 accumulation was observed in response to distinct inhibitors. Whereas 10 μM PSI was sufficient to moderately enhance the level of p53 protein, higher concentrations of lactacystin and MG-132 (20 μM) were necessary to induce the accumulation of RS p53. The same treatment of wt cells resulted in decrease of p53 level (Fig. 11) despite the equal protein loading as shown by immunoblotting with anti-actin-antibodies. These results show clearly that normal and PARP deficient cells exhibit quite different susceptibility to the three different proteasome inhibitors. To ensure that the great difference depends on the presence of functionally active PARP but not on other unknown factors, we reconstituted the PARP -/- cells with the corresponding human gene and tested respondency of the newly generated cell clones to the inhibitors. The reconstitution restored the normal sensitivity of cells to the inhibitors. In response to increasing concentrations of PSI and MG-132 enhanced cytotoxicity was observed and the cells died. Moreover, the application of low concentrations of the proteasome inhibitors was sufficient to induce strong accumulation of RS p53 (Fig. 12). These results unequivocally show that the resistance of the PARP deficient cells to treatment with proteasome inhibitors was a consequence of the inactivation of PARP gene.

DISCUSSION

The exact and thorough control of p53 activation is critical for normal cell growth and development. Not only the extent of p53 induction is of great importance but also the duration of p53 response. The level of activated p53 plays a key role in the consecutive decision between cell cycle arrest or apoptotic cell

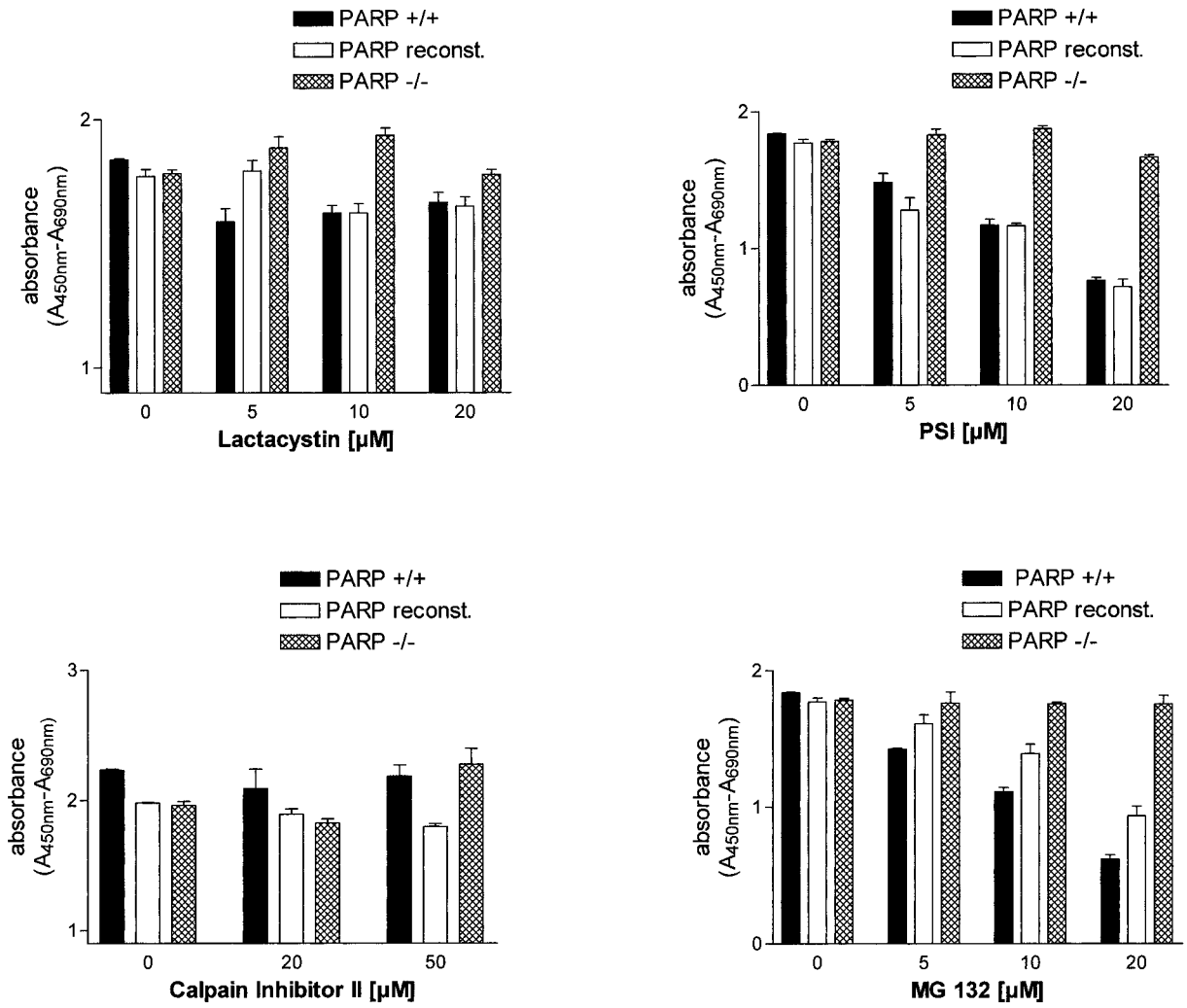

Fig. 9. Resistance of PARP deficient cells to cytotoxic action of proteasome inhibitors. Cells were treated with different concentrations of proteases inhibitors for 24 h. Thereafter medium was changed and cells were cultivated for further 72 h. Four h prior to cell harvesting, WST-1 was added. The absorbance was measured at 450 nm.

death. Some lines of evidence indicate that higher concentration of wt p53 promotes initiation of apoptosis. In the regulation of p53 level, the mdm-2 feedback loop seems to be of importance [Haupt et al., 1997; Kubbatat et al., 1997]. The interaction between wt p53 and mdm-2 takes place on two distinct levels. In one respect wt p53 functions as transcription factor and induces the expression of mdm-2 gene, which contains a cis-acting p53 responsive element in the first intron. On the other hand upregulated mdm-2 protein can bind to p53 via its NH_2-terminal domain and block the transcriptional activation function of p53 protein. Moreover, mdm-2 protein is able to target p53 for degradation. Mdm-2 mediated destabilization of p53 leads to its ubiquitination and finally to degradation by the proteasome.

In this study we tried to dissect two steps that are essential for p53 degradation: nuclear export and proteasome mediated cleavage. Treatment of mouse fibroblasts with three distinct proteasome inhibitors revealed marked differences in the susceptibility depending on the genotype of cells. Whereas the morphology and viability of PARP null genotype cells remained unaffected, the normal MEFs showed a strong reaction to the treatment. The cells shrinked, lost their adherence, underwent apoptosis and died. Proteasome inhibitors induced apoptosis has been previously reported [Lopes et al., 1997]. The events of apoptotic death

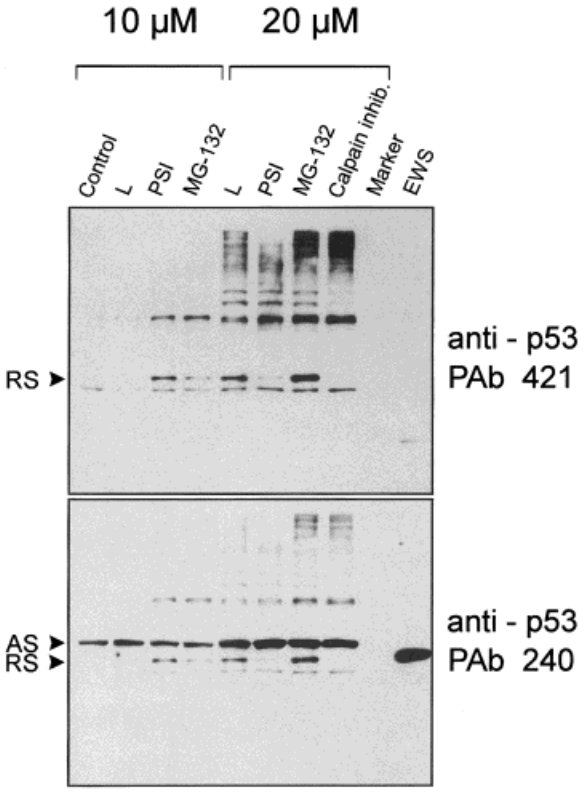

Fig. 10. Moderate accumulation of RS p53 in PARP -/- cells in response to higher doses of proteasome inhibitors. Inhibitors at a final concentration of 10 μM and 20 μM were applicated for 24 h. p53 was detected by sequential immunoblotting with PAb421 and PAb240.

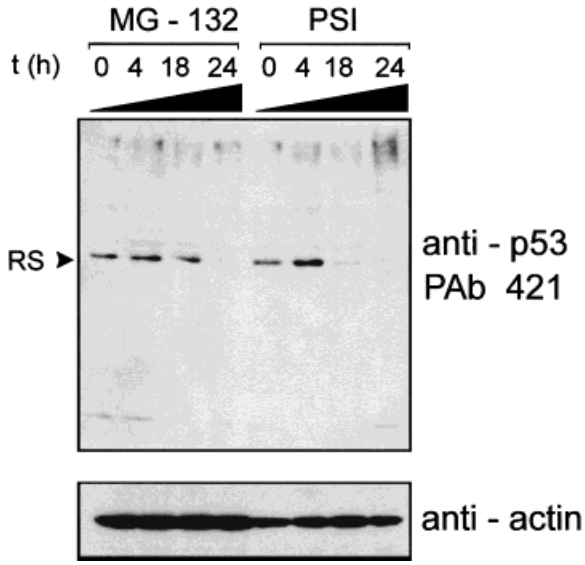

Fig. 11. Loss of wt p53 protein in normal MEFs in response to higher concentrations of proteasome inhibitors. Inhibitors were applicated at a final concentration of 20 μM for different periods of time.

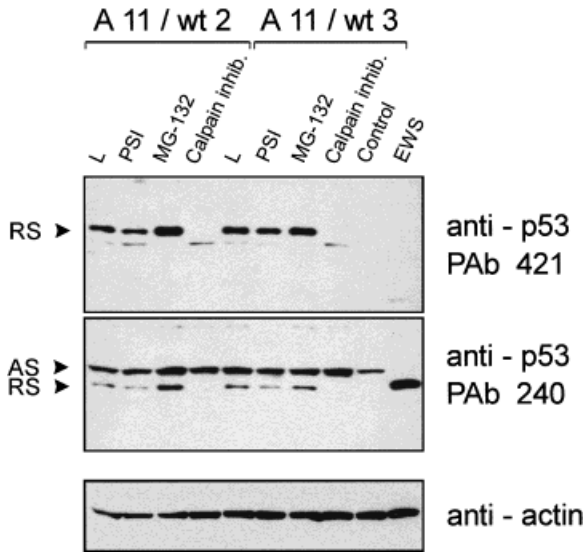

Fig. 12. Increased sensitivity of PARP reconstituted cells to proteasome inhibitors. Two different clones of PARP reconstituted cells (A11/wt2 and A11/wt3) were treated with proteasome inhibitors at a final concentration of 5 μM for 24 h. Total cell lysates were analyzed by immunoblotting for the accumulation of RS p53 protein.

were initialized by an increase of wt p53 level. Whether the same phenomenon is involved in the initiation of apoptosis in normal mouse fibroblasts is questionable, since no close correlation occurs between apoptosis rate and the extent of p53 increase. The highest elevation of p53 concentration was observed upon lactacystin application, whereas the highest cell death rate occurred upon MG-132. Surprisingly, cells lacking PARP remained resistant to proteasome inhibitors even at high concentrations and did not die. On the other hand, low concentrations of proteasome inhibitors decreased the DNA replication in both normal and PARP -/- cells and the kinetics of action as well as extent of inhibition was very similar.

The influence of proteasome inhibitors on the level of RS p53 in the tested cells was contrary to our anticipation. One could expect that inhibitors would elevate the amount of RS p53 protein just in PARP deficient fibroblasts since in these cells p53 exhibits eight-fold reduced half-life. However, no considerable effect of lower doses of proteasome inhibitors on the level of RS p53 could be detected in cells lacking PARP. The inefficacy of the lower doses of inhibitors in PARP KO cells can not be simply explained by their higher inhibitor require-

ment due to elevated proteasome activity. Normal MEFs as well as cells lacking PARP exhibited comparable activity of proteasomal proteases. Moreover, the proteasomes present in both normal and PARP deficient cells showed in an in vitro assay the same sensitivity to inhibitors. On the other hand, PARP deficient cells responded very well to the inhibition of nuclear export by LMB. The kinetics of p53 accumulation in these cells resembled that in normal counterparts. Interestingly, the LMB mediated increase of RS p53 protein was not accompanied by activation of the mdm-2 protein, suggesting that the accumulation of p53 protein is a consequence of its stabilization and did not represent activated form of RS wt p53 protein [Chernov et al., 1998]. Our above results show the different effects of proteasome inhibitors and LMB on cells lacking PARP.

What is the mechanism of LMB action on p53 ? Does this drug really inhibit p53 export or rather induce p53 stress response? It has been recently shown that LMB doesn't activate p53 stress response [Freedman et al., 1998]. In our study LMB had no obvious effect on nuclear p53 staining and level in normal MEFs. Otherwise, we have previously found that PARP deficient cells fail to stimulate RS p53 protein upon treatment with genotoxic agents. Therefore, the observed nuclear accumulation of p53 in PARP -/- cells is a consequence of the inhibition of nuclear export. How does LMB act? LMB isolated from Streptomyces extracts is able to interfere with the nuclear export signal (NES) at very low concentrations and completely blocks nuclear export of proteins possessing the required signal sequence. It inhibits in the nucleus the formation of ternary complexes consisting of CRM1, RanGTP, and proteins that contain a NES motif, thus unspecifically blocking nuclear export. It has been recently shown [Stommel et al., 1999] that wt p53 possesses a highly conserved leucine-rich NES located in its tetramerization domain and its export can be effective prevented by LMB.

Our present results show quite different susceptibility of normal and PARP -/- MEFs to proteasome inhibitors. On the one hand, normal MEFs showed great sensitivity to treatment with the inhibitors and died after treatment with MG-132 and PSI, whereas PARP deficient cells were completely resistant to them. On the other hand the inhibitors stabilized RS p53 protein in normal MEFs at lower concentrations, but not in PARP null genotype cells though the turnover of p53 protein is extremely rapid in the latter. The action of three distinct compounds was investigated in the present study. Lactacystin is characterized as an irreversible and very specific inhibitor of proteasome activity [Fenteany et al., 1995] whereas aldehyde peptides such as MG-132 and PSI can additionally inactivate at least to a low extent the lysosomal proteases [Traenckner et al., 1994].

In conclusion, our findings show that some biological effects exerted by proteasome inhibitors depends on the PARP status. Whereas the inhibition of DNA replication by inhibitors seems to be PARP independent, other effects e.g. high cytotoxicity of inhibitors is mediated by the functional PARP gene. The complete resistance of cells lacking PARP to cytotoxicity exerted by MG-132 and PSI resembles that shown by pancreatic beta-cells in response to streptozocin [Burkart et al., 1999; Pieper et al., 1999]. Preliminary results of our further studies indicate that cells lacking PARP develop a multidrug resistance and the inefficacy of proteasome inhibitors is due to this phenomenon.

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